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Effects of Lactobacillus rhamnosus Milk Isolate on the Production of Inflammatory Cytokines in Enterocytes

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by

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ABSTRACT

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In the gastrointestinal tract, probiotics have been shown to promote host immunity and to regulate immune signaling pathways. This study used Caco-2 cell line to examine the effects of a *Lactobacillus rhamnosus* isolate from *"amabere amaruranu"* a Kenyan traditional cultured milk, on the production inflammatory cytokines in enterocytes. Live *Lactobacillus rhamnosus* (MRS6AN), its cytoplasmic fraction (CF), filtered spent broth (FSB) or heat inactivated FSB (HIB) were used as treatments on differentiated Caco-2 cell monolayer in transwells. Cytokine content in the cell lysates, apical and basolateral supernatants were determined using ELISA. Caco-2 cell lysate treatments showed significantly increased anti-inflammatory TGF-β (ng/ml) levels on average about 100x more compared to the increase in pro-inflammatory IL-8 (pg/ml) levels. These levels were significantly reduced after inhibition of NF-κB. In conclusion, live *Lactobacillus rhamnosus*, its CF, FSB or HIB seemed to modulate the production of inflammatory cytokines in enterocytes partly via the NF-κB signaling pathway.

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DEDICATION

To my family in Kenya and my fiancé Billy Tanui

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CHAPTER 1

INTRODUCTION

Probiotics

"Probiotics are live microorganisms that confer health benefits to the host when administered in adequate amounts" (FAO/WHO 2001). The term probiotic was derived from the Greek word meaning "for life" (Reid et al. 2003). They are live commensal microorganisms that majorly colonize the gastrointestinal tract where they regulate intestinal epithelial development and function (Gaudana et al. 2010). Naidu et al. (1999), described probiotics as dietary adjuncts that benefits the physiology of the host. They modulate the immune system and improve the nutritional and microbial balance in the gastrointestinal tract.

Predominant microbes in the human intestinal tract that have been identified are lactic-acid producing gram positive *Lactobacillus* and *Bifidobacterium* genera (Gill and Cross 2002). Probiotics are widespread in nature. Lactic acid bacteria naturally occur in soil, water, sewage, silage and plants. They can be found in dietary supplements and fermented products: beverages, bakery products, vegetables, meat, and milk. They can also occur in mucous membranes of the human body such as the skin, mouth, urinary and genital organs, (Harzallah and Belhadj 2013) and the intestine where they enhance gastrointestinal system function (Schaafsma, 1996). They play an important role in the fermentations of food and feeds which help to increase their shelf life (Bhutada and Tambekar 2009).

For over a decade there has been an increase in the interest for use of probiotics for prevention and treatment of various human diseases (Metchnikoff 1907). This is due to the rise in number of pathogens that have multidrug resistance and the recognition of the role in which commensal bacteria play in health and disease (Britton and Versalovic 2008). Currently, some probiotic compounds or metabolites are being used in the management of gastrointestinal diseases such as in Inflammatory Bowel Disease (IBD) therapy (Madsen et al. 1999; Shibolet et al. 2002). National Health Interview Survey (NHIS) (2015), reported that 1.6% of US adults (3.9 million) and 0.5% of US children (294,000) use probiotics (Clarke et al. 2012). Inasmuch as many milk products containing probiotics are readily available in the market (Miyoshi, 2010), there is need for more information on the mechanisms of these probiotics in order to consider them for the treatment and prevention specific diseases (Ouwehand 2002).

The function of probiotics mainly occurs through their adaptation factors and probiotic factors - health beneficial effects (Lebeer et al. 2008). Adaptation factors include adherence to intestinal mucosa and mucus, stress tolerance and production of antimicrobial products whereas probiotic factors mainly include maintenance of microbial balance, epithelial protection and immune modulation (Medzhitov 2007).

Probiotic Potential in the Gastrointestinal Tract

Probiotic potential of microorganisms is dependent on their “viability, large scale reproducibility, stability during use and storage and the ability to survive in the intestinal ecosystem” (Boyiri and Onyango 2015). Probiotic adaptation factors include stress

tolerance, production of anti-microbial factors and impairment of pathogenic colonization in the gastrointestinal tract (GIT). Studies by Guandalini (2002), showed that probiotics produce adhesins which play a protective role by preventing the binding of pathogenic bacteria to the intestinal epithelia. *In vitro*, studies have illustrated that probiotics have lectin-like adhesive components that bind to carbohydrate form glyco-conjugate receptors on intestinal epithelial cells (IEC) which prevent pathogens from binding (Gourbeyre et al. 2010). Probiotics can also impair the growth and colonization of pathogenic strains by producing antimicrobial products such as hydrogen peroxide, bacteriocins (lantibiotics) and organic acids (lactic, acetic and propionic acids) (Gourbeyre et al. 2010; Balamurugan et al. 2014).

Apart from producing antimicrobial compounds, probiotics exert intestinal colonization by competing for receptors on the mucus layer or on intestinal epithelial cells and via bacteria-bacteria communication in biofilms or microenvironment through quorum sensing (Schiffrin and Blum 2002). Probiotics also stimulate enterocytes to produce cyto-protective substances such as heat shock proteins (Tao et al. 2006) which protect the intestinal epithelia from tissue damage. They also produce antimicrobial peptides like defensins (Salzman et al. 2010) which “directly kill or inhibit the growth of bacteria in the lumen” (Salim and Madsen 2014); and mucins (Mack et al. 1999) which may directly limit intestinal infection by adhering to pathogens (Linden et al. 2008). They also protect against secretion of gastric and duodenal acids (Purchiaroni et al. 2013).

In their ability to stimulate defensin production (Salim and Madsen 2014) and activity (Britton and Versalovic 2008), probiotics can modify the ability of pathogens to bind to or colonize colonic epithelial cells by seizing vital nutrients from pathogens or by

changing the pH making it unfavorable for pathogenic strains to survive (Schiffrin and Blum 2002). Probiotics have also been shown to promote mucosal integrity and to "act on tight junctions by increasing the expression of *zonula occludens* proteins and *occludins"* (Shen et al. 2006; Gareau et al. 2010). Studies have pointed out that a good number of probiotics are able to withstand the adverse environmental conditions of the GIT including low pH or high acidity, high temperature, presence of bile salts, antibiotics and they also play a major role in the promotion of the host immunity (Bhutada and Tambekar, 2009; Leeber et al. 2012). Apart from their adaptation factors, probiotics also confer health benefits to their hosts (Lebeer et al. 2008).

Health Effects of Probiotic Lactobacilli

Probiotics have beneficial effects in the intestinal tract in that they help to stabilize the native microbial population, protect against intestinal infection, alleviate lactose intolerance, reduce serum cholesterol levels and enhance non-specific immune systems (Gaudana et al. 2010). Several studies have shown how *Lactobacilli* exert health benefits to their hosts and how they have been used in the treatment and prevention of enteric infections like acute infectious diarrhea, reduction of relapse of *Clostridium difficile* associated diarrhea (Pillai et al. 2008) and prevention of antibiotic-associated diarrhea (Sazawal et al. 2006). For example, Basu et al. (2009) in their study reported that *Lactobacillus rhamnosus* doses (10^{10} and 10^{12} cfu) were both effective in reducing the duration and frequency of acute watery diarrhea. In another study at a daycare in Croatia by Hojsak et al. (2009), compared to the placebo group (n=142),

children who were given 100ml of fermented milk product with *Lactobacillus rhamnosus* GG (10^9 cfu; n=139) had a significant reduction in respiratory tract infections. Clinical studies have also reported the use of *Lactobacilli* in the treatment and prevention of colorectal cancer (Rafter et al. 2007), inflammatory bowel disease - IBD (Hedin et al. 2007), bacterial vaginosis and urogenital diseases in women (Falagas 2007), prevention of food hypersensitivity and atopic disease (Boyle et al. 2006), prevention of dental caries (Meurman and Stamatova 2007) and treatment of irritable bowel syndrome (Camilleri, 2006). However, it is important to consider the probiotic strain dosage and the host's health condition in determining the health benefits that probiotics may confer to the host. The lowest daily dose of *Lactobacillus rhamnosus* reported to benefit the host is 1 X 10^8 cfu/day (Kekkonen et al. 2009).

Immunological Importance of Probiotics in the GIT

Purchiaroni et al. (2013) stated that microbiota in the gut plays a crucial role in the maintenance and disruption of gut immune latency by interacting with both innate and adaptive immune system. Probiotics interact with a extensive variety of cells including enterocytes, dendritic cells, T-helper 1, T-helper 2 and T-regulatory in the gastrointestinal tract (GIT) and this enables them to either modulate or to promote pro- or anti-inflammatory immune responses (Gourbeyre et al. 2010). Intestinal *Lactobacilli* and *Bifidobacteria* have specific components which have been linked with health effects in the host such as the elevation of gut maturation and integrity, immune modulation and antagonisms against pathogens (Schiffrin and Blum 2002). Probiotics that have an

anti-inflammatory potential, may reduce bacteria-induced production of pro-inflammatory cytokines or cause a decrease in serum C-reactive proteins (CRP) levels (Galdeano et al. 2008; Mercola 2008). Gill and Cross (2002), in their research, found that probiotics were able to modulate the immune system via both immune-stimulatory and immune-regulatory mechanisms. However, selection of probiotic strain with immunological properties: their ability to influence cytokine expression or to favor a claimed immune response must be well defined (Perdigon et al. 2013). Immunological effects of probiotics may also result from soluble factors that may alter epithelial permeability, downregulates inflammatory cascade, or enhance activation, maturation, or survival of dendritic cells (Corthesy et al. 2007).

Probiotics could also maintain intestinal immune homeostasis by reversing immune dysbiosis and prevent inflammation (Schiffrin and Blum 2002). According to Britton and Versalovic (2008), probiotics compete for an ecological niche and play a vital role in the balance between homeostasis and dysbiosis. Consequently, deviations from appropriate development and regulation of innate and adaptive immunity could lead to dysbiosis, loss of metabolic and immune homeostasis. Probiotic bacteria have the potential to reverse dysbiosis and restore tolerance towards the microbiota (Zeuthen et al. 2008; Baarlen et al. 2013). Probiotics could therefore employ a twofold function; stimulation of mucosal defense mechanism and maintenance of immune balance (Schiffrin and Blum 2002). Delcenserie et al. (2008), in their studies, concluded that probiotics can act on a variety of intestinal cells thereby modulating the immune system towards a pro-inflammatory or anti-inflammatory responses depending on the strain

used, the types of cells being treated, the setting and the immunological factors being measured.

Enterocytes in Gastrointestinal Immunity

Enterocytes occur in a single monolayer forming a barrier that protects the gastrointestinal tract from nearly 400 species: approximately 10^{14} bacteria (Pinto et al. 2009). Human colonial epithelial cells form the bowel mucosal barrier which play an important role in the initiation and advancement of intestinal inflammation (Dimitrov and Gotova 2014). Delcensenrie et al. (2008), examined how the interaction between probiotics and enterocytes serve as an initiating event in immunomodulation and suggested that the interaction between probiotics and the intestinal epithelial cells is a central factor for production of cytokines by enterocytes before they interact with immune system cells. Further, Perdigon et al. (2002) supported that the activation of the secretory and systemic immune responses by lactic acid bacteria requires many multifaceted interactions among the different components of the intestinal environment.

Enterocytes facilitate a trilogue or communication between luminal bacteria, epithelial cells and immune cells in the lamina propria. Upon binding and colonization of the gut, many probiotic strains can influence the host's mucosal immune system through the secretion of immune mediators that affect different cell signaling pathways in mammalian cells (Hemaiswarya et al. 2013). Many immune mediators involved in innate immune responses are synthesized by intestinal epithelium (Zhang et al. 2010). Whether the response is pro-inflammatory or anti-inflammatory is dependent on the individual microorganism in the gut (Neish 2002). Enterocytes can release cytokines

such as IL-6, TNF-α and IL-8 when triggered by stimuli (Claud et al. 2003). *In vitro*, Caco-2 cells (human colon adenocarcinoma derivative) have been used as a model of intestinal epithelium because they display structural, morphological and functional properties that mimic that of intestinal epithelial cells. They spontaneously proliferate and differentiate, form tight junctions and express high levels of digestive brush border enzymes (Olejnik et al. 2003). Intestinal epithelial cells can sense microbes or their components through membrane bound Pattern Recognition Receptors (PRR) like Toll like receptors (TLR) and nucleotide binding oligomerization domain (NOD) like receptors. TLR recognize a variety of microbial compounds whereas NOD1 and NOD2 receptors majorly recognize peptidoglycan components (Kingma et al. 2011).

Possible Mechanisms of Gastrointestinal Immunity Stimulation by Probiotics

Major mechanisms of action for Probiotic *Lactobacilli* include inhibition of pathogens, maintenance of microbial and immune homeostasis, augmentation of epithelial barrier function and antimicrobial activity, prevention of inflammation and modulation of immune responses (Schiffrin and Blum 2002; Fedorak and Madsen 2004; Marco et al. 2006; Boirivant and Strober 2007; Leeber et al. 2008; Salim and Madsen 2014). Most probiotic *Lactobacilli* are gram positive, non-sporulating, anaerobic bacteria (Claeson et al. 2007). Being Gram positive bacteria, *Lactobacillus* possess conserved microbe-associated molecular patterns (MAMPs): cell wall exopolysaccharides, lipoprotein anchors, peptidoglycan, wall teichoic acids (WTA) and lipoteichoic acid (LTA)

in the cytoplasmic membrane (Lebeer et al. 2008; Baarlen et al. 2013) which are sensed by Pattern Recognition Receptor (PRRs) (Willing et al. 2010).

Lactobacilli has a thick peptidoglycan complexed with covalent and non-covalent interactions with teichoic acids, lipoteichoic acids, proteins and polysaccharides (Delcour, 1999). Teichoic acids which are covalently bonded to peptidoglycan or attached to cytoplasmic membrane, are “anionic cell wall polymers made up of polyglycerol phosphate or polyribitol phosphate repeating units” (Neuhas and Baddiley 2003). The cell wall of *Lactobacillus rhamnosus* and *Lactobacillus casei* has been shown to only contain LTA in their peptidoglycan (Perear-Velez et al. 2007). By use of single-molecule force spectroscopy, study by Francius et al. (2008), reported that *Lactobacillus rhamnosus* GG expressed two types of exopolysaccharides: short glucose-rich and long galactose-rich exopolysaccharides. *Lactobacillus rhamnosus* GG was also shown to possess cell surface proteins made up of glycoproteins (Francius et al. 2008).

The intestinal epithelium has diverse innate immune sensors like the Toll-like-receptors (TLRs) and Nod-Like Receptors (NODs) which are crucial for probiotic-host interaction (Claes et al. 2012). These receptors could detect ‘Molecular Associated Molecular Patterns’ (MAMPs) present on commensal bacteria (Ghosh, 2010; Purchiaroni et al. 2013). These MAMPs interact with TLRs, NODs and C-type lectin receptors (CLRs) via PRR signaling resulting in the induction of signaling cascades which drive innate immune responses including production of pro- and anti-inflammatory cytokines, antigen presentation, activation of immune responses and expression of antimicrobial factors (Claes et al. 2012; Baarlen et al. 2013). TLRs were the first pattern

recognition receptors associated with the recognition of commensal microorganisms (Rakoff-Nahoum et al. 2004; Slack et al. 2009). In mammals, TLR's are present on intestinal epithelial cells, dendritic cells, macrophages and neutrophils, (Purchiaroni et al. 2013). Muramyl peptides which are present in all *Lactobacilli* are recognized by NOD2 receptors (Delcour, 1999). In support, Petnicki-Ocwieja et al. (2009) reported that the NOD2 expression is dependent on the presence of non-pathogenic bacteria as mice deficient in NOD2 were found to have higher amounts of commensals. Consequently, the expression of NOD2 protein can be activated by bacterial peptidoglycan by triggering mitogen activated protein (MAP) kinase and Nuclear Factor-kappa B (NF-kB) signalling pathway leading to production of cytokines such as TNFα, IL-1β, IL-8 and antimicrobial peptides (Cheng and Huang 2013).

Gram-positive bacterial cell-wall components including lipoteichoic acid, peptidoglycan and muramyl dipeptide were shown to bind to leucocyte Pattern Recognition Receptors (PRRs), including the endotoxin receptor (CD14), TLR 2 and Type 1 macrophage scavenger receptor. This could present the possible mechanism through which probiotics directly stimulate immune responses (Gill and Cross 2002). Galdeano and Perdigon (2004), in their study demonstrated that probiotics or even their products could access the intestinal immune system and persist for a certain period of time and initiate a specific immune response. Interestingly, other beneficial effects of *Lactobacilli* on intestinal epithelial cells do not require direct cell contact.

Lactobacillus rhamnosus may secrete proteins into their spent supernatant thereby triggering specific signaling pathways in intestinal epithelial cells to stimulate or inhibit cytokine production (Yan and Polk 2002; Yan et al. 2007; Britton and Versalovic

2008). *Lactobacillus rhamnosus GG* have multiple cell surface structures and secreted factors that have been shown to promote protective role on intestinal epithelial cells via multiple signaling pathways (Lebeer et al. 2008). Particularly, *Lactobacillus rhamnosus GG* was shown to influence inflammatory gene expression via TGF-β and TNF cytokines. They also influence apoptosis, cell growth and differentiation, cell-cell signaling, signal transcription/ transduction and cell adhesion indicating its ability to modulate mucosal immune responses in the intestinal mucosa (Di Caro et al. 2005).

In vitro, some probiotic strains or their products (Fig.1a) may stimulate innate immune response by inducing TNF-α production by epithelial cells, thereby activating NF-kB in macrophages and inhibiting host immune response by influencing the production of IL-8 and subsequent recruitment of neutrophils to the site of infection. Some strains (Fig.1b) may stimulate the innate immune system by signaling dendritic cells, which then migrate to mesenteric lymph nodes where they induce T-reg cells resulting in the release or production of anti-inflammatory cytokines like IL-10 and TGF-β (Gareau et al. 2010 in Harzallah and Belhadj 2013).

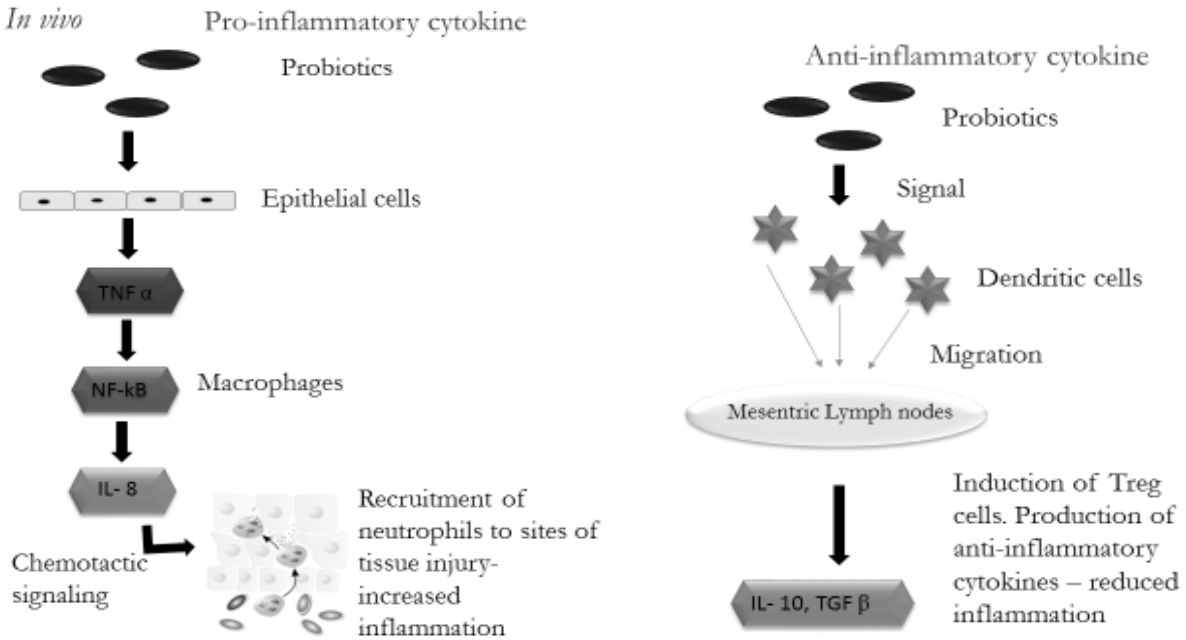


Figure 1 (a) and (b): Potential mechanism of action by probiotics

Probiotic Effects in Inflammatory Cytokine Production in Enterocytes

Cytokines are small molecular weight proteins or peptides, secreted by immune and non-immune cell and they regulate the duration and intensity of immune responses (Wenchao et al. 2013). Cytokines are able to alter the character of developing immune response or inflammatory reaction (Gill and Cross 2002). They act via specific receptors and, depending on the specific cytokine and the receptors they bind to, they can either up-regulate or down-regulate the activity of other immune cells. They may exert either pro-inflammatory or anti-inflammatory effects or both depending on their specific microenvironments (Su et al. 2011). Cytokine secretion is one of the first immune responses and can provide information on the nature of infection (Wenchao et al. 2013). They are extremely potent at low concentrations and their potency is partially due to

cascades and networks, whereby the presence of one cytokine leads to the secretion of others (Stadnyk 1994). Cytokines produced by epithelial cells are grouped as shown in Table 1.

Table 1. Cytokines Grouped by Function, made by Epithelial Cells

General Function	**Cytokine**
Inflammatory/ immune regulation	IL-1α, IL-1β, IL-6, IL-10, TNFα, LIF, TGF-β
Growth Factors/ differentiation	IL-1α, IL-1β,IL-3,IL -7, TNFα, LIF, TGFβ ,TGFα, PDGF, G-CSF and GM-CSF
Chemotactic Factors	IL-8, MGSA/gro, MCP-1, CINC
Epithelial Cell Transformation	TGF-α, TGF-β, IL-1, IL-6

Source: (Stadnyk, 1994)

Probiotics have been reported to regulate immune cell signaling pathways, modulate immunity and induce the production of anti-pathogenic factors by the host (Britton and Versalovic 2008). The secretion of cytokines from intestinal epithelial cells when they interact with probiotic bacteria is strain dependent (Kemgang et al. 2014). *In vivo*, cytokines released by epithelial cells may function as signals to other immune cells (Kurchazik et al. 1997). Sartor et al. (2014), stated that several probiotic species or their products induce the production of cytokines which are protective and critical mediators of intestinal homeostasis including IL-10 and TGF-β, and may suppress pro-inflammatory cytokines such as TNF-α (Baarlen et al. 2013). IL-10 is important in maintenance of immune homeostasis as mice deficient in IL-10 signaling were highly susceptible to colitis and the severity increased with deficiency in TGF-β signaling

(Sanjabi et al. 2009). However, it is important to note that inflammatory effects of probiotics are strain-dependent and their efficacy is dependent on conditions, quality, dose and concentrations of probiotic preparations (Delcenserie et al. 2008).

Lactobacillus rhamnosus GG, was shown to inhibit pro-inflammatory signaling and inflammatory cytokine-induced cell death in colonic epithelial cells through a mechanism that is dependent on epidermal growth factor receptor (EGFR) (Sartor et al. 2014). Previous study by Zhang et al. (2005) noted that *in vitro,* higher doses of *Lactobacillus rhamnosus* GG without TNF-α stimulation induced the production of IL-8. Both live and heat-killed *Lactobacillus rhamnosus GG* seemed to down-regulate LPS-induced pro-inflammatory immune mediators and up-regulate anti-inflammatory mediators in liver, lung and plasma (Nan et al. 2009). In another study, *Lactobacillus rhamnosus GG* secreted proteins diminished the production of TNF-α by LPS-activated macrophages (Pena and Versalovic 2003). *Lactobacillus casei* DN-114001 was found to increase mRNA expression of TGF-β and IL-10 in TLR4 knock out mice (Fukata and Abreu 2007). Studies by Kekkonen et al. (2008a) and Kekkonen et al. (2008b), reported that *Lactobacillus rhamnosus* GG appeared to have anti-inflammatory effects as they were able to reduce inflammatory cytokines and to decrease inflammatory mediators: sensitive CRP, inflammatory lipid-derived mediators such as lysophosphatidylcholines and sphingomyelins. Probiotic *Lactobacillus plantarum* increased the production of anti-inflammatory IL-10 and reduced the secretion of pro-inflammatory cytokines; TNF-α, IL-1β, IL-6 and IL-8 in TNBS-induced mice (Grangette et al. 2005). *In vitro* studies with Caco-2 enterocyte-like cells demonstrated that fermentative *Lactobacillus sakei* and

probiotic *Lactobacillus johnsonii* could stimulate the expression of anti-inflammatory TGF-β but not pro-inflammatory TNF-α or IL-1β mediators (Gill and Cross, 2002).

Further, the use of leucocytes on Caco-2 cells, *Lactobacillus* co-culture promoted the secretion of anti-inflammatory IL-10 and induced the production of pro-inflammatory mediators by epithelial cells (Gill and Cross 2002, Haller et al. 2000). Research has also shown that by use of Caco-2 cell culture model *in vitro*, different probiotics could stimulate the production of a variety of cytokines prior to interaction with immune system cells as shown in Figure 2 (Delcenserie et al. 2008).

Probiotic	Cytokine / Chemokine Produced	Cells	References
L. sakei	IL-1B, IL-8, TNFa	Caco2	(Haller et al. 2000)
L. johnsonii	TGF b	Caco2	(Haller et al. 2000)
L. rhamnosus GG	IL-8	Caco2	(Zhang et al. 2005)
L. casei DN 144 001	CXCL1, CXCL2, CCL20	Caco2	(Tien et al. 2006)
Non-pathogenic *E. coli*	IL 8	Caco2	(Parselak et al. 2014)
L. casei DN 114 001	TGF b and IL 10	Primary intestinal epithelial cells	(Chung et al. 2008)

Adapted from (Delcenserie et al. 2008)

Figure 2. Cytokines produced following interaction between probiotics and intestinal epithelial cells

In vivo, probiotics can modulate immune cells including dendritic cells and T-regulatory cells. Exposure of dendritic cells to some probiotics may induce anti-inflammatory responses and activation of IL-10 producing T-regulatory cells (Salim and

Madsen, 2014). T-regulatory cells through their IL-10 and TGF-β production are able to regulate immune responses by inhibiting T-helper 1 and T-helper 2 activities (Gourbeyre et al. 2010). Certain probiotics could stimulate T-helper1 or T-helper 17 cells to produce cytokines like IFNγ, IL-12, IL-6 and TNF-α from (Boirivant and Strober 2007) whereas others may induce T-regulatory cells to produce regulatory cytokines like IL-10 and TGF-β from (Belkaid and Tarbell 2009; Zhang et al. 2010). The intestinal epithelium is also capable of releasing some pro-inflammatory chemokines such as IL-8 (Hausmann et al. 2002). IL-10 can be produced by diverse cells including T Cells, monocytes, macrophages, dendritic cells and endothelial cells (Dimitrov and Gotova 2014) and they play a crucial role in inhibiting and diminishing immune and inflammatory responses (Moore et al. 2001). Dimitrov and Gotova (2014) also found that especially after stimulation with pro-inflammatory cytokines like TNFα, intestinal epithelium produced pro-inflammatory chemokine Interleukin 8 (IL-8). They also noted that some LAB strains with yoghurt origin, could inhibit the secretion of IL-8 from epithelial cells and this could have a beneficial potential against inflammation.

Transforming Growth Factor-B

Transforming Growth Factor β (TGF-β) is a pleiotropic cytokine with effective regulatory and/or inflammatory activity and is majorly produced by many immune and non-immune cells (Li and Flavell 2008; Sanjabi et al. 2009). It is an important anti-inflammatory cytokine that is alleged to play a majorly regulate intestinal barrier function and promote maintenance of peripheral tolerance or tolerance to commensal bacteria (Dignass and Podolsky 1993; Planchon et al. 1994; Sanjabi et al. 2009). It composes of

399 amino acids and it occurs as a homodimer with a total molecular mass of 25kDa; each monomer molecular mass of 12.5kDa. *In vitro*, TGF-β is produced by macrophages and can exert an anti-inflammatory effect during inflammation upon phagocytosis of apoptotic cells, (Fadok et al. 1998; Su et al. 2011). As an anti-inflammatory peptide (Said 1998), TGF-β acts as an immune suppressor. TGF-β has been shown to induce NF-κB inhibitor thereby inhibiting NF-κB activation (Arsura et al. 1996). It performs cellular functions such as promoting differentiation of IL-10 producing cells and T-reg cells (Sun et al. 2007; Zheng et al. 2007) and in combination with IL-4, TGF-β also promotes the differentiation of IL-10 producing cells (Dardalhon et al. 2008).

Interleukin 10

Interleukin 10 is pluripotent, anti-inflammatory cytokine (Asadullah et al. 2003). Its crystal structure occurs as a homodimer with a total molecular mass of 37kDa. IL-10 monomer is composed of 160 amino acids and has a molecular mass of 18.5kDa (Mocellin et al. 2004). IL-10 is known to be produced by many immune cells such as macrophages, monocytes, dendritic cells, T helper-1, T helper-2 and T-reg cells (Kamanaka et al. 2006). They can also be produced by non-immune cells such as epithelial cells and keratinocytes (Moore et al. 2001; Williams et al. 2004). As an immune suppressor, IL-10 is essential for balancing the immune system (Asadullah et al. 2003; Sanjabi et al. 2009). It transcriptionally controls activation of NF-kB transcription factor through a negative feedback inhibition and has been shown to suppress NF-κB nuclear translocation by inhibiting I-kappa B kinase IKK activity and

delaying the degradation of IκBα or inhibit DNA binding of p65/p50 heterodimers (Moore et al. 2001). Therefore, IL-10 may exert its anti-inflammatory activity by inhibiting the NF-κB transcription factor.

Interleukin 8

Interleukin 8 is a pro-inflammatory cytokine. It has a three dimensional structure with a molecular mass of 11kDa and is composed of 77 amino acids. It is a major mediator during sepsis (Asha et al. 2007). IL-8 is transcriptionally controlled by NF-κB transcription factor and it can be rapidly induced by other cytokines such as TNF-α or IL-1β or bacterial products (Hobbie et al 1997; Mastronade et al. 1998; Hoffman et al. 2002; Hsu-Wei et al. 2010). It primarily functions as a neutrophil chemo-attractant which stimulates the migration of neutrophils to injured intestinal sites where they induce inflammatory responses (Claud et al. 2003). According to Delcenserie et al. (2008), following an interaction between enterocytes and probiotics, IL-8 seems to be the major cytokine produced. However, probiotic strains differ in their ability to augment the expression of IL-8. Studies by Zhang et al. (2005) illustrated that the quality and dose of probiotic preparation may impact the production of IL-8 in enterocytes as higher doses of live *Lactobacillus GG* without TNF-α led to increased production of IL-8 in Caco-2 cell model.

Tumor Necrosis Factor α

Tumor Necrosis Factor α (TNFα) is a pro-inflammatory cytokine. Its crystal structure shows that it occurs as a homo-trimer with a total molecular mass of 51kDa and is composed of 233 amino acids. Each monomer has a molecular mass of 17kDa. TNF is widely expressed in granulocytes, fibroblasts, macrophages and epithelial cells (Spriggs et al. 1992; Parameswaran and Patial 2010). TNF-α is majorly involved in the activation of transcription factors, multiple signal transduction pathways, and regulation of many gene transcription via specific cell surface receptors (Shehnaaz et al. 1995). TNF-α primarily mediates inflammatory responses in various immune system functions including antimicrobial activity, antitumor activity and mediation of inflammation (Li et al. 2012). As inflammatory mediators, they activate the NF-κB transcription factor by promoting the phosphorylation and ubiquitination of inhibitor of kappa-B. TNF-α cytokines are also major mediators during the induction and progression of sepsis (Asha et al. 2007; Schulte et al. 2013) and they can lead to severe levels of inflammation in the case of immune dysbiosis (Round and Mazmanian 2009).

NF-κB signalling pathway

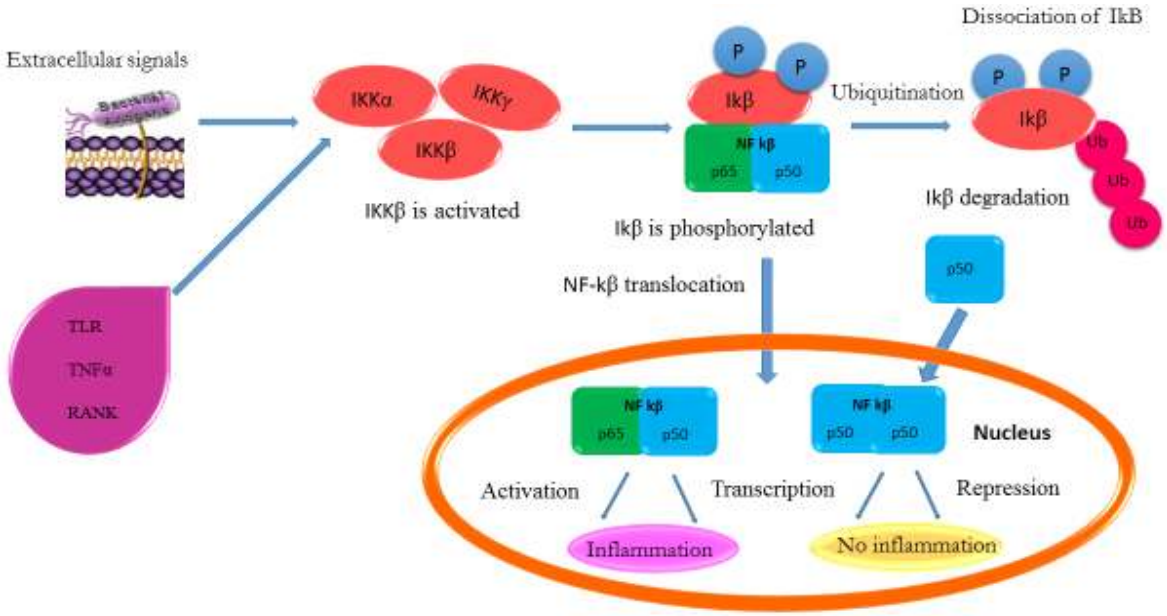
NF-κB is a transcription factor that plays an important role in immune regulation and inflammatory reactions (Lee et al. 2008). Nuclear factor-kappa B (NF-κB) signaling pathway is the key signaling channel (Fig 3). Intensive communication between the host intestinal epithelial cells; the initial point of contact and the commensal bacteria or probiotics greatly influences inflammatory signaling pathways in intestinal epithelial cells

(Kemgang et al. 2014). NF-κB is a heterodimeric transcription factor comprising of various subunits namely “p50/p105, p65/RelA, p52/p100, c-Rel and Rel-B” (Scaife et al. 2002). NF-κB is essential for transcriptional activation and regulation of inflammatory effectors such as IL-8, TNF-α, IL-6, Cox2 and iNOS (LeBlanc et al. 2011). In the gastrointestinal tract, inflammatory reactions mainly involves the IκB/NF-κB complex (Lopez et al. 2008).

Under non-stimulatory conditions or in the absence of stimuli, NF-κB exists in its inactive form in the cytosol where it is bound to its inhibitor molecule, the inhibitor of NF-kB (IκB), which inhibits NF-κB translocation into the nucleus thus preventing transcriptional activities (Beg et al. 1992). Under the influence of stimuli, NF-κB is activated when IκB is phosphorylated by IκB kinase (IKK) targeting it for ubiquitination and subsequent proteasomal degradation (Hay et al. 1999). Once dissociated from IκB, the NF-κB is able to migrate into the nucleus where it binds target promoters and activates transcription of effector genes like pro-inflammatory genes (Thomas and Versalovic 2010). Some probiotic strains are able to prevent the proteasomal degradation of IκB thereby inhibiting the relative expression of pro-inflammatory cytokines like IL-8 or TNF-α by intestinal epithelial cells.

It has been reported that various LAB strains such as *L. acidophilus, L. johnsonii* and *L. delbrueckii* can suppress inflammatory signals by inhibiting NF-κB activation in a strain dependent manner (Rocha et al. 2015). NF-κB has been noted to control the expression of nearly all pro-inflammatory cytokines such as IL-1 α, TNF-α, IL-6 and IL-12. They also transcriptionally control the expression of chemokines such as IL-8, cell surface adhesion molecules and immune receptors including inducible nitric oxide

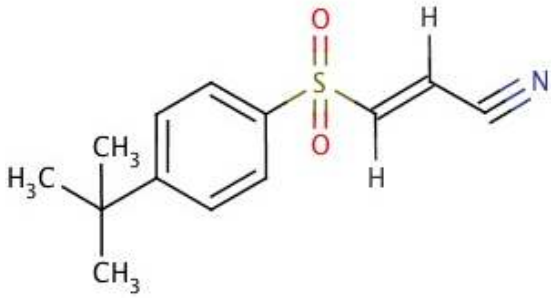
synthase, intracellular adhesion molecule 1, T-cell receptor and vascular cell adhesion molecule 1 (VCAM-1) (Berkes et al. 2003).



Adapted from (Schotellius et al. 1999)

Figure 3. NF-κB signalling pathway

NF-kB function can either be inhibited or altered, directly or indirectly by NF-κB inhibitors. Direct inhibition of NF-κB involves binding of the inhibitor to the NF-κB molecule whereas indirect inhibition means the IκB does not bind to NF-κB instead may reduce the expression, activation or transport of NF-κB to the nucleus by inhibiting IκB phosphorylation or translation of NF-κB (Kuwaka, 2011). Example of commercially available indirect NF-κB inhibitor is (2E)-3-[4-(tertiary butyl phenyl) sulfonyl]-2-propenenitrile also referred to as BAY-11-7085. IκB BAY 11-7085 has been shown to inhibit IκB phosphorylation and TNF-α induced NF-κB activation (Pierce et al. 1997).



Source: Santacruz Biotechnology

Figure 4. Structure of BAY-11-7085

Role of Pro- and Anti-inflammatory Cytokines in Gastrointestinal Disease

Asha et al. (2007), found that pro-inflammatory cytokines play a relevant role in the initiation and advancement of sepsis. Among those, TNF-α, IL-1β and IL-6 were important mediators during sepsis. The pro-inflammatory cytokines such as TNF-α and IL-1β appeared to trigger several events that led to “inflammation, tissue destruction and loss of function of the normal cellular activities” (Round and Mazmanian 2009). Duary et al. (2004), stated that by enhancing the immune system of gut, probiotics benefit in Inflammatory Bowel Disease (IBD) including Crohn's disease and Ulcerative colitis which are chronic inflammatory diseases of the intestines, characterized by deep inflammation with granulomas (Purchiaroni et al. 2013).

Crohn’s disease predominantly causes ulcerations which is the breaking of small and large intestinal lining while ulcerative colitis is a chronic disease of the colon (Duary et al. 2004). The immune system is tolerant to commensal microorganisms (Duchmann et al. 1995) and the breakdown of indigenous bacteria and epithelial barrier could lead to inflammation and result in IBD (Cheng and Huang 2013). There is therefore the need

to maintain a balance between tolerance towards commensals and reactivity towards pathogens as deviation from such a balance could lead to upregulation of inflammatory responses resulting in IBD (Medzhitov 2007). Activation of pro-inflammatory NF-κB signaling pathway has been thought to be a molecular event involved in IBD pathogenesis (Jobin and Sartor 2000; Schmid and Alder 2000). Increased expression of TNF-α resulting in increased NF-κB activation has been reported in IBD - Ulceritis colitis and Crohn's Disease (Kaser et al. 2010; Petersen and Round 2014).

Treatment of Crohn's disease includes drugs for suppressing inflammation or the immune system. The crucial role of IL-10 in the prevention of IBD has been demonstrated in IL-10 deficient mice where IL-10 downregulates the inflammatory cascades by suppressing the secretion of pro-inflammatory cytokines (Sanjabi et al. 2009). By strengthening the epithelial barrier and reducing the mucosal levels of pro-inflammatory cytokines, some probiotics may be effective in the treatment of IBD (Madsen et al. 2001). Also that probiotic bacterial colonization in the gut may lead to reduced contact of immune cells to the bacterial antigens which are believed to drive IBD (Purchiaroni et al. 2013). Also that by altering the microbial environment, some probiotic components could downregulate inflammation when supplemented to patients with gastrointestinal diseases (Isolauri et al. 2002). However, it is important to note that not all probiotics act in the same manner with regards to their anti-inflammatory effects for example the stimulation of IL-10 producing cells is a strain dependent trait and their effectiveness depends on the method of administration and the concentrations used (LeBlanc 2011).

Why Lactobacillus rhamnosus isolate?

Lactobacillus rhamnosus MRS6AN is a facultative anaerobic lactic acid gram positive bacteria. It was isolated from a local Kenyan cultured milk *"amabere amaruranu"* in Dr. Onyango's lab, Department of Health Sciences, ETSU, using 16sRNA and biochemical tests among other probiotic potential strains: *L. paracasei, L. casei, L. acidophilus, L. reuteri, and Acetobacter* (Boyiri and Onyango 2015).

In Kenya, pastoral communities mainly produce traditional cultured milk products and some of these fermented milk have been reported to confer health beneficial effects (Muigei et al. 2013) examples include *"kule nato"* produced by the Maasai community (Mathara et al. 2008), *"mursik"* by the Kalenjin community (Mathara et al. 1995) and *"amabere amarunanu"* by the Gusii community (Mokua 2004; Boyiri and Onyango 2015). However, there is no substantial information available concerning the effect of *L. rhamnosus* isolated from *"amabere amarunanu"* on the production of inflammatory cytokines in the gastrointestinal tract. In addition, the use of *Lactobacillus* as a probiotic for humans requires a fine-tuned selection of appropriate strains and a broad understanding of its action on immune system components (Kemgang et al. 2014).

Therefore, this study used *Lactobacillus rhamnosus* because previous research work in the lab found *Lactobacillus rhamnosus* to have a higher probiotic potential than the other isolates from *"amabere amarunanu"*. *Lactobacillus rhamnosus* did not degrade mucin, showed stability in acid/bile conditions, had the highest antimicrobial activity against bacterial test strains and was sensitive to antibiotics (Boyiri and Onyango 2015).

Hypothesis

Lactobacillus rhamnosus isolated *"amabere amarunanu"*, a Kenyan traditional cultured milk preparation will have effects on the production of pro-inflammatory and anti-inflammatory cytokines in enterocytes.

Objectives

To determine the production of pro- and anti-inflammatory cytokine by Caco-2 cells when co-cultured with *Lactobacillus rhamnosus* live culture.

To determine the production of pro- and anti-inflammatory cytokine by Caco-2 cells when treated with *Lactobacillus rhamnosus* cell extracts and its filtered spent broth.

To evaluate whether the production of these cytokines in enterocytes is dependent on NF-κB signaling pathway.

To determine the consequential effect of NF-κB inhibitor in the production of pro- and anti-inflammatory cytokines in enterocytes.

CHAPTER 2

MATERIALS AND METHODS

Experimental Materials

Probiotic Lactobacilli Live Cells

The potential probiotic microorganisms (LAB) isolated from a Kenyan traditional cultured milk *"amabere amaruranu"* in Dr. Onyango's lab included *L. rhamnosus, L. paracasei, L. casei, L. acidophilus, L. reuteri, and Acetobacter.* This study focused on *Lactobacillus rhamnosus* which had been isolated using 16sRNA and identified using biochemical tests. They were found to have a higher probiotic potential compared to the other isolates as they did not degrade mucin, showed stability in acid or bile conditions, had the highest antimicrobial activity against bacterial test strains and was sensitive to antibiotics (Boyiri and Onyango 2015).

Culture Media for Lactobacillus rhamnosus

The media used in for the growth of *Lactobacillus rhamnosus* was Thermo scientific Oxoid deMann Rogosa, Sharpe (MRS) broth (Cat No. CMO359).

Human Enterocyte like Caco-2 Cells

Caco-2 cells are cells that are derived from human colonic adenocarcinoma cells. After confluence they differentiate into small intestinal-like enterocytes. Their culture requires 21 day period to attain a differentiated monolayer (Olejnik 2003). Caco-2 cells

were cultured in Dulbeco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (Hyclone, Cat No. SH30088.03, GE Lifecare Healthsciences, Logan, Utah, 84321) and 5% penicillin-streptomycin (Corning Cellgro, Mediatech, Inc, Mannasas, VA 20109) at 37°c, 5% CO_2 until they reached 70% confluence. Cells were passaged 2 times and stored in liquid nitrogen in aliquots containing complete DMEM with Dimethyl Sulfoxide (DMSO) (Corning cellgro, Mediatech, Inc, Mannasas, VA 20109).

Human Enterocyte like Caco-2 Cells Culture Media

The media that was used for culturing Caco-2 cells was Dulbeco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (Hyclone, Cat No. SH30088.03, GE Lifecare Healthsciences, Logan, Utah, 84321) and 5% penicillin-streptomycin (Corning cellgro, Mediatech, Inc, Mannasas, VA 20109).

Lactobacillus rhamnosus Cell Extract

Lactobacillus rhamnosus seed culture was grown by inoculating one storage bead at -80^{o}c into 5ml MRS broth (Thermoscientific) in a test tube. It was then incubated at 37 ^{o}c under aerobic condition for 12 hours. Ten drops of seed culture were inoculated into 100ml of MRS broth in a 250ml flask and incubated at 37^{o}c on shaker for 24hrs under aerobic conditions. The OD_{550nm} of the culture was monitored until it reached OD_{550nm} reading of 2.08nm. Culture (50ml) was dispensed into 50ml centrifuge tubes and spun at 1100g for 15mins. Supernatant was decanted into clean 50ml screw

cork flasks and filtered using a 500ml pre-sterilized steritop and stericup vacuum filtration and storage system 0.22nm millipore millex filter (Fisher Scientific Co., Cat No. SCGPU05RE) to produce FSB (filtered spent broth). The cell pellets were washed and re-suspended in 30ml PBS pH 7.4 (Gibco, Invitrogen). The cell suspension was sonicated at amplitude 44% for 2 mins with 6min rest, repeated sonication 5 times. Sonicated suspension was spun at 1100g for 15mins and supernatant was decanted into clean flask. Supernatant was filtered using 0.22nm millipore millex filter to produce cytoplasmic fraction (CF). Samples were then stored in -20^{o}c freezer until they were ready to be used as treatments on Caco-2 cells (Boyiri and Onyango 2015).

Transwells Permeable Support

Caco-2 cell culture was seeded in Transwell permeable supports, 12mm diameter, 12-well inserts 0.4 μm nucleopore size polycarbonate membrane (Costar, Corning, Cat No. 29442-086, ME 04043). Transwell inserts have three openings for standard pipette tips to allow samples to be added or removed from the lower compartment. Dulbeco's Modified Eagle's Media (DMEM) containing 10% fetal bovine serum (Hyclone, Cat No. SH30088.03, GE Lifecare Healthsciences, Logan, UT 84321) and 5% penicillin-streptomycin (Corning cellgro, Mediatech, Inc, Mannasas, VA 20109) was added into the 12 multiple well plate (Costar, Corning, ME 04043). The transwell insert was then placed into the well. Then, the media was added into the inside compartment of the transwell insert. To improve cell attachment DMEM media was first added to the multiple well plate and then to the transwell insert. The plate was then

incubated for one hour at 37°c, 5% CO_2. Caco-2 cells at a density of 2.5×10^5 cells/ml on 12 mm in cell culture were added on inserts (0.4 µm nucleopore size; Corning, USA) and cultured 18–22 days at 37°c, 5% CO_2 in DMEM containing 10% fetal bovine serum and 5% penicillin-streptomycin. The media levels were checked periodically and changed every second day until the cells were fully differentiated at day 21.

Enzyme Linked Immuno-Sorbent Assay (ELISA) Kits

ELISA kits with pre-coated plates (Biolegend) were used to quantify cytokine produced in the cell culture lysates and the cytokines secreted into the cell culture supernatants. The kits were stored at 4^oc. Human IL-10 (Cat No. 430607) was composed of anti-human IL-10 pre-coated 96 well strip microplate, human IL-10 detection antibody, standard, avidin-HRP A, assay buffer A, wash buffer (20X), substrate solution F, stop solution and plate sealers. The human latent TGF-β (Cat No. 432907) was composed of anti-human latent TGF-β pre-coated 96 well strip microplate, human latent TGF-β detection antibody, standard, avidin-HRP D, assay buffer E, wash buffer (20X), substrate solution D, stop solution and plate sealers. Human IL-8 (CXCL8) (Cat No. 431507) was composed of anti-human IL-8 pre-coated 96 well strip microplate, human IL-8 detection antibody, standard, avidin-HRP A, assay buffer A, wash buffer (20X), substrate solution F, stop solution and plate sealers. Human TNF-α (Cat No. 430207) anti-human was composed of TNF-α pre-coated 96 well strip microplate, human TNF-α detection antibody, standard, avidin-HRP B, assay buffer A, wash buffer (20X), substrate solution D, stop solution and plate sealers.

Nuclear Factor-kappa B Antibody

The NF-κB antibody that was used in western blotting assay to detect the presence of NF-κB transcription factor in treated/ untreated Caco-2 cells was rabbit anti-human NF-κB p65 (C-20) Santacruz, Cat No. sc-372). The molecular weight of NF-κB p65 is 65kDa.

NF-κB Inhibitor

The NF-κB inhibitor that was used in this study was (E)-3-(4-t-Butylphenysufonyl) -2- propenenitrile (BAY 11-7085 (CAS 196309-76-9), Santacruz Biotechnology, Cat No. sc-202490). It has a molecular weight of 243.33. It inhibits the activation of NF-κB and the phosphorylation of IκB. It was solubilized in DMSO (25mg/ml) and stored at -20^{o}c.

Experimental Methods

Growth of Lactobacillus rhamnosus MRS6AN

Probiotic *Lactobacillus* are nutritionally fastidious and they grow in rich media containing carbohydrates, amino acids, peptides, fatty acid esters, salts, nucleic acid derivatives and vitamins (Kandler and Weiss 1986). *Lactobacillus rhamnosus* were grown at 37^{o}c overnight in de Mann Rogosa, Sharpe broth in anaerobic conditions to reach early stationery phase. *Lactobacillus rhamnosus* was also grown on MRS agar plate to confirm that it was a pure culture. Bacteria was harvested by centrifugation at 3000g for 5mins at 6^{o}c. They were then washed twice with sterile PBS (pH 7.2). Cells

were then re-suspended in DMEM media to reach a final concentration of 10^9 cfu/ml of media. Live *Lactobacillus rhamnosus* cells were ready to be transferred to Caco-2 cell culture.

Caco-2 Cell Culture in DMEM Media

Caco-2 cells were seeded in a biological safety II cabinet in sterile T75 flask and incubated at 37°C, 5% CO_2. The cell culture media was changed every second day until the cells reached 70% confluence.

Splitting Caco-2 Cells

The 70% confluent Caco-2 cells were then trypsinized with 3ml of Trypsin-EDTA (Gibco, Grand Island, NY) in 37°C for about 7mins until they were fully detached from the bottom of the flask. Fresh complete DMEM (5ml) was added to deactivate the trypsin-EDTA. The cells were then spun at 200g, 5mins in 50ml centrifuge tubes. The trypsin and media were aspirated from the centrifuged tubes and the cells were re-suspended in complete DMEM and were further split and cultured into 6 well plate and 12 transwells. The media was changed every second day until the cells were fully differentiated at 21day post-confluence.

Use of Transwells Permeable Support in Caco-2 Cell Culture

Caco-2 cells were seeded at density of 1x100 per well containing 2ml complete DMEM. Caco-2 cell culture requires 21 day period to attain a differentiated monolayer (Olejnik 2003). Therefore, the cells were washed and media changed in both the apical and basolateral side until they were fully differentiated - day 21 (Hallera et.al 2000).

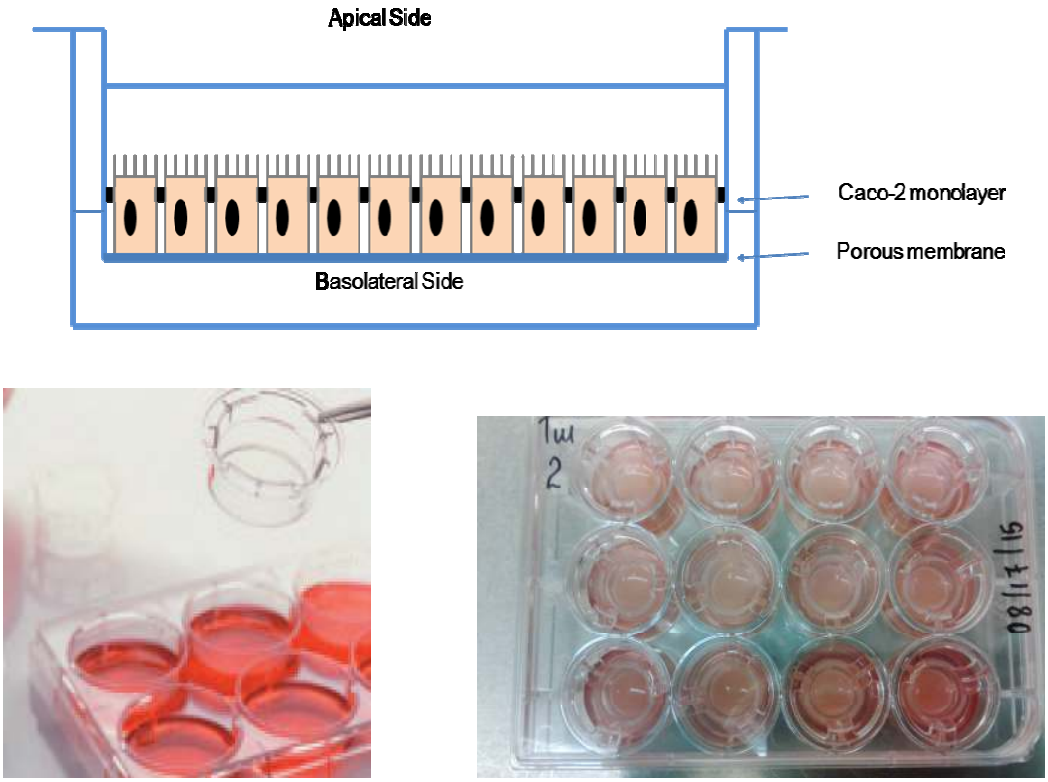


Figure 5. Transwell support

Storage of Caco-2 cells in Liquid Nitrogen

The Caco-2 cells grown in T75 flasks were trypsinized using 3ml trypsin in 37^{o}c for 7mins. Then DMEM was added to the trypsinized cells and spun at 200g for 5 mins in 50ml centrifuge tubes. The trypsin and media were aspirated. The cell pellets were

then rinsed twice with PBS pH7.4 and re-suspended in 5ml DMEM storage media containing 40% fetal bovine serum and 10% Dimethyl sulfonate (DMSO). Cells were counted using a hematocytometer and aliquoted into 1ml tubes for storage in liquid nitrogen.

Co-culture of Caco-2 Cells with Lactobacillus rhamnosus

Lactobacillus rhamnosus were grown at 37ºC in MRS broth in anaerobic conditions to reach the early stationary phase. They were harvested by centrifugation at 3000g for 5mins at 6ºC. After two washes with sterile PBS pH 7.2 were resuspended in DMEM to reach a final concentration of 1×10^9 cfu/ml of medium. Then the bacteria were added to the cell culture wells at appropriate dilutions. Caco-2 cells were grown in DMEM containing 10% fetal calf serum. When grown to confluence in single layer, cells were washed three times in PBS pH 7.2 to remove culture medium and non-adherent cells (Boyiri and Onyango 2015). The bacteria in culture medium were transferred into these wells.

Caco-2 monolayers were apically challenged by addition of 1×10^9 cfu/ml of *Lactobacillus rhamnosus* MRS6AN alone or by stimulation with TNF-α (10 ng/ml, invitrogen). PBS was used as a negative control. After stimulation for 18hrs (37°c, 5% CO_2), cell culture supernatants were separately collected from the apical and basolateral compartments, spun at 13,300g, 10mins and stored at −20°C until further analysis. Adherent Caco-2 cells were washed once with ice-cold PBS (1× pH 7.2) and lysed in denaturation solution (RIPA buffer + protease inhibitor). They were scrapped off

from the wells using cell scrapper and transferred to microfuge tubes. The microfuge tubes were then incubated in ice with agitation for 30mins at 4°c. The lysed cells were then spun at 13300g, 20mins, 4°c and the supernatants were collected in new microfuge tubes. The cellular lysates were then stored at −20°c for further analysis

Preparation of Lactobacillus rhamnosus Bacterial Extracts

Lactobacillus rhamnosus seed was cultured by inoculating one storage bead at -80°c into 5ml MRS broth (Thermoscientific) in a test tube. They were then incubated at 37 °c under aerobic conditions for 12 hours. Ten drops of seed culture were inoculated into 100ml of MRS broth in a 250ml flask and incubated at 37 °c on a shaker for 24hrs under aerobic conditions. The growth was monitored at optical density (OD_{550nm}) until they reached OD_{550nm} reading of 2.08nm. Culture (50ml) was dispensed into 50ml centrifuge tubes and spun at 1100g for 15mins. The supernatant was decanted into clean 50ml screw cork flasks and filtered using a 500ml pre-sterilized steritop and stericup vacuum filtration and storage system 0.22nm millipore millex filter (Fisher Scientific Co., Cat No. SCGPU05RE) to produce FSB (filtered spent broth). Cell pellets were washed and re-suspended in 30ml PBS pH 7.4 (Gibco, invitrogen). The cell suspension was then sonicated at amplitude 44% for 2 minutes with 6 minutes rest, the sonication repeated x5 times. Sonicated suspension was spun at 1100g for 15mins and supernatant was decanted into clean flask. The supernatant was then filtered with 0.22nm millipore millex filter to produce cytoplasmic fraction (CF). Samples were stored in -20 °c freezer until they were ready to be used as treatments on Caco-2 cells.

Treatment of Caco-2 Cells with Lactobacillus rhamnosus Bacterial Extracts

Caco-2 monolayers were apically challenged by addition of 100ul of *Lactobacillus rhamnosus* MRS6AN cell extracts: cytoplasmic fraction or 100ul of filtered spent broth. PBS was used as a negative control. After stimulation for 6-36 hrs (37°c, 5% CO_2), cell culture supernatants were separately collected from the apical and basolateral compartments, spun at 13300g, 10mins and stored at −20°C until further analysis. Adherent Caco-2 cells were washed once with ice-cold PBS (1× pH 7.2) and lysed in denaturation solution (RIPA buffer + protease inhibitor). They were scrapped off from the wells using cell scrapper and transferred to microfuge tubes. The microfuge tubes were then incubated in ice with agitation for 30mins at 4^{o}c. The lysed cells were then spun at 13300g, 20mins, 4°c and the supernatants were collected in new microfuge tubes. The cellular lysates were then stored at −20°c for further analysis.

ELISA: Enzyme Linked Immuno-sorbent Assay

Sandwich Enzyme Linked Immunosorbent Assays were used to measure the production of cytokine in the cell lysate and cytokine secretion in the apical and basolateral supernatants. All the reagents were brought to room temperature prior to use. All the standards and samples were ran in duplicate. According to the protocol described in the ELISA Kits: Human IL-10 (Biolegend, Cat No. 430607), Human TNF-α (Biolegend, Cat No. 430207), Human Latent TGF-β (Biolegend, Cat No. 432907) and Human IL-8 (Biolegend, 431507). Each of the lyophilized human cytokine (TGF-β, IL-10, IL-8 and TNF-α) standards were reconstituted by adding assay buffer to make the

required concentration standard stock solution. The reconstituted standard was allowed to sit at room temperature for 15-20mins and then vortexed to mix completely. The top standard was prepared by diluting stock standard stock solution assay buffer in a polypropylene microfuge tube. Then six two- fold serial dilutions were performed according to the kits instructions for each cytokine by mixing and transferring 250ul to the next microfuge tube.

The final human latent TGF-β standard concentrations in the tubes were 100ng/ml, 50ng/ml, 25ng/ml, 12.5ng/ml, 6.25ng/ml, 3.13ng/ml and 1.56ng/ml respectively. For IL-10 were 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml, 15.6pg/ml, 7.8pg/ml and 3.9pg/ml respectively. For IL-8 and TNF-α were 1000pg/ml, 500pg/ml, 250pg/ml, 125pg/ml, 62.5pg/ml, 31.3pg/ml and 15.6pg/ml respectively. Assay buffer served as the zero standard concentration. Wash buffer (20X) was diluted to 1X with deionized water. The plates were washed four times with 300ul of 1X wash buffer per well and any residual buffer was blotted by firmly tapping the plate upside down on absorbent paper. Assay buffer (50ul) was added to each well containing either the standards or the samples. Standards (50ul) and samples (50ul) were added to their appropriate wells, the plates were sealed and incubated for 2 hours at room temperature while shaking at 200rpm. The contents of the plates were discarded into the sink then the plate was washed 4 times with 1X wash buffer. The specified volume of detection antibody solution was added to each well, the plate was sealed and incubated at room temperature for 1 hour while shaking. The contents of the plates were discarded into the sink and the plate was washed 4 times with 1X wash buffer. The specified volume of Avidin-HRP solution was then added into each well, the plates

were sealed and incubated at room temperature for 30minutes while shaking. The contents of the plate were then discarded into the sink and the plate was washed 5 times with 1X wash buffer. For the fifth wash the wells were soaked in 1X wash buffer for 30 seconds to 1 minute to help minimize the background. The prescribed volume substrate solution was added to each well and incubated at room temperature for the time specified for each cytokine in the dark. Wells containing cytokines turned blue in color and their intensity was proportional to their concentration. The reaction was then stopped by adding 100ul of stop solution to each well. The absorbance was read at 450nm. A standard curve was generated and the concentration of TGF-β, IL-10, IL-8 and TNF-α per sample were determined.

Western Blot Assay for NF-kB Transcription Factor

Loading buffer was prepared by the addition of 50µl of beta mercaptoethanol to 950µl Laemli buffer. Cell lysate samples were prepared by centrifugation at 3000g, 4^{o}C for 5mins. The samples were loaded into 4-20% Mini-protean TGCTTM gel (Biorad, Cat No. 456-1096) and were separated by SDS-polyacrylamide gel electrophoresis in 1x Tris-Glycine SDS page running buffer (30.0g tris base, 144g glycine, 10g sodium dodecyl sulfate in 1 litre of dH_2O). After 1 hr of electrophoresis the gel was transferred to immunobiolin transfer membrane, pore size 0.45um (Millipore Corporation, Billerica, MA 01821, Cat No. IPVH00010) in 1x transfer buffer (30.0g tris base, 144g glycine, 200ml methanol in 700ml of dH_2O) for 1 hour 30 minutes. The membranes were blocked in TBS-T (88g NaCl, 2g KCl, 30g Tris base, 5ml tween-20 in I litre of dH_2O, pH

adjusted to 7.4 using HCl) containing 5% non-fat milk. The blots were incubated overnight at 4^{o}c on a shaker with primary antibody against NF-κB transcription factor p65 (rabbit anti-NF-kB p65 (C-20), Cat No. sc-372 Santacruz Biotechnology; www.scbt.com). The membranes was washed three times in 1x TBS-T (10mM Tris –Cl, pH 7.4, 150mM NaCl, 0.05% Tween-20) for 5 minutes each.

The membranes were then immunoblotted with corresponding secondary antibodies (Peroxidase-conjugated affinipure donkey anti-rabbit IgG (H+L), Jackson Immuno Research; www.jacksonimmno.com) on a shaker for 1 hour at room temperature. The membranes were subsequently washed three times in 1x TBS-T for 5 minutes per wash. Supersignal West Femto reagents (Thermofischer scientific, Cat No. 34095) was prepared by mixing in a ration 1:1 of the reagents in the kit. The mixture was then added to the membrane and allowed to sit for 5-10 minutes in the dark and chemiluminescence was developed by exposing the membrane to ultraviolet using Proteinsimple Flour Chem M. The images were quantified and analysed using Alpha-view software and the data was presented as density of the bands relative to the control bands.

Cell Adhesion Assay

Cells were dispersed and plated at 40,000cells per well in 96-well dishes. The cells were then treated with live *Lactobacillus rhamnosus*, its cytoplasmic fraction, filtered spent broth, heat inactivated broth with or without NF-κB inhibitor BAY 11-7085 for 8-36 hours. Untreated cells and cells only treated with IκB were used as negative

and positive control respectively. The cells were washed with 100ul/well of ice-cold PBS. The cells were fixed for 10 mins in 100% ice-cold methanol (100ul/well) then allowed to air-dry. The cells were stained with 100ul/well of 0.1% crystal violet in H_2O for 10 mins, then washed gently four times with ddH_2O and four times with PBS. The plates were then air-dried completely. The stained cells were solubilized in 1% sodium deoxycholate and the plates read at 590nm in a spectrophotometer. The absorption at 590nm is proportional to the number of cells attached (Scaife et al. 2002).

Treatment of Caco-2 Monolayers with NF-kB Inhibitor (BAY 11-7085)

Bay 11-7085 (10uM) (E)3-[(4-t-Butylphenyl)sulfonyl]-2-propenenitrile (Santacruz Biotechnology, Cat No. sc-202490) solubilized in dimethyl sulfoxide (DMSO) was used as a treatment on Caco-2 cell confluent monolayers for one hour before challenge with overnight live *Lactobacillus rhamnosus* or its cytoplasmic fraction or filtered spent broth. Caco-2 cells, bacterial treatments and NF-κB inhibitor BAY 11-7085 were then co-incubated for 8-36hours at 37^{o}c, 5% CO_2 incubator. After 30 hours of co-incubation, the plates containing treated Caco-2 cells were placed on ice and were washed with ice cold PBS, the cells monolayers were scrapped off, harvested and lysed using RIPA buffer with Protease inhibitor. The cell lysate was spun at 3,000g for 15mins at 4^{o}c. The samples were stored at -20^{o}c for further analysis.

Treatment of Caco-2 Monolayers with Heat Inactivated Broth

Filtered spent media from overnight *Lactobacillus rhamnosus* milk isolate was boiled at 90^{o}c for 15mins to denature any secreted proteins that may have been present in the spent media to produce heat inactivated broth (HIB). The heat inactivated broth (100ul) per well was then used as a Caco-2 cell treatment with the NF-kB inhibitor BAY 11-7085.

Statistical Analysis

Numerical data was analysed as completely randomized design using General Linear Models GLM in SAS 9.4 software. Means were separated using Least Square Means (LSM) and Tukey-Kramer methods. The differences in values were considered statistically significant at $P < 0.05$.

CHAPTER 3

RESULTS

Cytokine Profiles using ELISA

Caco-2 cells were treated with live *Lactobacillus rhamnosus* (MRS6AN) milk isolate, its cytoplasmic fraction (CF), filtered spent broth (FSB). Cell supernatants and lysates were analyzed using ELISA. The levels of TGF-β were determined in (ng/ml) and for IL-10, IL-8 and TNF-α in (pg/ml). Caco-2 cell lysate levels of TGF-β and IL-8 were significantly increased in most of the probiotic cell treatments. The various probiotic treatments did not stimulate production of significant levels of IL-10 and TNF-α in Caco-2 lysate. Caco-2 cell lysate levels of IL-10 when Caco-2 cells were treated with (PBS Control, CF, FSB or MRS6AN) were 7.1, 33.6, 31 and 44 pg/ml, respectively; and of TNF-α were 4.1, 33.1, 30.1 and 27.6pg/ml, respectively.

Generally, there were low levels of TGF-β, IL-8, TNF-α and IL-10 cytokines secreted into the cell supernatants. Levels of cytokine secretion in the apical supernatant cell treatments (CF or FSB) for TGF-β were 3.6 and 1.8 ng/ml, respectively. Live *Lactobacillus rhamnosus* did not stimulate the secretion of TGF-β into the supernatants. Levels of cytokine secretion in the apical supernatant cell treatments (CF, FSB, MRS6AN or MRS6AN + TNFα stimulation) for IL-8 were 96.4, 45.6, 39.0 and 39.6 pg/ml, respectively.

Anti-inflammatory TGF-β Cytokine

The levels of TGF-β produced when Caco-2 cells were variously treated with PBS Control, live *Lactobacillus rhamnosus* (MRS6AN), its cytoplasmic fraction (CF) and filtered spent broth (FSB) were 15.7, 162.97, 146.51 and 106.88 ng/ml, respectively (Figure 6). All probiotic treatments significantly increased the production of TGF-β in Caco-2 cells: MRS6AN (p<0.0001), CF (p<0.0001) and FSB (p<0.0004) when compared to the control. However, there was no significant mean difference in the production of TGF-β between the three bacterial treatments.

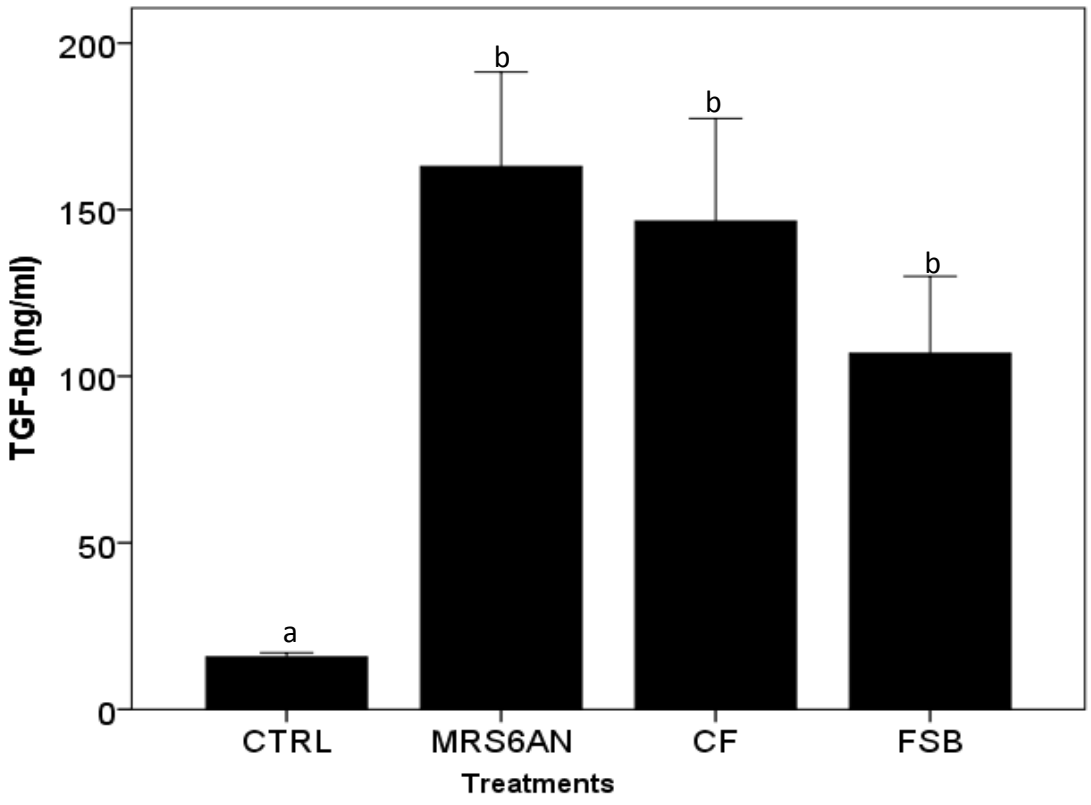


Figure 6. Cell lysate levels of TGF-β (ng/ml) produced by Caco-2 cells when treated with live *Lactobacillus rhamnosus* (MRS6AN), its cytoplasmic fraction (CF) or filtered

spent broth (FSB). Each bar represents mean ± SD of TGF-β levels in cell lysate. Means without a common letter differ, (P<0.05).

Pro-inflammatory IL-8 Cytokine

Caco-2 cells were treated with PBS Control, live *Lactobacillus rhamnosus* (MRS6AN), its cytoplasmic fraction (CF), filtered spent broth (FSB) or live *Lactobacillus rhamnosus* with TNF-α (10ng/ml) stimulation to induce the production of IL-8. The levels of pro-inflammatory IL-8 cytokine when Caco-2 cell were treated with PBS control, MRS6AN, CF, FSB or MRS6AN with TNF-α were 415.45, 887.09, 1453.33, 1849.36 and 2041.21 pg/ml, respectively (Figure 7).

CF, FSB, MRS6AN+TNF-α significantly increased the production of IL-8 in Caco-2 cells: CF (p<0.0002), FSB (p<0.0001) and MRS6AN+TNF-α (p< 0.0001) when compared to the control. Live *Lactobacillus rhamnosus* with no TNF-α stimulation did not induce significant production of IL-8. A significant mean difference was observed between the live probiotic treatment and the other treatments (CF, FSB, MRS6AN+TNFα).

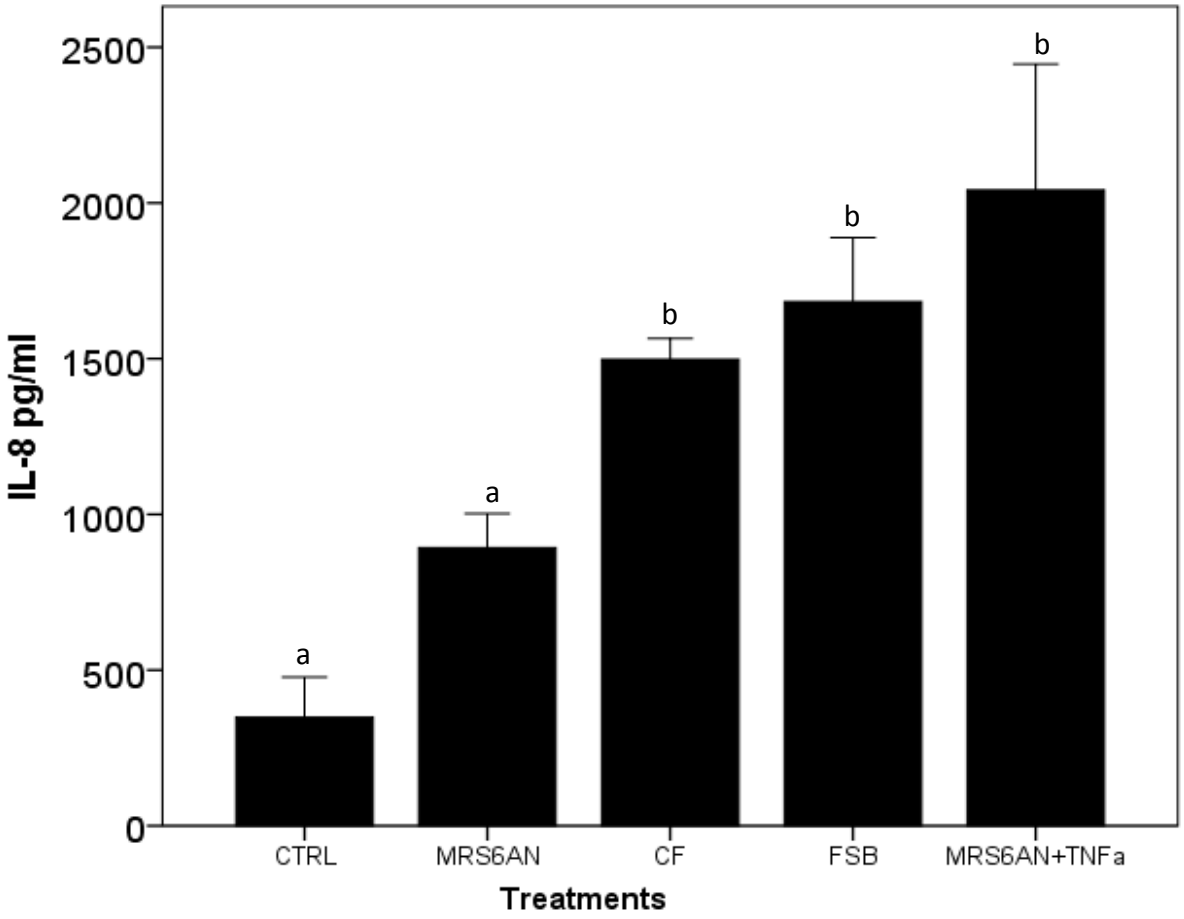


Figure 7. Cell lysate levels of IL-8 (pg/ml) produced by Caco-2 cells when treated with live *Lactobacillus rhamnosus* (MRS6AN), its cytoplasmic fraction (CF) or filtered spent broth (FSB). Each bar represents mean ± SD of IL-8 levels in cell lysate. Means without a common letter differ, (P<0.05).

Expression of NF-κB Transcription Factor

Protein expression of NF-κB was determined in Caco-2 cells lysates treated with live *Lactobacillus rhamnosus*, its cytoplasmic fractions or overnight filtered spent broth. The expression of transcription factor NF-κB p65 was then determined using western

blot assays (Figure 8a). This was done to ascertain whether the production of these cytokines in the various probiotic treatments was dependent on NF-κB signaling pathway. The expression of NF-κB in all the probiotic treatments was quantified and presented as fold change of band intensity relative to the control (Figure 8b) and Table 2. NF-κB was significantly expressed in Caco-2 cell lysate treatments with CF, FSB and MRS6AN+ TNFα. There was significant mean difference in NF-kB expression between live MRS6AN and CF, FSB and MRS6AN with TNF-α treatments.

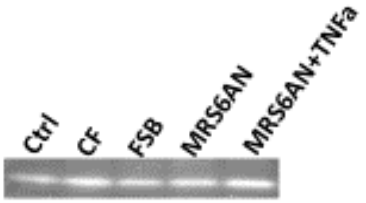


Figure 8a. Western blot assay image

Table 2. Effect of cytoplasmic fraction (CF), filtered spent broth (FSB), live *Lactobacillus rhamnosus*, live *Lactobacillus rhamnosus* with TNF-α stimulation on the protein expression of NF-κB p65 in Caco-2 cells.

Treatments	Relative expression[a]
Control	0
MRS6AN	19.0
CF	45.3
FSB	49.6
MRS6AN + TNFα	55.6

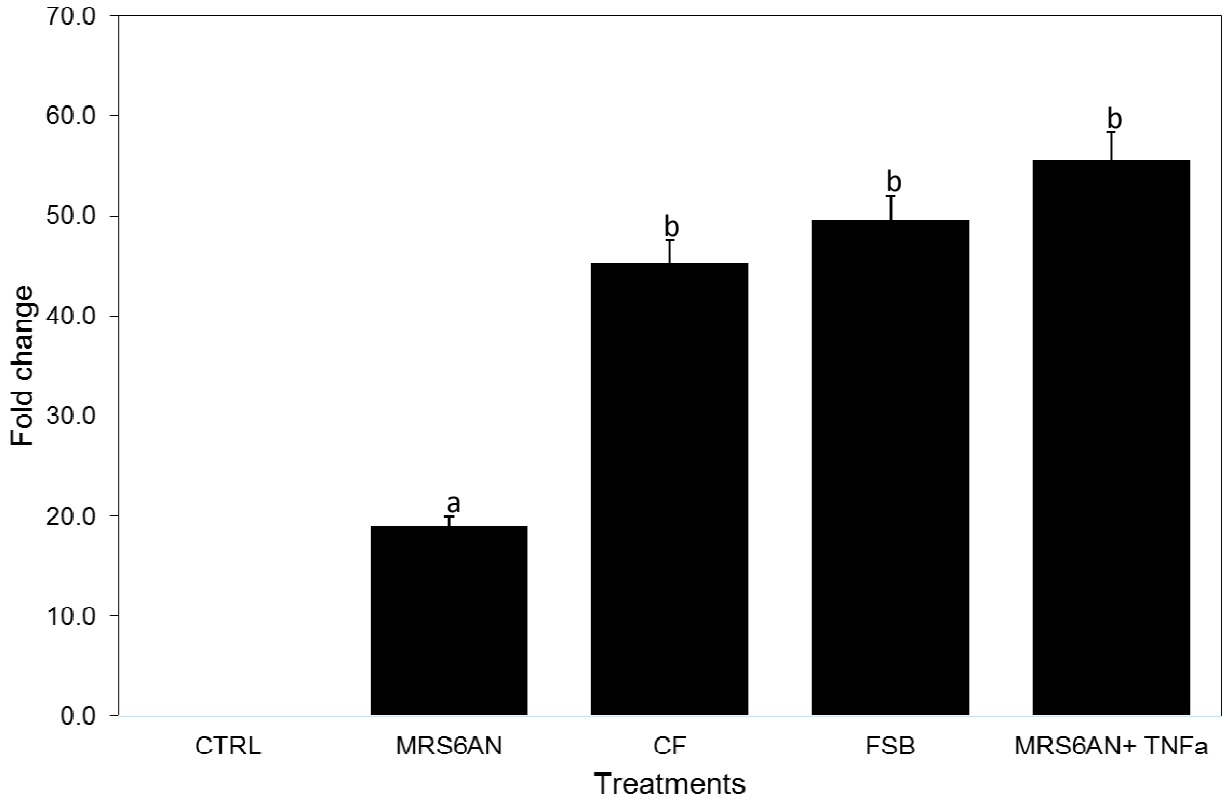


Figure 8b. Relative protein expression of NF-κB p65 transcription factor. Effect of treatments of Caco-2 cells with cytoplasmic fraction, filtered spent broth and live co-culture with *Lactobacillus rhamnosus* or with TNF-α stimulation on the expression of NF-κB p65 transcription factor. The expression is presented as fold change relative to the control where y-axis was set to 0 to normalize the graph to the control. Values are fold changes of means ± SD relative to the control, means without a common letter differ, (P<0.05).

Effect of NF-κB Inhibitor on Cytokine Production

To examine the possible effects of NF-κB inhibitor (IκB BAY 11-7085) on the production of pro-inflammatory and anti-inflammatory cytokines, fully confluent and differentiated Caco-2 cells were treated with IκB BAY 11-7085 (10um) for one hour before challenge with overnight live *Lactobacillus rhamnosus* culture, its cytoplasmic fraction, filtered spent broth or live *Lactobacillus rhamnosus* with TNF-α stimulation. All bacterial treatments in the presence of NF-kB inhibitor showed increased production of TGF-β and IL-8. The production of IL-10 and TNF-α cytokines after addition of NF-κB inhibitor BAY 11-7085 were below detection level. Generally, the presence of IκB seemed to decrease the production of IL-8 and TGF-β in all cell lysate treatments in comparison to cell treatments that had no NF-κB inhibitor BAY 11-7085 but the reduction was not to the baseline (PBS control).

Effect of NF-κB Inhibitor on the Production of TGF-β in Caco-2 Cells

In the presence of NF-kB inhibitor, there was significant production of TGF-β in all probiotic cell treatments: CF (p<0.0001), FSB (p<0.0001) and MRS6AN (p<0.0001) when compared to the control (Figure 9). There was however no significant difference of TGF-β levels between cytoplasmic fraction and live *Lactobacillus rhamnosus* treatments. FSB in the presence of IkB significantly increased the production of TGF-β in Caco-2 cells (p<0.001) in comparison to the other probiotic treatments.

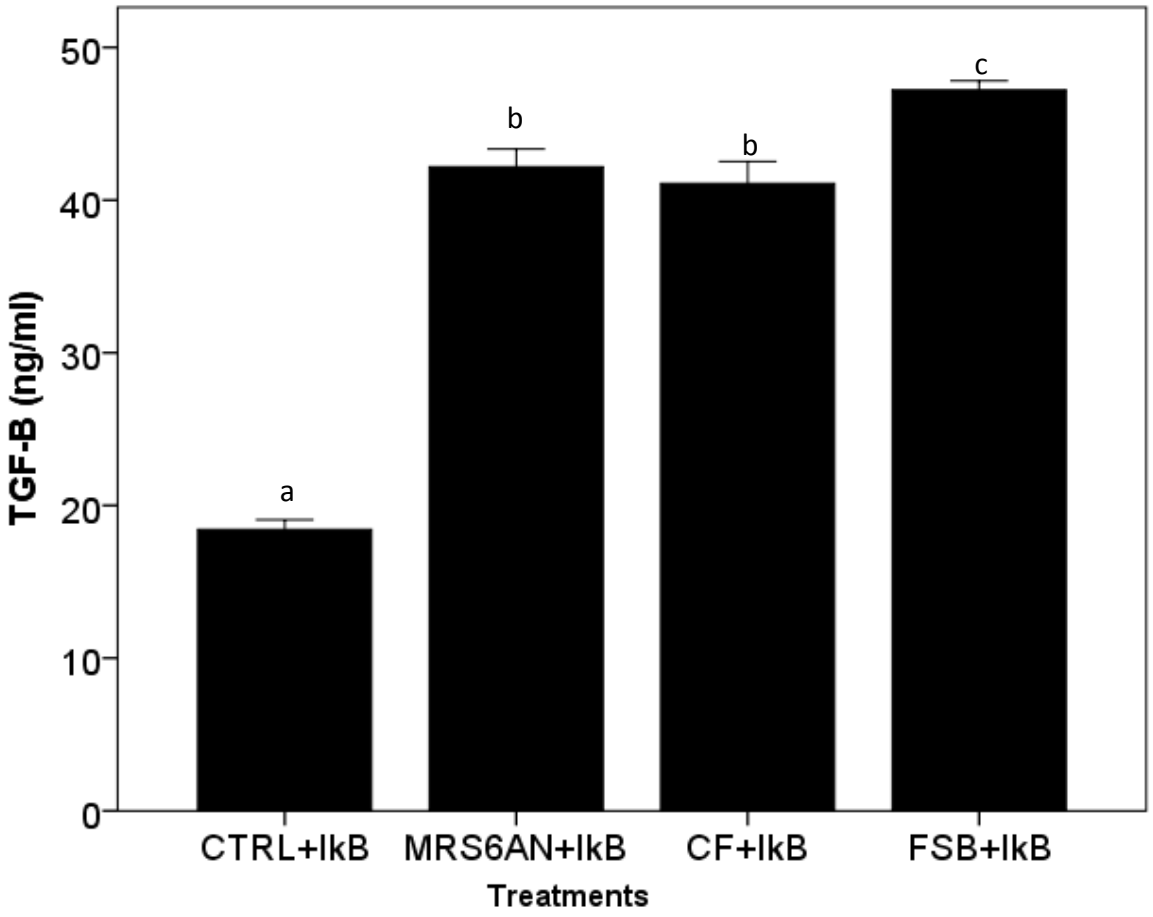


Figure 9. Cell lysate levels of TGF-β (ng/ml) when Caco-2 cells were treated live *Lactobacillus rhamnosus*, its cytoplasmic fraction or filtered spent broth in the presence of IkB. Each bar represents mean ± SD of TGF-β levels in cell lysate. Means without a common letter differ, (P<0.05).

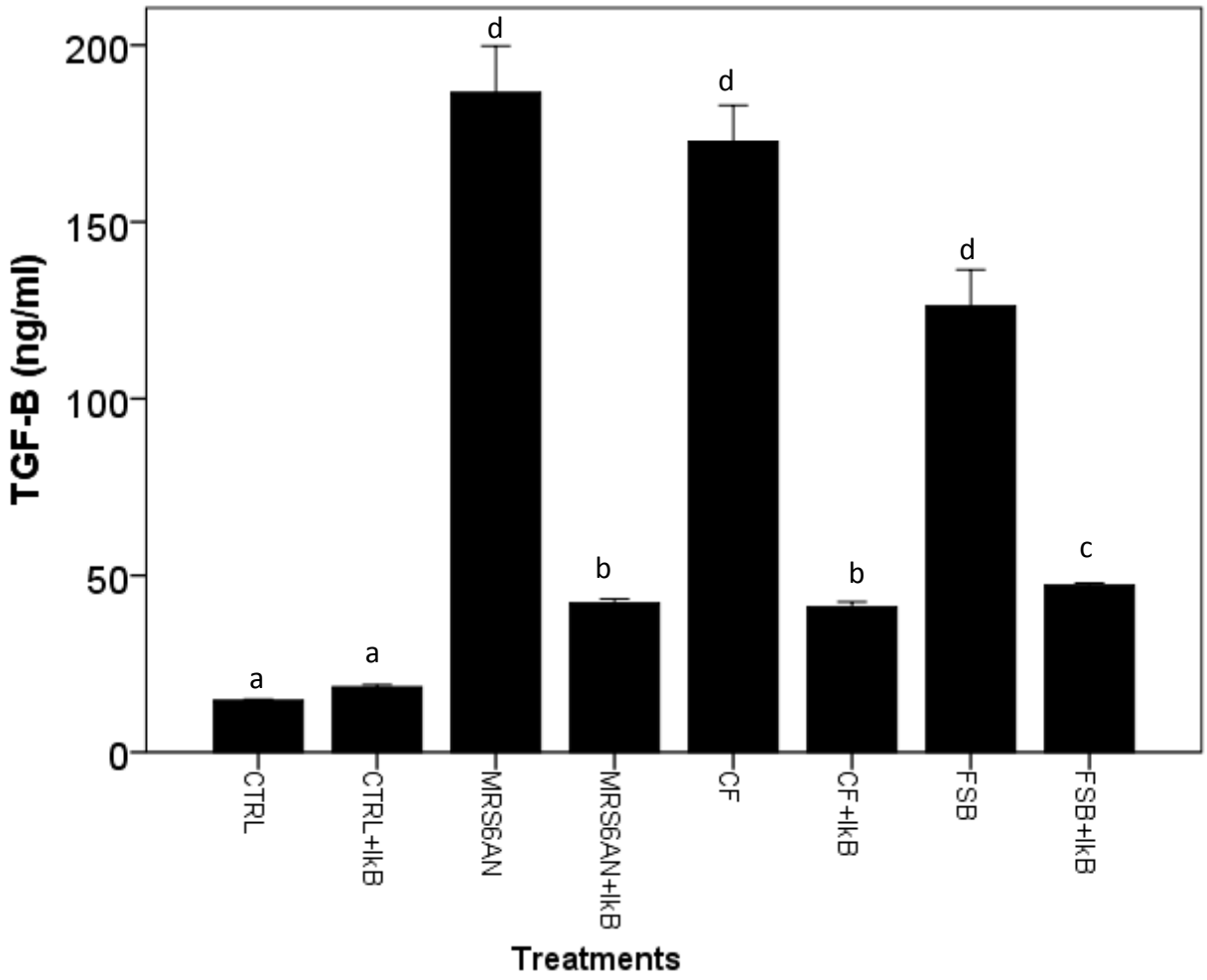


Figure 10. Comparison of the effect of the absence or presence of NF-κB inhibitor on the production of TGF-β (ng/ml) by Caco-2 cells when treated with live Lactobacillus rhamnosus, its cytoplasmic fraction, filtered spent broth. Each bar represents mean ± SD of TGF-β levels in cell lysate. Means without a common letter differ, (P<0.05).

Effect of NF-κB Inhibitor on the Production of IL-8 in Caco-2 Cells

All the probiotic treatments in the presence of inhibitor of NF-kB significantly increased the production of IL-8: MRS6AN ($p<0.005$), CF ($p<0.002$), FSB ($p<0.0001$)

and MRS6AN+TNFα (p< 0.0004) when compared to the control (Figure 11). In the presence of IkB BAY 11-7085, there was no significant difference in the production of IL-8 between Caco-2 cells treated with live *Lactobacillus rhamnosus,* its cytoplasmic fraction or live *Lactobacillus rhamnosus* with TNF-α stimulation. However, there was significant difference between cell lysate FSB treatments (p<0.0001) and the other probiotic treatments (CF, MRS6AN and MRS6AN+TNFα).

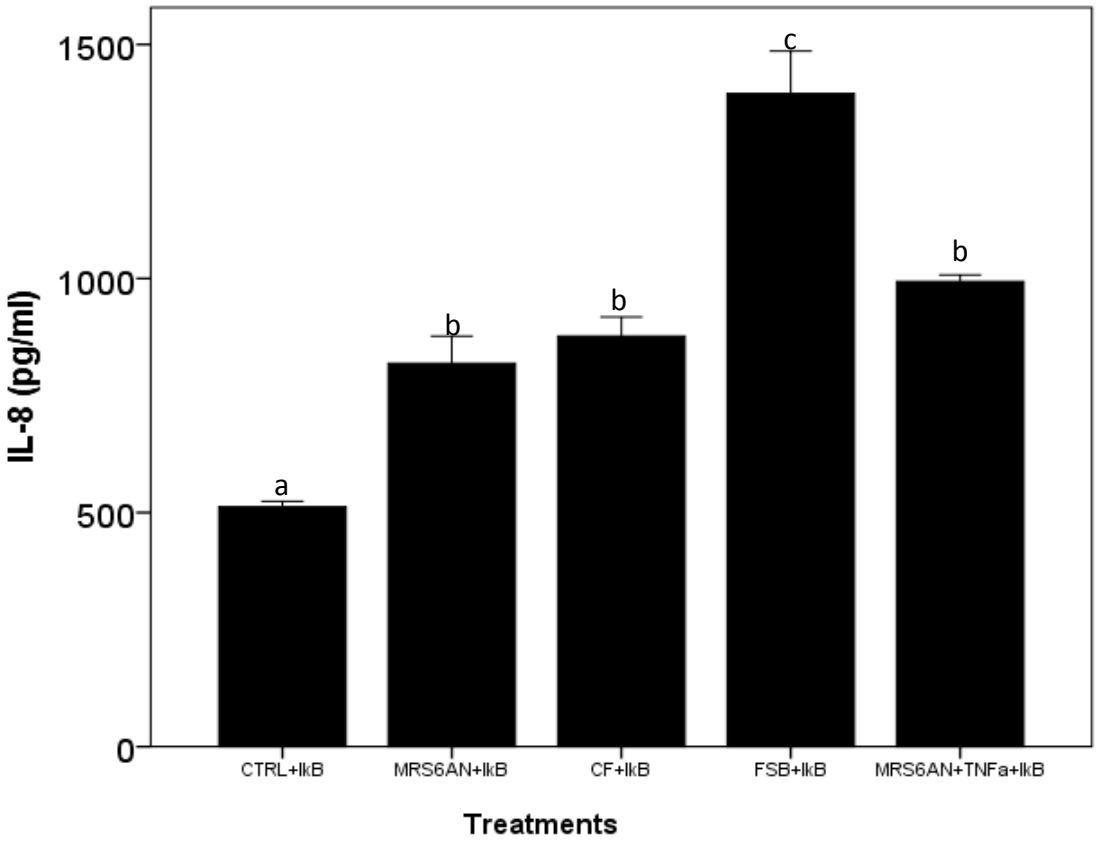


Figure 11. Cell lysate levels of IL-8 (pg/ml) when Caco-2 cells were treated with live *Lactobacillus rhamnosus*, its cytoplasmic fraction, filtered spent broth or live *Lactobacillus rhamnosus* with TNF-α stimulation in the presence of IkB. Each bar represents mean ± SD of IL-8 levels in cell lysate. Means without a common letter differ, (*P*<0.05).

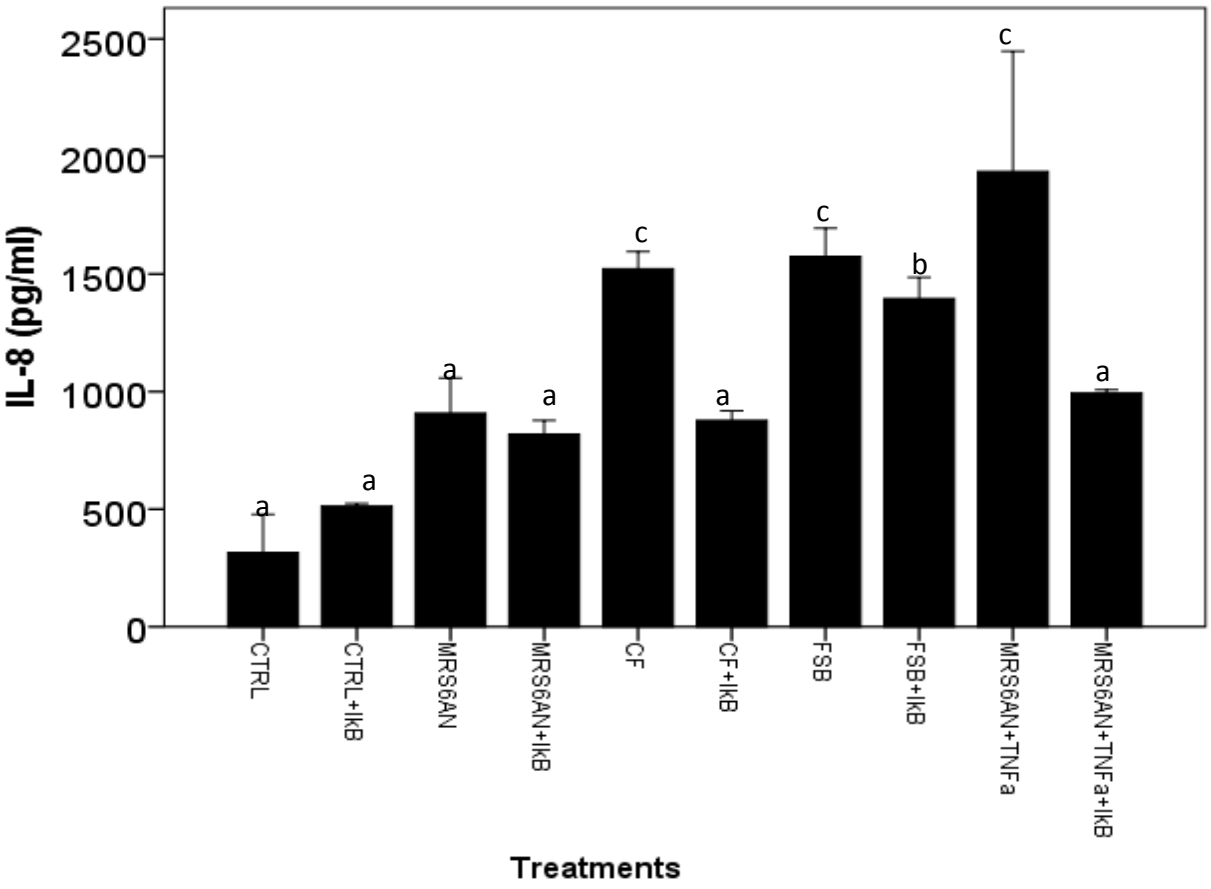


Figure 12. Comparison of the effect of the absence or presence of NF-κB inhibitor on the production of IL-8 (pg/ml) by Caco-2 cell when treated with live *Lactobacillus rhamnosus,* its cytoplasmic fraction, filtered spent broth or live *Lactobacillus rhamnosus* with TNF-α stimulation. Each bar represents mean ± SD of IL-8 levels in cell lysate. Means without a common letter differ, (P<0.05).

Effects of Heat Inactivated Broth on the Production of TGF-β and IL-8 Cytokines

Relative to the control, cell lysate levels of TGF-β and IL-8 were notably less reduced in Caco-2 cells treated with overnight *Lactobacillus rhamnosus* filtered spent

broth in the presence of NF-κB inhibitor. To determine whether the spent media had a heat labile biomolecule, the filtered spent broth was boiled to denature any secreted proteins that may have been present in the spent media. The heat inactivated broth (HIB) was then used as a Caco-2 cell treatment with IκB BAY 11-7085. In the presence of IkB, there was a significant difference in the production of TGF-β between the HIB+IκB ($p<0.0001$) when compared to the IkB control. There was also a significant difference in the production of IL-8 in HIB+IκB ($p<0.005$) when compared to the IkB control.

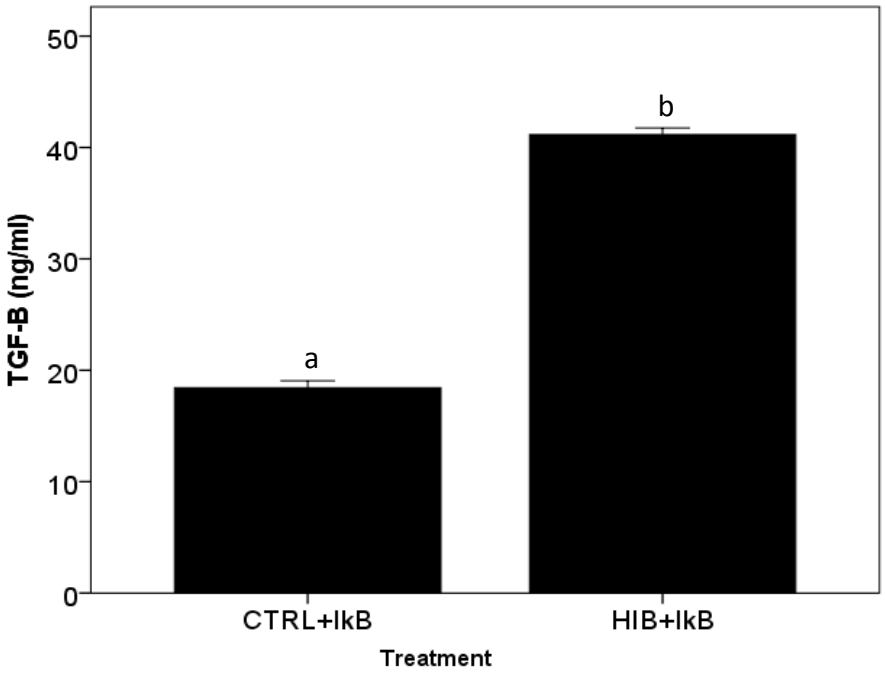


Figure 13. Cell lysate level of TGF-β (ng/ml) when Caco-2 cells were treated with heat inactivated broth in the presence of IkB. Each bar represents mean ± SD of TGF-β levels in cell lysate. Means without a common letter differ, ($P<0.05$).

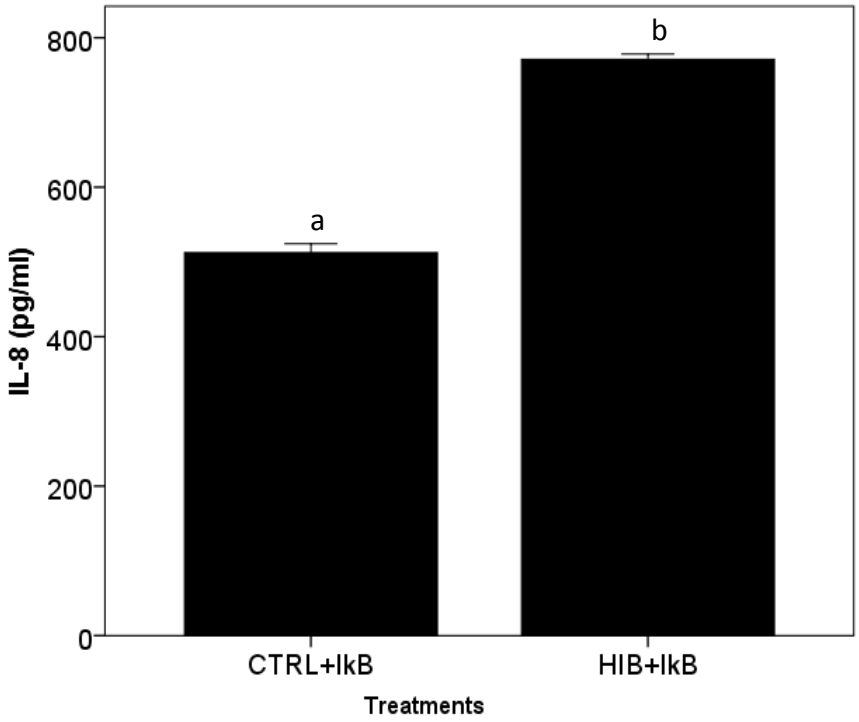


Figure 14. Cell lysate level of IL-8 (pg/ml) when Caco-2 cells were treated with heat inactivated broth in the presence of IkB. Each bar represents mean ± SD of IL-8 levels in cell lysate. Means without a common letter differ, (P<0.05).

Comparison of the Effects of FSB and HIB on TGF-β and IL-8 Cytokine Production

The levels of TGF-β and IL-8 were compared after treatments with filtered spent broth and heat inactivated broth to determine whether there were heat labile biomolecules that were responsible for the less reduced production of these cytokines in Caco-2 cells after addition of IkB BAY 11-7085. There was significant mean difference in TGF-β production between filtered spent broth and heat inactivated broth treatments ($p<0.0076$) in the presence of IkB. There was also significant mean difference in IL-8

production between filtered spent broth and heat inactivated broth treatments ($p<0.0001$) in the presence of IkB.

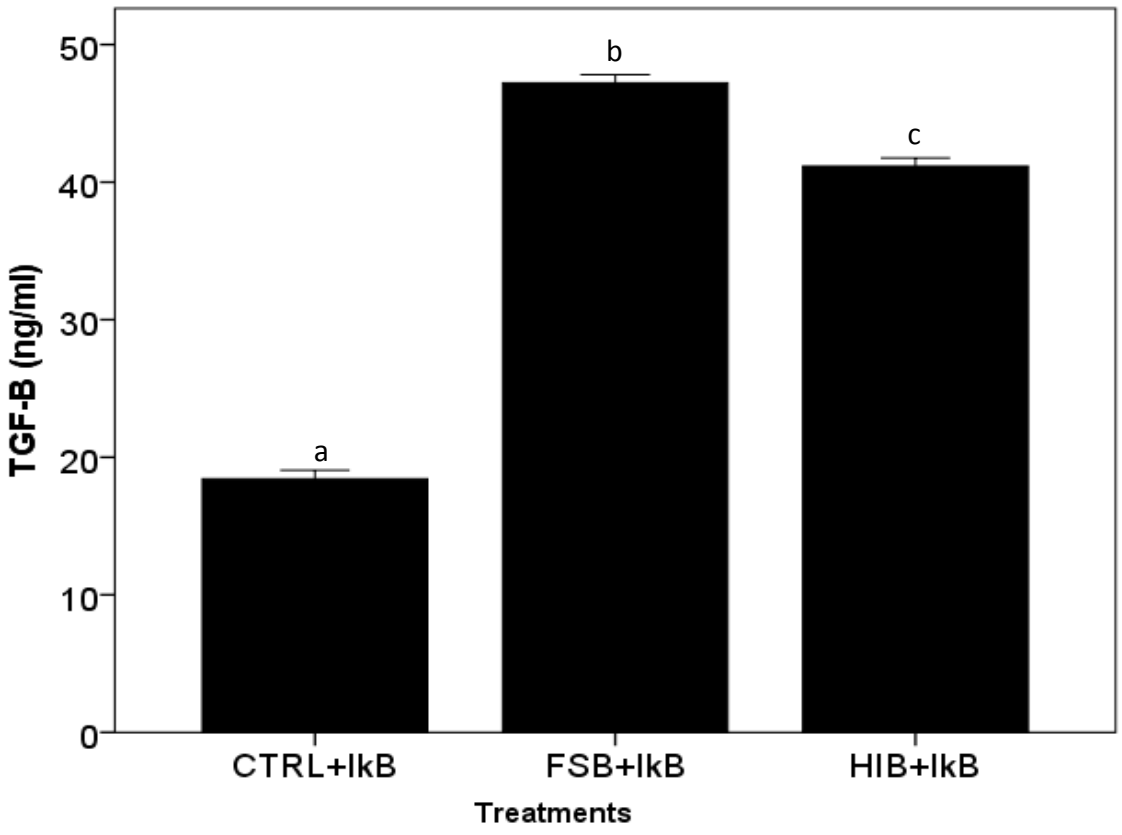


Figure 15. Comparison between the effects of HIB and FSB on the production of TGF-β (ng/ml) in Caco-2 cell lysate in the presence of IκB BAY 11-7085. Each bar represents mean ± SD of IL-8 levels in cell lysate. Means without a common letter differ, ($P<0.05$).

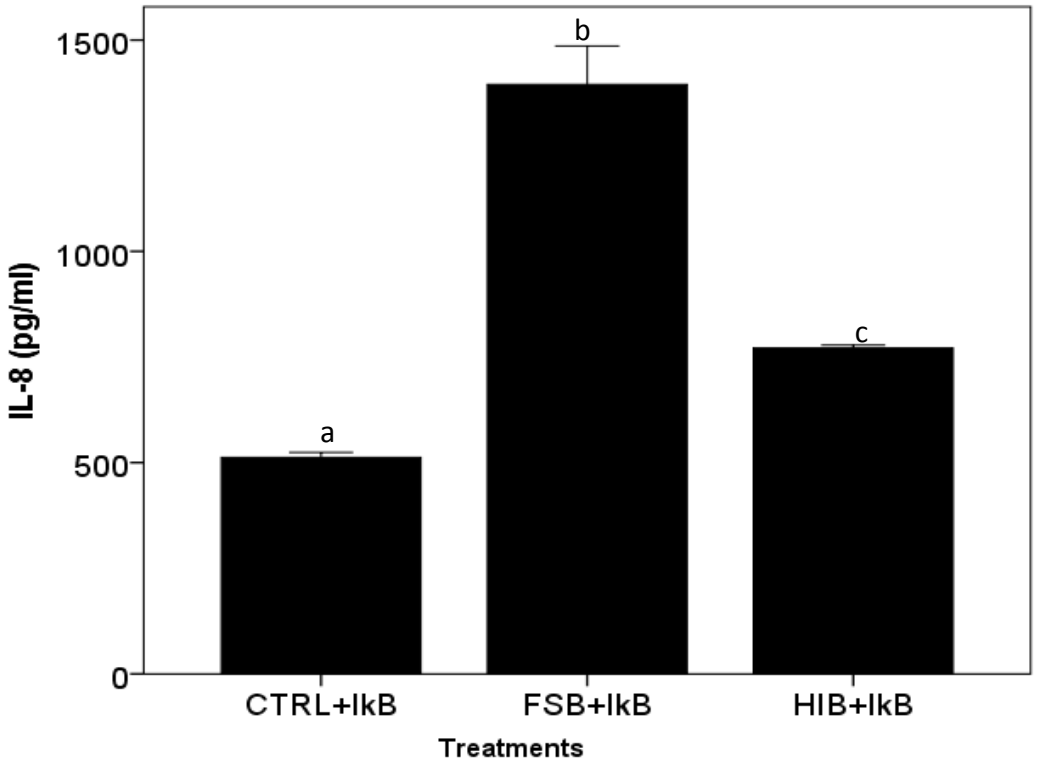


Figure 16. Comparison between the effects of HIB and FSB on the production of IL-8 in Caco-2 cell lysate in the presence of IκB BAY 11-7085. The bars represent the mean concentration of IL-8 (pg/ml). Values are means ± SD, means without a common letter differ, (*P<0.05*).

Effects of NF-κB Inhibitor (IkB BAY 11-7085) on Caco-2 Cell Adhesion

On addition of IκB to the fully confluent and differentiated Caco-2 cells, it was noted that the cell adhesion of Caco-2 cells was altered. Therefore, a cell adhesion assay was conducted to determine the varying effects of the presence or absence of IκB with the bacterial treatments. Caco-2 cells were cultured in a 96 well plate. The cells were then treated with live *Lactobacillus rhamnosus*, its cytoplasmic fraction, filtered

spent broth, heat inactivated broth with or without NF-kB inhibitor for 8-36 hours. The absorbance was taken at 590nm and each absorbance reading represented cell attachment. In the presence of IkB and probiotic treatments, it was observed that Caco-2 cell adhesion greatly decreased with a significant decrease in the FSB+IkB treatments ($p<0.03$) Probiotic treatments alone did not affect Caco-2 cell adhesion in comparison to the negative control (media alone) untreated cells or positive control (cells treated with IkB alone).

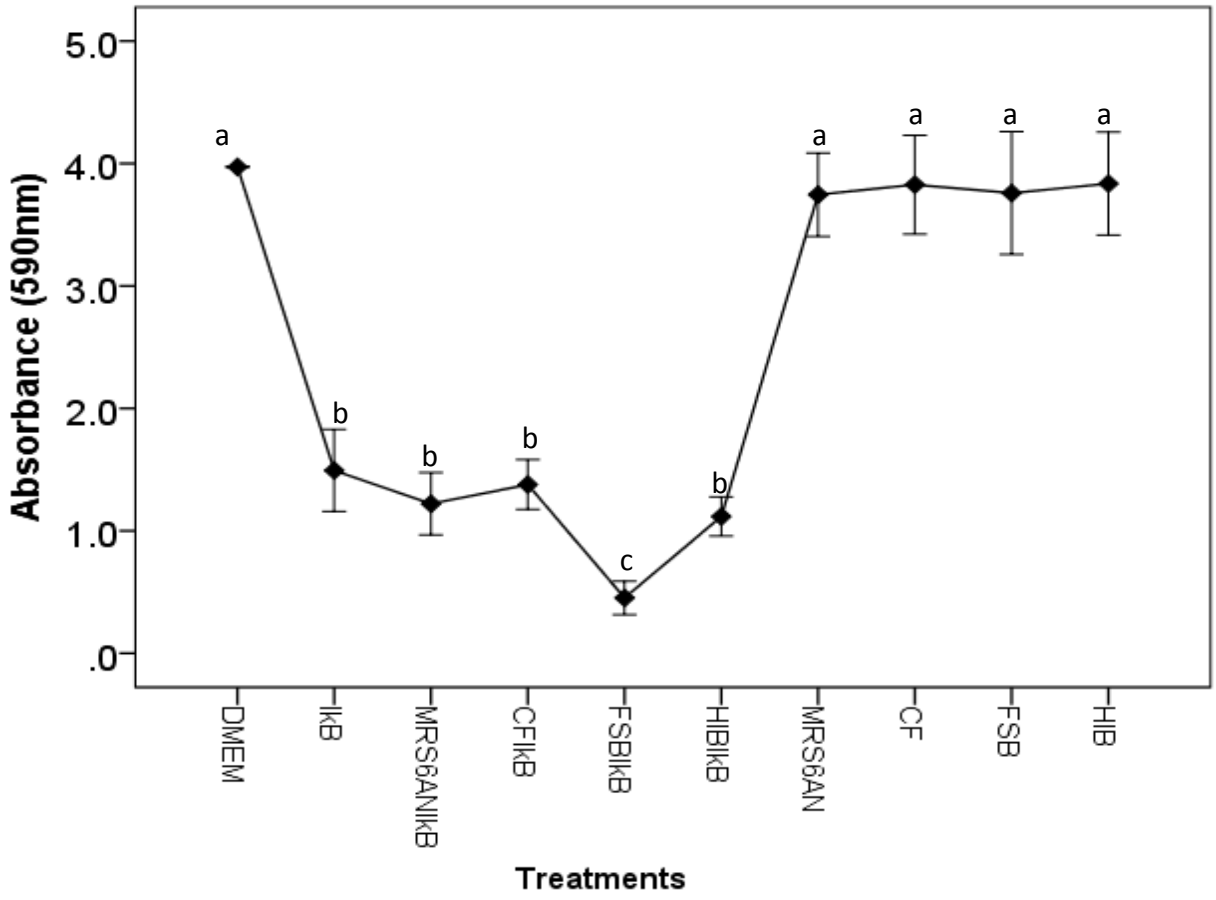


Figure 17. Effect of NF-kB inhibitor on adhesion of Caco-2 cells when treated with live *Lactobacillus rhamnosus*, its cytoplasmic fraction, filtered spent broth, heat inactivated broth with or without NF-κB inhibitor for 8-36 hours. Caco-2 cells with DMEM media was

used as a negative control while cells treated with IκB was used as positive control. A cell adhesion assay was carried out and the absorbance was taken at 590nm in each well. The absorbance reading is an equivalent of cell adhesion. The points on the line represent the mean absorbance for the various treatments. Values are means ± SD, means without a common letter differ, (p<0.05).

CHAPTER 4

DISCUSSION AND CONCLUSION

Discussion

Different studies have reported *Lactobacillus rhamnsosus* to have the potential of modulating immune responses through epithelial cells (Wallace et al. 2003; Zhang et al. 2005; Lopez et al. 2008). This study therefore examined the effects of *Lactobacillus rhamnosus* (MRS6AN) which was isolated using 16sRNA and biochemical tests from *"amabere amarunanu"* a Kenyan traditional cultured milk (Boyiri and Onyango 2015) on the production of pro-inflammatory and anti-inflammatory cytokines in enterocytes. This study hypothesized that probiotic treatments such as live *Lactobacillus rhamnosus*, its cell extracts, or filtered spent broth on enterocytes would have an effect on the production of pro-inflammatory cytokines; Interleukin 8 (IL-8) and Tumor Necrosis Factor–α (TNFα) and anti-inflammatory cytokines; Interleukin 10 (IL-10) and Transforming Growth Factor–β (TGF-β) in Caco-2 cells.

Probiotic cells extracts were used in this study because even though probiotics should be administered as live micro-organisms, yet isolated components from the probiotic cells have been shown to have therapeutic effects (Rachmilewitz et al. 2002). At single-celled level, epithelial cells mostly show characteristics of polarization, having unique apical and basal surface features with vectorial functions (Schoenenberger and Matlin 1991). Therefore, Caco-2 cell model was used in this study because they mimic important properties structural, morphological and functional properties of the mucosal epithelium. After confluence, they stop to proliferate and start to undergo a spontaneous polarized differentiation (Chantret et al. 1988).

Enzyme Linked Immunosorbent Assays (ELISA) showed very low levels of cytokine secretion in both basolateral and apical supernatants. Parlesak et al. (2004) and Haller et al. (2000) argued that basic stimulation of enterocytes with commensal bacteria without inducing cytokine secretion via LPS or cytokines or co-culture with leucocytes may result in low secretion of cytokine in cell supernatant. In this study, it was observed that secretion of both anti-inflammatory (TGF-β) and pro-inflammatory (IL-8) mostly occurred in apical cell supernatant with the various treatments. This could be due to the ability of intestinal epithelial cells to establish their response and to produce immune mediators in a bidirectional and vectorial manner (Sonnier, et al. 2010).

This study found that Caco-2 cells were directly responsive to challenge by live *Lactobacillus rhamnosus*, its cell extracts or overnight spent media as they had differential stimulation in the production of inflammatory cytokines in enterocytes. All probiotic treatments (MRS6AN, CF or FSB) showed significant increased levels of TGF-β in differentiated Caco-2 cells compared to the PBS control. *Lactobacillus rhamnosus* cell extract, its ovenight spent media or live *Lactobacillus rhamnosus* with TNF-α stimulation induced a significant increased production of IL-8 cytokines in differentiated Caco-2 cells compared with the PBS control and live *Lactobacillus rhamnosus* treatments. Live *Lactobacillus rhamnosus* alone did not stimulate significant production of IL-8 in Caco-2 cells. This coincides with Zhang et al. (2005) who found that both living and dead *Lactobacillus rhamnosus* GG inhibit IL-8 production by Caco-2 cells via interference with the NF-kB activation. This also supports previous study by Lopez et al.

(2008) who reported a decreased production of IL-8 in intestinal epithelium by live *Lactobacillus rhamnosus* GG.

It was observed that live *Lactobacillus rhamnosus*, its cytoplasmic fraction or filtered spent broth stimulated a significant increased production of TGF-β which was measured in nanograms/ml compared to the production of IL-8 which was measured in picograms/ml in the various probiotic treatments. This showed that *Lactobacillus rhamnosus*, its cell extracts or secreted products majorly stimulates the production of anti-inflammatory TGF-β cytokines. Taken together, these results indicate that the whole probiotic bacterial cell or its cell extracts or secreted products have differential immunomodulation properties and effects in the production of inflammatory cytokines in enterocytes.

Possible mechanisms through which probiotics influence differential immuno-modulatory activities have been discussed in different studies. *Lactobacillus rhamnosus* has been found to have different conserved microbe-associated molecular patterns (MAMPs) including the presence of lipoteichoic acid in their peptidoglycan (Perear-Velez et al. 2007), short glucose-rich and long galactose rich exopolysaccharides and cell surface glycoproteins (Francius, et al. 2008) which could bind to various Pattern Recognition Receptors on enterocytes such as TLR or NODs and trigger different immunomodulatory effects in enterocytes. Hardy et al. (2013), noted that probiotic bacterial products may exert their immuno-modulatory or stimulatory effects by direct interaction with the host cells or via secreted probiotic products. In this study, cell free supernatants or filtered spent broth were found to significantly increase the production of both TGF-β and IL-8 suggesting the capacity of probiotic *Lactobacillus rhamnosus* to

have immunomodulatory effects via the production and secretion of metabolites into its media. This agrees with Yan and Polk, (2002) and Yan et al. (2007) who reported that *Lactobacillus rhamnosus* secrete proteins into their spent supernatant and this may trigger specific signaling pathways in intestinal epithelial cells.

Further, multiple cell surface and secreted factors of *Lactobacillus rhamnsosus* GG were shown to exert protective effects on intestinal epithelial cells via multiple signaling pathway (Lebeer et al. 2008). Two secreted proteins; p40 and p75 (Yan et al. 2007) which are obtained from proteins marked as surface antigen and cell wall associated glycoside hydrolase have been reported to promote the survival and growth of intestinal epithelial cells (Claes et al. 2012). Kankainen et al. (2009) noted that the genome of probiotic *Lactobacillus rhamnosus* GG contains pilin encoding *spaBCA* operon which has been shown to bind to mucus and has been proposed to modulate IL 8 mRNA expression induced by surface molecules like LTA in Caco-2 cells (Lebeer et al. 2012). Also, *in vitro*, Illiev et al. (2005) reported that *Lactobacillus rhamnosus* GG chromosomal DNA to contain a specific, immunogenic structure that stimulated both human and murine lymphocytes indicating that not only live *Lactobacillus rhamnosus* GG but also its structural components may have immunomodulatory effects. A previous research exhibited that *Lactobacillus rhamnosus GG* influenced inflammatory gene expression via TGF-β cytokines (Di Caro et al. 2005).

In this study, it was observed that treatment with live *Lactobacillus rhamnosus* alone did not stimulate the production of IL-8 whereas live *Lactobacillus rhamnosus* with TNF-α stimulation showed a significant increased production of IL-8. This agrees with Stadnyk, (1994) who was studying the cascade effect of cytokines and stated that

epithelial cells may be induced to make cytokines by treatment with other cytokines. It has been reported that IL-8 can be synthesized by many different cell types and its production is mostly induced by a wide range of stimuli or ligands including TNF-α and bacterial products (Stadnyk 1994; Hoffman et al. 2002). Expression of IL-8 has been shown to be mostly regulated by stimuli like TNF-α (Xie 2001) but their expression may however be dose-dependent as Zhang et al. (2005) reported that when higher doses of *Lactobacillus rhamnosus* GG were co-incubated with Caco-2 cells, less IL-8 was produced via TNFα-stimulation.

Following an encounter with probiotic organism, IL-8 seems to be the major pro-inflammatory cytokine produced by enterocytes (Delcenserie et al. 2008) and its production has been reported to be transcriptionally controlled by NF-κB transcription factor (Hoffman et al. 2002; Smith et al. 2001). This study carried out western blot assays of NF-kB to ascertain whether cytokines production by enterocytes was dependent on NF-κB signal transduction pathway. Protein NF-κB p65 was expressed as band intensities in all the treatments with significant increased expression in CF, FSB and live *Lactobacillus rhamnosus* co-culture with TNF-α stimulation. These findings therefore support the study by Miettinen et al. (2000), who found that *Lactobacillus rhamnosus* GG activated transcription factor NF-κB, which is the central activator of the innate immune response and Toll-like receptors TLR1 and TLR2 which mediate bacterial recognition and cellular signaling. They further reported that *Lactobacillus rhamnosus* GG is able to induce a cascade of immunological events in the gut epithelial cells because it is recognized by the TLR2 receptor (Miettinen et al. 2008). Studies have also reported that significant IL-8 expression partly requires activation of NF-κB

transcription factor which in turn is sometimes regulated by protein kinase pathway (Hoffman et al. 2002). NF-κB signaling pathway is involved in the regulation of a wide spectrum of biological activities including regulation of inflammatory responses, cell proliferation, differentiation, apoptosis, lipid metabolism and coagulation (Li et al. 2012).

According to Pierce et al. (1997), NF-kB could be inhibited by two major inhibitors which are commercially available: BAY 11-7082 and BAY 11-7085 which have been shown to inhibit IκB phosphorylation and TNF-α induced NF-κB activation. To determine whether NF-κB signaling pathway had an effect on the production of these inflammatory cytokines, NF-κB inhibitor (BAY 11-7085) was added to Caco-2 cells one hour before treatment with the various probiotic treatments and the levels of TGF-β, IL-10, IL-8 and TNF-α, were measured using ELISA. After inhibition with IκB BAY 11-7085, it was observed that there was still significant production of TGF-B and IL-8 levels in Caco-2 cell lysate treatments compared to the control but their levels were remarkably decreased compared to their levels in the absence of NF-kB inhibitor. This suggested that apart from NF-kB signaling pathway, the production of TGF-B and IL-8 in Caco-2 cell lysate treatments could be transcriptionally regulated via other cell signaling pathways. Levels of IL-10 and TNF-α after treatment with IκB BAY 11-7085 were below their detection limits. Interestingly, concentration levels of TGF-β which is majorly regulated by SMAD pathway (Bitzer et al. 2000) were also affected by the inhibition of the NF-κB signaling pathway. This may suggest that immunomodulatory signaling pathways are interdependent or inter-related.

Compared to the other treatments, after inhibition of NF-kB by BAY 11-7085, it was observed that the concentration of TGF-β and IL-8 were less reduced in the filtered

spent broth treatments. This suggested that *Lactobacillus rhamnosus* ovenight spent media may have some secreted products that stimulate the production of TGF-β and IL-8 via a different signaling pathway. To determine whether the spent media had a heat labile biomolecule, differentiated Caco-2 cell monolayers were treated with heat inactivated broth (HIB). There was still significant production of TGF-β and IL-8 in Caco-2 cell lysate treatments with HIB indicating that the the presence of heat labile biomolecules with immunostimulatory properties. However, the levels of TGF-β and IL-8 in FSB treatments were significantly increased in comparison to their levels in HIB treatments. This suggested the presence of other immune stimulatory soluble factors in the overnight *Lactobacillus rhamnosus* spent media that are not basically heat labile. It also suggested that *Lactobacillus rhamnosus* secreted products in the spent media may regulate inflammatory responses partly via NF-κB signaling pathways. Study by Hoffman et al. (2002) who investigated the multiple signaling pathways that regulate the production or expression of IL-8 found that apart from NF-kB, there are three mitogen-activated protein kinase (MAPK) pathways that contribute to IL-8 gene expression including the extracellular-regulated protein kinase (ERK), JUN-N-terminal protein kinase (JNK) and p38 MAPK cascades.

Another important observation was that the presence of IκB (10uM) together with the various probiotic treatments significantly inhibited the adhesion of Caco-2 cells in comparison to probiotic cell treatments in the absence of IκB. The absorbance detected in the cell adhesion assay was an equivalent of adherent Caco-2 cells. This indicated a possible relationship between Caco-2 cell adhesion and NF-kB signaling pathway in mucosal enterocytes. Studies by patent Kumaka, (2011) and Scaife et al. (2002)

demonstrated that high concentrations of BAY 11-7085 was responsible for the rapid inhibition of cell adhesion in colon cancer cell lines (HT-29 cells) and suggested that NF-κB promoted cell adhesion. Transcriptional activator NF-kB has been found to regulate the genes of cell adhesion molecules, acute phase proteins cytokines, hemapoietic growth factors and transcription factors (Bitzer et al. 2000).

Conclusion

In conclusion, live *Lactobacillus rhamnosus*, its cell extracts or secreted products significantly increased the levels of TGF-β and IL-8 in Caco-2 cells. The increase in the levels of anti-inflammatory TGF-β was on average about 100x more compared to the increase in the pro-inflammatory IL-8 indicating that *Lactobacillus rhamnosus* treatments majorly stimulate anti-inflammatory responses in enterocytes. Inhibition of NF-κB significantly reduced the levels of TGF-β and IL-8 stimulated by the various *Lactobacillus rhamnosus* treatments on Caco-2 cells. However, the levels of TGF-β and IL-8 were not completely diminished after inhibition. This may be an indication that the transcriptional regulation of inflammatory cytokine production in enterocytes is partly via NF-κB signaling pathway.

After NF-κB inhibition, TGF-β and IL-8 levels were less reduced in FSB treatments (filtered overnight culture media of *L. rhamnosus*) compared to heat treated FSB (HIB) treatments. This may be an indication of the presence, in FSB, of other immune stimulatory soluble factors that are not heat labile. This study has also shown

that there is a possible relationship between Caco-2 cell adhesion and NF-κB signaling pathway because NF-κB inhibition led to a significant decreased Caco-2 cell adhesion.

Future Research

Since there was notable but varying inflammatory modulatory effects of whole live *Lactobacillus rhamnosus* milk isolate and its cytoplasmic fraction or filtered spent broth, there is therefore the need to characterize and further investigate the specific probiotic Commensal Associated Molecular Pattern (CAMPs) such as peptidoglycan, lipoteichoic acids, genes and their secreted products in the spent media responsible for such responses.

A further study should be carried out to determine the other signaling pathways that could transcriptionally regulate the production of inflammatory cytokines especially in the overnight spent media treatments on Caco-2 cells. The findings of this study were based on cell line experimental models which may not necessarily represent the actual *in vivo* situation, therefore it may be important to carry out this study in an animal model.

Also the co-culture of the milk isolate *Lactobacillus rhamnosus* and treatments with their extracts or filtered spent broth on Caco-2 cells could be carried out in a transwell model with leucocyte basolateral stimulation and or with cytokine/ lipopolysaccharide apical stimulation to further determine the effect of interaction or communication between the probiotic milk isolate, intestinal epithelial cells and immune cells on the production of various inflammatory cytokines.

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