4.0. DISCUSSION

4.1. *In-vivo* evaluation of the effect of *E. faecium* SF68 against *Salmonella typhimurium* DT 104

There are a number of possible means by which probiotics may alter health; one of those putative effects is the alteration of immune function. *E. faecium* SF68 affected both the local and the systemic immune system of the piglets. However, only alterations in specific immune parameters cannot be interpreted unidirectionally as beneficial or deleterious. Piglets fed with the probiotic *E. faecium* SF68 had a significant reduction in the CD8+ T lymphocytes in the IEL 24hrs after *Salmonella* infection. A former study using the same probiotic strain also reported significant decrease in the frequency of CD8+ T cells in the jejunal epithelium of piglets (Scharek et al., 2005). A parallel study on the piglets used in our study reported reduction in the frequency of the CD8+ T cells in the blood of the piglets before *Salmonella* infection took place (unpublished data). Thus, cumulatively, these data indicate that one of the biological effects of exposure to *E. faecium* SF68 is the reduction of the CD8+ T cells. However, the direction of this outcome on the health effect depends on the type of the infection model used and the clinical manifestation.

Using the same experimental animals, Szabó et al. (2009) reported a significantly higher number of *Salmonella* in the tonsils, colon and faeces as well as a slightly increased incidence of diarrhoea in piglets of the probiotic group when compared to the same control group as used in our study. This could possibly be due to the impairment of the cellular immunity against *S. typhimurium*; i.e., decrease in the CD8+ lymphocytes, attributed to the probiotic treatment that may have lead to an increased translocation and dissemination of the pathogen. By the same token, Emanuel et al. (2007) reported that *E. faecium* induced inflammation in feedlock steers when both the probiotic and the yeast were administered at the same time. In contrast, Scharek et al. (2005) and Pollmann et al. (2005) reported a remarkable decline in the colonization of piglets with other pathogens, namely ß-haemolytic and O141 serovars of *Escherichia coli* and reduced carryover infections of chlamydia from sows to piglets, as a result of the probiotic treatment. However, in their study, the pathogens were already established in the herd and could be isolated from many sows. Sows and piglets used in our study were proved to be negative for *Salmonella* before the experimental infection was performed. In the former studies, the feeding of *E. faecium* also lead to a reduction of CD8+ cells in the sows that may have resulted in to a refreshed infection of the sows with *E. coli* and

Chlamydia followed by an antibody boost against these pathogens. Piglets from the sows that were boosted during pregnancy could have benefited from the maternal antibodies that were transferred directly after birth with the colostrum. However, Szabó et al., (2009) also reported that despite the decreased CD8+ cells, piglets of the probiotic group used in our study had a stronger humoral immune response with significantly higher levels of IgM and IgA in the sera. Our results also showed that piglets of the probiotic group had a slightly higher proportion of CD4+ lymphocytes in the spleen and in the discrete PP. Parallel studies undertaken by other members of our research group observed a significantly increased frequency of CD16+ cells in the distal PP of the probiotic group (H. Mafamane, unpublished data). However, despite the increase in immune-responses attributed to the probiotic supplement, there were no significant differences observed regarding clinical signs in comparison to animals without probiotic feeding (Szabó et al. (2009).

It is clear that probiotics might have immunomodulatory effects; however, it is still unknown how these effects are achieved. A natural next step to analyze the effects of exposure to probiotics is to investigate various gene expression in the animals' immune cells. Such parameters too should be validated against clinical effects found in the disease models. Several studies have shown that probiotics can alter the expression of various genes of the immune system (Ukena et al., 2005; Ruiz et al., 2005; Ewaschuk et al., 2007). Most of our data on the effect of *E. faecium* SF68 on the gene expression profile were collected from the PBMC of the piglets. Although PBMC are only partially representative of immunocompetent cells, phenotypical similarities with respect to receptors involved in the recognition of bacterial antigens constitute the link between these cell population and other cells of the immune system, such as lymphocytes and macrophages. Thus studies on PBMC may provide important indications on functional aspects of the immune response to bacteria. There were individual variations in the expressions of genes of interests in each group (probiotic and control). Pig samples taken at the slaughterhouse show a wide inter-individual variety of immunological results (Saalmüller et al., 1987).

CD9 is associated with the production of anti-inflammatory cytokines such as TGF-ß, which also has a regulatory function during inflammation by initiating the production of IL-10 (Kitani et al., 2003; Ha et al., 2005). Our data showed that *E. faecium* SF68 can up-regulated the expression of CD9 and TGF-ß in the PBMC of the piglets (Fig. 20). Di Giacinto et al. (2005) also showed that probiotic effects are linked to the ability to induce TGF-ß bearing

regulatory cells. Suzuki et al (2009) reported that macrophage CD9 negatively regulates LPS response towards inflammatory cytokine production by preventing the formation of the LPS receptor complex. However, our data show that the expression of the pro-inflammatory genes for IL-1α, IL-1ß, IL-8 and TLR2 in the PBMC; and IL-1α in the distal PP of piglets of the probiotic groups were significantly up-regulated. *Salmonella typhimurium* has lead to a 15-fold increase in the expression levels of TLR mRNAs (Tötemeyer et al., 2005). Ewaschul et al (2007) reported that exposure of intestinal epithelial cells to DNA from pathogenic bacteria including *Salmonella* resulted in a significant increase in TLR mRNA expressions and IL-8 secretion. However, they reported no change in mRNA levels of TLR9 in response to lactic acid bacteria (Ewaschuk et al., 2007). Exposure of cells to commensal microorganisms has been shown not to affect the expressions of TLRs. Exposure of HT-29 epithelial cell to DNA from pathogenic organisms increased TLR9 expression, where as exposure of the cells to probiotic bacteria did not have this effect (Ewaschuk et al., 2007). On the other hand, the protective effect of probiotic DNA is mediated by the TLR9 signaling (Rachmilewitz *et al*., 2004) that leads to anti-inflammatory activities. Thus, our results showed the anti-inflammatory potential of *E. faecium* SF68 through the up-regulation of the genes for TLR9, CD9 and TGF-ß.

The pathogenesis of *Salmonella* also involves the production of inflammatory cytokines. Piglets of the probiotic group had a higher level of expression of the inflammatory genes for IL-1, IL-8 and TLR2. *Salmonella* induces multiple intracellular signalling events, including the activation of the nuclear factor-kB (NF-kB), which leads to the synthesis and release of a number of proinflammatory cytokines, such as IL-1 and IL-8 (Tötemeyer et al., 2005; Lundberg et al., 1999). Depending on the type of the strain used, a more pro-inflammatory or more immune-regulatory type of response could be stimulated by probiotics (Blum et al., 2002). However, while probiotic bacteria are able to induce an up-regulation of molecules involved in pro-inflammatory processes, it has been shown that these pro-inflammatory effects are likely to be transient (Ukena et al., 2005; Ruiz et al., 2005). The increased mRNA expressions of the inflammatory genes in the piglets of the probiotic group could be attributed to a higher rate of *Salmonella* infection in this group associated to the immunological disadvantage they have: reduction in CD8+ cells as a result of *E. faecium* supplementation. Our results indicate the biological potential of *E. faecium* to up-regulate the level of the inflammation regulatory genes, TLR9, CD9 and TGF-ß; however the up-regulation of these genes was not enough to favour a greater level of expression of the major anti-inflammatory

cytokine, IL-10 (Weckmann and Varela, 1996; Weiss et al., 2004) and to improve the clinical status of the piglets (Szabó et al., (2009).

Certain probiotic bacteria convey protection against only specific pathogens. *E. faecium* has been reported to protect against *E. coli* pathogens and chlostridia (Taras, 2006; Garcia Galaz, 2004), but not against *Salmonella* and *Campylobacter* (Vahjen and Männer, 2003). In contrary, Watthana et al. (2006) reported the potential of *E. faecium* and *E. gallinarum* strains isolated from a commercial probiotic product to inhibit the growth of *Salmonella enterica* serovar Enteritidis strain IF03313. This shows the strain specificity of the action of probiotics against different pathogens. Our results showed that *E. faecium* SF68 could not down-regulate *Salmonella*-associated inflammatory genes in piglets infected with 10^9 CFU *Salmonella typhimurium* DT104 /pig.

Finally, this study could show that *E. faecium* could modulate the immune system, particularly decrease the CD8+ lymphocytes in the IEL and up-regulate the anti-inflammatory genes for TLR9, CD9 and TGF-ß in the PBMC. Patterns of immune alteration due to the probiotics may be interpreted in the context of the clinical effects observed, so that eventually such patterns alone would be able to indicate possible beneficial or deleterious effects of probiotics. Information on patterns of effects indicative of positive or negative consequences of the use of probiotics will eventually form the basis of designing a protocol for the evaluation and application of probiotics to be put on the market.

4.2. *In-vitro* evaluation of the effect of *E. faecium* SF68 against TGEV

Since the availability of pig intestinal epithelial cells is limited, most of the data concerning the role of IEC in the innate immune response of the intestine have been obtained from *in vivo* experiments (Oswald, 2006). However, given the complex intercellular signalling that occurs within the gut, these experiments are more difficult to interpret than *in vitro* experiments on IEC.

The intestinal epithelium is composed of various cells; namely, absorptive epithelial cells, goblet cells, paneth cells, tuft cells, cup cells, M cells, crypt cells and villous cells. Like in goblet cells, the microvilli of both type I and type II cells were irregular in shape and sparse in number. The microvilli of goblet cells contain actin filaments (Kato and Owen, 2005; Fig. 38), which appeared to be absent in type II cells and observed in type I cells. However, both

type I and type II cells were negative for the expression of mucin and appeared morphologically different from goblet cells.

Goblet cells

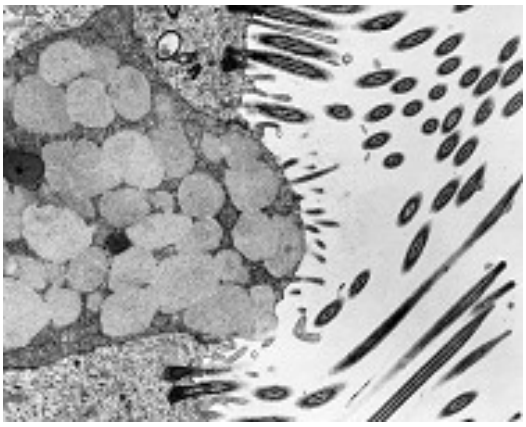

Fig. 38. *The microvilli of goblet cells: Atlas of electron microscopic pictures of cells, tissues and organs. http://www.uni-mainz.de/FB/Medizin/Anatomie/workshop/EM/EMMvilliE.html*

The sparse occurrence of organelles such as, smooth and rough ER in type I cells indicates the possibility of type I cells to be crypt cells (Kato and Owen, 2005). The fact that type II cells contain many secretory granules and that they express TNF-α may indicate that they could be paneth cells (Keshav et al., 1990). However, paneth cells have a characteristic pyramidal shape in contrast to the irregular shape of type II cells. Tuft cells or caveolated cells and cup cells are the other known cells of the epithelium of the intestine, whose function are not yet clear. Tuft cells are shown to have long microvilli (Blom and Helander, 1981) while cup cells have shorter microvilli but small mitochondria and few vesicular bodies (Madara, 1982). Besides, these cells have not yet been reported in swine.

Type II cells have many features similar to M cells, which are derived from intestinal epithelial cells (Bhalla and Owen, 1982). Like M cells (Kato and Owen, 2005), the apical cytoplasm of type II cells has closely packed mitochondria and numerous rounded tubular or oval microvesicles but few lysosomes (Fig. 29). The microvilli of M cells, like those of type I and type II cells, do not have or have very few microfilaments and the microvilli do not have a developed terminal web like those in the absorptive epithelial cells have (Fig. 39) (Kato and Owen, 2005). The location of the Golgi apparatus and ER just above the nucleus of type II cells (Regoli et al., 1995) (Fig. 29), the lack of mucin (Kato and Owen, 2005) and the low

lysosome content of the cytoplasm (Owen et al., 1986) are other similarities of type II cells with M cells.

Terminal web of the microvilli of absorptive epithelial cells

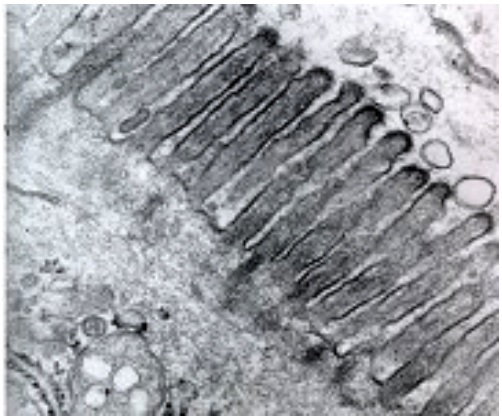

Fig. 39. *Terminal web of absorptive epithelial cells: Atlas of electron microscopic pictures of cells, tissues and organs.*
http://www.uni-mainz.de/FB/Medizin/Anatomie/workshop/EM/eigeneEM/26736.jpg

Many epithelial cells are characterized by having tight junctions (Schierack et al., 2006). The pig tight junction has been poorly characterised (Oswald, 2006). There was no clear cell-to cell contact detected in type II cells. This may be due to the expression of the genes for the cytokines TNF-α and IFN-γ in these cells. The independent and synergistic down-regulation of the transcription of occludin, one of the transmembrane sealing proteins localized to the tight junction, by TNF-α and IFN-γ has been demonstrated by Mankertz et al (2000). Type II cells share another common feature with M cells in that no connective junctions were ultrastructurally demonstrable like between M cells (Kato and Owen, 2005).

The expression of MHC class II could be detected in type II cells and not in type I cells (data not shown). MHC class I (SLA1) was expressed in both type I and type II cells (data not shown). Swine epithelial cells of both the villi and crypts express MHC Class I. In contrast, MHC class II is present only on epithelial cells of the villi (Oliver et al, 1994). Human IECs throughout the intestine constitutively express (MHC) class I, but they also constitutively express MHC class II predominantly in the small intestine (Lin et al., 2005; Hundorfean et al., 2007). The human M cells express MHC class II HLA-DR antigen, analogous to dendritic cells and macrophages, which are known to be APC (Nagura et al., 1991). This suggests the

ability of type II cells, like these cells, to function as APC. In addition, in humans only M cells in Peyer´s patch stain for HLA-DR but not colonic M cells (Kato and Owen, 2005). As compared to type II cells, type I cells shared few characteristics with M cells. Bye el. (1984) observed morphologically immature M cells with features shared by mature M cells and crypt cells in all regions of the follicle dome. Thus further characterization of the cells using M-cell markers would be of importance. In swine, cytokeratin 18 is a useful M cell marker (Gebert et al., 1994). No universal marker has been found to be used across species lines. In addition, studying the expression of sIgA receptors, which is another characteristic feature of M cells (Kato 1990), in type II cells could be the next step.

The observation that epithelial cells express and respond to cytokines, has put a new view on the epithelium as a functionally dynamic cell population. Type I and type II cells expressed a wide variety of cytokines (Table 5). Several authors have reported the constitutive expression of genes for several cytokines, including TGF-α, IL-1α, IL-1β, IL-10, IL-15 and IL-18, by the IEC (Dotan and Mayer, 2003; Oswald, 2006). Cytokines may play a role in the basal influx of immune cells into the mucosa, in epithelial cell growth and homeostasis. IEC are both a source and responder to IL-1. Other cytokines such as IL-6, IL-8 and TNF-α, are also expressed by normal epithelial cells but are markedly up-regulated in response to microbial infection (Dotan and Mayer, 2003; Schierack et al., 2005; Oswald, 2006). Panja et al. (1995) also reported that fresh human IEC could produce IL-6, which promotes T-cell growth and B-cell differentiation.

Type I cells did not express TNF-α while Type II cells did. Reports on other cytokines produced by IEC such as TNF-α have been inconsistent (Dotan and Mayer, 2003). The epithelial cell types lining the skin, digestive tract, urinary tract, reproductive tract and upper respiratory tract constitutively expressed IFN-ß (Bielenberg et al., 1998). However, only very few reports are available on the expression of IFN-γ by epithelial cells. The production of IFN-γ by a human epithelial colonic adenocarcinoma cell line, HCT-8, has been assessed using ELISA (Somaja Louis et al., 2004). Similarly, when oral epithelial cell cultures were infected with *C. albicans*, they produced significant amounts of IFN-γ after a 3h contact (Rouabhia et al., 2002). IFN-*γ* is also highly expressed by the trophectoderm of pig blastocysts, which represent a monolayer of polarized epithelial cells, during early pregnancy (Cencic and Bonnardière, 2002; Marcelo and Lefèvre, 2004). Our results, together with previously published data from other groups (Oswald, 2006; Dahan et al., 2007) emphasize

the ability of the IEC to influence its vicinity and to participate in immune regulation in the mucosa.

This study also showed that type II cells were sensitive to TGEV infection. TGEV is known to infect epithelial cells of the porcine small intestines; leading to potentially fatal gastroenteritis (Saif, 2004; Weiss and Martin, 2005). When examined under transmission electron microscopy, the enveloped virion of TGEV surrounded by the crown-like structure, corona, from which the name coronaviruses originated, was observed inTGEV-infected type II cells (Fig. 34). It has also been indicated that the virus can infect the epithelial cells of the upper respiratory tract (Saif, 2004). However, no cells other than epithelial cells have been reported to allow the growth of TGEV in it. This further supports to our hypothesis that type II cells are of epithelial nature.

One of the most promising areas of preventing viral diseases has been the use of probiotics and their role in health and disease. Various authors have indicated the potential of probiotics to boost antiviral activities (Cross, 2002; Tao, 2004; Cunningham-Rundles et al., 2000). In infants, *E. faecium* SF68 prevents episodes of diarrhea caused by rotavirus (Marteau et al., 2001; Bellomo et al., 1980). Our study is the first to show the antiviral potential of *E. faecium* SF68 against TGEV in a cell culture model. There have been hardly any biological studies of probiotic-virus-host interactions until Botić et al. (2007) designed the first cell model to study these interactions, whereby they showed that probiotic strains of lactobacilli can exert an antiviral activity against vesicular stomatitis virus (VSV).

In the present thesis, an increase in the survival of cells after TGEV infection and a drop in the TGEV yields were achieved when the monolayers had been treated either with the probiotic pellet or with its filter-sterilized culture supernatant prior to infection (Fig. 35, 36). This is in line with Botić et al. (2007), who reported that the pre-treatment of IPEC-J2 cells with probiotic bacteria pellets and supernatants decreased the yields of the rhabdovirus, VSV. These studies suggest a possible mechanism of antiviral activities of probiotics by interference with virus attachment and entry into the cells or with the viral multiplication, which could be mediated by the probiotic bacteria themselves and via a secreted antimicrobial product(s). Charteris et al. (1998) and Lee et al. (2000) stressed the importance of the viability and number of probiotic bacteria in the intestinal tract. Botić et al. (2007) reported that 10^5 CFU ml−1 is needed to exert an antiviral effect, with the maximal effect at 10^8 CFU ml−1.

Our data were inline with this observation; all the dilutions we tested 10^6 to 10^8 CFU ml−1 exerted an anti-viral effect, with the maximal effect at 10^8 CFU ml−1. Although the antiviral effect of the more diluted filter-sterilized culture supernatant of the probiotic bacteria was not strong, the one-to-one dilution of the supernatant with DMEM lead to a significant antiviral effect. Our results indicate that *E. faecium* SF68 produces metabolites with anti-viral activity against TGEV infection.

In addition to the effects of probiotics on cytokine responses of other cells of the immune system, probiotics may also influence cytokine production by epithelial cells themselves. In this study, the probiotic pre-treatment induced IL-6, IL-8 and IFN-γ expression in type II cells, which indicates that *E. faecium* SF68 alteres the state of the intestinal epithelial cells leading to an antiviral response. This is the first study to investigate the possible immunological alterations attributed to probiotic pre-treatment that could occur on epithelial cell cultures before and after a viral infection. Compared with the TGEV induced expression of the pro-inflammatory cytokines, the level of IL-6, IL-8 and IFN-γ expression induced by *E. faecium* SF68 was far lower. It is important that invasive pathogens induce the release of inflammatory cytokines from epithelial cells much stronger than non-invasive microbes stressing the role of the epithelial barrier integrity in regulating inflammation through signals that discriminate between enteropathogens and commensal bacteria (Shaykhiev and Bals, 2007). The probiotic *Lactobacillus sakei* also induced inflammatory cytokines, including IL-8, in Caco-2 cells (Blum et al., 2002). Nemeth et al. (2006) reported that various strains of probiotic lactobacilli induced significant but very low levels of IL-8 when compared with those induced by *Salmonella*. Several studies indicate that epithelial cells release abundant pro-inflammatory cytokines as a result of injury or infection by invasive pathogens (Shaykhiev and Bals, 2007). Human oral epithelial cell cultures infected with *Candida albicans* also produced significant amounts of IFN-γ (Rouabhia et al., 2002). Induction of PBMC IFN-γ by probiotic strains of *Lactobacillus* species has also been reported (Blum et al., 2002). In addition, in contrast to the non-pathogenic *E. coli*, enteropathogenic *E. coli* induced a long-lasting pro-inflammatory signal in CaCO-2 cells (Blum et al., 2002). Prolonged secretion of IL-6 by different epithelial cells as a result of persistent infection is currently thought to be critical for the maintenance of chronic inflammation (Bartoccioni et al., 2003; Kaplanski et al., 2003).

The probiotic strain of *E. faecium* used in our study significantly down-regulated the virus induced inflammatory cytokines. This could possibly be due to the reduced multiplication of the virus by the probiotic pre-treatment of the host cells. *In-vitro* pre-incubation of the epithelial cell line HT-29 with probiotics down-regulated the pro-inflammatory response induced by *Salmonella typhimurium* (O´Hara et al., 2006). Pre-treatment of a human epithelial cell-line with probiotic lactobacilli also resulted in an inhibition of *Salmonella* induced IL-8 expression (Nemeth et al., 2006; Silva et al., 2004; O´ Hara et al., 2006). Similarly, incubation of Caco-2 cells with the probiotic *Lactobacillus plantarum* reversed TNF-α -induced increases in intestinal permeability and IL-8 secretion (Ko et al., 2007). The study by Jin-Gang et al. (2005) also showed the down-regulation of *E-coli*-induced IL-6 production by probiotic *Lactobacillus* strain in primary epithelial cells. NF-κB is activated by proinflammatory stimuli through regulated phosphorylation, ubiquitination and proteolysis of a physically associated class of molecules called inhibitors of NF-KB function (IKBs). Non-virulent bacterial strains inhibit NF-κB by blocking the degradation of IκB, which thus remains associated with NF-κB, blocking its activation and thereby attenuating secretion of inflammatory cytokines by epithelial cells (Neish et al., 2000).

In summary, these results suggest possible mechanisms of the beneficial effect of probiotics to intestinal infections might be associated with the interference of the probiotic bacteria with the viral attachement, entry into the host cells or multiplication inside the cells or through the down-regulation of the expression of inflammatory genes induced by infecting agents. Although it remains unclear how these protective effects are achieved, it should be recalled that these effects required the pre-treatment of epithelial cells with probiotics; suggesting that the probiotic must initiate signalling in advance of a challenge to have an effect.