THE ROLES OF LACTIC ACID BACTERIA IN HOST INFLAMMATORY RESPONSES

WANG SHUGUI

DEPARTMENT OF MICROBIOLOGY

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WANG SHUGUI

(B.Sc., Sun Yat-Sen University)

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- Alexandra Are, Linda Aronsson, **Shugui Wang**, Gediminas Greicius, Yuan Kun Lee, Jan- Åke Gustafsson, Sven Pettersson, and Velmurugensan Arulampalam. *Enterococcus faecalis* from newborn babies enhance transcriptional activity of endogenous PPAR-gamma through phosphorylation. In Press in *Proceedings of the National Academy of Sciences*, 2008.
- **Shugui Wang**, Annelie Lundin, Linda Aronsson, Lydia Ng Hui Mei, Velmurugesan Arulampalam, Sebastian Pott, Sven Pettersson, Martin Hibberd, Yuan Kun Lee. Human intestinal *Enterococcus faecalis* modulates inflammation by attenuating JNK and p38 signaling pathways. (In preparation)
- Wai Ling Chow, **Shugui Wang**, Yuan Kun Lee. Modulation of cytokine gene expression in the intestinal tract of mouse by fucose. (Submitted)

Conference publications

- **Shugui Wang**, Yuan Kun Lee, 2005, Probiotics activate distinct signaling pathway involve in host-microbe interaction in intestinal epithelial cell. 7th World Congress on Inflammation, Melbourne, Australia. Aug 20-24, 2005
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ABBREVIATIONS

%	-	Percentage
°C	-	Degree Celsius
µg	-	Microgram
µl	-	Microliter
µm	-	Micrometer
µM	-	Micromolar
50x	-	50 times
APC	-	Antigen-presenting cells
bp	-	Base pair
BSA	-	Bovine serum albumin
cDNA	-	Complementary deoxyribonucleic acid
CFU	-	Colony forming unit
cm	-	Centimeter
CO_2	-	Carbon dioxide
ConA	-	Concanavalin A
CTL	-	Cytotoxic T lymphocytes
DC	-	Dendritic cells
DEPC	-	Diethylpyrocarbonate
DNA	-	Deoxyribonucleic acid
dNTP	-	Deoxynucleotide triphosphate
DTT	-	Dithiothreitol

dUTP	-	Deoxyuridine triphosphate
EC	-	*Enterococcus*
EDTA	-	Ethylenedia mine tetraaacetic acid
ELISA	-	Enzyme-linked immunosorbent assay
ETBR	-	Ethidium bromide
FACS	-	Fluorescence-activated cell sorting
FBS	-	Fetal Bovine Serum
g	-	Gram
GALT	-	Gut-associated lymphoid tissues
GI	-	Gastrointestinal
h	-	Hour(s)
HRP	-	Horseradish peroxidase
IBD	-	Inflammatory bowel disease
ICAM	-	Intercellular adhesion molecule
IECs	-	Intestinal Epithelial Cells
IgG	-	Immunoglobulin
IL	-	Iterleukin
INF	-	Interferon
kDa	-	Kilodalton
LAB	-	Lactic acid bacteria
LB	-	Luria-Bertani
LFA-1	-	Lymphocyte function-associated antigen 1
LPS	-	Lipolysaccharide
LRR	-	Leucine-rich repeat

M	-	Molar
MAPK	-	Mitogen-activated protein kinases
mg	-	Milligram
MHC	-	Major histocompatibility complex
min	-	Minute(s)
ml	-	Milliliter
mm	-	Millimeter
mM	-	Millimolar
MOI	-	Multiplicity of Infection
MOPS	-	3-[N-Morpholino] propanesulfonic acid
MRS	-	de Man, Rogosa and Sharpe
MRSA	-	Methicillin-resistant *Staphylococcus aureus*
ng	-	Nanogram
nm	-	Nanometer
PAMPs	-	Pathogen-associated molecular patterns
PBMC	-	Peripheral blood mononuclear cells
PBS	-	Phosphate buffer saline
PCR	-	Polymerase chain reaction
PGN	-	Peptidoglycan
PI	-	Propidium iodide
PMSF	-	Phenylmethylsulfonylfluoride
RANL	-	Receptor Activator of NF-κB Ligand
rDNA	-	ribosomal DNA
RNA	-	Ribonucleic acid

rpm	-	Rotation per minute
s/sec	-	Second(s)
SB	-	SB203580 iodo
SDS-PAGE	-	Sodium-Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SP	-	SP600125
succinyl ConA	-	Succinyl Concanavalin A
TBST	-	Tris buffer saline-Tween
TGF	-	Tumor growth factor
Th	-	T helper cell
TLRs	-	Toll like receptors
TNF	-	Tumor necrosis factor
Tollip	-	Toll inhibitory protein
TRAF6	-	TNF receptor associated factor 6
TSA	-	Tryptone Soy Agar
U/ml	-	Unit per millimeter
UC	-	Ulcerative colitis
UV	-	Ultra violet
v/v	-	Volume by volume
w/v	-	Weight by volume

SUMMARY

Intestinal epithelial cells (IECs) regulate the mucosal immune responses upon a variety of stimuli, including lactic acid bacteria (LAB). LAB are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. Lactobacilli, bifidobacteria, enterococci and streptococci are important members of this group. In infants, bifidobacteria, enterococci account for more than 90% of the total intestinal bacteria. Lactobacilli and streptococci are regularly present and increased in older infants when they are switched to a diet of cow's milk or solid food. However, the nature of the associations between human and their intestinal commensal bacteria is still unclear.

Usually, the host and bacteria are thought to interact in a dynamic manner. Both of them derive benefits from each other. The commensal bacteria derive from the host a supply of nutrients and a stable environment, while the host obtains from the normal flora certain nutritional benefits, stimulation of the innate and adaptive immune system, and colonization strategies that exclude potential pathogens at the site. Recently, clinical studies in infants showed that babies who developed allergy were less often colonized with enterococci during the first month of life and with bifidobacteria during the first year of life (Bengt Bjorksten, 2001). Thus these LAB may play a critical role in the regulation of the immune system even in the early stage of human life. With the growth of the human, LAB play an even more critical role in the maintenance of the intestinal immune system. There is accumulating evidence that

the imbalance of the intestinal bacteria might cause inflammatory disorders such as allergy, inflammatory bowel disease (IBD), and even colon cancer.

Studies showed that inflammation can be activated through many pathways. Toll like receptors (TLRs), which is important in the innate immune system, could respond to many pathogens and lead to the initiation of inflammation by secreting proinflammatory cytokines such as IL-8 and TNF-α. These cytokines might be expressed continuously in chronic inflammatory tissues. Also, there are some other signaling pathways that lead to proinflammatory cytokines production which thus activate inflammatory responses. Mitogen-activated protein kinase (MAPK) and NF-κB signaling pathways can be activated by responding to proinflammatory cytokines (e.g. IL-1β and TNF-α) and pathogens to further activate inflammation. Therefore, suppression of proinflammatory cytokines and pathways such as MAPK and NF-κB can inhibit inflammatory responses.

Since inflammation causes serious diseases in the intestine, many approaches have been used to suppress inflammatory responses in the intestine. Clinical studies have suggested that LAB or so-called probiotics may be an effective therapy in IBD. They could boost immune system in the early stage of life and also, they could regulate many inflammatory responses in the intestine through inner cellular signaling pathways. However, the molecular mechanisms of their regulation are still undefined. Moreover, the possible molecules on LAB which might be involved in the immunosuppressive responses are still unknown. Therefore, it would be very interesting to explore the underlying mechanisms and to demonstrate the possible molecules which participate in the immunosuppressive responses.

E. faecalis is the second most common bacterium in the human intestine. Its function in the human intestine remains unclear. It is nevertheless used as probiotics

in food industry but is also an opportunistic pathogen in immunodeficient hosts. Therefore, it is necessary to verify how *E. faecalis* interacts with the human intestine.

In the present study, 56 strains of human intestinal LAB isolated from 3 days and 1 month old infants together with LAB isolated from fermented milk and obtained from our culture collections, their anti-inflammatory effects were investigated in human colon cell lines (Caco-2, HT-29 and HCT116). It was demonstrated that *E. faecalis* was the main immune modulator among the intestinal LAB. However, the effect was limited to only a few strains of *E. faecalis*. Carbohydrates on bacterial cell surface were involved in both its adhesion to intestinal cells and regulation of inflammatory responses in the host. Also, *E. faecalis* from new born infants, which showed a strong ability to suppress inflammatory responses in human IECs, attenuates proinflammatory cytokine secretion especially IL-8 via distinct pathways. They suppressed JNK and p38 and further downregulated c-JUN protein expression to inhibit inflammatory responses. Furthermore, the inhibition of inflammatory responses by *E. faecalis* bypassed NF-κB signaling pathways. Cytokine receptors but not TLRs may play the key role in the modulation of immune responses in IECs. Some other signaling pathways like PIN1 and E2F1 might also be involved in the immune regulation by *E. faecalis* in the host. These findings highlight new cellular targets and approaches for therapeutic treatment of inflammatory diseases in human.

Chapter 1

INTRODUCTION

1.1 INTRODUCTION

Human gastrointestinal (GI) tract represents a complex ecosystem in which the intestinal microbes and the host maintain a delicate balance. A dynamic and huge number of microbes, which are mainly comprised of facultative anaerobes and obligate anaerobes, can be found in human GI tract. Prior to birth, the intestine is sterile. However, bacteria begin to colonize in the intestine during the birth process. Within 1 to 3 days of birth, large numbers of enterobacteria and streptococci could be found in infant's GI tract. Shortly thereafter, depending on the food ingested, the GI tract becomes populated with a variety of genera of bacteria. Studies showed that colons of breast-fed infants become dominated by bifidobacteria species (Gueimonde *et al*., 2007) while enterococci predominate in bottle-fed infants. During weanling, bifidobacteria and enterococci decrease as colon begins to adopt a more adult profile mostly based on diet (Topping *et al*., 2001; Shahani *et al*., 1980). The dominant microorganisms in GI are illustrated in Figure 1.1.

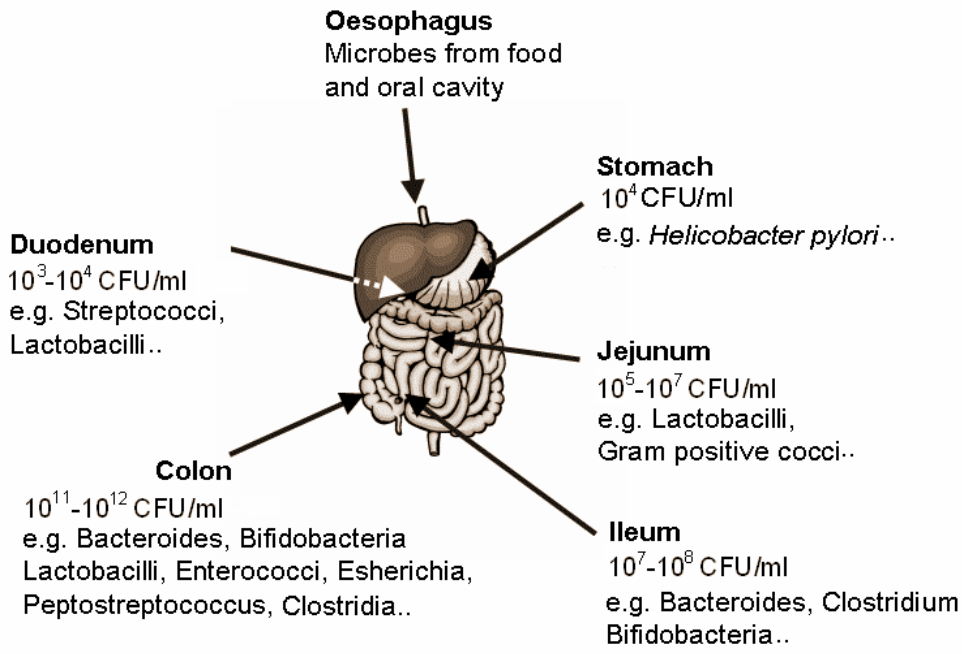


Figure 1.1 Micro-organisms in GI tract.

Colon, which accounts for the majority of the bacteria in GI tract, contains a range between 10^{11} to 10^{12} colony-forming units (CFU) per milliliter (ml) of bacteria (Figure 1.1). The adult colon's contents are about 35-50% (dry weight) bacteria. In fact, the population of microbes in the colon is estimated to be 10 times that of the total number of human cells (Topping *et al*., 2001; Backhed *et al*., 2005; Cummings *et al*., 1991). Genera that are prominently represented include *Bacteroides*, *Bifidobacterium*, *Lactobacillus*, *Enterococcus*, *Escherichia, Peptococcus*, *Peptostreptococcus*, *Clostridium*, *Fusobacterium*, *Eubacterium*, and *Veillonella* (Xu *et al*., 2003; Topping *et al*., 2001). More than 800 species (>7,000 strains) of bacteria are estimated to inhabit the colon. These include species that are commensal which cause no harm in the intestine, and those that are potentially pathogenic (Backhed *et al*., 2005). Among the microbes, most colonic microbes provide beneficial effects and are necessary for proper colon function. Some of these effects include absorption of water, electrolytes and other nutrients, synthesis of certain vitamins (e.g., vitamins K, B and biotin), and digestion of dietary fibers (Xu *et al*., 2003; Hill *et al*., 1997; Bengmark *et al*., 2000).

Other functions of the intestinal microbes are their importance for maturation of the immune system (Kelly *et al*., 2007), the development of normal intestinal morphology and maintaining immunological balanced responses. The microbes reinforce the barrier function of the intestinal mucosa, helping in the prevention of the attachment of pathogenic microorganisms and the entry of allergens. Alteration of the microbial flora of the intestine due to the antibiotic use, disease and aging, can negatively affect the host. In addition, an imbalance in microbial populations often favors the proliferation of pathogenic species, leading to infectious diseases, chronic inflammation diseases such as inflammatory bowel diseases (IBD) and even colon

cancer. Therefore, it is very important to maintain the balance of the microbial population especially the population of beneficial intestinal microbes.

When one considers beneficial intestinal microbes, lactobacilli and bifidobacteria are the two most common genera that come to mind. However, specific species and strains of other genera, even those generally considered to be potentially pathogenic, have been shown to provide healthful benefits. Some examples include *Enterococcus*, *Clostridium* and *Escherichia* (Kanauchi *et al*., 2005; Bondarenko *et al*., 2004). Among the "good" bacteria, lactobacilli, bifidobacteria and enterococci are Gram positive. They belong to lactic acid bacteria (LAB) which is an important group in maintaining human health.

The role of intestinal microflora, such as several strains of lactic acid bacteria (LAB) in priming the immune system during ontogeny to limit allergy and chronic inflammatory responses has been brought to attention in recent years (Cross *et al*., 2001; Marteau, 2002; Iwabuchi *et al*., 2007). Clinical and experimental studies have put forward that intestinal microflora is essential for the maintenance of intestinal homeostasis by altering microbial balance or by specifically interacting with intestine immune system (Kelly *et al*., 2004), and their influence may extend beyond the gut, modifying systemic immunity. However, the mechanisms of how they interact with the host and the role that they play in the intestine immune system especially in the context of chronic inflammation are still unclear.

LAB are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation. The production of lactic acid results a very low pH which is low enough to inhibit the growth of most other microorganisms including the most common human pathogens. Since these organisms lack many biosynthetic capabilities and generally have complex nutritional requirements, LABs are usually abundant only

in communities where these requirements can be provided such as intestines (e.g., *Enterococcus faecalis*), plant leaves (*Lactobacillus*) as well as decaying plant or animal matter. Studies also showed that metabolic and functional properties of probiotic LAB in the human intestinal ecosystem are related to many beneficial health effects (Haller *et al*., 2001). Lactic acid bacteria contribute to both the processes of decomposition and reconstruction in the intestinal tract. They supply digestive enzymes that help break down food, while simultaneously micro-activate vital nutrients. The genera *Lactobacillus, Bifidobacteria, Enterococus* and *Streptococcus* are important members of this group. These microbes are broadly used in the production of fermented food such as yogurt, cheeses, and sausage.

Most of *Lactobacillus* is rod-shaped, Gram positive, non-spore-forming, belonging to the family of lactobacillaceae. They can ferment glucose into lactose with the production of lactic acid. The most common application of *Lactobacillus* is industrial, specifically for dairy production like sour milk and yogurt. *Lactobacillus* is generally harmless to humans and rarely inciting harmful infections or diseases.

The other important group of LAB is *Enterococcus faecalis*. Prior to 1984, *E. faecalis* was known as *Streptococcus faecalis* (Schleifer, 1984). They were classified as Group D streptococci due to the fact that they have the Lancefield Group D antigen (glycerol teichoic acid antigen) in their cell walls. *E. faecalis* are nonmotile, Gram-positive, facultative anaerobic spherical bacterium of Streptococcaceae family. They are usually catalase negative, although sometimes tests can come out slightly positive. Most strains are non-hemolytic.

E. faecalis normally inhabit in the intestines of animals and humans. However, they also can be found in soil, vegetation, and surface water, probably due to contamination by animal excrement. In human, *E. faecalis* usually colonize the large

intestine (~10^7 organisms per gram of feces) and urinary tract. They can be observed singly, in pairs, or in short chains. *E. faecalis* have a fermentative metabolism in which they convert carbohydrates to lactic acid. Therefore, they belong to LAB. In addition, *E. faecalis* play a key role in the fermentation of nonabsorbed sugars in the gastrointestinal tract. They have a large number of sugar uptake systems, and considerably more than any other sequenced bacteria. Moreover, the cation homeostasis mechanisms of *E. faecalis* contribute to their resistance to pH, salt, metal, and desiccation. *E. faecalis* can grow at a range of temperatures from 10°C-45°C, and also capable of growing in hypotonic, hypertonic, acidic, and alkaline environments.

The genus *Enterococcus* especially *E. faecalis* is a controversial group of LAB. This controversial nature of *E. faecalis* has prompted an enormous pro/contra groups in scientific papers and reviews in recent years. In the following text, I will examine the properties of *E. faecalis* from these two sides. Currently, *Enterocuccus* and several other species of LAB, which are usually derived from human intestinal bacteria, are used for starter cultures in dairy products and are also marketed as medical probiotic preparations to enhance the host immune responses (Domann *et al*., 2007; Shimada *et al*., 2007). *E. faecalis*, one of the representative strains, has been utilized as live bacteria products with balancing activity of intestines since the 1950s. Studies on the microbes of many traditional cheeses have indicated that enterococci play an important role in the ripening of these cheeses, probably through proteolysis, lipolysis, and citrate breakdown, hence contributing to their typical taste and flavour (Foulquie´ Moreno *et al*., 2006). Enterococci are also present in other fermented foods, such as sausages and olives. They were reported to be dominant in freshly fermentation products however much less in spoil products (Johanna *et al*., 2004) although their roles in these products have not been fully elucidated. At the same time, *E. faecalis*

have been associated with a number of human infections. A number of the strains are natural antibiotic resistance and they are the leading cause of hospital-acquired secondary infections (Domanna *et al*., 2007). Several virulence factors have been described and the number of vancomycin-resistant *E. faecalis* is increasing.

In most cases, *E. faecalis* cause no infection. They were classically considered more as commensal bacteria of the gastrointestinal tract of humans and animals rather than a specialized human pathogen. In some people though, *E. faecalis* can cause clinical problems. These include opportunistic urinary tract infections and wound infections.

Interestingly, a new strain called "*Enterococcus faecalis* TH10" isolated from fermented food is proving to be highly effective against even the most deadly antibiotic-resistant bacterial strains, including methicillin-resistant *Staphylococcus aureus* (MRSA). *Enterococcus faecium* SF68 is another probiotic strain that has been used in the management of diarrheal illnesses. Bengt *et al* demonstrated that *E. faecalis* could be found in three-day old babies, and most importantly, babies who developed allergy were less often colonized with *Enterococcus* during the first month of life compared with healthy infants (Bengt *et al*., 2001). Therefore, *E. faecalis* might affect intestinal immune system development in the early stage of life. However, the possible underlying mechanism of *E. faecalis* in the modulation of intestinal immune responses is not clear.

Shimada *et al* showed that *E. faecalis* FK-23 could suppress inflammatory responses in IBD and other inflammatory diseases in the intestine (Shimada *et al*., 2004; 2007). They claimed that *E. faecalis* might have an effect on the immune balance between Th1 and Th2 immunity (Shimada *et al*., 2004; Kropec *et al*., 2005). Also, some clinical studies demonstrated that *E. faecalis* could compete with anti-

antiboitic strains in some serious condition of inflammatory diseases to eradicate the pathogenic strains. Although many clinical studies have been done on its effects on inflammatory diseases, none of them has shown how *E. faecalis* act on the intestine and how they communicate with the intestinal epithelial cells.

In the human intestine, epithelial cells provide the first barrier and contact with all kinds of bacteria. They express numerous receptors, adhesion molecules and proinflammatory mediators and act as the sentinel of the mucosal immune system. Therefore, they have a pivotal role in the bacteria-host communication and the functional changes of the epithelial cells may contribute to many pathological features of intestinal inflammation (Jijon, 2002). Disruption of the immune regulatory functions of the epithelial cells by an imbalanced microflora could lead to exacerbated effector responses and chronic inflammatory diseases. IBD is a chronic relapsing disorder involving a dysregulated host–microbe interaction. Evidence supporting the hypothesis that intestinal bacteria play a role in the pathogenesis of IBD includes the observation that inflammation and lesions generally occur in intestinal regions with the highest bacterial concentrations (i.e. the ileum and colon) (Thompson-Chagoyan *et al*., 2005). It has been shown that IBD patients possess an increased risk for the development of colorectal cancer (Mark *et al*., 2007). Many solutions for the therapeutic treatment of IBD have been studied. Recently, the focus has been placed on LAB or so called probiotics, which aims to restore balance to the intestinal microflora and to reduce intestinal inflammation. *In vitro/vivo* studies and some clinical trials have demonstrated the potential therapeutic benefits of these microbes that can suppress inflammatory responses in the intestine (Bai and Ouyang, 2006; Bengmark, 2007). LAB exert their beneficial effects through a variety of mechanisms that are unique to each strain (Fedorak *et al*., 2004; Sartor *et al*., 2004; De *et al*.,

2006). They competitively exclude undesirable bacteria through binding to mucosal border and thus prevent pathogenic bacteria from crossing the cell wall, because they align themselves closer to the microvillus than many pathogenic bacteria (Elmer, 2001; Zhong *et al*., 2004). They also prevent adhesion and translocation of pathogenic bacteria, which is probably important in preventing bacterial antigens from reaching the lamina propria and mucosal immune system. Enterocin, which is a product of *E. faecalis* showed antimicrobial properties. Research using *E. faecalis* and the combination of other four antimicrobial chemicals demonstrated that the inhibitory effect of *E. faecalis* contributed significantly to the overall anti-*S. aureus* effect (Kerry *et al*., 2006). In addition, LAB stimulate immunoglobulin release into the lumen. They change the mucosal immune system with a resultant less proinflammatory response and a greater anti-inflammatory response. Therefore, these LAB may be efficient means to treat inflammatory diseases in the human intestine.

LAB have not only been demonstrated to manipulate the intestinal microbes and reduce the inflammatory responses but also increase the natural resistance of the host to infections since a balance intestinal microflora could resist the invasion of pathogens. When we are considering LAB for the treatment of diseases, the safety should be ensured. Lactobacilli and bifidobacteria are two commonly used LAB (Biavati *et al*., 2000). They have a long history of safe use as microbial adjunct nutrition (Salminen *et al*., 1998) and are rarely implicated in human gastrointestinal and extraintestinal infections in the presence of predisposing factors (Charteris *et al*., 1997). However, the safety for consumption should still be considered. Many studies have been conducted to test their antibiotics resistance property. It is reported that most lactobacilli and bifidobacteria strains are susceptible to ampicillin, bacitracin, clindamycin, dicloxacillin, erytromycin, novobiocin, penicillin G and rifampicin,

however, resistant to aztreonam, cycloserin, kanamycin, nalidixic acid, polymyxin B and spectinomycin (D'Aimmo *et al*., 2007). Among all LAB species, *Enterococcus*, especially *E. faecalis*, is a controversial group. Therefore, the safety of LAB, especially *E. faecalis*, for their applications in food and pharmaceutical industries must be ensured.

To modulate human immunity, LAB must "talk" to the host that is endowed with recognition receptors or otherwise is sensitive to bacteria-derived products (e.g. metabolites and cell wall components). Much attention has been paid to the adhesive properties of these LAB (Vesterlund *et al*., 2006). Their ability to adhere to host cells or mucus is commonly considered as a requirement for LAB to conduct their anti-inflammation function. Some *in vitro* studies have shown that *E. faecalis* adhesive ability is strain dependent (Carlors *et al*., 1991). Interestingly, Morita *et al* showed that there was no correlation between adhesive capacity on human epithelium and cytokine induction (Morita *et al*., 2000). However, the importance of LAB-epithelial cells adhesion and its role in the inflammatory responses are not fully understood. Studies showed that carbohydrate residues present on the bacterial cell surface might mediate the adherence (Carlors *et al*., 1991). The determination of the structure of carbohydrates on bacterial surface is important for the understanding of their biological function such as anti-inflammatory responses in the host. Lectins are useful in the investigation of protein-carbohydrate interactions (Naeem *et al*., 2007). Moreover, it has been shown that carbohydrate and protein structures present on the bacterial surface are involved in several immune and biological responses in health and inflammatory conditions (Carlors *et al*., 1991; Mizoguchi and Mizoguchi, 2007).

Normal GI microflora populations are crucial for the maturation of acquired immunity. Beneficial intestinal bacteria can stimulate the synthesis and secretion of

IgA, the antibody that coats and protects mucosal surfaces against microbial infection (Forchielli and Walker, 2005). The GI microflora also assists in the development of normal immunity by its effects on antigen-presenting cells (APCs). APCs are macrophages, B lymphocytes and dendritic cells that process antigens and then present them to T helper (Th) cells for recognition and appropriate response. Dendritic cells are the APCs of the colon that reside in the lamina propria just beneath the mucosal surface and extend their dendrites through the epithelial cell layer into the lumen of the gut. When a dendrite comes in contact with an antigen, it releases the antigen by phogocytosis or endocytosis, and then presents the epitope to a Th cell that also resides within the lamina propria. The Th cell then determines whether or not the antigen requires further attention, and appropriate action can be initiated.

A study demonstrated the specificity of colonic dendrites regarding their reaction to various species of bacteria found in the gut. Undifferentiated human monocytes produced higher levels of interleukin (IL)-12 (p70) and tumor necrosis factor (TNF) in response to *Lactobacillus plantarum* and *Bifidobacterium adolescentis*, two healthful colonic bacteria, than to potentially pathogenic *Escherichia coli* and *Veillonella parvula* (John, 2006). On the other hand, human monocytes that had differentiated into dendritic cells in the gut secreted large amounts of IL-12 (p70), TNF, IL-6 and IL-10 in response to *E. coli* and *V. parvula*, but were practically non-responsive to *L. plantarum* and *B. adolescentis*. This evidence suggests that dendritic cells in the gut recognize and respond to potential pathogenic bacteria, but do not respond to healthful bacteria (Karlsson, 2004).

The means by which the intestinal microflora assists immune development are not fully understood, although it is now known that polysaccharides play a critical part in this process. Researchers using a human commensal bacterium, *Bacteroides*

fragilis, in germ-free mice showed that this bacterium was sufficient to correct T cell deficiencies normally seen in these animals. But more than that, investigators discovered that polysaccharide on the commensal bacterial cell wall was the critical factor in Th1/Th2 imbalances (Mazmanian, 2005). Thus, at least in this case, a bacterial carbohydrate is the requisite molecule for trigging the development of the immune system.

A balanced Th cell response is important in the modulation of inflammatory reactions. The Th1 subset of lymphocytes is responsible for many cell-mediated responses, and is associated with promotion of inflammation and tissue injury. The Th2 subset promotes the humoral response, and supports allergic reactions. Therefore, an imbalance favoring a Th1 response can contribute to inflammatory bowel diseases (Neuman, 2007), and an imbalance that favors the Th2 response can promote atopic diseases (Kuby, 1997). TH17 and T reg cells are also important in the regulation of host responses upon commensal and pathogenic bacteria. Appropriate colonization of GI microflora helps to maintain proper Th cell responses, thus reducing the risk of these diseases.

Inflammatory diseases have a close relationship with intestinal bacteria. Patients with IBD demonstrate immunological reactivity to their own commensal bacteria (Duchmann *et al*., 1995; Cohavy *et al*., 2000) and fecal diversion is effective in modifying intestinal inflammation (Rutgeerts *et al*., 1991; Janowitz *et al*., 1998; Sartor, 1998). Manipulation of the bacterial flora either through the use of antibiotics or probiotics is effective in ameliorating IBD (Sandborn *et al*., 2000; Sartor, 2000; Gionchetti *et al*., 2000; Rembacken *et al*., 1999; Prantera *et al*., 1996). Ulcerative colitis (UC) and Crohn's disease are one form of IBD. The most serious IBD is colon cancer. It has long been noted that cancer arises from regions of chronic inflammation

(Osawa *et al*., 2006). In cancer tissues, inflammatory cells and cytokines of the immune system are more likely to contribute to their growth and progression.

Cytokines are small secreted polypeptides or glycoproteins (≤ 30KDa) which mediate and regulate immunity, inflammation, and hematopoiesis. They generally act over short distances and short time spans and at very low concentration. Cytokines produce their actions by binding to specific high affinity cell surface receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter gene expression in the target cells. Responses to cytokines include increasing or decreasing expression of membrane proteins including cytokine receptors, proliferation, and secretion of effector molecules.

It is common for different cell types to secrete the same cytokines or for a single cytokine to act on several different cell types. Cytokines are redundant in their activity, meaning that different cytokines can do similar functions. Moreover, cytokines are often produced in a cascade, as one cytokine stimulates its target cells to make additional cytokines. Cytokines can also act synergistically or antagonistically. Several interested cytokines are illustrated in the following Table 1.1.

Table 1.1 Selected immune cytokines and their activities

Cytokine	Producing Cell	Target Cell	Function
IL-1β	monocytes macrophages B cells DC	Th cells	co-stimulation
		B cells	maturation and proliferation
		NK cells	activation
		various	inflammation, acute phase response, fever
IL-2	Th1 cells	activated T and B cells, NK cells	growth, proliferation, activation
IL-4	Th2 cells	activated B cells	proliferation and differentiation IgG_1 and IgE synthesis
		macrophages	MHC Class II
		T cells	proliferation
IL-5	Th2 cells	activated B cells	proliferation and differentiation IgA synthesis
IL-6	monocytes macrophages Th2 cells stromal cells	activated B cells	differentiation into plasma cells
		plasma cells	antibody secretion
		stem cells	differentiation
		various	acute phase response
IL-8	Macrophages, epithelial cells, endothelial cells	neutrophils	Chemotaxis; Inflammation
IL-10	Th2 cells	macrophages	*cytokine production*
		B cells	activation
IL-12	macrophages B cells	activated T cells	differentiation into CTL (with IL-2)
		NK cells	activation
IL-17	T cells	T cells	Activation
		various	inflammation
IL-18	keratinocytes, small intestine epithelial cells, adrenal cells, macrophages, pancrease, skeletal muscle, liver, lung and PBMC	activated T and B cells, NK cells	IFN-γ production

IFN-γ	Th1 cells, T cells, NK cells	various	*Viral replication*
		macrophages	MHC expression
		activated B cells	Ig class switch to IgG_{2a}
		Th2 cells	*proliferation*
		macrophages	pathogen elimination
TGF-β	T cells, monocytes	monocytes, macrophages	chemotaxis
		activated macrophages	IL-1 synthesis
		activated B cells	IgA synthesis
		various	*proliferation*
TNF-α	macrophages, mast cells, NK cells	macrophages	cytokine expression
		tumor cells	cell death
		tumor cells	cell death
ICAM1	Vascular endothelium, epithelial cells, macrophages and lymphocytes.	tumor cells	Proliferation
		Neurtophil	Inflammation
		Lymphocyte	Ligand for LFA-1

CTL: cytotoxic T lymphocytes; DC: dendritic cells; IL: interleukin; IFN: Interferon; TGF: Tumor Growth Factor; TNF: Tumor Necrosis Factor; MHC: Major histocompatibility complex; Th: T helper; PBMC: peripheral blood mononuclear cells; ICAM1: intercellular adhesion molecule 1; LFA-1: lymphocyte function-associated antigen 1. ** Italicized activities are inhibited.
Adapted from "Immunology Tutorials" online:
http://microvet.arizona.edu/Courses/MIC419/Tutorials/cytokines.html

Among the immune cells, I would like to emphasize that T helper cells have two important functions: one is to stimulate cellular immunity and inflammation, and the other is to stimulate B cells to produce antibody. Two functionally distinct subsets of T cells secrete cytokines which promote these different activities. Th1 cells produce IL-2 and IFN-γ, which activate T cells and macrophages to stimulate cellular immunity and inflammation. Th2 cells secrete IL-4, IL-5, IL-6 and IL-10, which stimulate antibody production by B cells. IL-4 stimulates Th2 activity and suppresses Th1 activity, while IL-12 promotes Th1 activities. Th1 and Th2 cytokines are antagonistic in activity. The balance between Th1 and Th2 activity may steer the

immune response in the direction of cell-mediated or humoral immunity. However, the imbalance between Th1 and Th2 cells might contribute to inflammatory responses thus leading to diseases. Evidence indicates that a dysregulation of mucosal immunity in the gut of IBD causes an overproduction of inflammatory cytokines and trafficking of effector leukocytes into the bowel, thus leading to an uncontrolled intestinal inflammation (Nakamura *et al*., 2006). IL-8 and TNF-α, which are important proinflammatory cytokines that were upregulated in IBDs, together with intercellular adhesion molecule-1 (ICAM-1), which is an important adhesion molecule which play a key role in many inflammatory diseases will be discussed in the following text. IL-18, which is emerged as a potential therapeutic target in inflammatory/autoimmune disorders recently, will also be further explored.

IL-8, which is a prototypic human chemokine, is found expressed in many inflammatory tissues. It is barely detectable in healthy tissues. However, it is rapidly induced by 10 to 100 folds in response to proinflammatory cytokines such as TNF-α or IL-1β, bacterial or viral products and cellular stress. In human intestine, IL-8 can be expressed by IECs in response of various stimulations. Hence, epithelial cell derived IL-8 may mediate neutrophil chemoattraction in infection or inflammation and also, by virtue of its angiogenic properties (Koch *et al*., 1992; Szekanecz *et al*., 1994), revascularization in the subsequent resolution of inflammation. In addition, malignant transformed colonic epithelial continually expressed IL-8 suggesting the involvement of IL-8 in carcinoma progression. Studies also showed that IL-8 is expressed in a majority of human colorectal carcinomas, which suggested that IL-8 may also play an autocrine role in the growth of colon carcinoma cells (Brew *et al*., 1996). Furthermore, IL-8 is elevated in the context of various other intestinal pathologies including Crohn's diseases, UC (Reddy *et al*., 2007), and possibly

intestinal neoplasia (Van, 1997; Eckmann, 1993). Therefore, the examination of signals that trigger IL-8 secretion is relevant to multiple cellular processes. In addition, IL-8 production was regulated by several well-known signaling pathways like NF-κB and MAPK pathways. Upon stimulation, NF-κB and JNK pathways are activated to induce transcription, and the resulting mRNA is rapidly stabilized by the p38 MAPK pathway (Hoffmann *et al*., 2002). In this way, cells are able to rapidly increase and at the same time to fine tune the amount of IL-8 secreted and thereby control the extent of leukocytes attracted to sites of tissue injury. Increased IL-8 production is thought to play important functions in inflammation and angiogenesis and consequently in healing and tumor development (Zheng and Martins-Green, 2007). Suppression of IL-8 might result in the inhibition of inflammation in the tissue and further tumor growth.

TNF-α is another important proinflammatory cytokine which is abundantly expressed in the gut of IBD (Murch, 1993; Breese, 1994; Braegger, 1992). It is synthesized by monocytes, macrophages, neutrophils, mast cells, natural killer (NK) cells and T cells (Table 1.1) (Tracey, 1997). During the inflammatory process, it orchestrates the initiation of further leukocytic infiltration via adhesion molecule upregulation, dendritic cell maturation and survival, macrophage activation, and driving Th1 cell responses within tissues in many experimental and clinical autoimmune diseases. Increased TNF-α expression in inflammatory cell has been seen in many experimental autoimmune diseases. TNF-α is also thought to facilitate on-going T cell effector responses, possibly through the activation of anti-apoptotic pathways dependent on TNF-induced nuclear factor NF-κB activation (Wang *et al*., 1998). In animal models of experimental colitis, treatment with anti-TNF-α antibody has been shown to be effective in the suppression of intestinal inflammation (Powrie

et al., 1994; Kosiewicz *et al*., 2001). As a result, this cytokine was considered to be an attractive target for the treatment of IBD and several anti-TNF reagents have thus been developed. All the anti-TNF reagents have been demonstrated to be effective in reducing intestinal inflammation. Therefore, reagents that could suppress TNF-α might be an effective "drug" in the therapy of inflammatory intestinal diseases like IBD.

Inflammation is a complex interaction between a noxious stimulus and leukocytes, vessel walls, and connective tissue with its cellular and noncellular constituents. A complex of cytokines and growth factors controls cell-to-cell communication during progress of the inflammatory process (Fuss, 2003). Whereas a distinct set of cell surface molecules, collectively designated cell adhesion molecules (CAMs), is required for the binding of leukocytes to tissue components and presumably also for the directional movement of leukocytes toward inflamed areas (Springer, 1990; 1994). Over the last few decades, extensive research on the expression and function of CAMs in chronic inflammatory disorders has resulted in the development and testing of drugs specifically aimed at interfering with the effects of these adhesion receptors (Van Assche, 2002). These include an antisense oligonucleotide inhibitor of intercellular adhesion molecule-1 (ICAM-1). The ICAM-1 molecule is an immunoglobulin-like glycoprotein which is constitutively expressed on the surface of a variety of cells including endothelial and epithelial cells, fibroblasts, lymphocytes and monocytes (Roebuck, 1999). The primary function of ICAM-1 is to promote adhesion between leucocytes and endothelial cells, in order to facilitate tissue-specific localisation of leucocytes for immune and inflammatory responses (Mazzone, 1995; Albelda, 1994; Springer, 1994; Bevilacqua, 1993). ICAM-1 plays an important role, not only in the stabilization of leukocyte interactions with

the vasculature, but also in signaling initiations (Van *et al*., 2007). Results show, however, that ICAM-1 is also involved in several of the subsequent steps of the inflammatory cascade. ICAM-1 activation has been well characterized in human umbilical vein endothelial cells (HUVECs) in response to stimulation with TNF-α, lipopolysaccharide(LPS) or oxidised low density lipoproteins (LDLs) (Niessen *et al*., 2002). Association between IBD and polymorphism in the gene encoding ICAM-1 on chromosome 19 has been demonstrated recently (Braun *et al*., 2001; Matsuzawa *et al*., 2003; Papa *et al*., 2004; Low *et al*., 2004) suggesting that ICAM-1 plays a specific role in the pathogenesis of IBD. The connection between neutrophil dominance during the active phases of UC, the interaction between ICAM-1 and neutrophils, and the linkage between UC and the ICAM-1 gene make UC an obvious target for anti-ICAM-1 treatment. In addition, a number of animal studies have shown evidence of anti-ICAM-1 antibodies providing protection against ischaemia reperfusion injury, effects associated with reduced neutrophil infiltration (Ma *et al*., 1992; Lefer *et al*., 2002).

Therefore, the crucial role of adhesion molecules in IBD and other inflammatory diseases makes them a very interesting target for drug development. Indeed, manipulating adhesion molecule is also being considered in multiple inflammatory diseases, including multiple sclerosis (O'Connor *et al*., 2004; Vollmer *et al*., 2004; Tremlett *et al*., 2005), rheumatoid arthritis (Maksymowych *et al*., 2002) and psoriasis (Elices *et al*., 2003; Gewirtz *et al*., 2001). Since leukocyte–endothelial adhesion plays a central role in leukocyte extravasations and it represents a key feature of tissue inflammation, disrupting such an interaction could be beneficial to intestinal inflammation. Therefore, any reagents that inhibited ICAM-1 expression in

inflammatory tissue would suppress the inflammatory responses and acted as therapy for the diseases.

Besides the adhesion molecules (ICAM-1) and proinflammatory cytokines (IL-8 and TNF-α), IL-18 is another emerging important cytokine that attract a profound interest as a potential therapeutic target in autoimmune/inflammatory diseases. It was originally termed interferon-inducing factor, which can be produced by various cell types (Table 1.1). IL-18 is a multi-functional cytokine with structural similarities to the IL-1 cytokine family (Okamura *et al*., 1995). Studies showed that the activation of caspase-1 could release IL-1β and IL-18 from the cells (Li *et al*., 2007). However, the mechanism for processing and secretion of IL-18 remains somewhat of a mystery. The activation of caspase-1 occurs independently of gene expression. IL-18 may also be processed by stimuli other than caspase-1. In addition, IL-18 has been shown to promote both Th1 and Th2 responses (Okamura *et al*., 1995). Increased IL-18 levels have been reported in both Th1 and Th2 dominated diseases. Moreover, the effects of IL-18 appear to depend on the presence of other cytokines. Co-administration of IL-18 and IL-12 was shown to inhibit IgE production in helminth-infected mice in an IFN-γ dependent fashion (Yoshimoto *et al*., 1997). In different animal studies, IL-18 caused increased production of IgE and Th2 cytokines (Kumano *et al*., 1999; Yoshimoto *et al*., 1999, 2000; Wild *et al*., 2000). Moreover, IL-18 alone in relatively high doses induced IgE production in vivo. Furthermore, IL-18 was found expressed at the site of many inflammation related disease like IBD (Pizarro *et al*., 1999), Rheumatoid arthritis (Gracie *et al*., 1999; Bombardieri *et al*., 2007). However, it is not very clear whether IL-18 is a marker of the diseased state or has an important effector function in acute or chronic diseases. Interestingly, administration of IL-18 to mice infected with a lethal dose of virulent strain of *S.*

typhimurium reduces the bacterial number in the tissues, rescuing them from death. On the other hand, macrophage infected with *S. typhimurium* exhibited reduced expression of IL-18, suggesting the ability of this pathogen to suppress production of IL-18 (Elhofy and bost, 1999; John *et al*., 2002). A more recent study showed that IL-18 has an effective anti-tumor effect by developing a long-lasting protective immunity including both innate and adaptive immunity in the host (Xu *et al*., 2007). Therefore, IL-18 might be involved in a complex regulation mechanism in the inflammatory responses.

Cytokines act on their target cells by binding to specific membrane receptors. The receptors and their corresponding cytokines have been divided into several families based on their structure and activities: Hematopoietin family receptors (e.g. GM-SF and IL-5 receptors), interferon family receptors (e.g. IFN receptors), tumor necrosis factor family receptors (e.g. TNF receptors) and chemokine family receptors (e.g. IL-8RA, and IL-8RB). Cytokine activity can be blocked by antagonists, molecules which bind cytokines or their receptors. During immune responses, fragments of membrane receptors may be shed and then compete for cytokine binding. Microbes also influence cytokine activities. They can affect the expression of the receptors and also the cytokine's binding. In the following text, I will further examine TNF receptor family and the chemokine family receptors.

The TNF receptor family include a variety of receptors (e.g. TNFR1, TNFR2 and TNFRSF11A) which mediate or initiate diverse biologic responses though TNF. TNFR1 is generally considered to be responsible for the majority of biologic actions of soluble TNF. Direct signaling through TNFR2 occurs less extensively and appears to be confined mainly to cells of the immune system and maybe important during cell-cell contact (Kriegler *et al*., 1988). TNFRSF11A, another TNF receptor which mainly

respond to TNFSF11, is a NF-κB activator. It is expressed on cells of the monocyte/macrophage lineage which include preosteoclasts. In addition, TNFRSF11A can be expressed in the colonic mucosa by subpopulations of activated macrophages or dendritic cells at higher levels in IBD compared to normal colon (Franchimont *et al*., 2004). However, most of the studies on TNFRSF11A were related to bone homeostasis (Nakao *et al*., 2007). Recent studies showed that the binding of the Receptor Activator of NF-κB Ligand (RANL also named as TNFSF11) to its receptor (TNFRSF11A), increases osteoclast development, osteoclast activity and survival upon the stimulation of proinflammatory cytokines (Walsh *et al*., 2003). Alternatively, TNFSF11 could also be produced in the gut and act in an endocrine manner on bone metabolism. In physiological conditions, TNFSF11 is not highly expressed in the intestine but TNFSF11 expression in both T-cells and osteoblastic cells is known to be tightly regulated (Anderson *et al*., 1997; Theill *et al*., 2002). Studies showed that the TNFRSF11A and TNFSF11 interaction results in an increase in dendritic cell (DC) survival and DC production of cytokines (Cremer *et al*., 2002; Josien *et al*., 1999). TNFRSF11A-expressing cells were mainly localized in the colon mucosa and had morphological features of large mononuclear cells suggestive of the monocyte-macrophage and/or dendritic cell lineages. Studies also showed that increased densities of TNFRSF11A expressing cells in CD patients in non-disease colon tissue (Franchimont *et al*., 2004). Therefore, inhibition of TNFRSF11A in inflammation intestine contributes to the host immune balance.

Chemokines/cytokines exert their function through binding to their receptors. All chemokine receptors are membrane-bound molecules composed of seven transmembrane domains and are coupled to G proteins at the carboxylterminal portion and possibly the third intracellular loop (Murphy *et al*., 2000; Murdoch, 2000).

Following ligand binding, chemokine receptors are presumed to be internalized and subsequently recycled, reappearing on the cell surface within 1h on the human IL-8 receptor system (Samanta *et al*., 1990). In the case of the human IL-8 receptor system, the inhibition of recycling reduced IL-8 mediated chemotaxis, suggesting its important role in the chemokine-mediated chemotaxis. IL-8 binds to two distinct types of receptors, IL-8RA and IL-8RB with similar high affinity (Murphy and Tiffany, 1991; Holmes *et al*., 1991). IL-8 activates the receptors IL-8RA and IL-8RB to induce chemotaxis in leukocytes, but only IL-8RA mediates cytotoxic and cross-regulatory signals. This may be due to the rapid internalization of IL-8RB (Richardson *et al*., 2003). In addition, IL-8RA but not IL-8RB mediated signals attenuated the response to other types of chemoattractants without any effects on their receptor expression (Richardson *et al*., 1998). However, IL-8RA and IL-8RB tune the responses to IL-8 in a coordinated manner. In intestinal epithelial cells (Caco-2, HT-29 and HCT116), all cells expressed IL-8RA however the expression of IL-8RB was weak (Brew *et al*., 2000). Both bind IL-8 with high affinity but IL-8RA is specific for IL-8, whereas IL-8RB also binds other chemokines with a similar affinity (Lee *et al*., 1992). Since epithelial cells express IL-8 receptors, IL-8 may have an autocrine function in epithelial regeneration, as has been proposed for Melanoma growth stimulatory activity factor (MGSA) in wound healing (Nanney *et al*., 1995). Therefore, the regulation of IL-8RA in intestine would also be important in the modulation of inflammatory responses especially those involved IL-8.

Since inflammation causes some serious diseases in the intestine, many approaches have been used to suppress inflammatory responses in the intestine. Crohn's disease and ulcerative colitis are two idiopathic IBD. So far, many drugs have been used to treat IBD in clinical trials, e.g. 5-aminosalicylic acid compounds,

corticosteroids, immunomodulators and calcineurin inhibitors. Emerging novel therapies—including biological therapies—directed at cytokines (e.g. infliximab, adalimumab, certolizumab pegol) (Franchimont *et al*., 2004) and receptors (e.g. visilizumab, abatacept) involve T-cell activation, selective adhesion molecule blockers (e.g. natalizumab, MLN-02, alicaforsen), anti-inflammatory cytokines (e.g. IL-10) and leucocyte apheresis. Many monoclonal antibodies, small molecules, recombinant growth factors, and MAP kinase inhibitors are targeting at various inflammatory cells and pathways (Baumgart and Sandborn, 2007). Besides the biochemicals that suppress inflammatory responses, clinical studies have suggested that probiotic bacteria may be effective therapeutic agents in IBD treatment although the molecular mechanisms of their regulation are still undefined. Probiotics have been used in the treatment of a number of inflammatory conditions including arthritis (Baharav *et al*., 2004), atopic eczema (Isolauri *et al*., 2000 and Viljanen *et al*., 2005), pouchitis (Gionchetti *et al*., 2000; Gionchetti *et al*., 2003; Katz, 2003; Kuisma *et al*., 2003; Gosselink *et al*., 2004 and Mimura *et al*., 2004), chemotherapy-induced mucositis (Tooley *et al*., 2006), ulcerative colitis (Rembacken *et al*., 1999; Bibiloni *et al*., 2005 and Furrie *et al*., 2005), Crohn's disease (Prantera *et al*., 2002 and Schultz *et al*., 2004; March, 2006), antibiotic-induced diarrhoea (Kim *et al*., 2003) and experimental colitis (Madsen *et al*., 1999; Steidler *et al*., 2000, O'Mahony *et al*., 2001; Shibolet *et al*., 2002; Dieleman *et al*., 2003; Moon *et al*., 2004; Rachmilewitz *et al*., 2004; Gaudier *et al*., 2005; Herias *et al*., 2005 and Moller *et al*., 2005, Foligne *et al*., 2007).

Studies suggest that altered bacterial species in the intestine may be a cause rather than a consequence of the IBD (Fedorak, 2007). Patients with IBD appear to have a genetically determined loss of immunologic tolerance, as evidenced by specific

cell-mediated and humoral immune responses targeting commensal gut bacteria (Duchmann *et al*., 1995; Macpherson *et al*., 1996). The mechanisms of action of probiotics treatment in IBD are currently a topic of much investigation. Probiotic strains exert their beneficial effects through a variety of mechanisms that are unique to each strain (Fedorak *et al*., 2004; Sartor *et al*., 2004). They competitively exclude undesirable bacteria through binding to mucosal border thus prevent pathogenic bacteria from crossing the cell wall, because they align themselves closer to the microvillus than many pathogenic bacteria. They also prevent adhesion and translocation, which is probably important in preventing bacterial antigens from reaching the lamina propria and mucosal immune system. In addition, probiotics stimulate immunoglobulin release into the lumen. They change the mucosal immune system with a resultant less Th1 proinflammatory response and a greater T-regulatory anti-inflammatory response. Furthermore, probiotics have antimicrobial activity. They are able to secrete defensins and other products like lactic acid that inhibit and kill pathogenic bacteria. Moreover, they enhance the barrier integrity by direct and indirect effects on tight junctions. Therefore, probiotics might be efficient and safer means to treat inflammatory diseases in human intestine. However, not all probiotics function via the same mechanisms; thus, not all probiotics are effective therapeutic agents in the treatment of all inflammatory diseases.

Given the diversity of inflammatory or immune responses that can be mounted by the intestinal epithelium, association of LAB with epithelial cells could be sufficient to trigger signaling cascades that ultimately activate underlying immune cells in the lamina propria. Alternatively, LAB may also release soluble factors that themselves trigger signaling cascades at the level of the epithelium or associated immune system (Madsen *et al*., 2001; Menard *et al*., 2004; Hoarau *et al*., 2006). Many

studies have been conducted to elucidate how pathogen virulence factors trigger inflammatory immune responses in the intestine. Little is known on how commensal bacteria control the innate immunity and inflammatory responses at the level of epithelial cells. Kelly *et al* using *Bacteroides thetaiotaomicron*, a prevalent anaerobe of the human intestine, showed that they could attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-γ and RelA (Kelly *et al*., 2004). Although the study demonstrated a new cellular target involved in commensal bacteria regulation, given the complex mechanisms defining host-bacteria communication, the possibility still exists that the immune system in the intestine is regulated by various bacteria at different levels. Studies using intestinal epithelial cell (IEC) lines have suggested that commensal bacteria may "program" IECs to prevent or down-modulate pro-inflammatory responses by interfering with Toll-like receptors (TLRs) expression (Abreu *et al*., 2001; Lan *et al*., 2005).

Toll-like receptors (TLRs) are a family of transmembrane receptors which are homologues to the *drosophila* Toll protein (Mushegian, 2001; Akira *et al*., 2001; Muzio *et al*., 2000). 13 mammalian TLRs have been identified so far: 10 human (TLR1–10) and 12 murine (TLR1–9 and TLR11–13) receptors, of which some are homologues and 2 NLRs (NOD1 and NOD2) (Mitchell *et al*., 2007; Rock *et al*., 1998). They are a class of pattern recognition molecules with unique functions in the innate and the acquired immune systems. The extracellular domain of Toll receptors contains leucine-rich repeat (LRR), whereas the cytoplasmic domain shows striking homology with that of interleukin receptors (IL-Rs), and is referred to as Toll/IL-R (TIR) domain. These domains are responsible for the recognition of pathogen-associated molecular patterns (PAMPs), present on diverse microbes including Gram-positive, Gram-negative bacteria, fungi, and viruses. TLR ligand recognition occurs via

interaction of the extracellulular TIR domain with its PAMP and results in the activation of multiple intracellular signaling events like the activation of NF-κB signaling pathway and the production of cytokines such as IL-8 and TNF-α.

TLR4 is the most extensively studied PRR and it recognizes a variety of ligands but is mostly known as the lipolysaccharide (LPS) receptor (Hoshino *et al*., 1999). TLR2 also recognizes a broad range of ligands, such as bacterial lipopeptides, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria. The variety of ligands recognized is believed to be due to heterodimer formation of TLR2 with two other TLRs, TLR1 or TLR6, which can discriminate subtle changes in the ligand structure (Bauer *et al*., 2001; Sugawara *et al*., 2003; Takeuchi *et al*., 1999). Mutations in TLR2 are associated with severe mycobacterial, staphylococcal infection and lepromatous leprosy (Takeuchi *et al*., 2000; Lorenz *et al*., 2000; Kang *et al*., 2001). TLR5 detects a conserved domain on flagellin monomers, the main structural protein that forms the flagella on Gram-negative and positive bacteria, thus to induce mobilisation of NF-κB to the nucleus and promote expression of proinflammatory cytokines in mammalian cells (Cristofaro and Opal, 2006). TLR9 recognizes nucleic acids such as hypomethylated CpG, motifs, which are common among bacterial and viral DNA (Cristofaro and Opal, 2006). Bacterial DNA stimulates proinflammatory cytokines, metric oxide and thus promotes B-cell activation and induces a Th1 type cytokine response (Cristofaro and Opal, 2006). TLR3 recognizes nucleic acids like TLR9, but confined to single-stranded (ss) and double-stranded (ds) RNA rather than DNA (Sen and Sarkar, 2005). TLR3 and TLR9 are reported to response to Gram-positive bacteria. The expression of TLRs differs with cell types and cellular localization where some have been found to be expressed primarily extracellularly (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) and others intracellularly (TLR3,

TLR7, TLR8 and TLR9) on numerous myeloid cells (e.g. macrophages, dendritic cells, neutrophils, T and B cells) and also on nonmyeloid cells (e.g. epithelial cells and fibroblasts).

Upon recognition of their cognate ligands, TLRs dimerize and initiate a signaling cascade that leads to the activation of a proinflammatory response (Akira, 2006). Ligand binding induces two signaling pathways, one is MyD88-dependent and the other is MyD88-independent inducing the production of proinflammatory cytokines and type I interferons (IFNs) (Kawai and Akira, 2006). These two distinct responses are mediated via the selective usage of adaptor molecules recruited to the TIR domains of the TLRs after ligand recognition and binding. Four adaptor molecules have been identified so far, MyD88, TIR-associated protein (TIRAP), TIR domain containing adaptor protein-inducing IFN-β (TRIF) and TRIF-related adaptor molecules (TRAM) (O'Neill *et al*., 2003; Wang *et al*., 2004; Yamamoto *et al*., 2002, 2003, 2003). MyD88 and TIRAP are responsible for the induction of proinflammatory genes and TRIF, and TRAM for the induction of the IFNs. A disturbance of anti-inflammatory and immunosuppressive mechanisms in the gut is believed to lead to colitis, driven by a hyperresponsiveness to the commensal flora. TLR signaling may have a dual role in gut homeostasis as it was shown to be required for commensal-dependant colitis in IL-10-deficient mice but not in mice deficient in IL-2 (Rakoff-Nahoum *et al*., 2004, 2006; Rakoff-Nahoum and Medzhitov, 2006). The mechanisms by which the intestinal microflora suppresses inflammatory signaling may be dependent on the noninvasive nature of these organisms, as it was recently reported that activation of TLR9 located in the apical surface domain resulted in tolerance to TLR activation, whereas activation of TLR9 in the basolateral region, only reachable by invasive bacteria caused immune activation (Lee *et al*., 2006; 2007). Previous

studies in human IBD showed increased TLR expression and NF-κB activity in lamina propria macrophages and the epithelium under conditions of chronic intestinal inflammation (Hausmann *et al*., 2002; Cario and Podolsky, 2000; Schreiber *et al*., 1998; Rogler *et al*., 1998; Andresen *et al*., 2005), suggesting that the continuous activation of downstream TLR effector systems including the activation of the transcription factor NF-κB could potentially lead to persistent inflammation. Therefore, the communication of bacteria with the host through TLRs is important in the initiation of inflammatory responses. Better understanding of these basic molecular mechanisms of TLRs and their downstream pathways regulated by commensal bacteria would have profound impacts on understanding of the inflammatory processes in human intestine.

TLRs could be activated by pathogens leading to the initiation of inflammation by secreting pro-inflammatory cytokines such as interleukin-8 (IL-8) and tumor necrosis factor (TNF)-α (Janeway, 2001). IECs are a source of IL-8 which recruits neutrophils into the infected intestine. Appropriate neutrophil recruitment results in prevention of bacterial invasion. However, excessive IL-8 expression or neutrophil influx into the intestine contributes to the development of chronic inflammation diseases. It was demonstrated in the animal and clinical trials that inhibition of pro-inflammatory cytokines production and the supplementation of anti-inflammatory cytokines reduced inflammation reaction (MacDermott, 1996). Recent studies have suggested that chronic inflammation can be instrumental in both initiation and progression of many epithelial tumors (Balkwill and Mantovani, 2001; Coussens and Werb, 2002; Greten *et al*., 2004; Hussain *et al*., 2003; Ying *et al*., 2005). Therefore, better understanding of the regulation of IL-8 production in the human

intestine can be useful in the development of effective therapies for inflammatory diseases and even cancers in the intestine.

In addition, emerging evidences suggest that changes in the homeostasis of bacteria- and host-derived signal transduction at the epithelial cell level may lead to functional and immune disturbances of the intestinal epithelium. It has become clear from numerous studies that LAB in the intestine are critical components in the development and prevention/treatment of chronic intestinal inflammation. Signal-specific activation of mitogen-activated protein kinases (MAPK), interferon-regulated factors (IRF) and the transcription factor NF-κB through pattern recognition receptor signaling effectively induce inflammatory defense mechanisms.

Mitogen-activated protein kinases (MAPKs) are another family of proteins able to transduce extracellular signals into inflammatory gene transcription. MAPK signaling pathway is one of the most important intracellular signaling cascades conserved from yeast to mammals. MAPKs are a family of serine/ threonine kinases in which three distinct groups of MAPKs have been identified in mammals, including the extracellular signal-regulated kinases (ERKs), the c-JUN NH_2 terminal kinases (JNKs), and p38. Of these, the most extensively studied are the ERKs, which are acutely stimulated by growth and differentiation factors, heterotrimeric G protein-coupled receptors, and cytokine receptors (Humberto, 2002). JNKs and p38 family members are generally implicated in responses to cellular stress, inflammation and apoptosis (Cobb, 1999; Lewis, 1998). Activation of any of the MAPK family members requires dual phosphorylation on serine/threonine and tyrosine residues by their upstream kinases termed as MAPK kinases (MKK). ERK, JNK and p38 family members are activated by MKK1/2, MKK4/7 and MKK3/6 respectively. MKKs, in turn, are activated by MKK kinases. To date, twelve member proteins of the MAPK

family have been identified in mammalian cells, and these can be grouped into five subfamilies on the basis of sequence homology and function (Garrington and Johnson, 1999). Seven MKKs and fourteen MKKKs have been functionally identified in mammalian cells. The combination of twelve MAPKs, seven MKKs and fourteen MKKKs, seems extraordinarily complex. However, the complex combination and diversity of regulatory domains in different MKKKs give the family of MAPK modules the flexibility to respond to a wide range of cellular stimuli. Moreover, beside the activation model of MKKK-MKK-MAPK, MAPK family members also can be autophosphorylated triggered by the interaction of MAP3K7IP1/TAB1 protein with the kinase.

Dephosphorylation of one or both of the regulatory residues (serine/threonine and tyrosine) induces deactivation of MAPK family members. MAPK dephosphorylation is carried out by some phosphatases, similar to the dual specificity MAPK phosphatases (DUSPs) also known as MKPs, which simultaneously dephosphorylate both serine/threonine and tyrosine residues (Tonks and Neel, 1996; Vicent *et al*., 2004; Liu *et al*., 2007). The first characterized member of this family was DUSP1 (also known as MKP1). It is encoded by an early response gene, which is transiently induced by mitogens and stress signals. Although ERK, JNK, and p38 are known substrates of DUSP1, the ability to dephosphorylate each one of these kinases varies depending on the cell system and its environmental conditions (Vicent *et al*., 2004).

MAPK signaling pathways can be activated by a variety of stimuli like proinflammatory cytokines. IL-1β could activate ERK, p38, and JNK. These three MAPK kinases may co-operatively mediate IL-1β stimulated receptor activator of nuclear factor-kappaB ligand (RANKL also know as TNFRSF11A) expression and its

activity in human periodontal ligament cells (Oikawa *et al*., 2007). Furthermore, JNK and p38 are the most important kinases implicated during inflammation mediated by activating cytokine gene expression (Kyriakis *et al*., 2001). JNK and p38 modulate gene expression by different mechanisms. They can target transcription factors directly by enhancing their ability to activate transcription or may modulate formation of new transcriptional factors as the activation protein-1 (AP-1) complex after phosphorylation of c-JUN and the activating transcription factor-2 (ATF-2). They control chromatin access by histone phosphorylation and then modulate gene expression by remodeling the structure of chromatin. P38 and JNK are also involved in posttranscriptional control of gene expression by stabilizing mRNAs as JNK for interleukin IL-2 transcripts (Chen, 1998) and p38 for IL-8 transcripts (Hoffmann *et al*., 2002). JNK, like NF-κB, are strongly activated under most conditions that induce IL-8. The observation that JNK are in fact essential for IL-8 (and IL-6) expression was made initially in human epithelial cells by blocking JNK activation by stable expression of antisense RNA or by dominant-negative mutants (Krause *et al*., 1998). The JNK inhibition was specific and did not affect NF-κB or p38 MAPK activation (Krause *et al*., 1998). The absolute requirement of JNK for IL-6 expression was later confirmed in JNK2-/- fibroblasts (Chu *et al*., 1999). In contrast to the findings above, the JNK inhibitor had no effect on IL-8 expression in lipopolysaccharide (LPS)-stimulated human monocytes (Bennett *et al*., 2001). Furthermore, MAPK activation would lead to the induction of other proinflammatory cytokines such as IL-1β, TNF-α, IFN-γ (Malamut *et al*., 2006) and IL-12 (Parrello *et al*., 2000) and the suppression of anti-inflammatory cytokines such as IL-10 (Foey *et al*., 1998) and IL-11 (Bamba *et al*., 2003). Thus, MAPK pathway appears as a central therapeutic target to control inflammation.

MAPK signaling pathways can be activated by a variety of stimuli like proinflammatory cytokines. JNK and p38 are the most important kinases implicated during inflammation mediated by activating cytokine gene expression (Kyriakis *et al*., 2001). JNK and p38 modulate gene expression by different mechanisms. They can target transcription factors directly by enhancing their ability to activate transcription or may modulate formation of new transcriptional factors as the activation protein-1 (AP-1) complex after phosphorylation of c-JUN and activating transcription factor-2 (ATF-2). MAPK activation would lead to the induction of proinflammatory cytokines such as IL-1β, TNF-α, IL-8 (Matsumoto *et al*., 2007), IFN-γ (Malamut *et al*., 2006) and IL-12 (Parrello *et al*., 2000) and the suppression of anti-inflammatory cytokines such as IL-10 (Foey *et al*., 1998), IL-11 (Bamba *et al*., 2003). NF-κB is another important signaling pathway involved in the inflammatory responses in the host.

In vertebrates, NF-κB transcription factor stand out as master regulators of innate and adaptive immunity, inflammatory responses, organ development and cell survival (Papa *et al*., 2006). They have also been recognized as a major regulator of pathogen- and inflammatory cytokine-inducible gene regulation (Baeuerle and Henkel, 1994). NF-κB is a dimeric transcription factor composed of a family of five subunits, namely NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), and c-Rel, RelA (p65), and RelB (Baeuerle and Baltimore, 1996). These five subunits are characterized by the so-called Rel homology domain (RHD), which mediates DNA binding, dimerization and association with inhibitory proteins of IκB group. Normally, NF-κB is retained in the cytoplasm in an inactivate state by its binding to inhibitory proteins (IκB). IκB phosphorylation by IκB kinases (IKK) complex which consists of three subunits, IKKα (IKKA), IKKβ (IKKB) and IKKγ/NEMO, results in

ubiquitination and rapid degradation of IκBs by the proteasome, allowing NF-κB to translocate to the nucleus where they activate expression of distinct sets of target genes encoding numerous cytokines, chemokines, adhesion molecules, growth factors, immune receptors and prosurvival proteins. Through the induction of these genes, NF-κB marshals and coordinates innate and adaptive immunity, inflammation, cell differentiation and cell survival. But enhanced NF-κB-induced transcriptional activity might additionally require phosphorylation of the subunits as well as binding of coactivators (Baeuerle and Baltimore, 1996; Schmitz *et al*., 2001). At the level of DNA, NF-κB synergizes with many additional transcription factors that may determine cell type-specific expression of genes and the input from other signal transduction pathways in their regulation.

There are two main pathways for NF-κB activation (Bonizzi *et al*., 2004). One is the canonical pathway, triggered in response to microbial products, stress and proinflammatory cytokines, depends on the activity of IKKβ and mainly results in the nuclear translocation of NF-κB1/RelA and NF-κB1/Rel dimers. The alternative pathway, activated by certain members of the TNF receptor (TNFR) family such as lymphotoxin (LT)β and BAFF receptors, depends instead on IKKα and causes activation of NF-κB2/RelB complexes by inducing the proteolytic processing of the NF-κB2/p100 precursor(Bonizzi *et al*., 2004; Hayden, 2004; Siebenlist *et al*., 2005). NF-κB signaling pathway can be activated by a wide spectrum of stimuli including microbial infection, chemicals, UV light (Pahl, 1999; Adachi *et al*., 2003) and proinflammatory cytokines like TNF-α and IL-1β. TNF-α is the key initiator of inflammation. Its deregulation causes many disorders including IBD, rheumatoid arthristis, and toxic shock syndrome. The binding of TNF-α to its receptor triggers a

series of intracellular events, resulting in the activation of transcription factors, including NF-κB, CEBR and AP-1 (Baud and Karin, 2001; Chen and Goeddel, 2002). However different cellular processes seem to be differentially regulated by specific signal transduction pathways. TNF-α causes activation of IKKs, kinases that phosphorylate IκB and induce its degradation, which results in activation of NF-κB. Importantly, a knock out IKKB is defective in signaling from TNF-α to NF-κB. Therefore, IKKB might play an important role in the regulation of NF-κB activation in response to proinflammatory cytokines. Experiments using chromatin immunoprecipitation, binding of p65 NF-κB to the endogenous IL-8 promoter and subsequent recruitment of RNA polymerase II are found rapidly, within one-half hour of IL-1 stimulation, underscoring the important role of NF-κB in IL-8 transcription (Hoffmann *et al*., 2002). Additional experimental evidence for the importance of NF-κB in directing proinflammatory processes under conditions of chronic inflammation was elegantly shown in trinitrobenzene sulfonic acid (TNBS)-treated mice (Neurath *et al*., 1996). On the other hand, but equally important, the inhibition of NF-κB activity with pharmacological inhibitors during the resolution phase of carrageenan-induced acute inflammation had adverse effects on the host (Lawrence *et al*., 2001), suggesting dual functions of activated NF-κB including protective and detrimental mechanisms during the course of inflammation.

They can be activated by a wide spectrum of stimuli including microbial infection, chemicals, UV light (Pahl, 1999; Adachi *et al*., 2003) and proinflammatory cytokines like TNF-α and IL-1β. NF-κB conducts its function by translocating to the nucleus where it activates expression of distinct sets of target genes encoding numerous cytokines, chemokines, adhesion molecules, growth factors, immune

receptors and pro-survival proteins. Through the induction of these genes, NF-κB coordinates innate and adaptive immunity, inflammation and cell survival.

However, unbalanced activation of these innate signaling pathways because of host genetic predispositions and/or the lack of adequate anti-inflammatory feedback mechanisms may turn a physiological response into a pathological situation including failure of bacteria clearance and development of chronic inflammation. Therefore, it is very important for a healthy crosstalk between the bacteria and the innate signaling network of the intestinal epithelium in order to shape the extent and duration of inflammatory processes. The progress of the inflammatory process is controlled by a complex of cytokines and growth factors during LAB-to-host communication (Fuss, 2003). IL-2 and interferon (IFN)-γ are efficient in activating cellular immune responses that promote inflammatory responses thus eliminate intracellular pathogens. IL-4, IL-5 and IL-10 could promote humoral immune responses further to inhibit inflammatory responses. IL-4 and IL-10 have been implicated in the inhibition of IL-8 formation (Kasama *et al*., 1994; Meda *et al*., 1994; Wertheim *et al*., 1993). IL-5 has long been associated with several allergic diseases including allergic rhinitis and asthma (Shen *et al*., 2003). TGF-β, which is an important inflammation regulatory cytokine, is important in the suppression of proinflammatory cytokines production. IL-12 could promote IFN-γ production, however, IFN-γ together with IL-2 augment IL-6 and TNF-α. Increased TNF-α expression in inflammatory cell has been seen in many experimental autoimmune diseases. In addition, IL-17 is characterized as cytokine-inducing cytokine that exhibits pleiotropic biologic activities on various cell types. It can activate IL-8 in IECs. In addition to inducing cytokines and chemokines, IL-17 presents itself with certain other unique functions. It enhances surface expression of intercellular adhesion molecule-1 (ICAM-1) in human fibroblasts.

ICAM-1 is also involved in several of the subsequent steps of the inflammatory cascade. It is usually found in inflamed colonic tissues, mediates the arrest and further migration of neutrophils. In addition, increased IL-18 levels have been reported in both Th1 and Th2 dominated diseases. Interestingly, administration of IL-18 to mice infected with a lethal dose of virulent strain of *S. typhimurium* reduces the bacterial number in the tissues, rescuing them from death. Therefore, suppression of pro-inflammatory factors/cytokines such as ICAM-1, IL-2, IL-6, IL-12, IL-17, TNF-α and IFN-γ and upregulation of IL-4, IL-10 and TGF-β could inhibit the inflammatory responses in the host. IL-18 may also be involved in the immune responses in the host upon the stimulation of bacteria.

Another important aspect in the network of cytokine actions during the inflammatory responses is the modulation of the level of cytokine receptors expression. The activation of cytokines can affect their own or other cytokines' receptors. However, the exact mechanism of cytokine receptors involving in the bacteria-host interaction and inflammatory responses has not been fully studied.

Although NF-κB and MAPK signaling pathways are the main pathways that regulate the inflammatory reactions inside the cells, there are some other signaling pathways that might be involved in the regulation. JAKs, Stats, SOCS and PIAS, which are involved in cytokine signaling, might also be important in the inflammatory responses. A recently identified signaling pathway related with MAPK and NF-κB might also involve in the inflammatory responses in the host. The prolyl isomerase PIN1 is a conserved enzyme that is intimately involved in diverse biological process and pathological conditions (Yeh and Means, 2007). By catalyzing cis-trans interconversion of certain motifs containing phosphorylated serine or threonine residues followed by a proline residue, PIN1 has profound effects on phosphorylation

signaling (Wulf *et al*., 2005). PIN1 can target the pTHR254-Pro motif in the p65/RelA subunit of NF-κB and prevent NF-κB from binding to its inhibitor IκB. This prevents p65/RelA nuclear export and its subsequent degradation by an ubiquitin-mediated pathway (Ryo *et al*., 2003). This leads to enhanced nuclear accumulation, protein stability and transcriptional activity of NF-κB towards its target genes such as cyclinD1 and IκB (Ryo *et al*., 2003). CyclinD1 is an important cell-cycle regulator that is known to have a key role in the development of many cancers (Yu *et al*., 2001; Lamb *et al*., 2003). PIN1 regulates cyclinD1 expression through multiple mechanisms. It stabilizes cyclinD1 through directly binding to its pThr286-pro motif thus inhibiting its nuclear export and ubiquitin-mediated degradation (Liou *et al*., 2002). PIN1 also inhibits negative feedback of MAPK signaling (Dougherty *et al*., 2005). Raf kinase is activated by Ras and then triggers a protein kinase cascade, leading to the activation of MAPKs. MAPKs can phosphorylate and inactivate Raf in a negative feedback mechanism (Dougherty *et al*., 2005). PIN1 prevents Raf kinase from been turned off after growth stimulation by promoting dephosphorylation of Raf. In addition, PIN1 is transcriptionally regulated by the E2F1 family of transcription factors in the response of growth factors and other stimulating conditions. E2F1 has been found to be released from Rb and activating its target genes in breast cancer (Zhang *et al*., 2000) and chronic inflammation disease such as IBD (Ying *et al*., 2005). Furthermore, PIN1 increases the protein half–life of p53 by inhibiting its binding to the MDM2 ubiquitin ligase, which regulates the degradation of p53. Therefore, PIN1, which is a specific phosphorylase, closely involves in an array of biological and pathological processes. It is involved in the regulation of NF-κB and MAPK signaling pathways thus might also regulate inflammatory responses in the host.

Above all, we know that the LAB-host interaction is pivotal in the regulation of host immune responses. The imbalance composition of microbes in the human intestine would cause inflammatory responses. The presence of some LAB would help to inhibit the inflammatory responses and thus maintain the intestine homeostasis. However, the population of microbes harbored in the human intestine is dynamic and varies with age. It was reported that infants who developed allergy were often found to possess less enterococci in the intestines than healthy infants during the first month of life (Björkstén *et al*., 1999; 2001; Kirjavainen *et al*., 2002). *Enterococcus faecalis* is a major commensal gastrointestinal flora in humans (Fuller, 1991; Lammers *et al*., 2002). It is one of the earliest bacterial strains which colonized the human intestine after birth. So far, it is not clear if the depletion of enterococci in allergic children is a cause or effect of allergy. Although there are some reports that *E. faecalis* caused infections in immune deficient mice (Ruiz *et al*., 2005), many studies also showed that *E. faecalis* could suppress the proliferation of intestinal pathogens and thus may prevent infection and the induction of inflammation (Giraffa, 1995; Harris *et al*., 1989; Lsolauri *et al*., 2002; McKay, 1990). Another study showed that *E. faecalis* lysate could relieve the clinical symptoms of Japanese cedar pollinosis (Shimada *et al*., 2004). However, the role and effect of *E. faecalis* in inflammation reactions are not clear. It is the intention of this study to shed light on the mechanisms of anti-inflammatory effect of *E. faecalis*. This knowledge could provide a therapeutic window and development of treatments or prophylaxis of intestinal infectious, inflammatory and allergic reactions and perhaps carcinogenesis.

1.2 AIMS OF THE STUDY

Knowing how commensal bacteria, especially LAB, interact with host has become very important for human health due to the increasing number of population who suffered from all kinds of intestinal diseases because of the acute or chronic inflammation. Recently, there are great interests in finding out how these bacteria can suppress the inflammatory responses in the human intestine. However, the mechanisms how these LAB regulate immune responses in human are still unclear.

The purpose of this study was to examine the immunomodulating effects of LAB, especially some newly isolated human intestinal *E. faecalis* strains from the human intestine and to further investigate their underlying cellular and molecular mechanisms in the immunemodulating effects in human. To understand the roles that LAB, especially *E. faecalis*, play in the human intestinal homeostasis, the following are described in this thesis:

1. How LAB, especially *E. faecalis*, interact with intestinal epithelial cells and their corresponding responses to them.
2. How *E. faecalis* regulates the inflammatory responses in intestinal epithelial cells and the possible molecules that may be involved.
3. How bacteria modulate MAPK signaling pathway during the host-bacteria interaction processes.
4. How bacteria modulate NF-κB signaling pathway.
5. How *E. faecalis* is involved in other possible signaling pathways during the communication with intestinal epithelial cells.

The study focused on the LAB, *E. faecalis*, which dominates the infant intestinal microflora. This study intended to show the possible mechanisms that *E.*

faecalis participates in the immunomodulation in the human intestine. These mechanisms could underpin the myth how LAB interact with host, thus to highlight new cellular targets and approaches for therapeutic intervention in the treatment of inflammatory diseases. Moreover, the identification of the effector molecules on *E. faecalis* involved in the immune regulation may open a new therapeutic window for human inflammatory bowel diseases and also other inflammatory conditions.

Chapter 2

MATERIALS & METHODS

2.1 BACTERIA STRAINS

2.1.1 Bacteria Culture

The origin of the bacterial strains used in the experiment is indicated in Table 2.1. All *Lactobacillus* strains were grown and subcultured weekly on de Man, Rogosa and Sharpe (MRS) Agar (Merck, USA) at 37°C in a 5% CO_2 incubator (Forma, USA), except for *Lactobacillus casei subsp casei* NCIMB 11970, which was maintained at 30°C. Enterococci were grown and subcultured monthly on Tryptone Soy Agar (TSA) (Merck, USA) at 37°C in a 5% CO_2 incubator. *Streptococcus thermophilus* NCIMB 10387 (10387) was grown on M17 medium (Oxoid, England). *S. typhimurium* 14028 (Salm) was maintained on Luria-Bertani (LB) Agar (BBL, Cockeyville, MD, USA). *Bifidobacterium* were grown on Reinforced Clostridial Medium (RCM) at 37°C in an aerobic chamber (Lee Hung, Singapore).

Table 2.1 The LAB tested for their anti-inflammatory properties

Lactic Acid Bacteria (LAB)	Origin	Remark
Bifidobacterium adolescentis ATCC15703	Infant intestine	-
B. brevi ATCC15700	Infant intestine	-
B. dentium scardori & crociani ATCC27534	Dental carries	-
B. longum ATCC15707	Adult intestine	-
Enterococcus faecalis EC1	Infant feces Age: 1 month	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Enterococcus faecalis EC3	Infant feces Age: 3 days	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Enterococcus faecalis EC15	Infant feces Age: 3 days	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Enterococcus faecalis EC16	Infant feces Age: 3 days	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Other 22 strains of enterococci and lactobacilli	Infant feces Age: 3 days to 1 month	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Lactobacillus acidophilus ATCC4356	Human	-
L. brevis K7	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. brevis T1	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. brevis T6	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. casei ATCC11578	Oral	-
L. casei IS7257	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. casei subsp. *casei* NCIMB11970	Cheese	-
L. casei Shirota	Human	Commercial probiotic
L. delbrueckii subsp. *bulgaricus* NCIMB11778	Bulgarian yogurt	Commercial probiotic
L. delbrueckii bulgaricus D1	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA

L. lactis subsp. *lactis* B4	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* B9	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* B12	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* K5	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* K6	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. lactis subsp. *lactis* K7	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. paracasei LP33	Fermented milk	Commercial probiotic
Lactobacillus paracasei Chamyto	Yogurt	Identified by phenotypic characterization and sequence analysis of 16S rDNA
L. paracasei subsp. *paracasei* ATCC11974	Dental carries	-
L. paracasei subsp. *paracasei* NCIMB8001	-	-
L. rhamnusus NCIMB6375	Oral	
L.rhamnosus GG	Human feces	Commercial probiotic
L. rhamnosus NCIMB 8690	-	-
Leuconostoc mesentroides K13	Fermented milk	Identified by phenotypic characterization and sequence analysis of 16S rDNA
Streptococcus thermophilus NCIMB10387	Yogurt	Commercial probiotic

2.1.2 Identification of Bacteria

The bacterial stains from healthy baby gut and fermented milk were identified by the following methods.

2.1.2.1 Phenotypic Characterization

Bacteria were first Gram stained. Carbohydrate fermentation patterns of the bacteria were determined using the API 50 CH test strips for rod-shaped bacteria and rapid ID 32 STREP test for the coccal shaped strains. The cocobacilli shaped strains were determined using two of the above tests. The data were analyzed by BioMerieux software (BioMerieux, Nurtingen, Germany).

2.1.2.2 Genotypic Characterization

2.1.2.2.1 Extraction of Total DNA

Cell cultures (3ml) in the late exponential growth phase were centrifuged at 8,000g for 10min and the cell pellets were washed and resuspended in 0.5ml of TE-buffer (10mM Tris–HCl, 1mM EDTA; pH 8.0). The suspension was transferred to a 2ml screw-capped tube containing 0.3g of sterile zirconium beads (diameter, 0.1mm), homogenized in a bead-beater (Biospec Products, Bartlesville, UK) at 5,000 rpm for 3min and cooled on ice. The homogenate was centrifuged at 13,000g for 5min and the supernatant fluid was stored at -20 °C until use.

2.1.2.2.2 PCR and Sequence Analysis of the 16s rDNA

PCR amplification was performed with Taq DNA polymerase Recombinant (Invitrogen-Life Technologies, UK) using primers 7f 5V-AGAGTTTGATC/TA/CTGGCTCAG-3V and 1510 rev 5V-ACGG(C/T) ACCTTGTTACGACTT-3V (Lane, 1991; Cinzia, 2004). DNA was amplified using the program as follow: 94°C for 3min; 30 cycles of 94°C for 30s, 52°C for 30s and 68°C for 1.5min; and finally, 68°C for 7min.

The amplified PCR products were purified with the Qiaquick PCR purification kit (Qiagen Hilden, Germany) for sequence analysis. 8µl of PCR product of the 16S rDNA were directly sequenced using primer 1100 reverse 5V-GGGTTGCGCTCGTTG-3V to obtain partial sequence of the 16S rDNA (Lane, 1991; Cinzia, 2004). Homology searches of the 16S rDNA sequences were performed using the Pubmed Nucleic acid sequence search service available in the internet. The website can be accessed at http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi.

2.1.2.3 Protease Analysis

Protease analysis was only carried on *E. faecalis* (EC1, EC3, EC15, EC16, ATCC 29212). DNA was harvested using DNA extraction kit according to manufacture's instruction (Qiagen Hilden, Germany).

Table 2.2 PCR Primers and the annealing temperatures used for protease PCRs

Gene Names	Primer	Polarity	Annealing Temp. (°C)	Length (bp)
EF0706	TGTCAACAAAATGAAGCAAC	Forward	45	402
	GGCGATTTAAGATTTCAATG	Reverse		
EF0771	CCAACAGTTATTGAACAATC	Forward	45	408
	GAGCAGCAATTTCAATTTCT	Reverse		
EF1590	GTCAGTATTTATTAGAGAGTGTACC	Forward	45	400
	ATGTTCCCAAACACCTAACC	Reverse		
EF1679	ATGAAGAACAAACGAACTGT	Forward	45	503
	TTACCTCGGACTTTTGAGAC	Reverse		
EF1917	ATGTACGACAATACGGATAA	Forward	45	517
	CATTATAGTCAGCAGATTGTAG	Reverse		
EF2355	ATTGAAAAAATGACAACCACGC	Forward	45	548
	CGACCAATGATTGGATCTTG	Reverse		
EF2380	TCACATTCATTATTGTCTTCGG	Forward	45	503
	AATGCGTTGCGATAATTTCG	Reverse		
EF2821	ATGAAGAAACAGGAAATTCG	Forward	45	403
	GGTCTGAACTATAATCCCAA	Reverse		
EF3027	ATGCAACGAAAAGATGTTAC	Forward	45	501
	GGTACCATCAGATAAAACAA	Reverse		
EF3282	TGGATGAACTATTTACAGAAAGTGC	Forward	45	525
	AAGAATCCAATGTTGGCGTC	Reverse		

PCR amplification was performed with Taq DNA Polymerase Recombinant (New England BioLabs, Beverly, MA) using primers as listed in Table 2.2. DNA was amplified with a total volume of 30µl using the program as follows: 94°C for 2min; 30 cycles of 94°C for 30s, 45°C for 30s and 72°C for 2min; and finally, 72°C for 5min.

2.1.2.4 Antibiotics Resistance Test

In this test, small filter paper disks (6 mm) impregnated with a standard amount of antibiotic are placed onto an agar plate to which bacteria have been swabbed. The plates are incubated overnight, and the zone of inhibition of bacterial growth is used as a measure of susceptibility. Large zones of inhibition indicate that the organism is susceptible, while small or no zones of inhibition (6mm) indicate resistance.

2.1.2.5 Gene Tree of the Bacteria

Phylogenetic tree calculation is based on a sequence distance method and utilizes the Neighbor Joining (NJ) algorithm of Saitou and Nei (Saitou, N. and Nei, M. 1987).

2.1.3 Conversion Factors

For each strain of bacteria, the conversion factor is shown in Apendix1. The conversion factors were derived using Miles and Misra techniques. Briefly, 3-5 colonies of freshly cultured bacteria were inoculated into 4ml bacteria specific broth and kept at 37°C for 24 hours without shaking. A 5% inoculum was then made by adding 1ml of the culture into 20ml appropriate broth and further incubating at 37°C

to reach their late exponential phases with shaking at a speed of 120rpm. 10ml of culture was then harvested and centrifuged at 5,000g at 4°C for 5 minutes. The pellet was washed three times and resuspended in 10ml saline (0.85% w/v NaCl). This suspension was diluted 2, 5, 10 and 20 times for measuring absorbance at 600nm. Each of these 4 dilutions was further serially diluted from 10^1 to 10^8 times, and 25µl of each serial diluted bacterial suspension was dropped onto two surface dried agar plates. Colony counts (Valid range from 5-50 colonies) were made after a 48h incubation at 37°C in a 5% CO_2 incubator, and the absorbance at 600nm (y-axis) was plotted against the average colony forming unit per ml (CFU/ml) (x-axis) to obtain a trend line with acceptable R^2 value greater than 0.96 (Appendix 1).

2.1.4 Preparation of Bacteria Strains

To prepare for the bacteria for experimental treatments, 3-5 colonies were taken from a fresh cultured plate and transferred into 10ml specific bacterial broth in a 15ml Falcon tube (BD bioscience, San Diego, CA). The broth was then cultured overnight at 37°C without shaking. The fresh cultured bacteria were further inoculated into 50ml broth in a 250ml flask with a 5% inoculum. To reach their exponential phases, the flask was shaken at a speed of 120rpm at 37°C. The bacteria were then pelleted down by centrifugation at 5,000g for 5min. Pellets were washed using serum-free medium three times and then resuspended in complete medium. To get the proper number of bacteria for experimental treatment, OD reading at 600nm was taken. According to the conversion factor we obtained in section 2.1.3, the number of the bacteria was estimated. The desired concentration of bacteria was achieved by proper dilution in complete medium.

2.2 CELL CULTURE

2.2.1 Intestinal Cell Lines and Monocyte Cell Line Culture

All solutions and media for cell culture were prepared under aseptic condition in a laminar flow cupboard (Gelman Science, Australia) or biohazard hood (Nuaire, USA). Cells were grown in sterile plastic tissue culture flasks (Nalge Nunc International, USA) or 24-well/6-well Multidishes Nunclon™ plates from (Nalge Nunc International, USA). They were cultured in a humidified incubator at 37°C with 5% CO_2.

Caco-2, HT-29, HCT116, THP1 and CRL-1790 cells were obtained from American Type Culture Collection (Manassas, VA). Caco-2 and HT-29 are human colonic adenocarcinoma cells. Differentiated Caco-2 are enterocyte-like cells (Amano, and Oshima. 1999) whereas HCT116 is human colon carcinoma cells. All cells were cultured at 37°C in a 5% CO_2 atmosphere. Caco-2 cells were cultured in MEM (GIBCO, Grand Island, NY) containing 20% fetal bovine serum (FBS) (Bioclot, Germany) and 1% nonessential amino acids (GIBCO). HT-29 and HCT116 cells were cultured in DMEM (GIBCO) containing 10% FBS (Bioclot, Germany). THP1 is human acute monocytic leukemia cell line. They were cultured in RPMI1640 (GIBCO) with 10% FBS at 37°C in a 5% CO_2 incubator. CRL-1790, which is a normal human colon cell line, were cultured in 1:1 Ham's F12 medium and DMEM mixture contained 0.02mg/ul insulin, 50μm Hydrocortisone, 1ng/ml epidermal growth factor, 0.5mM sodium pyrurate, 2mM L-glutamine and 10% FBS. All culture media were supplemented with 100U/ml penicillin and 100μg/ml streptomycin (GIBCO).

2.2.2 Preparation of Cell Lines

After the cells were 70%--80% confluent, the old medium was decanted off. Cells in the flask were washed with 10ml phosphate buffered saline (PBS) (Appendix 3) for three times. 3.5ml (Caco-2 and HT-29) or 1ml (HCT116) 1xTrypsin-EDTA (GIBCO) were added into the flask and then incubated at room temperature for 5min (HCT116 for 1min) to obtain single cells. Cells were then resuspended in 10ml fresh medium and transferred to a sterile falcon tube. For THP1 cell line, supernatants were taken out directly from the flask to a sterile falcon tube. After pelleted down by centrifugation at 800g for 5min, cells were resuspended in an appropriate amount of fresh medium. Cell viability was assessed by Hemo Cytometry method exclusion technique. In brief, 100µl cell suspension was mixed well with 100µl Trypan blue vital dye (GIBCO-BRL, USA). The mixture was loaded to chambers from sample loading point (Pic 1). Totally eight corners of the two chambers were counted (illustrated in Pic 2). The final cell number is n = N (total number)/8 x 2 x 10^4 cells/ml. Appropriate dilution were made to get the proper number of cells. 1ml of the culture medium containing 10^5cells/ml was then added into each well on a 24-well plate (Nalge Nunc International, USA). The plate was incubated at 37°C with 5% CO_2 for 24h and ready for use. To harvest protein, 10^6cells/ml were seeded into the 6-well plates (Nalge Nunc International, USA) and cultured at 37°C with 5% CO_2 for 24h.

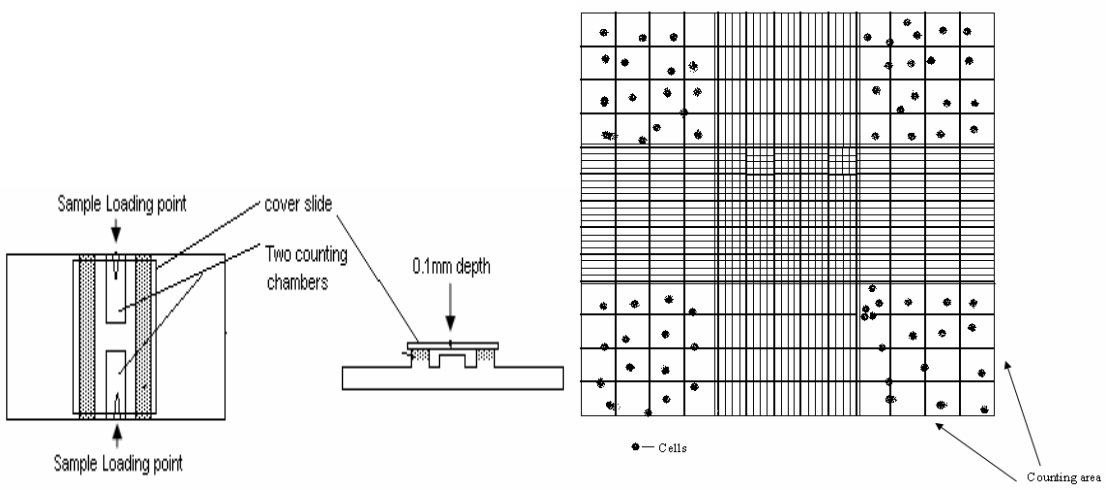


Figure 2.1 Left: overview of the Hemocytometer. Right: the picture of one counting chamber. The black dots on it represent cells to be counted. Only four big corner-squares were counted in this method.

2.3 TREATMENT OF CELLS

Biological triplicates were done for the following experiments.

2.3.1 Co-culture of Bacteria and Cells

To obtain a polarized monolayer of intestinal epithelial cells (IECs), $1x10^5$ Caco-2 cells were grown in a sterile 24-well plate (Nalge Nunc International, USA) to confluence and then for 2 additional weeks to reach full differentiation and polarization. The cultured medium was changed every two days. For HT-29 and HCT116 cell lines, $1x10^5$ cells were cultured in sterile 24-well flat-bottom plates (Nalge Nunc International, USA) for 24 hours before the infection experiments as described in section 2.2.2. For the infection study, Caco-2, HT-29 and HCT116 cells were incubated in fresh medium with bacteria at a multiplicity of infection (MOI) of 100 or 1,000 for 6 hours at 37°C with 5% CO_2. For protein harvesting, a MOI of 100 was applied to HCT116 and Caco-2 cells.

THP1 cells were treated with *E. faecalis, L.GG, S. thermophillus* 10387 (10387) with a MOI of 1, 10, 100 or 1000 for 30min, 1h, 2h, 4h, 6h and 24h. LPS were used at a concentration of 100ng/ml to stimulate the cells for 3h, *E. faecalis* strains were then added into them. In another experiment, *E. faecalis* strains were first added into the THP1 cells for 1h and then LPS at a final concentration of 100ng/ml was added and incubated for another 3h. Sonicated *E. faecalis* EC3 cells (Described in section 2.5.2) were also used to treat the THP1 cells at a MOI of 1 for 1h and a MOI of 100 for 6h. Supernatants were harvested for Enzyme-linked immunosorbent assay (ELISA) assay and cell lysates were harvested for protein measurement. RNAs were extracted using the method described in section 2.7.1 for gene expression analysis.

2.3.2 TNF-α and IL-1β Induced Cytokine Secretion and Protein Production

The Caco-2 and HCT116 cells were prepared as described in section 2.2.2, TNF-α (Preproteck, INC, rocky hill, NJ) was added at a concentration of 200ng/ml and IL-1β (Preproteck, INC, rocky hill, NJ) at a concentration of 2ng/ml. *E. faecalis* EC16 was prepared as section 2.1.4. Cells were then infected with *E. faecalis* EC16 with or without TNF-α / IL-1β. TNF-α and IL-1β were also used to treat the cells individually. The cells were then cultured at 37°C with 5% CO_2 for 30mins, 1h, 2h, 4h and 6h. Supernatants were collected and the concentration of IL-4, IL-10, IL-6, IL-8, TGF-β and IL-12(p70) were determined by ELISA. Other cytokines like IL-18, IFN-γ, TNF-α, IL-2, IL-5, IL-17 and ICAM-1 were determined using cytokine assay. The proteins were harvested for Western blotting assay.

2.3.3 *S. typhimurium* Induced Cytokine Secretion and Protein Production

The Caco-2 and HCT116 cells were prepared as in section 2.2.2. *E. faecalis* EC16 and *S. typhimurium* were prepared as in section 2.1.4. They were then added to cells individually with a MOI of 100. In order to see whether *E. faecalis* EC16 could suppress the inflammation activated by *S. typhimurium*, *E. faecalis* EC16 and *S. typhimurium* were added to the cells with a total bacteria number at a (MOI) of 100. Supernatants were collected for ELISA and cytokine assay. Proteins were harvested for Western blotting assay.

2.3.4 Protein Inhibitors Study

The Caco-2 and HCT116 cells were prepared as in section 2.2.2. Phosphorylated p38 inhibitor SB203580 iodo (SB), Phosphorylated JNK inhibitor SP600125 (SP) and DUSP1 inhibitor Triptolide (TRI) were purchased from Calbiochem (La Jolla, CA). A final concentration of 10μM of SB and SP was employed to the cells. For Triptolide, we used a final concentration of 1μM. Cells were cultured with these inhibitors or a combination of SB and SP for 30min, 1h, 2h and 6h in an incubator at 37°C with 5% CO_2. Moreover, inhibitors were also added into the cells with the presence of 2ng/ml IL-1β. Supernatants were harvested for ELISA assay and proteins were harvested for Western blotting analysis.

2.3.5 Adhesion Study

Monolayers of HCT116 were prepared by inoculating on 2-chamber slides (Lab-Tek Chamber Slide, Nunc, Inc., USA) with 1.4 x 10^5 cells per chamber in 2ml of complete DMEM 24h before the adhesion assay. After 24h, the monolayers were washed twice in 1ml of sterile PBS. Bacterial strains were prepared and 2ml of each suspension was added to a chamber of the slide and incubated at 37°C with 5% CO_2 for 1h. After incubation, the monolayers were washed twice with sterile PBS then Gram stained and examined microscopically. The numbers of adherent bacteria per 100 HCT116 cells were counted in 10 random microscope areas.

2.3.5.1 Gram Staining

After washing the cells, the slides were flooded with crystal violet and stood for 1min. The crystal violet was then washed away for 5s with running water. After that, the slides were covered with iodine solution and stood for 1min as well. When time has expired, the slides were rinsed with running water for 5sec and then applied with 95% ethanol to wash off crystal violet. The slides were then rinsed with running water to wash away ethanol. The final step involved the application of the counterstain safranin. The slides were flooded with the dye for 1min. And again the slides were rinsed with water to remove any excess dye. The slides were then blotted to dry and examined under microscope.

2.4 MEASUREMENT OF CYTOKINES

2.4.1 ELISA

Cytokines in cell culture supernatants were assayed by Enzyme Linked Immunosorbent Assays (ELISAs). After the infection (described in section 2.3), supernatants were harvested and centrifuged for 10min at 12,000g to remove the bacteria. IL-8, IL-4, IL-10, TGF-β, IL-6 and IL-12(P70) measurements were performed as the instructions in the ELISAs kits (BD bioscience, San Diego, CA). Individual samples were tested in triplicate and the concentration of cytokines was determined using the standard provided by the manufacture. To get the accurate results, samples with a high production of the cytokines were diluted to make the readings fall within the standard range. To test IL-8 expressed inside the cells using total protein harvested, a 50x dilution was done. In Brief, a 96 well plate (Nune, Roskilde. Denmark) was coated with 100µl per well of capture antibody diluted in coating buffer (Appendix 4). The plate was sealed and incubated overnight at 4°C. The wells were then washed 3 times with 300µl/well wash buffer (Appendix 4). For each wash, invert plate and blot on absorbent paper to remove any residual buffer. The plate was further blocked with 200µl/well assay diluents (Appendix 4). Incubate the plate at room temperature (RT) for 1h. And wash as above. The standard and sample were prepared in assay diluents. 100µl of each standard, sample, and control were added into appropriate wells. Again, the plate was sealed and incubated for 2h at RT. The plate was washed as described above but with 5 total washes. 100µl of prepared working detector (Detection Antibody + Avidin-HRP reagent) were applied to each well and incubate for 1h at RT. After this step, the plate was washed total 7

times with 30sec to 1min incubation with wash buffer for each wash. 100µl of substrate solution (BD bioscience, San Diego, CA) was then added into each well and plate was incubated for 30min at room temperature without sealer in the dark. After 30min, 50µl of stop solution (Appendix 4) were added into each well. And absorbance was read at 450nm within 30min of stopping reaction. An absorbance at 570nm was subtracted from absorbance 450nm to obtain the more accurate readings.

For TGF-β assays, 100µl supernatants were transiently acidified with 2µl 1M HCL for 1 hour at 4°C, and then neutralized with 2µl 1M NaOH. The supernatants were then applied to TGF-β ELISAs (BD bioscience, San Diego, CA) according to the manufacturer's instructions.

2.4.2 Cytokine Assay

The level of IL-18, ICAM-1, IFN-γ, TNF-α, IL-2, IL-5, and IL-17 in the culture supernatants was determined using Bio-Plex Cytokine Assay (Bio-Rad, USA) according to the manufacture's instruction. In brief, a 96-well filter plate was pre-wet with 150µl of Bio-Plex assay buffer. The buffer were then removed by vacuum filtration using MultiScreen Vaccum Manifolds (Millipore, USA) and the bottom of the filter plate was blotted to dry with a clean paper towel. 50µl of pre-mixed multiplex beads (Anti-cytokine conjugated) was added per well and the buffer was removed by vacuum filtration. The beads were then washed twice with 100µl of Bio-plex wash buffer. 50µl of sample obtained as described in section 2.3 was added into each well. The filter plate was sealed with plate sealing tape and wrapped with aluminum foil to protect the beads from light. Room temperature incubation was applied to the plate with shaking at 1,100rpm for the first 30sec and 300rpm for the next 30min. The buffer was then removed and the beads were washed three times

with 100µl of wash buffer before 25µl of 50x Bio-Plex detection antibody adding to each well. The plate was sealed, covered with aluminum foil and incubated at the same condition as described above for 30min. The buffer was then removed and beads were washed for three times with 100µl wash buffer. 100x Streptavidin-PE was diluted and vortexed before adding 50µl/well. The plate was then sealed and shaken at 1,100rpm for 30sec, then 300rpm for 10min in the dark. The buffer was then removed and beads were washed three times with 100µl wash buffer. The beads were then resuspended with 125µl of Bio-Plex assay buffer and the plate was shaken at 1,100rpm for 30sec immediately before reading the plate on Bio-Plex System (Bio-Rad, USA).

2.5 BACTERIA MACRO-MOLECULES ANALYSIS

2.5.1 Conditional Medium and Cell Inserts

In order to test for the location of the active components on the bacteria which might play a role in the suppression of inflammatory responses, the cell culture supernatants obtained from section 2.3.1 were harvested sterilely and conditional media were then added to the cell cultures prepared as described in section 2.2. In addition, bacteria themselves were also cultured with the control supernatants obtained from section 2.2 treatments. All the treated cells were then incubated at 37°C with 5% CO_2 for 6h. The supernatant were then collected for cytokines assay.

For cell inserts test, HCT116 cells were plated in wells of Transwell® (USA) multiple well plate and incubated at 37°C with 5% CO_2 for 24h. Accompanying

inserts were added 1h before co-culturing. 100µl of bacterial suspensions were added to each insert.

Triplicates of each condition were done and supernatants were harvested for cytokine assay.

2.5.2 Cell Wall Component and Crude Cell Extract Preparation

Before sonication, a protease inhibitior with 2mM phenylmethylsulfonylfluoride (PMSF), 1mM dithiothreitol (DTT) and 1mM sodium metavanadate was added to bacterial suspensions. The bacterial cells were then disrupted by sonication (MSE Soniprep 150, SANYO, Japan) on ice for 10 cycles with 30sec pulses and 1min rest (Shon *et al*., 2005). 100µl of the sonicated bacteria was then observed under microscope to ensure disruption of the cells. Cell debris was pelleted down by centrifugation at 15,000g for 60min at 0°C. The bacterial culture supernatant was aseptically kept on ice for use. The remaining pellet was resuspended in an appropriate volume of complete DMEM such that cell debris in each ml of pellet suspension was equal to the amount in 1ml of whole bacteria cells. 1ml of each supernatant, cell debris suspension and un-centrifuged sonicated extract were added to the cell culture. Biologically triplicates were done on this experiment.

2.5.3 Protein Digestion and Carbohydrate Oxidation of Bacterial Cell Wall

4ml of appropriate amount of bacterial cells were pelleted by centrifugation at 12,000g at 0°C for 10min. The bacterial cells were then washed once in PBS and then suspended in 1.5ml of one of the following solutions in Table 2.3:

Table 2.3 Chemicals and enzyme solutions for treatment of bacteria

	Solution	In buffer	pH
1	0.05M sodium m-periodate	A: 5.0M LiCl in water; 0.1M citrate-phosphate-0.1M NaCl	4.5
2	0.05M sodium iodate	A: 5.0M LiCl in water; 0.1M citrate-phosphate-0.1M NaCl	4.5
3	5.0mg of trypsin per ml of buffer	B: 0.05M Tris-HCl-0.1M NaCl	8.0
4	5.0mg of chymotrypsin per ml in buffer	B: 0.05M Tris-HCl-0.1M NaCl	8.0
5	5.0mg of pepsin per ml in buffer	C: 0.05M glycine-HCl-0.1M NaCl	2.2

The bacterial cells were incubated with the five solutions for 1h at 37°C. After that, the bacteria were centrifuged at 12,000g at 0°C for 10min, washed twice in 1ml of PBS and resuspended in 4 ml of complete DMEM. 1ml of the each re-suspension was added to 1 well containing cell culture. Biological triplicates of each condition were done.

2.5.4 Blocking of Specific Carbohydrate Ligands and Receptors on Cell Wall

3ml of bacterial cells and broth culture supernatant were separated by centrifugation at 12,000g at 0°C for 10min. The bacterial cells were then washed once in PBS and then suspended in 3ml of one of the following solutions-15μg/ml Concanavalin A (ConA) in PBS; 15μg/ml succinyl Concanavalin A (succinyl ConA) in PBS and 15μg/ml *Tetragonolobus purpurea* in PBS. The cells were incubated for 30min at 37°C, centrifuged at 12,000g at 0°C for 10min, washed twice in 1ml of PBS, and resuspended in 3ml of complete DMEM. Treatments were done as described in section 2.3.

The ability of carbohydrates to inhibit the anti-inflammatory response by binding to receptors specific for carbohydrates on the HCT116 cell surface was

assayed by adding different sugars – mannose, D-galactose, D-trehalose, N-acetyl glucosamine, D-glucose and D-fucose at a final concentration of 10mg/ml to the intestinal cells. Sugars were added to the HCT116 cells for 1h incubation at 37°C in 5% CO_2 before co-culturing with bacteria (Guzman, *et al*. 1991). When the bacteria were added, extra sugar was added to the bacterial suspension to ensure that the final concentration remained at 10mg/ml.

2.5.5 UV- and Heat-Killed Bacteria

3ml of bacterial suspensions were exposed to UV light for 5min (Conner-Kerr, *et al*. 1998) to make sure at least 99% bacteria were killed. To kill the bacteria by heat 3ml of bacterial suspensions were put on a heating block at 100°C for 20min. The killed bacteria were plated on their respective agar plates to ensure cell death. After killed the bacteria, treatments were performed as described in section 2.3.

2.6 APOPTOSIS ANYLISIS

Propidium iodide staining assay was applied to colon cancer cell line: HT-29, HCT116 cells and colon normal epithelial cells CRL-1790 (both floating and attached cells) to detect apoptotic cells after treatment with various LABs. Cells cultured with medium only were used as a control. In the propidium iodide (PI) staining assay, the supernatants and the trypsinised cells were collected in 15ml falcons' tube and pelleted. The pellet was washed twice with ice cold PBS and then resuspended in 500µl of PBS. 4.5ml of 70% ethanol was added while vortexing gently followed by incubation for at least 30min at −20°C. Fixed cells were washed twice and allowed to rehydrate in PBS. Prior to fluorescence-activated cell sorting (FACS) analysis, the

cells were resuspended in 500µl sample buffer (PBS containing 0.1% glucose) with 5µl 1mg/ml propidium iodide and 10µl 100µg/ml RNaseA and incubated for 30min. With this method, the percentage of apoptotic cells was determined by quantification of the sub-G_1 fraction of cells. In this assay, 20,000 fluorescent events were measured by a FACScalibur flow cytometer (Becton Dickinson Immunocytometry Systems; San Jose, CA) for each sample. 10^8cfu/ml bacteria only were used as a control in the flow cytometry analysis. Flow cytometric data were analyzed using WinMDIv2.8 software.

2.7 GENE EXPRESSION ANALYSIS

2.7.1 RNA Extraction

RNA was extracted after a 6h incubation of cell culture with 1 x 10^7 or 10^8cfu/ml of bacteria (Section 2.3), using the High Pure RNA Isolation Kit (Roche Applied Science, Germany). The wells were washed once with 1ml of ice cold PBS to remove excess bacterial cells, and resuspended in 200µl of PBS. Lysis-binding buffer was added to each well (400µl per well) and residual cells were scrapped off the well bottom before adding the cell lysate to the filter and collection tubes. The tubes were centrifuged at 10,000g for 15sec to remove the buffer while RNA remained bound to the glass fiber fleece on the filter tubes. DNase was added to each tube to digest contaminating DNA for 15min. The RNA was purified from salts, proteins and other cellular impurities using the wash buffer provided in the kit followed by centrifugation before elution from the glass fiber fleece. RNA was then kept at -70°C immediately.

2.7.2 RNA Quantification

RNA extracted was diluted 200 folds in sterile diethylpyrocarbonate (DEPC; Sigma, USA) treated water before measuring the optical density at 260nm using quartz cuvettes. The RNA was quantified using the following equation:

Amount of RNA present in sample (μg of RNA/ml) = OD_{260} x 40 x Dilution Factor

In addition, optical density was also measured at 280nm to quantify any contaminating proteins. A ratio of OD 260: OD 280 has to be between 1.8 and 2.0 to exclude any protein contamination in the RNA obtained.

The integrity and quality of RNA were determined by running formaldehyde gel. In brief, to form the formaldehyde gel, 87.5ml sterile distilled water, 1.5g agarose, 10ml 10xMOPS, 1.6ml formaldehyde were mixed and micro-waved for 2min to get a clear gel solution. Before casting of the gel, the solution was added 2μl ETBR and mixed well. To prepare the sample, 15.5μl master mixture (2μl 10x MOPS, 3.5μl formaldehyde, 10μl formamide) (Appendix 6) together with 4.5μl sample were mixed and incubated at 65°C for 10min. The samples were then cooled on ice for another 10min and loaded into the gel wells. To view the gel image, the gel was destained in deionized water for 45min and then viewed under UV light.

2.7.3 Reverse Transcription-Polymerase Chain Reaction

cDNA were synthesized from a template of 1μg total RNA using the Superscript III (Invitrogen Life Technologies, UK) according to the manufacture's instruction. In brief, 1μg of RNA, 1μl of 50μM oligo$(dT)_{20}$ primers were added to 1μl of a 10mM dNTP mix, and DEPC-treated water was used to make up a final volume of 10μl. The mixture was incubated at 65°C for 5min before placing on ice for at least

1min. A total volume of 10µl of the following cDNA synthesis mix was added to each reaction tube: 2µl of 10x RT buffer, 4µl of 25mM $MgCl_2$, 2µl of 0.1M DTT, 1µl of 40U/µl RNaseOUT™, and 1µl of 200U/µl SuperScript™ III Reverse Transcriptase enzyme. The cDNA synthesis reaction was carried out at 50°C for 50min, and terminated by heat inactivation at 85°C for 5min before chilling on ice. 1µl of *E. coli* RNase H (2U/µl) was then added and kept at 37°C for 20min, to remove the RNA template from the cDNA:RNA hybrid molecule after first strand synthesis, so as to increase the sensitivity of the subsequent PCR step.

DNA contamination was excluded by the use of a reverse transcriptase negative control for each sample and performing PCR with β-actin primers. Reverse transcriptase enzyme positive and negative samples were also run as an internal experimental control.

PCR were performed using 2µl of the cDNA as a template in a final volume of 50µl. The standard program used was as follows: denaturation for 2min at 94°C, 35 cycles of 30sec treatment at 94°C, 1min at 55°C or 60°C, and 1min at 72°C, followed by a final elongation for 7min at 72°C. 0.2 unit of Taq (Invitrogen Life Technologies, UK) was used for one PCR. H_2O was used as a negative control and plasmids containing the specific gene were used as a positive control in the PCR. After electrophoresis in a 1.2% agarose gel containing Ethidium Bromide, PCR products were quantified using the Gene Tool software (Syngene, UK). The primers used and annealing temperature are described in Table 2.4.

In this study, the expression of TLR1, TLR2, TLR3, TLR4, TLR5, TLR9, Tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) and Toll inhibitory protein (Tollip) were measured using RT-PCR. Cytokine gene expression like IL-8, IL-4, IL-10, TGF-β, TNF-α, were also detected using this method.

Table 2.4 PCR Primers and the annealing temperatures used for PCR

Gene Name	Primer	Polarity	Annealing Temp. (°C)	Length (bp)
β-actin	GGCGACGAGGCCCAGAGCAAGAGAGGCAT	Forward	55	460
	CGATTTCCCGCTCGGCCGTGGTGGTGAAGC	Reverse		
IL-4	AACACAACTGAGAAGGAAACCTTC	Forward	55	276
	GCTCGAACACTTTGAATATTTCTC	Reverse		
IL-8	ATGACTTCCAAGCTGGCCGTGGCT	Forward	55	289
	TCTCAGCCCTCTTCAAAAACTTCTC	Reverse		
IL-10	ATGCCCCAAGCTGAGAACCAAGACCCA	Forward	55	352
	TCTCAAGGGGCTGGGTCAGCTATCCCA	Reverse		
TGF-β	TGACAGCAGGGATAACACACT	Forward	55	288
	GTAGGGGCAGGGCCCGAGGCA	Reverse		
TLR1	CTATACACCAAGTTGTCAGC	Forward	55	219
	GTCTCCAACTCAGTAAGGTG	Reverse		
TLR2	GCCAAAGTCTTGATTGATTGG	Forward	55	346
	TTGAAGTTCTCCAGCTCCTG	Reverse		
TLR3	GATCTGTCTCATAATGGCTTG	Forward	55	304
	GACAGATTCCGAATGCTTGTG	Reverse		
TLR4	CTTTATCCAACCAGGTGC	Forward	50	650
	GGAATGCTGGAAATCCAG	Reverse		
TLR5	TAGCTCCTAATCTGATG	Forward	50	437
	CCATGTGAAGTCTTTGCTGC	Reverse		
TLR9	GTCCCCACTTCTCCATG	Forward	55	259
	GGCACAGTCATGATGTTGTTG	Reverse		
TNF-α	CAGAGGGAAGAGTTCCCCAG	Forward	55	324
	CCTTGGTCTGGTAGGAGACG	Reverse		
Tollip	CGCGGTACCGCCACCATGGCGACCACCGTCAGC	Forward	60	850

	GCCGGGCCCTGGCTCCTCCCCCATCTG	Reverse		
TRAF 6	GTCGGTACCGCCACCATGAGTCTGCTAAACTGTGAA	Forward	60	1600
	CGCGGGCCCCTATACCCCTGCATCAGTAC	Reverse		

2.7.4 Semi-Quantitative Gene Expression Analysis (Densitometry)

The bands obtained after gel electrophoresis were analyzed using a densitometry software (Gene Tools; Syngene, UK), a semi-quantitative method to compare gene expression intensities. The intensities were normalized by analysis of expression of the gene encoding β-actin which acts a loading control between samples. Thereafter, the samples were compared against the control well (without bacteria treatment) to obtain the gene expression fold difference.

2.8 MICROARRAY ANALYSIS

2.8.1 RNA Extraction and Probe Labeling

RNA was extracted according to the protocol described in section 2.7.1. First-strand cDNA was synthesized using AmpoLabeling-LPR Kit (Superarray, USA) according to the manufacture's instruction. Briefly, 1.5μg of total RNA was used for constructing the cDNA. RNA was mixed with 1μl RT primer and appropriate RNase free H_2O to get a total volume of 10μl. The contents were mixed well by gentle pipetting up and down 2 to 3 times followed by a brief centrifugation to collect the mixture at the bottom of the tube. The mixture was then incubated at 70°C for 3min

followed by a brief centrifugation. Tubes were placed on ice immediately and kept at 37°C for 10min. The cDNA Synthesis Master Mix were prepared as below (Table 2.5):

Table 2.5 cDNA Synthesis Master Mix

cDNA Synthesis Master Mix	**Per Array (µl)**
RNase free H2O	4
Buffer **BN**	4
Rnase Inhibitor **(RI)**	1
Reverse Transcriptase**(RE)**	1
Final Volume	10

The cDNA Synthesis Master Mix was mixed well and put on ice for use. For each array, transfer 10µl of the cDNA Synthesis Master Mix to the 10µl Annealing Mixture. They were mixed well but gently with a pipetting up and down 2 to 3 times followed by a brief centrifugation to collect the mixture at the bottom of the tube. The mixtures were then continued to be incubated at 37°C for 25min. After that, they were heated at 85°C for 5min to hydrolyze the RNA and to inactivate the reverse transcriptase. The finished RT Reactions were then held on ice until the next step.

To prepare for the LPR cocktail (Labeling reaction), the followings (Table 2.6) were mixed well in a sterile PCR tube per Array.

Table 2.6 LPR Cocktail

Master Mix	**Per Array**
Buffer **L**	18µl
Buffer **AF (BLUE tube)**	9µl
Biotin-16-UTP from Roche	2µl
DNA Polymerase(LE)	1µl
Final Volume	30µl

For each array, add 30μl of the LPR Cocktail to each RT reaction (20μl) and mixed well but gently with a pipettor. Program the thermal cycle for Liner polymerase reaction (LPR) as follows:

85°C 5min

85°C 1min, 50°C 1min, 72°C 1min for 30 cycles

72°C 5min

Before denaturing the probes for hybridization, the incorporation efficiency of dUTP with probe was checked according to the manufacture's instruction (Superarray, USA). The labeled cDNA probes were then denatured by heating the tube containing the LPR at 94°C for 2min, and quickly chilling on ice. The cDNA probe is now ready for hybridization.

2.8.2 Hybridization

cDNA microarrays (GEArray S Series Human Immunology Signaling Pathways Gene Array) were obtained from Superarray (USA). A total of 406 human immunology signaling pathway related cDNA clones were detected in the experiments.

Before pre-hybridization, membranes were pre-wet by adding roughly 5ml of deionized water to the hybridization tube. Warm the GEAhyb Hybridization Solution (in the kit) to 60°C, and then add 2ml of the GEAhyb Hybridization Solution into each tube. The membranes were then pre-hybridized in the oven at 60°C for 24h with continuous agitation at 5-10rpm.

The hybridization step was done according to the manufacture's instruction. The target hybridization solution was prepared by adding 5μg of biotin labeled cRNA to 1ml of prewarmed GEAhyb Hybridization solution. The solution was then mixed

well by pipetting and kept at 60°C till use. The pre-hybridization solution from the hybridization tube was discarded and the Target Hybridization Mix was added into the membrane in the hybridization tube. The membranes were then hybridized overnight at 60°C with continuous agitation (5-10rpm). After hybridization, the Target Hybridization Mix was poured off. The membranes were washed by Wash Solution 1 (2x SSC, 1%SDS) 10min twice at 60°C and Wash Solution 2 (0.1x SSC, 0.5%SDS) 7min twice at 60°C with 20-30 rpm agitation. The signals were then detected using Chemiluminenscent detection Kit (Superarray, USA) according to the manufacture's instruction. Image were acquired by exposing to X-ray film (Koda, USA), the images were then scanned using image scanner (Amersham, UK) and analyzed.

2.8.3 Data Analysis

The signals were analyzed using the web-based GEArray Expression Analysis Suite (Supperarray, US). Intensity of the spot equal to the signal strength of gene tested subtract average signal of the negative controls. All the signals were normalized against the average signal of positive controls i.e. GAPDH in our study. The genes with an up regulation or down regulation at least 1.5 folds were selected for the further study. In this experiment, five replicates were done for one treatment. Statistical significance was assessed by student t test to control the false-positive rate ($P < 0.05$ was considered significant). Scatter plot images and clustering analysis was generated by the software. To obtain more accurate data, GeneSpring GX 3.7.1 software was also used to analyze the data. The signals with "absent" flag were excluded. Volcano plots were generated by the software with a P value available ($P< 0.05$ was considered as significant regulation.).

2.9 SIGNALING PATHWAY VERIFICATION

2.9.1 TaqMan Low Density Array (TLDA)

The RNA was harvested as described in section 2.7.1. A 48-well format Taqman low density array was designed for a subset of genes that were differentially expressed in the array experiments including two endogenous controls 18S and β-actin (Applied Biosystems, USA). Gene and ABI assay IDs are listed in Table 2.7. 0.5μg total RNA was converted to cDNA using High-Capacity cDNA archive kit (Applied Biosystems, USA) and 10μg cDNA in 100μl TaqMan universal PCR master mix (Applied Biosystems, USA) was used for each port and run on an ABI 7900 system (Applied Biosystems, USA). Data was analyzed using the SDS2.2 software where baseline and threshold settings were automatically adjusted.

Table 2.7 Gene ID and Taqman Primers ID

GeneBank ID	Human Gene ID	Gene symbol	Assay ID
NM_053056	Hs.523852	CCND1	Hs00277039_m1
NM_000077	Hs.512599	CDKN2A	Hs00233365_m1
NM_004379	Hs.516646	CREB1	Hs00231713_m1
NM_004417	Hs.171695	DUSP1	Hs00610256_g1
NM_005225	Hs.96055	E2F1	Hs00153451_m1
NM_005252	Hs.25647	FOS	Hs00170630_m1
NM_000874	Hs.549042	IFNAR2	Hs00174198_m1
NM_001556	Hs.413513	IKBKB	Hs00233284_m1
NM_000572	Hs.193717	IL10	Hs00174086_m1
NM_014432	Hs.445868	IL20RA	Hs00205346_m1
NM_000589	Hs.73917	IL4	Hs00174122_m1
NM_000584	Hs.624	IL8	Hs00174103_m1
NM_000634	Hs.194778	IL8RA	Hs00174146_m1
NM_002228	Hs.525704	JUN	Hs00277190_s1
NM_006116	Hs.507681	MAP3K7IP1	Hs00196143_m1
NM_005204	Hs.432453	MAP3K8	Hs00178297_m1
NM_002751	Hs.57732	MAPK11	Hs00177101_m1

NM_002754	Hs.178695	MAPK13	Hs00234085_m1
NM_002749	Hs.150136	MAPK7	Hs00611114_g1
NM_002752	Hs.484371	MAPK9	Hs00177102_m1
NM_004759	Hs.519276	MAPKAPK2	Hs00358962_m1
NM_002392	Hs.369849	MDM2	Hs00242813_m1
NM_005919	Hs.153629	MEF2B	Hs00232232_m1
NM_003684	Hs.371594	MKNK1	Hs00374376_m1
NM_005373	Hs.82906	MPL	Hs00180489_m1
NM_172390	Hs.534074	NFATC1	Hs00232342_m1
NM_003998	Hs.431926	NFKB1	Hs00231653_m1
NM_002502	Hs.73090	NFKB2	Hs00174517_m1
NM_003629	Hs.170510	PIK3R3	Hs00177524_m1
NM_006221	Hs.465849	PIN1	Hs00749260_s1
NM_006244	Hs.75199	PPP2R5B	Hs00196561_m1
NM_005903	Hs.167700	SMAD5	Hs00195437_m1
NM_005585	Hs.153863	SMAD6	Hs00178579_m1
NM_005904	Hs.465087	SMAD7	Hs00178696_m1
NM_005905	Hs.528630	SMAD9	Hs00195441_m1
NM_138473	Hs.524461	SP1	Hs00412720_m1
NM_003120	Hs.502511	SPI1	Hs00231368_m1
NM_000660	Hs.1103	TGFB1	Hs99999918_m1
NM_003264	Hs.519033	TLR2	Hs00610101_m1
NM_003265	Hs.29499	TLR3	Hs00152933_m1
NM_003266	Hs.174312	TLR4	Hs00152939_m1
NM_017442	Hs.87968	TLR9	Hs00152973_m1
NM_000594	Hs.241570	TNF	Hs00174128_m1
NM_003839	Hs.204044	TNFRSF11A	Hs00187192_m1
NM_019009		TOLLIP	Hs00184085_m1
NM_004620	Hs.444172	TRAF6	Hs00371508_m1
NM_001101		ACTB	Hs99999903_m1
		18S	Hs99999901_s1

2.9.2 Western Blotting

2.9.2.1 Protein Harvesting

After the treatments (Section 2.3), the supernatants were removed and cells were washed twice with PBS. 2ml of PBS were added in to the wells. Cells were then scraped from the plates and transferred into a tube. Cells were pelleted down to the

bottom of the tube by centrifugation at 800g for 5min (Sigma, USA). PBS was removed thoroughly and cell pellets were resuspended in ice cold 1x cell lysis buffer (Cell signaling technology, USA) and transferred into a 1.5ml tube. The suspension was then briefly sonicated with a medium power (25W) of a sonicator (MSE Soniprep 150, SANYO, Japan) for 3 cycles with 5sec on and 15sec rest to shear genomic DNA on ice. The tube was centrifuged at 14,000g (Eppendorf, Germany) for 10min at 4°C. Supernatants were kept for Bradford assay and pellets were discarded. 40µg of protein (for two pieces of gel) was aliquot and mixed with 3x SDS sample buffer (Appendix 8) with a final volume of 24µl. The mixture was then boiled at 95°C for 2min and stored at -20°C. Before use, the mixture was thawed on ice and 10µl was loaded to an SDS PAGE gel after a 2min boil at 95°C. The rest of the samples were kept at -20˚C for next time use.

2.9.2.2 Bradford Assay

After harvesting the protein, Bradford assay were conducted. A standard curve using BSA (0, 100, 200, 400, 600, 800, 1000 µg/ml) were made from a stock solution of 12mg/ml. 10µl of standards were added into a 96-well flat bottom plate in duplicate. 1µl of samples were added into the wells in duplicate. An appropriate dilution of the samples in distilled H_2O was necessary if the protein concentration was too high. This is to make sure the sample concentration fell within the range of 0-1000µg/ml. 190µl of diluted Bradford assay dye (Bio-Rad, USA) was added into each of the standard wells and 199 µl of diluted Bradford assay dye was added into each of the sample wells. The plate was kept at room temperature for 15min and the bubbles were removed using a needle. The plate's bottom was wiped using a clean tissue paper with alcohol and the reading at the absorbance of 595nm was taken. A standard

curve was obtained by using the wavelength reading at 595nm against protein concentration. The sample protein concentration was determined according to the standard curve with a multiple of 10 (the volume of standard is 10 times of sample). 20µg of protein was aliquot and mixed with 3x SDS buffer (Appendix 8).

2.9.3.3 Sodium-Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium-dodecyle sulfate-polyacrylamide gel electrophoresis was performed on a 10% gel by using 20µg per lane of whole cell lysate. Briefly, 10% separation gel (Appendix 8) was cast between two glass plates. The gel mixture was poured to about 2cm below the top of the glass. Isopropanol was layered over the gel mixture to ensure a straight meniscus and to exclude any bubbles which inhibits gel polymerization reaction. The gel was allowed to polymerize for approximately 15min before decanting the isopropanol. Stacking gel solution (Appendix 8) was then mixed well and added on the top of the separation gel. A comb was quickly put in position after pouring the stacking gel and the gel was allowed to set for 15min. The comb was carefully removed after gel polymerization. The gel assembly was immersed into the electrophoresis chamber filled with running buffer (Appendix 8).

The samples prepared as in 2.8.3.1 were then loaded into each well and also 5µl of the Precision Plus Protein Kaleidoscope standards (Bio-rad, USA). Electrophoresis was carried out using consistent voltage of 75 volts for approximately one and a half hours. After electrophoresis, the gel was then transferred onto a 0.22µm Nitrocellulose membrane (Biorad, USA) by using transfer buffer (Appendix 8). Filter papers, nitrocellulose membrane and sponges for transferring sandwich were presoaked in transfer buffer for at least 15min. The separating gel was placed between

a wet nitrocellulose membrane and the pre-wet filter papers. One piece of sponge was placed outside the filter papers on each side. The transblot cell was then immersed into the transfer chamber filled with transfer buffer with the separating gel side adjacent to the negative electrode. The transfer was carried out at 85 volts for 2h in cold room.

2.9.3.4 Immunoblotting

After transfer, the membrane was immersed in Ponceau S dye (Sigma, USA) for 1-2 minutes and then washed with running tap water to make sure that the transfer was done properly. The membrane was then blocked in Tris buffer saline-Tween (TBST) (Appendix 8) containing 5% skim milk for at least 1h. Primary antibodies (Table 2.8) were then diluted (1:500) in TBST containing 5% BSA or 5% milk according to the supplier's instruction. The membrane with the primary antibody was incubated overnight at 4°C on an orbital shaker (Bellco, USA).

After the incubation with primary antibody, the membrane was washed three times for 10min each with 10ml TBST. Appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Table 2.8) diluted in TBST containing 5% skim milk were added into the membrane and incubated for 1 hour at 4°C on an orbital shaker (Bellco, USA). After that, the membrane was then washed three times for 10min each using TBST. Visualization of the immunolabeled bands was then carried out using ECL Plus Western Blotting Detection Reagents or ECL Advance Western Blotting Detection Kit (GE Healthcare, UK) according to manufacturer's instruction . The signals were exposed on X-ray film (Koda, USA) and scanned into computer for analysis.

2.10 STATISTICAL ANALYSIS

Data were expressed as means ± SD. Significance of differences was determined using the Student's T-test and analysis of variance. p values < 0.05 were considered to be statistically significant.

Table 2.8 Primary and secondary antibodies used for immunoblotting

Primary antibodies and dillution	Source	Secondary antibodies and dillution	Source
Phospho-p38 (1:500)	Cell Signaling Technology (CST) (USA)	HRP-conjugated anti-mouse IgG (1:2000)	Santa Cruz Biotechnology, Inc (Germany) (SCB)
Total p38 (1:500)	CST	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
Phospho-ERK (1:500)	CST	HRP-conjugated anti-mouse IgG (1:2000)	SCB
Total ERK (1:500)	CST	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
Phospho-JNK (1:500)	CST	HRP-conjugated anti-mouse IgG (1:2000)	SCB
Total JNK (1:500)	CST	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
Phospho-c JUN (1:500)	CST	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
Total c-JUN (1:500)	CST	HRP-conjugated anti-mouse IgG (1:2000)	SCB
P-65 (1:1000)	SCB	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
P-50/105 (1:500)	CST	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
Caspase 1	CST	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
E2F1	SCB	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
PIN1	R&D Systems (USA)	HRP-conjugated anti-mouse IgG (1:2000)	SCB
IL-8R1	SCB	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
DUSP1	SCB	HRP-conjugated anti-rabbit IgG (1:2000)	SCB
Cyclin D1	SCB	HRP-conjugated anti-mouse IgG (1:2000)	SCB
β-actin	Sigma-Aldrich (USA)	HRP-conjugated anti-mouse IgG (1:2000)	SCB

Chapter 3

SCREENING OF LACTIC ACID BACTERIA

3.1 RESULTS

3.1.1 MEASUREMENT OF CYTOKINE PRODUCTION

IL-8 is an inflammation marker. In healthy tissues, IL-8 is barely detectable. However, it could be rapidly induced in response to proinflammatory cytokines, bacterial and virus products and cellular stress. In some cancer tissues, IL-8 is expressed constitutively. It has been suggested that IL-8 generated by cancer cells may promote tumor progression by facilitating inflammation and tissue damage**.**

To assess the effects of LAB on IL-8 secretion in IECs, Caco-2, HT-29 and HCT116 cells were incubated with the respective LAB strains listed in Table 2.1. THP1 cells were treated with EC1, EC3, EC15, EC16, L.GG and *S. typhimurium* only. The experiments were repeated 2-5 times using the method described in Material and Methods (section 2.3).

3.1.1.1 IL-8 Production in Caco-2, HT-29 and HCT116

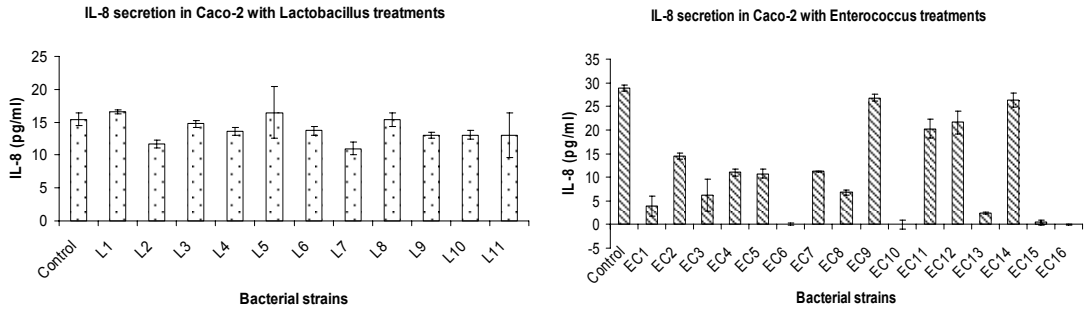
The anti-inflammation effects of the enterococci and lactobacilli isolated from infants were investigated by co-culturing them with intestinal epithelial cells (Caco-2, HT-29 and HCT116). Supernatants were harvested for cytokine assays as described in Materials and Methods.

Figure 3.1A-C showed that none of the *Lactobacillus* attenuated IL-8 secretion in Caco-2, HT-29 and HCT116 cells significantly. However, most of *Enterococcus* could suppress IL-8 production in Caco-2 and HCT116 cells (Figure 3.1A-C). Strikingly, only EC1, EC3, EC15 and EC16 showed the ability to suppresse IL-8 secretion in all these three cell lines (Figure 3.1A-C). We further tested these four

strains together with *L. rhamnosus GG* (L.GG) and *S. typhimurium* (Salm) with a multiplicity of infection (MOI) of 100 or 1000 for 2h, 4h, 6h and 24hours in HCT116 cells (Figure 3.1D). Data showed that the regulation of IL-8 production by ECs is dose dependent, the higher concentration of ECs the more suppression of IL-8 secretion (Figure 3.1D). L.GG, which is a commercial probiotics, could attenuate IL-8 either with 24 hours incubation or a higher concentration in HCT116 cells. *S. typhimurium*, as expected, stimulated IL-8 secretion in HCT116 at both high and low concentrations. The reduction of IL-8 secretion by *S. typhimurium* with MOI of 1000 at 24 hours might be due to the apoptosis of HCT116 cells

From the above data, we can see that EC1, EC3, EC15, and EC16 downregulated IL-8 secretion in all three IECs. With the treatment of other LAB listed in Table 2.1, most of them showed no regulation on IL-8 secretion in IECs. However, few strains showed the ability to inhibit IL-8 secretion. Together with EC1, EC3, EC15, EC16, these interesting LAB were treated with IECs for 6h with a MOI of 100 and 1000. Three independent experiments were done.

A



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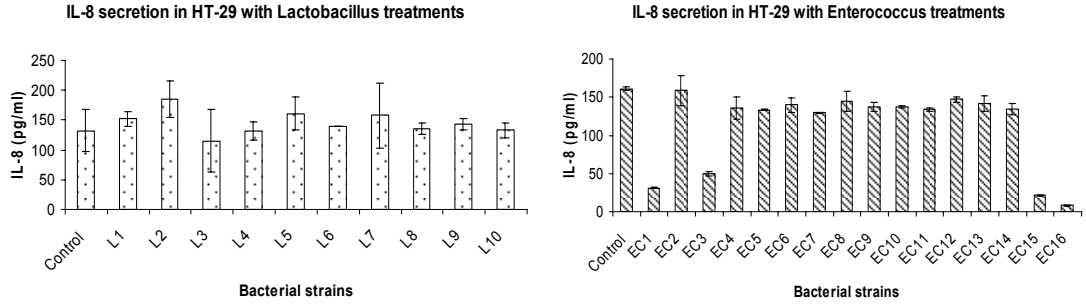

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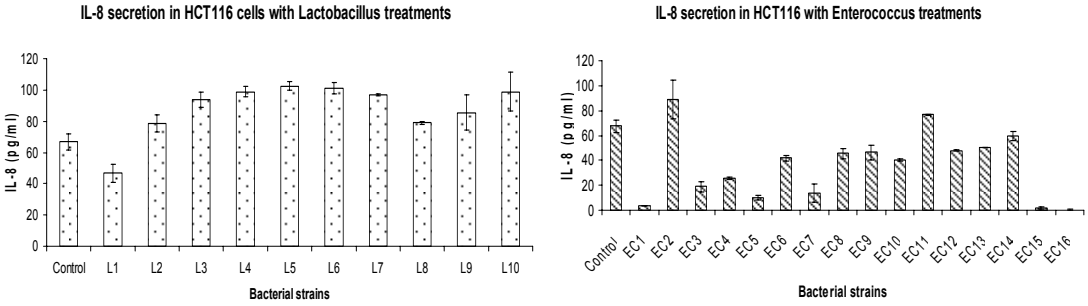

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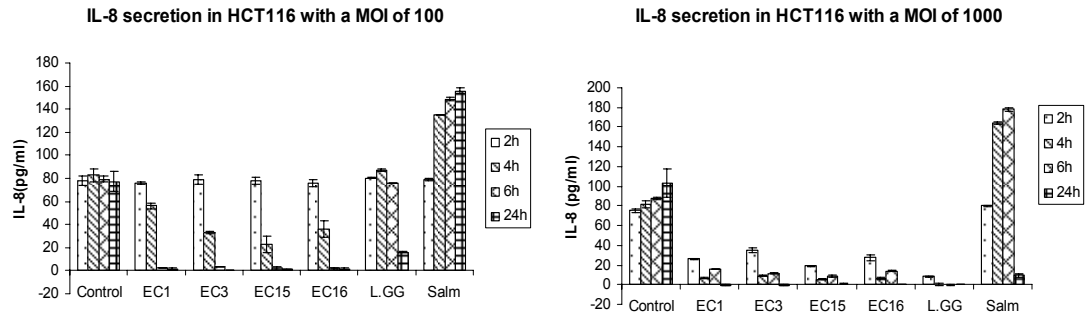
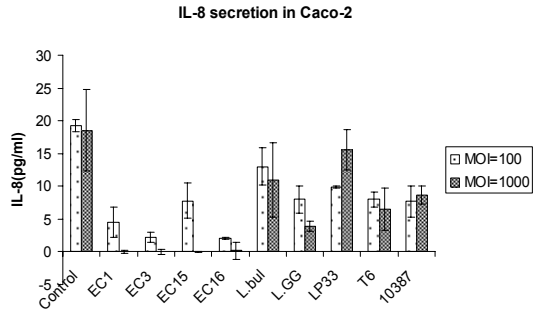


Figure 3.1 IL-8 secretions in Caco-2 (A), HT-29 (B) and HCT116 (C) with the treatment of *Lactobacillus* and *Enterococcus*. A total number of 10^7 cfu/ml bacteria were added into the cells for 6h. (D) HCT116 cells were co-cultured with EC1, EC3, EC15 and EC16 and *L. rhamnosus GG* (L.GG) and *S. typhimurium* (Salm) with a multiplicity of infection (MOI) of 100 or 1000 for 2h, 4h, 6h and 24h. Supernatants were harvested for cytokine assay as described in Materials and Methods. Three independent experiments were compiled to produce the data shown. Error bars indicated as the standard deviation.

A



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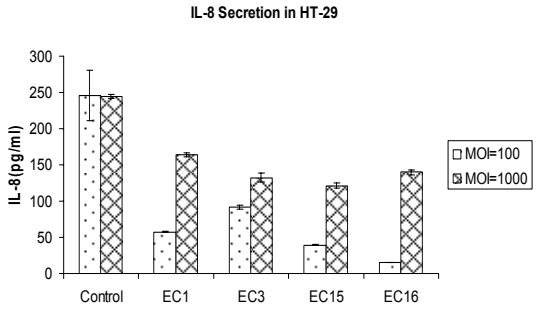

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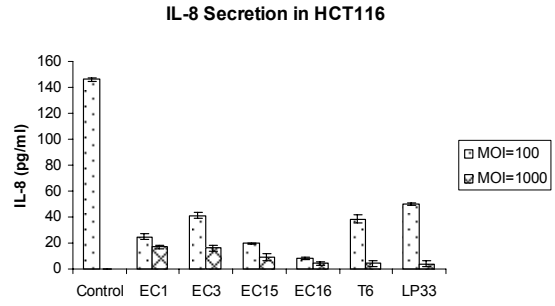


Figure 3.2 IL-8 secretions in Caco-2 (A), HT-29 (B) and HCT116 (C) cells treated with/without LAB for 6 hours. Bars represent mean cytokine production and the error bars represent standard deviation of mean. "Control" represents the consistent expression level of IL-8 without bacteria infection.

Figure 3.2 shows the IL-8 secretion in Caco-2, HT-29 and HCT116 treated with LAB. Most of the LAB tested (including strains of ECs) showed no significant regulation of IL-8 secretion (data not shown) in Caco-2, HT-29, and HCT116 cell lines. *Bifidobacterium,* which was reported to down-regulate TNF-α and IL-8 productions and inhibit NF-κB activation in inflamed mucosa of active ulcerative colitis (Bai *et al*., 2006), showed no effect on IL-8 secretion in all the three intestinal epithelial cell lines tested. However, EC1, EC3, EC15, EC16, *L. delbrueckii bulgaricus* D1 (L.bul), *L. rhamnosus GG* (L.GG), *L. paracasei* LP33 (LP33), *L. brevis* T6 (T6) and *Streptococcus themophillus* NCIMB10387 (10387) downregulated IL-8 secretion in Caco-2 cells (Figure 3.2A). In HT-29 cells, only EC1, EC3, EC15

and EC16 could downregulate IL-8 secretion (Figure 3.2B) among all the LAB tested. Whereas in HCT116 cell line, EC1, EC3, EC15, EC16, T6 and LP33 suppressed IL-8 secretion (Figure 3.2C).

Although all the 4 strains of ECs and other LAB inhibited the secretion of IL-8, it is interesting to note that different bacteria reduced IL-8 secretion to different levels after their exposure. ECs in general suppressed IL-8 production to a higher degree than other LAB. From Figure 3.2, we can see that the downregulation of IL-8 secretion was dose dependent in Caco-2 and HCT116. The higher the concentration of LAB, the less secretion of IL-8. However, 10^8cful/ml of LAB treated HT-29 cell secreted more IL-8 than 10^7cfu/ml of LAB treated cell did. This might be due to the competition of the adhesion site on the cell surface by ECs. The higher concentration of the bacteria might not adhere as well as the low concentration of ECs. Above all, these results demonstrate that ECs could suppress IL-8 secretion in human cancer cell lines and thus suppress inflammation. In this respect, our findings extend those of Ma *et al* findings who showed that *L.reuteri* inhibited constitutive synthesis and secretion of IL-8 in human cancer cell lines (Ma *et al*., 2004).

3.1.1.2 Production of Other Cytokines by Caco-2, HT-29 and HCT116

The balance between inflammatory and regulatory cytokines is of crucial importance in gut immune modulation. TGF-β is an important inflammation regulatory cytokine. In our study, TGF-β cytokine was greatly induced by EC3 and EC15 (10^7cfu/ml) in Caco-2 cells as shown in Figure 3.3. Other LAB, however, could not induce TGF-β cytokine secretion above the detection level (above 62.5pg/ml) in Caco-2 cells. In HCT116 and HT-29 cell line, no TGF-β cytokine were detected in the cell cultures (data not shown). TGF-β, which plays a key role in the control of

differentiation, proliferation, and state of activation of immune cells, is important in downregulating inflammatory cytokine response. These data suggest that the increase of TGF-β might account for the balance of the human intestinal immune system. Moreover, recent studies showed that TGF-β also plays an important role during malignant transformation (Pinkas *et al*., 2006). Tumor-suppressor activities of TGF-β are inactivated at a later stage of colon cancer. In addition, colon cancer usually arises from chronic inflammation. Therefore, upregulation of TGF-β would help the immune system to fight against inflammation to further inhibit malignant transformation.

In addition, IL-4 and IL-10, which are secreted by Th2 cells, are the dominant mediators of Th2 cell differentiation and proliferation. They have similar function in many anti-inflammatory and immunosuppressive activities. Both of them can inhibit the production of cytokines such as TNF-α, IL-1β, IL-6, IL-8 and IL-12. However, in our study, no IL-4 and IL-10 cytokine secretion were observed in Caco-2, HT-29 and HCT116 cells after all the LAB stimulation.

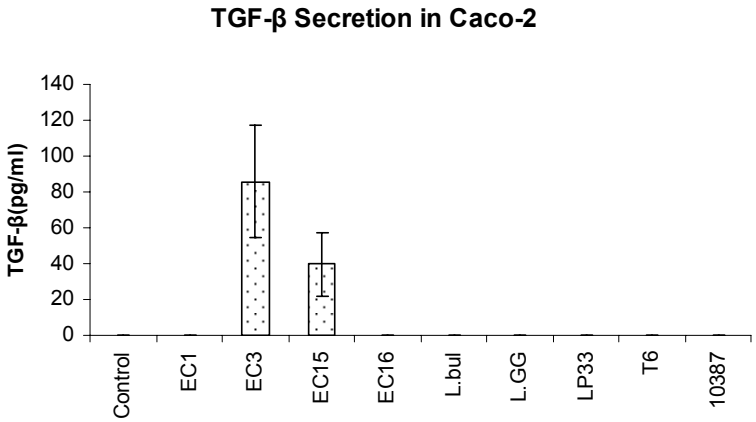
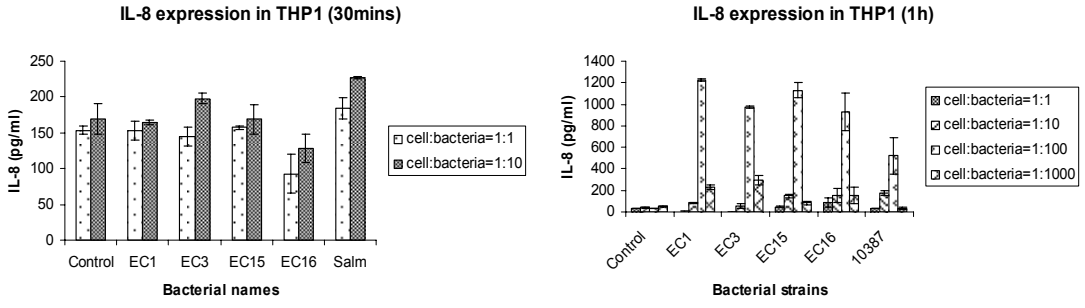


Figure 3.3 TGF-β production in Caco-2 cells treated with LAB for 6 hours. Bars represent mean cytokine production upon different strains treatments. The error bars represent standard deviation of mean. The ratio of cell: bacteria=1:100.

3.1.1.3 Production of IL-8 by THP1 cells

ECs suppressed IL-8 secretion in IECs, however, its regulatory function in macrophage is still unknown. THP1 is human acute monocytic leukemia cell line. We therefore co-cultured EC1, EC3, EC15 and EC16, *L. rhamnosus* GG (L.GG), and *S. thermophillus* NCIMB10387 (10387) with THP1 cells for 1h, 2h, 4h, 6h, 8h and 24h with a MOI of 1, 10, 100 and 1000. *S. typhimurium* (Salm) and EC1, EC3, EC15 and EC16 were also coculured with THP1 cells for 30mins at a MOI of 1 and 10. Supernatants were harvested for ELISA test. Figure 3.4A showed that ECs, which suppressed inflammatory responses in IECs, activated IL-8 secretion in THP1 cells. The effects are time and dosage dependent. IL-8 expression peaked with a MOI of 10 or 100 at various time points tested. Interestingly, L.GG and 10387 which are commercial probiotics also activated IL-8 secretion in THP1 cells (Figure 3.4A), suggesting that macrophage cells may not possess the ability to differentiate bacteria like IECs. Any bacteria in contact with macrophage would be considered as a foreign invader.

A



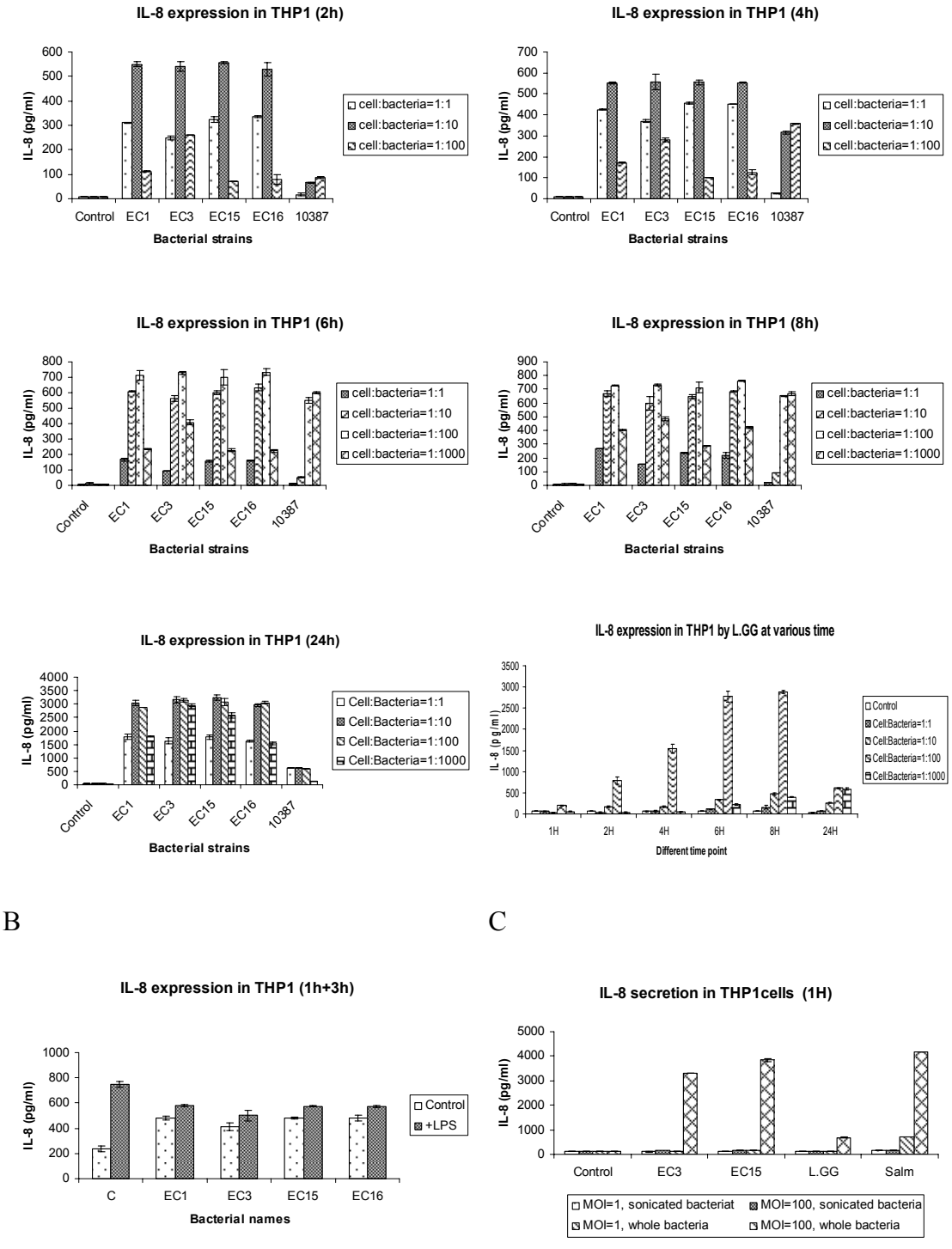


Figure 3.4 IL-8 production in THP1 cells. (A) IL-8 production in THP1 cells with the treatment of ECs, L.GG, Salm and 10387 for 30mins, 1h, 2h, 4h, 6h, 8h and 24h with a MOI of 1, 10, 100 and 1000. (B) IL-8 expression in THP1 with the treatment of LPS and ECs for 4 hours or LPS stimulated cells for 1h and then co-culture with ECs for 3 hours. (C) IL-8 expression in THP1 with the treatment of sonicated bacteria and whole cell with a MOI of 1 or 100 for 1h. Values are the averages of results obtained on three independent experiments.

With the stimulation of LPS, IL-8 went up to a very high level (Figure 3.4B). Although ECs stimulated IL-8 expression in THP1 cells, they suppressed IL-8 secretion stimulated by LPS (Figure 3.4B). More interestingly, live bacteria upregulated IL-8 secretion in THP1 cells, however, sonicated bacteria lost their ability to stimulate IL-8 expression in THP1 cells in one hour incubation (Figure 3.4C). From the above data, we can see that the suppression of IL-8 production by ECs is cell type dependent. THP1 responded to all types of bacteria tested, suggesting that any bacteria that enter the immune system would be a potential "pathogen". In addition, THP1 cells could only recognize bacteria in general but not differentiate them. The dead bacteria lost their ability to trigger IL-8 responses in THP1 cells however still had the ability to suppress IL-8 secretion in IECs, implicating the efficacy of using dead bacteria in suppressing inflammatory responses.

3.1.2 GENE EXPRESSION ANALYSIS

3.1.2.1 Semi-Quantitative Gene Expression Analysis

3.1.2.1.1 Cytokine Gene Expression Analysis

Since ECs modulated human intestinal immune balance through regulating IL-8 and TGF-β secretion, mRNA expressions of the inflammatory cytokines by the cell lines in response to ECs and other LAB treatments were studied. Each test was performed 2 times in triplicate. Data are expressed as mean value ± SD.

Differentiated Caco-2 monolayers were exposed for 6 hours with 10^7 and 10^8 cfu/ml EC1, EC3, EC15, EC16, L.GG, LP33, T6, 10387 and L.bul. Not consistent with IL-8 secretion, IL-8 mRNA expression was upregulated by 10^7cfu/ml EC1, EC3, EC15, EC16, L.GG and L.bul. Notably, T6 and L.bul could inhibit IL-8 mRNA

expression at 10^8cfu/ml (Figure 3.5), while other LAB had no effects on IL-8 gene expression at the concentration of 10^8cfu/ml (Figure 3.5).

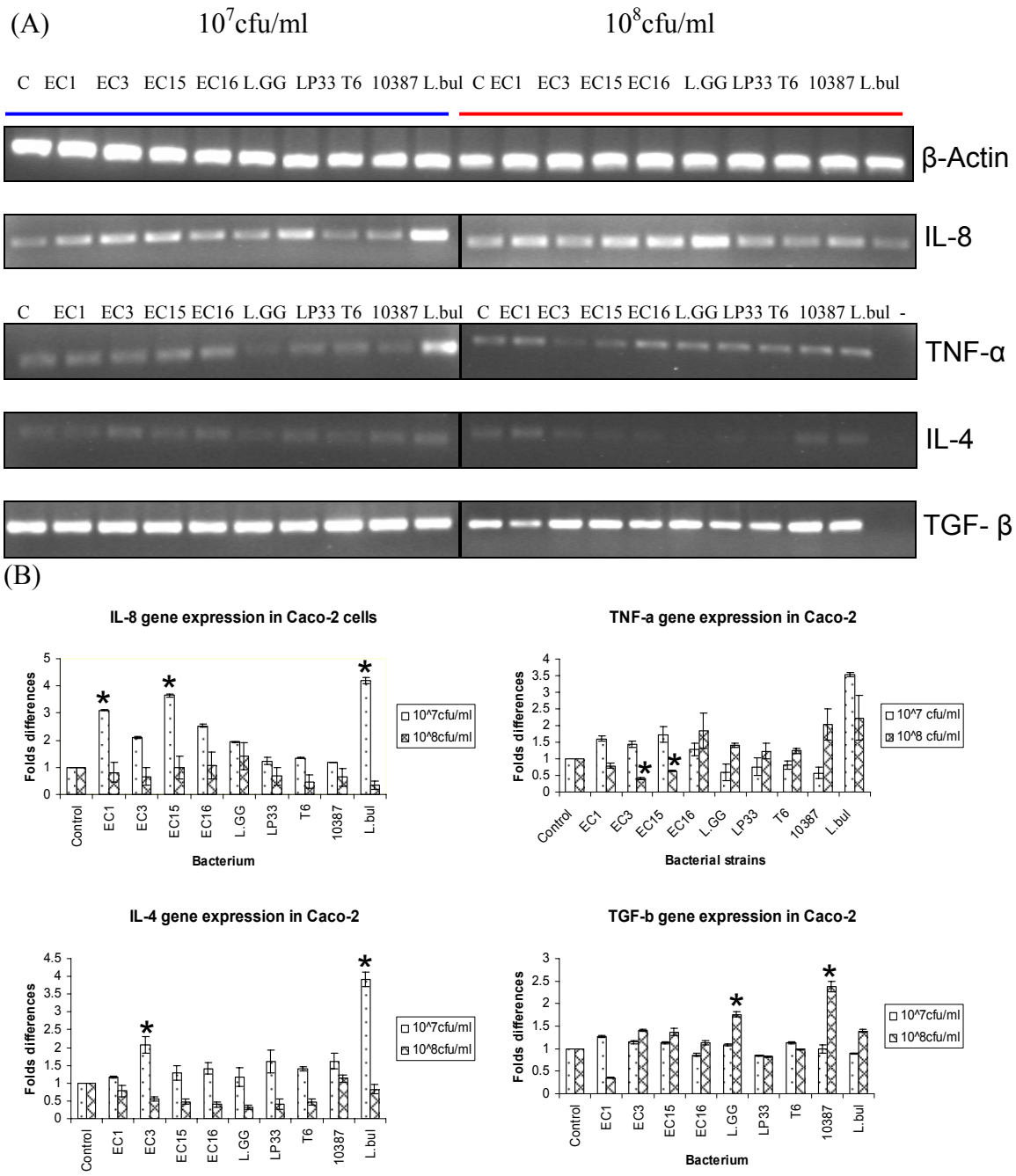


Figure 3.5 Different cytokine expressions in Caco-2 cells in response to LAB. RT-PCR analysis was used to determine IL-8, IL-4, TNF-α and TGF-β expression. (A) 10^7 and 10^8 cfu/ml EC1, EC3, EC15, EC16, L.GG, LP33, T6, 10387, and L.bul were treated to differenciated Caco-2. mRNA expressions of the cytokines were analyzed as described in Materials and Methods. Results represent one of three independent experiments. (B) Quantitative densitometry was carried out on EtBr-stained gels and the results from three independent experiments were compiled to produce the data shown. * Represent the significantly regulated genes.

Addition of EC1, EC3, EC15, EC16, LP33 and T6 (10^8cfu/ml) to HCT116 cells strongly suppressed IL-8 mRNA expression (Figure 3.6). However, 10^7cfu/ml EC1, EC3, EC16 and LP33 enhanced IL-8 mRNA expression in HCT116 cells. TNF-α is a primary mediator of immune stimulation and inflammatory responses. As shown in Figure 3.5B, TNF-α mRNA expression was stimulated by 10^7cfu/ml EC1, EC3, EC15 and *L.bul* in Caco-2 cells. However, TNF-α mRNA expression was suppressed at 10^8cfu/ml by EC1, EC3 and EC15. In HCT116 cells, TNF-α gene was expressed at a very low level with or without the treatment of LAB (data not shown). Interestingly, as we can see from Figure 3.6, TGF-β gene expression was greatly enhanced by 10^7cfu/ml EC1, EC3, EC15, EC16 and T6 in HCT116 cells. However, 10^8cfu/ml of LAB showed no regulation of TGF-β gene expression. In general, the above studies demonstrate that ECs could regulate the human intestinal immune system at mRNA level. The upregulation of TGF-β and downregulation of IL-8 mRNA by ECs in HCT116 cell lines might explain the suppression mechanism of the inflammatory responses in the human intestine. Given the evidence showing the downregulation of IL-8 secretion, the demonstrated ability of ECs to regulate IL-8 mRNA synthesis in HCT116 cell lines is particularly interesting. Hence, the regulation of IL-8 secretion might be at both transcriptional and post-transcriptional levels in HCT116 cell lines at the higher concentration of LAB treatment. However, at the lower concentration of LAB, the regulation of IL-8 secretion might be mainly at the post-transcriptional levels since IL-8 mRNA were enhanced by them. Furthermore, the mRNA level of the proinflammatory cytokine TNF-α was downregulated by ECs. TNF-α could amplify inflammatory responses by activating neutrophils, mononuclear phagocytes, eosinophils and stimulate IL-8 secretion. Thus, downregulation of TNF-α gene expression may further dampen the mucosal inflammatory response.

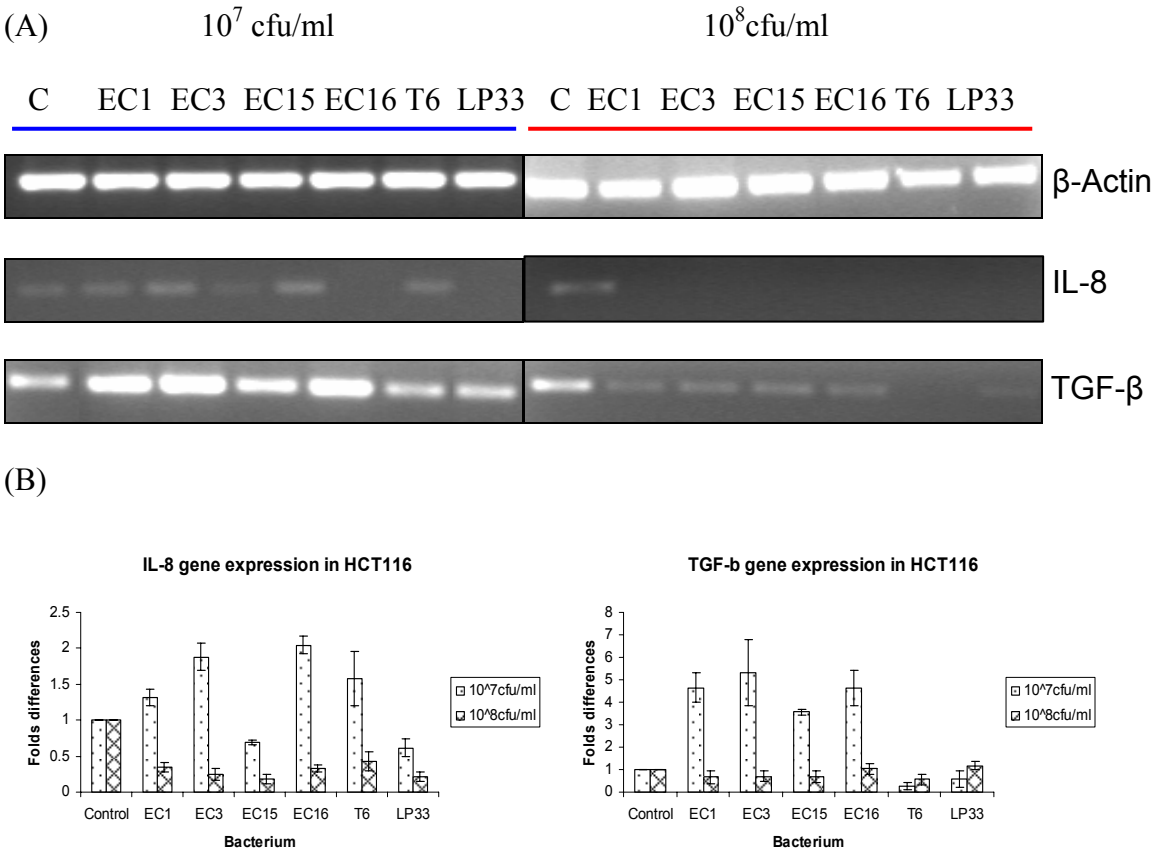


Figure 3.6 IL-8 and TGF-β expression in HCT116 cells in response to LAB. HCT116 cells were cultured for 6 hours in complete medium alone (control) or medium containing EC1, EC3, EC15, EC16, LP33 and T6 as described in Materials and Methods. (A) RT-PCR analysis was used for IL-8 and TGF-β after RNA was extracted. Results represent one of three independent experiments. (B) Quantitative densitometry was carried out on EtBr-stained gels and the results from three independent experiments were compiled to produce the data shown. The gel densities were normalized against β-actin.

3.1.2.1.2 TLR Signaling Pathway Gene Expression Analysis

Activation of signal transduction pathways by TLRs will lead to the induction of inflammatory cytokine and chemokine genes that function in host defenses and tumor progression. At least 13 TLRs have been identified in mammalian cells. They recognize a set of highly conserved pathogen-associated molecular patterns (PAMP). Among all the 13 members of TLRs, TLR3, TLR5, TLR9 are mainly expressed by epithelial cells and TLR2 is an important receptor in response to bacteria. In addition,

some findings suggest that epithelial cell derived TLR3 and TLR9 are capable of regulating proinflammatory cytokines. We therefore determined the expression of TLR1, TLR2, TLR3, TLR4, TLR5 and TLR9 in IECs upon ECs treatments in our study. The ability of the intestinal cell lines in response to ECs and other LAB at the level of TLRs mRNA expression was therefore assessed using semi-quantitative RT-PCR. Gene encoding TLR1, TLR2, TLR3, TLR4, TLR5, TLR9, TRAF6 and Tollip were analyzed.

TLR9 recognizes unmethylated CpG DNA in bacteria. Examination of the TLR9 gene expression showed that, 10^7cfu/ml EC1 and T6 downregulated TLR9 mRNA expression in Caco-2 cells (Figure 3.7). In HCT116 cells, although TLR9 mRNA was expressed very low, its expression was greatly inhibited by 10^8cfu/ml EC1, EC3, EC15, EC16, T6 and LP33 treatment (Figure 3.8).

TLR4 is an essential receptor that transduces the signals of bacterial cell wall lipopolysaccharide (LPS). In the TLRs signaling pathways, TLR4 could activate numerous cytokine genes via TRAF6. 10^7cfu/ml T6 and 10^8cfu/ml EC1 suppressed TLR4 expression in Caco-2 cells (Figure 3.7). In HT-29 cells, EC1, EC3 and EC15 but not EC16 (10^7cfu/ml) suppressed TLR4 mRNA expression (Figure 3.9). We could not detect TLR4 gene expression in HCT116 cells

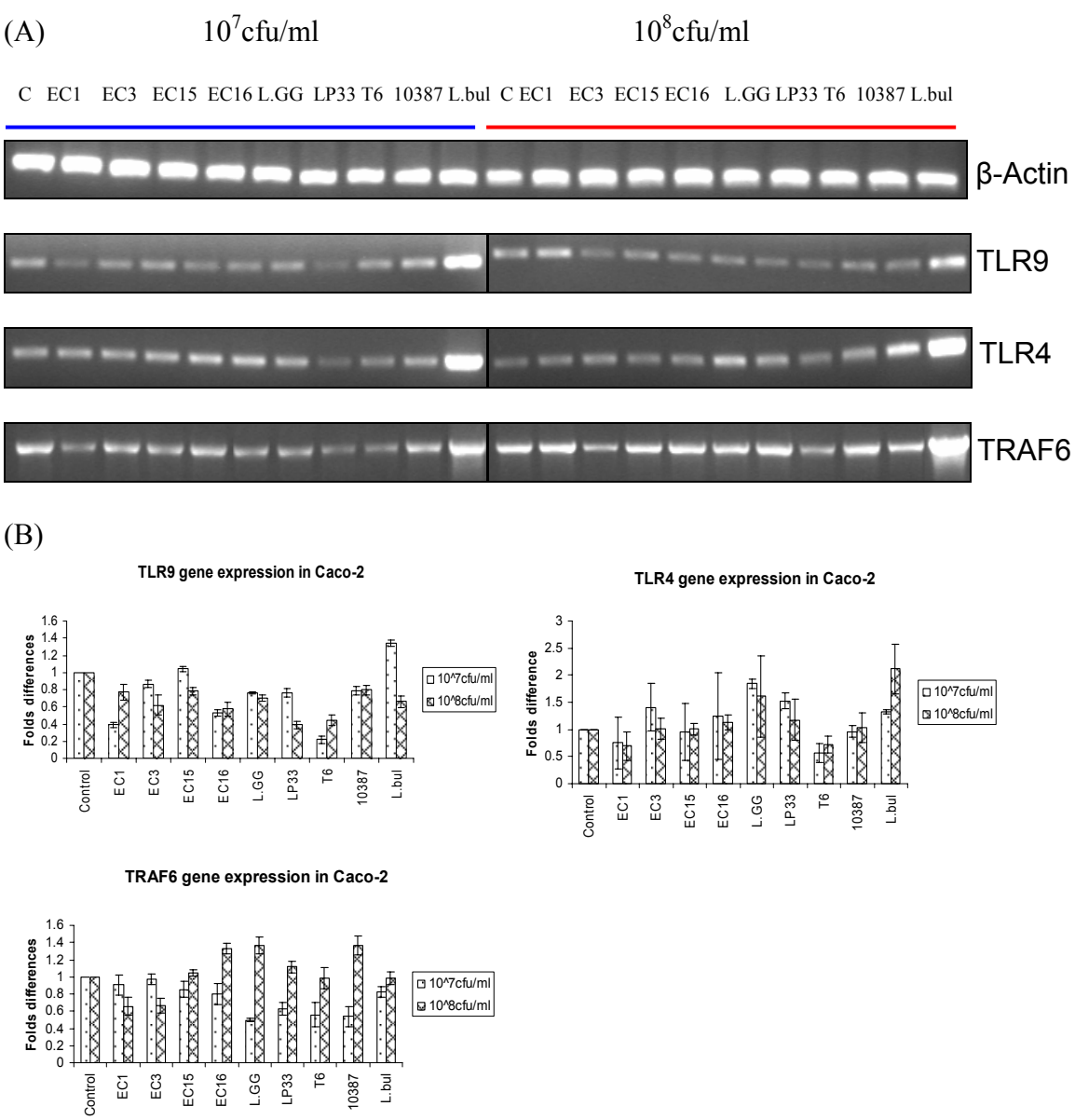


Figure 3.7 TLR9, TLR4 and TRAF6 mRNA expression in Caco-2 cell line treated with LAB. (A) RT-PCR analysis was used to determine their mRNA expressions after exposed to EC1, EC3, EC15, EC16, L.GG, LP33, T6, 10387 and L.bul for 6 hours with the concentration of 10^7cfu/ml and 10^8cfu/ml. "C" represents control using culture medium only. "+" represents the positive control (plasmid containing certain genes) used in the analysis. (B) Quantitative densitometry was carried out on EtBr-stained gels and the results from three independent experiments were compiled to produce the data shown. The gel densities were normalized against β-actin.

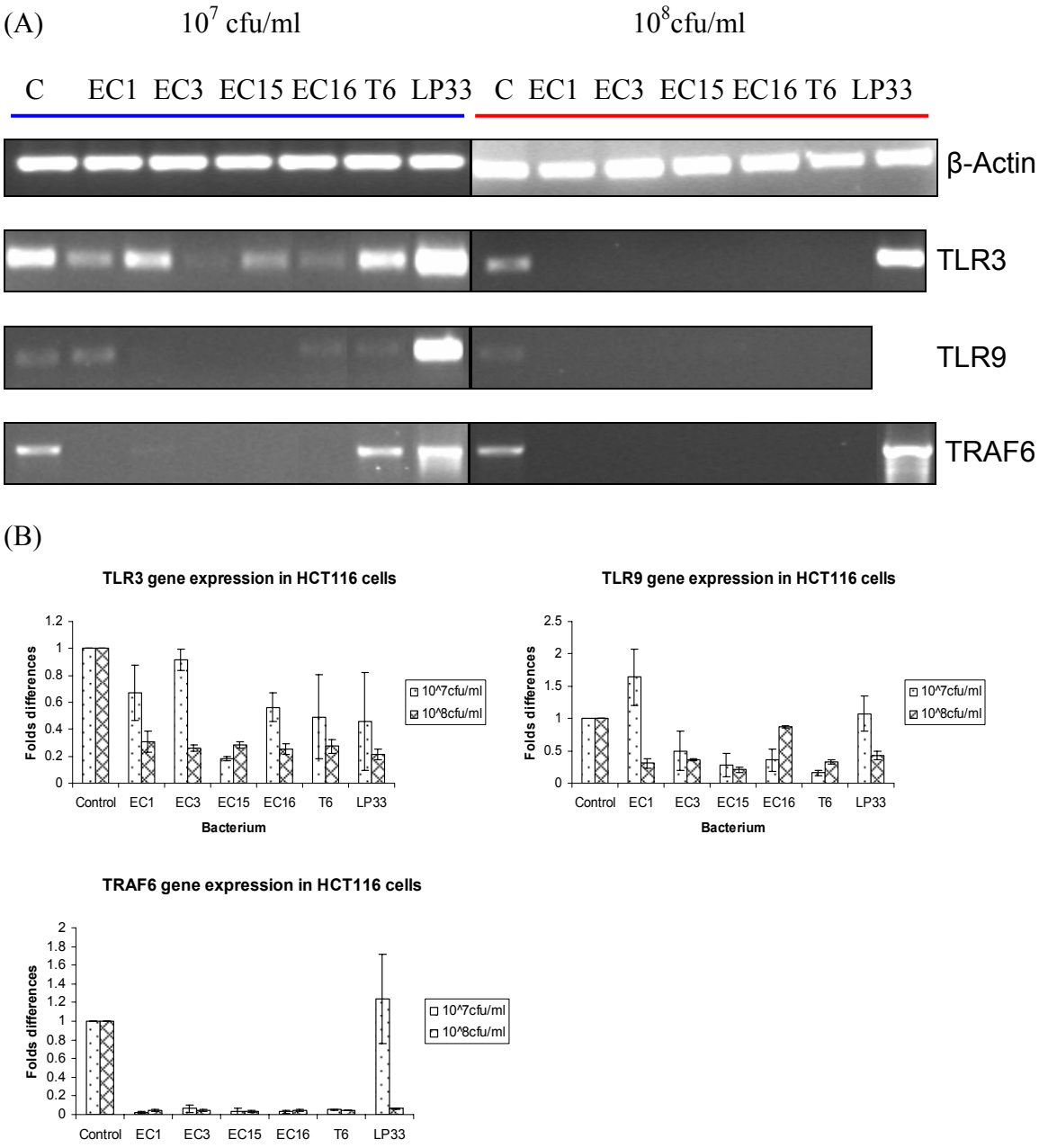


Figure 3.8 TLR3, TLR9 and TRAF6 mRNA expression in HCT116 cell line by EC1, EC3, EC15, EC16, T6 and LP33. (A) The above LAB were added to HCT116 cells as described in Materials and Methods. RT-PCR analysis was used to determine their mRNA expression. "C" represents control using culture medium only. "+" is positive control in PCR reactions using specific plasmid. (B) Quantitative densitometry was carried out on EtBr-stained gels and the results from three independent experiments were compiled to produce the data shown. The gel densities were normalized against β-actin.

Compared to the control and also their 10^8cfu/ml treatment, TRAF6 mRNA expression was greatly suppressed by 10^7cfu/ml L.GG, LP33, T6 and 10387 in Caco-2 cells (Figure 3.7). In HT-29 cells, 10^7cfu/ml EC1, EC3 and EC15 but not EC16

suppressed TRAF6 mRNA expression (Figure 3.9). This observation parallels the TLR4 regulation in HT-29 cells. Most interestingly, TRAF6 gene expression in HCT116 cells, was totally inhibited after treated with 10^7cfu/ml (except L.bul) and 10^8cfu/ml LAB (Figure 3.8).

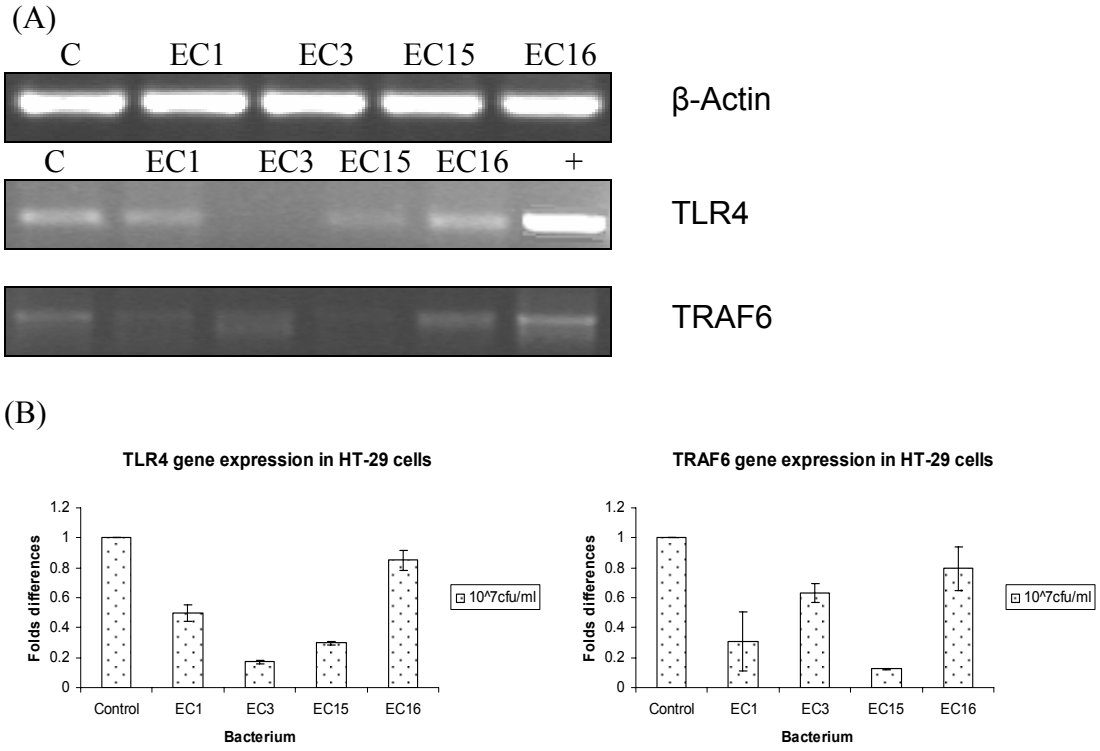


Figure 3.9 TLR4 and TRAF6 mRNA expression in HT-29 cell line with the treatment of EC1, EC3, EC15 and EC16. (A) The four strains were added to HT-29 cells as described in Materials and Methods. RT-PCR analysis was used to determine their mRNA expressions. Culture medium only was used as a control ("C"). (B) Quantitative densitometry was carried out on EtBr-stained gels and the results from three independent experiments were compiled to produce the data shown. The gel densities were normalized against β-actin.

TLR3 recognizes the double-stranded RNA produced by most viruses during replication. As shown in Figure 3.8, TLR3 mRNA expression was also greatly inhibited by 10^8cfu/ml EC1, EC15, EC16, T6 and LP33 in HCT116 cells. However, there was no significant mRNA expression regulation in TLR3, TLR9 and Tollip after co-culturing HT-29 with EC1, EC3, EC15, EC16 (data not shown). Moreover, LAB

did not modulate TLR2, TLR3 and Tollip mRNA expression in Caco-2 cells and Tollip mRNA expression in HCT116 cells (data not shown). In addition, we did not detect the expression of transcripts encoding TLR2 in HT-29 and HCT116 cells and TLR1 and TLR5 in Caco-2, HT29 and HCT116 cell lines.

3.2 DISCUSSION

In this study, 56 LAB strains, which were isolated from infants, fermented products and also from our lab collection, were investigated on their immune modulation effects on human IECs (Caco-2, HT-29 and HCT116). These IECs have been shown to constitutively produce IL-8 in the study. IL-8 is a potent chemotactic and activating peptide from leukocytes, macrophages and T lymphocytes that can induce neutrophil accumulation and activation at the site of production. Among the LAB tested, none of the bifidobacteria showed any anti-inflammatory properties in IECs. Bifidobacteria is an important group of LAB which is usually considered as probiotics. They were reported to dominate in breast-fed infants (Gueimonde *et al*., 2007). However they showed no suppression of IL-8 secretion in this study. This finding extended what others found that bificobacteria strains did not induce IL-8 in human IECs (Lammers *et al*., 2002). Thus, bifidobacteria may regulate human immune system through some other cytokines or pathways other than downregulation of IL-8 production. Interestingly, most *lactobacillus*, which is another major group of LAB usually used for food production, showed no ability to suppress inflammatory responses in the human intestine either. However, *Lactobacillus delbrueckii bulgaricus* D1 (L.bul) and *Lactobacillus rhamnosus* GG (L.GG) showed an inhibition of IL-8 secretion in Caco-2 cells but not in HT-29 and HCT116 cells. *Lactobacillus paracasei* LP33 (LP33) and *Lactobacillus brevis* T6 (T6) suppressed IL-8 secretion both in Caco-2 and HCT116 cells but not HT-29 cells (Figure 3.2). These data showed that lactobacilli exhibited distinct ways in the regulation of immune responses in human. L.GG, but not other strains of lactobacilli, was reported on its anti-

inflammatory properties in the human intestine (Frick *et al*., 2007; Camilleri, 2005). Pathogenic *S. typhimurium* was also tested for its property in the regulation of inflammatory responses in the IECs. As expected, *S. typhimurium* upregulated IL-8 secretion in the IECs (Figure 3.1D). This suggests that IECs could differentiate commensal bacteria from pathogenic bacteria and thus lead to different immune responses.

In this study, among the 16 strains of enterococci isolated from 16 different infants aged 3 days and 1 month tested for their anti-inflammatory properties, four strains of ECs showed strong ability to suppress the inflammatory cytokine IL-8 secretion in three IECs tested (Caco-2, HCT116 and HT-29) (Figure 3.1). Studies using *Lactobacillus reuteri* showed that this strain inhibited the constitutive synthesis of IL-8 and inhibited the synthesis and secretion of IL-8 induced by TNF-α in the human intestinal cell lines (Ma *et al*., 2004). Interestingly, most of LAB tested in this study including most strains of ECs (12 out of 16 strains isolated) did not show any effect on IL-8 production. However, IL-8 secretion was greatly downregulated after exposure to EC1, EC3, EC15, and EC16 in all the three IECs tested (Figure 3.1). This suggests that the immune reactions of an individual could be modulated by specific strains of a bacterial species. His healthy status is determined by the specific strains of LAB which were established during early childhood.

Interestingly, ECs induced IL-8 secretion in THP1 which is a human acute monocytic leukemia cell line. L.GG and *Streptococcus thermophilus* which are commercial probiotics also activated IL-8 expression as well as the pathogenic *S. typhimurium* (Figure 3.4A). Therefore, the suppression of IL-8 production by ECs is cell type dependent. ECs could boost the immune response however suppress the inflammatory reactions in the host. THP1 responded to all bacteria tested, suggesting

that any bacteria that encounters macrophage could be considered as a potential "pathogen" and THP1 cells could not differentiate bacteria types. In addition, dead bacteria (low concentration) were not able to trigger IL-8 secretion in THP1 cells (Figure 3.4C) but were still able to suppress IL-8 secretion in IECs. These data implicate that the effector molecules of ECs to suppress IL-8 secretion from intestinal cells might reside on the bacterial cell surface.

Paradoxically, IL-8 secretion level was not parallel with its mRNA expression level in the IECs in response to the LAB. IL-8 mRNA was upregulated by ECs in HCT116 cells and Caco-2 cells at the lower concentration treatment (Figure 3.5; Figure 3.6) even though they suppressed IL-8 secretion in the IECs (Figure 3.1). Upon the challenge of *S. typhimurium*, IL-8 mRNA remained unchanged but its secretion was upregulated in the IECs (Figure 3.1). This implies that ECs could boost the immune system to be ready for the challenge of pathogen. However, the inflammatory responses were suppressed in the absence of pathogen. On the contrary, *S. typhimurium* activated inflammatory responses without boosting the expression of immune genes. Thus, the regulation of IL-8 protein secretion by ECs could involve post-transcriptional processes in protein synthesis and secretion (Hoffmann *et al*., 2002). The upregulation of IL-8 mRNA in HCT116 cell might also be due to the negative feedback of IL-8 production because ECs suppressed IL-8 secretion to a very low level in HCT116 cells. Furthermore, the inhibition of IL-8 secretion in IECs is dose and time dependent. The higher concentration and longer incubation time of ECs, the lower concentration of IL-8 in the supernatants.

Interestingly, TGF-β, which is important in inflammatory disease recovery, was upregulated significantly by EC3 and EC15 in Caco-2 cells at protein level (Figure 3.3) and in HCT116 at the gene level (Figure 3.6). TGF-β is a potent ligand

that regulates carcinoma initiation, progression and metastasis through a broad and complex spectrum of interdependent interactions (Bierie *et al*., 2006). TGF-β is also a key factor implicated in the regulation of intestinal barrier function and is considered to mediate tolerance to the indigenous microflora. Compared with *Lactobacillus* and *Bifidobacterium, Enterocuccus* demonstrated stronger effects on TGF-β production. A previous study suggested that lysed *Enterococcus faecalis FK-23* (LFK) may have an effect on immune balance between Th1 and Th2 cells (Shimada *et al*., 2004). In this study, we observed that IL-8 cytokine production was suppressed and TGF-β was activated in the presence of ECs, therefore, ECs may also alter the balance among T cell responses leading to the prevention of inflammation in the intestinal cells.

Chapter 4

CHARACTERIZATION OF LAB AND THEIR EFFECTOR MOLECULES

4.1 RESULTS

4.1.1 CHARACTERIZATION OF LACTIC ACID BACTERIA

4.1.1.1 Phenotypic and Genotypic Characterization

A total of 56 strains of lactic acid bacteria (LAB) were tested for their ability to regulate immune responses in intestinal epithelial cell lines (IECs). Among those bacterial strains, 27 strains were isolated from 3-day and 1-month old healthy infants, 17 strains were isolated from fermented food, and 12 strains were from our lab collection (Table 4.1). The bacteria are listed in Table 4.1.

The 27 LAB were selected from infants' feces on MRS and Slanetz/Bartley agar plates. All of the 27 isolates were Gram positive and rod or coccal in shape. The BioMerieux software, based on carbohydrate fermentation patterns, assigned 9 strains to *Lactobacillus* and 16 strains to *Enterococcus*.

The partial sequence of the 16S rDNA demonstrated high identity (99%) of 13 strains of the enterococci with 16S rDNA sequences of *Enterococcus faecalis* from GenBank, 1 strain of *Enterococcus faecium* and 2 strains of other *Enterococcus*. The identity of these strains is listed in Table 4.1. The sequences of these strains are listed in the Appendix 9.

Table 4.1 Identification of 27 strains isolated from infants

Bacterial names	Bacterial source (Infants Age)	Strain ID obtained from 16S rDNA sequencing
L1	3day	*Staphylococcus hominis*
L2	3day	*L.casei or E. faecium*
L5	3day	*Lactobacillus casei*
L6	3day	*Lactobacillus casei*
EC3	3day	*Enterococcus faecalis*
EC6	3day	*Enterococcus faecalis*
EC9	3day	*Enterococcus avium*
EC14	3day	*Enterococcus faecium*
EC15	3day	*Enterococcus faecalis*
EC16	3day	*Enterococcus faecalis*
L3	1month	*Lactobacillus casei*
L4	1 month	*Lactobacillus casei*
L7	1month	*Lactobacillus casei*
L8	1month	*Lactobacillus casei*
L9	1month	*Lactobacillus casei*
L10	1month	*Yeast*
L11	1month	*Yeast*
EC1	1month	*Enterococcus faecalis*
EC2	1month	*Enterococcus faecalis*
EC4	1month	*Enterococcus faecalis*
EC5	1month	*Enterococcus faecalis*
EC7	1month	*Enterococcus faecalis*
EC8	1month	*Enterococcus faecalis*
EC10	1month	*Enterococcus faecalis*
EC11	1 month	*Enterococcus gallinarum*
EC12	1 month	*Enterococcus faecalis*
EC13	1 month	*Enterococcus faecalis*

To fully characterize the *E. faecalis*, protease analysis and antibiotics resistance test were conducted.

4.1.1.2 Protease Analysis

In this assay, protease EF0706, EF0771, EF1590, EF1679, EF1917, EF2355, EF2380, EF2821, EF3027, EF3282 were tested in *E. faecalis* EC2, EC3, EC16 and *E.*

faecalis ATCC 29212. No protease was detected in *E. faecalis* EC2, EC3 and EC16. The positive control *E. faecalis* ATCC 29212 produced all the ten proteases.

4.1.1.3 Antibiotics Resistance Test

Antibiotics resistance test using chloramphenicol, erythromycin, methicillin, tetracycline, neomycin and penicillin is listed in Figure 4.1.

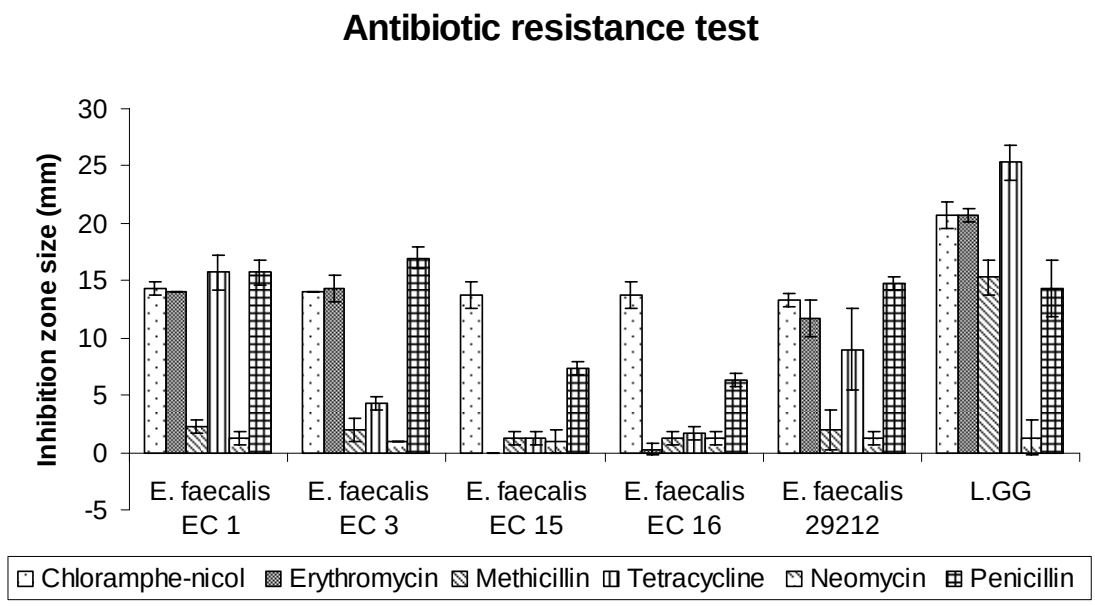


Figure 4.1 Antibiotic resistance properties of *E. faecalis*.

From the above data, we can see that most of the *E. faecalis* strains are susceptible to chloramphenicol, penicillin, but resistance to neomycin and methicillin. Interestingly, *E. faecalis* EC1 and EC3 were susceptible to erythromycin and tetracycline, but not *E. faecalis* EC15 and EC16. L.GG, which is a commercial probiotics, is susceptible to most of the antibiotics tested but resistant to neomycin which did not seem to inhibit any of the bacteria tested. *E. faecalis* ATCC 29212 was resistant to methicillin and neomycin and susceptible to other antibiotics tested. Taken all these data together, we can see that *E. faecalis* contained some antibiotics resistant genes. These results are consistent with recent studies which suggested that *E. faecalis*

contains antibiotic resistant genes (Poeta *et al.*, 2005). However, they are still susceptible to certain antibiotics.

4.1.1.4 Gene Tree of the Bacteria

25 strains (excluding yeast) of the bacteria were clustered according to their 16s rDNA sequences. The resulting gene tree is shown in Figure 4.2.

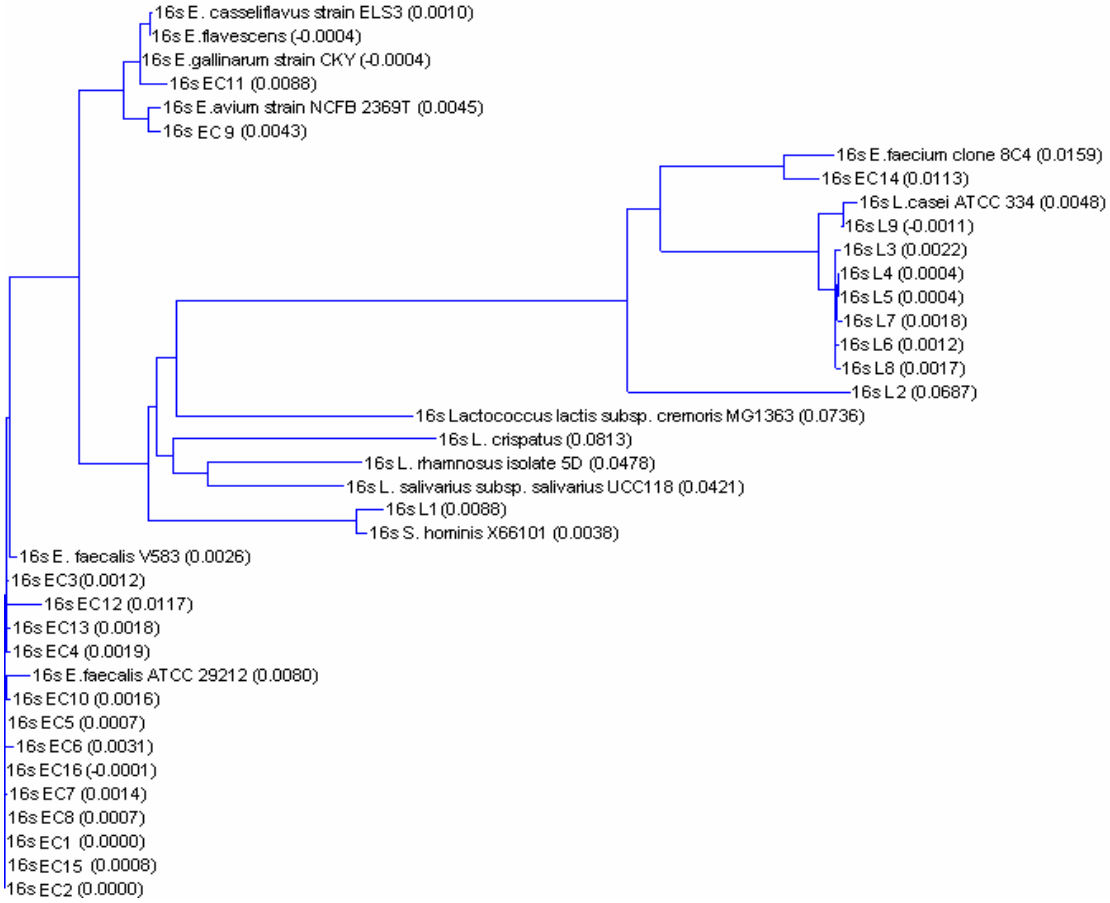


Figure 4.2 Gene tree of the 25 strains of bacteria isolated from new born infants.

From the phylogenetic tree, we can see that enterococci are closely related to lactobacilli. The recent study on *Lactobacillus* supertree, combining individual phylogenetic trees from each of 354 core proteins, showed that *Lactobacillus* supertree had four main branches comprising *L. salivarius*-*L. plantarum*; *L. sakei*; *E.*

faecalis; and *L. acidophilus-L. johnsonii* (Canchaya *et al*., 2006). Interestingly, *L. sakei* is close to *E. faecalis* than to *L. salivarius* (Canchaya *et al*., 2006).

4.1.2 IDENTIFICATION AND CHARACTERIZATION OF POSSIBLE EFFECTOR MOLECULES ON LAB

From the above, we know that some LAB, especially *E. faecalis*, inhibited IL-8 secretion in IECs. We further explored what are the possible molecules that play the important roles in the anti-inflammatory effects. To make the system simple, we chose *E. faecalis* EC1, EC3, EC15 and EC16 for this part's study. The components from the four bacteria were co-cultured with HCT116. IL-8 level was measured using ELISA method.

4.1.2.1 Cell Wall Components and Crude Cell Extracts

To investigate the effect of bacterial viability, bacterial cell wall structures and bacterial cell contents on the anti-inflammatory responses, the bacteria were sonicated and their cell wall components were separated from the crude cell extracts. Cell wall components were collected as cell debris after centrifugation at high speeds.

According to Figure 4.3A, cell wall components from all *E. faecalis* strains demonstrated similar abilities as whole cells in reducing IL-8 levels (Figure 4.3B). Cell debris of the positive control, *S. typhimurium*, showed the expected ability in upregulating IL-8 levels since lipopolysaccharide (LPS) could be released by *S. typhimurium* lysis. Co-culturing with L.GG cell debris caused no change in IL-8 concentration.

To study whether attenuation of IL-8 levels required bacterial cell contents, crude bacterial cell extract was co-cultured with HCT116 cells. In contrast to the results obtained using the cell debris, the crude cell extracts of all four *E. faecalis* strains showed no ability in reducing IL-8 levels when compared to the basal IL-8 levels (Figure 4.3C). In fact, the crude cell extract of *E. faecalis* EC16 significantly increased the IL-8 concentration. Surprisingly, *S. typhimurium* crude cell extract did not activate IL-8 secretion but reduced IL-8 levels. This phenomenon suggested that cell wall components but not cell contents of *S. typhimurium* was very important in stimulating the inflammatory responses. Again, L.GG crude cell extract showed no significant change in IL-8 secretion.

In order to confirm that only the cell debris of *E. faecalis* has an attenuating effect on IL-8 levels, both cell debris and crude cell extracts were combined and co-cultured with HCT116. As seen in Figure 4.3D, treatment using a mixture of debris and crude cell extracts from all four *E. faecalis* strains could significantly attenuate the IL-8 levels to the same extent as the cell debris alone. *S. typhimurium* cell debris and crude cell extract had a similar significant ability in upregulating of IL-8 levels as the bacterial whole cell and cell debris. L.GG cell debris and crude cell extract showed no significant ability in affecting IL-8 concentration as expected.

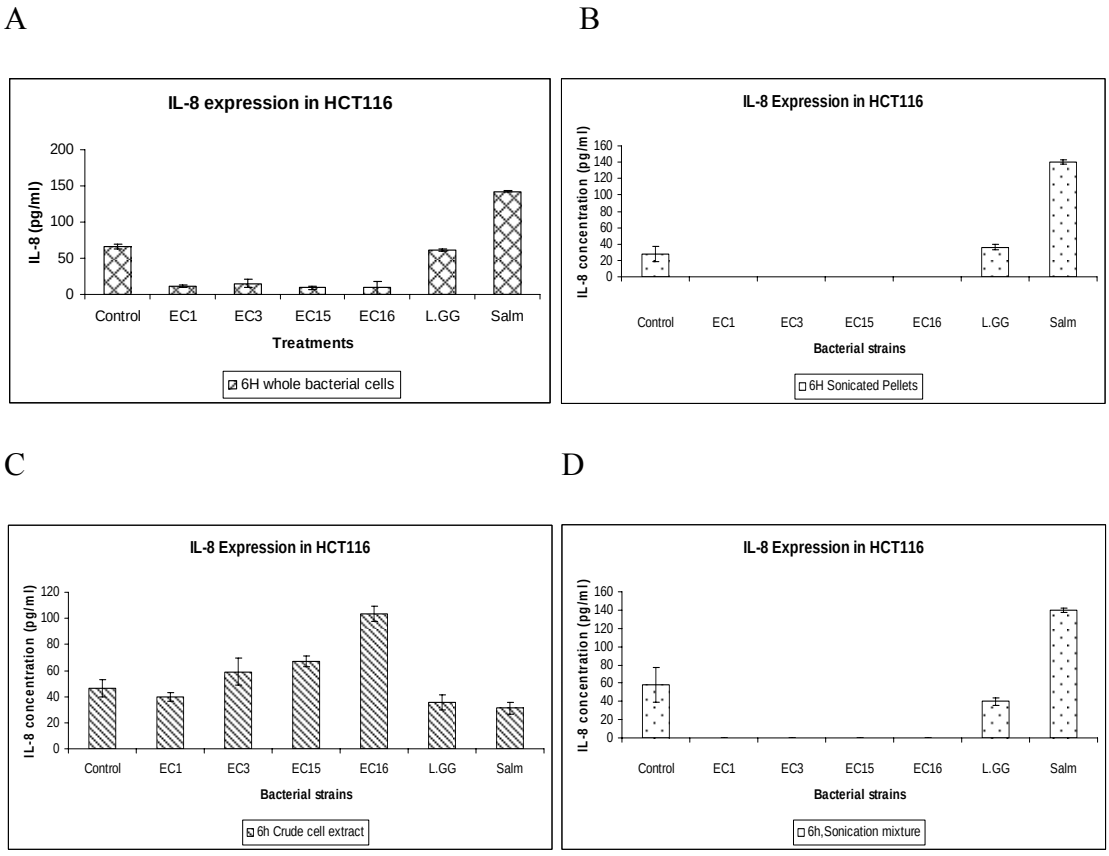


Figure 4.3 IL-8 concentrations obtained upon treatment of bacterial cell debris (A), whole bacterial cell (B), bacterial crude cell extracts (C), cell debris and crude cell extracts mixture(D) in HCT116 cells. Experiments were done three times. Standard deviations are indicated as error bars.

4.1.2.2 Conditional Medium and Cell Inserts

To further investigate the possible ability of *E. faecalis* cell wall components in stimulating a release of anti-inflammatory factors by the HCT116, conditional media using cell wall components was first obtained. Figure 4.4A demonstrates that the conditional media generated using cell debris was incapable of reducing IL-8 levels in HCT116 cells.

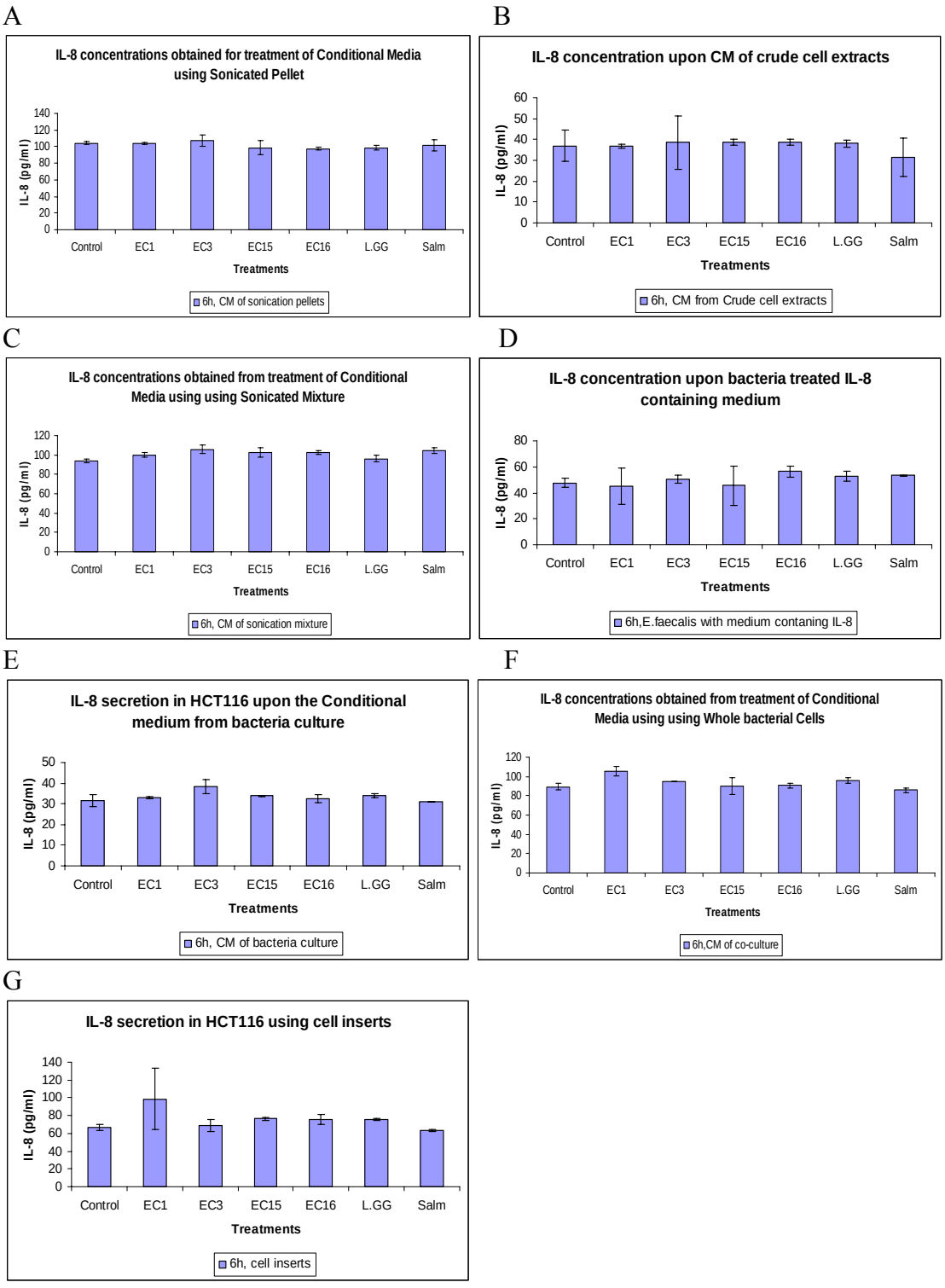


Figure 4.4 IL-8 secretion obtained upon the treatments of conditional medium on HCT116 cells. (A) Conditional media using cell wall components. (B) Conditional medium from crude cell extracts. (C) Conditional medium from a mixture of cell wall components and crude cell extracts. (D) *E. faecalis* strains co-culture with IL-8 containing medium. (E) Cells co-culture with conditional medium from *E. faecalis* strains. (F) Conditional medium from co-culture of *E. faecalis* strains with cells. (G) Cell inserts. Experiments were done three times. Standard Deviations are indicated as error bars.

These results were confirmed with the conditional medium obtained from crude cell extracts or a mixture of cell wall components and crude cell extracts which were co-cultured with HCT116 cells. The lack of IL-8 attenuation was also observed (Figure 4.4 B, C).

To determine whether reduction in IL-8 levels required the direct contact between HCT116 and bacteria or could be mediated by secreted products from either the bacteria or the intestinal epithelial cells, conditional media were made and added to HCT116 cells. Three experiments were designed to test this idea. In the first test involved, supernatants of the cell culture only were harvested and used in the subsequent treatment as conditional medium. *E. faecalis* strains were then added and incubated for 6h. Supernatants were tested for the IL-8 regulation. If the effector molecules were released by *E. faecalis* strains or cells or some proteases from *E. faecalis* strains, there should be a downregulation of IL-8 level. However, the IL-8 did not change in this conditional medium (Figure 4.4 D). For the second test involved, *E. faecalis* strains were co-cultured with cell medium for 6h, bacteria were removed before adding the medium into the cell culture. If molecules from bacteria or some factors from bacteria stimulated cells to release the molecules, there should be a reduction of IL-8 level compared to the control. However, there was still no change in IL-8 level (Figure 4.4 E). For the third test involved, the conditional media were generated using whole cell bacteria co-cultured with cells as described in Material and Methods. Figure 4.4 F demonstrated that the supernatants harvested from co-culturing bacterial cells with HCT116 were incapable of reducing IL-8 levels in HCT116 cells. From the above data, I hypothesized that the effector molecules that suppress IL-8 level could not be the factors secreted by *E. faecalis*.

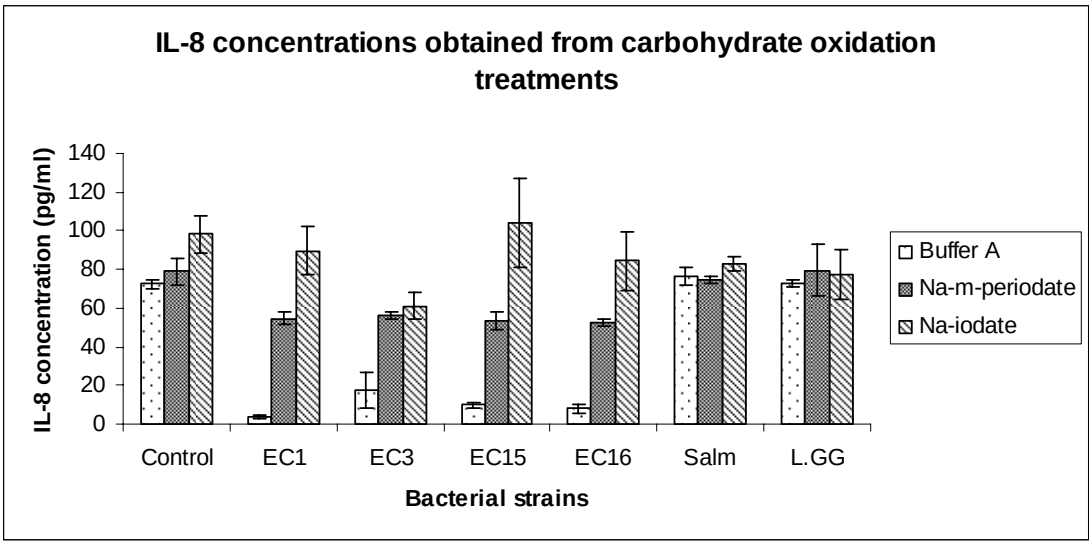
To further confirm the hypothesis, HCT116 cells cultures were incubated in the presence of bacterial strains housed in sterile plastic inserts. This ensured the physical separation between the HCT116 cells and bacteria while allowing secreted bacterial products to reach the HCT116 cells. Figure 4.4 G show that all the bacterial strains could not suppress IL-8 levels as well. Taken all the data together, the effector molecules that inhibited IL-8 secretion in IECs are unlikely to be the factors secreted by *E. faecalis* or cells.

4.1.2.3 Carbohydrate Oxidation and Protein Digestion of the Bacterial Cell Wall

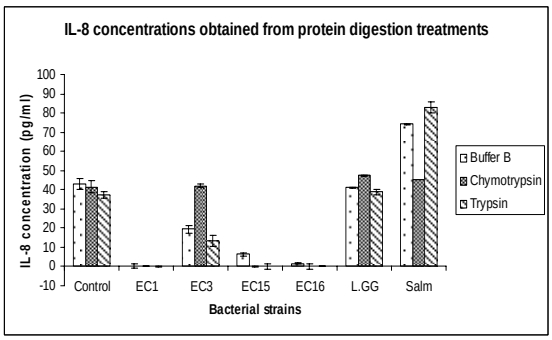
In order to study the importance of cell wall carbohydrates in attenuating IL-8 levels, cell wall carbohydrates were oxidized using mild periodate and iodate. This m-periodate oxidation at acid pH cleaves carbohydrate vicinal hydroxyl groups without changing polypeptide chain structures (Bobbitt, 1956). M-periodate was used because periodate oxidation analysis is one of the chemical methods for elucidation of carbohydrate structure. Periodate consumption and iodate formation, gives insight to the number of vicinal dihydroxyl groups (Honda, *et al*. 1989).

The four *E. faecalis* strains in buffer A showed an expected large decrease in IL-8 levels as seen in Figure 4.5A. The Na-iodate and Na-m-periodate treatments of the *E. faecalis* however, prevented an appreciable decrease in IL-8 levels (Figure 4.5A). Figures 4.5A also showed that Na-m-periodate treatment had no effect on L.GG's abilities in changing IL-8 concentrations. These results confirm the importance of carbohydrates on the bacterial cell wall on the suppression of IL-8 secretion on the IECs.

A



B



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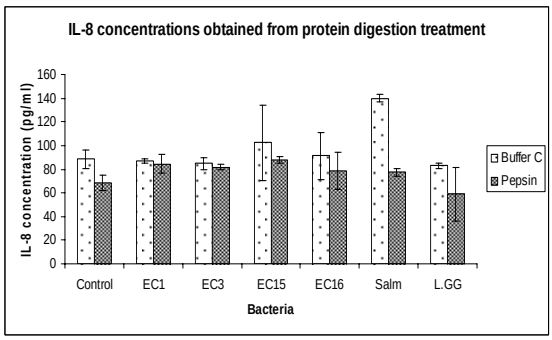


Figure 4.5 Regulation of IL-8 levels by carbohydrate oxidation and protein digestion of *E. faecalis* on IECs. (A) Carbohydrate oxidized by m-periodate and iodate. (B) Protein digested by chymotrypsin and trypsin in buffer B. (C) Protein digested by pepsin in buffer C. Treated bacteria were co-cultured with HCT116 cells for 6h and supernatants were harvested for IL-8 test using ELISA as described in Materials and Methods. Values are the averages of results obtained on three independent experiments.

Although carbohydrate plays a very important role in the suppression of IL-8, we can not exclude other components' importance especially the proteins on the bacterial cell wall. We therefore test their proteins in attenuating IL-8 levels. The proteins were digested using chymotrypsin and trypsin in buffer B and pepsin in buffer C as described in Materials and Methods. Figure 4.5 B show that buffer B did not change the abilities of the *E. faecalis* strains in attenuating IL-8 levels. Only *E.*

faecalis EC3 in buffer B did not show its usual reduction of IL-8. However, the drop in IL-8 levels is still statistically significant. Protein digestion via chymotrypsin and in buffer B caused no change in the abilities of the *E. faecalis* EC1, EC15 and EC16 in attenuating IL-8 levels. Chymotrypsin treated *E. faecalis* EC3 seemed unable to significantly reduce IL-8 levels as seen in Figure 4.5B.

Protein digestion via trypsin in buffer B caused no changes in the abilities of the *E. faecalis* in attenuating IL-8 levels. *S. typhimurium* treated with buffer B and also with trypsin in buffer B showed no change in its capacity to stimulate IL-8 secretion. However, with the treatment of chymotrypsin in buffer B, *S. typhimurium* lost its ability to stimulate IL-8 secretion (Figure 4.5 B). L.GG in all three conditions did not show any changes in IL-8 regulating ability in comparison to its typical action seen in Figure 4.5B.

Figure 4.5 C shows that buffer C prevented all four *E. faecalis* strains from attenuating IL-8 levels. Therefore, the effect of pepsin in buffer C in changing their abilities in decreasing IL-8 levels could not be determined.

4.1.2.4 Adhesion Study

From the cell inserts and conditional medium results, we can see that *E. faecalis* strains may need contact with cells to suppress IL-8 secretion in IECs. Moreover, the sonication pellets study and also the carbohydrate oxidation study suggested that bacterial cell wall carbohydrates played a role in the adhesion and further to inhibit IL-8 secretion in IECs. In order to study the importance of cell wall carbohydrates in adhering to HCT116 cells, carbohydrates on bacterial cell surfaces were oxidized using m-periodate. The results demonstrated that m-periodate treatment significantly reduced adhesion of all four *E. faecalis* strains to HCT116 cells (Figure

4.6 B, C). *E. faecalis* EC15 and EC16 were used as a representation here. Moreover, the secretion of IL-8 in HCT116 was greatly upregulated (Figure 4.5A). This study implied that carbohydrates on bacterial cell wall helped bacteria to adhere to cells and the carbohydrates may act as ligands to activate signaling pathways which led to the suppression of IL-8 secretion from IECs. The function of carbohydrates might not need the live bacteria since sonicated bacteria still suppressed IL-8 secretion (Figure 4.3). L.GG also adhered to HCT116 cells (Figure 4.6 E). However, they did not adhere as well as *E. faecalis*. This could explain why L.GG did not suppress IL-8 secretion as much as *E. faecalis* in my study. In addition, *S. typhimurium*, which is a pathogen, did not show a strong ability to adhere to HCT116 cells (Figure 4.6 F) suggesting that the adhesion ability of pathogen may not be important in the induction of IL-8 secretion.

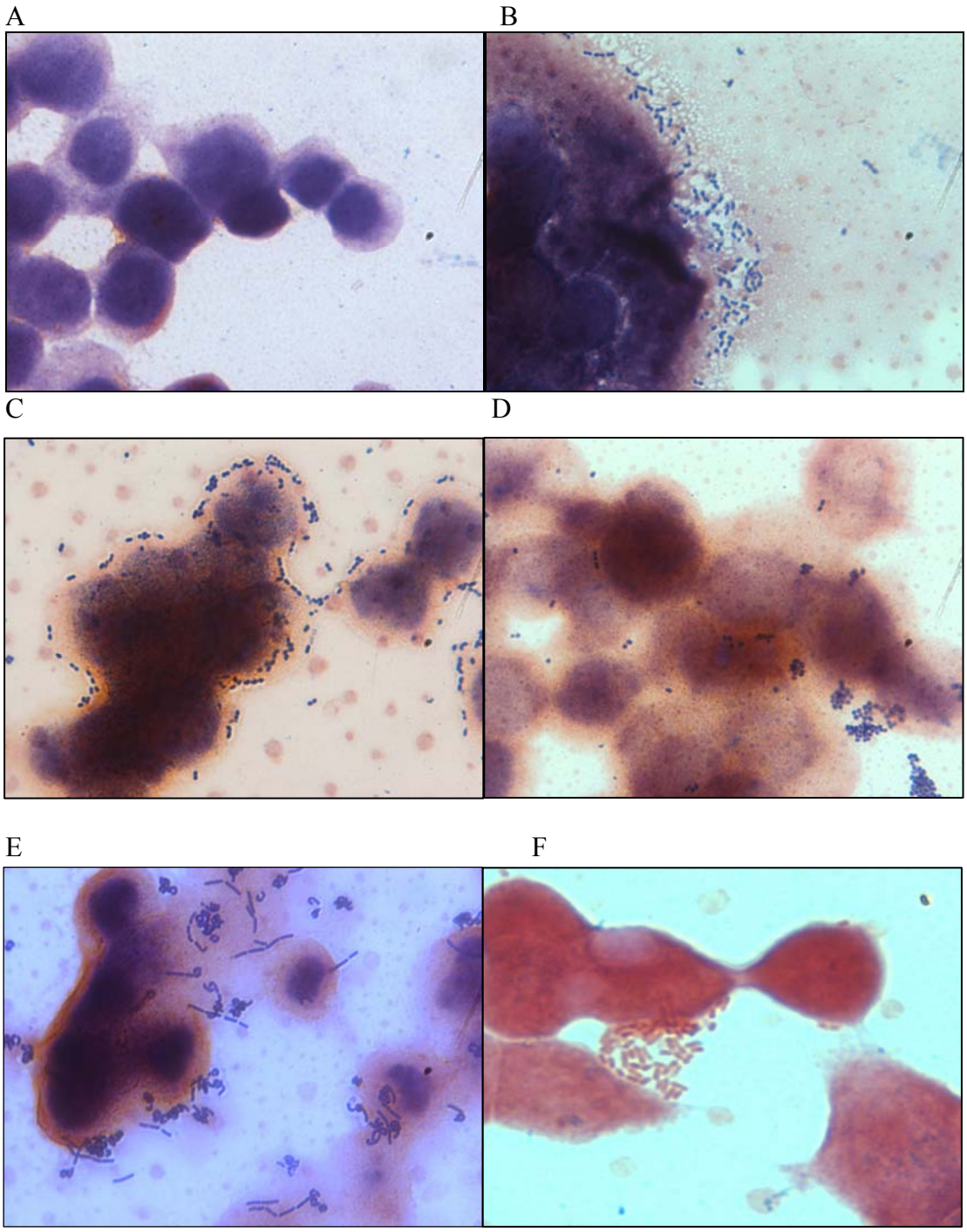


Figure 4.6 Pictures of bacteria adhesion on monolayer of HCT116 cells observed using light microscopy at 1000X. (A) HCT116 cell without bacteria treatment. (B) HCT116 cells treated with *E. faecalis* EC16. (C) HCT116 cells treated with *E. faecalis* EC15. (D) HCT116 cells treated with *E. faecalis* EC15 which was treated with m-periodate. (E) HCT116 cells treated with L.GG. (F) HCT116 cells treated with *S. typhimurium*.

4.1.2.5 UV- and Heat- Killed Bacteria

The need for the four *E. faecalis* strains to be alive to attenuate IL-8 levels was tested by co-culturing UV- treated and heat- killed bacteria with HCT 116 cells. The UV-killed *E. faecalis* maintained its ability to suppress IL-8 levels as shown in Figure 4.7A. However, heat-killed *E. faecalis* lost its ability to reduce IL-8 secretion as seen in Figure 4.7B**.** This might due to heat inactivation (20mins boiling) of some components on the bacterial cell wall. This further suggested that the effector molecules which are very important in regulation of IL-8 secretion might be heat sensitive. Interestingly, *S. typhimurium* lost its ability to stimulate IL-8 secretion in HCT116 cells after they were UV-killed or heat treated. This phenomenon indicates that UV and heat treatment of *S. typhimurium* might cause them to lose the ability to activate IL-8 secretion in HCT116.

A B

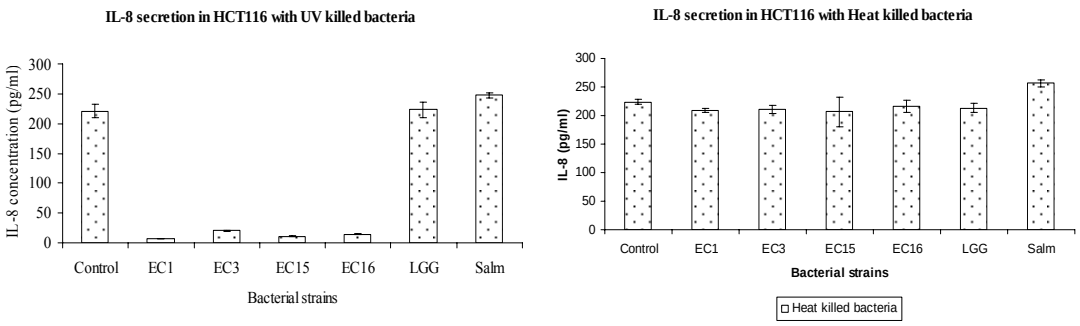


Figure 4.7 IL-8 secretion in HCT116 with UV-killed bacteria (A) and heat-killed bactera (B). The bacteria were killed using the method described in Materials and Methods and treated with HCT116 cells for 6h. Supernatants were harvested for IL-8 secretion test. Three independent experiments were compiled to produce the data shown.

4.1.2.6 Blocking of Specific Carbohydrate Receptors on Bacterial Cell Wall

From the above study, we know the importance of carbohydrates in the inhibition of IL-8 secretion in HCT116 cells. In order to find out which specific carbohydrates are important in reducing IL-8 levels, *E. faecalis* was treated with three different lectins (Succinyl-ConA, ConA and *Tetragonolobus purpureas*). These lectins would bind specifically to these residues and possibly change the overall carbohydrate structures, potentially affecting their roles in attenuating IL-8 secretion. Figure 4.8A showed that incubation of the *E. faecalis* in PBS did not have an effect on its ability to suppress IL-8 secretion. Figure 4.8A showed that the three lectins used also had no effect on depressing IL-8 secretion. Interestingly, with the employment of lectins, the ability to activate IL-8 by *S. typhimurium* was inhibited. This might imply that lectins may act on LPS or some other carbohydrates on *S. typhimurium* to eliminate its stimulation on IL-8 secretion.

In order to identify monosaccharides on the effector molecules, sugar inhibition studies were employed. Exogenous sugars seemed to be incapable of inhibiting all four *E. faecalis* strains from reducing IL-8 levels. Fucose, glucose, trehalose, N-acetyl-glucosamine, galactose and mannose incubated with the HCT116 cells prior to addition of bacteria did not prevent the attenuation of IL-8 by the four *E. faecalis* strains (Figure 4.8 B).

A B

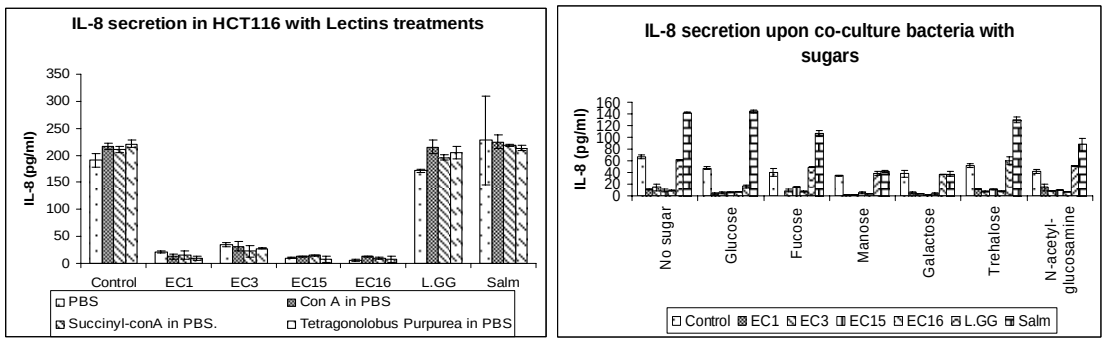
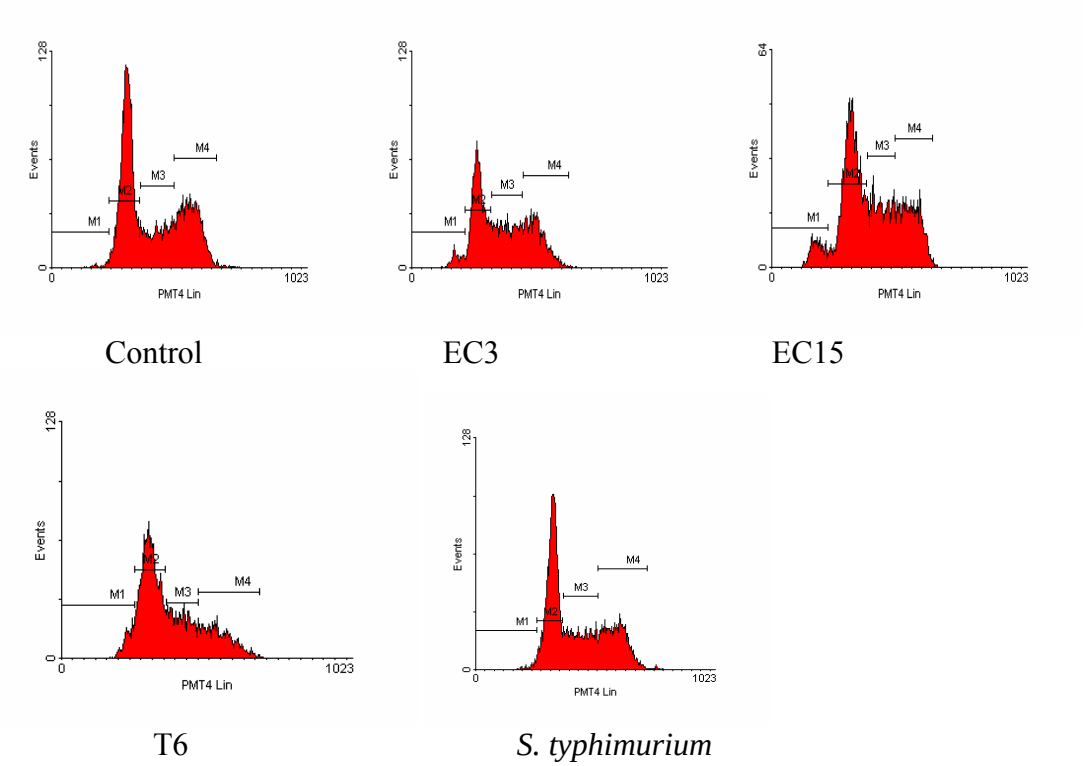


Figure 4.8 IL-8 secretion in HCT116 cells with three Lectins (A) and various sugar treatments (B). They were treated as described in Materials and Methods. Three independent experiments were compiled to produce the data shown.

4.1.3 APOPTOSIS ANALYSIS

From the above observations, LAB especially *E. faecalis* could inhibit the proinflammatory cytokine expression and secretion. We therefore examined the apoptosis feature that might be induced by LAB in IECs to see whether the suppressive effects of the bacteria are due to the cell death. In our study, flow cytometry method was used to quantify the apoptosis induced by LAB. The data showed that the LAB could not induce apoptosis at a concentration of 10^7cfu/ml in HCT116 with 6h incubation (data not shown). In HT-29 cells, 10^7cfu/ml EC3, EC15 and T6 could slightly induce apoptosis after 6 hours incubation (Figure 4.9). However, other LAB like EC1, EC16, L.GG, 10387, L.bul, and Lp33 did not induce any apoptosis (data not shown). Most interestingly, *S. typhimurium*, which is a pathogen, did not show any property to induce apoptosis (Figure 4.9). CRL-1790, which is a normal colon cell line, was used as the control in this study. There were no obvious apoptotic responses detected in CRL-1790 with the treatments of the LAB (data not shown).

A



B

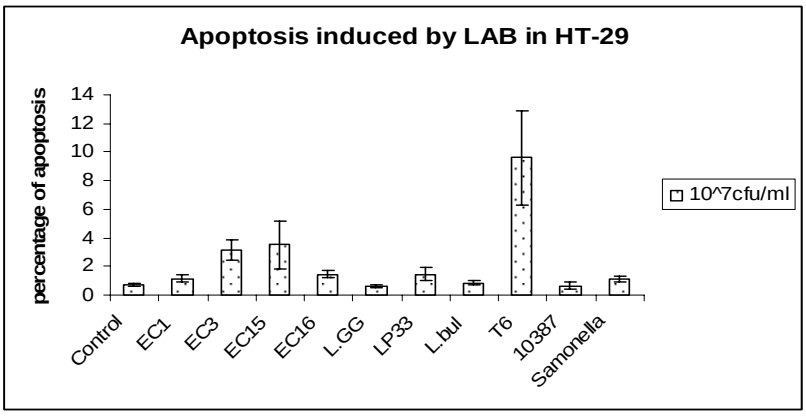


Figure 4.9 Apoptosis assay induced by 10^7cfu/ml LAB and *S. typhimurium* in HT-29 cells. HT-29 cells were cultured and treated with LAB as described in Materials and Methods. Cells were then harvested and assayed by flow cytometry. For each sample, 20,000 cells were measured. Flow cytometric data were analyzed using WinMDIv2.8 software. (A) The pictures showed represent one in three independent experiments. (B) Apoptosis percentage induced by LAB and *S. typhimurium*.

4.2 DISCUSSION

Among the 56 strains of LAB, 25 strains were isolated from 16 different infants aged 3 days and 1 month. Based on the carbohydrate fermentation profile and 16S rDNA sequencing, we assigned 9 strains to *Lactobacillus* and 16 strains to *Enterococcus.* Moreover, most of the lactobacilli are *Lactobacillus casei* with a homology of 99% and the enterococci are *Enterococcus faecalis* with a homology of 99% (Table 4.1). From the phylogenetic tree, we can see that the enterococci are closely related to lactobacilli (Figure 4.2). The recent study on *Lactobacillus* supertree, combining individual phylogenetic trees from each of 354 core proteins, showed that *L. sakei* branch was found phylogenetically closer to *E. faecalis* branch than to *L. salivarius* (Carlos *et al*., 2006). Moreover, they clustered *E. faecalis* as a branch of lactobacilli family. This further suggests that *E. faecalis* may be safer for human consumption than we imagined since it also belongs to lactobacilli. To further identify the properties of *E. faecalis* strains isolated from the infants, we tested their anti-antibiotics properties. It is known that *E. faecalis* has natural antibiotic resistance property and is the leading cause of hospital-acquired secondary infections. In our test, most of *E. faecalis* strains were susceptible to chloramphenicol and penicillin, but resistance to neomycin and methicillin (Figure 4.1). Interestingly, *E. faecalis* EC1 and EC3 were susceptible to erythromycin and tetracycline, while *E. faecalis* EC15 and EC16 were not. Thus, chloramphenicol and penicillin are the effective antibiotics for *E. faecalis* tested in the study. L.GG, which is a commercial probiotics also demonstrated resistance to some antibiotics tested. Therefore, these *E. faecalis* strains are potentially safe due to their susceptibility to most clinically relevant antibiotics. However, the safety of *E. faecalis* for human uses needs to be further verified.

We further characterize the effector molecules of *E. faecalis* that modulate inflammatory responses in IECs. When bacterial cell wall fragments were applied to the intestinal cells after sonication, there was a dramatic suppression of IL-8 secretion in HCT116 cells (Figure 4.3). In addition, the remaining supernatant, the crude cell extract containing bacterial cytoplasm, ribosomes, small membrane vesicles, and large membrane structures, did not suppress IL-8 secretion. The use of conditional medium showed that the effector molecules were not secreted by cells or bacteria or molecules activated after co-culturing the cells and bacteria. This finding was further confirmed by using cell inserts. No IL-8 downregulation was observed when the cells and bacteria were separated by cell inserts (Figure 4.4). Moreover, we tested 10 proteases that might be secreted by *E. faecalis*. It was demonstrated that none of the *E. faecalis* tested possessed these proteases, suggesting that it might not be due to the protease secreted by *E. faecalis* that downregulated IL-8 level in the IECs. Therefore, the effector molecules that suppressed IL-8 secretion from the IECs were not inside the bacteria or secreted by them but on the surface of bacterial cell walls. In addition, only bacterial cell walls could inhibit IL-8 secretion, which implies that the inhibition does not involve bacterial metabolites but some specific molecules present on the bacterial cell surface.

Results from oxidation of carbohydrate moieties of the whole cell surfaces of *E. faecalis* showed that the four *E. faecalis* strains lost their ability to depress IL-8 secretion (Figure 4.5 A). Thus, carbohydrates could be the main effector molecules on *E. faecalis* cell surface. This mild periodate oxidation at acid pH cleaves carbohydrate vicinal hydroxyl groups without changing polypeptide chain structures (Bobbitt, 1996), suggesting that vicinal dihydroxyl groups on *E. faecalis* cell surface possibly play a role in suppression of inflammation (Honda *et al*., 1989). Chymotrypsin-

mediated protein digestion of the effector molecules on *E. faecalis* EC3's cell surface stripped them of their ability to attenuate IL-8 levels (Figure 4.5 B). Thus, *E. faecalis* EC3's effector molecule probably has protein structure(s) necessary for suppressing IL-8 secretion. Similar chymotrypsin-mediated protein digestion of the three other *E. faecalis* strains and trypsin-mediated protein digestion on all four *E. faecalis* strains failed to alter their ability in reducing IL-8 levels (Figure 4.5 B and C), suggesting that proteins might not be the major components from the bacterial cell wall in suppressing IL-8 secretion.

The adhesion study of *E. faecalis* strains demonstrated that they adhered very well to the mucosal layer of epithelial cells (Figure 4.6). Oxidation of the carbohydrate moieties on the bacterial cell surface caused a decrease in their adhesive abilities and also a drop in the ability to suppress IL-8 secretion. This suggests that the cell surface carbohydrates are necessary for the adhesion of four *E. faecalis* strains to IECs. In addition, this is in congruence with Guzmàn *et al* who previously observed that enterococcal surface carbohydrate adhesins were necessary for binding of *E. faecalis* to Girardi heart human cells and urinary tract epithelial cells (Guzman *et al*., 1991). Another studies by Shorrock and Lambert also demonstrated that carbohydrate oxidation reduced binding abilities of *E. faecalis*, suggesting that surface adhesin with carbohydrate component is necessary for adhesion (Lsolauri *et al*., 2002). Since the same oxidation of carbohydrate moieties also stripped these *E. faecalis* strains of their IL-8 secretion suppressing abilities, adhesion of these bacteria via carbohydrate moieties could play a vital role in suppressing inflammation in IECs by *E. faecalis*.

Another study using UV-killed bacteria confirmed that the killed bacteria retained the ability to suppress IL-8 secretion in HCT116 (Figure 4.7). However, the heat-inactivated bacteria lost their ability to suppress IL-8 production in HCT116.

This might be due to heat decomposition of the carbohydrate structure on the bacterial cell surface (20mins at 100°C). Therefore, these results showed that live *E. faecalis* is not required to induce its IL-8 inhibitory effects and the carbohydrate structure which is important in IL-8 suppression might be heat sensitive. Taken all the data together, we can deduce that some structures of carbohydrate on the *E. faecalis* cell surface play the key role in the inhibition of IL-8 secretion in the host.

The specific carbohydrate monosaccharides involved in IL-8 level attenuation could not be determined from the sugar inhibition and lectin studies because sugar inhibition and lectin binding had no effects on the IL-8 regulation with the treatment of *E. faecalis*. Con A has an affinity for terminal α-D-glucosyl and α-D-mannosyl residues. Succinyl ConA has an affinity for mannose and glucose residues agglutination activity (Gunther *et al*., 1973). *Tetragonolobus purpureas* has an affinity for α-L-fucosyl residues and a high affinity for α-L-fucose residues on type II chain blood group oligosaccharides. These lectins can bind to their respective target monosaccharide and alter the overall carbohydrate structure of the effector molecules. *E. faecalis* pre-treated with ConA, succinyl ConA and *Tetragonolobus purpurea*, however, were unable to strip the *E. faecalis* strains of their inflammation suppressing ability. Therefore, Lectin studies suggest that α-L-fucosyl, α-L-fucose, D-mannosyl and D-glucosyl residues are probably not vital or possibly absent from the effector molecules. However they may be important in upregulating IL-8 secretion in *S. typhimurium* since blockage of carbohydrate residues by lectins on *S. typhimurium* cell surface stripped its ability to induce IL-8 secretion (Figure 4.8). In order to identify monosaccharide on the effector molecules, sugar inhibition studies were employed. Sugar inhibition studies showed that the blocking of potential receptors on the intestinal epithelia with the following sugars - fucose, mannose, glucose, N-acetyl-

glucosamine, galactose, and trehalose - did not prevent the four *E. faecalis* strains from suppressing inflammation (Figure 4.8). This suggests that these monosaccharides might not be associated with the *E. faecalis* effector molecules. Moreover, we further tested the possibility of *E. faecalis* to induce apoptosis leading to the reduction of IL-8 secretion. The results showed that *E. faecalis* did not induce apoptosis in human IECs at a MOI of 100 (Figure 4.9) suggesting that the suppression ability of *E. faecalis* in IL-8 secretion is not because of the apoptosis. Furthermore, IL-8 is stable at a pH of 2-9 (Mukaida *et al*., 2003). Therefore, the downregualtion of IL-8 level in the supernatant is not due to the degradation of IL-8 by the production of lactic acid by the *E. faecalis*.

Chapter 5

CHARACTERIZATION OF SIGNALING PATHWAYS

5.1 RESULTS

5.1.1 MICROARRAY ANALYSIS

The four strains of *E. faecalis* attenuated IL-8 cytokine secretion in the human intestinal cell lines. Their regulation on immune gene expression was tested using cDNA microarray and confirmed by Taqman low density array (TLDA) and Western blotting.

Microarray technology simultaneously providing information on expression levels of hundreds of genes has revealed new potential signaling pathways and molecular targets that *E. faecalis* regulated. In our experimental design, cDNA microarrays (GEArray S Series Human Immunology Signaling Pathways Gene Array) were obtained from Superarray (USA). A total of 406 human immunology signaling pathway related cDNA clones were detected in the experiments. The regulation of these genes' expression by *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG, *S. typhimurium*, T6 and *S. typhimurium* coculture with EC16 were tested in Caco-2 cells at a MOI of 100 for 6h. Acquired data were initially scanned into computer to obtain the image (Figure 5.1). Each of the bellow images is one representation of five independent experiments. The signals were then analyzed using the web-based GEArray Expression Analysis Suite provided by the supplier. Scatter plots were generated automatically by the software with a p value available (Figure 5.2). From the scatter plot, we can see the numbers and names of genes upregulated or downregulated by the bacteria. In addition, the clustergrams gave hints on the gene expression level and regulation pattern (Figure 5.3). To get more accurate data,

GeneSpring GX 7.3.1 (Agilent technologies, US) was further used to analyze the data, genes were accompanied by “absent” or “bad” flags were excluded. The regulated genes were in part listed in Table 5.1 and Table 5.2.

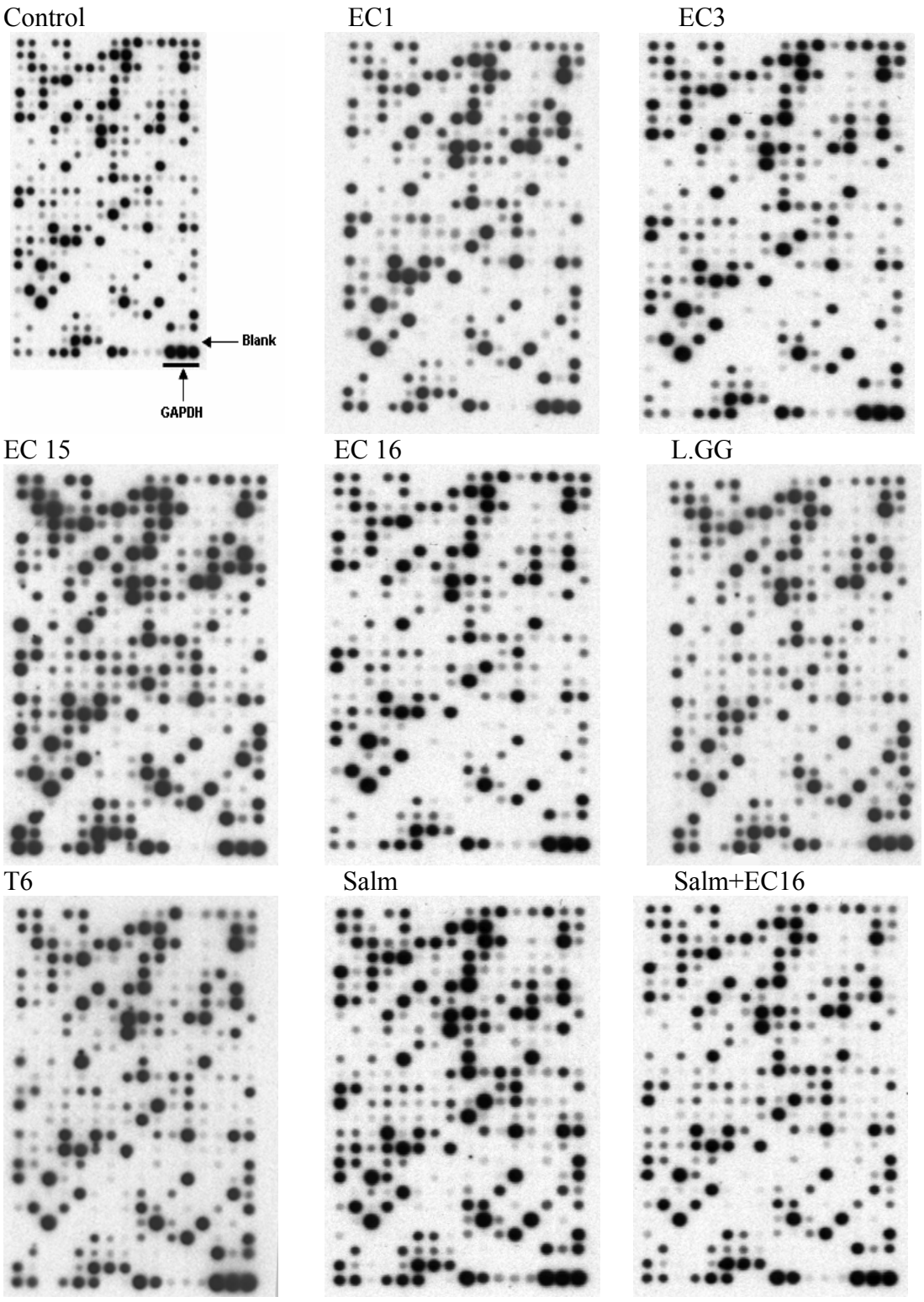
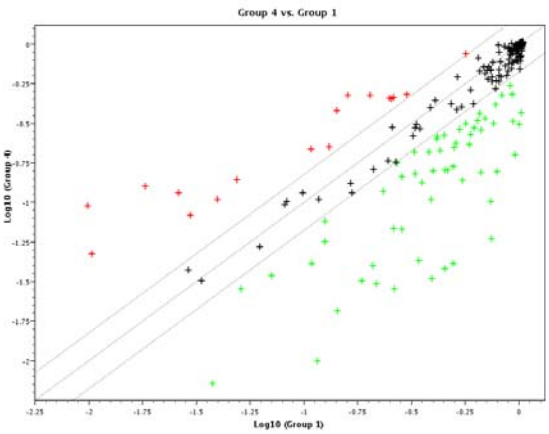


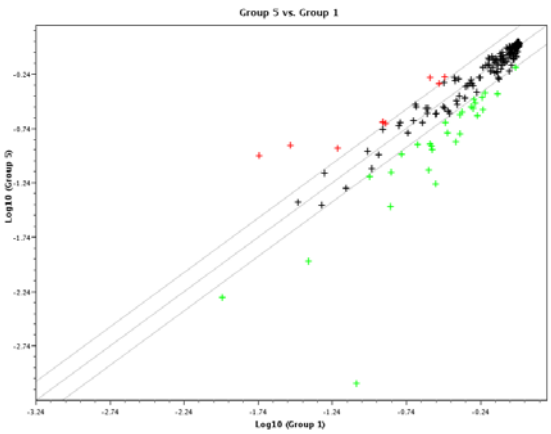
Figure 5.1 Original images of different treatments. Caco-2 was treated with *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG, *S. typhimurium*, T6 and *S. typhimurium* coculture with EC16 at a MOI of 100 for 6h. RNA was harvested and gene expression was tested using the method described in Materials and Methods. The image above showed one representation of five independent experiments.

In the original images of the treatments, GAPDH, which is the positive control used in this study, were expressed consistent in each treatments. Blank wells (Figure 5.1 control) detected nothing. Using the web-based GEArray Expression Analysis Suite provided by the supplier, gene regulation (>1.5 folds or < 0.67 folds) was expressed in scatter plots (Figure 5.2). Here are the labeling: Group1: Control; Group2: EC15; Group3: EC16; Group4: EC1; Group5: EC3; Group6: L.GG; Group7: Salm; Group8: T6; Group9: Salm+EC16.

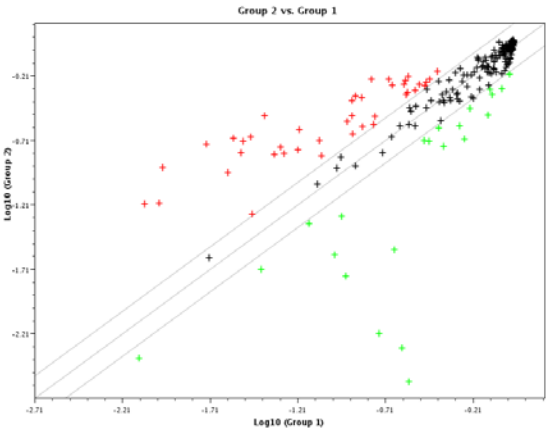
EC1 vs Control



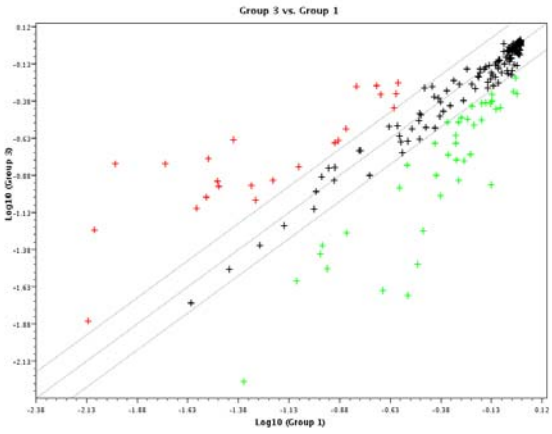
EC3 vs Control



EC15 vs Control



EC16 vs Control



L.GG vs Control

Salm vs Control

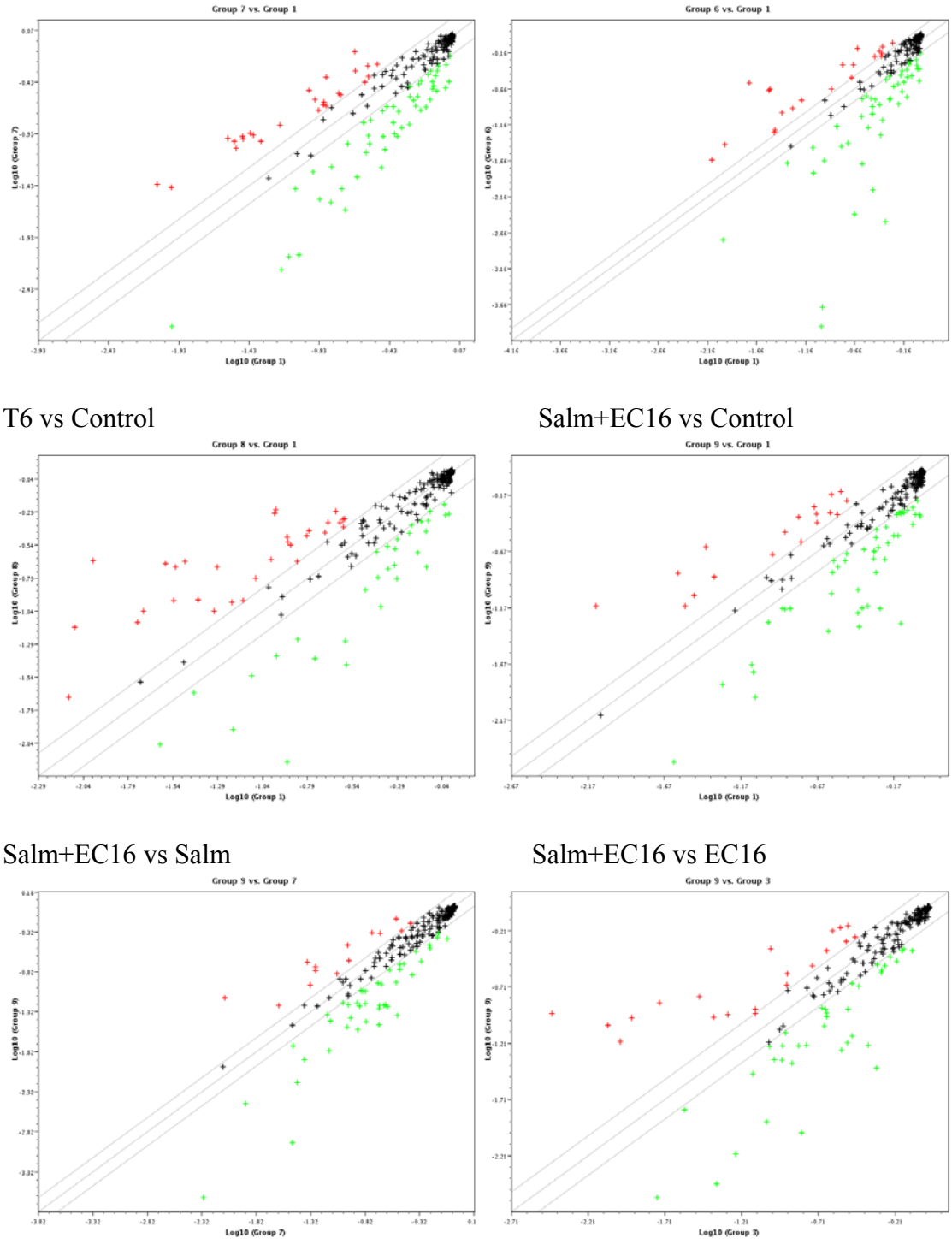


Figure 5.2 Scatter plot of different treatments. Red dots are those upregulated genes (>1.5 folds) and green dots are those downregulated genes (<1.5 folds). Black dots are those non-regulated genes (1-1.5 folds). Five individual experiments were conducted and analyzed.

From the scatter plots, some interesting genes are listed in the Table 5.1. The number in a bracket before the genes in each column is the number of genes regulated by the bacteria. These data were obtained according to the average of image density.

Table 5.1 Gene regulation in Caco-2 cells detected using cDNA microarray

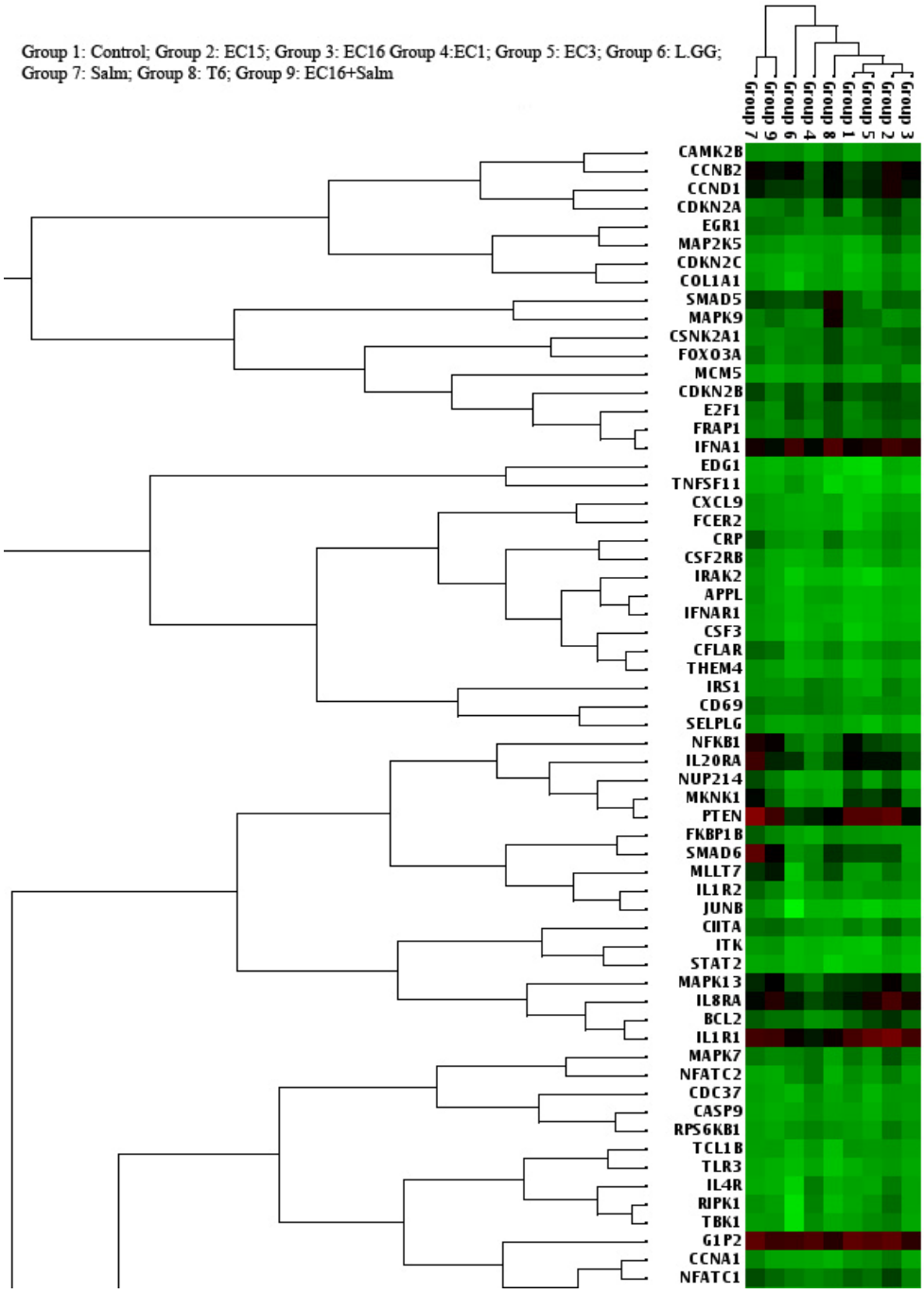
	Upregulation		Downregulation	
	1.5-5 folds	> 5 folds	0.2-0.66 folds	< 0.2 folds
EC1	(**17**) IFNAR2, SMAD5, DUSP1, CREB1, GATA3, MDM2, PPP2R5B, etc.	IL-10	**(59**) CDKN2B, FOS, IKBKB, IL8RA, SMAD9, MAP3K8, MAPK11, MEF2B, NFKB2, PIN1, TNFRSF11A, etc.	IL20RA, MAPK9, MAPK13, NFKB1, etc.
EC3	(**9**) IFNAR2, GATA3, E2F1, MDM2, CDKN2B, TNFAIP3 and etc.	DUSP1	(**29**) E2F, FOS, IKBKB, MAPK1, MEF2B, MKNK1, NFKB1, IL20RA, TNFRSF11A, PIN1, etc.	IL-1R2 PPP2R5B, etc.
EC15	(**41**) CREB, IFNAR2, IKBKG, JUN, MDM2, NFATC1, SMAD5, DUSP1, PPP2R5B, etc.		(**21**) E2F1, FOS, IL-20RA, MAPK7, MEF2B, NFKB1, SMAD7, TNFRSF11A, etc.	MPL, MAPKAPK2, TLR9, etc.
EC16	(**22**) IFNAR2, IL-10, CREB1, BRAF, E2F1, MDM2, JUN, **etc**.	PPP2R5B	(**40**) IKBKB, IL20RA, MAPK7, MAPK9, MAP3K8, MEF2B, NFKB1, NFATC1, NFKB2, PIN1, TNFRSF11A, etc	MKNK1
L.GG	(**20**) BRAF, CCNB2, CDK2, TRAF6, etc	E2F1	(**51**) IFNAR2, IKBKB, IL20RA, SMAD1, SMAD9, MAP3K8, MAPK11, MAPK13, MAPK7, MAPK9, MAPKAPK2, NFKB2, NFKBIA, NFKBIL1and etc.	MAP4K3, NFKB1

Salm	(**26**) CCND1, EIF4EBP1, GNB1, IKBKG, JUN, PPP2R5B, SMAD1, SMAD5, SMAD6, SMAD7and etc.		(**53**) DUSP1,GATA3, G1P3, GSK3B, IKBKB and etc.	
T6	(**30**) BRAF, CCND1, CDK2, SMAD5, MAPK9, MDM2, RAC2 and etc.	CSNK2A1, EIF4EBP1,	(**31**)ATF2, FCGR3B, IKBKB, IL20RA, MAP3K7IP, NFKB1, NFKB2, PIN1, PPP3CB, TRAF3 and etc.	PIK3CB
Salm+EC16	(**17**) EGR1, IFNAR2, SMAD1, CIITA and etc.	GNB1, PPP2R5B	(48) CCNE2, CTLA4, DLK1, G1P3, MAPK7, MEF2B, NFKB2, PIN1, TLR9, TNF and etc.	PPP3CB, TNFRSF11A,
SEC16 vs Salm	(**14**) DUSP1, GATA3, KPNA5, NCOA1 and etc.	IL22RA1	(**40**) IL1R2, IL20RA, IL2RG, JUN, MAPK7, NFKB2, NFKBIA, TLR9, TNFRSF11A and etc.	PPP3CB
SEC16 vs EC16	(**18**) ETS1, SMAD1, SMAD3, MAPK9, NFATC1, NFKB1, TNFAIP3 and etc.	CFLAR, SMAD4, MKNK1	(**37**) CDKN2B, CREB1, CTLA4, JUN and etc.	CCNE2, DLK1,

Note: EC1, EC3, EC15 and EC16: *E. faecalis*; L.GG: *L.rhamnosus* GG; Salm: *S. typhimurium;* SEC16: Salm+ EC16;

From the raw data of microarray, several common signaling pathways were found to be involved in the anti-inflammatory effects of *E. faecalis.* MAPK family members (e.g. MAP3K8 and MAPK7) were greatly downregulated with the treatment of *E. faecalis.* DUSP1, which is known as MAPK phosphatase 1 that can inactivate MAPK signaling pathway, was greatly upregulated with the treatment of *E. faecalis* but not L.GG and *S. typhimurium.* NF-κB members (e.g. NF-κB1, NF-κB2 and IKBKB), which are the master regulator of proinflammatory responses, were

downregulated upon *E. faecalis* treatments too. Interestingly, some cytokine receptors (e.g. TNFRSF11A, IL-20RA and IL-8RA), were also suppressed by the *E. faecalis*. In addition, some genes, which are involved in cell proliferation and tumor genesis such as PIN1, gene transcriptions such as MEF2B and MKNK1, were greatly downregulated too.



To be continued

Continued

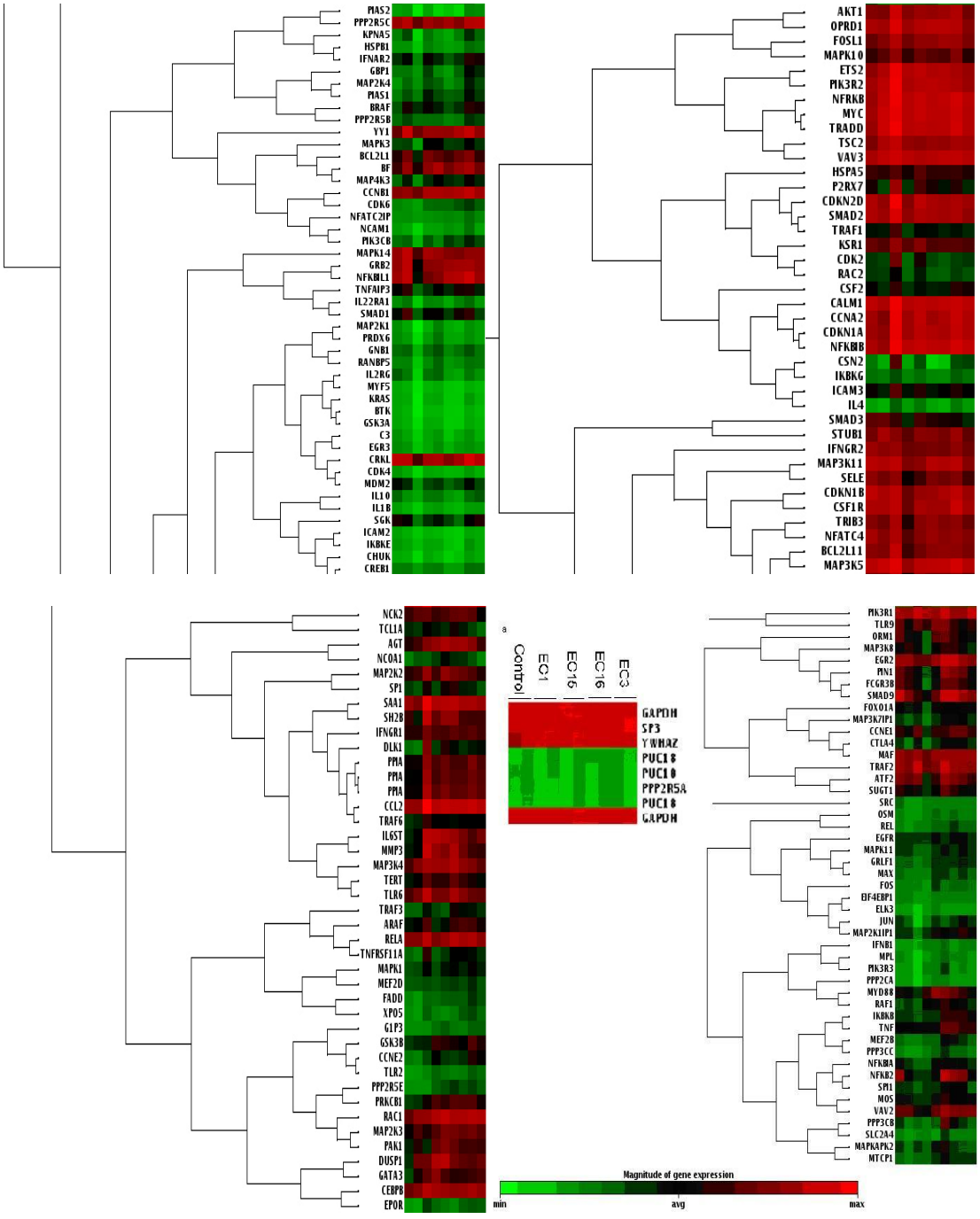


Figure 5.3 Clustergram of all the treatments obtained using GEArray Expression Analysis Suite. The inner small graph (a) demonstrated the consistency of same group and also the consistency of the positive control used.

Figure 5.3 demonstrated the genes regulation with the treatments of the bacteria in a clustergram form. The red color means the higher expression of the

genes and the green color means the lower expression of genes. These pictures gave us a direct impression of the regulation of the genes. Together with the results from Table 5.1, we can see that with the coculture of *E. faecalis* and *S. typhimurium* in Caco-2 cells, DUSP1 was greatly upregulated, however cytokine receptors (IL1R2, IL20RA, IL2RG, and TNFRSF11A) and MAPK/NF-κB family members (MAPK7, NFKB2 and NFKBIA) were suppressed (Table 5.1) implicating *E. faecalis* might suppress the inflammatory signals activated by pathogenic *S. typhimurium.*

Moreover, we used GeneSpring GX to analyze the data. The significantly differentially expressed genes were filtered with the Volcano Plot built by comparing "Treatments" with "Control". Using the Genes in the "Flags are present" gene list, genes were differentially expressed was defined by fold difference of 1.5 and a p-value cut-off of 0.05.

Table 5.2 significantly regulated gene (P<0.05) in Caco-2 cells treated with *E. faecalis* strains for 6h measured by cDNA microarray.

Functional category	Gene population
Response protein	***GBP1;*** SLC2A4, NPPB,
Receptors	***EGFR;*** IL8RA, TNFRSF11A, P2RX7
Transcription factors	***MLLT7, EGR1, ETS1;*** MAPKAPK2, MAPKAPK3, MAX, MEF2B, MKNK1, SP1
AKT& PI3K family	PIK3R3, TCL1A, TCL1B
MAKP famlily	***MAPK13;*** MAPK7, MAP4K1
Adaptor	***TNFAIP3, MDM2;***
Nuclear factors	NCOA1, SPI1
Signal transduction kinases	IKBKB, MAP3K7IP1, PRKCA
Rel/NF-kb	NFKB1, NFKBIL2, NFKB2
Others	***SMAD4, SMAD1, BCL2;*** NFATC2, NCK2, PPP3CB, PPP3CC, CTLA4, SMAD6.

Note: ***Bold italic*** labeled genes are those upregulated. The rest are downregulated genes.

The genes demonstrated in Table 5.2 were those significantly regulated by *E. faecalis* strains. From those data, we can see that MAPK and NF-κB are two main families that involve in the immune regulation of *E. faecalis*. Cytokine receptors are also important in the regulation. Interestingly, 10 transcription factors were modulated by *E. faecalis* in Caco-2 cells suggesting that *E. faecalis* regulates immune responses in the human intestine might through modulating all kinds of transcription factors. Moreover, cytokine gene expression such as ICAM-1 did not change significantly. JAK/ STAT family members which are involved in the signaling of cytokines did not change significantly either.

To further investigate the signaling pathways that *E. faecalis* regulated in IECs. We chose 46 genes for further study using TLDA. A 48-well format TLDA was designed for a subset of genes that were differentially expressed in the array experiments including two endogenous controls 18S and β-actin.

5.1.2 SIGNALING PATHWAY ANALYSIS

5.1.2.1 TaqMan Low Density Array Results

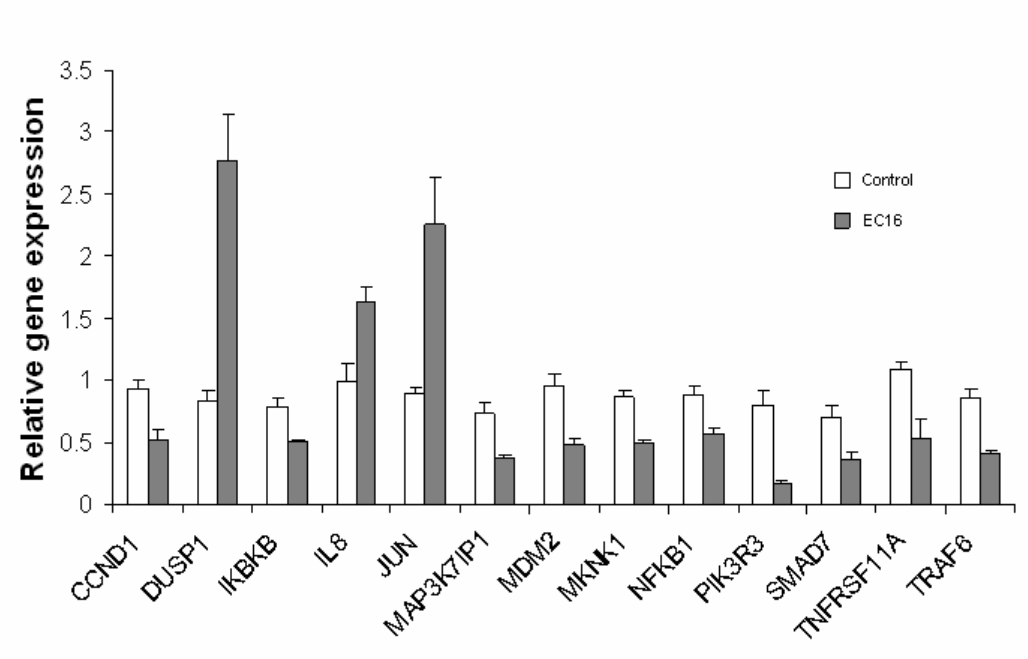
In this part, RNAs from HCT116 and Caco-2 with the treatment of *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium* for 6h at a MOI of 100 were harvested. cDNA was made using 0.5μg total RNA and further tested on TLDA cards. Data were analyzed using the SDS2.2 software where baseline and threshold settings were automatically adjusted. In this part, each test was done with four biological replicates. The gene expression in HCT116 with the treatment of EC16 was done with two technical replicates including the four biological replicates. Student T-test were applied to the results and $p<0.05$ were considered significant.

5.1.2.1.1 Results in HCT116 cells

The gene ID tested in the TLDA cards are listed in Table 3.7. In HCT116 cells, the four *E. faecalis* (EC1, EC3, EC15 and EC16) strains demonstrated a similar regulation pattern on the genes. We therefore use EC16 as an example to illustrate the results. Figure 5.4A showed the genes regulated by EC16 significantly in HCT116 cells.

Figure 5.4A shows that CCND1 (cyclin D1), IKBKB (Inhibitor of nuclear factor kappa B kinase beta subunit), MAP3K7IP1 (Mitogen-activated protein kinase kinase kinase 7 interacting protein 1), MDM2 (transformed 3T3 cell double minute 2, p53 binding protein) , MKNK1 (MAP kinase-interacting serine/threonine kinase 1), NF-κB1, PIK3R3 (Phosphoinositide-3-kinase, regulatory subunit 3 (p55, gamma)), SMAD7 (SMAD family member 7) , TNFRSF11A (Tumor necrosis factor receptor superfamily, member 11a, NFKB activator) and TRAF6 (TNF receptor-associated factor 6) were suppressed with the treatment of *E. faecalis* EC16 in HCT116, however DUSP1 (Dual specificity phosphatase 1) , IL-8 and JUN were upregulated.

A



B

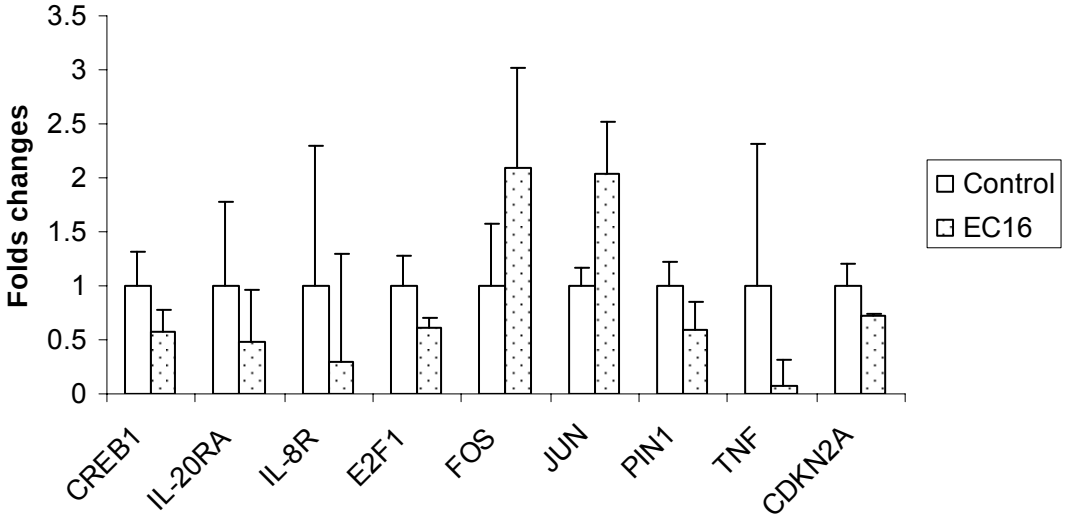


Figure 5.4 Gene expression in HCT116 with the treatment of EC16. (A) All the genes listed in this figure were regulated significantly. (B) The gene expression were not regulated significantly (except JUN) in HCT116 but with a fold change above 1.5. Experiments were done on 4 biological replicates and 2 technical replicates using 10μg cDNA. Folds changes > 1.5 and T-test with a $P < 0.05$ were considered significant.

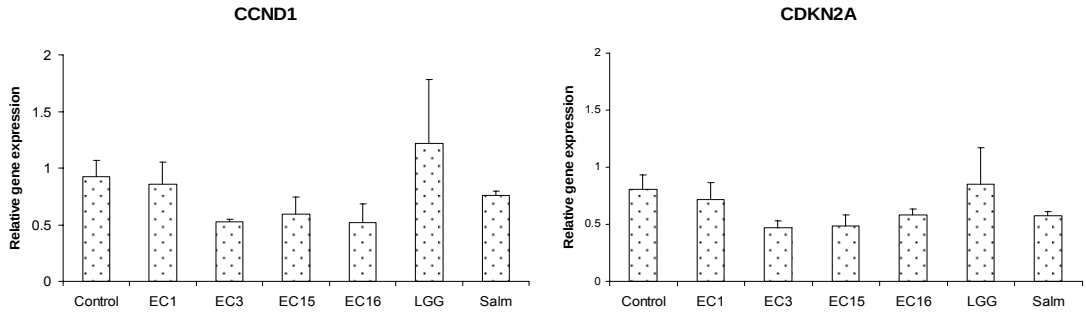
There are also other interesting genes regulated by *E. faecalis* in HCT116 cells. MDM2, which is a target gene of the transcription factor tumor protein p53, is a nuclear phosphoprotein that binds part of an autoregulatory negative feedback loop. It

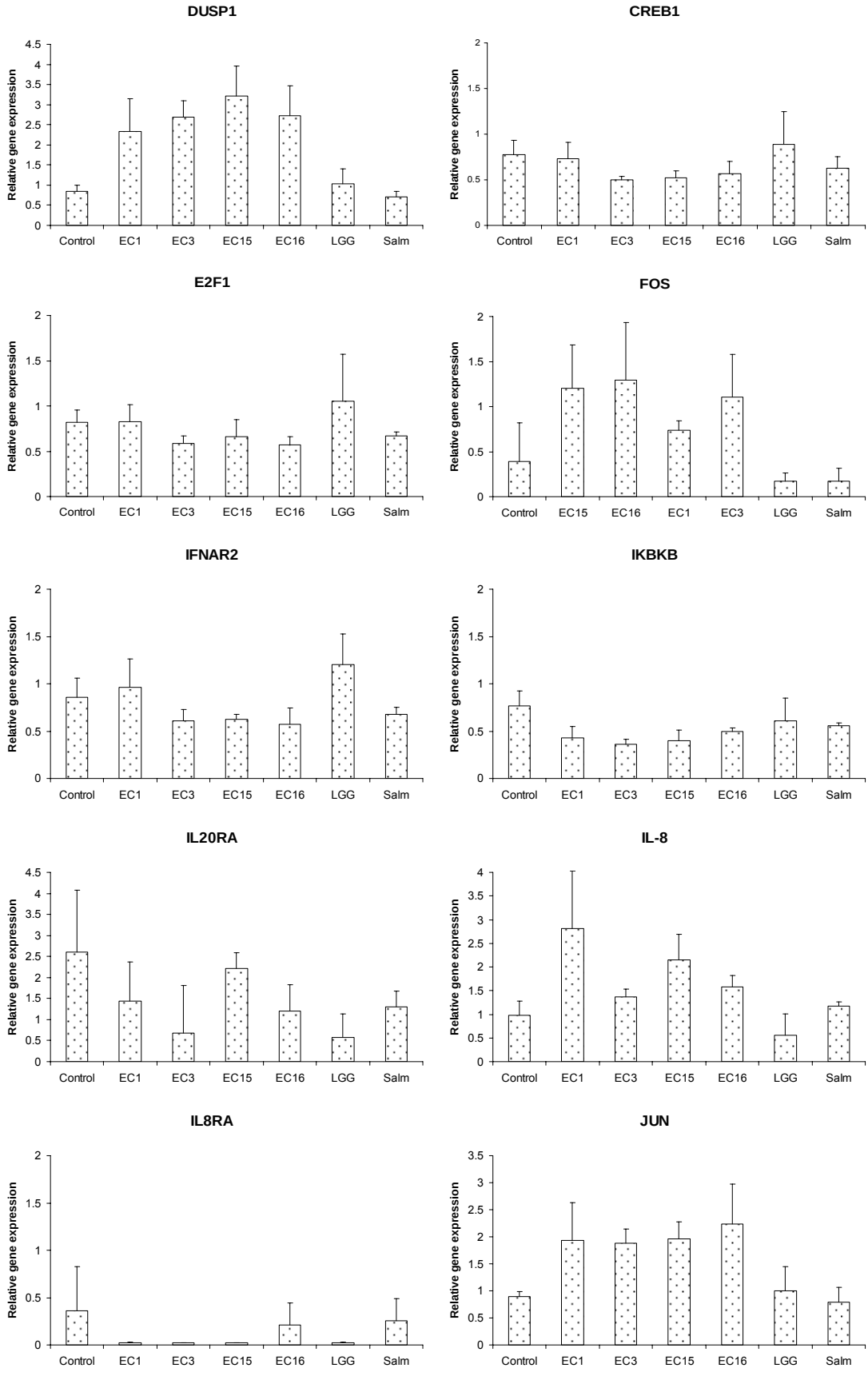
was greatly suppressed with the treatment of *E. faecalis* in our study (Figure 5.4A). Overexpression of this gene can result in excessive inactivation of tumor protein p53, diminishing its tumor suppressor function. MDM2 also has E3 ubiquitin ligase activity, which targets tumor protein p53 for proteasomal degradation. This protein also affects the cell cycle, apoptosis, and tumorigenesis through interactions with other proteins, including Rb1 (retinoblastoma 1). Another interesting gene is SMAD7, which is SMAD superfamily. SMAD7 was also suppressed by *E. faecalis* in our study (Figure 5.4A). It induces tumorigenicity by blocking TGF-β induced growth inhibition and apoptosis. However, SMAD7 has no effect on TGF-β induced activation of p38 and ERK. It blocks the phosphorylation of Akt by TGF-β and enhances TGF-β induced phosphorylation of c-JUN. Therefore, suppression of these proteins may suppress the tumor progression in colon cancer cell lines.

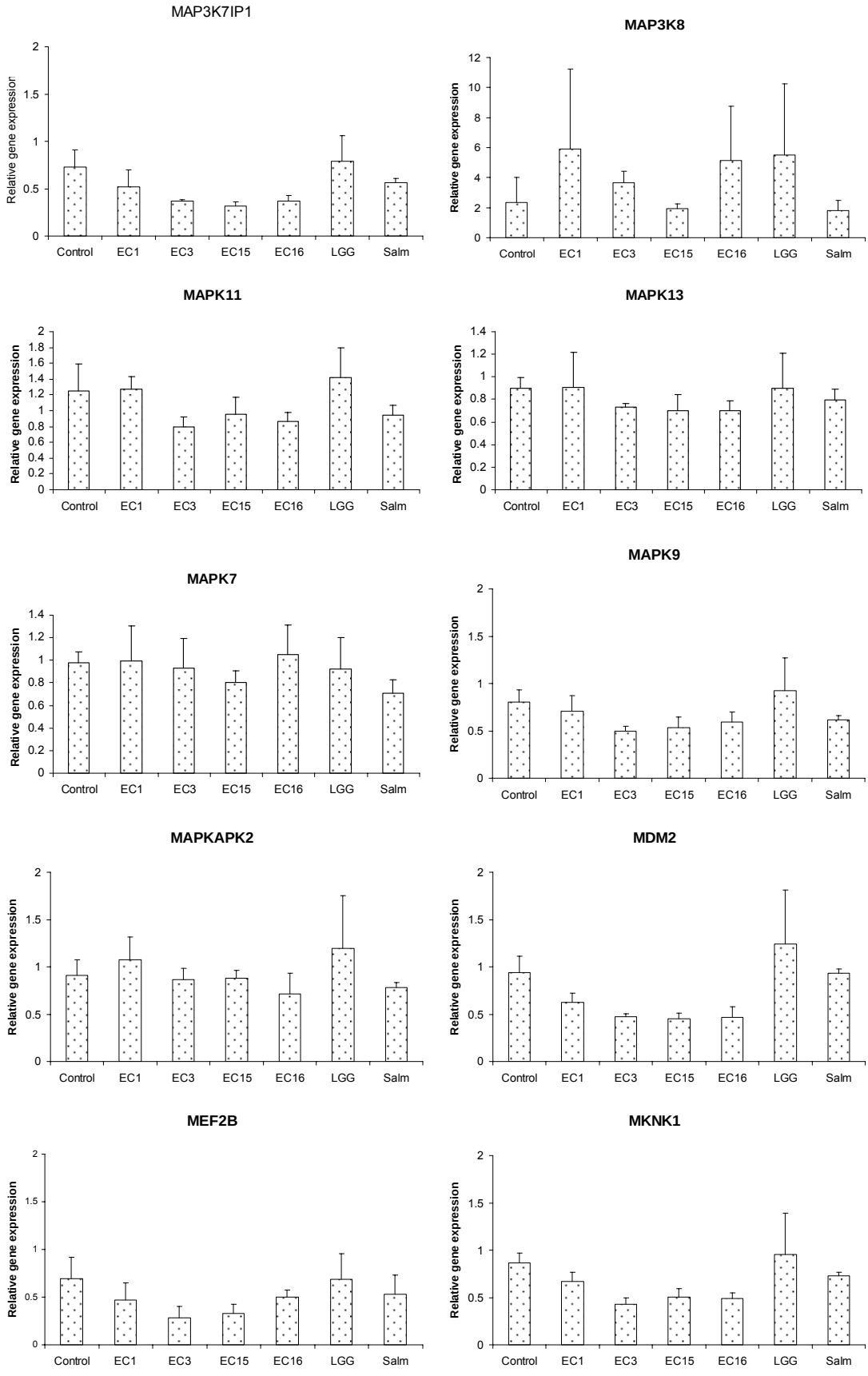
Figure 5.4B showed the trends of the gene regulation in HCT116 with the treatment of *E. faecalis* EC16. Cytokine receptors like IL-20RA, IL-8RA were suppressed. Unexpectedly, FOS and JUN, which can dimerize and form the transcription factor complex AP-1, were upregulated. However, CREB1 (cAMP responsive element binding protein 1), which is also an important transcription factor, was suppressed, suggesting a complex regulation system of the gene transcriptions in the host at the presence of *E. faecalis* strains. Conflicting with the upregulation of IL-8 gene expression, TNF was suppressed in HCT116 (Figure 5.4B) which hints the regulation of IL-8 protein production may involve a post-transcription regulation. Furthermore, PIN1 (Protein (peptidylprolyl cis/trans isomerase) NIMA-interacting 1), a conserved enzyme involved in diverse biological processes and pathological conditions, has profound effects on phosphorylation signaling pathways. It was found overexpressed in many human cancers (Gerburg *et al.*, 2005). However, it was

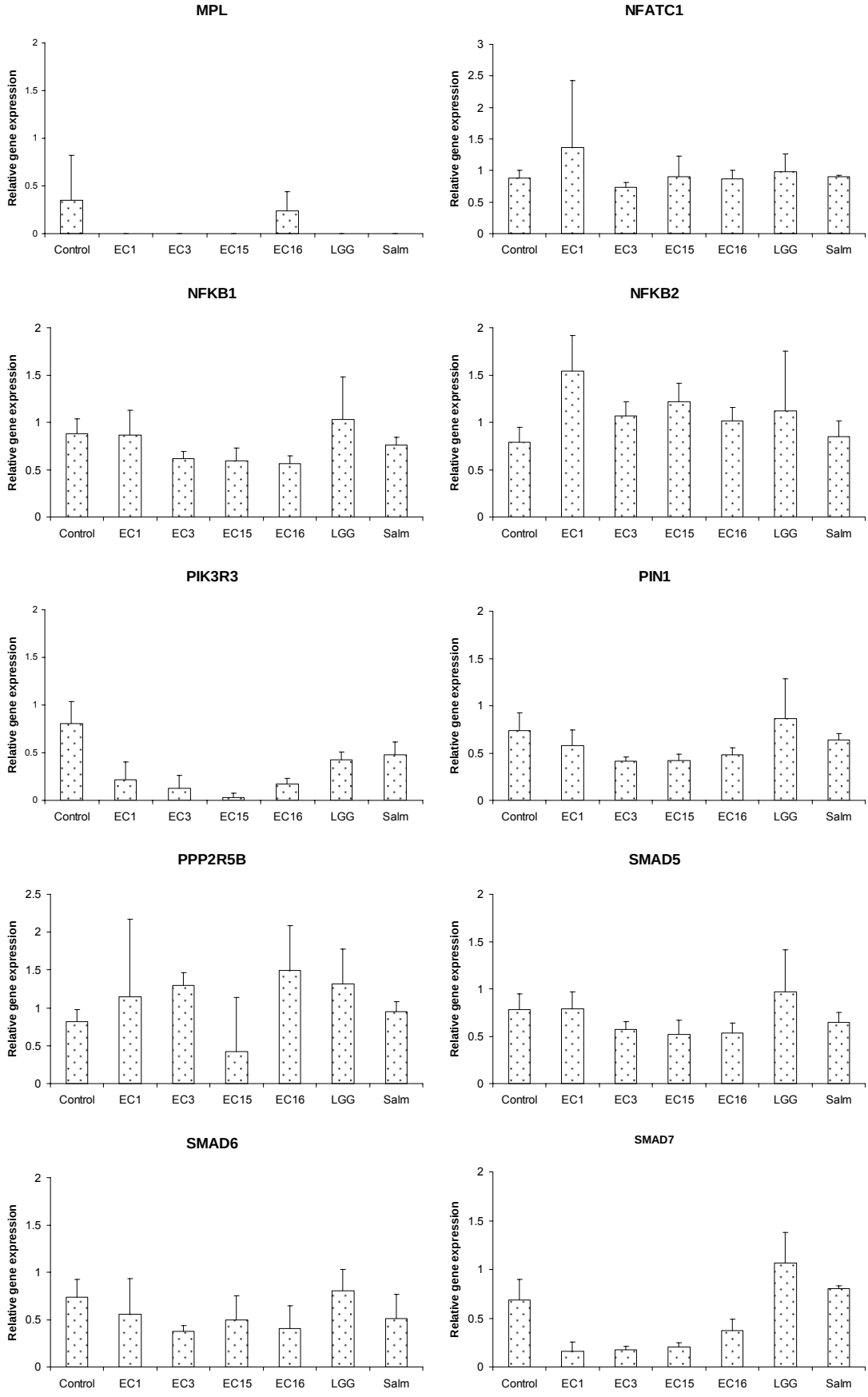
suppressed with the treatment of *E. faecalis* in human colon cancer cell lines. PIN1 is also a target gene of E2F1 in response to growth factors and other stimulating conditions (Ryo *et al*., 2002), and PIN1 in turn positively regulates cyclin D1 function at the transcriptional level and also through post-transcriptional stabilization. Moreover, PIN1 also inhibits negative feedback of MAPK signaling (Dougherty *et al*., 2005). This further suggests the inhibition of the overexpression of PIN1 in human colon cancer cell lines may put a brake on multiple oncogenic signal pathways at multiple levels. Interestingly, in our study, PIN1, cyclin D1 and E2F1 were all suppressed in the presence of *E. faecalis* in HCT116 cells (Figure 5.4), suggesting an overwhelming function of *E. faecalis* in the regulation of host response. In summary, the genes regulated by all bacteria tested were listed in Figure 5.5.

From Figure 5.5, we can see the regulation of all the 40 genes detected using TLDA cards. The gene expression of IL-4, IL-10, SPI1, TLR2, TLR4 and TLR9 were either not expressed or expressed in minute quantities in HCT116. *S. typhimurium* and L.GG did not regulate most of the genes tested. *E. faecalis* EC1 which was isolated from one month old infant exhibit some different regulation pattern compared with other *E. faecalis* isolated from three day old infants. EC1 did not inhibit NF-κB, MKNK1, MAP3K7IP and cyclin D1 expression as other *E. faecalis* suggesting a different regulation mechanism of EC1 on the genes.




DUSP1
CREB1
E2F1
FOS
IFNAR2
IKBKB
IL20RA
IL-8
IL8RA
JUN
Relative gene expression
Control
EC1
EC3
EC15
EC16
LGG
Salm


MAP3K7IP1
MAP3K8
MAPK11
MAPK13
MAPK7
MAPK9
MAPKAPK2
MDM2
MEF2B
MKNK1
Relative gene expression
Control
EC1
EC3
EC15
EC16
LGG
Salm


MPL
NFATC1
NFKB1
NFKB2
PIK3R3
PIN1
PPP2R5B
SMAD5
SMAD6
SMAD7
Relative gene expression
Control
EC1
EC3
EC15
EC16
LGG
Salm

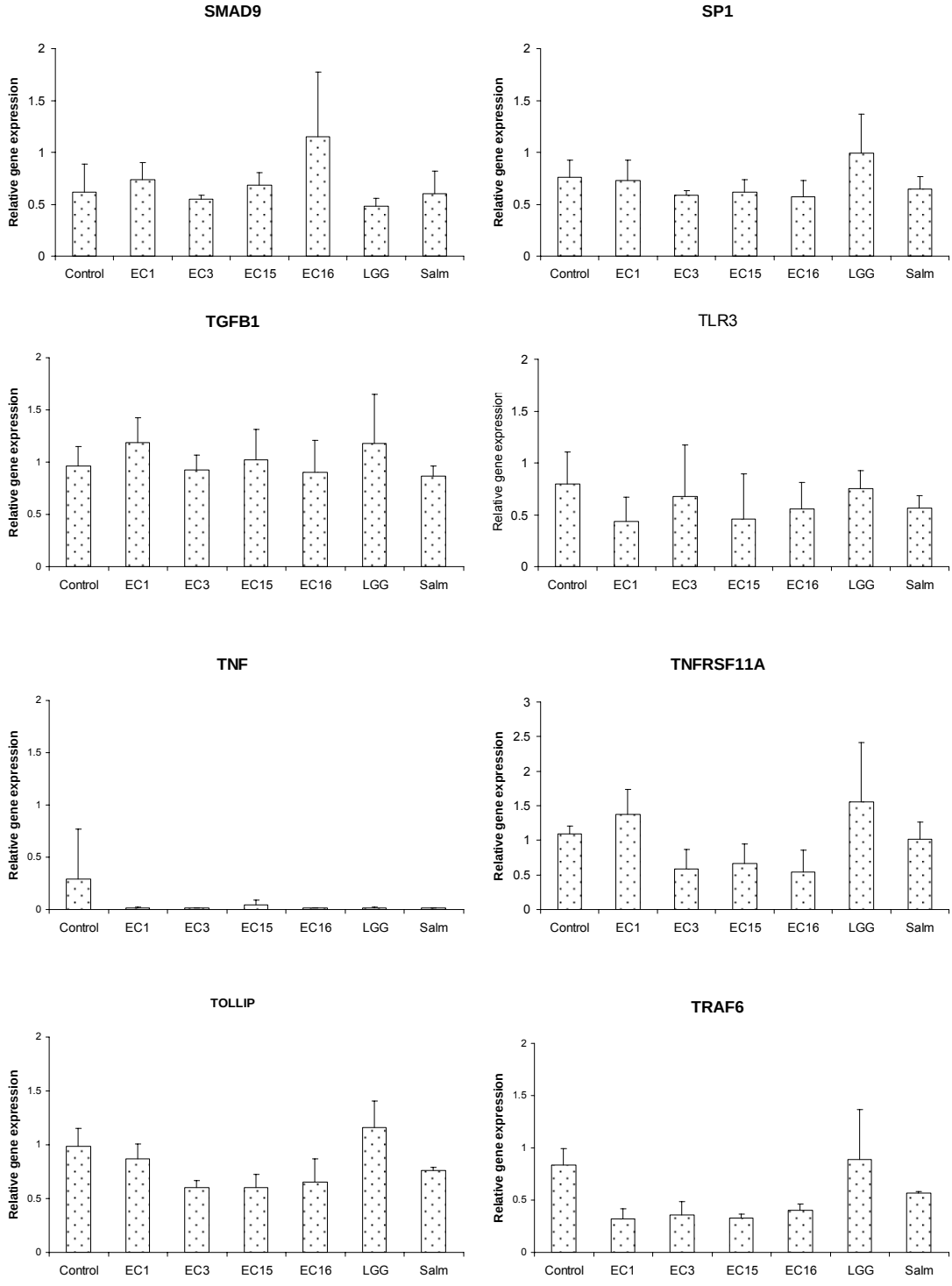


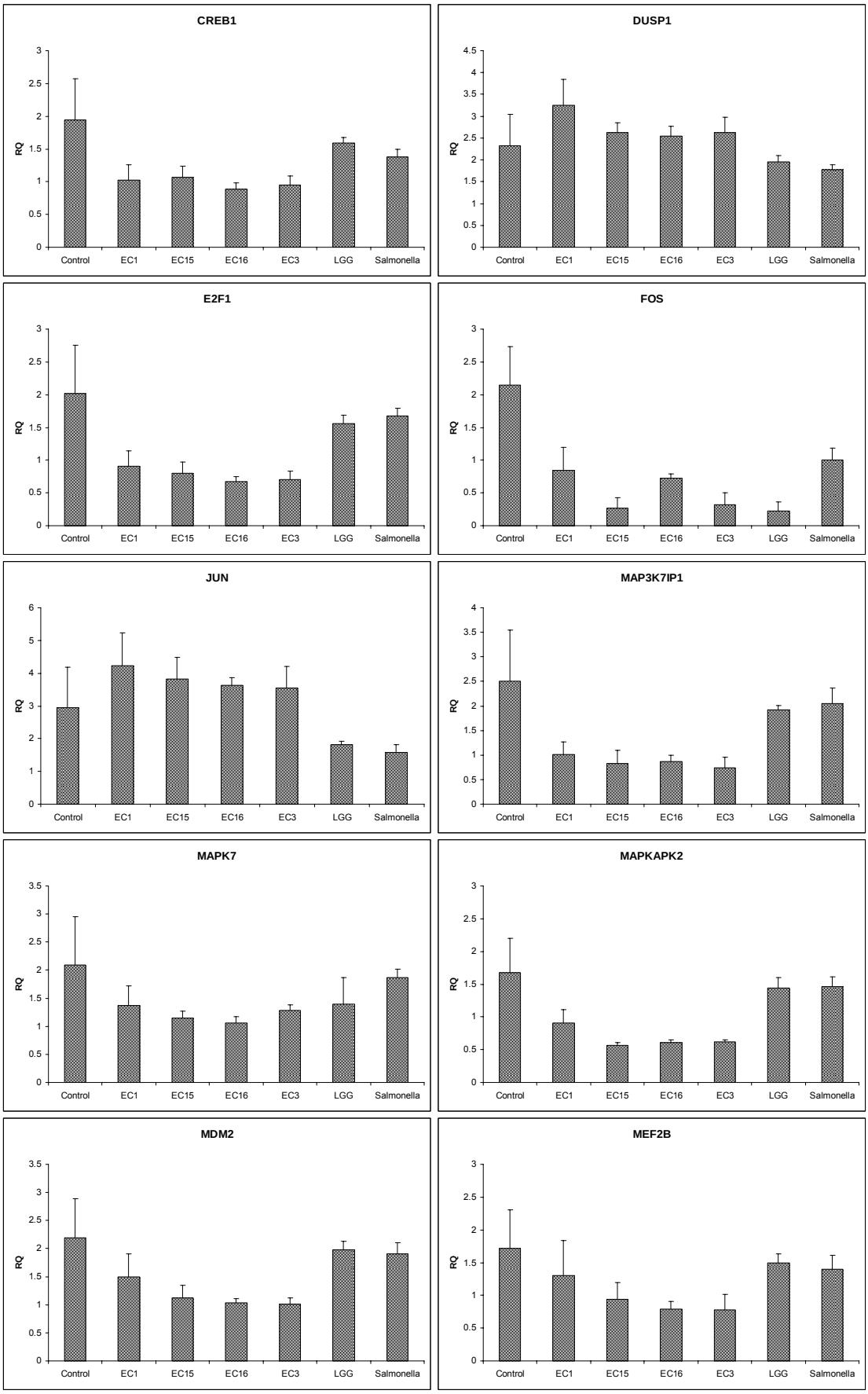
Figure 5.5 Gene expression in HCT116 with the treatment of *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium*. Experiments were done on 4 biological replicates using 10μg cDNA as described in Materials and Methods. Values are the averages of results obtained on four independent experiments.

5.1.2.1.2 Results in Caco-2 cells

To find out the common signaling pathways in the immune responses that *E. faecalis* regulates in the host, we tested the gene expression in Caco-2 cells as well. Figure 5.6 showed the results with the changes exceeding 1.5 folds compared with control in at least one treatment.

Consistent with the observations in HCT116 cells, NF-κB1, MAP3KPIP, MKNK1 as well as E2F1 and MDM2 were suppressed. DUSP1 was also upregulated in Caco-2 cells (Figure 5.6). This further suggested the importance of NF-κB and MAPK signaling pathways in the regulation of immune responses upon the treatment of *E. faecalis*. Interestingly, IL-8 was not upregulated significantly as we observed in HCT116 cells suggesting a multiple regulatory pathway of IL-8 protein production.

TLRs are important in the regulation of immune responses including modulating the cytokine production. TLR3 could not be detected or expressed low in Caco-2 but TLR2, TLR4 and TLR9 were expressed in Caco-2 cells. Taken the results from HCT116, in which, TLR2, TLR4 and TLR9 were not detected, we can see that TLRs might not play a key role in the regulation of immune responses in IECs in responses to *E. faecalis* since there were no common TLRs regulated in these two IECs. In addition, transcription factors like CREB1, FOS, E2F1 and MKNK1 were inhibited with the treatment of *E. faecalis*. The inhibition of these transcription factors might inhibit the gene transcription and also translation thus to inhibit the proinflammatory protein production. However, JUN was still upregulated by *E. faecalis* in the gene level. The different regulation pattern of transcription factors by *E. faecalis* again suggested the complex regulation mechanism of it in the host.


CREB1
DUSP1
E2F1
FOS
JUN
MAP3K7IP1
MAPK7
MAPKAPK2
MDM2
MEF2B
RQ
Control
EC1
EC15
EC16
EC3
LGG
Salmonella

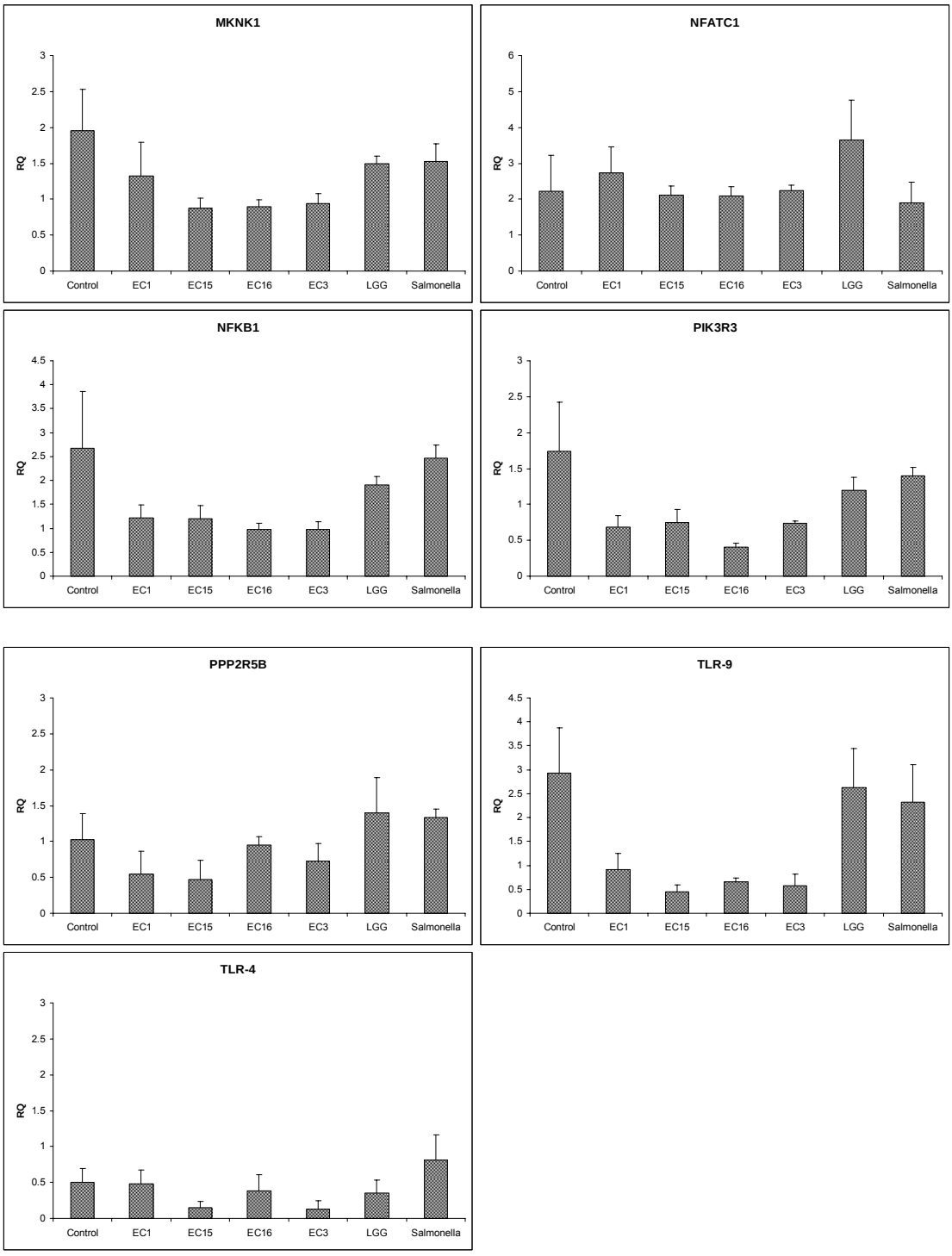


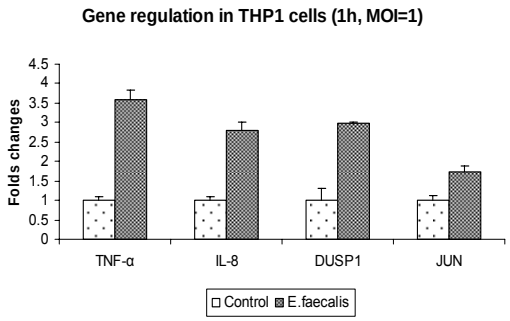
Figure 5.6 Gene regulation in Caco-2 cells with the treatment of *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium* for 6h. Experiments were done on 4 biological replicates using 10µg cDNA. Values are the averages of results obtained on four independent experiments.

5.1.2.1.3 Results in THP1 cells

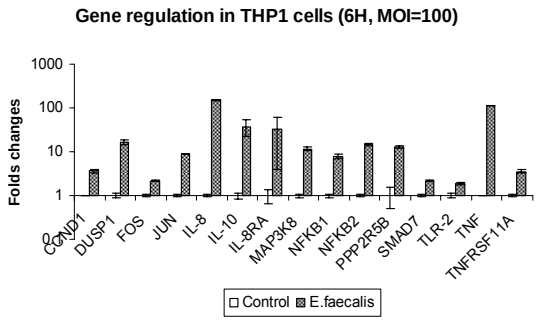
For simplicity, in this part, *E. faecalis* EC3 was used to treat with THP1 cells for 1 hour with a MOI of 1 and 6 hours with a MOI of 100. Experiments were repeated three times. All the data showed below are fold changes >1.5 with a P value< 0.05. All controls were set to 1.

E. faecalis stimulated IL-8 secretion in THP1 cell even at a low concentration (MOI=1) and short incubation time (1h) (Figure 3.4). The immune genes regulated by *E. faecalis* in THP1 cell were therefore investigated using TLDA cards. Figure 5.7A showed that TNF, IL-8, DUSP1 and JUN were quickly upregulated even with 1h incubation at a MOI of 1. The upregulation of DUSP1 might imply the negative feedback mechanism of MAPK signaling pathway which play a critical role in the inflammatory responses. JUN, however, was activated in both IECs and THP1 cells, suggesting that it is an important transcription factor in the inflammatory responses (Figure 5.4A and Figure 5.7A, B).

A



B



C

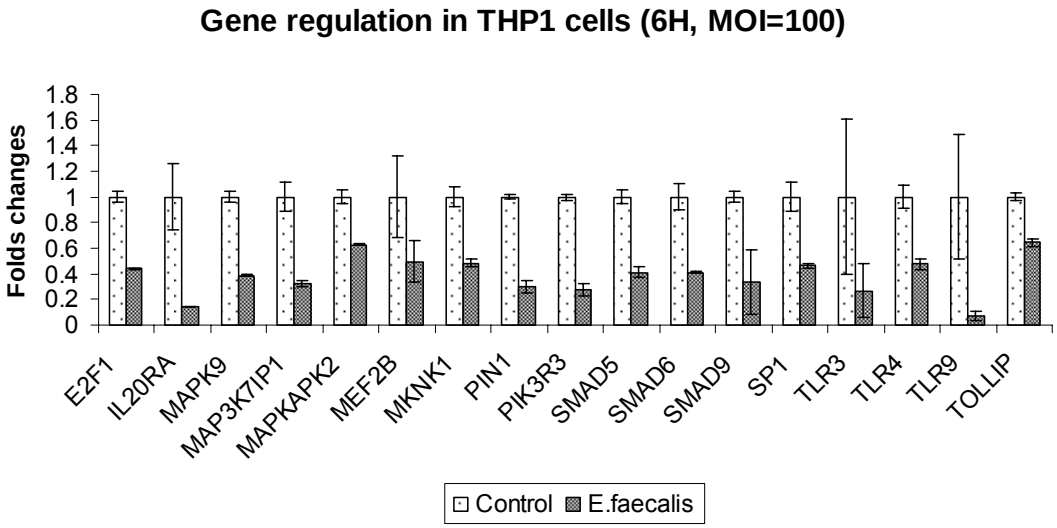


Figure 5.7 Gene regulation in THP1 cells with the treatment of *E. faecalis* EC3 for 1h at a MOI of 1 (A) and for 6h at a MOI of 100 (B, C). Experiments were done on 3 biological replicates using 10μg cDNA. Values are the averages of results obtained on three independent experiments.

More interestingly, with stronger stimulation of *E. faecalis* to THP1 cells, 15 genes were greatly upregulated and 17 genes were suppressed. Compared with the data obtained from HCT116 and Caco-2 with the treatment of *E. faecalis*, the results show that TLR3, TLR4 and TLR9 were all suppressed in the IECs and THP1 cells although not all of them were expressed in both Caco-2 and HCT116 cells. In our study, TLRs were merely detectable in HCT116 cells whereas TLR2, TLR4 and TLR9 were found expressed in Caco-2 cells. Only TLR3 were detected in HCT116

cells (Figure 5.4; Figure 5.5; Figure 5.6). Therefore, there are no common TLRs expressed by both HCT116 and Caco-2 cells. The low expression of TLRs in IECs implicated the mechanism of tolerance to the commensal bacteria in the intestine. In addition, IL-8 was suppressed in IECs but stimulated in THP1 cells. It is logical to reason that TLRs may not be involved in the regulation of IL-8 production or even inflammatory responses modulated by *E. faecalis* in intestinal epithelial cells. However, the upregulation of IL-8 secretion in THP1 cell might through TLR2 but not TLR3, TLR4 and TLR9.

Consistent with our cDNA microarray results, TLDA results showed that *E. faecalis* suppressed some cytokine receptors on the cell surface including IL-8RA and TNFRSF11A in IECs (Figure 5.4). Furthermore, using Western blotting, it showed that *E. faecalis* suppressed IL-8RA protein production in HCT116 cells (Figure 5.8). However, IL-8RA and TNFRSF11A were greatly upregulated in THP1 cells with the elevated level of IL-8 secretion (Figure 5.7B). Taken the data together, *E. faecalis* could regulate immune responses in the host through cytokine receptors like TNFRSF11A and IL-8RA. TLRs although are important in the innate immunity might not be the vital receptors on IL-8 regulation or inflammation suppression regulated by *E. faecalis*. In addition, NFKB1 and NFKB2 were greatly upregulated in THP1 cells suggesting their important roles in the inflammatory responses in macrophage. Other genes regulated in THP1 are listed in Figure 5.7. Several SMAD family members (SMAD5, 6, 9) were suppressed in THP1 by *E. faecalis* whereas SMAD7 was upregulated (Figure 5.7 B, C). Moreover, some of the MAPK family members (MAPK9, MAP3K7IP1 and MAPKAPK2) were also suppressed by *E. faecalis* in THP1 cells whereas MAPK8 was upregulated suggesting that *E. faecalis* regulates the

immune responses in the host in a very distinct way in both intestinal cells and immune cells (Figure 5.7 B, C).

5.1.2.2 Immunobloting Analysis

In response to *E. faecalis*, genes such as DUSP1, PIN1, E2F1, cylin D1 and IL8RA were regulated in HCT116 cells (Figure 5.4). To test protein expression encoded by these genes, we co-cultured *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium* with HCT116 cells for 6h with a MOI of 100 and analyzed the protein production using Western blotting as described in Materials and Methods.

Consistent with the gene expression in HCT116 cells, PIN1, cyclin D1 and E2F1 protein production were suppressed by *E. faecalis* (EC1, EC3, EC15 and EC16) but not L.GG and *S. typhimurium* (Figure 5.8). PIN1 has been shown to catalytically induce conformational changes in proteins following phosphorylation, thereby having profound effects on their catalytic activity, dephosphorylation, protein-protein interactions, subcellular location and/or turn over (Hsu *et al*., 2001; Liou *et al*., 2002; Lu *et al*., 1999; Ryo *et al*., 2001, 2002; Shen *et al*., 1998; Stukenberg *et al*., 2001; Wulf *et al*., 2001; Yaffe *et al*., 1997; Zhou *et al*., 2000). Thus, PIN1 is critical in phosphorylation signaling (Lu *et al*., 2002). Upregulation of PIN1 has been shown to elevate cyclin D1 gene expression by activating c-JUN, β-catenin and E2F1 transcription factors (Ryo *et al*., 2001; Wulf *et al*., 2001). Cyclin D1 has been shown to play a pivotal role in the development of cancer. Overexpression of cyclin D1 is found in many cancers (Bartkova *et al*., 1994; Gillett *et al*., 1994; Elsheikh *et al*., 2007). In contrast, inhibition of cyclin D1 expression causes growth arrest in tumor cells. Thus, PIN1 is an E2F1 target gene that is essential for transformation of mammary epithelial cells through activation of cyclin D1. In our study, the

suppression of PIN1, E2F1, cyclin D1 by *E. faecalis* at the same time implies the multiple control of tumor cell growth by the bacteria. This control might be mediated through inflammatory responses to *E. faecalis* in IECs, as tumor growth and metastasis are results of inflammation (Lu *et al*., 2006). However, results obtained from coculturing THP1 cells with *E. faecalis* demonstrated that even with a high secretion of IL-8, E2F1 protein production did not change (data not shown), suggesting the indirect regulation of E2F1 to IL-8 gene transcription and protein production.

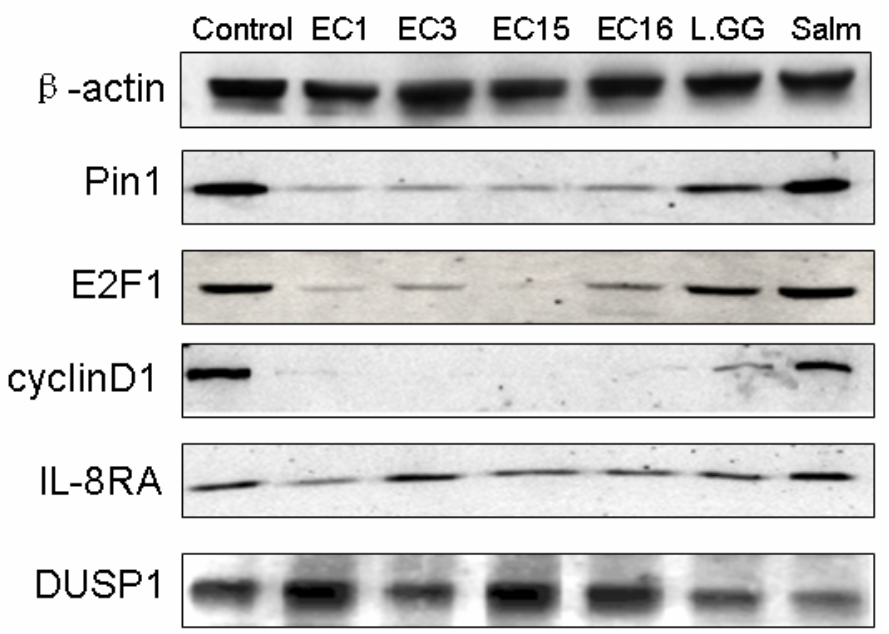


Figure 5.8 Protein expression in HCT116. *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium* (Salm) were treated with HCT116 cells for 6h with a MOI of 100. Total protein was harvested and protein production of PIN1, E2F1, cyclin D1, IL-8RA and DUSP1 were analyzed using Western blotting as described in Materials and Methods.

Recent study also showed that transcription factors like E2F1 are linked with central signaling pathways like MAPK (Tzippi, 2006). In addition, MAPK regulates cytokine production in response to a variety of stimuli. We therefore measured the phosphorylation of MAPK in IECs exposed to *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium* for 6h at a MOI of 100. In our study, DUSP1

protein was greatly stimulated upon the treatment of *E. faecalis* (EC1, EC15 and EC16). However, L.GG and *S. typhimurium* did not show any significant change on its expression (Figure 5.8). DUSP1, which dephosphorylates and inactivates MAPKs, is required for the inhibition of proinflammatory signaling pathways in mouse model (Sonya *et al*., 2006). As shown in Figure 5.9, phosphorylated ERK were suppressed by *E. faecalis* EC1, EC3 and EC15. *E. faecalis* EC16 seems have no effect on phosphorylated ERK production compared with other *E. faecalis* tested. Interestingly, phosphorylated p38 and JNK were not highly expressed with or without the bacteria stimulation. *E. faecalis* suppressed phosphorylated p38 and JNK1 at 6h incubation. L.GG and *S. typhimurium* surprisingly suppressed the phosphorylated p38 and JNK1 at 6h incubation too. The suppression of phosphorylated p38 and JNK by *E. faecalis* might due to upregulation of DUSP1, on the other hand, the suppression of phosphorylated p38 and JNK by L.GG and *S. typhimurium* might due to the negative feed back in the MAPK signaling pathway.

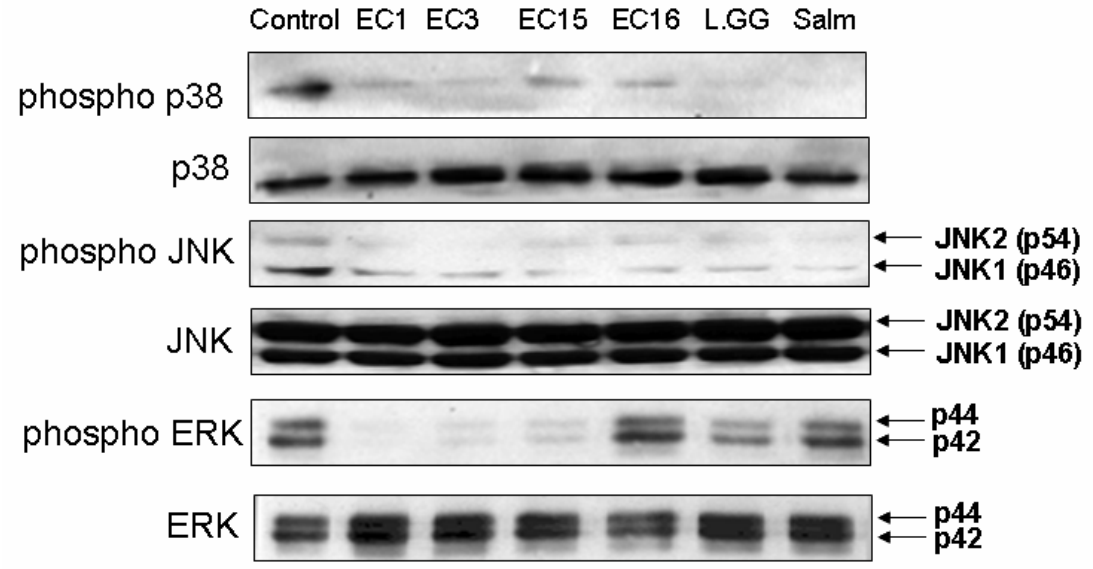
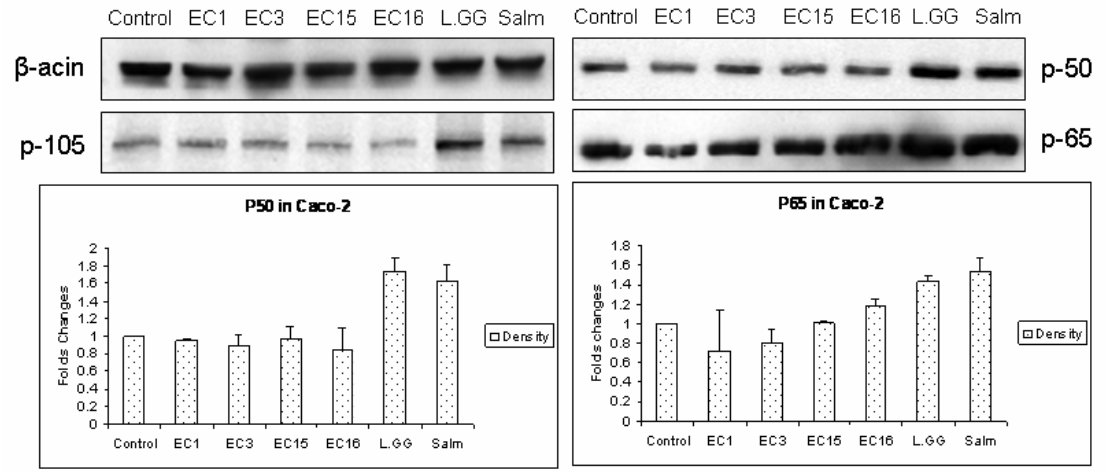


Figure 5.9 Protein expression in HCT116. *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium* (Salm) were administrated to HCT116 cells for 6h with a MOI of 100. Total protein was harvested and protein production of p38, JNK and ERK were analyzed using Western blotting as described in Materials and Methods.

Other protein tested such as p65, p105/p50 did not change significantly (data not shown) in HCT116 cells and in Caco-2 cells (Figure 5.10). Almost the same results were observed in Caco-2 cells as in HCT116 cells on the protein expression, such as cyclin D1, PIN1, E2F1, DUSP1 and MAPKs (data not shown).

A



B

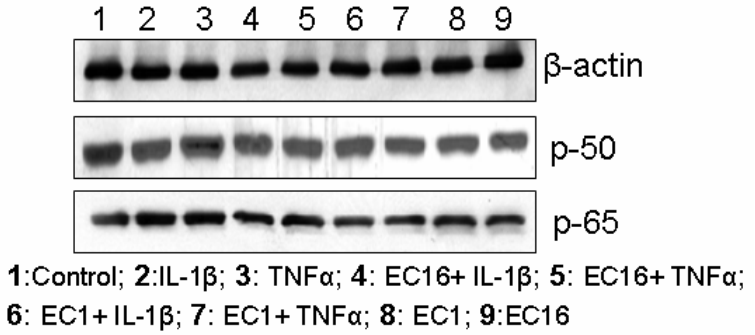


Figure 5.10 Protein expression in Caco-2. (A) p50 and p65 expression in Caco-2 cells with the treatment of *E. faecalis* (EC1, EC3, EC15 and EC16), L.GG and *S. typhimurium* (Salm) for 6h with a MOI of 100. (B) p50 and p65 expression in Caco-2 cells with the treatment of 2ng/ml of IL-1β, 200ng/ml of TNF-α, the coculture of 2ng/ml of IL-1β and 200ng/ml of TNF-α with *E. faecalis* EC1, EC16 respectively, and *E. faecalis* EC1, EC16 at a MOI of 100. Total protein was harvested and protein production was analyzed using Western blotting as described in Materials and Methods.

The data above show that *E. faecalis* did not change the expression of p105/p50 and p65 significantly even with the stimulation of IL-1β and TNF-α, implying the suppression of inflammatory responses in IECs by *E. faecalis* might

bypass NF-κB signaling pathway. However, L.GG and *S. typhimurium* seemed to upregulate p50 and p65 expression (Figure 5.10A). Furthermore, the results from EMSA experiment showed that *E. faecalis* did not activate NF-κB binding activities (data not shown), further suggested that *E. faecalis* regulates inflammatory responses might bypass NF-κB signaling pathways at first 6h tested.

Taken the results from HCT116 and Caco-2 cells, we can see that MAPK signaling pathway might be the main regulatory pathway involve in the inflammatory suppression by *E. faecalis* in IECs. More interestingly, other proteins (e.g. E2F1 and PIN1) might also be involved indirectly in the immune modulation by *E. faecalis*.

5.1.2.3 TNF-α, IL-1β and *S. typhimurium* Induced Cytokine Secretion and Protein Production

The above data showed that *E. faecalis* suppressed inflammatory responses in IECs by inhibition of IL-8 production. To investigate the effects of *E. faecalis* on inflammatory responses stimulated by proinflammatory cytokines and pathogen, we cocultured IECs (HCT116 and Caco-2) with 2ng/ml of IL-1β, 200ng/ml of TNF-α and *S. typhimurium* with or without the presence of *E. faecalis* EC16 at a MOI of 100 for 30mins, 1h, 2h, 4h and 6h as described in Materials and Methods. Supernatants were harvested for ELISA and cytokine assays. Proteins were harvested for Western blotting analysis. IL-8 concentrations inside cells were determined using ELISA method.

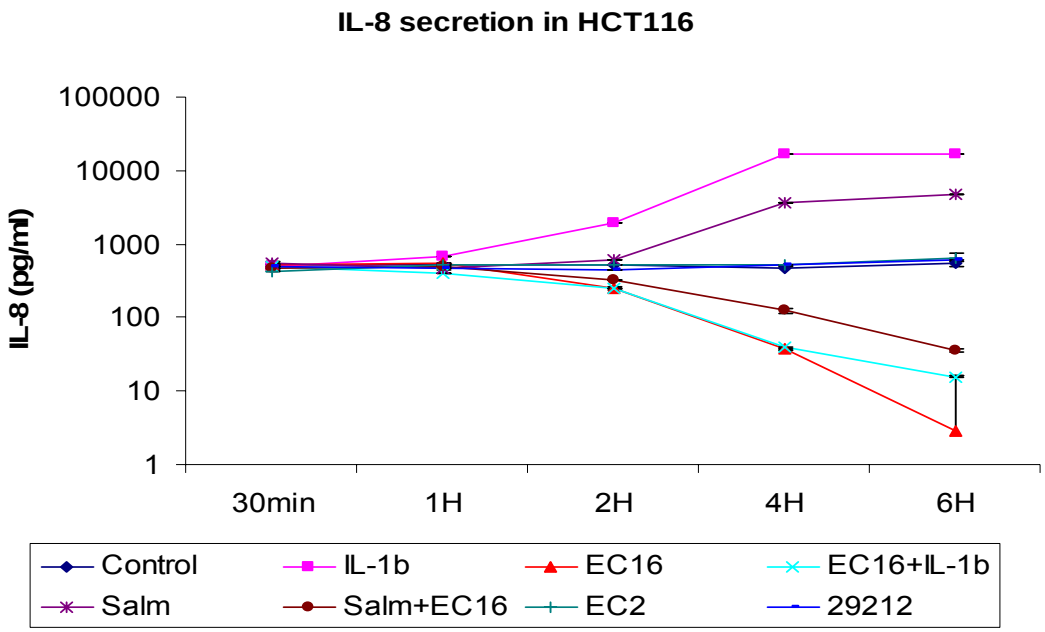
5.1.2.3.1 Cytokine Production

The results showed that IL-8 secretion by HCT116 cells increased from 30mins to 6 hours with IL-1β and *S. typhimurium* stimulation (Figure 5.11A). In contrast, IL-8 secretion into the supernatant remained suppressed with the

administration of EC16 to IL-1β and *S. typhimurium* stimulated culture and thereafter rapidly decreased from 4h to 6h. As shown in Figure 5.11A, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 did not show any effects on IL-8 secretion in HCT116 cells suggesting that only few strains of *E. faecalis* possessed the ability to suppress inflammatory responses in the host.

To further investigate how *E. faecalis* regulates IL-8 production, we examined the IL-8 protein expression inside the cells upon the stimulation with IL-1β and *S. typhimurium* with or without the presence of EC16. As shown in Figure 5.11B, IL-1β and *S. typhimurium* stimulated IL-8 production inside the cells from 30mins or even earlier. The attenuation of IL-8 inside HCT116 by EC16 was observed from 4h to 6h. Compared with the IL-8 production in IL-1β and *S. typhimurium* stimulated culture, EC16 suppressed the IL-8 activation from 30mins. As expected, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 did not show any effects on IL-8 production (Figure 5.11B).

A



B

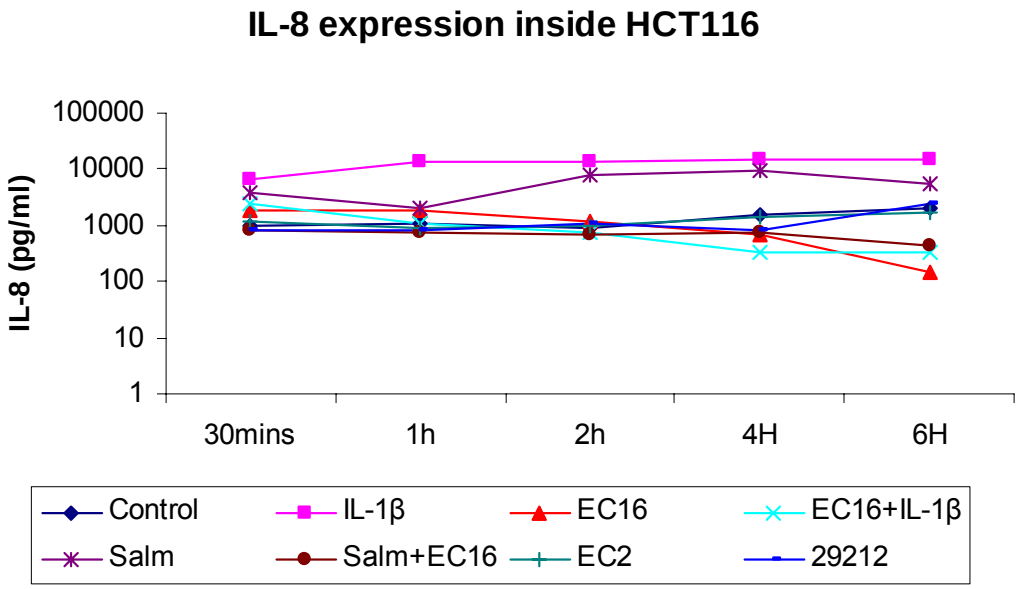
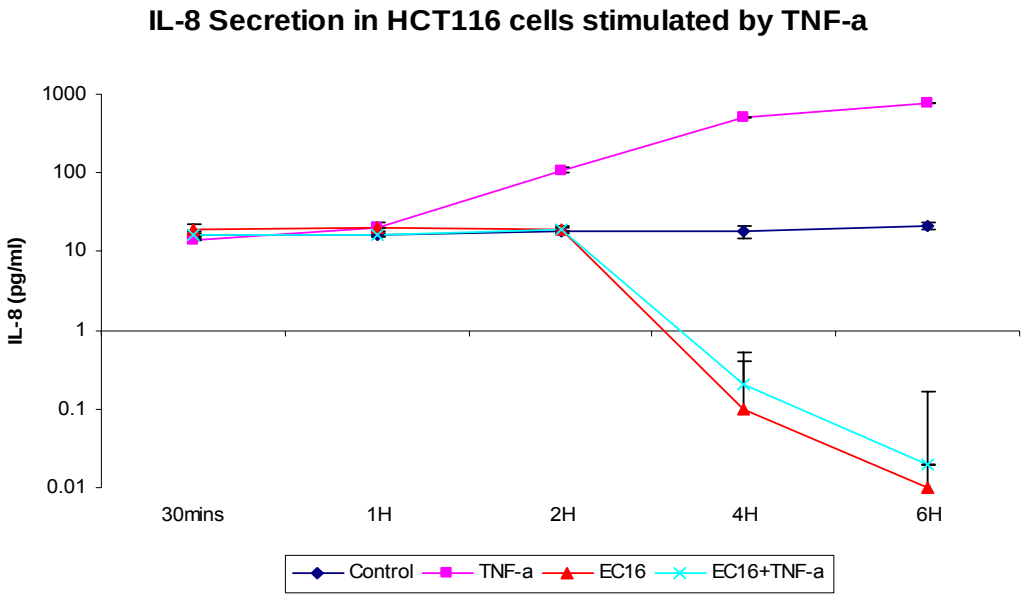


Figure 5.11 IL-8 expression in HCT116 with the stimulation of 2ng/ml of IL-1β, *E.faecalis* EC16, EC16 in the presence of 2ng/ml IL-1β, *S. typhimurium*, *S. typhimurium* coculture with EC16, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30mins, 1h, 2h, 4h and 6h. Control was added with culture medium only. (A) Supernatants were harvested and IL-8 secretion was tested using ELISA method as described in Materials and Methods. (B) Proteins were harvested and IL-8 expression was tested using ELISA with a 50 times dilution. Three independent experiments were compiled to produce the data shown.

A



B

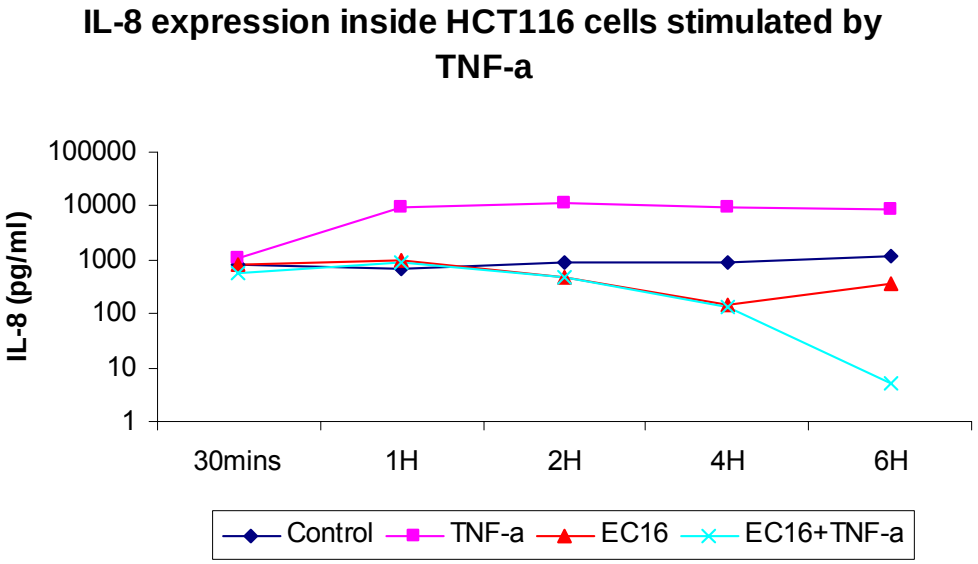


Figure 5.12 IL-8 expression in HCT116 with the stimulation of 200ng/ml of TNF-α, *E.faecalis* EC16, and EC16 in the presence of 200ng/ml TNF-α for 30min, 1h, 2h, 4h and 6h. Control was added with culture medium only. (A) Supernatants were harvested and IL-8 secretion was tested using ELISA method as described in Materials and Methods. (B) Proteins were harvested and IL-8 expression was tested using ELISA with a 50 times dilution. Three independent experiments were compiled to produce the data shown.

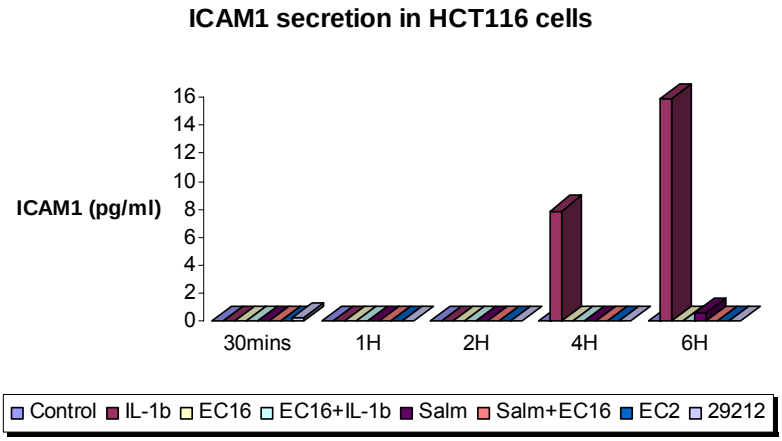
The same phenomena were also observed with the stimulation of TNF-α in HCT116 cells (Figure 5.12). IL-8 secretion was activated by TNF-α from 1h.

However, in the presence of EC16, the effects of TNF-α on inflammatory reaction was eliminated. Not surprisingly, EC16 also attenuated IL-8 stimulation by TNF-α inside HCT116 cells (Figure 5.12 B).

In the studies described above, we focused on IL-8 regulation by *E. faecalis* in IECs. However, the IL-8 attenuation effects may be only one of many important events regulated by *E. faecalis*. Therefore, we examined other cytokines and inflammation related molecules production (ICAM-1, IL-18, IL-2, IL-5, IL-17, IFN-γ and TNF-α) modulated by *E. faecalis* in IECs using cytokine assays. IL-12(P70), IL-6, IL-4, IL-10 and TGF-β expression were tested using ELISA.

Including numerous receptors, IECs also express adhesion molecules and proinflammatory mediators that allow IECs to communicate with the immune system. It is well established that activated endothelial cells in inflamed colonic tissue express intercellular adhesion molecule-1 (ICAM-1) (Vainer *et al*., 2000; 2002; 2006), however the function and expression pattern in IECs have not been fully illustrated. From Figure 5.13 A, we can see that ICAM-1, which is important in immune responses by mediating the arrest and further migration of neutrophils, was expressed in HCT116 cells. More interestingly, IL-1β upregulated ICAM-1 expression from 4h and *S. typhimurium* stimulate its expression from 6h. *E. faecalis* EC16 suppressed the ICAM-1 expression stimulated by IL-1β and *S. typhimurium* (Figure 5.13 A).

A



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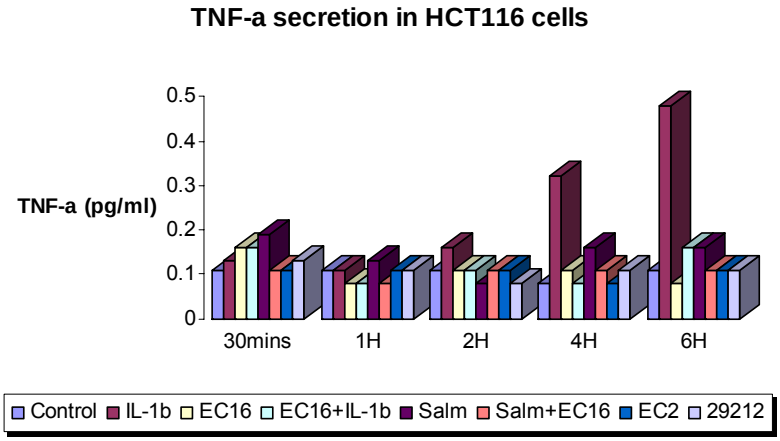

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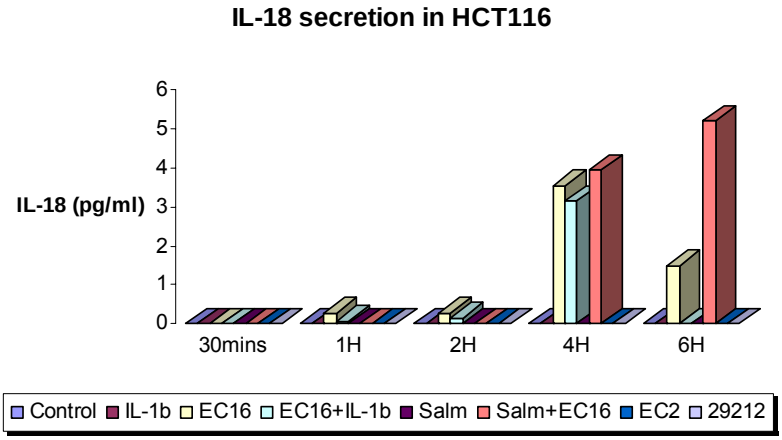


Figure 5.13 ICAM-1, TNF-α and IL-18 secretion in HCT116 with the stimulation of 2ng/ml of IL-1β, *E.faecalis* EC16, EC16 in the presence of IL-1β, *S. typhimurium*, *S. typhimurium* coculture with EC16, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30min,1h, 2h, 4h and 6h. Control was added with culture medium only. Supernatants were harvested for cytokine assay as described in Materials and Methods. (A) ICAM-1 secretion in HCT116. (B) TNF-α secretion in HCT116. (C) IL-18 secretion in HCT116. Three independent experiments were compiled to produce the data shown.

Another important regulator of epithelial function during inflammation is TNF-α. Its levels are elevated in both human inflammatory bowel diseases and animal models of intestinal inflammation. Therefore, therapies that target TNF-α may provide promising new alternative for the treatment of intestinal inflammation (Jijon *et al*., 2002). TNF-α, the important proinflammatory cytokine, was expressed at a very low level in HCT116 cells (Figure 5.13B). However, with the stimulation of IL-1β, TNF-α was enhanced to a higher level compared with control suggesting a positive feed back loop on the inflammatory responses in HCT116 cells upon proinflammatory cytokine stimulation. *E. faecalis* EC16, again, suppressed TNF-α secretion stimulated by IL-1β. *S. typhimurium*, however, had no significant effect on TNF-α secretion.

As we can see from Figure 5.13C, *E. faecalis* activated IL-18 secretion in HCT116 from 1 hour. IL-18 is a member of the IL-1 family. IL-1β and IL-18 are related closely, and both require the intracellular cysteine protease caspase-1 for biological activity. IL-18 is a proinflammatory cytokine. It plays an important role in immune and inflammatory reactions, and is notably present in several autoimmune disorders. It seems to exert its functions not by itself but rather in concert with other factor, promoting many of the biologic activities associated with these other cytokines. Different with most of the other cytokines, IL-18 is abundant in non-immune tissues including the gut and the skin and it likely provides an important role in the defense against mucosal and commensal organisms. Studies showed that activation of caspase-1 could release IL-18 from the cells (Li *et al*., 2007). However, the mechanism for processing and secretion of IL-18 remains somewhat of a mystery. The activation of caspase-1 occurs independently of gene expression. IL-18 may also be processed by stimuli other than caspase-1. Interestingly, administration of IL-18 to mice infected with a lethal dose of virulent strain of *S. typhimurium* reduced the

bacteria number in the tissues, rescuing them from death. On the other hand, macrophage infected with *S. typhimurium* exhibited reduced expression of IL-18, suggesting the ability of this pathogen to suppress production of IL-18 (Elhofy and Bost, 1999; John *et al*., 2002). A more recent study showed that IL-18 has an effective antitumor effect by developing a long-lasting protective immunity including both innate and adaptive immunity in the host (Xu *et al*., 2007). In our study, proinflammatory cytokine IL-1β and *S. typhimurium* could not activate IL-18 secretion in HCT116. However, *E. faecalis*, which suppressed several inflammatory cytokines production, activated IL-18 secretion in HCT116 even in the presence of *S. typhimurium*. Furthermore, IFN-γ, which was reported to increase in the presence of IL-18 in several cell lines (Bombardieri *et al*., 2007), was not detected in our system. Therefore, *E. faecalis* activated IL-18 expression in HCT116 may help in the suppression of inflammatory responses other than activation.

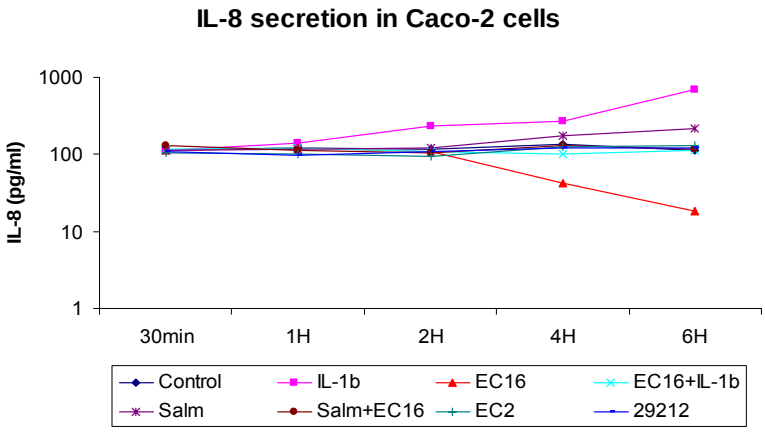
Other cytokines like, IL-4, IL-10, TGF-β, IL-12 (P70), IL-6, IL-2, IL-5, IL-17 and IFN-γ were not detected in HCT116 cells.

To confirm the regulatory effect of *E. faecalis* on IECs, Caco-2 was treated with 2ng/ml of IL-1β, *E. faecalis* EC16, EC16 in the presence of IL-1β, *S. typhimurium*, *S. typhimurium* co-culture with EC16, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30min, 1h, 2h, 4h and 6h. IL-8 secretion in the supernatants and its expression inside the cells were tested using ELISA as described in Materials and Methods. Other cytokines and adhesion molecules expression (ICAM-1, IL-18, IL-2, IL-5, IL-17, IFN-γ and TNF-α) were detected using cytokine assay at 30mins, 2h and 6h of the treatments.

Consistent with what we found in HCT116 cells, IL-1β and *S. typhimurium* triggered IL-8 secretion and also its expression inside the Caco-2 cells from 1h or

even earlier (Figure 5.14). When *E. faecalis* EC16 was added into the media, IL-8 activation by IL-1β and *S. typhimurium* was eliminated. In addition, *E. faecalis* EC16 suppressed IL-8 secretion in Caco-2 from 4h. However, they did not downregulate IL-8 expression inside the cells. As expected, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 had no effect on IL-8 production in Caco-2 (Figure 5.14).

A



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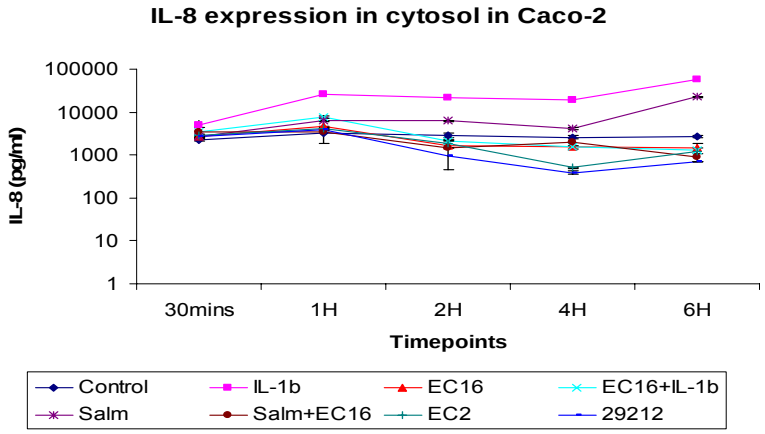


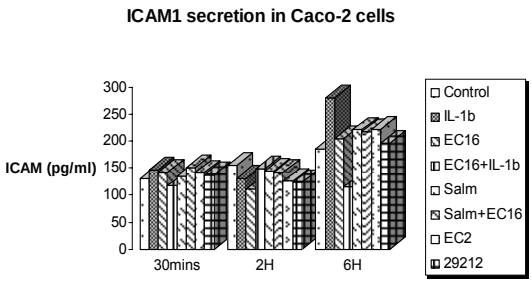
Figure 5.14 IL-8 expression in Caco-2 with the stimulation of 2ng/ml of IL-1β, *E.faecalis* EC16, EC16 in the presence of IL-1β, *S. typhimurium*, *S. typhimurium* coculture with EC16, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30mins, 1h, 2h, 4h and 6h. Control was added with culture medium only. (A) Supernatants were harvested and IL-8 secretion was tested using ELISA method as described in Materials and Methods. (B) Proteins were harvested and IL-8 expression was tested using ELISA with a 50 times dilution. Three independent experiments were compiled to produce the data shown.

We further tested other cytokines production in Caco-2 cells. As shown in Figure 5.15, ICAM-1 was stimulated by IL-1β at 6h however was suppressed by

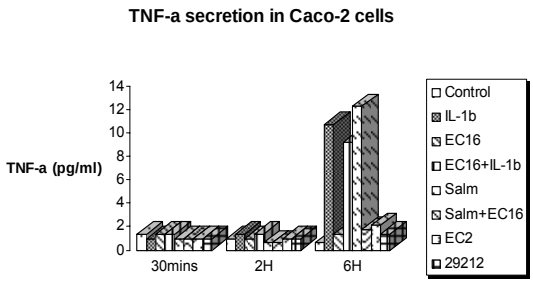
adding *E. faecalis* EC16. *S. typhimurium*, surprisingly did not activate ICAM1 at 6h in Caco-2 cells (Figure 5.15A). TNF-α was stimulated by IL-1β and *S. typhimurium* at 6h. Its secretion activated by *S. typhimurium* was attenuated by *E. faecalis* EC16. However *E. faecalis* EC16 did not inhibit TNF-α secretion with the stimulation of IL-1β suggesting the different mechanisms regulated by proinflammatory cytokines and pathogenic bacteria. As expected, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 did not regulate ICAM-1 and TNF-α expression in Caco-2 cells (Figure 5.15 B).

Cytokine action is contextual (Sporn and Roberts, 1998). As it was reported, IL-1β can activate IL-2, IL-6 and chemokines in various types of cells. IL-2 and IFN-γ can augment IL-1, TNF and IL-6 production. IL-2 can stimulate IFN-γ (Torres *et al*., 1982). In our study using Caco-2 cells, IL-2 was greatly augmented with the presence of IL-1β and *S. typhimurium* at 6 hours. *E. faecalis* EC16 suppressed IL-2 activation in Caco-2 cells (Figure 5.15 C). Confusingly, *E. faecalis* did not reduce IL-2 level stimulated by IL-1β. *E. faecalis* EC2 and *E. faecalis* ATCC 29212 activated IL-2 secretion at 6h. The same phenomena were observed in the regulation of IL-5, IL-17 and IFN-γ secretion in Caco-2 with the treatment of *E. faecalis*, IL-1β and *S. typhimurium*.

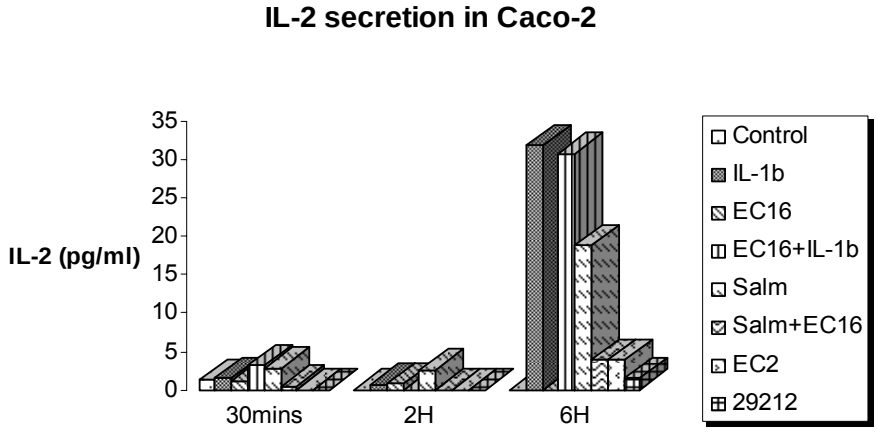
A


ICAM1 secretion in Caco-2 cells
ICAM (pg/ml)
300
250
200
150
100
50
0
30mins
2H
6H
Control
IL-1b
EC16
EC16+IL-1b
Salm
Salm+EC16
EC2
29212

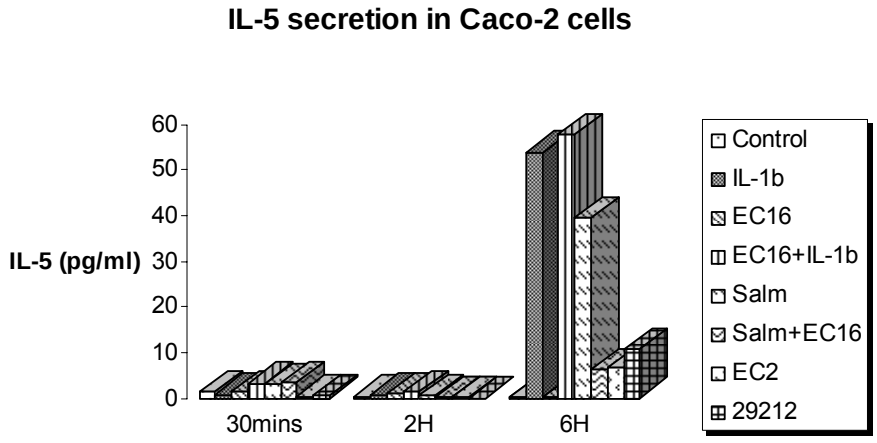
B


TNF-a secretion in Caco-2 cells
TNF-a (pg/ml)
14
12
10
8
6
4
2
0
30mins
2H
6H
Control
IL-1b
EC16
EC16+IL-1b
Salm
Salm+EC16
EC2
29212

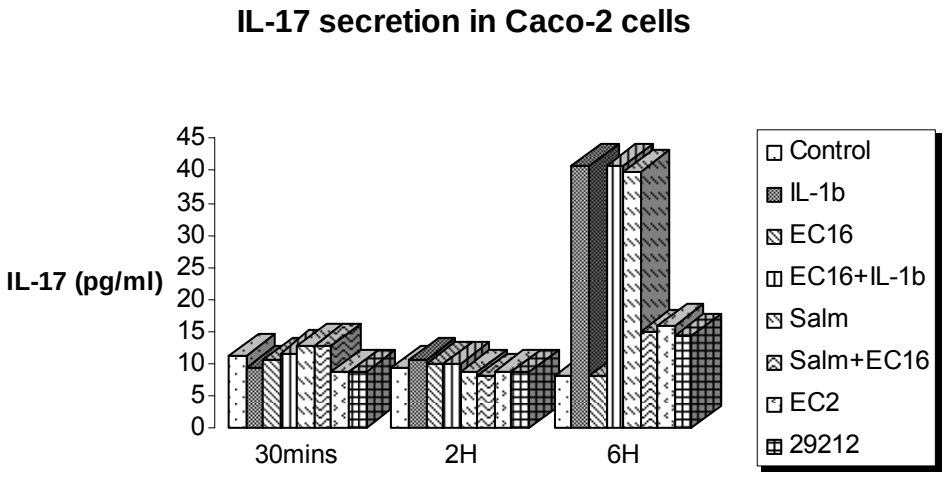
C


IL-2 secretion in Caco-2
IL-2 (pg/ml)
35
30
25
20
15
10
5
0
30mins
2H
6H
Control
IL-1b
EC16
EC16+IL-1b
Salm
Salm+EC16
EC2
29212

D


IL-5 secretion in Caco-2 cells
IL-5 (pg/ml)
60
50
40
30
20
10
0
30mins
2H
6H
Control
IL-1b
EC16
EC16+IL-1b
Salm
Salm+EC16
EC2
29212

E



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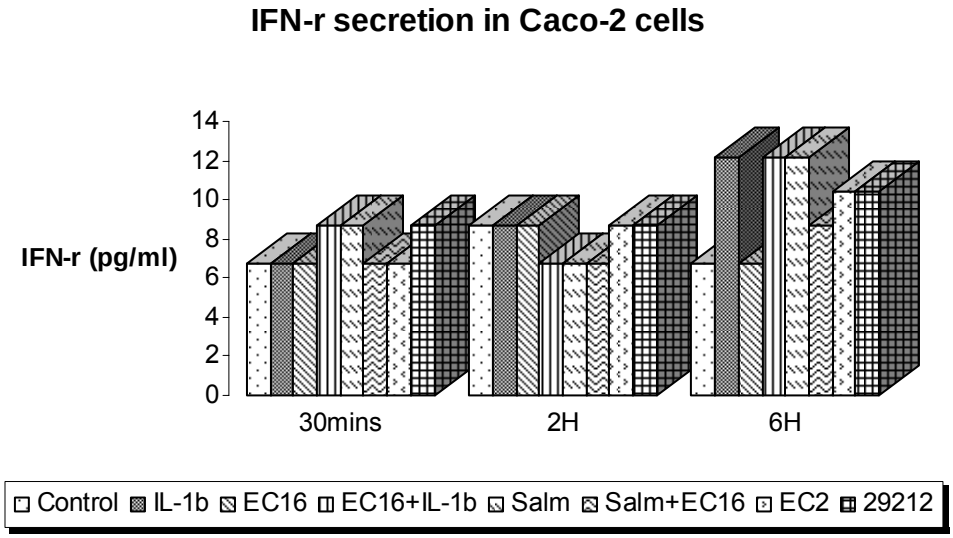


Figure 5.15 ICAM-1(A), TNF-α (B) and IL-2 (C), IL-5 (D), IL-17 (E) and IFN-γ (F) secretion in Caco-2 cells with the stimulation of 2ng/ml of IL-1β, *E. faecalis* EC16, EC16 in the presence of 2ng/ml IL-1β, *S. typhimurium*, *S. typhimurium* coculture with EC16, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30min, 2h and 6h. Control was added with culture medium only. Supernatants were harvested for cytokine assay as described in Materials and Methods. All experiments were done once.

IL-5 is a Th2 cytokine which promote humoral immune responses, especially IgE production leading to allergy. IL-17 is characterized as cytokine-inducing

cytokine that exhibits pleiotropic biologic activities on various cell types. It can activate IL-8 in IECs. In addition to inducing cytokines and chemokines, IL-17 enhances surface expression of ICAM-1 in human fibroblasts. Also, in human keratinocytes, IL-17 specifically and dose-dependently expression augmented IFN-γ induced ICAM-1 expression and the induction of IL-8 (Teunissen *et al*., 1998; Albanesi *et al*., 1999). IL-17, in addition to inducing NF-κB and AP-1 DNA binding activities, also regulates the activities of MAP kinases in IECs and therefore could be involved in enteric disorders (Andoh *et al*., 2001). In our study, IL-5, IFN-γ and IL-17 were induced by IL-1β and *S. typhimurium* but suppressed by *E. faecalis* with the stimulation of *S. typhimurium* (Figure 5.15 D, E, F).

From the above results, we can see that together with reduced production of inflammatory cytokine IL-8, *E. faecalis* EC16 inhibited several other proinflammatory cytokines production in IECs. This inhibition may contribute to human innate and adaptive immune homeostasis in response to infection and overexpression of proinflammatory cytokines in the intestine.

IL-4, IL-10, IL-6, IL-12 (P70), IL-18 and TGF-β could not be detected in Caco-2 with the treatments stated above.

5.1.2.3.2 Immunoblotting study

So far, we tested cytokine production in the IECs and also the gene expression of some interesting genes. We further examine the protein expression in IECs. In this part, Caco-2 and HCT116 were treated with 2ng/ml of IL-1β, *E. faecalis* EC16, EC16 in the presence of 2ng/ml IL-1β, *S. typhimurium*, *S. typhimurium* co-culture with EC16, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30min, 1h, 2h, 4h and 6h respectively. Proteins were harvested as described in Materials and Methods and their expression were determined using Western blotting.

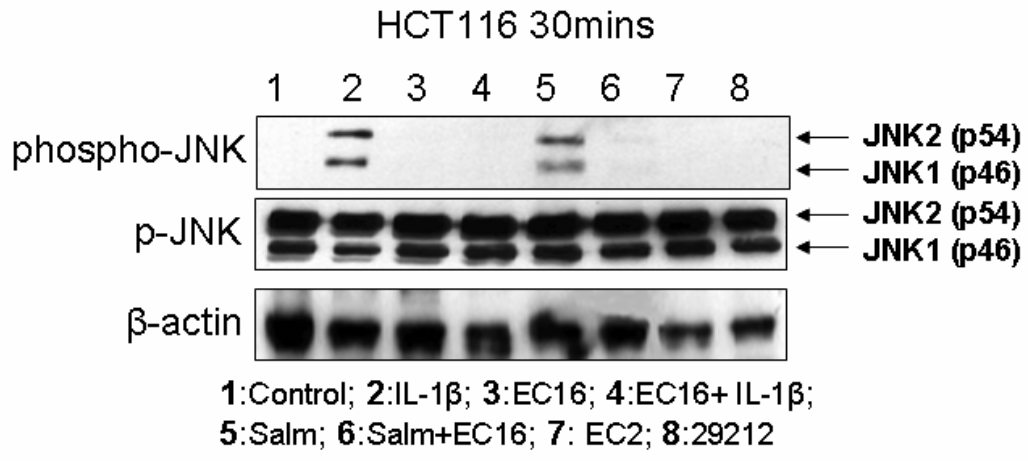
From the microarray and TLDA data, we can see that MAPKs signaling pathways were regulated by *E. faecalis*. In addition, a consequence of IL-1β or pathogen stimulation in IECs is the activation of mitogen activated protein kinases (MAKPs). At least three groups of MAPKs have been identified including the extracellular signal-regulated kinases (ERKs), the c-JUN NH_2-terminal kinases (JNKs) and p38. Of these, ERKs are responsible for growth and differentiation. JNKs and p-38 family members are generally implicated in responses to cellular stress, inflammation and apoptosis (Jijon *et al*., 2002; Cobb, 1999). C-JUN is a major component of the AP-1 transcription factor which regulates various aspects of cell proliferation and differentiation. In our study, activation of these kinases was assayed by using antibodies specific for the phosphorylated, active forms of these kinases by Western blotting. As shown in Figure 5.16 A and D, phosphorylated JNK was greatly activated by IL-1β and *S. typhimurium* at 30mins. Interestingly, *E. faecalis* suppressed the activated phosphorylated JNK in Caco-2 and HCT116 cells (Figure 5.16 A and D). phosphorylated JNK level activated by IL-1β and *S. typhimurium* then went down from 1 hour (Figure 5.16 B and E). p38, which was reported to stabilize IL-8 mRNA (Hoffman *et al*., 2002), was greatly upregulated by IL-1β and *S. typhimurium* at 1h, 2h and 4h incubation (Figure 5.16 D, E and F). Interestingly, *E. faecalis* EC 16 itself not only inactivate p38 expression but also suppress the activation stimulated by IL-1β and *S. typhimurium* (Figure 5.16 D, E and F) at first several hours incubation.

However co-culture of *E. faecalis* EC16 in the presence of IL-1β or *S. typhimurium* activated phosphorylated JNK (Figure 5.16 B, E, G, and H) even though there was no activation of IL-8 production from 1h (Figure 5.14). Unexpectedly, *E. faecalis* EC16 itself activated phosphorylated JNK expression from 2h and peaked at 4h incubation in Caco-2 cells (Figure 5.16 F, G and H) with the suppression of IL-8

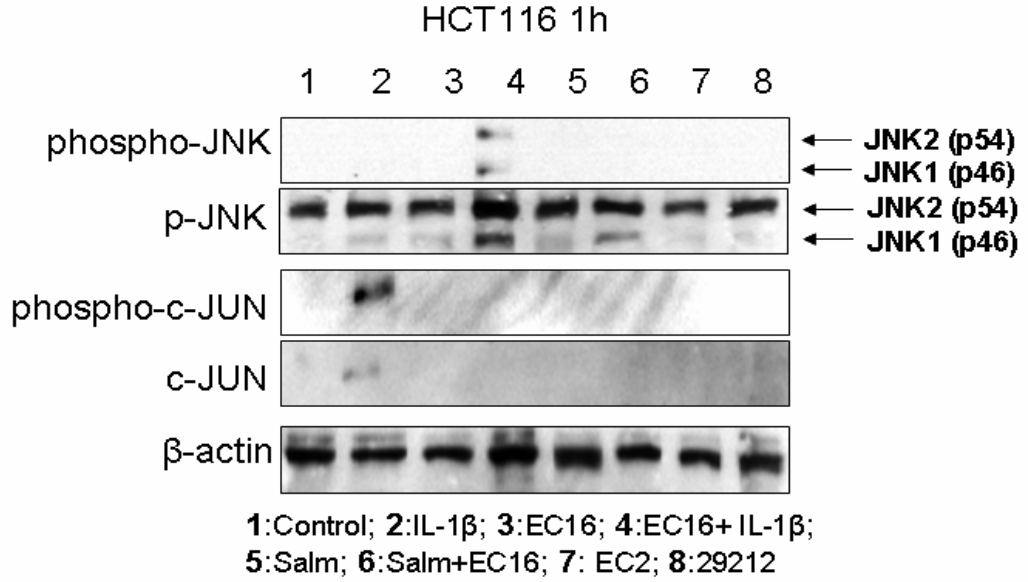
secretion (Figure 5.14A). The same was observed with phosphorylated p38 expression from 4h (Figure 5.16 G and H). These data conflicted with what we observed in HCT116 at 6h incubation with *E. faecalis* (Figure 5.9) suggesting that the immune regulation by *E. faecalis* may be cell line dependent. The different regulation pattern in JNK and p38 by *E. faecalis* in Caco-2 and HCT116 might explain the different modulation ability of *E. faecalis* in the inflammatory cytokines production (Figure 5.12; Figure 5.13; Figure 5.14; Figure 5.15).

From Figure 5.16 (B, C, E and F), we can see that c-JUN activated by IL-1β and *S. typhimurium* but suppressed by the co-culture of *E. faecalis* EC16 at 1h and 2h in both Caco-2 and HCT116 cells. Interestingly, *E. faecalis* EC2 and 29212 also activated c-JUN in Caco-2 cells (Figure 5.16E and F), which might explain the upregulation of IL-2 in Caco-2 with the treatment of *E. faecalis* EC2 and 29212 (Figure 5.15 C). C-JUN expression could not be detected in either HCT116 or Caco-2 cells with the treatments after 2h. Recent studies also showed that AP-1 especially c-JUN are important in regulation of IL-8 expression (Matsumoto, 2007; Choi, 2007). Moreover, we found that ERKs in IECs did not change upon the treatment of IL-1β, *S. typhimurium* and *E. faecalis* from 30min to 6h (Figure 5.16 H), suggesting that ERKs are not involved in the regulation of inflammatory responses in IECs upon *E. faecalis* treatment. Taking all the data together, we can see that *E. faecalis* suppresses inflammatory responses through suppression of JNK and p38 signaling pathway and further suppress AP-1. JNK and p38 might have negative feedback mechanisms since the longer incubation time of *E. faecalis* activated their phosphorylation.

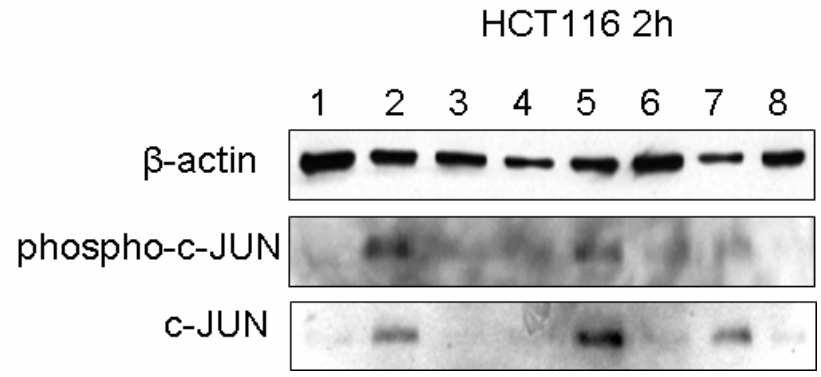
A


HCT116 30mins
1 2 3 4 5 6 7 8
phospho-JNK
p-JNK
β-actin
JNK2 (p54)
JNK1 (p46)
JNK2 (p54)
JNK1 (p46)
1:Control; 2:IL-1β; 3:EC16; 4:EC16+ IL-1β;
5:Salm; 6:Salm+EC16; 7: EC2; 8:29212

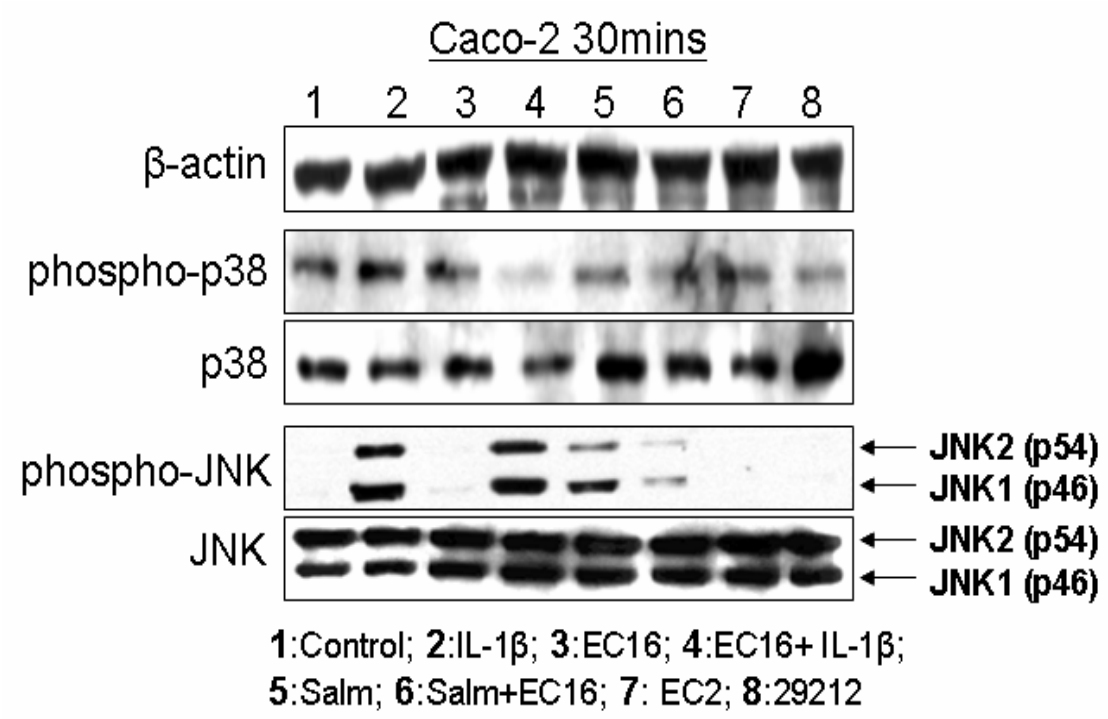
B


HCT116 1h
1 2 3 4 5 6 7 8
phospho-JNK
p-JNK
phospho-c-JUN
c-JUN
β-actin
JNK2 (p54)
JNK1 (p46)
JNK2 (p54)
JNK1 (p46)
1:Control; 2:IL-1β; 3:EC16; 4:EC16+ IL-1β;
5:Salm; 6:Salm+EC16; 7: EC2; 8:29212

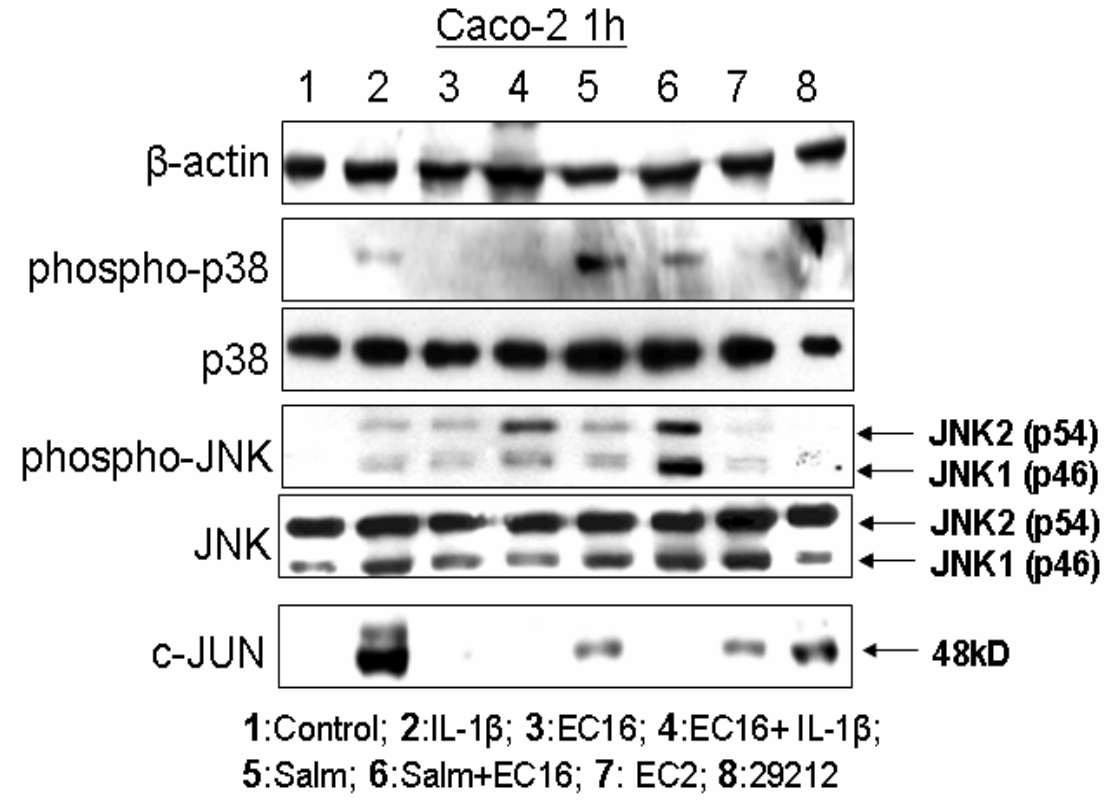
C


HCT116 2h
1 2 3 4 5 6 7 8
β-actin
phospho-c-JUN
c-JUN

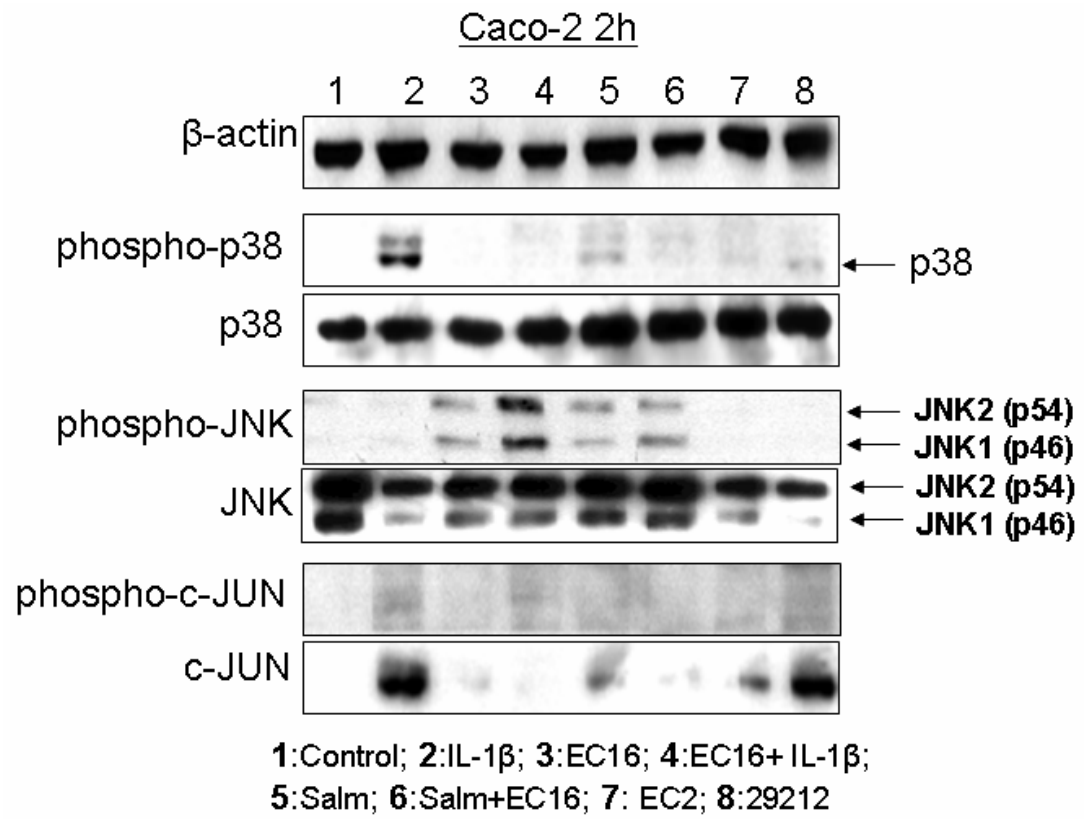
D


Caco-2 30mins
1 2 3 4 5 6 7 8
β-actin
phospho-p38
p38
phospho-JNK
JNK
JNK2 (p54)
JNK1 (p46)
JNK2 (p54)
JNK1 (p46)
1:Control; 2:IL-1β; 3:EC16; 4:EC16+ IL-1β;
5:Salm; 6:Salm+EC16; 7: EC2; 8:29212

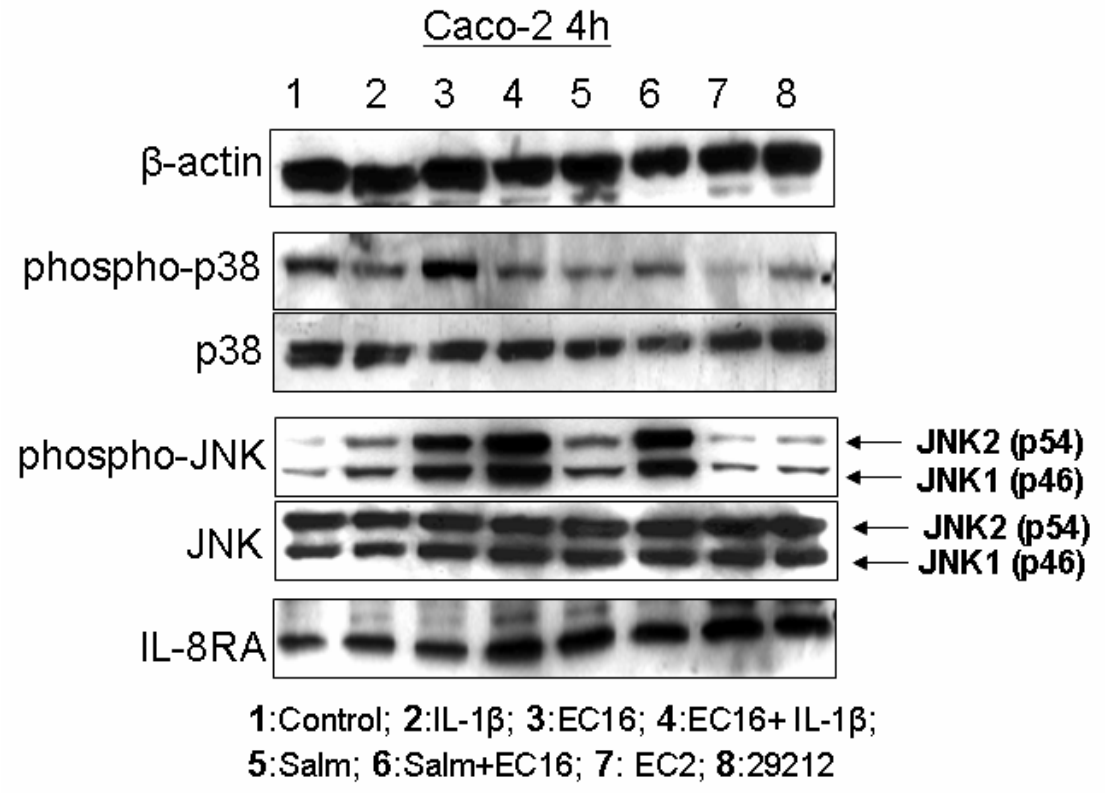
E


Caco-2 1h
1 2 3 4 5 6 7 8
β-actin
phospho-p38
p38
phospho-JNK
JNK
c-JUN
JNK2 (p54)
JNK1 (p46)
JNK2 (p54)
JNK1 (p46)
48kD
1:Control; 2:IL-1β; 3:EC16; 4:EC16+ IL-1β;
5:Salm; 6:Salm+EC16; 7: EC2; 8:29212

F


Caco-2 2h
1 2 3 4 5 6 7 8
β-actin
phospho-p38
p38
p38
phospho-JNK
JNK2 (p54)
JNK1 (p46)
JNK
JNK2 (p54)
JNK1 (p46)
phospho-c-JUN
c-JUN
1:Control; 2:IL-1β; 3:EC16; 4:EC16+ IL-1β;
5:Salm; 6:Salm+EC16; 7: EC2; 8:29212

G


Caco-2 4h
1 2 3 4 5 6 7 8
β-actin
phospho-p38
p38
phospho-JNK
JNK2 (p54)
JNK1 (p46)
JNK
JNK2 (p54)
JNK1 (p46)
IL-8RA
1:Control; 2:IL-1β; 3:EC16; 4:EC16+ IL-1β;
5:Salm; 6:Salm+EC16; 7: EC2; 8:29212

H

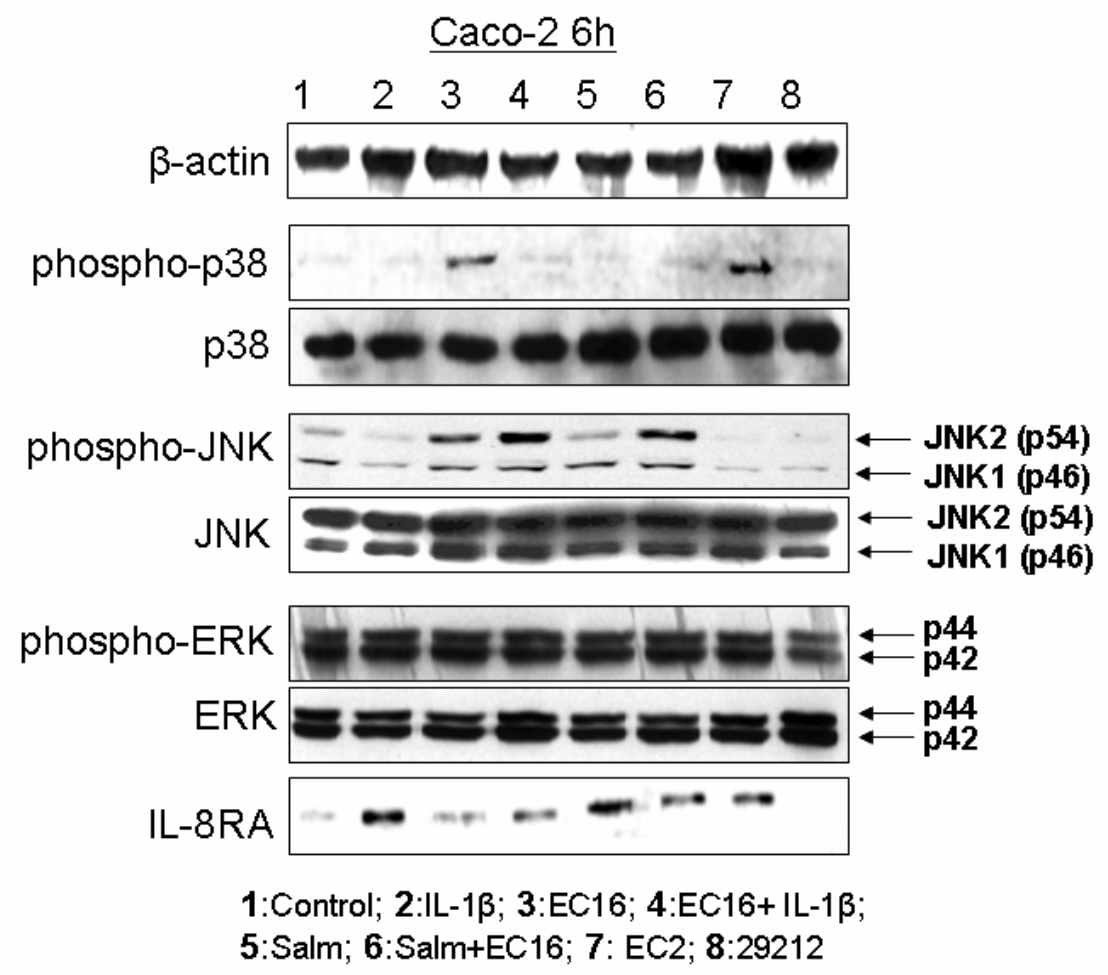


Figure 5.16 Protein expression in HCT116 and Caco-2 cells with the stimulation of 2ng/ml of IL-1β, *E. faecalis* EC16, EC16 in the presence of 2ng/ml IL-1β, *S. typhimurium*, IL-1β and *S. typhimurium* coculture with EC16, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30min, 1h, 2h, 4h and 6h. Control was added with culture medium only. Protein expression was detected using Western blotting method as described in Materials and Methods. Only the interested proteins expressions were listed above. (A) JNK expression in HCT116 cells at 30min with the treatments. (B) JNK and c-JUN expression in HCT116 cells at 1h with the treatments. (C) c-JUN expression in HCT116 cells at 2h with the treatments. (D) p38 and JNK expression in Caco-2 cell at 30min with the treatments. (E) p38, JNK and c-JUN expression in Caco-2 cell at 1h with the treatments. (F) p38, JNK and c-JUN expression in Caco-2 cell at 2h with the treatments. (G) p38, JNK and IL-8RA expression in Caco-2 cell at 4h with the treatments. (H) p38, JNK, ERK and IL-8RA expression in Caco-2 cell at 6h with the treatments.

IL-8RA expression was also detected using Western blotting. From Figure 5.16 G and H, we can see that IL-8RA expression was enhanced with the treatment of IL-1β and *S. typhimurium* at 6h, and suppressed with the administration of *E. faecalis*

EC16. However, there is no change in IL-8RA expression detected before 4h (data not shown). These data implied the importance of the IL-8RA in the inflammatory responses and its regulation might be at late phase compared with MAPK signaling pathway.

From Figure 5.13, we found that IL-18 was activated by *E. faecalis* in HCT116 cells. Caspase-1, which could release IL-18 from cells, is an important enzyme in inflammatory responses. We therefore tested its active form (p20) expression in HCT116 cells using Western blotting.

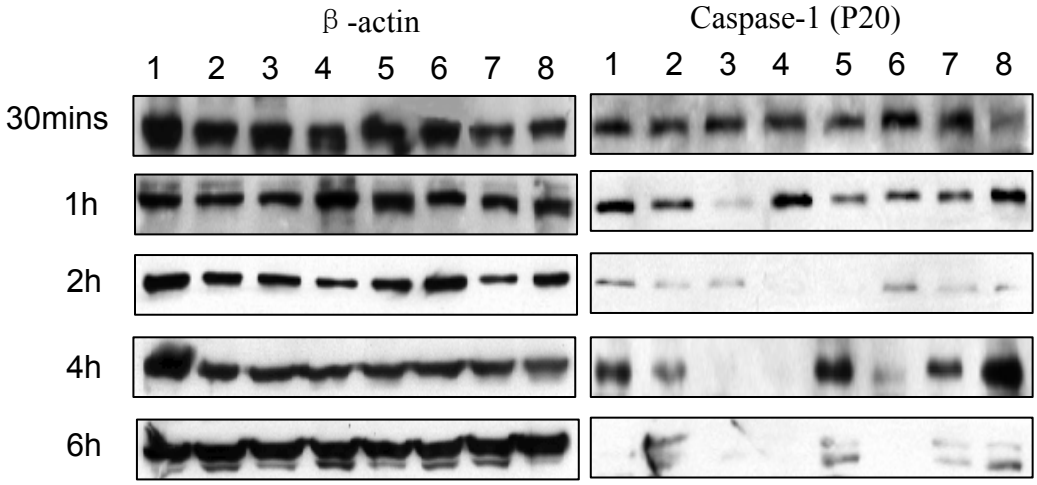


Figure 5.17 Caspase-1(p20) expression in HCT116 cells with the stimulation of 2ng/ml of IL-1β, *E.faecalis* EC16, *S. typhimurium,* EC16 in the presence of *S. typhimurium* and 2ng/ml IL-1β, *E. faecalis* EC2 and *E. faecalis* ATCC 29212 at a MOI of 100 for 30min, 1h, 2h, 4h and 6h. Control was added with culture medium only. Caspase-1 expression was detected using Western blotting method as described in Materials and Methods.

Figure 5.17 showed that *E. faecalis* suppressed the active form of caspase-1 from 1h. Interestingly, IL-1β and *S. typhimurium* activated p20 at 6h incubation. However, *E. faecalis* suppressed its enhanced expression activated by IL-1β and *S. typhimurium.* Together with the data that *E. faecalis* activated IL-18 expression, we

can see that the activation of IL-18 might be independent of caspase-1 in HCT116 in response to *E. faecalis*.

5.1.2.4 Protein Inhibitors Study

The above results showed that *E. faecalis* suppressed inflammatory responses in IECs by suppression of JNK and p38 signaling pathways. To mimic the suppression function of *E. faecalis* in IECs on these pathways, we therefore used p38 inhibitor (SB203580 iodo), JNK inhibitor (SP600125) and DUSP1 inhibitor (Triptolide) to treat the cells. SB203580 is a specific p38 inhibitor that inhibits the catalytic activity of p38 MAPK by competitive binding in the ATP pocket. This compound has been shown to inhibit p38 *in vivo* in a variety of animal models of inflammatory diseases (Kumar *et al*., 2003). SP600125, which is a reversible ATP-competitive inhibitor, preferentially inhibits JNK *in vitro* (Ki 0.19μM) and at concentrations above 5 μM *in vivo*. However, at higher concentrations (50 μM), it also inhibited p38 MAPK (Bennett *et al*., 2001). In our study, a final concentration of 7.5μM of p38 inhibitor (SB), JNK inhibitor (SP) was employed to the cells. For Triptolide (TRI), we used a final concentration of 1μM. Caco-2 cells were tested on SB, SP and TRI incubations. Supernatants were harvested for ELISA test and total proteins were harvested for Western blotting analysis and also IL-8 expression inside the cells.

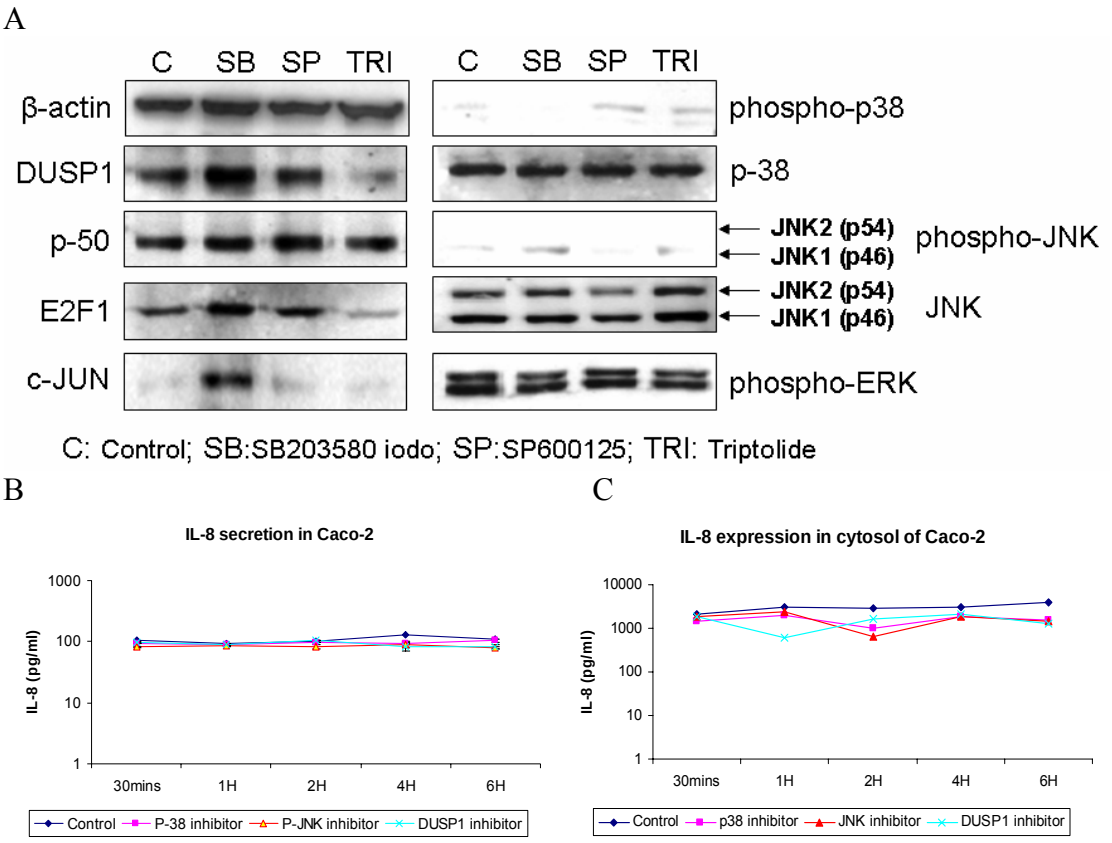


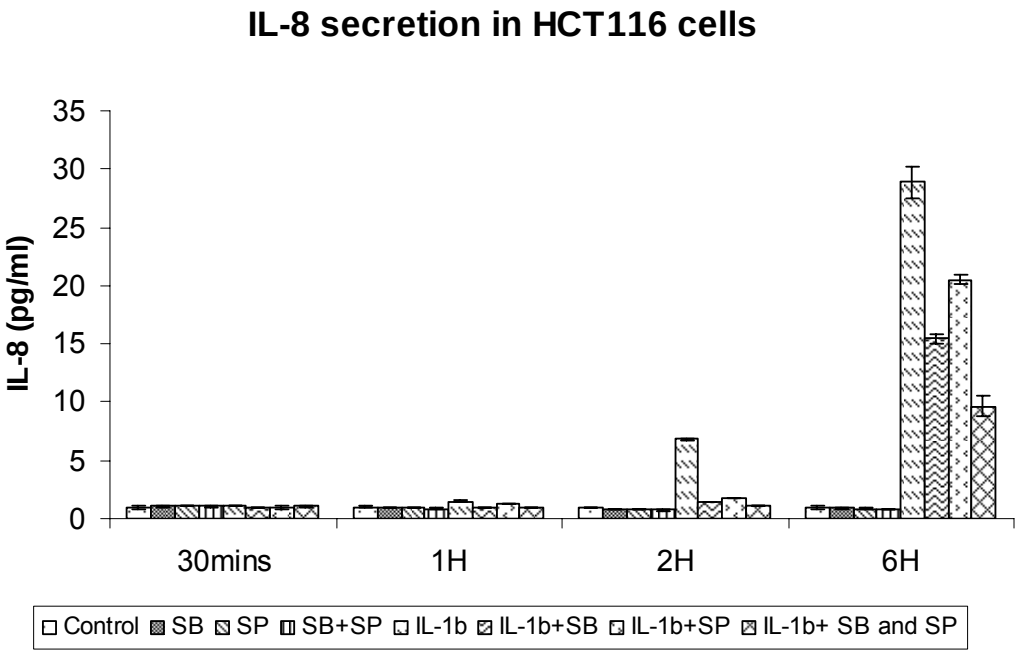
Figure 5.18 Protein expression in Caco-2 cells with the treatment of p38, JNK and DUSP1 inhibitors. A final concentration of 7.5μM of p38 inhibitor (SB), JNK inhibitor (SP) and 1μM of DUSP1 inhibitor, Triptolide (TRI) was employed to Caco-2 cell and incubated for 30min, 1h, 2h, 4h and 6h. (A) Western blotting results on 6h incubation. (B) IL-8 secretion in Caco-2 cells. (C) IL-8 secretion inside Caco-2 cells. Experiments were done three times independently. Error bars indicated as the standard deviation (B, C).

From Figure 5.18A, we can see that these three inhibitors had their effects at 6h incubation. With the inhibition of phosphorylated p38, E2F1, c-JUN and phosphorylated JNK expression were increased. However, p50, and phosphorylated ERK did not change significantly. Almost the same results were observed with the inhibition of phosphorylated JNK, E2F1, and phosphorylated p38 expressions were enhanced, suggesting the interplay between phosphorylated JNK and p38 suppression/activation in Caco-2 cells. In addition, with the inhibition of DUSP1, phosphorylated p38 production was upregulated (Figure 5.18A), which is consistent with previous suggestion that DUSP1 is the phosphatase of p38.

Surprisingly, with the inhibition of p38 or JNK, consistent IL-8 secretion in Caco-2 was not suppressed as expected (Figure 5.18B). The IL-8 production inside the cells tested using ELISA showed that IL-8 producing level was inhibited although not that much (Figure 5.18C). Unexpectedly, with the inhibition of DUSP1, IL-8 secretion was not upregulated (Figure 5.18B and C). The phenomenon observed above may be due to the interplay of JNK and p38 signaling pathways. When one of them was inhibited, the other was activated. In addition, other proteins like E2F1 and c-JUN may also be activated to "balance" the system. NF-κB and ERK seemed not to be involved in the regulation since they were not changed upon JNK and p38 inhibitors. However, JNK, p38, c-JUN, and E2F1 were suppressed in IECs with the treatment of *E. faecalis* (Figure 5.8; Figure 5.16). We therefore deduce that *E. faecalis* could put the brake at multiple levels of the inflammatory responses and inhibit the phosphorylation of JNK and p38 and even some other inflammation related proteins at the same time.

To confirm our hypothesis, we used the combination of p38 and JNK inhibitors to treat the cells. A final concentration of 7.5μM of phosphorylated p38 inhibitor (SB) and phosphorylated JNK inhibitor (SP) was employed. HCT116 cells were cultured with the respective inhibitors or a combination of SB and SP for 30mins, 1h, 2h, and 6h with or without the presence of 2ng/ml IL-1β.

A



B

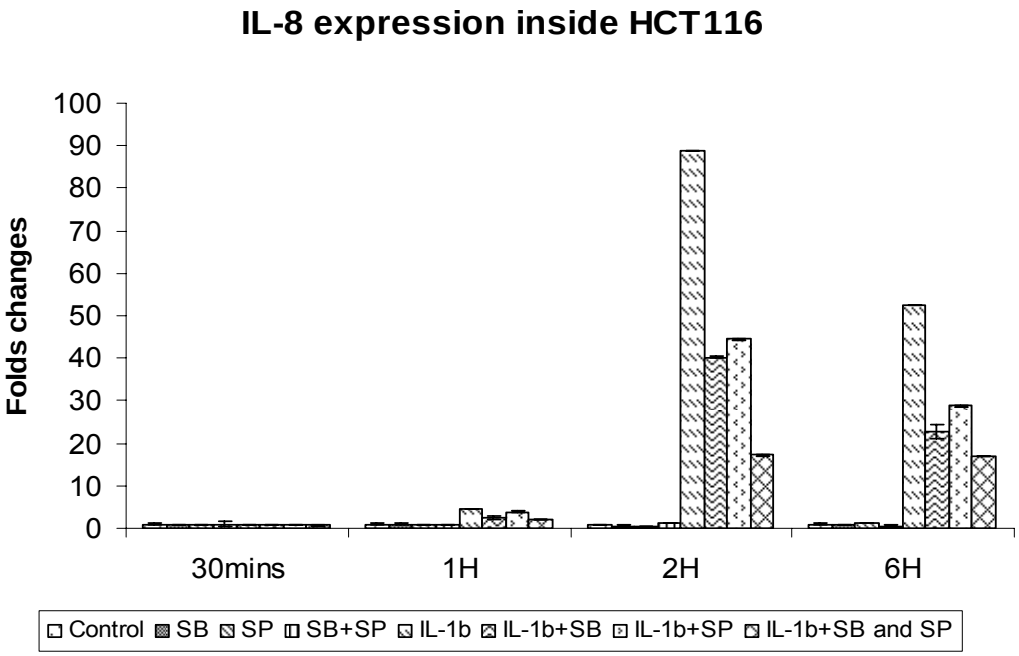


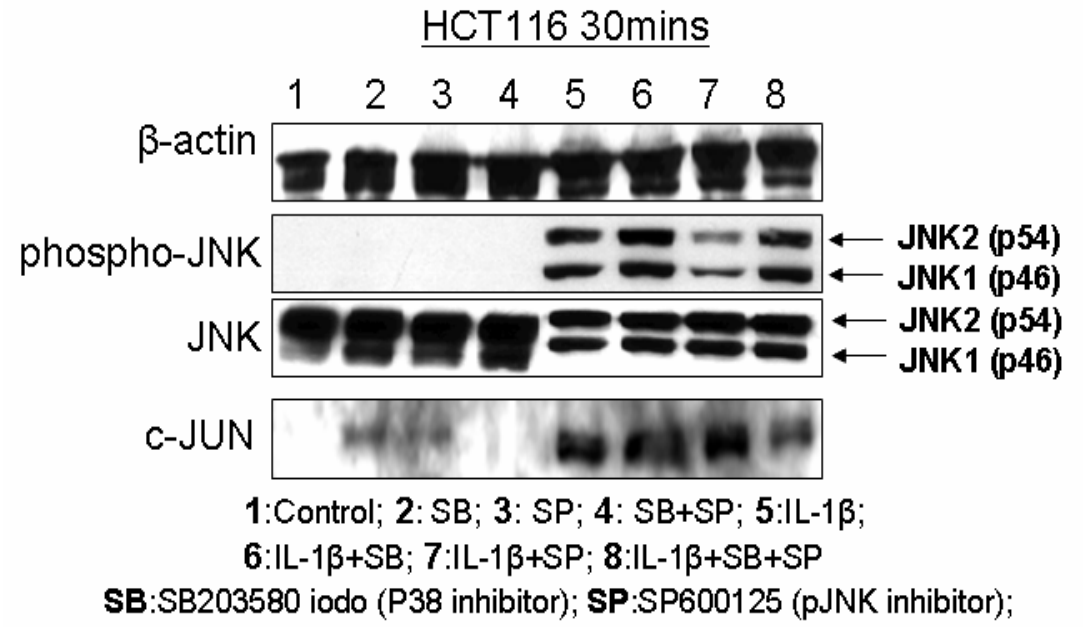
Figure 5.19 IL-8 production in HCT116 after incubating with p38 inhibitor (SB), JNK inhibitor (SP), p38 inhibitor combined with JNK inhibitor (SB+SP), 2ng/ml IL-1β, IL-1β with SB, IL-1β with SP, IL-1β with SB+SP for 30mins, 1h, 2h and 6h. (A) IL-8 secretion in the supernatants. (B) IL-8 expression inside the cells. Three independent experiments were done. Error bars indicated as the standard deviation.

From Figure 5.19 A, we can see that SB (p38 inhibitor) and SP (JNK inhibitor) inhibited IL-1β activated IL-8 secretion from 2h. The combination of these two inhibitors had a stronger effect in the suppression. Compared with the SB, SP did not exhibit the same ability to suppress IL-8 secretion in HCT116 cells. Interestingly, IL-8 expression inside the cells was inhibited by the inhibitors from 1h. Moreover, the IL-8 expression level peaked at 2h inside the cells (Figure 5.19 B). Taken the above data together, we can see that the combination of the two inhibitors had a much stronger suppression effects on IL-8 production.

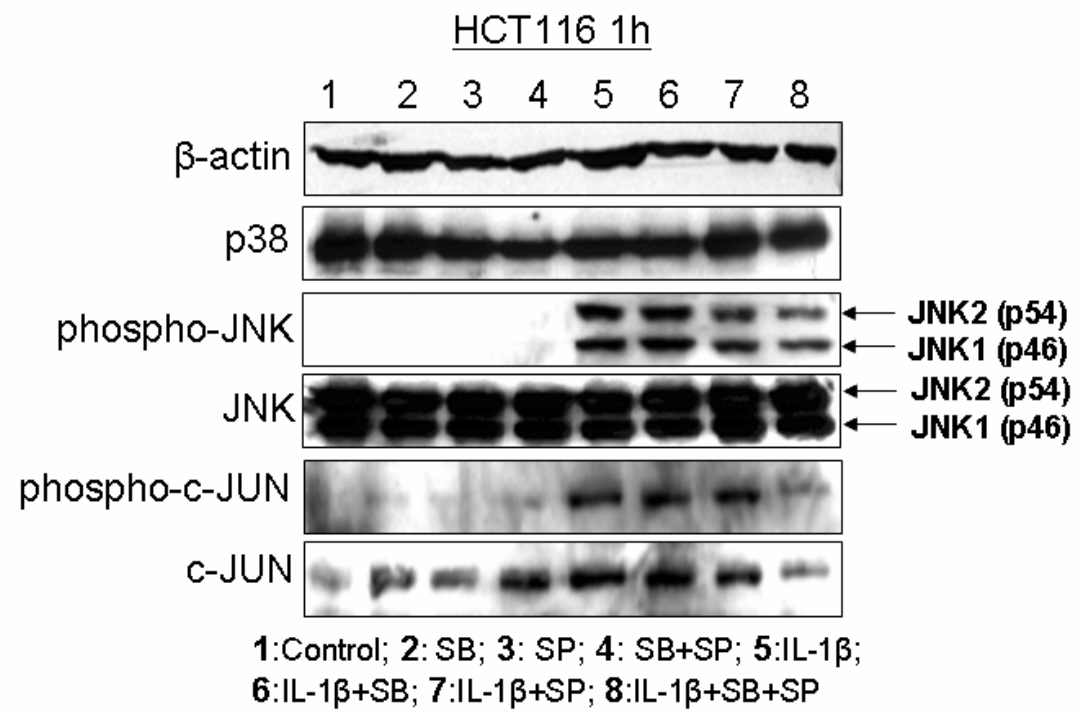
Immunoblotting assay was then performed to check for the expression of JNK, p-38 and c-JUN protein (Figure 5.20). Total protein from 30min incubation subjected to Western blotting revealed a suppression of phosphorylated JNK expression activated by IL-1β with JNK inhibitor. However, phosphorylated JNK appeared to be activated with the application of p38 inhibitor (Figure 5.20 A) which is consistent with what we had observed using p38 inhibitors in Caco-2 cells (Figure 5.18). With the combination of these two inhibitors, phosphorylated JNK was also activated which further suggested the interplay between p38 and JNK.

c-JUN is a main target protein of JNK. It can be greatly upregulated with the treatment of IL-1β in HCT116 cells. However, c-JUN expression was suppressed with the administration of JNK inhibitor but not p38 inhibitor (Figure 5.20 A).

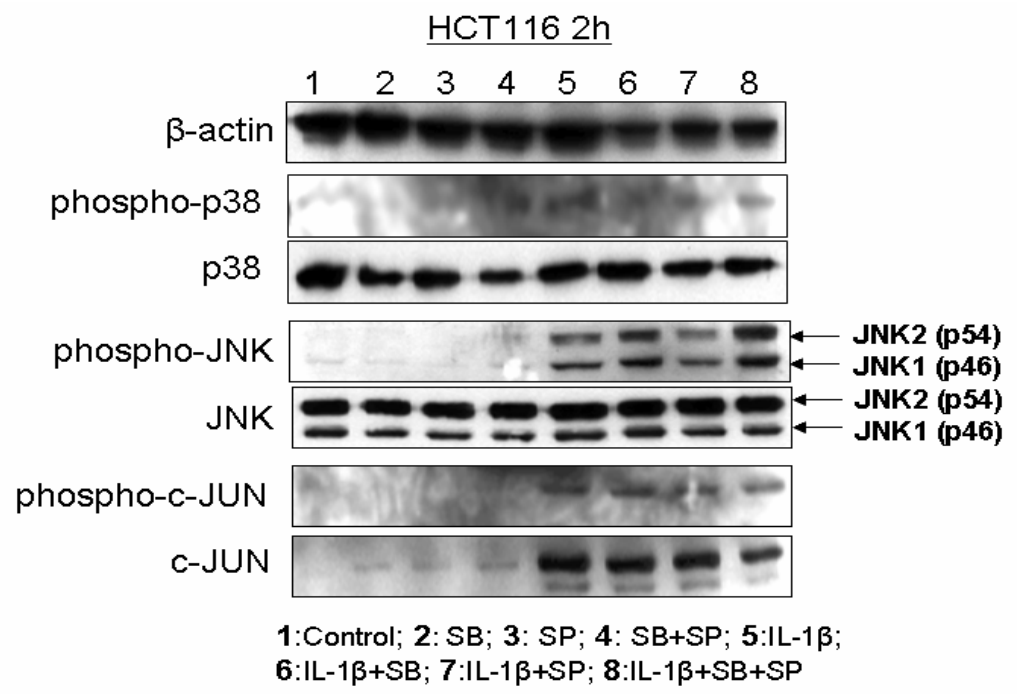
A


HCT116 30mins
1 2 3 4 5 6 7 8
β-actin
phospho-JNK
JNK2 (p54)
JNK1 (p46)
JNK
JNK2 (p54)
JNK1 (p46)
c-JUN
1:Control; 2: SB; 3: SP; 4: SB+SP; 5:IL-1β;
6:IL-1β+SB; 7:IL-1β+SP; 8:IL-1β+SB+SP
SB:SB203580 iodo (P38 inhibitor); SP:SP600125 (pJNK inhibitor);

B


HCT116 1h
1 2 3 4 5 6 7 8
β-actin
p38
phospho-JNK
JNK2 (p54)
JNK1 (p46)
JNK
JNK2 (p54)
JNK1 (p46)
phospho-c-JUN
c-JUN
1:Control; 2: SB; 3: SP; 4: SB+SP; 5:IL-1β;
6:IL-1β+SB; 7:IL-1β+SP; 8:IL-1β+SB+SP

C



D

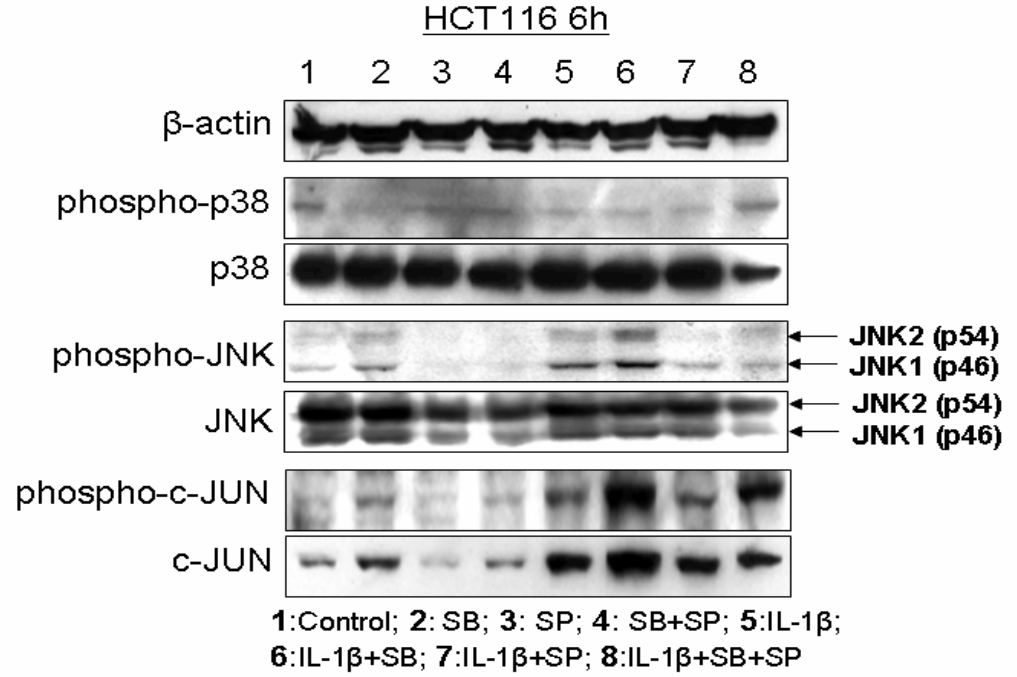


Figure 5.20 Protein expression in HCT116 after incubating with p38 inhibitor (SB), JNK inhibitor (SP), p38 inhibitor combine with JNK inhibitor (SB+SP), 2ng/ml IL-1β, IL-1β with SB, IL-1β with SP, IL-1β with SB+SP for 30min, 1h, 2h and 6h. (A) JNK and c-JUN expression in HCT116 cells at 30min treatment. (B) JNK, p38 and c-JUN expression in HCT116 cells at 1h treatment. (C) JNK, p38 and c-JUN expression in HCT116 cells at 2h treatment. (D) JNK, p38 and c-JUN expression in HCT116 cells at 6h treatment.

Almost the same results were observed in 30min, 1h and 2h treatment in HCT116 cells with the inhibitors (Figure 5.20 A, B, C). The combination of the two inhibitors suppressed the phosphorylated c-JUN and also total c-JUN expression activated by IL-1β (Figure 5.20 B, C). At 6h incubation with treatments described above, phosphorylated p38 and phosphorylated JNK was inhibited with their inhibitors respectively. As expected, when phosphorylated p38 was inhibited, phosphorylated JNK expression was greatly upregulated and vice versa (Figure 5.20 D). In addition, with the application of p38 inhibitor, phosphorylated and total c-JUN was increased which might be due to the upregulation of phosphorylated JNK treated with the p38 inhibitor (Figure 5.20 D). With the combination of p38 and JNK inhibitors at 6h, phosphorylated JNK was inhibited. Furthermore, c-JUN was also suppressed compared with the treatment with phosphorylated p38 inhibitor only (Figure 5.20 D).

Taking together all these results, the regulation of p38 and JNK appears to be linked. The suppression of them might reduce the stimulated IL-8 production but not consistent IL-8 production in IECs. With a combination of these two inhibitors, a stronger IL-8 suppression was observed. However, even with the combination of these two inhibitors, the suppression effects were not as strong as observed with *E. faecalis*. This suggests a complicated signaling pathway in the regulation of IL-8 production by *E. faecalis* and also the complex mechanism of communication of different MAPK family members.

5.2 DISCUSSION

Using human Immunology Signaling Pathway cDNA Microarray assay, we detected the regulation of immune genes modulated by *E. faecalis*. It showed that several common signaling pathways may be involved in the anti-inflammatory effects of *E. faecalis*. MAPK and NF-κB are two main families that are involved in the regulation by *E. faecalis*. MAPK7, MAP4K1 and MAP3K7IP1 (Mitogen-activated protein kinase kinase kinase 7 interacting protein 1) were suppressed by *E. faecalis* (Table 5.2). In addition, NFKB1, NFKBIL2 (Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor-like 2), NFKB2 and IKBKB (Inhibitor of nuclear factor kappa B kinase beta subunit) were greatly suppressed by *E. faecalis* (Table 5.2). MAPK and NF-κB family are important in innate and adaptive immune responses in the host. The activation of these two signaling pathways would cause inflammatory responses in the host. Suppression of the gene expression of these two families by *E. faecalis* may further inhibit inflammatory responses. Furthermore, microarray data also showed that cytokine receptors are important in the regulation. *E. faecalis* suppressed cytokine receptors expression in IECs (Table 5.1; Table 5.2; Figure 5.3). Moreover, quite a number of transcription factors were also regulated by *E. faecalis* (Table 5.2) which might indicate that they regulate immune responses in the host mainly through regulating gene transcription and translation.

To verify the signaling pathways regulated by *E. faecalis* in the host, we further confirmed the gene expression using Taqman low density array (TLDA) and their protein expression using Western blotting method. 48-well TLDA cards were designed according to the data we obtained from microarray assay. The results from

TLDA demonstrated that a similar regulation pattern of different *E. faecalis* in two different cell lines (Caco-2 and HCT116) (Figure 5.4; Figure 5.5; Figure 5.6). Consistent with what we found in microarray assay, MAPK and NF-κB families are still the main genes regulated by *E. faecalis* (Figure 5.4). DUSP1 (Dual specificity phosphatase 1), the important phosphatase of MAPK family, was greatly upregulated by *E. faecalis* however other MAPK members such as MAP3K7IP1 and MKNK1 (MAP kinase-interacting serine/threonine kinase 1) were suppressed (Figure 5.4). NF-κB1 and IKBKB were also inhibited in IECs with the treatment of *E. faecalis*. The regulation of MAPK signaling pathway was confirmed using Western blotting by detection of p38, JNK and ERK expression. It showed that *E. faecalis* suppressed JNK and p38 expression but not ERK (Figure 5.9) in HCT116 cells. These results implicated the importance of suppression of MAPK in the inhibitory effects of inflammation by *E. faecalis* and also the different functions of the individual MAPK members. Interestingly, other genes that are involved in cell proliferation such as CCND1 (cyclin D1), PIN1 (Protein (peptidylprolyl cis/trans isomerase) NIMA-interacting 1), E2F1 and MDM2 (transformed 3T3 cell double minute 2) were also inhibited by *E. faecalis* (Figure 5.8) in the host suggesting an overwhelming function of *E. faecalis* in the regulation of host responses.

CyclinD1 (CCND1) belongs to the highly conserved cyclin family, which function as regulators of CDK kinases. Cyclin D1 forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activity is required for cell cycle G1/S transition. Moreover, mutations, amplification and overexpression of this gene are observed frequently in a variety of tumors and may contribute to tumorigenesis. Hence, suppression of cyclin D1 in cancer cell lines might suppress the tumor proliferation or even tumorigensis.

Cyclin D1 can be regulated by MAPK family members which are critical to the immune responses. MAPK are negatively regulated via their dephospohrylation by DUSP1. The bacterially expressed and purified DUSP1 protein has intrinsic phosphatase activity, and specifically inactivates mitogen-activated protein (MAP) kinase *in vitro* by the concomitant dephosphorylation of both its phosphothreonine and phosphotyrosine residues. Furthermore, it suppresses the activities of MAP kinase to regulate both pro- and anti- inflammatory cytokines (Chi *et al*., 2006). Thus, the upregulation of DUSP1 (Figure 5.4A) may play an important role in the human cellular response to environmental stress as well as in the regulation of human immunity through regulation of MAPK signaling pathways.

MAP3K7IP was identified as a regulator of the MAP kinase kinase kinase MAP3K7/TAK1, which is known to mediate various intracellular signaling pathways, such as those activated by TGF-β, IL-1 and WNT-1. This protein interacts and thus activates MAP3K7. Also, it can interact with and activate MAPK14/p38alpha (Mitogen-activated protein kinase 14), and thus represents an alternative activation pathway. Attenuation of MAP3K7IP may suppress the activation of the MAPKK pathways, which contributes to the biological responses of MAPK14 to various stimuli. Interestingly, MKNK1 (MAP kinase-interacting serine/threonine kinase 1), a down stream of MAPK protein which phosphorylates human eukaryotic translation initiation factor 4G (eIF4G) thus to regulate protein translation in human, was suppressed in HCT116. Studies using MKNK1 inhibitors showed that TNF-α production is inhibited in a concentration-dependent manner by CGP57380 (MKNK1 inhibitor). The inhibition targets posttranscriptional regulation and is paralleled by inhibition of the phosphorylation of eukaryotic initiation factor 4E (eIF4E) (Andersson *et al*., 2006). Moreover, MKNK1 inhibitor blocked soluble IL-1β and IL-

8 production in THP1 cells, suggesting that the regulation of cytokine expression in part through activation of MAPK cascades, activation of MNK1, and phosphorylation of eIF4E (Cherla *et al*., 2006). Taken together, *E. faecalis* suppressed the inflammatory responses through attenuating MAPK signaling pathways.

Besides MAPK signaling pathway, NF-κB is another important signaling pathway in the regulation of immune responses in the host. NF-κB is a transcription factor which plays an evolutionarily conserved and critical role in the triggering and coordination of both innate and adaptive immune responses. NF-κB1 (p-50/p-105) or NF-κB2 (p49/p100) is bound to REL, RELA (p-65), or RELB to form the NF-κB complex. The NF-κB complex is inhibited by IκB proteins, which inactivate NF-κB by trapping it in the cytoplasm. Phosphorylation of serine residues on IκB proteins by IKBKA or IKBKB marks them for destruction via the ubiquitination pathway, thereby allowing activation of the NF-κB complex. Activated NF-κB complex translocates into the nucleus and binds DNA at NF-κB-binding motifs thus activating inflammatory gene transcription. In our study, NF-κB1, and IKBKB were suppressed with the application of *E. faecalis* to HCT116 cells (Figure 5.4A), suggesting that *E. faecalis* might inhibit NF-κB signaling pathway through inhibition of IκB kinase and also the attenuation NF-κB family members' genes expression.

More interestingly, NF-κB can be activated through cytokine receptors such as TNF-receptor superfamily (Papa *et al*., 2006). TNFRSF11A, which belongs to tumor necrosis factor receptor superfamily, interacts with various TNF receptor associated factor (TRAF) family proteins, through which this receptor induces the activation of NF-κB and MAPK8/JNK and thus the inflammatory responses in the host (Papa *et al*., 2006). TRAF6, which is a member of the TRAF protein family, is associated with,

and mediates signal transduction from members of the TNF receptor superfamily. This protein mediates the signaling not only from the members of the TNF receptor superfamily, but also from the members of the Toll/IL-1 family. Signals from receptors such as TNFRSF11 and IL-1 have been shown to be mediated by this protein. TRAF6 also interacts with various protein kinases including IRAK1/IRAK and SRC, which provides a link between distinct signaling pathways. In the NF-κB signaling pathway, it functions as a signal transducer that activates IκB kinase (IKK) in response to proinflammatory cytokines. In the study using *E. faecalis* co-culture with HCT116, the TNFRSF11A, TRAF6 as well as NF-κB family members were suppressed (Figure 5.4A) suggesting a strong inhibition mechanism by *E. faecalis* on inflammatory responses in HCT116 cells.

From the above results and discussion, we can see that some strains of *E. faecalis* have the distinct properties to suppress IL-8 secretion in IECs in a dose- and strain- dependent manner. The inhibitory effects of *E. faecalis* could be conducted through the carbohydrates on the cell surface. Furthermore, *E. faecalis* may act through MAPK and NF-κB signaling pathways to suppress the inflammatory responses in the host. However, *E. faecalis* induced IL-8 production in THP1 cells (Figure 3.4). We thus tested the genes regulation in THP1 cells upon *E. faecalis* treatment. Opposite to what we observed in IECs, *E. faecalis* upregulated IL-8 and TNF gene expression to a very high level (Figure 5.7) suggesting a multiple regulation mechanisms of IL-8 production by *E. faecalis* in different cell lines. Furthermore, MAPK (MAP3K8), NF-κB (NFKB1 and NFKB2) and cytokine receptors (TNFRSF11A and IL-8RA) family members were greatly upregulated (Figure 5.7B). Interestingly, most of the Toll-like receptors (TLRs) were suppressed

irrespective of the activation of IL-8 secretion by *E. faecalis*. The results suggested that MAPK and NF-κB are the most possible signaling pathways regulated by *E. faecalis* to the immune responses in the host. TLRs and cytokine receptors might also be important in the suppression of inflammatory responses upon *E. faecalis* exposure. Some other proteins like cyclin D1, PIN1 and E2F1 may be involved in the regulation indirectly. However, to fully illustrate the signaling pathways, co-culture of *E. faecalis* with other well known inflammation activators is important. We further confirm these ideas through co-culture of *E. faecalis* with pathogenic *S. typhimurium* and the proinflammatory cytokines TNF-α and IL-1β. Their protein productions were tested by Western blotting and their cytokine productions were measured using ELISA and cytokine assays.

Results showed that these four *E. faecalis* strains inhibited the synthesis and secretion of IL-8 protein triggered by TNF-α, IL-1β and pathogenic *S. typhimurium*. TNF-α, IL-1β and pathogenic *S. typhimurium* greatly stimulated IL-8 secretion in IECs from 1h (Figure 5.11; Figure 5.12; Figure 5.14). In addition, they activated accumulation of IL-8 inside the cells even earlier. *E. faecalis* inhibited TNF-α, IL-1β and pathogenic *S. typhimurium* induced IL-8 protein production both inside and outside of the cells (Figure 5.11; Figure 5.12; Figure 5.14). Considering the crucial role of IL-8 in inflammatory processes and implication of the IBD, the findings that *E. faecalis* attenuated IL-8 production at baseline and also in the response to proinflammatory cytokines and pathogenic bacteria in IECs are particularly interesting. *E. faecalis* could be a potential therapeutic agent to treat inflammation diseases such as IBD in the human intestine and also in the suppression of inflammatory responses activated by pathogen and other components in infants' intestine.

Beside IL-8, other cytokines or factors such as IL-4, IL-10, TNF-α, IL-2, IL-5, IL-6, IL-12 (P70), IL-17, IL-18, ICAM-1 and IFN-γ are also important in the regulation of immune responses in human. Among these cytokines, IL-4 and IL-10 have been implicated in the inhibition of IL-8 formation (Kasama *et al*., 1994; Meda *et al*., 1994; Wertheim *et al*., 1993). However, these cytokines were not detected in the supernatants of IECs in the study. Therefore, the inhibitory role for these agonists could be excluded from the current experimental system. IL-2 and IFN-γ can augment IL-1, TNF, IL-6 production. Moreover, IL-2 can stimulate IFN-γ (Torres *et al*., 1982) and IL-8 secretion (Wei *et al*., 1994). IL-12 induces IFN-γ production in T and NK cells and appears to be a major regulator of IFN-γ production in the intact organism (Trinchieri, 1995). IL-17 is characterized as cytokine-inducing cytokine that exhibits pleiotropic biologic activities on various cell types. It can activate IL-8 in IECs. In addition to inducing cytokines and chemokines, IL-17 presents itself with certain other unique functions. It enhances surface expression of ICAM-1 in human fibroblasts. Also, in human keratinocytes, IL-17 specifically and dose-dependently augmented IFN-γ induced ICAM-1 expression and the induction of IL-8 (Teunissen *et al*., 1998; Albanesi *et al*., 1999). However, IL-12 and IL-6 was not detected in IECs in this study. Other proinflammatory cytokines like TNF-α, IL-2, IL-5, IL-17 and IFN-γ were suppressed by *E. faecalis* in Caco-2 cells even with the stimulation of pathogenic *S. typhimurium* (Figure 5.15 B, C, D, E and F). TNF-α was also suppressed by *E. faecalis* in HCT116 cells (Figure 5.13B). Therefore, *E. faecalis* exhibits its distinct anti-inflammatory ability to suppresse multiple inflammatory cytokines and further to inhibit inflammatory responses.

ICAM-1, which is found in inflamed colonic tissues, mediates the arrest and further migration of neutrophils. Clinical studies also showed that a sustained increase

in ICAM-1 occurs during acute stress in children which may contribute to morbidity in patients with traumatic brain injury (Briassoulis *et al*., 2007). In the study, *E. faecalis* was found to suppress IL-1β and *S. typhimurium* induced ICAM-1 expression in IECs (Caco-2 and HCT116) (Figure 5.13A; Figure 5.15A). Therefore, the suppression of ICAM-1 by *E. faecalis* may inhibit inflammatory responses in infants activated by Gram negative bacteria or other stimuli.

E. faecalis induced IL-18 secretion in HCT116 was unexpected (Figure 5.13C). IL-18 is a member of the IL-1 family, which plays a role in immune and inflammatory reactions, and is notably present in several autoimmune disorders. Different with most of the other cytokines, IL-18 is abundant in non-immune tissues including the gut and the skin and may involve in the defense against mucosal organisms. Interestingly, administration of IL-18 to mice infected with a lethal dose of virulent strain of *S. typhimurium* reduced the bacteria number in the tissues and rescued them from death. On the other hand, macrophage infected with *S. typhimurium* exhibited reduced expression of IL-18, suggesting the ability of this pathogen to suppress production of IL-18 to facilitate its survival in the host (Elhofy and bost, 1999; John *et al*., 2002). A more recent study showed that IL-18 has an effective antitumor effect (Xu *et al*., 2007). In the study, *E. faecalis*, which suppressed several inflammatory cytokines production, activated IL-18 secretion in HCT116 even with the presence of *S. typhimurium*. Therefore, *E. faecalis* induction of IL-18 expression in HCT116 without corresponding production of other proinflammatory cytokines may serve to enhance removal of pathogens without the involvement of inflammatory responses. In addition, studies showed that the release of IL-18 from the cells could be activated by caspase-1 (Li *et al*., 2007). In the study, caspase-1 was not activated by *E. faecalis* but

suppressed (Figure 5.17). Therefore, the activation of IL-18 by *E. faecalis* in HCT116 cells involved in a caspase-1 independent pathway.

E. faecalis regulated inflammatory responses through the network of cytokines. Studies using commensal bacteria suggested that there are a variety of pathways involved in the anti-inflammatory effects of these bacteria, not all of which depend on signaling by TLRs (Rachmilewitz *et al*., 2004). TLRs are important in regulating inflammatory responses and Th1/Th2 immunities in the host. In the study, most of TLRs were suppressed in both IECs and THP1 by *E. faecalis* irrespective of the production of IL-8 (Figure 5.4; Figure 5.5; Figure 5.6; Figure 5.7). No TLRs except TLR3 were detected in HCT116 cells. TLR2, TLR4 and TLR9 were found expressed in Caco-2 cells but not in HCT116 cells. Expression of TLR3, TLR4 and TLR9 were suppressed in THP1 cells upon *E. faecalis* treatment even though a super high IL-8 and TNF expression were detected (Figure 5.7). Recent studies suggest that TLR3 and TLR9 signaling could be used for therapeutic treatment of inflammatory diseases including cancer, asthma and allergy (Salaun *et al*., 2006; Bhattacharjee *et al*., 2006). In addition, a link was found between TLR4 signaling, inflammation and tumor growth (Kelly *et al*., 2006). TLR4 was usually lowly expressed in healthy intestine resulting in tolerance of the large quantities of luminal LPS (Nomura *et al*., 2000). Moreover, we found that tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) mRNA was suppressed by L.GG, LP33, T6 and 10387 in Caco-2 cells (Figure 3.7), *E. faecalis* (EC1, EC3 and EC15) in HT-29 cells (Figure 3.9), *E. faecalis* (EC1, EC3, EC15, EC16), T6, L.bul in HCT116 (Figure 3.8; Figure 5.4). TRAF6 is an adaptor in TLRs signaling pathway. It is also involved in cytokine signaling pathways through MAPK. TRAF6 could bind TAB1, TAK1 and TAB2 to form a complex leading to the activation of IKK, thus the activation of NF-κB. Activation of

TAK1 also results in the activation of MAP kinases (Akira and Sato, 2003). Moreover, we have observed that TLR2 was expressed by Caco-2 cells and Tollip was expressed by all the intestinal cells. Both TLR2 and Tollip were not affected significantly by LAB (Figure 5.5) even at a high concentration at a MOI of 1000 (data not shown). From this we can deduce that TLR2 and Tollip might not be involved in the inflammation modulation by LAB. Interestingly, cytokine receptors such as IL-8RA and TNFRSF11A were greatly inhibited on their expression upon the treatment of *E. faecalis* in IECs with the suppression of multiple proinflammatory cytokines production (Figure 5.4; Figure 5.5). However, they were greatly upregulated in THP1 cells with a high induction of IL-8 secretion (Figure 5.7). In addition, IL-8RA protein expression was upregulated by IL-1β and *S. typhimurium* treatment but suppressed in the presence of *E. faecalis* (Figure 5.16 D). Taken these data together, the inhibitory effects of *E. faecalis* appear to mediate through cytokine receptors instead of TLRs. The low expression of TLRs in intestinal epithelial cells has important implication in the mechanism of tolerance to the commensal bacteria in intestine. The regulation of inflammatory response by *E. faecalis* may not be mediated through TLRs but cytokine receptors. However, the modulation of intestinal immunity by *E. faecalis* may involve TRAF6.

NF-κB is another important pathway which regulates inflammation reaction in the human. Inhibition of NF-κB pathway can block specific gene expression such as IL-8 (Hoffmann *et al*., 2002). However, Lawrence *et al* suggested that the NF-κB pathway may also be an important anti-inflammatory pathway in the resolution of inflammation (Lawerence *et al*., 2001). In the study, we showed that NF-κB1, NF-κB2 and IKKB gene expression were suppressed by *E. faecalis* in IECs (Table 5.2;

Figure 5.4; Figure 5.6). However, their protein productions did not change significantly (Figure 5.10). Furthermore, EMSA experiments on the NF-κB DNA binding analysis showed that *E. faecalis* did not inhibit its DNA binding activity, suggesting that *E. faecalis* uses a different anti-inflammatory signaling pathway other than inhibiting NF-κB protein expression or their DNA binding activities. However, NF-κB may be involved in late regulation mechanisms since their gene expression were suppressed by *E. faecalis*. The mechanisms utilized may vary between different commensal species since Kelly *et al* demonstrated that the anti-inflammatory activity of the commensal gut bacterium *Bacteroides thetaiotaomicron* does not involve inhibition of IκB degradation but selectively antagonizes NF-κB via a new mechanism (Kelly *et al*., 2004).

Activation of mitogen-activated protein kinase (MAPK) is important in the regulation of cytokines production and inflammation. Inhibition of MAPK would cause widespread suppression of cytokine network *in vivo* (Sheryanna *et al*., 2007). *E. faecalis* inhibited IL-1β and *S. typhimurium* activated phosphorylated JNK and p38 but not ERK during the first two hours of incubation (Figure 5.16 A, B, C, D, E and F). The transcriptional regulator AP-1 is downstream of MAPKs signaling pathway. It is a homo- or hetero-dimer composed of c-JUN, JUN D, JUN B, ATF-2, c-FOS, FRA-1, FRA-2 and other family members. It is important to note that JNK are the only c-JUN kinases identified to date. The gene expression of c-JUN was upregulated by *E. faecalis*. However the phosphorylated form of c-JUN was suppressed by *E. faecalis* (Figure 5.16 B, C, E and F). These data suggest the importance of c-JUN in the regulation of IL-8 production. Moreover, JNK was enhanced by *E. faecalis* but not IL-1β and *S. typhimurium* after 2h of incubation (Figure 5.16 G and H) in Caco-2

cells. This might explain the upregulation of c-JUN gene expression but not protein production. Furthermore, JNK1 and JNK2 have different function in the regulation of inflammatory responses. Thus the upregulation of JNK expression might be involved in a new mechanism of *E. faecalis* in the suppression of inflammatory responses which needs further verifications. Using inhibitors of JNK and p-38, the roles of JNK and p38 in the modulation of stimulated IL-8 expression were further confirmed. JNK and p38 inhibitors suppressed the IL-1β stimulated IL-8 production inside and outside of HCT116 cells (Figure 5.19). The combination of these two inhibitors showed stronger suppression ability compared to single inhibitor (Figure 5.19). The suppression of JNK by specific JNK inhibitor activated p38 expression and the suppression of p38 by specific p38 inhibitor activate JNK expression (Figure 5.18A; Figure 5.20) suggesting the interplay between JNK and p38 signaling pathways. This result extended the finding of Krause *et al* which suggested that the specific inhibition of JNK did not affect p38 activation (Krause *et al*., 1998). Unexpectedly, inhibitors did not affect significantly on the constitutive expression of IL-8 in IECs (Figure 5.18 B, C; Figure 5.19 A, B). Therefore, inhibition of MAPK signaling pathway by inhibitors could be effective in the suppression of stimulated IL-8 secretion but not constitutive expression of IL-8. A previous study showed that inhibitors of JNK had no effect on IL-8 expression in LPS stimulated human monocytes (Hoffmann *et al*., 2002). Studies also showed that treatment with p38 MAPK inhibitor did not suppress renal levels of IL-1β or IL-6 *in vivo* (Sheryanna *et al*., 2007). In addition, the activation of p38 and JNK protein expression during the late hours of incubation with *E. faecalis* might be one of the unknown mechanisms of the suppression of inflammation or the negative feed back of this signaling pathway. Further studies need to be carried out to prove this.

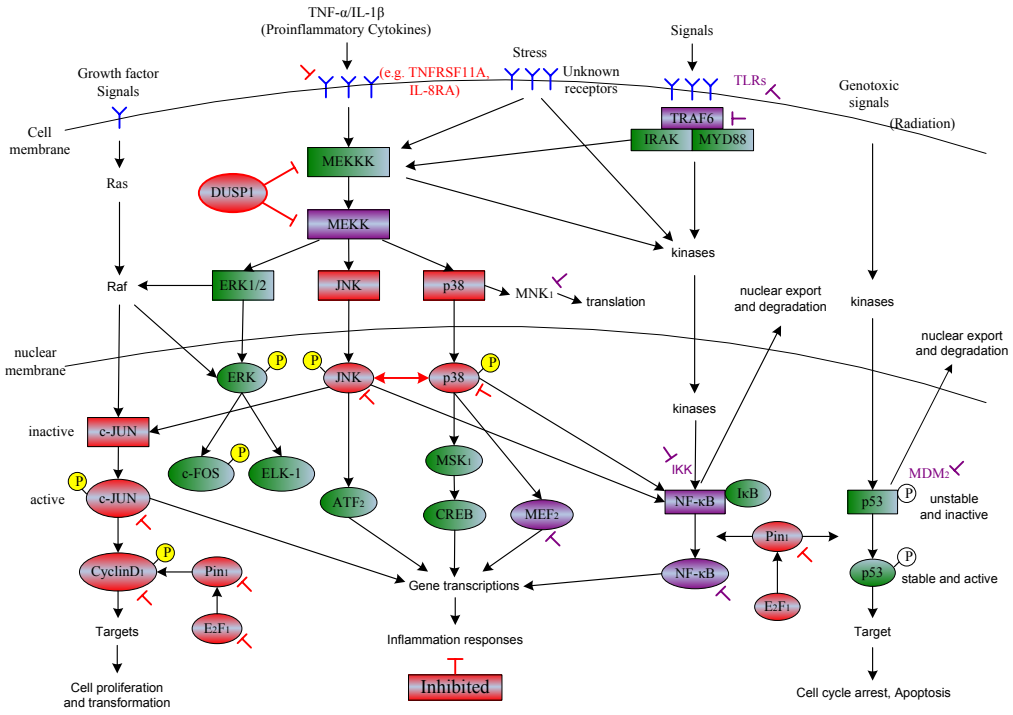


Figure 5.221 Signaling pathways that *E. faecalis* may be involved in regulating anti-inflammatory responses in the human intestine. The red colors labeled are those regulated by *E. faecalis* at protein levels, and purples color labeled are those regulated at gene levels. "T" means the suppression of the proteins/genes by *E. faecalis*. In brief, *E. faecalis* inhibited cytokine receptors like TNFRSF11A and IL-8RA on the cell surface, further inhibited JNK and p38 by suppressing their protein phosphorylation and upregulating DUSP1, and thus inhibiting c-JUN gene transcription and translation. PIN1 and E2F1 were also suppressed. NK-κB family members were suppressed on gene levels but not protein level.

Overall, *E. faecalis* isolated from infants demonstrated strong anti-inflammation properties in the human IECs by inhibiting several proinflammatory cytokines especially IL-8. In addition, the study has demonstrated for the first time that *E. faecalis* is the major human intestinal LAB in modulating expression of host immune genes that participate in inflammation. The immune regulatory mechanism may involve the adhesion of *E. faecalis* on intestinal cells and suppression of several inflammation related signaling pathways via carbohydrates mediated adhesion-receptor interaction. Infant intestine usually undergo acute inflammation when they are exposed to Gram negative bacteria. The presence of *E. faecalis* would help the

intestine to maintain the immune balance by suppressing the inflammation activated by Gram negative bacteria thus enhanced the removal of pathogens and maintain immune homeostasis. IL-8 synthesis and secretion could be regulated by multiple signaling pathways as JNK, c-JUN, PIN1 and E2F1 expression was suppressed by *E. faecalis* (Figure 5.21). JNK and p38 appear to be important in the suppression of IL-8 production since blockade of these pathways at early inflammatory stage significantly suppressed IL-8 secretion. The signaling pathways involved in the suppression of inflammatory responses by *E. faecalis* are illustrated in Figure 5.21.

Chapter 6

CONCLUSIONS

6.1 Conclusions

The immunomodulation effects of lactic acid bacteria (LAB) on human intestine and the possible mechanisms of how *E. faecalis* regulates human intestinal inflammatory responses were investigated in the thesis. The results demonstrated for the first time that specific strains of human intestinal *E. faecalis* suppressed inflammatory responses in the human intestinal epithelial cell lines (IECs).

Commensal bacteria are key regulators of mucosal immune responses, which are determined by the host genetic background. Lactic acid bacteria (LAB) are a very important group in human gut bacteria population. They help in gut immune homeostasis. Studies derived from different experimental model systems showed that *Enterococcus* is a critical component in regulation of chronic intestinal inflammation and gastrointestinal dysfunction. However, little is known about the molecular mechanisms of bacteria-specific crosstalk and the host cells with respect to inflammatory or protective immune responses.

It becomes apparent that various pathogenic bacteria or other stimuli trigger an innate immune response in the intestine that is tightly controlled in the normal host. A failure to terminate these responses may lead to persistent inflammation and potentially chronic inflammation in a susceptible host. In infants, because the gut microflora population is very unstable in early neonatal life, it is often difficult for their gut to maintain the delicate balance between harmful and beneficial bacteria, thus reducing the ability of the immune system to function normally. Therefore, the presence of the bacteria with anti-inflammation properties in the infants is crucial for them to maintain the balance of the gut. In the study, we found four *E. faecalis* strains, which are from healthy newborn infants' stool, among 56 strains of LAB of all kinds

of sources. They demonstrated a very strong ability to suppress inflammatory responses in the human intestinal epithelial cells. These *E. faecalis* strains inhibited several proinflammatory cytokines and factors such as IL-8, ICAM-1 and TNF-α at the basal level and also the level activated by IL-1β and pathogenic *S. typhimurium* (Figure 6.1). Among the four interesting *E. faecalis* strains, three were from 3 day old infants and 1 was from 1 month old infants, suggesting that the wellness of the infants could be determined by the composition of their gut bacteria.

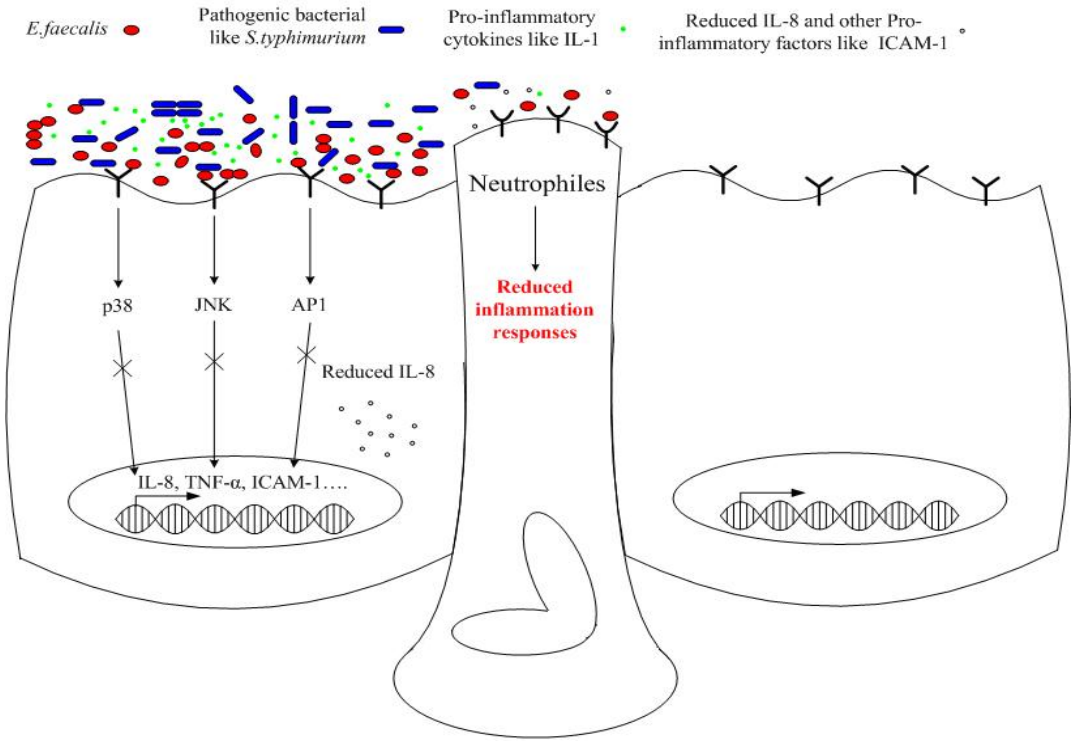


Figure 6.1 The cartoon demonstrated how *E. faecalis* suppressed inflammatory responses in the host with the stimulation of proinflammatory cytokines like IL-1β and pathogenic bacteria such as *S. typhimurium*. *E. faecalis* adheres to the cell surface very well and thus blocks the receptors to be accessed by proinflammtory cytokines and pathogens. It further inhibits the signaling pathways like p38, JNK and AP-1, and block the translation and possible transcription of IL-8, TNF-α and also ICAM-1. The reduced production of IL-8 and other proinflammatory cytokines lower the response of immune cells like neutrophils thus inhibit the inflammatory responses in the intestine.

In the study, we also demonstrated the importance of the adhesion ability of *E. faecalis* and its cell surface carbohydrates in the regulation of anti-inflammatory responses. It suppressed MAPK signaling pathway, through downregulation of JNK and p38 expression leading to inhibition of c-JUN and its downstream signals thus inhibit inflammatory responses in the host (Figure 6.1). Moreover, *E. faecalis* could regulate multiple cellular signaling pathways including cell proliferation, and even oncogenic signal pathways through the inhibition of PIN1 and E2F1 protein expression (Figure 5.21). Furthermore, *E. faecalis* may modulate the immune responses through cytokine receptors rather than TLRs. Its regulation could bypass NF-κB in the regulation of inflammatory responses in the host. Therefore, *E. faecalis* exhibits its unique but broad regulation pattern on multiple biological evens in the host. These unique properties could contribute to immune homeostasis both in the early childhood and possibly also in adult. The understanding of signal transduction mechanisms by *E. faecalis* in the IECs will help to develop new strategies to terminate immunopathology of intestinal inflammation. Exploitation of such findings may lead to the development of new therapeutics for human IBD and other inflammatory conditions.

6.2 Future Work

Because of the complexity of the human immune system, further studies might include the investigation of the receptors on the cell surface. The involvement of TLRs in response to *E. faecalis* needs to be further confirmed. TLRs play a key role in the host innate immune system. They were regulated by *E. faecalis* at the gene levels. Further studies using TLRs mutants in the IECs could investigate the role of TLRs in

response to *E. faecalis* and other commensal bacteria. In addition, other new signaling pathways might also be involved in the regulation of inflammatory responses by *E. faecalis* since IL-18 which was reported as a proinflammatory cytokine was greatly upregulated but other proinflammatory cytokines were greatly suppressed.

New technologies such as proteomics (expression, functional and structural), genomics and bioinformatics will likely contribute to the discovery of the components involved in the interaction of bacteria with the host under normal and pathological conditions of inflammation. Isolation and identification of specific components can lead to the isolation of these compounds for therapy in gut inflammatory conditions.

For *in vivo* studies, a novel colitis related mouse model initiated with azoxymethane (AOM) or dextran sodium sulfate (DSS) could be used. The incidence and syndrome ponderance of colitis induced by AOM/DSS in mice treat with or without *E. faecalis* would be evaluated.

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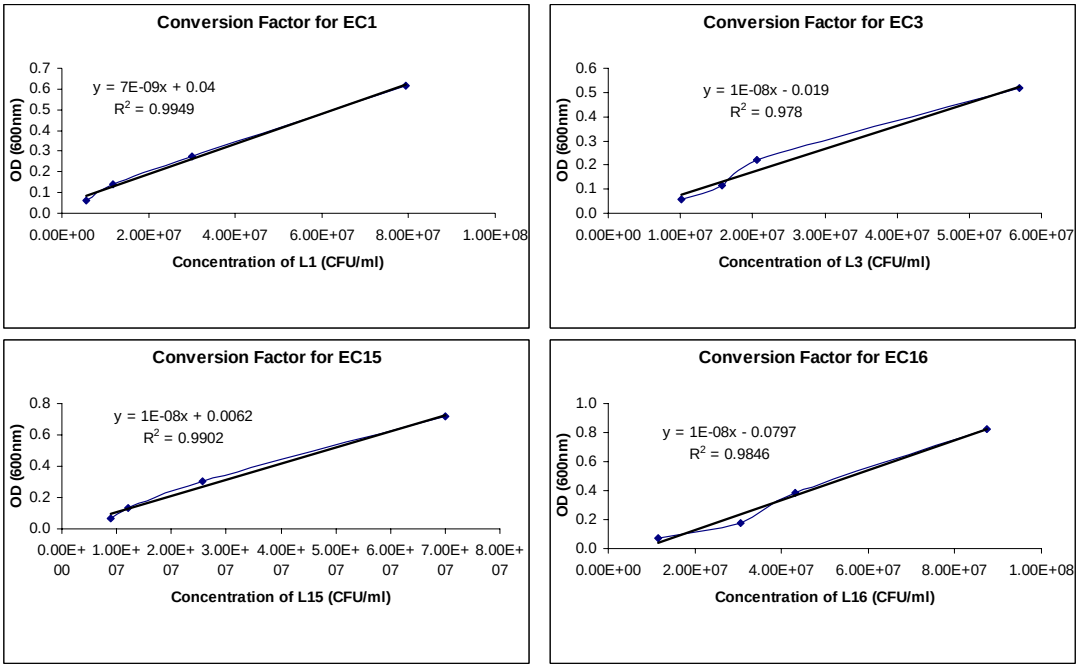
APPENDIX 1

CONVERSION FACTORS OF BACTERIA

Lactic Acid Bacteria (LAB)	Conversion Equations (Y=OD reading at 600nm; X= CFU/ml bacteria)
Bifidobacterium adolescentis ATCC15703	y = 1E-10x + 0.2475
Bifidobacterium. brevi ATCC15700	y = 2E-10x + 0.1559
B. dentium scardori & crociani ATCC27534	y = 2E-07x + 0.0043
Bifidobacterium. longum ATCC15707	y = 5E-08x - 0.0063
Enterococcus faecalis EC1	y = 7E-09x + 0.04
Enterococcus faecalis EC2	y = 2E-10x + 0.0983
Enterococcus faecalis EC3	y = 1E-08x - 0.019
Enterococcus faecalis EC5	y = 6E-11x + 0.0047
Enterococcus faecalis EC7	y = 5E-11x + 0.1456
Enterococcus faecalis EC15	y = 1E-08x + 0.0062
Enterococcus faecalis EC16	y = 1E-08x - 0.0797
Lactobacillus acidophilus ATCC4356	y = 1E-08x + 0.1408
L. brevis K7	y = 6E-09x + 0.0479
L. brevis T1	y = 5E-09x + 0.2843
L. brevis T6	y = 4E-06x - 0.0089
L. casei ATCC11578	y = 6E-10x + 0.5116
L. casei IS7257	y = 1E-10x + 0.3184
L. casei subsp. *casei* NCIMB11970	y = 5E-11x + 0.542
L. casei Shirota	y = 3E-09x + 0.2131
L. delbrueckii subsp. *bulgaricus* NCIMB11778	y = 8E-09x - 0.3748
L. delbrueckii bulgaricus D1	y = 1E-11x + 0.4361
L. lactis subsp. *lactis* B4	y = 4E-08x - 3E-06
L. lactis subsp. *lactis* B9	y = 8E-08x - 1E-05
L. lactis subsp. *lactis* B12	y = 8E-07x + 0.0685
L. lactis subsp. *lactis* K5	y = 3E-09x + 0.3222
L. lactis subsp. *lactis* K6	y = 7E-06x + 0.0407
L. lactis subsp. *lactis* K7	y = 6E-09x + 0.0479
L. paracasei LP33	y = 8E-10x + 0.2244
Lactobacillus paracasei Chamyto	y = 6E-09x + 0.1245
L. paracasei subsp. *paracasei* ATCC11974	y = 3E-09x + 0.4289
L. paracasei subsp. *paracasei* NCIMB8001	y = 2E-10x + 0.2283
L. rhamnusus NCIMB6375	y = 6E-10x + 0.369
L.rhamnosus GG	y = 2E-11x + 0.7368
L. rhamnosus NCIMB 8690	y = 4E-07x - 0.0682
Leuconostoc mesentroides K13	y = 2E-10x + 0.3348

Streptococcus thermophilus NCIMB10387	y = 3E-08x - 0.0184
S. typhimurium 14028	y = 4E-10x + 0.169
L. plantarum 1	y = 7E-09x + 0.1965
L. plantarum 2	y = 4E-09x + 0.4011
L. plantarum 3	y = 2E-10x + 0.3886
L.casei 705	y = 1E-08x - 0.1479
L. Indenesia	y = 1E-10x + 0.3149
L. casei I2775	y = 3E-09x + 0.1987

The following four graphs are the conversion factor curve for *E. faecalis* EC1, EC3, EC15 and EC16.



APPENDIX 2

MATERIAL FOR BACTERIA CULTURE

1. Saline

Add 0.85g NaCL (Merck, Germany) in 100ml Nano water and autoclave at 121°C for 15min (Hirayama, Japan), store at room temperature or 4°C.

2. de Man, Rogosa and Sharpe (MRS) broth/agar

MRS Broth

52.2g of MRS broth powder (Merck, USA) were added into 1L distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and store at room temperature or 4°C.

MRS Agar

68.2g of MRS Agar powder (Merck, USA) were added into 1L distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and cooled to 60°C before pouring plates, stored at 4°C.

3. Reinforced Clostridial Medium (RCM) broth/agar

RCM Broth

33g of RCM broth powder (Merck, USA) were added into 1L distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and store at room temperature or 4°C.

RCM Agar

52.5g of RCM Agar powder (Oxoid, UK) were added into 1L distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and cooled to 60°C before pouring plates, stored at 4°C.

4. Luria-Bertani (LB) broth/agar

Luria-Bertani (LB) Broth, PH 7.0

Items	Amount	Source
Bacto-tryptone	10g	BD biosciences, USA
Bacto-yeast extract	5g	BD biosciences, USA
NaCL	10g	Merck, Germany

The above components were dissolved in 1 liter of distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and store at room temperature or 4°C.

Luria-Bertani (LB) Agar

Items	Amount	Source
Bacto-tryptone	10g	BD biosciences, USA
Bacto-yeast extract	5g	BD biosciences, USA
NaCL	10g	Merck, Germany
Agarose	15g	Oxoid, UK

The above components were dissolved in 1 liter of distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and cooled to 60°C before pouring plates, stored at 4°C.

5. M17 broth/agar

M17 Broth

37.25g M17 broth powder (Oxoid, UK) were added into 950ml distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and cooled to 50°C before adding 50ml 10% (W/V) Lactose solution. Store at room temperature or at 4°C.

M17 Agar

37.25g M17 broth powder (Oxoid, UK) and 15g Agarose (Oxoid, UK) were added into 950ml distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and cooled to 50°C before adding 50ml 10% (W/V) Lactose solution. Pouring plates after adding lactose then store at 4°C.

10% Lactose (L70)

10g Lactose (L70) was added into 100ml distilled water autoclaved at 121°C for 15min (Hirayama, Japan) or filter sterile for use.

6. Tryptone Soy (TS) broth/agar

TS Broth

30g of TS broth powder (Merck, USA) were added into 1L distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and store at room temperature or 4°C.

TS Agar

30g of TS broth powder (Merck, USA) and 15g Argrose (Oxoid, UK) were added into 1L distilled water. The solution was autoclaved at 121°C for 15min (Hirayama, Japan) and cooled to 60°C before pouring plates, stored at 4°C.

7. TE buffer

The final concentration of TE buffer is 10 mM Tris–HCl and 1 mM EDTA; adjusted pH to 8.0

APPENDIX 3

MATERIALS FOR CELL CULTURE

All materials prepared for cell culture were either sterile or sterilized. Nano water (Corning, USA) was used for all dilutions.

1. Phosphate Buffered Saline (PBS)

Items	Amount (g)	Source
KH_2PO_4	0.24	Merck, Germany
Na_2HPO_4	1.44	Merck, Germany
NaCL	8	Merck, Germany
KCL	0.2	Merck, Germany

The materials were dissolved in 1 liter of Nano water. The pH was adjusted to 7.4 using either 1M HCL or 1M NaOH. The solution was aliquoted and sterilized using autoclave machine (Hirayama, Japan) for 15min at 121°C. Store at 4°C.

2. Trypsin-EDTA

10 ml 10X Trypsin-EDTA (GIBCO) was dilluted in 90ml PBS buffer. Filter sterilization and store at 4°C.

3. 0.5% Trypan Blue Solution

Items	Amount	Source
Trypan Blue	0.5g	Merck, Germany
PBS	100ml	Appendix 3

The solution was autoclaved (Hirayama, Japan) at 121°C for 15min and filtered before use.

4. 200mM L-glutamate

2.923g L-glutamate was disolved in 100ml Nano water and store at 4°C for use.

5. 0.1M Na-pyrurate

1.1g of Na-pyrurate was disolved in 100ml Nano water and store at 4°C for use.

6. 7.5% Sodium bicarbonate

18.75g of $NaHCO_3$ was disolved in 250ml Nano water and store at 4°C for use.

7. MEM medium for Caco-2

9.39g MEM (Minimal Essential Medium) powder (Giboco, USA) was dissolved in 750ml of Nano water and autoclaved (Hirayama, Japan) at 121°C for 15min before use.

Items	Amount (ml)	Source
MEM	750	Appendix 3
Fetal Bovine Serum	200	Bioclot, Germany
7.5% Sodium bicarbonate	20	Appendix 3
0.1M Na-pyrurate	10	Appendix 3
200mM L-glutamate	10	Appendix 3
10mM Non-essential amino acid	10	GIBCO, USA
Penicillin/Streptomycin	1	GIBCO, USA

The above solutions were then filter sterilized and store at 4°C.

8. DMEM medium for HT-29 and HCT116

One package of DMEM powder (GIBCO, USA) was dissolved in 900ml Nano-water.

Items	Amount (ml)	Source
DMEM	900	Appendix 3
7.5% Sodium bicarbonate	20	Appendix 3
Fetal Bovine Serum	100	Bioclot, Germany
Penicillin/Streptomycin	1	GIBCO, USA

The above solutions were then filter sterilized and store at 4°C.

9. RPMI 1640 for THP1

One package of RPMI1640 powder (GIBCO, USA) and 1.5 sodium biocarbonate and 4.5g glucose was dissolved in 880ml Nano-water.

Items	Amount (ml)	Source
RPMI1640	880	Appendix 3
0.1M Na-pyrurate	10	Appendix 3
200mM L-glutamate	10	Appendix 3
Fetal Bovine Serum	100	Bioclot, Germany
Penicillin/Streptomycin	1	GIBCO, USA

The above solutions were then filter sterilized and store at 4°C.

APPENDIX 4

MATERIAL FOR CYTOKINE ASSAY

1. Coating buffer (0.1m Sodium Carbonate, pH 9.5)

Items	Amount (g)	Source
$NaHCO_3$	8.40	Merck, Germany
Na_2CO_3	3.56	Merck, Germany

The materials were dissolved in 1 liter of Nano water. The pH was adjusted to 9.5 using either 1M HCL or 1M NaOH. The solution was freshly prepared or use within 7 days of preparation, stored at 2-8°C.

2. PBS* for ELISA

Items	Amount (g)	Source
KH_2PO_4	0.2	Merck, Germany
Na_2HPO_4	1.16	Merck, Germany
NaCL	8	Merck, Germany
KCL	0.2	Merck, Germany

The materials were dissolved in 1 liter of Nano water. The pH was adjusted to 7.0 using either 1M HCL or 1M NaOH. The solution was aliquoted and sterilized using autoclave machine (Hirayama, Japan) for 15min at 121°C. Store at 4°C.

3. Assay Dilluent

PBS*(Appendix 4) with 10% FBS (Bioclot, Germany), pH was adjusted to7.0. Freshly prepared or use within 3 days of preparation and stored at 2-8°C.

4. Washing buffer

PBS* (Appendix 4) with 0.05% Tween-20 (Merck, Germany), freshly prepare or use within 3 days of preparation, with 2-8°C storage.

5. Substrates

Tetramethylbenzidine (TMB) and Hydrogen Peroxide. We bought from the BD Pharmingen™ TMB Substrate Reagent Set (Cat.No. 555214). Mix them at the equal volume before use.

6. Stop buffer (1M H_3PO_4)

12.55ml of original H_3PO_4 was added in to 200ml H_2O. Mix well and keep at 4°C.

Appendix 5

MATERIAL FOR APOPTOSIS ANALYSIS

1. **Sample buffer**

1g Glucose was added into 1L PBS buffer (Appendix 3).

2. **10mM Tris-HCL buffer**

1.576g Tris-HCL and 0.877g NaCL were dissolved in 1L distilled water and adjusted pH to 7.5.

3. **1000U/ml RNAse**

0.012g of RNase A were dissolved in 1 ml 10mM Tris-HCL buffer and read to use.

4. **Propidium Iodide**

1mg of Propidium Iodide was dissolved in 1ml of PBS buffer (Appendix 3). Keep in the dark at 4°C.

APPENDIX 6

MATERIAL FOR RNA/DNA PREPARATION

For RNA

1. Diethyl pyrocarbonate (DEPC) treated water

0.5ml of DEPC (Sigma, USA) was added into 1L autoclaved Nano water and mixed overnight, then autoclave 15min (Hirayama, Japan) to destroy DEPC by causing hydrolysis of DEPC.

2. Formadehy gel

Items	Amount	Source
Formaldehyde	1.6ml	Sigma, USA
10xMOPS	10ml	Appendix 6
Agarose	1.5g	Merck, Germany
Ethidium Bromide	2 ul	Sigma, USA
H_2O	87.5ml	Corning, USA

3. 10x MOPS (1L)

MOPS 41.8 g

DEPC water 700ml

↓

Adjust PH 7.0

↓

20ml 1M sodium acetate

20ml 0.5 M EDTA (PH 8.0)

↓

Make 1 L then filter

4. Gel loading buffer/dye (RNA)

50% glycerol	25ml
1mM EDTA (PH 8.0)	
0.25% bromophenol blue	
0.25% xylene cyanol FF	
DEPC water	25ml
Total	50ml

For DNA

5. 1.2% agarose gel

Items	Amount	Source
Agarose	0.3g	Seakem, USA
10x TBE	25ml	Prepared
Ethidium Bromide	2µl	Sigma, USA

6. DNA loading buffer

Items	Amount	Source
Bromophenol Blue	25mg	Sigma, USA
Sucrose	4g	Merck, Germany
H_2O	10ml	Corning, USA

Appendix 7

MATERIALS FOR MICROARRAY

1. **20x SSC**

Dissolve 175.3 g NaCl and 88.2 g sodium citrate dihydrate into 900 ml H_2O. Adjust pH to 7.0 with 1M HCl. Dilute to 1 L with H_2O. Autoclave for 15mins (Hirayama, Japan) and store at room temperature for up to 2 months.

2. **20% SDS**

Dissolve 200 g sodium dodecyl sulfate in 1 L H_2O. Heat to 65 °C if necessary to dissolve. Store at room temperature.

3. **Wash Solution 1& 2**

Wash Buffer:	20X SSC (ml)	20% SDS	dH2O	Total Volume
1. 2X SSC, 1% SDS	10	5	85	100
2. 0.1X SSC, 0.5% SDS	0.5	2.5	97	100

Appendix 8

MATERIAL FOR WESTERN BLOTTING

1. **Stacking gel (5%)**

Items	Amount	Source
Water	2.7ml	Nano water (Corning, USA)
30% acrylamide	0.67ml	BioRad,
1.0M Tris-base (pH6.8)	0.5ml	Merck, Germany
10% SDS	0.04ml	Merck, Germany
10% ammonium persulphate*	0.04ml	Merck, Germany
TEMED	0.004ml	BioRad,

*prepare fresh

2. **Running gel (10%), 2 gels.**

Items	Amount	Source
Water	5.3ml	Nano water (Corning, USA)
30% acrylamide	2.0ml	BioRad,
1.5M Tris-base (pH8.8)	2.5ml	Merck, Germany
10% SDS	0.1ml	Merck, Germany
10% ammonium persulphate*	0.1ml	Merck, Germany
TEMED	0.008ml	BioRad,

*prepare fresh

Pour isopropanol over the running gel (let gel solidify for ~20min). Decant away the isopropanol just before adding the stacking gel.

3. **Running buffer (10x)**

Items	Amount	Source
Tris base	30.2g	Merck, Germany
Glycine	188g	Merck, Germany
10% SDS	100ml	BioRad, USA
Water	Top up to 1L	

4. Transfer buffer (Prepare fresh)

Items	Amount	Source
Tris base	3.03g	Merck, Germany
Glycine	14.4g	Merck, Germany
Methanol	200ml	Merck, Germany
Water	Top up to 1L	

5. Ponceau S dye

Items	Amount	Source
Ponceau S dye	0.5g	Sigma, USA
Acetic acid	25ml	Merck, Germany
Water	475ml	Corning, USA

6. Tris buffer saline-Tween (TBST), pH 7.6

Items	Amount	Source
5M NaCl	27ml	Appendix 8
1M Tris-HCl pH 7.4	12ml	Appendix 8
Tween-20	900ul	Merck, Germany
Water	900ml	Corning, USA

pH was adjusted to 7.6 and then top up to 1 liter. The solution was then autoclaved at 121°C for 15min (Hirayama, Japan) and store at room temperature.

5M NaCl : 292.2g NaCl (Merck, Germany) was dissolved in 1 liter Nano water for use.

1M Tris-HCl pH 7.4: 157.6g Tris-HCl (Merck, Germany) was dissolved in 1 liter Nano water and pH was adjusted to 7.4 for use.

7. 5% skim milk

5g of skim milk (Diploma, singapore) was dissolved in 100ml TBST (Appendix 8). Mix well and ready for use.

8. 3X SDS Sample Buffer

187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% (w/v) SDS, 30% glycerol and 0.03% (w/v) bromophenol blue. (Store at room temperature.)

9. 30X Reducing Agent

1.25 M dithiothreitol (DTT) (Store at –20°C.) Add 30X DTT at **1:10** to 3X SDS sample buffer

10. 1X Cell Lysis buffer

20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin. Add 1 mM PMSF (Sigma, USA) immediately before use.

APPENDIX 9

The sequence of the strains

The ID of the strains can be obtained in the gene bank http://www.ncbi.nlm.nih.gov/blast/Blast.cgi with blasting

Enterococci

EC1:

```
   1 ctgcgcgtgc tatacatgca gtcgaacgct tctttcctcc cgagtgcttg cactcaattg
  61 gaaagaggag tggcggacgg gtgagtaaca cgtgggtaac ctacccatca gaggggata
 121 acacttggaa acaggtgcta ataccgcata acagtttatg ccgcatggca taagagtgaa
 181 aggcgctttc gggtgtcgct gatggatgga cccgcggtgc attagctagt tggtgaggta
 241 acggctcacc aaggccacga tgcatagccg acctgagagg gtgatcggcc acactgggac
 301 tgagacacgg cccagactcc tacgggaggc agcagtaggg aatcttcggc aatggacgaa
 361 agtctgaccg agcaacgccg cgtgagtgaa gaaggttttc ggatcgtaaa actctgttgt
 421 tagagaagaa caaggacgtt agtaactgaa cgtcccctga cggtatctaa ccagaaagcc
 481 acggctaact acgtgccagc agccgcggta atacgtaggt ggcaagcgtt gtccggattt
 541 attgggcgta aagcgagcgc aggcggtttc ttaagtctga tgtgaaagcc cccggctcaa
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1261 ctcttaaagc ttctctcagt tcggattgca ggctgcaact cgcctgcatg aagccggaat
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EC2:

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EC3:

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1261 tctcttaaag cttctctcag ttcggattgc aggctgcaac tcgcctgcat gaagccggaa
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EC5:

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EC6:

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EC7:

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Lactobacilli

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L5:

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 301 gacacgagct gacgacaacc atgcaccacc tgtcattttg cccccgaagg ggaaacctga
 361 tctctcaggt gatcaaaaga tgtcaagacc tggtaaggtt cttcgcgttg cttcgaatta
 421 aaccacatgc tccaccgctt gtgcgggccc ccgtcaattc ctttgagttt caaccttgcg
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1201 ggatttatgc ggtattagca tctgtttcca aatgttatcc cccacttaag ggcaggttac
1261 ccacgtgtta ctcacccgtc cgccactcgt tcaaaattaa atcaagatgc aagcaccttt
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1321 caataatcag aactcgttcg acttgcatgt atagcacgcg cag

L6:

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1141 cgggtccatc caaaagcgat agcttacgcc atctttcagc caagaaccat gcggttcttg
1201 gatttatgcg gtattagcat ctgtttccaa atgttatccc ccacttaagg gcaggttacc
1261 cacgtgttac tcacccgtcc gccactcgtt caaaattaaa tcaagatgca agcacctttc
1321 aataatcaga actcgttcga cttgcatgta tagcacccgc ccgcc
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L7:

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 241 taaatgctgg caactagtca taagggttgc gctcgttgcg ggacttaacc caacatctca
 301 cgacacgagc tgacgacaac catgcaccac ctgtcatttt gcccccgaag gggaaacctg
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 421 aaaccacatg ctccaccgct tgtgcgggcc cccgtcaatt cctttgagtt tcaaccttgc
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1321 tcaataatca gaactcgttc gacttgcatg tatagcacgc gcctc

L8:

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1261 acccacgtgt tactcacccg tccgccactc gttcaaaatt aaatcaagat gcaagcacct
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L9:

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1321 catcaacgag aactcgttcg acttgcatgt atagcacgcc gcagcc