Probiotic modulation of immune responses in an *in vitro* mucosal co-culture model

By

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A thesis submitted to the University of Plymouth

In partial fulfilment for the degree of

Doctor of Philosophy

School of Biomedical and Biological Sciences

Faculty of Science and Technology

This project funded by Ministry of Higher Education

Baghdad, Iraq

February 2013

Copyright Statement

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Abstract

Probiotics confer health benefits through many mechanisms including modulation of the gut immune system. Gut mucosal macrophages play a pivotal role in driving mucosal immune responses. The local environment and macrophage subset determine immune response: tolerance, associated with an M2-like, regulatory macrophage phenotype and inflammatory activation with an M1-like phenotype. The aims of this study were firstly to investigate the immunomodulatory effects of a panel of heat-killed (HK) probiotic bacteria and their secreted proteins (SP) of *Bifidobacterium breve (BB)*, *Lactobacillus rhamnosus GG* (LR), *L. salivarius* (LS), *L. plantarum* (LP), *L. ferrmentum* (LF), and *L. casei strain Shirota* (LcS) on cytokine production and TLR expression in monocultures of monocytes, macrophage subsets, and intestinal epithelial cells. Normally, mucosal gut macrophages resemble the M2 subset and fail to express CD14, a co-receptor for LPS signalling. Therefore, probiotic modulation of LPS-induced NF-kB activity and cytokine expression was investigated using a THP-1 monocyte-derived reporter cell line, model of $CD14^{hi}/^{lo}$ M1 and M2 macrophages. Secondly, a transwell co-culture system was developed to investigate probiotic modulation of macrophage-influenced epithelial barrier function. Parameters investigated included cytokine, TLR and hBD-2 expression, TEER and IHC staining of the tight junction protein, ZO-1. Probiotics selectively modulated monocyte and macrophage subset cytokine expression. Probiotics (HK and SP) suppress $CD14^{lo}$, augment $CD14^{hi}$ M1, and differentially regulated TNF-α production in M2s. M2 macrophage IL-6 production was suppressed by both HK and SPs, and differentially regulated in $CD14^{lo}$ and $CD14^{hi}$ M1s. NF-κB activation failed to parallel probiotic regulation of TNF-α and IL-6. Probiotics (HK-LF and HK-LcS) selectively modulated both endogenous and exogenous TNF-α and IL-10, as well as their induction of epithelial cell expression of TLR and hBD-2. Epithelial expression of TEER, ZO-1 and the endogenous TLR signal regulator, Tollip, were suppressed upon co-culture with pro-inflammatory M1 macrophages paralleled by a suppression of IL-10 and up-regulation of TNF-α and IL-8. In the presence of LPS, HK-LF enhanced TEER, ZO-1 and partially rescued Tollip expression, whereas HK-LcS had no effect on TEER and ZO-1 and displayed a weaker rescue effect on Tollip compared with LF. In the M2/epithelial cell co-culture, both probiotics enhanced TEER and ZO-1 in the presence of LPS, whilst displaying a differential modulation of Tollip, dependant on the format of probiotic (HK or SP). In conclusion, probiotic strains can differentially exert immune activatory or suppressive functions and immunomodulation is determined by strain, inflammatory environment, and mucosal macrophage effector phenotype. Future probiotic development must consider prophylactic use in healthy individuals or therapeutic treatment of defined pathological conditions, strain-specific effects, gut mucosal integrity, and immune phenotype of mucosal macrophages.

Acknowledgments

I would like to express my sincere thanks and gratitude to my first supervisor Dr Andrew D. Foey for his patience, advices, enthusiasm, encouragement, and supervision of the research project. I would like to extend my sincere thanks to my second supervisor Dr Jane Beal for her guidance and encouragement throughout my study. The research would not be possible without funding support, therefore, huge thanks to the Ministry of Higher Education and Scientific Research-Republic of Iraq for providing the financial support for this study, in addition, thanks to the Iraqi cultural attaché in London for their kind support. Special thanks to Dr Wondwossen Abate Woldie, Dr Michele Kiernan, and Dr Tracey Madgett for their valuable assistance in molecular biology. Sincere thanks go to Ms Lynne Cooper, Mr Matthew Emery, and Miss Sarah Jamieson for their valuable comments and advice during my lab work. So many wonderful people have been instrumental with their friendship, thus I would like to express my sincere gratitude to my colleagues and friends such as Lorna Dallas at the School of Biomedical and Biological Sciences for their support. I would also like to express my appreciation to all those people, though their names are not listed, who supported me. Finally, I am indebted to my parents who instilled in me moral discipline, respect for societal value, confidences to face obstacles, as well as provided me with everything they could, especially for good education. My late father in particular, had been an example of simplicity and higher thinking for me and taught me the values of a good education. Last, but no least, I am grateful to my husband, brothers, and sisters who always showed on me their love and helped me in pursuing careers and higher studies.

Author Declaration

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Word count of the main body of the thesis without references: **70,134**

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List of Publications

International peer review publications

(Please refer to appendices for the abstract manuscripts)

- **Habil, N**., W. Al-Murrani, J. Beal and A.D. Foey: (2011): Probiotic bacterial strains differentially modulate macrophage cytokine production in a strain-dependent and cell subset-specific manner. Beneficial Microbes 2(4):283-293.
- **Habil, N**., Beal, J. and Foey, A.D. (2012): *Lactobacillus casei strain Shirota* selectively modulates macrophage subset cytokine production. Int. J. Probiotics & Prebiotics 7(1):1-12.
- **Habil, N**., Abate Woldie, W., Beal, J. and Foey, A.D. (2012): Probiotic bacterial species selectively modulate gut epithelial cell beta-defensin-2. (Submitted).
- **Habil, N**., Abate Woldie, W., Beal, J. and Foey, A.D. (2012): Probiotic modulate epithelial cell barrier properties influenced by co- culture with macrophages (Manuscript in preparation).

Conference publications

Published Abstracts

- **Habil, N**., Al-Shamgani, H., Beal, J. and Foey, A. (2011): Probiotics modulate epithelial cell barrier properties influenced by co-culture with macrophages. Immunology 135 (S1): 131.

Unpublished abstracts / conference proceedings

- Sattar, A., **Habil, N**., Jackson, S., Foey, A**.** and Bradley, G. (2012): Endotoxin activity of Bacteroides lipopolysaccharides from contaminated marine bathing waters. American Society for Microbiology, Annual Conference, San Francisco, USA, 18th -22nd June.
- **Habil, N**., Beal, J. and Foey, A**.** (2011): Probiotic bacterial species selectively modulate gut epithelial cell expression of beta defensin 2. International Probiotic Conference, Slovakia, 13th - 15th June.
- **Habil, N**., Beal, J. and Foey, A. (2010): The immunomodulatory effects of *Lactobacillus casei strain Shirota* on monocyte, M1- and M2-macrophage cytokine production. International Probiotic Conference, Slovakia, 15th - 17th June.

Presentation and Conferences Attended

- Mucosal Immunology and Intestinal Microflora, University of East Anglia UK, 9th June 2009.
- International Probiotic Conference (IPC), Slovakia 15th-17th June 2010. **Poster**
- International Probiotic Conference (IPC), Slovakia 12th-16th June 2011. **Oral presentation**
- British Society of Immunology (BSI) Congress, 5th -8th December 2011, Arena and Convention Centre in Liverpool, UK. **Poster**
- Annual Research Day, 5th April 2011, Centre for Research in Translation Biomedicine (CRTB) of University of Plymouth/UK. **Poster**
- University of Plymouth, 21th March 2012. **Oral presentation**

- Annual Research Day, 14th July 2012, Centre for Research in Translation Biomedicine (CRTB) of University of Plymouth/UK. **Poster**

Symposium

- Probiotic Progress: Health Implications for the Gut and Beyond 12th October 2010, London.
- Gastro-intestinal Models for the study of Probiotic and Prebiotic Scientific Symposium, Slovakia 14th June 2010.

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Abbreviations

AAD- Antibiotic Associated Diarrhoea

AD- Atopic Dermatitis

AMPs- Antimicrobial Peptides

APCs- Antigen presenting cells

ATF- Activating Transcription Factor Protein

BAFF- B-cell activating factor

BB- *Bifidobacterium breve*

bp - Base pair

BSA- Bovine Serum Albumin

cAMP- Cyclic Adenosine Monophosphate

CARD15- Caspase Activating and Recruiting Domain 15

CD- Cluster of Differentiation

CDI- Clostridium Difficile Infection

cDNA- Complementary Deoxyribonucleic Acid

CFU- Colony Forming Unit

CIA- Collagen Induced Arthritis

CO_2- Carbon Dioxide

DAMP- Damage Associated Molecular Patterns

DCs- Dendritic Cells

DNA- Deoxyribonucleic Acid

dNTP- Deoxynucleotide Triphosphate

DSS- Dextran Sulphate Sodium

DW- Distilled Water

EDTA– Ethylene-Diamine Tetra-Acetic Acid

ELISA- Enzyme Linked IMmune-Absorbent Assay

ERK- Extracellular Signal Related Kinase

FACS- Fluorescence Activated Cell Sorting

FCS- Fetal Calf Serum

Flag- Flagellin

g - Gram

GALT- Gut Associated Lymphoid Tissue

GFP- Green Fluorescent Protein

GIT- Gastrointestinal Tract

GM-CSF- Granulocyte Macrophage Colony-Stimulating Factor

hBD-2- human Beta Defensin -2

HK- Heat- killed

hr- Hour(s)

HRP- Horseradish Peroxidase

IBD- Inflammatory Bowel Disease

IBS- Irritable Bowel Syndrome

ICAM- Intracellular Adhesion Molecule

IECs- Intestinal Epithelial Cells

IFN-γ- Interferon Gamma

IgG- Immunoglobulin G

IHC- Immunohistochemical

IKK- Inhibitor of κB Kinase

IL- Interleukin

iNO- inducible Nitric oxide

IRAK- IL-1 Receptor Associated Kinase

IRF- IFN-Regulatory Factor

IκB -Inhibitor of κB

KDa- Kilo Dalton

LAB- Lactic acid bacteria

LBP- LPS Binding Protein

LcS- *Lactobacillus casei* strain Shirota

LF- *Lactobacillus fermentum*

LGG- *Lactobacilus rhamnosus Gorbach and Goldin*

LP- *Lactobacillus plantarum*

LPS- Lipopolysaccharide

LR- *Lactobacillus rhamnosus* GG

LRR- Leucine-Rich Repeat

LS- *Lactobacillus salivarius*

LTA- Lipoteichoic acid

M– Molar

M cells- micro fold cells

M1- Pro-inflammatory Macrophage

M2- Anti-inflammatory Macrophage

MAPK- Mitogen-Activated Protein Kinases

MCP-1-Macrophage Chemoattractant Protein-1

M-CSF- Macrophage Colony Stimulating Factor

MDP- Muramyl Dipeptide

MFI- Mean Fluorescence Intensity

mg- milligram

MHC- Major Histocompatibility Complex

min- Minute(s)

MLCK-Myosin Light Chain Kinase

MLNs-Mesenteric Lymph Nodes

MRS- de Man, Rogosa and Sharpe

MyD88- Myeloid Differentiation Factor 88

MΦ- Macrophages

NCBI- National Centre of Biotechnology Institute

NCIMB- National Collection of Industrial, Marine, and Food Bacteria

NF-kB- Nuclear Factor kappa B

ng – Nanogram

NODs- Nucleotide Oligomerisation Domains

OD- Optical Density

PAMPs- Pathogen Associated Molecular Patterns

PBMCs- Peripheral Blood Mononuclear Cells

PBS- Phosphate Buffered Saline

PCR- Polymerase Chain Reaction

PE- Phycoerythrin

PGN– Peptidoglycan

PI3K- Phosphatidylinositide 3-kinases

PKC- Protein kinase C

PMA- Phorbol-12-Myristate Acetate

POXP3- Forkhead Box P3

PPAR-γ- Peroxisome Proliferator Activated Receptor Gamma

PPs- Peyers' Patches

PRRs- Pattern Recognition Receptors

R10- RPMI supplemented with 10% v/v foetal calf serum (FCS)

RA- Retinoic Acid

RPMI- Roswell Park Memorial Institute

RT- PCR- Reverse Transcriptase Polymerase Chain Reaction

RT- Room Temperature

SCFA- Short Chain Fatty Acid

SDS-PAGE- Sodium-Dodecyl Sulphate-Polyacrylamide Gel electrophoresis

SE- Standard Error

SIGIRR – Single Immunoglobulin Interleukin I Receptor Related

SP- Secreted Protein

SP-1- Salmonella Pathogenicity Island 1

SSC - Side Scatter

STAT - Signal Transducers and Activators of Transcription

TACE -TNF-α Converting Enzyme

TAK1- Transforming Growth Factor Activated Kinase

TCR- T Cell Receptor

TEER- Transepithelial Electrical Resistance

TGF-β-Transforming Growth Factor Beta

Th T- helper cell

TIMP3- Tissue Inhibitor of Metalloproteinase 3

TJ-Tight Junction

TLR- Toll-Like Receptor

TNBS-Trinitrobenzene Sulfonic Acid

TNF-α- Tumour Necrosis Factor Alpha

Tollip- Toll Inhibitory Protein

TRAF6 - TNF Receptor Associated Factor 6

Treg-regulatory T cells

TREM-1-Triggering Receptor Expressed on Myeloid Cells-1

TSLP- Thymic Stromal Lymphopoietin

UC- Ulcerative Colitis

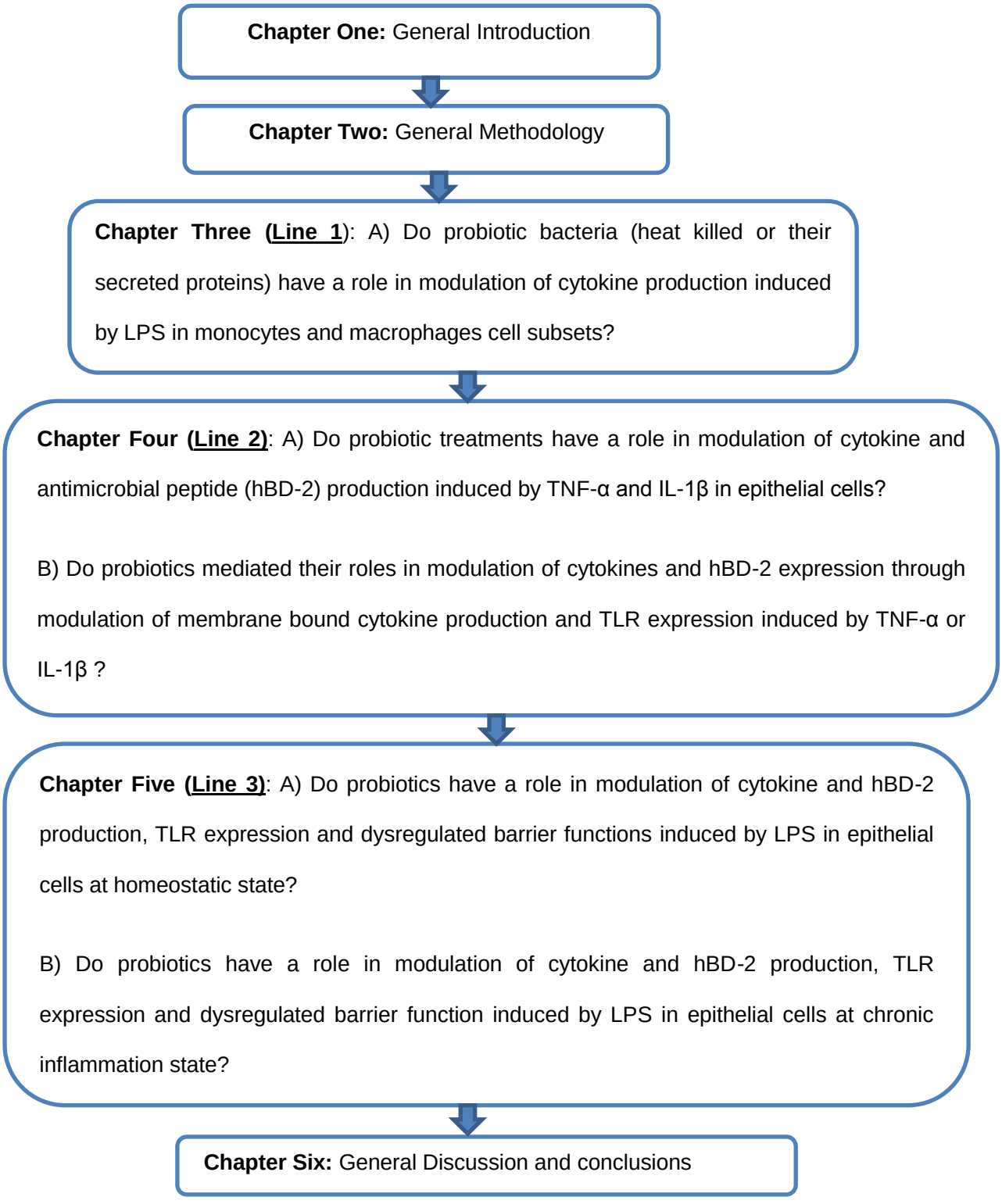
UEA- Ulex Europaeus Agglutinin

UV- Ultra Violet

v/v - Volume by volume

w/v - Weight by volume

ZO-1- Zonula Occludens -1



Thesis outline and aims of each line experimental investigation

Chapter 1

General Introduction and literature review

Chapter 1: General Introduction and literature Review

1.1. Introduction

Over a century ago, it was claimed that the intestinal microflora express an array of functions on human health (Iacono et al., 2011). Interactions of gut microbiota with gut mucosa are vital in maintaining immune homeostasis (Wells et al., 2011a). Commensal microbiota of the gastro-intestinal tract (GIT) show a significant role in governing infectious residence of enteropathic bacteria and their possible injurious effects to the host (MacDonald and Monteleone, 2005). Breakdown of gut homeostasis can lead to a loss of tolerance (Bamias et al., 2005). Owing to this breakdown of tolerance, Inflammatory Bowel Diseases (IBD) such as ulcerative colitis and Crohn's disease are becoming more prevalent (Molodecky et al., 2011).

IBD is generally a chronic relapsing inflammatory disorder of the GIT. Approximately 0.1% western people suffer from IBD; the incidence is highest in developed countries (Goh and Xiao, 2009, Shanahan, 2001). The most generally accepted opinion on the pathogenesis of IBD is that it results from the irregular immune response to enteric bacteria, resulting in overproduction of pro-inflammatory cytokines such as TNF-α and IL-1β, promoting tissue injury of the gut mucosa (Baumgart and Carding, 2007).

Modulation of the mucosal immune response by naturally occurring substances is one possible approach for the prevention and therapy of IBD (Fedorak, 2008). Probiotic bacteria could be an effective alternative to the use of pharmacological substances (e.g. antibiotics) in nutrition and medicine, because synthetic therapeutics has numerous side effects (Bomba et al., 2002). Despite a wealth of

knowledge obtained, the mechanisms of probiotic bacterial actions are still poorly understood (Tsai et al., 2010, Walker, 2008). Since the data regarding the mechanisms of probiotics are often contradictory, it will be important to obtain additional knowledge on their mode of action, and searching for ways to improve probiotic bacterial efficacy in the treatment of IBD.

1.2. Gut associated lymphoid tissue (GALT)

GALT is structured into discrete lymphoid follicles or Peyers' Patches (PPs) situated underneath the epithelial cell layer along the whole length of the gut. The epithelium overlying the PPs is composed of cells that differ from the surrounding enterocytes; these cells are called micro-fold (M) cells. M cells lack microvilli, have no glycocalyx coating, and are designated to interact directly with antigens. They are indented; basolateraly, forming a pocket that contains T, B cells, dendritic cells (DCs), monocytes, and macrophages (Figure1.1). GALT is constantly exposed to foreign matter (e.g. food, drinks), and an enormous amount of commensal microorganisms about $1x10^{14}$ CFU/ml (Alain, 2004). The mucosal immune system of the gut has developed sophisticated mechanisms by which it can selectively sample the luminal contents in response to these microbes and the food-antigen-rich environment.

Antigens from the lumen are taken up by two defined ways: endocytosis (transport across the M cells in vesicles and release at the basolateral mucosal surfaces) followed by binding to DCs or macrophages; or phagocytosis by DCs (direct microbial sample), which is dependent on active polymerization of the actin cytoskeleton and independent of a clathrin based mechanism. Processing and presenting antigens to the T cells through MHC class II, in addition to co-

stimulatory molecules such as CD86, is the main step in maintaining innate and adaptive immunity via the antigen presenting cells (APCs) such as DCs and macrophages. Subsequently, T cells decide on either an amplifying inflammatory or anergy immune response depending on the type of antigens presented by APCs. There is constant interplay between the host cells and the microflora that resident on the gut mucosa. The interaction between the epithelium and gut microflora could play a role in determining the fate of the subsequent immune responses (activation or tolerogenic mechanism) (Bibiloni and Schiffrin, 2010).

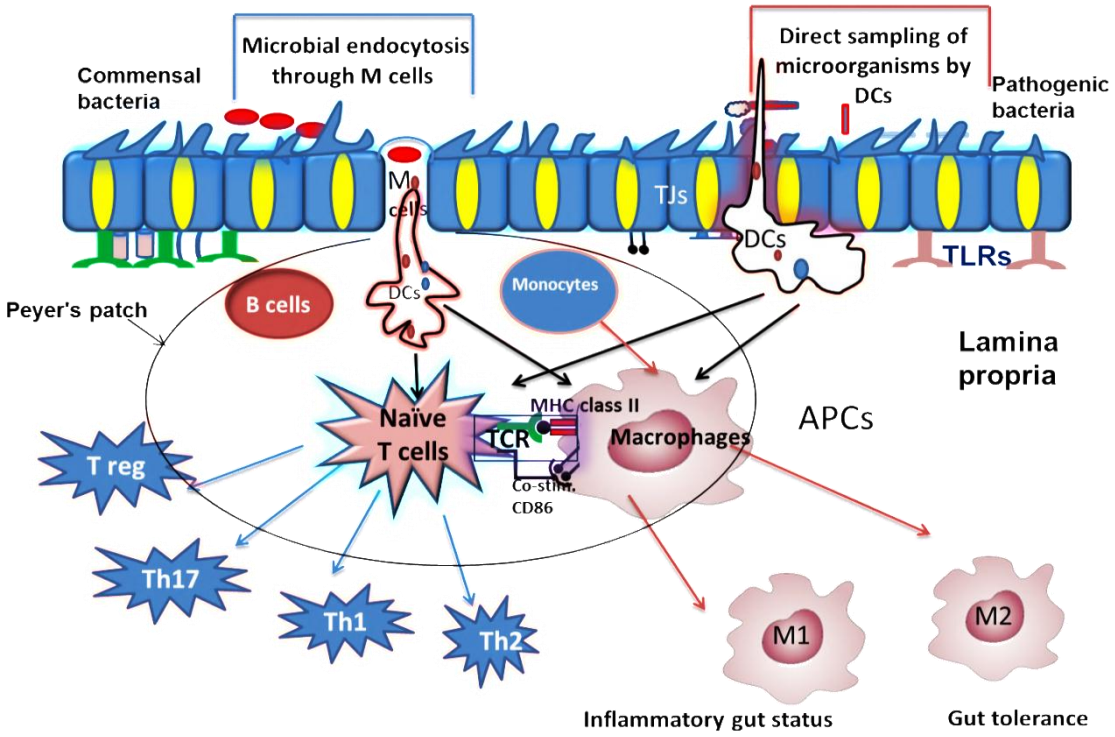


Figure 1. 1: Gut associated lymphoid tissue (GALT).

The GALT consists of Peyers' Patches (PPs), which contain follicles consisting of Micro-fold cells (M cells), T, B cells, Dendritic cells (DCs), monocytes, and macrophages. T cells differentiated into Th1, Th2, Th17, and Tregs. Macrophages differentiated into either M1-pro-inflammatory or M2-anti-inflammatory subsets. Antigens are taken up by M cells through

endocytosis or by DCs through phagocytosis. Antigens processing and presentation is performed by antigen presenting cells (APCs) such as DCs and macrophages. APCs presented antigens to T cells through MHC class II, recognised by T cells through the T cell receptor (TCR). T cells, in response to antigen presentation decide upon immune activation or mucosal tolerance, dependent on the type of stimuli.

1.3. Mucosal tolerance

Hypo-responsiveness or lack of immunity against food antigens and commensal microbiota is referred to as mucosal tolerance. In normal physiological conditions, food and commensal microbiota are tolerated, while the potential to initiate an immune response against invading pathogenic microorganisms is maintained. There are several active mechanisms of tolerance including mechanisms initiated by low dose antigens via Tregs, which promote tolerance by suppression of immune response. Alternatively, at high dose of antigens, tolerance is initiated by clonal deletion or anergy of effector T cells present in the inductive sites of the gut such as PPs (Duchmann et al., 1995). Intestinal DCs, the most potent APCs, are central in controlling this immunological tolerance (Bibiloni and Schiffrin, 2010). DC tolerance is controlled by epithelial expression of thymic stromal lymphopoietin (TSLP), transforming growth factor beta (TGF-β), and retinoic acid (RA), which results in the specific DCs phenotype (DCs $CD103^{+}$) that induce gut tolerance by promoting proliferation and differentiation of Treg $Foxp3^{+}$ (Zivny et al., 2001). However, macrophages acting as APCs also control the mucosal immune tolerance in the GIT. Elicited immune responses by macrophages are dependent on tissue environment and the resulting effector cell subset, where homeostatic macrophages resemble M2 and inflammatory macrophages resemble M1 macrophage cells (Mosser and Edwards, 2008). IBD is induced by breaking down mucosal tolerance whereby the immune response is initiated against food and the

non-pathogenic commensal microbiota in vulnerable subjects (Silvia and Kim, 2009).

1.4. Gut microbiota

Gut microbiota is composed of species within four bacterial divisions: *Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria* (Sartor, 2008). It is well known that the GIT microbiota is established from birth. During the first month of life, the microbiota change and become more complex, reaching the composition of an adult by the age of one to two years (Palmer et al., 2007). The establishment of the gut microbiota is usually characterised by specific stages of development, early colonisation by facultative anaerobes such as *Bifidobacterium* species, whereas later stages of life, are associated with suppression of *Bifidobacterium* species and augmentation of *Clostridia and Bacteroides* species. The microbiota stays relatively stable over time depending on many factors such as genetic factors, age, diet, and antibiotic treatment (Tlaskalová-Hogenová et al., 2004, Zoetendal et al., 1998). The dominance of the microorganisms depends on the region of the GIT, e.g. *Helicobacter pylori* in the stomach, *Streptococcus*, and *Lactobacillus* in the duodenum, *Lactobacillus,* Gram-positive cocci in the jejunum, *Bacteroides*, *Clostridium*, and *Bifidobacterium* in the ileum, and finally *Lactobacillus*, *Enterococcus*, *Escherichia coli*, *Peptostreptococcus* and *Clostridium* in the colon (Sartor, 2008, Winkler et al., 2007) (see Fig.1.2). A balanced ecosystem of intestinal flora is essential for microbial cell survival. Changing the diet and antibiotic use are the main factors associated with the alteration of normal microbial ecosystem (Vanhoutvin et al., 2009). Indeed, one of the main factors that are associated with gut diseases, particularly IBD, is the alteration or dysbiosis of the complex microbial ecosystem (Bibiloni and Schiffrin, 2010).

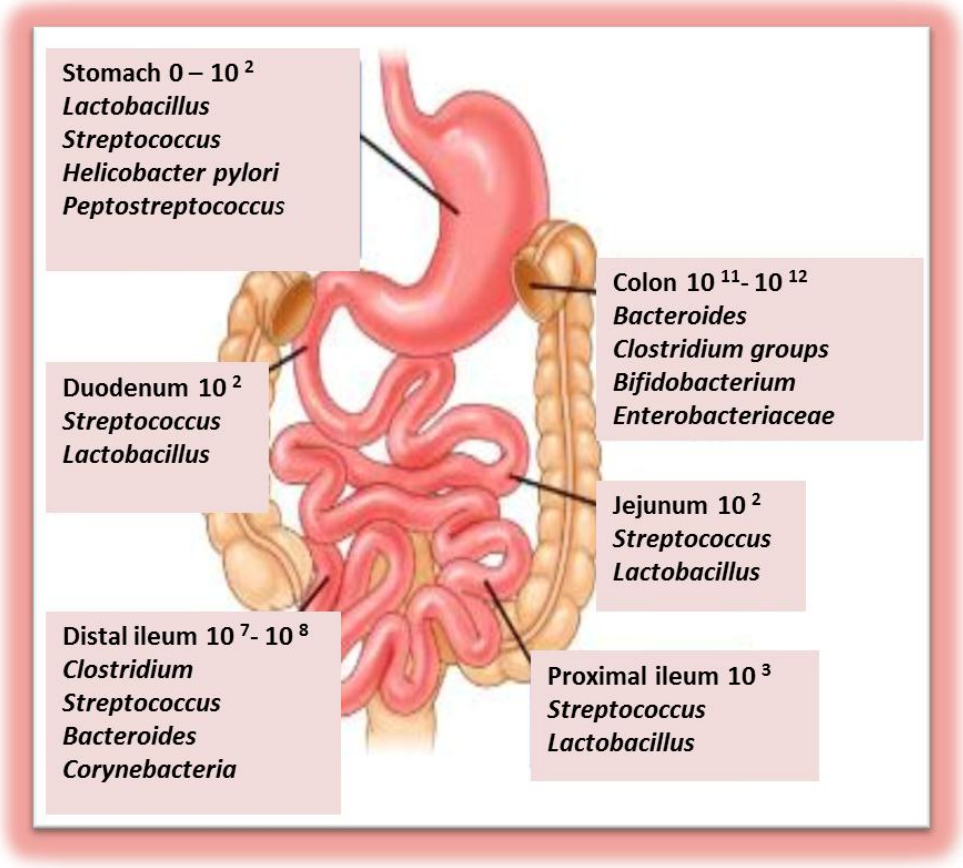


Figure 1. 2: Relative composition of dominant microbial species in various regions of the gastrointestinal tract (Sartor, 2008)**.**

1.4.1. Main functions of the gut flora

The population of microorganisms in the gut has more than 800 species, classified as normal commensal, and pathogenic microorganisms (Backhed et al., 2005). The beneficial role of the gut commensal microbiota can be grouped as are metabolic, trophic, and protective. Normally, gut microbiota ferment non-digestible components by two defined ways: saccharolytic pathway resulting in acetic, propionic and butyric acid, and proteolytic pathway resulting in nitrogenous metabolites such as phenols and heterocyclic amines. Short chain fatty acids (SCFAs) such as butyrate modulate the growth and differentiation of the intestinal epithelium. Epithelial cells of the caecum and colon depend on butyrate for their

carbon and energy (Vanhoutvin et al., 2009), facilitating epithelial cell growth, maintaining a healthy gut mucosa and reducing the risk of inflammation and cancer (Peter et al., 1992). In addition, colonic microflora participate in vitamin synthesis (e.g. vitamin K, B, and Biotin), and the absorption of calcium, magnesium, and iron (Hill, 1997).

Commensal microbiota performs several tasks against pathogens via numerous mechanisms include; colonisation resistance or barrier effector mechanisms (competitive colonization, the ability of physical adherence, and competition for nutrients available in the gut) (van der Waaij et al., 1971). Another aspect of commensal microbiota against pathogens is to compete with pathogenic bacteria through antagonistic mechanisms. It is well reported that surface components of many enteric bacteria are important for their virulence, which include capsular polysaccharide (CPS) and lipopolysaccharide (LPS) (Moran et al., 1997). The Lipopolysaccharides is composed of biphosphorylated lipid (lipid A) forming the matrix of the outermost membrane leaflet and a hydrophobic polysaccharides, extending outward from the bacterium. The polysaccharides moiety consists generally of two distinct regions, a core oligosaccharides containing 10 to 12 sugars and a polysaccharide chain of repeating units, the O-specific chain. The core oligosaccharide is covalently bound to lipid A through an acidic sugar, usually 3-deoxy-Dmanno-oct- 2-ulopyranosonic (Kdo). Backhed et al (2003) reported that the hexa-acylated lipid A in the commensal bacteria performed as an antagonist of hypo-acylated lipid A, which is the main feature of several microflora causing chronic infection such as pathogenic *Salmonella* species (Guo et al., 1998). These mechanisms are one of the tolerogenic features of commensal microorganism

when acting as competitive inhibitors against pathogenic microorganisms (Weintraub et al., 1989).

One of the protective functions of the microbiota against pathogens is by inhibition of inflammatory cell signalling caused by pathogenic microorganisms. Evidence supports that the commensal microbiota of the GIT play a significant role in controlling infection of enteropathogenic bacteria and their potential harmful effects to the host by inhibiting the transcriptional factor nuclear factor kB (NF-kB). This master regulator of epithelial cell function is induced and up-regulated by pathogens (Tien et al., 2006).

In terms of maintaining epithelial gut barrier function, microbiota have a role in reinforcing the different intracellular junctions, which include tight junctions (TJs), adherence junctions, and desmosomes by up-regulation of Zonula occludens-1 (ZO-1) protein expression (Aijaz et al., 2006). Gut microbiota are also maintain gut barrier function through the secretion of polysaccharides, facilitating the initiation of adhesion to epithelial cells. Shah and Lankaputhra (1997) reported that the colon wall provides an ecological site for bacterial attachment and proliferation.

As a part of innate immunity of the GIT, goblet cells secrete mucus composed of mucin glyco-proteins. Mucous layer coated gut epithelium forming the glycocalyx, provides protection in the GIT from invading microorganisms. Andrew et al (1997) indicated that the bioactive factors including microbes, microbial products, toxins, cytokines, hormones and growth factors regulates the mucosal layer composition, positively or negatively. Deplancke et al (2000) reported that the alteration of mucus composition, or mucus secretion in response to intestinal microbes or host-derived inflammatory mediators was characteristic in the majority of gut diseases,

particularly IBD. Additionally, gut flora have significant roles in maturation of the mucosal immune system (Hart et al., 2002). They can enhance the immune response by increasing the secretion of immunoglobulin A (IgA) (Sekirov et al., 2010). sIgA binds to the mucus layer overlying the gut epithelium, leading to the neutralisation of pathogens and their toxins (Mantis and Forbes, 2010).

1.5. Gut epithelial cells

In addition to absorption of nutrients, electrolytes, and water, gut epithelial cells exhibit a protective function through specialised cells called intestinal villous or M cells, associated with the expression of Ulex europaeus agglutinin (UEA^+)-1. Both intestinal villous and M cells have been observed to take up green fluorescent protein (GFP) expressing *Salmonella*, as well as gut bacterial antigens (Jang et al., 2004). These cells have a pivotal role in scanning the gut environment for microbial threats, interpreting signals from luminal contents, presenting this information to professional immune cells in the lamina propria, and thereby cooperating with GALT to mount a response to certain antigenic stimuli (Bibiloni and Schiffrin, 2010, Wells et al., 2011a).

The gut epithelial cells can function as antigen presenting cells (APCs), expressing receptors, and antigen presenting molecules (MHC class II & I) (Mayer, 1998). These receptors, which include pattern recognition receptors (PRRs) such as Toll like receptors (TLRs) and Nod like receptors (NLR), allow the epithelial cells to sense microbial components (Wells et al., 2011a). In addition to antigen presenting molecules and PRR expression, enterocytes express the adhesion molecules such as intercellular adhesion molecule (ICAM-1), and CD58 (Huang et al., 1996). However, they do not express co-stimulatory molecules (CD80, CD83,

and CD86) required for T-cell activation, suggesting that they are good candidates for tolerogenic APCs in *vivo* (Cario and Podolsky, 2005, Melmed et al., 2003).

Presentation of antigens by enterocytes to adjacent $CD4^+$T cells might help to explain local tolerance but not systemic tolerance, because naive $CD4^+$T cells are rare in the Lamina propria, in addition, lamina propria T cells do not migrate out of the gut (MacDonald and Monteleone, 2005). Therefore, it remains possible that the presentation of antigens to lamina propria $CD4^+$T cells by enterocytes could be involved in maintaining the survival and activity of previously primed regulatory or and effector T cells, thereby maintaining local tolerance to environmental antigens. Enterocyte tolerance induction demonstrates that the rules of the mucosal immune system are different from those of the systemic immune system (Cario and Podolsky, 2005a). Normally, commensal microbiota should not elicit an inflammatory response; however, some investigators have shown that a constant TLR stimulation and low level NF-kB activation may be necessary for intestinal health as a part of the surveillance system of the gut (Rakoff et al., 2004, Jijon et al., 2004, Rachmilewitz, 2002).

Epithelial cells still indirectly communicate with other cells of the immune system; they achieve this through releasing cytokines and antimicrobial peptides (AMPs). Upon pathogenic activation, epithelial cells release pro-inflammatory cytokines/chemokines (IL-8) that recruit pro-inflammatory cells from circulation such as neutrophils, or release MCP-1 to recruit monocytes to the site of infection. In addition to secreting cytokines, they secrete AMPs, particularly human β defensin-2 (hBD-2) (Ganz, 2003). Basically, there are many types of AMPs, such as lactoferrin, cathelicidins (LL37), hepcidins, dermicidins, histamine, thrombocidins, and defensins (Klüver et al., 2006).

Defensins are an important natural innate immune defence; these peptides display broad-spectrum activity killing against both Gram-positive and Gram-negative bacteria, yeast, fungi and enveloped viruses. During the first attack, epithelial cells secrete AMPs particularly hBD-2, to kill pathogenic microorganisms. The main mechanism of defensins- mediated microbial killing is by binding the microbial cell membrane (which has negative charges) and disturbing the microorganism cell wall by forming a large hollow polymer (permeabilized microbial membrane), these results in lysis of bacterial cells. Defensins may also translocate across the cell membrane, bind directly with microbial DNA and disturbing the synthesis of DNA and/or protein leading to pathogen killing (Cleveland et al., 2001). Defensins are grouped into α and β definsin based on the connectivity of disulphide bounds between cysteine pairs, for example, hBD-2 has three bridges of disulphide bounds (cyst.1-cyst.5), (cyst.2-cyst.4) and (cyst.3-cyst.6). Paneth cells of the small intestine and neutrophils are the main produces of human α- defensins (3.5-4 K Da), whereas hBD-2 (4-6 K Da) is produced by epithelial cells (Ganz, 2003) and macrophages (Romano et al., 2009). They are stored in the granules in the Paneth cells situated beside the proliferative crypt stem cells, and secreted into the luminal space in response to microbial molecules e.g. LPS stimulation. The release of hBD-2 is controlled by pattern recognition receptors signalling particularly NOD-2-mediated signalling as mutation in NOD-2 is associated with low hBD-2 expression, resulting in deficiency of pathogenic microbial killing and potential dysbiosis (Grimm and Pavli, 2004). They are stored as an inactive pro-peptide form. The activation of defensins in humans is by trypsin isozymes expressed in Paneth cells, which cleave the pro-peptide at the Arg55-Ala56 and Arg62-Thr63 sites (Ghosh et al., 2002); therefore, trypsin exhibits a significant role in regulation of innate immunity. Defensins are either constitutively (e.g.hBD-1) or

inducibly (e.g.hBD-2) expressed by epithelial cells (Vora et al., 2004). hBD-2 is induced by two different activation pathways: firstly, indirect endogenous stimuli such as pro-inflammatory cytokines (IL-1β, TNF-α, IL-22, IL-17and IL-23), which are predominantly produced by macrophages, DCs, and Th17 by the host in response to microbial stimulation. The most important cytokines controlling hBD-2 production are IL-10 and TNF-α (Kanda et al., 2011, Marian et al., 2009). Secondly, direct exogenous stimuli such as microbial pathogenic associated molecular patterns (PAMPs) of LPS, PGN, and microbial DNA (Mondel et al., 2008, Schlee et al., 2008). Therefore, the expression of hBD-2 provides a first line of defence against potentially pathogenic gut microbes. Moreover, hBD-2 has a role in the regulation of immune responses and inflammation (Klüver et al., 2006). Several studies described the potential role of defensins in augmenting pro-inflammatory cytokines such as IL-8 (Chaly et al., 2000, Van Wetering et al., 1997), TNF-α and IL-1 at the site of microbial infection. This augmentation of pro-inflammatory cytokines seems likely to amplify local inflammatory responses in order to clear pathogens (Chaly et al., 2000). Deficiency of hBD-2 is associated with defects in the innate immune response to pathogens which correlate with gut diseases and pathogenesis of IBD, particularly Crohn's disease (Baumgart and Carding, 2007, Wehkamp et al., 2005).

1.5.1. Gut epithelial permeability

The main factor controlling epithelial cell barrier function is the formation of epithelial tight junctions (TJs) which are the most apically situated cell-cell junctions holding epithelial cells together in a way that prevents leaking between cells. TJs regulate diffusion of molecules through the para-cellular pathway. They are composed of different types of protein including Zonula Occludens ZO-1, ZO-2,

and Claudins. They are responsible for the formation of tight junction strands connected with the actin cytoskeleton mediated by ZO-1 (Schneeberger and Lynch, 1992).

ZO-1 is described as a linkage protein between trans-membrane proteins (Occludin and Claudine) and the actin cytoskeleton. It belongs to a family of membrane-associated guanylate kinases (MAGUK) involved in signal transduction during cell-cell contact, which depends on the phosphorylation of myosin light chain kinase (MLCK) (Yamamoto and Gaynor, 2001). ZO-1 plays an important role in stabilising the barrier of intestinal epithelial cells, functionally linking it to the actin-myosin cytoskeleton, and limiting permeability of the cell layer to large solutes (Gilmore, 2006), where the permeability to solutes is measured in terms of trans-epithelial electrical resistance (TEER). Furthermore, microbiota have a significant role in maintaining the barrier function via modulation of ZO-1 by modulation of heat shock proteins (Hsps), particularly Hsp70. Normally, Hsps can regulate intracellular signalling processes in response to cellular stress (Pratt and Toft, 2003). Hsp70 acts as an activator of ZO-1-associated nucleic acid-binding protein (ZONAB) (Tsapara et al., 2006); the domain of ZO-1 interacts with signalling proteins to regulate para-cellular permeability (Balda and Matter, 2000). Under stress conditions, up-regulation of Hsp70 leads to augmented ZO-1 expression, thereby maintaining the epithelial cell barrier by sealing the tight junctions.

A variety of both exogenous and endogenous factors can affect the permeability of the intestinal epithelial tight junctions. These include pathogenic bacterial toxins, dietary glucose, cytokines, cellular stress, and growth factors (Nusrat et al., 2000). Studies have shown the role of inflammation in affecting the tight junctions and

increasing their permeability through up-regulation of pro-inflammatory cytokines, such as TNF-α. Walsh et al. (2000), based on evidence from several studies, postulated that IFN-γ alters para-cellular permeability by several mechanisms, including possible direct effects on ZO-1 and/or via changes in the perijunctional actin cytoskeleton. In contrast, Bruewer et al. (2005) reported that TNF-α affects the localisation and expression of ZO-1 in T84, a human intestinal epithelial cell line, in addition, Schmitz et al. (1999) showed that TNF-α induced decrease in TEER of the intestinal epithelial cell lines HT29 and Caco-2 correlated with suppression of ZO-1 expression. On the other hand, anti-inflammatory cytokines promote the barrier function in human enterocytes (Capaldo and Nusrat, 2009). Mazzon et al. (2002) observed that IL-10 knockout mice, a model of spontaneous colitis, have augmented levels of pro-inflammatory cytokines TNF-α, IL-1, and IL-6, they also pointed out that IL-10 ablation correlates with miss-localisation of ZO-1 protein tight junction.

Although, the relationship between epithelial cell survival, their permeability and endogenous cytokine expression related to ZO-1 expression is not fully understood. Several researchers such as Yang et al. (2003) and Jin et al. (2010) reported that the increasing intestinal epithelial cell permeability to small molecules has been linked to the suppression of endogenous IL-6 expression, which leads to TJ ZO-1 instability. This explains the vital role of endogenous cytokine expression in maintaining epithelial cell integrity and homeostasis. Stuart and Nigam (1995) reported that TJ protein biogenesis, particularly ZO-1, is regulated by protein kinase C (PKC) in response to luminal bacteria and the activation of PKC via its substrate myristolytated alanine rich C kinase (MARCKS), which is mediated directly via TLR2 and its ligand (PGN) (Cario et al., 2004). Therefore, TLR2

expression is vital in maintaining the epithelial cell barrier through it is a role in ZO-1 translocation to seal tight junctions. However, the molecular mechanism underlying ZO-1 protein expression mediated epithelial gut permeability is not fully clear.

1.6. Molecular mechanism of microbial recognition by gut mucosal cells

Induction of innate immune response by microorganisms is mediated through the families of pattern recognition receptors (PRRs) such as TLRs and NLRs. These families contain a number of soluble (collectines), surface-expressed (TLR2, 4, 5), endosomal (TLR9, 3, 7) and cytosolic (NOD-2) receptors that recognise pathogen associated molecular pattern (PAMPs). The main role of PRRs is defence against pathogens, on the other hand, gut epithelial PRR recognition of normal commensal PAMPs are converted into signals for anti-microbial peptide expression (hBD-2), barrier strengthening (ZO-1 re-distribution), and proliferation of epithelial cells (Cario and Podolsky, 2005a). Therefore, the impairment of cell signalling resulting from polymorphism in TLR and NLR genes is involved in many diseases e.g. IBD (Franchimont et al., 2004, Hedl et al., 2007).

Until now, eleven TLRs (Takeda et al., 2003) and 20 NLRs (Inohara and Nunez, 2003) have been identified in humans. The TLR family comprises human homologues of the *Drosophila* Toll protein. The extracellular domain of Toll receptors contains a leucine-rich repeats (LRR), whereas the cytoplasmic domain displays homology with that of interleukin-1 receptor (IL-1R), and is referred to as Toll/IL-R (TIR) domain. The recognition of microbes or microbial by-product by the external portion of TLR receptor will result in the activation of a signalling cascade inside the cells that culminates in the production of the inflammatory mediators.

However, TLRs recognise and respond to not only pathogen associated molecular patterns, but also bind to non-pathogenic antigens such as heat shock proteins (Osterloh and Breloer, 2008). Cytoplasmic NLR recognise a wide range of microbial ligands and toxins as well as certain damage-associated molecular patterns (DAMP) of the host cell. NODs and NALPs are main groups well characterised within NLR family (Williams et al., 2010).

The localisation of TLRs and NLRs has a vital role in controlling inflammation and maintaining the normal gut mucosa. The expression of TLRs varies within cell types and cellular localisation. TLRs are expressed on many of the myeloid cells such as macrophages and DCs; and on non-myeloid cells such as epithelial cells and fibroblast. This expression is either extracellular such as (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11), intracellular (TLR3, TLR7, TLR8, TLR9), or both (TLR4, TLR9). The immune response occurs after microbial PAMPs are recognised via PRRs (interaction of the extracellular LRR domain with microbial PAMPs) followed by recruitment of cytoplasmic adaptor (MyD88), resulting in the activation of NF-kB (Takeda et al., 2003).

A key inducer of the inflammatory response to Gram-negative bacteria is LPS. LPS is a large molecule consisting of O antigen, core, and lipid A. It has two types; smooth and rough (Moreno et al., 1979); acts as endotoxin, and elicits a strong immune response through the Lipid A portion (Raetz and Whitfield, 2002). In fact, the loss of O antigen resulting in rough LPS, which makes the bacterial cell membrane more permeable and easier to destroy by AMPs. LPS is mostly recognised through TLR4 (Beutler, 2000), particularly binding the lipid A portion of rough LPS in the presence of CD14 and MD-2 (Taniguchi et al., 2009), whereas smooth LPS binds LPS binding protein (LBP) in the presence of CD14 and MD2

(Wright et al., 1990). MD2 protein has been revealed to form a complex with TLR4, it is essential for surface expression and LPS responsiveness; therefore, MD-2 mediates the signal between LPS and TLR4 (Shimazu et al., 1999).

CD14 is a co-factor for LPS signalling, described as a glycosylphosphatidylinositol (GPI)-linked membrane protein, which is unable to initiate a trans-membrane signal, because of lacking the cytoplasmic domain, and therefore must interact with other proteins such as TLR4 to mediate signalling. CD14 expression has multiple functions. Jean et al. (2001) reported that LPS is cross-linked precisely to TLR4 and MD-2 only when co-expressed with CD14. In addition to its role in LPS recognition, CD14 also recognises bacterial PAMPs; PGN, mycobacterial lipoarabinomannan and streptococcal cell wall polysaccharides (Pugin et al., 1994, Soell et al., 1995, Weidemann et al., 1997, Wright et al., 1990). Thus having the capability to recognise and bind components of both Gram negative and Gram positive bacteria and serving as a co-receptor for TLR4 and TLR2. Furthermore, CD14 facilitates innate responses to infectious non-self-molecules as well as interacting with apoptotic cells, serving as a scavenger receptor in macrophage cells (Devitt et al., 1998, Pradhan et al., 1997). CD14 has two forms, soluble and membrane bound protein, expressed by many cells such as monocytes/macrophages and epithelial cells (Jean et al., 2001). Up to date studies have verified that endogenous molecules or damage-associated molecular patterns (DAMP) released from damaged tissues are able to activate the co-receptor of TLR4 and TLR2 (CD14) (Yu et al., 2011).

TLR2 recognises microbial PAMPs for both Gram-negative and Gram-positive bacteria e.g. bacterial lipopolypeptides, peptidoglycan (PGN), lipoteichoic acid (LTA), and zymosan from yeast. It is involved in recognition of Gram-positive

bacterial products (PGN) coupled with CD14 (Meyenburg et al., 2004). TLR2 expression has a significant role in maintaining gut mucosa. Recently, researchers showed that the induction of TLR2 by epithelial cells, in response to normal non-pathogenic microbiota, has a role in the induction of the regulatory cytokine, IL-10, by macrophages and Tregs resident in the lamina propria leading to promoting gut tolerance (Round et al., 2011). In addition, Cario (2008), based on evidence from recent animal model and human studies, has reported that the deficiency of TLR2 signalling may cause discrepancies of commensal-dependent intestinal epithelial barrier defences, resulting in mucosal injury. Evidence showed that the polymorphism of TLR2 gene is associated with severe bacterial infections such as *Staphylococcus* and *Mycobacterium* infection. This suggests that mutation of TLR2 gene could enhance the impairment of the host response to a certain range of microbial pathogens (Lorenz et al., 2000, Texereau et al., 2005), leading to initiation of intestinal injury (Candia et al., 2012, Hartmann et al., 2012).

TLR5 recognises flagellin, the main structural protein of the flagella on Gram-negative and Gram-positive bacteria (Oshima et al., 2010). TLR5 has a vital role in maintaining the gut mucosa by inducing anti-microbial peptides, particularly hBD-2 (Schlee et al., 2007); consequently, the lack of it is associated with gut injury (Carvalho et al., 2011).

TLR9 recognises unmethylated CpG bacterial DNA (Minns et al., 2006). The expression of TLR9 by epithelial cells has a crucial role in regulating innate immunity through its roles in degranulation of antimicrobial peptides such as defensins by Paneth cells (Rumio et al., 2004). Normally, TLR9 is expressed basolateraly of epithelial cells; however, it is also expressed on the apical cell surface in response to pathogenic microbial infection (Ewaschuk et al., 2007,

Takeshita et al., 2001). Apical expression of TLR9 enables the cells to recognise pathogenic bacterial DNA; whereas; basolateral expression recognises commensal non-pathogenic bacteria (O'Hara et al., 2012).

TLR3 recognises double stranded viral RNA (Alexopoulou et al., 2001), expressed by mature colonic epithelium against viral infection. The activation of TLR3 expression is followed by intracellular activation downstream of the cells independent of the myeloid differentiation factor 88 (MyD88) pathway, which differs from other TLR cell activation pathways (Yamamoto et al., 2003).

Nucleotide Oligomerisation domains (NODs) are a family of cytosolic proteins that act as PRRs and trigger a signalling cascade leading to inflammatory responses. NOD-2, also named Caspase activating and recruiting domain (CARD15), is one of the NOD family and recognises intra-cytoplasmic PAMPs of muramyl dipeptide (MDP), the smallest bioactive components of PGN (Girardin et al., 2003a). NOD-2 is expressed by monocytes/macrophages, dendritic and intestinal epithelial cells in response to intracellular microorganisms. The activation of NOD-2 by invading intracellular pathogenic microorganisms such as *Salmonella* and *Shigella* is followed by amplification of inflammation by up-regulating the production of pro-inflammatory cytokine (Girardin et al., 2003b). However, commensal bacteria like *Lactobacillus casei strain Shirota* (*LcS)* is also recognised through NOD-2, but they exhibit beneficial effects through their suppression of inflammatory cytokines leading to an improved gut mucosa in the IBD model (Matsumoto et al., 2009). NOD-2 has a crucial role in maintaining the gut mucosa (van Heel et al., 2005); NOD-2 gene polymorphism causes defects in PGN signalling, when recognition of commensal PGN results in amplifying the immune response associated with up-regulation of the inflammatory cytokines.

1.6.1. Toll like receptor (TLR) signalling pathways

Recognition of microbial ligands by epithelial TLRs is considered the first step in controlling innate immune responses. Upon recognition of microbial ligands, TLRs are dimerised and trigger a signalling cascade, leading to the activation of the pro-inflammatory cytokines. Five adaptor proteins mediate TLR signalling; MyD88, TIR-associated protein (TIRAP), TIR domain containing adaptor protein inducing IFN-β (TRIF), TRIF-related adaptor molecules (TRAM), and Sterile-alpha and Armadillo motif containing protein (SARM) (Belinda et al., 2008, ONeill and Bowie, 2007, Yamamoto et al., 2002).

Two signalling pathways are induced by microbial ligands; MyD88-dependent which involves the activation of (TLR 2, 4, 5, and 9) and MyD88-independent (TLR3 and 4) (Akira and Takeda, 2004, Muzio et al., 2000, Takeda and Akira, 2005). Binding of TLR and MyD88 recruits IL-1 receptor-associated kinase 4 (IRAK-4), allowing the association of IRAK-1. After phosphorylation by IRAK-4, IRAK-1 binds with TNF receptor activated factors 6 (TRAF-6), an event blocked by the negative regulator, IRAK-M (Palsson and O'Neill, 2004). TRAF-6 activation is triggered, transforming growth factor-β activated kinase (TAK-1), which phosphorylates MAP kinases and the inhibitory kB kinase (IKK) complex leading to activated IKK complexes. The activation of IKK complexes will phosphorylate IkB, liberating NF-kB from the inhibition and allowing nuclear translocation of NF-kB to the nucleus, resulting in transcription of genes for inflammatory cytokines and co-stimulatory molecules (Banerjee and Gerondakis, 2007), see Fig.1.3. Diverse mechanisms seem to be contributing in controlling TLR activation by the intestinal epithelium, because continued and extreme activation of TLRs can prime uncontrolled inflammation. These mechanisms contain the shared effects of

several negative regulators that include IRAK-M, Tollip, SIGIRR, A20, and NOD-2 (Shibolet and Podolsky, 2007). Tollip has been described as a negative regulator of NF-kB signalling, triggered by different stimuli such as LPS (Burns et al., 2000). The main mechanism of Tollip, which is localised at the Golgi apparatus (Tao et al., 2004), is to negatively regulate TLR signalling by suppressing the activity of IL-1 receptor-associated kinase (IRAK). The inhibition of the activity of IRAK lead to suppression of inflammation associated with the up-regulation of TLR2 and TLR4 expression (Zhang and Ghosh, 2002). In addition, studies of animal and human cell line models also showed that Tollip controls the magnitude of inflammatory cytokine production in response to IL-1β (Didierlaurent et al., 2006). Therefore, keeping stable intestinal homeostasis at gut epithelial cell setting require several mechanisms of epithelial cell hypo-responsiveness to LPS, and expression of Tollip is one of these mechanisms.

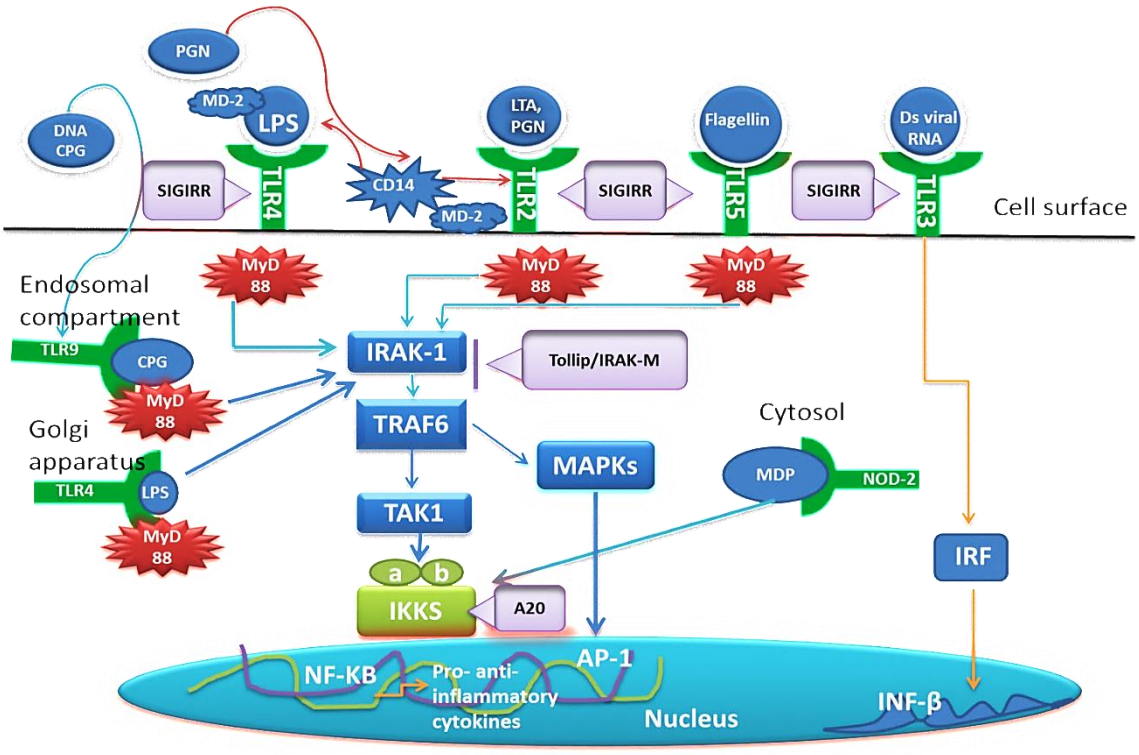


Figure 1. 3: TLR cell signalling at gut mucosa.

LPS recognised by the complex of surface expression of TLR4, CD14 and MD-2, flagellin by TLR5, LTA, PGN by TLR2, and dS viral DNA by TLR3. Intracellular CPG of microbial DNA is recognised by TLR9, MDP of Gram (+) bacteria by NOD-2 and intracellular LPS by TLR4. The adaptor protein MyD88 associated with TLR4, TLR2, TLR9, and TLR5. After phosphorylation of IRAK1 and binds with TRAF-6, this process is followed by activation the TAK-1, which phosphorylates MAP kinases and the inhibitory kB kinases (IKK) complex, liberating NF-kB from inhibition, and allowing nuclear translocation of NF-kB resulting in transcription genes for inflammatory cytokines, (Adapted from Takeda et al., 2003).

1.6.2. Nuclear factor K B (NF-kB)

NF-kB is a master regulator of innate, adaptive immunity, and inflammatory responses, localised in the cytoplasm and translocate to the nucleus upon stimulation with different stimuli after a series of phosphorylation. It is a dimer composed of five subunits called NF-kB1 (p50 and it is precursor p105), NF-kB2 (p52 and it is precursor p100), c-Rel, RelA (p65), and RelB (Baeuerle and Henkel, 1994). Phosphorylation and ubiquitin mediated degradation of inhibitory kB (IkB) control NF-kB activation.

There are two main pathways for NF-kB activation; a canonical classical and alternatively non-canonical (Hoffmann et al., 2006). Canonical classical pathway involves the activation of the inhibitors of kB kinase (IkB) complex, which phosphorylates and enables the ubiquitin-mediated destruction of inhibitory kB, which masks nuclear localisation sequences on NF-kB. IKK consist of subunits: IKKα, IKKβ and a regulatory subunit called NEMO. The classical pathway is triggered in response to microbial ligands, and pro-inflammatory cytokines dependent on IKKβ activation. The activation of the classical pathway mostly results in the nuclear translocation of p50/RelA dimers, while the alternative pathway activates p52/RelB, which is dependent on IkBα. In fact, the alternative pathway has two types, one of them depends on proceeding through an IKK

complex that contains IKKα, and the second one is when the Ikβ-like co-activator Bcl-3 interacts with p50 (or p52) homodimers (BcL-3/kβ) after entering the nucleus and becomes a transcriptional activator by virtue of this interaction (Gilmore, 2006). The members of the TNF receptor (TNFR) family such as lympho-toxin β, B-cell activating factor (BAFF), and CD40 are involved in the alternative NF-kB activation pathway. In all three pathways, a variety of post-translational modifications (e.g., phosphorylation, acetylation, and poly-isomerization) of the NF-kB subunits may alter their transcriptional activity resulting in an array of inflammatory molecules.

1.7. Antigen presenting cells (APCs) at gut mucosa

APCs can be defined as cells able to process and present antigen complexes with MHC class II on their surfaces, resulting in T cell activation and initiation of immune responses, determined by type of antigenic stimulation. They are classified into two categories: professional APCs (DCs, B cells, macrophages) or non-professional APCs (such as fibroblast, thymic epithelial, thyroid epithelial, glial, pancreatic beta, and vascular endothelial cells) (Amsen et al., 2004).

APCs are efficient in internalising antigens by several ways, such as phagocytosis, or endocytosis, they have diverse mechanisms of processing the antigens using an arsenal of weapons. Following processing of the antigens, APCs present the antigen by binding it to the MHC class II molecules on their surfaces, in addition to expressing co-stimulatory molecules facilitating T cell activation in tightly regulated manner. The main characteristics of professional APCs are expression of MHC class II and co-stimulatory molecules, whereas non-professional APCs expressed MCH class II but fail in the expression of co-stimulatory molecules. The main member of the APC family is DCs. These cells reach the gut lumen by cytoplasmic

cell extension through epithelial cell tight junctions without disrupting their assembly via the creation of tight junction-like complexes between DCs and epithelial cells (Rescigno et al., 2001). They directly sample microbial cells by phagocytosing antigens in the gut contents. After phagocytosis, antigen processing and presentation to the T cells through MHC class II will be the second task of DCs as APCs. In addition to direct microbial sampling, DCs indirectly perform their duties in microbial sampling through cross talk with M cells at PPs (Wilson and Villadangos, 2005). Normally, DCs in the gut recognise and respond to the potential pathogenic bacteria by presenting their broadest array of antigens and expression of the co-stimulatory molecules such as CD86, CD80, but do not respond to the beneficial commensal bacteria (Niess and Reinecker, 2006).

Macrophages are foremost among the cells that "present" antigens, a crucial role in initiating an immune response. Macrophages differentiated from monocytes derived from bone marrow, are found in the lamina propria. They mediate innate immunity by direct killing of pathogen (phagocytosis), or indirect killing, via secreting mediators, that kill pathogens at the breakdown gut barrier, and adaptive immunity via secretion of cytokines that have a crucial role in the differentiation of T cells such as IL-4 (Th2) and IL-12 (Th1). They are professional phagocytes, kill pathogens, and degrade microorganism debris, and process and present antigens to T cells resulting in initiation of adaptive immunity. They express an array of molecules such as MHC MHC II and I and co-stimulatory molecules, B7-1 (CD80) and B7-2 (CD86), effectively directing adaptive immunity (Mosser and Edwards, 2008). Depending on their functions, macrophages are classified as classically activated M1 pro-inflammatory macrophages and alternatively activated anti-inflammatory M2 macrophages (Mantovani et al., 2007). M1 pro-inflammatory

macrophages promote inflammation and destructive effects on tissues, whilst alternatively activated M2 anti-inflammatory/regulatory macrophages promote constructive effects on the tissues and resolve inflammation (Mantovani et al., 2007, Mosser and Edwards, 2008); both of the phenotypes are important in innate and adaptive immunity. At the early stage of infection, macrophages are recruited to the infected site in response to the attracting microbial signal recognised by macrophage TLRs; they engulf microbes or ingest neutrophils (which mediated the first step of inflammation). This results in the production of pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-8 and recruitment of additional macrophages, and neutrophils, if needed to eliminate the pathogens. Macrophages can digest more than 100 bacteria before they finally die due to their own digestive compounds through physiological apoptosis; however, some bacteria, such as *Mycobacterium tuberculosis*, are resistant to these methods of digestion, and can survive inside the macrophage phagolysosome. Overproduction of pro-inflammatory cytokines by pro-inflammatory M1 macrophages in response to stimulation by pathogenic microorganisms leads to tissue destruction. On the other hand, at the late stage of infection, the macrophages exhibit another phenotype (resemble regulatory anti-inflammatory M2 macrophages) that contributes to the resolution of inflammation and facilitates tissue repair through production of anti-inflammatory cytokines such as IL-10 and TGF-β. Therefore, macrophages exhibit a wide range of functions, which are determined by differentiation and activation factors dependent on the tissue environment. Generally, macrophages are professional in firstly, inflammation (producing pro-inflammatory cytokines), secondly, phagocytosis (expression of membrane bound scavenger receptors such as mannose receptor [MR], related to high capacity for endocytic clearance of mannosylated ligands represented by

microbes). Thirdly, tissue repair (producing anti-inflammatory cytokines and promoting extracellular matrix [ECM]). Fourthly, priming of adaptive immune responses (producing pro and anti- inflammatory cytokines), and finally, antimicrobial killing (production of reactive oxygen and nitrogen species, hydrolytic enzymes, and lysozyme to destroy the remaining of microorganisms in the inflammatory loci). In addition to their roles as APCs, the resident macrophages remove dead cell material (apoptotic cells) at strategic locations, which is important in chronic inflammation (Baeten et al., 2002, Mantovani et al., 2007, Mosser and Edwards, 2008). The diversity and plasticity of macrophages depend on many factors such as the stimuli presented by the tissue environment and the cytokines produced by other immune cells, such as T helper cells. Granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) have been concerned in the differentiation of M1 and M2-like macrophages, respectively (Verreck et al., 2004), in addition, Signal Transducer and Activator of Transcription (STAT) 1& 3 are involved in differentiation of M1 and M2 macrophage cells, respectively (Sica and Bronte, 2007). Furthermore, Gordon and Martinez (2010) reported that the M1 macrophage cell differentiation requires priming by IFN-γ and triggered with microbial LPS, or GM-CSF. These events are followed by dramatic alteration in the profile of the cells and the cells become the phenotype like IL-12^{hi} , IL-23^{hi}, IL-10^{lo}, TNF-α^{hi}, CD14^{+}, CD86^{+}, STAT-1^{+} , iNOS^{+} (after utilization of the amino acid L. arginine), and professional in degrading extracellular components such as collagen, elastin, and fibrinogen (by producing nitric oxide and proteolytic enzymes including matrix metalloproteinase MMP-1,-2,-7,-9, and -12). Consequently, M1 pro-inflammatory macrophages involved in tissue destruction. Up-regulation of pro-inflammatory cytokines (TNF-α) lead to enhance cell death program, resulting

in apoptosis of several cell types such as epithelial cells, promote chronic inflammation, tumour regression, autoimmune diseases, and IBD. Alternatively, M2 macrophage cell differentiation and activation is mediated by IL-4, IL-13, IL-10, TGF-β, M-CSF, vitamin D_3, immune complexes, IL-1β, and TLR ligation. Recently Foey (2012) reviewed that M2 macrophages have subgroups listed as M2a induced by IL-4 and IL-13, M2b induced by combining exposure to immune complexes and TLRs exert immune-regulatory function, and M2c induced by IL-10 repress immune responses and mediate tissue remodelling. In addition, Hesse et al. (2001) reported that M2 macrophages promote extracellular matrix construction and tissue repair by up-regulating the enzyme arginase-1 after utilisation of the amino acid L arginine resulting in production of proline and spermine (see Fig.1.4). Ym1 and Fizz1 are secreted proteins that have been identified in a variety of Th2-mediated inflammatory settings. They are induced and up-regulated by M2 macrophages in response to nematode infection prior to pathogenic clearance (Nair et al., 2005). Therefore, the main phenotype of M2 macrophage includes IL-12^{lo}, IL-23^{lo}, IL-$10^{hi,}$ TNF-α^{lo}, TGF-β^{+}, IL-1ra, arginase-1^{+}, MR^{+}, $CD86^{+}$, $CD163^{+}$, SR^{+}, STAT-3^{+}, STAT-6^{+}, M-CSF, and MMP^{+} , Ym1+, Fizz1**+** (Mantovani et al., 2007), see Fig.1.5.

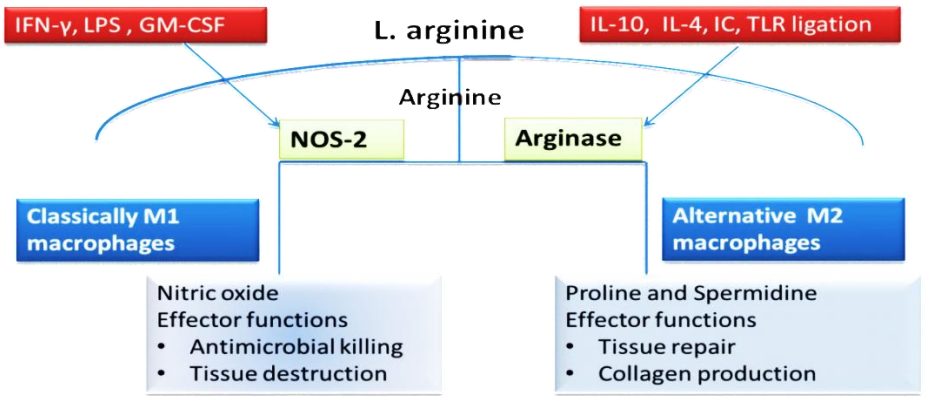


Figure 1. 4: Catabolism of arginine by classical M1 or alternative M2 macrophages.

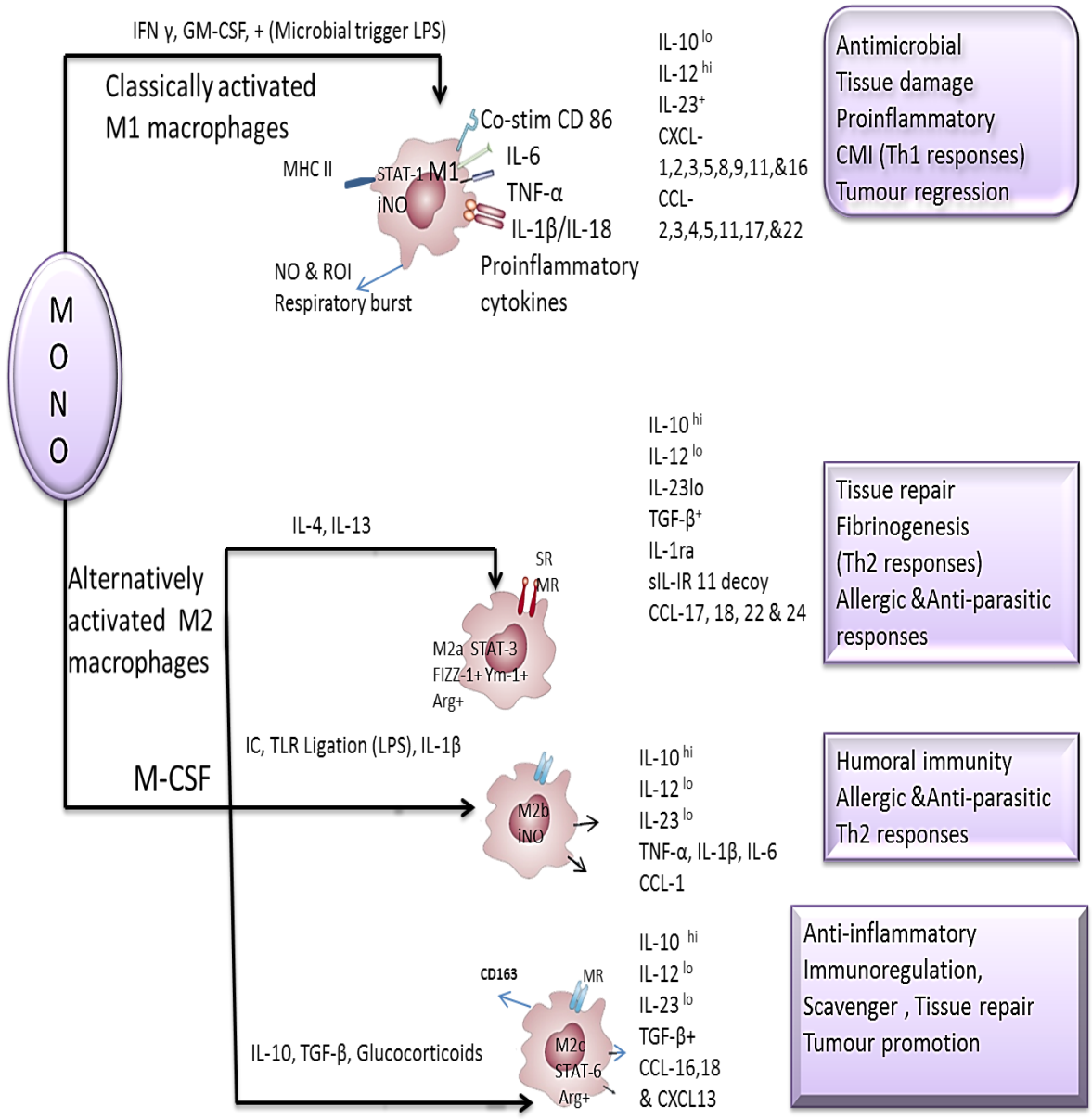


Figure 1. 5: Functional phenotypes of macrophage cell subsets.

Monocytes can be differentiated into different macrophage effector phenotypes by a range of signals: M1, pro-inflammatory macrophages are differentiated by GM-CSF, IFNγ, and LPS. M2, anti-inflammatory macrophages are differentiated by M-CSF, IL-4/IL-13, immune complexes (IC) +TLR ligation, IL-10, TGF-β and glucocorticoids. Macrophage functions determined by many factors such as expression of arginase (Arg), inducible nitric oxide synthase (iNOS), transcriptional factors (STAT-1, 3, 6), Ym-1 and FIZZ-1, scavenger receptors (SR) such as mannose receptors MR, MHC II, co-stimulatory ligands (CD80, CD86) and cytokines (IL-12, IL-23, IL-10, TGF-β), chemokines (CC-and CXC) expression. This figure is adapted from information presented in (Foey et al., 2002, Gordon, 2003, Mantovani et al., 2004).

The immune cells of innate and adaptive immunity regulate each other in reciprocal relationships by secreting an array of cytokines. In response to different stimuli, M1 macrophages release cytokines leading to differentiation of Th1 cells (mainly through their production of IL-12), or (IL-23 to stimulate differentiation of Th17). On the other hand, M2 macrophages release IL-4 (differentiation of Th2), TGF-β (differentiation of T regs), and IL-6, IL-1β (differentiation of Th17), refer to Fig.1.6. Normally, T cells can be classified by the type of glycoprotein expressed on their surfaces (CD4 or CD8) (Mosmann et al., 1986), as cytotoxic cells (Tc) ($CD8^+$) and T helper ($CD4^+$) cells. Naïve $CD4^+$ T cells differentiate into distinct cell phenotypes determined by the pattern of signals they receive during their initial interaction with antigens or in response to signals from other immune cells such as macrophages and DCs. They are differentiated into: Th1 in the presence of IL-12 and transcriptional factor T bet-/ STAT-4, Th2 in the presence of IL-4 and GATA-3/STAT-5 (Mosmann et al., 1986), Th17 in the presence of IL-1β, IL-6, IL-23, TGF-β and RORγt /STAT-3 (Stockinger and Veldhoen, 2007) and T regs in the presence of TGF-β, retinoic acid, Foxp3/STAT-5 (Hori et al., 2003). In addition, the microbiota present in the gut has a role in Th17 cell differentiation related to the presence of Cytophaga-Flavobacterium-Bacteroides (CFB) phylum members (Ivanov et al., 2008). T cells play a central role in adaptive immunity by stimulating B cells to release antibodies via IL-4, inducing macrophages to develop the enhanced microbicidal activity via IFN-γ, recruiting neutrophils via IL-8 to the sites of infection and inflammation and producing TGF-β and IL-10 to induce oral tolerance (Zhu and Paul, 2008). Abnormal activation of Th1 is involved in the autoimmune diseases and Th2 cells are responsible for allergic inflammatory diseases and asthma. Th17 cells have been recognised recently to play a critical in protecting against microbial challenges (particularly extracellular bacteria and

fungi) (Weaver et al., 2006), however, autoimmune responses attributed to Th17 are implicated in collagen-induced arthritis (CIA) and IBD. T helper cells play critical roles in orchestrating the adaptive immune response. They exert such functions mainly through secreting cytokines that activate and/or recruit target cells such as macrophages. Th1 cells secrete (IL-2, INF-γ, IL-6, IL-1β, TNF-α); Th2 (IL-4, IL-5, IL-6, IL-13, IL-21, IL-10); Th17 (IL-17, IL-21, IL-22), and Tregs (TGF-β, IL-10) (Romagnani, 1992, Zhu et al., 2010).

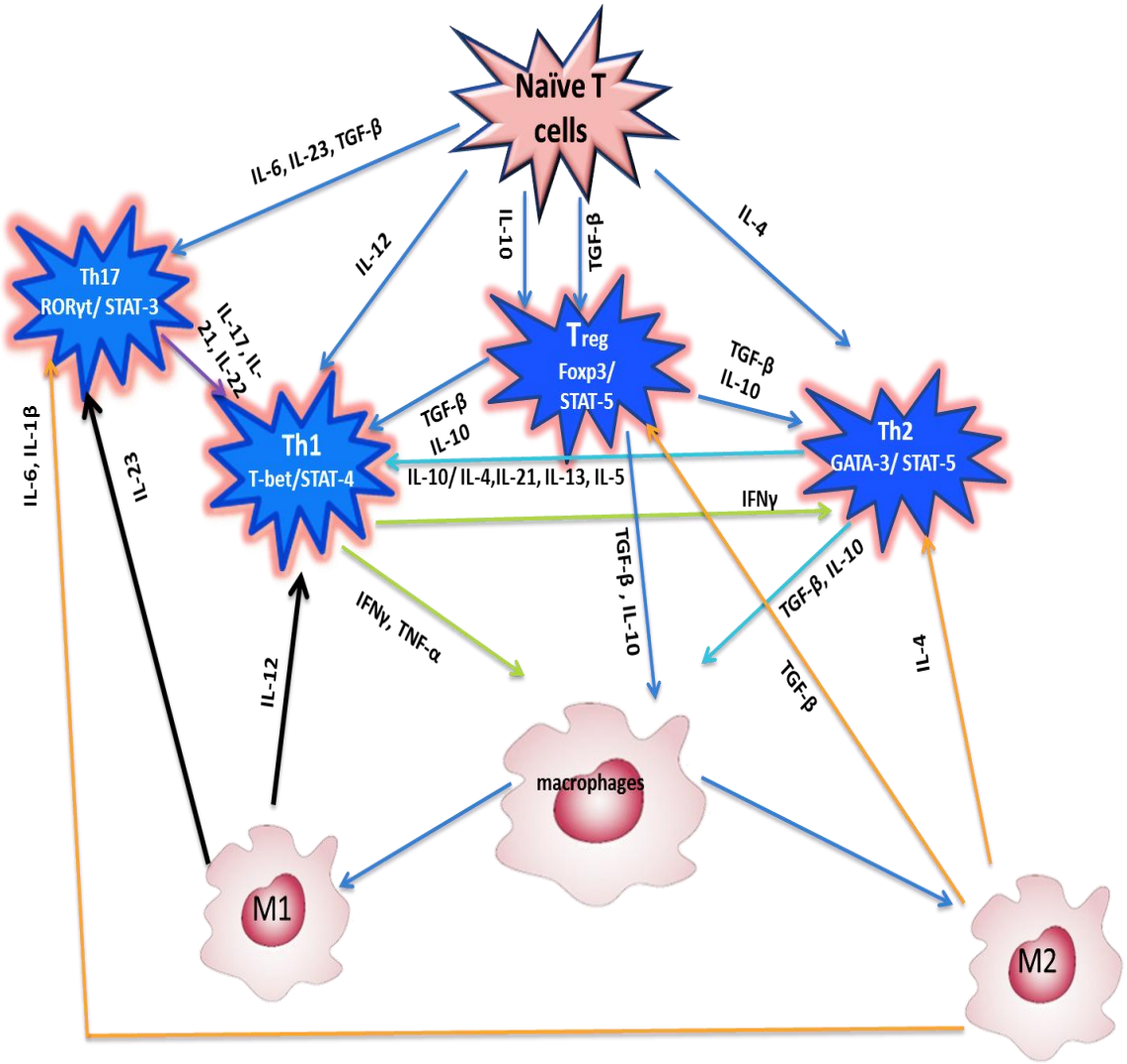


Figure 1. 6: Schematic representation of mucosal immune cells intercommunication through cytokines

1.7.1. Macrophages and intestinal immune responses

Gut mucosal macrophages are found in the lamina propria, usually nearby to the basal membrane of the epithelium. Because of the constant exposure of antigens and food borne antigens to the gut mucosa, gut macrophages are central to deciding immune responsiveness to luminal antigens and bacteria as an activatory or tolerogenic response by communicating with antigens, and at the same time with other immune cells found in the lamina propria.

Under a homeostatic condition, the predominant macrophage phenotype resembles the M2 subset, described by less easily activated in acute inflammatory responses. As a function, the M2 gut macrophage subset expressed the scavenger receptors (CD33, CD36, and CD68) due to a high level of professional phagocytic clearance of mannosylated ligands. In addition, they express inducible nitric oxide (iNOS), reactive oxygen species (ROS), lysozyme, acidification of phagolysosomes, acid hydrolyses and nutrient competitors such as lactoferrin. Expression of these factors facilitates the phagocytic uptake and microbial killing. Furthermore, they do not express chemokines, fail to produce significant levels of pro inflammatory cytokines (such as TNF-α, IL-1β, IL-6, IL-8, IL-12, IL-18, IL-23), and down-regulate the expression of antigen presentation molecules such as MHC II and co-stimulatory molecules CD86 and CD40. The intestinal macrophages exhibit hypo-responsiveness to TLR ligands through lack of or low expression of CD14, TLR2, TLR4, TLR5, and TLR9, and/-or express distinct TLR negative regulators that include IRAK-M, Tollip, SIGIRR, A20, and NOD-2. In addition to down-regulation of the expression of MyD88, TRAF6, TRIF, IRAK1 and IRAK4 adaptor molecules, and expression of membrane receptors (CD200R, TGF-β RI, and TREM-2), membrane bound ligands (IL-10, TGF-β) (Platt and Mowat, 2008,

Smith et al., 2001, Smythies et al., 2005, Triantafilou et al., 2001, Shibolet and Podolsky, 2007, Smythies et al., 2010).

At acute inflammatory reactions, Triggering receptor expressed on myeloid cells-1 (TREM-1) is expressed by monocytes, macrophages, and neutrophils lead to enhanced secretion of inflammatory cytokines and chemokines. TLR ligands of LPS and lipoteichoic acid (LTA) and pro-inflammatory cytokine such as TNF-α are the main factors involved in inducing TREM-1 expression by inflammatory immune cells. TREM-1 expression is suppressed in gut macrophages (Schenk et al., 2007). This suppression is regulated by commensal non-pathogenic microbiota through microbial induction of IL-10 (Ueda et al., 2010). Breakdown of gut mucosal tolerance is followed by alteration of macrophage phenotype to $CD14^{hi}$ M1 pro-inflammatory macrophages (Segura et al., 2002) which exhibit increased TLR responsiveness and expression of the pro-inflammatory cytokines such as IL-1β, IL-8, IL-12 and TNF-α (Platt and Mowat, 2008).

1.8. Immunomodulatory effects of probiotic bacteria

The first theory about gut microbial effects on human health was by Metchnikoff (1845-1916) who proposed his theory of longevity that ‘‘the intake of yoghurt leads to decreases in the toxic effect of colonic flora by inhibiting the growth of putrefactive bacteria in the large intestine’’. In fact, the probiotic field grew explosively in human and animal application therapy when Shirota and Kellogg in 1930s and German nutritionists in 1950s used probiotics in the treatment of several diseases (Vasiljevic and Shah, 2008). In 2001, the FAO/WHO defined probiotics as ‘Live microorganisms which when administered in adequate amounts confer a health benefit on the host’ (FAO/WHO, 2001). The most important criteria

for probiotic designation include a strain of human origin, safe for human use, stable in acid and bile, and adherence to the intestinal mucosa (Gueimonde and Salminen, 2006).

Generally, probiotic bacteria are Gram positive, lactic acid bacteria (LAB). They are an important group in maintaining human health, because they produce lactic acid through their metabolism resulting in a low pH, which inhibits the growth of most pathogenic microorganisms. Probiotic bacteria are different in their metabolism, for example, *Lactobacillus fermentum* can ferment glucose compounds into lactose with the production of lactic acid, whereas *Bfidobacteria* are efficient in the production of short chain fatty acids such as butyrate, which also have a crucial role in maintaining a healthy gut mucosa and essential for mucosal homeostasis (Vanhoutvin et al., 2009).

Numerous investigations in animals and humans have already shown that how a single probiotic strain or combinations of strains may modulate gut function (O'Mahony et al., 2001, Gackowska et al., 2006, Malai et al., 2009). Many of these investigations have generated promising results concerning the use of probiotics in the treatment of acute gastroenteritis, *Clostridium difficile*-associated diarrhoea or colitis, irritable bowel syndrome (IBS), necrotizing enterocolitis, and others (Preidis and Versalovic, 2009). At the same time, other investigations have shown either no effect or slight effects by probiotics. Verdú et al. (2009) reported a review based on evidence from animal models that the intestinal content is critical in determining the natural gastrointestinal physiology and the modification in luminal content by dietary, antibiotic or probiotic manipulation can result in alterations in gut function. IL-10 gene-deficient mice (BALB/c) were studied to determine whether increased intestinal permeability occurs as a primary deficiency before the onset of mucosal

inflammation or is secondary to mucosal injury. Madsen et al. (1999) showed that an enhance in ileal and colonic permeability in the absence of any histological injury, was associated with increased mucosal secretion of IFN-γ and TNF-α. While IL-10 gene-deficient mice raised under germ-free conditions have no inflammation, and demonstrate normal permeability and cytokine levels, which suggested that the intestinal permeability defect in IL-10 gene-deficient mice occurs due to a dysregulated immune response to normal enteric microflora. In terms of probiotic bacterial role in the treatment of intestinal inflammation de Moreno de LeBlanc and Perdigón (2010) demonstrated, using a TNBS-induced murine colon cancer model, that yoghurt consumption repressed tumour growth by diminishing the inflammatory response via increasing IL-10-secreting cells, reducing IL-6, cellular apoptosis and reducing procarcinogenic enzymes. Jeon et al. (2012) treated BALB/c mice for three months with *Lactobacillus casei* and *Bifidobacterium breve* in order to test the effects of these probiotics on the intestinal homeostasis by investigating their effects in T cell development in the intestine, and they found that. *B. breve*, but not *L. casei*, induced development of IL-10-producing Tr1 cells that express IL-21, in addition to *B. breve* activates intestinal $CD103^+$ DCs to produce IL-10 and IL-27 via the TLR2/MyD88 pathway.

Probiotics have been shown to influence both innate and adaptive immunity. They can exert their effects on a wide array of mucosal immune cells including T, B, and natural killer (NK) cells (Takeda and Okumura, 2007), DCs (Foligne et al., 2007), monocytes/macrophages and epithelial cells (Ciorba et al., 2012, Zhang et al., 2006). This immunomodulation by probiotics is partly attributable to the mucosal cell type being studied, and the strain of probiotic being used. Probiotic bacteria have been demonstrated to exert their immunomodulatory effects through the

bacterial cell wall, bacterial DNA, soluble secreted proteins (Frick et al., 2007, Sanchez et al., 2009, Yan and Polk, 2002), and bacterial cell metabolites such as SCFAs (Foey, 2011, Vanhoutvin et al., 2009). Indeed, probiotic bacteria have been demonstrated to modulate phagocytosis by macrophage cells (stimulator to phagocytosis in healthy subjects, and suppressor in allergic patients) (Isolauri et al., 2001).

Cytokines are messengers that regulate the fate of the immune signal decisions (activation or tolerance) that are made by immune cells. It is well documented the aptitude of probiotic bacteria to modulate the profile of cytokine production by an array of cell system in the human and animal models. Indeed modulation of cytokine expression is one of the main targets to manipulate the immune responses by probiotic bacteria and represents a good field in the treatment and prophylaxis of immunopathology. LAB, such as *L. rhamnosus*, have been documented to modulate macrophage function by both suppressing and enhancing IL-12 production (Foligne et al., 2007, Shida et al., 2006, Shida et al., 2011), which will impact on Th1 development and activation. LAB has been described to suppress mucosal TNF-α during inflammation leading to limiting of inflammation (Borruel et al., 2003) and augmentation of the anti-inflammatory cytokine IL-10, resulting in maintaining gut mucosa (de Moreno de LeBlanc et al., 2011, Madsen et al., 2001). The cell wall-derived component of polysaccharide/PGN complex in LAB was demonstrated to suppress LPS-induced IL-6 production by peripheral blood mononuclear cells (Matsumoto et al., 2005) or induced high levels of IL-6, TNF-α and IL-12 (Christensen et al., 2002). *Lactobacillus casei strain Shirota* (LcS) has been demonstrated to facilitate the development of Th1 cells through the induction of IL-12 (Shida et al., 2006) and

augment NK cell cytotoxicity (Takeda and Okumura, 2007). Furthermore, *Lactobacillus* and *Bfidobacteria* species induce expression of a wide range of cytokines including pro-inflammatory and anti-inflammatory cytokines refer to table 1.1.

Table 1. 1: Probiotic stains differentially modulate pro-and anti-inflammatory cytokines.

Probiotic strain	Cell system	Main findings	References
L. acidophilus, *L. casei* strain Shirota	Peyer's Patches (PPs) after the oral administration to BALB/c mice, MΦ U937 cell line	All strains increased the number of IFN-γ and TNF-α cells, but not of IL-10(+) cells in the total population of PPs IL-10 and TNF-α ↑ in response to LTA stimulation in a dose dependent way. Whole probiotic bacteria activate the transcriptional factor NFAT and TLR9 not LTA.	(Dogi et al., 2009)
L. reuteri ATCC 6475 and ATCC PTA 5289	THP-1 cell line (human)	↓ LPS induced TNF-α.	(Jones and Versalovic, 2009)
B.breve, *B. longum,* *B. adolescentis,* *Enterococcus faecalis*	MΦ RAW264,7 (murine)	IL-10 mRNA levels were significantly decreased after exposure to *E. faecalis* compared with exposure to *Bifidobacterium* species, whereas IL12p40, TNF-α, and IL-1β were decreased by co-culture with *B. breve* and *B. longum*. *Bifidobacterium* significantly inhibited phosphorylation of IκB-α induced by LPS associated with up-regulation of SOCS1 and SOCS3 resulting in suppression of cytokine expression.	(Okada et al., 2009)

L. salivarius Ls33 *L. rhamnosus Lr32* *L. casei B123* *L. acidophilus NCFM* *L. acidophilus IPL908* *L. plantarum NCIMB8826* *B. animalis, B. lactis*	PBMCs from murine model of acute TNBS-induced colitis	All strains TNF-α↑. *L. salivarius* Ls33, *L. rhamnosus, B. animalis, B.Lactis* the most potent inducer of IL-10. *L. plantarum, B. animalis*, *L. acidophilus* and *B. lactis* the most potent inducer of IL-12. IL-10/IL-12 ratio ↑in *L. acidophilus* and *L. salivarius.*	(Foligne et al., 2007)
L. paracasei 1688	PBMCs from human healthy individuals	IL-12, IFN-γ ↑with single strains and their combination	(Castellazzi et al., 2007)
L. gasseri, L. johnsoni, L.reutri	PBMCs derived myeloid DCs from human healthy individuals	↑ IFN-γ and IL-12	(Mohamadzadeh et al., 2005)
non-pathogenic *E coli,* *Lactobacillus sakei*	Caco-2 co-culture with PBMCs from human	↑ TNF-α, IL-1β, IL-8, TGF-β by Caco-2 cells co-cultured with PBMCs	(Haller et al., 2000b)

	healthy individuals	↑TNF-α, IL-1β, MCP-1 and IL-10.by PBMCs	
Gram-positive bacteria including *Lactobacilli* and *Bifidobacteria*	PBMCs from human patients with grass-pollen allergy and healthy subjects	Intracellular IFN-γ/IL-4 ratio↑ in lymphocyte in allergic patients but not in healthy subjects	(Rasche et al., 2007)
L.rhamosus GG, *L. rhamosus E509,* *L.plantarum E98,* *L.acidophilus* E507	PBMCs from human healthy blood donor subjects	All strains TNF-α and IL-6 ↑ , *L. rhamnosus* E509, *L. rhamosus* GG, *B. animalis* E508, *L. acidophilus* E507, were the best inducers of TNF-α, *L. rhamosus* E509, *L. rhamosus* GG, *B. animalis* E508 were the best inducers of IL-6. Live bacteria induced cytokine production better than glutar-aldehyde-fixed bacteria	(Miettinen et al., 1996)
L. rhamnosus, L. casei	PBMCs, Lymphocyte from human healthy individuals	Anti-proliferative effects, IL-4↓	(Sütas et al., 1996)
L. acidophilus, *L. brevis*, *L. bulgaris,*	PBMCs from human healthy individuals	All strains TNF-α↑ *B. bifidum, B. infantis, L. casei, L. lactis* the most potent inducers of IL-10, *B. breve, ,L. rhamnosus and L.*	(Timmerman et al., 2007)

L. casei, *L. helveticus, L.paracasei*, *L. paracasei, L. plantarum, L. salivarius, B. bididum, B. breve, B.infantis, B.lactis, B.longum*		*salivarius* did not induce IL-10, *B. breve, L. brevis and L. rhamnosus* induced high concentrations of pro-inflammatory cytokines	
L. acidophilus LA201, L. plantarum L. salivarius LA302, B. lactis LA303 and combination of them	PBMCs from human healthy individuals	All strains IL-12 and IL-10↑ *L. salivarius and B. lactis* IL-10/IL-12 ratio↑	(Drouault et al., 2006)
L. salivarius UCC118, B. infantis 35624,	Human mononuclear cells and DCs from MLNs and PBMCs isolated from patients with active colitis	PBMCs and PBMC-derived DCs secreted TNF-α, IL-12 in response to the *Lactobacillus, Bfidobacteria*, and *Salmonella* strains, whereas MLN cells and MLN-derived DCs secreted TNF-α, IL-12 only in response to *Salmonella* challenge. PBMCs secreted IL-10 in	(O'Mahony et al., 2006)

		response to the *Bifidobacterium* strain but not in response to the *Lactobacillus* or *Salmonella.* MLN cells secreted IL-10 in response *to Bfidobacteria and Lactobacilli but not in response to Salmonella.*	
L. casei strain Shirota*, L. rhamnosus, L.zeae, L. fermentum, L. johsonii*	Mouse peritoneal macrophages	↑ IL-12. Phagocytosis of lactobacilli was necessary for IL-12 induction. Only intact cell wall of LcS induced IL-12.	(Shida et al., 2006)
L. acidophilus, *L. delbrueckii*, *L. bulgaricus* LbY-27, *B. bididum* Bb12 and combination of them	PBMCs from human healthy individuals	All strains and combination (TNF-α and IL-10) ↑ *L. acidophilus* the strongest IFN-γ and IL-12 inducer of cytokine production. Bacterial combination lower inducer than in single strain	(Gackowska et al., 2006)
B .breve Y8, B. infantis Y1, B .longum Y10, E. coli Nissle 1917, L. acidophilus MB443, L. rhmnosus GG, L. casei MB451, L. delbuecekii MB453, L. plantarum MB452, whole bacteria and	PBMCs from human healthy individuals	IL-10, IL-1β and TNF-α ↑ by *Bifidobacterium* and *E. coli* more than by *Lactobacillus* IL-10, IL-1β, and TNF-α ↑ by *L. bulgaricus* and *L. plantarum* more than by *L. casei.*	(Helwig et al., 2006)

genomic DNAs			
Genomic DNAs of *L. acidophilus LA14, L. delbueckii spp. L. bulgaricus LB31, B. infantis B107*	PBMCs from human healthy individuals	IL-10↑by *Bifidobacterium* more than by *Lactobacillus* IL6 ↑by *Lactobacillus* more than by *Bifidobacterium*	(Lammers and Campieri, 2003)
L. johnsonii, L. sakei, Escherichia coli	PBMCs and Lymphocytes from human healthy individuals	IL12 and IFN-γ↑. Proliferation of PBMCs and the strongest proliferative response was observed *with L. johnsonii.* *E.coli* preferentially induced IL-10. Up-regulation of CD69 and CD25 on $CD56^+$ NK cells	(Haller et al., 2000a)
L. rhmnosus, *L. palantraum,* *L. paracasei*	PBMCs from human healthy individuals	IL-12↑by *L. paracasei more than by L. plantarum or L. rhamnosus.* IL-10↑by *L. rhamnosus more than by L. plantarum.* Whole killed *lactobacilli* were potent stimulators of IL-12	(Hessle et al., 1999)
L. rhmnosus, *L. bulgaricus*	PBMCs from healthy individuals	All strain TNF-α, IL-1β, IL-6, IL-18, IL-10, IFN-γ and IL-12↑ *by L. rhmnosus* more than by *L. bulgaricus* (mRNA and protein). IL-10 and IL-4 production was induced weakly.	(Miettinen et al., 1998)
L. gasseri	Spleen-macrophages	↑IFN-γ (mRNA and proteins)	(Haruki et al., 1994)

	(SP-M03B8) and PPs -adherent cells		
L. johnsonii La1	NK cells from health individuals	Activation of CD3- CD16+ CD56+ NK cells, including expression of the activation antigen CD69 and secretion of IFN-γ. Phagocytosis of bacteria required for their stimulation.	(Haller et al., 2002)
L. rhamnosus Lcr35	Healthy Human monocyte-derived immature DCs	↑ TNFα, IL-1β, IL-12p70, IL-12p40 and IL-23, and only a low increase in IL-10 concentration. Maturation of DCs via up-regulation of the membrane expression of CD86, CD83, HLA-DR and TLR4, associated with a down-regulation of DC-SIGN, MR and CD14.	(Evrard et al., 2011)
L. plantarum	Caco-2 (human)	After stimulation with LPS for 48 h, IL-23 ↑ (mRNA and protein level) as TLR2 dependent. Adding *L. plantarum* ↓ IL-23. Suppression of TLR2 correlated with suppression of IL-23 and hBD-2	(Paolillo et al., 2009)
L. reuteri	THP-1 cell line (human)	Produced histamine that suppressed TNF-α production via stimulation the increasing levels of cAMP, which inhibited downstream MEK/ERK MAPK signalling via protein	(Thomas et al., 2012)

		kinase A (PKA), and resulting in suppression of TNF-α production by transcriptional regulation	
B. lactis strain BB12, *Bacteroides vulgatus*	Germ-free Fisher F344 rats, mouse epithelial cell (IEC) line Mode-K	↑ NF-kB (RelA),and p38 MAPKs in native IEC resulting in production of IL-6 and TLR2 dependent, whereas *Bacteroides vulgatus* failed in up-regulation of IL-6 through their failing in augmentation of p38	(Ruiz et al., 2005)
L.casei CRL 431, L.helveticus R389	BALB/c female mice small intestinal epithelial cells	Up-regulated IL-6 using live and heat killed probiotic bacteria. IL-6 is necessary for B-cell differentiation and IgA production	(Vinderola et al., 2005)
L. rhamnosus GG (L GG) L. plantarum 299v,	HLA-B27 transgenic (TG) rats	*L.rhamnosus GG not L. plantarum* prevented colitis relapse in antibiotic treated rats associated with suppression of IL-1β, TNF-α and up-regulation of IL-10.	(Dieleman et al., 2003)
L. rhamnosus GG	Mouse colon epithelial cells	*L*. rhamnosus secreted proteins activated Akt, inhibited cytokine-induced epithelial cell apoptosis and promoted cell growth.	(Yan et al., 2007)
L. casei Shirota (LcS)	lamina propria mononuclear cells isolated from murine	Inhibited LPS-induced IL-6 via suppression of STAT-3 and inhibition of NF-kB.	(Matsumoto et al., 2009)

	IBD		
L. fermentum	PBMCs from very young children with atopic dermatitis (AD)	↑ TNF-α and IFN-γ, ↓ IL-10 and IL-13	(Prescott et al., 2005)
L.casei Shirota (LcS)	PBMCs from human healthy older volunteers	↑ NK cell activity, ↑ ratio of IL-10/IL-12.	(Dong et al., 2013)
B. longum SP 07/3, L.rhamnosus GG (L.GG) and L. casei Shirota (LcS).	PBMCs from human healthy older volunteers	↑ NK cell activity by probiotics except for L.GG, probiotics ↑ IFN-γ and IL-6. *B. infantis* ↑ IL-10/IL-12 ratio.	(You and Yaqoob, 2012)

Several studies have demonstrated that immune modulatory role of the probiotics such as Dong et al. (2010) who reported that LcS differentially induced expression of CD69 and CD25 on NK cells (CD56+) and cytotoxic T cell (CD8+) subsets, leading to activation of these cells. This suggested that LcS could potentiate the destruction of infected cells in the body. Indeed, other probiotic strains such as *Lactobacillus fermentum* CECT5716 and *Lactobacillus salivarius* CECT5713, isolated from human breast milk mediated the activation status (CD69 and CD25 expressions) of NK cells (CD56+), total T cells (CD3+), cytotoxic T cells (CD8+) and Th1 (CD4+) cells in addition to Tregs (CD4+CD25+Foxp3+) cells (Pérez-Cano et al., 2010). The immune system undergoes substantial change with age in both humans and animals, which is linked to alterations in both the strength and quality of the immune response (Hodes, 2005). Probiotic modulation and activation of aged NK and T cell subsets (CD8+,CD4+ and Tregs) can lead to a substantial enhancement of both natural and acquired immune responses (Dong et al., 2010). Questions have been raised about probiotic bacterial immuno-regulatory effects through augmentation of regulatory cytokines such as IL-10, Medina et al. (2007) revealed that the level of guanine cytosine (GC) in probiotic bacterial DNA has a significant role in inducing the regulatory cytokines such as IL-10 by PBMCs stimulated by *B. longum* strains. However, it is difficult to explain the underlying mechanism by which probiotic bacteria exert such multifunctional activities as immuno-regulatory or immuno-stimulatory. Indeed, ingested probiotic bacteria are considered to affect the intestinal immune system through several routes. Shida et al. (2009) reported that the digested LcS cell wall augmented the pathway of CREB, which is known for its suppression of pro-inflammatory cytokines such as IL-12 and up-regulation of anti-inflammatory cytokines such as IL-10. In addition,

TLR2 recognition of peptidoglycan and NOD2 recognition of the by-product of peptidoglycan (PGN) digestion, muramyl dipeptide (MDP), leads to inhibition of macrophages IL-12 production stimulated by different probiotic bacteria. Additionally, Yasuda et al. (2008) reported that polysaccharide moiety of the cell wall of LcS regulates cytokine production by RAW 264.7 macrophages and spleen cells via suppression of LPS-induced IL-6 production. On the other hand, the intact rigid cell wall of LcS that is resistant to intracellular digestion effectively stimulates macrophages to secrete a large amount of IL-12 in mouse peritoneal macrophages (Shida et al., 2006). It seems to be that the fate of probiotic bacterial immunomodulation is determined by the type of phagocytic cell, sensitivity to intracellular digestion by phagolysosomal enzymes (Silverstein, 1995), potential routes of phagolysosome escape and the end product of the cell wall digestion (Pitt et al., 1992). Therefore, probiotic immunomodulation can be either regulatory/anti-inflammatory or activatory/pro-inflammatory (Shida et al., 2011) dependent on the environmental pathological condition and type of immune cells that are stimulated by probiotics.

An important element of probiotic bacteria for the treatment or prevention of disease is the organism's ability to secrete useful concentrations of proteins and other metabolites that regulate intestinal homeostasis (Dotan and Rachmilewitz, 2005). Extracellular proteins from probiotic bacteria exist in two formats: surface associated membrane bound and free secreted proteins. They could diffuse through the mucus layer that shields the intestinal mucosa, allowing interaction with epithelial and immune cells (Sanchez et al., 2010). Probiotic secreted proteins (SP) play an important role in modulation of the host immune response (Sanchez et al., 2010). It is well reported that probiotic SP have beneficial effects on human

health through their roles in modulating the immune response (Bauerl et al., 2010, Hoarau et al., 2008, Sanchez et al., 2009). Probiotic SP exhibit different functions such as inhibition of pancreatic and neutrophil elastases by Serpin secreted from *B. longum* (Ivanov *et al.*, 2006), and growth promotion in human colon epithelial cell line HT-29 by p40 (homologous to gi|116493594) secreted from *L. rhamnosus* GG, via inhibition cytokine induced epithelial cell apoptosis (Yan et al., 2007). Secreted proteins (SP) also reduce the injuries caused by TNF-α overproduction after treatment with hydrogen peroxide through p75 (homologous to gi|116493849) secreted from *L. rhamnosus* GG*.* These SP (P40 and P75) prevented the disruption of tight junctions and barrier function in Caco-2 cell monolayers via preventing hydrogen peroxide-induced redistribution of Occludin, ZO-1, E-cadherin, and β-catenin by translocation of PKCβI (Seth et al., 2008). Indeed, Bauerl et al. (2010) reported that *Lactobacillus casei/paracasei* carrying two genes encoding homology of P40 and P75 from *L rhamnosus* GG, which are characteristic of proteins with cell-wall hydrolase activity. These proteins were secreted to the growth medium and were located at the bacterial cell surface. Both proteins (P40 and P75) bound to mucin, collagen and to intestinal epithelial cells, stimulated epidermal growth factor receptor phosphorylation in mouse intestine *ex vivo.*

Studies showed that secreted proteins such as flagellin secreted from *E. coli Nissle* 1917 has a significant role in inducing hBD-2 and IL-8 expression by Caco-2 cells (Schlee *et al*., 2007). Furthermore, hBD-2 production in epithelial cells was induced by unidentified secreted proteins secreted from *L. acidophilus*, *L*. *fermentum*, *L. paracasei* (Schlee et al., 2008). Additionally, S-layer protein (ASlpA) secreted from *L. acidophilus* regulates immature DCs and T cell functions via inducing IL-10 (Konstantinov et al., 2008). Accumulating evidence demonstrates

that probiotics communicate with the host by modulating key signalling pathways, such as NF-κB and MAPK, via bacterial cell wall or their secreted protein to either enhance or suppress the activation and influence downstream pathways refer to table 1.2.

Table 1. 2: Probiotic modulation of signalling pathways in intestinal epithelial cells and macrophages.

Types of probiotic bacterial species	Model system	Signalling pathway	Probiotic effects	References
L. reuteri	Caco-2 and HT-29	NF-KB	Prevents IκBα degradation	(Ma et al., 2004)
Bacteroides thetaiotaomicron	Caco-2, HT-29	NF-kB	Enhances RelA nuclear export via PPARγ	(Kelly et al., 2004)
Faecalibacterium prausnitzii DSM 17677	Caco-2 cells, epithelial cells from TNBS-induced colitis in mice, PBMCs	NF-kB	Inhibits NFκB activation associated with suppression of IL-12, IL-8 and IFN-γ and up-regulation of IL-10.	(Sokol et al., 2008)
B. lactis	Naïve epithelial cells from F344 rats, small intestine, Caco-2, HT-29	NF-kB	Activates NF-κB RelA and p38 MAPK phosphorylation associated with up-regulation of IL-6 TLR2 dependent	(Ruiz et al., 2005)

L. rhamnosus GG ATCC 53103	PBMCs differentiated into macrophages by GM-CSF	NF-kB, STAT-1/3	Induces NFκB and STAT-3/1 DNA-binding activity	(Miettinen et al., 2000)
L. casei Shirota	THP-1 cells derived macrophages (M1)	NF-kB	Inhibits IκBα phosphorylation	(Watanabe et al., 2009)
B.breve, Streptococcus thermophilus	THP-1 cell line	NF-kB	↓ NF-kB, decreases LPS binding to CD14	(Menard et al., 2004)
Lactobacillus fermentum YIT	RAW264.7 (murine)	MAPKs	Activation of JNK associated with ↑ TNF-α	(Matsuguchi et al., 2003)
Supernatant from *Bifidobacterium*	PBMCs derived DCs	MAPK and PI3K	↑ PI3K, ↓ p38MAPK and GSK3	(Hoarau et al., 2008)
Supernatant from *Lactobacillus rhamnosus GG*	Human and mouse intestinal epithelial cells	NF-kB and MAPKs	p75 and p40 activated Akt, inhibited cytokine-induced epithelial cell apoptosis (TNF-α)	(Yan et al., 2007)
Lactobacillus plantarum	Intestinal epithelial cell (YAMC) and macrophage (RAW 264.7) and murine DCs	NF-kB	↓ NF-kB	(Petrof et al., 2009)
DNA from *VSL#3*	HT-29	NF-kB and MAPKs	↓ NF-kB, p38	(Jijon et al., 2004)

Gómez-Llorente et al. (2010) reported a review based on evidence that probiotics modulate TLR expression by epithelial cells. In addition to the probiotic cell wall and probiotic-secreted proteins, probiotic DNA also played a vital role in probiotic therapy through the TLR9 signalling pathway. DNA from probiotic bacteria has been shown to protect mice from experimental colitis in a TLR9 dependent manner (Rachmilewitz et al., 2004).

The gut barrier function is one of the probiotic bacterial targets. They strengthened the mucosal barrier function by augmentation of TEER and ZO-1 protein associated with TJs (Fanning et al., 1998, Johnson et al., 2008). Epithelial cell survival and restitution are also influenced by probiotic bacteria. Probiotics exerted a significant role in the renewal of epithelial cells, and reduced epithelial cell apoptosis through up-regulation of endogenous cytokines associated with epithelial cell turnover (Banasaz et al., 2002, Resta and Barrett, 2003). Finally, a less exploited but very promising field is the use of probiotics or some of their components as adjuvants for vaccines, as well as using genetically modified strains for delivering regulatory molecules such as IL-10 or encoding proteins from pathogenic microorganisms for immunization (Nagy et al., 2005).

1.8.1. Probiotic roles in the treatment of IBD

IBD is defined as chronic relapsing inflammatory disorders of the GIT. The most common types of IBD are Ulcerative colitis and Crohn’s disease, which have become more prevalent diseases (Molodecky et al., 2011). Crohn’s disease is a type of IBD affecting anywhere along the GIT, with dense infiltration of lymphocytes particularly Th1, Th17 and the M1 macrophage phenotype. Overproduction of TNF-α and IL-1β, IL-17, IL23 is the main characteristics of the acute phase of Crohn’s disease (Baumgart and Carding, 2007).

TNF-α production and the bioavailability of its receptors on the cell surface are regulated by TNF-α converting enzyme (TACE), also named ADAM17 a pleiotropic metalloproteinase. TACE is mainly regulated by the tissue inhibitor of metalloproteinase 3 (TIMP3) (Blaydon et al., 2011). ADAM17 role in activating TNF-α is by cleavage of the trans-membrane TNF-α precursor. Crohn's disease is associated with a high expression of TACE correlated with down-regulation of TIMP3 (Cesaro et al., 2009).

IL-1β is primarily synthesised as a pro-IL-1β, then converted to the bioactive form, through protease belonging to the cysteine-dependent protease family, named IL-1β converting enzyme (ICE or Caspase-1). Caspase-1 is the intracellular protease that cleaves the precursors of IL-1β, in addition to, IL-18. The role of IL-1β in intestinal inflammation depends on both the up-regulation of IL-1β production as well as the level of its naturally occurring inhibitor, the IL-1 receptor antagonist (IL-1Ra), therefore the balance of IL-1 and IL-1Ra may affect the disease outcome (Burns et al., 2000).

Ulcerative colitis (UC) is another disease belonging to IBD, where the inflammation affects the mucosal layer of the colon and may be extended to the rectum with infiltration of lymphocytes and macrophages, loss of goblet cells and the presence of ulceration (Bamias et al., 2005, Lennard-Jones, 1989, Strober and James, 1986). Among infiltrative cells in the lamina propria in the case of UC are Th2 cells resulting in an abundance of IL-4 and IL-13 cytokine production, also there are an abundance of a specific macrophage phenotype which have $CX3CR1^{+}TLR2^{+}$ $CD33^{+}$ (Candia et al., 2012).

IBD is described as a collection of multi-factorial diseases; mainly the hypothesis of IBD initiation is that it results from the unusual immune response to enteric bacteria in individuals with vulnerability due to polygenic defects (Bibiloni and Schiffrin, 2010). Evidence supporting microbial agents involving in the hypothesis of IBD initiation include; infection with pathogenic bacteria (e.g. *Mycobacterium avium paratuberculosis*, *Listeria*, in Crohn's disease, and *E.coli* in UC (El-Zaatari et al., 1999), delicate alteration in bacterial population, function, colonization, and composition, especially after antibiotic therapies. Alteration in the mucosal barrier, (e.g. defective mucus, weakening of tight junctions, low rate of epithelial turnover and less epithelial cells restitution) (Silvia and Kim, 2009), mutations in genes (such as NOD-2 associated with Crohn's disease) (Hedl et al., 2007), and defective regulation of mucosal immune responses associated with innate and adaptive immunity have also contributed to the initiation of IBD.

Several studies have revealed that the administration of probiotics has a role in the treatment of IBD (Fedorak, 2008, Rogler and Andus, 1998, Shida et al., 2009, Wallace, 2009). Several studies noted that a dysbiosis and a marked reduction in the numbers of *Firmicutes* found in the GIT of patients with Crohn's disease, including the beneficial commensal *Faecalibacterium prausnitzii* and *Bifidibacteria* (Sokol et al., 2008). Oral administration of the live bacterium of *Faecalibacterium prausnitzii* lead to reduced evidence of experimental colitis in mice (Sokol et al., 2008). Jia et al. (2010) reported that a marked increase in IL-10 secretion and significant reduction in IFNγ and IL-12 production was seen in PBMCs exposed to *Faecalibacterium prausnitzii* and lead to the suggested use as a potential therapeutic approach in the treatment of Crohn's disease. As UC is driven by Th2 immunopathology, skewing of the immune response to a more tolerogenic tissue

environment may alleviate the damaging effects of a dysregulated inflammatory response. Mouse models of UC showed that *Lactobacillus* strains modulate the pro-inflammatory responses. *L. plantarum* 299v in IL-10 knockout mice (Schultz et al., 2002) as well as *L. rhamnosus* GG in transgenic mice (Dieleman et al., 2003) have been shown to decrease levels of pro-inflammatory cytokines including IL-1β, TNF-α, IFN-γ, IL-12, while augmenting the anti-inflammatory cytokine (IL-10). A model of TNBS-induced colitis in rats showed that VSL#3 remodels bacterial composition through enhancing species richness and diversity index (Uronis, 2011). This study correlated with human clinical trials which showed that this probiotic cocktail decreased tissue inflammation, enhanced maintenance of remission in children with ulcerative colitis (Miele et al., 2009), and induced remission in patients with mild-to-moderately active ulcerative colitis in adult subjects (Sood et al., 2009). Furthermore, Kruis et al. (2004) reported that *E coli Nissle* 1917 maintained remission in patients with UC, whereas Malchow (1997) reported a similar effect on remission in Crohn's disease. It seemed to be that the multifaceted immunomodulation actions exerted by probiotics have a crucial role in the treatment of these diseases. Generally, the type of modulation required to down regulate the chronic inflammation in Crohn's disease is quite different from the one required for the treatment of UC. In Crohn's disease, there is an enhanced Th1 response against their own microbiota, which is characterised by a high level of pro-inflammatory cytokines, therefore, probiotics able to increase the level of cytokine such as IL-10 that suppress TNF-α production, may be used as therapeutic supplementation for Crohn's disease treatment. However, in UC the opposite may be required, since probiotics that mediate enhancement of pro-inflammatory cytokines such as IL-12 and down-regulated regulatory cytokines such as Th2 cytokine production are suitable in the treatment of UC (Shida et al.,

2011). Therefore, modulation of cytokine expression by probiotics is one of the possible methods of prevention and therapy for these diseases.

Thus far, studies have focused on the role of commensal bacteria (probiotics) in modulation of the immune responses induced by specific inflammatory signal in single cell types. In contrast, the present work used a developed *in vitro* co-culture system to investigate the use of immunoregulatory probiotics (both probiotic bacterial cells or their secreted proteins) on modulation of macrophages and epithelial cell immune responses induced by a specific signal (comparing two statuses: normal homeostasis and chronic inflammation) in order to choose such probiotic strains that might be boosting mucosal tolerance. These models mimic the interaction of gut epithelial cells with immune cells (Caco-2/M1 to resemble a chronic inflammation model, and Caco-2/M2 to resemble the normal homeostatic model). This knowledge could highlight the important gaps, which need to be filled in terms of the probiotic role in driving mucosal tolerance through modulation of the mucosal immune response. This immunomodulation by probiotics would provide a therapeutic window, and potentially develop the treatment for intestinal inflammation, particularly IBD.

1.9. Aims and objectives of this study

Although many studies have been conducted on the effects of probiotics on human health, there are still a lot of unanswered questions, because the mechanisms of these bacteria, which regulate the immune response in humans, are still unclear. The aims of this project were to find a suitable *in vitro* model to investigate possible immunomodulatory effects of probiotics, in order to choose a specific probiotic strain that might boost mucosal tolerance via modulation of immune responses induced by specific stimuli using THP-1 monocytes, THP-1 derived macrophage subsets and Caco-2 epithelial cells. In this study, a cell line model was developed that mimics the cell-to-cell cross talk between epithelial cells and immune cells, that is vital to the immune fate decision in the gut mucosa. Tolerance (Caco-2/M2) versus activation (Caco-2/M1) was used to investigate the immunomodulatory effects of a panel of probiotics and their secreted proteins (*Bifidobacterium breve, Lactobacillus salivarius, Lactobacillus rhamnosus, Lactobacillus casei strain Shirota, Lactobacillus fermentum, and Lactobacillus plantarum*), refer to Fig.1.7. The specific study objectives in this thesis are:

1- The immunomodulatory capacity of probiotics (heat-killed and secreted proteins) on monocytes, and macrophage cell subset cytokine production induced by enteropathic LPS as a pro-inflammatory stimulus (Chapter 3), Fig.1.7 line 1.
2- The effect of a range of potentially immunoregulatory probiotics on the modulation of macrophage subset LPS-induced NF-κB activation and cytokine production, using a stably transfected NF-κB-reporter cell line

model of $CD14^{lo}$/$CD14^{hi}$ mucosal resident homeostatic and infiltrating inflammatory macrophages (Chapter 3), Fig.1.7 line 1.

3- Probiotic immunomodulation of cytokines, hBD-2 and TLRs by monocultures of epithelial cells induced by TNF-α and IL-1β (Chapter 4), Fig.1.7 line 2. In addition, modulation of epithelial cell functionality in Caco-2/M1 and Caco-2/M2 co-culture models (Chapter 5), Fig.1.7 line 3.

4- The role of probiotics in recovering dysregulated barrier functions (TEER, ZO-1) induced by enteropathic LPS in Caco-2/M1 and Caco-2/M2 (Chapter 5), Fig.1.7 line 3.

5- Probiotic immunomodulation of PRR in the context of endogenous inhibitor expression (Tollip) in macrophage subsets, monocultures of Caco-2 and Caco-2/M1, and a Caco-2/M2 co-culture models (Chapter 3, 4 and 5 respectively), Fig.1.7 line 1, 2 and 3.

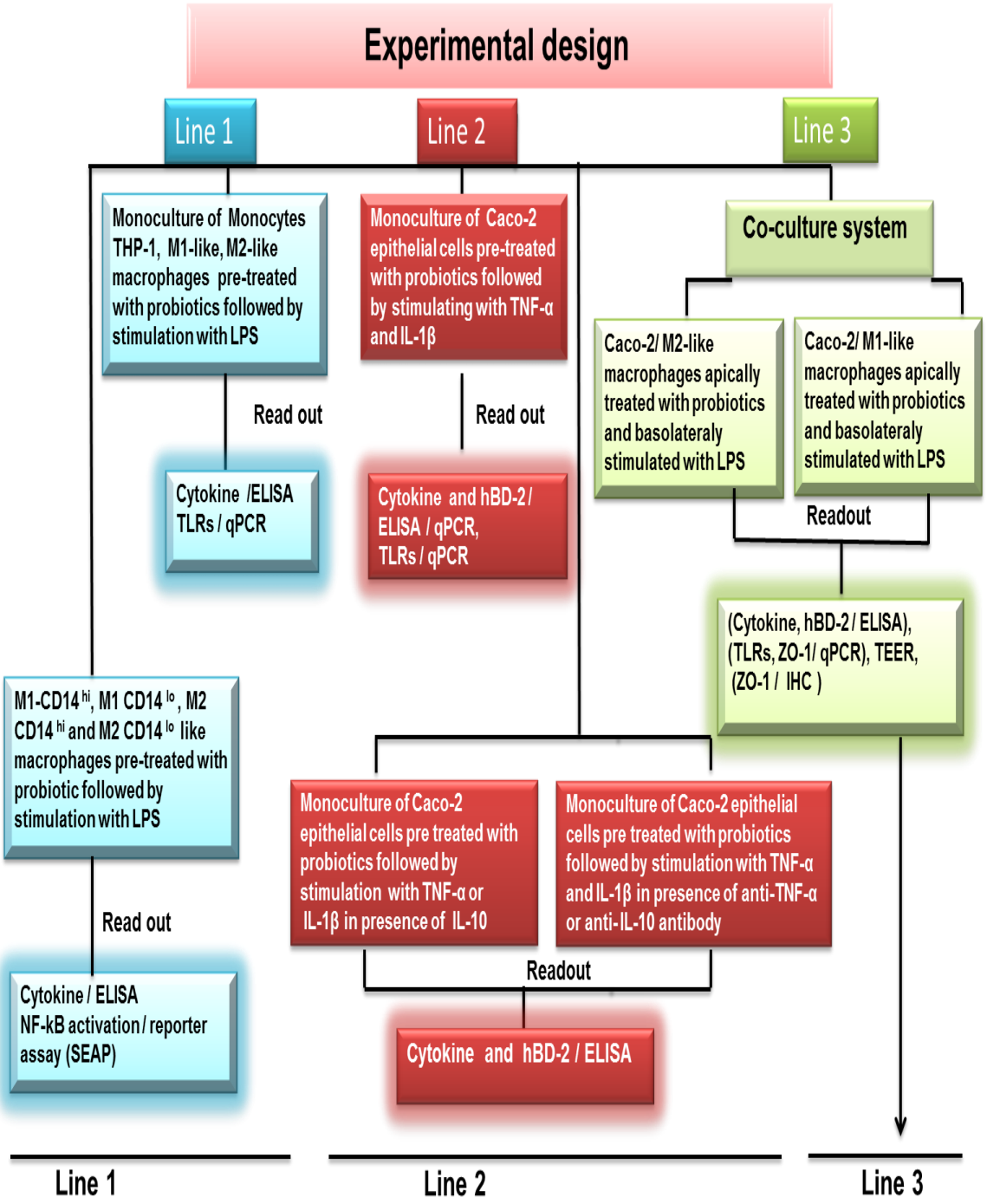


Figure 1. 7: The lines of research investigations planned for the whole project Line3-chapter 5, line 2 –chapter 4, line 1 chapter 3.

Chapter 2

Materials and Methods

Chapter 2: Materials and Methods

2.1. Materials

See the appendices

2.2. Methods

2.2.1. Microorganisms

Bifidobacterium breve strain NCIMB 8807 (BB), *Lactobacillus rhamnosus* GG strain NCIMB 8824 (LR), *Lactobacillus salivarius* strain NCIMB 41606 (LS) and *Lactobacillus plantarum* strain NCIMB 41605 (LP) were obtained from National Collections of Industrial, Marine and Food Bacteria (NCIMB), Aberdeen, Scotland. *Lactobacillus fermentum* strain MS15 (LF) was isolated from the crop of a chicken (Savvidou, 2009) and obtained from internal microbiology stocks at the University of Plymouth. *Lactobacillus casei* strain Shirota (LcS) was obtained from a commercially available Yakult probiotic drink (Yakult, UK). These probiotic bacterial strains were cultured aerobically in deMan Rogosa Sharp (MRS) broth (Oxoid Ltd., Basingstoke, Hampshire, UK) at 37^{o}C for 24 hr. For checking microorganisms' purity, they were streaked on MRS agar media and Gram-stained to check identity. Subsequently a single colony was used to inoculate broth media. Cultures were incubated overnight with shaking (120 rpm) according to the optimum conditions and the growth time. For storage, volumes of cultures (0.5 ml) were mixed with 0.5 ml of sterile glycerol, cryo-protectant and stored at -80ºC or in liquid nitrogen. For maintaining bacterial growth, bacteria were grown in MRS agar and kept at 4ºC, and sub-cultured every 10-15 days.

2.2.1.1. Preparation of heat-killed bacterial cells

Heat killed (HK) bacterial cells were prepared according to the modified method described by (Young et al., 2004). In brief, bacterial cells were grown in MRS broth until stationary phase was achieved (10-14 hr at 37°C with shaking 120 rpm) as monitored by turbidity, measured at an absorbance 600 nm (see Fig.2.1). The bacterial cells were then harvested by centrifugation at 5000 g for 10 min (Rotina 46 centrifuge, Hettich Zentrifugen, Germany) washed three times with sterile phosphate buffered saline solution (PBS) pH7. The bacterial numbers were adjusted by dilution in sterile PBS to give an OD 600 nm reading of 1.2; the corresponding bacterial viable count for the resulting bacterial suspension was determined by colony forming unit (CFU) counting on MRS agar plates, where OD 600 nm 1.2 was found to equate to $1x10^{10}$ CFU/ml. After adjusting the bacterial concentration at $1x10^{10}$ CFU/ml, bacterial cells were heated for 2 hours at 90^{o}C. Bacterial cell death was confirmed by plating on MRS agar and incubation for a minimum of 18 hr. Bacterial cell integrity was checked by Gram stain and light microscopy.

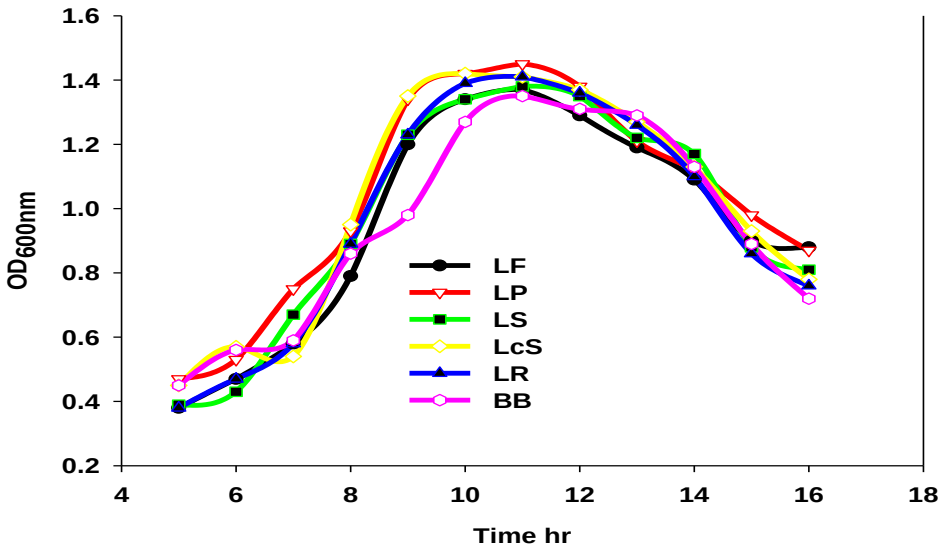


Figure 2. 1: Probiotic bacterial growth curve.

Probiotic bacteria *Lactobacillus fermentum (LF), L. plantarum (LP), L. salivarius* (LS), *L. casei strain Shirota* (LcS), *L. rhamnosus* GG *(LR), and Bifidobacterium breve* (BB), were cultured aerobically at 37°C for 16 hr with shaking 120 rpm in MRS broth. In order to determine the bacterial growth phases, each bacterial strain was monitored by optical density (OD_{600} nm) to determine the bacterial cell density and streaked on MRS agar to determine the viable bacterial count at each time point starting from lag phase (5), exponential phase (6-10), stationary phase (10-14) then ended by the decline phase. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments.

2.2.2. Protein analysis methods

2.2.2.1. Preparation of secreted protein extracts by probiotic bacteria

A single colony of fresh culture of each probiotic strain was used to inoculate 10 ml of MRS broth and incubated overnight at 37°C with shaking (120 rpm). The culture (0.5 ml) was used to inoculate 50 ml of fresh MRS medium and incubated overnight as before until reaching the stationary phase (10-14 hr culture) as monitored by absorbance at 600 nm (see Fig 2.1). According to the modified protocol of (Sanchez et al., 2009), stationary phase bacterial culture supernatants or fresh MRS broth (control) were harvested by centrifugation at 3500 g for 10 min

at 4ºC. The cell supernatant was filtered through nitrocellulose membranes 0.45 µm (Fisher, UK) followed by adding 10 ml of 5%v/v sodium deoxycholate to the resulting filtrate to exclude salts that might exist in the bacterial cell supernatant, then incubated at 4^{o}C for 30 min. After which chilled trichloroacetic acid (TCA) was added to a final concentration of 60 g/L and proteins were allowed to precipitate for 2 hr at 4ºC. Proteins were recovered by centrifugation (9300 g for 10 min at 4ºC). Protein pellets were washed twice with 2 ml of chilled acetone and allowed to dry at room temperature, proteins were re-solubilised in an ultra-sonic bath (35kHz.frequency, Fisher brand FB 11010) for 2 min in 40 µl of Laemmli buffer for SDS-PAGE analysis, or in 1 ml of sterile LPS free PBS for treating the immune cells. Proteins were resolved by SDS-PAGE in a 12.5%w/v polyacrylamide gel and molecular weight determined by the retention factor (RF) (a common abbreviation for relative protein mobility throughout the gel, which related to the protein molecular mass) compared to a defined protein standard. The Bradford method was used to estimate the concentration of protein in each bacterial secreted extract (refer to section 2.2.2.2).

2.2.2.2. Protein quantification

The Bradford method (Bradford, 1976) modified for the micro-plate reader was employed for protein quantification. The Bradford method is recommended for general use, especially determination of protein content of cell fractions and secreted extractions. The main concept of this assay depends on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when engagement with protein occurs (Bradford, 1976). Bovine Serum Albumin (BSA) protein standards between 7 µg/ml and 5 mg/ml were prepared by dissolving BSA in distilled water; following that, an equal

volume of dye reagent (Bradford) was added and incubated for 5 min at room temperature (RT). The absorbance was determined in a micro-plate reader (Molecular Devices, Manchester, UK) at 595 nm. A standard curve was produced between absorbance value and protein concentrations, and the test sample was calculated based on comparison to the standard curve (see Fig. 2.2).

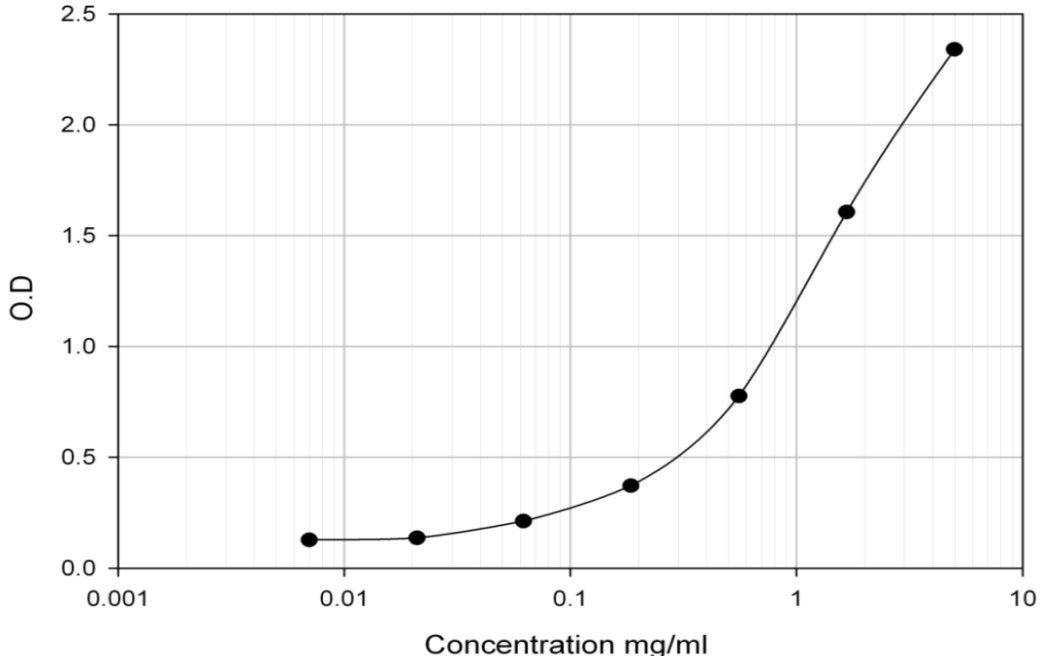


Figure 2. 2: Standard calibration curve of BSA by Bradford assay.

Bovine serum albumin (BSA) protein standard was prepared with the range of 7 µg/ml to 5 mg/ml. The absorbance (OD) was determined in a micro-plate reader at 595 nm. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments.

2.2.2.3. Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS–PAGE)

SDS-PAGE is widely used as an analytical technique for resolving complex mixtures of proteins. It was performed using a Laemmli discontinuous buffer system. Glass plates (BIO-RAD, UK) were cleaned with decant detergent and washed with deionised water, followed by 70 %v/v ethanol and air dried before using. The final concentration of the resolving gel was 12.5%w/v (see appendices

section 1.1.3). The required resolving gel solutions were firstly mixed followed by adding fresh 1%w/v ammonium per sulphate (APS) and Tetramethylethylene diamine (TEMED) for polymerisation and immediately poured into the performed space between the glass plates and filled to two thirds the height of the largest glass plate. Two ml of 10%v/v water saturated isopropanol was added immediately on top of the resolving gel making a layer on top of the gel to liberate the air bubbles. The gel was left for at least 30 min at RT for polymerisation. After polymerisation of the resting gel, the isopropanol layer was removed, then the stacking gel 3%w/v was prepared according to the recipe mentioned in (appendices, section 1.1.3), APS and TEMED were added, then immediately poured on top of the resolving gel. In order to make wells for loading the protein sample, a comb was inserted directly into the stacking gel. The gel was left for at least 30 min for polymerisation and transferred to the electrophoresis tank. The electrophoresis tank was filled with running buffer, covering the top of the gel. The comb was removed carefully and the wells were washed with running buffer to clean the wells from residing stacking gel materials. A volume of 12 µl of defined concentration protein sample and 3 µl of loading buffer were boiled for 3 min to denaturation the protein, quenched on ice for 2 min, briefly vortexed, centrifuged at 12000 rpm for 30 sec then loaded into each well of the gel. The gel was run at 100 V for 2 hr and monitored by observing the dye marker (Hyper Page pre-stained protein Marker, Bio line, UK). The gel was removed from the electrophoresis system tank, plates were removed, and the gel was stained with Coomassie blue (see appendices section 1.1.4) overnight then the gel was transferred to the de-staining buffer until the protein bands appeared.

2.2.2.4. Western blotting (WB)

Western blotting (WB) is widely used for detecting specific proteins stained with specific antibodies in a given complex sample protein. After resolving the proteins by SDS-PAGE, proteins in gels were transferred to polyvinylidene fluoride (PVDF) membranes using an electro-blotter system (Criterion blotter, BIO-RAD, UK). After electrophoresis and prior to blotting, the gel was placed in 100 ml of transfer buffer (500mM Glycine, 50mM TrisHCl, 0.01% SDS, 20% methanol) (refer to appendices, section 1.1.6) for 5 min. Pieces of PVDF were cut to the same size as the gel, along with two pieces of What man filter papers. The PVDF blotting papers were soaked in methanol (100%v/v) for 15s, DW for 2 min and the transfer buffer for 5 min. The blotting apparatus blotting system was assembled as follows: black plastic cassette plate at the bottom, gauze, filter papers, SDS-PAGE gel, PVDF blotting paper, filter papers, gauze, and red plastic cassette at the top (see Fig.2.3). The air bubbles were removed with a roller, and the cassette was firmly closed and placed in the tank with the black side towards the cathode (black electrode). The tank was filled with cold transfer buffer and an ice pack was placed at the bottom. In addition, a magnetic stirrer was placed in the bottom of the tank for cooling distribution, to avoid the overheating of the buffer, which might affect the blotting system.

When blotting system was connected to the power, the gel was electro blotted at 100 V for 35-45 min. After blotting, the PVDF membrane was blocked with 5%w/v BSA / tris buffered saline with Tween 20 (TBST) solution at RT for 1hr on the roller. Membranes were exposed overnight to the primary antibody in 1% BSA in TBST (see appendices, section 1.1.6) at 4°C on the roller. After that, the antibody was placed into an empty falcon tube, and the PVDF membranes were washed with TBST three times (5 min each at RT on the roller). After that, the membranes were

incubated with appropriate secondary antibodies coupled to horseradish peroxidase (HRP) for 2h at RT. The membranes were washed three times for 5 min with TBST at RT on roller followed by washing once for 5 min with TBS (appendices, section 1.1.6) on roller to remove traces of Tween-20.

Developing colour for protein detection was performed by using enhanced chemiluminescence (ECL) (GE Healthcare Life Sciences, Buckinghamshire, UK) to detect peroxidase activity from HRP-conjugated antibody. According to the manufacturer's instructions, the solution for developing colour was prepared in a falcon tube protected from light. The membrane was removed after washing with TBS, dabbed dry on a paper towel and laid out on a plastic sheet. The developing solution was carefully pipetted over the entire membrane, and left for 5 min. The protein bands were visualized by a gel documentation system using EC3 imaging system (UVP ultra violet product, Ca, UK).

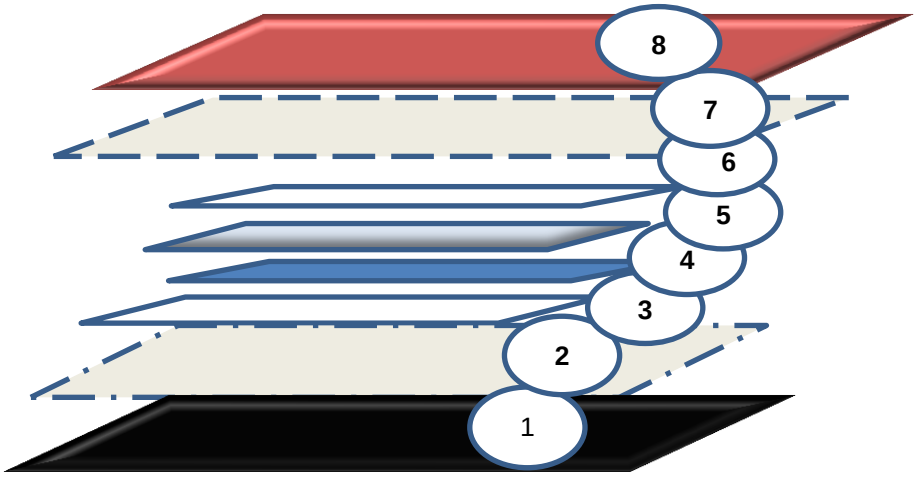


Figure 2. 3: Western blotting apparatus assembly.

A schematic showing the order of western blot apparatus,1) black plastic cassette plate, 2) gauze, 3) filter paper, 4) gel, 5) PVDF membrane, 6) filter paper, 7) gauze, 8) red plastic cassette plate.

2.2.2.5. Separation of bacterial secreted proteins by gel chromatography filtration

Gel filtration is widely used to separate protein molecules based on protein size (molecular weight). The type of gel used were porous, where the small proteins can penetrate the pores and eluted slowly, in comparison with large protein molecules which cannot enter the beads were travelling around them and eluted quickly. The protein fractions were collected into a series of tubes with a fraction collector, where the volume in each tube is called the elution volume (Ve), and the fraction collected in contains the separated protein. Blue dextran dye (see appendices, section 1.1.7) was used to determine the void volume (Vo), which is the volume of the eluent collected from the beginning of the run until the blue dye begins to elute. Bacterial secreted protein extracts were subjected to gel chromatography filtration under non-denaturing conditions using Sephacryl S-200 HR (1-80 KDa MW range (dextran), 5-250 kDa MW range (globular protein). In order to separate the protein mixture, the steps were performed following the method described by Hagel (2001). In brief, the Sephacryl S-200 HR medium was poured into a column (2.4×24 cm) to form packed beads, 100 ml of 0.15 M sodium phosphate buffer (pH 7.2) was added and left for casting at 4^{o}C for three days. According to Delmas et al. (2001), the column was first calibrated with a standard protein marker mixture before being used for the samples. The standard protein marker mixture (1 ml) containing 5 mg apoferritin (443 KD), 2 mg β-amylase (200 KD), 2.5 mg alcohol dehydrogenase (150 KD), 5 mg bovine serum albumin (BSA) (66 KD), and 1.5 mg Carbonic anhydrase (29 KD) was added to the column, followed by 100 ml of 0.15 M sodium phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min. The protein concentration in each eluted fraction was determined by absorbance at 280 nm. In order to plot the standard curve to determine the

molecular weight of the protein sample, the optical density (OD) value of the peaks, which corresponded to the different proteins in the standard protein mixture, was divided by void volume (Vo), and plotted against the natural log molecular weight (Log MW) of each protein in the standard protein mixture. Calculation of molecular weight (MW) of the eluted protein was performed using the known protein standard curve (see Fig 2.4).

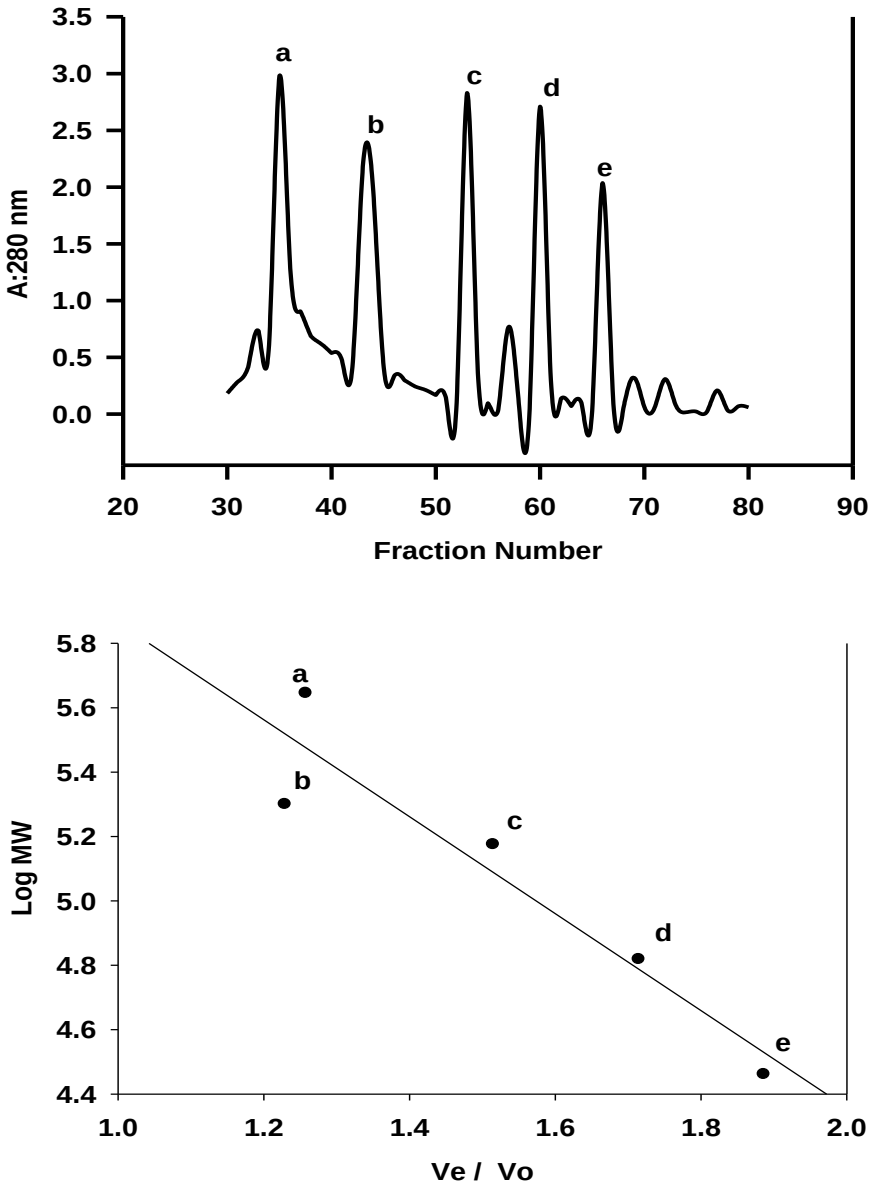


Figure 2. 4: Calibration curve for gel permeation determination.

One ml of protein mixture containing of **a**) 5 mg apoferritin (443 kD), **b**) 2 mg β-amylase (200 kD), **c)** 2.5 mg alcohol dehydrogenase (150 kD), **d**) 5 mg BSA (66 kD), and **e**) 1.5 mg Carbonic anhydrase (29 kD) were subjected to a Sephacryl S-200 column (2.4×24 cm) that had been washed with 100 ml of 0.15 M sodium phosphate buffer (pH 7.2) at a flow rate of 0.5 ml/min. Dextran blue was used to determine the void volume (Vo), while (Ve) is the elution volume. Fraction size 0.5 ml protein concentration was determined by absorbance at 280 nm for each fraction that referred to elution volume (Ve). The optical density value of the peaks which labelled **a**, **b**, **c**, **d**, **e** corresponded to the different protein in the standard mixture were divided by void volume (Vo), plotted against the log molecular weight (Log MW) of each protein in a standard protein mixture.

2.2.3. Human cell line culture methods

2.2.3.1. THP-1 human monocytic cell line

The human pre-monocytic cell line, THP-1, was obtained from European Collection of Cell Cultures (ECACC, UK), and routinely used for these studies between passages 7 and 35. THP-1 cells were maintained in R10 medium composed of Roswell park memorial institute-1640 medium (RPMI-1640) medium supplemented with 10%v/v foetal calf serum (FCS), 2 mM L-glutamine and 100 U/ml penicillin/100µg/ml streptomycin, and sub-cultured every 3 days (Tsuchiya et al., 1980, Yea Ping et al., 2008). THP-1 Blue ($CD14^{lo}$) and THP-1 Blue-CD14 ($CD14^{hi}$) (Invivogen, Calne, UK) were maintained in R10 medium in the presence of the selection antibiotics, 200 µg/ml zeocin or 200 µg/ml zeocin and 10µg/ml blastocidin (Invivogen, Calne, UK), respectively. Generally, the cells were maintained in R10 medium and plated out for experimentation at a final density of $1x10^5$cells/100µl/well in 96 flat-bottomed well tissue culture plates (Sterllin, Newport, UK) in a humidified atmosphere of 5% CO2 incubator set at 37ºC. For maintaining the cells, they were sub-cultured every 3 days at a ratio of 1:4 using R10 for cell dilution.

2.2.3.2. Caco-2 intestinal epithelial cell line

Caco2 epithelial cells (Human colon adenocarcinoma cell line) (kind gift from Dr. Maria O'Connell HNR, Cambridge, UK) were maintained in D10 medium composed of Dulbecco's Modified Eagles' Medium (DMEM) supplemented with 10%v/v FCS, 2 mM L-glutamine and 100 U/ml penicillin, 100 µg/ml streptomycin. Cells were plated out at a density of $5x10^5$ cells/ml/well in 6 well plates and incubated in a humidified atmosphere of 5% CO_2 at 37ºC for 21 days for full cell differentiation (Hilgers et al., 1990). For cell maintenance, they were sub-cultured every 3 days when a confluence reached up 70%. The sub-culturing of cells was performed by pouring off the old medium. Cells were washed with Dulbecco's Phosphate-Buffered Saline (DPBS) twice and trypsinised by adding 2-3 ml of 0.25% v/v versene/trypsin EDTA (TE), the excess of TE was removed and cells were incubated for (10-15 min) at 37ºC. The cells were monitored by light microscopy, and gently agitated until detached. Trypsin was deactivated by adding D10 medium (Walgren et al., 1998). Cells re-suspended in an appropriate volume of D10 medium prior to seeding depending on the experimental design.

2.2.3.3. Determination of total cell number and cell viability

Assessment of cell viability and cell count was determined microscopically using vital staining with (0.2% (w/v) trypan blue in 0.9%NaCl) and counting in a Neubauer haemocytometer chamber.

2.2.3.4. Storage of human cell lines

THP-1 and Caco-2 cells were grown in appropriate media. THP-1 cells were centrifuged at 1200 rpm for 5 min, the supernatant was discarded and cells re-suspended in 1 ml of storage medium refer to (appendices section 1.2), and kept at -80°C. Caco-2 cells were grown to 80% confluence, trypsinised, and harvested by centrifugation at 1200 rpm for 5 min, the supernatant was aspirated, and cell

pellets were re-suspended gently in 1 ml of storage medium, and then kept at -80°C.

2.2.3.5. Macrophage cell differentiation

According to the protocol of Schwende et al. (1996), THP-1 human monocytic cells were differentiated into pro-inflammatory M1-like and anti-inflammatory M2-like macrophage cells. Pro-inflammatory M1-like, M1-CD14^{hi}, or M1-CD14^{lo} macrophages were differentiated by 25 ng/ml Phorbol-12-myristate acetate (PMA) for 3 days, whereas anti-inflammatory M2-like, M2-CD14^{hi}, or M2-CD14^{lo} macrophages were differentiated by 10 nM 1,25-$(OH)_2$-vitamin D_3 for 7 days (refer to Fig. 2.5).

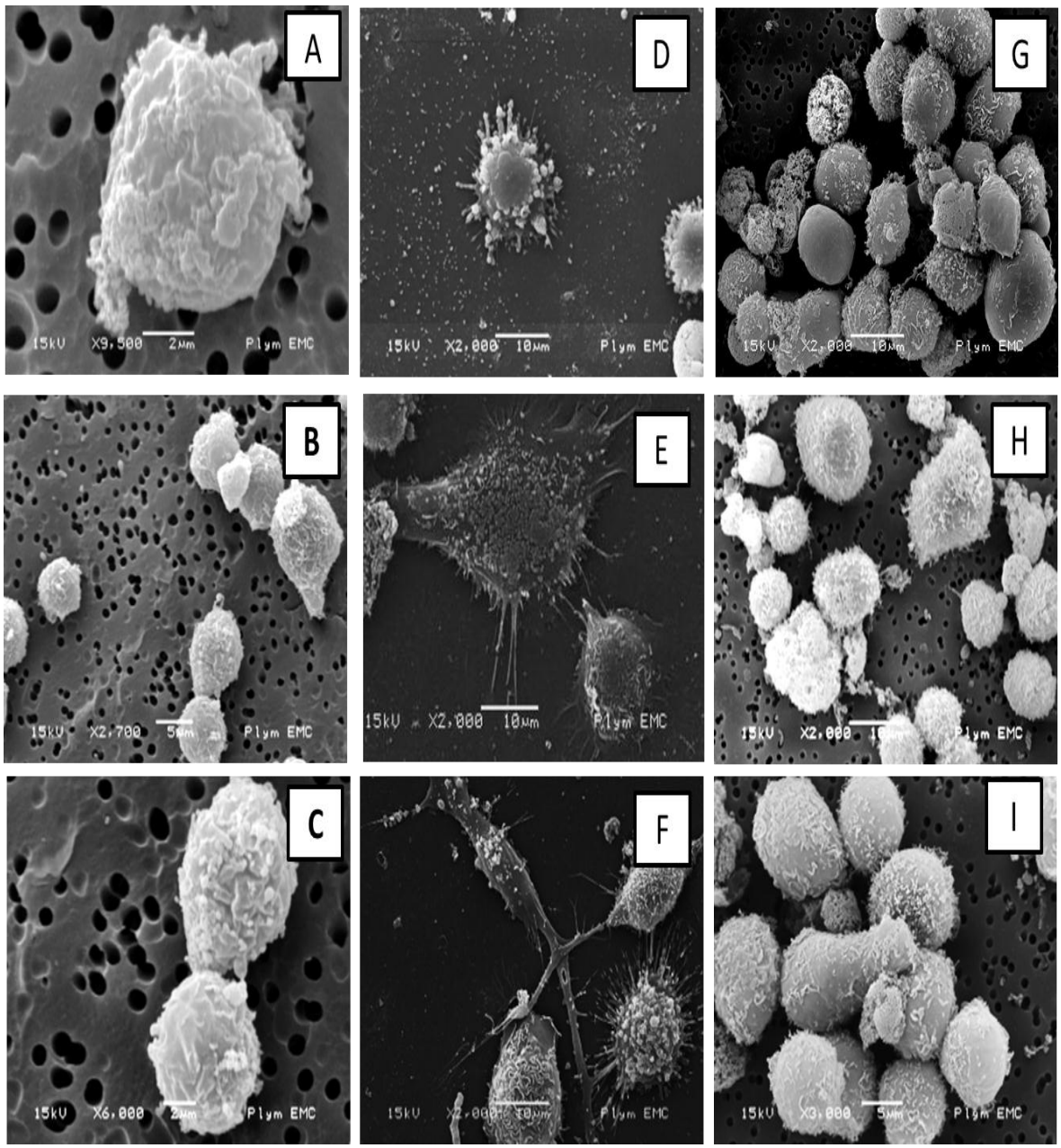


Figure 2. 5: M1-like and M2-like macrophage differentiation.

This figure showed pictures comparing cells treated with 25 ng/ml PMA to differentiate THP-1 into M1 pro-inflammatory macrophages and 10 nM 1,25-(OH)2-vitamin D3 to differentiate THP-1 into M2-anti-inflammatory macrophages to the control treatment THP-1 cells at different time points. **A, B** and **C** scanning electron microscopy (SEM) pictures are the control group taken after 0, 3 and 7 days respectively. **D**, **E** and **F** are the (THP-1 +PMA) treatment after 1, 2 and 3 days respectively with pictures **G**, **H** and **I** showing the (THP-1+Vitamin D_3) treatment after 1, 3 and 7 days. **D** shows M1 has lost the original ruffles shown in **A** and has begun to develop pseudopodia whereas when the cells are treated with vitamin D_3 (picture **G**) the ruffles on M2 appear smaller. After 2 days (**E**) M1 appears globular and lumpy, when compared to

the control (**B**) as opposed to the vitamin D_3 treatment after 3 days (**H**) where M2 is spindly with fine, small ruffles. After 3 days with PMA treatment (**F)** M1 has developed long, branching pseudopodia compared with the control (B). **I** shows the final stage of vitamin D_3 treatment where M2 has spinier ruffles compared to the control treatment (**C**). The macrophage sample cells were fixed with glutaraldehyde followed by dehydration in an ethanol series then dried with liquid carbon dioxide in a critical point dryer. Samples were sputter coated in gold and examined under a Jeol JSM 6500 SEM 6500 and the total magnification is shown in each figure.

2.2.3.6. Epithelial cell differentiation

Caco-2 cells differentiated into the small intestine enterocyte-like cells were performed by long term culturing for 21 days (Amano and Oshima, 1999). They spontaneously grew reaching the maturation stage with regular homogenous glycocalyx brush-border despite their colonic origin (Costa de Beauregard et al., 1995), see Fig.2.6.

2.2.3.6. Scanning Electron Microscopy (SEM)

Samples of macrophage cell subsets and epithelial cells were analysed using SEM by following standard protocols. The samples were washed with PBS to remove any associated particles of the medium, before being fixed for 1 hr in 2.5% glutaraldehyde (Agar Scientific, U.K. R1102) in 0.1 M sodium cacodylate buffer (Agar Scientific, R1012) at 4ºC. The samples were rinsed in 0.1 M sodium cacodylate three times for 15 minutes each. All samples were dehydrated in an ethanol series including 50%, 70%, 90%, and 100% for 15 minutes each with 2 absolute ethanol changes. The samples were then dried with liquid carbon dioxide in a critical point dryer (Samdri-780, Tousimis, USA). Samples were sputter coated in gold (K550, Emitech, U.K.) and examined under a Joel JSM 6500 (University of Plymouth, UK) SEM operated at 15 kV.

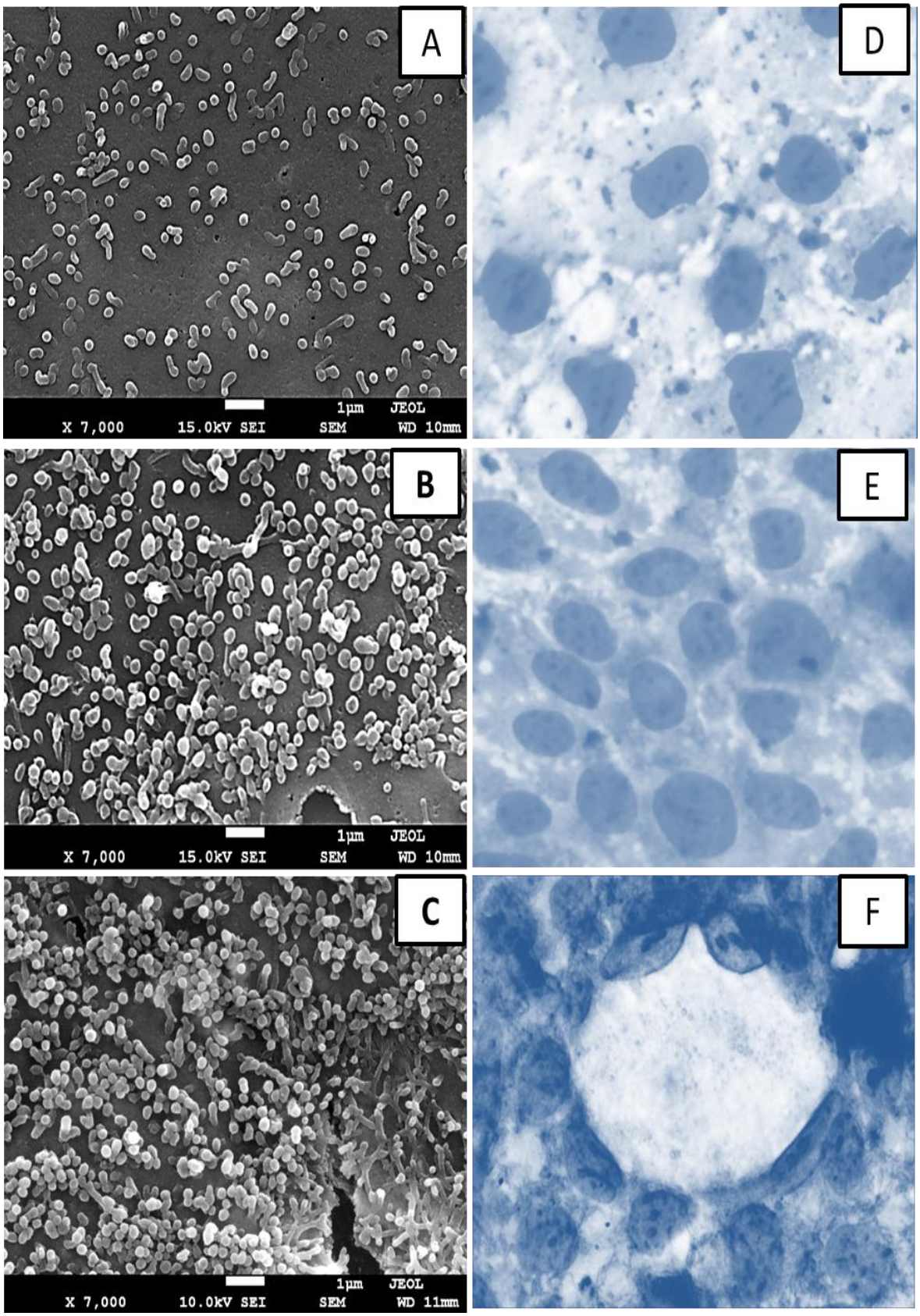


Figure 2. 6: Caco-2 epithelial cell differentiation.

A) Monolayer of Caco-2 cells after 3 days, showing the initial stages of microvilli growth, B) monolayer of Caco-2 cells; microvilli begin to grow vertically after 7 days, C) monolayer of Caco-2 cells with regular homogenate brush border formed after 21 days. Cells were fixated with glutaraldehyde followed by dehydration in an ethanol series then dried with liquid carbon dioxide in a critical point dryer. Samples were sputter coated in gold and examined under a Jeol JSM 6500 SEM operated at 15KV, and the total magnification was x7, 000 for A, B and C. D, E and F monolayer of Caco-2 cell after 3, 7 and 21 days observed with a light microscope, showing increasing in cell growth associated with the number of cells and cell density. Cells were fixed with 70% methanol and then stained with Giemsa, the image was observed using light microscopy at a total magnification x1000.

2.2.3.7. Activation of monocytes, macrophage cell subsets and epithelial cells

Monocytes, and macrophage cells were stimulated with the bacterial pathogen associated molecular pattern (PAMP); 100 ng/ml *E. coli* strain K12 lipopolysaccharides (LPS) (expressed by enteropathic Gram-negative bacteria and detected by TLR4) (Beutler and Poltorak, 2001). These cells were cultured in the presence of a stimulus for 18 hr as the optimal time for cytokine release by macrophages in a humidified environment at 37^{o}C, 5% CO_2. Caco-2 cells were stimulated with cytokines (10 ng/ml TNF-α, or 5 ng/ml IL-1β) and cultured for 18 hr. Cell supernatants were harvested and stored at-20^{o}C until required for assay by sandwich enzyme-linked immunosorbent assay (ELISA) and the cell lysate for detection of gene expression by quantitative polymerase chain reaction (qPCR).

2.2.3.8. Cell viability test by MTT assay

To confirm that the acids and other bacterial metabolites were not toxic for cultured immune cells, cell viability was assessed using the 3-4-5-dimethy-2.5 thiazol-2.5 diphenyltetrazolium bromide (MTT). The main principle of the MTT assay is a measurement of mitochondrial function where the metabolically active cells reduce yellow tetrazolium MTT to the purple formazan product by dehydrogenase enzymes. According to the modified protocol by Schlee et al

(2007), Caco-2 cells, monocytes, or macrophage cells were seeded in 96-well plates. Treatment of cells was performed using a suspension of live bacteria or heat killed bacteria at cell concentrations of $3x10^3$, $3x10^6$ – and $3x10^9$ CFU / ml, and secreted protein at concentrations of 0.3, 3 and 30 µg/ml. Cells with medium alone were used as control. After 6, 12 and 24 hr post treatment, the cell supernatant of each time point for each treatment was replaced with D10 or R10, and 50 µg/ml of MTT for each well, and incubated for 4 hr at 37ºC. Living cells degrade MTT through mitochondrial succinate dehydrogenase, resulting in the production of MTT formazan. The converted dye was solubilised with acid isopropanol (0.04 M HCl in absolute isopropanol). Colorimetric development was measured spectrophotometrically by an OPTIMax tuneable micro-plate reader at 570 nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA). The MTT assay data were confirmed by counting treated and untreated cells using Trypan blue.

2.2.4.1. Assessment of the probiotic bacterial role in modulating the immune response by monocytes, macrophage subsets and epithelial cells

In *vitro* studies are useful for establishing cellular and molecular mechanisms for the immunomodulatory action of probiotic bacteria; however, the main limitation of the *in vitro* system is the degree to which they reflect *in vivo* condition. THP-1 monocytic cell line, and THP-1 derived macrophage subsets were used as a suitable model for immune cells (monocytes, M1-like and M2-like macrophage subsets, refer to Fig.2.5), whereas Caco-2 epithelial cell line was used as a suitable model for intestinal epithelial cells (refer to Fig.2.6) to investigate the immunomodulatory effects of probiotics.

Two types of probiotic preparation were used; secreted proteins (SP) and heat killed (HK), to treat immune cells and epithelial cells. Indeed, during the last few years, a substantial body of scientific evidence has accumulated suggesting that certain extracellular components such as proteins produced by probiotic bacteria could be responsible for some of their mechanisms of action. Extracellular proteins include proteins that are actively transported to the bacterial surroundings through the cytoplasmic membrane, as well as those that are simply shed from the bacterial surface. These SP would be able to directly interact with the host mucosal cells leading to modulation of signal transduction pathways. Therefore, the current study focused on the potential immunomodulatory effects of SP in different cell types (immune cells and epithelial cells). There is no reliable evidence of the literature suggested the concentration of SP relative to the various parts of the GIT or relative for each probiotic bacterial strain, therefore, a series of optimization had been done to choose the concentration that induced detectable cytokine expression in various cell types used in this study.

One of the main limitations in using live bacteria in cell culture technique is the accumulation of the lactic acid during bacterial cell growth, leading to subvert immune cell. In addition, lactic acid itself has a direct effect on immune cells (Peluso et al., 2007). Indeed, Zhang et al. (2005) reported that HK-probiotic bacteria are able to prevent intestinal inflammation without the potential pro-inflammatory effect; therefore, to exclude any side effects, heat killed bacterial cell format was used in this study.

The ranges of bacterial concentrations were used according to the relative composition of the dominant microbial species in various regions of the GIT (see Fig.1.2) and based on the information from the literature (Okada et al., 2009).

Probiotic strains used in this study were *B. breve* (BB), *Lactobacillus rhamnosus* (LR), *L. fermentum* (LF), *L. casei strain Shirota* (LcS), *L. salivarius* (LS), and *L. plantarum* (LP). Based on evidence from several studies that probiotic bacteria are different in their sensitivity of lysozymes digestion, *Lactobacillus fermentum* (LF) probiotic bacteria was chosen as probiotic bacteria sensitive to lysozyme digestion (Šimelyte et al., 2000), whereas *Lactobacillus casei strain Shirota* (LcS) bacteria are resistant with rigid cell wall (Shida et al., 2006).

Cytokine and TLR expression are useful readout for the immunomodulation by probiotic bacteria. According to the modified protocol by Foey (2011), probiotic preparations were used to investigate a potential regulatory role of probiotics in modulating the immune responses induced by inflammatory signal (LPS) in monocytes and macrophage cell subsets. In brief, THP-1 monocytes, M1-like, M1-CD14^{hi}, M1-CD14^{lo}, M2-like, M2-CD14^{hi}, M2-CD14^{lo} like macrophages derived from THP-1 at a cell density of 1x10^5 cells/100µl/well cultured in 96 well plates were pre-treated with probiotics as a final CFU/ml equivalents of 3x10^8 HK probiotic or 3 µg/ml of SP extracts for 18 hr followed by stimulation with 100 ng/ml K12-LPS and incubated for 18 hr in a humidified environment at 37^{o}C, 5% CO_2 (see Fig.1.7 line 1).

Regarding Caco-2 cells, they cultured in 6 well plates at a cell density of 5x10^5 cells/ml, pre-treated with probiotics (HK or SP) at a final CFU/ml equivalents of 3x10^8 HK probiotic for 18 hr followed by stimulation with cytokines (10 ng/ml,

TNF-α or 5 ng/ml IL-1β) for 18 hr in a humidified environment at 37^{o}C, 5% CO_2 (refer to Fig.1.7 line 2). The cell supernatant of monocytes, macrophage subsets, and Caco-2 cell were collected and stored at -20°C for cytokine detection by ELISA, and cell lysate for detecting mRNA level of the gene of interest using qPCR.

2.2.4.2. Detection of IL-10 membrane bound protein by macrophage subsets

Full cell differentiation of macrophage subsets at a cell density of 1x10^{5} cells/100µl/well cultured in 96 well plates were stimulated by 100 ng/ml K12-LPS in the presence or absence of 10 µg/ml purified anti-human IL-10 antibody (clone JES3-9D7) and incubated for 18 hr in a humidified environment at 37^{o}C, 5% $CO_{2.}$ Macrophage cell supernatant was collected and stored at -20°C for cytokine detection by ELISA.

2.2.4.3. Detection of pSTAT-3 and total STAT-3 in macrophage cells.

Macrophage subsets at a cell density of 1x10^{6} cells/ml cultured in 6 well plates were stimulated by 0.1, 1, or 100 µg/ml K12-LPS and incubated for 18 hr in a humidified environment at 37^{o}C, 5% $CO_{2.}$ Macrophage cell supernatant was collected to determine the cytokine level for TNF-α, IL-6, IL-8 and IL-1β by ELISA, whereas macrophage cells were washed gently with PBS, then lysed using lysis buffer, supplemented with a protease inhibitor cocktail (1:20) and phosphate inhibitor cocktail (1:100) (refer to appendices, section 1.1.6). Cell lysates were centrifuged at 3,500 rpm at 4°C for 10 minutes to spin down the cell debris into a pellet. Cell supernatants were carefully removed without disturbing the pellet and put into new eppendrofs. Protein cell concentration was determined by the Bradford method. Defined protein concentration in each sample was mixed with 5X Laemmi loading buffer, boiled for 3 min, quenched on ice for 2 min, briefly

vortexed, centrifuged at 12000 rpm for 30 sec then loaded into each well of the SDS-PAGE prior to electrophoresis, followed by the routine procedure of WB (refer to section 2.2.2.4). For detection of phosphorylated STAT-3 (pSTAT3) protein (Tyr 705) (see the appendices section 1.3), the primary antibody was prepared in 1:500 in 5 ml of blocking solution (5%BSA). The secondary antibody (anti-Rabbit HRP conjugated) solution was prepared in 1:2500 in antibody solution (50:50 blocking solution: TBS-Tween), refer to appendices section 1.1.6.

In order to detect total STAT-3 protein from macrophage cells, stripping of the membrane was performed by incubating the membrane (which had been used for pSTAT-3 protein detection) in stripping off buffer (refer to appendices, section 1.1.6) at RT on the roller for 5-10 minutes. Subsequently, the buffer was discarded and the membrane was incubated again with fresh stripping off buffer for 10 min at RT on the roller. After discarding the buffer, the membrane was incubated twice with PBS for 10 min, followed by incubation twice with TBST for 5 min. After stripping off the membrane, detection of total STAT3 protein was performed as follows: the PVDF membrane was incubated with the primary antibody overnight at 4ºC on the roller (the primary antibody was prepared in 1:1000 in 5 ml of blocking solution). The PVDF membrane was then incubated with the secondary antibody, which was prepared in 1:2500 in antibody solution (50:50 blocking solution: TBS-Tween), followed by developing colour and visualization with ECL, as in the pSTAT-3 detection.

2.2.4.4. Time-dependent cytokine and hBD-2 secretion by epithelial cells

For detecting the optimal time point for cytokine and AMPs release of epithelial cell over time, the cytokines TNF-α or IL-1β were used as stimuli. A concentration of 10 ng/ml of TNF-α was used to treat epithelial cell using the

pulse chase method for detecting TNF-α cytokine release by epithelial cells. The pulse chase method was performed by stimulating the cells with TNF-α for 6 hr, followed by washing the cells with fresh medium and incubating them for 24 hr, the cell supernatant was collected at each time point (0, 1, 2, 4, 6, 9, 12, 18, and 24 hr) for detection TNF-α cytokine release. For detecting other cytokines such as IL-6, IL-8 and IL-10, cells were treated with TNF-α at (0, 1, 2, 4, 6, 9, 12, 18, and 24 hr), cell supernatant was collected at each time point and kept at -20°C until used for cytokine detection by ELISA. IL-1β at the concentration of 5 ng/ml was used to treat cells at each time point of (0, 1, 2, 4, 6, 9, 12, 18, and 24 hr); the cell supernatant was collected at each time point to detect cytokine and antimicrobial peptide hBD-2 release using ELISA method and cell lysate for detecting mRNA level of the gene of interest using qPCR.

2.2.4.5. Assessing the role of secreted protein extract fractions on modulation LPS-induced macrophage subset cytokine production

To investigate the role of secreted protein extract fractions, which were separated by gel chromatography on modulation of the LPS induction of cytokine production by macrophage cell subsets, LcS secreted protein fractions, was used to treat immune cells. In brief, after separation the LcS secreted protein as fractions by gel chromatography based on protein size, the fractions were collected in sterile 1 ml Eppendrof tubes. LcS protein extract fractions (100 µl) were used to treat THP-1 derived M1-or, M2-like macrophages at a cell density of $1X10^5$ cells/100µl/well cultured in 96 well plates for 18 hr followed by adding 100 ng/ml k12-LPS in the presence or absence of LcS secreted protein fractions and incubated for a further 18 hr. The cell supernatant of macrophage cell subsets were collected and stored at -20^{o}C until used to detect cytokine production by ELISA technique.

2.2.4. 6. Assessing the role of probiotics in modulation of TNF-α or IL-10 induced hBD-2 expression in Caco-2 cells

It is well reported that cytokine produced by immune cells such as macrophages induce or suppress the expression of hBD-2 by Caco-2 cell as a part of innate immunity (Albanesi et al., 2007, Chen et al., 2006). In order to find out the probiotic role in the modulation of cytokine-induced hBD-2, Caco-2 cells were seeded in six well plates at a cell density of $5x10^5$cells/well and cultured for 21 days. Cells were pre-treated with heat killed probiotic bacteria LcS, or LF at cell concentration of $3x10^8$CFU/ml for 18 hr, followed by stimulation with either 10 ng/ml TNF-α, or 5 ng/ml IL-1β in the presence or absence of 10 ng/ml IL-10 and further incubated for 18 hours. The cell supernatant was collected to detect hBD-2, IL-10, or TNF-α protein released by ELISA, and cell lysate for detecting mRNA level of the gene of interest using qPCR.

2.2.4.7. Modulation of cytokines TNF-α or IL-1β induced hBD-2 after neutralisation the bioactivity of TNF-α or IL-10 by probiotic bacteria in Caco-2 cells

Probiotic bacterial modulation of the inflammatory signal induced cytokine and hBD-2 expression have been investigated in an earlier section (see 2.2.4.4). In order to find out whether the immune response was associated with membrane bound or endogenous cytokine, IL-10 or TNF-α to induce hBD-2 in Caco-2 cells and what the probiotic role in this situation is, neutralization of IL-10 or TNF-α cytokine bioactivity were performed. To achieve the task mentioned earlier, purified anti-human IL-10 antibody clone JES3-9D7 (Biolegend, San Diego, Ca) to neutralise 10 ng/ml of IL-10 (Parry et al., 1997), or chimeric anti-TNF-α monoclonal antibody clone cA2 (MACS Miltenyi Biotec Ltd, UK) which neutralises TNF-α in a number of *in vitro* bioassays at concentrations of 1 to 4 µg/ml (Foey et al., 1998) were used. According to the modified protocol of Tsutsumi et al .(2003), Caco-2

cells were differentiated for 21 days, and cells were pre-treated with probiotic for 18 hr followed by stimulation with inflammatory cytokines (10 ng/ml TNF-α, or 5 ng/ml IL-1β) in the presence or absence of 10 µg/ml of anti-IL-10 or anti-TNF-α antibody and incubated for a further 18 hr. The cell supernatants were collected to detect cytokines and hBD-2 protein and cytokine released by ELISA, and cell pellets to detect expression of genes of interest by qPCR.

2.2.4.8. Epithelial cell- macrophage co-culture system

A co-culture model was carried out to mimic the inflammatory gut pathology (homeostatic and inflammatory), when epithelial cells cross-talked with other immune cells such as macrophage subsets in the presence of probiotics (see Fig 2.7). According to the modified protocol by Watanabe et al. (2004), the co-culture system was performed using Caco-2 human epithelial cells and THP-1 monocyte derived macrophage cell subsets M1 or M2-like macrophages. In brief, three groups of Caco-2 cells were seeded on transwell cell culture insert (Becton Dickinson, NJ USA) plates (1.0 µm pore size) at a cell density of $5x10^5$ cells/500µl/well and cultured in D10 medium in a humidified 5% CO_2 incubator at 37ºC for 21 days allowing for full cell differentiation. Two groups of Caco-2 cells grown in transwell inserts were incubated with THP-1 derived M1- or M2-like macrophages for 18 hr in the co-culture system, and a third group of Caco-2 cells was left without co-culture as a control. Epithelial cells were apically applied with heat- killed (HK) probiotic at a concentration of $3x10^8$ CFU/ml, or 3 µg/ml secreted proteins (SP) for 18 hr. For mimicking chronic inflammatory gut pathology, the epithelial cells in co-culture system were basolateraly stimulated with 100 ng/ml K12-LPS, for 18 hr. The apical cell supernatant was collected and stored at -20^{o}C for ELISA assay, total mRNA extracted from Caco-2 cells for gene expression

analysis, Trans epithelial electrical resistance (TEER) assay to test barrier function, and immunohistochemistry (IHC) staining for ZO-1 protein expression were performed for each treatment group (see Fig.1.7 line 3).

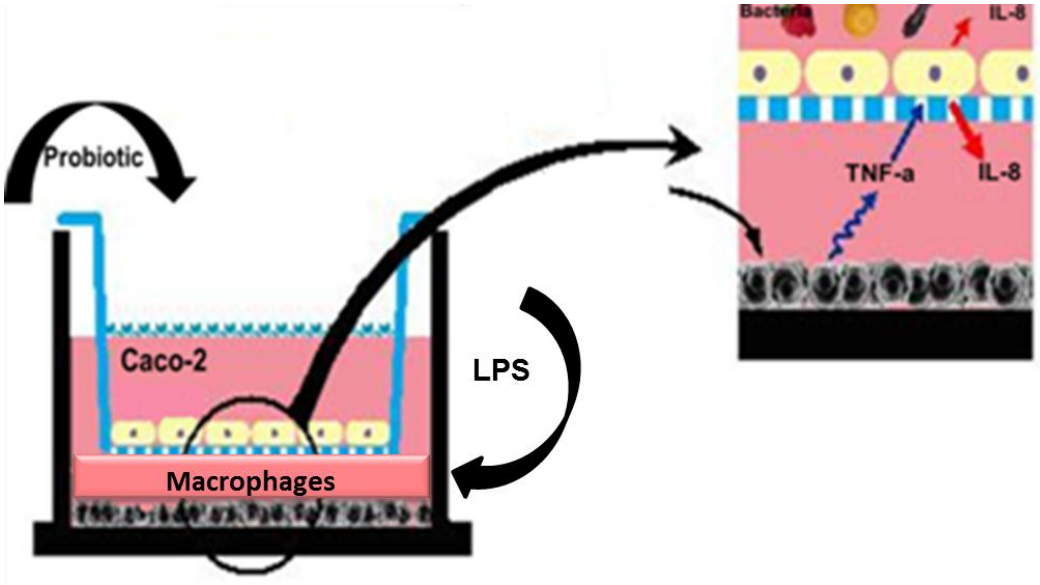


Figure 2. 7: Epithelial cell- macrophage co-culture system.

For mimicking chronic inflammatory gut pathology, Caco-2 cells were cultured in transwell insert, co-cultured with THP-1 derived M1-like macrophages (chronic inflammation model) or M2-like macrophages (normal homeostatic model), apically treated with probiotics, and stimulated with LPS basolateraly.

2.2.5. Cytokine measurement

Monocytes, macrophages and epithelial cell production of the inflammatory cytokines; TNF-α, IL-1β, IL-6, IL-8, and anti-inflammatory cytokine IL-10, in addition to, the antimicrobial peptide hBD-2 were quantified by using sandwich ELISA. The 96 immunoabsorbance well plates (Nunc, Fisher scientific, UK) were coated with commercially available capture antibodies from R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK, anti-TNF-α (0.5 µg/ml),

anti-IL-1β (1 µg/ml), anti-IL-8 (2 µg/ml), anti-IL-10 (0.5 µg/ml), anti-IL-6 (1 µg/ml) or anti-hBD-2 (1 µg/ml) (PeproTech EC, UK) and incubated overnight at 4^{o}C. The plates were washed twice with PBS/Tween-20 (PBS/0.05%v/vTween-20), before being blocked with 2%w/v BSA/ PBS for 4 hr at RT. The plates were then washed three times, incubated with serially diluted recombinant cytokine, and hBD-2 standards, test samples, and left overnight at 4^{o}C. Plates were washed three times followed by incubation with biotinylated anti-TNF-α (0.5 µg/ml), anti-IL-1β (100 ng/ml), anti-IL-8 (5 ng/ml), anti-IL-10 (1 µg/ml), anti-IL-6 (10 ng/ml), or anti-hBD-2 (0.5 µg/ml) for 4 hr at RT. Plates were washed with (x3 PBS/Tween-20) and incubated with 50 µl/well biotinylated-streptavidin horseradish peroxidase (HRP) at 1/250 dilution in 2%w/vBSA/PBS and incubated for 1 hr at RT. Finally, the plates were washed with (x3 PBS/Tween-20), followed by the addition of a colour reagent (Tetramethylbenzidine-TMB), for 30 minutes at RT. The reaction was stopped with 1.8 M sulphuric acid. Colorimetric development was measured spectrophotometrically by an OPTIMax tuneable microplate reader at 450 nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA). Protocols were followed according to manufacturer's instructions and compared to standard curves, between the ranges (7 to 5000 pg/ml), using the recognised international standards available from (NIBSC, Potter's Bar, UK) to determine the cytokine concentration. The highest level of cytokine detection was 5 ng/ml and lower level of detection was < 7 pg/ml.

Inter assay % coefficient of variance (CV) is determined by (standard error mean [(SEM) of three replicate experiments) divided by the mean] multiply by 100, whereas [(intra assay %CV is SEM of three replicate of the same experiment)]

multiply by 100. The inter assay %CV values were not included, since the high variability of cytokine amplitude between experiments depended on passage number, concentration of stimuli, and type of stimuli, therefore, all data are presented as a representative experiment of replicate experiments and the intra assay % CV was 2.1- 5.2.

2.2.6. NF-kB activity measurement

NF-kB activity was measured by colorimetric reporter gene assay for secreted embryonic alkaline phosphatise (SEAP) under the control of an NF-kB promoter sequence associated with the stably-transfected reporter gene cell lines, THP-1Blue ($CD14^{lo}$) and THP-1Blue-($CD14^{hi}$). Activation of NF-kB increases the secretion of SEAP, which is readily detectable using QUANTI-blue (Invivogen, Calne, UK). Quantiblue colorimetric reagents turned purple/blue in the presence of SEAP, hence, is directly proportional to NF-kB activity. Briefly, 50 µl of fresh cell supernatant was added to 150 µl of fresh quantiblue followed by incubation for 30 minutes at $37^{o}C$/ 5% CO_2. Colorimetric development was measured by an OPTIMax tuneable microplate reader at 620 nm and analysed by Softmax Pro version 2.4.1 software (Molecular Devices Corp., Sunnyvale, CA, USA).

2.2.7. Molecular biology methods

In order to find out the molecular basis of biological activity of the target gene of interest, several methods have been used; the polymerase chain reaction (PCR) is one of them. PCR is an *in vitro* technique established in 1983 in molecular biology laboratories and rapidly became one of the most reliable and sensitive tools used in different medical and biological studies. The main principle of the PCR is to generate an enormous number of copies from one single copy of the target gene

of interest. The amplification process of the PCR can selectively amplify a single copy of a desired sequence that exists in a complex mixture containing optimized concentrations of the DNA template, Taq polymerase enzyme, primers and deoxynucleotides (dNTPs). DNA polymerase will add complimentary to the DNA template generating a new DNA molecule. The PCR technique involved several steps including extraction of total RNA, CDNA synthesis followed by conventional PCR amplification using specific primers (see Fig.2.8). The resulting RT-PCR product was then subjected to agarose gel electrophoresis to check the molecular weight of the product, and qPCR was performed using normalisation with a housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which determined the threshold cycle (CT) value as a fold change.

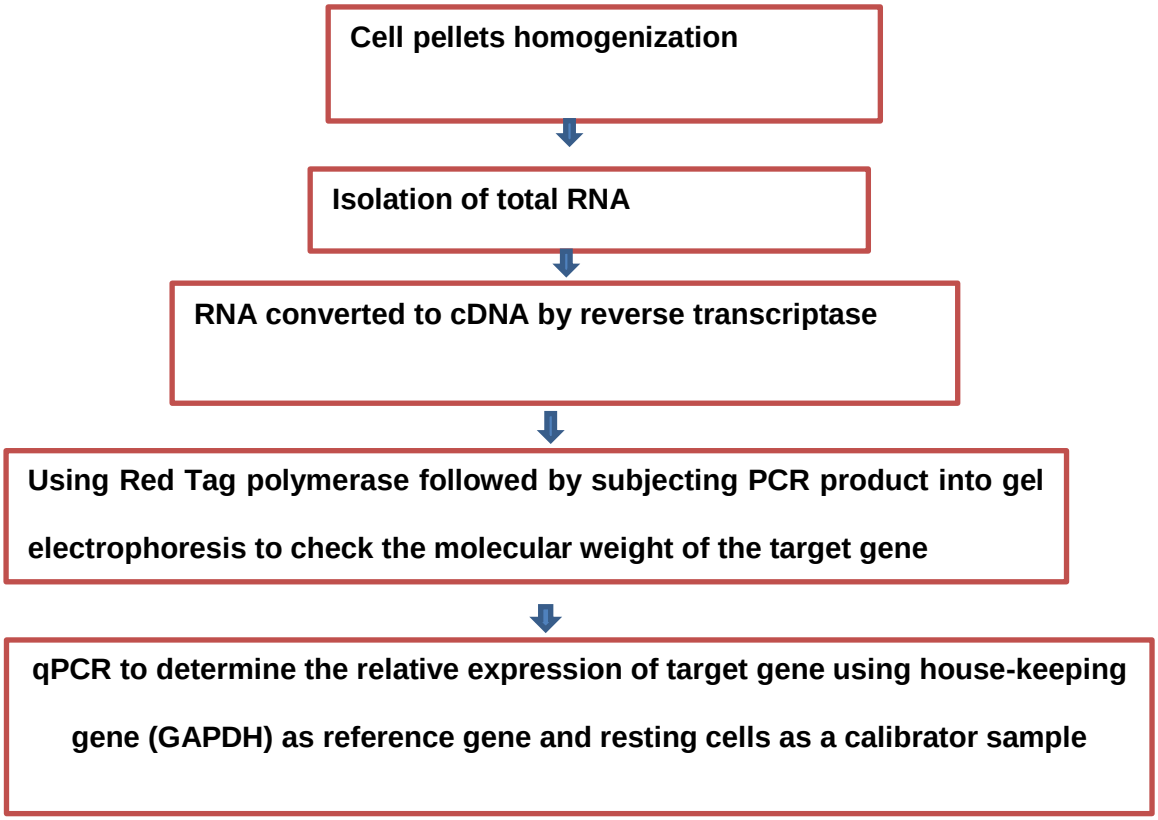


Figure 2. 8: Main steps of the polymerase chain reaction (RT-PCR) assay.

2.2.7.1. Total RNA extraction

Total RNA was extracted using a Sigma GenElute mammalian total RNA extraction kit (RTN70, Sigma, Poole, UK) following the manufacturer's procedure for each treatment of the cells. Caco-2 adherent cells were washed twice with sterile PBS free of Ca+2/Mg+2 to remove the excess of bacteria or secreted protein then detached by TE (see section 2.2.3.2). M2 cells were collected by centrifugation at 1200 rpm for 5 min; whereas adherent M1-macrophages were washed twice with PBS then harvested by sterile rubber cell scraper (Sterllin, UK). After washing, the cells were re-suspended in 500 µl of lysis buffer provided by the kit supplemented with 5 µl 0.02%v/v 2-mercaptoethanol for denaturation of RNases (reducing disulfide bonds and destroying the enzyme functionality of

RNases released during the lysis step of the RNA isolation). Ultrasonic cell disruption (Microson^{Tm,} PGC scientific, USA) for 10 sec at 5 watts (RMS) was used to help the cell lysing and liberate the cell contents. To remove potential DNA contamination, DNase digestion with RNase-Free DNase set was embedded in the protocol. It is performed by adding 10 µl of DNase and 70 µl of digestion buffer to exclude any contamination of DNA on the top of each filter of the column used for RNA isolation and the columns were incubated for 15 minutes at room temperature.

2.2.7.2. RNA quantification and evaluation

The RNA extracted from each sample was eluted in 50 µl of elution buffer provided with the RNA extraction kit. The RNA was quantified using spectrophotometer, NanoVue™ (GE Healthcare, Germany), measuring both concentration of RNA and purity (contamination of protein and DNA). A ratio of OD_{260}/OD_{280} has to be between 1.8 and 2.0 to exclude any contamination in the RNA sample extracted (Liu et al., 2003). Purified RNA was immediately stored at -20^{o}C until required for gene expression analysis. The integrity of RNA sample was checked by running 1% formaldehyde agarose gel according to the protocol of (Van et al., 2004). In brief, the formaldehyde agarose gel was prepared by mixing 87.5 ml sterile distilled water, 1.5 g agarose, 10 ml 10xMOPS in a flask and heated in a microwave for 2 min to dissolve all the agarose. Subsequently, 7 µl of SAYBER safe or red gel was added into small gel set followed by shaking to mix all the components of agarose liquid then cooled to 60°C prior to adding 26 ml formaldehyde with stirring in a fume hood. The mixture was poured out into the BIO-RAD gel set and left for casting. The gel was mounted in an electrophoresis tank and overlaid with 1x MOPS electrophoresis buffer. For loading buffer the mixture was prepared as

follows (2 µl 1x MOPS, 3.5 µl formaldehyde, 10 µl formamide, 0.4%w/v bromophenol blue). A volume of 15.5 µl of loading buffer and 4.5 µl of sample were mixed together and incubated for 10 min at 65^{o}C to control RNAase contamination and denaturation, quenched on ice for 10 min, followed by brief centrifugation and loaded into the gel well. The gels were run at 40-60 volts for 60 min, de-stained by using deionised water for 45 min, and viewed under UV light using the documentary gel viewer (Gel Doc ^{TM} .XR, BIO-RAD,CA).

2.2.7.3. Reverse Transcription (RT) of total RNA

Total RNA was reverse transcribed to complementary DNA (cDNA) using M-MLV Reverse Transcriptase reaction Kit (Sigma, UK). Following a modified manufacturer's protocol, 10 µl containing 1 µg/ml RNA of sample and 1 µl of 10 mM dNTP mixture (deoxyribonucleotide triphosphates), 1 µl of random nanomer and topped up with nuclease free water (Applied Biosystems, Lingley House, Warrington, UK) to make cDNA from a template of 1 µg/ml total RNA. The mixture was incubated at 70^{o}C for 10 min, after that the mixture was placed on ice for at least 2 min. A total of 10 µl containing 1 µl of M-MLV enzyme, 2 µl of buffer, 0.5 µl of RNAase inhibitor and 6.5 µl of nuclease free water were added to each sample. The cDNA synthesis was performed at 37^{o}C for 50 min and reaction terminated by heat inactivation for 10 min at 95^{o}C before chilling at 4^{o}C.

2.2.7.4. Real Time PCR (RT-PCR)

2.2.7.4.1. Design of primers

The full-length sequences of the genes of interest were identified using the National Centre of Biotechnology Institute (NCBI). Primer express software provided with Step one PCR machine (Applied Biosystems, Lingley House, Warrington, UK), was used to design the primers based on the sequence number

of nucleotides, and melting temperature. The following settings were used for primer design (Primer T_m: 60-65°C, Primer length: 20-28 bp, Amplicon size: 100-160 bp (see table 2.1). To ensure that the primers were designed to match the same gene map sequenced, the primers were blasted by using NCBI blast software at http://blast.ncbi.nlm.nih.gov/, and the primers characteristics were assessed using Sigma DNA calculator website http://www.sigma-genosys.com/calc/DNACalc.asp. The primers were synthesised by Eurofin MWG/ Operon (Germany). The lyophilized primers were dissolved in the appropriate volumes of nuclease free water to prepare 10 pmol of each primer depended on MWG instructions and then stored at -20°C.

Table 2. 1: Summary of primers used in this study and the product size.

Target name	Forward primer 5'-	Size base pair (bp)	Revers primer 3'-	Size bp	Product size (bp)
GAPDH	CTGCTCCTCCTGTTCGACAGT	21	CCGTTGACTCCGACCTTCAC	23	100
hBD-2	CACCTGTGGTCTCCCTGGAA	20	CTCTGATGAGGGAGCCCTTTC	17	100
IL-6	TGGCTGCAGGACATGACAAC	20	TGAGGTGCCCATGCTACATTT	20	100
TNF-α	ACATCCAACCTTCCCAAACG	20	GCCCCCAATTCTCTTTTTGAG	22	151
IL-8	TCAGAGACAGCAGAGCACACAA	22	GGCCAGCTTGGAAGTCATGT	20	100
IL-10	AGGAGGTGATGCCCCAAGCTGA	22	TCGATGACAGCGCCGTAGCCT	21	110
TLR2	GGCATGTGCTGTGCTCTGTT	20	GGAGCCAGGCCCACATC	17	100
TLR4	AGCCCTTCACCCCGATTC	18	TAGAAATTCAGCTCCATGCATTG	23	100
MD-2	TGCACATTTTCTACATTCCAAGGA	24	ATAACTTCTTTGCGCTTTGGAAGA	24	100
CD14	ACCCTAGCGCTCCGAGATG	19	AGCTTGGCTGGCAGTCCTTT	20	100
TLR9	GGACCTCTGGTACTGCTTCCA	21	AAGCTCGTTGTACACCCAGTCT	22	151
ZO-1	GCAATGGAGGAAACAGCTATATGG	24	TGAGGATTATCTCGTCCACCAGAT	24	104
NOD-2	CAGAATTTCAAACGGCCTCACTA	23	ATGAAATGGAACTGCCTCTTGTG	23	102
Tollip	TCTCATGCCGTTCTGGAAAAT	21	TCACATCACAAAATGCCATGAA	22	110

2.2.7.4.2. Conventional polymerase chain reaction (PCR)

In general, the PCR allows *in vitro* synthesis of large amounts of DNA by primer sequence-specific polymerisation of nucleotide triphosphates (dNTPs), catalysed by DNA polymerase. A gene's amplicons of interest were amplified using total cDNA prepared in (Section 2.2.7.3) and primers (Section 2.2.7.4.1). Following the modified protocol by (Sigma-Aldrich, Poole, UK), total volume of 25 µl (1 µl cDNA, 0.75 µl of 10 pmol forward primer, 0.75 µl of 10 pmol of reverse primer, 2.5 µl PCR reaction buffer, 1.25 µl Red Taq DNA polymerase, 1 µl random nanomer and 17.5 µl nuclease free water) were mixed in 200 µl nuclease free tubes for each treatment sample. Standard PCR reaction steps were performed using an applied biosystem thermo cycler under the following conditions; pre-heating for 95°C for 10 min (stage 1), followed by (stage 2) 40 cycles start at 95°C for 30 sec, 60°C for 1 min and 72°C at 1 min for individual cycle, and (stage 3) final elongation for 10 min at 72^{o}C and holding the samples at 4°C. The PCR product samples were run in 2%w/v agarose gels in TAE buffer (0.4 MTris, 0.04M Na acetate, 0.01M EDTA) (Invitrogen, UK) using SYBER safe or gel red along with a 100 bp ladder (Invitrogen, UK). DNA bands were visualised in a gel documentation system using UV light whereby images were captured.

2.2.7.4.3. DNA agarose gel electrophoresis

DNA agarose gel electrophoresis was used to determine the size molecular weight of genes interest. It is performed by dissolving 1.4 g agarose (Sigma-Aldrich, Poole, UK) in 70 ml TAE buffer using a microwave oven for 2-3 min until the agarose completely dissolved, then 7 µl of SYBER safe or gel red (Sigma-Aldrich, Poole, UK) was added to the molten agarose solution once the solution became cooler (40°C) and mixed well. The molten agarose was poured into a tray of

agarose kit (BIO-RAD, UK); followed by inserting comb in order to create wells. The gel was left for 30 min for casting, then placed into an electrophoresis tank (BIO-RAD, UK) and submerged in 350 ml of TAE buffer. After the comb was removed, the RT-PCR DNA samples were loaded into the wells of the gel. The gel was run at 100 V for 45 min, and DNA was visualised using UV light by gel documentation system (EC3, UVP ultra violet products, UK) whereby images were captured.

2.2.7.4.4. Quantitative Polymerase Chain Reaction (qPCR)

Using the housekeeping gene in the PCR assay relies on the assumption that their levels of expression remain constant in different cells, samples, and treatments. Therefore, they have been used to normalise the data. Following a modified manufacturer's protocol (Applied Bio systems, Warrington, Cheshire, UK), a total volume of 12 µl composed of 1 µl cDNA of each sample, 0.25 µl of 10 pmol of specific primers for each target (forward and reverse), 3.5 µl of Power SYBER Green® (Applied Biosystem, UK) and 7.0 µl of nuclease free water was plated out into 96-well plates (Applied Bio system, UK), and then sealed with adhesive film. The qPCR was performed using Step One Plus thermal cycler whereby the amplification of target was carried out under the following conditions; pre-heating at 95^{o}C for 10 minutes, followed by 40 cycles at 95^{o}C for 30 seconds, 60^{o}C for 1 minute and 72^{o}C for 1 minute and hold the samples at 4°C (see Fig.2.9). The qPCR data were analysed following $2^{-\Delta\Delta Ct}$ method as described by Livak et al.(2001) using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an endogenous control and resting cells as a reference sample. Thus, the relative quantity of the target transcript is described as fold change relative to the reference sample (resting cells) and GAPDH using the following equation:

ΔΔC t = ΔCt interest gene (resting cells) –ΔCt endogenous control

Relative quantitation (RQ)= $\mathbf{2^{-\{\Delta\Delta CT\ sample\ -\ \Delta\Delta Ct\ control\ (\ resting\ cells)\}}}$

Where CT is threshold cycle

In this study the expression of hBD-2, TNF-α, Il-6, IL-8, IL-10, TLR2, TLR4, CD14, MD-2, TLR9, NOD-2, ZO-1, and Tollip gene expression were quantified using qPCR.

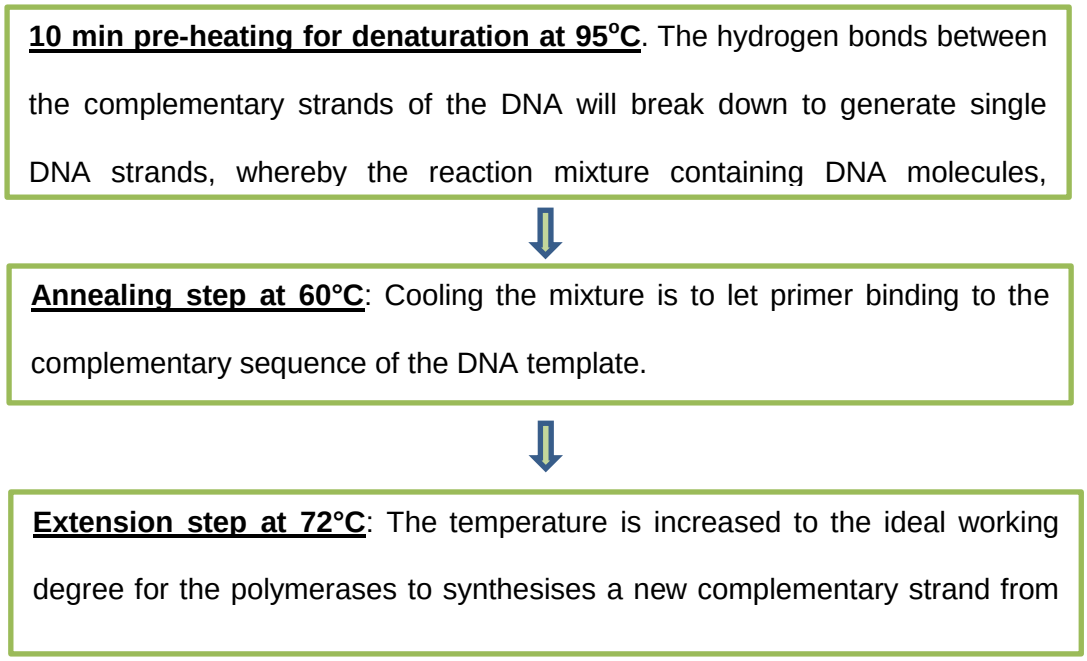


Figure 2. 9: The main steps of the quantitative polymerase chain reaction (qPCR) protocol.

2.2.8. Trans epithelial electrical resistance (TEER) assay

Transepithelial electrical resistance (TEER) measurement has been routinely used to study the paracellular transport properties (permeability) of epithelial cells grown on permeable filters and barrier integrity. TEER measurement was performed

according to the modified protocol by Teoh et al. (2000). In brief, Caco-2 cells were cultured in transwell inserts for 21 days, incubated with macrophage cells either with M1 or with M2-like macrophage cells. After the treatment, the cells were washed twice with DPBS and 0.5 ml of the DPBS was added to the inner and 1 ml to the outer of the transwell. The electrical resistance was calculated using EVOM Epithelial Voltammeter (Pharma, UK), where each well reading multiplied by the surface area of the transwell (0.33 cm^2) to calculate the final value in Ωcm^2.

2.2.9. Detection of ZO-1 protein expression by immunohistochemistry (IHC) staining

It is well documented that cell permeability increases in IBD due to the disruption of the tight junction, leading to a down-regulation barrier of epithelial cell. Zona Occulin-1 (ZO-1) is one of the main proteins found in the intracellular junction associated with cell barrier; therefore, IHC staining was performed to determine the modulatory effect of probiotic treatments on the disruption of ZO-1 induced by LPS at co-culture system using a fluorescent label anti-ZO-1 antibody. According to the modified protocols of (Montalto et al., 2004), Caco-2 cells were cultured in transwell insert plated at a cell density of $5X10^5$ cells/ml. M1-like or M2-like macrophages were generated from THP-1 human monocytic cell line. Caco-2 cells were incubated either with M1 or with M2-like macrophages, apically treated with probiotics and basolateraly with 100 ng/ ml K12-LPS. After that, the transwell supernatants were collected, and epithelial monolayer in transwell inserts was washed apically by PBS, and then cells were fixed with 3%v/v paraformaldehyde in CS buffer (0.1M NaOH, 0.1M HEPES, 1mM EGTA, pH 6.8) for 20 minutes, followed by washing (x3 CS each wash for 5 min) and permeabilised with 0.1%v/v TritonX-100 (Ouwendijk et al., 1998). Cells were then blocked with 1%w/v

BSA/PBS for 1 hr followed by incubation with 2.5 µg/ml Rabbit polyclonal anti ZO-1 in 1%BSA/PBS overnight at 4^{o}C. Consequently, cells were washed three times by CS buffer followed by incubation with the 0.5 µg/ml secondary antibody Alexa flour 488-conjugated anti Rabbit IgG in 1%BSA/PBS for 1hr. Afterward all monolayers were washed three times by CS buffer then mounted with DPX (Sigma-Aldrich, Poole, UK) on slides and visualised by a Nikon Eclipse 80i epifluorescence microscope equipped with Qi1Mc camera using NIS-Elements Software (Nikon DS- (BR 3.0, Nikon, USA).

2.2.10. Detection of Toll like Receptors (TLRs) protein expression by flow cytometry analysis

Volume of 35 µl aliquots containing 1x10^{5} cells was washed twice with sterile PBS and incubated for 30 min on ice with 1%w/v BSA/(Ca^{+2}/Mg^{+2} free buffer PBS) for blocking non-specific binding of the antibodies. Cells were washed by FACS buffer (2%v/v FCS in a Ca^{+2}/Mg^{+2} free buffer PBS) and incubated with the appropriate flurochrome-conjugated antibodies (PE) specified for (anti-TLR4 Clone HTA125, anti-TLR2 Clone TL2.1, and isotype controls) (eBioscences, UK) for 30 min at 4^{o}C in the dark. The antibodies for targets and isotype controls were used at a 1:200 dilution. The cells were washed twice with FACS buffer to remove the unbound antibody and re-suspended in 500 µl of FACS buffer, filtered and analysed on FACS calibre flow cytometry (Becton Dickinson, San Jose, CA) using BD FACS Diva Software v.6.0. Forward scatter (FSC) and side scatter (SSC) were adjusted to allow gating on live cells. Unstained samples were used as a control for auto-fluorescence cell staining. A total of 10,000 events were routinely collected and the expression of the target was determined by a Mean fluorescence Intensity (MFI) by subtracting from the isotype control (see Fig 2.10).

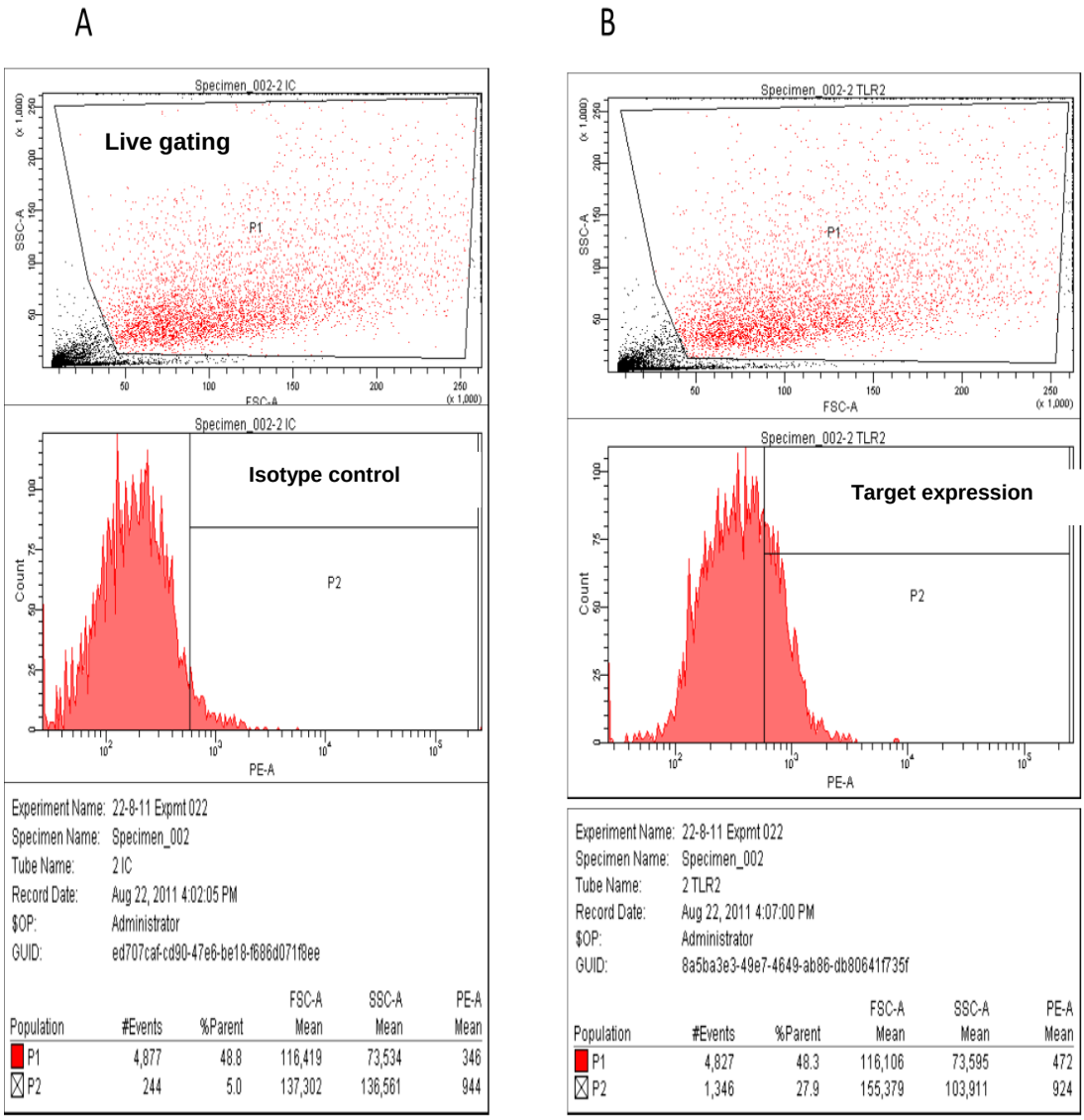


Figure 2. 10: Mean fluorescence Intensity (MFI) of TLR expression.

Cells were prepared and stained with antibody against interest target such as TLR2 .**A**) Cells stained by Isotype antibody, **B**) cells stained with anti-TLR2 labelled antibody. FSC and SSC were gating on live cells (P1), and then adjusted at confidence intervals 5% of live gating cells (P2). Mean Fluorescence Intensity (MFI) was determined by subtracting isotype from target (TLR2).

2.2.11. Statistical analysis

All values are presented as means and standard error. Data were analysed using Minitab version 16. Significant differences among treatments were evaluated by balanced analysis of variance, one or two-way analysis of variance (ANOVA) when applicable. The criterion for statistical significance was defined as $P< 0.05$, 0.01, and 0.005.

Chapter 3

Modulation of inflammatory responses in a THP-1 cell model of M1 and M2 macrophages by probiotic bacteria

Chapter 3: Modulation of inflammatory responses in a THP-1 cell model of M1 and M2 macrophages by probiotic bacteria.

3.1. Introduction

Mucosal tolerance is fundamental to efficient GIT function, i.e. tolerating food and commensal bacteria and at the same time maintaining immune responsiveness to pathogens (Foey, 2011). Gut microbiota are vital in maintaining immune homeostasis through their interactions with gut mucosal epithelial cells and also with underlying immune cells in the lamina propria such as macrophages (Bibiloni & Schiffrin, 2010). Mucosal macrophages play a pivotal role in tolerance; whereas in IBD dysfunctional macrophages initiate the breakdown of tolerance, whereby commensals perpetuate inflammation. Gut macrophages regulate immune homeostasis, mounting tolerogenic responses to food and commensal bacteria or immune inflammatory responses to pathogens. It is well documented that macrophages are highly plastic cells (Smith et al., 2011). Local environment and macrophage subset determine tolerance, associated with an M2-like phenotype, or inflammatory activation, associated with an M1-like phenotype. The macrophage subset that predominates in a healthy gut mucosa is the M2-like macrophage phenotype. Disruption of tissue homeostasis by pathogenic microbial infection leads to activation of macrophage cells, activation is associated with alterations in macrophage cell phenotype from M2s to M1s depending on the signals received from the tissue environment.

The immune system recognises pathogen associated molecular patterns (PAMPs) expressed by the gut microbiota through a series of pattern recognition receptors (PRRs) such as TLRs and NLRs expressed by specific cells such as

macrophages. Normally, gut mucosal macrophages exhibit hypo-responsiveness in a healthy homeostatic functioning gut through low or absent expression of PRRs, whereas an inflammatory immune response is elicited upon pathogenic challenge through augmentation of the expression of PRRs (Smythies et al., 2005). Therefore, resident macrophages (M2s) in a healthy gut mucosa are described by a reduction in responsiveness to microbial PAMPs (i.e. M2 macrophages fail to express CD14 and selected TLRs). Whereas, in an inflammatory situation, M1 macrophages are express high levels of CD14 and selected TLRs (Platt & Mowat, 2008; Smythies *et al*., 2005). Thus, gut mucosal macrophages have been characterised as $CD14^{lo/absent}$, $TLR^{lo/absent}$ M2-like phenotype in a healthy gut mucosa or $CD14^{hi}$, TLR^{hi} M1 phenotype in the inflammatory gut mucosa (Smith et al., 2001, Smythies et al., 2005, Zareie et al., 2001). As a result, CD14 has been suggested as an indicator molecule of tolerogenic or inflammatory macrophage cell phenotype. Probiotic modulation of these functionally distinct macrophage subsets will be determined by CD14 expression. Macrophage subset predominance will determine immunomodulatory effects of probiotic species.

It is generally recognised that signal transducer and activator of transcription 3 (STAT-3) is required to mediate the anti-inflammatory activity of IL-10 (Williams et al., 2004). STAT family members are phosphorylated by the receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This transcription factor is latent in the cytoplasm until it is activated by extracellular signalling proteins (mainly cytokines and growth factors) that bind to specific cell-surface receptors. Biethahn et al. (1999) reported that STAT-3 has two isoforms, STAT-3a (86 kDa) and STAT-3b

(79 kDa). STAT-3 is activated by phosphorylation at Tyrosine705, which induces dimerization, and is further phosphorylation at Serine 727, followed by nuclear translocation and DNA binding (Darnell et al., 1994). O'Rourke and Shepherd (2002) demonstrated that transcriptional activation of STAT-3 was regulated by phosphorylation at Tyrosine 705 and Serine 727 in macrophages via the MAPKs. Indeed STAT-3 is involved in M2 (anti-inflammatory) macrophage cell differentiation (Sica and Bronte, 2007). Usually, the duration and degree of gene activation are strictly regulated by a series of negative acting proteins. There are several types of negative regulators of STAT proteins in the cell cytoplasm such as suppressors of cytokine signalling (SOCS proteins) which block further STAT activation in the cell cytoplasm (Bromberg, 2002). Liu et al. (2008) demonstrated that SOCS-3 is essential for the classical M1 macrophage function, which involves the suppression of the anti-inflammatory action of IL-10/IL-6, and knockout of SOCS-3 results in an alternative M2-like phenotype with increased expression of SOCS-1. Cheng et al., (2003) demonstrated that manipulation of STAT-3 signalling in either direction (blockade or stimulation) influenced immune responses, explaining that STAT-3 has a role in determining immune activation versus immune tolerance.

The signal pathway transcription factor, NF-kB plays a dominant role in inflammatory responses; it has been well established that it regulates macrophage inflammatory cytokine production such as TNF-α, IL-6, IL-8, and IL-1β (Bondeson et al., 1999, Yamamoto and Gaynor, 2001). NF-kB activation is controlled by IκB kinase (IKKβ) Integral to the polarised macrophage phenotypes is the expression and activity of NF-kB. Inhibition of NF-kB activity by overexpression of IKBα was demonstrated to change macrophage phenotype to an anti-inflammatory M2-like

effector function (Wilson et al., 2005). Fong et al. (2008) demonstrated that IKKβ inhibits the classically activated M1-like macrophages, and deletion of IKKβ was associated with increased expression of IL-12, inducible nitric oxide synthase (NOS2), and MHC class II by macrophages, which reverse macrophages to M1 phenotype through STAT-1. This manipulation of macrophage functionality via controlling its plasticity would be of great benefit for controls of both homeostatic and pathological macrophages.

Human THP-1 cells were used as a source of macrophages in the current study. Schwende et al. (1996), Auwerx (1991) reported that after treatment of THP-1 cells with PMA, the cells differentiated into macrophage-like cells, which mimic native monocyte-derived macrophages in several respects. This provides a valuable model for studying the mechanisms involved in macrophage differentiation and regulation. Several studies have been done to characterize markers for macrophage subsets differentiated by PMA or Vit.D_3 see table 3.1.

Table 3.1. 1: Markers involved in THP-1 derived macrophage subsets.

Marker	M1-like macrophages (+PMA)	M2-like macrophages (+Vit.D3)	Reference
LPS-stimulated TNF-α (protein)	↑	↓	(Schwende et al., 1996)
LPS stimulates IL-10 (MRNA)	↓	↑	(Chanput et al., 2010)
LPS stimulated IL-β and IL-8 (mRNA)	↑	↓	(Chanput et al., 2010)
iNOs (mRNA)	↑	ND	(Chanput et al., 2010)
Arginase (mRNA)	ND	↑	(Chanput et al., 2010)
Morphological changes compared with THP-1 cells	Associated with pseudopodia	Associated with fine ruffled	(Daigneault et al., 2010)
Phagocytic capacity	High phagocytic capacity related to increased numbers of mitochondria and lysosomes in compared with THP-1 cells	↑ however in M2 more than in M1	(Daigneault et al., 2010)
Resistance to apoptosis	↓	↑	(Daigneault et al., 2010)

TLR2, CD206 (mRNA) expression			
Yim-1, Fizz-1, MRC-1, Dectin-1 (mRNA)	↓	↑	(Chanput et al., 2013)
IL-12p40 (mRNA)	↑	↓	(Chanput et al., 2013)
CD36, PPAR-γ, CD204, PKC delta isoform	↑ in compared with THP-1 cells	ND	(Barilli et al., 2011)

Note: "↑", "↓", "↔"and ND means up-regulation, down-regulation, no-modulation and not determined of the indicated target, respectively.

Based on the variance in marker expression, it has been concluded that THP-1 cells treated with PMA or Vit.$D_{3,}$ results in different macrophage subsets (M1-like and M2-like, respectively). This new macrophage polarizing model was used to compare the immunomodulatory action of probiotics associated with gut homeostasis (M2) or inflammatory status (M1) for the first time. The present study aimed to determine the effect of probiotic treatments on modulation of the immune responses induced by LPS in monocytes and macrophage subsets. It is well reported the endotoxin LPS is released after bacterial digestion by macrophages, followed by lipid A release via macrophage exocytosis. Lipid A then circulates into the blood, binds to the immune cell surface via interaction with TLR4/MD-2/CD14, triggering the immune responses associated with fever, diarrhoea, and septic shock. Indeed, LPS stimulation of macrophages results in activation of the NF-kB transcription factor, which orchestrates a gene expression schedule leading to the activation of inflammation, cell proliferation (Th1), differentiation (Th17), which are mediated through the release of chemokines and cytokines. Furthermore, an increase in the population of activated macrophages expressing LPS recognition

PRRs (TLR2, TLR4 and CD14) that infiltrates the intestine in IBD has been reported by several researchers such as Candia et al. (2012), and Mahida et al. (1989). Therefore, a range of probiotic bacteria were used to investigate the potential efficacy of probiotic treatments in modulation of LPS induced cytokine expression by monocytes and macrophage subsets.

It is well documented that microbes that colonize the GIT produce diverse extracellular proteins, such as p40/p75 from *Lactobacillus rhamnosus* GG (Yan et al., 2007), which modulate specific host immune and physiological responses (Clarke and Sperandio, 2005) in epithelial cells. Unfortunately, the majority of these proteins have not been characterized immunologically. Thus far, no data exist in this area of research, therefore this study focused on the effects of both probiotic associated cell wall bacterial cells as heat killed (HK) and their secreted proteins (SP) on modulation of LPS induced cytokine production by monocytes and macrophage subsets.

In this chapter, the investigations focused on determining the potency of probiotic modulation of LPS induced cytokine production. This was assessed in monocytes and macrophage subsets derived from THP-1 human monocytic cell line resembling either mucosal resident homeostatic macrophages (M2s), or infiltrating inflammatory macrophages (M1s). In addition, it investigated the roles of probiotics in modulation of macrophage subset NF-kB activation and their cytokine production induced by LPS using transfectant human monocytic THP-1 NF-κB reporter cell lines, THP-1Blue ($CD14^{lo}$) and THP-1CD14Blue- ($CD14^{hi}$) acting as internal controls for observations of CD14 expression responses of the inflammatory pathological and homeostatic macrophages.

The specific hypotheses tested in this investigation are:

Hypothesis 1: Secreted proteins (SP) and heat-killed (HK) probiotic strains are able to induce cytokine production by monocytes and macrophage subsets (refer to section 3.2.1)

Hypothesis 2: SP and HK probiotic strains modulate LPS induced cytokine production in monocytes and macrophage subsets (refer to section 3.2.2).

Hypothesis 3: SP and HK probiotic strains modulate LPS induced TLR expression in macrophage subsets (refer to section 3.2.3).

Hypothesis 4: SP and HK probiotic strains differentially modulate LPS induced cytokine production by macrophage subsets dependant on CD14 expression status (either high or low; refers to 3.2.4)

Hypothesis 5: SP and HK probiotic strains differentially modulate LPS induction of NF-kB activity, dependant on macrophage subset and CD14 status (see 3.2.5).

3.2. Results

3.2.1. LPS induced different markers in M1-like and M2-like macrophages derived from THP-1 cells.

THP-1 human monocytic cells were used as a source to generate macrophage cell subsets. In addition to available data from several studies (see table 3.1), validation of this model was achieved by several techniques (refer to sections 2.2.4.2, 2.2.4.3 and 2.2.10). Firstly, cytokine production in response to stimulation with three concentrations of k12-LPS (0.1, 1 and 10 µg/ml) was used to determine the differences between these subsets. Results showed that, at the sub-optimal LPS concentration 1 µg/ml, M1s produced IL-1β higher than M2s, increasing by

17.2 fold, respectively. TNF-α was 12.10 fold increased in M1s, and IL-8 increased by 1.2 fold. Whereas M2s produced IL-6, more than M1s by 24 fold (Fig.3.2.1). Control (un-stimulated cells) routinely failed to secrete cytokines above the lower level detection of the ELISA (7 pg/ml).

Based on the hypothesis that the M2 macrophage subset (anti-inflammatory) is resident in a healthy gut mucosa, and is able to release anti-inflammatory cytokines (such as IL-10), cell supernatant concentration of IL-10 was measured. This showed that the THP-1 derived M2-like macrophage model failed to release detectable levels of IL-10. This suggested that M2 cells might be expressing IL-10 as membrane bound protein, therefore, experiments were undertaken to determine whether M2 produced membrane bound protein (see methodology section 2.2.4.2). As shown in Fig. 3.2.2, addition of neutralising anti-IL-10 antibody up-regulated TNF-α by 94% in resting M2-like macrophages, whereas it failed to cause up-regulation in M1-like macrophages. This method indirectly measures IL-10 activity via its suppression of TNF-α production. This result suggested that M2-like macrophages constitutively express IL-10 as a membrane bound protein. Adding anti-IL-10 antibody also up-regulated TNF-α by 30% in LPS stimulated M2-like macrophages only, suggesting that M2-like macrophages also express inducible IL-10 membrane bound protein.

It is well documented that pSTAT-3 mediates the anti-inflammatory activity of IL-10 (Williams et al., 2004), therefore, the next experiment was undertaken to determine if macrophage subsets express pSTAT-3. Figure 3.2.3 shows that both macrophage subsets express total STAT-3, however, the significant evidence was that only M2-like macrophages express pSTAT-3 in response to LPS stimulation (10 µg/ml). In M2-like macrophages STAT-3 is activated by phosphorylation at

Tyrosine705 in response to LPS stimulation, this induces dimerization followed by nuclear translocation and DNA binding. Indeed, results showed that the pSTAT-3 expression level was dependent on the macrophage cell phenotype and concentration of ligand (LPS), refer to Fig.3.2.3.

Secondly, TLR expression was used to determine the differences between macrophage subsets in response to LPS stimulation. Results showed that M1 TLR2 surface expression was down-regulated by LPS whereas, TLR4 highly up-regulated; however, LPS stimulation up-regulated M2s TLR2 and TLR4 (Fig.3.2.4). The noteworthy observation was that the predominant TLR expression in response to LPS stimulation by M1-like macrophages was TLR4, whereas it was TLR2 in M2-like macrophages. Therefore, data from these two methods of validation showed that the macrophage cells generated from monocytes were differentiated into distinct cell phenotypes (M1s and M2s).

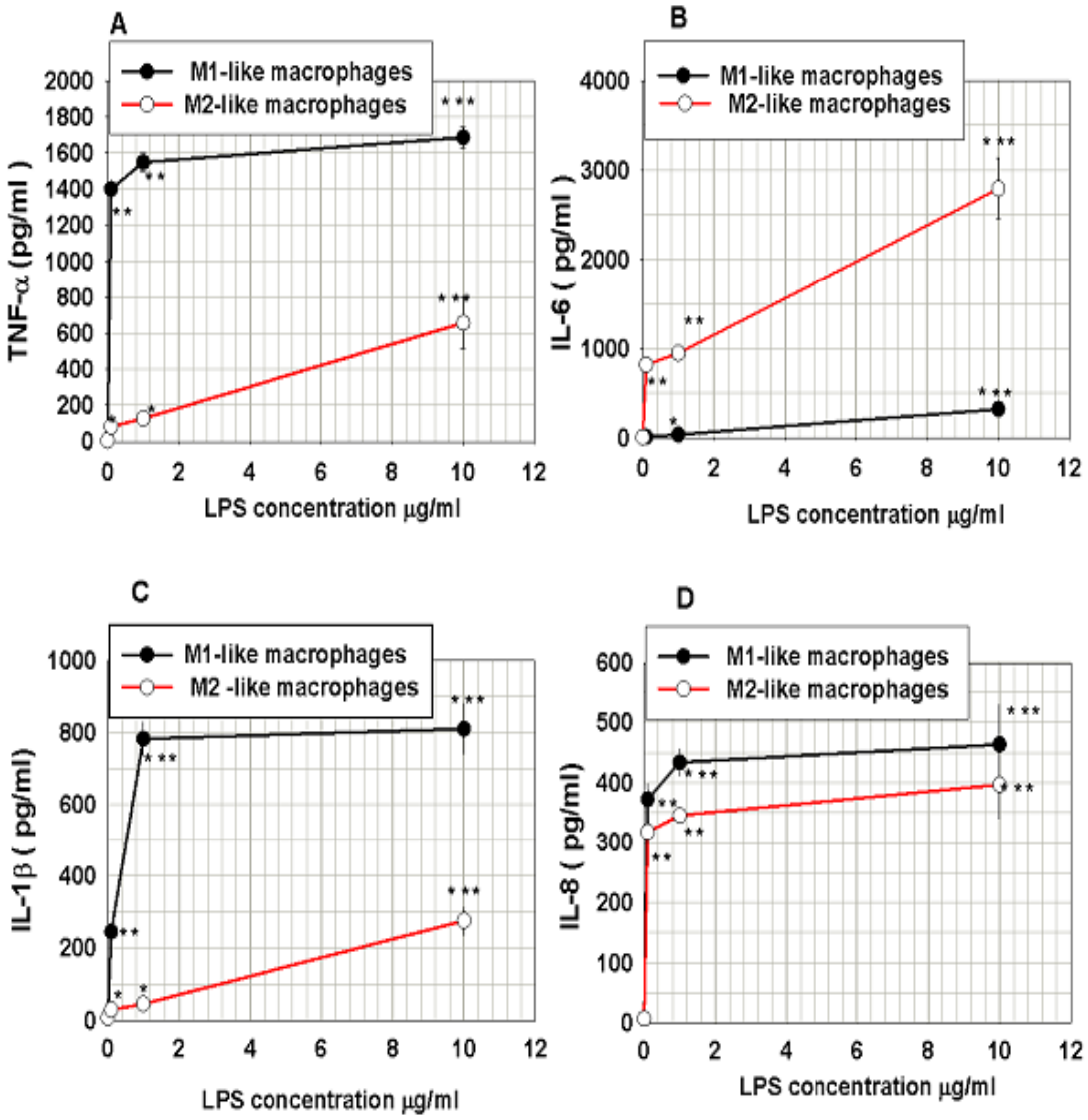


Figure 3.2. 1: LPS induction of cytokine production by macrophage cell subsets.

A) TNF-α, **B**) IL-1β, **C**) IL-6), **D**) IL-8. M1 and M2 like macrophage cell subsets were generated by differentiating THP-1 monocytes with either 25 ng/ml Phorbol-12-myristate-13-acetate (PMA) for 3 days or 10 nM 1, 25-$(OH)_2$ vitamin D_3 for 7 days, followed by stimulation of cells with 0.1, 1 or 10 µg/ml of *E.coli* K12 LPS. Cytokine production is expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples (n=5). Significant effects compared to the control (un-stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

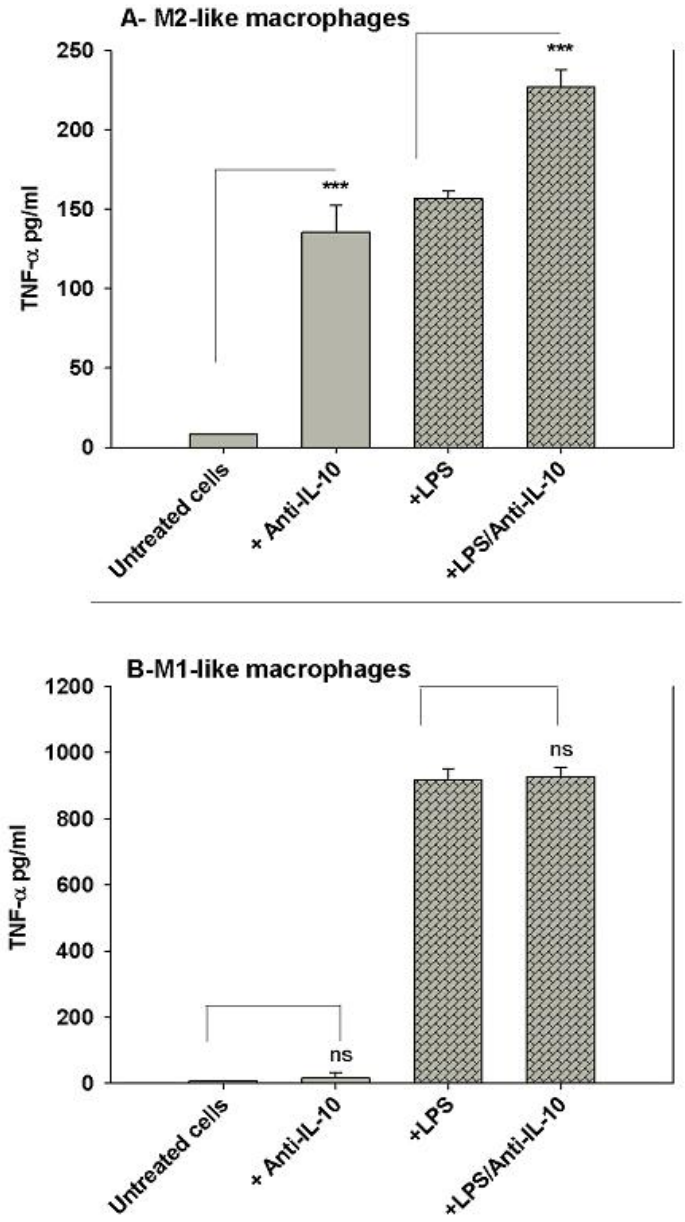


Figure 3.2. 2: M2-like macrophages express IL-10 membrane bound protein.

Full macrophage subset differentiation **A)** M2-like macrophages and **B**) M1-like macrophages at a cell density of $1x10^5$ cells/ml cultured in 96 well plates were stimulated by 100 ng/ml K12 LPS in the presence or absence of 10 µg/ml of purified anti-human IL-10 antibody clone JES3-9D7. Cell supernatant was collected to determine the level of TNF-α by ELISA which indirectly measure the IL-10 bioactiviy. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as * P<0.05, ** P<0.01 *** P<0.005 and NS (non-significant).

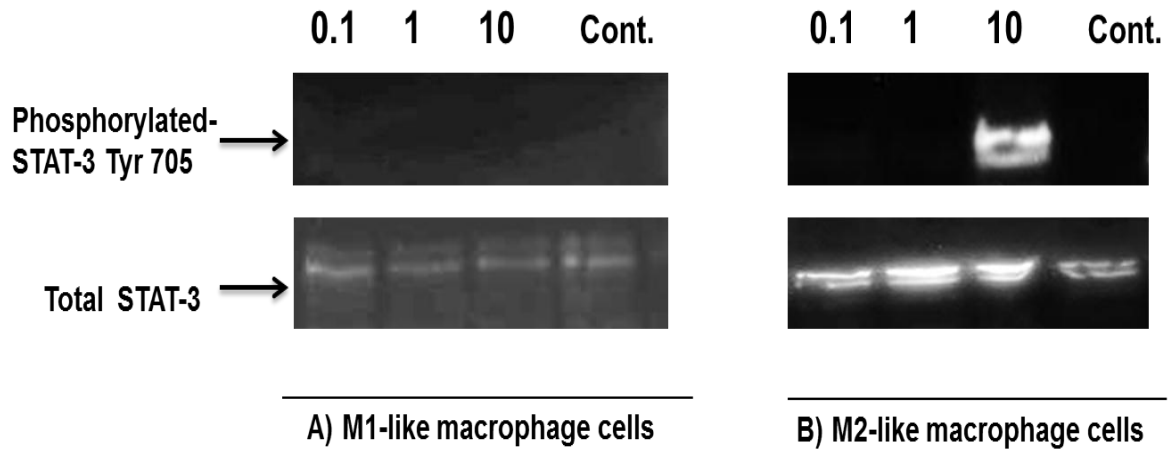


Figure 3. 2. 3: Only M2-like macrophages express phosphorylated STAT-3 in response to LPS stimulation.

Representative western blot for phosphorylated STAT-3 Tyr (705) and total STAT-3 from full cell differentiated macrophage subsets following culture in the presence of 0.1, 1 or 10 µg/ml k12-LPS for 18 hr. Data is representative of three independent experiments.

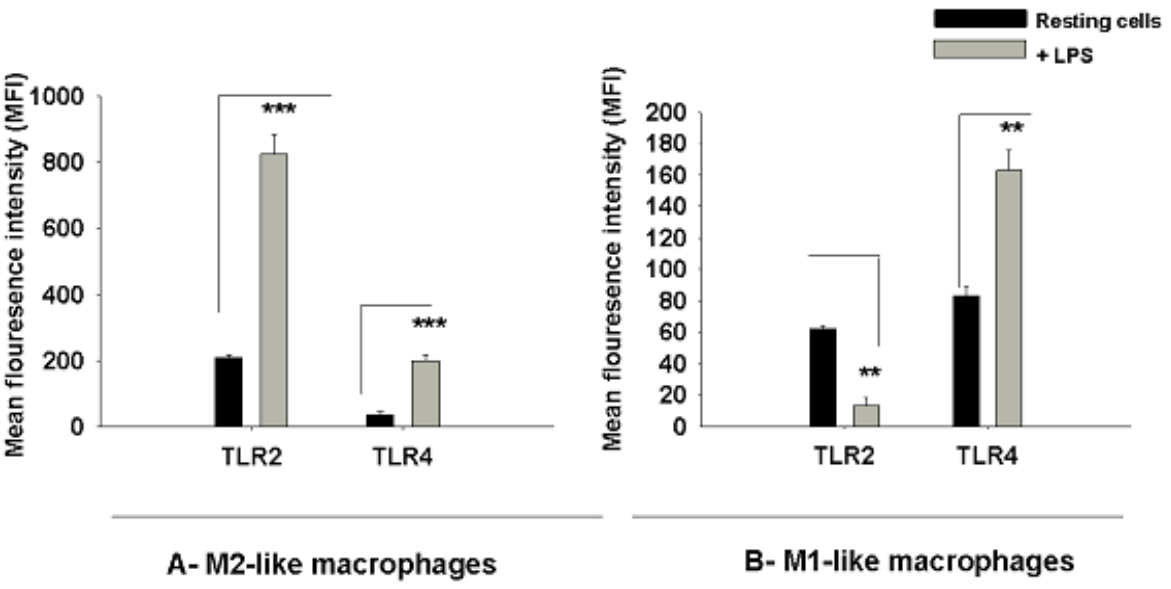


Figure 3.2. 4: TLR2 and TLR4 expression by macrophage subsets.

M1- or **M2**-like macrophage cells stimulated with 100 ng/ml *E.coli* K12 LPS. Surface expression of TLR2 or TLR4 was assessed by flow cytometry. Data displayed is a representative experiment with triplicate samples from n=3 replicate experiments. TLR

expression is expressed as the mean±SE in MFI. Significant effects compared to the control (un-stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

3.2.2. SP and HK probiotic strains induced cytokine production by monocytes and macrophage cell subsets

Probiotic bacteria have been shown to modulate macrophage effector functions by a mechanism, which is not well understood. In order to test probiotic bacterial roles in inducing cytokines in THP-1 monocytes and macrophage cell subsets (derived from THP-1 cells), *Lactobacillus casei strain* Shirota (LcS) probiotic bacteria were used either live (L) or heat killed (HK) at different concentrations ($3x10^6$ to $3x10^9$CFU/ml). As shown in Fig.3.2.5, LcS probiotic bacterial stimulation induced TNF-α cytokine release by monocytes and macrophage cell subsets dependent on bacterial cell dose, and type of immune cells. Results showed that at $3x10^6$ CFU/ml of live LcS, monocytes released the highest level of TNF-α (1563 pg/ml), whereas TNF-α peaked at $3x10^8$ CFU/ml (4921 pg/ml) for M1-like macrophages and at $3x10^9$ CFU/ml (201 pg/ml) for M2-like macrophages. TNF-α induced by HK probiotic bacteria steadily increased with bacterial cell dose in monocytes (7 to 611pg/ml) and in M1-like macrophages (7 to 4645 pg/ml) and peaked at $3x10^8$ CFU/ml in M2-like macrophages (678 pg/ml). These results also showed that pro-inflammatory macrophages (M1s) released significantly more TNF-α than anti-inflammatory M2s.

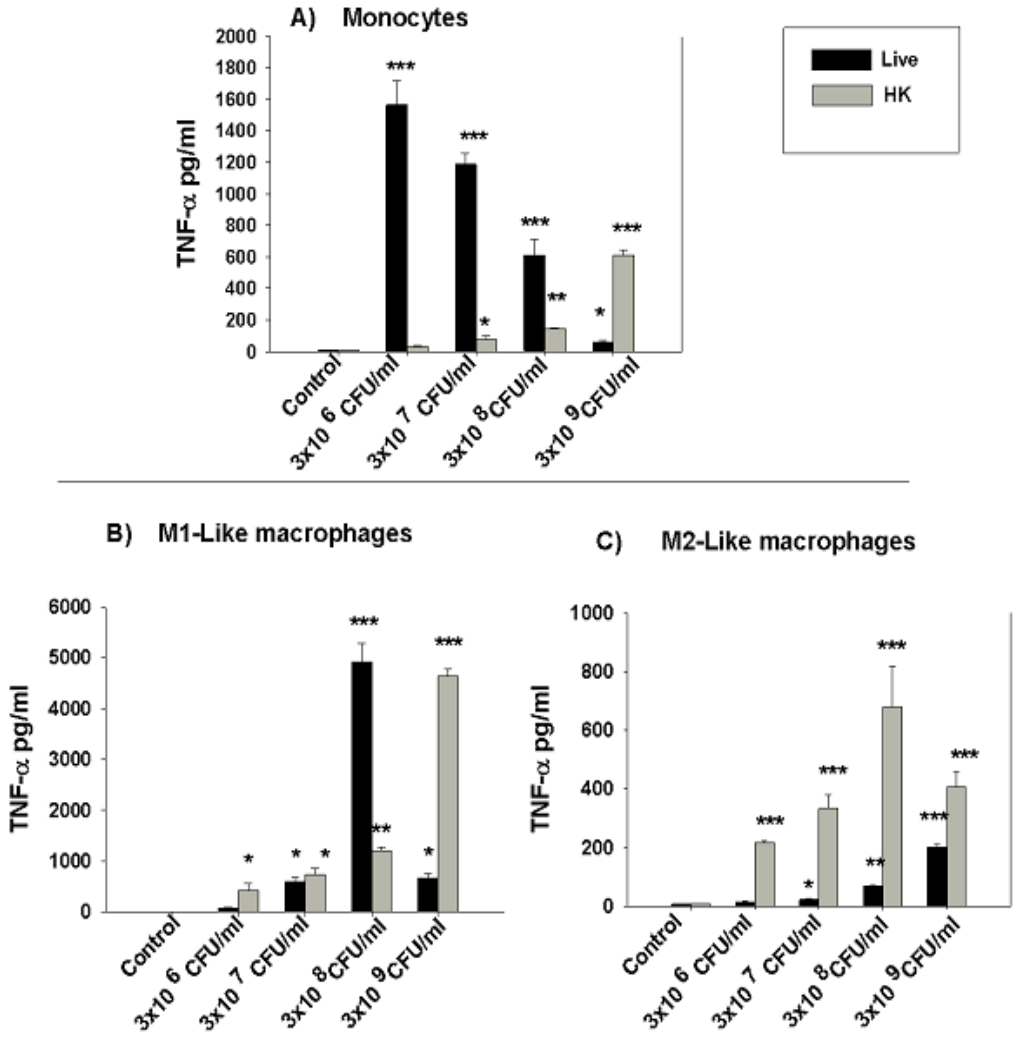


Figure 3.2. 5: *Lactobacillus casei* strain Shirota (*LcS*) induction of TNF-α cytokine production by monocytes and macrophage cell subsets.

A) Monocytes, **B**) M1-like macrophage cells, **C**) M2-Like macrophage cells. Monocytes, M1s,or M2s were co-cultured with *Lactobacillus casei* strain Shirota (LcS) as Live (L) or heat killed (HK) at cell concentration of $3x10^6$, $3X10^7$, $3X10^8$, and $3x10^9$ CFU/ml for 18 hr followed by detection of cytokine production by ELISA. TNF-α cytokine production is expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=5 replicate experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

In an attempt to find out the immunomodulatory effects of secreted proteins (SP) by probiotic lactic acid bacteria on the cytokine production by immune cells, bacterial growth cell medium from the stationary phase (see Fig.2.1) was used to

obtain proteins released by probiotics through their metabolism. Proteins were precipitated using TCA (see section 2.2.2.1). The precipitated proteins of each bacterial sample were resolved by SDS-PAGE, resulting in different profile of protein bands dependent on the strain of probiotic bacteria. Results showed that each bacterial sample displayed different protein bands with different molecular weight (KD) (see Fig. 3.2.6).

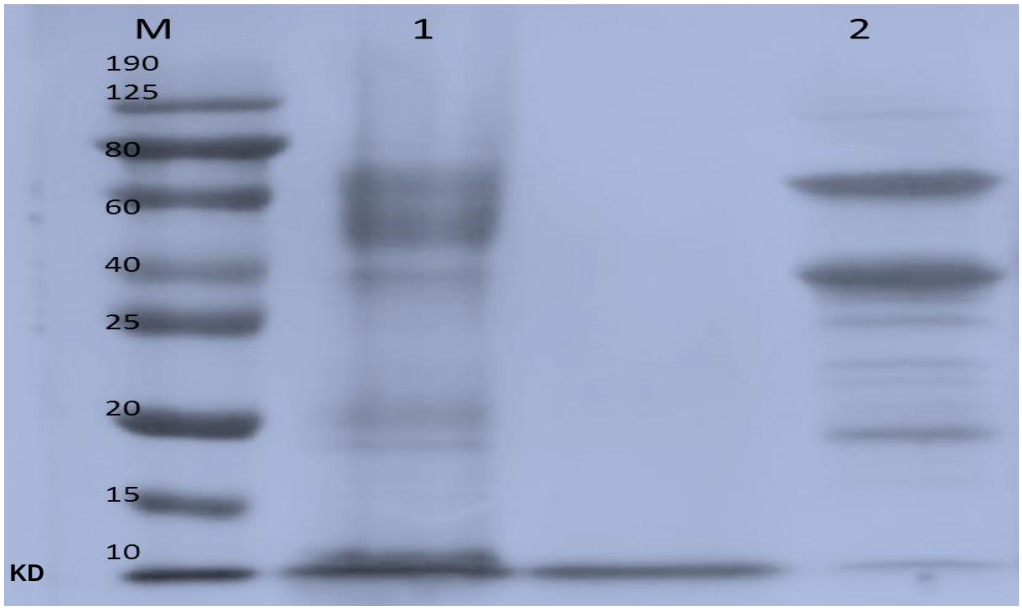


Figure 3.2. 6: Resolving secreted proteins released by probiotic Lactic acid bacteria.

Marker (M), lane 1. Lactobacillus fermentum, lane 2 L. casei strain Shirota. Both probiotic bacterial strains were grown at 37°C for 18 hr in MRS broth. The cell supernatant was collected by centrifugation, and secreted proteins of lactic acid bacteria precipitated using trichloroacitic acid (TCA) followed by resolving in 12.5% SDS-PAGE and stained by Coomassie blue.

Following the LcS probiotic SP precipitation, the LcS-SP extract was used to stimulate cultures of monocytes and macrophage cell subsets in order to find out the potency of this extract in inducing cytokines by immune cells. As shown in Fig.

3.2.7, LcS-SP extract induced TNF-α in monocytes and macrophage cell subsets. TNF-α cytokine release was LcS-SP dose dependent. Cell viability > 90% suggested that LcS-SP have no effects on cell viability in the concentration used in this study. Data indicated that LcS-SP induced TNF-α cytokine release, this ranged between 7 and 36 pg/ml for monocytes, 7 to 1842 pg/ml for M1-like macrophages and 7 to 352 pg/ml for M2-like macrophages. As shown by stimulating immune cells with LcS bacteria (HK, and live), M1-like macrophages released significantly more TNF-α than anti-inflammatory M2s and monocytes.

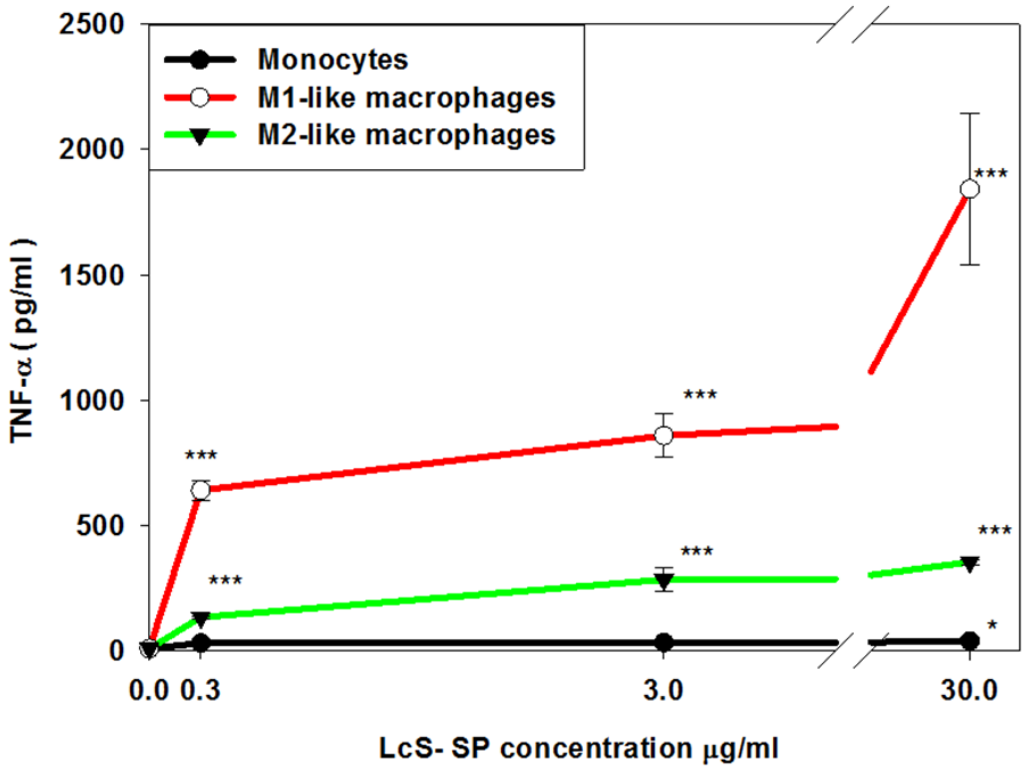


Figure 3.2. 7 : LcS-SP induces TNF-α in monocytes and macrophage cell subsets.

Monocytes, M1-like macrophages, and M2-like macrophages treated with *Lactobacillus casei strain* Shirota (LcS) secreted proteins (SPs) at a concentration of 0.3, 3 or 30 µg/ml and incubated for 18 hr. The cell supernatants were collected and stored at -20ºC to determine the cytokine level by ELISA. Bacterial secreted proteins were precipitated with trichloro acetic acid (TCA) washed twice with acetone then re-suspended in sterile LPS free

phosphate buffer saline (PBS, pH 7.4). The protein concentration was determined by Bradford assay. Cytokine production was expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

3.2.3. SP and HK probiotic bacterial strains selectively modulate LPS-induced cytokine production by monocytes and macrophage cell subsets

The first set of analyses examined the impact of K12-LPS in inducing cytokine production by THP-1 monocytes, and THP-1 derived M1 and M2-like macrophages, in addition to assessing probiotic potency in inducing cytokines by these cells (Fig.3.2.1 & 3.2.2). These results showed that either LPS or probiotics induced a range of cytokines; however, these results raised questions as to whether probiotic treatments are able to modulate LPS induced cytokine in these cells. Therefore, this experiment was undertaken to establish whether HK or SPs extract from each of *Bifidobacterium breve* (*BB), L.rhamnosus GG (LR), L.fermentum* (LF), *Lactobacillus casei strain Shirota* (*LcS), L.salivarius* (LS), *L. plantarum (LP)* probiotic bacteria (in the absence of any non-specific effects of lactic acid produced) exerted immunomodulatory effects on cytokine expression induced by LPS in THP-1 monocytes, THP-1-derived M1-like or M2-like macrophages see (Section 2.2.4.1). Results indicated that SPs or HKs of probiotic strains differentially regulated LPS induction of TNF-α cytokine expression in monocytes, M1 and M2-like macrophage cell subsets. HK-LcS, HK-LP, LS-SP, and LP-SP up-regulated LPS induced monocyte TNF-α cytokine, whereas, all the rest of probiotic treatments suppressed LPS induced monocyte TNF-α cytokine production (Fig.3.2.8A). All probiotic treatments suppressed LPS induced M1-like macrophage TNF-α cytokine production (Fig.3.2.8B). Groups of HK-BB, HK-*LR*, HK-LS, BB-SP, LR-SP, *LF*-SP and LcS-SP suppressed LPS

induced M2-like macrophage TNF-α production, whereas other groups of HK-LF, HK-LcS, HK-LS, and LP-SP results in up-regulation (Fig. 3.2.8C).

Both HK and SP of BB and LR suppressed monocyte IL-1β production whereas HK and SP of LF, LcS, LS, and LP up-regulated LPS induction of monocyte IL-1β production (Fig.3.2.8D). M1-like macrophage IL-1β induced by LPS was selectively regulated by probiotic treatments as HK-BB suppressed LPS induced IL-1β, whereas all up-regulated LPS induced M1 IL-1β production (Fig 3.2.8E). Groups of HK-BB, HK-LR, HK-LF, HK- LcS, HK-LS, HK-LP, BB-SP, LF-SP, LcS-SP, and LP-SP up-regulated LPS induced M2-like macrophage IL-1β production, whereas treatments of LR-SP and LS-SP suppressed LPS induced M2-like macrophage IL-1β production (Fig 3.2.8F).

Probiotic treatments dramatically suppressed LPS induced monocyte IL-6 cytokine production, (Fig 3.2.9A) and M2-like macrophage IL-6 production, (Fig.3.2.9C). M1-like macrophage IL-6 induced by LPS was selectively modulated by probiotic treatments, HK-BB, HK-LR, HK-LF, HK-LcS, HK-LS, BB-SP, LR-SP, and LcS-SP suppressed LPS induced IL-6 production, whereas HK-LP, LF-SP, LS-SP, and LP-SP up-regulated LPS induced M1 IL-6 production, (Fig 3.2.9B).

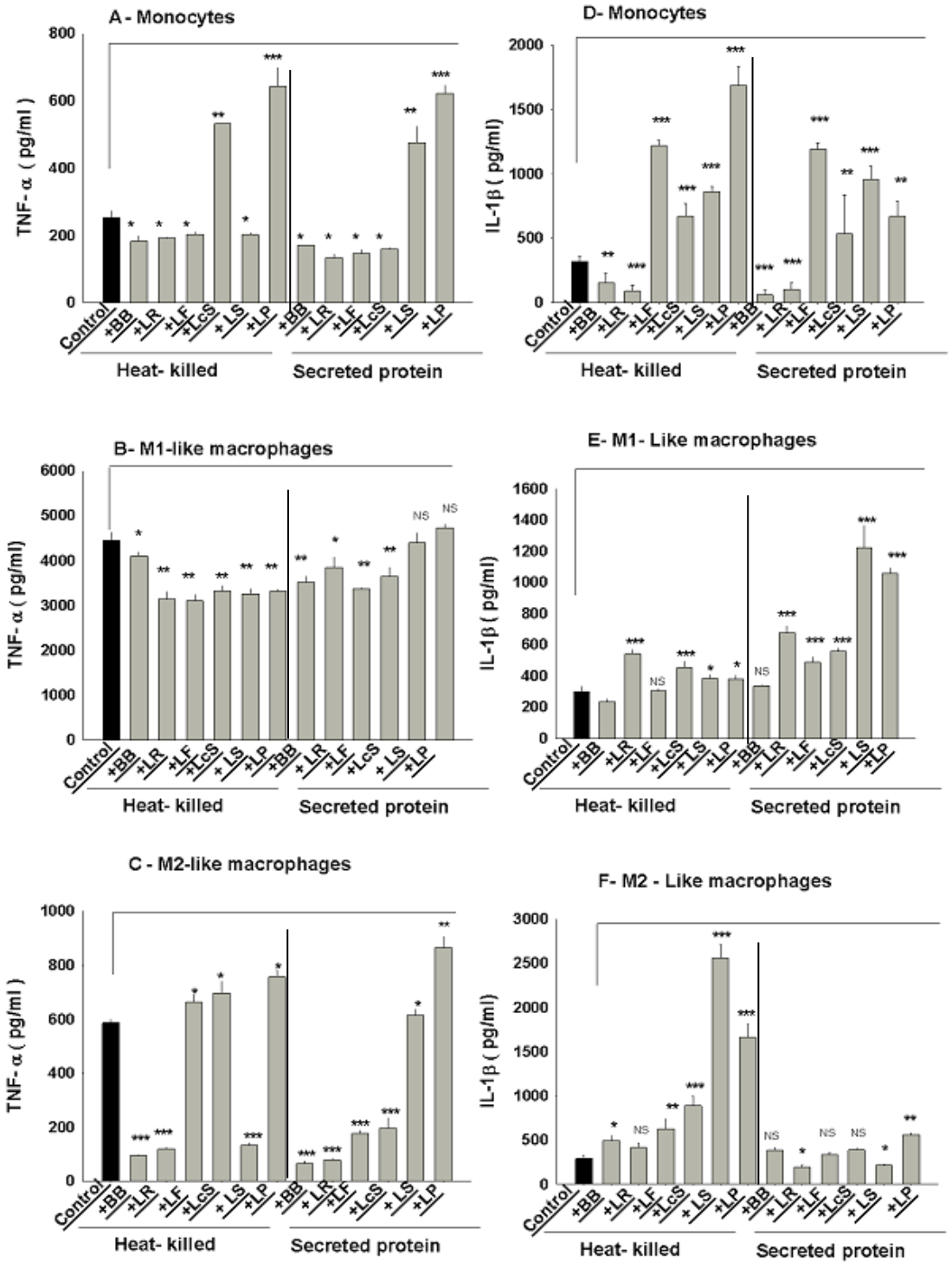


Figure 3.2. 8: Secreted proteins and heat-killed probiotic strains selectively modulate LPS induced monocyte and macrophage subset TNF-α and IL-1β cytokine production.

A, D) THP-1 monocytes, **B, E**) M1-like macrophages and, **C, F)** M2- like macrophages. Monocytes, M1, or M2-like macrophage cell subsets stimulated with 100 ng/ml *E.coli* K12 LPS in the presence or absence of $3x10^8$ cfu/ml heat-killed (HK) of *B.breve* (*BB*), *Lactobacillus rhamnosus GG* (*LR*), *L.fermentum* (*LF*), *L. casei strain* Shirota (LcS), *L.salivarius* (*LS*), *L.plantarum* (*LP*), or 3 µg/ml secreted proteins (SP) extracted from each of these probiotic strains supernatant. M1 and M2 macrophages generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. Cytokine production is expressed as the mean±SE in pg/ml for TNF-α and IL-1β. Data displayed is a representative experiment with triplicate samples of n=5 replicate experiments. Significant effects compared to stimulus control for the indicated monocytes and macrophage subset are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

Probiotic strains differentially regulated IL-8 cytokine expression induced by LPS in monocytes, M1, and M2-like macrophages. Monocyte IL-8 induced by LPS was up-regulated by both HK and SP probiotic treatments, Fig.3.2.9D. M1-like macrophage IL-8 induced by LPS was suppressed by BB and LR as HKs and SPs, and also by LS-SP, whereas the rest of probiotic treatments up-regulated LPS induced M1 IL-8 expression (as HKs and SPs) (Fig 3.2.9E). Probiotic treatments of HKs and SPs of BB, LR, LcS, LS, HK-LF, HK-LP exhibit significant suppression of LPS induced M2-like macrophage IL-8 production whereas LF-SP and LP-SP up-regulated LPS induced M2 IL-8 production (Fig 3.2.9F).

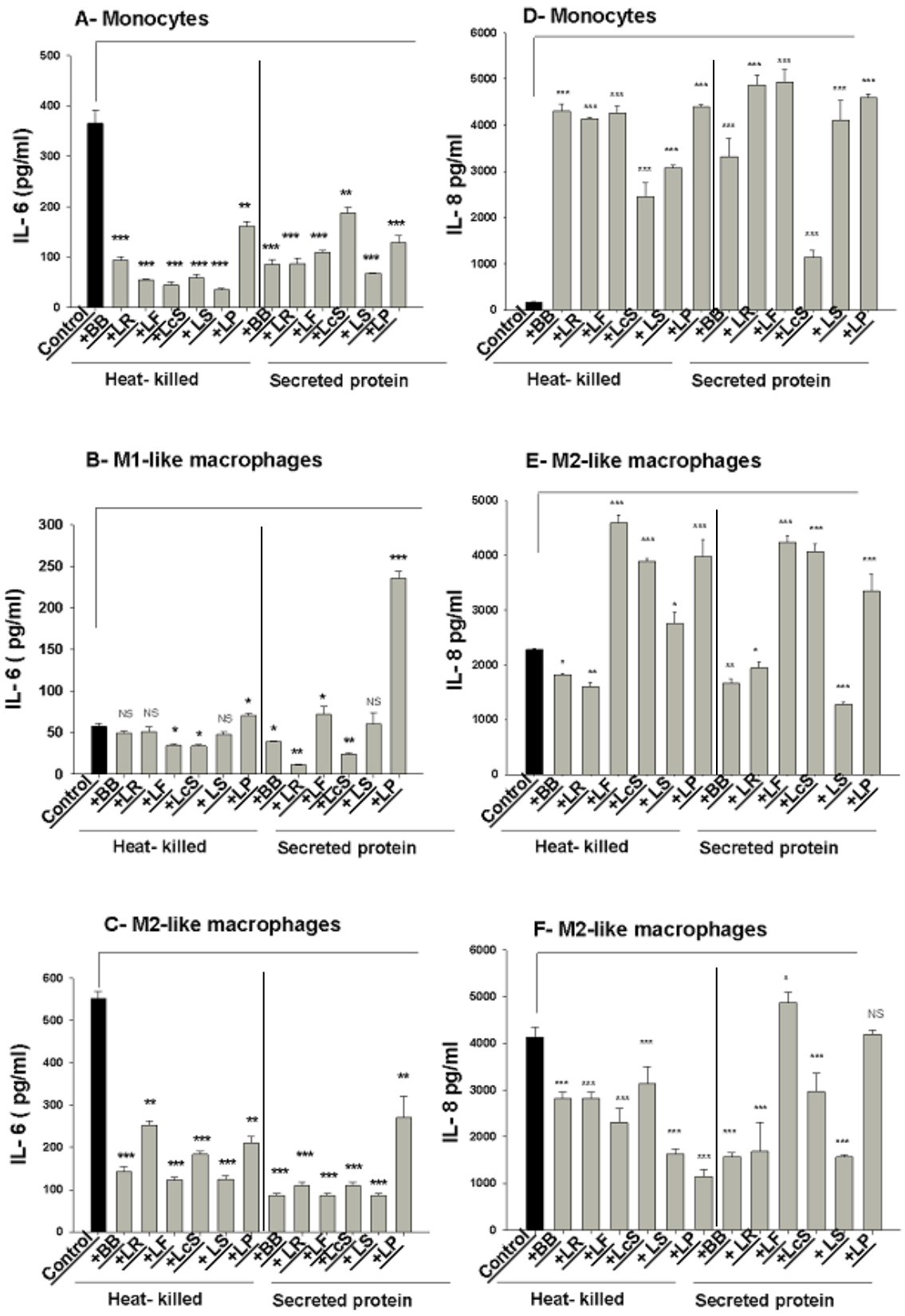


Figure 3.2. 9: Secreted proteins and heat-killed probiotic strains selectively modulate LPS induced monocyte and macrophage subsets IL-6 and IL-8 production.

A, D) THP-1 monocytes, **B, E**) M1-like macrophages and, **C, F**) M2- like macrophages. Monocytes, M1-like, and M2-like macrophage cell subsets stimulated with 100 ng/ml *E.coli* K12 LPS in the presence or absence of $3x10^8$ cfu/ml heat-killed (HK) of *B.breve* (*BB*), *Lactobacillus rhamnosus GG* (*LR*), *L.fermentum (LF*), *L. casei strain* Shirota (*LcS*), *L.salivarius* (*LS*), *L.plantarum* (*LP*), or 3 µg/ml secreted proteins (SP) extracted from each of these probiotic strains supernatant. M1 and M2 macrophages generated by differentiating THP-1 monocytes with either 25 ng/ml PMA for 3 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. Cytokine production expressed as the mean±SE in pg/ml for IL-6 and IL-8. Data displayed is a representative experiment with triplicate samples of n=5 replicate experiments. Significant effects compared to stimulus control for the indicated monocytes and macrophage subset are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

The above results clearly showed that probiotic secreted proteins exhibit different immunomodulatory effects in modulating LPS induced cytokine production in immune cells. Resolving of proteins by SDS-PAGE showed that each probiotic strain secretes different types of proteins through their metabolism (see Fig. 3.2.6). These secreted proteins exhibit different modulation of LPS induced cytokine expression in immune cells. Therefore it is proposed that each single protein might have selective immunomodulatory effects in macrophage subsets. Thus, the separation of the LcS-SP mixture using Sephacryl S-200 HR gel chromatography was performed (refer to section 2.2.2.1), followed by using these protein fractions to regulate LPS induced cytokine production in macrophage subsets (refer to section 2.2.4.5). The LcS-SP were separated by fraction number dependent on protein size; high molecular weight proteins were separated first and small molecular weight proteins separated in the late fractions (see Fig.3.2.10). After resolving all the LcS protein fractions by SDS-PAGE, the fraction number 80, 81 and 84 have protein bands with a molecular weight of 40,257 KD, 36,097KD, and 26,021KD respectively (see Fig 3.2.11). Following the separation of LcS secreted proteins, each fraction was used to treat cultures of macrophage subsets previously stimulated with LPS. As shown in Fig 3.2.12, the fraction number 80

and 81 suppressed LPS induced M1 TNF-α, whereas fraction number 84 up-regulated LPS induced M1 TNF-α. However, fraction number 80 and 81 up-regulated LPS induced M2-like macrophage TNF-α, whereas fraction number 84 suppressed LPS induced M2-TNF-α production. These findings suggested that the different types of LcS secreted proteins exhibit different effects on the modulation of LPS induced cytokine production by macrophage subsets.

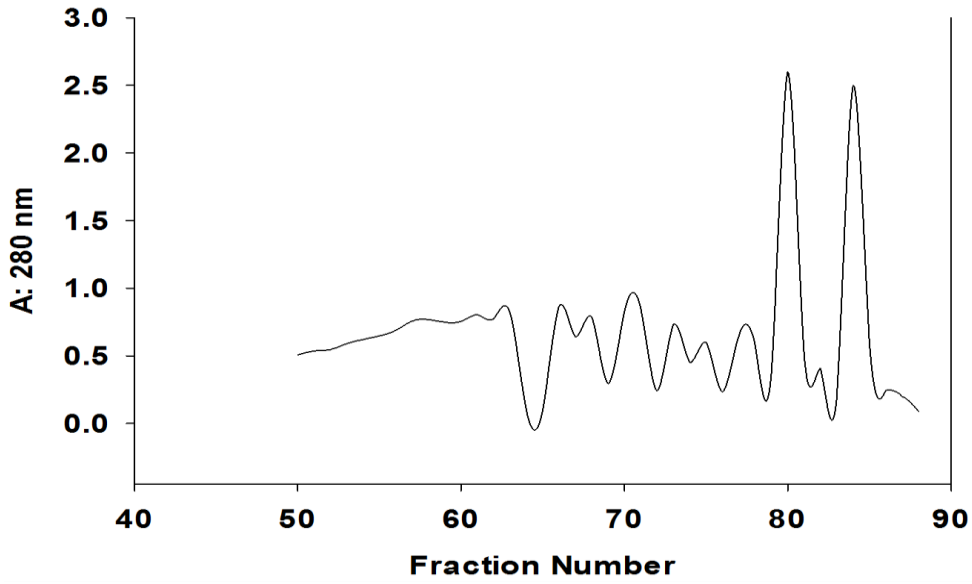


Figure 3.2. 10: A typical elution profile for the LcS-SP chromatography.

Lactobacillus casei strain Shirota (LcS) secreted proteins were separated using Sephacryl S-200 HR column (2.4 × 24 cm) at a flow rate of 0.5 ml/min. The concentration of protein in each fraction was determined using spectrophotometer at absorbance (A: 280). Data displayed is a representative experiment of triplicate experiments n=3.

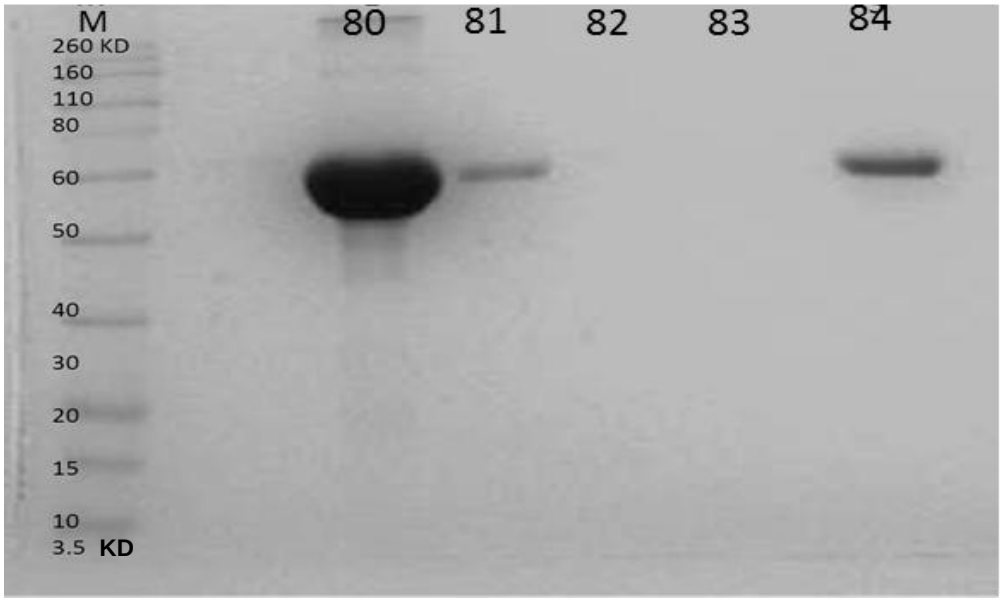


Figure 3.2. 11: Resolving pattern of protein fractions by LcS-SP.

Lactobacillus casei strain Shirota (LcS) secreted protein separation by gel chromatography resulting in a series of protein fractions were subjected to SDS-PAGE; then gel stained with Comassie blue. Lanes 80, 81, 82, 83, and 84 labelled for each protein fraction number.

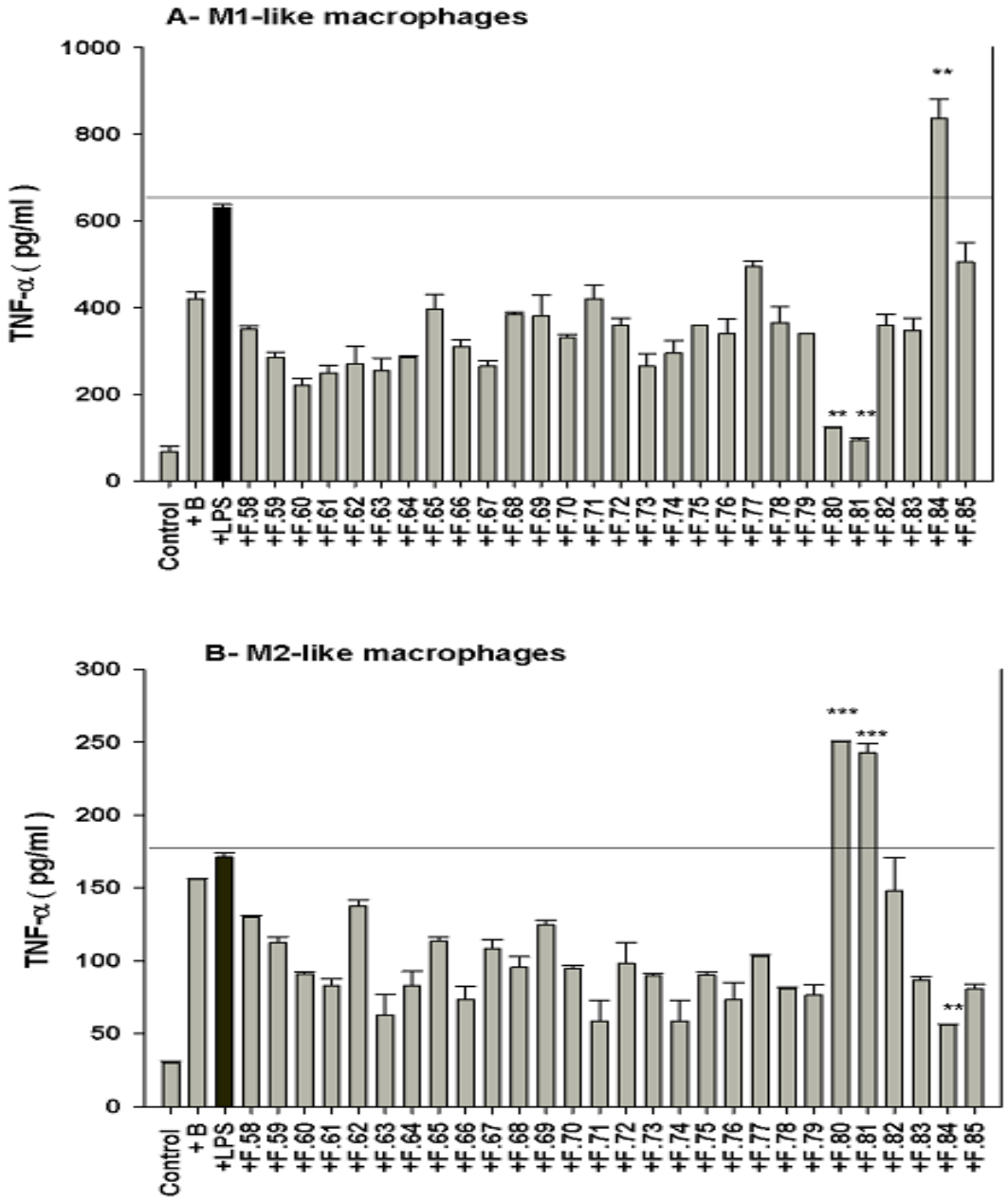


Figure 3.2. 12 : LcS protein fractions selectively modulate LPS induced TNF-α macrophage subset cytokine production.

LcS secreted proteins were separated by gel chromatography resulting in a series of LcS protein fractions. M1-like or M2-like macrophages were pre-treated with LcS protein fractions followed by stimulation with 100 ng/ml K12-LPS. TNF-α protein is expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control (+LPS) are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

3.2.4. Probiotic treatments selectively modulate LPS-induced TLR expression by macrophage subsets

The previous data clearly demonstrated that probiotic treatments have a crucial role in modulating the immune responses of macrophage subsets depending on probiotic bacterial strain. As cell signalling controls the outcomes of the immune response, the immunomodulation could occur via modulation of macrophage PRR expression including TLRs and NLRs. Information from the literature suggests that probiotic bacteria have different levels of Lipoteichoic acids (LTA) and peptidoglycan (PGN) on their cell surface (Matsuguchi et al., 2003), and different sensitivities to N-acetymuramidase digestion (e.g. LcS is resistant and LF is sensitive) (Shida et al., 2009, Turner et al., 2004, Shida et al., 2006b, Šimelyte et al., 2000). Therefore LcS or LF were chosen to determine the role of probiotic treatments in modulation of LPS induced macrophage PRR expression (refer to section 2.2.4.1). Results indicated that M1-like macrophage TLR4 induced by LPS was up-regulated by HK-LcS, HK-LF, LcS-SP and LF-SP (Fig.3.2.13A). M2-like macrophage TLR4 induced by LPS was suppressed by HK-LcS, LcS-SP and LF-SP, and up-regulated by HK-LF (Fig.3.2.13B). The clear observations were that TLR4 expression in M1s was elevated compared to M2s by 129-fold in cells stimulated with LPS, and HK-LF treatment exhibited an immune activatory role by the up-regulation of LPS induced TLR4 in both macrophage subsets.

M1-like macrophage TLR2 induction by LPS was up-regulated by HK-LcS, HK-LF, LcS-SP, and LF-SP (Fig.3.2.13C), whereas M2-like macrophage TLR2 induction by LPS was suppressed by HK-LcS, LcS-SP, and LF-SP (Fig.3.2.13D).

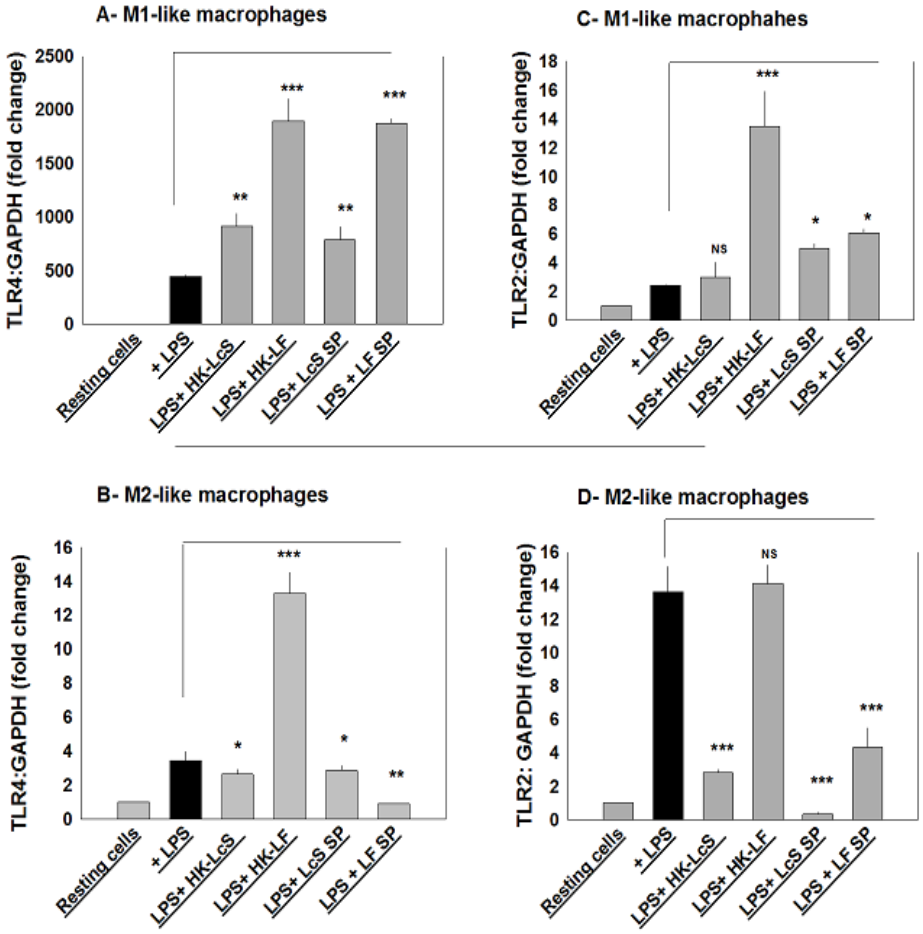


Figure 3.2. 13: Secreted proteins and heat-killed probiotic treatments selectively regulate LPS induced TLR4 and TLR2 expression in macrophage subsets.

Macrophage subsets stimulated with 100 ng/ml E.coli K12 LPS in the presence of 3x108 cfu/ml heat-killed (HK) Lactobacillus casei strain Shirota (LcS), or *L. fermentum* (LF), or 3 µg/ml secreted proteins (SP) extracted from each of these probiotic strain growth medium to test TLR expression by qPCR. TLR4 and TLR2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

CD14 (a co-receptor for LPS) induced by LPS, was suppressed by probiotic treatments in both macrophage subsets (Fig.3.2.14A and Fig.3.2.14B). CD14 up-regulation in M1s was significantly higher than in M2s and probiotic treatments exhibit anti-inflammatory regulatory effects by suppression of LPS induced CD14 in both macrophage subsets.

MD-2 (a linkage of TLR4 and TLR2 receptor for LPS signalling) induced by LPS was up-regulated by HK-LF, LcS-SP, and LF-SP, whereas it was suppressed by HK-LcS in M1-like macrophages (Fig.3.2.14C). M2-like macrophage MD-2 expression induced by LPS was up-regulated by HK-LF and LcS-SP and suppressed by HK-LcS and LF-SP (Fig.3.2.14D). This immunomodulation profile suggests that HK-LcS exhibit anti-inflammatory effects by suppression of LPS induced TLR4, TLR2, CD14 and MD-2 expression in M2-like macrophages, the opposite of HK-LF treatment.

LPS-induced NOD-2 expression was significantly up-regulated by probiotic bacterial treatments for both macrophage subsets (Fig.3.2.15A and Fig.3.2.15B), whereas M1-like macrophage TLR9 induced by LPS was selectively modulated by probiotic bacterial treatments. HK-LcS, LcS-SP and LF-SP up-regulated LPS induced TLR9 expression and HK-LF treatment suppressed LPS induced TLR9 (Fig.3.2.15C). In contrast, M2-like macrophage TLR9 expression was up-regulated by HK-LcS, HK-LF, LcS-SP and LF-SP (Fig.3.2.15D).

It is hypothesized that the up-regulation of TLRs can lead to up-regulation of cytokine expression as a result of immune activation in response to different stimuli. Probiotic regulated cytokine production by M1s (in particular, TNF-α, IL-1β, and IL-8 induced by LPS) is similar to the pattern of TLR4 and TLR2 expression,

suggesting synergistic effects of these TLRs might be driving cytokine expression in M1-like macrophages. In M2-like macrophages, the up-regulation of TLR9 might be driving IL-1β induction; TLR2 expression might be driving IL-6 and IL-8 cytokine production, and TLR4 drives TNF-α.

The adaptor protein Tollip is associated with TLR expression and plays an inhibitory role in TLR-mediated cell activation. Figure 3.2.16A showed that M1-like macrophage Tollip expression induced by LPS was suppressed by HK-LF, LcS-SP, and LF-SP, whereas it was augmented by HK-LcS. LPS-induced M2-like macrophage Tollip expression was suppressed by most probiotic treatments and augmented only by HK-LcS (Fig.3.2.16B). Comparing profiles of TLR2, TLR4 and Tollip expression suggested that probiotic regulation of these TLRs (leading to modulated cytokine expression) is largely independent of Tollip expression modulation. This suggests that there is another mechanism, which might be mediating probiotic immunomodulation in macrophage subsets, such as regulation of IRAK-M expression, which need to be investigated in the future.

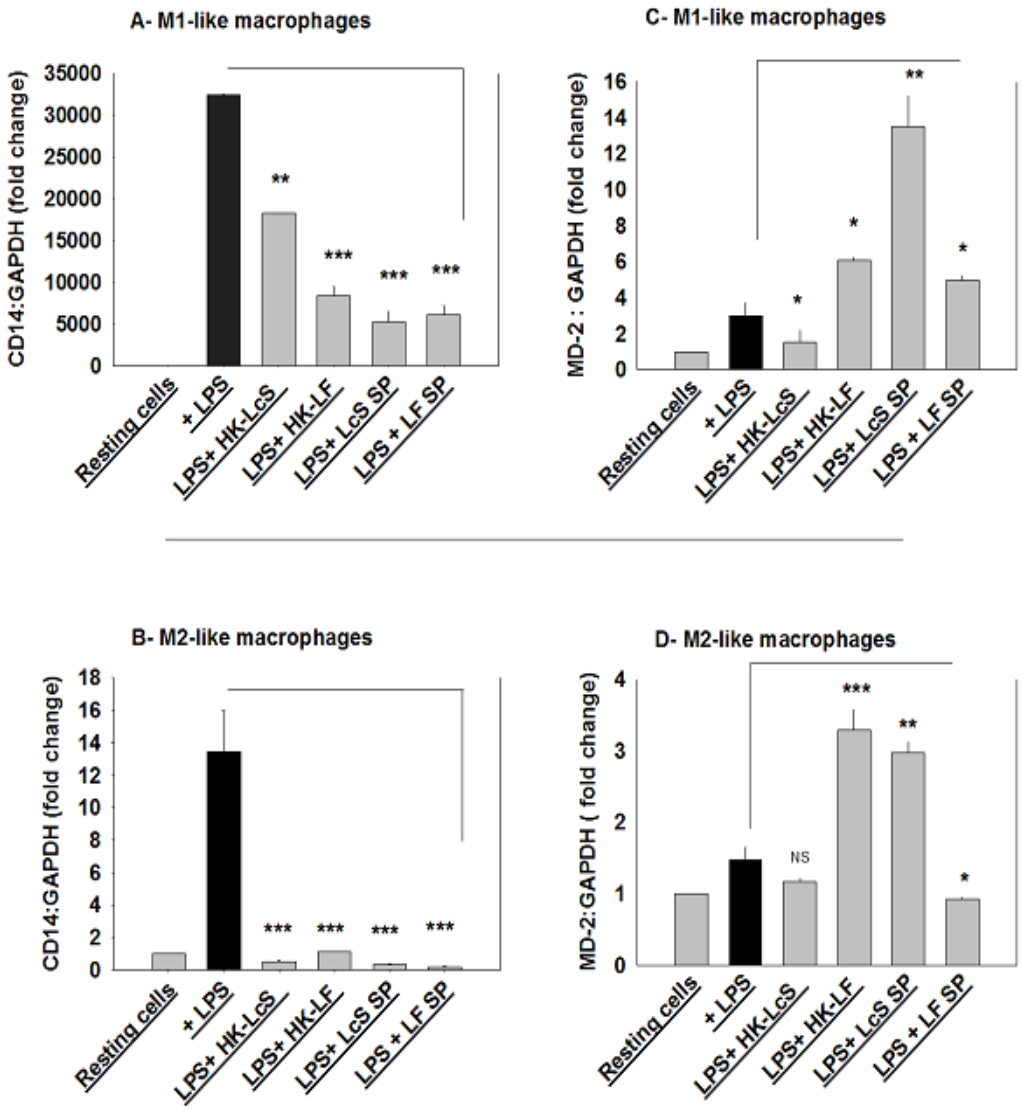


Figure 3.2. 14 : Probiotic treatments selectively regulate LPS induced CD14 and MD-2 expression in macrophage subsets.

Macrophage subsets stimulated with 100 ng/ml *E.coli* K12 LPS in the presence of $3x10^8$ cfu/ml heat-killed (HK) *Lactobacillus casei strain* Shirota (LcS), or *L. fermentum* (LF), or 3 µg/ml secreted proteins (SP) extracted from each of these probiotic strains' growth medium to test the mRNA expression of CD14 and MD-2 by qPCR. CD14 and MD-2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$**.** Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control (+LPS) are indicated as * P<0.05, ** P<0.01 *** P<0.005 and NS (non-significant)

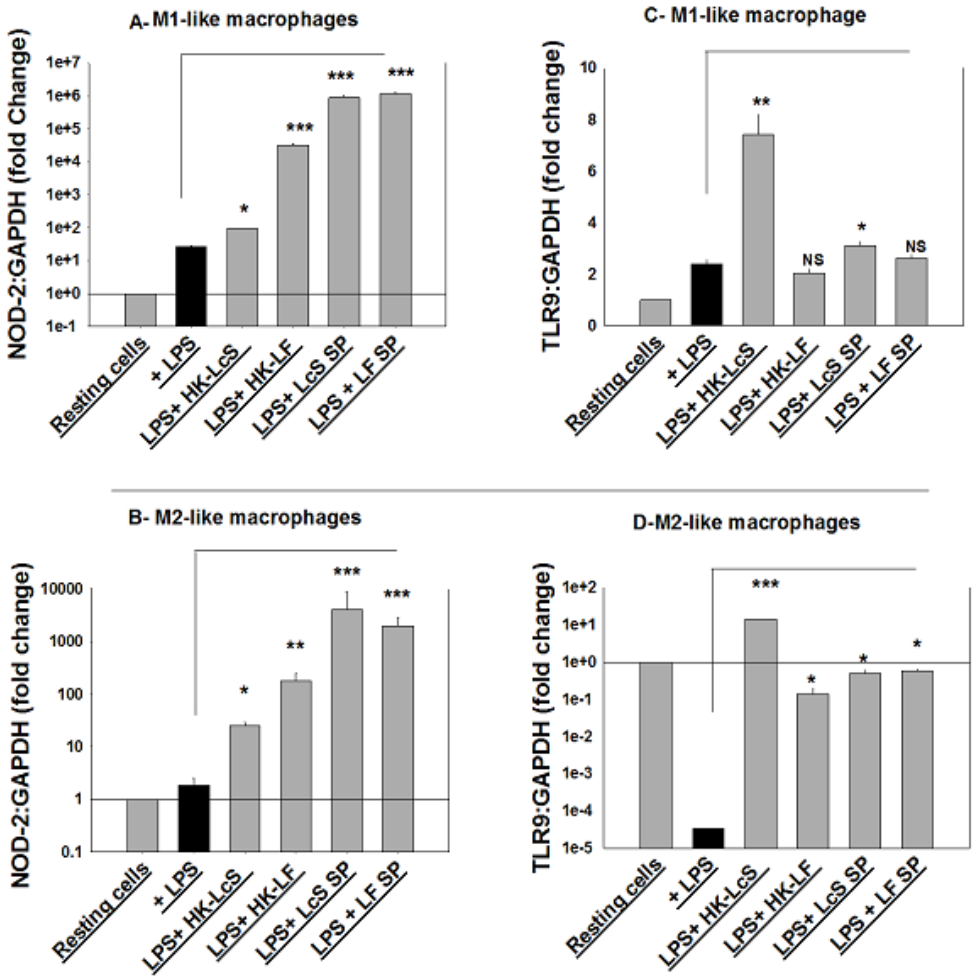


Figure 3.2. 15 : Probiotic treatments selectively regulate LPS induced NOD-2 and TLR9 expression in macrophage subsets.

Macrophage subsets stimulated with 100 ng/ml *E.coli* K12 LPS in the presence of $3x10^8$ cfu/ml heat-killed (HK) of *Lactobacillus casei strain* Shirota (LcS), or *L. fermentum* (LF), or 3 µg/ml secreted protein (SP) extracted from each of these probiotic strains' growth medium to test the mRNA expression of NOD-2 and TLR9 by qPCR. NOD-2 and TLR9 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control (+LPS) are indicated as * P<0.05, ** P<0.01 *** P<0.005 and NS (non-significant).

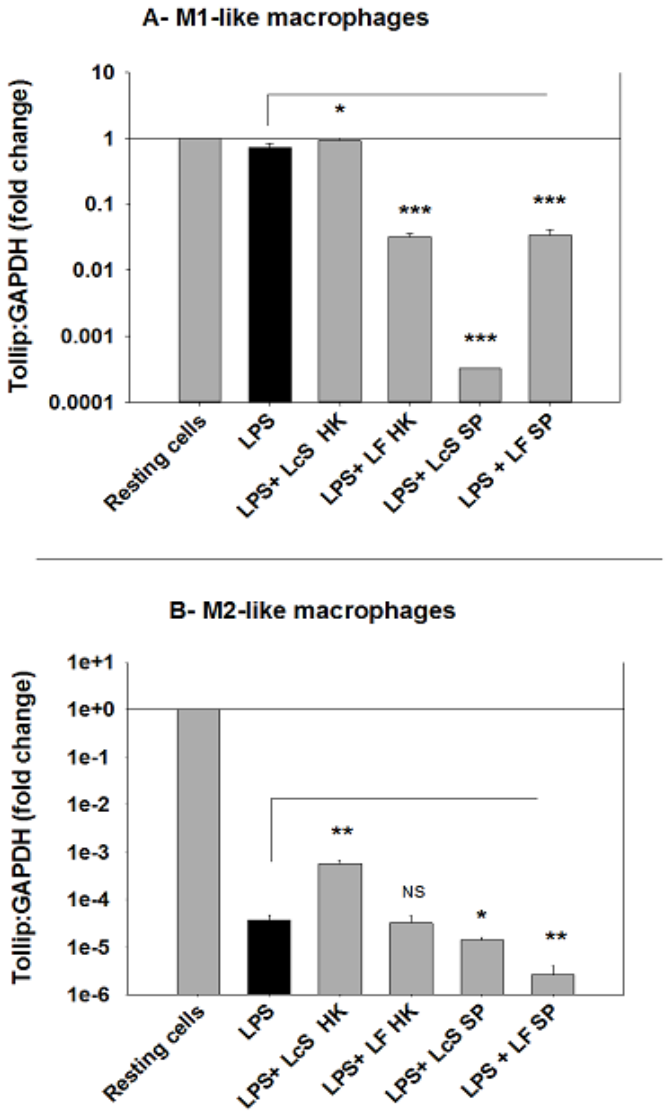


Figure 3.2. 16: Probiotic treatments selectively modulate LPS induced Tollip expression in macrophage subsets.

A) M1-like macrophage, **B**) M2-like macrophages. Macrophages stimulated with 100 ng/ml *E.coli* K12 LPS in the presence of $3x10^8$ cfu/ml heat-killed (HK) *Lactobacillus casei strain* Shirota (LcS) and *L. fermentum* (LF), or 3 µg/ml secreted protein (SP) extracted from each of these probiotic strains' growth medium. Tollip gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control (+LPS) are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant)

3.2.5. Probiotic treatments selectively modulate macrophage subset inflammatory mediator's production depending on CD14-status

Gut mucosal macrophage effector phenotypes and functions are different from other tissue macrophages. They are generally hypo responsive to microbial PAMPs (e.g. $CD14^{lo}$) when present in a homeostatic healthy gut mucosa whereas, under inflammatory conditions (they are $CD14^{hi}$) and express inflammatory mediators. In an attempt to extrapolate earlier data to gut mucosal macrophages in inflammatory or tolerogenic conditions, $CD14^{hi}$ and $CD14^{lo}$ stable transfectant were driven towards M1 and M2 macrophage subsets then stimulated with LPS in the presence or absence of HK or SP preparations from a range of probiotic bacterial strains of BB, LR, LF, LcS, LS and LP (refer to section 2.2.4.1). Results indicated that HK- and SP-probiotic bacterial samples differentially regulated LPS-induced M1 and M2 TNF-α production; modulation of TNF-α being dependent on macrophage subset, CD14 expression, and probiotic bacterial strain. The most obvious effect observed for $CD14^{hi}$ macrophages, was in $CD14^{hi}$ M1s (representative of inflammatory infiltrating macrophages) where HK probiotics up-regulated LPS-induced TNF-α by ×4, ×3.2, ×3.6, ×3.9, ×4 and ×3.7 of the control (1,161±148 pg/ml) and SP by ×4, ×4.1, ×4.1, ×1.8, ×3 and ×3.5 for BB, LR, LF, LcS, LS and LP, respectively (Fig.3.2.17A). $CD14^{hi}$ M2s displayed a differential sensitivity to HK compared to SP (Fig.3.2.17C). HKs augmented TNF-α production resulting in increases of ×4.9, ×12, ×3.6, ×7-fold for LR, LF, LS and LP respectively (control 14±1pg/ml) whereas HK-BB or HK-LcS did not modulate LPS induced TNF-α. SP extracts, in comparison, only weakly modulated cytokine production where LS suppressed TNF-α production by 3.6% (control 14±1 pg/ml to 9±1 pg/ml) and LP augmented it by 86% (control 14±1 pg/ml to 26±3 pg/ml). Generally, in the case of $CD14^{lo}$ macrophages, HK and SP probiotics partially

suppressed LPS-induced TNF-α production. In the case of $CD14^{lo}$ M1s, HK suppressed TNF-α cytokine production (control levels of 1,454±94 pg/ml) by 51% (709±4 pg/ml), 19% (1,184±98 pg/ml), 40% (876±175 pg/ml), 57% (628±316 pg/ml), and 72% (396±28 pg/ml) for BB, LF, LS, LP and LcS, respectively. SPs suppressed TNF-α cytokine production by 47% (770±120 pg/ml), 59% (590±6 pg/ml) and 52% (704±119 pg/ml), 81% (264±20 pg/ml) for LR, LF, LP and LcS (Fig.3.2.17B). Finally, in the case of $CD14^{lo}$ M2 macrophages (representative of homeostatic healthy gut mucosal macrophages), HK suppressed TNF-α (control 21±2 pg/ml) by 62%, 38%, 33%, 57% and 52% for BB, LR, LF, LS and LP. The exception was LcS which augmented TNF-α from control 21±2 pg/ml to 619±17 pg/ml. SP extracts suppressed TNF-α cytokine production by 19%, 62%, 24% and 67% for BB, LF, LS and LP respectively, whereas LR-SP, or LcS-SP augmented TNF-α by 38%, 37% respectively (Fig.3.2.17D).

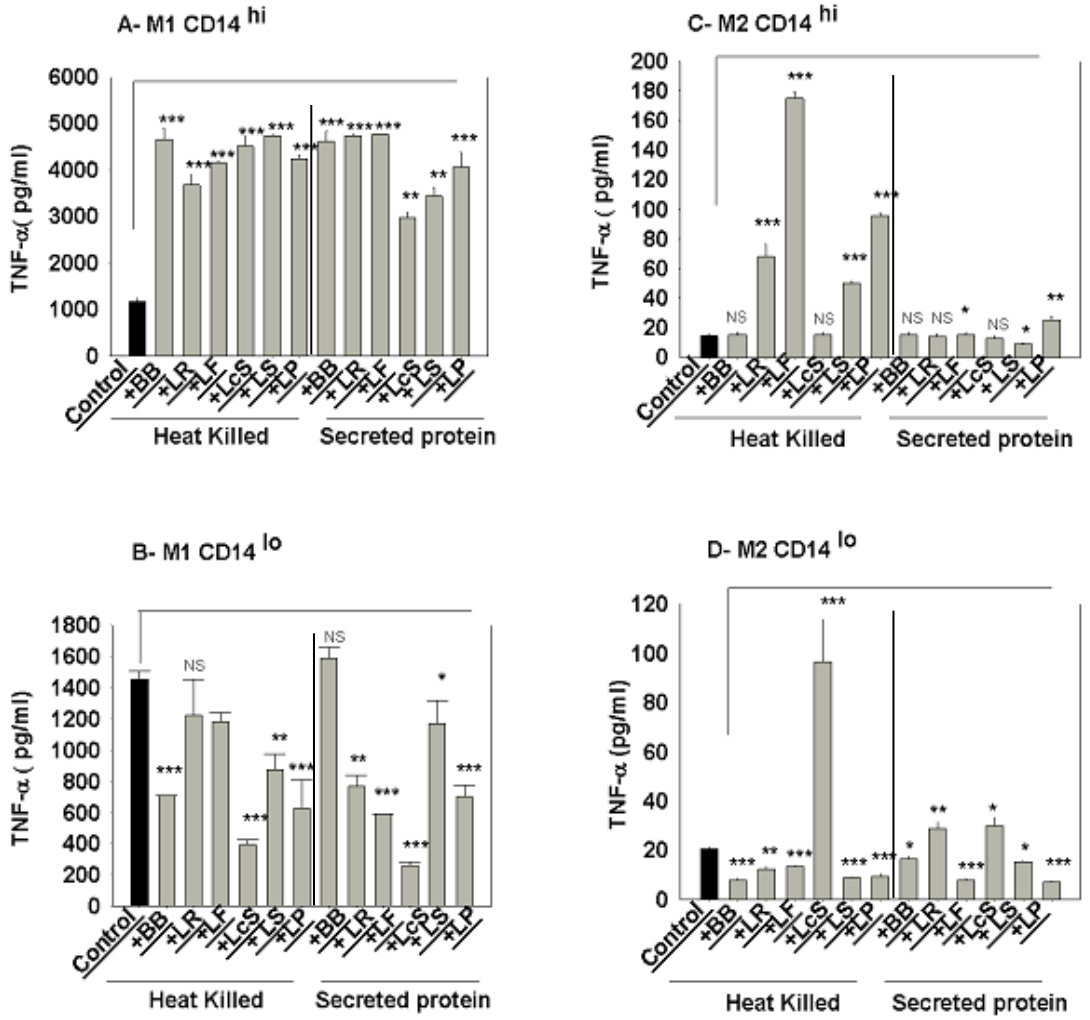


Figure 3.2. 17: Secreted proteins and heat-killed probiotic strains selectively modulate macrophage subset TNF-α production.

THP-1-derived $CD14^{hi}$ and $CD14^{lo}$ macrophage subsets were stimulated with 100 ng/ml *Escherichia coli* K12 LPS in the presence or absence of 3×10^{8} cfu/ml heat-killed (HK) probiotic bacterial strains *Bifidobacterium breve* (BB), *Lactobacillus rhamnosus* (LR), *Lactobacillus fermentum* (LF), *Lactobacillus casei strain* Shirota (LcS), *Lactobacillus salivarius* (LS) and *Lactobacillus plantarum* (LP), or 3 µg/ml secreted protein extracted from each of these probiotic strains. M1 and M2-like macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1- NF-κB reporter monocytes with either 25 ng/ml PMA for 3 days or 10 nM 1,25-$(OH)_2$ vitamin D_3 for 7 days, respectively. TNF-α pro-inflammatory cytokine production is expressed as the mean±SE in pg/ml for (**A**) $CD14^{hi}$ M1, (**B**) $CD14^{lo}$M1, (**C**) $CD14^{hi}$ M2, and (**D**) $CD14^{lo}$M2 like- macrophage subsets. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to stimulus control (Black bar) for the indicated macrophage subset are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

The above data clearly demonstrates an immunomodulatory role for both HK and probiotic-SP with respect to the expression of the pro-inflammatory cytokine TNF-α by pro-inflammatory (M1) and anti-inflammatory/regulatory (M2) macrophage subsets. These macrophage subsets have been described to express different cytokine profiles, which underlie their effector function; one such differential cytokine, which exhibits both pro-inflammatory and anti-inflammatory properties, is IL-6. This experiment was undertaken to establish whether HK and SP probiotics exerted any selective immunomodulatory effects on IL-6 by M1 and M2 macrophage subsets. Results indicated that HK and SP probiotic treatments differentially regulated IL-6 cytokine expression by M1 and M2 macrophage subsets. Both HK and SP preparations suppressed LPS induced IL-6 production by M2 macrophages, the potency of the suppression being regulated by the level of CD14 expression. With respect to $CD14^{lo}$ M2 macrophages, HKs and SPs suppressed IL-6 (Fig.3.2.18C). IL-6 production by $CD14^{hi}$ M2s was highly sensitive to suppression from both HKs and SPs (Fig.3.2.18D). Pro-inflammatory M1 macrophages exhibit a different IL-6 regulatory profile upon exposure to HKs and SPs of the probiotics. In $CD14^{lo}$ M1 macrophages, HK-BB, HK-LR, HK-LcS, HK-LS and HK-LP augmented LPS-induced IL-6 (control 216±5 pg/ml) by ×1.3, ×2.6, ×1.8, ×1.04, and ×2.1 respectively, whereas HK-LF suppressed IL-6 production by 29% (Fig.3.2.18A). LR-SP, LS-SP and LP-SP suppressed LPS induction of IL-6 by 30%, 26% and 49%, whereas, LF-SP, LcS-SP augmented LPS induction of IL-6 production by ×1.4, ×1.02 and BB-SP failed to modulate IL-6 expression. The $CD14^{hi}$ M1 subset displayed an intriguing profile upon introduction of HK or SP probiotic. HK-LF, HK-LS and HK-LP suppressed LPS induction of IL-6 expression by 61%, 61% and 74%, respectively, whereas SP preparations from the same strains augmented LPS induced IL-6 production by 55%, 26% and 11%,

respectively. HK-LR, LR-SP, and LcS-SP were suppressed IL-6 by 17% and 11%, and 69% respectively, and finally, BB failed to modulate LPS-induced IL-6 in these pro-inflammatory macrophages in comparison to the control (62±8 pg/ml) (Fig.3.2.18B). Thus, CD14 is an important co-receptor for LPS signalling by macrophage subsets and has a crucial role in modulating the immune response induced by LPS.

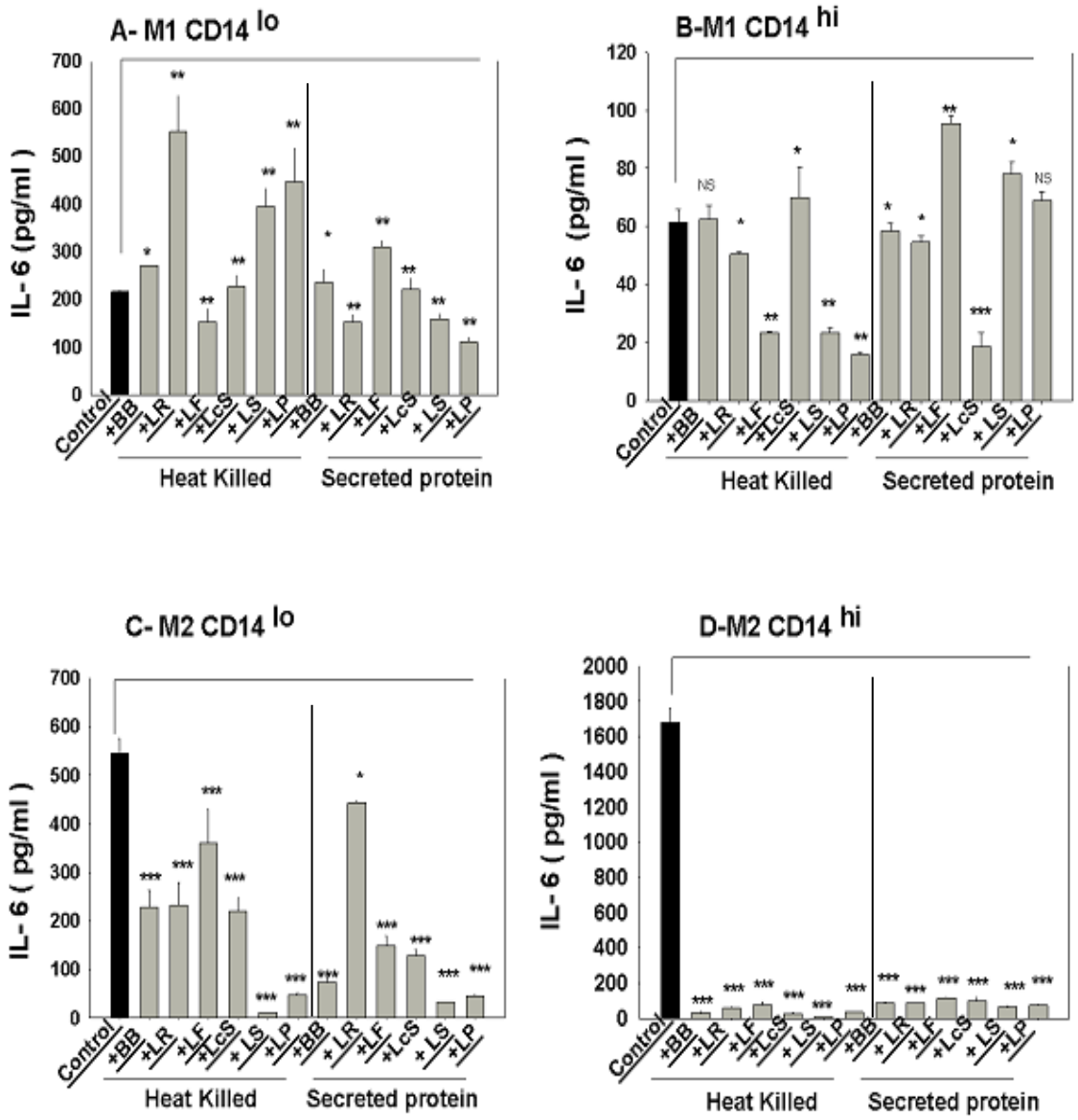


Figure 3.2. 18: Secreted proteins and heat-killed probiotic strains selectively modulate macrophage subset IL-6 production.

THP-1-derived $CD14^{hi}$ and $CD14^{lo}$ macrophage subsets were stimulated with 100 ng/ml *Escherichia coli* K12 LPS in the presence or absence of 3×10^8 cfu/ml heat-killed (HK) probiotic bacterial strains of *Bifidobacterium breve* (BB), *Lactobacillus rhamnosus* GG (LR), *Lactobacillus fermentum* (LF), *Lactobacillus casei* strain Shirota (LcS), *Lactobacillus salivarius* (LS) and *Lactobacillus plantarum* (LP), or 3µg/ml secreted protein (SP) extracted from each of these probiotic strains. M1 and M2 macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1- NF-kB reporter monocytes with either 25 ng/ml PMA for 3 days or 10 nM 1, 25-(OH) 2 vitamin D_3 for 7 days respectively. IL-6 is expressed as the mean±SE in pg/ml for (**A**) $CD14^{lo}$ M1, (**B**) $CD14^{hi}$ M1, (**C**) $CD14^{lo}$ M2, and (**D**) $CD14^{hi}$ M2 like-macrophage subsets. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to stimulus control for the indicated macrophage subset are indicated as * P<0.05, ** P<0.01 *** P<0.005 and NS (non-significant)

3.2.6. SP and HK probiotic strains selectively modulate macrophage subset NF-κB activity

NF-kB transcription factor stands out as master regulator of innate immunity, and major regulator of pathogen-and inflammatory cytokine inducible gene regulation, therefore any regulation of pro-inflammatory cytokine expression was expected to be as a consequence of the modulation of NF-kB activity. Both IL-6 and TNF-α are pro-inflammatory cytokines induced in response to microbial infection and exhibit-binding consensus sequences in their respective promoter regions. Any regulation of the expression of these inflammatory cytokines by probiotic bacteria was expected to be as a consequence of modulation of NF-κB activity. When comparing profiles between TNF-α, IL-6 and NF-κB activation, these data are suggestive that probiotic regulation of these pro-inflammatory cytokines are largely independent of NF-κB activity. One clear observations were that the probiotic-secreted protein augmented NF-κB activation in $CD14^{lo}$ M1, $CD14^{lo}$ M2, and $CD14^{hi}$ M2 macrophages (Fig 3.2.19). Secreted protein preparation treatments failed or only partially suppressed NF-κB activity upon LPS stimulation of $CD14^{hi}$ M1s. Secreted proteins augmented NF-kB in $CD14^{lo}$ M1s (LPS control level of 0.530±0.063 arbitrary units) by 53%, 32%, 69%, 47% and 60%, for BB-SP, LR-SP,

LF-SP, LS-SP, and LP-SP, respectively, whereas LcS-SP suppressed LPS induced NF-kB activation by 21% (fig 3.2.19A). In $CD14^{lo}$ M2s (LPS control 0.427±0.009 arbitrary units) probiotic-secreted proteins (SP) up-regulated LPS induction of NF-KB activation by 92%, 116%, 63%, 21%, 83% and 156% (Fig.3.2.19C) and LPS induction of NF-kB activation in $CD14^{hi}$ M2s by 36%, 97%, 138%, 48%, 58% and 144% (LPS control 0.363±0.005 arbitrary units), for BB-SP, LR-SP, LF-SP, LcS-SP, LS-SP, and LP-SP respectively (Fig. 3.2.19D). With the exception of LF-SP, where no modulation of NF-κB was observed, the probiotic SP only partially suppressed $CD14^{hi}$ M1 NF-κB (LPS control 1.198±0.084 arbitrary units) by 13%, 23%, 30% 20%, and 27% for BB, LR, LS LP and LcS, respectively (Fig.3.2.19B). The heat-killed preparations seemed to be partially suppressed M1 NF-κB activation whereas they augmented NF-kB activation in the M2 macrophage subset. The HK-LABs suppressed NF-kB in $CD14^{lo}$ M1s induced by LPS by 42%, 25%, 28% 31% 47% and 27% (Fig.3.2.19A) and in $CD14^{hi}$ M1s by 28%, 32%, 96% 28% and 27% for LR, LF, LS, LP, and LcS, respectively (Fig.3.2.19B). HK-LABs generally augmented LPS-induced NF-kB activity in M2 macrophages, however heat-killed preparation of BB failed to modulate NF-κB activity. HK-probiotics augmented NF-kB activity in $CD14^{hi}$ M2s by 29%, 65%, 8.1%, 125% and 59% for LR, LF, LcS, LS, and LP respectively (Fig.3.2.19D). Finally, in the case of $CD14^{lo}$ M2s, HKs from LF, LS, and LP weakly modulated NF-κB activity, augmenting by 26%, 20%, and 33%; respectively, whereas HK-LcS suppressed LPS induced NF-kB by 10% (Fig.3.2.19C). The data clearly demonstrated that probiotics exhibit their roles in modulation of LPS induced pro-inflammatory cytokines partially on NF-kB activation, suggesting that another transcriptional factor might be involved in this process such as AP-1; this might be worth investigating in future.

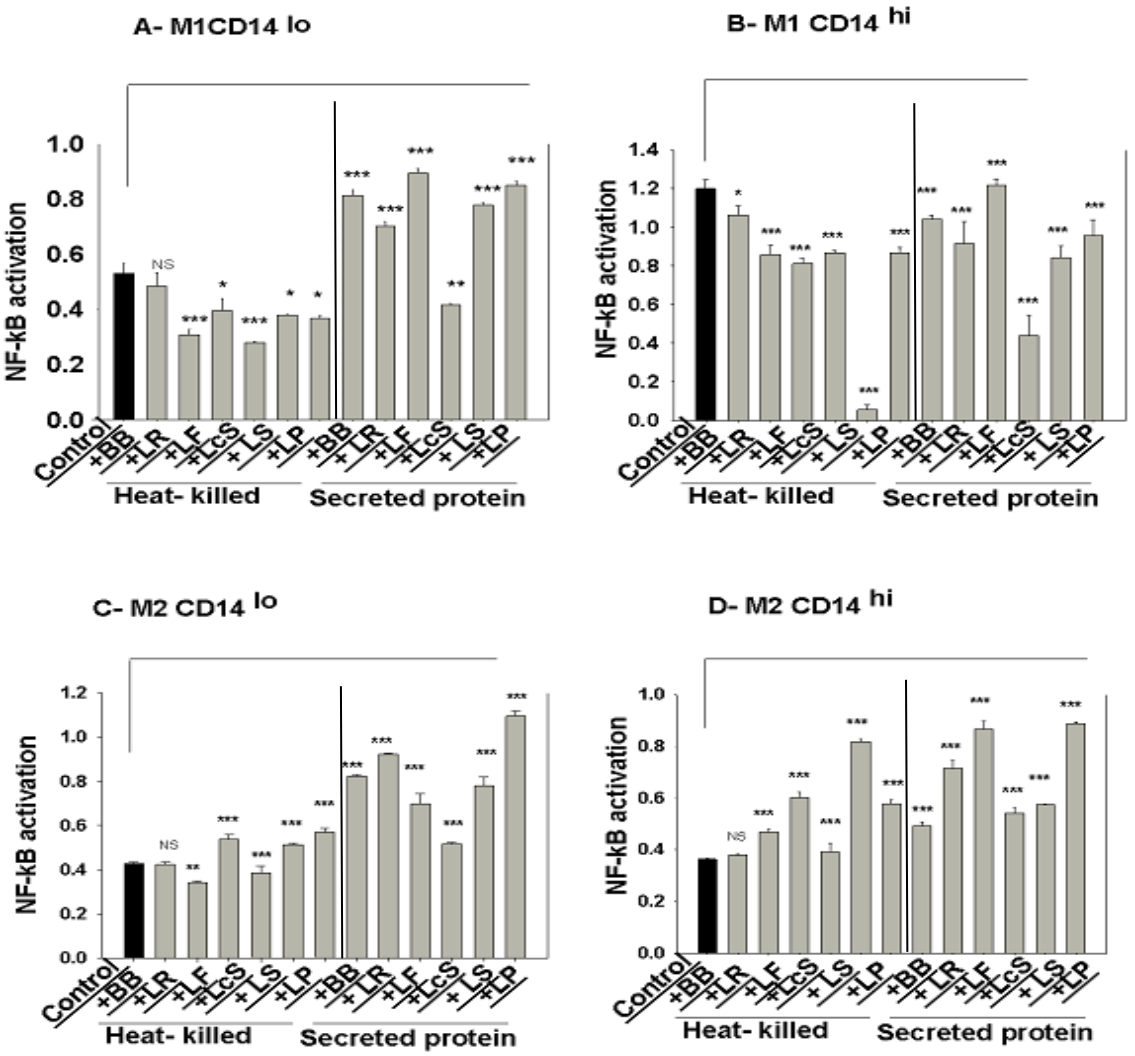


Figure 3.2. 19: Secreted proteins and heat-killed probiotic strains selectively modulate macrophage subset NF-κB activity.

THP-1-derived $CD14^{hi}$ and $CD14^{lo}$ macrophage subsets were stimulated with 100 ng/ml *Escherichia coli* K12 lipopolysaccharides (LPS) in the presence or absence of 3×10^8 cfu/ml heat-killed (HK) probiotic bacterial strains of *Bifidobacterium breve* (BB), *Lactobacillus rhamnosus GG* (LR), *Lactobacillus fermentum* (LF), *Lactobacillus casei strain* Shirota (LcS), *Lactobacillus salivarius* (LS) and *Lactobacillus plantarum* (LP) or 3µg/ml secreted protein extracted from each of these probiotic strains. M1 and M2 macrophages were generated by differentiating $CD14^{hi}$ and $CD14^{lo}$ THP-1-NF-κB reporter monocytes with either 25 ng/ml PMA for 3 days or 10 nM 1, 25-(OH) 2 vitamin D_3 for 7 days, respectively. NF-KB reporter activity is expressed as the mean±SE in arbitrary absorbance units (A:620 nm) for (**A**) $CD14^{lo}$ M1, (**B**) $CD14^{hi}$ M1, (**C**) $CD14^{lo}$ M2 and (**D**) $CD14^{hi}$ M2 like- macrophage subsets. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to stimulus control (Blue bar) for the indicated macrophage subset are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

3.2.7. Summary of chapter 3 results

Table 3.2. 1: Summary of probiotic immunomodulation of cytokine production induced by LPS in monocytes and macrophage subsets.

	BB-HK	LR-HK	LF-HK	LcS-HK	LS-HK	LP-HK	BB-SP	LR-SP	LF-SP	LcS-SP	LS-SP	LP-SP
Mono-TNF-α	↓	↓	↓	↑	↓	↑	↓	↓	↓	↓	↑	↑
M1-TNF-α	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↔	↔
M2-TNF-α	↓	↓	↑	↑	↓	↑	↓	↓	↓	↓	↓	↑
Mono-IL-1β	↓	↓	↑	↑	↑	↑	↓	↓	↑	↑	↑	↑
M1-IL-1β	↓	↑	↔	↑	↑	↑	↔	↑	↑	↑	↑	↑
M2-IL-1β	↑	↔	↑	↑	↑	↑	↔	↓	↔	↔	↓	↑
Mono-IL-6	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
M1-IL-6	↔	↔	↓	↓	↔	↑	↓	↓	↑	↓	↔	↑
M2-IL-6	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Mono-IL-8	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
M1-IL-8	↓	↓	↑	↑	↑	↑	↓	↓	↑	↑	↓	↑
M2-IL-8	↓	↓	↓	↓	↓	↓	↓	↓	↑	↓	↓	↔

Note: "↑", "↓,"and "↔"means up-regulation, down-regulation, and no-modulation of the indicated target, respectively

Table 3.2. 2: Summary of probiotic immunomodulation of TLR expression induced by LPS in macrophage subsets.

	HK-LcS	HK-LF	LcS-SP	LF-SP
M1-TLR4	↑	↑	↑	↑
M1-TLR2	↓	↑	↓	↓
M2-TLR2	↔	↑	↑	↑
M2-TLR2	↓	↔	↓	↓
M1-CD14	↓	↓	↓	↓
M2-CD14	↓	↓	↓	↓
M1-MD-2	↓	↑	↑	↑
M2-MD-2	↔	↑	↑	↓
M1-NOD-2	↑	↑	↑	↑
M2-NOD-2	↑	↑	↑	↑
M1-TLR9	↑	↔	↑	↔
M2-TLR9	↑	↑	↑	↑
M1-Tollip	↑	↓	↓	↓
M2-Tollip	↑	↔	↓	↓

Table 3.2. 3: Summary of probiotic treatments on LPS induced NF-kB activation.

	BB-HK	LR-HK	LF-HK	LcS-HK	LS-HK	LP-HK	BB-SP	LR-SP	LF-SP	LcS-SP	LS-SP	LP-SP
$M1^{hi}$ TNF-α	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
$M1^{lo}$ TNF-α	↓	↔	↓	↓	↓	↓	↔	↓	↓	↓	↓	↓
$M2^{hi}$ TNF-α	↔	↑	↑	↔	↑	↑	↔	↔	↓	↔	↓	↑
$M2^{lo}$ TNF-α	↓	↓	↓	↑	↓	↓	↓	↓	↓	↓	↓	↓
$M1^{hi}$ IL-6	↔	↓	↓	↑	↓	↓	↓	↓	↑	↓	↑	↔
$M1^{lo}$ IL-6	↑	↑	↓	↑	↑	↑	↑	↓	↑	↑	↓	↓
$M2^{hi}$ IL-6	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
$M2^{-lo}$ IL-6	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
$M1^{hi}$ NF kB	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
$M1^{lo}$ NF-KB	↔	↓	↓	↓	↓	↓	↑	↑	↑	↓	↑	↑
$M2^{hi}$ NF-kB	↔	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
$M2^{lo}$ NF-kB	↔	↓	↑	↓	↑	↑	↑	↑	↑	↑	↑	↑

3.3. Discussion

3.3.1. THP-1 monocytes differentiation into M1-like and M2-like macrophages.

Several studies have demonstrated that gut macrophages have at least two subsets classically activated pro-inflammatory M1-, and alternative anti-inflammatory M2-macrophages (Edwards et al., 2006, Gordon, 2003, Mosser and Edwards, 2008). Using THP-1 human monocytic cell line to generate macrophage cell subsets as a model in this study resulted in the development of different distinct macrophage cell phenotypes representative of M1, and M2-like macrophages, which varied in morphological features (Fig.2.5) and functionality (Fig.3.2.1, Fig.3.2.2, Fig.3.2.3 and Fig.3.2.4). The cytokines IL-1β, TNF-α, IL-6 and IL-8 which are a major cytokines of gut pathology (Funakoshi et al., 1998, Zuo et al., 2010) were used as markers to distinguish between macrophage subsets in response to LPS (a component of the enteropathic bacterium, *E. coli* strain K12) stimulation. Indeed, cytokine readout showed that monocytes differentiated by PMA (M1-like) differed from monocytes differentiated by Vit.D_3 (M2-like), expressing different cytokine levels, and profiles.

Generally, LPS signal transduces its signal via TLR4 (Beutler, 2000) resulting in cytokine expression through activation of mitogen activated protein kinase (MAPK) and NF-kB signalling pathways (Libermann and Baltimore, 1990, Yamamoto and Gaynor, 2001). The significant observations here were the production of IL-1β, TNF-α and IL-8 after stimulation with LPS in M1s was higher than by M2s, and *vice versa* with IL-6 expression. Of particular interest was the expression of IL-6 by M2-like macrophages. IL-6 is a pleotropic cytokine that can exhibit pro and anti-inflammatory effects via its ability to induce suppression of cytokine signalling

proteins (SOCS) expression (Xing et al., 1998). The regulation of SOCS and their downstream targets such as STATs play an important role in macrophage subset differentiation and determination of effector responses. Transcription factor STAT-3 mediates cytokine signalling and is implicated in a variety of immune responses. STAT-3 is one of the selected transcriptional factors expressed by alternative activation of macrophages (M2s) (Gordon, 2003; Mosser & Edwards, 2008). It is well established that STAT-3 mediates IL-10R signalling and exerts anti-inflammatory activity by inhibiting pro-inflammatory cytokines such as TNF-α and IL-1β (Bromberg, 2002 , Williams et al., 2007, Williams et al., 2004). In the absence of IL-10 signalling, macrophages are induced to produce pro-inflammatory cytokines (M1) associated with up-regulation of SOCS-3. SOCS-3 is essential for the function of classical M1 macrophages (Liu et al., 2008), and suppresses anti-inflammatory effectors that result from IL-10/IL-6 activation of M2 macrophages, thus controlling macrophage plasticity; knockout of SOCS-3 results in an alternative M2-like phenotype with increased expression of arginase, mannose receptor (MR), anti-inflammatory cytokines and SOCS-1. Accordingly, there is a reciprocal relationship between SOCS-3 and SOCS-1 regulation of STAT-3 and STAT-1 controlling the differentiation of macrophage subsets. STAT-3 itself induces the anti-apoptotic factors bcl-2 and bcl-xL (Fukada et al., 1996, Kovalovich et al., 2001). Interestingly, only treatment with Vit.D3 (M2) results in phosphorylation of STAT-3 at Tyr 705 in response to LPS stimulation. These results are in agreement with Daigneault et al. (2010) when they showed that treatment of THP-1 cells with Vit.D_3 results in macrophage cell phenotype resistance to apoptosis. THP-1 cells treated with Vit D_3 resulted in cells expressing membrane bound IL-10 constitutively in resting cells and inducibly in response to LPS stimulation. It is possible that phosphorylation of STAT-3 in M2 cells mediates

the expression of membrane bound IL-10. The STAT-3 signalling pathway is shared by IL-6 through gp130 a common signal transducer for the IL-6 cytokine (Fukada et al., 1996). Yasukawa *et al.* (2003) demonstrated that IL-6 is produced after activation of STAT-3 and inhibits SOCS-3. Taken together, M2-like macrophages differentiated from THP-1 monocytes are like anti-inflammatory macrophages when expressing pSTAT-3 results in the up-regulation of IL-6 in response to LPS higher than by M1s. LPS induced higher levels of TNF-α and IL-1β production in THP-1 cells treated with PMA (M1-like), which in agreement with Schwende et al. (1996) and Chanput et al. (2010), depended on different activation level of NF-kB leads to increased pro-inflammatory cytokine levels (Collart et al., 1990). It is well established that M1, which represent pro-inflammatory macrophages expressing levels of CD14, TLR2, TLR4, TLR5, CD89, and CD16, mediate the induction of pro-inflammatory cytokines (Gordon, 2003). The data of this study are consistent with other research groups (Mantovani et al., 2002) with respect to differential cytokine profiles expressed by macrophage subsets in response to LPS.

Further study was performed to investigate TLR expression by macrophage subsets in response to LPS stimulation. Both macrophage subsets showed that the TLR4 surface expression was up-regulated after LPS stimulation. The findings of the current study are consistent with those of Mantovani et al. (2004) who found that TLR4 expression is predominant in M1 macrophages in response to LPS stimulation. This finding supports previous research into this macrophage area, which links TLR4 and TNF-α expression (Oshima et al., 2004); consequently, M1s expressed TNF-α higher than M2-like macrophages by virtue of this relationship. This study confirms that TLR4 expression is associated with production of pro-

inflammatory cytokines by M1-like macrophages (TNF-α, IL-1β, and IL-8). On the question of TLR2 expression by macrophage subsets, this study found that the predominant expression is TLR2 in M2-like macrophage subset in response to LPS stimulation. The most interesting finding was that IL-6 expression by M2-like macrophages was greater than by M1-like macrophages. This finding corroborates the ideas of Biswas et al. (2008), who suggested that IL-6 expression is related to TLR2 expression. These results are consistent with those of other studies and suggest that the different profiles of cytokine expression by macrophage subsets matched with the TLR profiles (Fairweather and Cihakova, 2009). This finding has important implications for developing model macrophage subsets. Therefore, it can be assumed that the cells differentiated from THP-1 are different distinct macrophage subsets.

3.3.2. SP and HK of probiotic strains induced cytokine production by monocytes and macrophage subsets

The second question in this research was the probiotic bacterial role in inducing cytokine expression by macrophage subsets. The results of this study show significant increases in cytokine expression by macrophage subsets in response to LcS stimulation, as live and heat killed formats. Probiotic bacteria (LcS) are gram positive, characterised by external-facing lipoteichoic acid, polysaccharides, and exposed PGN. PGN has been described to induce macrophage production of TNF-α, IL-1β and IL-6 (Gupta et al., 1999, Weidemann et al., 1997); and all of these cytokines possess NF-kB, AP-1 and CREB binding sites in their promoters resulting in the transcription of the downstream genes of these cytokines. Interestingly, PGN activates AP-1 and CREB/ (ATF) in M2-like vitamin D_3 differentiated THP-1 macrophages (Gupta *et al*., 1999). It seems to be that LcS-

polysaccharide induced cytokine expression by macrophage cell subsets, might be depending on the activation of the cAMP response element binding protein, which is lead to activation of the transcriptional factors CREB/ATF-1 (Foey et al., 2003), thus it is possible that the immunostimulatory effects of probiotic bacteria may be dependent on the appropriate recognition of Gram-positive Lactic acid bacterial cell wall of peptidoglycan-polysaccharides (PGN-PS) by macrophage cells. In addition to PGN, LTA has a significant role in inducing cytokine expression in a range of immune cells such as monocytes and macrophage subsets (Nilsen et al., 2008, Morath et al., 2001). LTA induced differential cytokine release via activation of NF-κB and AP-1, depending on the differences in LTA structure (Finney et al., 2012). Bacterial DNA also has a significant role in inducing cytokine expression, and level of GC determines the type of cytokine induction; high level of GC (as found in *Bfidobacteria)* tend to induce anti-inflammatory cytokines such as IL-10, whereas low level GC (as found in *Lactobacilli*) tend to induce pro-inflammatory cytokine such as IL-12 and TNF-α (Medina et al., 2007). Taken together, cytokine production by different immune cells after recognition of LcS was dependent on level of PGN recognition, LTA structure of the bacterial cell wall, and GC level in bacterial DNA. These induce different levels of TLR, differential type of PRRs that lead to different levels of NF-kB activation and consequent induction of different types and levels of cytokine production, which in turn mediate infection/inflammation or intestinal homeostasis (Ginsburg, 2002, Claes et al., 2012a).

Live probiotic bacteria can produce metabolites through their metabolism of carbohydrate and proteins; these bacterial metabolites include proteins and short chain fatty acids, which can modulate the immune responses of cells in the gut

mucosa (Walker, 2008). From preliminary investigation of the secreted protein extracts from LcS and LF, denaturation SDS-PAGE analysis showed the presence of protein bands which differ in molecular mass for each probiotic bacterial strain. This study set out with the aim of assessing the importance of secreted proteins on cytokine expression by monocytes and macrophage subsets. One unanticipated finding was that LcS-SP treatments successfully induced cytokine release resulting in different profiles of TNF-α augmentation in monocytes and macrophage subsets, explaining that these extracts contain proteins that interact with monocyte and macrophage PRRs resulting in augmentation of TNF-α cell signalling. This finding is in agreement with Yan et al. (2007) who showed that *Lactobacillus fermentum* and *L. rhamnosus* GG secrete immunomodulatory proteins which modulate signaling pathways driving pro-inflammatory cytokine production. There are similarities between the mechanism expressed by LcS-SP in this study and those described by Hoarau et al. (2008) when they showed that the fermentation product of a *Bifidobacteria* can differentially activate MAPKs, Glycogen synthase kinase (GSK3) and Phosphatidylinositide 3-kinases (PI3K) resulting in induction of cytokine expression in DCs when probiotic metabolites modulate cytokine expression. However, the findings of the current study displayed a role of other probiotic metabolites (SP) in the mediation of the induction of cytokine signaling in immune cells. Interestingly, this suggests that *Lactobacillus casei* stain Shirota (LcS) probiotic bacteria secrete extracellular proteins which interact with monocytes, M1-like and M2-like macrophages resulting in induction of TNF-α. Profiles of TNF-α produced by monocytes, M1-like and M2-like macrophages induced by LcS-SP was protein-dose-and immune cell type- dependent. The observed differences between immune cell TNF-α level expression indicate the differences in PRR expression. Future studies on the

current topic of the probiotic role in inducing cytokine expression as HK and SP in different immune cells are therefore recommended.

3.3.3. Probiotics selectively modulated LPS induction of cytokine production by monocytes and macrophage subsets

Several studies, including this study, have described that probiotics have a role in induction of cytokine production by immune cells such as monocytes and macrophages, but the reports are often contradictory; observation being determined by cell source, level of differentiation, bacterial strain, stimulus used and local environment (Gackowska et al., 2006, Ivec et al., 2007, Lin et al., 2008, Malai et al., 2009, Matsumoto et al., 2005, Miettinen et al., 1996, Zeuthen et al., 2006) (see table 1.1).

The third question in this research was focused on the probiotic bacterial role in the modulation of LPS induced cytokine production by monocytes, and macrophage subsets. It is interesting to note that in all probiotic treatments of this study, probiotic strains selectively modulated pro-inflammatory cytokine production by monocytes and macrophage subsets. In addition, the format of probiotics, whether heat killed (HK) whole bacteria or live and their secreted proteins (SP), displayed a differential modulation of cytokine expression of these effector immune cells. HKs and SPs of probiotic strains differentially modulated cytokine expression dependent on maturation degree of monocytes/macrophage cells and type of probiotic strains. As an example HK-LcS augmented monocyte LPS-induced TNF-α, IL-1β and IL-8 whereas it suppressed IL-6 production. M1 IL-1β and IL-8 production were augmented by HK-LcS whereas TNF-α was suppressed. LcS-SP augmented LPS-induced monocyte IL-1β and IL-8 whereas suppressed TNF-α and IL-6. M2 macrophages exhibit a different modulation profile by LcS-SP with

respect to these pro-inflammatory cytokines. LcS-SP failed to modulate TNF-α production, augmented IL-1β, whereas it suppressed IL-6 and IL-8 production in response to *E. coli* K12 LPS. A strong relationship between bacterial cell wall structure associated with intracellular digestion by monocytes/macrophages and modulation of cytokine expression has been reported in the literature. Šimelyte et al. (2000) and Turner et al. (2004) reported that *L. fermentum* cell wall was lysozyme sensitive, resulting in induction of anti-inflammatory cytokine such as IL-10, whereas Shida et al. (2006), and Matsumoto et al. (2009) reported that LcS has a rigid cell wall resistant to lysozymes and intracellular digestion induced pro-inflammatory cytokine such as IL-12. Comparing LcS and LF immunomodulation showed only monocyte TNF-α induced by LPS was different, when LcS up-regulated LPS induced TNF-α whereas LF suppressed it. This suggested that despite the differences between these strains in their cell wall structure (sensitivity for lysozyme digestion), they exhibit the same trend of immunomodulation for LPS induced cytokine expression by monocytes and macrophage subsets because they are sharing the protective effects via the similarly in LTA (Setoyama et al., 1985). Screening of probiotic immunomodulation showed that there was similarly between HK-BB and HK-LR immunomodulation of LPS induced cytokine expression and the only difference was, IL-1β production induced by LPS in macrophage subsets (refer to table 3.2.1). They exhibit profound anti-inflammatory effects via suppression of a range if LPS induced cytokines, which is in agreement with other research groups (Okada et al., 2009, Ciszek-Lenda et al., 2011). There was similar between HK-LS and HK-LP, when they suppressed LPS induced M1-TNF-α, monocyte IL-6, M2-IL-6 and M2-IL-8, which is in agreement with Díaz-Ropero et al. (2007) and Kim et al. (2013). Indeed, there are several possible explanations for the immunomodulatory action of probiotics in the current study.

LPS induces the pro-inflammatory cytokines by activating transcription factors such as NF-kB, cJun and activating transcription factor 2 (ATF) family members; probiotics exhibit different roles in modulating these transcriptional factors resulting in different profiles of cytokine production by these immune cells. Okada et al. (2009) demonstrated that probiotic strains inhibited LPS induced pro inflammation cytokines via inhibiting the phosphorylation of IκB-α induced by LPS, associated with up-regulation of SOCS-3. Several studies highlighted that probiotics mediated anti-inflammatory activity through enhancement of IL-10 expression by anti-inflammatory macrophage subset (Steidler et al., 2000, Madsen et al., 2001, Galdeano and Perdigón, 2006, Shida et al., 2011).

MAPKs have been shown to play a role in driving LPS-induced TNF-α and IL-1β production (Foey et al., 1998), modulation of these cytokines by probiotics may well target MAPKs activity. Indeed, the probiotic *Lactobacillus reuteri* was demonstrated to suppress monocyte and macrophage TNF-α production by inhibiting the activation of MAPK-regulated transcription factors, cJun and AP-1 (Lin et al., 2008). Bcl-3 is one of IκB family members, which attenuates LPS induced inflammatory responses in macrophages (Wessells et al., 2004), therefore, HK-LcS down regulated LPS induction of TNF-α may be through induction of Bcl-3 protein, or suppressed IKK activity and inhibition of NF-κB DNA binding (Schottelius et al., 1999). Another possible explanation for this result is that probiotic modulation of LPS induction of cytokine production is by enhancing the anti-inflammatory cytokine production such as IL-10 leading to inhibition of pro-inflammatory cytokine (Konstantinov et al., 2008). On the other hand, the up-regulation of LPS induces TNF-α production by M1s as pro-inflammatory macrophages may be through up-regulation of the expression of TLRs such as

TLR4 or NLRs (such as NOD-2). This leads to increases of the NF-kB heterodimer p50/RelA or MAPKs phosphorylation resulting in increasing of pro-inflammatory cytokine production (Petrof et al., 2009).

SP extracts selectively modulated LPS induced cytokine expression by monocytes and macrophage subsets. Screening of SP extract immunomodulation of cytokine induced by LPS in monocytes and macrophage subsets showed there was similarity between BB-SP and LR-SP regarding with modulation of LPS induced cytokines (TNF-α, IL-1β, IL-8 and IL-6) by monocytes and macrophage subsets, which is in agreement with Menard et al. (2004) who showed that protein extract from *Bifidobacterium breve* supernatant were suppressed LPS induced TNF-α in THP-1 cells, however, the differences between them was IL-1β production induced by LPS in macrophage subsets. BB-SP suppressed LPS induced M1-IL-1β, whereas LR-SP augmented it, and *vice versa* with M2 IL-1β induced by LPS. LcS-SP and LF-SP also exhibited the same trend of immunomodulation of LPS induced cytokine expression when suppressed LPS induced TNF-α, IL-1β and IL-8 by monocytes and macrophage subsets, except the M1-IL-6 and M2-IL-8 induced by LPS. Moreover, LS-SP and LP-SP were also exhibited similar effects in modulation of LPS induced cytokine expression except M2-TNF-α, M1-IL-1β, IL-8 M1 and IL-8 M2-like macrophage subset. Indeed, Hoarau et al. (2008) showed that proteins from *Bifidobacterium breve* C50 supernatant were differentially regulated cytokines associated with modulation of transduction signaling pathways (NF-kB, PI3K) in a healthy human DCs and Claes et al. (2012b) reported that protein extract from *lactobacillus rhamnosus* GG promote survival and growth of intestinal cells shared homology with cell wall hydrolysis. Taken together, data suggested that the crude SP might have the same proteins that exhibit the same

effects in modulation of LPS induced cytokine expression by monocytes and macrophage subsets (Sanchez et al., 2010).

These findings further support the idea of identifying the immunomodulatory effects of LcS-SP extract, fractionating single proteins of the crude LcS-SP and examining the effect of these secreted proteins on modulation of LPS-induced cytokine expression by monocytes and macrophage subsets. This investigation was performed by gel filtration to separate proteins based on protein molecular weight (MW). Regulation of the immune responses induced by LPS with LcS-SP fractions resulted in different profile of immunomodulation as fraction number 80 and 81 up-regulated LPS induced M2-like macrophage TNF-α expression, whereas down-regulated M1-TNF-α cytokine expression, and *vice versa* with fraction number 84. This result may be explained by the fact that the specific protein in each fraction modulates the LPS macrophage interaction process resulting in modulation of TNF-α expression as the outcome of the immune response. However, the crude LcS-SP extract exhibit different effects, when they suppressed LPS induced TNF-α in macrophage subsets. Indeed there were several explanations of this particular result, it might be the concentration of specific protein needed to up-regulate LPS induced TNF-α was less than to suppress LPS induced TNF-α, or it might be the antagonistic effects between these proteins lead to suppressed LPS induced TNF-α expression by macrophage subsets. Fraction number 80 and 81 exhibit anti-inflammatory effects by suppression of TNF-α induced by LPS in M1-like macrophage, whereas fraction 84 in M2-like macrophages may represent future therapeutic agents that could serve to suppress chronic inflammation associated with M1-like macrophages. Because of the lack of the well-defined molecular signals that are produced by probiotics,

the discovery of these microbial metabolites will enable the development of new drugs that limit inflammation. This study corroborated the findings of a great deal of previous work in this field e.g. Thomas et al. (2012), who showed that histamine derived from probiotic *Lactobacillus reuteri* suppresses TNF-α. In addition, Sanchez et al. (2009) showed that the fractionation of a conditioned medium extract produced by *Lactobacillus rhamnosus* GG identified several protein bands of which a cell wall-associated hydrolase, Serpin B1, could play a significant role in modulating host immunity. Taken together, the macrophage cytokine profiles obtained upon LPS stimulation following probiotic regulation were different dependent on the type of probiotic strain and type of macrophage subsets. These results give a new insight on the fine-tuned balance between the maintenance of normal mucosal homeostasis to commensal bacteria and the specific inflammatory responses elicited by pathogenic bacterial PAMPs, such as LPS, which might be achieved through of modulation of macrophage cytokine production. This is an important issue for future research in order to identify the types of proteins mediating immunomodulation by immune cells using MS/MS technique followed by PCR cloning.

3.3.4. Probiotic treatments selectively modulated LPS-induced TLR expression in macrophage subsets

Innate immune responses exhibit the first line of defense, detect pathogenic microorganisms, and mount a fast defensive response through a group of proteins called TLRs. The main role of TLRs is detecting exogenous stimuli such as microbial PAMPs, and they are also able to sense endogenous signals, such as heat shock protein 60, fibronectin, fibrinogen, unknown factors from the injured tissue and necrotic cells, and facilitate antigen clearance. This mechanism provides a tight immune surveillance system displayed by TLRs (Abreu et al.,

2005). Indeed, macrophage recognition of microbial PAMPs via TLRs, convert to the signals downstream of the cell cytoplasm, resulting in a variety of activities such as cytokine production.

Among TLRs, the expression of TLR2 and TLR4 and the co-receptor CD14 and MD-2 are important factors in recognition of LPS in macrophage cells. The present study was designed to determine the effect of probiotic treatments on modulation of LPS induced immune responses. In this study, probiotic treatments selectively modulated macrophage subset immune responses induced by LPS. What is surprising is that the HK-LcS suppressed LPS induced TLR4, TLR2, and co-receptor CD14, whereas, HK-LF augmented LPS induced TLR2, TLR4, and suppressed co-receptor CD14 expression. The present findings seem to be consistent with other research groups, which found differences in immunomodulation of TLR expression in macrophage cell subsets depending on the bacterial cell structure (Shibata et al., 2011). CD14 was highly expressed in M1s associated with pro-inflammatory cytokine production in this macrophage effector phenotype. This factor may explain the relatively good correlation between CD14 expression and ability of M1 macrophages to express high levels of pro-inflammatory cytokines (Mosser and Edwards, 2008, Smith et al., 2011). Probiotic treatments suppressed LPS induced CD14 expression in macrophage subsets, hence, it could conceivably be hypothesised that the probiotics exhibit an anti-inflammatory role in this setting by dampening down LPS signalling. The profiles of TLR expression were partially matched with cytokine expression in macrophage subsets. For example the profile of TLR expression induced by LPS and regulated by probiotics was matched with IL-6 expression but not with TNF-α in M2s, suggesting that the LPS signalling was achieved via a complex of TLR2/CD14

rather than through a complex of CD14/TLR4 (refer to table 3.2.1, and 3.2.2). These results are consistent with those of other studies such as Meyenburg et al. (2004) who suggested that TLR4 functions as the true LPS receptor, and TLR2 is also involved in recognition of Gram-positive bacterial products.

Intestinal TLR signaling has a dual role, including maintaining intestinal homeostasis and protection from injury, as well as initiates inflammatory responses. It is well documented that unmethylated CpG motifs present in bacterial DNA stimulates a rapid and vital innate immune response. Bacterial DNA is recognised by TLR9 which is one of the PRRs expressed intracellularly in macrophages. The expression of TLR9 mediates anti-inflammatory effects through induction of type 1 IFN (Lee et al., 2006); however, up-regulation of TLR9 mediates the activation of macrophages to produce significant levels of pro-inflammatory cytokines leading to initiation of tissue injury. Both CpG-DNA and LPS modulate expression of cell surface receptors, transcription factors, and proteins related to cell differentiation suggested that TLR9 sharded homology with TLR4 in macrophage cells (Gao et al., 2003, Gao et al., 2002). The findings of the current study are consistent with those of An et al. (2002) who found that LPS stimulation up-regulated gene expression of TLR9 and initiated inflammation via NF-kB, ERK, and p38 MAPKs signal pathways. Indeed, augmentation of TLR9 expression induced by LPS in macrophages facilitates responding to occupied bacteria more efficiently. The data of this study show that the genomic structure of LcS up-regulated LPS induced TLR9 in both macrophage cell subsets. It is seemed to be that the probiotic up-regulation of TLR9 induced by LPS in M1s is to enhance the production of pro-inflammatory cytokines, whereas enhances the anti-inflammatory cytokines expression in M2-like macrophages. This finding

corroborates the ideas of Zhang et al. (2010), who suggested that the up-regulation of TLR9 induced by LPS is to up-regulate Arg1 and FIZZ1 mediating M2 macrophage function. It can therefore be assumed that probiotic DNA exhibits two sides in modulating the immune responses in macrophage cell subsets.

NOD-2 is one of the PRRs expressed intracellularly in macrophages and exhibits a surveillance system to detect microorganisms that occupy and reside in the cytoplasmic infected cells, such as *Shigella* (pathogenic bacteria) or LcS (non-pathogenic bacteria) (Hasegawa et al., 2006). Specifically, it recognises a breakdown product of the bacterial cell wall component, PGN, namely muramyl dipeptide (MDP) (Girardin et al., 2003a). This receptor has been demonstrated to have both pro-inflammatory and anti-inflammatory function in response to microbial stimuli. NOD-2 up-regulation leads to activated defiance signaling pathways resulting in provoking pro-inflammatory mediators such as TNF-α (Kim et al., 2008). In this study, probiotic treatments were found to up-regulate LPS induction of NOD-2 expression in macrophage cell subsets. A possible explanation for this might be that probiotic treatments manipulated the adaptor RICK in macrophage subsets (Bernardo et al., 2012). It is possible, therefore, that probiotics maintain innate immunity via eliminating the pathogen bacterial occupation. Synergistic effects between TLR9 and NOD-2 mediate intestinal homeostasis (van Heel et al., 2005). One of the issues that emerges from these findings is the synergistic effects between TLR9 and NOD-2 when probiotics up-regulated LPS induction of TLR9 and NOD-2 expression in macrophage subsets.

Maintaining the immunological balance is one of the main aspects of a healthy gut mucosa in terms of keeping a balance between activation and tolerance to avoid detrimental and inappropriate inflammatory responses. The sustained activation of

TLR expression may prime the development of chronic inflammatory disorders and autoimmune diseases. Another important finding was that probiotics have a critical role in modulating the immune response in macrophage cell subsets through modulation of PRRs in context of modulation of endogenous inhibitor expression such as Tollip. This modulation might open a new window in the treatment of IBD targeting TLR expression, since the uncontrolled TLR expression have been implicated in several autoimmune and inflammatory gut diseases such as IBD (Wehkamp et al., 2004, Steenholdt et al., 2009). Various negative regulatory mechanisms have evolved to attenuate TLR signalling, and Tollip expression is one of these mechanisms. The first observation was reported that the identification of Tollip initially as an intermediate in IL-1 signalling. IL-1 is a pro-inflammatory cytokine that elicits its pleiotropic effects through activation of the transcription factors NF-κB and AP-1 (Sims et al., 1994). IL-1 receptor consists of two different chains IL-1R1, IL-1RAcP. In resting cells, Tollip forms a complex with IRAK and inhibits IL-1 induced signalling by blocking IRAK phosphorylation, whereby recruitment of Tollip-IRAK complexes to the activated receptor complex occurs through association of Tollip with IL-1RAcP (Burns et al., 2000). Therefore, over-expression of Tollip results in reduced NF-κB activation and limits the inflammation by decreasing cytokine production. Zhang and Ghosh (2002) reported that Tollip associates directly with TLR2 and TLR4 and plays an inhibitory role in TLR-mediated cell activation, explaining that the inhibition by Tollip is mediated through its capability to suppress the activity of IL-1 receptor-associated kinase (IRAK) after TLR activation. Probiotics have been demonstrated to inhibit the inflammation (Rautava et al., 2005, Thomas and Versalovic, 2010), but the mechanisms behind their effects are still poorly understood. The results of this study showed that the probiotic treatments selectively modulated LPS induction of Tollip expression in

macrophage cell subsets. The results of this study indicated that HK-LcS treatment augmented LPS induced Tollip expression in M1-like macrophages leading to inhibition further amplification of inflammation. Whereas, HK-LF treatment suppressed LPS induced Tollip expression; facilitating more chance to initiate inflammation by enhancing IRAK phosphorylation after the activation of TLR expression. Several reports highlighted the anti-inflammatory effects of LcS-PS via down-regulation of pro-inflammatory cytokines in the lamina propria (Matsumoto et al., 2005, Matsumoto et al., 2009, Shida et al., 2009). One unanticipated finding was that LcS treatment augmented LPS induced Tollip expression. This finding has important implications for developing new tools in the inhibition of pro-inflammatory cytokines leading to diminishing the inflammation associated with overproduction of pro-inflammatory cytokines by M1-pro-inflammatory macrophage cell subset.

3.3.5. HK and SP probiotic bacterial strains selectively modulate M1 and M2 macrophage subset production of inflammatory mediators: CD14-dependency

Resting gut mucosal macrophages are characterised by lacking CD14 expression in contrast with pro-inflammatory macrophages which highly express CD14, mediate expression of pro-inflammatory cytokines in response to stimulation with pathogenic bacteria and their PAMPs (Platt & Mowat, 2008). The current study found that pro-inflammatory cytokines, TNF-α and lL-6, expressed by macrophages M1, and M2 whether $CD14^{hi}$ or $CD14^{lo}$ in response to LPS stimulation, were differentially modulated by probiotic bacterial treatments. Probiotic immunomodulation was dependent on the macrophage subset; CD14 expression and bacterial strain preparation (HK or SP), refer to table 3.2.3. Probiotic bacteria used in this study exhibit a strong pro-inflammatory effect on

$CD14^{hi}$ M1 macrophages which mimic infiltrating, inflammatory mucosal macrophages. Both HK and SP preparations augmented LPS-induced TNF-α production in these macrophages.

Generally, TNF-α expression of $CD14^{lo}$ M1 macrophage was partially inhibited by these probiotic preparations with the exception of HK-LR, BB-SP and LS-SP. A clear observation was noticed in the contrasting data between $CD14^{hi}$ and $CD14^{lo}$ M1s, this indicates an important role for CD14 expression in probiotic immunomodulation. With regard to $CD14^{hi}$, M2 macrophage TNF-α production was differentially modulated by HK and SP probiotics. M2 macrophages produced higher levels of IL-6 than M1s. Both $CD14^{hi}$ and $CD14^{lo}$ M2 macrophage IL-6 production was suppressed by both HK- and SP probiotic treatments used in this study. For the first time in this study, BB demonstrated a clear immunoregulatory capacity, where both SP and HK extracts suppressed IL-6 in both $CD14^{hi}$ and $CD14^{lo}$ M2s. Thus, probiotics differentially modulate the pro-inflammatory cytokines TNF-α and IL-6 in LPS-stimulated M1 and M2 macrophages. Macrophages characterised by CD14 expression were associated with Crohn's disease through vast expression of pro-inflammatory cytokines particularly TNF-α, IL-6 and IL-1β which participate in the gut tissue destruction (Kamada et al., 2008). CD14 has been defined to be both pro-inflammatory, via oligomerisation with the LPS receptor, TLR4 (Wright et al., 1990), and anti-inflammatory through its action as a scavenger receptor for apoptotic cells (Devitt et al., 1998). In fact, cytokine production acts as a useful readout for probiotic immunomodulation. The relative suppression of TNF-α in $CD14^{lo}$ macrophages would suggest that modulation is as a consequence of down-regulation of the CD14/TLR4/TLR2 pro-inflammatory complex rather than a downstream suppressive function of IL-10 or TGF-β induced

by the recognition and phagocytosis of apoptotic cells. M2 IL-6 production was suppressed by probiotics with a stronger suppression evident in $CD14^{hi}$ M2s; regarding the fact that IL-6 is both pro- and anti-inflammatory properties, probiotic suppression of this cytokine may exhibit inflammatory and tolerogenic/suppressive functions. Modulation of M1 IL-6 production was less clear and exhibited both suppressive and augmentation responses for both $CD14^{hi}$ and $CD14^{lo}$ which appeared to be strain selective and dependent on probiotic bacterial preparation (HK or SP).

Pro-inflammatory cytokines may be modulated indirectly by probiotic bacteria through the production of anti-inflammatory/regulatory cytokines. Anti-inflammatory activity by IL-10 induces SOCS proteins, which suppress the activity and expression of several cytokines; one such example includes IFN-γ, which has a significant role in activation and differentiation of pro-inflammatory M1 macrophages. Interestingly, like for IL-10 regulation, IL-6, when acting as an anti-inflammatory mediator, induces SOCS-1 expression that inhibits Th1 cytokine expression (Diehl et al., 2000). This negative feedback mechanism may partially explain the contradictory probiotic regulation observed between IL-6 and TNF-α production for $CD14^{hi}$ M1 and M2 macrophages. Moreover, there is a reciprocal relationship between IL-6 and TNF-α, which observed in conditions whereby TNF-α is augmented or highly expressed; IL-6 expression is low or suppressed (Ahmed and Ivashkiv, 2000). Therefore, probiotic bacteria are capable of both driving immune responses towards predominant Th1 and Th2 responses and suppressing such responses. This suggests probiotics can manipulate and redress immunopathological mechanisms; Th1-driven pathologies such as Crohn's disease, may benefit from probiotics that either suppress harmful immune

reactions or induce type II cytokines (IL-10, IL-4, IL-13) and conversely, Th2-driven pathologies such as ulcerative colitis, may benefit from immunosuppressive probiotics or those that induce type I cytokine expression (IFN-γ, IL-12 and TNF-α). Combinations of probiotics will allow the development of disease-group specific treatments (Th1 or Th2-driven) based on a thorough understanding of the immunopathogenic mechanisms. These data must be interpreted with caution because some probiotics may be inappropriate in inflammatory pathologies where $CD14^{hi}$ M1 subset predominate, since probiotic treatment may enhance inflammation by augmentation of TNFα production: a cautionary approach to their usage is recommended. On the other hand, probiotic treatment of $CD14^{lo}$ M2s, resembling homeostatic/regulatory mucosal macrophages (Platt & Mowat, 2008; Smith et al., 2001; Smythies et al., 2005), fails to augment inflammatory cytokines and may well induce expression of the regulatory cytokines, IL-10 and TGF-β, resulting in tolerance/immune hypo responsiveness. The present results are significant in at least major two respects; CD14 expression associated with cytokine expression in macrophage cell subsets and the differential role of probiotics in modulation of macrophage subset cytokine expression. Further research should be done to investigate the downstream cell signalling targeting CD14 expression in order to understand the probiotic immunomodulation at the cellular level.

3.3.6. SP and HK probiotic bacterial strains selectively modulated LPS induced macrophage subset NF-κB activity

It is well established that LPS induction of monocyte/macrophage pro-inflammatory cytokine expression depends on the regulation of NF-kB activation (Bondeson et al., 1999). Libermann and Baltimore (1990) reported that the

promoter region of the IL-6 gene has a putative NF-kB-binding site. Collart et al. (1990) reported that the stimulation of macrophages with LPS resulting in increased TNF-α transcription related with an increased content of NF-kB. Thus, it was predicted that NF-kB activation would match probiotic regulation of TNF-α and IL-6 cytokine production by macrophage subsets. However, the results of this study showed that the only profile that partially parallels NF-kB activation is the regulation of IL-6 production by $CD14^{hi}$ M1s (refer to table 3.2.3). M2 macrophage NF-kB activation is partially augmented by probiotic bacteria, nevertheless, regulation of IL-6 resulted in the opposite effect, suppression: this suggested that probiotic modulation of IL-6 was NF-kB- independent. Furthermore, probiotic up-regulation of TNF-α by pro-inflammatory $CD14^{hi}$ M1 macrophages were also NF-kB-independent, as NF-kB was either partially suppressed or unaltered. Based on this poor level of association of NF-kB activation with cytokine production, it is probable that other signalling pathways are involved in probiotic regulation such as MAPKs and NOD-2. NOD-2 exhibits a vital role in preventing IBD (Natividad et al., 2012), through expression of short and long splice variants can positively or negatively regulate NF-kB activity (Girardin et al., 2003b, Rosenstiel et al., 2006). Therefore, it is possible to hypothesise that the immunomodulation by probiotics are likely to occur via modulation of NOD-2 expression leading to modulated NF-kB activity.

There are, however, other possible explanations of probiotic immunomodulation related with NF-kB activation mediating the up-regulation or suppression of cytokine expression, such as induction of endotoxin tolerance to microbial PAMPs leading to regulated NF-kB activation. In this study, tolerance may be initiated via chronic LPS stimulation or cross-toleration through NOD2, TLR2, TLR-4, TNF-R,

and IL-1βR (Foey and Crean, 2013, Ferlito et al., 2001) signalling. In general, therefore, it seems that regulation/tolerance is likely to be dependent on environmental stimuli, macrophage lineage, and CD14 expression. Future research will focus on the mechanisms of probiotic modulation of endotoxin tolerance; facilitate a comprehensive mechanistic understanding of probiotic immunomodulatory functions (either as immune activatory or suppressive functions) leading to improved tools in the treatment of such diseases (IBD).

Chapter 4

Modulation of inflammatory responses by probiotic bacteria in a Caco-2 cell line model

Chapter 4: Modulation of inflammatory responses by probiotic bacteria in a Caco-2 cell line model

4.1. Introduction

It is becoming increasingly difficult to ignore the role of the mucosal surface in aspects of microorganisms' interaction with the gut mucosa. Gut epithelial cells represent the main sites at which environmental microorganisms and antigens interact with the host (Madara, 2004). They are one of the most important groups of surveillance systems; monitoring gut contents via sensing of microbial PAMPs through a group of PRRs leading to maintenance of the gut mucosa. Consequently, epithelial cells participate in or regulate both innate as well as adaptive immune responses (Flintoft, 2010). Epithelial cells recognise the microorganisms that are present in the gut lumen by a discriminatory system including TLRs and NLRs, which transduce signals from the gut lumen to the adjacent immune cells resident at the lamina propria such as the macrophages, DCs and lymphocytes (Abreu et al., 2005, Janssens and Beyaert, 2003, Kim et al., 2008, Takeda et al., 2003). They perform this task via molecules expressed on the cell surface, such as MHC I, MHC II, TLRs and cytokine cell receptors (Cario and Podolsky, 2000b). The outcome of the epithelial cell interactions with various stimuli is the release of many mediators such as cytokines and antimicrobial peptides (AMPs). Epithelial cells express a range of cytokine receptors that activate after binding with specific cytokines, followed by activation of specific transcriptional factors such as NF-kB, AP-1 and the STAT family, resulting in DNA binding and production of a range of cytokines (Bahrami et al., 2011b). Normally, cytokines can regulate each other; for example, TNF-α can induce IL-10 in monocytes (Foey et al., 1998) and IL-8 in epithelial cells (Eckmann et al., 1993a). Dignass and Podolsky (1993) reported

that mouse epithelial cells release TGF-β, promoting epithelial restitution and repair of tissue injury. In fact TGF-β is one of the main mediators promoting gut tolerance in mammalian species (Duchmann et al., 1995). Indeed, the epithelial-mucosal barrier of the gut is integral to the maintenance of tolerance to luminal bacteria and food-borne antigens. The cellular interactions between epithelial cells and immune cells of the GALT are pivotal to this mucosal tolerance. Over the past century, there has been a dramatic increase in IBD as a result of losing tolerance to the gut flora (Molodecky et al., 2011). Recently, researchers have shown an increased interest in AMP expression particularly, hBD-2 because they kill pathogenic microorganisms, and at the same time modulate the mucosal immune response (Huttner and Bevins, 1999, Klüver et al., 2006). Therefore, the determining factors controlling the hBD-2 expression are very important in terms of maintaining gut mucosa associated with barrier function. Central to the entire discipline of hBD-2 expression is the concept of a clinical association between disease, especially between IBD and deficiency in hBD-2 production (Baumgart & Carding, 2007; Wehkamp *et al*., 2005). Based on the main proposed mechanisms of probiotic action exerted in the host which include modulation of the gut microbial content; maintenance of the integrity of the gut barrier (prevention of bacterial translocation) and modulation of the local immune response by the gut-associated immune system, recent developments in the field of probiotics have led to a renewed interest in the probiotic role in the treatments of gut diseases (Klaenhammer et al., 2012). Kotzampassi et al. (2012) reported that satisfactory evidence from randomised clinical trials (RCTs) is available to support the therapeutic use of probiotics in decreasing the incidence of antibiotic-associated diarrhoea (AAD) and *Clostridium difficile* infection (CDI) and acute gastroenteritis. Probiotic treatments shorten the duration of symptoms when administered in

paediatric populations with acute gastroenteritis. However, it should be highlighted that all analyses RCTs is considered by the type of probiotic species. Generally, probiotics are beneficial in the treatment and prevention of GIT diseases, for example, probiotics had a positive significant effect on pouchitis, infectious diarrhoea, irritable bowel syndrome (IBS), *Helicobacter pylori*, CDI, and ADD. Despite this, similar efficacy was not observed for traveller's diarrhoea or necrotizing enterocolitis associated with the use of probiotic species *L. acidophilus*, *L. plantarum*, and *B. infantis* (Ritchie and Romanuk, 2012). Therefore, choice of probiotics is essential in the treatment or prevention of GIT disease; two factors need to be taken into consideration; type of probiotic stain and type of disease.

Caco-2 cells were used as a model for intestinal epithelial cells in this study. Caco-2 cells after full differentiation become enterocyte/M-like cell, and they express markers such as Ulex europaeus agglutinin (UEA)-1 mediated endocytosis (Gabor et al., 1998), CD155 mediated *Poliovirus* transcytosis (Ouzilou et al., 2002), and *Salmonella* pathogenicity island 1 (SP-1) mediated *Salmonella* translocation (Martinez-Argudo and Jepson, 2008). They express sophisticated responses to inflammatory stimuli. TNF-α and IL-1β are the main cytokines mediate gut pathology, leading to destruction of epithelial tissue in IBD (Cesaro et al., 2009, Cominelli and Pizarro, 1996). Numerous studies showed that treating Caco-2 cells with TNF-α induced a range of mediators including lL-8, ICAM-1, IP-10, MCP-1, TNF-α and MMP-1 (Sonnier et al., 2010, Treede et al., 2009), and treating cells with IL-1β also induced lL-2 and RANTES receptors, CCR1 (Rodríguez-Juan et al., 2001). Several studies showed that probiotics have a significant role in inducing cytokine expression in Caco-2 cells (Hosoi et al., 2003, Bahrami et al., 2011b). In addition, studies showed that probiotics regulate cytokine production in Caco-2

induced by inflammatory cytokines; probiotics, *L. paracasei or L. plantarum,* regulated IL-6 production in Caco-2 cells treated with IL-1β (Reilly et al., 2007). In addition to their roles in inducing cytokines, probiotics exhibit a significant role in inducing hBD-2 (Wehkamp et al., 2004). However, probiotic role in the regulation of cytokine (exogenous, membrane bound and intracellular) induced hBD-2 by human intestinal epithelial cells/ Caco-2 is so far not known. This study aimed to investigate the probiotic bacterial role in regulation of cytokines associated with hBD-2 expression, (refer to sections 2.2.4.1, 2.2.4.3, 2.2.4.5, 2.2.4.6), in order to build up a strong platform of knowledge about probiotic bacterial mechanisms in maintaining gut mucosa.

The main questions addressed in this investigation are:

Hypothesis 1: Probiotic bacterial strains are able to induce cytokine and hBD-2 expression by intestinal epithelial cells (Caco-2 cells).

Hypothesis 2: Probiotics regulate inflammatory signal (TNF-α and IL-1β)-induced cytokines and hBD-2

Hypothesis 3: Probiotics regulate inflammatory signal (TNF-α and IL-1β)-induced epithelial TLR and NLR expression.

4.2. Results

4.2.1. Probiotic induction of IL-8 and hBD-2 production in epithelial cells

To assess probiotic bacterial role in inducing cytokines and hBD-2 expression, LcS and LF probiotic bacteria whether heat killed or live bacterial cell format were used to treat the cultures of fully differentiated Caco-2 epithelial cells as described in section 2.2.4. The results, as shown in Fig.4.2.1, showed that both live and heat

killed probiotic bacteria induced IL-8 cytokine production. Live bacteria up-regulated IL-8 by 714% and 285% of the control (7±0.0 pg/ml) at 12 hours, diminishing to 285% and 214% at 24 hours for LcS and LF respectively, at a density of $3x10^3$ cfu/ml. This induction was both time-and bacterial density-dependent, reaching maximal levels of 7500% (LcS) and 5600% (LF) at 12 hours for $3x10^9$ cfu/ml and persisting at appreciable levels to 24 hours (Fig. 4.2.1A&B). HK cell format also induced IL-8 up to 214%, 142% at 12 hr, 285%, 814% at 24 hr at a density of $3X10^3$ cfu/ml, augmenting to 685%, 342% at 12 hr, 842%, 1000% at 24 hr at a density of $3x10^6$ cfu/ml by LcS and LF respectively. Results showed that the induction of IL-8 was both time-and bacterial density-dependent, reaching maximal levels of 342%, 442% at 6 hr, 1371%, 1300% at 12 hr, 1928%, and 1500% at 24 hr at a density of $3x10^9$cfu/ml (Fig. 4.2.1C&D). Live bacteria induced hBD-2 up to 616% and, 533% of the control (12.1±.9 pg/ml) at a density of $3x10^3$ cfu/ml, diminishing to 416% and 408% at a density of $3x10^6$ cfu/ml, and further diminishing to 358% and 333% at 24 hr for LcS and LF respectively, at a density of $3x10^9$ cfu/ml (Fig.4.2.2A). Using HK format, hBD-2 production was augmented (from control, 12.1±.9 pg/ml) by 408% and 366% at a density of $3x10^3$ cfu/ml, augmenting to 491% and 408% at a density of $3x10^6$ cfu/ml, and further augmentation of 725% and 575% at 24 hr by LcS and LF respectively a density of $3x10^9$ cfu/ml (Fig.4.2.2B).

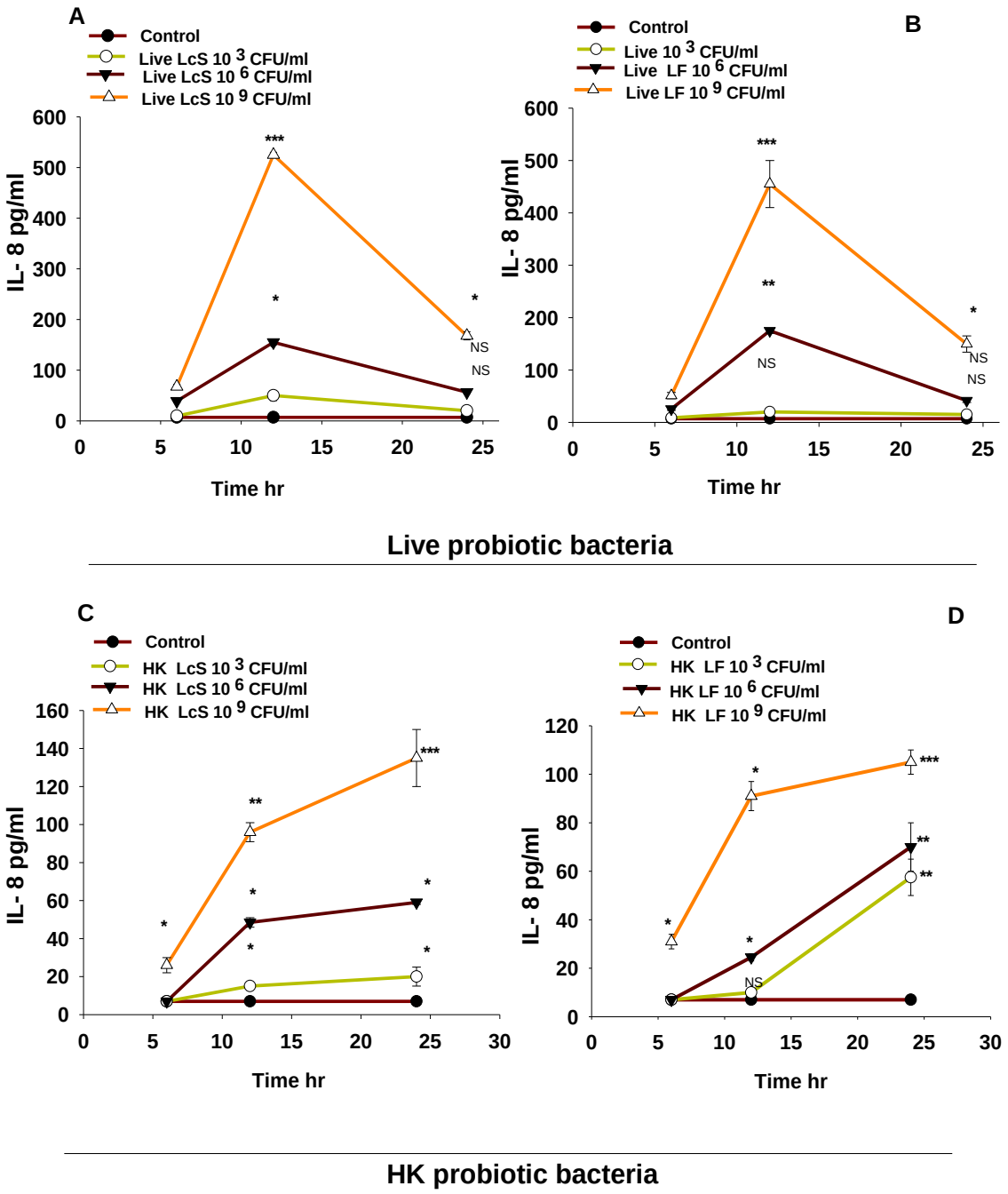


Figure 4.2. 1 : Probiotics induce IL-8 by Caco-2 cells.

Caco-2 epithelial cells were treated with different concentration of *Lactobacillus casei strain* Shirota (LcS) or *L.fermentum* (LF) as a live cell format (**A**, **B**) or heat killed format (**C**, **D**) at a density of $3x10^3$, $3x10^6$, and $3x10^9$ cfu/ml for 6 hr,12 hr, and 24 hr. IL-8 cytokine production is expressed as mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to the control to the indicated epithelial cells are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

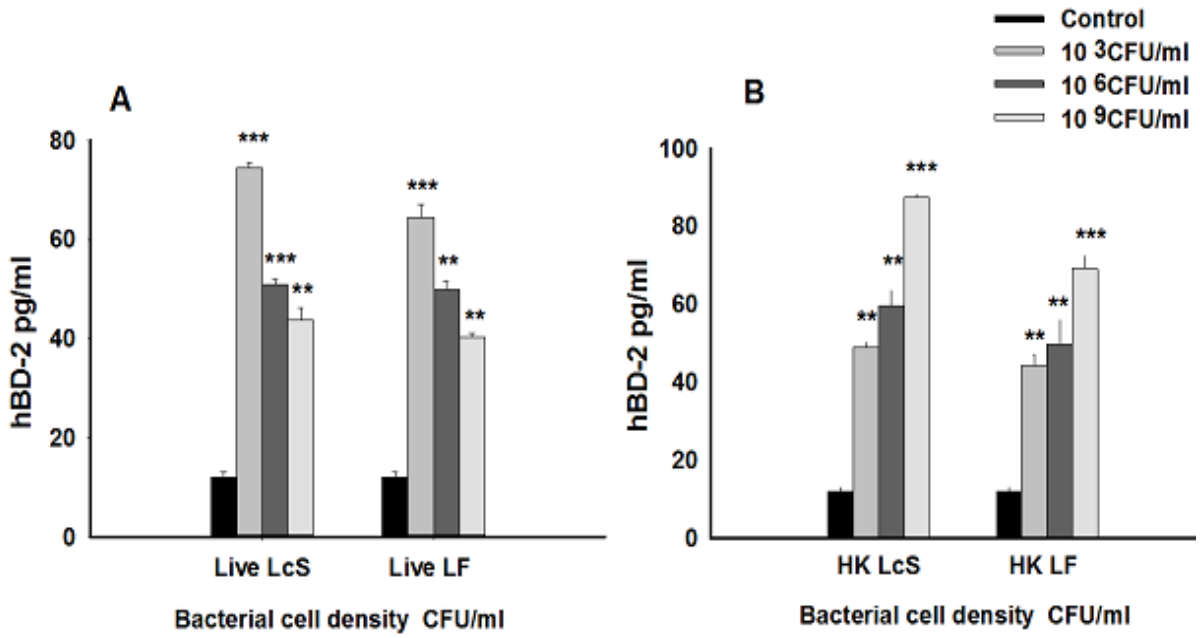


Figure 4.2. 2: Epithelial hBD-2 induction by probiotic bacteria.

Caco-2 epithelial cells treated with different concentration of *Lactobacillus casei strain* Shirota (LcS) or *L.fermentum* (LF) as heat killed (HK) (**B**) or live bacterial cell format (**A**) at cell densities of $3x10^3$, $3x10^6$, and $3x10^9$ CFU/ml. hBD-2 production expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to the control for the indicated probiotic treatment are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

4.2.2. Role of probiotic bacteria in the modulation of cytokine expression induced by TNF-α and IL-1β in epithelial cells

Simple statistical analysis was used to determine the level of cytokine expression during TNF-α and IL-1β stimulation as a time course (please see section 2.2.4.3), using one way analysis of variance (ANOVA). Fig.4.2.3 shows that the stimulation of epithelial cells with TNF-α and IL-1β induced an array of cytokines. These cytokines differ in time to reach peak expression dependent on the type of stimuli. The peak of IL-8 cytokine production induced by TNF-α was at 12 hr (Fig.4.2.3A), TNF-α at 18 hr (Fig.4.2.3B), IL-10 at 9 hr (Fig.4.2.3C), IL-6 at 4 hr (Fig.4.2.3D). IL-

8 induced by IL-1β reached its peak at 6 hr, TNF-α at 12 hr, IL-10 at 4 hr and IL-6 at 9 hr. What is interesting in this data is that the kinetics of cytokine production by epithelial cells was stimulus dependent. As the epithelial cytokine release was time and-stimulus dependent, hBD-2 release was also timely and stimuli dependent. hBD-2 reached peak production at 12 hr using IL-1β, whereas it peaked at 18 hr using TNF-α (Fig. 4.2.4). A significant positive correlation was found between TNF-α (R= 0.95), IL-10 (R = 0.68) and the hBD-2 expression using TNF-α to stimulate epithelial cells, whereas only TNF-α (R=0.78) and hBD-2 were positively correlated using IL-1β as a stimulus. Interestingly, this correlation is related to the significant role of TNF-α in inducing hBD-2 expression whether using TNF-α or IL-1β as stimuli.

Uncontrolled expression of TNF-α and IL-1β are the main factors associated with gut pathology. Using these cytokines to induce cytokine expression in Caco-2 epithelial cells resulted in different profiles of cytokine expression as shown earlier, therefore, this experiment was undertaken to establish whether heat-killed probiotics (in the absence of any nonspecific effects of lactic acid produced) from a panel of probiotic bacteria exert immunomodulatory effects on cytokine expression of IL-8, TNF-α, IL-6, and IL-10. In addition, hBD-2 release by epithelial cells induced by TNF-α or IL-1β was quantified (please refer sections 2.2.3.7 and 2.2.4.1). It is apparent from this data that probiotic treatments selectively regulated cytokine expression induced by TNF-α or IL-1β in Caco-2 cells. Results showed that the probiotic treatments augmented TNF-α induced IL-8 mRNA levels, and among the probiotic treatments, LR exhibited the highest induction of TNF-α induced IL-8 (Fig.4.2.5A). Probiotic treatments also augmented TNF-α induced TNF-α mRNA levels, however, LcS exhibited the highest induction of TNF-α

induced TNF-α mRNA level (Fig.4.2.5B). Probiotic treatments selectively regulated IL-10 expression, BB, LS, LR, LcS, and LP augmented TNF-α induced IL-10 mRNA levels, and among them BB treatment exhibited the highest induction of TNF-α induced IL-10. LF treatment, however, had a significant suppressive effect on TNF-α induced IL-10 (Fig.4.2.5C). IL-6 expression induced by TNF-α was also selectively modulated by probiotic bacteria - BB, LS, LR, LF or LP augmented IL-6, whereas LcS exhibited no effects. Among the probiotic treatments, LR exhibited the highest induction of TNF-α induced IL-6 (Fig.4.2.5D). Results showed that LR exhibited the highest induction of TNF-α induced IL-8 and IL-6, whereas, LcS induced the highest induction of TNF-α, and BB induced the highest induction of IL-10 mRNA level.

At the protein level, IL-8 cytokine production induced by TNF-α was significantly augmented by probiotic treatments: LS treatment exhibited the highest induction of TNF-α induced IL-8 (Fig.4.2.5A1). For TNF-α, results indicated that BB, LS, LR, LcS down-regulated TNF-α induced TNF-α, and among them, LR treatment exhibited the highest suppressive effects on TNF-α (Fig.4.2.5B1). LF and LP treatments augmented TNF-α induced TNF-α, whereas LP treatment exhibited the highest induction of TNF-α (Fig.4.2.5B1). IL-10 production was up-regulated by BB, LS, LR or LcS, and again BB treatment exhibited the highest induction of TNF- α induced IL-10. LF and LP treatments suppressed TNF-α induced IL-10 (Fig.4.2.5C1). IL-6 induced by TNF-α was up-regulated by LR more than by LS whereas, BB, LcS, LF or LP exhibited no effects in modulation of TNF-α induced IL-6 (Fig.4.2.5D1).

Further analysis showed that probiotics selectively modulated cytokine production induced by IL-1β. BB, LS, LR, LcS, LF and LP augmented IL-1β induced IL-8

mRNA levels, and among probiotic treatments, LR exhibited the highest induction of IL-8 mRNA (Fig.4.2.6A). LcS treatment exhibit the highest induction of TNF-α mRNA level, whereas LF and LP had no effect on regulation of IL-1β induced TNF-α levels (Fig.4.2.6B). LF and LP had significantly augmented IL-10, whereas BB, LS, LR, LcS suppressed IL-1β induced IL-10 (Fig.4.2.6C). IL-6 mRNA level induced by IL-1β was suppressed by BB, LS and LR, and augmented by LcS and LF (Fig.4.2.6D). At the protein, level LF probiotic treatment exhibited the highest induction of IL-1β induced IL-8, whereas only LP significantly suppressed IL-1β induced IL-8 (Fig.4.2.6A1). TNF-α expression induced by IL-1β was suppressed by LR, LcS and LP, whereas BB, LS, and LF exhibited no modulatory effect on IL-1β induced TNF-α (Fig.4.2.6B1). IL-10 induced by IL-1β was down-regulated by BB, LS, LR, and LcS, whereas LF or LP augmented IL-1β induced IL-10 (Fig.4.2.6C1) Among probiotic treatments, LcS exhibited the highest suppressive effects on IL-1β induced IL-10, whereas, LF treatment exhibited the highest augmentation of IL-10. IL-6 induced by IL-1β was up-regulated by BB, LS, LR and LcS, whereas suppressed by LF and LP (Fig.4.2.6D1). Overall, probiotic treatments exhibit two faces in modulating the immune responses by Caco-2 epithelial cells in the presence of two types of inflammatory factors (TNF-α and IL-1β).

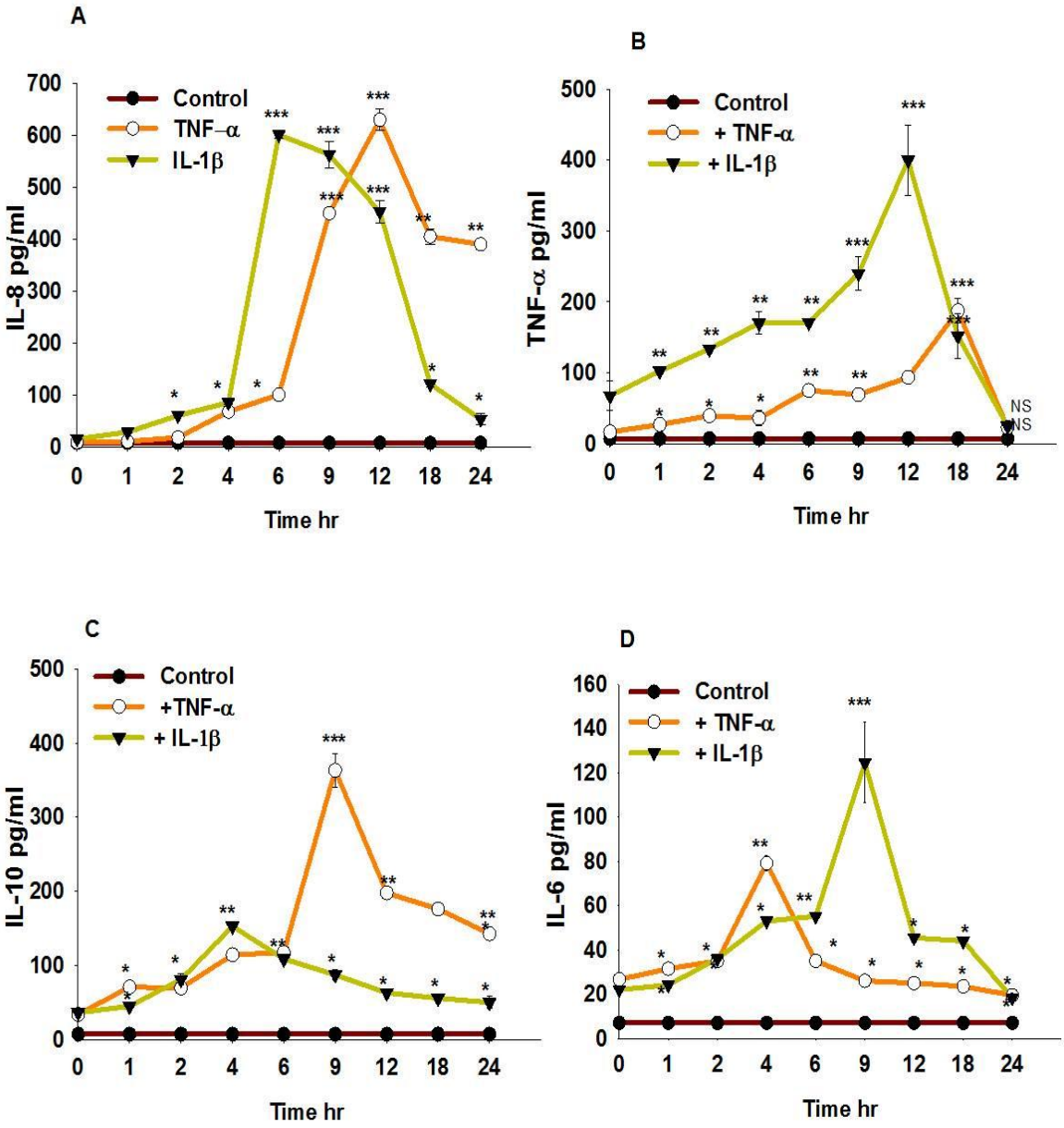


Figure 4.2. 3: TNF-α and IL-1β induce a range of epithelial cytokine production.

Caco-2 epithelial cells were treated with 10 ng/ml of TNF-α or 5 ng IL-1β at each time point (0, 1, 2, 4, 6, 9, 12, 18, and 24) hr. Cytokine IL-8 (**A**), TNF-α (**B**), IL-10 (**C**), and IL-6 (**D**) production is expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to the control (un-stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

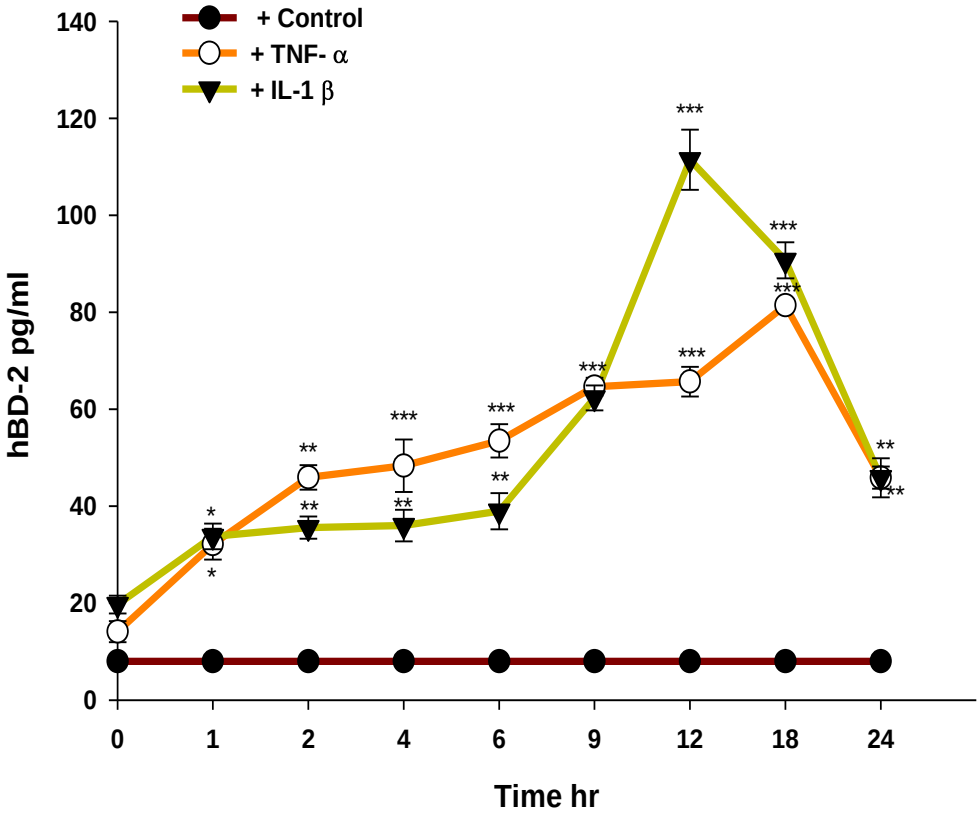


Figure 4.2. 4: Pro-inflammatory cytokines TNF-α and IL-1β induce hBD-2 in epithelial cells.

Caco-2 epithelial cells treated with 10 ng/ml of TNF-α or 5 ng IL-1β at each time point (0, 1, 2, 4, 6, 9, 12, 18, and 24) hr. hBD-2 production is expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to the control (un-stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

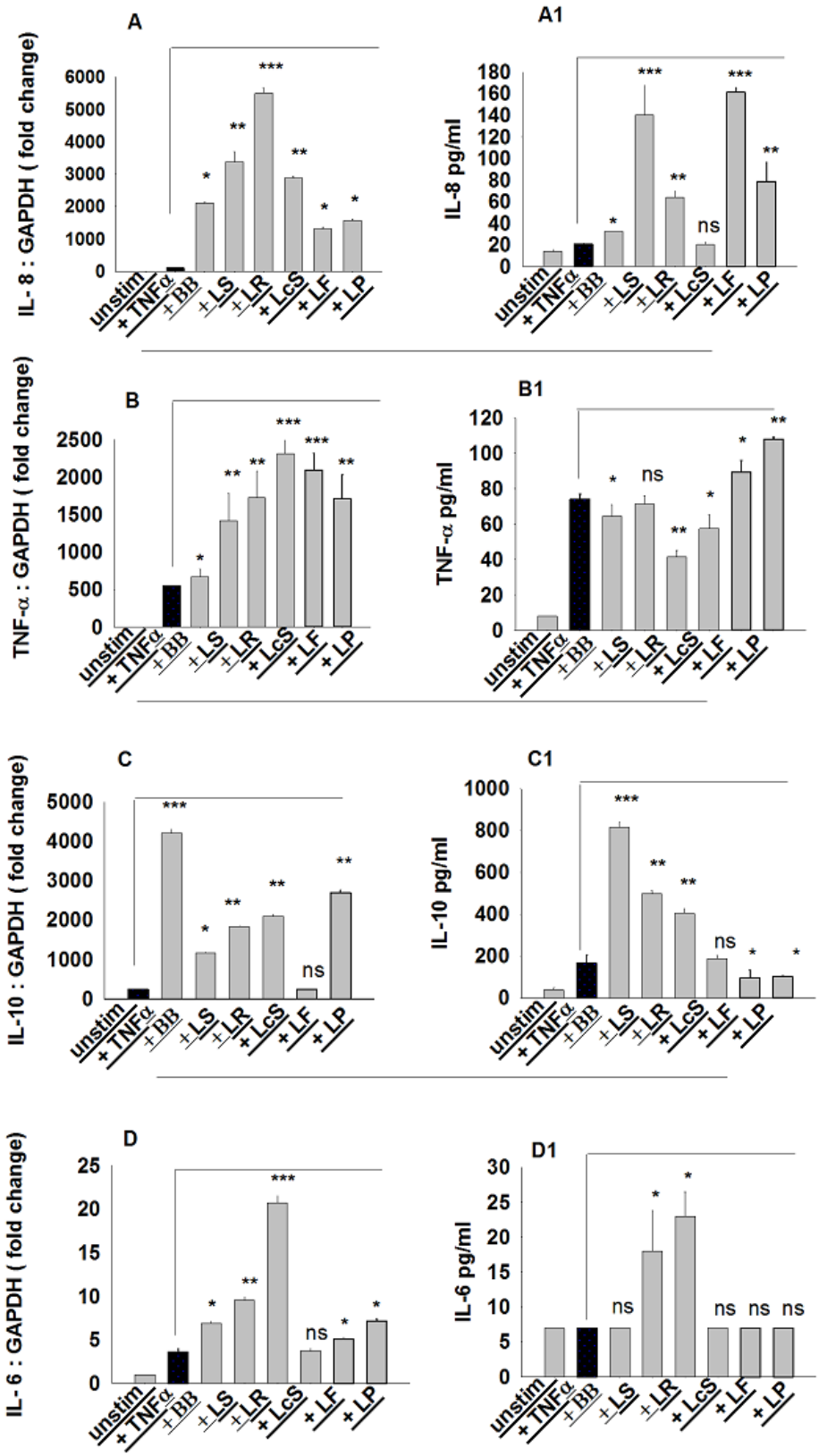


Figure 4.2. 5 : Heat killed probiotic bacterial strains selectively modulate epithelial cytokine expression induced by TNF-α.

Caco-2 cells pre-treated with heat killed (HK) of *B.breve* (BB), *L.salivarius* (LS), *L. rhamnosus GG* (LR), *L.casie strain* Shirota (LcS), *L.fermentum* (LF), and *L. plantarum* (LP) at a density of $3x10^8$ cfu/ml for 18hr, followed by stimulation with 10 ng/ml TNF-α. Cytokine production of IL-8 (**A1**), TNF-α (**B1**), IL-10 (**C1**) and IL-6 (**D1**) are expressed as the mean±SE in pg/ml and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al.(2001) using $2^{-\Delta\Delta Ct}$ (**A**, **B**, **C, D**). Data displayed is a representative experiment with triplicate samples of n=3 independent experiments. Significant effects compared to the control (+TNF-α) are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

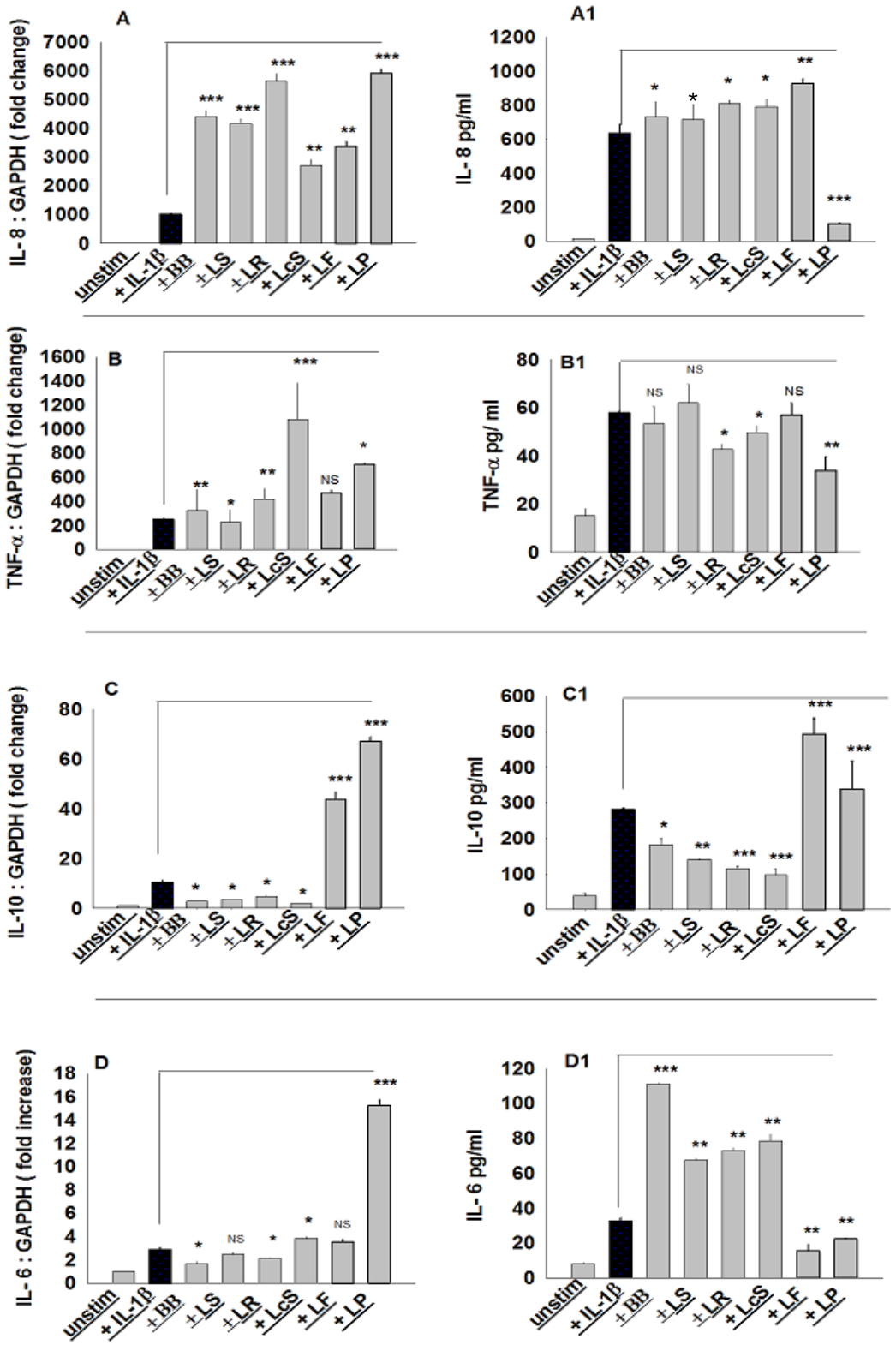


Figure 4.2. 6: Heat killed probiotic bacterial strains selectively modulate epithelial cytokine expression induced by IL-1β.

Caco-2 cells pre-treated with heat killed (HK) of *B.breve* (BB), *L.salivarius* (LS), *L. rhamnosus* GG (LR), *L.casei strain* Shirota (LcS), *L.fermentum* (LF), and *L. plantarum* (LP) at a density of

$3x10^8$ cfu/ml for 18hr, followed by stimulation with 5ng/ml IL-1β. Cytokine production of IL-8 (**A1**), TNF-α (**B1**), IL-10 (**C1**) and IL-6 (**D1**) are expressed as the mean±SE in pg/ml and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. (**A**, **B**, **C,** and **D**). Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control (+IL-1β) for the indicated epithelial cells are indicated as * P<0.05, ** P<0.01 *** P<0.005 and NS (non-significant).

4.2.3. Probiotics selectively regulated TNF-α or IL-1β induced hBD-2 in epithelial cells

To assess levels of hBD-2 expression in epithelial cells induced by TNF-α or IL-1β in the presence of probiotic treatments, fully-differentiated Caco-2 cells pre-treated with HK of BB, LR, LS, LcS, LF and LP followed by stimulation with 10 ng/ml TNF-α, (please refer section 2.2.4.1 and Fig.1.7 line 2). Results indicated that hBD-2 mRNA expression induced by TNF-α was differentially regulated by probiotics, LF and LP had significant effects on the augmentation of hBD-2 expression, whereas BB, LS, LR or LcS suppressed TNF-α induced hBD-2. Among probiotic treatments, LF exhibited the highest effects in augmentation of TNF-α induced hBD-2 Fig.4.2.7. At the protein level, BB, LS, LR, and LcS suppressed hBD-2 expression, whereas LF and LP augmented TNF-α induced hBD-2 (Fig.4.2.7A1). IL-1β was used to induce hBD-2 expression in Caco-2 epithelial cells; results showed that probiotic treatments selectively modulated IL-1β induction of hBD-2. *BB, LS, LR, LcS* augmented IL-1β induced hBD-2 expression, whereas LF and LP had no modulating effect on IL-1β induced hBD-2 mRNA (Fig.4.2.7B). At the protein level, BB, LS, LR and LcS augmented IL-1β induced hBD-2; whereas LF treatment suppressed IL-1β, induced hBD-2 and LP had no effect (Fig.4.2.7B1). The most striking result to emerge from the data is that probiotic treatments exert two sides in modulation of stimuli that induced hBD-2 expression.

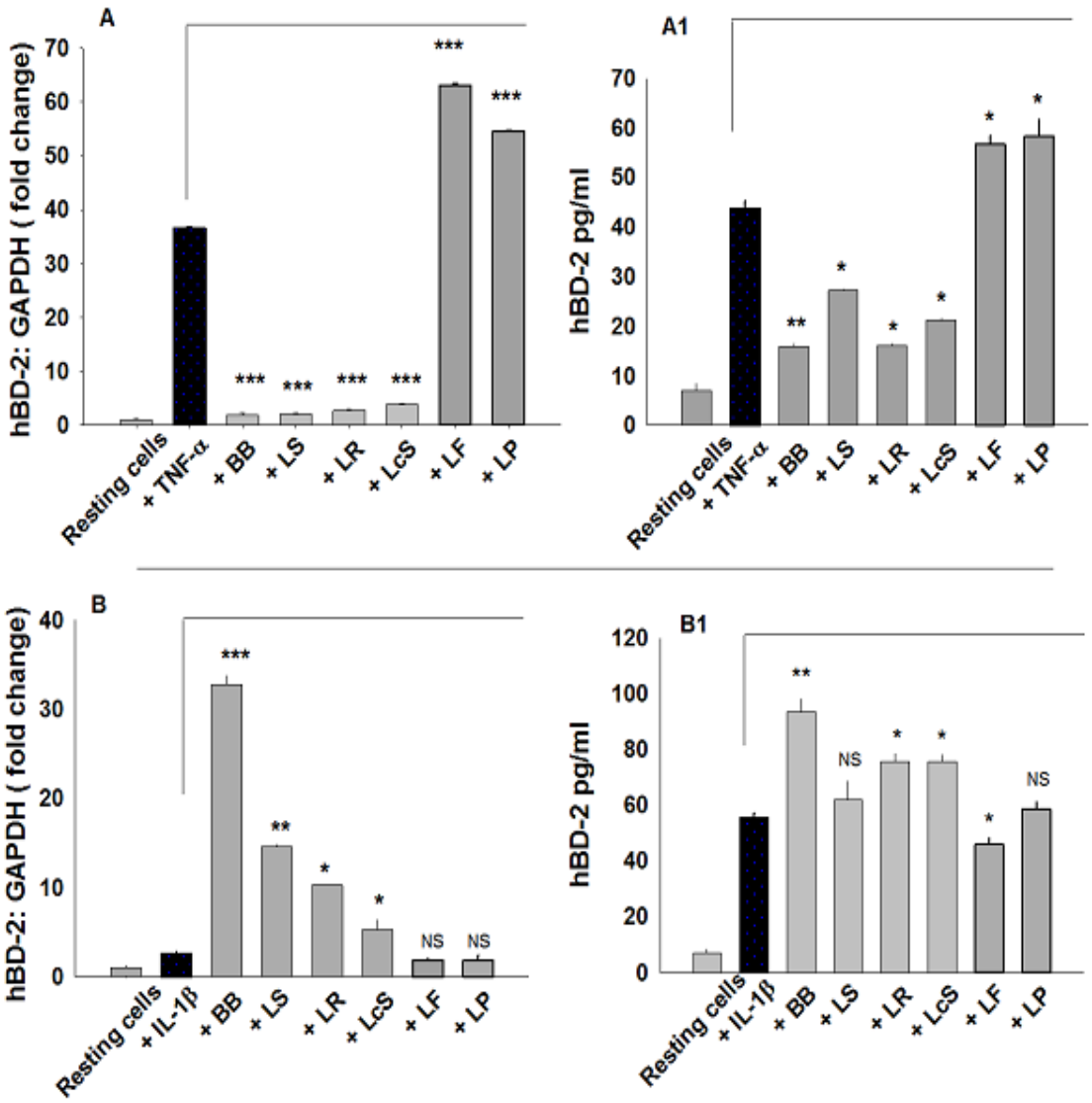


Figure 4.2. 7 : Heat killed probiotic bacterial strains selectively modulate epithelial hBD-2 expression induced by TNF-α or IL-1β.

Caco-2 epithelial cells pre-treated with heat killed (HK) *B.breve* (BB), *L.salivarius* (LS), *L.rhamnosus* GG (LR), *L. casei strain* Shirota (LcS), *L.fermentum* (LF), and *L.plantarum* (LP) at a density of $3x10^8$ cfu/ml for 18hr, followed by stimulation with 10 ng/ml TNF-α(**A**) or 5 ng/ml IL-1β (**B**). hBD-2 production is expressed as the mean±SE in pg/ml (**A1**, **B1**), and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. (**A**, **B**,). Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control (+IL-1β or +IL-1β) for the indicated epithelial cells are indicated as * P<0.05, ** P<0.01 *** P<0.005 and NS (non-significant).

4.2.4. Probiotic bacterial strains selectively modulate IL-1β or TNF-α induced cytokines after neutralisation of the bioactivity of TNF-α or IL-10 in epithelial cells

The first set of analyses examined the impact of probiotic bacterial treatments on modulation of the release of cytokines (cell associated or exogenous expression) induced by TNF-α and IL-1β (Fig.4.2.5& Fig.4.2.6). To assess the probiotic bacterial role in modulation of TNF-α or IL-1β induced epithelial cytokine expression after neutralisation of TNF-α or IL-10 bioactivity. The first set of analyses used cultures of Caco-2 cells pre-treated with probiotics in the presence or absence of anti-TNF-α or anti-IL-10 neutralising antibodies, and the second set of analyses was performed by pre-treating Caco-2 cells with probiotics followed by stimulation of cells with IL-1β or TNF-α in the presence of anti-TNF-α or anti-IL-10 antibodies (please refer section 2.2.4.6 and Fig.1.7 line 2).

In Fig.4.2.8, there is a clear trend of cytokine modulation induced by different stimuli in the absence of IL-10 or TNF-α. Results showed that treating resting cells with anti-IL-10 antibody results in up-regulation of TNF-α mRNA and protein levels Fig.4.2.8 A & A1. Stimulation of cells with IL-1β induced more TNF-α (mRNA and protein) than stimulation of cells with TNF-α. Treating cells stimulated with TNF-α with anti-IL-10 antibody caused up-regulation of TNF-α (mRNA and protein) Fig.4.2.8B & B1, whereas treating IL-1β-stimulated cells with anti-IL-10 antibody suppressed TNF-α (Fig.4.2.8C & C1). Determination of free cytokine TNF-α is indirectly determining the IL-10 bioactivity. Figure 4.2.8 showed that Caco-2 intestinal epithelial cells exhibit different profiles of IL-10 bioactivity, which is stimulus dependent. Stimulating cells with IL-1β suppressed the IL-10 bioactivity, whereas stimulating cells with TNF-α augmented IL-10 bioactivity in epithelial cells.

Data from Fig.4.2.5 can be compared with the data in Fig.4.2.6 which shows that probiotics exhibit either pro-inflammatory or anti-inflammatory effects via suppression or augmentation of the exogenous TNF-α and IL-10 expression, therefore, LcS (as a probiotic bacteria with a rigid cell wall resistant to lysozyme digestion) was chosen from one group, and LF (as a probiotic bacteria sensitive to lysozyme digestion) was chosen from another one (refer to section 2.2.4.1) to test the ability of probiotics in modulation of cytokines after neutralisation of IL-10 or TNF-α bioactivity. Results showed that treating resting cells with LcS and LF up-regulated TNF-α (mRNA and protein) levels. LcS suppressed TNF-α after neutralisation of IL-10, whereas LF treatment up-regulated it as mRNA or protein (Fig.4.2.9A & A1). Where cells were pre-treated with probiotics followed by stimulation with TNF-α in the presence of anti-IL-10 antibody, results showed that TNF-α mRNA and protein levels were up-regulated by LcS and LF after neutralisation of IL-10 bioactivity (Fig.4.2.9B & B1). In contrast, cells stimulated with IL-1β previously treated with probiotics in the presence of anti-IL-10 antibody, showed TNF-α augmented by probiotic (Fig.4.2.9C), whereas at protein level probiotics suppressed TNF-α induced by IL-1β after neutralisation of IL-10 bioactivity (Fig.4.2.9C1). Figure 4.2.9 showed that probiotic treatments negatively regulated IL-10 bioactivity when cells were stimulated with TNF-α and positively regulated IL-10 bioactivity when cells were stimulated with IL-1β. For assessing IL-10 expression after neutralisation of TNF-α bioactivity in Caco-2 cells (refer to section 2.2.4.6 and Fig.1.7 line 2), results indicated that neutralisation of TNF-α bioactivity suppressed IL-10 mRNA, and had no effect on protein level in resting cells. Treating resting cells with LcS or LF augmented IL-10 expression (mRNA and protein), suggesting that probiotics exhibited a significant role in the augmentation of IL-10 in the absence of TNF-α (Fig.4.2.10A & A1). Where cells

were pre-treated with probiotics then stimulated with TNF-α in the presence of anti-TNF-α antibody, IL-10 mRNA and protein levels were augmented by LcS and LF treatments (Fig.4.2.10B & B1). Similarly, stimulation of cells with IL-1β in the presence of anti-TNF-α antibody, the probiotics augmented IL-1β induced IL-10 (Fig.4.2.10C & C1). The data demonstrated that probiotic treatments exhibited significant effects in up-regulation of IL-10 induced by TNF-α or IL-1β in the absence of TNF-α.

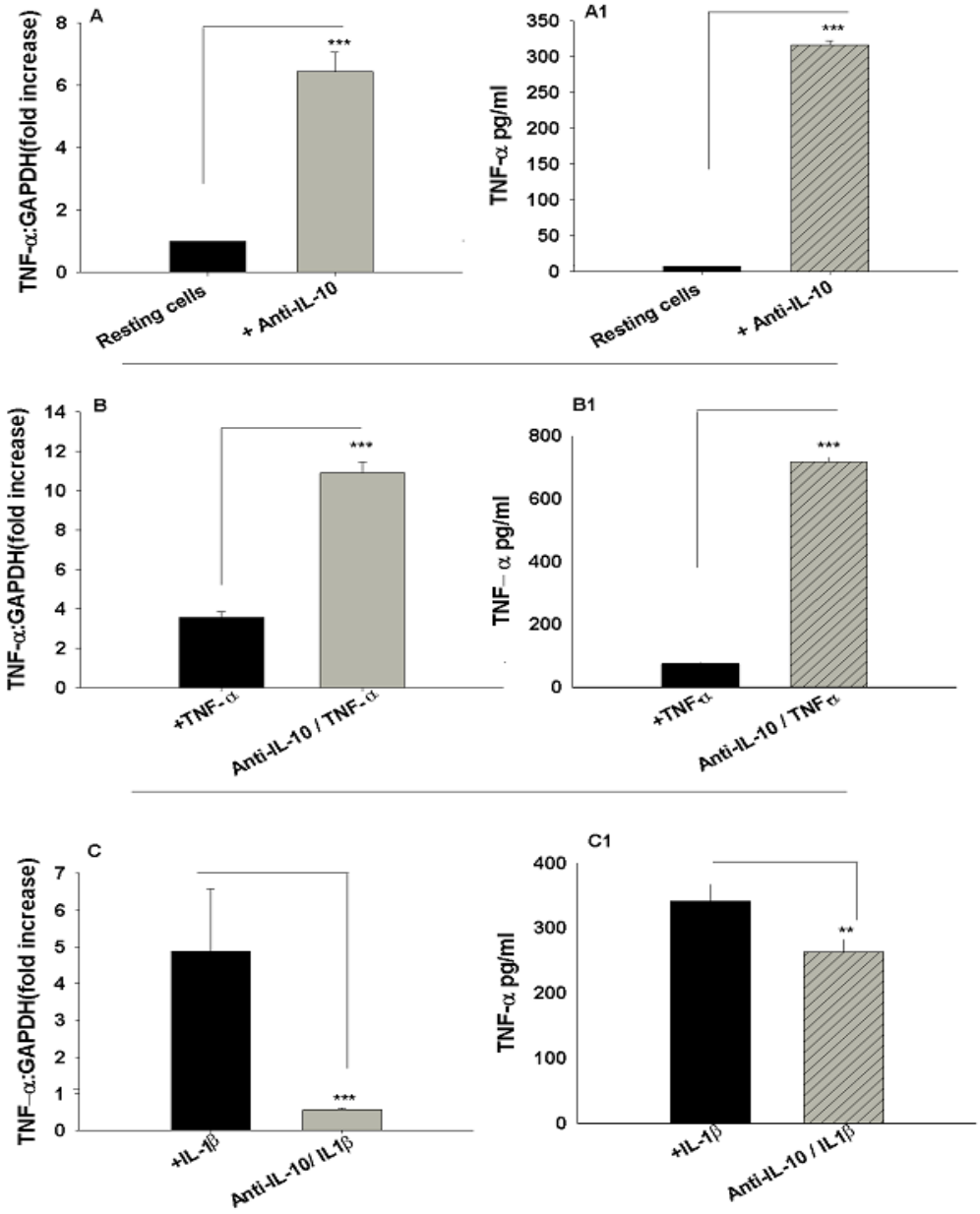


Figure 4.2. 8 : Caco-2 express membrane bond IL-10 selectively regulated by pro-inflammatory cytokines (TNF-α and IL-1β).

Caco-2 cells stimulated with 10 ng/ml TNF-α or 5 ng/ml IL-1β for 18hr followed by treating with 10 µg/ml of anti-IL-10 antibody for 18hr. Cytokine production is expressed as the mean±SE in pg/ml (**A1**, **B1**, **C1**) and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (A, B, C). Data displayed is a representative experiment with triplicate sample of n=5 independent experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

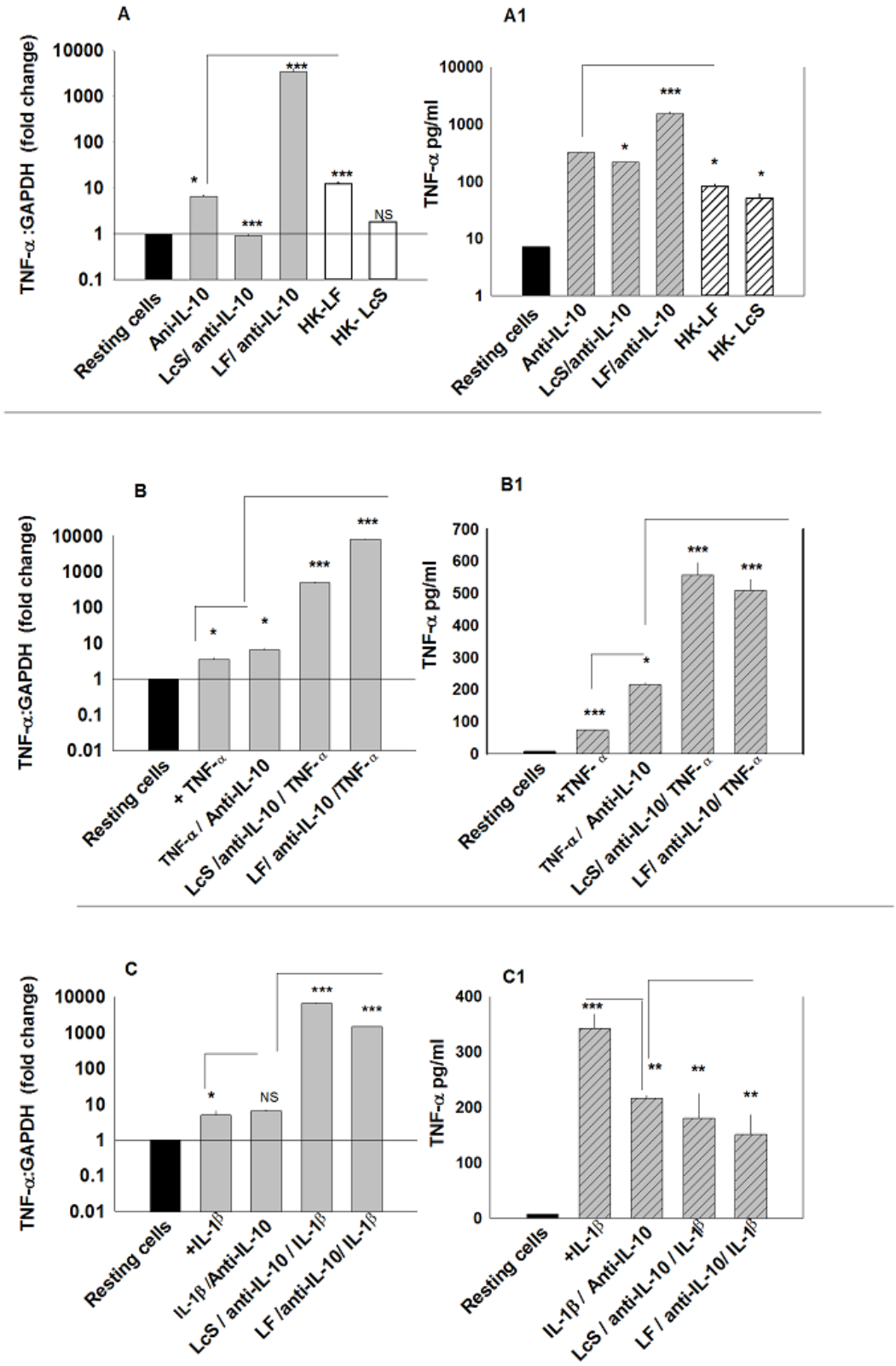


Figure 4.2. 9: Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-β induced TNF-α after neutralisation the IL-10 bioactivity in epithelial cells.

Caco-2 cells treated with heat killed of *Lactobacillus casei strain* Shirota (LcS) or *L. fermentum* (LF) at a cell density of $3x10^8$ CFU/ml for 18hr, followed by stimulating cells with 10 ng/ml TNF-α or 5 ng/ml IL-1β in the presence or absence of 10 µg/ml of anti-IL-10 antibody for 18hr. Cytokine production is expressed as the mean±SE in pg/ml (**A1**, **B1**, **C1**) and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A**, **B**, **C**). Data displayed is a representative experiment with triplicate sample of n=4 independent experiments. Significant effects compared to the control (stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

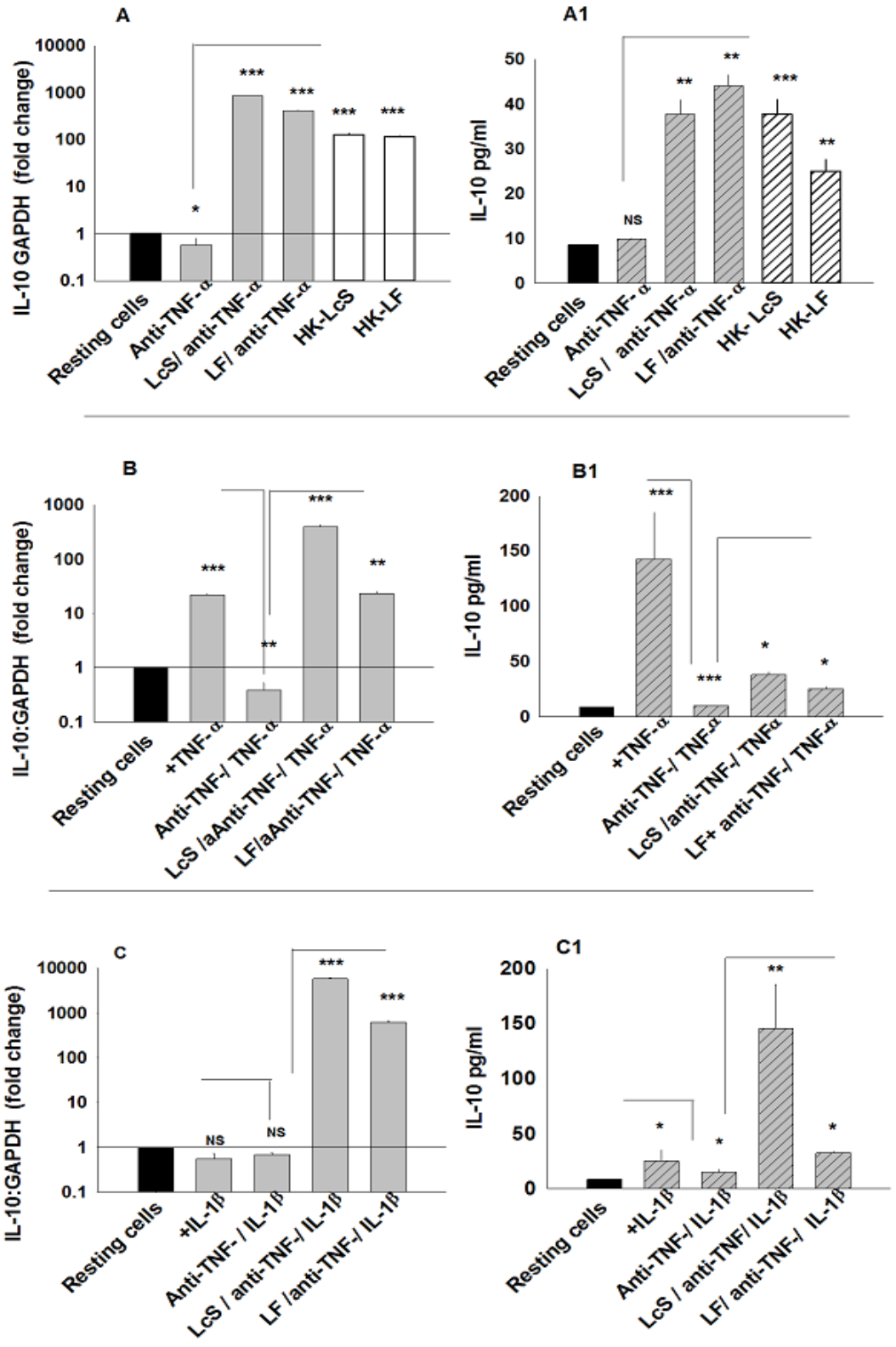


Figure 4.2. 10: Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-1β induced IL-10 after neutralisation of TNF-α bioactivity in epithelial cells.

Caco-2 cells treated with heat killed (HK) *Lactobacillus casei strain Shirota* (LcS) or *L.fermentum* (LF) at a cell density of $3x10^8$ CFU/ml for 18hr followed by stimulation with 10 ng/ml TNF-α or 5ng/ml IL-1β in the presence or absence of 10μg/ml of anti-TNF-α antibody for 18hr. Cytokine production is expressed as the mean±SE in pg/ml (**A1**, **B1, C1**), and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A**, **B**, **C**). Data displayed is a representative experiment with triplicate samples of n=4 independent experiments. Significant effects compared to the control (stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

4.2.5. Probiotic treatments selectively modulate TNF-α or IL-1β induced hBD-2 after neutralisation of the bioactivity of IL-10 or TNF-α in epithelial cells

Data in Fig.4.2.4 shows that cytokine (TNF-α, IL-β) treatments exhibited significant effects in inducing hBD-2 expression, therefore this experiment was undertaken to establish whether hBD-2 was regulated by TNF-α or IL-1β in the presence or absence of TNF-α or IL-10 (refer to section 2.2.4.6 and Fig.1.7 line 2). Results showed that the neutralisation of TNF-α, results in suppression of hBD-2 mRNA and no effect at the protein level, whereas neutralisation of the IL-10 bioactivity results in augmentation of hBD-2 as mRNA and protein (Fig.4.2.11A & A1). Stimulating cells with TNF-α followed by treating cells with anti-TNF-α antibody showed that hBD-2 was suppressed at mRNA and protein levels, whereas treating cells with anti-IL-10 antibody results in up-regulation of hBD-2 at mRNA and protein levels (Fig.4.2.11B & B1). The more surprising correlation is with TNF-α, in the absence of TNF-α there is no hBD-2 expression in epithelial cells. Similarly, hBD-2 suppressed at mRNA and protein levels in cells stimulated with IL-1β followed by treating cells with anti-TNF-α antibody. Whereas hBD-2 expression was augmented in cells pre-stimulated with IL-1β followed by treating cells with anti-IL-10 antibody at mRNA level, and suppressed at protein level (Fig.4.2.11C & C1).

Data in Fig.4.2.7 shows that probiotic bacterial treatments significantly modulated hBD-2 expression induced by TNF-α or IL-1β. To determine probiotic effects on the modulation of hBD-2 expression induced by TNF-α or IL-1β, the first set of analyses was performed by pre-treating Caco-2 cells with probiotics followed by treating them with anti-TNF-α or anti-IL-10 to assess probiotic bacterial roles in modulation of hBD-2. Second set of analyses was performed by pre-treating Caco-2 cells with probiotics followed by stimulation with IL-1β or TNF-α in the presence or absence of anti-IL-10 or anti-TNF-α antibody to assess the probiotic bacterial role in modulation of IL-1β or TNF-α induced hBD-2 expression (refer to section 2.2.4.6 and Fig.1.7 line 2). Concerning the first set of analyses, results showed that the presence of probiotics selectively modulated hBD-2 expression, LcS treatment up-regulated hBD-2 mRNA and LF treatment did not significantly modulate it, however, both LcS and LF non-significantly (P-value 0·59) modulated hBD-2 at protein levels after neutralisation of TNF-α bioactivity. In cells pre-treated with probiotics followed by treating with anti-IL-10 antibody, hBD-2 was up-regulated by LcS, whereas protein was suppressed. In contrast, LF suppressed hBD-2 mRNA level, whereas protein was augmented (Fig.4.2.12A & B). Data showed that LF treatment negatively regulated IL-10 bioactivity at the protein level, thereby up-regulating hBD-2 expression, in contrast with LcS treatment, which positively regulated IL-10 bioactivity resulting in suppression of hBD-2 in epithelial cells.

Referring to the second analysis set, the comparisons between different inflammatory scenarios where TNF-α or IL-1β is predominant, probiotic treatments selectively modulated hBD-2 expression (Fig.4.3.13). Results indicated that hBD-2 mRNA expression by cells pre-treated with probiotics then stimulated with TNF-α

in the presence of anti-TNF-α antibody was not significantly modulated by LcS treatment, whereas LF treatment up-regulated hBD-2 mRNA levels suggesting that LF treatment augmented hBD-2 mRNA induced by TNF-α after neutralisation of the TNF-α bioactivity, whereas LcS treatment failed (Fig.4.2.13A). Where cells were pre-treated with probiotics followed by stimulation with TNF-α in the presence of anti-IL-10 antibody, hBD-2 mRNA was suppressed by LcS, and augmented by LF suggesting that LcS suppressed TNF-α induced hBD-2 mRNA, whereas LF treatment augmented TNF-α induced hBD-2 mRNA after neutralisation of IL-10 bioactivity. At the protein level, probiotics failed to modulate TNF-α induced hBD-2 after neutralisation of TNF-α bioactivity regulated hBD-2 expression, whereas LF treatment augmented TNF-α induced hBD-2, and LcS suppressed TNF-α induced hBD-2 after neutralisation of the IL-10 bioactivity (Fig.2.3.13B). Results showed that at the breakdown of tolerance where IL-10 bioactivity was absent, LF treatment exhibited significant anti-inflammatory effects via up-regulation of TNF-α induced hBD-2 facilitating a good chance for bacterial clearance leading to maintenance of the gut barrier. The more surprising correlation is with TNF-α regulating hBD-2 expression, when probiotics failed to induce or regulate hBD-2 expression when TNF-α was absent after its neutralisation.

Where cells were pre-treated with probiotics followed by stimulation with IL-1β in the presence of anti-TNF-α antibody, probiotics augmented IL-1β induced hBD-2 mRNA after neutralisation of the TNF-α bioactivity. Whereas, IL-1β induced hBD-2 selectively modulated after neutralisation of IL-10 bioactivity, LcS up-regulated IL-1β induced hBD-2 and LF non-significantly suppressed IL-1β induced hBD-2 mRNA. At the protein level, LcS augmented IL-1β induced hBD-2 at the neutralisation TNF-α bioactivity, whereas LF treatment suppressed it. Both

probiotic strains suppressed IL-1β induced hBD-2 at the neutralisation IL-10 bioactivity (Fig.4.2.13B). Data showed that probiotics selectively modulated the inflammatory signal induced hBD-2, which depended on the background setting of the cells, either homeostatic status (when IL-10 was available) or inflammatory status when IL-10 was absent. Data showed that probiotic treatment modulated hBD-2 induced by either TNF-α or IL-1β via modulation of membrane bound of IL-10 or TNF-α.

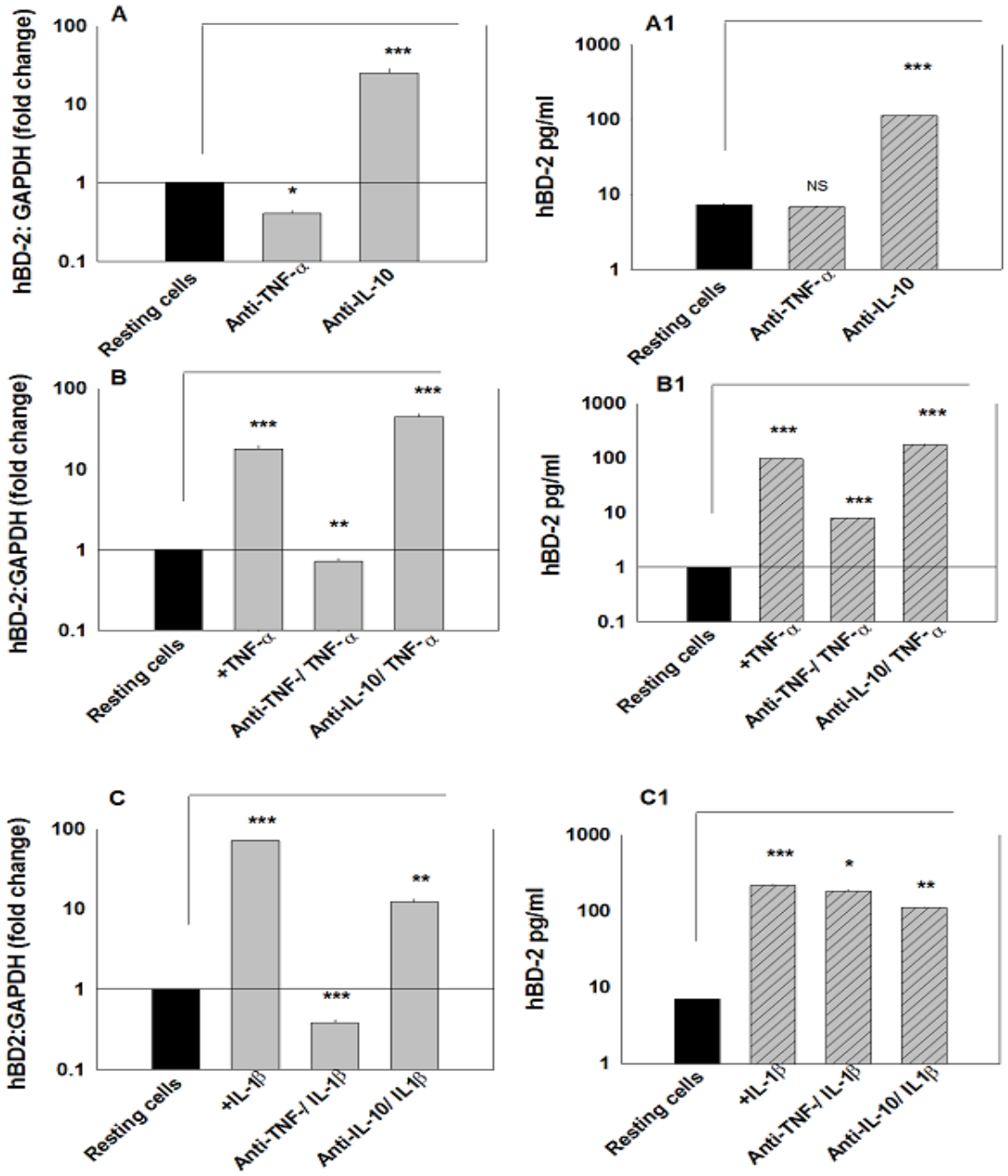


Figure 4.2. 11 : hBD-2 expression induced by TNF-α or IL-1β selectively modulated by membrane bound of TNF-α or IL-10 in epithelial cells.

Caco-2 epithelial cells treated with 10 µg/ml of anti-TNF-α, or anti-IL-10 antibody in the presence or absence of 10 ng/ml TNF-α or 5 ng/ml IL-1β for 18hr. hBD-2 production is expressed as mean±SE in pg/ml (**A1**, **B1, C1**), and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A**, **B**, **C**). Data displayed is a representative experiment with triplicate samples of n=4 independent experiments. Significant

effects compared to the control (stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

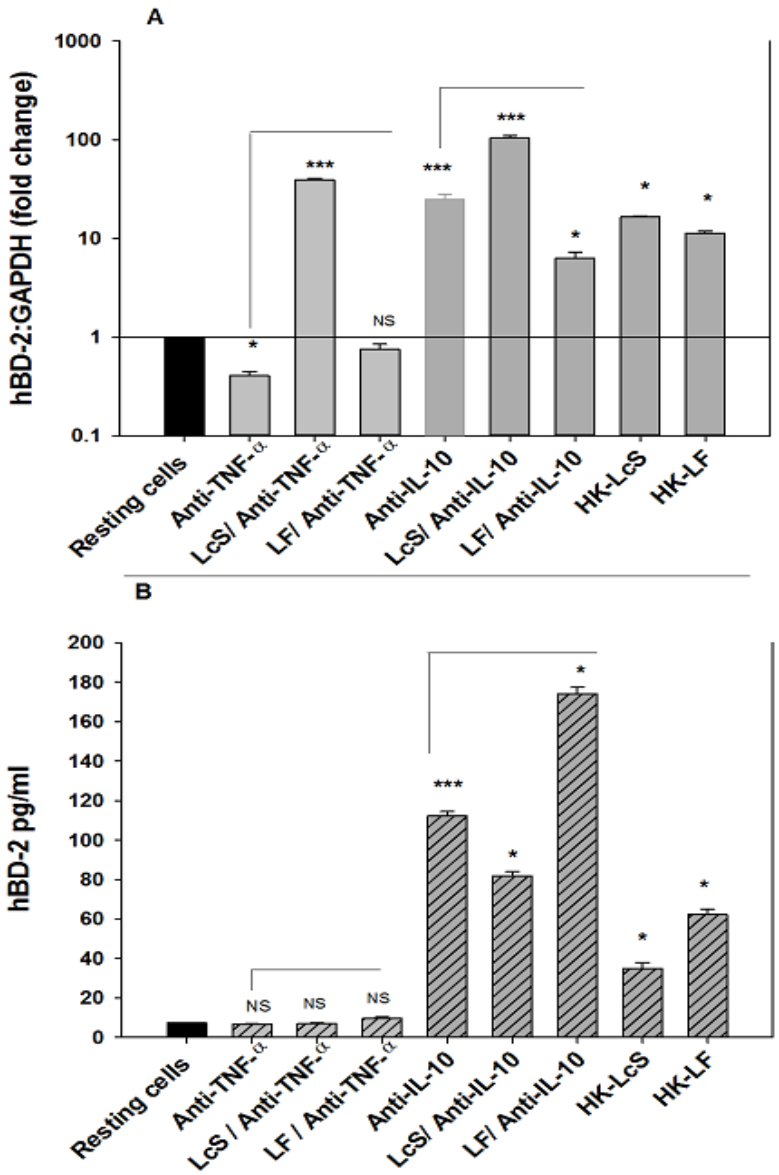


Figure 4.2. 12: Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-1β induced hBD-2 after neutralisation of the TNF-α or IL-10 bioactivity in epithelial cells.

Caco-2 epithelial cells treated with 10 µg/ml of anti-TNF-α or anti-IL-10 antibody in the presence or absence of $3x10^8$ CFU/ml of probiotic bacteria *Lactobacillus casei strain* Shirota (LcS) or *L.fermentum* (LF) for 18hr. hBD-2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A**) and protein level is expressed as the mean±SE in pg/ml **(B)**. Data displayed is a representative experiment with triplicate samples of n=4 independent

experiments. Significant effects compared to the control (stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

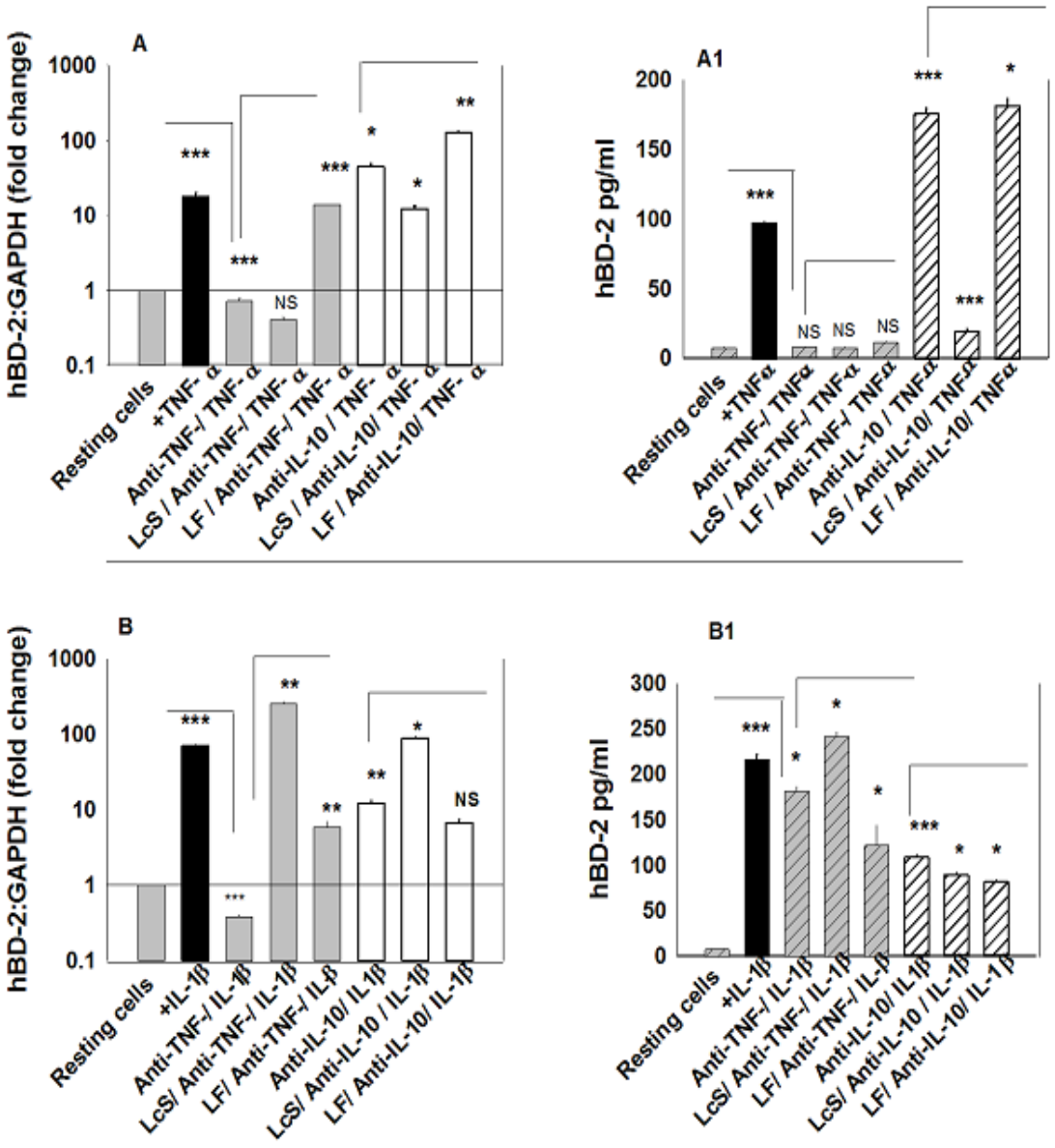


Figure 4.2. 13: Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-1β induced hBD-2 after neutralisation of the TNF-α or IL-10 bioactivity in epithelial cells.

Caco-2 cells treated with 10µg/ml of anti-TNF-α, or anti-IL-10 antibody in the presence or absence of $3x10^8$CFU/ml of probiotic bacteria *Lactobacillus casei strain* Shirota (LcS) or *L. fermentum* (LF) in the presence or absence of 10 ng/ml TNF-α or 5 ng/ml IL-1β for 18hr. hBD-2 production is expressed as the mean±SE in pg/ml **(A1, B1),** and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a

calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A, B)**. Data displayed is a representative experiment with triplicate samples of n=4 independent experiments. Significant effects compared to the control (stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

4.2.6. Probiotic bacterial role in modulation of the suppressive activity of exogenous IL-10 regulated hBD-2 expression induced by TNF-α or IL-1β.

Strong evidence of IL-10 bioactivity was found in controlling hBD-2 expression (Fig.4.2.13), therefore, further investigation was performed to determine the effect of exogenous IL-10 on hBD-2 expression (refer to section 2.2.4.6). Analysis of variance (ANOVA) was used to analyse the relationship between exogenous IL-10 and hBD-2 expression (P-value 0·005). Interestingly, for those cells treated with IL-10, hBD-2 was up-regulated by 7.3 fold change (from control 1±0), while there was no significant effect at the protein level. Whereas, treating cells previously stimulated by TNF-α or IL-1β with IL-10 suppressed hBD-2 expression (mRNA and protein level) (Fig.4.2.14A & A1). Probiotic presence (LF and LcS) up-regulated hBD-2 mRNA levels. At the protein level, where cells were stimulated with IL-1β followed by treating with IL-10, probiotic treatments up-regulated TNF-α induced hBD-2 whereas, they selectively modulated IL-1β induced hBD-2; LcS treatment augmented IL-1β induced hBD-2 while LF treatment suppressed it (Fig.4.2.14B1).

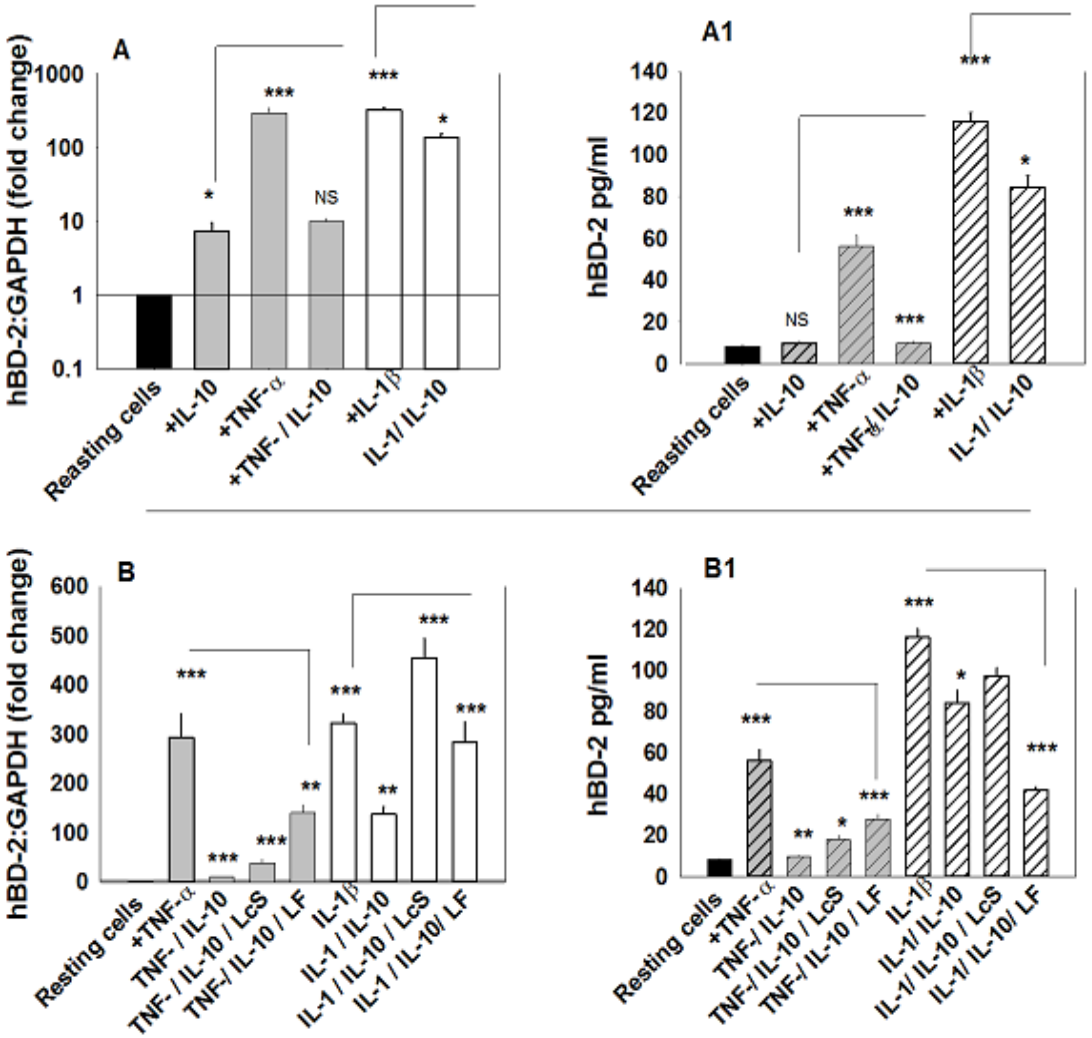


Figure 4.2. 14 : Exogenous IL-10 suppressed epithelial hBD-2 expression: Probiotic modulation of suppressive activity.

Caco-2 epithelial cells pre-treated with $3x10^8$ CFU/ml of probiotic bacteria LcS or LF followed by stimulation with 10 ng/ml TNF-α or 5 ng/ml IL-1β in the presence or absence of 10 ng/ml IL-10 for 18r. hBD-2 production is expressed as mean±SE in pg/ml **(A1, B1),** and gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A, B)**. Data displayed is a representative experiment with triplicate samples of n=4 independent experiments. Significant effects compared to the control (stimulated cells) are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

4.2.7. Probiotic bacterial role in modulation of TLR expression induced by TNF-α or IL-1β after neutralisation of IL-10 or TNF-α bioactivity

On the question of the probiotic bacterial role in the modulation of cytokine induced hBD-2 expression, this study found that probiotic treatments selectively modulated cytokine induced hBD-2 expression. One possible cause of these effects exerted by probiotic treatments is via modulation of TLR expression. Results indicated that treating cells with anti-TNF-α antibody suppressed TLR4 expression, and probiotic presence resulted in further suppression of TLR4 under these conditions, whereas treating cells with anti-IL-10 antibody up-regulated TLR4, and probiotic presence resulting in further up-regulation of TLR4 expression (Fig.4.2.15A). Stimulating cells with TNF-α resulted in up-regulation of TLR4, whereas, treating cells previously stimulated by TNF-α with anti-TNF-α antibody resulted in suppression of TLR4, besides that, probiotics presence resulted in the further suppression of TLR4. In contrast, treating cells stimulated by TNF-α with anti-IL-10 antibody resulted in augmentation of TLR4, and probiotic presence resulted in augmentation of TLR4 (Fig.4.2.15B). Stimulating resting cells with IL-1β up-regulated TLR4, and treating these cells with anti-TNF-α antibody resulted in up-regulation of TLR4, however, probiotic presence resulted in suppression of TLR4. Nevertheless, treating cells previously stimulated by IL-1β with anti-IL-10 antibody up-regulated TLR4, and with probiotic presence TLR4 was suppressed in these cells (Fig.4.2.15C).

TLR2 was suppressed after treating cells with anti-TNF-α antibody whereas, probiotics in this setting, up-regulated it. In contrast, treating resting cells with anti-IL-10 antibody up-regulated TLR2; probiotic presence suppressed it (Fig.4.2.15D). Stimulating cells with TNF-α up-regulated TLR2, whereas neutralisation of TNF-α bioactivity suppressed TLR2 and probiotic presence resulted in up-regulation

TLR2 expression. Stimulating cells with TNF-α followed by neutralisation of IL-10 bioactivity resulted in up-regulation of TLR2 expression, and probiotic presence resulted in suppression of TLR2 (Fig.4.2.15E). Stimulating cells with IL-1β up-regulated TLR2, whereas treating cells with anti-TNF-α antibody previously stimulated with IL-1β resulted in suppression of TLR2, and probiotic presence resulted in up-regulation of TLR2 expression. However, treating cells with anti-IL-10 antibody previously stimulated with IL-1β up-regulated TLR2 expression, besides that, presence of probiotics suppressed TLR2 induced by IL1β (Fig.4.2.15F).

Treating resting cells with anti-TNF-α antibody suppressed CD14, and probiotic presence resulted in the further suppression of CD14 expression. In contrast, treating cells with anti-IL-10 antibody up-regulated CD14 probiotic presence resulted in suppression of CD14 (Fig.4.2.16A). Stimulating cells with TNF-α resulted in up-regulation of CD14, however, treating these cells with anti-TNF-α antibody resulted in suppression CD14 expression, and probiotic presence in this setting selectively modulated TNF-α induced CD14 i.e. less suppressed IL-1β-induced CD14 expression, whereas LF treatment up-regulated it. On the other hand, treating cells stimulated by TNF-α with anti-IL-10 antibody resulted in augmentation of CD14 and probiotics presence in this setting suppressed CD14 expression (Fig.4.2.16B). Stimulating cells with IL-1β up-regulated CD14 expression, whereas, treating stimulated cells with anti-TNF-α antibody resulted in suppression of CD14 expression, and probiotic presence resulted in the further suppression of CD14 expression. Nevertheless, stimulating cells with IL-1β followed by treating with anti-IL-10 antibody resulted in up-regulation of CD14

expression and probiotic presence resulted in the further suppression of CD14 (Fig.4.2.16C).

MD-2 expression was suppressed after neutralisation of the TNF-α bioactivity, and probiotic presence resulted in augmentation of it. In contrast, treating resting cells with anti-IL-10 antibody up-regulated MD-2 expression, and probiotic presence resulted in suppression of MD-2 (Fig.4.2.16D). Stimulating cells with TNF-α augmented MD-2, however, treating cells stimulated by TNF-α with anti-TNF-α antibody suppressed it, and probiotic presence at this setting resulting in suppression MD-2 expression. Comparing the LcS and LF treatment (where cells treated with anti TNF-α followed by stimulation with TNF-α), data showed that LcS treatment exhibited more suppression of MD-2 than LF. Similar effects were exhibited by LcS when cells were stimulated by TNF-α then treated with anti-IL-10 antibody (Fig.4.2.16E). Stimulating cells with IL-1β up-regulated MD-2, whereas treating cells stimulated by IL-1β with anti-TNF-α antibody suppressed MD-2 and probiotic LcS augmented it, whereas LF failed to modulate TNF-α induced MD-2 after neutralisation of the TNF-α bioactivity. Treating cells stimulated by IL-1β with anti-IL-10 antibody suppressed MD-2 expression and probiotic presence caused further suppression of MD-2 induced by IL-1β after neutralisation of the IL-10 bioactivity (Fig.4.2.16F).

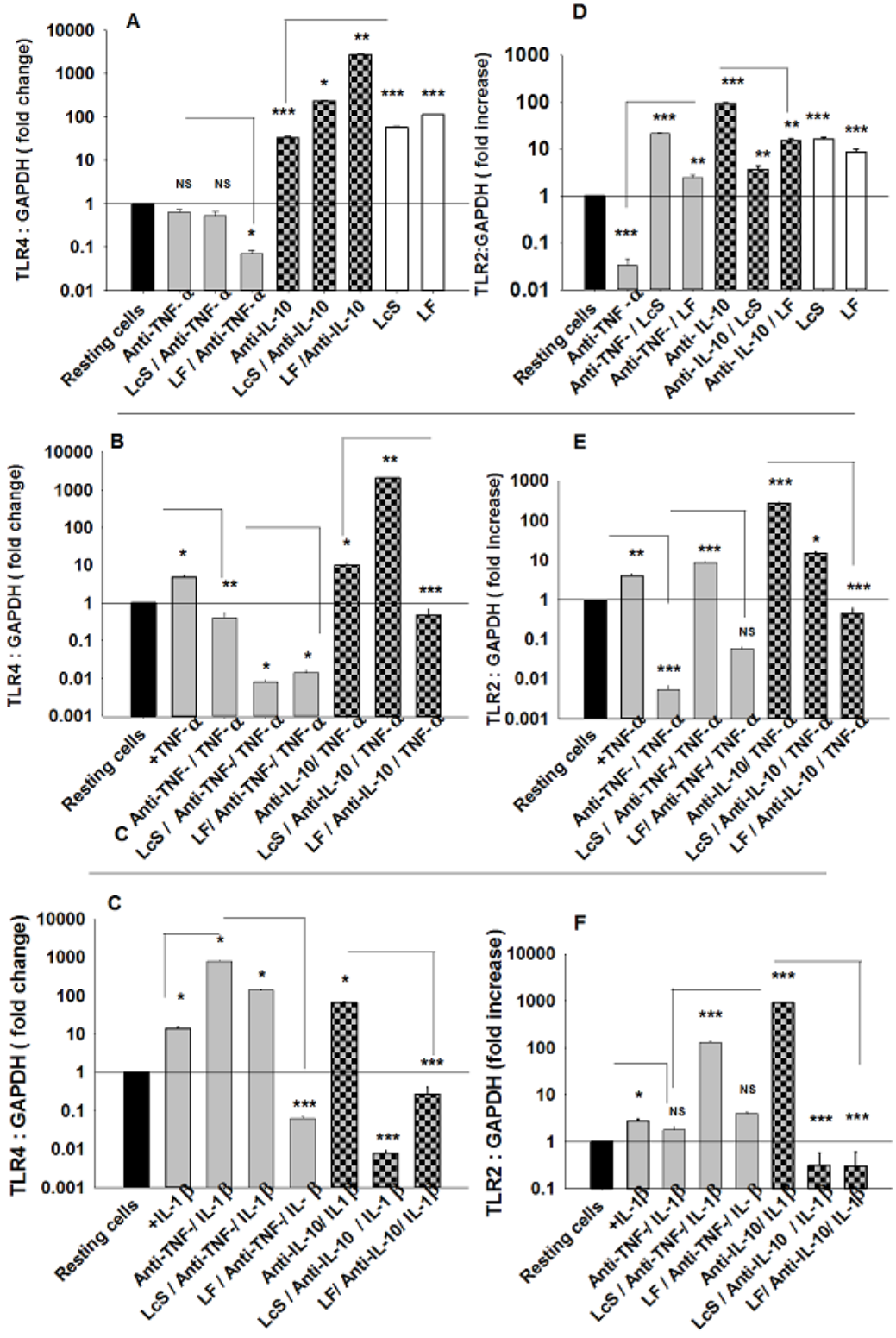


Figure 4.2. 15: Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-1β induced TLR4 and TLR2 expression after neutralisation of TNF-α or IL-10 bioactivity in epithelial cells.

Caco-2 cells pre-treated with $3x10^8$ CFU/ml of heat killed probiotic bacteria *Lactobacillus casei* strain Shirota (LcS) or *L. fermentum* (LF) for 18hr, followed by treating cells with 10 µg/ml of anti-TNF-α, or anti-IL-10 antibody for 18hr (**A, D**). Cells pre-treated with probiotic for 18hr, followed by stimulation with 10 ng/ml TNF-α in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**B, E**), or stimulation with 5 ng/ml IL-1β in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**C, F)** for 18hr. TLR4 or TLR2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$**.** Data displayed is a representative experiment with triplicate samples of n=3 independent experiments. Significant effects compared to the control are indicated as * $P<0.05$, ** $P<0.01$ and *** $P<0.005$, and NS (non-significant).

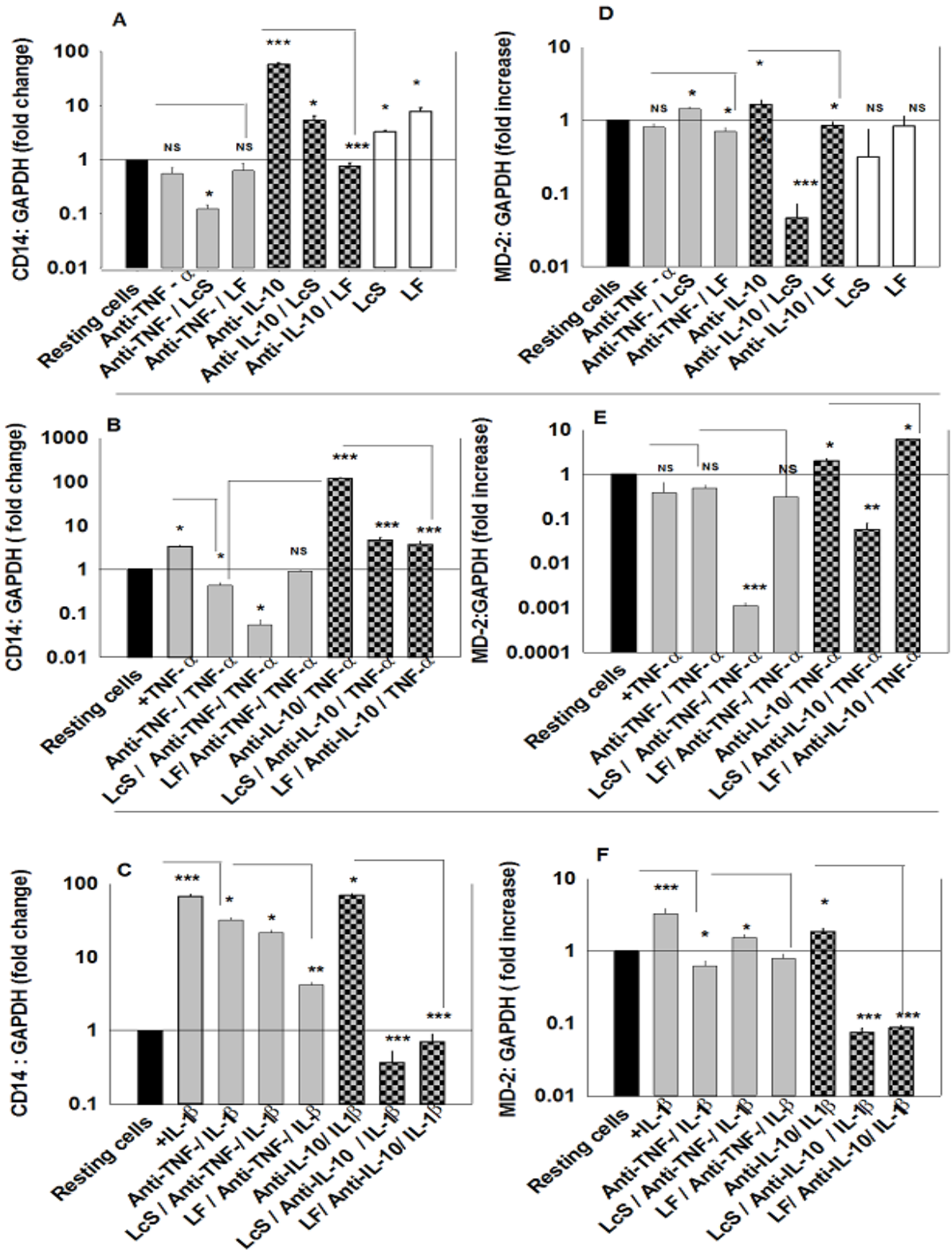


Figure 4.2. 16 : Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-1β induced CD14 and MD-2 expression after neutralisation of TNF-α or IL-10 bioactivity in epithelial cells.

Caco-2 cells pre-treated with $3x10^8$ CFU/ml of heat killed probiotic bacteria *Lactobacillus casei strain* Shirota (LcS) or *L. fermentum* (LF) for 18hr, followed by treating cells with 10 µg/ml of anti-TNF-α, or anti-IL-10 antibody for 18hr (**A, D**). Cells pre-treated with probiotic for 18hr, followed by stimulation with 10 ng/ml TNF-α in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**B, E**), or stimulation with 5 ng/ml IL-1β in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**C, F)** for 18hr. CD14 or MD-2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 independent experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005, and NS (non-significant).

Treating cells with anti-TNF-α antibody augmented NOD-2 expression and probiotic presence resulted in suppression of it. Treating cells with anti-IL-10 antibody also up-regulated NOD-2 expression and NOD-2 expression was suppressed by probiotic treatments (Fig.4.2.17A). Stimulating cells with TNF-α up-regulated NOD-2 expression. Treating cells stimulated by TNF-α with anti-TNF-α antibody up-regulated NOD-2, and probiotic presence selectively modulated NOD-2 induced by TNF-α after neutralisation of TNF-α bioactivity; LcS treatment up-regulated NOD-2 induced by TNF-α, whereas LF treatment suppressed it. In contrast, treating cells stimulated by TNF-α with anti-IL-10 antibody, NOD-2 expression was augmented, and probiotic presence resulted in suppression of NOD-2 after neutralisation of IL-10 bioactivity (Fig.4.2.17B). Stimulating cells with IL-1β up-regulated NOD-2. Treating cells stimulated by IL-1β with anti-TNF-α antibody up-regulated NOD-2 expression and probiotic presence selectively modulated NOD-2 induced by IL-1β after neutralisation of the TNF-α bioactivity, i.e. LcS up-regulated TNF-α induced NOD-2, whereas LF treatment suppressed TNF-α induced NOD-2 expression at this setting. Treating cells stimulated by IL-1β with anti-IL-10 antibody up-regulated NOD-2 and probiotic presence suppressed NOD-2 induced by IL-1β after neutralisation of the IL-10 bioactivity (Fig.4.2.17C).

Treating resting cells with anti-TNF-α antibody up-regulated TLR9, and probiotic presence suppressed it. Treating cells with anti-IL-10 antibody also up-regulated TLR9 expression and combined with probiotic presence, resulted in suppression of TLR9 (Fig.4.2.17D). Stimulating cells with TNF-α up-regulated TLR9 whereas, treating cells stimulated by TNF-α with anti-TNF-α antibody suppressed TLR9, besides that probiotic presence resulted in the further suppression of TLR9 induced by TNF-α after neutralisation of the TNF-α bioactivity Treating cells stimulated by TNF-α with anti-IL-10 antibody resulted in up-regulation of TLR9 expression and again probiotic presence resulted in the further suppression of TLR9 induced by TNF-α after neutralisation of the IL-10 bioactivity (Fig.4.2.17E). Stimulating cells with IL-1β up-regulated TLR9, treating cells with anti-TNF-α antibody up-regulated TLR9, and probiotic presence resulted in suppression of TLR9 induced by IL-1β after neutralisation of the TNF-α bioactivity. Similarly, treating cells stimulated by IL-1β with anti-IL-10 antibody up-regulated TLR9 and probiotic presence suppressed TLR9 induced by IL-1β after neutralisation of the IL-10 bioactivity (Fig.4.2.17F). Indeed probiotic treatments exhibit significant effects in modulation of TNF-α or IL-1β induced PRRs in epithelial cells after neutralisation of the bioactivity of IL-10 or TNF-α.

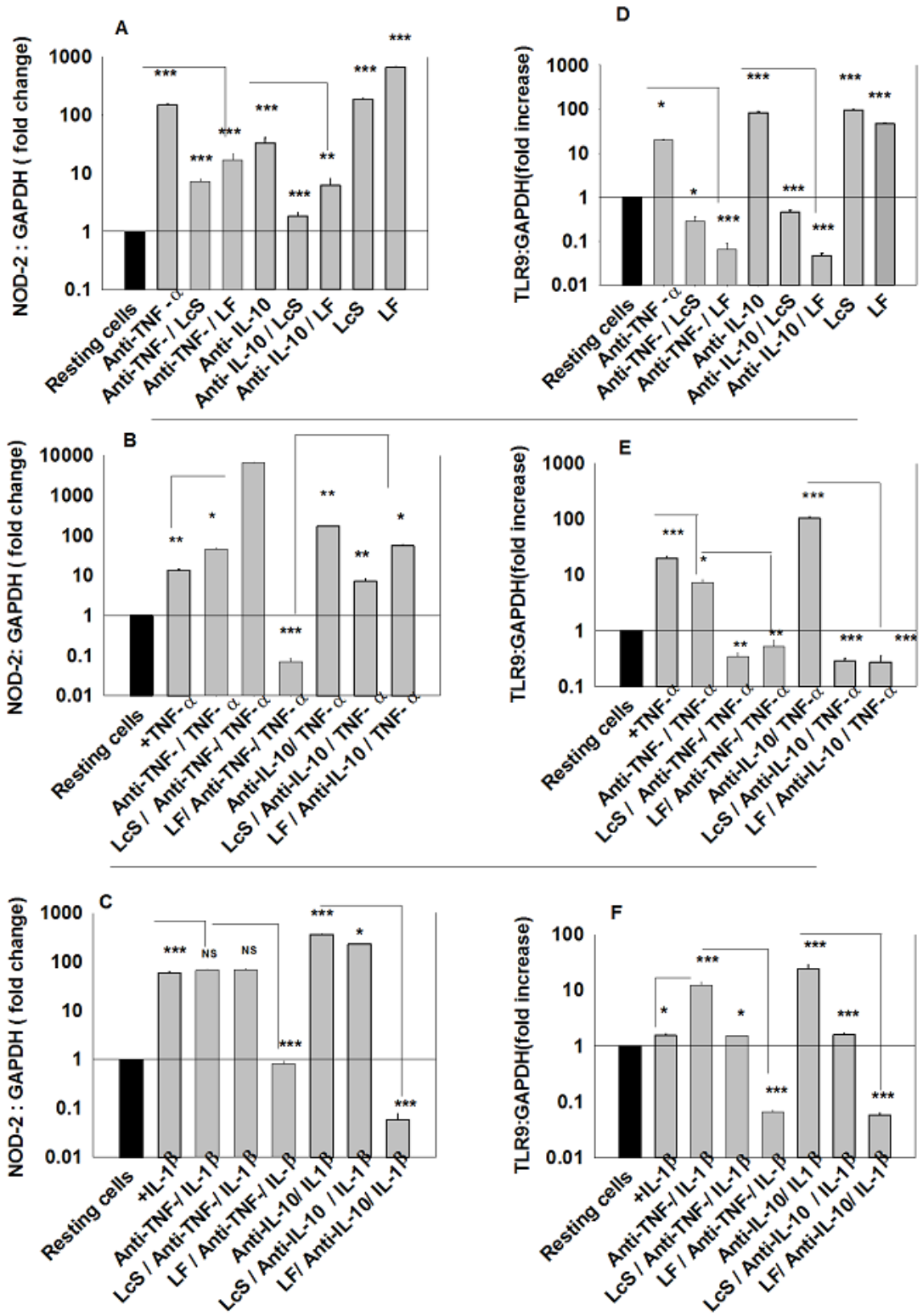


Figure 4.2. 17: Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-1β induced NOD-2 and TLR9 expression after neutralisation of the TNF-α or IL-10 bioactivity in epithelial cells.

Caco-2 cells pre-treated with $3x10^8$ CFU/ml of heat killed probiotic bacteria *Lactobacillus casei strain S*hirota (LcS) or *L. fermentum* (LF) for 18hr, followed by treating cells with 10 µg/ml of anti-TNF-α, or anti-IL-10 antibody for 18hr (**A, D**). Cells pre-treated with probiotic for 18hr, followed by stimulation with 10 ng/ml TNF-α in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**B, E**), or stimulation with 5 ng/ml IL-1β in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**C, F)** for 18hr. NOD-2 or TLR9 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 independent experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005, and NS (non-significant).

Treating resting cells with anti-TNF-α antibody up-regulated Tollip, besides that presence of probiotics resulted in the further suppression of it. Treating cells with anti-IL-10 antibody suppressed Tollip and probiotic presence resulted in augmentation of Tollip expression (Fig.4.2.18A). Figure 4.2.18B showed that stimulating cells with TNF-α suppressed Tollip, treating cells stimulated by TNF-α with anti-TNF-α antibody resulted in up-regulation of Tollip expression, and probiotic treatments selectively modulated Tollip; LcS treatment augmented TNF-α induced Tollip whereas LF treatment suppressed TNF-α induced Tollip after neutralisation of TNF-α bioactivity. Treating cells stimulating by TNF-α with anti-IL-10 antibody resulted in augmentation of Tollip expression, and probiotic presence augmented TNF-α induced Tollip after neutralisation of the IL-10 bioactivity. Stimulating cells with IL-1β suppressed Tollip expression, besides that, treating cells stimulated by IL-1β with anti-TNF-α antibody resulted in up-regulation of Tollip and probiotic treatments suppressed IL-1β induced Tollip after neutralisation of the TNF-α bioactivity. In addition, treating stimulated cells with anti-IL-10 antibody augmented Tollip and probiotic presence selectively modulated IL-1β induced Tollip expression, when LcS augmented IL-1β induced

Tollip whereas LF suppressed it after neutralisation of IL-10 bioactivity (Fig.4.2.18C).

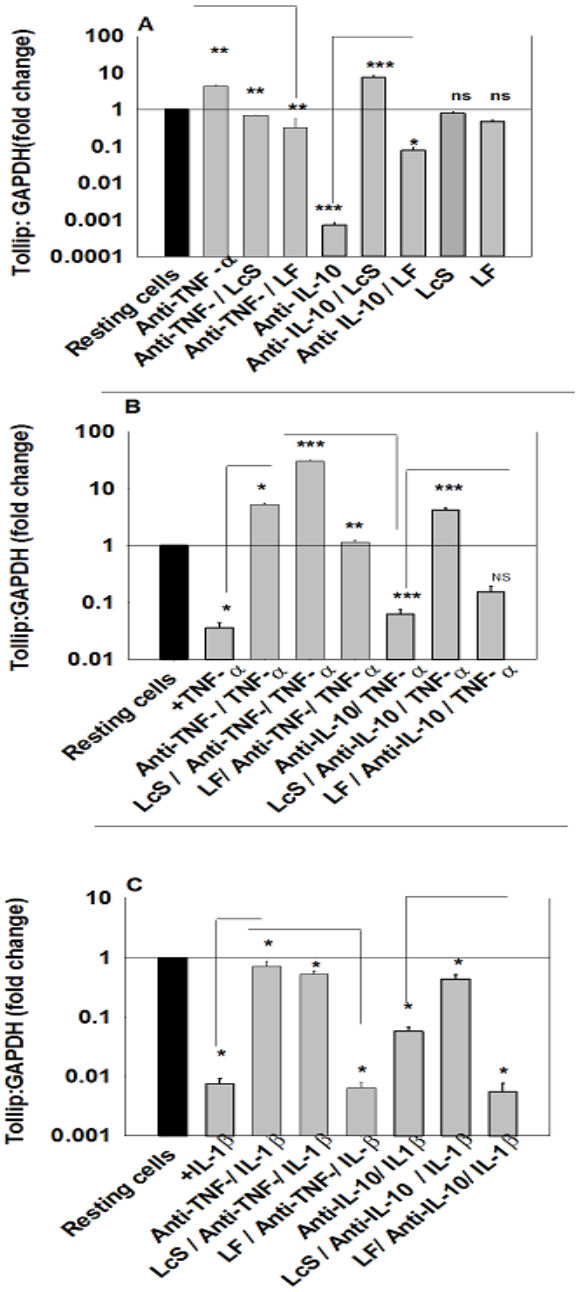


Figure 4.2. 18: Heat killed probiotic bacterial strains selectively modulate TNF-α or IL-1β induced Tollip expression after neutralisation of the TNF-α or IL-10 bioactivity in epithelial cells.

Caco-2 cells pre-treated with $3x10^8$ CFU/ml of heat killed probiotic bacteria *Lactobacillus casei* strain Shirota (LcS) or *L. fermentum* (LF) for 18hr, followed by treating cells with 10

µg/ml of anti-TNF-α, or anti-IL-10 antibody for 18hr (**A, D**). Cells pre-treated with probiotic for 18hr, followed by stimulation with 10 ng/ml TNF-α in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**B, E**), or stimulation with 5 ng/ml IL-1β in the presence or absence of 10 µg/ml anti-TNF-α or anti-IL-10 (**C, F)** for 18hr. Tollip gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 independent experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005, and NS (non-significant).

4.2.8. Summary of chapter 4 results

Table 4.2. 1: Probiotic treatments selectively modulate TNF-α and IL-1β induced cytokines and hBD-2 expressed by Caco-2 intestinal epithelial cells.

	mRNA expression						Protein expression (pg/ml)					
	BB	LS	LR	LcS	LF	LP	BB	LS	LR	LcS	LF	LP
IL-8 induced by TNF-α	↑	↑	↑	↑	↑	↑	↑	↑	↑	↔	↑	↑
TNF-α induced by TNF-α	↑	↑	↑	↑	↑	↑	↓	↓	↓	↓	↑	↑
IL-10 induced by TNF-α	↑	↑	↑	↑	↔	↑	↑	↑	↑	↑	↓	↓
IL-6 induced by TNF-α	↑	↑	↑	↔	↑	↑	↔	↑	↑	↔	↔	↔
IL-8 induced by IL-1β	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↔
TNF-α induced byIL-1β	↑	↑	↑	↑	↔	↑	↔	↔	↓	↓	↔	↓
IL-10 induced by IL-1β	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↑	↑
IL-6 induced by IL-1β	↓	↔	↓	↑	↔	↑	↑	↑	↑	↑	↓	↓
hBD-2 induced by TNF-α	↓	↓	↓	↓	↑	↑	↓	↓	↓	↓	↑	↑
hBD-2 induced by IL-1β	↑	↑	↑	↑	↔	↔	↑	↑	↑	↑	↓	↓

Table 4.2. 2: Probiotic treatments of HK-LcS and HK-LF selectively modulated TNF-α or IL-1β induced cytokines, hBD-2 and PRRs mRNA expression after neutralisation of the IL-10 or TNF-α bioactivity in Caco-2 epithelial cells.

mRNA	LcS						LF					
	+ Ant-IL-10	+Anti IL-10/ TNF-α	+ Anti-IL-10/ IL-1β	Anti-TNF-α	Anti-TNF-α/ TNF-α	Anti-TNF-α/ IL-1β	+ Anti-IL-10	+ Anti-IL-10/ TNF-α	Anti-IL-10/ IL-1β	Anti-TNF-α	Anti-TNF-α/ TNF-α	Anti-TNF-α/ IL-1β
TNF-α	↓	↑	↑	ND	ND	ND	↑	↑	↑	ND	ND	ND
IL-10	ND	ND	ND	↑	↑	↑	ND	ND	ND	↑	↑	↑
hBD-2	↑	↓	↑	↑	↓	↑	↓	↑	↑	↓	↑	↓
TLR4	↑	↑	↓	↔	↓	↓	↑	↓	↓	↓	↓	↓
TLR2	↑	↓	↓	↑	↑	↑	↓	↓	↓	↑	↑	↑
CD14	↓	↓	↓	↓	↓	↓	↓	↓	↓	↑	↑	↓
MD-2	↓	↓	↓	↑	↓	↑	↑	↑	↓	↓	↑	↓
NOD-2	↓	↓	↓	↓	↑	↔	↓	↓	↓	↓	↓	↓
TLR9	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
Tollip	↑	↑	↑	↓	↑	↑	↑	↔	↓	↓	↓	↓

Table 4.2. 3: Probiotic treatments of HK-LcS and HK-LF selectively modulated TNF-α or IL-1β induced cytokines and hBD-2 production after neutralisation of IL-10 or TNF-α bioactivity in Caco-2 epithelial cells.

pg/ml	LcS						LF					
	+ Anti-IL-10	+ Anti-IL-10/ TNF-α	+ Anti-IL-10/ IL-1β	Anti-TNF-α	Anti-TNF-α/ TNF-α	Anti-TNF-α/ IL-1β	+ Anti-IL-10	+ Anti-IL-10/ TNF-α	Anti-IL-10/ IL-1β	Anti-TNF-α	Anti-TNF-α/ TNF-α	Anti-TNF-α/ IL-1β
TNF-α	↓	↑	↓	ND	ND	ND	↑	↑	↓	ND	ND	ND
IL-10	↑	↑	↑	ND	ND	ND	↑	↑	↑	ND	ND	ND
hBD-2	↑	↓	↓	↔	↔	↑	↓	↑	↓	↔	↔	↓

Note: "↑", "↓", "↔"and ND means up-regulation, down-regulation no-modulation and not determined of the indicated target, respectively.

4.3. Discussion

4.3.1: Probiotic strains selectively induced cytokine and hBD-2 expression in Caco-2 intestinal epithelial cell model

The majority of the information on the role of epithelial cells in immune defence at the gut mucosa is derived from studies on columnar epithelial cell lines from human intestinal tracts. Indeed, cell models are earning an increasing interest among the scientific research community. They are becoming more realistic and representative of the *in vivo* physiological model of human mucosa, and therefore offer a suitable alternative for *in vivo* animal model. Cell culture models can support enormous screening in contrast to the limited screening capacity of animal models.

Since the gut is a complex system with many cooperating cell types and the microbiota, any models that is used to study this system should take into consideration as many of these factors as possible. Indeed, *in vitro* cell models of the gut should functionally resemble the *in vivo* situation. There are several cell line model of the gut such as Caco-2 and HT-29 (human epithelial cells originated from colon adenocarcinoma), and HIEC-6 (normal human epithelial cells originated from small intestine) (Cencic and Langerholc, 2010). Between these cell lines, Caco-2 cells are widely used as a model of human intestinal epithelial cells. They undergo in culture a process of spontaneous differentiation that leads to the formation of a monolayer of cells, expressing several morphological and functional characteristics of the mature enterocyte (Sambuy et al., 2005). Costa de Beauregard et al. (1995) reported that Caco-2 cells differentiated into enterocyte-like cells with regular homogenous glycocalyx brush-border, express markers such as Ulex europaeus agglutinin (UEA)-1 mediated endocytosis (Gabor et al., 1998)

and *Salmonella* pathogenicity island 1 (SP-1) mediated *Salmonella* translocation (Martinez-Argudo and Jepson, 2008), which is similar to the marker of the M cells, after 21 days of culture. In addition, Caco-2 cells differentiate into small intestinal-like cells after confluence (Engle et al., 1998). This cell line, because of all these attributes, represent a good *in vitro* model for human intestinal epithelial cells, therefore, it is used as a model in this study to investigate the immunomodulatory effects of probiotics. The gut epithelial cells are also actively involved in the defence of the gut by releasing cytokines, which orchestrate the recruitment (IL-8) of the immune cells into the gut mucosa. In addition other epithelial cells -derived cytokines appear to have important roles immunoregulation (e.g. IL-10 and TGF-β), and tissue repair (e.g. TGF-β) (Wells et al., 2011a). Thus, in the present study, we investigate the effect of probiotics on epithelial cells derived cytokines. Although epithelial cells release an array of cytokines in response to different stimuli, there are contradictory reports about the spectrum of cytokine release either during normal homeostasis or in inflammation (Ohkusa et al., 2009, Bahrami et al., 2011b). Among the cytokines released by epithelial cells during normal homeostasis and up-regulated at inflammation is IL-8. It is well known that epithelial cells constitutively expressed IL-8 (Eckmann et al., 1993a); however, the expression of IL-8 by epithelial cells is augmented after bacterial attack (Eckmann et al., 1993b). This is in order to recruit immune cells such as neutrophils to the site of infection prior to amplifying a proper immune response mediated by the clearing of pathogen via direct killing by neutrophil phagocytosis, or indirect killing by cytokines and antibodies. However, increasing infiltrating of immune cells will lead to chronic inflammation and tissue destruction.

In this study, two strains of probiotic bacteria were used to investigate the potential of probiotics in inducing IL-8 by Caco-2 cells. The results of this study showed that both live and heat killed bacteria successfully induced IL-8 dependent on the strain and bacterial cell density. These findings are in agreement with Candela et al. (2008) when they showed that live *Lactobacillus acidophilus*, *L. plantarum*, *Bifidobacterium longum* and *B. lactis* induced IL-8 in monolayer Caco-2 cells, and Lammers et al. (2002) were also reported that live probiotic bacteria *E. coli Nissle* 1917 strain induced IL-8 in a dose-dependent way in HT-29 intestinal epithelial cells. In addition to IL-8 production by epithelial cells, Bahrami et al. (2011a) reported that treating Caco-2 and HT-29 epithelial cell lines with *Lactobacilli* and *Bifidobacteria* obtained from healthy people results in different profiles of pro-inflammatory (IL-1β, IL-6, IL-18 and TNFα) and anti-inflammatory cytokines (TGF-β, IL-4 and IL-10). Indeed, Cario and Podolsky (2005a) have speculated that epithelial cell stimulation by bacteria and their products results in the release of an array of cytokines, such as IL-8, which results from the interaction between bacteria and epithelial cells. Hence, it could conceivably be hypothesised that the probiotic bacteria used in this study (live, HK) expressed PAMPs that interacted with pattern recognition receptors on epithelial cells results in triggering active immune response. The preparation of probiotic bacteria has a significant effect on their ability to induce cytokine expression by intestinal epithelial cells; Wong and Ustunol (2006) reported that five heat-killed lactic acid bacterial strains induced IL-8 in Caco-2 cells, whereas the same irradiated strains attenuated IL-8 cytokine production, explaining that the same probiotic bacteria with different preparation used in the same cell culture could provide opposite cytokine production and immune modulation. However, the findings of the current study do not support this idea because both live and heat-killed probiotic bacteria induced IL-8 by Caco-2

cells, which indicated that there is a similar ligands found at both live and heat-killed bacteria induced IL-8 by epithelial cells

In this study, probiotic stimulation was also found to induce the hBD-2 production in intestinal epithelial cells (Caco-2). Data showed live as well as heat-inactivated probiotic bacteria successfully induced hBD-2 in the epithelial cells. The factors behind this stimulation effect are still a matter of debate. Some authors such as (Wehkamp et al., 2004) have found that *E. coli Nissle* 1917 have a significant role in inducing hBD-2 mRNA expression in Caco-2 cells and Schlee et al. (2007) provided evidence that *E. coli Nissle* 1917 exhibit their role in inducing hBD-2 expression through flagellin protein found in bacterial flagella. However, data of this study showed that other factor mediated hBD-2 induction in epithelial cells. In fact, there are several possible explanations for these results, for example, bacterial cell wall, genomic bacterial DNA and bacterial cell metabolites may all exhibit a vital role in inducing hBD-2 expression. The findings of this study are in agreement with Schlee et al. (2008) when hBD-2 was induced by different probiotic bacteria even in the absence of flagella. Therefore, it might be suggested that (heat-killed/live probiotic) bacteria and their metabolites have unique molecular patterns responsible for inducing hBD-2 as well as IL-8 expression in Caco-2 intestinal epithelial cells.

4.3.2. Probiotic treatments selectively modulate TNF-α or IL-1β induced hBD-2 and cytokine expression in epithelial cells.

Cytokines are the messengers that control innate, adaptive, and also epithelial cell functions. Uncontrolled production of cytokines TNF-α and lL-1β by immune cells, such as macrophages, induces gut pathology such as IBD (Strober and James, 1986). Numerous studies used Caco-2 epithelial cells as an in *vitro* model to

investigate the characteristics of some important factors associated with pathophysiology of IBD, such as barrier function and microbial cell interaction leading to modulated immune responses associated with cytokine expression (Zeuthen et al., 2008, Bahrami et al., 2011a, Eckmann et al., 1993a). This study set out with the aim of assessing the importance of probiotic bacteria in modulation of the immune responses induced by TNF-α or IL-1β in epithelial cells. The results of this study showed that stimulation of epithelial cells with TNF-α or IL-1β resulted in releasing different profiles of cytokines (TNF-α, IL-6, IL-8, and IL-10), dependent on the type of stimuli. In fact, epithelial cells express a range of cytokine receptors up-regulated in response to stimulation by cytokines, leading to triggering of the immune response via activation of a complex of transcriptional factors (Reinecker and Podolsky, 1995, Jung et al., 1995). Basically, the binding of the cytokine to its receptor, or bacterial PAMPs with PRRs on the epithelial cells results in a message, which will be transferred downstream resulting in the activation of a multiple cell signalling such as NF-kB. Consequently, in response to stimulation with bacteria or cytokines, epithelial cells release cytokines and AMPs (Gilmore, 2006, Wehkamp et al., 2005). Evidence was provided by researchers that epithelial cells represent APCs that can respond to stimuli through the production of cytokines and chemokines (Wells et al., 2011b). Cytokine networking controls the fates and the outcomes of the epithelial cell immune response. In this study, TNF-α, IL-6, IL-8, IL-10 and hBD-2 are released in different profiles (stimulus and time- dependent) in Caco-2 epithelial cells upon stimulation with TNF-α or IL-1β. Fundamentally, cytokines are always produced by specific cells in a cascade manner (Brennan and Feldmann, 1992), however, the interesting finding is that the cytokine mediated intestinal homeostasis, tissue repair and immunoregulation (IL-

10, and IL-6) reached their production peak first whether using TNF-α, or IL-1β for stimulation, whereas TNF-α was relatively delayed to get it to the maximum level.

IL-6 has a vital role in the regeneration of epithelial cells; Jin et al (2010) demonstrated an animal model (mice) showing that the loss of IL-6 resulted in augmentation of the activation of pro-apoptotic and necrotic pathways in epithelial cells after injury. The main properties of IL-10 as an anti-inflammatory cytokine is to inhibit inflammation through down-regulation of MHC class II and B7-1, B7-2 co-stimulatory molecule expression and decrease the production of IL-1β, TNF-α, and IL-8 (Herfarth and Schölmerich, 2002). The decreasing of TNF-α cytokine production by IL-10 is performed by suppressing the activation of p38 MAPKs pathways (Williams et al., 2004). IL-8 cytokine expression enhances epithelial cell restitution and controls epithelial cell turnover (Dignass and Podolsky, 1993). The biological effects of TNF-α are exerted through the expression of its receptors on cell membranes; p55 (associated with cell apoptosis), and p75 (associated with cell survival) (Peschon et al., 1998). It seems that epithelial cells respond to the TNF-stimulation directly, via activated TNF receptor, and indirectly, via the sequential release of other cytokines (Janes et al., 2006). Hence, it could conceivably be hypothesised that the results of this study are due to the different roles of these cytokines in responding to immune stimulation, with the anti-inflammatory (IL-10) and restorative (IL-6 and IL-8) cytokines expressed prior to the apoptotic cytokines (TNF-α). This allows for enhanced wound repair by monolayer epithelial cells through restitution of the cells (Dignass and Podolsky, 1993). Foey *et al*.(1998) reported that TNF-α has a vital role in inducing macrophage IL-10 expression. Interestingly, the novel findings reported above show that TNF-α and IL-1β induced IL-10 expression in epithelial cells. In addition

to cytokine release, hBD-2 was released after stimulation of cells with TNF-α or IL-1β. Data showed that the release kinetics of hBD-2 were the same as that of TNF-α (after both TNF-α or IL-1β stimulation), suggesting a strong positive link between the expression of hBD-2 and TNF-α.

Regarding a probiotic bacterial role in modulation of the immune responses induced by TNF-α or IL-1β, data showed that probiotic treatments selectively modulated TNF-α or IL-1β induced epithelial cytokine and hBD-2 expression (cytokine, and bacterial strain specific dependent), refer to table 4.2.1. A number of reports showed that probiotic bacteria have potential effects in the modulation of cytokine production induced by pro-inflammatory cytokines such as TNF-α or IL-1β which mediates destructive effects on the epithelial gut mucosa. Indeed, de Moreno de LeBlanc et al. (2011) reported a review showing that probiotic bacteria enhanced epithelial IL-10 cytokine production, based on evidence from human and animal models; *L. casei* reduced TLR4 and IL-1β mRNA levels and significantly increased mucosal IL-10 in ulcerative colitis patients (D'Incà et al., 2011), *L. casei* BL23 increased IL-10/IL-12 cytokine ratio leading to protection against colitis in TNBS-induced mice (Foligne et al., 2007). In addition, de Moreno de LeBlanc and Perdigón (2010) demonstrated that milk fermented with *Lactobacillus helveticus* R389, administered to the mouse model, attenuated the inflammation of the gut mucosa by decreasing IL-6 and increasing IL-10 in serum and in the mammary glands, therefore, it can be speculated that the consumption of fermented milks can modulate the immune system. In a cell line model, Resta–Lenert and Barrett (2006) reported that probiotic DNA of *Streptococcus thermophilus* and *Lactobacillus acidophilus* reversed TNF-α and IFN-γ induced epithelial dysfunction

(barrier function and cytokine expression) in HT-29 monolayer cells via suppression of the activation of SOCS-3 and STAT-1.

Focusing on specific strains (such as LF and LcS), Matsumoto et al. (2005) reported that LcS exhibits an anti-inflammatory effect by suppression of pro-inflammatory cytokine (IL-6) in LPS stimulated large intestinal lamina propria mononuclear cells isolated from mice with TNBS-induced colitis, and in murine macrophages (RAW264·7 cells) and in peripheral blood mononuclear cells (PBMCs) derived from patients with ulcerative colitis. LcS exhibited their roles in suppression of LPS induced pro-inflammatory cytokines via suppression of NF-kB through a polysaccharide-peptidoglycan complex (PSPG) derived from LcS, suggesting that LcS might be a useful probiotic for the treatment of human IBD. Prescott et al. (2005) reported that heat killed probiotic LF have pro inflammatory properties by suppressing the anti-inflammatory cytokines such as IL-10 and up-regulate the Th1 cytokines (TNF-α and IFN-γ) in PBMCs isolated from children with atopic dermatitis (AD). In this study, probiotic bacteria selectively modulated cytokine TNF-α or IL-1β induced hBD-2 and cytokine production at mRNA and protein levels. Data showed that probiotics selectively modulated TNF-α or IL-1β-induced hBD-2 and cytokine expression. Based on their effects on the modulation of cytokines and hBD-2 production, probiotics are partially listed in two groups; group one suppressed TNF-α induced hBD-2 production, whereas group two augmented TNF-α induced hBD-2 production, and *vice versa* associated with IL-1β induced hBD-2 regulated by probiotic treatments (refer to table 4.2.1).

Focussing on LcS treatment on modulation of cytokines and hBD-2 at protein levels, it suppressed TNF-α induced TNF-α, IL-8, and hBD-2, augmented TNF-α induced IL-10 and exhibited no modulation on TNF-α induced IL-6. On the other

hand, LF treatment augmented TNF-α induced TNF-α, IL-8 and hBD-2, suppressed TNF-α induced IL-10, and no modulation of TNF-α induced IL-6. In contrast, using IL-1β to induce cytokine and hBD-2, LcS treatment augments IL-1β induced IL-6, IL-8 and hBD-2, whereas suppressed IL-1β induced TNF-α and IL-10. LF treatment augmented IL-1β induced IL-8, IL-10; suppressed IL-1β induced TNF-α, IL-6 and hBD-2. Screening of this data suggesting that the inhibition of pro-inflammatory cytokines such as TNF-α lead to inhibition of the expression of hBD-2, whereas up-regulation of IL-10 lead to suppressed hBD-2 expression. Albanesi et al. (2007) reported that hBD-2 expression is induced by TNF-α or IFN-γ via activation of NF-kB and STAT-1 signalling negatively regulated by anti-inflammatory Th2 cytokines (IL-4, IL-13) via activation of STAT-6 and induction of SOCS-1 and SOCS-3 in keratinocytes. Alternatively, Howell et al. (2005) reported that hBD-2 suppression in atopic dermatitis (AD) and psoriasis was associated with elevated IL-10; neutralization of the IL-10 bioactivity augmented the production of TNF-α, IFN-γ and hBD-2 by peripheral blood mononuclear cell from AD patients and in keratinocytes. Data from this study suggested that probiotic treatments indirectly regulated hBD-2 expression in intestinal epithelial cells via modulation of cytokine regulated hBD-2 expression (IL-10 and TNF-α). Taken together, the group of probiotic bacteria that suppressed TNF-α induced hBD-2 expression, have a unique molecular structure to exert anti-inflammatory properties, whereas the other group that augmented hBD-2 expression induced by TNF-α have a unique molecular structure to exert pro-inflammatory properties. Interestingly, the pattern of TNF-α induction of cytokines modulated by probiotics differed from that of IL-1β induction of cytokine production. The two faces of probiotic immunomodulation were dependent on bacterial strain and type of inflammatory signal. These data are in agreement with Shida et al. (2011) which

showed that *LcS* exhibit two immunomodulation faces through induction of different types of cytokines; *LcS* can be a potent IL-12 inducer (pro-inflammatory properties) and also a potent IL-10 inducer (anti-inflammatory properties) leading to regulated hBD-2 expression. Probiotic bacteria used in this study belong to the lactic acid bacteria (LAB), have a thick layer of PGN coupled with LTA and a thin layer of LPS, presenting a G+C content in their DNA lower than 55% except BB which have more than 55%, vary in their sensitivity to lysozyme activity, where LF is sensitive (Šimelyte et al., 2000), and LcS bacteria are resistant (Shida et al., 2006). Several studies highlighted the importance of LTA in modulation of the immune responses induced by different stimuli in various cell types including epithelial cells, DCs and macrophages (Claes et al., 2012a, Ginsburg, 2002, Henneke et al., 2005). Bacterial PGN also has significant effects in modulation of the immune responses (Xu et al., 2001, Gupta et al., 1999). The differences between LF and LcS on their effects on the modulation of the cytokine expression induced by TNF-α or IL-1β might depend on the differences on their cell wall structure (types of LTA, and types of peptides that formed PGN), which caused different effects on the modulation of cytokine production. The complex of polysaccharide-peptidoglycan in LcS cell wall suppressed LPS-induced pro-inflammatory cytokine in a macrophage murine model (Matsumoto et al., 2005), and LTA from LF prevented atopic disease via up-regulation of the pro-inflammatory cytokines in a randomised placebo-controlled trial (Kalliomaki et al., 2001). Therefore, the sensitivity of LTA and/or PGN to either TNF-α or IL-1β will determine the fate of the immune response initiated in response to stimulation with cytokine and regulation of probiotic bacteria. Indeed, it seemed to be that cytokines and bacteria through their activation of epithelial cells appear to act through the same pathways, namely tyrosine kinases, p38 MAP kinases, and NF-

kB (McDermott et al., 2003), and manipulation of these pathways by probiotic treatments lead to modified outcomes of the immune responses either suppress or augment cytokines induced-hBD-2 expression. Several possible explanations of probiotic immunomodulation regulating cytokine expression include modulating the activity of STAT-1, STAT-6, SOCS-1 and SOCS-3 signalling pathway (Albanesi et al., 2007) leading to modulated TNF-α or IL-10 expression in different ways (suppression or activation) and regulated hBD-2 expression. Focussing on TNF-α expression, ADAM17 expression controls TNF-α release (Cesaro et al., 2009), therefore, probiotics might regulate the expression of ADAM17 leading to regulated TNF-α expression, consequently controlled hBD-2 expression. Data showed that the production of hBD-2 was linked to expression of IL-10 and TNF-α (inducer for TNF-α or suppressor for IL-10), which was in agreement with other research groups (Kanda et al., 2011, Marian et al., 2009). Consequently, data of this study showed that hBD-2 induction in epithelial cells was bacterial strain and type of cytokine dependent; LF up-regulated TNF-α induced hBD-2, whereas LcS up-regulated IL-1β induced hBD-2.

Enhancement of hBD-2 at the gut mucosa is not exclusively for killing pathogenic microorganisms, in addition, hBD-2 augments TNF-α, IL-1, and IL-8 leading to amplifying local immune responses prior to clearing pathogens (Van Wetering et al., 1997, Chaly et al., 2000, Niyonsaba et al., 2004). In addition, Niyonsaba et al. (2007) reported that hBD-2 is an important mediator of cytokine release, including release of IL-6, MIP-3α, MCP-1, and RANTES through activation of G protein and phospholipase C-dependent pathways, and also promote epithelial cell migration and proliferation, angiogenesis, chemotaxis, and wound repair in epidermal keratinocytes cells. One of the major roles of hBD-2 at the gut mucosa is linking

innate and adaptive immunity, mediating the recruitment of immature DCs and memory T cells via binding to the chemokine receptor CCR6 (Yang et al., 1999). Therefore, enhancement of defensin expression via modulation of cytokines induced hBD-2 by probiotic bacteria might represent a new therapeutic strategy for reduction of infection in human diseases associated with hBD-2 deficiency, such as Crohn's disease.

4.3.3. Probiotics selectively modulate TNF-α or IL-1β induced cytokines and hBD-2 expression after neutralisation of IL-10 or TNF-α bioactivity.

Due to their central roles in the mucosal immune system and accessibility to luminal agents, there is a growing interest for modulating epithelial cell's activities in various intestinal pathological contexts. Intestinal epithelial cells are active participants in the mucosal immune system (Jung et al., 1995), mainly through the production of cytokines (Bahrami et al., 2011b). Normally, gut epithelial cells produced a range of cytokines include; cytokine mediated gut tolerance (TGF-β and IL-10), trigger the active immune response (IL-8), tissue damage (TNF-α) and tissue repair (IL-6) in response to different stimuli. Cytokine expression has two faces, endogenous (membrane bound and or intracellular) or exogenous (free released into the medium) (Stadnyk, 2002). In a normal physiological context, endogenous cytokine expression enhances epithelial cell restitution, proliferation and migration of the intestinal epithelial cells mediates epithelial cell turnover (Dignass and Podolsky, 1993). In this study, Caco-2 epithelial cells expressed endogenous cytokines including TNF-α and IL-10, which in agreement with other research groups (Jarry et al., 2008), and probiotic treatments clearly modulated the induction of endogenous cytokine production. In addition, probiotic treatments were also modulated cytokine production induced by either TNF-α or IL-1β dependent on the strain and type of stimulus. In reviewing the literature, no data

was found on the association between probiotic treatment, hBD-2, cytokine stimulation of TNF-α or IL-1β, and endogenous cytokine expression of IL-10 and TNF-α in epithelial cells. The current study demonstrated that probiotic treatments of LcS or LF failed to up-regulate hBD-2 expression after neutralisation of TNF-α bioactivity, suggesting that the crucial role of endogenous TNF-α in mediating the induction of hBD-2 expression. Data showed that after the neutralisation of TNF-α bioactivity, hBD-2 was not expressed even in the presence of probiotic bacteria. In contrast, upon neutralisation of IL-10 bioactivity, hBD-2 was augmented and probiotic treatments differentially modulated hBD-2 expression suggesting a vital role of IL-10 in controlling hBD-2 expression (refer to table 4.2.3). Several lines of evidence support both pro- and anti-inflammatory roles for hBD-2 promoting the release of both pro-inflammatory (IL-6, IL-18) and anti-inflammatory (IL-10) cytokines from epithelial cells as shown by several in vitro studies (Niyonsaba et al., 2005). Donnarumma et al. (2007) reported that human lung cell line A549 expressed LPS-induced hBD-2 reduced the expression of IL-1β. Therefore, data of this study suggested at the breakdown of tolerance after neutralisation of the bioactivity of IL-10, probiotic treatments that augment hBD-2 exhibit significant effects in modulation of the immune responses via up-regulation of hBD-2 expression. It was therefore proposed that probiotics play an important role in immunomodulation of endogenous cytokine-induced hBD-2 expression. The current study demonstrated that after neutralisation of IL-10 bioactivity, constitutive TNF-α expression would drive hBD-2 expression. Probiotics exerted differential roles in modulation of the immune responses induced by TNF-α or IL-1β after neutralization of the bioactivity of TNF-α or IL-10. Indeed the immunomodulation scenario driven by probiotics was strain and type of inflammatory signal dependent. It seemed to be that the differences in PGN level and specific polysaccharide

component in a LAB bacterial cell wall might be playing a crucial role in modulation of endogenous as well as exogenous epithelial cytokine expression (Matsumoto et al., 2009), and consequently in modulation of hBD-2 induction. Nevertheless, *in vivo* studies should be done to confirm the hypothesized probiotic effects in IBD models, such as mice suffering from chemical-induced colitis {induced with oxazolone, trinitrobenzen sulfonic acid, or dextran sodium sulphate or spontaneous colitis (e.g. IL-10 knockout mice)} (Ekstrom, 1998, Fichtner-Feigl et al., 2007, O'Mahony et al., 2001, Rachmilewitz, 2002).

4.3.4. Probiotics selectively modulate TNF-α or IL-1β induced TLR expression after neutralisation of the IL-10 or TNF-α bioactivity

TLRs are pattern recognition receptors which recognise microbial (Abreu, 2010) and non-microbial (Vabulas et al., 2001) components, that play a key role in innate immunity. These TLRs bind and convey the signals from the gut contents into the underlying immune cells. These interactions lead to the promotion of an appropriate immune response to the microbial events, leading to the induction of cytokines and AMP expression. Contradictory reports were found about the expression of TLR in epithelial cells (Cario and Podolsky, 2005a). Indeed, the upsurge of TLR expression has been reported during the course of microbial infections (Furrie et al., 2005), or in IBD (Cario and Podolsky, 2000a). Very little information was found in the literature on the question associated with probiotic effects, cytokine stimulation (exogenous and or endogenous), hBD-2 and epithelial TLR expression. It proposed that the intestinal epithelial cells hyporesponsivness are related to the absence or low level of TLR4 expression, which recognises LPS. The current study demonstrated that TLR4 expression suppressed after neutralisation of the TNF-α bioactivity even in the presence of probiotic bacteria, suggesting that probiotic bacteria might be failing to deliver the immune signal

through TLR4. This failing was confirmed by failing in hBD-2 expression. Whereas after the neutralisation of the IL-10 bioactivity, TLR4 was up-regulated and probiotic treatments significantly further up-regulated (refer to table 4.2.2), suggested that probiotic bacteria can deliver the signal of TLR4 after neutralisation of IL-10 bioactivity not TNF-α. Data showed that after neutralisation of IL-10 bioactivity, LF treatment up-regulated TLR4 expression correlated with up-regulation of hBD-2 protein expression was more than by LcS treatment, suggesting that the lysozyme sensitivity and cell wall structure of LF has significant effects in up-regulation of TLR4. Goto et al. (2008) reported that stimulation of epithelial cells with specific TLR4 ligands induced a range of cytokines including pro-inflammatory cytokines (TNFα, G-CSF, IL-1α, and IL-6) and anti-inflammatory cytokines such as IL-10. It seemed to be that probiotic bacterial treatments have significant roles in the up-regulation of epithelial TLR expression. These data are in agreement with Vizoso Pinto et al. (2009), who showed that stimulation of HT-29 with *L. plantarum* or *L. rhamnosus GG* modulated epithelial TLR expression, which correlated with modulation of cytokine expression. The up-regulation of TLR4 by probiotics might be contributing in either initiation of inflammation via up-regulation of pro-inflammatory cytokines (Niyonsaba et al., 2007), or maintaining the gut mucosa via inhibiting pro-inflammatory cytokines via up-regulation of anti-inflammatory cytokines such as IL-10 (Donnarumma et al., 2007) which might be induced by hBD-2. Data showed that treating cells with TNF-α or IL-1β augmented TLR4, which is in agreement with Abreu et al. (2002), when they showed that Th1 cytokines (TNF-α or IFN-γ) perpetuate intestinal inflammation by altering TLR expression and bacterial reactivity in cell lines (Caco-2, HT-29, and T84) via suppression of SOCS-3 expression. However, probiotic treatments selectively modulated TNF-α or IL-1β induced TLR4 expression after neutralisation of the IL-

10 or TNF-α bioactivity. A number of reports showed that the up-regulation of TLR4 expression in gut epithelial cells mediated gut pathology (Cario, 2010, Guillot et al., 2004). The significant observation was the suppression of TLR4 induced by TNF-α after neutralisation of IL-10 bioactivity by LF treatment. It seemed the treatment with LF exhibited significant effects in suppression of TLR4 via up-regulation of IL-10 (Mueller et al., 2006). Probiotic treatments exhibit more sensitivity to TLR4 expression induced by IL-1β, as they suppressed TLR4 expression after neutralisation of TNF-α or IL-10 bioactivity via up-regulation of IL-10 as shown by this study. This suppression by probiotic treatments might be leading to suppression of the pro-inflammatory mediators associated with gut diseases such as Crohn's disease, suggesting a new therapeutic strategy in the treatment of IBD.

TLR2 is normally present at gut epithelial cells and has a significant role in stabilizing epithelial barrier function via its roles in ZO-1 translocation (Cario et al., 2007). However, uncontrolled expression of TLR2 plays a key role in triggering gut inflammation mediated tissue destruction via up-regulation of pro-inflammatory cytokines such as TNF-α and IL-1β (Vinderola et al., 2005). Data of this study showed that the TLR2 expression was suppressed after neutralisation of TNF-α bioactivity, and up-regulated in probiotic presence. It seemed to be that LTA in LF or LcS was recognised by TLR at this setting, might be resulting in maintaining gut homeostasis through their roles in maintaining the barrier function of epithelial cells (Cario et al., 2004). Data showed that neutralisation of IL-10 bioactivity results in augmentation of TLR2 expression. However, probiotic treatments suppressed TLR2 after neutralisation IL-10 bioactivity. This suppression by probiotics was not correlated with hBD-2 expression suggesting that hBD-2

expression was independent of TLR2 expression and associated with TLR4, which is in agreement with Vora et al. (2004). TLR2 induced by TNF-α or IL-1β was suppressed by probiotic treatments after neutralisation of TNF-α or IL-10 bioactivity. This suppression of TLR2 expression at the breakdown of tolerance or after stimulation with pro-inflammatory cytokines might be via augmentation of anti- inflammatory cytokines. Since the intestinal epithelium is continually exposed to a large variety of commensal bacteria, they constitutively express several members of the TLR family *in vitro* and *in vivo* (Cario et al., 2000a). Based on the surveillance system performed by gut mucosa, the up-regulation of TLR4 or TLR2 in the presence of TNF-α or IL-1β are characterised as the main task achieved by gut epithelial cells in response to these alarm signals. Indeed, epithelial cells monitor of gut contents resulting in amplifying an immune response in response to stimulation by a variety of stimuli started from up-regulation of TLR expression. The presence of TNF-α or IL-1β as a pro-inflammatory cytokine in this scenario will send warning signals to the gut epithelial cells. Gut epithelial cells respond to these signals (cytokines of TNF-α and IL-1β or bacterial PMAPs) by up-regulation of the TLR signals, subsequently the signal will be transferred downstream of the cells as activatory or tolerogenic immune response (dependent on the type of signal). The role of probiotics in the modulation of TLR expression, particularly TLR2, at either normal physiological or pathological level is still a matter of debate, especially the immunomodulation of cytokine induced TLR expression and regulation by probiotics after neutralisation of the TNF-α or IL-10 bioactivity. In the presence of a pro-inflammatory background (TNF-α or IL-1β), TLR4 and TLR2 expression is suppressed by probiotics, consequently, the inflammation induced by the up-regulation of these PRRs might be limiting leading to maintain gut tolerance (Murphy et al., 2009).

Human intestinal epithelial cells are capable not only to express CD14 but also to release the soluble form (Funda et al., 2001). This study and other research groups (Bocker et al., 2003) showed that CD14 is constitutively expressed in Caco-2 epithelial cells. CD14, a co-receptor for LPS signalling, is selectively modulated by probiotic treatments after neutralisation of the TNF-α bioactivity, when LcS exhibit suppression effects in contrast with augmentation by LF treatment. These differences in CD14 expression were strain-dependent. The up-regulation of CD14 by LF might be increasing epithelial cell sensitivity to LPS facilitating more opportunity to trigger inflammation, where epithelial cells should exhibit hyporesponsivness as a front line of gut mucosa toward a massive complex community of microorganisms found in gut contents. At a background inflammation after neutralisation IL-10 bioactivity, CD14 was significantly up-regulated (10 fold) in comparison with CD14 expression in resting cells; however, probiotic treatments suppressed CD14. This suppression by probiotic treatments might limit the inflammation where tolerance was broken after neutralisation of the IL-10 bioactivity; therefore, it was proposed that probiotic treatments might be maintaining gut mucosa via suppression of CD14 at this setting. One unanticipated finding was that TLR4 and CD14 expression disappeared after neutralisation of TNF-α bioactivity, even in the presence of LcS which correlated with suppression of TNF-α, hBD-2 and up-regulation of IL-10, suggesting that LcS suppressed TNF-α induced hBD-2 through up-regulation of IL-10 was via suppression of TLR4/CD14 expression (Mueller et al., 2006). Therefore, it was proposed that LcS probiotic bacteria are a good candidate to use when tolerance is broken. Several reports highlighted that TLR expression was up-regulated by cells at gut mucosa such as macrophages (Hausmann et al., 2002), and epithelial cells (Cario and Podolsky, 2000b). CD14 induced by TNF-α and regulation by probiotics was

suppressed by LcS and augmented by LF in comparison with CD14 after neutralisation TNF-α bioactivity, which correlated with suppression of hBD-2 and up-regulation of IL-10. These results suggested that LcS suppressed expression of TLR4/CD14 at an inflammatory background {directly by suppression of pro-inflammatory cytokines that induce TLR4/CD14 expression after adding TNF-α (exogenous) or indirectly by inducing factors after neutralisation of IL-10 bioactivity (endogenous)}. Nevertheless, probiotic treatments suppressed CD14 induced by IL-1β after neutralisation of the IL-10 or TNF-α bioactivity associated with up-regulation of IL-10, suggesting that CD14 expression induced by IL-1β after neutralisation TNF-α or IL-10 bioactivity was more sensitive to probiotic treatments when suppressed at both conditions (tolerance breakdown and inflammatory background). In addition to LPS recognition, CD14 expresses other functions such as scavenging for apoptotic cells (Devitt et al., 1998, Pradhan et al., 1997). Evidence showed that endogenous molecules or damage-associated molecular patterns released from damaged tissues are able to activate CD14 expression (Yu et al., 2011). Therefore, the data of this study suggested that probiotic treatments might modulate the scavenger ability (endocytosis) by epithelial cells (Hershberg and Mayer, 2000).

MD-2 protein has been revealed to be expressed as a linkage between TLR4 and LPS (Shimazu et al., 1999), as well as with TLR2/LPS (Dziarski and Gupta, 2000). One of the main tolerance mechanisms against LPS responsiveness of epithelial cells is the low or lack of expression of MD-2 (Abreu et al., 2002). Data of this study showed that neutralisation of IL-10 bioactivity had a critical role in up-regulation of MD-2 expression, suggesting a vital role of IL-10 expression in maintaining gut tolerance. In this study, probiotic bacteria exhibit anti-inflammatory

effects of suppression of MD-2 leading to limited responsiveness to LPS by epithelial cells after the cell tolerance was broken (Mocellin et al., 2003). This suppression was correlated with suppression of TLR4/TLR2/CD14 and augmentation of IL-10 and confirmed the immunoregulatory role of IL-10 in maintaining the gut mucosa (Mueller et al., 2006). Data also showed that LcS augmented MD-2 after neutralisation of TNF-α bioactivity, however, this augmentation did not reflect the augmentation of TNF-α or hBD-2, since TLR4/TLR2/CD14 was suppressed, explaining the fact that MD-2 expression alone did nothing unless associated with augmentation of other TLRs (TLR2/TLR4/CD14). MD-2 as a part of PRRs was significantly augmented after stimulating cells with pro-inflammatory cytokines; however probiotic treatments selectively modulate MD-2 induced by TNF-α after neutralisation either TNF-α or IL-10 bioactivity, when LcS suppressed MD-2 whereas LF treatment augmented MD-2, which not associated with TLR4/TLR2/CD14 induced TNF-α, IL-10 or hBD-2 suggesting other mechanisms might be involved in regulation of hBD-2, TNF-α or IL-10. However, the anti-inflammatory effects exhibited by probiotic treatments when IL-1β- induced MD-2 expression was suppressed after neutralisation of IL-10 bioactivity. In stark comparison, probiotic treatments exhibit selective effects in modulation of IL-1β-induced MD-2 after neutralisation of the TNF-α bioactivity. LcS treatment augmented IL-1β induced MD-2 after neutralisation of the TNF-α bioactivity (which was not -associated with up-regulation of TLR4/TLR2/CD14), linked with hBD-2 expression, suggesting that hBD-2 induced by other signalling such as NLRs or TLR9 at these settings. Results showed that both TLR9 and NOD-2 expression were up-regulated after neutralisation of both IL-10 and TNF-α and probiotic presence suppressed their expression suggesting the anti-inflammatory role of probiotics in driving mucosal tolerance associated with

expression of NOD-2 and TLR9 at inflammatory and tolerance breakdown. Indeed, intestinal PRR cell signalling like extracellular PRRs have a dual role involved in maintaining intestinal homeostasis and protection from injury, as well as initiating inflammation in response to warning signals. Normally, TLR9 stimulation enhances innate immune responses after recognition of CpG bacterial DNA, and NOD-2 stimulation enhances innate immunity through recognition of MDP, the bioactive component of PGN of the bacterial cell wall. The synergy between TLR9 and NOD2 in enhancing the innate immune system is very important at the normal gut mucosa. The loss of this synergistic relationship is a factor associated with IBD, in particular Crohn's disease (van Heel et al., 2005). The findings of this study clearly demonstrated that TNF-α or IL-10 have a critical role in TLR9 and NOD-2 expression. It seemed that the NOD-2 expression is controlled by TNF-α which is in agreement with Rosenstiel et al. (2003). Indeed, the up-regulation of these PRRs at the scenario when TNF-α was absent represents one of the main factors in maintaining intestinal homeostasis (van Heel et al., 2005). However, the up-regulation of TLR9 and NOD-2 are associated with gut diseases (Girardin et al., 2003b, Bamias et al., 2005), therefore, probiotic suppression might be enhancing the mucosal tolerance leading to maintaining gut mucosa through suppression of amplified inflammation initiated by augmentation of these receptors. In addition, at the inflammatory background of TNF-α or IL-1β stimulation, probiotics also exerted anti-inflammatory effects by suppressing the expression of TLR9 induced by either TNF-α or IL-1β after neutralisation of the TNF-α or IL-10 bioactivity which is not associated with TNF-α, IL-10 and hBD-2 expression. Whereas, selectively modulated TNF-α induced NOD-2 after neutralisation of the TNF-α bioactivity, when LcS up-regulated TNF-α induced NOD-2, and LF suppressed it after neutralisation of TNF-α bioactivity. Neutralisation of TNF-α did not modulate NOD-

2 induced by IL-1β and probiotic treatments of LcS (non-significant suppressed), and LF (significant suppression) exhibit anti-inflammatory effects via suppression of NOD-2 expression was also not associated with TNF-α, IL-10 or hBD-2 suggesting that the intracellular PRRs are not involved in the regulation of hBD-2, TNF-α or IL-10. In fact, TLR signalling pathways have suggested a promising mechanism for boosting vaccine responses (van Duin et al., 2006). Further studies with more focus on probiotic bacterial role in modulation of TNF-α or IL-1β induced TLR expression after neutralisation of TNF-α or IL-10 bioactivity is therefore suggested to establish the basis for development of mucosal vaccine adjuvants.

Tollip is one of the main proteins expressed in intestinal epithelial cells, as a negative regulator of TLR expression and polymorphisms in Tollip expression are associated with susceptibility to several gut diseases such as IBD (Abreu et al., 2005, Shah et al., 2012). Very little was found in the literature on the question of the probiotic bacterial role in modulation of TNF-α or IL-1β induced Tollip expression after neutralisation the TNF-α or IL-10 bioactivity. The current study demonstrated that Tollip expression was highly up-regulated at the scenario of TNF-α absence, suggesting that there is a critical role for TNF-α in controlling Tollip expression consequently, suppression of TLR activation. These data are in line with Melmed et al. (2003) who showed that TLR2 suppressed in human intestinal epithelial cells correlated with high expression of Tollip. Data showed that the presence of probiotics resulted in suppression of Tollip expression even in the absence of TNF-α. This suppression might be via recognition of microbial PAMPs by epithelial PRRs, which induce production of pro-inflammatory cytokines, consequently, suppression of Tollip expression (autocrine effect). In addition to TNF-α, IL-10 also exhibited a critical role in controlling Tollip expression. Data

showed that neutralisation of IL-10 bioactivity resulted in suppression of Tollip expression, which is associated with up-regulation of pro-inflammatory cytokines. These data are in agreement with Didierlaurent et al. (2006) who showed that Tollip suppressed pro-inflammatory responses induced by LPS and IL-1β was associated with suppression of NF-kB and MAPKs in animal model (mice). One unanticipated finding was that LcS treatment augmented Tollip expression even in the absence of IL-10. This enhancement by LcS might be through augmentation of TGF-β, the potent regulatory cytokine mediated gut homeostasis (Rautava et al., 2005). TGF-β has a vital role in limiting inflammation by suppression of TLR expression (Gómez-Llorente et al., 2010). Tollip expression induced by TNF-α was up-regulated by LcS treatment after the neutralisation of the TNF-α bioactivity suggesting that LcS up-regulated Tollip might be via up-regulation of IL-10 and vice versa with LF (refer to table 4.2.2 & 4.2.3). These data are in agreement with Shimazu et al. (2012) who showed that *Lactobacillus jensenii* exhibit anti-Inflammatory effects on porcine intestinal epithelial cells by modulating Tollip signalling pathway via down-regulating TLR4-dependent NF-kB MAPKs pro-inflammatory activation. Taken together, the scenario of probiotic modulation of epithelial TLR expression induced by TNF-α at the absence of IL-10 provides a new insight into the mechanisms of positive regulation of Tollip by IL-10, and negative regulation of TNF-α. These regulations of Tollip expression will lead to the modulation of the dynamic balance between controlled surveillance and appropriate responses to mucosal challenge by luminal flora. Further research to investigate mechanisms is required and human trials are needed to confirm these immunomodulatory effects of these probiotic strains (LcS and LF) in modulation of endogenous cytokine expression *in vivo*.

Chapter 5

Effects of probiotics on Inflammatory responses in an epithelial cell: macrophage co-culture model

Chapter 5: Effects of probiotics on inflammatory responses in an epithelial cell: macrophage co-culture model

5.1. Introduction

In the GIT, epithelial cells display a variety of functions including the physical barrier to separate the external environment of microbiota and food antigens from the internal environment where the cells of the lamina propria such as macrophages are resident. Elicited macrophage responses are dependent on tissue environment and the resulting cell subsets, with inflammatory macrophage resembling M1 subset and homeostatic macrophages resembling an M2 macrophage subset (Mantovani et al., 2007). Uncontrolled immune responses of macrophages induced by different stimuli are implicated in gut diseases particularly, IBD which is associated with loss of gut tolerance. The precise aetiology of IBD is still a matter of debate. However, understanding the pathophysiology of IBD has advanced, and the typical features of these diseases shown in various studies by using *in vivo* and *in vitro* models.

To resemble the *in vivo* situation of the gut physiology, *in vitro* cell models of the gut using a combination of different cell types was used to investigate the immunomodulation of probiotics on the barrier cell function. Expression of tight junction proteins is necessary for the formation of epithelial barrier, integrity, and polarity. Epithelial cells undergo a series of developments to form a monolayer with a tightly packed selectively permeable membrane with measurable trans-epithelial resistance. Epithelial cells in combination with other cell lines to build up a model resembling gut physiology, should respond to environmental factors such as cytokines and inflammatory molecules. Of these, the most suitable model for

intestinal epithelial cells is Caco-2, and THP-1 human monocytic cell line for monocyte derived M1-like and M2-like macrophage cell subsets. Therefore these two cell lines were used to build up a model resembling gut physiology, where epithelial cells (Caco-2) interacted with M1-like macrophages to model inflammatory gut physiology or with M2-like macrophages as a normal homeostasis model. Caco-2 cells upon reaching confluence, express characteristics of enterocytic differentiation and functionality, with feature include; microvilli, large vacuolated mitochondria, smooth and rough endoplasmic reticulum (Lee et al., 2009). Lo et al. (2004) reported that transwell based Caco-2/Raji B cell culture model resulted in differentiating enterocytes into M cell-like cells. Comparing functional features in M-cell like Caco-2 cells to M cells in follicle-associated epithelium (FAE) of normal Peyer's patch tissue which include; gene expression of laminin β_3 (a matrix metalloproteinase), a tetraspan family member, and C. perfringens enterotoxin receptor (CPE-R) in UEA-1^+ M cells, suggested that M like Caco-2 cells shared features with M cells in FAE. Bacterial transport of *Salmonella* (either live or killed) was also examined using these models in order to confirm model functionality for investigating transcytosis and cell signalling pathways in M like cells (Martinez-Argudo and Jepson, 2008). However, Caco-2 cells are polyclonal nature and this high diversity has resulted in the need for standardised protocols (concerning passage number, time of usage post-seeding) to be strictly followed. The interests of Caco-2 were also co-cultured with other cell types such as PBMCs and macrophages. Parlesak et al. (2004) reported that co-culturing of Caco-2/PBMCs modulate the cytokine kinetics during challenge with bacteria (non-pathogenic *Escherichia coli*) in a compartmentalized co-culture model by Caco-2. Adding bacteria were applied apically to the Caco-2 cell layer, the production of TNF-α, IL-12, IL-1β, IL-8, IL-6, IL-10, and TGF-β was markedly

lower as compared to the bacterial stimulation of leucocytes beneath the Caco-2 cells. Modulation of cytokine expression by Caco-2 cells supports the concept of leucocyte-epithelial cell crosstalk roles in modulating cytokine responses in the gut mucosa. Similar findings were found by Haller et al. (2000b) when Caco-2 co-cultured with human blood leucocytes and profiles of TNF-α, IL-1β, IL-8, monocyte chemoattracting protein 1 (MCP-1), and IL-10 were modulated in response to stimulation Caco-2 cells with non-pathogenic (*Lactobacillus sakei*) and enteropathogenic bacteria.

Not only cytokine expression is important at gut mucosa, AMPs such hBD-2 are also important mediators at gut mucosa, lead many researchers to focus on the main mechanisms that control their expression. Tsutsumi and Nagaoka (2003) established a co-culture model of A549 pulmonary epithelial cells with mononuclear phagocytes (Mono-Mac-6 monocytic cells) stimulated with LPS to investigate the modulation of hBD-2 in pulmonary epithelial cells. Presence of LPS was markedly up-regulated hBD-2 promoter activity, whereas A549 alone did not respond to LPS to activate the hBD-2 promoter. IL-1β and TNF-α in the culture supernatants from LPS-stimulated macrophages activated the hBD-2 promoter in A549 cells associated with NF-kB activation. Epithelial cell barrier function was also addressed by this co-culture model. Tanoue et al. (2008) established a co-culture system of Caco-2 cells (apical side) and macrophage RAW264.7 cells (basolateral side) for assessing the anti-inflammatory effect of food factors. In this study the stimulation of RAW264.7 cells with LPS was followed by a decrease in trans-epithelial electrical resistance (TEER) associated with an increase in TNF-α production from RAW264.7 cells and IL-8 mRNA expression in Caco-2 cells.

Treatment with anti-TNF-α antibodies suppressed TNF-α production and IL-8 mRNA expression.

THP-1 cells are used in co-culture systems with different cell types, for instance, adipocytes, T-lymphocytes, platelets and intestinal cells. It is well reported that adipocytes obesity-associated inflammation enhances macrophage infiltration in adipose tissue by inflammatory cytokine production, such as TNF-α and IL-6 (Berg and Scherer, 2005). Keuper et al. (2011) established an *in vitro* model system for human adipose tissue by either incubation of SGBS adipocytes (pre-adipocyte cell line) with THP-1 cells in indirect incubation with conditioned medium from THP-1 cells or direct co-culture of SBGS adipocytes with THP-1 cells. Spencer et al. (2010) also established co-culture system by co-culturing primary human adipocytes and pre-adipocytes with THP-1 cells using a transwell method to examine the gene expression pattern of M1, M2a, M2b, and M2c macrophages. Data of this study indicated that co-culture of adipocytes with either M1 or M2 macrophages led to an overall shift of macrophage gene expression via secreting soluble factors from adipocytes, which promote a shifting of the M1 to the M2-phenotype cell during co-culture. Jurkat T cells are often used to study T cell signalling. Fuentes et al. (2002) established an *in vitro* co-culture model by co-culturing THP-1 cells with Jurkat cells to study the interaction between macrophages and T-cells.

Studies using THP-1 derived macrophages co-cultured with intestinal epithelial cells include; study by Watanabe et al. (2004) which showed that Caco-2 cells barrier function could be disrupted by co-culturing with THP-1 macrophages differentiated by PMA due to the secretion of TNF-α by THP-1 derived macrophages. A similar finding was reported by Moyes et al. (2010) when Caco-2

cells co-cultured in a transwell system with underlying THP-1 macrophages differentiated by PMA, produced a lower, less organised epithelium and greater microparticle uptake.

Over the past century, there has been a dramatic increase in attention on probiotic use in animal and human fields. Probiotic bacteria have long since displayed immunomodulatory properties of the gut immune system but the mechanisms are not well understood (Vasiljevic and Shah, 2008). From research into food factors with anti-inflammatory properties against intestinal inflammation {using animal intestinal inflammation models that are induced by administration of either dextran sodium sulphate (DSS) (Okayasu et al., 1990), or 2, 4, 6,-trinitrobenzene sulfonic acid (TNBS) (Morris et al., 1989) and IL-10 knockout mice (Kühn et al., 1993)}, the administration of probiotic bacteria has been shown to result in an improvement of mucosal appearance (Matsumoto et al., 2005). However, the modulatory effects of probiotic bacteria are still poorly understood at the cellular level. Because probiotic treatments exhibit different immunomodulation effects which depended on probiotic bacterial strain, probiotic cell preparation (live, heat-killed, irritated, cell lysate), probiotic concentrations, type of target cells (macrophage, DCs, NK cells, T cells, epithelial cells), type of cell model (monoculture or co-culture), type of cells in co-culture model, the experimental data are rather controversial, and there is no general agreement about the probiotic bacterial role in modulation of immune responses associated with epithelial cell barrier function. Therefore, a more precise *in vitro* assessment model of modulatory effects is required to elucidate these mechanisms by developing an *in vitro* co-culture system. In this chapter of this thesis, for the first time, a developed transwell co-culture system was established comparing two different status of gut physiology; inflammation (Caco-

2/M1) or homeostatic (Caco-2/M2) to investigate the immunomodulatory effects of probiotics on barrier function influenced by LPS-stimulated macrophages. Culturing fully differentiated Caco-2 at apical compartment and THP-1 cells differentiated by PMA (M1-like macrophages), or cells differentiated by Vit D_3 (M2-like macrophages at lower compartment, apically treated with probiotics, and basolateraly stimulated with LPS was performed to resemble gut pathology (inflammatory) and normal homeostasis model, respectively (refer to section 2.2.4.8). The investigations focused on probiotic regulation of epithelial cytokine, hBD-2 and TLR expression, TEER (as an index of intestinal epithelial barrier function), (refer to section 2.2.8, section 2.2.10, section 2.2.5) and ZO-1 protein associated with barrier function expression tracked by gene expression (refer to section 2.2.7.4) and immunohistochemistry at homeostatic and chronic inflammation model (refer to section 2.2.9).

The main issues addressed in this chapter were found suitable model to investigate:

Hypothesis 1: SP and HK probiotic strains can modulate epithelial cytokine and hBD-2 expression induced by LPS in different co-culture models.

Hypothesis 2: Probiotics can modulate epithelial barrier function (TEER, ZO-1) in different co-cultures models.

Hypothesis 3: Probiotics are able to modulate epithelial TLR expression induced by LPS in different co-culture models.

5.2. Results

5.2.1. Cytokine expression and barrier function are influenced by macrophage subset in epithelial cells.

To assess probiotic bacterial role in modulation of the epithelial barrier function in the normal homeostasis or chronic inflammation status, developing co-culture models of Caco-2/ M2 (normal homeostasis), and Caco-2/ M1 (chronic inflammation) was used to performing this task (refer to 2.2.4.8, Fig.1.7 lines 3). The first readout was identifying levels of epithelial cytokine production influenced by macrophage subsets.

Pro- and anti-inflammatory cytokine production was analysed in epithelial cells co-cultured with M1-like or with M2-like macrophages. In this analysis, protein level of the pro-inflammatory cytokines IL-8, IL-6 and TNF-α, and of the anti-inflammatory cytokine IL-10 were determined. Results showed that level of IL-8 produced by cells co-cultured with M1 was more than cells co-cultured with M2 at 3 fold, TNF-α at 59 fold, IL-6 at 51 fold, and finally IL-10 at 3 fold from control level 7±0.0 pg/ml (un-stimulated cells) (Fig.5.2.1A). The second readout was TEER in both co-culture models. Results showed that the TEER of resting cells (914.5±13.78) Ω/cm^2 dropped down after culturing with macrophage subsets, however, TEER value of Caco-2 cells co-cultured with M2-like macrophages was more than cells co-cultured with M1-like macrophages at about 3 times (Fig.5.2.1B). The results above raised a crucial question about the sort of cytokines that might be produced in the lower compartment by macrophage subsets, which exhibit theses impacts on epithelial cell integrity. To address this question, different cytokines including IL-1β, TNF-α, IL-8, and IL-10 were used to treat epithelial cells basolateraly in the absence of macrophage subsets. The readout TEER was performed at each

treatment. Results showed that the cytokine treatment of the epithelial cells has a critical role in modulation of TEER value (Fig.5.2.2A). TEER suppressed by 87%, 71%, and 52% for TNF-α, IL-8 and IL-1β respectively, whereas, IL-10 treatment up-regulated TEER value by 10.93% from control 904.5±13.7886 Ω/cm^2. TEER value generally represents the epithelial cell barrier, which is dependent on epithelial cell tight junctions. Epithelial tight junction composed of proteins, and ZO-1 is one of the main proteins associated with epithelial cell tight junctions. Results showed that the pro-inflammatory cytokines selectively down-regulated ZO-1 expression by 95% (IL-1β), 76% (TNF-α), and 91% (IL-8), whereas IL-10 cytokine treatment up-regulated ZO-1 cell expression by 121% from control 1±0 fold change (Fig.5.2.2B).

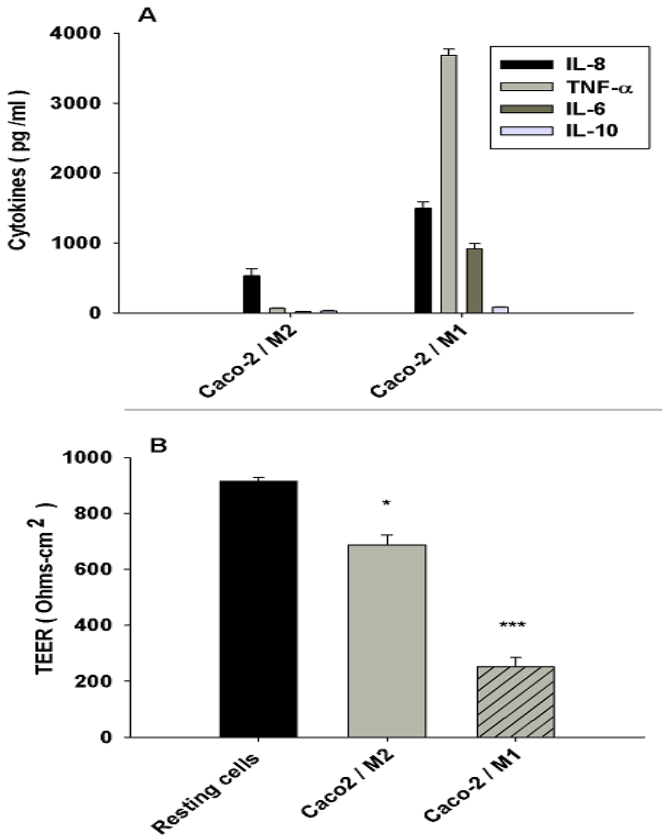


Figure 5.2. 1: Macrophage subsets selectively influenced cytokine production and epithelial cell integrity of Caco-2 cells.

Transwell inserts on which Caco-2 cells had been cultured were inserted into multiple plate well containing either M2-like macrophages, or M1-like macrophages. (**A**) Cytokine production is expressed as the mean±SE in pg/ml for IL-8, TNF-α, IL-6 and IL-10 secreted into apical compartment. (**B**) TEER measurements were performed using EVOM epithelial voltmeter for cells co-cultured with M2 or cells co-cultured with M1-like macrophages. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

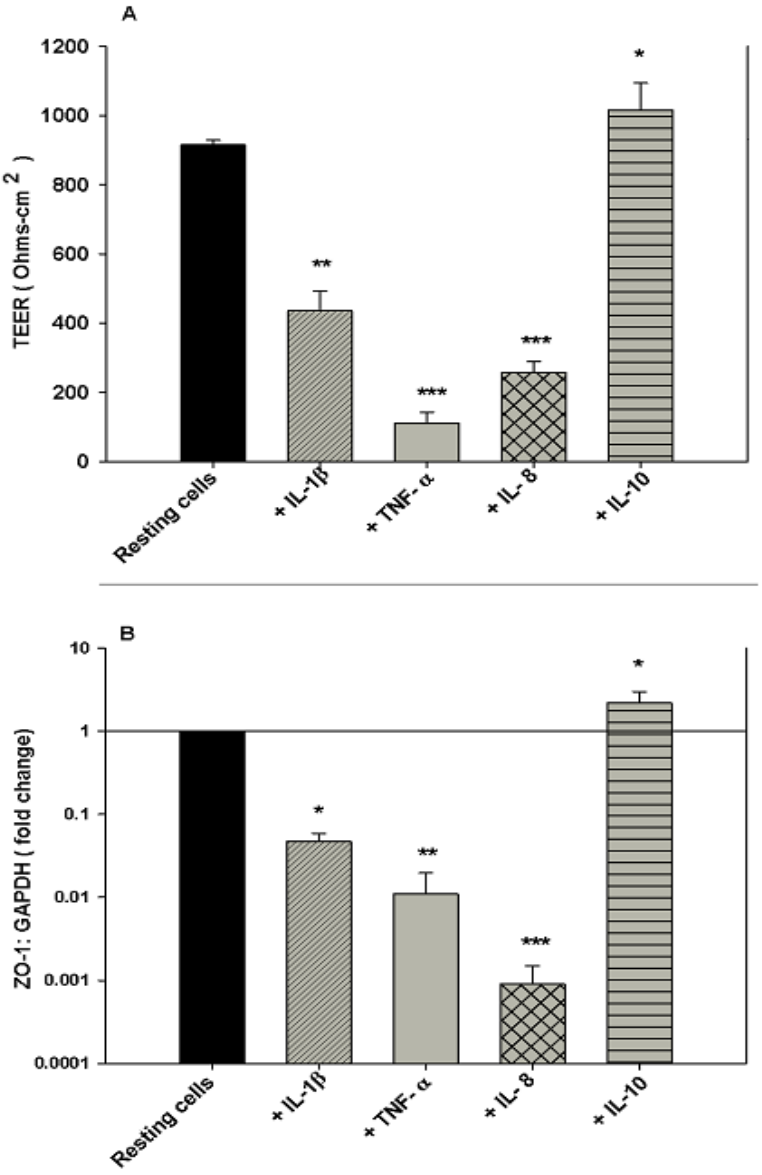


Figure 5.2. 2: Cytokines selectively modulate epithelial cell barrier (TEER and ZO-1).

Caco-2 cells were grown in transwell inserts for 21 days, treated with either 5ng/ml IL-1β, 10ng/ml TNFα, 10ng/ml IL-8, or 10ng/ml IL-10. TEER measurements were performed using an EVOM epithelial voltmeter (**A**). ZO-1 gene expression (mRNA level) is expressed as fold

change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**B)**. Data displayed is a representative experiment with triplicate samples of n=4 replicate experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005.

5.2.2. Role of SP and HK probiotic strains in modulation of epithelial cytokine expression and barrier function in different co-culture models.

In order to find out the role of probiotic treatments in the modulation of cytokine in epithelial cells co-cultured with either M1 or M2-like macrophages, HK or SP of LcS or LF were applied apically, then supernatant in apical compartments were used to determine pro-and anti-inflammatory cytokine production by ELISA (refer to Fig.1.7 line 3). Data presented in Fig. 5.2.3 show the effect of probiotic treatments on modulation epithelial cytokine production in Caco-2/M2, and Caco-2/M1. IL-10, IL-6 and TNF-α were up-regulated by treatment with all probiotics tested in the Caco-2/M2 co-culture model, whereas IL-8 was down-regulated by all the probiotic treatments in this model. In contrast, in the Caco-2/M1 co-culture model IL-10 and IL-6 were markedly down-regulated by all probiotic treatments, TNF-α selectively modulated (up-regulated by LF-HK and LcS-SP; down-regulated by LF-SP, and non-significantly up-regulated by HK-LcS) and IL-8 also showed selective modulation by probiotics (suppressed by LcS-HK and up-regulated by all others). hBD-2 was analysed in Caco-2/M2 and Caco-2/M1 co-culture models. Data represented in figure 5.2.4 show there was a clear immunomodulation of hBD-2 expression by probiotic treatments in both co-culture models. In normal homeostatic (Caco-2/M2) model, hBD-2 selectively modulated (up-regulated by LcS-SP and LF-SP, suppressed by HK-LcS, and non-significantly up-regulated by HK-LF (Fig.5.2.4A). hBD-2 up-regulated by HK-LF, suppressed by HK-LcS, and LF-SP, and non-significantly up-regulated by LcS-SP in Caco-2/M1 co-culture model (Fig.5.2.5B).

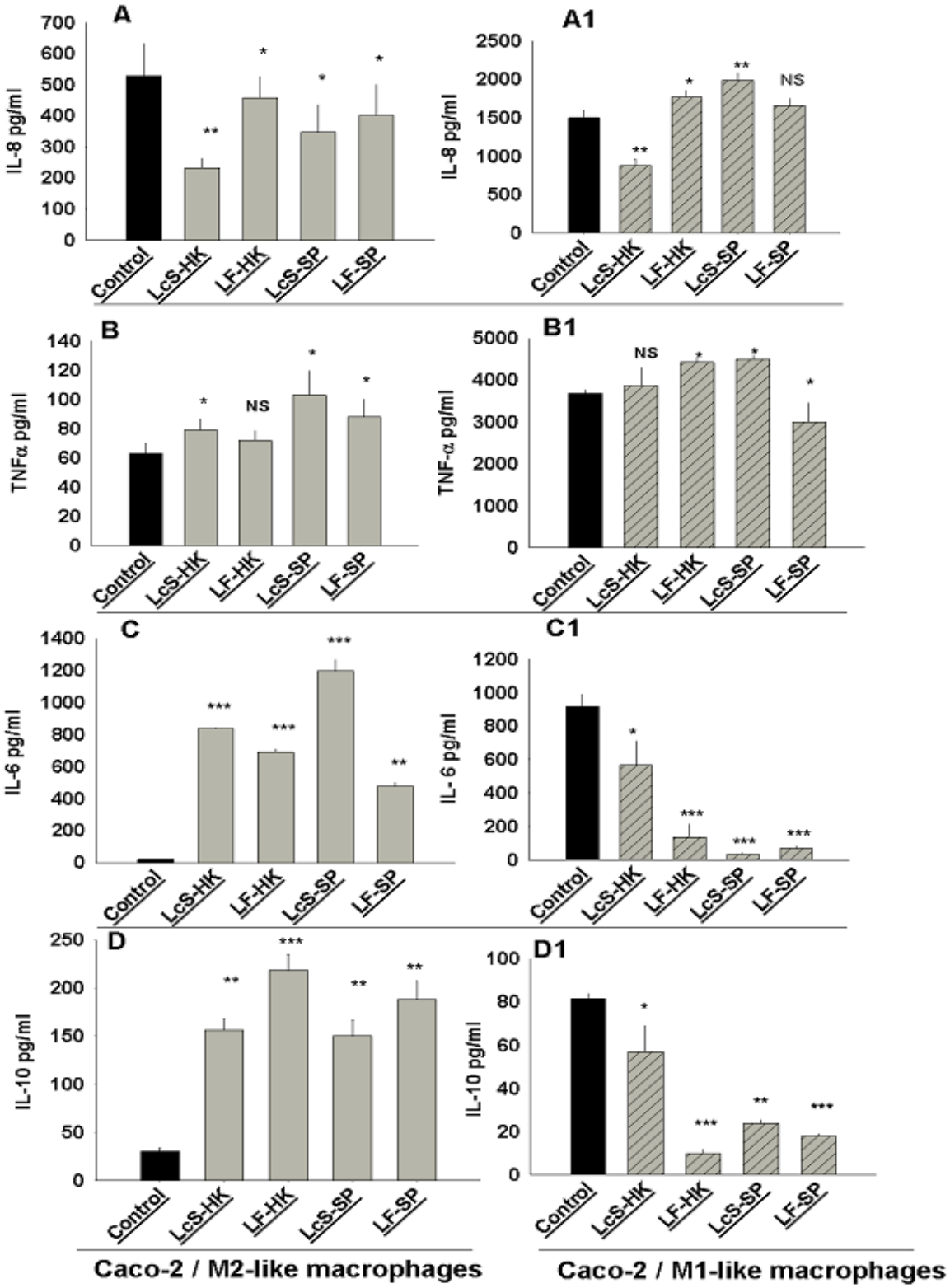


Figure 5.2. 3: SP and HK probiotic strains selectively modulate epithelial cytokine production in Caco-2/ M2 and Caco-2/M1 co-culture models.

Caco-2 cells grown in transwell inserts were inserted into multiple plate well containing either M2-like macrophages, or M1-like macrophages. Probiotic *Lactobacillus casei* strain Shirota (LcS) or *L. fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein (SP) extracts by them at 3.0µg/ml were added apically. Cytokine production is expressed as the mean±SE in pg/ml of IL-8 (**A, A1**), TNF-α (**B, B1**), IL-6 (**C, C1**), IL-10 (**D, D1**). Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

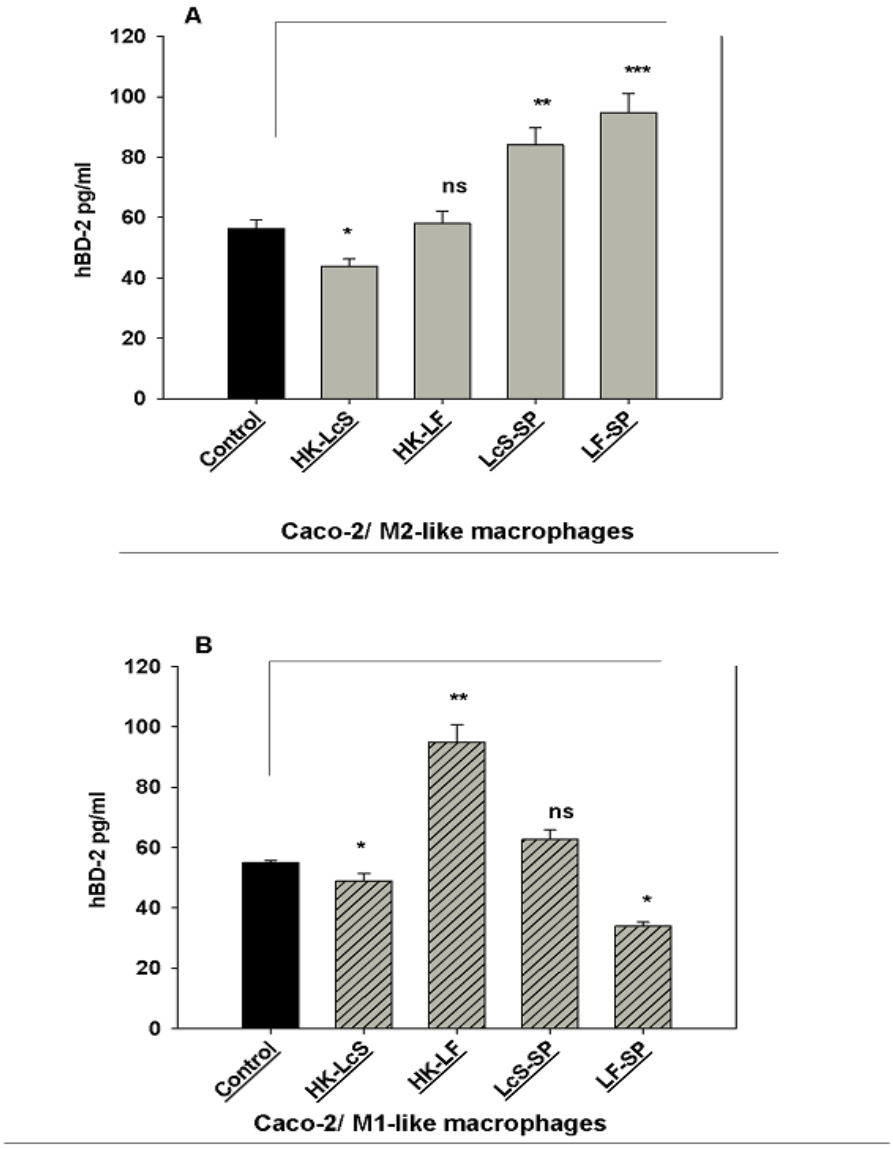


Figure 5.2. 4: SP and HK probiotic strains selectively modulate epithelial hBD-2 production in Caco-2/ M2 and Caco-2/M1 co-culture models.

Caco-2 cells grown in transwell inserts were inserted into multiple plate well containing either M2-like macrophages (shaded bars), or M1-like macrophages (hatched bars). Probiotic *Lactobacillus casei* strain Shirota (LcS) or *L. fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml were added apically. hBD-2 production is expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005, NS (non-significant).

5.2.3: Role of SP and HK probiotic strains in the modulation of cytokine and hBD-2 expression induced by LPS in Caco-2/M2 and Caco-2/M1 co-culture models

To imitate the pathogenic bacterial invading where the epithelial cell barrier was broken, LPS was applied basolateraly in co-culture systems, whereas probiotics added apically to pretend the probiotic administration as supplements to confer the human health (refer to 2.2.4.8). Data in fig.5.2.5 show the immunomodulatory effects of probiotic treatments on LPS induced pro-& anti-inflammatory cytokine in Caco-2/M2 and Caco-2/M1 co-cultured models determined by ELISA. IL-8 induced by LPS was suppressed by treatment with all probiotics tested in the Caco-2/M2 co-culture model and Caco-2/M1 co-culture model (Fig.5.2.5A, Fig.5.2.5A1), whereas IL-6 induced by LPS was augmented with the treatment of all probiotics tested in the Caco-2/M1 co-culture model (Fig.5.2.5C1). TNF-α induced by LPS was selectively modulated (suppressed by HK-LcS, augmented by HK-LF, LcS-SP, and non-significantly augmented by LF-SP) (Fig. 5.2.4B), IL-6 induced by LPS was augmented with all probiotics except LcS-SP, which suppressed LPS, induced IL-6 (Fig.5.2.4C). IL-10 induced by LPS was also selectively modulated (augmented by HK-LF, suppressed by all other probiotic treatments) (Fig.5.2.4D) in the Caco-2/M2 co-culture model. TNF-α induced by LPS was selectively modulated (augmented by HK-LcS, LcS-SP and LF-SP, suppressed by HK-LF) (Fig.5.2.4B1), IL-10 induced by LPS selectively modulated (suppressed by HK-LcS and LcS-SP,

non-significantly augmented by HK-LF and LF-SP) (Fig.5.2.4D1) in Caco-2/M1 co-culture model. In response to LPS stimulation, hBD-2 was selectively modulated by probiotic treatments. hBD-2 induced by LPS augmented by HK-LcS and LF-SP, and non-significantly augmented by HK-LF and LcS-SP in Caco-2/M2 co-culture model (Fig.5.2.6A), whereas hBD-2 induced by LPS was augmented with all probiotic treatments in Caco-2/M1 co-culture model (Fig.5.2.6B).

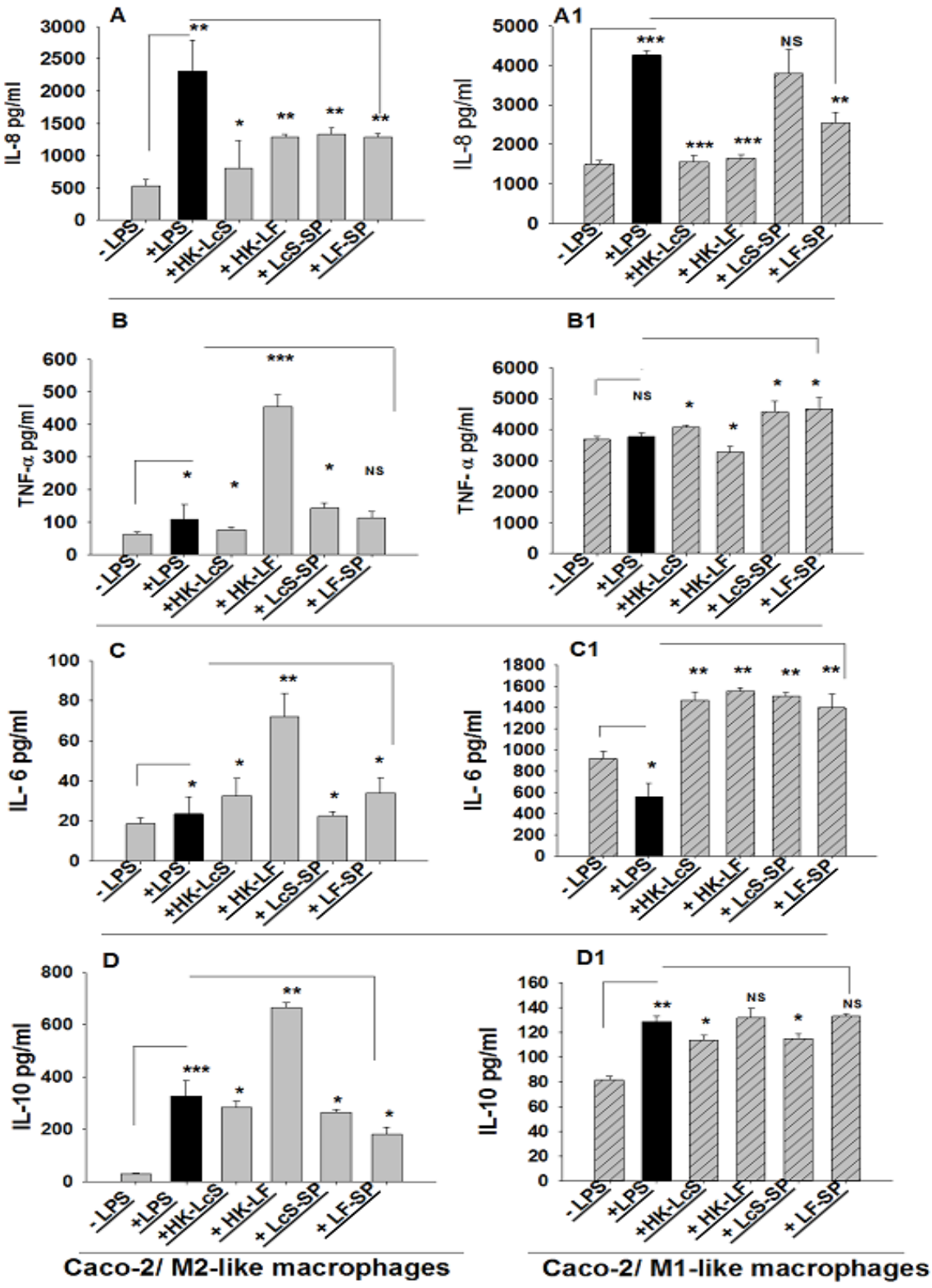


Figure 5.2. 5: SP and HK probiotic strains selectively modulate LPS-induced epithelial cytokine production.

Cultures of Caco-2 cells were inserted into multiple plate well containing either M2-like macrophages, or M1-like macrophages, 100 ng/ml of LPS was added to the basolateraly compartment. Whereas, probiotic *Lactobacillus casei strain* Shirota (LcS) or *L.fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein extracts (SP) by them in 3.0µg/ml were added apically. Cytokine production is expressed as the mean±SE in pg/ml of IL-8 (**A, A1**), TNF-α (**B, B1**), IL-6 (**C**, **C1**), IL-10 (**D**, **D1**). The data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control (+LPS) are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

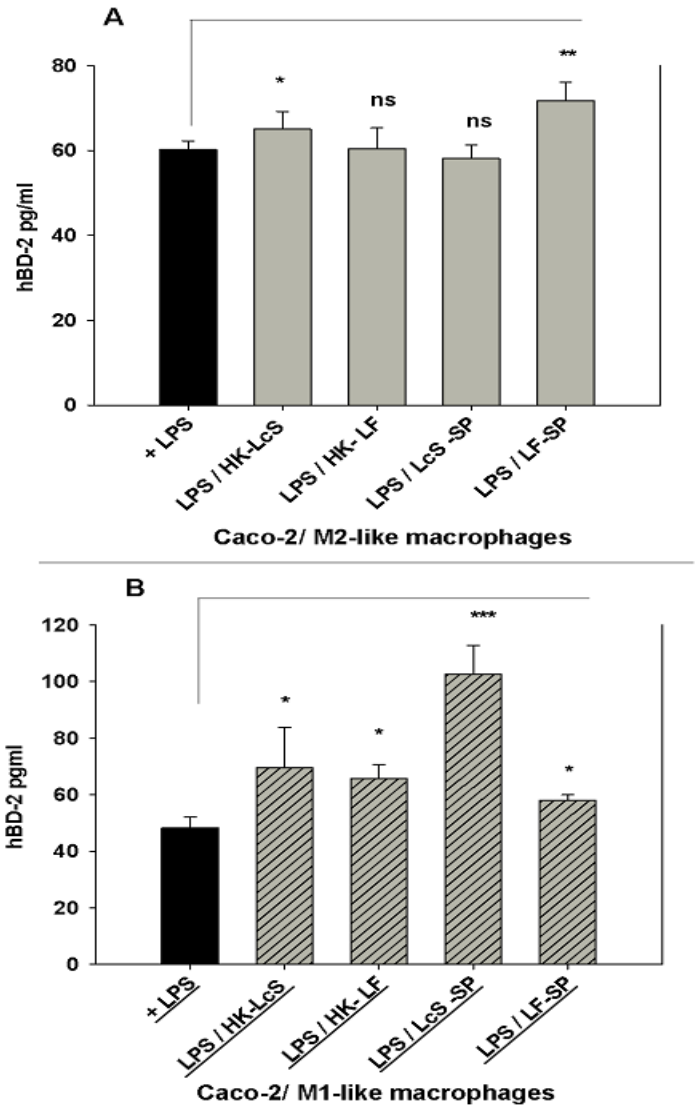


Figure 5.2. 6: SP and HK probiotic strains selectively modulate LPS-induced epithelial hBD-2 production in Caco-2/M2 and Caco-2/M1 co-culture models.

Caco-2 cells grown in transwell inserts were inserted into multiple plate well containing either M2-like macrophages (**A**), or M1-like macrophages (**B**), 100 ng/ml of LPS added to the

basolateraly compartment. Whereas, probiotic *Lactobacillus casei strain* Shirota (LcS) or *L. fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml added apically. hBD-2 is expressed as the mean±SE in pg/ml. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to the control are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

5.2.4. Role of probiotic bacteria in modulation of the epithelial barrier function in Caco-2/M2 and Caco-2/M1 co-culture models

TEER measurement was used to determine the epithelial cell integrity as a part of the epithelial cell barrier function (refers to 2.2.8 and 2.2.4.8). Data showed that TEER was selectively modulated (up-regulated by HK-LcS, LcS-SP, suppressed by HK-LF and non-significantly modulated by HK-LF) in Caco-2/M2 co-culture model (Fig.5.2.7A). In Caco-2/M1 co-culture model, TEER was suppressed by all probiotic treatments (Fig.5.2.7B). After activation by the inflammatory stimulus, LPS, probiotics selectively modulated TEER in both co-culture systems. What is interesting is that probiotic treatments successfully recovered damage induced by LPS in both co-culture models. Results showed that TEER value suppressed after LPS stimulation by 28.3%, then augmented by all probiotic treatments (Fig.5.2.7A). In chronic inflammation model TEER value suppressed by 93% after LPS stimulation, and augmented by all probiotic treatments (Fig 5.2.7B). ZO-1 is one of the main proteins associated with the epithelial cell barrier function described as a linkage protein between trans-membrane proteins Occludin, Claudine and actin cytoskeleton. In this analysis, mRNA expression level of the ZO-1 gene relative to GAPDH was determined in Caco-2/M2 and Caco-2/M1 co-culture models by RT-qPCR. Results indicated that ZO-1 mRNA level was augmented by probiotic treatments in cells of both co-culture models (Fig.5.2.7D). After LPS stimulation, ZO-1 induced by LPS was up-regulated with probiotic treatments in cells of both co-culture models (Fig.5.2.7D). The up-regulation of ZO-1 cell expression was

paralleled with the up-regulation of TEER value suggested that probiotics mediates their roles in maintaining epithelial barrier function via up-regulation of ZO-1. TEER measurements and ZO-1 cell expression strongly suggested that cytokines produced by macrophages in co-culture system differentially affect the paracellular permeability of the epithelial cells. Thus, the alteration of tight junction associated with protein ZO-1 expression was tracked by an immunohistochemistry approach (refer to 2.2.9). Results showed that the untreated monolayer cells displayed continuous ZO-1 labelling around the cell periphery (Fig. 5.2.8A). Co-cultured cells with M1s induced ZO-1 staining discontinuity and complete disorganization of ZO-1 expression (Fig.5.2.8B). Stimulation of cells with LPS caused marked disruption of ZO-1 expression, resulting in an absence of labelling of many cells crossing area and a complete destruction of a cellular network structure associated with ZO-1 expression (Fig. 5.2.8C). This morphological analysis showed that the co-cultured cells with M1-like macrophages followed by LPS stimulation had a pronounced effect on the barrier integrity of the Caco-2 epithelial cells. Treatment with probiotics slightly restored ZO-1 expression, Fig.5.2.8D, E, F& G for HK-LcS, HK-LF, LcS-SP, and LF-SP respectively. In stark comparison, co-cultured cells with M2-like macrophages slightly affect ZO-1 continuity (Fig.5.2.8B1). Stimulation of macrophages by LPS in the lower basolateral compartment sharply affect continuity of ZO-1 expression (Fig.5.2.8C1), and treatment with probiotics commendably restored the continuity of ZO-1 expression, Fig.5.2.8D1, E1, F1 & G1 for HK-LcS, HK-LF, LcS-SP, and LF-SP respectively. ZO-1 immunolabelling in the setting of cells co-cultured with M1s, and M2s were correlated with ZO-1 cell intensity which determined by Neuron J tracing tool for Image J software (Fig.5.2.8H & H1).

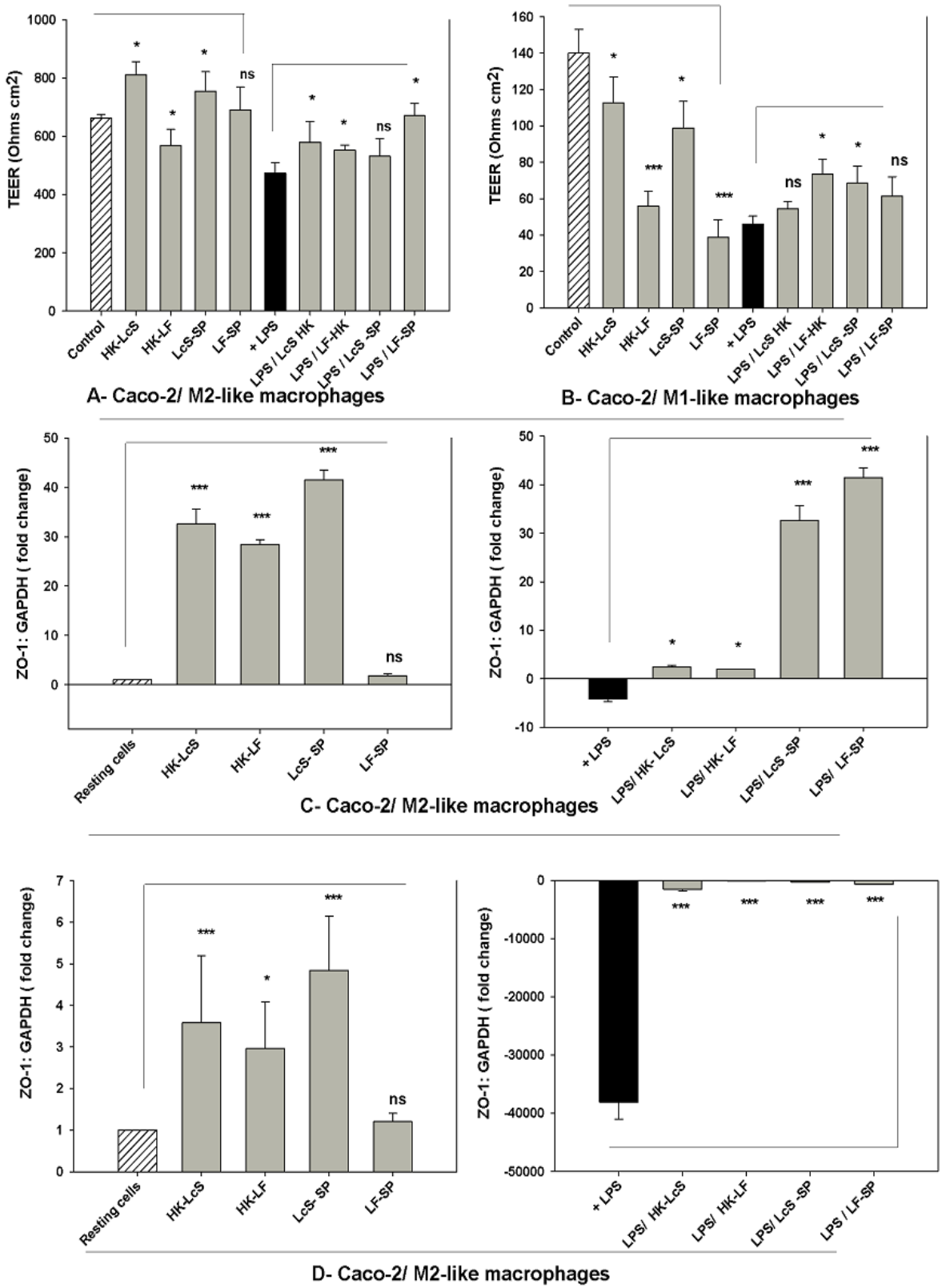
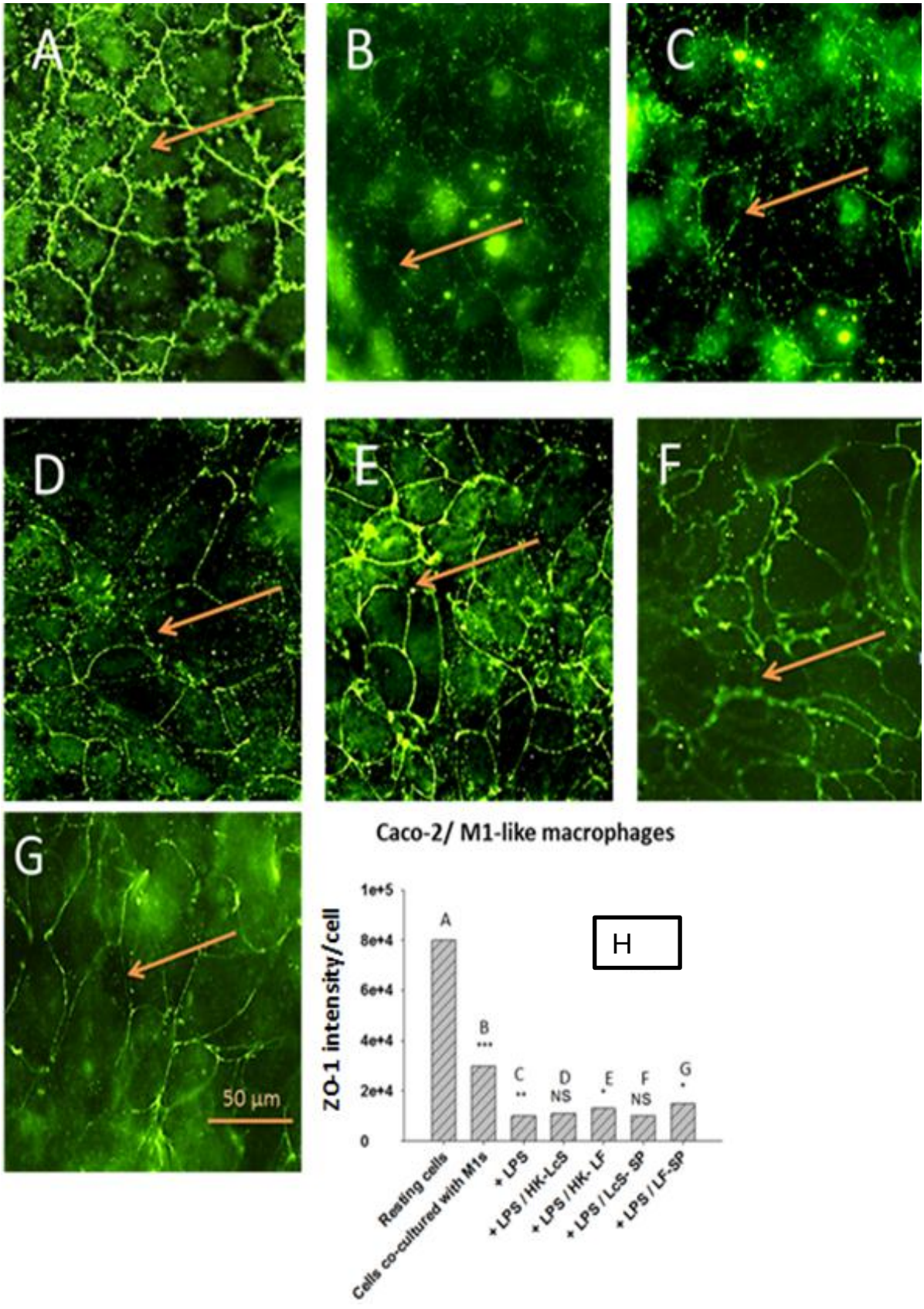


Figure 5.2. 7 : Probiotics selectively regulate TEER and ZO-1 induced by LPS in co-culture models.

TEER Ω/cm^2 of Caco-2 epithelial cells co-cultured with M2-like macrophages (**A**), or cells co-cultured with M1-like macrophages (**B**), ZO-1 expression of epithelial cells co-cultured with M2-like macrophages(**C**), or co-cultured with M1-like macrophages (**D**), 100 ng/ml of LPS added to the basolateraly compartment, whereas probiotic *Lactobacillus casei* strain Shirota (LcS) or *L.fermentum* (LF) either heat killed (HK) at cell density of 3x10^8 CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml added apically. TEER measurements were performed using an EVOM epithelial voltmeter. ZO-1 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).


A
B
C
D
E
F
G
50 µm
Caco-2/ M1-like macrophages
H
ZO-1 intensity/cell
1e+5
8e+4
6e+4
4e+4
2e+4
0
A
B
***
C
**
D
NS
E
*
F
NS
G
*
Resting cells
Cells co-cultured with M1s
+ LPS
+ LPS / HK-LcS
+ LPS / HK- LF
+ LPS / LcS- SP
+ LPS / LF-SP

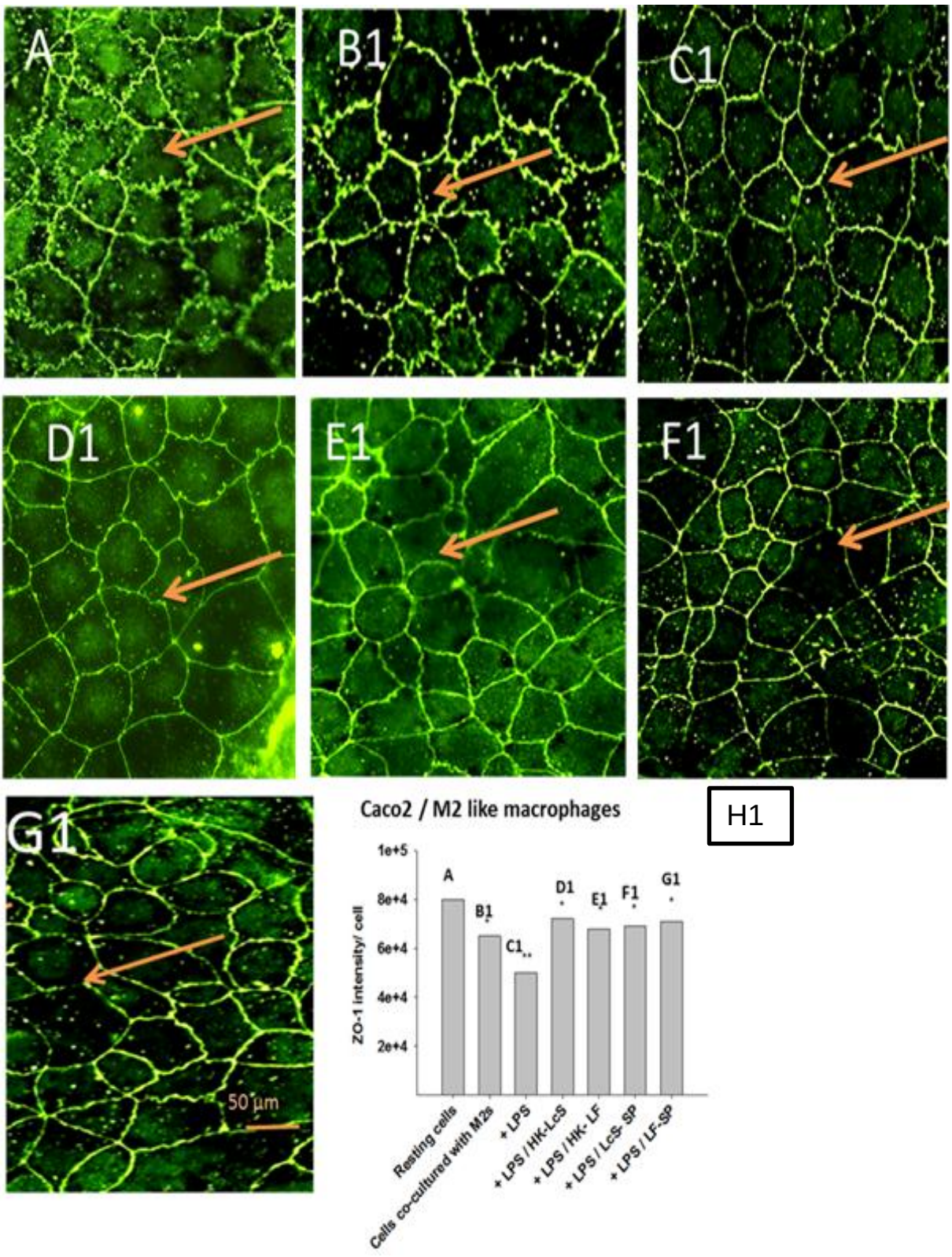


Figure 5.2. 8: Immunolocalization of Zonula Occluden (ZO-1) in a monolayer of Caco-2 epithelial cells induced by LPS in co-culture systems.

A) Immuno-staining for ZO-1 in untreated cells showed continuous labelling around the cell periphery in the region of the cell-cell junctional complex. Co-cultured cells with M1-like

macrophages (**B**), and followed by stimulation by 100 ng/ml K12-LPS caused complete disorganization of ZO-1 (**C**), which repaired by treating cells with heat killed (HK) probiotic *Lactobacillus casei strain Shirota* (LcS) (**D**), *L. fermentum* (LF) (**E**), LcS- secreted protein (SP) (**F**), and LF-SP (**G**). Cells co-cultured with M2-like macrophages (**B1**), and followed by stimulation with 100 ng/ml K12-LPS caused slight disorganization of ZO-1 (**C1**), which positively repaired by treating cells with HK-LcS (**D1**), HK-LF. (**E1**), LcS-SP (**F1**), and LF-SP (**G1**). The immuno-staining shown is representative of three different cultures Scale bar, 50 µm. ZO-1 cell intensity was analysed using Neuron J tracing tools for Image J software (**H, H1**). Date represented as mean±SE, n= 10. Significant effects compared to stimulus control are indicated as * P<0.05, ** P<0.01 and *** P<0.005 and NS (non-significant).

5.2.5. Role of probiotics in modulation of epithelial TLR expression induced by LPS in Caco-2/M2 and Caco-2/M1 co-culture models

In this analysis mRNA level of the TLR4, CD14, MD-2, TLR2, TLR9, NOD-2 and Tollip gene relative to GAPDH were determined in both Caco-2/M2 and Caco-2/M1 by RT-qPCR, TLR4, and TLR2 protein surface expression were determined by flow cytometry (refer to 2.2.10 and 2.2.7). TLR4 was up-regulated with probiotic treatments in Caco-2/M2 and Caco-2/M1 co-culture model of protein and MRNA level. TLR4 mRNA level induced by LPS was selectively modulated (suppressed by HK-LcS, HK-LF, and LF-SP, and augmented by LcS-SP) in Caco-2/M2 co-culture model (Fig.5.2.9A). TLR4 induced by LPS in Caco-2/M1 co-culture model was also selectively modulated (suppressed by HK-LcS, LcS-SP, and LF-SP, augmented by HK-LF) (Fig.5.2.9C). At the protein level, TLR4 induced by LPS was suppressed by all probiotic treatments except LcS-SP, which is non-significantly, augmented TLR4 induced by LPS in Caco-2/M2 co-culture model (Fig.5.2.9B). In the chronic inflammation model, probiotic treatments selectively modulated LPS induced TLR4 protein (suppressed by HK-LcS, LcS-SP, and LF-SP and augmented by HK-LF) (Fig.5.2.8D).

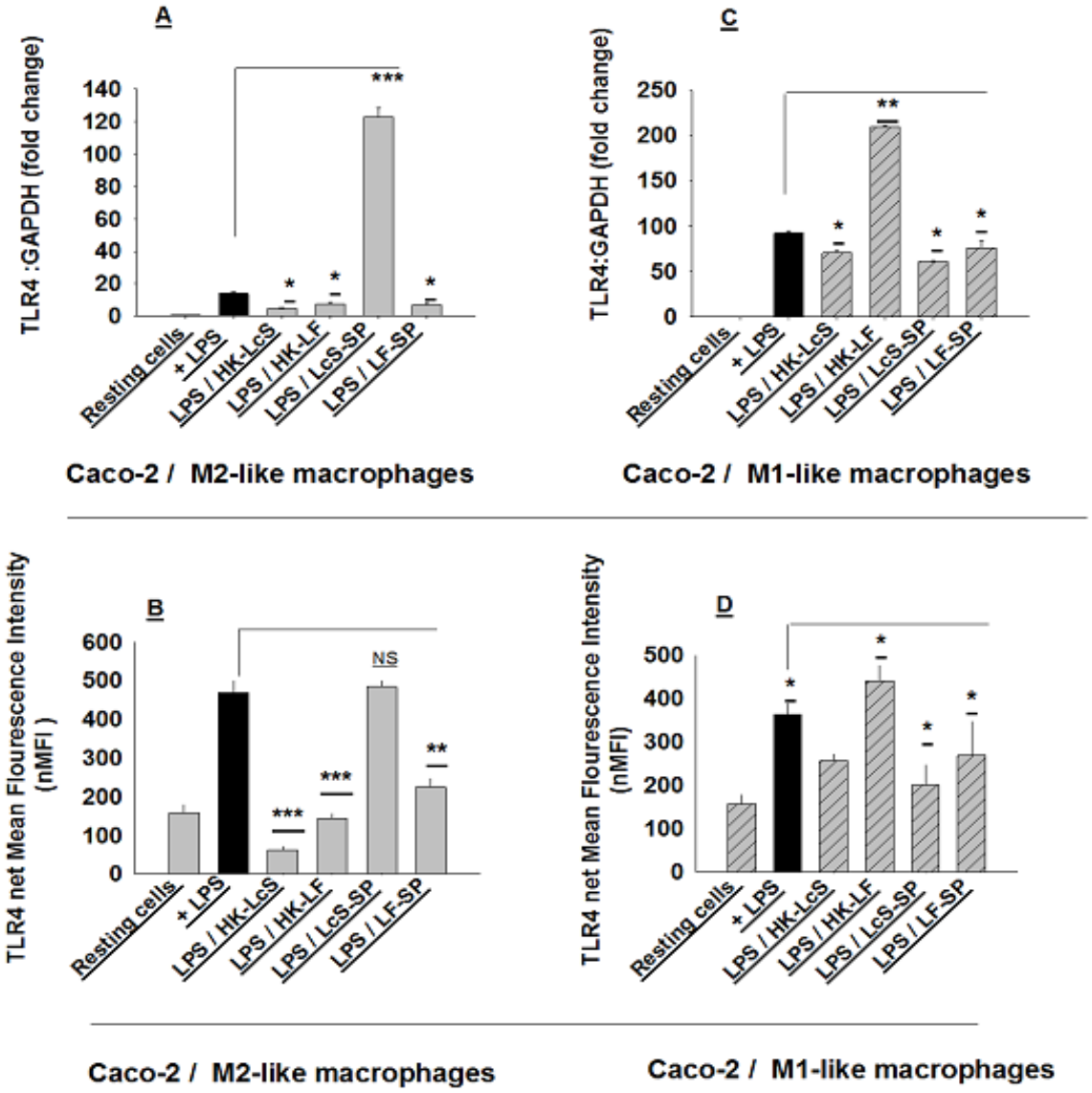


Figure 5.2. 9: Probiotics selectively regulate epithelial TLR4 expression induced by LPS in Caco-2/M2 and Caco-2/M1 co-culture models.

Cultures of Caco-2 cells were inserted into multiple well plates containing either M2 macrophages, or M1-like macrophages, 100 ng/ml of LPS added to the basolateral compartment, whereas probiotic *Lactobacillus casei* strain Shirota (LcS) or *L. fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml added apically. TLR4 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A**, **B**), and protein level by MFI (**C**, **D)**. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control are indicated as * P<0.05, ** P<0.01, *** P<0.005, NS (non-significant).

TLR2 mRNA and protein were augmented by all probiotic treatments in both co-culture models. After LPS stimulation, TLR2 mRNA level induced by LPS was selectively modulated (suppressed by HK-LcS, HK-LF, and LF-SP and non-significantly augmented by LcS-SP), in Caco-2/M2 co-culture model (Fig.5.2.10A). In Caco-2/M1 co-culture model, TLR2 mRNA level induced by LPS was also selectively modulated (augmented by HK-LcS, HK-LF, suppressed by LcS-SP, and LF-SP) (Fig.5.2.10B). At the protein level, LPS-induced TLR2 was suppressed by all probiotic treatments except HK-LF, which significantly augmented LPS induced TLR2 in Caco-2/M2 co-culture model (Fig.5.2.10C). TLR2 protein level induced by LPS in cells of Caco-2/M1 co-culture model was also selectively modulated (suppressed by HK-LcS, HK-LF, and LcS-SP, and augmented by LcS-SP) (Fig.5.2.10D).

CD14 mRNA level was selectively modulated by probiotic treatments (augmented by HK-LF and LcS-SP, suppressed by HK-LcS, and LF-SP) in Caco-2/M2 co-culture model, whereas all probiotic treatments augmented CD14 expression in Caco-2/M1 co-culture model. In LPS stimulation of the Caco-2/M2 co-culture model, probiotic treatments selectively modulated LPS induced CD14 (suppressed by HK-LcS, and HK-LF, augmented by LcS-SP and LF-SP) (Fig.5.2.11A). In the chronic inflammation (Caco-2/M1) model, HK-LcS suppressed LPS induced CD14; whereas all the rest of probiotic treatment augmented, LPS induced CD14 mRNA level (Fig.5.2.11B).

Probiotic bacterial treatments deferentially regulated MD-2 expression (suppressed by HK-LcS and LcS-SP, augmented by HK-LF and LF-SP) in normal homeostatic (Caco-2/M2) model, whereas augmented by all probiotic treatments in Caco-2/M1 co-culture model. In LPS stimulation of the normal homeostatic model,

HK-LF augmented LPS induced MD-2 expression, whereas HK-LcS, LcS-SP, and LF-SP suppressed it (Fig.5.2.11C). However, only HK-LcS treatment suppressed LPS induced MD-2 expression and all the rest of probiotic treatments augmented LPS induced MD-2 in Caco-2/M1 co-culture model (Fig.5.2.11D).

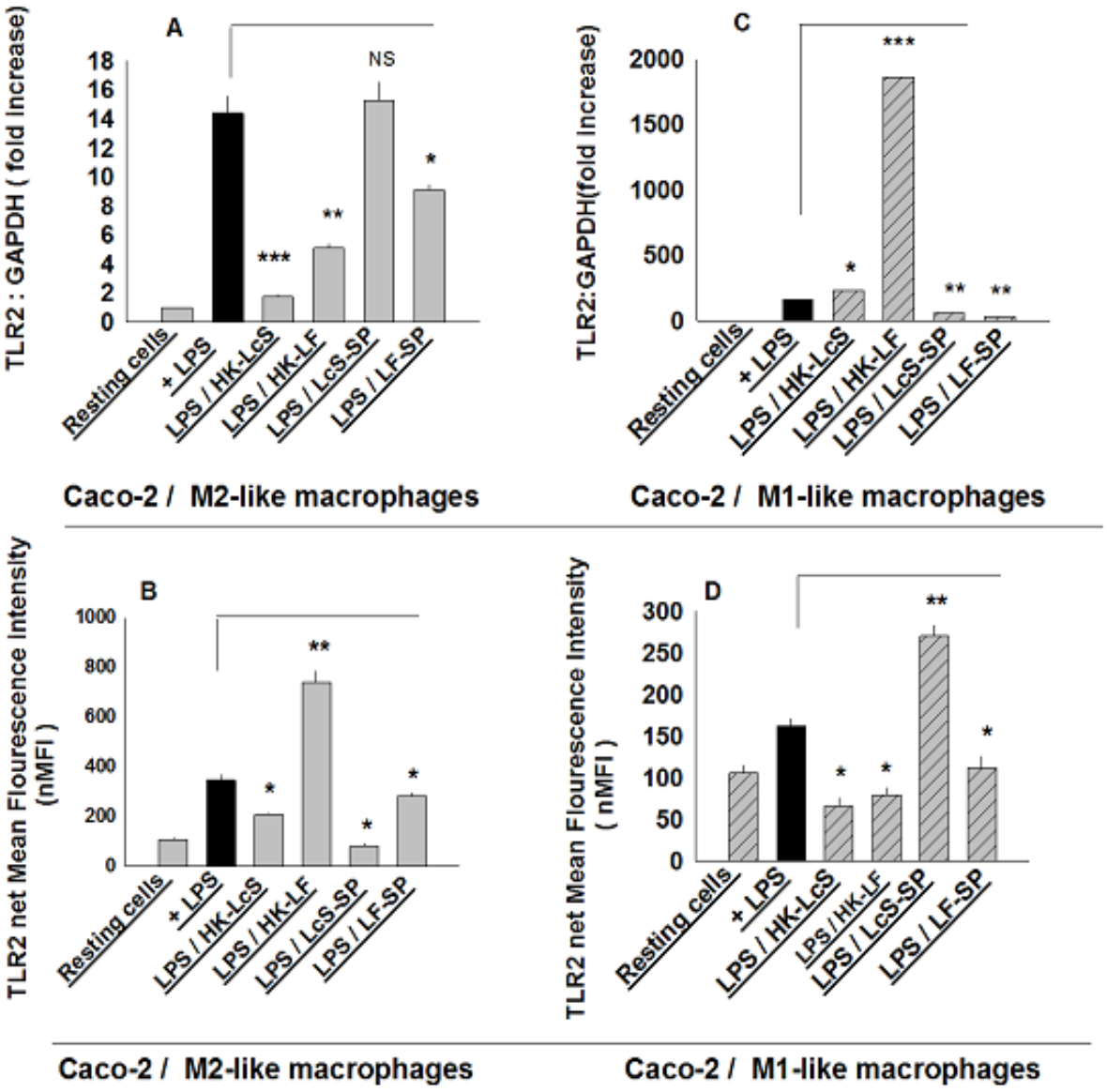


Figure 5.2. 10: Probiotics selectively regulate epithelial TLR2 expression induced by LPS in Caco-2/M2 and Caco-2/M1 co-culture model.

Cultures of Caco-2 cells were inserted into multiple well plates containing either M2 macrophages, or M1-like macrophages, 100 ng/ml of LPS added to the basolateral compartment, whereas probiotic *Lactobacillus casei* strain Shirota (LcS) or *L. fermentum* (LF)

either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml added apically. TLR2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$ (**A**, **B**), and protein level by MFI (**C**, **D)**. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control are indicated as * P<0.05, ** P<0.01, *** P<0.005, NS (non-significant).

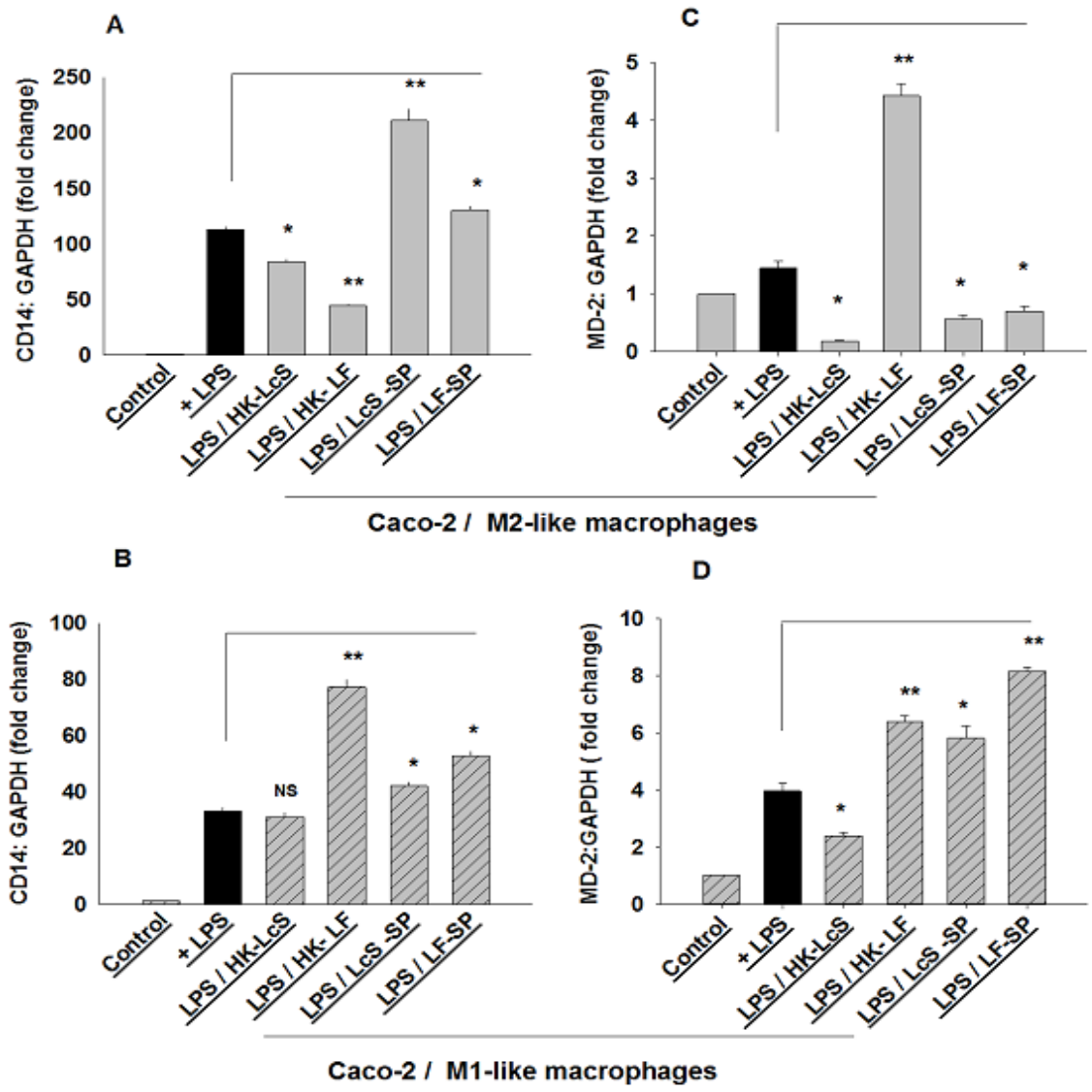


Figure 5.2. 11: Epithelial cell CD14 and MD-2 expression induced by LPS selectively modulated by probiotics in Caco-2/M2 and Caco-2/M1 co-culture models.

Cultures of Caco-2 cells were inserted into multiple well plates containing either M2 macrophages (**A, C**), or M1-like macrophages (**B, D**), 100 ng/ml of LPS added to the basolateral compartment, whereas, probiotic *Lactobacillus casei* strain Shirota (LcS) or *L.fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml added apically. CD14 and MD-2 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control are indicated as * P<0.05, ** P<0.01, *** P<0.005 and NS (non-significant).

Data showed that HK-LF probiotic treatment exhibited significant effects in up-regulation of LPS induced MD-2, TLR4, TLR2, CD14, whereas HK-LcS suppressed MD-2, non-significantly suppressed CD14, suppressed TLR4, and up-regulated TLR2 in Caco-2/M1 co-culture model. In contrast, HK-LF suppressed LPS induced MD-2, CD14, TLR4, TLR2, whereas HK-LcS suppressed LPS induced TLR4, TLR2, and CD14, augmented MD-2 expression in Caco-2/M2 co-culture model. Secreted proteins of probiotic treatments augmented LPS induced CD14, suppressed MD-2, TLR4 and TLR2 in Caco-2/M1 co-culture model, however, secreted proteins of probiotic treatment exhibited differential effects on LPS induced TLR4, TLR2, augmented CD14 and MD-2 in Caco-2/M2 co-culture model.

Probiotic treatments also selectively modulated intracellular PRR expression of TLR9 and NOD-2 in both co-culture models. Results indicated that in both Caco-2/M2 and Caco-2/M1 co-culture models, NOD-2 was augmented with all probiotic treatments. After LPS stimulation of cells in the normal homeostatic (Caco-2/M2) co-culture model, NOD-2 induced by LPS was suppressed by all probiotic treatments (Fig.5.2.12A), whereas only HK-LcS augmented LPS induced NOD-2 in the chronic inflammation (Caco-2/M1) co-culture model (Fig.5.2.12B).

TLR9 was augmented by all probiotic treatments in the normal homeostatic model, whereas selectively modulated (augmented by HK-LcS and LF-SP, suppressed by LcS-SP and HK-LF) in Caco-2/M1 co-culture model. After LPS stimulation of Caco-2/M2 co-culture model, TLR9 induced by LPS was selectively modulated (augmented by HK-LcS and LF-SP, suppressed by HK-LF, and non-significantly modulated by LcS-SP) (Fig.5.2.12C). In the chronic inflammation (Caco-2/M1) co-culture model, TLR9 induced by LPS was also selectively modulated (suppressed by HK-LcS, and HK-LF, augmented by LcS-SP and LF-SP) (Fig.5.2.12D). Data in Fig.5.2.12 shows that probiotics selectively modulate the intracellular PRR expression induced by LPS which associated with hBD-2 and cytokine expression in both co-culture models.

Tollip (the adaptor protein that inhibits TLR signal transduction) expression was selectively modulated by probiotic treatments, augmented with HK-LcS, whereas suppressed by HK-LF, LcS-SP, and LF-SP in Caco-2/M2 co-culture model. However, Tollip expression was suppressed with all probiotic treatments in the chronic inflammation model. After LPS stimulation of Caco-2 cells in the normal homeostatic model, Tollip induced by LPS was selectively modulated (non-significantly augmented by HK-LcS, and HK-LF, suppressed by LcS-SP and LF-SP) (Fig.5.2.13A). Whereas augmented with all probiotic treatments in cells of chronic inflammation model (Fig.5.2.13B).

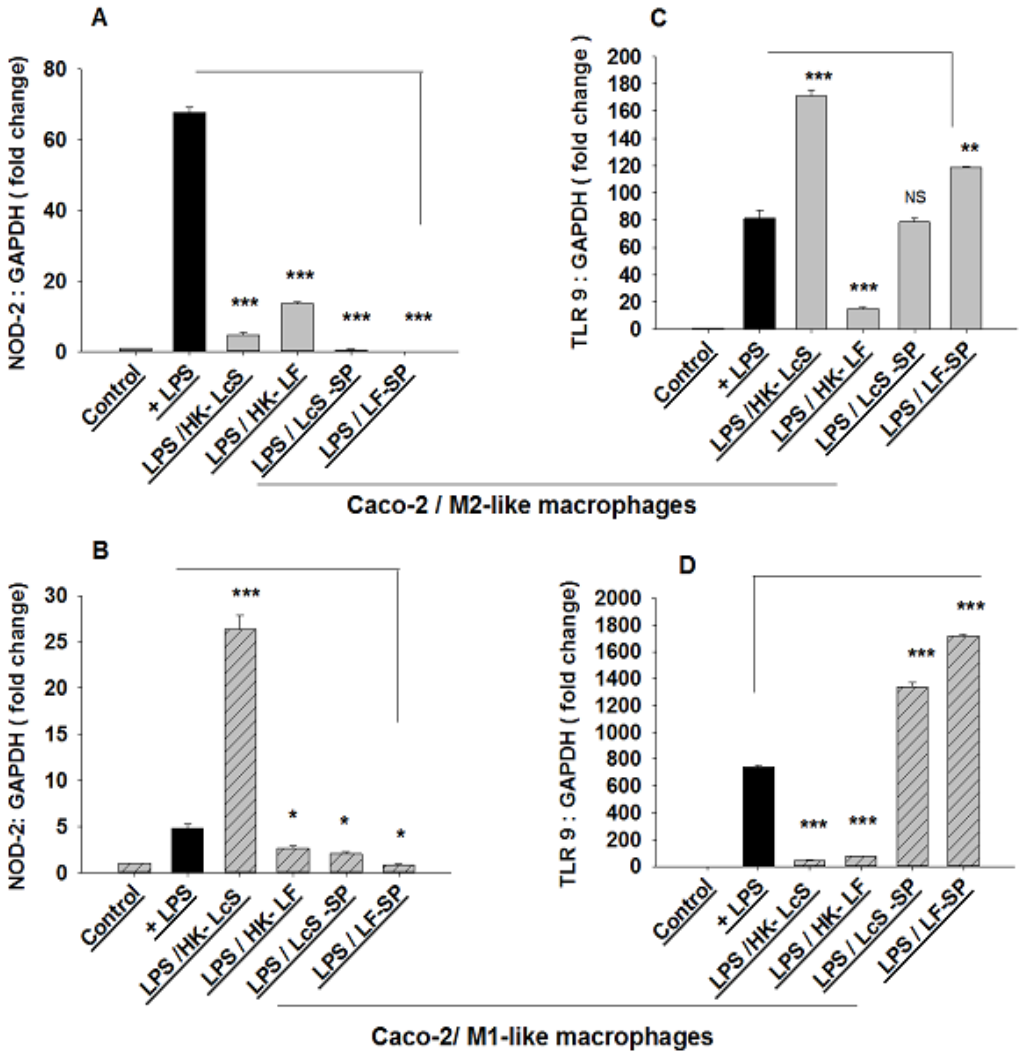


Figure 5.2. 12: Epithelial cell NOD-2 and TLR9 expression induced by LPS selectively modulated by probiotics in Caco-2/M2 and Caco-2/M1 co-culture models.

Cultures of Caco-2 cells were inserted into multiple well plates containing either M2 macrophages (**A, C**), or M1-like macrophages (**B, D**), 100 ng/ml of LPS added to the basolateral compartment, whereas, probiotic *Lactobacillus casei* strain Shirota (LcS) or *L.fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml added apically. NOD-2 and TLR9 gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control are indicated as * P<0.05, ** P<0.01, *** P<0.005 and NS (non-significant).

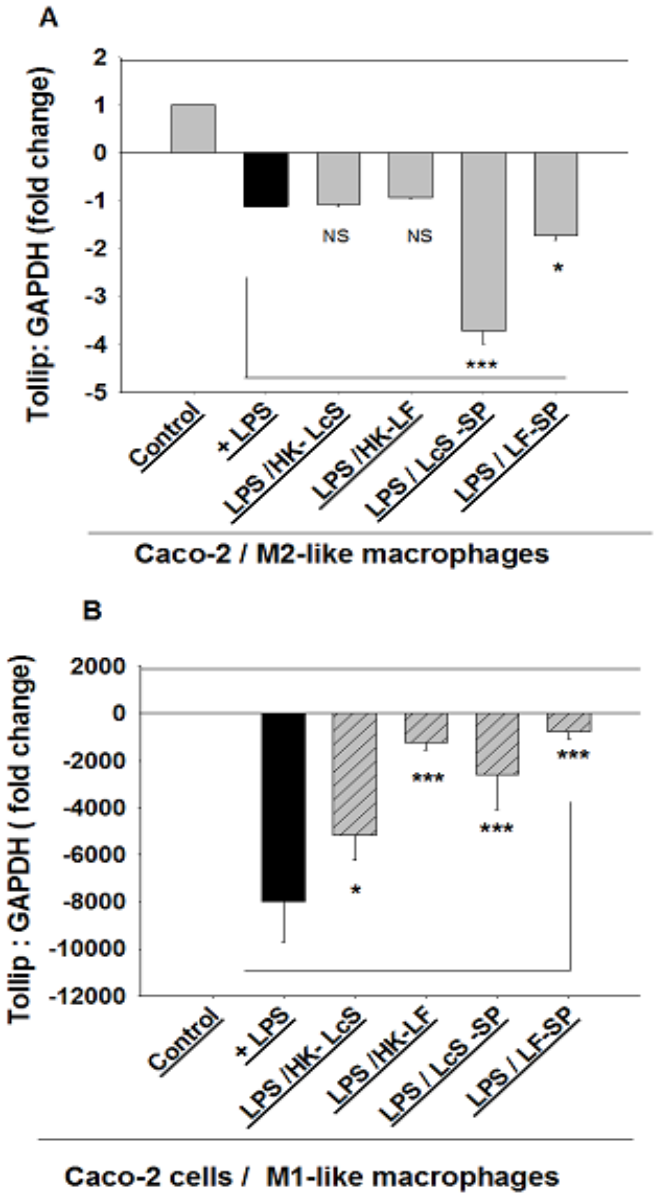


Figure 5.2. 13: Tollip expression induced by LPS selectively modulated by probiotics in Caco-2/M2 and Caco-2/M1 co-culture models

Cultures of Caco-2 cells were inserted into multiple well plates containing either M2 macrophages (**A**), or M1-like macrophages (**B**), 100 ng/ml of LPS added to the basolateral compartment, whereas probiotic *Lactobacillus casei* strain Shirota (LcS) or *L.fermentum* (LF) either heat killed (HK) at a cell density of $3x10^8$ CFU/ml or secreted protein (SP) extracts by them at 3.0 µg/ml added apically. Tollip gene expression (mRNA level) is expressed as fold change using GAPDH as reference gene and resting cells as a calibrator sample as described by Livak et al. (2001) using $2^{-\Delta\Delta Ct}$. Data displayed is a representative experiment with triplicate samples of n=3 replicate experiments. Significant effects compared to stimulus control are indicated as * P<0.05, ** P<0.01, *** P<0.005 and NS (non-significant).

5.2.6 Summary of chapter 5 results

Table 5.2. 1: Probiotic induction of cytokines (IL-8, TNF-α, IL-6, IL-10) and hBD-2 production in Caco-2/M2 and Caco-2/M1 co-culture models.

pg/ml	Caco-2/M2				Caco-2/M1			
	HK-LcS	HK-LF	LcS-SP	LF-SP	HK-LcS	HK-LF	LcS-SP	LF-SP
IL-8	↓	↓	↓	↓	↓	↑	↑	↔
TNF-α	↑	↔	↑	↑	↔	↑	↑	↓
IL-6	↑	↑	↑	↑	↓	↓	↓	↓
IL-10	↑	↑	↑	↑	↓	↓	↓	↓
hBD-2	↓	↔	↑	↑	↓	↑	↔	↓

Table 5.2. 2: Probiotic modulation of cytokine, hBD-2 and TLR expression induced by LPS in Caco-2/M2 and Caco-2/M1 co-culture models.

	Caco-2/M2				Caco-2/M1			
	HK-LcS	HK-LF	LcS-SP	LF-SP	HK-LcS	HK-LF	LcS-SP	LF-SP
IL-8 (pg/ml) induced by LPS	↓	↓	↓	↓	↓	↓	↔	↓
TNFα (pg/ml) induced by LPS	↓	↑	↑	↔	↑	↓	↑	↑
IL-6 (pg/ml) induced by LPS	↑	↑	↓	↑	↑	↑	↑	↑
IL-10 (pg/ml) induced by LPS	↓	↑	↓	↓	↓	↑	↓	↔
hBD-2 (pg/ml) induced by LPS	↑	↔	↔	↑	↑	↑	↑	↑
TLR 4 mRNA induced by LPS	↓	↓	↑	↓	↓	↑	↓	↓
TLR4 protein induced by LPS	↓	↓	↔	↓	↓	↑	↓	↓
TLR2 mRNA induced by LPS	↓	↓	↔	↓	↑	↑	↓	↓
TLR2 protein induced by LPS	↓	↑	↓	↓	↓	↓	↑	↓
CD14 mRNA induced by LPS	↓	↓	↑	↑	↔	↑	↑	↑
MD-2 mRNA induced by LPS	↓	↑	↓	↓	↓	↑	↑	↑
NOD-2 mRNA induced by LPS	↓	↓	↓	↓	↑	↓	↓	↓
TLR9 mRNA induced by LPS	↑	↓	↔	↑	↓	↓	↑	↑
Tollip mRNA induced by LPS	↔	↔	↓	↓	↑	↑	↑	↑

Table 5.2. 3: Probiotic modulation of TEER and ZO-1 expression in Caco-2/M2 and Caco-2/M1 co-culture models.

	Caco-2/M2				Caco-2/M1			
	HK-LcS	HK-LF	LcS-SP	LF-SP	HK-LcS	HK-LF	LcS-SP	LF-SP
TEER	↑	↑	↔	↑	↔	↑	↑	↔
ZO-1	↑	↑	↑	↑	↑	↑	↑	↑

Note: "↑", "↓,"and "↔"means up-regulation, down-regulation, and no-modulation of the indicated target, respectively.

5.3. Discussion

5.3.1. Probiotic bacteria and their secreted proteins selectively modulate LPS-induced cytokine expression in different co-culture models

The present study was designed to determine the immunomodulation of probiotic bacteria on epithelial cell barrier function (hBD-2, ZO-1, cytokine production and TEER) induced by LPS, comparing two status; normal and chronic inflammation. Very little was found in the literature on the question of how probiotic bacteria modulate the mucosal epithelial cells in normal homeostasis and chronic inflammatory environments. Indeed several studies were performed to investigate the immunomodulatory effects of probiotics using a combination of different cell types (co-culture model), focussed on the immunomodulatory effects of probiotics into a range of gut mucosa such as DCs and epithelial cells. Yeun et al. (2012) reported that heat-killed probiotic bacteria, *Bifidobacterium lactis* AD011, *Bifidobacterium bifidum* BGN4, *Lactobacillus casei* IBS041 and *Lactobacillus acidophilus* AD031 modulated mouse DCs co-cultured with mouse epithelial cell monolayers cytokines (IL-6, TNF-α and IL-10) expression. Haller et al. (2000b) reported that *Lactobacillus sakei* modulated profiles of TNF-α, IL-1β, IL-8, monocyte chemoattracting protein 1 (MCP-1), and IL-10 in Caco-2 cells co-cultures with human blood leucocytes. Regarding THP-1 derived macrophages using in a co-culture system as *in vitro* model to screen and predict the bioactivities from a variety of food components, Watanabe et al. (2004) established a co-culture model of Caco-2/M1-like macrophages to investigate the immunomodulatory effects of caffeine as food factors in the modulation of the disruption caused by macrophages into the Caco-2 cells. The macrophage cytokine expression of TNF-α during the incubation time has a significant effect on decreasing Caco-2 cell barrier function, suggested that this phenomenon (TNF-α

causing a decrease in barrier function) is similar to that observed with IBD. In Chapter 3 of this thesis, differentiation of THP-1 with PMA resulted in a macrophage subset expressing markers of M1-like macrophages associated with high production of pro-inflammatory cytokines in response to LPS stimulation. Whereas, using Vit.D3 to differentiate THP-1 cells resulted in macrophage subset with M2 markers associated with the production of anti-inflammatory cytokines and less production of pro-inflammatory cytokines. Basically, the interaction between epithelial cells as a front line of the gut mucosa with underlying macrophage subsets (M1 or M2) determine the fates and the outcome of the mucosal immune responses to the gut microbiota (probiotics); initiating an inflammatory environment or maintaining the gut mucosa. Therefore, two *in vitro* models were developed; one of them imitated a normal homeostatic model where Caco-2 cells co-cultured with M2-like macrophages, whereas the second one represented a chronic inflammation model where Caco-2 cells co-cultured with M1-like macrophages.

The first question in this research of this chapter was investigating the role of probiotic treatments in the modulation of epithelial cytokine expression influenced by macrophages stimulated by LPS in different co-culture models. The results showed that M2 or M1 sensitised Caco-2 cells *in vitro* during the incubation time after stimulation with LPS, resulted in inducing different cytokine profiles of cytokine expression in these models. The data demonstrated that epithelial cells act as APCs respond to the signals (soluble factors) from macrophage cells in lower compartment resulting in releasing an array of cytokines such as IL-8, IL-6, IL-10, and TNF-α dependent on type of macrophage subset. A number of reports showed that epithelial cells act as APCs, participate in the initiation and regulation of the mucosal immune response to bacteria and their products. They express

MHC class II molecules (Hershberg et al., 1998), MHC class I (Blumberg et al., 1991), the adhesion molecule ICAM-1 (Huang et al., 1996), complement, and cytokine receptors (Andoh et al., 1993, Reinecker and Podolsky, 1995). In this study, it seemed to be that epithelial cells acclimate themselves by changing some of their characterisation (cytokine and hBD-2 production) consequently to different stimulations in different co-culture models. Epithelial cells are distinguished between signals delivered by co-culturing with M2-like macrophages from signals co-culturing with M1-like macrophages, in addition to, distinguished signals delivered by LPS in cells co-cultured with M1s, from signal induced by LPS in cells co-cultured with M2s. These signals triggered the immune response leading to transducing of a discriminating signal by epithelial cells to underlying macrophage cell phenotypes and so on to create feedback loop stimulation between epithelial cells in the upper compartment and macrophage cells in lower compartment through soluble cytokines and their receptors. It is possible to hypothesise that these conditions are likely to occur in normal homeostasis and chronic inflammation.

Adding probiotic bacteria to the co-culture system resulted in the induction of an array of cytokines by epithelial cells. Moreover, the type and level of cytokine produced by epithelial cells in different co-culture systems is dependent on the type of probiotic strain (as HK or SP) and type of macrophage subsets. Since this difference in cytokines has been found in different co-culture models (Haller et al., 2000b, Yeun et al., 2012), it is probably due to the difference in probiotic bacterial cell wall and probiotic bacterial DNA and types of proteins in probiotic-secreted proteins.

LPS stimulation has induced different cytokines level in Caco-2 cells co-cultured with either M1s or M2s, and again the level of cytokine production was dependent on the type of macrophage subset in co-culture systems. It is well established that LPS stimulation induces TNF-α in macrophages (Björkbacka et al., 2004). TNF-α itself induces IL-8 expression in virtually all types of cells including intestinal epithelial cells (Baggiolini et al., 1995). Data presented here show that IL-8 expression was up-regulated in both co-culture systems. These data are in line with findings of other research groups which showed the regulatory effect of TNF-α in inducing IL-8 by epithelial cells in a co-culture model of Caco-2/RAW264.7 cells (Tanoue *et al.*, 2008), and Caco-2/leukocytes (Haller *et al.*, 2000b). It was therefore proposed that TNF-α secretion in lower compartment is necessary to up-regulate IL-8 production in Caco-2 cells in upper compartment by the virtue of this relationship.

In response to stimulation with LPS, epithelial cytokines (TNF-α, IL-8, IL-10) were up-regulated in both co-culture models, whereas only IL-6 was suppressed in the chronic inflammation model. The cytokine signalling network in response to LPS is composed of diverse cytokines and their receptors, which regulate each other at the gut mucosa. Consequently, function and fate of the mucosal cells are determined by the outcomes of the overall function of cytokines. Understanding the cytokine network is complicated in part because many cytokine receptors generate competing, antagonistic, or synergistic signals. For example, TNF-α promotes cell death by inducing activation of the cysteine proteases caspase-8 and caspase-3 through the expression of TNFR p55 (Thornberry, 1998), however, TNF-α also promotes cell survival by activating the NF-kB transcription factor through the expression of TNFR p75 (Karin and Ben, 2000). In addition, as part of

cytokine network, the expression of cytokine may enhance the expression of other cytokines such as TNF-α expression enhancing IL-10 expression (Foey et al., 1998), and at the same time IL-10 represses TNF-α cytokine expression (Fiorentino et al., 1991). Indeed, the effect of TNF-α expression extended to other cytokines such as IL-6, which is suppressed by TNF-α through cross-regulation of cytokine signalling mechanisms (Radtke et al., 2010). In this study, it seemed to be that connection between signals and cell fate in the microenvironment of the co-culture model is even more complex, because cells are exposed to multiple cytokines that act together in synergistic or antagonistic combinations. Conflicting stimuli often arise when cells are exposed to paracrine cytokines from neighbouring cells together with autocrine cytokines secreted by the cell itself, therefore the complicated scenario of the epithelial cytokine production depend on the consideration of paracrine and autocrine cytokine effects. However, the scenario of epithelial cytokine production in a co-culture system will be further complicated in the presence of probiotics, whereby probiotics induced different profiles of epithelial cytokine production, and again dependent on type of macrophage cell phenotype and type of probiotic strain in co-culture system. Probiotics exhibit anti-inflammatory effect of suppressing the induction of LPS induced IL-8 cytokine production in cells co-cultured with M2s and cells co-culture with M1s. IL-8 is potent chemotactic and an activator peptide produced by epithelial cells, macrophages, and T lymphocytes that can induce neutrophil accumulation and activation at the site of production. Over production of IL-8 will creates a flooding with infiltrating neutrophils and pro-inflammatory cells, especially pro-inflammatory macrophages (Baggiolini et al., 1995). The accumulation of the inflammatory cells (macrophages and neutrophil) will initiate tissue destruction such as in IBD (Strober, 1998). Therefore, the suppression of IL-

8 expression by probiotics will prevent the pro-inflammatory cytokine flooding produced by infiltrating cells. This suggests that destruction of tissues might be prevented through suppression of TNF-α induced IL-8. The findings of this study were supported by findings of Tanoue et al. (2008), when they showed that neutralization of TNF-α resulted in abolishing IL-8 cytokine expression in Caco-2 cells co-cultured with RAW264.7 cells. Therefore, these data suggested that probiotics mediated their roles through suppression of TNF-α induced IL-8 in cells in co-culture systems.

In terms of probiotic modulation of LPS induced TNF-α production in cells at co-culture systems, they exhibited different effects in different settings. In cells co-cultured with M2s, for example HK-LF treatment was significantly up-regulated LPS-induced TNF-α, whereas HK-LcS suppressed LPS-induced TNF-α in normal homoeostatic model. LF bacteria belong to the LAB with Gram-positive cell wall structured from a thick, multi-layered peptidoglycan decorated with proteins, teichoic acids and polysaccharides and high amount of Lipoteichoic acid (LTA), highly sensitive to the lysozyme digestion (Šimelyte et al., 2000, Logardt and Neujahr, 1975). LcS bacteria also belong to the LAB, but they have a rigid cell wall, resistance to the lysozyme digestion with a high amount of a complex of lipoteichoic acid and polysaccharide-peptidoglycan (PS-PG) (Matsumoto et al., 2009, Shida et al., 2009, Yasuda et al., 2008). Therefore, it could be proposed that the differences in cell wall structure of LcS and LF are the main cause in modulation of epithelial cytokine production. The findings of the current study are consistent with those of Prescott et al. (2005) who found that administration of probiotics LF resulted in significant clinical improvement atopic dermatitis (AD) in very young children associated with up-regulation of Th1 cytokines such as TNF-α

and INF-γ and polysaccharide moiety of LcS suppressed LPS-induced TNF-α in macrophages via inducing IL-10 (Yasuda et al., 2008). Taken together, LF probiotic bacteria tend to be a pro-inflammatory inducer, whereas LcS bacteria are anti-inflammatory inducer in this specific scenario. It seemed to be that LF probiotic bacteria enhanced the pathways that mediated TNF-α production such as up-regulation of TLR4 or up-regulation of TNF-α through manipulation of ADAM17 expression (Cario, 2005a; Cesaro *et al.*, 2009; Eckmann, Kagnoff & Fierer, 1993) resulting in activation of MAPKs or NF-kB pathways. Comparing two scenarios (chronic inflammation and normal models), probiotics (LcS and LF) exhibited opposite effects. HK-LcS treatment augmented LPS-induced TNF-α in cells in a chronic inflammatory model, whereas LF suppressed it. In the chronic inflammation model, M1-like macrophages express high amounts of TNF-α in response to LPS stimulation. It seemed to be that level of TNF-α have an effect in directing probiotic immunomodulation of the immune responses, since the difference in this scenario is the level of TNF-α and other pro-inflammatory (IL-1β, IL-6, and IL-8) that induced in response to LPS stimulation. These findings suggest that probiotics can worsen inflammation of an inflamed tissue via further up-regulation of pro-inflammatory cytokines such as TNF-α. These findings are supported by other research groups such as Tsilingiri et al. (2012), which showed that *Lactobacillus paracasei* have an inflammatory activity in both healthy and IBD tissue tested in an organ culture system of human health and IBD intestinal mucosa developed by his group. Therefore, data of this study suggested the cautionary use of probiotic administration during acute inflammatory responses.

However, up-regulation of TNF-α in the normal homeostatic environment might be contributing in maintaining barrier function at the gut mucosa via eliminating of

pathogenic bacteria. Some bacteria invade and intracellular residents such as *Salmonella* and *Shigella* (Bennish and Wojtyniak, 1991, Malik-Kale et al., 2012). Like these pathogenic bacteria, *LcS* probiotic bacteria resident intracellular in the epithelial cells; therefore, LcS probiotic bacteria might be suppressing the elimination of the intracellular bacteria such as *Salmonella* and *Shigella* via suppression of TNF-α. LcS augmented IL-6 in both co-culture systems. The data of this study were supported by the observation of Shida et al. (2011) and demonstrated that LcS can exhibit anti-inflammatory or pro-inflammatory effects dependent on its environmental situation. These findings are in line with findings of other research groups, which showed that *Lactobacillus* species have been shown to prevent colitis in IL-10 gene deficient mice (Madsen et al., 1999, Matsuzaki, 1998) by augmentation of anti-inflammatory cytokine expression mediated intestinal homeostasis and maintaining barrier function. The current study supported the hypothesis that the cell signalling initiated by probiotic bacterial cells in the mucosal surface requires a network of cellular interactions. According to these results, sensitisation of Caco-2 cells by neighbouring immuno-competent cells is considered as a crucial step in probiotic bacterial cell recognition of epithelial cells.

Although TNF-α is released at the start of inflammation in order to eradicate the pathogens, over-expression of it has been implicated in cross cytokine regulation, mediating the inhibition of IL-6 production induced by LPS (Ahmed and Ivashkiv, 2000, Radtke et al., 2010) leading to dysregulated barrier function as seen by a decrease in TEER value in this study. These observations suggested that pro-inflammatory cytokines such as TNF-α and pro-inflammatory mediator (LPS) in cells co-cultured with M1s, mediated to induce SOCS-3, the negative regulator of

IL-6 cytokine expression (Bode *et al.*, 1999). Nevertheless, the data suggested that neither LPS nor TNF-α mediated expression of SOCS-3 in cells co-cultured with M2s, and LPS induced IL-6 production is independent of the expression of SOCS-3 (Peter *et al.*, 2003).

Matsumoto et al. (2010) reported that trans-signalling phenomena is caused by the soluble IL-6 (sIL-6R) and IL-6 receptor (gp130, IL-6Rα shedding) derived from the macrophages resulting in chronic inflammation in epithelial cells in mouse colitis-associated premalignant cancer. Development of co-culture model (Caco-2/M1) in this study showed that epithelial cells suffered from chronic inflammation (up-regulation of TNF-α and IL-8, and suppression of IL-6 associated with suppression of TEER). Probiotic treatments enhanced LPS induced IL-6 cytokine production in cells co-culture with M2s as well as cells co-cultured with M1s suggesting that the probiotic treatments modulated the trans-signalling phenomena in colonic epithelial cells in co-culture system. IL-6 has a dual role as anti-inflammatory and pro-inflammatory activity (Tilg et al., 1994). The expression of IL-6 is vital for gut intestinal epithelial cell homeostasis by down-regulation of apoptotic signals and mediates epithelial cell survival (Kovalovich et al., 2001). However, the overproduction of IL-6 mediated tissue damage such as in IBD (Mudter and Neurath, 2007). Therefore, the findings of this study suggested that probiotic treatments are vital in maintaining epithelial cell homeostasis by up-regulation of anti-inflammatory classical IL-6 pathway (IL-6/IL-6RJAK/STAT-3) and inhibit the pro-inflammatory (sIL-6-gp130-STAT-3) pathway (Stefan, 2012) in the chronic inflammation model lead to inhibit the epithelial cell apoptosis rate and enhance epithelial cell survival. Probiotic bacteria selectively modulated LPS induced IL-10. Among probiotic treatments, HK-LF treatment augmented LPS induced IL-10 in

cells in both co-culture systems. Up-regulation of IL-10 lead to suppress the pro-inflammatory cytokines that cause tissue destruction (Ming and Shao, 2004), promote survival and activation of regulatory T cells mediating gut tolerance (Hara et al., 2001). Therefore, the data of this study suggested that LF probiotic bacteria are good candidate regarding issues required up-regulation of IL-10. In fact, these findings are in agreement with Lammers et al. (2003) when they showed that LF bacterial DNA up-regulated IL-10 in human peripheral blood mononuclear cells. HK-LcS treatment suppressed LPS induced IL-10 which correlated with augmentation of LPS induced TNF-α in the chrononic inflammation model which emphasises the cautionary use of probiotics. In the scenario of the chronic inflammation model, where M1-like macrophages are predominant, expressing high levels of pro-inflammatory cytokines in response to LPS stimulation, after the epithelial barrier failed to prevent the LPS crossing (phenomenon is similar to that observed with IBD), LcS treatment was augmented the inflammation via up-regulation of LPS-induced TNF-α and suppressed LPS-induced IL-10. Consequently, the apoptotic rate might be increased leading to subvert epithelial barrier function facilities more chance to trigger more inflammatory responses, suggested that probiotic use might be detrimental in inflamed IBD.

5.3.2. Probiotic treatments selectively modulate LPS-induced hBD-2 by epithelial cells in different co-culture models

A strong relationship between pathogenic infection and an upsurge in hBD-2 production by gut epithelial cells has been reported in the literature. Epithelial gut protection against pathogenic microorganisms is achieved through different mechanisms involving physical barrier such as TJs, mucus, pH, digestive enzymes, AMPs, sIgA, and bile acid. Once the physical barrier is pierced, the fast immune response will be initiated through recognition of invading microbiota as the first

step, resulting in augmentation of AMPs and cytokine production (Takeda et al., 2003). In the GIT, defensins are supposed to be a critical component of both innate and adaptive immune responses through their roles in eradication of pathogens (Wehkamp et al., 2004) and modulation of immune responses (Oppenheim et al., 2003) via chemotactic properties involved in recruiting polymorphonuclear leukocytes (PMN) to the site of infection. The expression of hBD-2 is controlled by pro-inflammatory cytokines such as IL-1β and TNF-α and anti-inflammatory cytokine IL-10 as shown in (chapter 4). The second question in this research of this chapter was investigating the role of probiotic treatments in modulation of hBD-2 expression induced by LPS in different co-culture models. In this study, level expression of hBD-2 induced by LPS in different co-culture systems was dependent on the type of macrophage subset. These data in part are in line with findings of Tsutsumi-Ishii et al. (2003) which showed that the IL-1β and TNF-α produced by mononuclear phagocytes in the co-culture system are main inducer of hBD-2 transcription in A549 pulmonary epithelial cells. In addition, stimulation of epithelial cells with probiotics and their secreted proteins in co-culture systems, resulting in different profiles of hBD-2 expression is dependent on the type of probiotic strain, probiotic preparation (HK or SP), and macrophage subset. Among the probiotic bacteria used in this study, SP of LcS and LF significantly enhanced hBD-2 expression in the normal homeostatic model, whereas HK-LF treatment enhanced hBD-2 expression in chronic inflammation model. These treatments of probiotics exhibit anti-inflammatory role by up-regulation of hBD-2 expression.

In LPS stimulation of cells in a chronic inflammation model, the hBD-2 release was suppressed. This suppression demonstrates of hBD-2 deficiency. Indeed, the

scenario of the chronic inflammation model is similar to that observed with IBD, when a deficiency of hBD-2 production is one of the main pathological signs (Wehkamp et al., 2003). In terms of probiotic treatments, they selectively modulated LPS induced hBD-2 in both co-culture systems. In the normal homeostatic model, probiotic treatments augmented LPS induced hBD-2. This augmentation of hBD-2 might up-regulate the barrier function against invading pathogenic microorganisms and maintaining gut mucosa (Yang et al., 2002). Focusing on HK-LcS probiotic treatment, it's up-regulated LPS induced TNF-α production, which in turn up-regulated hBD-2 (Albanesi et al., 2007) in chronic inflammation model, whereas HK-LF probiotic treatment down-regulated LPS induced TNF-α, but up-regulated hBD-2 in chronic inflammation model. These effects of HK-LF suggested that the up-regulation of hBD-2 expression might be through up-regulation of LPS induced IL-1β not up-regulation of LPS induced TNF-α (McDermott et al., 2003), or up-regulation of LPS induced IL-17 and IL-22 in macrophages in lower compartment that induce hBD-2 expression (Brand et al., 2006, van Baarlen et al., 2011), which in turn up-regulated the induction of LPS induced hBD-2 in epithelial cells. Secreted protein of LcS or LF up-regulated the induction of LPS induced TNF-α and again they up-regulated the induction of LPS induced hBD-2 in cells co-cultured with M1s and M2s. Taken together, it is possible that the discriminatory responding strategies of epithelial cells in a complicated environmental niche in the co-culture system explain why probiotic bacterial treatments are may be effective in the treatment of the IBD.

5.3.3. Probiotic bacterial treatments selectively modulated the dysregulated barrier function induced by LPS in co-culture models

The third question in this research of this chapter was investigating the role of probiotics in modulation of dysregulated barrier function induced by LPS in

different co-culture models. Probiotic bacterial role in the modulation barrier function of the gut mucosa at normal environment compared with chronic is not well understood. Methods to determine the epithelial cell monolayer integrity and barrier function, (which is mainly influenced by TJs within the epithelium) including trans-epithelial electrical resistance (TEER) measurements, which determine the Trans-and para-cellular permeability (Schulzke et al., 2006). Disruption of tight junctions leads to subvert the epithelial cell integrity causing leak flux. In the current study, two types of co-culture settings were developed, Caco-2/M1s resembling of the chronic inflammation model, and Caco-2/M2 resembling of the normal intestinal homeostatic model. Data showed that in Caco-2 co-cultured with M1s, TEER value was sharply down-regulated in comparison with resting single monoculture cells and stimulation with LPS resulted in a further decrease of TEER.

In Caco-2/M1 co-cultured model, M1s stimulated directly by LPS and indirectly by soluble factors released by Caco-2 cells during co-culturing time (paracrine factors). Increase macrophage stimulation resulting in increasing of pro-inflammatory cytokine production such as TNF-α, IL-6, IL-8, and IL-1β leading to suppress TEER and epithelial cell integrity (Schulzke et al., 2006). It is well known that the increase in TNF-α production leading to increased epithelial cell apoptosis through the up-regulation of TNFRp55 (Grell et al., 1999). IL-6 is vital for epithelial cell survival and epithelial cell turnover (Grivennikov et al., 2009). Increasing in TNF-α production causes the suppression in IL-6 expression (Radtke et al., 2010). Consequently, apoptotic rate is increased causing leak flux, making cytokine-induced apoptosis functionally far more relevant than is spontaneous apoptosis thereby perpetuating inflammatory responses. Indeed the model of Caco-2 cells co-cultured with M1s is similar to that observed with IBD, where the apoptotic rate

is increased leading to perpetuated inflammatory responses (Baumgart & Carding, 2007).

In the chronic inflammation model, TEER was decreased in comparison with cells in a single culture, and stimulation of cells with LPS strongly decreased TEER. This suppression is due to the pro-inflammatory cytokine production increasing during co-culturing time and stimulating macrophages with LPS. Probiotic treatments in chronic inflammation model failed in repairing the induced injuries by pro-inflammatory cytokines and LPS and full restoration of epithelial barrier. This data, in part, is in line with other research groups, which have documented the effects of pro-inflammatory cytokines on decreasing of TEER (Haller et al., 2000b, Parlesak et al., 2004). In fact, therapeutically, neutralising TNF-α-antibodies (infliximab) can restore barrier function in Crohn's disease by down-regulating epithelial cell apoptosis (Schulzke et al., 2006).

TNF α induced protein 3 (TNFAIP3) is a cytosolic protein that acts in a negative feedback loop to regulate cell signalling induced by TLR ligands and TNF-α mediated the regulation of the intestinal barrier. Suppression of TNFAIP3 increases epithelial cell permeability (Kolodziej et al., 2011). It is therefore proposed that the expression of TNFAIP3 was suppressed in epithelial cells in co-culture system especially, after LPS stimulation, and probiotic treatments failed to up-regulate TNFAIP3 that recovered the suppression of barrier function induced by pro-inflammatory cytokines and LPS in cells of chronic inflammation model.

Normally, M2-like macrophages secrete high levels of regulatory cytokines (IL-10 and TGF-β), and low levels of TNF-α in response to LPS stimulation (Mantovani, Sica & Locati, 2007). In normal homeostasis (Caco-2/M2) model, it is predicted

that the level of pro-inflammatory cytokine was less than in cells of chronic inflammation (Caco-2/M1) model. Therefore, the disruption of epithelial barrier in this scenario (Caco2/M2) was less than in cells of Caco-2/M1 co-culture model even after LPS stimulation. Interestingly, probiotic treatments positively repaired the damaged induced by cytokines and LPS, but without full recovery. This data is in agreement with other research groups such as Anderson et al. (2010) who showed that *Lactobacillus plantarum* reduced the suppressive effect of pathogenic bacteria *Escherichia coli* on TEER. The findings of this study suggested that probiotic treatment repair of epithelial damaged might be through inducing Hsp70, resulting in attenuation of the effects of pro-inflammatory cytokines (Koninkx et al., 2010) or by up-regulation of TNFAIP3 (Kolodziej et al., 2011) leading to mantained intestinal barrier function.

Tight junction (TJs) assembly and permeability are regulated by a network of signalling pathways including protein kinase C; suppression of protein kinase C resulted in a decrease in TEER (Chen et al., 2002). Therefore, probiotics exerted their effects in modulation of epithelial barrier function might be through modulation of the expression and/or activity of protein kinase C. These findings are in agreement with Zyrek et al. (2007) when they showed that *E.colli* Nissle 1917, restored the disrupted epithelial barrier via PKC in T84 epithelial cells induced by enteropathogenic bacteria *E. coli*.

It is well known that the important factor to determine TEER value is TJs: TJs are composed of different types of proteins including ZO-1, ZO-2 and Claudine (Schneeberger & Lynch, 1992), participating in forming the epithelial cell monolayer tight junctions, therefore, ZO-1 tight junctional protein expression was tracked by fluorescent-labelled antibody. In cells co-cultured with M1s (chronic

inflammation model), ZO-1 showed less intensity and discontinuous labelling around the cell periphery indicative of a disorganization process of the tight junction proteins (Pyrgos et al., 2010), and this is similar to that observed with IBD, when tight junctional intercellular changes resulting in reorganization of tight junction protein structure and subsequent changes in barrier properties (Capaldo and Nusrat, 2009). Indeed, over expression of pro-inflammatory cytokines, leading to activation of the inflammatory process which is associated with suppression of zinc finger protein A20 (Prasad et al., 2004). Al-Sadi et al. (2010) reported that activation of the inflammatory process through NF-kB increased tight junction permeability. Chen et al. (2012) reported that phosphorylation of the myosin L chain kinase (MLCK) induced a contraction in actin-myosin filaments associated with reorganisation of ZO-1 tight junction protein. This process is thought to be central to losing barrier function in IBD (Shen et al., 2009). These changes in ZO-1 structure were observed in this study by immuno-fluorescent staining and confirmed by ZO-1 gene expression. Disruption in ZO-1 protein expression leads to a complete loss of tight junction integrity associated with ZO-1, which is correlated with the destruction of the intercellular contacts, especially after LPS stimulation, when the lack of ZO-1 continuity around the cell periphery was predominant. The findings of the current study suggested that the cells co-cultured with M1s suffered from the pro-inflammatory cytokine produced by M1s. In an attempt to determine the type of cytokine mediating the down-regulation of ZO-1 expression, a single culture of Caco-2 cells was treated with pro and anti-inflammatory cytokines, resulting in suppression of ZO-1 expression in response to the pro-inflammatory cytokines IL-1β, TNF-α, and IL-8, whereas IL-10 treatment was augmented ZO-1 expression. These findings in part are in line with previous reports of Wang et al. (2006), who showed the decrease in TEER value after TNF-

α stimulation of Caco-2 cells stimulated with IFN-γ or TNF-α which correlated with suppression of TNF-α-induced MLCK phosphorylation. Probiotic bacterial treatments enhanced TEER and ZO-1 expression might be via regulation of Hsp70 (Tsapara et al., 2006). Overall, the data of this study suggested that probiotic bacterial treatments mediated their roles in maintaining the intestinal epithelial barrier through different mechanisms.

5.3.4. Epithelial cell PRR induced by LPS selectively modulated by probiotic treatments in different co-culture systems

It was hypothesized that probiotic bacteria modulated epithelial cell immune responses via modulation of PRR receptors. Epithelial cells are known for their effectiveness against invasion of luminal bacteria, and also their polarity has a major role in colonic homeostasis. This barrier normally does not react to commensal bacteria, but triggers pro-inflammatory signalling pathways in the presence of pathogenic bacteria or in response to pro-inflammatory cytokines (Cario and Podolsky, 2005a). In fact, the interaction of commercial non-pathogenic microbiota with intestinal epithelial cells results in inducing a program of epithelial cell homeostasis and repair through promoting epithelial cell proliferation, secretion of IgA into the gut lumen and expression of antimicrobial peptides (Cario, 2005b). However, in IBD, the barrier fails in discrimination between commensal non-pathogenic- and pathogenic bacterial recognition, resulting in increasing epithelial cell permeability and later changing the barrier properties (Bamias et al., 2005).

It is known that LPS is a potent inducer of TLR4 expression (Beutler, 2000) and binding to its receptor (TLR4) will initiate signalling cascades leading to activated NF-kB resulting in triggering the inflammatory process associated with up-

regulation of the expression of TNF-α, IL-1β, IL-8, and IL-6 (Taniguchi et al., 2009). TLR4 has been found to be expressed by epithelial cells in a very low level in the healthy intestine but highly up-regulated in IBD (Hausmann et al., 2002), whereas TLR2 is expressed in a healthy intestine (Cario et al., 2004) to enhance specific functions mediating barrier integrity of epithelial cells. TLR2 stimulation effectively conserves tight conjunctions associated barrier assembly against stress-induced injury through the promotion of PI3K/Akt-mediated cell survival via MyD88 (Cario et al., 2004). In addition to its roles in maintaining gut barrier function, TLR2 exhibits a vital role in promoting gut tolerance via Tregs. Round et al. (2011) reported that metabolites from commensal microbiota such as polysaccharide A of *B. fragilis* trigger signals through TLR2 directly on $Foxp3^+$ regulatory T cells to promote immunologic tolerance. However, over-stimulation of TLR2 mediated mucosal inflammation solely through its effects on epithelial cells (Cario et al., 2007).

Caco-2 cells constitutively express CD14, which was up-regulated after LPS stimulation. CD14 recognises a variety of bacterial PAMPs. It has the ability to recognise and bind components of both gram negative and gram positive bacteria, consequently, It serves as a co-receptor for TLR2 and TLR4 (Meyenburg et al., 2004).

MD-2 is necessary for LPS signalling of TLR4 (Abreu *et al*., 2002) and TLR2 (Schröder et al., 2003). It forms a complex with TLR4 or TLR2 with CD14 (Dziarski et al., 2001). NOD2 is strongly expressed in colonic epithelial cells (Girardin *et al*., 2003a), which identifies its ligand in the cytoplasm of the cells, and consequently interacts with its target molecules causing activation of the innate immune system through recognition of MDP (Chen et al., 2004). In a healthy intestine, activation of

NOD-2 results in activation of the transcription factor NF-kB followed by an increase in the expression of pro-inflammatory cytokines, such as TNF-α or IL-1β (Chen et al., 2004) and antimicrobial peptides such as hBD-2 (Voss et al., 2006), however, expression of NOD-2 mediates apoptosis through induction of caspase system (Ogura et al., 2001). Expression of the pro-inflammatory cytokines TNF-α or IFN-γ mediates to up-regulation of NOD-2 (Rosenstiel et al., 2003). A sensor for bacterial DNA in epithelial cells is TLR9 (Takeshita et al., 2001). Apical and basolateral surface TLR9 activation (de Kivit et al., 2011) results in activation of a wide range of pathways including NF-kB leading to release pro-inflammatory cytokines and chemokines (Gao et al., 2003, Rachmilewitz et al., 2004).

Stimulation of cells with LPS in different co-culture systems were sufficient to increase the expression of IL-8, TNF-α, IL-6 and IL-10, and the antimicrobial peptide hBD-2 in Caco-2 cells. The up-regulation of IL-8, IL-6, and TNF-α expression after stimulation with LPS in this study was reflected in TEER, and ZO-1 changes. Conflicting reports showed a hypo-responsiveness of Caco-2 cells to LPS stimulation (Bocker et al., 2003) which was described through a lack of MD-2 expression (Shimazu et al., 1999). It was shown that the basolateral stimulation of epithelial cells by macrophages during co-culturing time and treating Caco-2 cells apically with probiotics resulting in inducing paracrine (factors from macrophages) and autocrine (factor from epithelial cells) effects on the Caco-2 cells. Consequently, a different range of epithelial cell receptor expression might lead to activate a series of cell signalling resulting in the production of pro- and anti-inflammatory cytokines in addition to hBD-2. While LPS is recognised by TLR4 or TLR2 incorporation with CD14 and MD-2 resulting in up-regulation of cytokine production such as TNF-α and IL-1β and hBD-2 in lower compartment by

macrophages, epithelial cells on the other hand, in upper compartment recognised probiotic bacterial PAMPs; due to this, the fate of the immune response will be determined by an array of factors. In fact, the cooperation between these signals (upper and lower) will determine the outcomes of epithelial cell interactions with probiotic PAMPs, the subsequent downstream signalling pathway will be conveyed, which is dependent on the type and level of the signal being delivered to the cells.

Although several cell receptors are classified in one superfamily (O'Neill and Bowie, 2007) and suggested to share a common pathway in the activation of inflammation, specific MyD88-independent and dependent pathways may exist for the LPS signalling cascade which leading to trigger different downstream signalling pathways (Baeuerle and Henkel, 1994) resulting in different profiles of immune cell outcomes. In the chronic inflammation model of this study, stimulation of cells with LPS up-regulated a panel of receptors including extracellular receptors of TLR4, TLR2, MD-2, and CD14 and intracellular receptors of TLR9, NOD-2 and intracellular TLR4-dependent and -independent MyD88 pathways selectively regulated by probiotics.

In the chronic inflammation model, HK-LF probiotic treatment up-regulated LPS induced TLR4, CD14 and MD-2, resulting in up-regulation of IL-6, IL-10, hBD-2, whereas, HK-LcS probiotic treatment down-regulated LPS induced TLR4, TLR2, TLR9, CD14, MD-2, but up-regulated LPS induced NOD-2, resulting in up-regulation of LPS induced TNF-α, IL-6, and hBD-2 (refer to table 5.2.2). The data of this study demonstrated that HK-LF treatment up-regulated IL-6, IL-10, and hBD-2 through up-regulation of TLR4/CD14/MD-2, suggesting that HK-LF/TLR4/CD14/MD-2 ligation of epithelial cells drive this inflammatory response. These data are in agreement with de Kivit et al. (2011) who showed that apical

TLR4 ligation of intestinal epithelial cells drives an inflammatory response of epithelial cells (HT-29 and T84), that grew in trans-well inserts co-cultured with PBMCs resulted in enhancing the production of TNF-α associated with decrease numbers of Foxp3+ regulatory T cells, however, neutralisation of TSLP abrogated TLR4 -enhanced TNF-α secretion. Intestinal epithelial cells are a major source of TSLP. TSLP has a vital role in maintaining gut tolerance via its role in promoting mucosal $CD103^+$ DC that induce $Foxp3^+$ regulatory T cells (Wells et al., 2011a). Therefore, it might be proposed that epithelial cell responsiveness associated with up-regulation of TLR4, enhanced TNF-α production via suppression of TSLP and probiotic treatment of HK-LF might have a role in suppression of TSLP that lead to up-regulated TLR4 induced TNF-α in this system. However, HK-LF up-regulated LPS induced hBD-2. As mentioned earlier, hBD-2 linked innate and adaptive immunity, therefore, HK-LF might be a good candidate to prevent and/or treat disease associated with hBD-2 deficiency such as Crohn's disease.

HK-LcS treatment up-regulated LPS induced TNF-α and hBD-2 associated with up-regulation of NOD-2 expression in chronic inflammation model. These findings are in agreement with Hisamatsu et al. (2003) who showed that NOD-2 (mRNA and protein) were up-regulated by TNF-α in SW480 and Caco-2 cells, where in Caco-2 cells only up-regulated by *S. typhimurium*. Voss et al. (2006) reported that NOD2 mediates the induction of hBD-2 associated with activation of NF-kB and AP-1. Taken together, it might suggest that HK-LcS is a good candidate to enhance hBD-2 expression via enhancing NOD-2 in diseases associated with mutation in NOD-2 such as Crohn's' disease (Grimm and Pavli, 2004).

Probiotic secreted proteins of LF and LcS, also exhibited a significant role in modulation of LPS induced cytokines, and hBD-2, associated with modulation of

LPS induced epithelial PRR receptors. Secreted proteins of LcS and LF up-regulated TNF-α, IL-6, hBD-2, TLR2, CD14, MD-2, NOD-2 and TLR9, but suppressed IL-10 and IL-8. These data are in agreement with Frick et al. (2007), when they showed that LF supernatant inhibited IL-8 induced by *Yersinia enterocolitica* infection in HeLa cells associated with suppression of NF-kB and p38 MAPKs.

In a homeostatic model, HK-LF probiotic treatment up-regulated LPS induced TNF-α, IL-6, IL-10 through up-regulation of TLR2/MD-2, whereas HK-LcS treatment up-regulated IL-6 and hBD-2 through up-regulation of LPS induced TLR9. LF-SP treatment up-regulated LPS induced IL-6 and hBD-2 through up-regulation of LPS induced TLR9 and TLR2, whereas LcS-SP treatment up-regulated LPS induced TNF-α, TLR4 and CD14 and failed to up-regulate LPS induced hBD-2. The up-regulation of LPS induced hBD-2 and failing in up-regulation of TNF-α associated with up-regulation of TLR9 in cells of Caco-2/M2 is in agreement with the findings of de Kivit et al. (2011) when they showed that apical TLR9 augmentation of HT-29 and T84 cell lines enhanced IFN-γ and IL-10 secretion. Regarding HK-LcS treatment, it might be HK-LcS up-regulated LPS induced hBD-2 via up-regulation of LPS induced IFN-γ associated with up-regulation of TLR9 (Albanesi et al., 2007).

Data showed that HK-LcS with rigid, resistance to lysozyme digestion of the cell wall, enhanced LPS induced hBD-2 and cytokine production, via enhancing intracellular PRRs (NOD-2 and TLR9) expression, whereas HK-LF with sensitive of lysozyme digestion cell wall enhanced LPS induced hBD-2 and cytokine production via enhancing extracellular PRRs (TLR2, TLR4, CD14 and MD-2) expression suggested that the chemical structure of peptidoglycan is important in

determining the immunomodulation of the immune response induced by LPS in epithelial cells.

Continuity of TLR activation will lead to trigger more inflammatory response, and Tollip is one of the main adaptor proteins that mediate inhibition of TLR activation leading to switching off the signals being triggered in response to specific stimuli (Zhang and Ghosh, 2002, Shibolet and Podolsky, 2007). The inhibitory exploits of Tollip is mediated via repression of auto-phosphorylation and kinase activity of IRAK (Zhang and Ghosh, 2002). Studies showed that Tollip manages the magnitude of inflammatory cytokine production in response to IL-1β and LPS (Didierlaurent et al., 2006). As shown earlier in this study, different responses of epithelial cells, which sensitised by different macrophage subset occurred in normal and chronic inflammation model. In LPS stimulation of the chronic inflammation model, Tollip expression was down-regulated, suggesting that LPS and pro-inflammatory cytokines such as IL-1β and TNF-α produced by macrophages exhibit high influences in sensitised epithelial cells in co-culture system, resulting in suppression of Tollip expression. One unanticipated finding was that Tollip expression in the normal homeostatic model was more than 1000 times higher than cells in the chronic inflammation model due to the level of pro-inflammatory cytokine production. These data suggested that the level of pro-inflammatory cytokines in chronic inflammation model has significant effects in inhibiting Tollip cell expression. Data showed that probiotic treatments selectively modulated LPS-induced Tollip expression in both co-culture models. For example, the suppression of LPS-induced Tollip was associated with up-regulation of TLR4/CD14/MD-2 enhanced production of IL-6 and hBD-2 in response to stimulation with HK-LF in cells of Caco-2/M1 co-culture model. Moreover, among

the probiotic treatments in the chronic inflammation model, HK-LF and LF-SP were more effective that other treatments in augmentation of Tollip induced by LPS, due to the fact that they engaged with TLR4 or TLR2. Indeed, decreasing Tollip expression was associated with increasing TLR expression (Pimentel-Nunes et al., 2012). Treatment with probiotics in the chronic inflammation model showed that probiotic treatments enhanced LPS induced Tollip in an attempt to limit the inflammation as a part of the probiotic bacterial role in maintaining gut mucosa. It seemed to be that titration of signalling cascades by Tollip overexpression leading to blockade the signals was dependent on the type of probiotic bacterial treatments and type of macrophage subset involved in co-culture model. Further research should be done to investigate the immunomodulation of probiotic bacteria on epithelial TLR adaptor molecules (A20, IRAK-M, and SIGIRR) lead to limit the inflammation in GIT using in *vitro*, in *vivo* or ex *vivo* studies.

Chapter 6

General Discussion

Chapter 6: General Discussion

Dietary habits have been associated with the aetiology and prevention of diseases (such as IBD) which are a serious health problem for civilization (Molodecky et al., 2011, Singh et al., 2001). One possible factor, which contributes to initiate IBD, is the disruption of microbial sensing by the gut mucosal immune system leading to miscommunication between gut microbiota and intestinal cells (Sartor, 2008). This process results in the over-production of pro-inflammatory cytokines such as TNF-α and IL-1β, which contribute to tissue damage of the gut mucosa.

Macrophage cells are implicated in the initiation of IBD through their dysregulated production of pro- and anti-inflammatory cytokines as a result of activation of an array of endogenous and exogenous stimuli (microbial PAMPs and cytokines) (Butcher et al., 2005, Pull et al., 2005, Smith et al., 2011). Modulation of the mucosal immune response is one of the possible methods of prevention and therapy for IBD using substances of natural origin (Fedorak, 2008).

6.1. THP-1 cell line: a reliable cell model?

Owing to either financial or ethical constraints related to animal or human *in vivo* studies, *ex vivo* or *in vitro* studies become more applicable to the improvement of specific applications. *Ex vivo* systems have the benefit of their natural origin; however, the high individual variation among donors can make analyses and understanding of the results more complex. Human cell lines are valuable *in vitro* tools to investigate the cellular functions, mechanisms and responses, as well as signalling pathways, nutrient and drug transport/absorption. A disadvantage in the use of cell lines is that the malignant background and the cultivation of cells under controlled conditions (outside their usual environment) might, perhaps result in

different sensitivity and responses compared with standard cells in the body. Likewise, possibly relevant interactions between the target cells and neighbouring cells, as in normal tissues, cannot be simply mimicked. However, the correlation between *in vitro* results and predicted *in vivo* responses has been described e.g. evaluation of probiotics. Foligne et al. (2007) investigated the correlation between *in vitro* (human peripheral blood mononuclear cells) and *in vivo* (a murine model of acute TNBS-induced colitis) immunomodulation potential of the probiotic strain and its ability to prevent experimental colitis in mice. Dai et al. (2012) examined the effects of VSL#3 probiotics on colonic epithelium permeability *in vivo* (acute colitis induced by dextran sodium sulphate) and *in vitro* (HT-29 cell line). An extra benefit of using human cell lines for the *in vitro* investigations is that the outcomes can be used to investigate the cell signalling which can support the definite *in vivo* proven health promoting effectiveness of selected compounds for human intake.

The THP-1 cell line is isolated from the peripheral blood of patient suffering from acute monocytic leukemia; they are non-adherent cells, express Fc and C3b receptors (Tsuchiya et al., 1980). The THP-1 cell line has been widely used to investigate the immune responses not only while cells are in the monocytice state but also in the macrophage-like state, because of their ability to differentiate into a macrophage-like phenotype after exposure to PMA or to Vit.D3 (Auwerx, 1991, Chanput et al., 2010, Schwende et al., 1996). Technically, based on the homogeneous genetic background minimizes the degree of variability in the cell phenotype, which facilitates reproducibility of findings, THP-1 cells have some advantages over freshly isolated PBMCs from both animal and human (ex *vivo*) (Rogers et al., 2003). However, it is important to understand that THP-1 monocytes and differentiated THP-1 macrophages are differing from those derived

from PBMCs, such as the regulation of apoptosis. THP-1 is an immortalized cell line that can cultivate and divide forever *in vitro* under appropriate culture conditions, while human PBMCs monocytes need inflammatory mediators, for instance, IL-1β, TNF-α or LPS, to function as survival factors to avoid apoptosis (Mangan and Wahl, 1991). A number of publications have compared the similarities between THP-1 derived macrophages and human PBMCs derived macrophages with respect to various aspects such as the expression of macrophage receptors (CD11b, CD14 and CD36) (Schwende et al., 1996, Daigneault et al., 2010) polarizing ability (plasticity), macrophage morphology and adherence (Tsuchiya et al., 1982) macrophage function (phagocytosis, accumulation of lipids and antigen presentation) (Gupta et al., 2005). Sharif et al. (2007) concluded that THP-1 cells provide an accurate and valid cell model system for evaluating the LPS response in macrophages. Data in chapter 3 and 5 indicated that THP-1 derived macrophage subsets are useful tools for drug and compound screening purposes such as immunomodulation by probiotics. Taken together with the results of chapter 3 and 5, and from the literature studies, it can be concluded that THP-1 and THP-1 derived macrophage subsets cells appear to represent a simplified, suitable, and reliable model to investigate monocytes and macrophages functions/responses, macrophage differentiation and possible immunomodulatory effects of probiotics.

6.2. Caco-2 cell line represent gut intestinal epithelial cells?

Intestinal epithelial cells separate the mucosal immune system from the external milieu. The intestinal epithelium shows a key role in maintaining gut tolerance via its roles in interacting with the mucosal cells resident in the lamina propria (such as macrophages) and in the same time interacting with microbiota and food born

antigen in gut luminal contents, the mechanisms behind this crosstalk are largely unknown. In fact, the differentiation process of intestinal cells, pathologies related to inflammatory conditions in the intestine, and also the adaptation of intestinal cells to firm nutritional conditions are often investigated by an *in vitro* method using intestinal epithelial cell models (Hodin et al., 1997). Caco-2 cell line is a commonly used model, representing features of the human intestinal epithelium, they were derived from a human colon adenocarcinoma, differentiated spontaneously *in vitro* under proper culture conditions, thereby exhibiting enterocyte-like structural and functional characteristics (Lenaerts et al., 2007). After differentiation, they mimic typical characteristics of the human small intestinal epithelium, with well-developed brush border as seen in Fig.2.6. They express TLRs as shown in chapter 4 and 5, and also by other studies (Cario et al., 2000b), produce IL-8, IL-6, TNF-α, IL-10 and hBD-2 (chapter 4), TGF-β and TSLP in response to Gram-positive and Gram-negative bacteria (Zeuthen et al., 2008) and also in response to stimulation with pro-inflammatory cytokines such as TNF-α or IL-1β as shown by this study either as monoculture or as co-culture with macrophage subsets. Caco-2 cells modify themselves depending on the type of macrophage subsets in a co - culture model by changing their profiles of cytokine, hBD-2, and TLR expression (macrophage cell subset co-cultured dependent). Data in chapter 5 indicated that Caco-2 cells can discriminate between signals either from a co-culture with M1 (Caco-2/M1) or from M2 (Caco-2/M2). Therefore, it can be concluded that Caco-2 cells appear to represent a suitable and reliable model to study the interaction of epithelial cells with macrophage subsets representing normal or chronic inflammation model, and possible immunomodulatory effects of probiotics in both co-culture models.

6.3. Screening tool for probiotic immunomodulation

Regarding potentially protective foods, increasing attention has been paid to probiotics (Fuller, 1991, Vasiljevic and Shah, 2008). Probiotic bacteria and their products represent a novel therapeutic or preventive treatment option of IBD in humans (Bomba et al., 2006), because synthetic therapeutics have numerous side effects unfavourably influencing human health and at the same time inducing new problems. This study was designed to look for and identify a number of possible modulations of mucosal immune responses by probiotic bacteria induced by specific stimuli using a combination of THP-1 derived macrophage subsets, and Caco-2 intestinal epithelial cells to develop co-culture model of normal homeostasis (Caco-2/M2) or chronic inflammation model (Caco-2/M1). Three lines of investigations were designed (monoculture of macrophage subsets, monoculture of Caco-2 epithelial cells, and co-culture models of Caco-2/M1 or Caco-2/M1, refer to Fig.1.7) to build up a good platform of knowledge looking at the probiotic bacterial role in modulation of the immune response induced by specific stimuli in specific model. Firstly, the role of a panel of probiotic strains (*Bifidobacterium breve*, *Lactobacillus rhamnosus, L. salivarius, L. plantarum, L. fermentum, and L. casei strain Shirota*) as heat killed (HK) and secreted protein (SP) was identified in the modulation of monocyte and macrophage cell subset cytokine production induced by LPS as a pathogenic inflammatory signal. This immunomodulation by probiotic strains occurs by either suppression of pro-inflammatory cytokines, leading to limitation of inflammation, or by up-regulation of anti-inflammatory cytokines, leading to maintenance of the gut mucosa. This information allows the selection of appropriate probiotic bacteria for the treatment of conditions such as IBD. In addition, a stably transfected NF-κB-reporter cell line

model of $CD14^{lo}$/$CD14^{hi}$ mucosal resident homeostatic and infiltrating inflammatory macrophages was used to investigate the relationship between a range of potentially immunoregulatory probiotics (both bacterial cell and secreted protein preparations) and their effects on macrophage subset NF-κB activation. Moreover, the immunomodulation of probiotics on corresponding cytokine effector phenotype ($CD14^{hi/lo}$) was determined, of relevance to mucosal macrophages found in the GIT.

The overproduction of TNF-α and IL-1β by uncontrolled macrophage stimulation contributes to initiation of tissue damage in IBD (Strober and Fuss, 2011). Therefore, the second aim was to determine the role of probiotics in modulation of epithelial cell cytokine and hBD-2 expression induced by TNF-α and IL-1β. Thirdly, the probiotic bacterial role in modulation of epithelial cytokine, hBD-2 production, and the epithelial cell barrier, during either normal homeostasis or chronic inflammation, was identified using a developed *in vitro* co-culture system, which mimics the interaction of gut epithelial cells with immune cells (M2-like or M1-like macrophages). Investigation of the epithelial cell barrier was focused on determining TEER and ZO-1 cell expression associated with dysregulated epithelial cell integrity induced by LPS in co-culture models. Each line of research in this study provides productive and fruitful knowledge about the role of probiotic bacteria in modulation of the mucosal immune response.

6.4. Is probiotic administration always beneficial for human health?

The difference between monocytes and macrophages is monocytes are blood-circulating cells, whereas macrophages can be found only at the site of inflammation, therefore called inflammatory-monocyte derived macrophages or

resident in tissue such as lamina propria, and called tissue-resident macrophages. Therefore, THP-1 monocytes were differentiated into M1 or M2-like macrophages in order to use them for screening possible immunomodulation by probiotics either macrophage monoculture or using them to influence epithelial cells in co-culture models. Normally, mucosal gut macrophages resemble the M2 subset and fail to express CD14, a co-receptor for LPS signalling. Thus, probiotic modulation of LPS-induced NF-kB activity and cytokine expression was investigated using a THP-1 monocyte-derived reporter cell line, model of $CD14^{hi/lo}$ M1 and M2 macrophages. Inducing an inflammation, LPS acts via extracellular receptors of TLR4 or TLR2 coupled with CD14 and MD-2 as the main suggestive pathway to amplify the immune response. Accordingly, production of pro-inflammatory cytokines such as TNF-α, IL-8, IL-6, and IL-1β are amplified, which can consequently be used as markers to measure the inflammatory reaction (Backhed et al., 2003). Both HK-LcS and LcS-SP suppressed IL-6 and differentially regulated TNF-α and IL-8 expression dependent on macrophage subset. HKs and SPs of BB, LF and LR suppressed monocyte TNF-α expression induced by LPS, and both HK-BB and BB-SP suppressed LPS induced IL-1β expression in monocytes, M1, and M2-like macrophages. It seemed to be that the signals induced by probiotics whether HK or SP differentially regulate LPS induction in monocytes and macrophage cell subsets, leading to modulated cytokine production of TNFα, IL-1β, IL-6 and IL-8. HK probiotics suppressed $CD14^{lo}$ and augmented $CD14^{hi}$ M1 TNF-α production whereas SPs augmented $CD14^{hi}$ M1 TNF-α production. M2 macrophage IL-6 production was suppressed by both HK and SPs, and was differentially regulated in $CD14^{lo}$ and $CD14^{hi}$ M1s. HK-LcS augmented LPS induced epithelial TNF-α expression in Caco-2/M2 model, whereas non-significantly augmented LPS induced TNF-α in Caco-2/M1 co-culture

model, up-regulated LPS induced TNF-α in $M1^{hi}$, suppressed TNF-α in $M1^{lo}$, non-significantly augmented TNF-α in $M2^{hi}$, and up-regulated TNF-α in $M2^{lo}$, augmented TNF-α in monocytes and M2-like macrophages, whereas suppressed TNF-α in M1-like macrophages. In contrast, HK-LF augmented LPS induced TNF-α in Caco-2/M2, suppressed LPS induced TNF-α in Caco-2/M1, up-regulated TNF-α in $M1^{hi}$, suppressed TNF-α in $M1^{lo}$, up-regulated TNF-α in $M2^{hi}$, and suppressed TNF-α in $M2^{lo}$, suppressed TNF-α in monocytes and M1s and augmented TNF-α in M2-like macrophages. Comparison between these two probiotics in modulation of LPS induced TNF-α (as the principal cytokine that mediates regulation of infectious, inflammatory and autoimmune phenomena) (Pasparakis et al., 1996) in different types of cell lines showed that the probiotic immunomodulation was CD14 expression level, type of macrophage subset, and type of co-culture model (Caco-2/M2 or Caco-2/M1)-dependent. In spite of the differences between LcS and LF probiotic bacterial strains regarding sensitivity to lysozyme digestion and cell wall structure, they exhibited quite similar effects in modulation of LPS induced TNF-α in macrophage subsets, however, the differences were found only in modulation of LPS induced TNF-α in epithelial cell in co-culture models.

This work showed that HK and SP probiotic bacteria differentially regulated cytokine and NF-kB activation in a subset-dependent manner and suggested a cautionary approach to probiotic treatment of mucosal inflammation through the up-regulation of M1s $CD14^{hi}$ TNF-α macrophage cell expression, which leads to amplified inflammation resulting in tissue injury. High CD14 expression is one of the main pathological signs in IBD associated with pathophysiology in macrophages resident in the lamina propria (Kamada et al., 2008), and up-regulation of TNF-α in these subsets will worsen the inflammation; suggesting that

probiotics are not always beneficial and can also be detrimental in inflamed IBD. Up-regulation of LPS induced IL-1β and IL-6 by probiotic bacteria might enhance the proliferation and differentiation of Th17 subset, which is one of the main cells involved in IBD (Brand, 2009), therefore probiotic treatments might again increase the inflammation of the gut mucosa. Using HK-LcS; augmented LPS induced TNF-α and suppressed LPS induced IL-10 in Caco-2/M1 co-culture model (refer to table 5.2.2). During chronic inflammation, the up-regulation of TNF-α will increase epithelial cell apoptosis and suppress epithelial cell barrier. Taken together, probiotics can have inflammatory activities of both macrophages and epithelial cells (Tsilingiri et al., 2012).

Evidence suggests that macrophages play a central role in both innate and adaptive immune responses. In aged subjects the innate and adaptive arms of the immune system undergo changes associated with alterations in both the strength and quality of the immune response. Reduced TNF-α level in aging is associated with reduction in macrophage phagocytosis (Renshaw et al., 2002). MHC class II, TLR4, TLR2, p38, and ERK MAPKs signalling, JAK/STAT signalling and production of cytokines are also suppressed during aging (Gomez et al., 2005), resulting in a breakdown of the epithelial barriers of the skin, lung and GIT, which enables invasion of delicate mucosal tissues by pathogenic organisms. *In vivo* evidence showed that an abundance of pro-inflammatory cytokines associated with healthy young subjects (Franceschi et al., 2000), whereas dysregulation of the immune system referred to as immunosenescence is related to aging subjects (Hansen et al., 2013). Therefore, probiotic strains associated with immune activation may be appropriate for the aging stage, whereas probiotic strains

associated with immune regulation may be appropriate for young adulthood dependent on environmental tissue status.

6.5. Probiotic immunomodulation of cytokine (TNF-α or IL-1β) induction of epithelial cytokines and hBD-2 production

Epithelial cells represent a first line of defence; perform the first step in antigen processing and presentation by expressing a range of receptors in response to different stimuli to the other cells resident at the gut mucosa, such as macrophage cell subsets. They respond to the external and internal signals by releasing cytokine and antimicrobial peptides (AMPs) such as hBD-2. It is hypothesised that epithelial cells exhibit hypo-responsiveness toward gut microflora through suppression or absence of PRR expression. However, epithelial cells express a range of PRRs at the physiological level to keep normal homeostasis throughout their role in the surveillance and monitoring of gut contents (Cario, 2002). Probiotic strains used in this study are Gram-positive bacteria with cell wall enriched with muraemic acid, LTA, polysaccharides (PS) and PGN. It seemed to be that the differences in LTA and PGN structure between these strains might mediate the differences in the modulation of IL-1β or TNF-α induced hBD-2 and cytokine production. hBD-2 is an important antimicrobial peptide that links innate and adaptive immunity and functions as one of the main factors involved in maintaining the gut barrier.

TNF-α and IL-10 are the master cytokines regulating infection, inflammation and autoimmunity. In fact, there is a reciprocal relationship between them, up-regulation of IL-10 results in suppression of TNF-α, and *vice versa*, in addition, TNF-α play a significant role in inducing IL-10 produced by monocyte derived

macrophages (Foey et al., 1998). Data showed that treating epithelial cells with TNF-α or IL-1β induced IL-10, TNF-α, IL-6, IL-8, and hBD-2 (time and type of stimuli dependent). It will be useful to draw a link between extracellular and or (membrane bound or intracellular) cytokine expression of TNF-α and IL-10 associated with hBD-2 expression at each treatment using anti-TNF-α or anti-IL-10 antibody. Indeed, neutralising the TNF-α bioactivity leads to inhibition of hBD-2 expression, whereas neutralising the IL-10 bioactivity leads to up-regulation of hBD-2 expression. This confirmed the fact that hBD-2 expression is controlled by the expression of cytokines (TNF-α and IL-10). However, probiotic treatments modulated the cytokine induced hBD-2 (TNF-α or IL-1β), TNF-α and IL-10 after neutralisation of IL-10 or TNF-α. This immunomodulation by probiotics in hBD-2 production might lead to modulated DC maturation via modulation of CCR7 expression. Moreover, probiotic treatments modulated membrane bound and secretable IL-10 which might lead to the modulation of adaptive immunity via B cell activation. To identify the molecular mechanisms controlling cytokine and hBD-2 expression during the absence of TNF-α or IL-10, the expression of selected TLRs and NLRs in the presence or absence of the anti-TNF-α and anti-IL-10 antibody was studied. TLR4, TLR2, and CD14 were down-regulated after treating cells with anti-TNF-α antibody, suggesting that there is a crucial role of TNF-α expression in the regulation of expression of these PRRs. Probiotic bacteria exhibit a significant role in modulating TLR expression after treating cells with anti-TNF-α or anti-IL-10 antibody via direct (bacterial PAMPs) or indirect effects (inducing cytokines which modulate TLRs). TNF-α treatment up-regulated the expression of NOD2 in the intestinal epithelial cells, which might lead to increasing epithelial cell LPS susceptibility (Rosenstiel et al., 2003), and possibly to disruption of mucosal barrier function as it is observed in IBD, therefore, probiotic treatments exhibit anti-

inflammatory effects by suppression of TNF-α induced NOD-2 expression. The up-regulation of IL-1β is one of the clinical observations associated with IBD pathophysiology. Current investigations showed that only TLR4 expression was up-regulated upon treating cells with IL-1β in the presence of anti-TNF-α antibody, whereas both TLR2 and CD14 expression were down-regulated at this scenario. What is surprising is that the up-regulation of TLR4 expression alone, without engagement with CD14 and MD-2 expression, was not enough to trigger the successful immune response resulting in the up-regulation of either pro-inflammatory cytokines such as TNF-α or anti-inflammatory such as IL-10 by epithelial cells. However, treating cells with anti-IL-10 antibody induced the up-regulation of TLR expression triggering an active immune response. This explains that the absence of IL-10 will result in a breakdown of tolerance. Expression of Tollip by epithelial cells is one of the main mechanisms of epithelial cell hypo-responsiveness toward the continuous exposure to gut microbiota (Abreu, 2010, Didierlaurent et al., 2006). It has been hypothesised that the up-regulation of TLR expression in response to specific signals resulting in up-regulation of cytokines and hBD-2 is associated with the down-regulation of Tollip expression. This current study showed the crucial roles of TNF-α and IL-10 in Tollip expression, i.e. Tollip is up-regulated in the absence of TNF-α and suppressed at the absence of IL-10. Modulation of the IL-10 and TNF-α expression by probiotics resulted in different profiles of Tollip expression. Targeting molecules that mediate the tolerogenic mechanisms (suppression of TLR activation) might open a new window to the treatment of IBD. In future, PCR cloning is one of the suggested approaches for identifying the bioactive components of probiotic bacteria and their secreted proteins mediating the suppression of PRRs expression and limiting inflammation at gut mucosa.

6.7. Why co-culture model?

The monoculture of Caco-2 cells represents the normal mucosa with intact tight junctions and strong barrier properties. However, such a test system does not imitate the pathophysiological alterations happening in an inflamed region of IBD. Moreover, a single cell line can never represent the complex interplay of different cell types during an inflammatory process. Therefore, the developed *in vitro* co-culture systems, which mimics the interaction of gut epithelial cells with immune cells of the GALT, allows us to elucidate the role of commensal bacteria in driving mucosal tolerance and how it can be boosted by the use of immunoregulatory probiotics.

Based on the concept that the intestinal epithelium performed as an essential barrier between the gut lumen and the lamina propria, Caco-2 epithelial cells were used as the first layer exposed to the probiotics in this model of this study. Gut mucosal macrophages represented a large pool of APCs, playing a pivotal role in driving mucosal immune responses, resulting in either activation of inflammatory immune responses to pathogenic challenge or tolerance to beneficial luminal contents. Elicited macrophage responses are dependent on tissue environment and the resulting macrophage cell subsets, where homeostatic macrophages resemble the M2 macrophage subset and inflammatory macrophages resemble M1s.

6.8. Do probiotics modulate epithelial barrier function in chronic and normal homeostasis model?

It is difficult to find suitable cell lines fit to represent normal homeostasis and chronic inflammation status. However, the models that have been developed by

co-culturing Caco-2 cells at the upper compartment and M2- or M1- like macrophage cells at the lower, apically treated with probiotic bacterial treatments such as HK or SP, and then basolateraly stimulated with LPS to represent a normal homeostatic or chronic inflammation model. Application of this system might help the understanding of the cellular mechanisms of the interaction between macrophage subsets and epithelia cells at different environment.

This study was designed to look for the direct interaction between probiotic bacteria and their secreted proteins with epithelial cells in different co-culture models. Gut intestinal epithelium is known for its effectiveness against the invasion of luminal microorganisms, which may be present at concentrations of more than 10^{14}CFU/ml (Gigante et al., 2011). This barrier does not normally react to commensal bacteria, but triggers the pro-inflammatory signalling pathway only in the presence of pathogenic microorganisms (Sartor and Mazmanian, 2012). Triggering of the immune response by epithelial cells needs the activation of TLRs. Although TLR4 has been found at low levels in a healthy intestine (Cario, 2010), however, it is highly up-regulated in IBD (Abreu et al., 2005). Contrasting reports show the weakness of immune response results from LPS stimulation in Caco-2 epithelial cells, which was described as having a lack of MD-2 cell expression, a partner molecule for TLR4 signalling complex (Dziarski et al., 2001). This study and other research groups (Taniguchi et al., 2009) shown that TLR4 induced by LPS was expressed by epithelial cells at different inflammation status and is selectively modulated by probiotic treatments.

In a chronic inflammation model, TLR4/CD14/MD-2 expression was up-regulated by HK-LF, whereas these bacteria failed to up-regulate these TLR-related molecules by cells in the normal homeostatic model (refer to table 5.2.2). It

seemed to be that the inflammatory cytokines produced by stimulated M1s upon co-culture with epithelial cells enhanced epithelial cell TLR expression. The up-regulation of TLR4/CD14/MD2 expression correlated with a decrease in the epithelial cell barrier (TEER). In fact, TEER, a measurement of epithelial cell integrity, was sharply reduced in the chronic inflammation model.

For monitoring epithelial cell integrity, ZO-1 protein associated with epithelial cell integrity was tracked as mRNA by qPCR and as protein by immunohistochemistry studies. The localisation of ZO-1 in epithelial cells in the chronic inflammation model was remarkably different from cells in the normal homeostatic model, where the expression of ZO-1 in the first case was highly suppressed in comparison with the second case. TEER, ZO-1, and Tollip expression by epithelial cells in the chronic inflammation model were highly suppressed and treating cells with probiotic bacteria, whether HK or SP, only slightly recovered this suppression of intestinal epithelial cell integrity. However, in cells of the normal homeostatic model TEER, ZO-1, and Tollip expression induced by LPS were up-regulated by probiotic treatments. This confirmed the vital role of probiotic bacterial treatments in maintaining epithelial cell integrity, especially in Caco-2/M2 co-culture model though their roles in recovering the dysregulated barrier function induced by LPS via up-regulation of TEER and ZO-1 (refer to table 5.2.3). Damage induced by an array of factors including cytokines has been produced during co-culturing with macrophage subsets in addition to LPS stimulation. Several mechanisms have been postulated for the roles probiotic bacteria exert in recovering the gut epithelial barrier. These include the up-regulation of heat shock protein (hsps) such as hsp70 resulting in attenuating the effects of pro-inflammatory cytokines (Koninkx et al., 2010) or the up-regulation of TNFAIP3 (Kolodziej et al., 2011)

leading to enhanced barrier function. In addition, probiotic treatments might up-regulate the anti-inflammatory cytokines such as IL-10 and TGF-β leading to suppressed pro-inflammatory mediator expression induced dysregulated barrier function. Indeed, selective roles were performed by probiotics in modulation of mucosal immune response in the co-culture models, dependent on probiotic bacterial strain. HK-LF modulated the extracellular molecular mechanism induced by LPS. Modulation of TLR4/CD14/MD-2 leads to enhanced expression of IL-10 and hBD-2 and suppressed TNF-α in the chronic inflammation model; whereas TLR2/CD14 modulation in the normal homeostatic model resulted in up-regulation of IL-10, subsequently enhanced epithelial cell integrity. TNF-α and IL-1β enhance TJ permeability by stimulating MLCK gene expression via NF-κB in Caco-2 (Al-Sadi et al., 2011). In chronic inflammation model HK-LF, suppressed LPS induced TNF-α. This suppression performed by these bacteria might be via suppression of the MLCK phosphorylation leading to decrease cell permeability by decreasing of reorganisation of tight junction induced by TNF-α (Yu et al., 2010). HK-LcS failed to up-regulate LPS induced TLR4/CD14/MD-2 in both co-culture models, whereas it up-regulated NOD-2 expression in the chronic inflammation model and TLR9 expression in the normal homeostatic model, resulting in augmentation of TNF-α and hBD-2 release. Overproduction of TNF-α might be leading to up-regulated NOD-2 expression (Rosenstiel et al., 2003) via an autocrine effect, resulting in increasing mucosal permeability (D'IncÀ et al., 2006). LcS probiotic bacteria can be intracellular in addition to its extracellular resident mode in epithelial cells (Shida et al., 2006, Tien et al., 2006). This leads to enhanced intracellular PRRs, which results in up-regulation of the expression of cytokines and hBD-2. Probiotic bacteria secrete a range of extracellular metabolites through their metabolism. Probiotic extracellular proteins could diffuse through the mucus layer that covers

the intestinal mucosa, enabling the interaction with epithelial and immune cells at the gut mucosa. Probiotic secreted proteins exhibited significant effects in up-regulation of LPS induced Tollip associated with up-regulation of LPS induced ZO-1 and TEER in both co-culture systems which might be a promising approach leading to limit the inflammation induced by LPS at gut mucosa.

Chapter 7

Conclusions and future work

Chapter 7: Conclusions and Future Work

The studies described in this thesis investigated the possible immunomodulatory effects of a panel of probiotic bacterial strains *B. breve* (BB), *L.rhanonosus* (LR), *L. salivarius* (LS), *L.casei* strain Shirota (LcS), *L.fermentum* (LF), *L. plantarum* (LP) as heat killed (HK) and secreted proteins (SP) on the immune responses of monocytes, M1-like, M2-like macrophages and epithelial cells induced by different stimuli (microbial PAMPs and cytokines) using THP-1 monocytes, THP-1 monocyte derived M1, M2-like macrophages and Caco-2 epithelial cells. In addition, probiotic modulation of LPS-induced NF-kB activity and cytokine expression was investigated using a THP-1 monocyte-derived reporter cell line, model of $CD14^{hi/lo}$ M1 and M2 macrophages. Furthermore, these cells were used to develop *in vitro* co-culture models whereby Caco-2 epithelial cells were co-cultured with M1-like macrophages, resembling a chronic inflammation model, and Caco-2 co-cultured with M2-like macrophages, resembling a model of normal gut homeostasis, were used to investigate probiotic modulation of macrophage-influenced epithelial barrier function. The key findings are summarized below:

1- Probiotics selectively modulated monocyte and macrophage subset cytokine expression based on the ability of monocytes/macrophages to recognise and digest probiotics. They suppressed LPS induced monocyte IL-6, M1 TNF-α and M2 (IL-6, IL-8) expression, whereas selectively modulated monocyte (IL-8, IL-1β, TNF-α), M1 (IL-1β, IL-8, IL-6), M2 (TNF-α, IL-1β) cytokine expression, which might be lead to manipulated Th17 proliferation and differentiation via modulation of IL-1β and IL-6 expression (chapter 3).

2- Probiotic preparations (HK and SP) suppress $CD14^{lo}$, augment $CD14^{hi}$ M1 TNF-α, and differentially regulated M2 TNF-α production, suggestive of need for a cautionary approach to the use of probiotic in inflammatory pathology (chapter 3).

3- M2-like macrophage IL-6 production was suppressed by both HK and SPs, and differentially regulated in $CD14^{lo}$ and $CD14^{hi}$ M1s, which might lead to inhibit Th1 differentiation by elevating SOCS-1 that disrupt the IFN-γ signalling and development of Th1 (chapter 3).

4- NF-κB activation failed to parallel probiotic regulation of TNF-α and IL-6 (chapter 3).

5- Caco-2 epithelial cells express membrane bound (endogenous) as well as a secretable form (exogenous) of IL-10 and TNF-α (chapter 4).

6- hBD-2 expression positively regulated by TNF-α and negatively by IL-10 (chapter 4).

7- Probiotics (HK-LF and HK-LcS) selectively modulated both membrane bound or extracellular expression of TNF-α- and IL-10-induced hBD-2, as well as epithelial TLR expression, which might be lead to modulation of DCs maturation (up-regulation of CCR7) via modulation of hBD-2 expression and B cell activation via modulation of IL-10 (chapter 4).

8- Epithelial expression of TEER, ZO-1 and the endogenous TLR signal regulator (Tollip) were suppressed upon co-culture with pro-inflammatory M1-like macrophages paralleled by a suppression of IL-10, IL-6 and up-regulation of TNF-α and IL-8 (chapter5).

9- In the presence of LPS, HK-LF enhanced TEER, ZO-1 and partially rescued Tollip expression, whereas HK-LcS had no effect on TEER and

ZO-1 and displayed a weaker rescue effect on Tollip compared with LF in epithelial cells at co-culture models (chapter5).

In conclusion, probiotic strains differentially exert immune activatory or suppressive functions in monocytes, macrophage subsets, and epithelial cells. The immunomodulation by probiotics was determined by the probiotic bacterial strain, inflammatory environment, and mucosal macrophage effector phenotype. The studies described in this thesis demonstrate that probiotic has marked impact on the modulation of the immune responses in monocytes/ macrophages and epithelial cells for example; augment monocyte IL-1β, monocyte IL-8, M1 IL-1β, M2 IL-1β, and suppress M1 TNF-α and M2 IL-8, M2 IL-6 induced by LPS.

Probiotics are not always beneficial, especially in the case of probiotics augmentation of M1 $CD14^{hi/lo}$ TNF-α production with either HK or SP, and TNF-α induced by LPS in Caco-2/M1 co-culture model, therefore, further studies are required before taking any probiotics into the clinic. However, probiotics could be an alternative for the treatment of the inflammatory conditions when HK-LF up-regulated LPS induced IL-10 and hBD-2 which is correlated with up-regulation of TEER and ZO-1 expression in Caco-2/M1 co-culture model and treatments of BB and LR as HK and SP suppressed LPS induced monocytes TNF-α, M1TNF-α, M2TNF-α, monocyte IL-1β and suppression of $M1^{hi}$ NF-kB activation.

Since the current study reported that SP and HK probiotics differentially regulated LPS induced cytokine expression largely independent of NF-kB activation, future work is required to investigate other pathways which might be regulated by probiotics such as STAT-3/SOCS-3/SOCS-1, PI3K/AKT, PPAR-γ, and MAPKs.

It is clear that macrophages can be programmed via differentiation into specific subsets (M1 and M2 macrophages) in response to specific differentiation factor GM-CSF/PMA/IFNγ or M-CSF/Vit.D3/IL-4 respectively, the reversion of macrophage differentiation may represent a suitable macrophage cell based therapy, it is of interest to investigate the probiotic bacterial role in modulation of LPS induced iNOS, Arg, FIZZ-1, Ym-1, SR (CD33, CD36, CD68, CD163), FCR (CD16, CD32, CD64, CD89), CR (CR3, CR4) MR (CD206, CD204), and TREM-1.

Negative regulation of specific macrophage subsets and effector phenotype will allow manipulation of pro-inflammatory macrophages via manipulation of both surface bound negative regulator receptors (CD200R, TREM-2), and cytoplasmic molecules (MyD88s, IRAK-M) which suppress macrophage inflammatory cytokine production and SIGIRR which suppresses TLR expression, therefore, may be worth investigating probiotic bacterial role in modulation of the expression of these molecules induced by PAMPs or cytokines.

In the topic of immunomodulation, future work could also be directed at characterising the underlying signalling pathways when cell-cell interactions, for example, how probiotics modulate the expression of co-stimulatory molecules (CD86, CD80, CD40), antigen presenting molecules (MHC class I & II) and adhesion molecules (CD58, ICAM-1) in macrophage subsets, epithelial cells and T cells (CD28/CTLA-4) at cross talk between different cell types comparing chronic and homeostasis status.

In the current study, M1-like macrophage subset express high levels of pro-inflammatory cytokines (TNF-α, IL-8, IL-1β) that have an impact on proliferation, differentiation and apoptosis of other cells such as epithelial cells (survival via

TNF-α p75, and apoptosis via TNF-α p40). What's more, it will be useful to investigate the expression of other pro-inflammatory cytokines such as IL-12, that drive Th1 cell differentiation and NK cell activity (secretion of IFN-γ and CD69 expression) by M1-like macrophages. On the other hand, it would also be useful to investigate IL-4 and TGF-β expression by M2-like macrophages that drive Th2, and Tregs differentiation and proliferation, and determine if they are modulated by probiotics (HK or SP)?

Since the current study showed that epithelial cells expressed membrane bound IL-10 and TNF-α, it is of interest to investigate the factors that mediate cleavage of these membrane bound proteins such as ADAM17/TIPM3 or MMPs.

The main pathological signs of IBD vary with specific disease states, i.e. up-regulation of anti-inflammatory cytokines and suppression of hBD-2 in ulcerative colitis, in contrast to up-regulation of pro-inflammatory cytokines and suppression of hBD-2 in Crohn's disease (Kucharzik et al., 1995, Aldhous et al., 2009). Bearing this in mind, the data in this study suggest that HK-LcS is a good candidate in the treatment of UC, and HK-LF in the treatment of Crohn's diseases, and therefore it is important to confirm their effects using animal and *ex vivo* models.

LcS and LF differentially regulated cytokine and hBD-2 induced by LPS in epithelial cells in a co - culture model, it is of importance to investigate their role in modulating TGF-β, TSLP, APRIL, BAFF and RA that drive tolerance of $CD103^+$ DCs-class switching from IgM to IgA (humoral immunity), differentiation of T regs ($Foxp3^+$) (immune regulation), that mediate the maintaining of gut mucosal function comparing normal homeostasis versus chronic inflammation models.

In both the chronic inflammation and normal homeostatic models, LF-SP, and LcS-SP differentially regulated LPS induction of cytokines, hBD-2, and PRR expression. Therefore, it would be useful to determine the types of proteins released by probiotic bacteria using 2D gels following by running MS/MS for protein identification. The study looking for secreted protein immunomodulation requires further biochemistry research studies for protein separation and purification by high-performance liquid chromatography (HPLC) in order to determine each single protein's immunomodulation.

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Appendices

Appendices

1. Materials

1.1. Protein analysis

1.1.1. Protein extraction

5% w/v sodium deoxycholate

60 g/L w/v/Trichloroacetic acid

Acetone

1.1.2. Protein separation

Sephacryl S-200 HR (1-80 KDa MW range (dextran), 5-250 kDa MW range (globular proteins)

0.15 M sodium phosphate buffer (pH 7.4)

1.1.3. Protein quantification

Bradford reagent

Coommassie Brilliant Blue G-250	100 mg
95% v/v ethanol	50 ml
85% v/v phosphoric acid	100 ml

Dissolved and top up to 1Liter with distilled water (DW)

1.1.4. Protein resolving

SDS-PAGE reagents

Acrylamide, electrophoresis grade

Bis- acryl amide (N.N –methylenebisacrylamide)

Tris (2-hydroxymethyl-2-methyl-1, 3-propanediol)

SDS (sodium dodecyl sulphate or sodium lauryl sulphate)

TEMED (N, N, N, N,-tertamethylene-ethylenediamine)

APS (Ammonium per sulphate)

2-mercaptoethanol

Glycerol

Bromophenol blue

Glycine

HCl (Hydrochloric acid)

Solution A (separating gel buffer)

Acrylamide Stock Solution, 100 ml 30% (w/v) acrylamide (w/v) bis- acrylamide

Solution B (separating gel buffer):

1.125 M Tris base 68.14 g per 500 ml

0.3%w/v SDS 1.5 g per 500 ml

Dissolved in DW to 450 ml adjusted to pH 8.8 with HCl. Top up to 500 ml with DW.

Solution C (Stacking buffer)

140 mM Tris 8.42 g per 500 ml

0.11%w/v SDS 0.55 g per 500 ml

Dissolve in DW to 450 ml. Adjust to pH 6.8 with concentrated HCl 100%. Top up to 500 ml with DW

10%w/v APS (0.1g APS dissolved into 1ml DW)

Electrophoresis buffer (Running buffer)

Tris 3 g

Glycine 14.4 g

SDS 1 g

Dissolved in DW 1L, pH 8.3

5X Laemmli loading buffer (Reducing/ Denaturing)

1 M Tris –HCl (pH 6.8) 0.6 ml

50% v/v glycerol 5 ml

10% w/v SDS 2 ml

2-mercaptoethanol 0.5 ml

1% w/v Bromophenol blue 1 ml

0.9 ml DW

10 ml

Running gel (for semi-midi- gel)

6.3 ml (solution A)

8.6 ml (solution B)

70 µl (10%w/v APS)

7.5 µl TEMED

Staking gel

2.3 ml DW

0.67 ml solution A

1.0 ml solution C

30 µl 10% w/v APS

5 µl TEMED

Coomassie Gel stains (1L.)

Coomassie blue R-250 1.0 g

100%Methanol 450 ml

DW 450 ml

100 ml 100 % glacial acetic acid

De-staining Coomassie Gel stain solution (1L.)

Methanol 100% 100 ml

Glacial acetic acid 100% 100 ml

DW 800 ml

Protein Ladder (Hyper Page pre-stained protein Marker, Bio line, UK)

1.1.5. Western Blotting

Lysis Buffer

150 mM Nacl	0.87 g	
1% v/v Triton X	1ml	
50mM Tris with DW.	2.5ml	Dissolved in DW, pH 8.0 top up to 100ml

Inhibitors

Add to lysis buffer before use	Protease Inhibitor Cocktail	1:20 = for 200µl lysis buffer add 10µl
	Phosphatase Inhibitor Cocktail	1:100 = for 200µl lysis buffer add 2µl

Transfer buffer stock (10X Exc. Methanol)

Tris base	30.3g
Glycine	144g
DW	1L.

Transfer buffer working solution

10X transfer buffer	100 ml
Methanol	200 ml
DW	700 ml

Washing solution tris buffered saline with tween 20 (TBST)

TBS-Tween-20 (0.1% v/v)/TBS

10xTBS (tris buffered saline)

NaCl	80g
Tris	24.4g
DW to 1L with DW	Mix in 800ml of DW, adjust to pH 7.6, and make up

TBS-Tween-20 (TBST)

10x TBS	40ml
DW	959ml
Tween-20	1ml

Blocking solution

TBST	100 ml
5% w/v BSA	5g

Polyvinylidene fluoride (PVDF)

Whatman filter Filter papers

Stripping buffer

Mild stripping

15 g glycine

1 g SDS

10 ml Tween 20

Adjust pH to 2.2 Top up to 1 L with DW

1.2. Tissue culture

Media and supplementation materials

All reagents and media were obtained from (Lonza, Wokingham, UK)

Roswell Park Memorial Institute medium (RPMI-1640)

Dulbecco's Modified Eagles' Medium (DMEM)

Penicillin, Streptomycin

L-glutamine

Foetal calf serum (Bio sera, UK)

Zeocin, Blastocidin (Invivogen, Calne, UK)

Subculture materials

Dulbecco's Phosphate-Buffered Saline (DPBS) (Sigma –Aldrich, Pool UK)

0.25% v/v versene/ trypsin EDTA (TE) (Sigma –Aldrich, Pool UK)

Storage materials

20%v/v FBS/10% v/v dimethyl sulfoxide (DMSO)/ MDEM or RPMI

Differentiation of cell materials

All materials were obtained from (Sigma-Aldrich, Poole, UK)

Phorbol 12-myristate 13-acetate (PMA)

1, 25-$(OH)_2$-vitamin D_3

Activation of immune cells

K12-LPS (monocytes, macrophage subsets)

TNF-α and IL-1β (Caco-2 epithelial cells)

1.2.5. Cell viability

3-4-5-dimethy-2.5 thiazol-2.5 diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, Poole, UK)

1.3. Antibodies

Table 1: Summary of all antibodies used in this study

Specificity	Clone	Function	Source
Anti-IL-10	Clone JES3-9D7	Neutralization the bioactivity of IL-10 cytokine protein	(Biolegend, San Diego,Ca, USA)
Anti-TNF-α	Clone cA2 (MACS	Neutralization the bioactivity of TNF-α cytokine protein	Miltenyi Biotec Ltd, UK)
Mu anti-human TNF-α Mab I IgGI	Mab1	ELISA capture antibody for TNF-α protein	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Biotinylated mouse anti-Human TNF-α antibody	Mab11	ELISA detect antibody for TNF-α	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Rat anti-human IL-6 Mab MQ2-13A5 IgGI	MQ2-13A5	ELISA capture antibody for IL-6	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Biotinylated Rat anti-HuIIL-6	Mab MQ2-39C3IgG2a	ELISA detect antibody for IL-6	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Murine anti-IL-1β	Mab1 IgG 2805	ELISA capture antibody for IL-1β	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Biotinylated Goat anti-Hu lL-1β		ELISA detect antibody for IL-β	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Mu anti-human IL-8	Mab G 265-5 IgG2b	ELISA capture antibody for IL-8	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Biotinylated Mu anti-HuIL8	Mab G256-8IgG2b	ELISA detect antibody for IL-8	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK)
Rat anti-IL-10	Mab 1 IgGI JES3-9D7	ELISA capture antibody for IL-10	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK
Biotinylated Mu anti-HuIL-10	JES3-12G8	ELISA detect antibody for IL-10	R&D Systems UK Ltd., Abingdon, UK and BD-Pharmingen, Oxford, UK
Purified polyclonal antibody Goat anti-human BD-2		ELISA capture antibody forhBD-2	PeproTech EC, UK
Biotinylated Goat anti-Human BD-2		ELISA detect antibody for hBD-2	PeproTech EC, UK
Biotinylated - streptavidin horseradish peroxidase		ELISA	Sigma-Aldrich, Poole, UK
Rabbit polyclonal anti ZO-1		IHC	Invitrogen, UK
Alexa flour 488-conjugated anti Rabbit IgG		IHC	Invitrogen, UK
Anti-TLR4	Clone HTA125	Flow cytometry	eBioscences, UK
Anti-TLR2	Clone TL2.1	Flow cytometry	eBioscences, UK
Isotype antibody	Mouse IgG2a, κ	Flow cytometry	eBiosciences, UK
pSTAT-3		WB	New England Biolabs Ltd, (UK)
Total STAT-3		WB	New England Biolabs Ltd, (UK)
Anti-Rabbit HRP conjugated secondary antibody		WB	New England Biolabs Ltd, (UK)

2. Copies of the published papers associated with this thesis

- Habil, N., W. Al-Murrani, J. Beal and A.D. Foey: (2011): Probiotic bacterial strains differentially modulate macrophage cytokine production in a strain-dependent and cell subset-specific manner. Beneficial Microbes 2(4):283-293.
- Habil, N., Beal, J. and Foey, A.D. (2012): Lactobacillus casei strain Shirota selectively modulates macrophage subset cytokine production. Int. J. Probiotics & Prebiotics 7(1):1-12.

Beneficial Microbes, December 2011; 2(4): 283-293

Wageningen Academic Publishers

Probiotic bacterial strains differentially modulate macrophage cytokine production in a strain-dependent and cell subset-specific manner

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Received: 19 August 2011 / Accepted: 15 November 2011

Abstract

Gut mucosal macrophages play a pivotal role in driving mucosal immune responses, resulting in either activation of inflammatory immune responses to pathogenic challenge or tolerance to beneficial luminal contents such as food and commensal bacteria. Macrophage responses elicited are dependent on tissue environment and the resulting cell subset, where homeostatic macrophages resemble the M2 macrophage subset and inflammatory macrophages resemble M1s. Probiotics can modulate macrophage function with outcome dependent on subset present. Using a THP-1 monocyte cell line-derived model of $CD14^{high/low}$ M1 and M2 macrophages, the aim of this study was to investigate the immunomodulatory effects of a panel of heat-killed probiotic bacteria and their secreted proteins on the subset-specific inflammatory marker profile of TNFα, IL-6 and NFκB. M1 and M2 cells were generated by differentiation of monocyte stable transfectants for high and low CD14 expression with phorbol 12-myristate 13-acetate and vitamin D_3, respectively, where the resulting $CD14^{lo}$ M2 and $CD14^{hi}$ M1s mimicked homeostatic and inflammatory mucosal macrophages. Subsets were stimulated by enteropathic lipopolysaccharides in the presence or absence of heat-killed (HK) or secreted proteins (SP) from a panel of probiotic bacteria. Regulation of cytokine expression was measured by ELISA and NFκB activity by reporter assay. HK probiotics suppress $CD14^{lo}$ and augment $CD14^{hi}$ M1 and M2 production of TNFα whereas SPs augmented $CD14^{hi}$ M1 TNFα and were generally suppressive in the other subtypes. M2 macrophage IL-6 production was suppressed by both HK and SPs and differentially regulated in $CD14^{lo}$ and $CD14^{hi}$ M1s. NFκB activation failed to parallel the regulatory profiles for TNFα and IL-6 which is suggestive of probiotic bacteria exerting their regulatory effects on these cytokines in an NFκB-independent manner. In conclusion, HK and SP probiotics differentially regulate macrophage cytokines and NFκB activation in a subset-dependent manner and suggest a cautionary approach to probiotic treatment of mucosal inflammation.

Keywords: macrophage, probiotics, cytokines, inflammation

1.Introduction

The gut mucosal immune system is a major site of defence against potential pathogenic organisms that gain entry into the human body via the gastrointestinal tract (GIT). By its very nature, the GIT is full of microbes, their antigens and potential antigens released by the chemical breakdown of food. The mucosal immune system of the gut, in response to this microbe and food-antigen-rich environment has developed sophisticated mechanisms by which it can selectively taste luminal contents and respond to these signals; either by initiation of an immune inflammatory response or by a homeostatic mechanism resulting in tolerance or non-responsiveness of the immune system, whereby we tolerate useful products in the gut. This mechanism is referred to as oral tolerance, allowing the host to gain benefit from food/nutrients and beneficial commensal organisms.

These commensal microbes present in the gut afford health benefits through a variety of ways which include provision of metabolites which regulate host nutrition, epithelial

International Journal of Probiotics and Prebiotics Vol. 7, No. 1, pp. 1-12, 2012
ISSN 1555-1431 print,

LACTOBACILLUS CASEI STRAIN SHIROTA SELECTIVELY MODULATES MACROPHAGE SUBSET CYTOKINE PRODUCTION

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[Received September 26, 2011; Accepted October 29, 2011]

ABSTRACT: *Probiotics confer health benefits through many mechanisms including modulation of the gut immune system. Gut macrophages regulate immune homeostasis, mounting tolerogenic responses to food and commensal bacteria or immune inflammatory responses to pathogens. Local environment and macrophage subset determine immune response and tolerance, associated with an M2-like phenotype and inflammatory activation with an M1-like phenotype. Subset predominance will determine immunomodulatory effects of probiotic species such as Lactobacillus casei strain Shirota (LcS). The aim of this study was to investigate differential regulatory effects of LcS on M1 and M2 macrophage subsets. PMA or vitamin D_3 differentiated THP-1 human monocytic cells were used to investigate heat-killed LcS and secreted protein immunoregulation of M1 and M2 cytokine production, respectively. Additionally, regulation of $CD14^{hi}$ M2 and $CD14^{lo}$ M1 function was investigated. Cytokine expression was measured by ELISA and NFkB activity by reporter assay. Both HK-LcS and SP-LcS augmented IL-1β, suppressed IL-6 and differentially regulated TNFα and IL-8, dependent on macrophage subset. HK-LcS and LcS-SP augmented $CD14^{hi}$ M1 TNFα whereas suppressed $CD14^{hi}$ M2 IL-6 and $CD14^{lo}$ M1 NFkB. In conclusion, LcS differentially regulates macrophage cytokines and NFkB activation, is subset-dependent and suggests a cautionary approach to probiotic treatment of mucosal inflammation.*

KEY WORDS: Cytokines, Inflammation, Macrophage, Monocyte, Probiotics

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INTRODUCTION

The benefits of maintaining a healthy commensal microbiota has long since been recognised in the probiotic field, where supplementation by ingestion of lactic acid bacteria (LAB) facilitates a balanced microbial environment beneficial to the host. More recently, a health benefit of probiotics in the treatment of allergies has suggested that these probiotic supplements have a pivotal role to play in modulating the host's immune system. Probiotic investigations have indeed demonstrated LAB to modulate immune responses elicited by T, B, NK cells and monocyte/macrophages. These effects however, serve to confuse as they often display contradictory findings from study to study. This is partly attributable to immune cell phenotype being studied, its environment and the strain of probiotic being used. The Yakult bacteria, *Lactobacillus casei* strain Shirota (LcS) has been documented to be immune activator by inducing high levels of IL-6, TNFα and IL-12 (Christensen et al., 2002; Matsuguchi et al., 2003). In contrast, Borruel et al., 2003 found LcS to suppress the production of TNFα by human intestinal mucosa cells, preventing inflammatory effects. LcS has also been demonstrated to facilitate the development of Th_1 cells through the induction of IL-12 (Shida et al., 1998) and to augment NK cell cytotoxicity (Takeda et al., 2006). Thus, in the case of LcS, modulation of immune responses can be both regulatory/anti-inflammatory and activatory/pro-inflammatory. The same ambiguity exists when investigating another LAB, *L. Rhamnosus*, which has been shown to promote development of regulatory DCs (Foligne et al., 2007a), suppress epithelial cell production of IL-8 (Zhang et al., 2005), induce IL-12 production (Shida et al., 2006; Ichikawa et al., 2007) and to increase NK cell numbers (Gill et al., 2001). Again, as with LcS, *L. Rhamnosus* exhibits both immune-activatory and -regulatory function. The desired effect of the probiotic is determined by cell phenotype, recognition receptor expression and stimuli present in the local environment.

Commensal bacteria play an important role in gut mucosal development and function (Rhee et al., 2004). Inappropriate recognition of conserved commensal pathogen associated

3. Posters Presentation

The immunomodulatory effects of *Lactobacillus casei strain shirota* on monocyte, M1- and M2- macrophage cytokine production

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INTRODUCTION

- Probiotics have been widely used with the aim of improving human health.
- Macrophages exhibit different phenotypic subsets: M1 and M2.
- M1 macrophages are proinflammatory, and are crucial for the eradication of invading microorganisms, whereas M2-macrophages are anti-inflammatory and immunoregulatory.
- The dynamic balance between M1 and M2 macrophages is critical to mucosal immune fate, i.e. immune activation versus tolerance induction.
- **Aim** : This study aimed to investigate the potential immunomodulatory effects of the probiotic *Lactobacillus casei strain shirota* (LcS) on monocyte, M1- and M2-macrophage production of the proinflammatory cytokines TNF-α and IL-6.

METHODS

Macrophage cell differentiation

THP-1 monocytes were maintained in RPMI-1640/10% V/V FCS medium. Macrophages were generated by differentiation of monocytes into M1-like macrophages in the presence of 25ng/ml Phorbol Myristate Acetate (PMA) for 3 days, and M2-like macrophages in the presence of 10nM 1,25-$(OH)_2$-vitamin D_3 for 7 days (1).

Immune cell stimulation

To initiate the inflammatory response, the resulting monocytes and macrophages were stimulated with 0.1 µg /ml K12-LPS (LPS derived from enteropathic *E. coli* strain K12) for 16 h (2)

Immunoregulation by the probiotic

The probiotic LcS is thought to mediate modulation of immune response. To facilitate this effect, heat killed(HK) LcS and LcS secreted protein (SP) were added to monocyte, M1- and M2-macrophage cell cultures and incubated for 18 h at 37°C in a humidified 5% CO_2 incubator in the presence of K12-LPS (3).

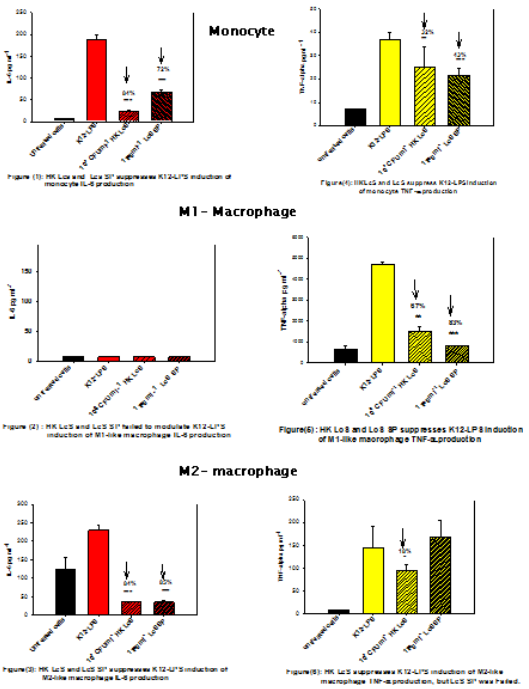
Cytokine assay

The supernatants were assessed by sandwich ELISA for pro-inflammatory cytokines TNF-α and IL-6.

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA) for comparison between groups. All results were expressed as mean ± SE and significance at $p<0.05$ (*), $p<0.01$,(**), $p<0.001$(***).

RESULTS



CONCLUSIONS

- Probiotic HK LcS and LcS SP differentially regulated K12-LPS induction of monocyte, M1- and M2-macrophage cytokine production.
  - Suppress IL-6 production by monocytes and M2-macrophages .
  - Suppress TNF-α production by monocytes and M1-like macrophages.
  - Weakly suppress M2-macrophage TNF-α production.
- The probiotic LcS exerts selective anti-inflammatory/regulatory effects on monocytes,M1 and M2 macrophages.

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Probiotics modulate epithelial cell barrier properties influenced by co-culture with macrophages

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BSI, Liverpool 2011
A-427-0004-00317

INTRODUCTION

- Macrophages exhibit different phenotypic subsets: M1 and M2.

M1 macrophages are proinflammatory, and are crucial for the eradication of invading microorganisms, whereas M2-macrophages are anti-inflammatory and immunoregulatory.

- Gut macrophage-derived cytokines are instrumental to mucosal immune responses: tolerance or inflammation.
- Probiotic bacteria are widely used in improving human health, but may or may not be of clinical benefit in the mucosal breakdown associated with inflammatory bowel disease.
- Probiotic bacteria may modulate macrophages, either beneficially by enhancing intestinal barrier function or detrimentally by perpetuating inflammatory destruction of epithelial barrier and underlying mucosal tissue.

- **Aim**: **to investigate modulation of macrophage-influenced epithelial barrier function by probiotic bacteria in the presence of inflammatory stimuli.**

METHODS

Macrophage cell differentiation

THP-1 monocytes were maintained in RPMI-1640/10% v/v FCS. Macrophages were generated by differentiation of monocytes into M1-like macrophages in the presence of 25ng/ml PMA for 3 days, and M2-like macrophages in the presence of 10nM 1,25- $(OH)_2$-vitamin D_3 for 7 days (1).

Epithelial cells differentiation

Transwell co-culture system was used to culture Caco-2 epithelial cells which were maintained in DMEM /10% v/v FCS ,for 21 days (2).

Preparation of heat killed probiotic bacteria

Probiotic *L.fermentum* and *L.casei Shirota* were cultured in MRS broth for 18h then killed by boiling at 90°C for 2 hrs.

Epithelial and macrophage co-culture model

Caco-2 epithelial cells were incubated in transwells in the presence of basolateral M1-like (pro-inflammatory) and M2-like (regulatory) macrophage subsets, probiotics were applied apically and inflammatory stimuli (5ng/ml IL-1β or 1 µg/ml K12-LPS) basolaterally (3).

Transepithelial electrical resistance (TEER)

TEER was used to assess Caco-2 cells permeability (4).

RNA extraction and quantitative RT-PCR analysis

ZO-1 gene expression was determined by extracting RNA from Caco-2 cells with GAPDH as the house keeping gene (5).

Immunohistochemistry staining of ZO-1 tight junction protein

After treatment with probiotic and stimulation with inflammatory signals, co- cultured Caco-2 cells were stained by fluorescence label antibody for ZO-1 protein tight junction.

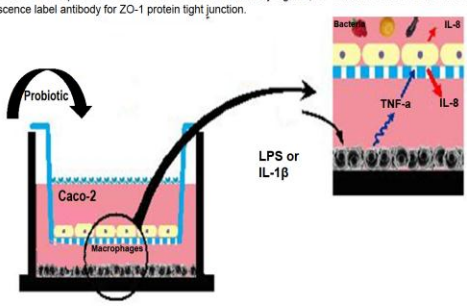

Figure (1): Caco-2 epithelial co-culture model with macrophage cells

Statistical analysis

Statistical analysis was performed using one way analysis of variance (ANOVA). All results were expressed as the mean of at least three independent experiments ± SE and significance at $p<0.05$ (*), $p<0.01$,(**), $p<0.001$(***).

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5. Zyrek et al.,2007.Cellular Microbiology,9,804-816.

RESULTS

Probiotics failed to repair the destructive effects induced by M1-like macrophages

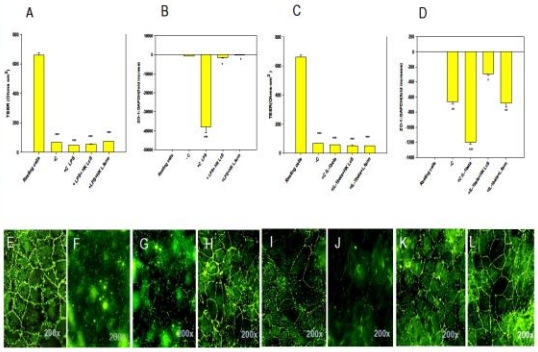

Figure (2):Probiotic effects on modulation of M1-like macrophages effects in Caco-2 epithelial barrier function in co-culture model.(A) TEER of epithelial cells pre-treated with probiotic bacteria followed by stimulation with LPS. (B) ZO-1 expression by epithelial cells pre-treated with probiotic bacteria followed by stimulation with LPS.(C) TEER of epithelial cells pre-treated with probiotic followed by stimulation with IL-1β.(D) ZO-1 expression by epithelial cells pre-treated with probiotic bacteria followed by stimulation with IL-1β.(E) Immunohistochemical staining of ZO-1 protein of untreated cells.(F) cells co-cultured with M1-like macrophages.(G) Cells stimulated with LPS in co-culture system.(H) cells apically treated with *L.fermentum* followed by stimulation with LPS.(I) Cells apically treated with *LcS* followed by stimulation with LPS.(J) cells stimulated with IL-1β in co-culture model.(K) cells apically treated with *L.fermentum* followed by stimulation with IL-1β.(L) cells apically treated with *LcS* followed by stimulation with IL-1β in co-culture model.

Probiotics differentially modulate the destructive effects induced by LPS and IL1-β after co-culture with M2- like macrophages

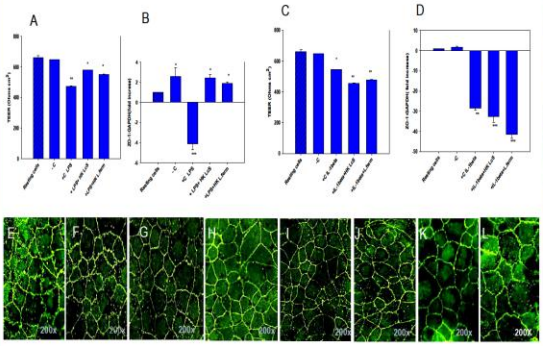

Figure (3): Probiotic effects on modulation of M2-like macrophages effects in Caco-2 epithelial barrier function in co-culture model.(A) TEER of epithelial cells pre-treated with probiotic bacteria followed by stimulation with LPS. (B) ZO-1 expression by epithelial cells pre-treated with probiotic bacteria followed by stimulation with LPS.(C) TEER of epithelial cells pre-treated with probiotic followed by stimulation with IL-1β.(D) ZO-1 expression by epithelial cells pre-treated with probiotic bacteria followed by stimulation with IL-1β.(E) Immunohistochemical staining of ZO-1 protein of untreated cells.(F) cells co-cultured with M2-like macrophages.(G) Cells stimulated with LPS in co-culture system.(H) cells apically treated with *L.fermentum* followed by stimulation with LPS.(I) Cells apically treated with *LcS* followed by stimulation with LPS.(J) cells stimulated with IL-1β in co-culture model.(K) cells apically treated with *L.fermentum* followed by stimulation with IL-1β.(L) cells apically treated with *LcS* followed by stimulation with IL-1β in co-culture model.

CONCLUSIONS

- TEER and ZO-1 tight junction protein are main barrier parameters influenced by co-culture system, they were dropped down after co-culture with proinflammatory M1-like macrophage cells, in compare with anti-inflammatory M2-like macrophage cells.
- Probiotic modulation of mucosal barrier properties is determined by strain, inflammatory environment and mucosal macrophage effector phenotype.

4. Membership

- Society for Applied Microbiology (SFAM)
- British society for Immunology (BSI)

5. Postgraduate Skills Training

- English language support for international students (ELSIS) feature of academic style 12-26th October 2009.
- Postgraduate Research Skills and method coursework (BioL5124), 2nd October 2009- 1st December 2009.
- General Teaching Associates Course (GTA) 1st October 2009- 1st December 2009.
- Env (demonstrating) 1st October – 1st December 2009.
- Greating basic web pages with Microsoft office share point designer 2007 (20th October 2009).
- Excel 2007/ Lookup tables and pivotal table reports (11th November 2009).
- Working with literature: electronic resources and advanced searching (16th November 2009).
- Introduction to endnote- for the science, technology, medical, and health disciplines (1st December 2009).
- Creating graphics using paint shop pro photo X2 (4th November 2009).
- Getting started in office 2007 (3rd October 2009).
- Research ethics (20th October 2009).
- Excel 2007- Database and filtering (21st October 2009).
- Methodology in science (23rd October 2009).
- PowerPoint 2007: Enhancing your presentation (27th October 2009).
- Safety and risk assessment field work (27th October 2009).
- Introduction to pebble pad (28th October 2009).
- Excel 2007 Introduction to essential features (29th October 2009).
- Safety and risk assessment laboratory (30th October 2009).
- Preparing for your viva (2nd November 2009).
- Scientific writing (3rd November 2009).
- Publication strategies (3rd November 2009).
- Critical review of writing (5th November 2009).
- Latex introduction (12th November 2009).
- Biostatistics 1 (24th November 2009).
- Biostatistics 2 (27th November 2009).
- The transfer process (27th November 2009).
- Careers in Biology (4th December 2009).
- Biostatistics 3 (8th December 2009).
- A stress management workshop for research student (23rd October 2009).
- Oral presentation (15th December 2009).
- Public communication of science (18th December 2009).
- Microsoft PowerPoint 2007 Enhancing your Presentation (23rd march 2010).
- A stress management workshop for research students (19th November 2010).
- Introduction to qualitative research (15th January 2010).
- Introduction to Spss part 1 (22nd January 2010).
- Essential tools for the viva: debating skills (26th January 2010).
- Introduction to Qualitative Research Methods 15th January 2010.

- Developing Professional Writing Skills (3rd February 2010).
- Power point-creating a presentation (10th February 2010).
- Endnote under clinic (18th February 2010).
- Going Global (19th February 2010).
- Developing Professional Writing Skills (24th march 2010).
- SPSS part 2 (27th April 2010)
- Impact factor (30th April 2010
- Effective reading (10th November 2010).
- Introduction to Endnot X3 (30th November 2010).
- Microsoft excel 2007 conditional formatting & charts (10th June 2010).
- Creating Graphics for paint shop pro (3rd December 2010).
- Words: creating form (10th December 2010).
- Presenting to an Audience (part 1) (9th March 2011).
- Professional writing skill (6th April 2011).
- Microsoft excel conditional formatting and charts (2nd February 2011).
- Plymouth consortium Student Associate Scheme (SAS), 5th June -17th 2011
- Ettan 2-D DIGE Course arranged by GE healthcare /Davy 211, 212 6th -8th March 2012
- Practical Technique in molecular biology workshop 16th -19th July 2012.
- Multiplexing & Luminex Technology /One day workshop, 14th September 2012
- Bioinformatics Work Shop- 16S rRNA gene Analysis 7th November 2012.