1.0. INTRODUCTION

Low dose dietary antibiotics, used as growth promoters, have been widely incorporated into livestock production since the 1950s, and their advantages for weanling pigs include improvement in average daily weight gain and feed efficiency. However, such use of low dose dietary antibiotics has been questioned in recent years because of concerns that their use in livestock feed may lead to antibiotic resistant strains of pathogens that pose both a risk to livestock and (or) a public health risk. Direct-fed microbials, known as probiotics, are potential alternatives to low dose in-feed antibiotics.

The application of probiotics requires the understanding of the gut-associated mucosal immunology and the role of gastrointestinal epithelial cells in protection against enteropathogens. The following literature gives an overview on the mucosal immunology and the involvement of probiotics in gastrointestinal immunity with special emphasis to the probiotic stain used in this study, *Enterococcus faecium* SF68 (NCIMB 10415). In addition, the pathogenicity of the enteropathogens used in this study, *Salmonella typhimurium* DT104 and transmissible gastroenteritis virus (TGEV), is also addressed so that the health outcomes attributed to the probiotic used could be seen in disease models. Attention will also be given to the expression of various genes and cytokines during gastrointestinal infections.

1.1. The intestinal immune system

The gastrointestinal tract (GIT) is home to roughly 500 to 1,000 species of bacteria establishing a community of approximately 10^{12} resident microorganisms (Sonnenburg et al., 2004). The primary function of the small intestine is absorbing nutrients. During the absorption, the intestine is exposed to a wide variety of antigens thus requires a powerful defense system. It is essential that the gastrointestinal system differentiate between pathogenic and commensal microbes in order to avoid over stimulation of the immune system yet maintain a disease free status. The GIT provides barrier protection against commensal and pathogenic invaders, mediates crosstalk to more conventional immune cells and is generally considered as the host's first line of defense against pathogenic microbes (Eckmann et al., 1995). The intestinal immunological barrier is composed of the gut associated lymphoid tissue; and 25% of the intestinal mucosa is considered lymphoid tissue housing 70 to 80% of all immunogenic secreting cells (Langkamp-Henken et al., 1992). Peyer's patches (PP), intestinal epithelial cells (IEC) including specialized epithelial microfold (M) cells, and the

lamina propria (LP) are some of the immune cell compartments and cells associated with the gut mucosa (Müller et al., 2005).

Within the small intestine there are three main lymphocyte populations, which reside in the epithelium (Intraepithelial lymphocytes, IEL), in the lamina propria (Lamina propria lymphocytes, LPL) and in the Peyer´s patches (Peyer´s patches lymphocytes, PPL). IEL are cells located above the epithelial basement membrane between the mucosal columnar epithelial cells. The phenotype of most IELs is $CD3^+CD8^+$ and therefore they basically have cytotoxic function. IEL also functions in surveillance of the intestinal epithelial layer for the detection of microbial pathogens, removal of damaged or transformed epithelial cells, maintenance of epithelial integrity via secretion of trophic factors important for epithelial cell growth and differentiation and regulation of local cell-mediated or humoral immune responses (Brandtzaeg and Pabst, 2004; Muller et al., 2005; Manzano et al., 2002). In lamina propria, large numbers of T-cells are available, of which the majority have $CD3^+CD4^+$ phenotype. However, in contrast to IEL, LPL interact freely with each other and with macrophages, which may result in activation of different pathways (Rothkötter, 2009). The most important cytokines that regulate the functional status of the mucosal lymphocytes are interleukin (IL)-7 and IL-15 (Shaykhiev and Bals, 2007).

PPL play a crucial role in the induction of intestinal immune responses. Peyer´s patches (PP) are primarily typical B cell lymphoid organs. Generally, residents of the jejunal (proximal or discrete) PP include populations of lymphocytes, dendritic cells, macrophages, villous epithelium and the specialized follicle-associated epithelium, which along with M cells, are considered the main site of antigen uptake (Beier and Gebert, 1998). M cells are cells of the modified dome epithelium overlaying the PP that sample intestinal antigens and transport them to lymphoid cells that underlie the epithelium in PP (Ohl and Miller, 2001). The jejunal PP within the pig populate both the jejunum and proximal ileum, are small, persist throughout life and number between 25 to 35 PP (Stokes et al., 1994). The ileal (distal continuous) PP in the pig is a large single ileocecal patch which extends along the terminal ileum (2.5m in length) but involutes with age (Stokes et al., 1994). T and B cell populations residing in each PP location vary with age and infection (Stokes et al., 1994). The predominant role of T cells in PP is to aid in the differentiation of B cells, which is the main subpopulation of this level, toward IgA-producing cells through the production and secretion of several cytokines by CD4 positive T cells (Manzano et al., 2002). The M cells, most prominent in the follicle-associated

epithelium (FAE) of the PP, are considered the primary site of antigen sampling in the gut lumen, but both the villus epithelium and the PP of the small intestine are also participants of antigen sampling (Neutra et al., 1999).

Since a number of applied experimental studies in different areas of physiology and medicine were performed in pigs, understanding of porcine immunology and gaining insight into the function of both the innate and the systemic mucosal immune system is of importance. The mucosal associated immune components assist in both the innate and adaptive immune responses. The function of the innate immune system is to quickly identify and respond to foreign microorganisms, to incapacitate pathogens, and act as an adjuvant for the acquired immune system (Yuan and Walker, 2004). Essential components of the intestinal innate immune system include the epithelial cell monolayer (a mechanical barrier), secretory IgA, antimicrobial peptides and proteins, commensal intestinal microbes, and biochemicals such as gastric acid, biliary and pancreatic secretions and mucins (Yuan and Walker, 2004). Active antigen sampling occurs at the mucosal surface followed by delivery to the lymph node tissues, whereupon systemic immune activation occurs (Brandtzaeg and Pabst, 2004).

1.1.1. The intestinal epithelial cells (IECs)

Sensing the presence of pathogenic bacteria by epithelial cells is one of the integral mechanisms of host response to pathogenic microorganisms at mucosal sites. For several years, intestinal epithelial cells (IEC) were believed to be exclusively involved in the absorptive process of digestion. The barrier function of the IEC to separate the inside from the outside and to protect the sensitive immune system from continuous contact with external microorganisms is critical. The IEC barrier function is maintained by a well-organized polarity so that the apical surface faces the lumen or outside of the intestine, and basolateral surfaces are in continuous contact with the body interior. Apical surfaces of IEC are covered with microvilli, which increase the surface area for the transport of substances across the membrane (Shaykhiev and Bals, 2007). Tight junctions, which are also located apically, separate the lumen from the tissue compartment and prevent paracellular diffusion of fluids, electrolytes, macromolecules and luminal microorganisms (Schneeberger and Lynch, 2004).

However, since IECs are located between the lumen and a wide array of mucosal lymphocytes in the mucosa associated lymphoid tissue (MALT) (Fig 1.), the concept that IECs played a role in immune regulation evolved. Apart from being a potential physical barrier, IECs can

receive signals and interpret and transmit this information to the underlying LP (Dahan et al., 2007). The immune cells within the MALT and numerous dendritic cells (DCs) and lymphocytes that reside within the healthy epithelium and subepithelial areas acquire antigens via the IEC-mediated mechanism. Induction of immune responses in the MALT is coupled to uptake of the luminal antigens. This uptake is mediated by microfold or membranous (M) cells, a specialized population of IECs present in the FAE, which overlies the subepithelial lymphoid follicles in the gastrointestinal tracts. Microbes taken up by these cells are then delivered directly to intraepithelial lymphoid cells, subepithelial areas, and underlying lymphoid follicles containing DCs, T and B cells. This close intercellular collaboration is determined by a unique structure of M cells: a deep invagination of the basolateral surface forms a so-called intraepithelial "pocket" into which lymphocytes migrate. The other morphological feature that helps to identify M-cells is their irregular, short and relatively sparse microvilli (Turner, 2003). The transepithelial transport of many pathogens, including *Salmonella* and HIV-1, is mediated by M cells (Shaykhiev and Bals, 2007).

IECs are prominent sources of chemokines and cytokines which lead to the recruitment of macrophages, lymphocytes, and polymorphonuclear leukocytes and can therefore further initiate both the innate and adaptive immune responses (Jung et al., 1995; Maaser and Kagnoff, 2002). The ability of IEC to respond to numerous microorganisms relies upon a set of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) that recognize conserved molecular patterns characteristic for bacterial and viral motifs, such as peptidoglycan (TLR2 in cooperation with TLR1 and TLR6), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), viral RNA (TLR7 and TLR8) and bacterial DNA (TLR9) (Akira et al., 2006; Dahan et al., 2007). Although there is substantial evidence confirming the functionality of epithelial PRRs *in vitro*, it is currently unknown to which extent innate immune recognition by IECs contributes to induction of immune responses *in vivo.* IECs also promote the transport of immunoglobulins (Igs), such as secretory IgA (SIgA), produced by the lamina propria B cells to the lumen. Luminal SIgA prevents the adhesion and entry of antigens to the epithelium (Shaykhiev and Bals, 2007).

Components of the network between IECs and leukocytes (Shaykhiev and Bals, 2007)

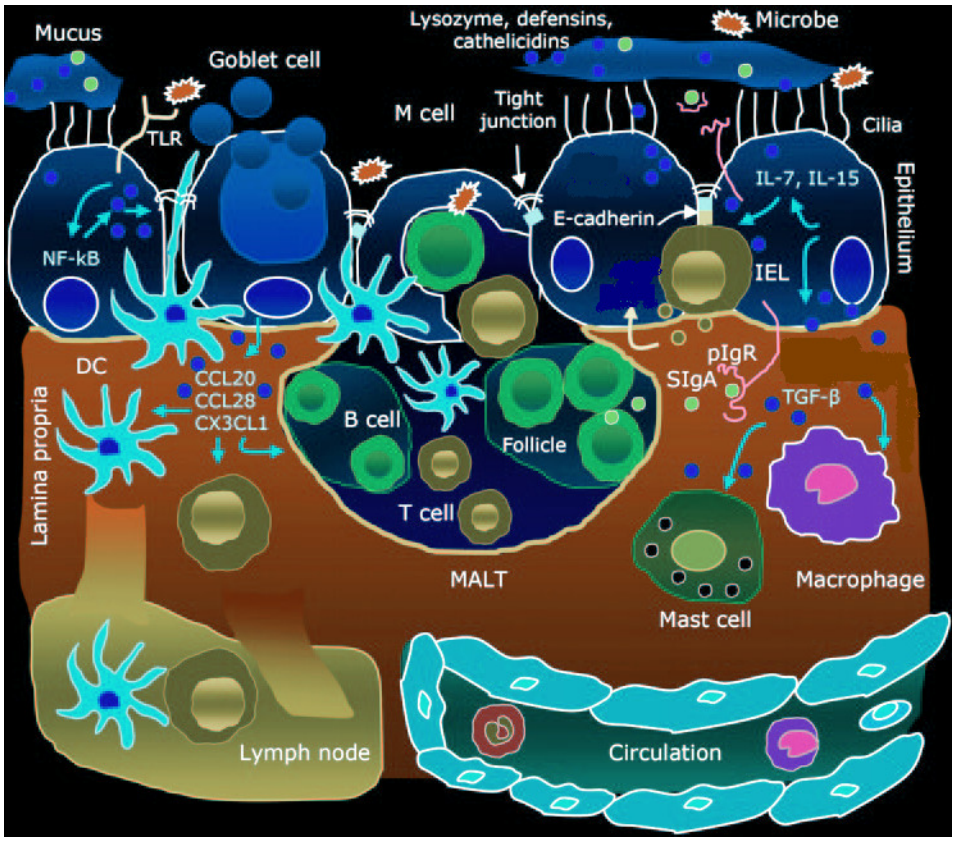

Fig.1. *IECs provide a mechanical barrier through their tight junctions (TJs) and mucociliary escalator and produce several host defense molecules such as mucin and substances with a direct antimicrobial activity (lysozyme, defensins, cathelicidins and others). IECs express pattern-recognition receptors (PRRs) necessary for innate immune detection of microbes and generation of proinflammatory mediators including cytokines and chemokines. In addition, IECs are actively involved in translocation of Ig: Epithelial polymeric Ig receptor (pIgR) is necessary for transfer of secretory IgA (SIgA). Different types of leukocytes—dendritic (DCs), macrophages, mast cells (MCs), and lymphocytes—are localized adjacent to IECs and interact continuously with them. The clusters of immune cells are posed immediately beneath the epithelium forming mucosa-associated lymphoid tissues (MALT), for which M cells serve as portals of antigen entry. E-cadherin expressed by IECs is an adhesion point for many subtypes of lymphocytes including intraepithelial lymphocytes (IELs). Chemokines CCL20, CCL28, and CXCL1 play a role in compartmentalization of DCs and lymphocytes within mucosa; cytokines IL-7 and IL-15 regulate homeostasis of IELs and other mucosal leukocytes; constitutively expressed TGF-ß control local inflammation.*

Jung et al., (1995) demonstrated that after challenge with invasive bacterial strains, human colon epithelial cell lines (T84, HT29 and Caco-2) expressed the proinflammatory cytokines IL8 and TNFα. Cyclooxygenase-2 (a rate limiting enzyme for prostaglandin production) and NO synthase (controls NO production) are also up-regulated after infection with invasive bacteria (Elewaut et al., 1999). The porcine intestinal epithelial cell line IPEC-J2 expresses mRNA for IL1α, IL6, IL7, IL8, IL12p40, IL18 and tumour necrosis factor-α (TNF-α) (Schierack et al., 2005; Skjolaas et al., 2006). Proinflammatory cytokine production in epithelial cells in response to microorganisms is accomplished by activation of the nuclear factor-κB (NF-κB), a transcriptional effector required for the synthesis of numerous cytokines, chemokines, adhesion proteins and other molecules critical for normal inflammatory function (Neish et al., 2000; Müller et al., 2005; Madsen, 2006). Certain probiotic strains can elicit anti-inflammatory responses in epithelial cells (Madsen, 2006). Although the mechanisms by which the IEC differentiate the normal flora from pathogens is not yet clearly understood, Neish et al. (2000) observed that the direct interaction of non-virulent *Salmonella* strains with human epithelia attenuated synthesis of inflammatory effector molecules elicited by diverse proinflammatory stimuli through inhibition of NF-κB.

In addition to the indirect transfer of information by IECs to the MALT via the secretion of chemokines and cytokines, IECs can directly also process antigens and present them to antigen-specific lymphocytes. This is largely mediated by M cells, which transport macromolecules without significant cellular processing to the underlying lymphoid tissue containing professional antigen presenting cells (APCs), including macrophages, dendritic cells (DCs) and B cells. IECs can also take antigen by receptor-mediated endocytosis (Ohl and Miller, 2001). IECs throughout the intestine constitutively express major histocompatibility complex (MHC) class I, but they also constitutively express MHC class II predominantly in the human small intestine (Lin et al., 2005; Dahan et al., 2007; Hundorfean et al., 2007). Although little is known about the MHC class II associated antigen processing mechanism in IEC, it is clear that IEC may process soluble antigens for presentation to T-cells (Dotan and Mayer, 2003). In addition IECs express a number of non classical MHC I molecules, such as CD1d, that enable them to transmit luminal signals to LPL and make them a unique APC in the gut. The M cells of the epithelium in pigs are known to express MHC class II antigens. In swine, MHC Class I are present on epithelium cells of both the villi and crypts. In contrast MHC class II is present only on epithelial cells of the villi (Oliver et al, 1994; Dotan and Mayer, 2003). The expression of these molecules plays a key role in

activating populations of T cells in the LP that can either regulate or promote inflammation (Dahan et al., 2007).

1.2. The involvement of probiotics in gastrointestinal immunity

Probiotics are defined as direct feed microbials or microbial cell preparations with the beneficial effect on the health and well-being of the host (Nemcová, 1997). The hypothesis that beneficial bacterial communities in the gut lead to positive effects on host health has led to the development of therapeutics that are based on the consumption of beneficial bacterial cultures (Nava et al., 2005) that are marketed for both human and animal health benefits. In addition to the health benefit hypothesis, the use of probiotics in human and animal health has increased in recent years due to additional concerns about the failure to identify and generate new antibiotics, the impending ban on the use of low dose antibiotics as growth promotants in animal feed, and the emergence of pathogenic microbes with antibiotic resistance genes (Hong et al., 2005). The mechanisms by which probiotic bacteria could mediate changes in the GIT are not well understood. However, it is suggested that probiotics may mediate their action by competitive exclusion or by changing the metabolic conditions in the gut (Sakata et al., 2003). The immuno-stimulatory effect of some probiotics is another hypothesis that explains the mechanism of action of probiotics. The cellular and molecular mechanisms by which non-pathogenic bacteria directly influence the intestinal epithelium in order to limit the activation of the immune system are not fully understood. However, these effects are suggested to be attributed to changes in the gastrointestinal microecology, which can lead to stimulation of the gut associated lymphoid tissue resulting in cytokine and antibody production, phagocytic activity and increased T cell and NK cell activity (Meydani and Ha, 2000).

Lactic acid bacteria (LAB), *Bacillus* spp., Enterococci, Stereptococci as well as yeasts such as Saccharomyces are among the common probiotics seen in use today. Among the Enterococci, *Enterococcus faecium* is the most frequently used species in commercial probiotics (Ozawa et al., 1983). Bacilli are primarily used in their spore form, and have demonstrated beneficial effects in the prevention of gastrointestinal disorders (Hong et al., 2005). Several studies have been completed with pigs using the BioPlus® 2B supplement (containing *Bacillus licheniformis* and *Bacillus subtilis* spores), which contributed to improved sow and piglet performance (Alexopoulos et al., 2004). Most probiotic LAB are reported to have immunostimulatory properties (Meydani and Ha, 2000) and alleviate gastrointestinal

disorders (diarrhea, inflammatory bowel disease, lactose intolerance, salmonellosis or shigellosis (Goldin, 1998; Madsen et al., 2001). *Lactobacillus reuteri* (LR) has also demonstrated direct anti-inflammatory activity in human epithelial cells through inhibition of TNF-α and *Salmonella* induced IL-8 expression (Ma et al., 2004). Oral ingestion of LR by healthy adult subjects also significantly increased CD4+ T cell populations in the ileum which may assist in maintaining gut health (Valeur et al., 2004). LR JCM 1081 and *Lactobacillus crispatus* JCM 8779, both of which are highly adhesive to Caco-2 cells, are able to reduce adhesion of pathogenic *Escherichia coli* and *Salmonella typhimurium*, and offer bactericidal substances (Todoriki et al., 2001). *Lactobaillus rhamnosus* also demonstrated increased host protection within BALB/c mice challenged with either a single oral dose or repeated daily doses (5d) of *Salmonella typhimurium*, whereby probiotic fed mice exhibited higher health scores, higher serum and intestinal tract anti-*Salmonella* antibody titers, decreased pathogen burdens in visceral organs, and higher overall survival rates (Gill et al., 2001). Coconnier et al., (2000) also demonstrated that cell-free culture supernatants from *Lactobacillus acidophilus* could affect intracellular *Salmonella* residing in Caco-2 cells, which included decreased transcellular passage, inhibition of intracellular growth and inhibition of adhesion-dependent *S. typhimurium*-induced IL-8.

There is no doubt that probiotics are important in supporting a functional yet balanced immune systems and further employment of immunomodulatory bacteria in health care can be seen in combating microbial pathogens, including viruses. The potential of using probiotic bacteria as viral inhibitors has been recently indicated in treatment of HIV-associated diseases (Chang et al., 2003), rotavirus gastroenteritis (Reid and Burton, 2002) and vesicular stomatitis in a pig intestinal epithelial cell line (Botić et al., 2007). However, understanding the role of probiotics against viral infections and their role in modulating the immune system of the host needs to be investigated in more detail.

1.3. *Enterococcus faecium*

Enterococci, Gram-positive spherical bacteria, are important inhabitants of animal intestine and are widely used in probiotic products because of their good growth, adhesive ability, lactic acid production and the stability of their enterocins, which have antimicrobial properties. *Enterococcus faecium* belongs to the common flora of porcine intestine (Macha et al., 2004). *E. faecium* strains used as probiotics efficiently protect animals from diseases caused by *E. coli* and clostridia (Maia et al., 2001). *E. faecium* SF68 (NCIMB10415) is a probiotic bacterium, originally isolated from a healthy Swedish baby, with inhibitory effects against important enteropathogens including enterotoxigenic *Escherichia coli*, shigellae, and clostridia (Lewenstein et al., 1979). The ability of the probiotic strain to reduce the rate of carryover infections of piglets by obligate intracellular pathogens, such as chlamydiae, has been reported (Pollmann et al., 2005). The mechanisms by which the probiotic strain interferes with infections are suggested to include exclusion of pathogens by means of competition for attachment and stimulation of host-cell immune defenses. *E. faecium* SF68 antagonizes giardia by enhancing both the humoral and cellular immune response in mice (Benyacoub et al., 2005). Similarly, it has been reported that *E. faecium* SF68 stimulates both the mucosal and the systemic immune system in dogs (Benyacoub et al., 2003). Scharek et al. (2005) reported a significant reduction of the levels of cytotoxic T cells (CD8+) in the jejunal epithelium of piglets treated with *E. faecium* SF68. They also showed a remarkable decline in the frequency of β-haemolytic and O141 serovars of *Escherichia coli* in the intestinal contents of piglets as a result of treatment with *E. faecium* SF68.

E. faecium it is known colonizes the porcine gut to a cell count of 10^5/g digesta (Macha et al., 2004; Vahjen et al., 2007), while other enterococci are far more frequent in the porcine intestinal tract. This implies that neither metabolic interactions nor the competitive occupation of intestinal receptors seem to be mechanisms that could be responsible for possible probiotic effects attributed to *E. faecium*. Subtle modification of the immune system appears to be a more likely mode of action that could even work with low numbers of bacteria present in the intestinal tract. Previous studies using this probiotic showed reduced frequency of ß-haemolytic and O141 serovars of *Escherichia coli* and reduced carryover infections of chlamydia in sows and piglets (Scharek et al., 2005; Pollmann et al., 2005). In contrast, under a different experimental setup, the immunological data obtained by Szabó et al. (2008) indicate a more severe infection with *Salmonella* in piglets treated with *E. faecium* SF68. Similarly, Scharek et al. (2005) reported the immune suppressive properties of *E. faecium*

SF68 observed with the reduction in the frequency of CD8+ T cells in the jejunal epithelium of piglets fed with this probiotic.

1.4. Salmonellosis

Salmonellosis is a disease characterized by a self-limiting diarrhea, vomiting, abdominal cramps and fever, but also contributes to approximately 15,000 hospitalizations and roughly 500 deaths in the United States (Mead et al., 1999). *Salmonella* spp, which are characterized as Gram-negative, facultative anaerobic, motile, non-lactose fermenting rods belonging to the family of *Enterobacteriaceae,* are the causative agents of the disease. *Salmonella* infects humans and animals generally by the oro-fecal route. The microorganisms can be frequently found in sewage, sea and river water and they can contaminate a variety of food. Salmonellae have been isolated from many animal species including pigs, cows, chickens, turkeys, sheep, dogs, cats, horses, donkeys, seals, lizards and snakes (Mastroeni et al., 2000). Disease is usually seen with 10^{8} to 10^{11} CFU of *Salmonella* via the oral route of infection and pigs can shed up to 10 million *Salmonella* per gram of faeces (Fedorka-Cray et al., 2000). Many *Salmonella* spp. have the ability to survive, remain viable and maintain infectivity for long periods in the environment (Gray and Fedorka-Cray, 2001).

Salmonella infections are an ongoing serious medical and veterinary problem world-wide and cause great concern in the food industry. In European countries the consumption of contaminated pork was found to be associated with 20% of human salmonellosis (Sakata et al., 2003). Similarly, in the United States an estimated 1.4 million persons are infected annually with *Salmonella*. Among the poultry, swine and feedlot cattle industries within the United States, 50% or more of the flocks and herds have been reported positive for *Salmonella* at various points within the production cycle (Hurd, 2004). Young pigs exposed to *Salmonella typhimurium* can be carriers for up to 28 weeks after exposure (Wood et al., 1989) and sub-clinically infected hosts increase the risk for infecting others and contaminating the food chain. Costs that account for human illness and lost production within the United States are estimated to be as high as 2.3 billion dollars annually (Hurd, 2004). Antibiotic treatment of the infection has been successful in the past but new multi-drug-resistant *Salmonella* strains are rapidly emerging. There are also a lot of vaccines to prevent *Salmonella* infections. However, the efficacy of the vaccines currently available is not always optimal (Cherayil and Antos, 2001).

1.4.1. Pathogenesis of *Salmonella*

Public health risks and economic losses associated with bacterial infections have led to the aim of understanding the mechanisms of bacterial pathogenesis and host immunity throughout the various stages of infection. *S. typhimurium* infects a wide range of animal hosts, including pigs, poultry, cattle, and humans. After entering the small bowel, salmonellae must traverse the intestinal mucus layer before encountering and adhering to cells of the intestinal epithelium. Salmonellae express several fimbriae that contribute to their ability to adhere to intestinal epithelial cells. Microscopic studies reveal that salmonellae invade epithelial cells by a morphologically distinct process termed bacterial-mediated endocytosis. Shortly after bacteria adhere to the apical epithelial surface, profound cytoskeletal rearrangements occur in the host cell; disrupting the normal epithelial brush border and inducing the subsequent formation of membrane ruffles that reach out and enclose adherent bacteria in large vesicles (Fig. 2) (Ohl and Miller, 2001). Salmonellae appear to preferentially adhere to and enter the M cells of the intestinal epithelium, although invasion of normally nonphagocytic enterocytes also occurs (Beier and Gebert, 1998).

Bacterial mediated endocytosis

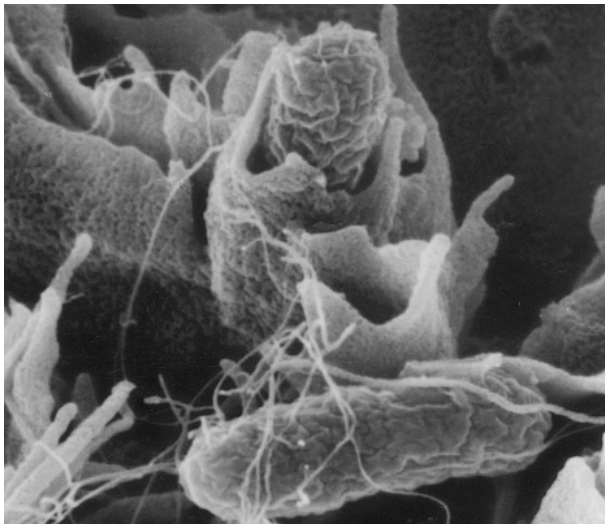

Fig. 2. *Scanning electron micrograph showing Salmonella typhimurium entering a Hep-2 cell through bacterial mediated endocytosis. Membrane ruffles extend from the cell surface, enclosing and internalizing adherent bacteria.*

In addition to invasion of the intestinal epithelial barrier, *Salmonella* induce a secretory response in the intestinal epithelium and initiate recruitment and transmigration of neutrophils into the intestinal lumen associated with production of several cytokines. Specifically, epithelial cell production of the potent neutrophil chemokine interleukin-8 (IL-8) appears to be important in recruiting neutrophils to the submucosal space, and factors not yet defined promote transmigration of neutrophils across the epithelial barrier (Lundberg et al., 1999).

Once across the intestinal epithelium, salmonellae encounter another obstacle of innate immunity, the submucosal macrophages. *Salmonella* serotypes that cause systemic infection enter macrophages and activate virulence mechanisms that allow evasion of the microbicidal functions of the phagocyte, permitting survival and replication in the intracellular environment (Mastroeni et al., 2000). Migration of infected phagocytes to other organs of the reticuloendothelial system probably facilitates dissemination of bacteria in the host (Ohl and Miller, 2001). Although residence in the macrophage shields the bacterium from elements of humoral immunity, it also exposes the bacterium to the nutrient-poor and microbicidal environment of the phagosome. Antimicrobial activities of the macrophage include production of reactive oxygen and nitrogen species, as well as the antimicrobial peptides and hydrolytic enzymes that enrich the mature phagolysosome. Salmonellae express several enzymes that directly inactivate reactive oxygen and nitrogen species produced by the macrophage. *Salmonella*'s resistance to nitric oxide (NO) and related reactive nitrogen compounds found in the macrophage is mediated partly by synthesis of homocysteine, an antagonist of NO (De Groote et al, 1996). Salmonellae also produce superoxide dismutases that can inactivate reactive oxygen species (Fang et al., 1999). Inactivation of these enzymes leads to decreased macrophage survival and attenuated virulence.

Analysis of the genetic structure of bacterial pathogens reveals that virulence genes often cluster in localized regions of the chromosome, termed pathogenicity islands (Groisman and Ochman, 1996). *Salmonella* pathogenicity island 1 (SPI-1) encodes genes necessary for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory responses. In contrast, *Salmonella* pathogenicity island 2 (SPI-2) encodes genes essential for intracellular replication, and is necessary for establishment of systemic infection beyond the intestinal epithelium. *Salmonella* causes SPI-2-dependent activation of the extracellular signal-regulated kinase ½ (ERK1/2) signaling pathway that leads to cylooxygenase-2 (COX-2) expression, resulting in the upregulation of the main prostaglandins (PGs) that activate

protein kinase A (PKA) signalling pathway and the prostaglandins PGE2 and PGI2 (Jackson et al., 2000). PGE2 or PGI2 may participate in the inhibition of the host defense by deactivating immune responses against many types of infection through suppression of production of proinflammatory cytokines and nitric oxide (NO) radicals or enhancement of the synthesis of anti-inflammatory cytokines (Uchiya and Nikai, 2004). PKA down-regulates the superoxide-dependent mechanisms of killing ingested bacteria in phagocytes. Activation of the PKA signalling pathway via these prostanoids is important for the intramacrophage survival of *Salmonella* and could be a critical mechanism that *Salmonella* uses to survive within macrophages (Uchiya and Nikai, 2004).

In summary, induction of cytoskeletal rearrangements that lead to membrane ruffling and bacterial internalization, induction of transmembrane fluid and electrolyte fluxes, and synthesis of cytokine and prostaglandin mediators of inflammation are among the major mechanisms of pathogenesis by salmonellae (Ohl and Miller, 2001; Hueck, 1998).

1.4.2. Host defence against salmonellosis

Once a pathogen enters and moves through its animal host, the host innate immune system detects the presence of microbial pathogens using receptors capable of sensing and distinguishing microbial signature structure such as the lipopolysaccharide, lipoproteins, flagellin, peptidoglycan, and bacterial DNA (Tötemeyer et al., 2005; Kirschning et al., 1998). This ultimately leads to stimulation of innate immune functions of epithelia and phagocytes. LPS has been shown to initiate multiple intracellular signaling events, including the activation of NF-κB, which ultimately leads to the synthesis and release of a number of proinflammatory cytokines; such as IL-1, IL-6, IL-8 and TNF-α as well as inducible enzymes; such as, inducible nitric oxide synthase (iNOS) (Tötemeyer et al., 2005).

Cellular invasion by *Salmonella* triggers responses that dictate the outcome of infection. Most often, activation of these innate immune functions eliminates the microbe from the host, leading to an asymptomatic resolution of infection. This plateau phase limits the primary infection and depends upon the release of proinflammatory mediators leading to the infiltration of neutrophils and macrophages leading to the development of granulomatous lesion (Cherayil and Antos, 2001; Mastroeni et al., 2000). Alternatively, bacterial pathogens that survive the innate immune effectors may persist in the host, allowing continued recognition of microbial signature molecules and ongoing activation of cytokine production

and inflammation. These persistent host responses lead to signs and symptoms of disease. In lethal infections, the early growth of the microorganisms in the tissues results in high bacterial numbers that lead to the death of the animal.

The molecular interactions that determine the outcome of this dynamic encounter between host and *Salmonella* is known partially (Ohl and Miller, 2001). Immune cells that play a key role in controlling and clearing *Salmonella* include the gut epithelial cells, monocytes/macrophages, neutrophils, and dendritic cells. Host resistance to *Salmonella* relies initially on the production of inflammatory cytokines leading to the infiltration of activated inflammatory cells in the tissues. Thereafter, T- and B-cell dependent specific immunity develops allowing the clearance of *Salmonella* from the tissues and the establishment of long-lasting acquired immunity to re-infection. The increased resistance that develops after primary infection requires T-cells and cytokines such as TNF-α, interferon-γ (IFN-γ), IL-12, IL-15 and IL-18 (Ohl and Miller, 2001; Mastroeni et al., 2000). Lack of the anti-inflammatory cytokines IL-4 and IL-10 is protective against Salmonellea (Eckmann and Kagnoff, 2001). TNF-α is involved primarily in granuloma formation, whereas IFN-γ contributes to the formation of focal granulomas in the tissues and is crucial for macrophage activation. Both IL-12 and IL-18 positively regulate IFN-γ production presumably by NK cells and contribute to down-regulate IL-10 expression. IL-15 is involved in the emergence of natural killer (NK) cells and IFN-γ production as well as in resistance to infection (Mastroeni et al., 2000).

1.5. Transmissible gastroenteritis virus (TGEV)

Transmissible gastroenteritis (TGE) is a very important and highly infectious disease of pigs caused by a corona virus called transmissible gastroenteritis virus (TGEV). Based on antigenic or genetic relatedness, coronaviruses are classified into three major groups, of which Group I includes TGEV among other related viruses (Saif, 2004). Coronaviruses are enveloped single-stranded RNA viruses with a genome ranging 28-32 kb encoding four major structural proteins, i.e. nucleocapsid (N) protein surrounding the genome and three membrane proteins: the surface spike (S) glycoprotein, membrane (M) glycoprotein and the envelope (E) protein. Hemagglutinin-esterase (HE) is another structural protein that forms a short-spike surface projections in group-II coronaviruses. The S protein is a critical determinant of viral attachment and fusion, species-specificity, and pathogenicity (Saif, 2004). Aminopeptidase N is known to be the functional receptor for TGEV and is abundant in the epithelium of the porcine intestine (Delmas et al., 1990). The general mechanism of replication of coronaviruses is explained by Weiss and Martin (2005) (Fig. 3).

Replication of coronaviruses

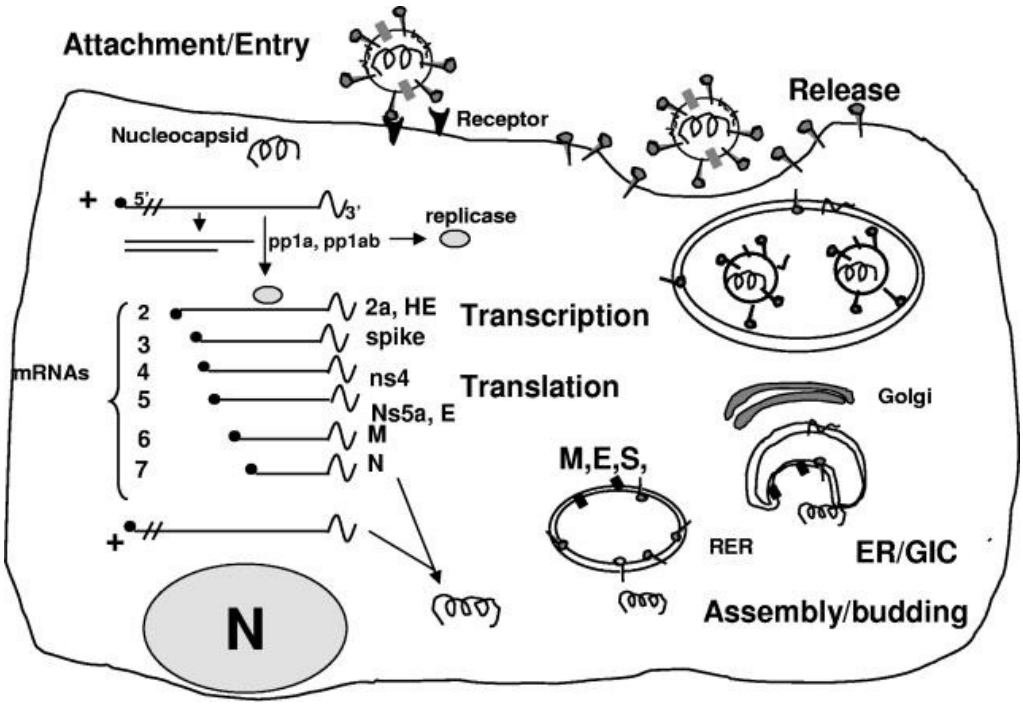

Fig. 3. *Coronaviruses attach to specific cellular receptors via the spike protein; this triggers a conformational change in spike which then mediates fusion between the viral and cell membranes which results in the release of the nucleocapsid into the cell. After entry, virus-specific RNA and proteins are synthesized in the cytoplasm. Proteins are translated and new virions are assembled by budding into intracellular membranes and released through vesicles by the cell secretory mechanisms. RER, rough endoplasmic reticulum; ER/GIC, endoplasmic reticulum/Golgi intermediate compartment (Weiss and Martin, 2005).*

The porcine coronavirus is a major cause of viral enteritis and fetal diarrhea in swine, which is most severe in neonates, with mortality resulting in significant economic loss (Enjuanes et al., 1995). TGEV replicates in and destroys the mature absorptive epithelial cells of the small intestine causing villous atrophy (Kemeny et al., 1975). The virus is shed in large numbers in the pig faeces that are the major source of feco-oral transmission. The virus can survive for long periods outside the pig in cold or freezing conditions (http://www.thepigsite.com/pighealth/article/301/transmissible-gastroenteritis-tge).

It has been suggested that several factors may contribute to the severity of TGEV in neonates. These include the relatively low replacement rate of enterocytes in newborn pigs (Moon, 1973); the presence of enterocytes of fetal origin, containing a prominent tubulo-vacuolar system which might facilitate virus replication, on the villi of newborn pigs (Wagner et al., 1973); and the lack of natural killer activity in the intraepithelial lymphocytes of newborn pigs (Cepica and Derbyshire, 1984). The preferential tropism of the virus for the villous enterocytes of newborn pigs could also be related to the presence of specific high-level virus-binding sites on these enterocytes, which are lacking on the cryptal enterocytes and on the enterocytes of older piglets (Weingartl and Derbyshire, 1993).

Although the virus is recognized in 1946 (Doyle and Hutchins, 1995), occurrences of TGE have become more sporadic and the disease is still reported from parts of Europe, North America and Asia (OIE, 2001). There is no specific treatment for TGE. Various types of vaccines have been evaluated for protection against TGEV but were unable to induce either passive or active protection against the virus (Weiss and Martin, 2005). The main reason proposed for TGEV vaccine failures was their inability to stimulate high levels of secretory IgA (SigA) antibodies in the milk of sows naturally infected with TGEV (Saif and Wesley, 1992). Furthermore, these vaccines did not adequately protect the sow against TGE, causing illness in the sow and failure to passively protect her piglets. Experimental studies on the available vaccines against TGEV have shown a lack of efficacy (Paton and Brown, 1990) or only partial cross-protection (Cox et al., 1993; Van Cott et al., 1994).

1.6. A closer look at selected receptors, enzymes, cytokines and inhibitors

The modulation of gastrointestinal inflammation is multi-factorial as previously discussed. Here we will look a bit more closely at the role of some receptors, enzymes and cytokines that function as assistants in the protection and repair of the GIT.

1.6.1. Receptors

Gut associated participation in the innate immune response is mediated through pattern recognition receptors (PRRs), which are molecules that recognize conserved pathogen associated molecular patterns (PAMPS). In this study, the effect of probiotic treatment on the expression of three different receptors, namely toll like receptors (TLR), CD9 and CD36, during *Salmonella* infection is measured. TLRs are one such pattern recognition receptor whose expression has been identified on intestinal epithelial cells (Abreu et al., 2005). The function of these TLRs is to convert the gut recognition of pathogen associated molecular patterns into signals for enhanced immune function, including the increase in anti-microbial peptide expression, barrier fortification and proliferation of epithelial cells (Abreu et al., 2005).

Activation of LPS-responsive cells, such as monocytes and macrophages, occurs rapidly after the binding of LPS to the 55-kd glycoprotein protein, CD14. Binding of LPS to CD14 requires the serum factor, LPS binding protein (LBP), which delivers LPS to CD14-expressing monocytes/macrophages (Kirschning et al., 1998). In CD14-negative cells such as endothelial cells, granulocytes and lymphocytes, soluble CD14 (sCD14) found in serum is thought to functionally replace membrane-bound CD14. Since CD14 is not a transmembrane protein, it lacks the ability to transduce cytoplasmic signals (Chow et al., 1999) and before the recent discovery of Toll-like receptors (TLRs), the identity of a transmembrane protein that could relay LPS-induced signals across the cell-surface membrane remained elusive. Several lines of evidence suggest that one or more members of the TLR family is the cell-surface receptor for LPS (Chow et al., 1999). TLR4, in association with the protein CD14, is the dominant LPS receptor in mammals and activates NF-κB–controlled genes for the inflammatory cytokines IL-1, IL-6 and IL-8 (Chow et al., 1999; Tötemeyer et al., 2005). In addition to TLR4, TLR2 and TLR9 play a role in controlling infection, particularly in the later stages of bacterial infection. Development of the plateau phase during sublethal *Salmonella* infection correlates with up-regulation of TLR2 and TLR9 mRNA expression (Tötemeyer et al., 2005). TLR2 is known to recognize bacterial lipoproteins and lipoteichoic acid

(Tötemeyer et al., 2005) and TLR9 is activated by bacterial DNA (Hemmi et al., 2000). The anti-inflammatory effect of probiotics is mediated by TLR9-probiotic DNA interaction and is not induced by TLR2 or TLR4 signaling (Rachmilewitz et al., 2004).

The other receptor indicated in this study is CD9, a tetraspannin protein, with a molecular mass of 22 to 28kDa (Lanza et al. 1991; Ovalle et al., 2007) that binds with other transmembrane proteins such as CD4, CD8, major histocompatibility complex class (MHC) I and II proteins and integrins. It also binds to intracellular signalling molecules including phosphatidylinositol 4-kinase, phosphatases and small guanosine triphosphate (GTP)-binding proteins. Yubero et al. (2003) have shown that CD9 is ubiquitously expressed in pigs with high levels of expression on platelets, intestine, lung, spleen, lymph node, alveolar macrophages and the heart. They also showed its abundant presence in peripheral mononuclear leukocytes and smooth muscles and lower levels of expression in liver, skin, polymorphonuclear granulocytes, testis and bone marrow.

Although the definitive biological functions of CD9 still remains elusive, tetraspanins are considered as adapter or facilitating proteins. By facilitating the formation of multimolecular complexes, they modulate key cellular functions like cell activation, proliferation and differentiation, cell adhesion, motility and migration, cell fusion, apoptosis, and signal transduction (Boucheix and Rubinstein, 2001; Ovalle et al., 2007). Tetraspanins interact with many proteins that play critical roles in the immune system, like the B cell receptor (BCR) and the T cell receptor (TCR), class I and class II MHC antigens and co-receptors such as CD4 and CD8 (Ovalle et al., 2007). The role of CD9 in the fusion of gametes, myoblasts and virus-infected cells has been reported (Miyado et al., 2000; Tachibana and Hemler, 1999). CD9 is also essential to prevent the fusion of mononuclear phagocytes. Given that multinucleation endows cells with more resorptive capacity for extracellular components such as infectious agents, functional alterations of CD9 may contribute to the progression of infection and diseases (Takeda et al., 2003). Activation of platelet aggregation is also among the known functions of CD9 (Yubero et al., 2003). A role for CD9 in the regulation of tumour progression by means of its association with the protein motility-related protein-1 (MRP-1) has also been reported (Higashiyama et al., 1997). This protein is related to cellular mobility, and its interaction with CD9 seems to be related to the invasion capacity of tumour cells since this complex acts as a suppressor of cellular mobility, thus reducing the potential metastasis of the cells (Yubero et al., 2003). In animals, CD9 has also been found to be a putative cellular

receptor for various viruses (Willet et al., 1994; Hosie et al., 1993). Finally, CD9 plays a role in the function of B cells as it potentiates their migration. It also enhances the signals from the B cell receptor (BCR) and thus lowers the threshold for B cell activation (Ovalle et al., 2007).

The third important receptor addressed in this study is CD36. The immuno-modulatory role of CD36 in inflammatory diseases such as diabetes has been reported (Aitman et al., 1999). All CD36+ cells produce IL-10. One function of CD36 is in the uptake of apoptotic bodies by macrophages and DC and subsequent modulation of immune responses (Barrett et al., 2007). The relationship between high CD36 expression and high IL-10 production could preferentially deliver apoptotic bodies to IL-10-producing cells, favoring immune suppression over inflammation. Murine influenza down-regulates CD36 antigen (Sakai et al., 2002). Local delivery or production of IL-10 may be the mechanism by which CD36 engagement, in some cases, dampens inflammatory responses (Bzowska et al., 2002).

1.6.2. Enzymes

Among the killing mechanisms employed by phagocytic cells is the generation of highly reactive molecular species derived from either superoxide (O_2^-) or from nitric oxide (NO). These reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) inactivate the protein, lipid and/or nucleic acid components of the engulfed microbe. The production of ROIs is under the control of phagocyte NADPH oxidase (phox), a multi-subunit protein complex that becomes associated with the phagosomal membrane, where as, NO is produced from arginine in a reaction that is catalyzed by the cytosolic enzyme nitric oxide synthase (NOS). NO increases epithelial permeability to macromolecules and to luminal bacteria thereby contributing to the clinical manifestations associated with acute *Salmonella* gastroenteritis in humans. In any event, the secretory response could be considered as a primitive, nonimmune defense mechanism allowing the intestine to flush out the unwanted bacteria. ROI are required for host resistance as early as day-one post infection, whilst RNI play a crucial role at a later stage of the infection (Mastroeni *et al.*, 2000).

There are three isoforms of NOS. NOS1 and NOS3 are constitutively expressed in many cell types and are activated by a rise in intracellular calcium. NOS2, the predominant form found in phagocytic cells, is inducibly expressed in response to a variety of stimuli, particularly bacterial products and inflammatory cytokines. The major mechanism regulating NOS2 activity is transcriptional upregulation of its gene. Based on its inducible expression and its

relative independence from calcium concentration, NOS2 is also known as iNOS (Cherayil and Antos, 2001).

iNOS expression and NO production are likely to be increased in salmonellosis. Infective gastroenteritis has been shown to be associated with elevated plasma levels of nitrate derived from NO (Forte et al., 1999). Similarly, rectal biopsy specimens from patients with acute shigella dysentery have been found to display increased staining for iNOS in the epithelial cells, as well as in mononuclear cells infiltrating the lamina propria (Raqib et al., 2000). A similar study indicated that iNOS is induced in human intestinal epithelial cell lines by *Salmonella* infection (Witthoft et al., 1998). The increased iNOS expression and RNI generation is attributed to the direct or indirect effects of cytokines produced at the site of infection. IL-12 produced by activated macrophages can stimulate natural killer (NK) cells and helper T cells to produce IFN- γ, which in turn can act on macrophages to upregulate iNOS expression (Cherayil and Antos, 2001). Growth of *Salmonella* can be inhibited *in vitro* by compounds that generate NO or its metabolites. Pharmacological inhibitors of iNOS are also indicated to enhance the survival of the organism (Cherayil and Antos, 2001). This indicates that RNIs produced by macrophages and other cells would help to limit *Salmonella* at the later stages of infection.

The other enzyme associated with infection with *Salmonella typhimurium* is cylooxygenase (COX). Prostaglandins (PGs) produced in various types of cell are important mediators of inflammation or immune responses. The rate-limiting step in PG synthesis is catalyzed by cyclooxygenase (COX). COX converts arachidonic acid to prostaglandin H2 (PGH2), the common precursor to all PGs (Jackson et al., 2000). There are two isoforms of COX enzyme, encoded by distinct genes. COX-1 is constitutively expressed in most cell types and plays a role in gastrointestinal and reproductive function. COX-2 is normally expressed at very low levels but is strongly induced by various stimuli, including cytokines, hormones and lipopolysaccharide (LPS) (Uchiya and Nikai, 2004). Cox-2 expression is responsible for increased epithelial ion transport in infected tissue, which likely underlies secretory diarrhea associated with *Salmonella* infection (Bertelsen et al., 2003). Levels of this enzyme increased rapidly after infection of human intestinal xenografts with *Salmonella typhimurium*. In contrast, Cox-1 was constitutively expressed and *Salmonella* infection did not alter expression of this gene (Bertelsen et al., 2003). Similarly, cholera toxin enhanced Cox-2 levels and not Cox-1 levels in infected rat jejunum (Beubler et al., 2001). These data suggest that Cox-1 is

likely not involved in the basal regulation of transport, whereas induction of prostaglandin synthesis secondary to Cox-2 expression can rapidly up-regulate ion and likely fluid secretion. COX-2 expression is increased in the livers and spleens of mice infected with *Salmonella*, suggesting that the COX-2 pathway may play an important role in the establishment of systemic infection of *Salmonella* (Takano et al., 1998).

1.6.3. Cytokines

Most of the knowledge on the mechanism of immune modulation by probiotics comes from profile analyses of cytokines produced by a wide variety of immune cells in response to probiotic treatment. Cytokines mediate profound biological effects. Their multiple activities are regulated mainly at the level of their secretion, by expression of their receptors, by the concomitant action of several cytokines and by the occurrence of inhibitory proteins and specific carrier molecules. The biological activity of cytokines depends on the proportion of cytokines and inhibitory molecules. Cytokines are involved in some pathophysiological states because of excessive production or inadequate inhibition (Weckmann and Varela, 1996). Based on the type of T helper (Th) cells that produce them, cytokines are classified into Th1 and Th2 cytokines. Th1 cytokines include the proinflammatory cytokines IFN-γ, TNF-α, IL-1 and IL-2 and are important for cellular immunity against intracellular pathogens. Th2 cytokines; such as, IL-4, IL-10 and IL-6, are important for humoral immunity against extracellular pathogens (Delcenserie et al., 2008). An imbalance between the Th1/Th2 responses leads to inflammatory or immune suppressive conditions. The strain, dose and experimental setup dependent modulation of the expression of pro- and anti-inflammatory cytokines by probiotics is well reviewed by Delcenserie et al. (2008). However, extrapolations of effects of probiotics on cytokine response should be supported by properly conducted clinical studies.

IL-10 is anti-inflammatory cytokine produced by a variety of hematopoietic cells, including Th2 cells, monocytes, mast cells, regulatory T cells (Tr), dendritic cells (DC), and B cells (Weiss et al., 2004). However, Th2 cells are common sources of IL-10. Healthy individuals have peripheral blood mononuclear cells (PBMC) constitutively producing IL-10; suggesting that IL-10 could play a role in homeostatic immune regulation. IL-10 down-modulates adaptive and innate immune responses (Moore et al., 2001). It blocks antigen presentation and production of pro-inflammatory cytokines (Weckmann and Varela, 1996). IL-10 suppresses inflammation by inhibiting production of IL-1, TNF, and a variety of chemokines by antigen

presenting cells (APC), T cells, and neutrophils (Katsikis et al., 1994). IL-10 inhibits Th1 responses indirectly by blocking IL-12 production and MHC Class II up-regulation by APC (Weckmann and Varela., 1996).

Although the regulatory role of IL-10 in various pathological states has been well described, there has been little investigation of IL-10 as a potential homeostatic regulator of the immune response. Dominance of anti-inflammatory cytokines such as IL-10 is associated with reduced immune responsiveness and susceptibility to persistent infection while conditions such as cardiovascular disease and diabetes are linked to chronic inflammation and lower IL-10 levels. Conversely, elevated IL-10 levels are associated with chronic bacterial infection (Barrett et al., 2007). Thus, the broad range of IL-10-mediated immunoregulation constitutes a double-edged sword with the beneficial effect of limiting immunopathology, counterbalanced by increased susceptibility to infectious disease. An appropriate threshold for immune activation is critical for optimal protection from infection and side-effects of immune effector mechanisms.

Transforming growth factor-ß (TGF-ß), like IL-10, constitute an adaptive immunosuppressive response, which can appropriately protect against autoimmunity and inappropriately increase susceptibility to infection. TGF-ß is cytokine produced by platelets, monocytes, macrophages, lymphocytes and synovial fibroblasts. TGF-ß plays a role in a number of inhibitory effects on immune cells. It blocks the growth and differentiation of lymphocytes, NK activity, B-cell proliferation, IgG production, production and effects of TNF-α and IL-1 in response to LPS, IL-1 receptor expression and acute inflammation. On the other hand, TGF-ß promotes IL-1 production, IL-l receptor antagonist production by monocytes and TNF production (Weckmann and Varela, 1996). TGF-ß and TNF-α are mutual antagonists. The injection of TGF-ß or anti-TNF-α antibody protects rats against collagen type II-induced arthritis. In contrast, TNF-α or anti-TGF-ß antibody injection enhances this arthritis (Takabayshi et al., 2006). TGF-ß is also able to oppose IL-1 activities *in vitro* and *in vivo*. It does not interfere with IL-1 binding to its receptor, but induces reduction in the expression levels of the IL-1 receptors (ILR), IL-1RI and IL-1RII (Weckmann and Varela, 1996). It also alters IL-l-induced functions, such as lymphocyte proliferation. TGF-ß is able to induce IL-1 mRNA in monocytes and also to reduce IL-1R expression. The receptor inhibition prevents the cells from responding to IL-1. This explains how TGF-ß, minimizes inflammatory responses and promotes the repair process (Dubois et al., 1990).

IFN-γ is a well known antagonist of IL-4 in B-lymphocytes, by blocking the IL-4-induced expression of MHC class II as well as IgG1 and IgE secretion. IFN-γ induces proinflammatory cytokine production and inhibition of IL-10 production by monocytes. Because IL-10 blocks antigen presentation and production of proinflammatory cytokines, the blocking effect of IFN-γ over IL-10 production may promote autoimmune disease. IFN-γ is strongly inhibited by IL-4 and IL-10. Its slight anti-inflammatory effects may include its ability to block TNF-α-mediated activities (Weckmann and Varela, 1996).

IL-4 seems to exhibit a coordinated anti-inflammatory action. It exerts different effects on cytokines, according to cell type, mode of activation and type of culture. Among the effects caused by IL-4 is, suppression of IL-1, IL-6, IL-8 and TNF-α production by monocytes. IL-4 reduces auto-induction of IL-4, and increases IL-1 receptor antagonist (IL-lra) synthesis (induced by IL-1) (Weckmann and Varela, 1996). IL-4 and IL-10 have similar effects on cytokine expression by monocytes. It appears that IL-10 serves mainly as a suppressor of immune functions and that IL-4 has anti-inflammatory actions, because IL-10 inhibits the effects of several proinflammatory cytokines (Miossec, 1993).

Various types of bacteria have been shown to stimulate the release of IL-8. These bacteria include those that remain inside phagosomal vacuoles (*Salmonella* spp.) and those that enter the cytoplasm (*Listeria monocytogenes*) (Eckmann et al., 1993b). However, not all bacteria or bacterial products elicit an IL-8 response, for example, LPS or non-invasive bacteria including *E. coli* and *Enteroccocus faecium* were found to be unable to induce an IL-8 response (Eckmann et al., 1993b). Functionally, IL-8 is considered to have an important role in the initiation of inflammatory immune responses and the induction of IL-8 by inflammatory stimuli (e.g., LPS, TNF-α or IL-1β) aids in recruitment and activation of other immune cells (Harada et al., 1996; Miller and Krangel, 1992). With regards to IL-8 produced from IEC, Eckmann et al. (1993b) have shown that epithelial cells from the intestine and cervix function to serve as an early signaling system to the host immune system by releasing IL-8 after bacterial invasion. Probiotic strains differ on their effects on the expression of the various cytokines.

1.6.4. Inhibitors

In biological fluids or supernatants of cell cultures, there are factors called inhibitors, which show specific action on cytokines. Inhibitors may function at different levels: by interfering with the cytokine-receptor complex and thus inhibiting signal transduction; by altering transcription or translation processes, by interfering with secretion or degradation of cytokines or by neutralizing their ligand (Weckmann and Varela, 1996).

IL-1 receptor antagonist (IL-lra) was formerly called IL-1 inhibitor. IL-1ra is synthesized as a propeptide of 177 amino acids and the pure protein weighs 18-25 kD. IL-1ra is produced by monocytes and diverse tissue macrophages. It is also produced by neutrophiles, keratinocytes and epithelial cells. It binds to IL-1 receptors and competes with IL-lα or IL-lß. It has the same affinity to the IL-1 receptor, IL-1RI, as IL-1 but it does not induce any biological response. It does not activate any protein kinase normally activated by IL-1, and it is not internalized after its binding to IL-1RI (Dinarello and Thompson, 1991). The existence of a natural IL-1ra suggests that the body mounts its own response against inflammation and that IL-lra synthesis is a natural component for the resolution of the disease process. IL-1ra blocks multiple IL-1-induced effects such as inflammation, synthesis of IL-1, TNF and IL-6 by monocytes, nitric oxide production by smooth muscle and IL-8 production by mononuclear cells stimulated with LPS or IL-1 (Arend et al., 1990; Dinarello and Thompson, 1991). Adherent IgG induces peripheral blood monocytes to produce IL-1ra. The use of several cytokines; such as, IFN-γ, IL-4, TGF-ß, IL-2, IL-6 and IL-10, may enhance IL-lra production, which would block IL-1 (Weckmann and Varela, 1996).