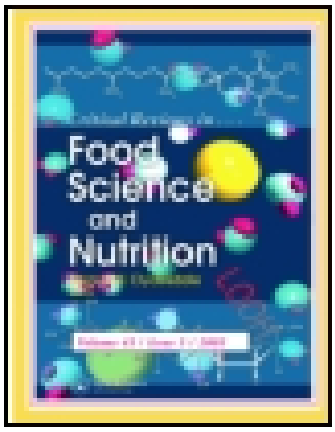

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Modulation of Intestinal Epithelial Defense Responses by Probiotic Bacteria

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ABSTRACT

Probiotics are live microorganisms, which when administered in food confer numerous health benefits. In previous studies about beneficial effects of probiotic bacteria to health, particularly in the fields of intestinal mucosa defense responses, specific probiotics, in a strain-dependent manner, show certain degree of potential to reinforce the integrity of intestinal epithelium and/or regulate some immune components. The mechanism of probiotic action is an area of interest. Among all possible routes of modulation by probiotics of intestinal epithelial cell–mediated defense responses, modulations of intestinal barrier function, innate and adaptive mucosal immune responses as well as signaling pathways are considered to play important role in the intestinal defense responses against pathogenic bacteria. This review summarizes the

beneficial effects of probiotic bacteria to intestinal health together with the mechanisms affected by probiotic bacteria: barrier function, innate and adaptive defense responses such as secretion of mucins, defensins, trefoil factors, immunoglobulin A (IgA), Toll–like receptors (TLRs), cytokines, gut associated lymphoid tissues and signaling pathways.

Keywords: Probiotics, intestinal epithelia, mucosal immunity, innate immunity, adaptive immunity

INTESTINAL EPITHELIUM – THE MAIN PLACE WHERE PROBIOTICS TAKE ACTIONS

As an important part of the digestive system, the gastrointestinal (GI) tract principally performs a role in digestion and absorption of ingested nutrients and excretion of waste. In addition, with the largest surface area (200 to 300 m^2) of the human body that is in direct contact with the outer environment (Lievin-Le Moal et al., 2006) and an abundance of multiple immune cells residing underneath the epithelial layer, the GI tract is considered as the largest immune interface with the environment and involves in the host defense responses (MacDonald et al., 2011).

Under normal conditions, the intestinal epithelia can display non-specific defense to maintain the coexistence with the microbiota. Upon exposure to unfriendly intrusion, the intestinal epithelium could be at high risk of pathogenesis and subsequent induced diseases. As the frontier of intestinal lumen, the epithelium functions not merely as a passive barrier but also sensitive indicators of virulence that correspondently activate a panoply of coordinated defensive responses which generally consist of barrier function, innate and adaptive immunity to prevent the entry of the external virulence (Lievin-Le Moal et al., 2006; Maldonado-Contreras et al.,

2011; Ohland et al., 2010). As the main habitat for indigenous bacteria, intestinal epithelium is also a most important place where probiotics interact with commensal bacteria and the host and exert their modulatory effects.

PROBIOTIC BENEFITS AND POSTULATED MECHANISMS OF PROBIOTICS ACTION

Probiotic organisms are defined as 'live microorganisms which when administered in an adequate amount confer a health benefit' (WHO, 2001). Beneficial effects of probiotic bacteria to GI tract have been extensively studied. Probiotics, either as food supplements or components of the microbiota were able to directly inhibit virulence in strain-specific manners by competitive exclusion (e.g. competing for binding sites, binding pathogen and impeding pathogenic internalization and competing for nutrients, etc.), direct antagonism (e.g. lowering pH environment by producing acids, secreting bacteriocin or bacterion-like substance, exerting de-toxification effects etc. (Reissbrodt et al., 2009; Woo et al., 2011)) and interfering the quorum sensing (Gillor et al., 2008; Ng et al., 2009). Moreover, probiotics were also reported to participate in the modulation of intestinal epithelial defense responses (Ohland et al., 2010). As

the outer layer of the intestine lumen, intestinal epithelial cell layer together with the overlying mucus was the frontier against the external virulent offense. Upon entry of pathogens, host initialized intestinal epithelial defense responses, which include the barrier function, innate and adaptive defense responses (such as secretion of mucins, defensins, trefoil factors, immunoglobulin A (IgA), Toll–like receptors (TLRs), cytokines, gut associated lymphoid tissues, signaling pathways etc.) (Duerr et al., 2012; Galdeano et al., 2007; Maldonado-Contreras et al., 2011). This review summarizes modes of action by probiotics against intestinal pathogens. The proposed modulation of intestinal barrier function is outlined in Figure 1.

PROBIOTIC MODULATION OF INTESTINAL BARRIER FUNCTION

The intestinal epithelium is a single layer of epithelial cells that functions as an integral defensive barrier and is under constant disturbances of external virulence. Tight junctions (TJs) in between the intestinal epithelial cells are membrane-associated proteins that seal intercellular space and the most important junction complexes that modulate the integrity of intestinal epithelium and prevent the entry of virulence. In general, TJs can be grouped into four types of proteins, namely trans-membrane proteins, adaptor proteins, regulatory proteins and

transcriptional and post-transcriptional regulators (Lodemann, 2010). These proteins work in a coordinating manner to help maintain the intestinal integrity, which can be well reflected by indicators like paracellular permeability and trans-epithelial electrical resistance (TEER, a measure of paracellular ion permeability) of cell monolayer (Oswald, 2006).

Unluckily, opportunistic pathogens might still be able to break the barrier by translocation through enterocytes and/or cleavaging the junction complexes between adjacent cells, which leads to leakiness of the intestinal tract by reduced TEER, increased paracellular permeability and subsequently, induces intestinal diseases such as inflammatory bowel diseases (IBD) (Clayburgh et al., 2004; Eun et al., 2011; Ewaschuk et al., 2008; Mennigen et al., 2009; Ohland et al., 2010).

Specific strains of probiotics have shown some potential in reserving the intestinal barrier function after impairment by cytokines, chemicals, pathogens, or stress (Ahrne et al., 2011), which might be worthwhile in the application of probiotics in combating the pathogenic challenges and relevant diseases (Figure 2). In *in vitro* studies, for example, *E.coli* Nissle 1917 (EcN) and VSL#3 (a commercially available mixture of eight strains of probiotic bacteria) were shown to be able to affect the regulation of TEER in T84 and HT-29 cells (Otte et al., 2004);

DNA microarray analysis also indicated the modulation by EcN in the expression and distribution of zonula occludens-2 (ZO-2) protein (Zyrek et al., 2007). In Caco-2 cell line model, *Lactobacillus casei* reversed the reduced TEER and increased epithelial permeability induced by cytokines, and modulated the ZO-1 expression (Eun et al., 2011). *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus farciminis* and *Lactobacillus plantarum* RO403 differentially prevented the disruption of ZO-1 tight junction protein in cytokine-challenged Caco-2bbe monolayer (Donato et al., 2010). In the above study, LGG also seemed to exert an modulating effect on barrier function via restoration of the TEER level which was originally reduced due to possible induced-damage by pro-inflammatory cytokines (Donato et al., 2010).

In *in vivo* studies, dextran sulfate sodium (DSS) has been used in rodents to induce acute and chronic colitis that mimics the clinical and histopathological conditions of human ulcerative colitis (Cooper et al., 1993; Okayasu et al., 1990). It was recorded that mice models appeared initially with a loss of ZO-1, and subsequently an enhancement in intestinal permeability, high disease activity index (DAI) and also inflammation (Poritz et al., 2007). VSL#3 was shown to reduce clinical disease activity indicated by DAI and histological inflammations visualized by microscopic appearance of crypts, and also reduce the increase in colonic epithelial permeability by measuring Evans blue uptake. In the investigation into a variety of junctional complexes

proteins including tight junction proteins and also adherens junction proteins E-cadherin and β-catenin, VSL#3 prevented the alterations in distribution and also expression of some tight junction proteins especially occludin, ZO-1, claudin-1, and -4. It was also indicated in the study that VSL#3 could significantly reduce the severity of epithelial apoptosis (Mennigen et al., 2009). In studies using single strains of probiotics such as *L. plantarum*, *L. casei*, *L. brevis* and *B. infantis,* probiotics were also proven to be efficacious to prevent DSS-induced damage (Osman et al., 2008; Osman et al., 2004; Ueno et al., 2011; Zakostelska et al., 2011) . A recent study was conducted to evaluate the effects of probiotics in the intestinal barrier maturation using neonatal mice models and it was found that *L. rhamnosus* GG regulated the expression of a multitude of intestinal TJ protein genes, with the most significant effects 2 to 3 weeks after they were born and simultaneous start of exposure to LGG. The time was coincident with that for the initial formation of enteric microbiota in new born mice, and at this presumable critic time point, claudin 3 yielded a most dramatic induction of gene expression and also protein re-localization. Therefore, it was proposed that LGG might take effects in an immature gut lacking indigenous bacteria by fostering maturation of intestinal barrier and modifying the claudin 3 – mediated intestinal paracellular permeability (Patel et al., 2012).

While most of the existing studies preferred to focus on probiotic capability in preservation

of tight junctions and restoration of epithelial integrity in pre-damaged or disease models, and restricted their attention to only several tight junction bridging proteins like ZO-1 and -2, recent studies began to look at the effects of probiotics in cell models without pre-disturbance, and also to expand the spectrum of target junctional proteins. For example, *Lactobacillus plantarum* MB452 induced an increase in TEER of Caco-2 cell monolayers in a dose-dependent fashion. By using global gene analysis of Caco-2 cell, *L. plantarum* MB452 was found to significantly modulate nineteen tight junction-related gene expression (e.g. the genes encoding occludin and plaque proteins) and to decrease the gene expression levels of eight cytoskeleton tubulin proteins and seven protein degrading proteasomes (Anderson et al., 2010). This study is a good basis for the future unraveling of possible beneficial effects of diverse probiotics to healthy individuals and also a good example to learn from in the field of related gene profile inspection.

MODULATION OF INNATE DEFENSE RESPONSES BY PROBIOTICS

There is increasing evidence that probiotic bacteria can affect both innate and adaptive components of the host immune system (Walker, 2008). Some probiotics act in the lumen of the gut by increasing the production of innate immune molecules, including goblet cell–derived

mucins, trefoil factors and defensins produced by intestinal Paneth cells, while others modulate the production of TLRs, secretory immunoglobulin A, heat shock proteins and P-glycoproteins (Figure 3).

Probiotic Modulation of Mucins

Outlying the intestinal epithelium, there is a transparent viscoelastic, semi-permeable and dynamic mucosal gel layer whose main components are hydrated mucins (Byrd et al., 2004; Hansson, 2012; Thornton et al., 2008). This mucus layer separates the host luminal content from the outer environment and plays an important role in various aspects. In addition to gut physiological function like lubrication and modification of the pH environment (Oswald, 2006), this mucus blanket serves important function in host defense responses: it constitutes a selective diffusion barrier permeable to nutrients but not to macromolecules including the external microbes (Oswald, 2006) and is able to trap these intrusive microbes due to its unique viscoelasticity. It also offers matrix for the retention of defensive compounds such as antimicrobials that result from regulation in defense responses (Cone, 2009; Linden et al., 2008). The mucus turnover is maintained by the dynamic balance of mucin synthesis and secretion into the gut lumen against degradation and shedding, which further assists in the clearance of unwelcome foreign substances. Last but not least, due to the richness of nutrient complex that

mucus matrix provides, the intestinal mucosa is also the habitat of commensal microbiota and plays a key role in their establishment, activity and maintenance of host–microbial homeostasis (Barnett et al., 2012; Duerr et al., 2012). A gradient increase in the thickness (from 150 - 300 μm thick in stomach, to 700 μm in the colon) of mucus gel accompanied by an increase in amount of endogenous bacteria present along the intestinal tract might be one of the signs for the correlation between intestinal mucus and the commensal bacteria.

Previous studies showed that probiotics have the capability to regulate mucin synthesis. For instance, experiments showed an increase in MUC2 gene expression in HT-29 cells by *Lactobacillus plantarum* strain 299v and a possibility to reduce the adherence of *Escherichia coli* O157:H7 *in vitro* (Mack et al., 1999). This probiotic agent with the ability to adhere to HT29 cells led to an increase in both gene expression level and extracellular secretion of MUC3 mucin and a reduced adherence of enteropathogen *E coli* E2348/69 (Mack et al., 2003). In a Caco-2 cell-culture model, *Lactobacillus rhamnosus* GG was claimed to up-regulate the expression of MUC2 mucin gene, but the conclusion is doubtful since if using the traditional way to calculate the amount of RNA extracted from cell treated with live probiotic bacteria, the total RNA would include the proportion from bacteria and it rendered different starting levels of RNA amount from cells in control and test groups. Therefore, new approaches to avoid this problem are yet to

be developed to help investigate modulation by live probiotic or other bacteria in all gene related assays addressing the live bacteria-cell interaction.

In addition to single strain of probiotic bacteria, mixture of probiotic agents, like VSL#3，were shown to increase MUC2, MUC3 and MUC5AC mucin secretion and also mucin gene expression level in HT29 cell lines (Otte et al., 2004). In another study, VSL#3 were shown to induce colonic mucin (MUC) secretion and MUC2 gene expression *in vivo* using Wistar rats model but effects in the modulation of MUC1 and MUC3 gene expression were not obvious. However, in an *in vitro* study conducted by the same investigators, it was shown that rather than VSL#3 bacteria which induced effect on mucin secretion, the bacterial conditioned media to a great extent stimulated the secretion of mucin secretion (Caballero-Franco et al., 2007).

All these finding rose interesting questions about how probiotics may exert effect on pathogenic virulence when they both appeared to target on the modulation of mucin as a first step, and also about whether probiotic agent works a potentiator or an attenuator. Up to date, studies about the interaction between probiotic and mucin still stress more on the adhesion ability of probiotic to mucin, since the adhesion ability is rendered as one of the standard for bacteria to be elected as probiotic (Van Tassell et al., 2011) and probiotic agents might be effective

competitors against the pathogens for the binding sites to the intestinal epithelium so that they might be rendered as promising pathogen antagonist based on some scientific evidence (Duary et al., 2012). Comparatively, few approaches have tried to address the potential role of secreted molecules by probiotics, which might at the same time influence the modulation of mucin into the mucus matrix where the interaction between probiotic and the host, or more precisely and crosstalk among probiotics, external environment and the host. Detailed pictures on how different probiotic bacteria influence the mucin behavior, especially in both gene expression and protein secretion need further clarification so as to help describe the role of probiotics in the interaction with mucin and correlation with innate immune responses.

Stimulation of Trefoil Factor 3

Trefoil factors (TFFs) are components that are co-secreted with mucins in the mucus gels. Together with mucins, TTFs act as the first barrier in the gut mucosal defense against intruding pathogens (Kindon et al., 1995; Plaut, 1997). In addition to being mucosal constituents, TFFs maintain the epithelial surface integrity by performing modulatory roles in cell migration (important activity for healing or ‘restitution’) and apoptosis (Dignass et al., 1994; Taupin et al.,

2000).

Trefoil factors (TFFs), including TFF1, TFF2 and TFF3, are characterized as ‘secretory products typical of the gastrointestinal tract’ (Hoffmann et al., 2001). While the stomach mucosa is the major origin of TFF1 and TFF2, TTF3 is more commonly found to be secreted by intestinal goblet cells (Hoffmann et al., 2001). TFF3 is suggested to be a feature of GI inflammation, since in most cases of GI inflammatory conditions like necrotizing enterocolitis and IBD, an over-production of TFF3 was found (Kjellev et al., 2007; Renes et al., 2002). Yet, a report has pointed out that it was not a potential makers of disease activity in patients with ulcerative colitis or Crohn’s disease (Grønbæk et al., 2006).

Probiotics are considered as promising therapy against GI inflammation. However, only few studies have addressed how probiotics associate with TFFs in the host defense. For example, in neonatal necrotizing enterocolitis (NEC) rat models, increased TFF3 positive cells were presented apically in NEC rat ileum but the amount of TFF3 positive cells was lowered down to normal level in NEC rats that were orally fed with live *Bifidobacterium bifidum* (Khailova et al., 2009). A recent study indicated that LGG supplementation in the diet did not show significant regulatory effects in the gene expression of TFFs in mice with DSS-induced colitis (Jiang et al.,

2011). Conversely, supplementation of LGG supernatant restored the down-regulated level of intestinal trefoil factor (namely TFF3) in alcohol damaged Caco-2 cell and also in mice model, and at the same time prevented alcohol-induced barrier dysfunction in colon cell monolayer and improved liver function (Wang et al., 2011). It was interesting that both over- and abated-production of TFF could disturb intestinal integrity. Therefore, as for the modulation by probiotics, though there showed a different direction of regulation upon TFF level, the roles of probiotics appear to be consistent in tuning the abnormal level, either higher or lower, back to normal status. Further studies are needed for better understanding of the interactions between role of probiotics and TFFs and how the interactions might affect the host defense.

Induction of Antimicrobial Peptides Secretion

One of the mechanisms of epithelial innate defense is secretion of antimicrobial properties (AMPs). AMPs are small cationic peptides with 12 – 50 amino acids, positively charged due to Arginine (Arg) and Lysine (Lys) residues and processing with amphipathic structures (Auvynet et al., 2009). Some are found to be secreted to the lumen by several innate leukocytes (such as monocytes, macrophages, mast cells and natural killer (NK) cells) and epithelial cells, or stored

in the intracellular compartment like neutrophil granules or Paneth cells (Auvynet et al., 2009). There are two main families of antimicrobial peptides, namely defensins and the cathelicidins (Oswald, 2006). From the perspective molecular structure, defensins contain six Cysteine (Cys) residues that form disulfide bonds and are sub-grouped into α-, β-, θ- defensins according to the location of Cys residues and sequence identity, while cathelicidins present a signal peptide at N-terminus and a C-terminal antimicrobial peptide with variable structures (Auvynet et al., 2009). When exposed to pathogens or microbial molecules like oligonucleotides and LPS, intestinal epithelial cells together with multiple immune cells secrete AMPs, which exert direct bactericidal activity by breaching the microbial membranes through interrupting its electrical charge (Auvynet et al., 2009; Oswald, 2006). Cathelicidins can kill both Gram- positive and - negative bacteria. As for human β-defensins (hBDs), hBD1 and hBD2 are predominantly antagonized against Gram-negative bacteria and some fungi, while hBD3 has antibacterial activity against a wider range of both Gram- positive and - negative bacteria.

Host-derived AMPs participate in innate immunity by enhancing phagocytosis and dendritic cell maturation, promoting neutrophil recruitment, stimulating the production of pro-inflammatory cytokines and modulating anti-inflammatory mediators to prevent excessive inflammatory response or to terminate immune response (Auvynet et al., 2009; Yang et al., 2002).

A deficiency in AMPs such as defensins has been determined as one of the causes of IBD like Crohn's disease. Such deficiency might lead to a defect in innate immunity and subsequent invasion of luminal microbe to the mucosa and trigger inflammation (Gersemann et al., 2008)

Some probiotics, apart from synthesizing AMPs, were shown to have the possibility of modulating intestinal epithelium-derived antimicrobial synthesis. In Caco-2 cell model, *Escherichia coli* Nissle 1917 (EcN) induced the secretion of human β-defensin 2 (hBD-2) (Schlee et al., 2007). A similar increase in hBD-2 level was also induced by *Lactobacillus plantarum* (Paolillo et al., 2009). These two studies proposed that the increase of hBD-2 might correlate with the expression of TLR2 and TLR5 in the intestinal cells, suggesting TLRs might also participate in the monitoring of probiotic induced effects. In a clinical trial, it was revealed that Symbioflor 2 (mixture of several *E. coli* strains) induces hBD-2 fecal peptide secretion; and in the following *in vitro* study, it showed that a 10- to 15-fold induction of hBD-2 was mediated by a tested *E. coli* genotype compared to probiotic EcN. This indicates a potency of probiotic *E. coli* in modulating the synthesis of AMPs from the host and the extent of modulation could be varied in different models and among different strains (Möndel et al., 2009). Studies also found that the induction of host AMPs by probiotics might be mediated by pro-inflammatory pathways. One of the examples is that *lactobacilli* and VSL#3 culture up-regulated the gene expression of

hBD-2 via activation of inflammatory pathways including nuclear factor kappa B and activator protein-1 as well as mitogen-activated protein kinases (MAPKs) (Schlee et al., 2008), which appeared to share the same modulatory pathway on the regulation of hBD-2 by EcN in a previous investigation (Wehkamp et al., 2004).

Stimulation of Secretory Immunoglobulin A Production

Immunoglobulin A (IgA) is featured as the most abundantly presented noninflammatory immunoglobulin antibodies that function as pathogen receptors in the gut (Maldonado-Contreras et al., 2011). IgA is secreted by intestinal B cells, and it can bind polymeric Ig receptor (pIgR) which is expressed on the basolateral surface of intestinal epithelium as an antibody transporter and facilitates the translocation of IgA dimmers to the luminal surface of epithelial cells. When secreted to luminal environment, IgA is generated into secretory IgA (SIgA) that sticks to the mucus covering the epithelial cells. Upon pathogenic invasion, it can aid in the prevention of tempting contact of pathogens with epithelia by forming a hydrophilic and epithelial-glycocalyx - repelling against the shell outlying the pathogens and thereby maintaining appropriate bacterial communities in specific area of intestinal lumen (Ohland et al., 2010; Sherman et al., 2009).

SIgA can also interfere with intracellular pathogens by deactivating bacterial lipoproteins and transporting them out to epithelial lumen during epithelial transcytosis. For the bacteria that have breached the epithelium, they might be cleared away by local IgA in the lamina propria via two routes: either sending back to the lumen via plgR or immune exclusion induced by the binding of IgA to receptor Fc_RI (CD89) on immune cells such as dendritic cells, monocytes, and some macrophages and activating downstream antimicrobial activity and inflammatory signaling (Cerutti et al., 2008).

Probiotics have shown potency in stimulating the production of IgA and thereby enhancing barrier function (Ohashi et al., 2009). For example, in a clinical trial in which 26 young healthy women eating a defined diet with supplementation of probiotic yogurt containing *Bifidobacterium lactis* Bb12, it was found that the IgA level was significantly increased in the fecal content during the probiotic feeding period compared to that of basal level samples, which showed an enhancement in immunogenic defense responses upon probiotics administration (Kabeerdoss et al., 2011). It was also indicated that some DNA motifs of probiotics and cell wall components of probiotic bacteria like lipoteichoic acids and peptideglycan can induce the production of IgA via the recognition by different TLRs (reviewed in Ohashi and Ushida 2009). Therefore, it is postulated that stimulation of IgA production might be one of the modes of

probiotic health beneficial action.

Stimulation of Toll-like Receptors

In order to protect the intestinal lumen against pathogens, the intestinal epithelium evolve to generate alertness against possible pathogen intrusion. The intestinal epithelial cells can express pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), to recognize different types of pathogenic bacteria according to pathogen-associated molecular patterns (PAMPs). TLRs exert their effects by binding with MyD88 (an adaptor protein), the stimulation of which will responsively recruit IL-1 receptor-associated kinase (IRAK) and thereby initiate signaling pathways via transcription factors such as nuclear factor κB (NF-κB), c-Jun N-terminal kinase (JNK), and p38 (Isono et al., 2007). The initiation of TLRs signaling was found to regulate diverse antimicrobial immune responses such as synthesis of pro-inflammatory cytokines, chemokines and antimicrobial peptides (Artis, 2008; Uehara et al., 2007), recruitment of B cells and production of IgA in lamina propria (Shang et al., 2008), which suggested their essential role in the innate immune responses.

Constitutive expression of many PRRs (such as TLR2, TLR3, TLR4 and TLR7) and NODs (NOD1 and NOD2) has been found in several intestinal cell lines (Uehara et al., 2007). Different TLRs can sense a range of bacterial motifs: for example, TLR-4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, whereas TLR-2 can bind to bacterial lipoproteins and lipoteichoic acid and are always found to work in conjunction with TLR-1 and TLR-6 (van Aubel et al., 2007). TLR-5 is involved in the recognition of flagellin (Hayashi et al., 2001), while TLR-9 senses the CpG (Vizoso Pinto et al., 2009). The PAMPS recognition by other TLRs can be seen in a previous review (Gómez-Llorente et al., 2010). However, when the pathogens such as gram-negative enteropathogens bypass the TLR signaling, NOD1, a cytosolic PRR that constitutively expressed by intestinal epithelial cells, can recognize microbial cell-wall peptidoglycans and work as an alternative activator of NF-κB signaling pathway (Kim et al., 2004).

PPRs also appear to participate in the discrimination of pathogens and commensal bacteria. Under normal situation they have limiting expression of PPRs like TLR2, TLR4 with CD14 on the apical surfaces of intestine to recognize bacterial LPS while some PPRs such as TLR3, TLR7, TLR8 and TLR9 are expressed and located within cellular compartment or on the basolateral surfaces to prevent direct contact with commensal bacteria and to antagonize internalized

pathogens. Upon pathogenic invasion, the host may initiate TLRS-mediated inflammatory responses to alert the host of the appearance of pathogens.

However, it was reported that some non-hazardous commensal bacteria can also induce the pro-inflammatory signal pathway. For example, intestinal epithelial cells can recognize and bind with the pathogenic flagellins from *E. coli* MG1655 via TLR-5, and subsequently recruit MyD88 adaptor protein and stimulate the NF-κB pro-inflammatory pathway (Bambou et al., 2004). Apart from commensal bacteria, probiotic bacteria are also considered to have potential in modulating TLRs in the intestinal epithelial cells, which might suggest a possible mode of action that probiotics could maintain 'a state of awareness' in the host for the surveillance of pathogenic intrusion (Vizoso Pinto et al., 2009). In a study using HT29 colon cell model, it was shown that *lactobacilli* (*L. rhamnosus* GG or *L. plantarum*) can stimulate the expression of TLR-2 and TLR-9 mRNA transcription level but such stimulation was absent when incubated with *S. typhimurium* only. *Lactobacilli* were also shown to up-regulate TLR-2 but not TLR-9 protein level, which in the authors' perspective, might be due to the short half-life of TLR-9 or downstream interference in translation of mRNA to protein. Interestingly, the protein expression of TLR-5, which is sensitive to flagellin, was also enhanced by *lactobacilli* which possess no flagella structures. This might be explained in a way that *lactobacilli* activate the common signal

pathways that involved in the transduction of TLR signals (Vizoso Pinto et al., 2009). Another *lactobacillus* strain *johnsonii* N6.2 was recently determined to induce the expression of TLR-7 and TLR-9 in differentiated Caco-2 cells (Kingma et al., 2011). Conversely, a reduction of TLR-4 in both mRNA and protein level was observed in *Clostridium butyricum* TO-A culture supernatant treated HT-29 human colon cells and it was demonstrated that the down-regulation in TLR-4 might be strongly correlated to the butyrate content in the supernatant (Isono et al., 2007), which might suggest that some probiotic species are more effective in restraining excessive or continuous inflammatory responses. This might also suggest that different probiotic bacteria have different abilities in maintaining the balance of antigen responsiveness and tolerance in the host response to pathogen invasion.

Studies have suggested that modulation in TLRs by probiotics can influence the activation and monitoring of innate defense responses such as production of antimicrobial peptides and cytokines by the host. As mentioned above, the induction of defensins in Caco-2 cell model by *Escherichia coli* Nissle 1917 (EcN) (Schlee et al., 2007) and *Lactobacillus plantarum* (Paolillo et al., 2009) might correlate with the expression of TLR2 and TLR5. As for the TLRs interaction with cytokines, for example, *L*. johnsonii N6.2 was shown to up-regulate type-1 interferon (IFN) and IFN regulators Stat1 and IRF7 in a TLR9-dependent way (Kingma et al., 2011). Incubation

with TLR-2 antagonists diminished the up-regulation of interleukin-23 (IL-23) expression in human colon cell model by LPS (Paolillo et al., 2009). However, the studies of interaction between probiotics and TLRs are inconclusive and further study on how probiotics modulate the expression of TLRs and the downstream components in the signaling pathway are needed to contribute to the understanding of the whole picture of probiotic modes of action.

Enhancement of Heat Shock Protein Production

Heat shock proteins (HSPs), which are ubiquitously found and highly conserved molecules in cellular life, mainly function as molecular chaperones to maintain cytoprotection, including protein folding, stabilization and translocation. Upon stress conditions like heat shock, oxidative stress, infection or inflammation, host will tend to produce more HSPs to prevent aggregation of misfolded proteins and to promote re-shaping or degradation of polypeptide substrates (Stewart et al., 2004; van Eden et al., 2005; Zügel et al., 1999). Apart from chaperone activity, the own HSPs from mammalian immune system are considered as a signal linking danger to recognition of pathogenic virulence and also participate in host innate and adaptive immune responses involved in bacterial infections (Osterloh et al., 2008; Srivastava, 2002; Stewart et al., 2004).

Probiotics were reported to induce HSPs in the intestinal epithelium. For instance, soluble factors from *Lactobacillus rhamnosus GG* conditioned media (CM) up-regulated the expression of both HSP25 and HSP72, which was postulated to be modulated through MAPK pathway (Tao et al., 2006). Similarity, CM of *Bacillus subtilis*, *Bifidobacterium breve*, LGG, *E. coli* Nissle, and *Lactobacillus plantarum* were shown to induce the expression of HSP27 in colonic Caco2bbe cells. It was found that competence and sporulation factor, a quorum-sensing peptide from *B. subtilis* was the efficacious component within the CM and the peptide might probably be involved in activating the phosphorylation of Akt and p38 MAPK (Fujiya et al., 2007). Heat-killed *L. brevis* SBC8803 induced HSP. The induction of HSP was probably negated by p38 MAPK inhibitor and was considered as one of the factors that ameliorate intestinal impairments and boost the survival rate in DSS mice suffering from lethal colitis (Ueno et al., 2011). In a follow up study, the supernatant of *L. brevis* SBC8803 was found to induce HSP27 secretion in human colon Caco2/bbe cells, and the HSP-induced component of probiotic secretion was determined as polyphosphate (poly P), which was expected to associate with intestinal integrin β1-p38 MAPK (discussed in 'Probiotics and Signaling Pathway') (Segawa et al., 2011). In another study, the level of HSP10, HSP60, HSP70, and HSP90 was brought down

after conventional therapies (mesalazine and probiotics) in ulcerative colitis patients, indicating HSPs are possible biomarker for intestinal bowel diseases (Tomasello et al., 2011).

Modulation of P-glycoprotein

P-glycoprotein (P-gp) is a well-studied transmembrane protein distributed in human body. It is encoded by multidrug resistance 1 (MDR1) and acts as ATP-binding cassette (ABC)-transporters. In the GI tract, high concentration of P-gp is found on intestinal epithelium of the luminal surface (Ho et al., 2003). From the perspective of intestinal epithelial defense, P-gp is suggested to be critical mediators of the efflux of drugs/xenobiotics and bacterial toxins from the intestinal mucosa into lamina propria (Ho et al., 2003). It was implicated that decreased expression and dysfunction of P-gp occurred in the pathogenesis of intestinal bowel diseases (Blokzijl et al., 2007; Langmann et al., 2004; Saksena et al., 2011).

A recent study firstly proposed a novel mode of action that probiotics might ameliorate intestinal inflammatory disorders through P-gp up-regulation (Saksena et al., 2011). In Caco-2 cell model, the culture supernatant (CS) of *Lactobacilli* strains *acidophilus* and *rhamnosus* enhanced P-gp activity which was indicated by measurement of verapamil-sensitive [^{3}H] digoxin

flux and the promoted P-gp activity that was shown to be mediated by an increase in MDR1/P-gp mRNA and protein expression. In addition, the CS of *L. acidophilus* and *L. rhamnosus* also improved MDR1 promoter activity. In an *in vivo* study, the healthy mice gavaged with live bacteria of *L. acidophilus* or *L. rhamnosus* exhibit an increased P-gp gene expression. Also, in another study, it was found that application of *L. acidophilus* to DSS mice appeared to lower the colonic MPO activity (an index of neutrophil accumulation), alleviate inflammation, and to boost the P-gp expression that was formerly reduced by DSS. To conclude, these findings envisaged a possibility of probiotic mechanism via P-gp. However, more studies are required to further validate the interaction between P-gp and the modulation of epithelial defense by probiotics.

MODULATION OF ADAPTIVE IMMUNE RESPONSES BY PROBIOTICS

Aside from innate defense responses, probiotic bacteria appear to participate in the modulation of adaptive immune responses mediated by intestinal epithelial cells such as the production of cytokines by enterocytes and the M cell-mediated gut associated lymphoid tissue (GALT) defense responses (Figure 4).

Modulation of Cytokines by Probiotics

Immunomodulatory effects of different species of probiotics were shown both *in vitro* and *in vivo* by their contribution to the induction or inhibition of specific cytokines, usually in the way of antagonizing the effects introduced by pathogens against the health of intestine. So far many studies have emphasized on the investigation of probiotic modulation on IL-8 secretion or gene expression, and it was found that in many cases, probiotic strains belong to *Lactobacilli*, *Bifidobacterium*, *Bacillus* and also probiotic mixture like VSL#3 could mitigate the augmentation of IL-8 in the induced models. For instance, *L. reuteri* exerted inhibitory effects against the increased IL-8 secretion and/or mRNA expression triggered by TNF-α or *Salmonella* in HT-29 and T84 human colon cell models (Ma et al., 2004). However, some studies showed converse tendency of modulation by probiotics such as EcN (Lammers et al., 2002; Otte et al., 2004) and *L. sakei* (Haller et al., 2000), the cause of which might be due to the fact that probiotic bacteria was applied without pre-induction or different cell models were used. The modulatory effects were also indicated in the synthesis of other cytokines. For example, LGG could up-regulate IFN-γ as well as IL-10 from mononuclear cells in an *in vitro* study (Kopp et al., 2008) and in the case of infants with cow's milk allergy or with IgE-associated dermatitis (Pohjavuori et al., 2004). IL-10 level was also increased in bone marrow-derived dendritic cells after

stimulation of cells by *Lactobacillus casei* and probiotic *E. coli* (Wells, 2011). EcN decreased growth related oncogene alpha (Gro-α) and IL-8 level in Caco-2 cells after bacterial challenge with Crohn's disease-associated *E.coli* strain LF82 (Huebner et al., 2011). In a co-culture cell model of dendritic cells with epithelial cells, two strains of lactic acid bacteria, *Lactobacillus casei* IBS041 (LC), and *Lactobacillus acidophilus* AD031 (LA), were shown to decrease the secretion level of IL-6 and TNF-α, respectively (Kim et al., 2012), prevent steatohepatitis and alleviate the inflammation in livers of alcohol-fed animals, which might be due to the inhibitory effects against the production of tumor necrosis factor alpha (TNF-α). In an *in vivo* experiment, pre-oral administration of EcN in gnotobiotic pigs posed interfering effect on translocation of *Salmonella* into mesenteric lymph nodes (MLN) and systemic circulation, which might be the cause that led to an absence of IL-10 in plasma and a decrease of TNF-α in plasma and ileum (Trebichavský et al., 2010). In clinical trials in patients with ulcerative colitis, the use of *Lactobacillus delbruekii* and *Lactobacillus fermentum* significantly ameliorated the inflammatory effects by reducing IL-6 concentration and expression of TNF-α and NF-κB p65 in colon (Hegazy et al., 2010).

As can be seen from previous experiment designs, most studies preferred to use ‘stimulated’ cell models which mimic a situation of infection or inflammation to test whether certain strains

of probiotics have an alleviating potential by measuring the level of target cytokines. However, few studies have addressed the modulatory effects of probiotics on non-stimulated intestinal epithelial cell model, a cell model that represents the normal status of intestine. Furthermore, the range of target cytokines was not broad enough to visualize the complete picture of modulation by probiotic bacteria since a variety of cytokines are working simultaneously to serve the immune function. Therefore, research on how probiotics influence the cytokine network and cytokines formation behavior which is highly correlated to the initiation of adaptive immune response seems necessary for the thorough understanding of the potential beneficial effects by probiotics in promoting the health of intestine.

Modulation of Gut Associated Lymphoid Tissues Defense Responses

Probiotic bacteria show their potency in modulating the adaptive defense responses by interacting with the immune cells in the Peyer's Patch (PP) in the lamina propria (Galdeano et al., 2007). Probiotic bacteria can be internalized into the PP via M cells mediation or through the paracellular transport by follicle-associated epithelial cells (Galdeano et al., 2007). Within the PPs, probiotics might be able to promote the IgA cycle which refers to the switch from IgM to

IgA B cells and in this way, increases the amount of B lymphocyte IgA+ in the mucosal sites distant to the intestine via their migration to MLN and then circulation to the other immune apparatuses (Galdeano et al., 2007). Other possible roles associated with adaptive immunity for probiotic bacteria may be shown in their capacity in ameliorating inflammation by modulating the behavior of immune cells in the lamina propria. For example, *Saccharomyces boulardii* treatment appeared promising in the inhibition of IBD in immune-compromised severe combined immunodeficiency (SCID) mice triggered by $CD4^+$ T cells transfer since it trapped the T-helper 1 cells in MLN and confined their entry to the inflamed colon and subsequent inflammation amplified by pro-inflammatory cytokines (Dalmasso et al., 2006). Probiotic modulation was also indicated in regulatory T cells. For example, administration of VSL#3 after the first course of trinitrobenzene sulfonic acid (TNBS)-induced colitis (TNBS-colitis) was shown to ameliorate the severity of recurrent colitis in mice with a simultaneous reduction of IFN-γ and IL-10 secretion, and that the protective effects by probiotics were mediated by the expression of IL-10 and were also associated with an increase of $CD4^+LAP^+$ TGF-β-bearing Treg cells that are from the lamina propria mononuclear cells (Di Giacinto et al., 2005). Another example is that *Lactobacillus casei* DN-114001 supplementation mitigated colitis and prompted the suppressed function of $Foxp3^+CD4^+$ regulatory T cells by controlling effector/ memory $CD8^+$ T cells in the lamina

propria of mice colon (Hacini-Rachinel et al., 2009). A recent study showed that *Bifidobacterium breve*, a probiotic strain that ameliorated intestinal inflammation in SCID mice, induced the mice colonic IL-10-producing type 1 regulatory T (Tr1) cells (Jeon et al., 2012), a major type of $CD4^+$ T cell subset and a Treg cell type that is as important as $Foxp3^+$ Treg cells in the reinforcement of intestinal homeostasis (Barnes et al., 2009). To conclude, apart from the interplay between host and pathogens, the interactions of host with microbes in the gut system including commensal bacteria and supplemented probiotic bacteria, from the perspective of adaptive immunity have also been an intriguing topic. Further understanding of the mechanism behind the interaction is necessary to approach the prevention and therapy to numerous immunologic disorders (Lee et al., 2010).

MODULATION OF SIGNALING PATHWAYS

Current studies on modes of action by probiotics also emphasized on their roles of modulation in the epithelial cell signal transduction pathways, which are crucial in monitoring the inflammatory responses in the host. Nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) are main factors that direct bacteria-derived signaling in immunity and

inflammation (Figure 5) (Sherman et al., 2009). The improved defense responses by probiotic bacteria have been reported to correlate with probiotic modulation on these inflammatory factors (van Baarlen et al., 2010).

Upon microbial infection, the host cells respond with an initiation of NF-κB pathway and induce the expression of a large reservoir of genes, many of which serve for the following innate immune responses and inflammatory reactions (Magalhaes et al., 2007). The signal cascades induced by microbes or derived products lead to the activation of the IκB kinase (IKK) complex and subsequent phosphorylation and degradation of inhibitory κB (IκB) proteins (Kim et al., 2004). Under such circumstances, NF-κB is allowed to translocate from cytoplasm to the nucleus, where it stimulates the transcription of a series of pro-inflammatory genes such as those encode cytokines and chemokines and also some growth factors that modulate the proliferation of immune cells (Kim et al., 2004; Magalhaes et al., 2007). In addition, NF-κB also monitors the synthesis of antimicrobial peptides such as β-defensins and immunoregulatory molecules such as CD40, which further illustrate its crucial role in this signaling pathway and relevant inflammatory responses (Magalhaes et al., 2007). It was reported that *L. reuteri* inhibited IκB degradation and IL-8 expression in TNF-α-induced T84 cells and NF-κB translocation to the nucleus in HeLa cells (Ma et al., 2004). Similarly, LGG lessened the translocation of NF-κB to

the nucleus, restored the decreased cytoplasmic IκB and limited IL-8 secretion in Caco-2 cell model (Zhang et al., 2005).

MAPKs such as extracellular regulated kinase (ERK1/2), p38 and c-Jun N-terminal kinase (JNK) are protein kinases that regulate a series of cell behaviors such as proliferation, differentiation, apoptosis and also gene expression upon challenges by stimuli such as mitogens, heat shock and cytokines (Pearson et al., 2001). It was indicated that improvement of epithelial integrity (indicated by an enhancement in TEER) of T84 cells by the spent culture medium (SCM) for probiotic mixture VSL#3 was associated with p42/44 MAPK and p38 activities, suggesting a possible role of soluble factors in VSL#3 SCM in modulating the cellular MAPK signaling (Otte et al., 2004). In a recent study, VSL#3 maintained the epithelial integrity by up-regulating the expression of the tight junction protein expression not only in intestinal epithelial HT29 models but also in DSS rats with acute colitis. The modulation by VSL#3 showed a strong correlation with the activating of the p38 and ERK signaling pathways (Dai et al., 2012). However, the modulations of signaling cascades are often multi-directional. For instance, *Bifidobacterium lactis* strain BB12 triggered both NF-κB RelAh and p38 MAPK phosphorylation in intestinal epithelial cell models; stimulation of the transcriptional factors was shown to correlate the augmentation of IL-6 synthesis (Ruiz et al., 2005). Another example is

that the increase in hBD-2 by *lactobacilli* and the VSL#3 was mediated by stimulation of pro-inflammatory pathways, including NF-κB and activator protein-1 as well as MAPKs (Schlee et al., 2008).

Studies also unveil the involvement of probiotics in modulation of other cellular signaling pathways. For example, viable probiotic agent *Lactobacillus helveticus* R0052 restored the level of IFN-γ-stimulated signal transducer and activator of transcription (STAT)-1 activation that was disrupted by EHEC O157:H7 within the epithelial cell signal transduction responses (Jandu et al., 2009). Dissecting the mechanisms by which probiotic bacteria interact with the signal pathways in epithelial cells will be pivotal to elucidate the modes of action by probiotics in host defense responses.

SUMMARY

Despite many studies that have shown that probiotic bacteria modulate intestinal epithelial cells mediated defense responses via modulation of intestinal barrier function, innate and adaptive defense responses, as well as signaling pathways, further validation is still needed to substantiate the efficacy of probiotics and strain-specific and dose-dependent modulatory effects

by probiotic bacteria for safety-proved application. The experimental validation of probiotic efficacy in promoting intestinal epithelial defense capability might possibly be regarded as one of the scientific basis for the future detailed study of benefits of probiotics towards the health of intestine and also dietary intervention against intestinal exposure to pathogens.

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Figure 1. Proposed modulations of intestinal epithelial defense responses by probiotic bacteria

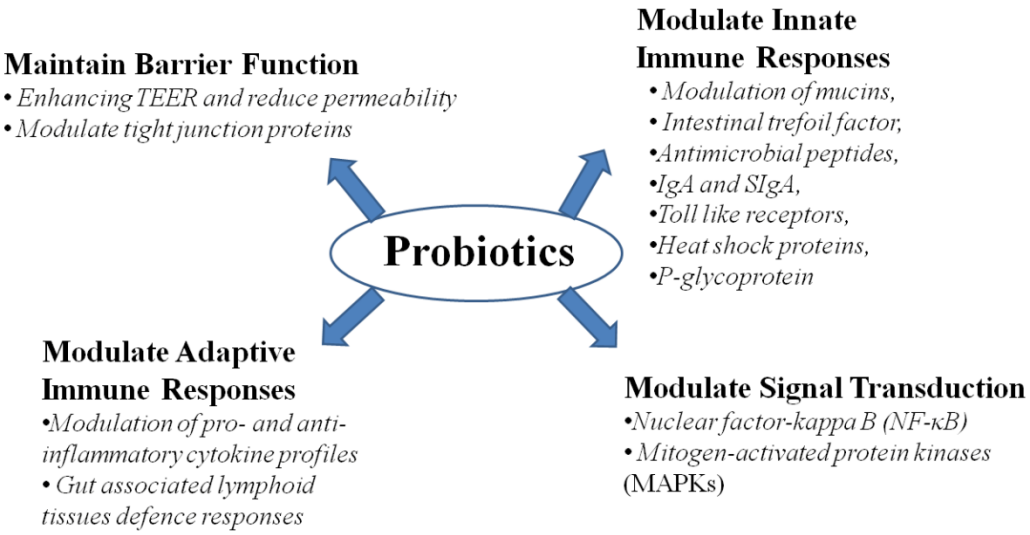

Figure 2. Modulation of intestinal barrier function by probiotics

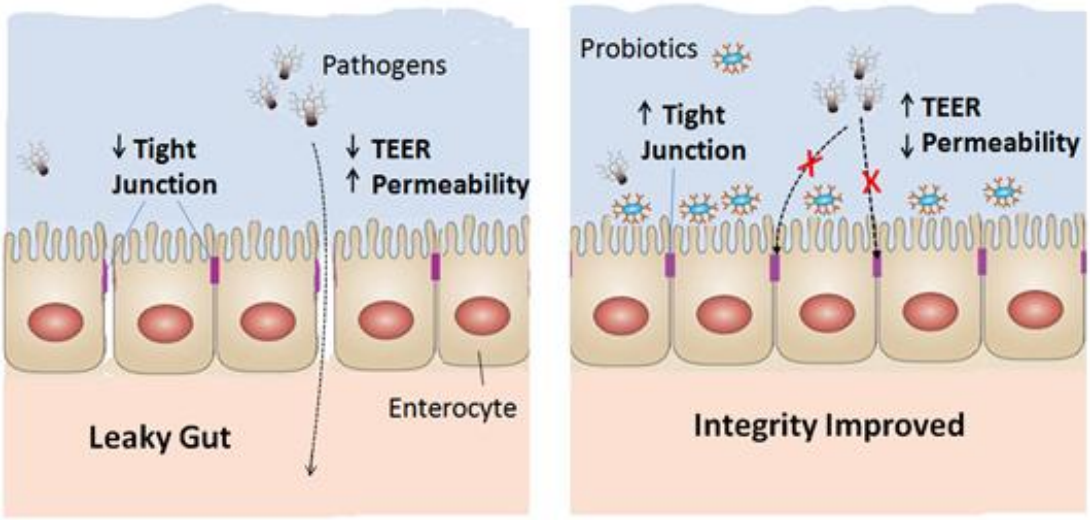

Figure 3. Modulation of innate defense responses by probiotics

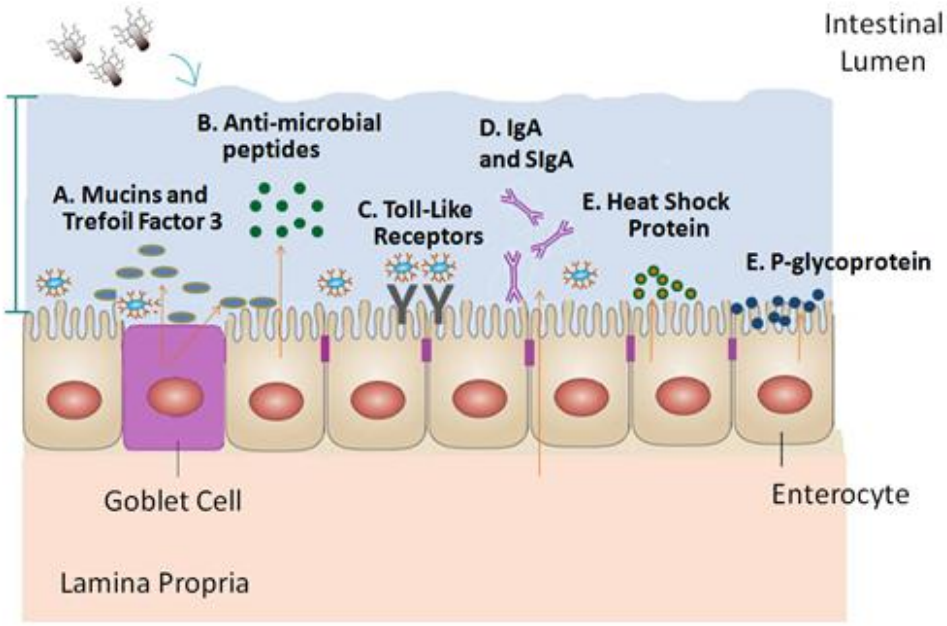

Figure 4. Modulation of adaptive defense responses by probiotics

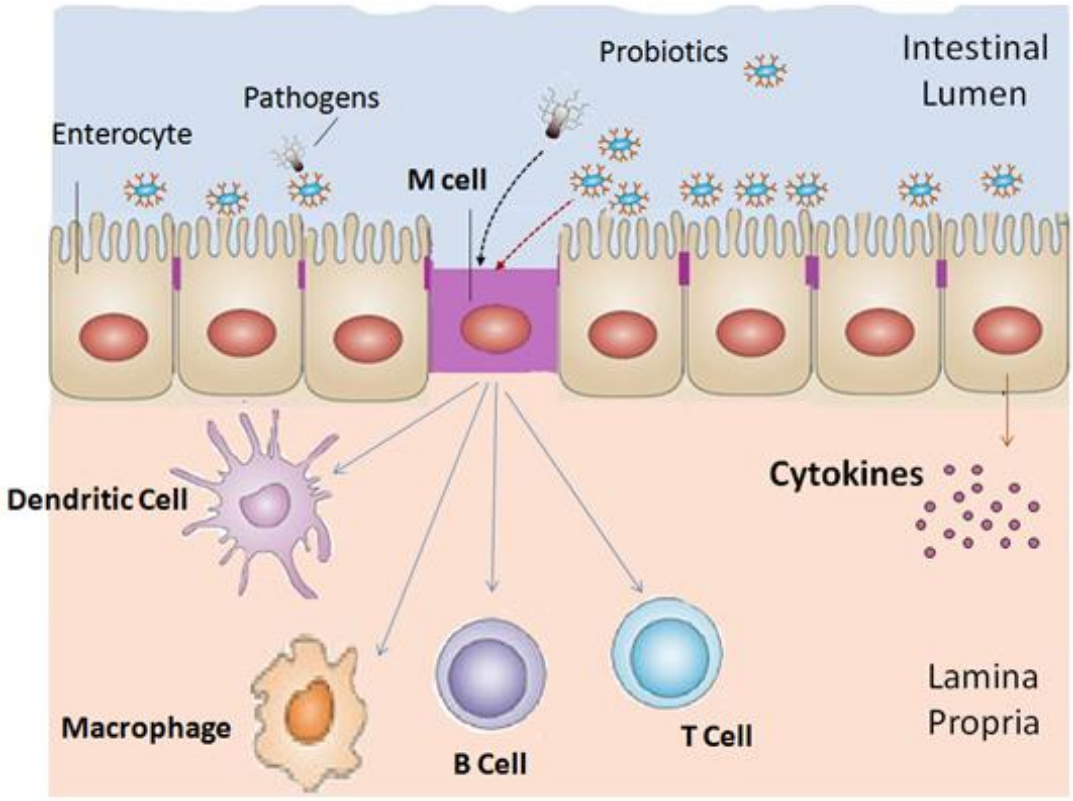

Figure 5. Modulation of epithelial signaling pathways by probiotics