Aus dem Institut für Veterinär-Physiologie
des Fachbereiches Veterinärmedizin
der Freien Universität Berlin

Probiotic effects on epithelial barrier properties and inflammatory response in an infection model of the porcine intestine

Inaugural-Dissertation
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Martina Kern
Tierärztin aus Wertheim

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Meiner Familie

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List of abbreviations

AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a caspase-recruitment domain
ATP	Adenosine triphosphate
Caco-2	Human epithelial colorectal adenocarcinoma cells
cAMP	3′,5′-cyclic adenosine monophosphate
CARD	Caspase-recruitment domain
CD	Cluster of differentiation
CFTR	Cystic fibrosis transmembrane conductance regulator
DAMPs	Damage-associated molecular patterns
E. coli	*Escherichia coli*
E. faecium	*Enterococcus faecium* NCIMB 10415
ETEC	Enterotoxigenic *Escherichia coli*
GALT	Gut-associated lymphoid tissues
IBD	Inflammatory bowel disease
IL	Interleukin
IPEC-J2	Intestinal porcine epithelial cell line J2
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LT	Heat-labile enterotoxin
MoDC	Monocyte-derived dendritic cells
NBD	Nucleotide-binding domain
NLRP	Nucleotide-binding oligomerization domain-like receptor protein
PAMPs	Pathogen-associated molecular patterns
PRR	Pattern recognition receptor
PWD	Post-weaning diarrhea
PYD	Pyrin domain
ROS	Reactive oxygen species
R_t	Transepithelial electrical resistance
ST	Heat-stable enterotoxin
TJ	Tight junction
TNF	Tumor necrosis factor
ZO	Zonula occludens protein

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Chapter 1: Introduction

One of the most critical periods in piglet rearing is the time post-weaning (Campbell et al. 2013). In this phase, piglets are most susceptible to infections since they are exposed to multiple stress factors (Campbell et al. 2013). These factors include separation from the mother sow and littermates, adaption to a new environment, and changes in diet and feeding (Pluske et al. 1997, Niekamp et al. 2007).

A common infection in the post-weaning period of piglets is post-weaning diarrhea (PWD) (Luppi et al. 2016). PWD is a multifactorial disease (Laine et al. 2008). Enterotoxigenic *Escherichia coli* (ETEC) are frequently isolated in piglets suffering from PWD (Kyriakis et al. 1999). The infection with ETEC provokes severe diarrhea by elevating the secretion of electrolytes into the intestinal lumen (Nagy et al. 1999). It often results in the death of newborn piglets and piglets in the post-weaning period (Francis 2002).

Until the end of the year 2005, antibiotic growth promoters were commonly administered in the swine industry (Heo et al. 2013). They were used to improve health, body weight gain, and feed conversion and to reduce morbidity, mortality, and subclinical infections (Cromwell 2002). Since 2006, antibiotic growth promoters have been banned in the EU as stated in EU Regulation (EC) No 1831/2003 and Commission Regulation (EC) No 429/2008 (The European Parliament and the Council of the European Union 2003, The Commission of the European Communities 2008). The Scientific Steering Committee recommended this ban in its opinion of 28 May 1999 because of the increasing prevalence of pathogenic microorganisms resistant to antimicrobial agents, a prevalence that has serious implications for both human and animal health (The European Parliament and the Council of the European Union 2003).

A suggested alternative to antibiotic growth promoters is the use of probiotics (Dowarah et al. 2017). Probiotics improve pig health and are thought to decrease the risk for certain diseases (Alexopoulos et al. 2004, Ouwehand et al. 2016). Hence, probiotics might help to reduce the need for antibiotic use and to minimize the risk of antimicrobial resistance development (Ouwehand et al. 2016, Tang et al. 2017). Several studies have demonstrated positive probiotic effects on diarrhea-associated infections in post-weaning piglets (Kyriakis et al. 1999, Zeyner et al. 2006, Zhang et al. 2010, Barba-Vidal et al. 2017, Pan et al. 2017). Supplementation of the licensed probiotic feed additive *Enterococcus faecium* NCIMB 10415 (*E. faecium*) reduces the frequency and severity of diarrhea and improves daily weight gain in post-weaning piglets (Zeyner et al. 2006, Busing et al. 2015). However, the underlying cellular mechanisms are still unknown or only partly understood.

The aim of this study was to examine the effects of *E. faecium* on epithelial barrier properties and cytokine expression in the porcine intestine during an infection with ETEC. The porcine inflammasome was investigated as a potential mediator of probiotic effects.

The study design comprised experiments *in vitro* in the intestinal porcine epithelial cell line J2 (IPEC-J2)*, in vivo* in piglets, and *ex vivo* in porcine jejunum. The *in vitro* experiments focused on probiotic effects on intestinal barrier properties and cytokine expression during an ETEC challenge. *In vivo*, piglets were supplemented with *E. faecium*, and the expression of a variety of inflammasomes and inflammasome components was examined in the jejunum, ileum, and colon of these animals. In addition, the influence of age on the expression of inflammasome components was investigated in pigs of various ages. To examine the porcine inflammasome as a potential mediator of probiotic effects during infection conditions within intestinal tissue, an ETEC infection trial was conducted in porcine jejunum *ex vivo*.

Chapter 2: Literature review

2.1 Probiotics

2.1.1 Definition of probiotics

Probiotics were first defined by Lilly and Stillwell in 1965 as “growth-promoting factors produced by microorganisms” (Lilly and Stillwell 1965). However, the first discovery of probiotic mechanisms is attributed to Elie Metchnikoff who found out that “the dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes” (Metchnikoff 1908). Elie Metchnikoff is regarded as the grandfather of modern probiotics and revealed that the regular intake of lactic acid bacteria resulted in health benefits (Anukam et al. 2007). To date, a widely accepted definition of probiotics is “live microbial food ingredients that are beneficial to health” (Salminen et al. 1998). The latter definition has been complemented by the Expert Consultation of Food and Agriculture Organization and the World Health Organization (2001) which defined probiotics as “live microorganisms which when administered in adequate amounts confer a health benefit on the host”.

2.1.2 Probiotics in pig nutrition

Probiotics are widely used as preventive and therapeutic agents for humans and animals (Gaggia et al. 2010, Singh et al. 2013). According to the regulation (EC) No 1831/2003 of the European Parliament and of the Council, probiotics have to be approved as feed additives. The most frequently used probiotics in animal nutrition are *Bacillus*, *Enterococcus*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, and *Saccharomyces* strains (Anadon et al. 2006, Liu et al. 2018). They are used in all stages of pig farming (Barba-Vidal et al. 2019). In piglets, probiotics improve growth performance, reduce mortality, and decrease the incidence and severity of diarrhea (Kyriakis et al. 1999, Zeyner et al. 2006). In lactating sows, probiotics increase litter size and vitality and improve colostrum quality and milk quality and quantity (Chaucheyras-Durand et al. 2010). The faecal microbiota is modified in probiotic-fed sows and is carried over to their offspring (Starke et al. 2013). In weaning pigs, body weight gain and food digestibility are enhanced, whereas noxious gas emissions, i.e. ammonia (NH_3) and hydrogen sulphide (H_2S), are diminished (Lan et al. 2016). Probiotic supplementation also increases the diversity and richness of gut microbiota in weaned pigs (Shin et al. 2019). In growing and finishing pigs, positive effects on body weight gain, noxious gas emissions and meat quality have been detected (Liu et al. 2013, Liu et al. 2018).

2.1.3 Mode of action of probiotics

The beneficial health effects of probiotics are associated with the probiotic strain used (Ohashi et al. 2009). They are also affected by dosage, feed formula and the age of the animals (Liu et al. 2018). Although the underlying mechanisms are still only partially understood, the following strategies have been proposed to confer beneficial effects to the host:

- **Immunomodulation**

Probiotics can interact with the host immune system by modulating nonspecific cellular immune responses such as the release of cytokines and the activation of macrophages, natural killer cells, and cytotoxic T-lymphocytes (Ashraf et al. 2014). Many studies have revealed protective probiotic effects with regard to the expression and release of inflammatory cytokines during gastrointestinal infections (see section 2.4.6) and have provided evidence for limited pathogen-induced immune responses (Hegazy et al. 2010, Roussel et al. 2017).

- **Enhancement of intestinal barrier properties**

Probiotics have been demonstrated to improve intestinal barrier function in numerous studies (Rao et al. 2013). Beneficial effects of probiotics have been detected on gastrointestinal diseases such as Inflammatory bowel disease (IBD), which typically impair the intestinal barrier (Ukena et al. 2007, Eeckhaut et al. 2013). The probiotic-mediated modulation of tight junction (TJ) proteins, the inhibition of epithelial apoptosis, and an increase in mucus production are suggested to be responsible for the barrier-enhancing effects (Mennigen et al. 2009).

- **Modulation of the composition of the gut microbiota**

The intestine is inhabited by a large number of bacteria, called the intestinal microbiota, which colonize the intestinal tract stepwise at the beginning of life (Salminen et al. 2006, Frese et al. 2015). The intestinal microbiota support various crucial physiological functions such as host metabolism, nutrition, and immune function (Guinane et al. 2013). Perturbation of the intestinal microbiota has been linked to inflammatory disease phenotypes such as IBD (Hold et al. 2014). Various probiotics have been shown to restore the composition of the gut microbiota and to mediate beneficial functions to gut microbial communities (Hemarajata et al. 2013, Butel 2014). Since the microbiota influence the brain *via* the gut-brain axis, the use of probiotics also has a neuromodulatory function (Hemarajata et al. 2013, Emge et al. 2016).

- **Direct antagonism to pathogens**

In direct antagonism, probiotics kill or decrease the growth of pathogens or reduce pathogenic virulence factors, such as adhesins and toxins (Roussel et al. 2017, Liu et al. 2018). Probiotics also exhibit pathogen-inhibiting effects by competition for nutrients and binding sites on the

intestinal surface (Fuller 1991). Furthermore, they confer beneficial effects by inducing intestinal mucin expression and by producing antimicrobial substances (Mack et al. 1999, Mandal et al. 2014).

2.1.4 The effects of the probiotic *E. faecium* in pigs

E. faecium is a licensed feed additive for piglets and sows in the EU (The European Union, 2020). In porcine feeding trials, supplementation with *E. faecium* provides beneficial effects on health and growth performance and reduces the incidence and severity of PWD (Taras et al. 2006, Zeyner et al. 2006). In addition, the infection rate with chlamydiae is lower, and mucosa-adherent *Escherichia coli* (*E. coli*) pathotypes are reduced in piglets fed with *E. faecium* (Pollmann et al. 2005, Bednorz et al. 2013). The underlying mechanisms are still largely unknown, but several modes of action have been described in literature.

Modulatory effects of *E. faecium* affecting the immune system have been detected (Siepert et al. 2014, Klingspor et al. 2015). Interleukin (IL)-8, IL-10 and co-stimulatory molecule CD86 are reduced in piglets supplemented with *E. faecium* (Siepert et al. 2014). Furthermore, microRNA-432-5p, which regulates immune-relevant genes, is altered in piglets fed with *E. faecium* (Kreuzer-Redmer et al. 2016). In IPEC-J2 cells, *E. faecium* decreases IL-8 expression and release during infection with ETEC (Klingspor et al. 2015).

Intestinal barrier properties are also modulated by *E. faecium* (Klingspor et al. 2013, Klingspor et al. 2015). The probiotic enhances intestinal barrier integrity in jejunum (Klingspor et al. 2013) and in IPEC-J2 cells during an ETEC infection when pre-incubated with *E. faecium* (Klingspor et al. 2015).

Another mechanism involving *E. faecium* is the alteration of the gut microbiota (Starke et al. 2013). The composition of gut bacteria is modified during the *E. faecium* supplementation of sows, which then possess increased fecal lactobacilli cell numbers (Starke et al. 2013). This modification in fecal bacterial groups is transferred to their litters, however, the quantitative composition of the bacterial groups in the mother sow is not be reflected in the litters (Starke et al. 2013).

2.2 The effects of ETEC in pigs

ETEC is a common cause of PWD in piglets (Francis 2002, Goswami et al. 2011). Other farm animals are also susceptible to ETEC infections especially during the first few days of life, and, as a result, experience severe watery diarrhea (Nagy et al. 1999). In pigs, ETEC infection is a crucial cause for economic losses during the post-weaning period (Luppi et al. 2016). However, pigs aged more than approximately 8 weeks seem to be resistant to *E. coli* infection (Francis 2002).

2.2.1 Mode of action of ETEC

ETEC is a gram-negative bacterium that expresses the heat-labile enterotoxin (LT), the heat-stable enterotoxin (ST), or both enterotoxins (Nataro et al. 1998, Nagy et al. 1999, Luppi 2017). The toxins are released locally on enterocytes after adherence of ETEC to the microvilli of the small intestine (Nagy et al. 1999). The attachment of ETEC to the enterocytes is mediated by fimbriae on the bacterial surface such as F4 (K88), F5 (K99), F6 (987P), F7 (F41), F42, F165, F17 and F18 which are the most common adhesins of ETEC (Nagy et al. 1999, Luppi 2017). Porcine ETEC isolates only produce F4, F5, F6, F7 and F18 (Wilson et al. 1986). The enterotoxins result in the increased secretion of anions, especially chloride (Cl^-) and hydrogen carbonate (HCO_3^-), and the decreased absorption of sodium (Na^+) and Cl^- by absorptive cells (Nataro et al. 1998). These alterations cause intestinal loss of water and clinical diarrhea (Evans et al. 1996). The increased secretion of Cl^- is mediated *via* upregulation of 3′,5′-cyclic adenosine monophosphate (cAMP) levels and the activation of the cystic fibrosis transmembrane conductance regulator (CFTR) channel (Roussel et al. 2017). Furthermore, ETEC affects intestinal barrier properties by the loss of cell-cell contact and the modulation of TJ proteins, cell viability, and transepithelial electrical resistance (R_t) (Klingspor et al. 2015, Liu et al. 2015, Lodemann et al. 2016, Wu et al. 2016). ETEC also provokes the expression of numerous inflammatory cytokines such as IL-1α, IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α and upregulates the inflammasome component called nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) (Shimazu et al. 2012, Finamore et al. 2014, Klingspor et al. 2015, Liu et al. 2016, Lodemann et al. 2016, Wu et al. 2016).

2.2.2 Probiotics and ETEC infection

Probiotic supplementation has been shown to reduce piglet PWD, which is most frequently caused by infection with ETEC (Zeyner et al. 2006). Schroeder et al. (2006) have revealed that piglets supplemented with *E. coli* Nissle 1917 are not affected by diarrhea after experimental infection with ETEC at weaning. Clinical signs of ETEC infection can also be prevented or at least alleviated by the oral administration of LSP 122 comprising viable spores of *Bacillus licheniformis* to piglets (Kyriakis et al. 1999). Various underlying cellular effects of probiotics have been described in the literature and convey these clinical benefits during an ETEC infection in piglets.

Since probiotics are suggested to reduce proinflammatory responses (Donato et al. 2010), probiotic effects on the prevention of ETEC-induced elevated levels of proinflammatory cytokines have been investigated in several studies.

In vivo, IL-6 serum concentrations were lower in ETEC-infected piglets pre-treated with *Lactobacillus rhamnosus* GG than in piglets infected with ETEC and pre-treated with sterile

physiological saline (Zhang et al. 2010). The effect was seen within 12 h after the oral administration of the bacterial strains. However, IL-1β and TNF-α concentrations were not downregulated in the group with the probiotic pre-treatment (Zhang et al. 2010). TNF-α expression levels were also not reduced in an ETEC challenge trial by the probiotics *Pediococcus acidilactici* and *Saccharomyces cerevisiae* in the ileum of piglets (Daudelin et al. 2011).

In vitro, the study of Finamore et al. (2014) determined that co-incubation with *Lactobacillus amylovorus*, and with its cell-free supernatant, ameliorated the ETEC-induced increased release of IL-1β and IL-8 in human epithelial colorectal adenocarcinoma cells (Caco-2)/TC7 cells (Caco-2 cells at late passage). During ETEC infection, the probiotic also decreased the mRNA levels of IL-8 and TNF-α in IPEC-J2 cells (Wu et al. 2016). The same effect of *Lactobacillus plantarum* on IL-8 and TNF-α expression in ETEC-infected IPEC-J2 cells has been shown in the study of Wang et al. (2018). In addition, that study revealed that also the secretion of IL-8 and TNF-α, and mRNA and protein expression of IL-6 were decreased (Wang et al. 2018). Furthermore, the ETEC-induced expression and protein release of IL-8 were prevented by the pre-incubation of IPEC-J2 and Caco-2 cells with *E. faecium* (Klingspor et al. 2015).

In addition to studies analyzing the modulation of proinflammatory cytokines by probiotics, diverse other protective probiotic effects have been identified during ETEC infection.

The adhesion of ETEC to the small intestinal mucus of piglets has been shown to be inhibited by *E. faecium* 18C23, *Lactobacillus crispatus* and *Lactobacillus fermentum* (Blomberg et al. 1993, Jin et al. 2000). Konstantinov et al. (2008) have also detected that piglets supplemented with *Lactobacillus sobrius* have reduced levels of ETEC in the ileum. Furthermore, the decrease in the mRNA expression of zonula occludens protein (ZO)-1 and in the mRNA and protein expression of occludin by ETEC in the jejunum of piglets can be ameliorated by supplementation with *Lactobacillus plantarum* (Yang et al. 2014). Since ZO-1 and occludin are essential for the intestinal barrier (see section 2.3.1), this indicates a protective barrier-enhancing effect of the probiotic.

In vitro, *Lactobacillus sobrius* and *Lactobacillus reuteri* also diminish membrane barrier damage in IPEC-1 cells and reduce the adhesion of ETEC to the epithelium (Roselli et al. 2007, Wang et al. 2016). *Lactobacillus plantarum* prevents the ETEC-provoked decreases of R_t, and of claudin-1, occludin and ZO-1 expression in IPEC-J2 cells (Wu et al. 2016, Wang et al. 2018). It also reduces bacterial growth and adhesion of ETEC to IPEC-J2 cells and activates host defense proteins (Wang et al. 2018). *E. faecium* inhibits the ETEC-induced decrease in R_t in IPEC-J2 and Caco-2 cells and counteracts the expression of heat shock protein 70 in Caco-2 cells (Klingspor et al. 2015).

2.3 The intestinal barrier

The intestinal barrier separates luminal contents comprising nutrients, commensal bacteria, and pathogens from the underlying immune cells (Ohland et al. 2010). It consists of a single cell layer (Groschwitz et al. 2009). The barrier function is mediated by desmosomes, adherens junctions, and TJ that form protein-protein networks linking neighboring epithelial cells and sealing intercellular spaces (Groschwitz et al. 2009). Physiological mucosal barrier function is crucial for the gastrointestinal health of animals (Clayburgh et al. 2004, Wu et al. 2016). The intestinal barrier is a selectively permeable barrier and is responsible for the absorption of nutrients, electrolytes, and water, while providing an effective defense against intraluminal toxins, antigens, and the enteric microbiota (Groschwitz et al. 2009).

2.3.1 TJ proteins

TJ proteins are essential for the intestinal barrier and are located at the apical region of the lateral membranes (Tsukita et al. 2001, Gonzalez-Mariscal et al. 2003). If TJ function is impaired, the paracellular permeability alters dramatically and, can result in dysregulated immune responses and chronic inflammatory diseases such as IBD (Walsh et al. 2000, Xavier et al. 2007). TJ proteins are linked to the actin cytoskeleton and comprise transmembrane proteins such as claudins, junctional adhesion molecules and occludin, adaptor proteins such as the cytoplasmic ZO-1, ZO-2 and ZO-3, regulatory proteins, and transcriptional and post-transcriptional regulators (Matter et al. 2003).

To date, the protein family of claudins includes more than 20 members (Gonzalez-Mariscal et al. 2003). They can either enhance the intestinal barrier or form channels across TJs thereby increasing permeability (Gunzel et al. 2012). Claudin-1 has sealing functions and its expression has been reported in the heart, lung, liver, brain, kidney, testis, and intestine (Tsukita et al. 2001, Gunzel et al. 2012, Yang et al. 2015). Claudin-2 serves as a permeability-mediating TJ protein inducing the formation of cation-selective channels in the TJ of epithelial cells and is upregulated in IBD patients (Amasheh et al. 2002, Barmeyer et al. 2017). However, in IPEC-J2 cells, claudin-2 is not expressed at the mRNA and protein level (Zakrzewski et al. 2013). Claudin-3, -4, -5, and -8 represent TJ proteins with sealing properties (Amasheh et al. 2011, Markov et al. 2014). Claudin-5 and -8 are typically decreased in patients suffering from Crohn's diseases (Barmeyer et al. 2017). The function of claudin-7 is ambiguous (Amasheh et al. 2011). The overexpression of claudin-7 elevates the paracellular Na^+ conductance and decreases the paracellular Cl^- conductance (Alexandre et al. 2005). However, knockdown of claudin-7 expression also increases paracellular Na^+ permeation and depresses paracellular Cl^- permeation (Hou et al. 2006). The integral protein occludin enhances intestinal barrier function (Mankertz et al. 2000, Saitou et al. 2000).

The expression and distribution of TJ proteins are regulated by various factors. The intestinal microbiota are involved in fortifying the intestinal barrier *via* the regulation of intestinal epithelial cell turnover, epithelial regeneration and TJ proteins (Yu et al. 2012). Furthermore, cytokines can influence TJ proteins (Capaldo et al. 2009). They can act directly or modulate TJ proteins indirectly by influencing the underlying actin cytoskeleton (Youakim et al. 1999, Nusrat et al. 2000). The proinflammatory cytokines TNF-α and interferon (IFN)-γ have been shown to decrease occludin expression (Mankertz et al. 2000). These cytokines are elevated during Colitis ulcerosa and Crohn's disease, indicating an association of occludin with barrier defects during IBD (Roberts-Thomson et al. 2011).

2.3.2 Probiotics and the intestinal barrier

Probiotics are involved in the modulation of TJ proteins (Thomas et al. 2010). They affect two regulator control elements of TJ proteins, namely cytokine expression patterns and the composition of the microbiota in the gut (see section 2.1) (Walsh et al. 2000, Chaucheyras-Durand et al. 2010, Thomas et al. 2010, Yu et al. 2012).

Probiotic effects on the different TJ proteins contributing to an enhancement of the intestinal barrier have been described, for example, for *Lactobacilli* and *Escherichia coli* Nissle 1907 during a pathogenic challenge (Ukena et al. 2007, Seth et al. 2008, Yang et al. 2014, Wu et al. 2016, Wang et al. 2018). In the jejunum of piglets, the mRNA expression of ZO-1 and the mRNA and protein expression of occludin are decreased by infection with ETEC, but this can be inhibited by supplementation with *Lactobacillus plantarum* (Yang et al. 2014). *In vitro*, *Escherichia coli* Nissle 1907 enhances the intestinal barrier integrity *via* the upregulation of ZO-1 expression during dextran sodium sulfate-induced colitis in colonic intestinal epithelial cells of mice (Ukena et al. 2007). In Caco-2 cells, *Lactobacillus rhamnosus* attenuates the hydrogen peroxide (H_2O_2)-induced decrease in R_t, and the redistribution of occludin and ZO-1 (Seth et al. 2008). In IPEC-J2 cells, the ETEC-induced decrease in claudin-1, ZO-1 and occludin expression has been ameliorated by the probiotic *Lactobacillus plantarum* (Wu et al. 2016, Wang et al. 2018).

The following studies have not analyzed the different TJ proteins but have investigated probiotic effects on R_t. R_t measurements are a useful method for analyzing barrier functions *in vitro* (Chen et al. 2015). A high R_t reflects a low ionic conductance of the paracellular pathway (Srinivasan et al. 2015). R_t measurements have been applied as a widely accepted method for measuring TJ integrity (Ding et al. 2011). The probiotic mixture #VSL3 (consisting of the bacterial strains of *Bifodobacteria*, *Lactobacilli*, and *Streptococcus salivarius* subsp. *thermophilus*) improves intestinal epithelial barrier properties in the colon of mice by enhancing R_t (Madsen et al. 2001). This has been supported by other studies in which *Lactobacillus*

acidophilus, *Streptococcus thermophiles,* #VSL, and *Bifidobacterium infantis*, *bifidum,* and *breve* have increased R_t in human intestinal epithelial cells (Resta-Lenert et al. 2003, Otte et al. 2004, Ewaschuk et al. 2008, Lopez et al. 2012). Furthermore, treatment with *Lactobacillus farcimis* ameliorates impaired colonic barrier integrity as measured *via* a radioactivity assay during trinitrobenzene sulphonic acid-induced colitis in rats (Lamine et al. 2004). In IPEC-J2 cells, *Lactobacillus plantarum* diminished the increase in gut permeability to fluorescein isothiocyanate-dextran induced by ETEC (Wang et al. 2018).

Despite knowledge of the probiotic effects on intestinal barrier properties and TJs, interactions between the probiotic *E. faecium* and TJ proteins have as yet been elucidated only rarely. Claudin-4 protein expression is significantly decreased in the ETEC-incubated jejunum of control piglets, but not in the ETEC-infected jejunum derived from piglets pre-fed with *E. faecium* (Lodemann et al. 2017). This indicates a protective effect of *E. faecium*, since claudin-4 has barrier-sealing properties (Markov et al. 2014). Claudin-1, -2, -3 and -7 are not affected by bacterial treatment, as shown by the study of Lodemann et al. (2017).

2.4 Cytokines

Cytokines are small secreted proteins enabling communication between cells (Arango Duque et al. 2014). They can act in an autocrine, paracrine or endocrine manner triggering proinflammatory or anti-inflammatory pathways (Zhang et al. 2007). Most cytokines are produced by immune cells, although intestinal epithelial cells can also be a source of cytokines (Jung et al. 1995, Liles et al. 1995, Stadnyk 2002). One of the main functions of cytokines is the coordination of immune and inflammatory responses (Arai et al. 1990). Cytokines comprise lymphokines, monokines, interleukins, interferons, and a variety of growth factors (Liles et al. 1995). In the present study, the cytokines TNF-α and IL-1α, IL-1β, IL-6, and IL-18 have been investigated in the porcine intestine. Therefore, these cytokines are described under the following subheadings in more detail.

2.4.1 TNF-α

TNF-α (cachectin) is biologically closely related to IL-1α and belongs to the proinflammatory cytokines (Gordon et al. 1991, Dinarello 1994, Zhang et al. 2007). The activation of TNF-α expression is mostly induced by viruses and bacteria (Tchorzewski 1994). TNF-α mediates programmed cell death, cell survival, inflammation and immunity (Dinarello 2002, van Horssen et al. 2006). Within the intestine, it induces intestinal epithelial cell shedding and impairs intestinal barrier properties in IBD patients (Watson et al. 2012, Slebioda et al. 2014). TNF-α was discovered in 1984 and received its name because it mediates anti-tumor activity both *in vitro* and *in vivo* (Gaur et al. 2003). It exerts its biological effects *via* interaction with TNF-

receptors TNFR1 and TNFR2, which activate different signal cascades (Naude et al. 2011). TNFR1 is associated with the prevention and triggering of cell death processes, whereas TNFR2 is involved in anti-apoptotic reactions (Cabal-Hierro et al. 2012). TNF-α can exist as a membrane-bound form (mTNF-α) or as a soluble cytokine (sTNF-α) (Ardestani et al. 2013). mTNF-α is the predominantly produced form and can be released rapidly (Kriegler et al. 1988, Wajant et al. 2003). Activation of sTNF-α is mediated by TNF-α converting enzyme, which cleaves sTNF from its membrane-bound precursors (Black 2002). Whereas mTNF-α can stimulate both TNFR1 and TNFR2, sTNF-α primarily activates TNFR1 (Wajant et al. 2003).

2.4.2 IL-1α

The proinflammatory cytokine IL-1α is mostly released by immune cells but can also be secreted by epithelial cells, endothelial cells, smooth muscle cells, fibroblasts, and keratinocytes (Liles et al. 1995). It is a member of the IL-1 family and signals through IL-1 receptor type I, which is present in most cells (Sims et al. 2010). Deregulated IL-1α exerts potent inflammatory signals resulting in diseases with severe acute or chronic inflammation (Di Paolo et al. 2016). It is constitutively expressed in many cell types (Arend et al. 2008). However, the production of the precursor pro-IL-1α can be upregulated by growth factors and proinflammatory or stress-associated stimuli (Di Paolo et al. 2016). Upon cell death, IL-1α is often released, functions as an alarmin and recruits neutrophils (Rider et al. 2011, Garlanda et al. 2013, Yazdi et al. 2013). Vasoactive intestinal polypeptides are also elevated by IL-1α (Eskay et al. 1992). The effects of the members of the IL-1 family are tightly regulated by receptor antagonists, decoy receptors, and signaling inhibitors (Garlanda et al. 2013).

2.4.3 IL-1β

IL-1β is classified as a proinflammatory cytokine (Al-Sadi et al. 2007). It is crucial for resistance to pathogens, but can also mediate auto-inflammatory diseases, provoke acute tissue injury and cause damage in chronic diseases (Zhu et al. 2006, Dinarello 2009, Lopez-Castejon et al. 2011). IL-1β belongs to the IL-1 family of cytokines and is released by cleavage of pro-IL-1β *via* the protease caspase-1 upon inflammasome activation (Sims et al. 2010, de Veerdonk et al. 2011) (see also section 2.5). It is produced extremely rapidly by cells of the immune system such as monocytes, macrophages, and dendritic cells, but also by intestinal epithelial cells (Matsushima et al. 1986, Waterhouse et al. 1999, Lopez-Castejon et al. 2011). In the intestine, IL-1β increases epithelial permeability and plays a crucial role in intestinal inflammation (Al-Sadi et al. 2007, Coccia et al. 2012). The biological function of IL-1β is exerted *via* binding to the IL-1 receptor type I and can be antagonized by the IL-1 receptor antagonist (Dinarello 1994, Wang et al. 2010).

2.4.4 IL-6

IL-6, also known as interferon-β2, B-cell stimulatory factor-2, and hybridoma/plasmacytoma growth factor, is a multifunctional proinflammatory cytokine (Ataie-Kachoie et al. 2014). It has a crucial function in both initiating and resolving inflammation (Scheller et al. 2011). IL-6 is produced quickly by several cell types such as macrophages, dendritic cells, B-cells, fibroblasts, endothelial cells, mast cells, and epithelial cells in response to infections or tissue damage (Hedges et al. 1992, Liles et al. 1995, Diehl et al. 2002, Tanaka et al. 2014). It mediates acute phase responses, hematopoiesis, and immune reactions (Tanaka et al. 2014). Within the intestine, IL-6 has been shown to stimulate intestinal epithelial proliferation and regeneration after injury (Kuhn et al. 2014). Generally, IL-6 mediates inflammation *via* the soluble IL-6 receptor, whereas signaling *via* the membrane bound IL-6 receptor mostly resolves inflammation (Schaper et al. 2015).

2.4.5 IL-18

IL-18 belongs to the group of proinflammatory cytokines and plays a role in several autoimmune diseases, emphysema, myocardial function, metabolic syndromes, psoriasis, IBD, hemophagocytic syndromes, macrophage activation syndrome, sepsis, and acute kidney injury (Dinarello 2000, Dinarello et al. 2013). However, it can also have a protective function (Dinarello et al. 2013). It is released by cleavage of pro-IL-18 *via* caspase-1 (de Veerdonk et al. 2011), an event that is mediated by the inflammasome (see section 2.5). Like IL-1α and IL-1β, it belongs to the IL-1 family of cytokines (Sims et al. 2010). IL-18 is thought to maintain the healthy composition of the gut microbiota (Elinav et al. 2011). It can promote epithelial regeneration indirectly by the downregulation of IL-22-binding protein, thereby elevating the levels of IL-22 (Chen 2014). These elevated IL-22 levels induce epithelial proliferation and repair (Perusina Lanfranca et al. 2016). Furthermore, IL-18 activates IFN-γ, which regulates antitumor responses (Bohn et al. 1998, Chen 2014). In contrast to IL-1β, the precursor of IL-18 is constitutively present in nearly all cell-types (Dinarello et al. 2013). The activity of IL-18 can be regulated by constitutively expressed and secreted IL-18-binding protein (Dinarello 2000).

2.4.6 Interactions of probiotics and cytokines

The immunomodulatory effects mediated by probiotics through their alteration of cytokine expression and release represent an important mode of probiotic action (Hemarajata et al. 2013) and have been widely studied.

In many investigations, probiotics have been shown to modulate basal cytokine levels. In the duodenum of dogs, incubation *ex vivo* with *E. faecium* decreased basal TNF-α expression (Schmitz et al. 2014). However, in rainbow trout fed with *Lactobacillus rhamnosus* GG or

E. faecium, TNF-α and IL-1β expression was upregulated in the spleen, and IL-1β expression levels were also increased in the kidney compared with control animals (Panigrahi et al. 2007). In the ileum of piglets supplemented with the probiotic *Lactobacillus salivarius*, IL-6 expression was elevated, although this effect was not seen in the duodenum, and no effects were detected in the duodenum and ileum with regard to the expression of TNF-α (Zhang et al. 2011). On the contrary, the supplementation of suckling piglets with *E. faecium* EF1 decreased the production of IL-1β, IL-6, and IL-12 in the jejunum and ileum, whereas TNF-α release was downregulated in the ileum but upregulated in the jejunum (Huang et al. 2012). Furthermore, IL-8 expression was modulated in piglets supplemented with *E. faecium* and showed reduced levels of IL-8 in the spleen of 12-day-old piglets and in ileal Peyer's patches of 34-day-old piglets compared with control animals (Siepert et al. 2014).

In vitro, various effects have also been detected on basal cytokine expression. The expression of IL-1α and TNF-α was decreased by probiotic treatment with *Bifidobacterium longum*, *Bifidobacterium bifidum, Bifidobacterium breve, Lactobacillus rhamnosus, Lactobacillus acidophilus*, and *Lactobacillus paracasei* in human colon cancer cells (HT-29 cells) and in Caco-2 cells (Bahrami et al. 2011). IL-18 mRNA was upregulated by *Bifidobacterium longum, Bifidobacterium breve*, and *Lactobacillus plantarum* in Caco-2 cells and by *Bifidobacterium adolescentis, Bifidobacterium bifidum*, and *Lactobacillus rhamnosus* in HT-29 cells and Caco-2 cells (Bahrami et al. 2011). However, IL-6 expression levels were not affected by any of the *Bifidobacterium* and *Lactobacillus* strains as tested in the study of Bahrami et al. (2011).

Furthermore, the protective probiotic effects on proinflammatory responses induced by various pathogens or noxes have been investigated. The probiotic mixture VSL#3 ameliorated lipopolysaccharide (LPS)/D-galactosamine-induced TNF-α and IFN-γ expression and secretion in the colon (Ewaschuk et al. 2007). In addition, LPS/D-galactosamine increased IL-6 expression, although this expression was downregulated in the hepatic tissue of VSL#3-treated mice (Ewaschuk et al. 2007).

In vitro, the probiotic *Lactobacillus rhamnosus* GG attenuated the elevated mRNA and protein levels of IL-8 and counteracted the increased protein release of IL-11 in IFN-γ- and TNF-α-stimulated Caco-2bbe cells (Donato et al. 2010). In primary bovine mammary epithelial cells and primary bovine endometrial epithelial cells pretreated with *Lactobacillus rhamnosus*, the IL-6, IL-8, and TNF-α expression induced by *E. coli* was reduced (Wu et al. 2015, Liu et al. 2016). In addition, *E. faecium* abolished the swine influenza-virus-induced expression of IL-6 at 2 h and 6 h post infection, the expression of TNF-α at 6 h and 24 h post infection, and the increased levels of anti-inflammatory IL-10 at 24 h post infection in the porcine macrophage cell line 3D4/21 (Wang et al. 2013). In IPEC-J2 cells challenged with ETEC, *Lactobacillus plantarum* reduced IL-6, IL-8 and TNF-α expression and release (Wang et al. 2018). ETEC-induced IL-8 expression and release were also prevented in IPEC-J2 cells pre-incubated with

E. faecium, as previously mentioned in section 2.2.2 ‘Probiotics and ETEC infection’ (Klingspor et al. 2015).

Effects on cytokines have also been detected on applying culture supernatants of probiotics. The H_2O_2-induced expression of IL-8 and TNF-α was prevented by the simultaneous incubation with culture supernatant of *Lactobacillus plantarum,* but not with culture supernatant of *E. faecium* CECT 4515, *Lactobacillus casei, Bacillus amyloliquefaciens*, and *Bifidobacterium animalis subsp. lactis* in IPEC-J2 cells (Paszti-Gere et al. 2012). Indeed, the latter two probiotics increased the H_2O_2-induced TNF-α expression levels, and *Bacillus amyloliquefaciens* also potentiated H_2O_2-mediated IL-8 expression (Paszti-Gere et al. 2012). However, cell-free supernatants of *Lactobacillus acidophilus, Lactobacillus casei, Lactococcus lactis, Lactobacillus reuteri,* and *Saccharomyces boulardii* decreased IL-8 expression in LPS-stimulated HT-29 cells (De Marco et al. 2018).

Probiotic effects on IL-1β and IL-18 as an output of inflammasome activation are described in section 2.5.3 ‘Interactions of probiotics and inflammasomes’.

Despite knowledge that probiotics exert protective immunomodulatory effects, studies analyzing the effects of the probiotic *E. faecium* NCIMB 10415 have only been conducted on cytokine expression in the porcine intestine in Peyer’s patches, on IL-8 levels in IPEC-J2 cells (Siepert et al. 2014, Klingspor et al. 2015), and on TNF-α and IL-8 levels in the porcine jejunum (Lodemann et al. 2017). After the manuscript of this present thesis had been published, additional research has been conducted at our institute on the expression of IL-1β, IL-18, IL-8, and TGF-β in IPEC-J2 cells incubated with *E. faecium* (Loss et al. 2018, Loss et al. 2019).

2.5 Inflammasomes

Interactions have been described between probiotics and inflammasomes (Miettinen et al. 2012). Inflammasomes are intracellular multiprotein complexes that play a key role in innate immunity (Lissner et al. 2011, Zaki et al. 2011). A subgroup of pattern recognition receptors (PRR), namely the nucleotide-binding domain, leucine-rich repeat containing (NLR) proteins, are key intracellular mediators participating in the formation of inflammasomes (Takeuchi et al. 2010, Davis et al. 2011). Several types of inflammasomes are known, such as the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 1 (NLRP1), NLRP3, NLRP6, the NLR family caspase recruitment domain containing 4 (NLRC4), and the absent in melanoma 2 (AIM2) inflammasome (Broz et al. 2016). Inflammasomes influence the permeability of the intestinal barrier, the release of cytokines, and the composition of the gut microbiota (Zaki et al. 2010, Gagliani et al. 2014, Lei-Leston et al. 2017). They regulate intestinal homeostasis and the immune response to a wide range of microbial pathogens (Gagliani et al. 2014). Inflammasomes play an important role in bacterial infections, metabolic

syndrome, autoimmune disorders, and inflammatory diseases (Strowig et al. 2012, Vladimer et al. 2013). Their dysregulation might elevate the risk of tumorigenesis (Zaki et al. 2011). Most experiments in inflammasome research have involved investigations into effects in humans, mouse models, or immune cells (Guo et al. 2015). Data concerning inflammasomes in pigs or in the intestinal epithelium are currently available to an only limited extent.

2.5.1 The NLRP3 inflammasome

To date, the most frequently studied and best characterized inflammasome in mouse and human is the NLRP3 inflammasome, which is also known as cryopyrin, NALP3, CIAS1, PYPAF1, and CLR1.1 (Hoffman et al. 2001, Feldmann et al. 2002, Willingham et al. 2009, Jo et al. 2016). Upon stimulation, NLRP3 activates caspase-1, which induces the cleavage of pro-IL-1β and pro-IL-18 into their active forms IL-1β and IL-18 (Strowig et al. 2012, Alcocer-Gomez et al. 2014).

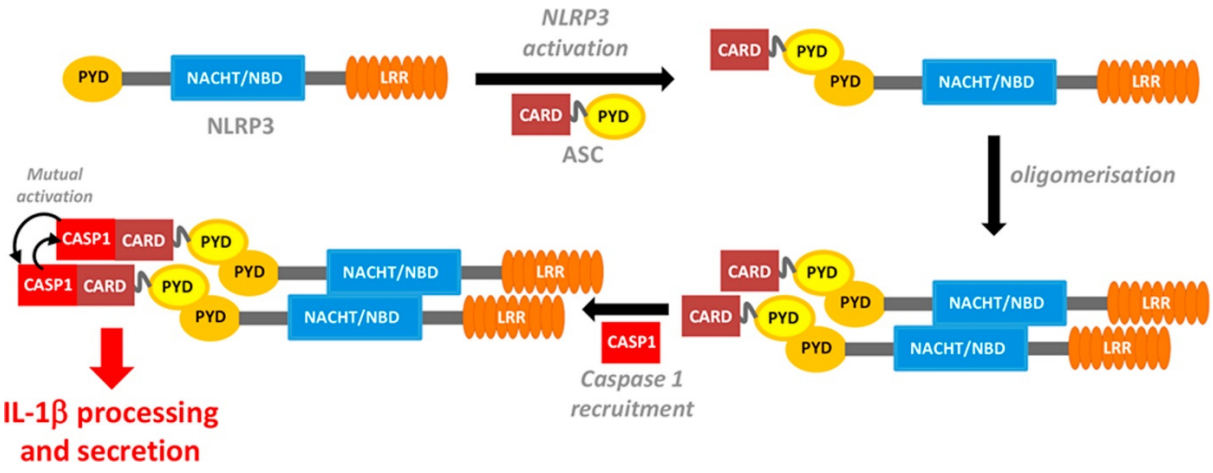

Figure 1: Assembly of the NLRP3 inflammasome. CARD, caspase recruitment domain; LRR, leucine-rich repeat; NACHT/NBD, nucleotide-binding domain; PYD, pyrin domain; CASP1, caspase-1 (Abderrazak et al. 2015).

NLRP3 consists of a pyrin domain (PYD), a nucleotide-binding domain (NBD), and a leucine-rich repeat (LRR) domain that associates with the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) when it is activated (see Figure 1) (Leavy 2014). ASC comprises PYD and a caspase-recruitment domain (CARD) (Lissner et al. 2011). The stimulated NLRP3:ASC complex oligomerizes and binds the enzyme caspase-1 *via* the CARD domain resulting in the active NLRP3 complex (NLRP3, ASC and caspase-1) (Abderrazak et al. 2015). The active NLRP3 complex induces the secretion of IL-1β and IL-18 *via* the activation of caspase-1 (Franchi et al. 2009).

A “priming signal” or “signal 1” is required for the synthesis of pro-IL-1β and the upregulation of NLRP3 expression (Dowling et al. 2012, Kawana et al. 2014). The “priming signal” is mediated, for example, by LPS, pathogen-associated molecular patterns (PAMPs), and damage-associated molecular patterns (DAMPs) *via* the activation of toll-like receptor 4 (TLR4) (Lissner et al. 2011, Nadatani et al. 2016).

The “activation signal” or “signal 2” is needed for the assembly of the active NLRP3 complex (Franchi et al. 2012, He et al. 2016). It is mediated by various stimuli such as adenosine triphosphate (ATP), crystals, reactive oxygen species (ROS), cholesterol, silica, aluminum salts, asbestos, fatty-acids, PAMPs, and DAMPs (Franchi et al. 2009, Lissner et al. 2011, Franchi et al. 2012). Since the activators of NLRP3 are chemically and structurally diverse, an activation mediated by an intermediate cellular signal that is stimulated by all these activators has been suggested (Franchi et al. 2012, Yang et al. 2019).

The NLRP3 inflammasome plays a crucial role in the regulation of intestinal homeostasis (Hirota et al. 2011). Defective NLRP3 is associated with a variety of diseases such as Alzheimer’s disease, type 2 diabetes, colon cancer and IBDs, i.e. Crohn’s disease and ulcerative colitis (Zaki et al. 2011, Yang et al. 2019). NLRP3 is mostly expressed in immune cells such as dendritic cells and macrophages (Mariathasan et al. 2006, Laudisi et al. 2013); however, it is also expressed in epithelial cells, e.g., human lung epithelial cells, retinal pigment epithelial cells, and intestinal epithelial cells (Peeters et al. 2013, Tseng et al. 2013, Song-Zhao et al. 2014). Both the hypo- and hyperfunction of the inflammasome can promote intestinal disease depending on the cell type in which it is activated (Lissner et al. 2011). In the intestinal epithelium, the inflammasome regulates permeability and epithelial self-renewal, whereas the activation of the inflammasome within immune cells of the *lamina propria* mediates severe intestinal inflammation (Lissner et al. 2011). Thus, inflammasome activation appears to have both destructive and enforcing effects on the intestinal barrier (Lissner et al. 2011).

In pigs, expression of NLRP3 mRNA has been detected in the gut-associated lymphoid tissue (GALT), spleen, small intestine, colon (Tohno et al. 2011, Yu et al. 2017), aortic tissue (Li et al. 2013), blood monocytes (Ye et al. 2016), alveolar macrophages (Li et al. 2015), monocyte-derived dendritic cells (MoDC), and IPEC-J2 cells (Loss et al. 2018, Loss et al. 2018, Loss et al. 2019).

2.5.2 The NLRP6 inflammasome

The NLRP6 inflammasome (also called PYPAF5) belongs to the NLR-family and is highly expressed in the intestine (Kanneganti et al. 2007, Chen et al. 2011). However, it has also been detected in hematopoietic cells (Chen et al. 2011). It plays an important role in intestinal homeostasis, intestinal epithelial regeneration, the maintenance of colonic microbiota,

colorectal cancer tumorigenesis, antiviral protection, and intestinal inflammation such as the development of colitis (Chen et al. 2011, Elinav et al. 2011, Normand et al. 2011, Wang et al. 2015, Yin et al. 2019). The cellular mechanisms by which NLRP6 influences the host remain unclear. However, NLRP6 has been demonstrated to be associated with IL-18 production (Elinav et al. 2011). Furthermore, evidence exists that NLRP6 expression in inflammatory monocytes is upregulated during dextran sulfate sodium-induced colitis in mice (Seregin et al. 2017). These monocytes infiltrate into the colon and mediate the NLRP6-dependent and IL-18-dependent production of TNF-α that protects against chronic inflammation (Seregin et al. 2017). Birchenough et al. (2016) have reported that NLRP6 was activated in so-called 'sentinel' goblet cells and induced mucin 2 production, which protected the gut lumen against infectious agents. NLRP6 influences the composition of the gut microbiome, and *vice versa*, microbiota-associated metabolites can also modulate NLRP6 signaling (Levy et al. 2015).
In pigs, NLRP6 mRNA expression has been detected in the jejunum and ileum of 35-day-old piglets (Yu et al. 2017).

2.5.3 Interactions of probiotics and inflammasomes

Probiotics affect the expression of cytokines and have a beneficial impact on the composition of the gut microbiota (Hemarajata et al. 2013, Plaza-Diaz et al. 2014). Inflammasomes can also affect health and disease *via* regulation of the gut microbiome and modulation of cytokines (Chen 2016). Thus, a few studies have investigated interactions between probiotics and inflammasomes.
Ex vivo and *in vivo* treatments with *E. faecium* did not influence the expression of inflammasome components NLRP3, caspase-1, IL-1β, and IL-18 in the duodenum of dogs with chronic enteropathy or in control animals (Schmitz et al. 2015). However, in pigs, *Lactobacillus delbrueckii subsp. bulgaricus* and *Lactobacillus gasseri* upregulated NLRP3 mRNA expression in GALT (Tohno et al. 2011). Pre-treatment with *Lactobacillus rhamnosus* decreased the mRNA expression of NLRP6, caspase-1, and IL-18, and ASC protein release in the ileum of piglets challenged with *Salmonella enterica* serovar Typhimurium (Yu et al. 2017). Significant modulatory effects of *Lactobacillus rhamnosus* on IL-1β expression and NLRP3 mRNA and protein expression were not apparent in the study of Yu et al. (2017).
In vitro, Lactobacillus rhamnosus decreased the *E. coli*-induced NLRP3 and caspase-1 protein expression and IL-1β and IL-18 mRNA expression in primary bovine mammary epithelial cells independently of ASC expression (Wu et al. 2015). The *E. coli*-induced expression of NLRP3, ASC, caspase-1, IL-1β, and IL-18 in primary bovine endometrial epithelial cells was ameliorated by *Lactobacillus rhamnosus* (Liu et al. 2016). Supplementation with *Lactobacillus plantarum* counteracted increased serum levels of IL-1β during LPS-induced acute systemic

inflammation in rats (Vilahur et al. 2015). It also attenuated NLRP3 gene expression in the ileum of Zucker diabetic obese rats, a model of subclinical chronic inflammation in the gut (Vilahur et al. 2015). No protective effects of the probiotic *E. faecium* on NLRP3 expression have been found in porcine MoDCs challenged with ETEC (Loss et al. 2018).

Chapter 3: Aims and objectives of the thesis

The main purpose of the present studies was to decipher the effects of *E. faecium* on intestinal barrier properties and inflammatory responses during an infection with ETEC. The porcine inflammasome was characterized and analyzed as a potential mediator of probiotic effects.
Studies were conducted *in vitro, in vivo*, and *ex vivo. In vitro*, IPEC-J2 cells were used in order to analyze the effects of the probiotic *E. faecium* on inflammatory cytokines, TJ proteins, R_t, cell structure, and cAMP concentrations during an ETEC infection. For the *in vivo* experiments, various parts of the intestine of *E. faecium*-supplemented piglets and of differently aged pigs were examined for effects on diverse inflammasome components. Ussing chamber experiments were employed to examine jejunum incubated with ETEC and *E. faecium* with focus on the different inflammasome components *ex vivo*.

The following hypotheses were tested in the present studies:

- *E. faecium* attenuates the inflammatory response of the intestinal epithelium by reducing the ETEC-induced increases of proinflammatory cytokine release during infection with ETEC.
- *E. faecium* acts protectively on intestinal barrier properties by modulation of TJ proteins.
- The inflammasome acts as a mediator of probiotic effects *via* the modulation of proinflammatory cytokines in the porcine intestine.

In the experiments, the following questions were examined:

a) Influence of the probiotic *E. faecium* on epithelial barrier properties and cytokine expression during infection with ETEC
    - Is the expression of proinflammatory cytokines upregulated by ETEC in the porcine intestine, and is this effect attenuated by *E. faecium*?
    - Is the ETEC-induced impairment of the intestinal barrier integrity diminished by *E. faecium*?
        - Are TJ protein expression and localization modulated by *E. faecium* during an infection with ETEC?
        - Is the ETEC-induced impairment of the physiological cell structure reduced by *E. faecium*?

b) The inflammasome as a potential mediator of probiotic effects
    - Are inflammasome components expressed in the porcine intestine?
        - Does the expression of inflammasome components differ in the various parts of the porcine intestine?

- Does the expression of the porcine inflammasome components differ in differently aged pigs?

- Is the expression of porcine inflammasome components modulated by *E. faecium* and ETEC?

Chapter 4: Altered cytokine expression and barrier properties after *in vitro* infection of porcine epithelial cells with enterotoxigenic *Escherichia coli* and probiotic *Enterococcus faecium*

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contribution to study design, preparation and repeated conduction of the *in vitro* infection experiments, cultivation of IPEC-J2 cells, cultivation of bacterial strains (*E. faecium*, ETEC), determination of bacterial growth curves, transepithelial electrical resistance (R_t) measurements, preparation of cells of the *in vitro* infection experiments for confocal laser-scanning microscopy and western blot analysis, RNA isolation, RNA quantification, RNA quality analysis, preparation of cDNA, RT-qPCR, ELISA assays, apoptosis and cytotoxicity assays, calculations and statistical analysis, evaluation of experimental results, preparation of the first draft of the manuscript, contributions to revision and editing of the manuscript.
The co-authors contributed either to the study design, specific parts of cell analysis (e.g. confocal laser-scanning microscopy, western blot analysis), or supported the conduction of the *in vitro* infection experiments. All co-authors participated in revising and editing of the manuscript.

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Research Article

Altered Cytokine Expression and Barrier Properties after In Vitro Infection of Porcine Epithelial Cells with Enterotoxigenic *Escherichia coli* and Probiotic *Enterococcus faecium*

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The aim of the present study was to elucidate the effects of the probiotic feed additive *Enterococcus faecium* NCIMB 10415 (*E. faecium*) on porcine jejunal epithelial cells (IPEC-J2) during an in vitro challenge with enterotoxigenic *Escherichia coli* (ETEC). Cells were incubated with *E. faecium*, ETEC, or both, and the effects on barrier function and structure and intra- and intercellular signaling were determined. Coincubation with *E. faecium* abolished the ETEC-induced decrease in transepithelial resistance (R_t) ($p \leq 0.05$). No differences were seen in the expression levels of the intercellular connecting tight junction proteins examined. However, for the first time, a reorganization of the monolayer was observed in ETEC-infected cells but not in coincubated cells. ETEC induced an increase in cytotoxicity that was prevented by coincubation ($p \leq 0.05$), whereas apoptosis rates were not affected by bacterial treatment. ETEC increased the mRNA expression and release of proinflammatory cytokines TNF-α, IL-1α, and IL-6 which could be prevented by coincubation for TNF-α mRNA expression and IL-6 protein ($p \leq 0.05$). Likewise, cAMP concentrations elevated by ETEC were reduced in coincubated cells ($p \leq 0.05$). These findings indicate a protective effect of the probiotic *E. faecium* on inflammatory responses during infection with ETEC.

1. Introduction

The gut microbiota has an important impact on immune responses and barrier function of the intestinal mucosa and, thereby, influences overall gut health. Several recent studies tried to assess the complex cross talk between microbiota, intestinal barrier, immune system, and the gut-brain axis [1, 2]. The high relevance of such cross talk has been shown for various disease phenotypes such as inflammatory bowel disease, which can be attributed, in part, to perturbations in the composition of the intestinal microbiota [3, 4]. To support the establishment or restoration of a healthy gut microbiota, probiotics have been proposed as preventive and therapeutic measures [3]. They have been successfully applied as a therapy in dysbiosis-associated diseases relevant in human medicine such as ulcerative colitis [5]. In animal rearing, probiotics have become valuable tools to prevent diarrhea in critical production phases and, thereby, exert positive effects on the performance and health status [6–8].

A very critical period in piglet rearing is weaning where diarrhea attributable to infections with enterotoxigenic *Escherichia coli* (ETEC) represents a major problem [9–11].

The probiotic strain *Enterococccus faecium* NCIMB 10415 (*E. faecium*), used in the present study, is a licensed feed additive for piglets and sows in the EU, and its effects on diarrhea incidence and performance have been studied in various feeding trials [7, 12, 13]. It has been found to reduce diarrhea incidence in piglets in the pre- and post-weaning period [7, 13], and when applied to sows, it diminishes piglet loss after birth [13]. It has further been shown to affect gut immunology [12, 14]. In a previous study, in vitro coincubation with *E. faecium* reduced the decrease in transepithelial resistance (R_t) induced by ETEC in human colonic and porcine jejunal epithelial cells [15]. The focus of the present study was to examine the effects of *E. faecium* on barrier properties and immunological readout values in a porcine intestinal epithelial cell model in vitro.

The structural barrier of the intestinal epithelium is formed by the inner lining of intestinal epithelial cells, which are connected by intercellular junctions. The paracellular permeability is regulated by tight junction (TJ) structures. Claudins are the main components of tight junctions. Some of these proteins, such as claudin-1, claudin-3, and claudin-5, have sealing functions, whereas others form channels with selectivity for cations or anions or are permeable to water [16]. TJ proteins and their localization have been shown to be regulated by cytokines [17], for example, by inducing the redistribution of various TJ proteins via internalization [18] or by regulating the transcription level of TJ proteins [19].

Based on these prior observations, we examined whether the protective effects of *E. faecium* during an ETEC challenge are attributable to an enhancement of barrier function by modulating TJ protein expression and localization, the cell structure, or the vitality of the cells. Furthermore, it was hypothesized that *E. faecium* reduces the release of inflammatory cytokines during an ETEC challenge. As the heat-labile toxin of ETEC enhances cAMP production, which can regulate the expression of proinflammatory cytokines [20], we also assumed that the intracellular concentrations of cAMP during infection of host cells may be modulated by the probiotic.

2. Material and Methods

2.1. Cell Culture. Intestinal porcine epithelial cells (IPEC-J2) were kindly provided by Prof. Dr. Anthony Blikslager (North Carolina State University, USA). They were cultivated as described in Klingspor et al. [15] in DMEM-Ham's F12 medium (Biochrom, Berlin, Germany) supplemented with 5% fetal bovine serum (FBS, Biochrom), 2.5 mmol/l L-glutamine (Biochrom), 5 ng/ml epidermal growth factor (Biochrom) and ITS (insulin [5 μg/ml], transferrin [5 μg/ml], and selenium [5 ng/ml]; Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany), and penicillin-streptomycin (100 units penicillin and 100 μg streptomycin/ml, Sigma-Aldrich Chemie GmbH). Cells were split at a ratio of 1 : 3 every 7 days.

For experiments, cells were either seeded in 24-well cell culture plates (15 mm diameter, TPP, Faust Lab Science, Klettgau, Germany) or on cell culture inserts (Transwell Clear, 12 mm diameter, 0.4 μm pore size [Corning B.V., Schiphol-Rijk, The Netherlands], Millicell cell-HA (12 mm diameter, 0.45 μm pore size [Merck Millipore Ltd., Darmstadt, Germany]) collagenized with rat tail collagen I (Serva Electrophoresis GmbH, Heidelberg, Germany) at a concentration of 10^5 cells per well or per cell culture insert. For apoptosis and cytotoxicity assays, cells were cultivated on 96-well cell culture plates (34 mm^2, Lumox multiwell, Sarstedt, Nümbrecht, Germany) at a concentration of 10^4 cells per well. Before being used in an experiment, the cells were cultivated for 14–21 days. On the day prior to experiments, the cells were supplied with serum- and antibiotic-free media.

2.2. Bacterial Strains. Enterotoxigenic *E. coli* IMT4818 (ETEC, isolated from a 2-week-old piglet with enteritis, O149:K91:K88 (F4), positive for the existence of virulence genes *est*-1a, *est*-2 [genes coding for heat stable enterotoxins I and II], and *elt*-1a/b [gene coding for heat labile enterotoxin I] by polymerase chain reaction [PCR]) was grown in LB medium (Luria/Miller) containing 10 g/l tryptone, 5 g/l yeast extract, and 10 g/l NaCl, at a pH of 7.0 (Roth, Karlsruhe, Germany).

The probiotic *E. faecium* NCIMB 10415 strain (from Cylactin®, DSM, Kaiseraugst, Switzerland) was cultivated in brain-heart infusion (BHI) broth (OXOID GmbH, Wesel, Germany). The bacteria were incubated overnight at 37°C and subcultured for approximately 180 min until midlog phase on a shaker at 37°C, centrifuged, and washed twice with phosphate-buffered saline (PBS, Biochrom). Bacterial cells were then resuspended in DMEM-Ham's F12 medium without penicillin/streptomycin or FBS. The concentration was adjusted to 10^8 colony-forming units (CFU)/ml based on optical density (*E. faecium* OD = 0.9, 600 nm, ETEC OD = 1.3, 600 nm) measured in the Heilos™ Epsilon spectrophotometer (Thermo Scientific, Waltham, USA). The bacterial concentration was confirmed by serial dilution followed by determination of viable counts on LB-plates.

2.3. Incubation of Cells with Bacterial Strains. Bacteria (*E. faecium* or ETEC) were added to the apical side of cell culture inserts, 24-well plates, or 96-well plates. The intestinal cells were infected with 10^6 bacteria per cell culture insert or well, corresponding to a multiplicity of infection (MOI) of approximately 10 bacteria per seeded cell. The 96-well cell culture plates were infected with 10^5 bacteria per well, which also equates to an approximate MOI of 10. To test the effect of *E. faecium* on ETEC infection, the cells were coincubated with both ETEC and *E. faecium* strains. In the coincubation studies with the probiotic and the pathogenic strains, the cells were first preincubated with *E. faecium*, and the ETEC strain was added 2 h later. In the following, this setup will be called "coincubation." The incubation times given in the legends were calculated based on the duration of the incubation with the ETEC strain. After 2 h of incubation with the respective strains, gentamycin (50 μg/ml medium, Biochrom) was added to kill the bacteria as described in Klingspor et al. [15].

For analysis of cyclic adenosine monophosphate (cAMP), the incubation time of the respective strains was 4 h and samples were taken after 4 h of incubation.

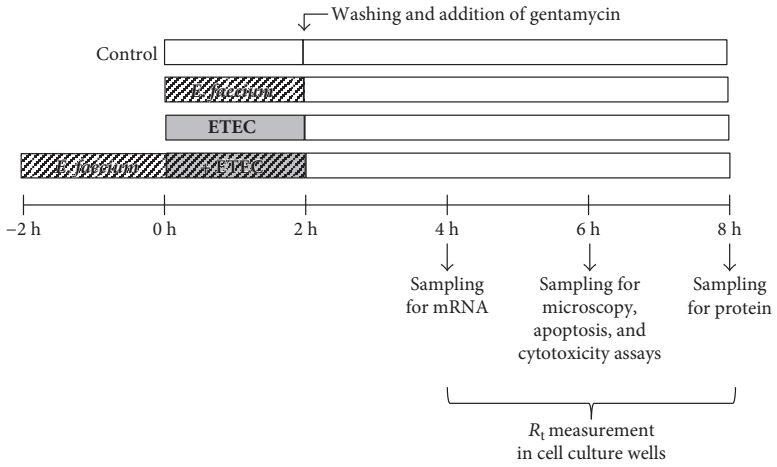

Figure 1: Experimental set-up.

For mRNA expression, protein expression, and confocal laser scanning microscopy (CLSM), samples were taken at various time points as indicated in Figure 1.

2.4. Resistance Measurements (R_t). R_t was determined for cells grown in cell culture inserts with a Millicell-ERS (Electrical Resistance System; Millipore GmbH, Schwalbach, Germany). The blank value (cell culture insert and medium without cells) was subtracted from the measurements, and the values were corrected for the membrane area. The R_t values before the beginning of experiments were $3509 \pm 117\ \Omega \cdot \text{cm}^2$ [mean ± SEM].

2.5. Real-Time Quantitative Polymerase Chain Reaction. Samples for analysis of mRNA expression were taken from 24-well plates. Cell layers were rinsed twice with PBS, and the cells were harvested in PBS with a cell scraper and centrifuged (5 min, 200 *g*). RNAlater (Qiagen GmbH, Hilden, Germany) was added to avoid RNAse degradation, and the samples were frozen at −20°C. Three wells of a culture plate were pooled per sample.

The following protocol is described in more detail by Lodemann et al. [21]. The nucleospin RNA II Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) was used to isolate total RNA. The RNA concentration was quantified by NanoDrop (PEQLAB Biotechnologie GmbH, Erlangen, Germany). The RNA quality was determined with a 2100 Bioanalyzer (Agilent Technologies, Böblingen, Germany), and only samples with an RNA integrity number above 7 were included in further analyses. cDNA was synthesized with the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories GmbH, Munich, Germany) by reverse-transcribing 100 ng total RNA from the IPEC-J2 cells in a final volume of 200 μl in an iCycler iQ™ Real-Time PCR Detection System (Bio-Rad Laboratories GmbH).

Information about primers for the targets is given in Table 1. Three reference genes were used for normalization (GAPDH, TBP, and YWHAZ) as described in Klingspor et al. [15]. Primers were synthesized by Eurofins MWG Synthesis GmbH (Ebersberg, Germany).

RT-qPCR was conducted in the iCycler iQ Real-Time PCR Detection System (Bio-Rad Laboratories GmbH) by using SYBR green I detection. The samples were analysed in duplicate; the final volume of the wells (25 μl) contained iQ SYBR Green Supermix (Bio-Rad Laboratories GmbH), primers (0.5 μl of 20 pmol/μl each), and 5 μl cDNA. Controls included transcription reactions without reverse transcriptase to prove the absence of genomic DNA contamination. iQ5 software was used for the analysis of the relative amount of the target genes (Bio-Rad Laboratories GmbH).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

2.6.1. IL-6. Supernatants of IPEC-J2 cells were collected from 24-well plates after 8 h (see Figure 1), centrifuged (5 min, 4000 *g*, 4°C), and frozen at −80°C until use. Porcine IL-6 ELISA (Raybiotech, Norcross, USA) was performed according to the manufacturers' instructions. Samples were analysed in duplicate.

2.6.2. IL-1α. Cell-culture supernatants were harvested from 24-well plates after 8 h, centrifuged (5 min, 4000 *g*, 4°C), and frozen at −80°C until use. IL-1α concentrations were determined with the Pig Interleukin 1 α (IL-1α) ELISA Kit (Cusabio, Wuhan, China) according to the manufacturers' instructions. Assays were performed in duplicate.

2.6.3. TNF-α. Supernatants of IPEC-J2 cells were taken from 24-well plates after 8 h, and cell debris was removed by centrifugation at 4000 *g* and 4°C. Samples were frozen at −80°C until use. Quantikine ELISA specific for porcine TNF-α (R&D systems, Minneapolis, United States) was performed according to the manufacturers' instructions. Assays were performed in duplicate. To enhance the protein content, the sample volume was increased to 250 μl per well. Optical density was determined within 30 min by using a microplate reader (EnSpire, Multimode Plate Reader, Perkin Elmer, Rodgau, Germany). Readings at 540 nm were

Table 1: Oligonucleotide primers and amplicon length of PCR products.

Gene information	Primer sequence	Amplicon length	Accession number	Reference
TNF-α (tumor necrosis factor α, *Sus scrofa*)	(S) 5′-GCT GTA CCT CAT CTA CTC CC-3′ (AS) 5′-TAG ACC TGC CCA GAT TCA GC-3′	291 bp	NM_214022.1	[22]
IL-6 (interleukin 6, *Sus scrofa*)	(S) 5′-ATG CTT CCA ATC TGG GTT CA-3′ (AS) 5′-GTG GTG GCT TTG TCT GGA TT-3′	198 bp	M80258.1	
IL-1α (interleukin 1α, *Sus scrofa*)	(S) 5′-CAA GGA CAG TGT GGT GAT GG -3′ (AS) 5′-TCA TGT TGC TCT GGA AGC TG-3′	167 bp	NM_214029.1	

Table 2: Primary and secondary antibodies (WB = Western blot, IF = immunofluorescence).

Primary antibodies					
Antigen	Species	Company (catalog #)	Dilution WB	Dilution IF	
Claudin-1	Rabbit	Invitrogen/Thermo Fisher Scientific (51-9000)	1 : 1000		
Claudin-3	Rabbit	Invitrogen/Thermo Fisher Scientific (34-1700)	1 : 1000	1 : 200	
Claudin-4	Mouse	Invitrogen/Thermo Fisher Scientific (32-9400)	1 : 5000	1 : 200	
Claudin-5	Mouse	Invitrogen/Thermo Fisher Scientific (35-2500)	1 : 1000		
Claudin-7	Rabbit	Invitrogen/Thermo Fisher Scientific (34-9100)	1 : 2000		
Claudin-8	Rabbit	Invitrogen/Thermo Fisher Scientific (40-2600)	1 : 1000		
Occludin	Rabbit	Invitrogen/Thermo Fisher Scientific (71-1500)		1 : 200	
β-actin	Mouse	Sigma (A5441)	1 : 10,000		
Secondary antibodies					
Antigen	Species	Company (catalog #)	Conjugate	Dilution WB	Dilution IF
Mouse IgG	Goat	Jackson Immuno Research (115-225-146)	Cy2		1 : 600
Rabbit IgG	Goat	Jackson Immuno Research (111-175-144)	Cy5		1 : 600
Mouse IgG	Goat	Jackson Immuno Research (115-035-003)	HRP	1 : 10,000	
Rabbit IgG	Goat	Jackson Immuno Research (111-036-003)	HRP	1 : 10,000	

subtracted from the readings at 450 nm and blank corrected. A four parameter logistic (4-PL) curve fit was generated for the calculation of the results.

2.6.4. cAMP. cAMP concentrations were determined by using the Cyclic AMP XP Assay Kit #4339 (Cell Signaling Technology, Danvers, United States). On the day prior to experiments, confluent 24-well plates of IPEC-J2 cells were washed twice with warm PBS and supplied with serum- and antibiotic-free media. On the experimental day, cells were lysed with Roche cOmplete™ Lysis-M (Sigma-Aldrich, Munich, Germany) after 4 h of bacterial treatment and centrifuged (5 min, 600 *g*, 4°C), and cAMP ELISA was performed following the manufacturers' instructions. Assays were performed in duplicate.

2.7. Apoptosis and Cytotoxicity Assays. Apoptosis and cytotoxicity were assessed at 6 h after the addition of the bacterial strains by using the ApoTox-Glo™ Triplex Assay (Promega, Madison, USA) according to the manufacturer's instructions. This assay measures apoptosis by quantifying caspase-3/7 and cytotoxicity by quantifying dead-cell protease. Excitation and emission settings for cytotoxicity measurements were set to excitation 485 nm and emission 520 nm. Apoptosis was determined by luminescence measurements.

2.8. Western Blots. Western blot (WB) analyses were performed by using standard techniques as described in detail by Amasheh et al. [23]. Primary and secondary antibodies are given in Table 2. The Lumi-LightPLUS Western Blotting Kit (Roche, Grenzach Wyhlen, Germany) was used to detect relevant protein bands via the Fusion FX 7 image acquisition system (Vilber Lourmat, Eberhardzell, Germany). Densitometric signal analysis was performed by using AIDA software (Raytest, Berlin, Germany), and β-actin was used as loading control.

2.9. Confocal Laser Scanning Microscopy. IPEC-J2 cells grown on membrane supports were washed with PBS with Ca^{2+} and Mg^{2+} and then incubated for 15–20 min in a 2% paraformaldehyde solution at room temperature. The paraformaldehyde was subsequently deactivated in a 125 mmol/l glycine solution. After subsequent rinsing with PBS containing Ca^{2+} and Mg^{2+}, preparations were stored in PBS at 4°C.

For staining, cells were permeabilized in 0.5% Triton-X100 in PBS (10 min) and incubated with primary antibodies (1 : 200 in PBS, 4°C, overnight). After the cells had been thoroughly washed, incubation with secondary antibodies (Jackson ImmunoResearch, Newmarket, UK, Cy2 Fab goat anti-mouse, Cy5 Fab goat anti-rabbit) was carried out at a

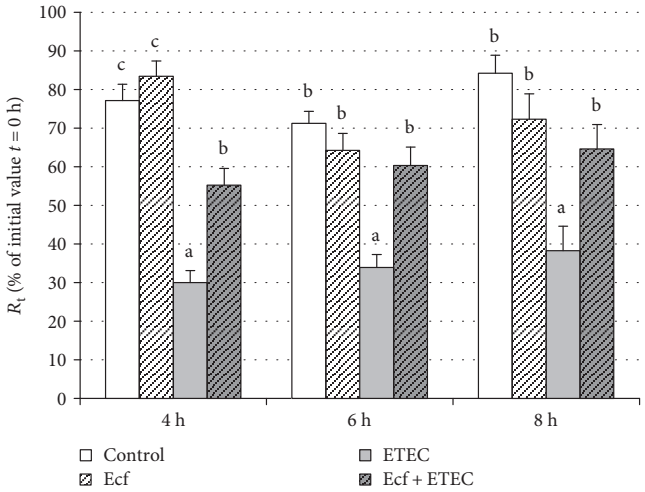

FIGURE 2: R_t in cells incubated with bacterial strains (Ecf, ETEC, and Ecf + ETEC) and in control cells not exposed to bacteria [means ± SEM]. Groups differ significantly ($P \leq 0.05$) when marked with different letters, (e.g. a, b) in the figure. $N = 5$ independent experiments.

concentration of 1 : 600 together with DAPI (2-(4-amidinophenyl)-1H-indole-6-carboxamidine, final concentration 1 μg/ml, for 45 min at room temperature, Roche Grenzach Wyhlen, Germany). Preparations were rinsed thoroughly and embedded in ProTaqs Mount Fluor (Biocyc, Luckenwalde, Germany). Confocal images were obtained with a confocal laser scanning microscope (Zeiss LSM780, Jena, Germany) by using excitation wavelengths of 405 nm, 488 nm, and 633 nm.

2.10. Calculations and Statistical Analysis. The statistical analyses and the plotting of graphs were carried out by means of the SPSS program for Windows, version 23 (Jandel, Chicago, IL, USA) and Microsoft Excel 2010 (Microsoft Corporation, Redmond, US).

The statistical significance of differences was assessed by variance analysis. For R_t values, the expression of cytokines, measurement of cAMP concentrations, and apoptosis and cytotoxicity assays, a variance analysis with the fixed factor "treatment" ("control," "*E. faecium*," "ETEC," and "*E. faecium* + ETEC") was conducted. If ANOVA indicated a significant difference, a post hoc Scheffé or least significant difference (LSD) test was conducted per time point to isolate the means that differed. For the protein expression of TJ proteins, an unpaired Student's *t*-test was used. The differences between groups were considered statistically significant for $p \leq 0.05$. Statistical significance is marked in the figures using letter coding; means are not different if they share the same letter and are different if they do not share at least one common letter. Results are given as means ± SEM. At least three independent experiments were conducted, as indicated in the figure legends.

3. Results

3.1. R_t. ETEC addition significantly reduced the R_t of IPEC-J2 cells ($p \leq 0.05$). This decrease in R_t was reduced or even prevented by coincubation with *E. faecium* as indicated by measurements taken at 4 h, 6 h, and 8 h ($p \leq 0.05$) (Figure 2).

3.2. Tight Junction Proteins. The changes in R_t indicated effects on barrier function. To elucidate whether these changes were attributable to the altered abundance of TJ proteins, we examined the expression of selected TJ proteins. However, in Western blots, no significant differences were detected in the expression of the TJ proteins claudin-1, claudin-3, claudin-4, claudin-5, claudin-7, or claudin-8 between bacterial treatments (Figure 3).

3.3. Confocal Laser Scanning Microscopy. Our previous study had demonstrated the presence of claudin-1, claudin-3, claudin-4, claudin-5, claudin-7, and claudin-8 in IPEC-J2 cells [24]. However, in IPEC-J2 cell layers cultured under the present conditions, only claudin-3 and claudin-4 showed continuous staining in TJs of all cells. In addition, occludin appeared to be a reliable TJ marker in these cell layers. In the present study, we therefore concentrated on the localization of claudin-3, claudin-4, and occludin.

By CLSM, no redistribution of claudin-3, claudin-4, and occludin localization was observed under any experimental condition. However, for the first time, a reorganization of the epithelial layer was detected in the ETEC-incubated cells that was largely inhibited by coincubation with *E. faecium* (Figures 4 and 5).

3.4. Apoptosis and Cytotoxicity. To investigate the changes in the epithelial architecture detected by CLSM, the rate of apoptosis and cytotoxicity was examined. No significant differences were observed with regard to apoptotic events between the bacterial treatments of the cells (Figure 6(a)). However, cytotoxicity was significantly increased in the ETEC-incubated cells compared with either coincubated

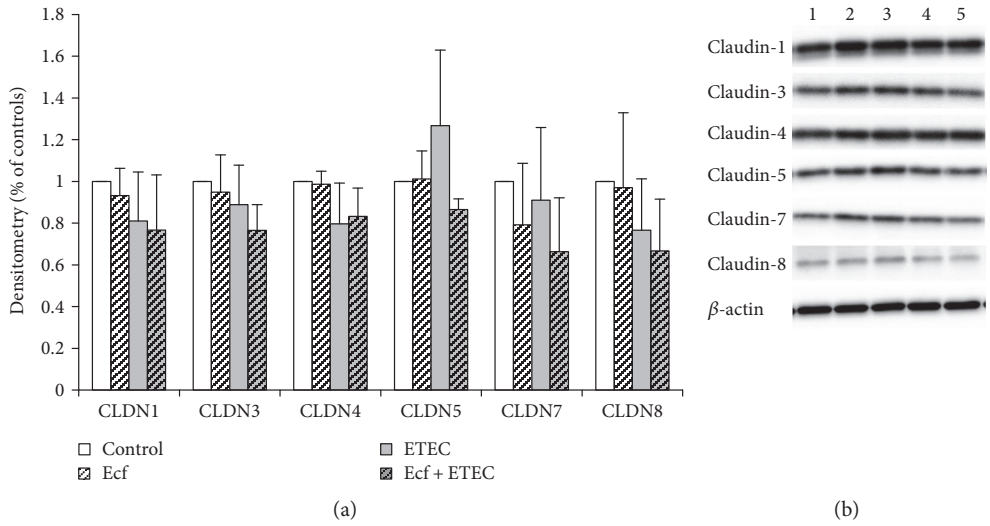

Figure 3: Protein expression (Western blot) of claudin-1, claudin-3, claudin-4, claudin-5, claudin-7, and claudin-8 after treatment with bacterial strains (Ecf, ETEC, and Ecf + ETEC). (a) Protein expression relative to respective controls [means ± SEM]. Samples were taken after 8 h. No differences were observed between treatment groups. $N = 3$ independent experiments per bar. (b) Exemplarily, data of one Western blot is shown. 1 = control (8 h), 2 = Ecf (8 h), 3 = ETEC (8 h), 4 = Ecf (8 h) + ETEC (6 h), and 5 = Ecf (10 h) + ETEC (8 h). Sample 4 was included as a control to rule out effects of longer total bacterial incubation (preincubation) and was not included in the statistical analysis shown in (a).

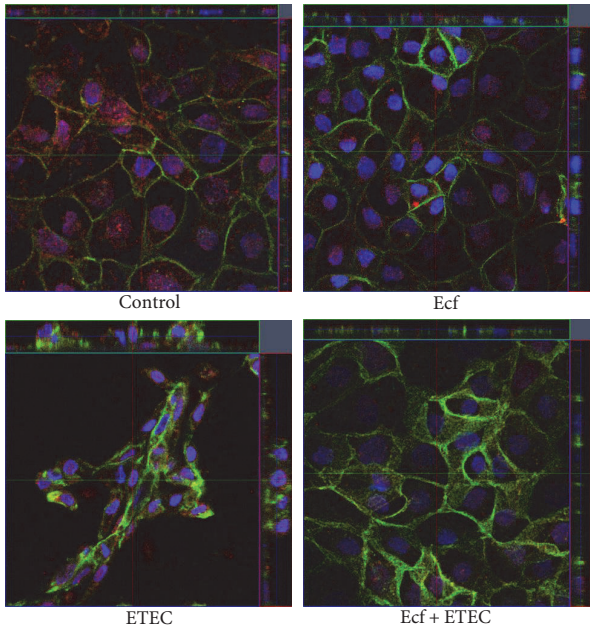

Figure 4: Confocal images of postconfluent IPEC-J2 cells at 6 h after treatment with bacterial strains (Ecf, ETEC, and Ecf + ETEC) and controls (red: claudin-3, green: claudin-4). Under all conditions, claudin-4 was localized in the tight junction and in the lateral membrane. $N = 5$ independent experiments.

cells, *E. faecium*-incubated cells, or control cells ($p \leq 0.05$) (Figure 6(b)). This represents a novel protective mechanism of *E. faecium* during ETEC infection.

3.5. Cytokine Expression. Since the epithelial barrier function can be influenced by inflammatory cytokines, such as TNF-α [25], the mRNA expression and release of several

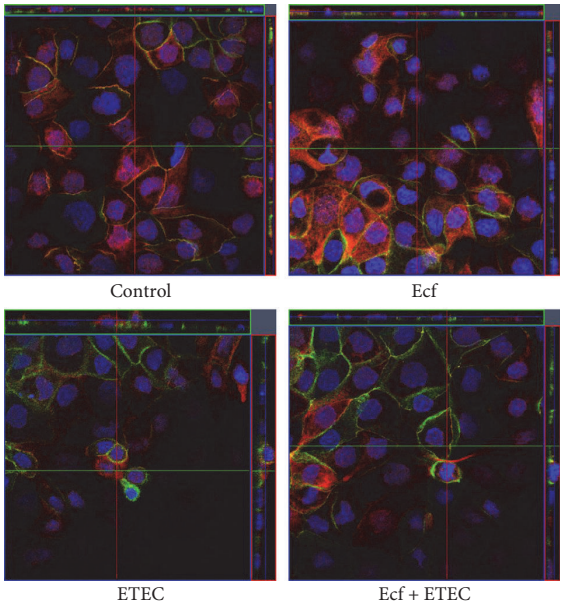

Figure 5: Confocal images of postconfluent IPEC-J2 cells 6 h after treatment with bacterial strains (Ecf, ETEC, and Ecf + ETEC) and controls (red: occludin, green: claudin-4). $N = 5$ independent experiments.

cytokines was assessed. IL-6 mRNA expression did not differ between bacterial treatments (Figure 7(a)). However, IL-6 protein release into the incubation medium was elevated in ETEC-incubated IPEC-J2 cells ($p \leq 0.05$) (Figure 7(b)). This effect could be prevented when cells were coincubated with *E. faecium* ($p \leq 0.05$) (Figure 7(b)).

IL-1α showed only a numerical increase in ETEC-incubated cells at the mRNA level, but a clear effect was detected at the protein level ($p \leq 0.05$) (Figures 7(c) and 7(d)). The ETEC-induced increase of IL-1α protein was not reduced by coincubation with *E. faecium* (Figure 7(d)).

TNF-α was significantly upregulated at the mRNA level in cell lysates and at the protein level in the incubation medium when cells were incubated with ETEC ($p \leq 0.05$) (Figures 7(e) and 7(f)). Increased release of TNF-α protein was not inhibited by coincubation with the probiotic strain; however, upregulation of TNF-α at the mRNA level was prevented when cells were coincubated with *E. faecium* ($p \leq 0.05$) (Figures 7(e) and 7(f)).

3.6. cAMP ELISA. Intracellular concentrations of cAMP were examined, since the heat-labile toxin of ETEC stimulates intracellular cAMP production, which can regulate the expression of proinflammatory cytokines [26]. Our data showed that the addition of ETEC increased the cAMP concentration in IPEC-J2 cells at 4 h after commencement of incubation with ETEC ($p \leq 0.05$) (Figure 8). This increase was attenuated in cells coincubated with *E. faecium* ($p \leq 0.05$) (Figure 8).

4. Discussion

In the present study, the effects of *E. faecium* during a challenge with ETEC were examined in a porcine intestinal epithelial cell culture model. The effects on epithelial cell function were analyzed by examining epithelial resistance, the expression of various TJ proteins, the epithelial cell structure, cytotoxic cell damage, and apoptosis. Furthermore, the release of inflammatory cytokines and intracellular cAMP concentrations was assessed.

One important step in revealing the underlying mechanisms of beneficial probiotics on epithelial barrier function is the investigation of changes in TJ protein expression and localization. We have shown recently that coincubation with *E. faecium* can reduce the effects of ETEC on R_t [15]. Since TJ proteins are crucial components of the intestinal barrier, changes at the TJ protein level appeared to be an attractive explanation for the probiotic effects detected. The intestinal epithelium represents a single epithelial cell layer which is interconnected by desmosomes, adherens junctions, and TJ proteins [27, 28]. TJs are the most apically located and determine the paracellular permeability. They can either seal the intercellular space or form selective paracellular pores [16]. The pathogenic ETEC strain has previously been shown to impair intestinal barrier integrity in IPEC-J2 cells through the loss of cell-cell contact, decreased cell viability, and a broken lining of the TJ adaptor protein zonula occludens-1 (ZO-1) [29] as well as changes in the expression and localization of claudin-1 [30, 31]. The present study has focused on claudin-1, claudin-3, claudin-4, and claudin-5, which are barrier-forming proteins, claudin-8 conveying a barrier for cations, and claudin-7, which is involved in changes in Cl^- and Na^+ conductance [16, 32–34]. Intriguingly, in Western blots, no consistent differences in the expression levels of the barrier-sealing proteins claudin-1, claudin-3, claudin-4, claudin-5, and claudin-8 or of claudin-7 were found after bacterial treatment (Figure 3). This was unexpected, because ETEC produced profound effects on electrical tissue resistance (R_t), and coincubation with *E. faecium* ameliorated and reversed this effect (Figure 2). A protective effect on R_t had also been observed in vitro for other probiotic strains such as *Lactobacillus plantarum*, which protected against the barrier disruption of intestinal epithelial cells challenged with ETEC [31]. Furthermore, treatment with *Lactobacillus casei* and *Butyricicoccus pullicaecorum* prevented a TNF-α-induced decrease in R_t in Caco-2 cells [35, 36]. As a novel finding, however, our data indicate that the protective effects of *E. faecium* on R_t are not based on changes in the expression of several TJ proteins but rather depend on changes in epithelial architecture.

Interestingly, in monolayers incubated with ETEC, a reorganization of the epithelial layer could be observed. Alterations in ETEC-infected cell layers ranged from single cells that protruded from the cell layer to dome formation or a stacked arrangement that seemed to compose a second epithelial cell layer in some areas. These cells were still attached to the main cell layer, as judged by their inclusion into an intact TJ pattern. The observed alterations are suggestive of increased cell shedding in the presence of ETEC.

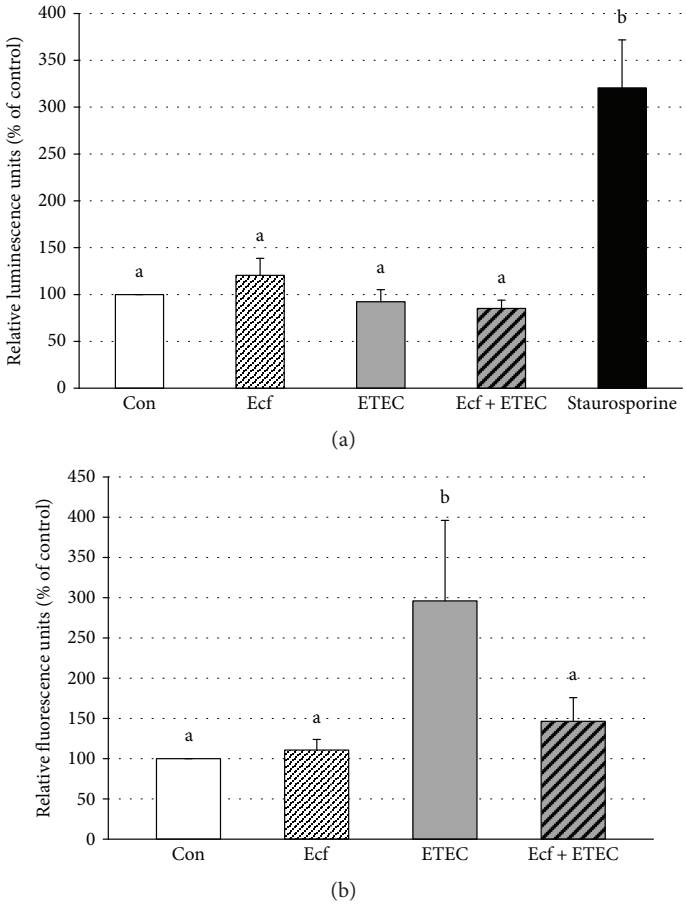

Figure 6: Apoptosis (a) and cytotoxicity (b) of IPEC-J2 cells after treatment with bacterial strains (Ecf, ETEC, and Ecf + ETEC) or without bacteria (Con = control) as assessed by caspase-3/7 activity in luminescence assay for apoptosis and by a dead cell protease fluorescence assay for cytotoxicity [means ± SEM]. Measurements were taken after 6 h. Groups differ significantly ($P \leq 0.05$) when marked with different letters, (e.g. a, b) in the figure. $N = 4$ (apoptosis assay) and $N = 5$ (cytotoxicity assay) independent experiments.

Epithelial rearrangement was greatly reduced or completely prevented in cells that had been preincubated with the probiotic strain before ETEC application revealing a novel protective probiotic mechanism during ETEC infection (Figures 4 and 5). Intestinal epithelial cell shedding and apoptosis can be induced, for example, by LPS or TNF-α [37–39]. To elucidate the underlying cellular mechanism, we examined the rate of apoptosis and cytotoxicity in the cells and the release of TNF-α and other cytokines. Our results revealed that differences in the epithelial structure are not attributable to apoptotic events but rather seem to depend on the cytotoxic effects of ETEC (Figure 6). In agreement with our findings, Lai et al. [40] have described increased cytotoxicity without apoptosis in ETEC-infected J774 macrophages. This commonly indicates that incubation with ETEC results in the necrosis of individual epithelial cells, an event that leads to localized barrier failure. Presumably, such focal necrosis and barrier failure is ameliorated and/or rapidly repaired in *E. faecium*-treated cells.

Since epithelial barrier function can be influenced by proinflammatory cytokines [25], changes in the cytokine response could further explain the finding that *E. faecium* prevents the ETEC-induced decrease in R_t.

Cytokines are able to mediate communication between cells of the immune system, hematopoietic cells, and other cell types [41]. They are expressed not only by immune cells, but also by intestinal epithelial cells [42, 43]. In the present study, we have focused on a well-established set of inflammatory cytokines including IL-6, IL-1α, and TNF-α [44]. Assuming the probiotic *E. faecium* to have protective effects, we hypothesized that the probiotic strain would decrease the inflammatory cytokine expression caused by an ETEC infection. IL-6 is one of the most crucial inflammatory cytokines. However, IL-6 also has a protective function within the intestine. It prohibits epithelial apoptosis during ongoing inflammation and is necessary for epithelial proliferation [45]. In intestinal epithelial cells, IL-6 is required for the regeneration and for the maintenance of the barrier [46, 47]. At the TJ level, the release of IL-6 can

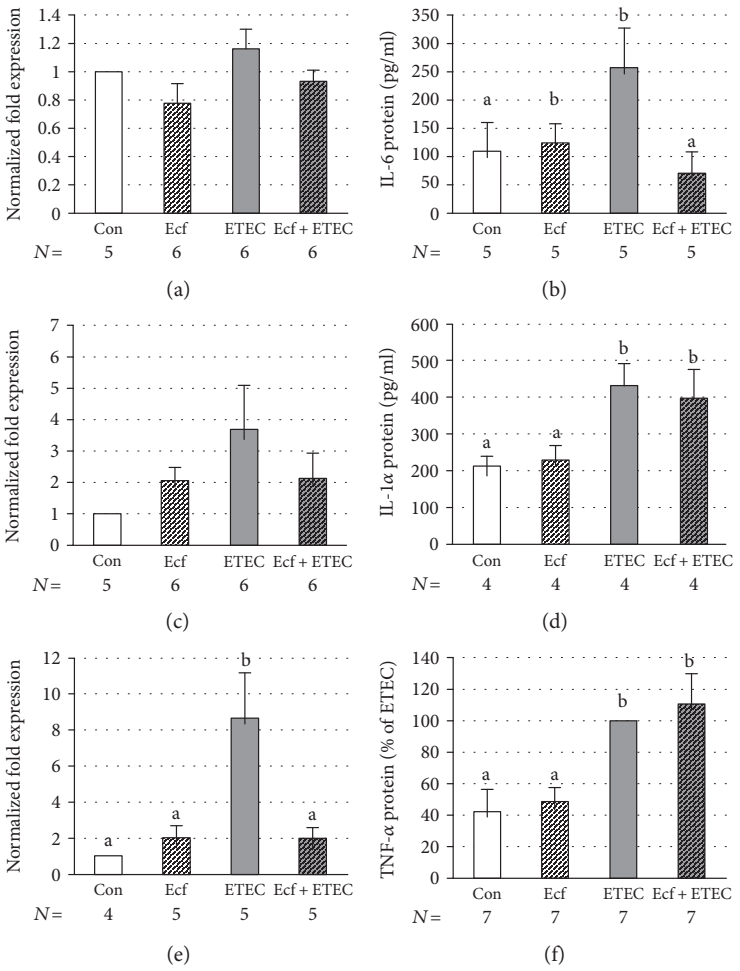

FIGURE 7: mRNA expression of IL-6 (a), IL-1α (c), and TNF-α (e) and cytokine release of IL-6 (b), IL-1α (d), and TNF-α (f) from IPEC-J2 cells after treatment with bacterial strains (Ecf, ETEC, and Ecf + ETEC) or without bacteria (Con = control) [means ± SEM]. Samples were taken after 4 h (mRNA) or 8 h (protein release). Groups differ significantly ($P \leq 0.05$) when marked with different letters, (e.g. a, b) in the figure. N = number of independent experiments as indicated in the figure caption.

lead to increased epithelial TJ permeability in Caco-2 cells [48–50]. On the other hand, the in vitro study of Suzuki et al. has revealed that claudin-1, claudin-3, and claudin-4 are not affected by IL-6 in Caco-2 cells. Our results indicate that the release of IL-6 is significantly reduced in coincubated cells compared with cells infected with ETEC alone (Figure 7(b)). Since IL-6 mostly functions as a proinflammatory cytokine, *E. faecium* might reduce inflammation during an ETEC infection. However, IL-6 might also be released in ETEC-incubated cells as a self-protective mechanism of the cells directed, for example, at the inhibition of apoptosis [45]. In either case, *E. faecium* seems to have a protective effect on the cells, as the stimulus for the release of IL-6 is inhibited by coincubation with this probiotic strain.

IL-1α exerts proinflammatory signals [51, 52] and is often released upon cell death [26]. It is physiologically attached to the cell nuclei, but translocates to the cytosol in the case of cell necrosis [53]. It provokes potent proinflammatory effects and is involved in neutrophil recruitment [52]. Moreover, IL-1 augments IL-8 expression and production in various cells [54]. In the present study, we could not detect any significant effects of the various bacterial treatments on the IL-1α mRNA level (Figure 7(c)). However, the release of IL-1α is significantly increased in ETEC-incubated and coincubated cells (Figure 7(d)). The latter indicates that the protective effects of *E. faecium* most likely do not depend on the modulation of IL-1α release.

TNF-α is an inflammatory cytokine that can promote apoptosis or the proliferation of cells [55]. Two forms of biologically active TNF-α are known: the membrane-bound and the soluble TNF-α [56]. In inflammatory bowel disease (IBD) patients, TNF-α is a crucial player that causes the loss of intestinal epithelial barrier integrity and stimulates the release of other proinflammatory cytokines [57]. TNF-α increases epithelial cell shedding, which can reduce intestinal barrier function [38, 58]. As such, TNF-α decreased R_t in T84

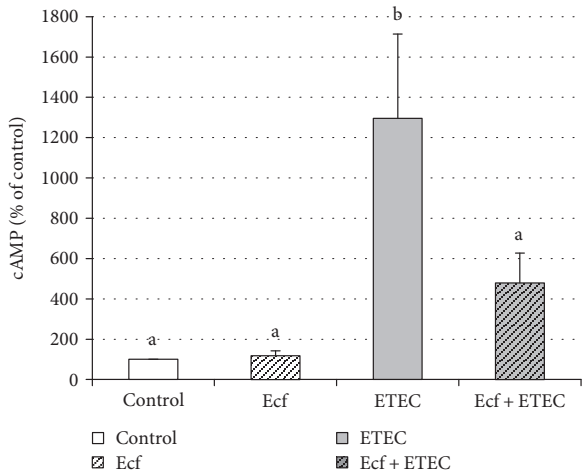

Figure 8: Intracellular cAMP concentrations in lysates of IPEC-J2 cells measured by ELISA at 4 h after commencement of incubation with bacterial strains (Ecf, ETEC, and Ecf + ETEC) compared with control [means ± SEM]. Groups differ significantly ($P \leq 0.05$) when marked with different letters, (e.g. a, b) in the figure. $N = 5$ independent experiments.

cells and Caco-2 cells [59, 60]. At the TJ level, TNF-α redistributes various TJ proteins [39, 61, 62]. In the present study, the mRNA expression of TNF-α is significantly increased 4 h after ETEC infection, whereas coincubation with *E. faecium* abolished this effect (Figure 7(e)). Wu et al. [31] obtained similar findings for TNF-α expression in IPEC-J2 cells incubated with the probiotic strain *Lactobacillus plantarum* and challenged with ETEC. Intriguingly, in our study, we could not detect a similar effect at the protein level (Figure 7(f)). The latter observation suggests that the release of TNF-α was rather small and could only be detected in the ELISA by increasing the sample volume to improve sensitivity. At such a low release level, the applied test may easily miss a possible decrease of TNF-α production when cells are coincubated with the probiotic strain (Figure 7(f)).

Cyclic AMP is a second messenger whose intracellular concentrations are adjusted by adenylate cyclase (AC) and cyclic nucleotide phosphodiesterase (PDE). AC heightens the level of cAMP, whereas PDE reduces its concentration in the cell. Cyclic AMP mainly activates protein kinase A (PKA), the GTP exchange protein directly activated by cAMP (EPAC) and cyclic nucleotide-gated ion channels [63]. Transcription factors modulating cytokine expression can be also regulated by cAMP [63]. Many triggers influence cAMP levels via AC and PDE, such as nutrients, hormones, neurotransmitters, pheromones, calcium, bicarbonate, CO_2, and cAMP itself [64]. The heat-labile toxin (LT) released by ETEC stimulates AC, and the resulting increases in cAMP concentrations and PKA activity lead to opening of chloride channels such as cystic fibrosis transmembrane conductance regulator (CFTR) to induce intestinal fluid secretion and diarrhea [65, 66]. In the present study, the intracellular levels of cAMP are significantly increased in cells incubated with ETEC, thus indicating the release of LT by ETEC (Figure 8). When the cells are coincubated with *E. faecium*, however, the increase in cAMP concentrations is alleviated (Figure 8). The latter firstly indicates that *E. faecium* protects the cells at an early state of ETEC infection, since the release of LT, which upregulates the cAMP response, is one of the early events of ETEC pathogenesis upon its adherence to the intestinal mucosa.

5. Conclusions

The present study reveals that ETEC infection of IPEC-J2 cells leads to barrier failure via cytotoxic insults that trigger the necrosis of individual cells and subsequently alter epithelial architecture. Preexposure to the probiotic *E. faecium* has beneficial effects on subsequent ETEC infection via the downregulation of certain inflammatory cytokines, reduced cytotoxicity, and decreased concentrations of the second messenger cAMP. Our study has further confirmed results of a former investigation in which protective effects of *E. faecium* were observed on the R_t decrease induced by ETEC. We now demonstrate for the first time, however, that these effects cannot be attributed to changes in the expression levels of several tested TJ proteins but might be based on the ability of *E. faecium* to prevent cell death and to preserve the physiological epithelial cell structure in coincubated cells.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Chapter 5: Characterization of Inflammasome Components in Pig Intestine and Analysis of the Influence of *Enterococcus Faecium* during an *Escherichia Coli* Challenge

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contribution to study design, sampling of porcine tissues, preparation and repeated conduction of the *ex vivo* infection experiments (Ussing chamber experiments), cultivation of bacterial strains (*E. faecium*, ETEC), determination of bacterial growth curves, RNA isolation, RNA quantification, RNA quality analysis, preparation of cDNA, RT-qPCR, ELISA, calculations and statistical analysis, evaluation of experimental results, preparation of the first draft of the manuscript, contributions to revision and editing of the manuscript.
The co-authors contributed either to the study design, to specific parts of tissue analysis (e.g. ELISA), or supported the conduction of the *ex vivo* infection experiments. All co-authors participated in revising and editing of the manuscript.

Chapter 6: Effects of *Ex Vivo* Infection with ETEC on Jejunal Barrier Properties and Cytokine Expression in Probiotic-Supplemented Pigs

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Chapter 7: Discussion

PWD in pigs is often caused by an ETEC infection and can produce significant losses in pig production (Nagy et al. 1999, Melkebeek et al. 2013). Therefore, an investigation of the substances that might reduce the incidence of diseases caused by ETEC (i.e. PWD), or that might reduce the severity of PWD during ETEC infection in piglets is of great interest. The use of probiotics has turned out to be a promising strategy in the prevention of ETEC infections (Roussel et al. 2018). With regard to clinical signs of ETEC infection in piglets, beneficial effects of probiotics have been described for the incidence and severity of diarrhea, mortality, and weight gain (Kyriakis et al. 1999, Alexopoulos et al. 2004, Schroeder et al. 2006, Breves et al. 2013). Positive effects have previously been shown in piglets treated *in vivo* with the probiotic *E. faecium* (Zeyner et al. 2006). However, the underlying cellular mechanism have not as yet been clarified.

As probiotics are known to influence the immune system (Isolauri et al. 2001), and as diarrhea/ETEC infection is often associated with increased expression of proinflammatory cytokines (Devriendt et al. 2010, Rana et al. 2012, Klingspor et al. 2015), the first aim of the present work was to elucidate whether the expression of proinflammatory cytokines is upregulated by ETEC in the porcine intestine, and whether this effect can be attenuated by *E. faecium*. Since the elevated release of proinflammatory cytokines often results in impaired barrier function (Bruewer et al. 2003), the second aim was to examine effects of *E. faecium* on intestinal barrier properties. A very new approach was the investigation of the inflammasome as a potential mediator of probiotic effects. The reason for this new approach was as follows. In a former study at the Institute of Veterinary Physiology of the Freie Universität Berlin, ETEC induced an increased expression and release of the cytokine IL-8, which could be counteracted by *E. faecium* in intestinal epithelial cells (Klingspor et al. 2015). Another study showed that cigarette smoke released IL-8 from human bronchial epithelial cells *via* TLR4 and 9 and inflammasome activation (Mortaz et al. 2011). Thus, the third aim of the present work was to examine whether the expression of porcine inflammasome components is modulated by *E. faecium* and ETEC. As the porcine inflammasome has been investigated to an only limited extent in literature, the present study examined initially the following basic questions. Are inflammasome components expressed in the porcine intestine? Does the expression of inflammasome components differ in various parts of the porcine intestine? Does the expression of the porcine inflammasome components differ in pigs of different ages? As a main result, interactions between inflammasomes, *E. faecium*, and ETEC infection were elucidated in the porcine intestine.

7.1 Effects of *E. faecium* on the intestinal barrier during an ETEC challenge

The intestinal barrier separates luminal toxins, antigens, microorganisms, and food from the internal milieu (Groschwitz et al. 2009, Martinez et al. 2012, Konig et al. 2016). It represents a crucial component for homeostasis of the gut (Turner 2009, Garrett et al. 2010). As a measure of intestinal barrier function, R_t and TJ protein expression were analyzed during an ETEC infection *in vitro* in the present study. IPEC-J2 cells were used for the *in vitro* experiments. *Ex vivo*, R_t was measured in porcine jejunum challenged with ETEC in Ussing chamber experiments.

In the present study, ETEC decreased R_t significantly at 4 h, 6 h, and 8 h post ETEC infection in IPEC-J2 cells. The drop in R_t was ameliorated by pre-incubation of the cells with *E. faecium*. Since R_t can be used for an evaluation of intestinal barrier function, a barrier-stabilizing effect of *E. faecium* can be proposed as one of the protective probiotic mechanisms during ETEC infection. This result confirms a former study conducted at our institute showing the same protective impact of *E. faecium* on an ETEC-induced decrease in R_t in porcine IPEC-J2 and human Caco-2 cells (Klingspor et al. 2015). Within the scope of the present dissertation, the reproducibility of the *E. faecium*-mediated protective effects on the barrier integrity in IPEC-J2 cells was proven. In agreement with these results, enhancing effects on R_t have also been reported for other probiotics such as *Lactobacillus acidophilus*, *Streptococcus thermophilus*, the probiotic mixture #VSL, and *Bifidobacterium bifidum, breve*, *and infantis in vitro* (Resta-Lenert et al. 2003, Otte et al. 2004, Ewaschuk et al. 2008, Lopez et al. 2012).

In many studies, changes in R_t accompany modulations in TJ protein expression (Anderson 2001, Anderson et al. 2009, Yasumatsu et al. 2010, Liu et al. 2015, Wu et al. 2016). On the assumption that TJ proteins are associated with the decrease in R_t detected in the present study, selected TJ proteins were analyzed, namely claudin-1, -3, -4, -5, -7, and -8. Intriguingly, at the protein expression level, the TJ tested in the present study showed no differences after any bacterial incubation compared with control cells. This was unexpected since modulatory effects on TJ proteins have been reported for various other probiotics during ETEC infection (Roussel et al. 2017). *Lactobacillus plantarum* inhibited the ETEC-induced decreased mRNA expression of claudin-1, ZO-1, and occludin in IPEC-J2 cells (Wu et al. 2016, Wang et al. 2018). In the study of Wang et al. (2018), protein expression of claudin-1, ZO-1, and occludin was also reduced in ETEC-infected IPEC-J2 cells pre-incubated with *Lactobacillus plantarum*, whereas in the study of Wu et al. (2016), protective effects on protein expression have only been detected for occludin. Roselli et al. (2007) have reported that *Lactobacillus sobrius* prevented the ETEC-induced delocalization of ZO-1 and decreased protein expression of occludin in IPEC-1 cells. In jejunal tissue, occludin was also downregulated at the protein and mRNA level (Yang et al. 2014).

Since the protein expression of TJ proteins analyzed in the present study was not altered in IPEC-J2 cells *in vitro*, one might conclude that *E. faecium* mediates its protective barrier-enhancing properties *via* an alternative pathway. Indeed, confocal laser-scanning microscopy revealed that the structure of ETEC-challenged IPEC-J2 cells was reorganized. Certain cells seemed to form a second epithelial cell layer in some areas. They were arranged on top of each other, indicating increased cell shedding in ETEC-infected cells. This effect could be prevented by *E. faecium*. The aforementioned alterations in epithelial cell structure have not been found in *E. faecium*-mono-incubated cells, cells pre-incubated with *E. faecium* prior ETEC infection, or control cells. Increased cell shedding is described to decrease the intestinal barrier function (Williams et al. 2015). Various mechanisms can be considered that could have provoked the ETEC-induced alterations on epithelial cell structure as detected in the present study. In the literature, increased intestinal epithelial cell shedding is described following treatment with LPS or TNF-α (Marchiando et al. 2011, Watson et al. 2012, Williams et al. 2013). Mice injected with LPS intraperitoneally showed increased cell shedding in the small intestine, but this has not been found in mice deficient in TNF-receptor 1 (Williams et al. 2013). These data suggest that TNF-receptor 1 is essential for the formation of epithelial cell shedding. In the present study, TNF-α mRNA expression was upregulated in ETEC-incubated IPEC-J2 cells. This strengthened the assumption of a correlation between increased TNF-α concentrations and epithelial cell shedding. However, at the protein level, no differences of TNF-α could be detected between ETEC-incubated cells and cells co-incubated with *E. faecium* and ETEC. Cytotoxicity and apoptosis assays were performed to examine whether the rearrangement of the cells was correlated with apoptotic or necrotic events. The number of apoptotic cells was not influenced by bacterial incubation; however, cytotoxicity significantly increased in ETEC-incubated cells. This suggests that the non-physiological epithelial cell structure detected in ETEC-incubated cells in the present study is provoked by necrotic events. Lai et al. (1999) have also detected ETEC-induced cytotoxicity in J774 macrophages without the induction of apoptosis. Since pre-incubation with *E. faecium* prevents ETEC-induced cytotoxic effects and maintains the physiological epithelial cell structure, the findings presented in the present thesis strongly suggest a novel protective mechanism of the probiotic *E. faecium*. Protective probiotic effects on ETEC-associated impairment of porcine epithelial cell structure and cytotoxicity have previously not been analyzed *in vitro* and have only rarely been studied in porcine intestinal tissue so far. Yang et al. (2014) revealed that *Lactobacillus plantarum* inhibited ETEC-induced membrane damage in porcine jejunal tissue. Trevisi et al. (2017) provided evidence that the activation of genes such as the trefoil factor 3 gene, which affects the impairment of the jejunal mucosa of piglets, is limited by *Saccharomyces cerevisae*. *In vitro* studies examining probiotic effects on cytotoxicity during infection have to date only been conducted with pathogens other than ETEC. In Caco-2 cells, *Lactobacillus delbrueckii spp.*

bulgaricus inhibited the cytotoxic effects of *Clostridium difficile* infection (Banerjee et al. 2009). *Lactobacillus rhamnosus* GG reduced *Salmonella*-induced cytotoxicity in Caco-2 cells (Burkholder et al. 2009). The cytotoxicity mediated by the toxins of *Clostridium difficile* and *Bacillus cereus* were prevented by co-incubation of vero and Caco-2 cells with the supernatant of the probiotic *Bacillus clausii* (Ripert et al. 2016). *Lactobacillus rhamnosus, oris* and *reuteri* provided protection against the *Streptococcus pyogenes*-induced cytotoxicity in human pharyngeal epithelial cells (Maudsdotter et al. 2011). Lactobacilli are thought to mediate their anti-cytotoxic effects *via* the production of lactic acid, which degrades the toxic lipoteichoic acid of *Streptococcus pyogenes* (Maudsdotter et al. 2011). A protease secreted by *Bacillus clausii* is suggested to prevent the cytotoxic events of *Clostridium difficile* and *Bacillus cereus* infection (Ripert et al. 2016). The underlying mechanisms of the anti-cytotoxic impact of *E. faecium* during ETEC infection remain to be elucidated and will provide an interesting topic of further research.

In the present study, the effects of *E. faecium* on the intestinal barrier were also examined in the complex tissue of porcine jejunum during an ETEC challenge *ex vivo* in Ussing chamber experiments. Intriguingly, R_t measurements following incubation with ETEC revealed no significant reduction in R_t within 180 min post infection. Indeed, a numerical decrease of R_t in control epithelia could be detected that was not seen or only seen to a lesser extent in epithelia incubated with ETEC and *E. faecium*. This trend is in accordance with a former Ussing chamber experiment that was conducted at our institute and that analyzed the effects of ETEC infection on the jejunum of probiotic-supplemented piglets (Lodemann et al. 2017). In that former study, an increase in R_t was found during incubation of the jejunum with ETEC compared with control epithelia, peaking at 2 h after infection (Lodemann et al. 2017). At about 4 h post ETEC infection, R_t decreased back to its prior value, indicating an initial barrier-enforcing effect that leveled out during the time course of ETEC infection (Lodemann et al. 2017). At the TJ level, the protein expression of claudin-4 was decreased at 280 min post ETEC infection, possibly leading to ETEC-induced barrier failure in the longer term (Lodemann et al. 2017). The present study strengthens the suggestion that a tightening effect on the jejunal intestinal barrier occurs as an early effect of ETEC infection, with barrier decreasing effects taking place later on.

When comparing different *in vitro* and *ex vivo* studies that investigated barrier properties during ETEC infection, findings appear contradictory. In IPEC-J2 cells, ETEC induced a decrease in R_t whereas ETEC fortified the intestinal barrier in the porcine jejunal tissue (Klingspor et al. 2015, Lodemann et al. 2017). In IPEC-J2 cells, the ETEC-induced reduction of R_t mostly occurred between 1 h and 8 h post infection (Klingspor et al. 2015, Liu et al. 2015, Lodemann et al. 2015). In the present study, ETEC decreased R_t at 4 h, 6 h, and 8 h post infection in IPEC-J2 cells. By contrast, R_t increased at 2 h and 3 h after ETEC infection in the jejunal tissue.

The latter seemed to be limited to early infection with ETEC, since R_t peaked at 2 h post infection and decreased afterwards (Lodemann et al. 2017). An early barrier-enhancing effect could also be detected in an LPS infection trial in Yorkshire pigs (Albin et al. 2007). At 4 h after LPS exposure *in vivo*, the pigs were euthanized, and samples of the small intestine were analyzed in modified Ussing chambers revealing increased R_t values in LPS-exposed tissues (Albin et al. 2007). The R_t enhancing effect during the first few hours of ETEC infection *ex vivo* probably cannot be found in cell monolayers *in vitro* since cell lines are limited in their suitability to mimic complex intestinal functions (Kim et al. 2013). 3D-strucures, differentiated cell types and underlying immune cells do not exist in cell monolayers (Haller et al. 2000, Susewind et al. 2016). However, they may play a crucial role in mediating this initiate protective barrier-enhancing effect.

In advanced states of ETEC infection, studies on both intestinal cells *in vitro* and on complex intestinal tissue showed impaired barrier function (Gao et al. 2013, McLamb et al. 2013, Yang et al. 2014, Klingspor et al. 2015, Liu et al. 2015, Lodemann et al. 2015). *In vitro*, ETEC-induced impairment of the intestinal barrier started in IPEC-J2 cells as early as 1 h after infection, whereas this effect was postponed to 3 days post infection in jejunal tissue *in vivo* (Yang et al. 2014, Liu et al. 2015). The study of Wu et al. (2018) revealed that the amount of the TJ proteins occludin, ZO-1, and claudin-1 increased in the jejunum of mice even at 1 day post ETEC infection indicating a barrier-fortifying effect. This suggests that the reduced intestinal barrier properties caused by an ETEC infection may occur between 1 day and 3 days post infection in complex jejunal tissue. Studies investigating barrier properties in intestinal tissue in the period between 1 day and 3 days post infection are still lacking.

During advanced ETEC infection *in vivo*, the impairment of intestinal barrier properties has also been found by other authors. At 4 days post ETEC infection, the intestinal barrier was impaired in ileal tissue, and at 11 days post infection, the mRNA expression of occludin, ZO-1, and ZO-2 decreased in jejunal tissue (Gao et al. 2013, McLamb et al. 2013).

Taken together, the results suggest that the intestinal barrier in porcine jejunal tissue increases as a first response to ETEC infection. This effect cannot be detected in intestinal cell lines, probably, because of the limitations inherent in cell culture models. The early decrease in R_t seen in the *in vitro* cell culture studies occurs at a later point of time in the complex jejunal tissue. The decrease in R_t during ETEC infection of IPEC-J2 cells *in vitro* has been associated with an ETEC-induced cytotoxic effect. Both the ETEC-induced drop in R_t and the cytotoxic effect can be ameliorated by the probiotic *E. faecium*. For the first time, a novel protective mechanism of *E. faecium* on cell structure has been detected in the present thesis: *E. faecium* maintains the physiological epithelial cell structure in the intestinal cells during ETEC infection.

7.2 Effects of *E. faecium* on cAMP levels during ETEC infection

The present study analyzed the levels of cAMP during an ETEC infection since an increase in intracellular cAMP concentration is one of the first reactions in intestinal epithelial cells upon ETEC infection (Roussel et al. 2017). After ETEC adheres to the small intestinal mucosa, it releases LT, which activates the adenylate cyclase and raises cAMP concentrations in the cell (Roussel et al. 2017). The main functions of cAMP in the cell are activation of protein kinase A, the cyclic nucleotide-gated ion channels, and the guanosine triphosphate (GTP) exchange protein (Fimia et al. 2001). The expression of proinflammatory cytokines can also be regulated by cAMP (Hershko et al. 2002). ETEC-induced watery diarrhea is mediated by the cAMP-induced activation of protein kinase A, which opens Cl^- channels such as the CFTR channel (Kopic et al. 2010).

We hypothesized that *E. faecium* modulates cAMP concentrations during ETEC infection. In accordance with this hypothesis, results of the present study revealed that the cAMP concentration was increased in ETEC-incubated IPEC-J2 cells. Pre-incubation with *E. faecium* abolished the ETEC-induced increase in cAMP concentration. Wang et al. (2012) also detected elevated cAMP levels after ETEC infection in intestinal adenocarcinoma HCT-8 cells. Presumably, increased cAMP levels were mediated by ETEC-released LT as the upregulation of cAMP concentration was significantly lower when the cells were infected with an ETEC strain deficient in LT (Wang et al. 2012). In human T84 colonic epithelial cells, an increase of cAMP concentration can be found by incubation with LT (Read et al. 2014). Probiotic effects on cAMP levels during a pathogenic challenge have previously been analyzed by Czerucka et al. (1994, 1995). Medium conditioned with *Saccharomyces boulardii* inhibited cholera-toxin-induced cAMP levels in rat intestinal epithelial cells (Czerucka et al. 1994). Interestingly, the effect is thought to be mediated by a specific yeast protein that occurs in the medium and that activates an receptor that is negatively coupled to adenylate cyclase (Czerucka et al. 1994). Another study of Czerucka at al. (1999) has revealed that receptor-mediated (cholera toxin, prostaglandin E_2, and vasoactive intestinal polypeptide) and nonreceptor-mediated (forskolin) cAMP synthesis is counteracted in human intestinal epithelial T84 cells by medium conditioned with *Saccharomyces boulardii*. A protective effect on cAMP-induced Cl^- secretion has also been detected for *Bifidobacterium breve* B50 and its conditioned medium, and for *Lactobacillus rhamnosus*, which decreased Cl^- secretion in forskolin-stimulated human intestinal epithelial cells (Heuvelin et al. 2010).

Taken together, counteraction of ETEC-induced increases of cAMP levels have been revealed as a protective mechanism of *E. faecium* in the present thesis for the first time. As mentioned above, cAMP elevation is one of the first reactions in ETEC pathogenesis (Roussel et al. 2017).

This suggests that the protective probiotic effect of *E. faecium* occurs at an early stage of ETEC infection.

7.3 Effects of *E. faecium* on inflammatory cytokines during ETEC infection

Cytokines are crucial contributors to immune reactions (Liles et al. 1995). They act as messengers for numerous signals resulting in cell growth, cell differentiation, and the activation of various pathways (Commins et al. 2010). Cytokines are mainly produced by immune cells; however, they can also be released by epithelial cells (Jung et al. 1995, Liles et al. 1995, Stadnyk 2002). On the one hand, proinflammatory cytokines such as TNF-α and IL-1β are known to impair intestinal barrier properties (Al-Sadi et al. 2007, Slebioda et al. 2014). On the other hand, inflammatory cytokines such as IL-6 and IL-18 convey protective effects upon infection with pathogenic bacteria (Dinarello et al. 2013, Kuhn et al. 2014). Since inflammatory cytokines are often released upon infection and play an essential role in modulating the reaction of the organism during pathogenic offense, a well-established set of inflammatory cytokines has been analyzed in the present study. Notably, in a former study conducted at our institute, ETEC has been shown to increase the expression and release of IL-8, both of which can be inhibited by *E. faecium* in IPEC-J2 cells (Klingspor et al. 2015). The present study investigated the effects of *E. faecium* during an ETEC infection on the following cytokines:

7.3.1 Effects of *E. faecium* on TNF-α during ETEC infection

ETEC elevates the mRNA expression of proinflammatory TNF-α in IPEC-J2 cells; this elevation can be ameliorated by pre-incubation of the cells with *E. faecium*. In agreement with these findings, Wu et al. (2016) and Wang et al. (2018) revealed that the upregulated expression of TNF-α in IPEC-J2 cells post ETEC infection can be reduced by pre-treatment with *Lactobacillus plantarum*. The effect is thought to be mediated through the modulation of TLRs, nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kappaB), and mitogen-activated protein kinase (MAPK) pathways (Wu et al. 2016). Increased release of TNF-α by ETEC was also detected at the protein level in the present study, however, a protective probiotic effect has not been found at that level. Zhang et al. (2015) and Wang et al. (2018) have also reported an increased protein release of TNF-α in IPEC-J2 cells incubated with ETEC. These studies even revealed a protective effect by the probiotic *Lactobacillus rhamnosus* resp. *Lactobacillus plantarum* on the elevated TNF-α release during ETEC infection (Zhang et al. 2015, Wang et al. 2018). However, the present study and the study of Zhang et al. (2015) and Wang et al. (2018) differ in their study design and the probiotic strains used. In the present study, the release of TNF-α was determined at 8 h after ETEC infection. ETEC strains were killed by the addition of gentamicin after 2 h, and the cells were washed

twice with gentamicin-conditioned cell culture medium. In the study of Zhang et al. (2015) and Wang et al. (2018), TNF-α protein was measured 3 h after ETEC infection without previous devitalization of bacterial strains. The pre-incubation time with the probiotic (i.e. 2 h) was the same in the study of Zhang et al. (2015) compared with the present study, whereas IPEC-J2 cells in the study of Wang et al. (2018) were pre-incubated for 6 h with *Lactobacillus plantarum*. Since the incubation time with viable ETEC varied and since the early released TNF-α was not washed out, the concentrations of TNF-α might have been higher in the study of Zhang et al. (2015) and Wang et al. (2018). In the present study, the concentrations of TNF-α were low and could only be detected by elevating the sample volume to increase the amount of protein in the samples. The latter suggests that the applied test in the present study might have missed a protective effect of *E. faecium* on TNF-α release because of low values of TNF-α protein in the samples. The prolonged pre-incubation time with *Lactobacillus plantarum* in the study of Wang et al. (2018) might have also modulated the probiotic effect. Moreover, different probiotic strains may convey their effects *via* different mechanisms. This might explain the dissimilar results on comparing the three studies.

7.3.2 Effects of *E. faecium* on IL-6 during ETEC infection

IL-6 expression was not regulated by ETEC infection at the mRNA level in the present study. In the literature, an elevated mRNA expression of IL-6 by ETEC was described in a porcine intestinal epitheliocyte cell line (PIE cells) at 12 h post infection (Shimazu et al. 2012). PIE cells are similar to IPEC-J2 cells, but were isolated from a different neonatal piglet (Berschneider 1989, Moue et al. 2008). Consequently, inter-individual differences might occur in the responsiveness of these cells to stimuli such as ETEC. IPEC-J2 cells were originally isolated in 1989 at the University in North Carolina (Berschneider 1989). The PIE cells used in the study of Shimazu et al. (2012) were originally isolated in 2007 at the Graduate School of Agricultural Science in Japan (Moue et al. 2008). In addition, PIE cells are not described as being derived specifically from the jejunum (Moue et al. 2008). They were scraped from the lumen of the intestine, but the part of the intestine was not specified precisely (Moue et al. 2008). This might also explain the different responses to stimuli of PIE cells in comparison with IPEC-J2 cells. However, ETEC-induced upregulated mRNA expression of IL-6 might have also been missed in the present study because of the time point of measurement. The mRNA expression can be assumed to have been upregulated before 8 h post ETEC infection, as the protein release of IL-6 was increased at this time point in ETEC-incubated cells in the present study. The upregulation of IL-6 mRNA in the ETEC-incubated cells at 12 h in the study of Shimazu et al. (2012) might indicate a second peak in IL-6 expression. However, the different findings might also be associated with the different cell lines used. In accordance with our results, Wang et

al. (2016) have not found an increase in IL-6 mRNA expression at 1.5 h post ETEC infection in IPEC-1 cells. However, Wang et al. (2018) detected an increase in IL-6 mRNA expression at 3 h post ETEC-infection in IPEC-J2 cells which was counteracted by preincubation with *Lactobacillus plantarum*. The different results may be explained by differences in the study design of the study of Wang et al. (2018) compared with the present study as already discussed in the previous section 7.3.1 'Effects of *E. faecium* on TNF-α during ETEC infection'. Although no effects on IL-6 mRNA levels were detected in the present study, ETEC increased the protein release of IL-6, which could be prevented by pre-incubation with *E. faecium*. At the protein level, Devriendt et al. (2010) also described a heightened release of IL-6 in IPEC-J2 cells at 26 h after ETEC infection. In their study, cells were incubated for 2 h with ETEC at a multiplicity of infection (MOI) of around 75, followed by washing with phosphate buffered saline (PBS) and incubation for another 24 h with differentiation medium containing gentamicin. Increased IL-6 protein release was also detected in ETEC-infected IPEC-1 cells at 1.5 h and IPEC-J2 cells at 3 h, an effect that could be counteracted when the cells were pre-incubated with *Lactobacillus reuteri* resp. *Lactobacillus plantarum* (Wang et al. 2016, Wang et al. 2018). Since *E. faecium* mediated the amelioration of ETEC-induced IL-6 release, a protective effect of the probiotic was clearly indicated in the present study.

7.3.4 Effects of *E. faecium* on IL-1α during ETEC infection

The proinflammatory cytokine IL-1α was numerically affected at the mRNA level, and IL-1α protein was significantly elevated by ETEC. However, pre-incubation with *E. faecium* could not abolish the ETEC-induced increase of IL-1α protein release. These findings suggest that ETEC regulates IL-1α protein release and mRNA expression in porcine intestinal epithelial cells, as previously described by Shimazu et al. (2012) and indicated by van der Aa Kuhle (2005) for mRNA levels of IL-1α. However, *E. faecium* might not mediate protective effects *via* alterations of this cytokine.

7.3.5 Effects of *E. faecium* on IL-1β and IL-18 during ETEC infection

In the present study, the effects of *E. faecium* on selected cytokines were also analyzed in complex jejunal tissue during an ETEC challenge trial *ex vivo* in Ussing chambers. We examined the cytokines IL-1β and IL-18 in jejunal tissue. The latter cytokines are released upon activation of the inflammasome NLRP3 (Zaki et al. 2011). IL-18 protein expression was affected by incubation with neither ETEC nor *E. faecium*. However, a significant effect was detected with regard to IL-1β protein expression. ETEC increased IL-1β release, which could be counteracted by pre-incubation with *E. faecium*. The results for IL-1β and IL-18 protein

expression during the ETEC challenge trial are discussed in more detail in section 7.4 ‘Expression of inflammasome components in the intestine of pigs’.

In summary, *E. faecium* mediates protective effects on IPEC-J2 cells during ETEC infection by the downregulation and decreased release of specific proinflammatory cytokines. In the present work, a protective effect of *E. faecium* was detected on ETEC-induced TNF-α mRNA expression and on IL-1β and IL-6 protein expression.

7.4 Expression of inflammasome components in the intestine of pigs

One of the aims of the present studies was to investigate, for the first time, whether the protective effects of *E. faecium* during ETEC infection are mediated *via* an activation of inflammasomes.
Since almost nothing can be found in literature about the existence and relevance of inflammasomes in pigs, the expression of inflammasome components NLRP3, NLRP6, ASC, caspase-1 and the cytokines IL-1β and IL-18 were analyzed in the jejunum, ileum, and colon of control pigs as a first step. Next, the effects of age on the expression of inflammasome components were analyzed in order to complete the characterization of basal inflammasome components in pigs. After this characterization, the inflammasome components were examined in the intestine of pigs supplemented with the probiotic *E. faecium*. Finally, to analyze the effects of *E. faecium* on components of the inflammasome during a pathogenic challenge with ETEC, an *ex vivo* infection trial was conducted using the Ussing chamber technique.

7.4.1 Basal expression of inflammasome components in the porcine intestine of control pigs of different ages

As stated above, almost nothing can be found about inflammasomes in pigs to date. In the present study, evidence for the expression of NLRP6 and ASC in the jejunum, ileum, and colon of pigs has been provided for the first time. Furthermore, a characteristic oral-aboral expression pattern was identified for NLRP6, caspase-1, and ASC. These inflammasome components showed a generally decreasing expression in an oral-aboral direction in the intestinal tissues of 29-day-old piglets. In the jejunum, expression of NLRP6, caspase-1, and ASC was highest, indicating that the functional role of these inflammasome components is most important in this part of the intestine. Interestingly, the number of the immune cells expressing cluster of differentiation (CD)2, major histocompatibility complex (MHC) class II, and CD172 is greater in the jejunum than in the ileum of 18- to 25-day-old piglets (Brown et al. 2006). Since inflammasomes play an important role in innate immunity, and since inflammasome components are often described to be present in immune cells (Mariathasan et al. 2004,

Laudisi et al. 2013, Guo et al. 2015), the increased expression of NLRP6, caspase-1, and ASC in the jejunum of the 29-day-old-piglets in the present study might be associated with the conceivably higher number of immune cells. Differences in the basal mRNA expression of IL-1β and IL-18 in the porcine jejunum, ileum, and colon could not be detected, suggesting that these cytokines are equally relevant in all these intestinal parts.

Comparing the expression pattern of inflammasome components in the intestine of pigs in the present study with the expression of inflammasome components in the human intestine, the following similarities could be revealed: NLRP3 and IL-1β appear to be expressed at almost the same levels in the small intestine and in the colon (Uhlen et al. 2010, The Human Protein Atlas 2020). NLRP6, ASC, and caspase-1 are expressed at higher levels in the small intestine than in the colon (Uhlen et al. 2010, Gremel et al. 2015, The Human Protein Atlas 2020). Hence, the present work reinforces the idea of using pigs as an animal model for translational and preclinical inflammasome research in humans. The expression patterns of inflammasome components in the intestine of humans and pigs resemble each other impressively.

An age effect could be revealed in the ileum of control piglets for NLRP3, which was significantly higher at the age of 29 days compared with that in 70-day-old pigs. This indicates that the activity of NLRP3 in the ileum of 29-day-old pigs has a specific function at this age. The expression of NLRP6, ASC, caspase-1, and IL-18 tended to be the lowest in the colon of both 29-day-old and 70-day-old pigs. However, this effect was only significant for NLRP6 and ASC expression in the 70-day-old fattening pigs. The findings suggest that the investigated inflammasome components are constantly expressed in the colon at a low level. Consequently, they may have a minor functional relevance in the colon.

The previously mentioned oral-aboral mRNA expression pattern of NLRP6, caspase-1, and ASC in the jejunum, ileum, and colon of 29-day-old piglets leveled off in 70-day-old fattening pigs. Intriguingly, other authors have identified a similar pattern for the existence of immune cells in the jejunum and ileum of pigs (Brown et al. 2006). As previously stated, Brown et al. (2006) have reported that the number of immune cells in 18- to 25-day-old piglets is significantly higher in the jejunum than in the ileum. With increasing age post-weaning, this difference in the number of immune cells in these parts of the intestines can no longer be found. The reason for this effect is the greater number of CD2 cells in the ileum of piglets later in life (Brown et al. 2006). Thus, the expression of NLRP6, caspase-1, and ASC might mainly derive from the different number of immune cells at different ages in the intestine of pigs.

Significant age effects for the expression of the cytokines IL-1β and IL-18 could not be detected in the jejunum, ileum, and colon. Hence, these cytokines may not be affected by age-dependent alterations such as increased CD2 expression in the ileum or decreased villus length in the jejunum of older pigs (Cera et al. 1988, Brown et al. 2006).

7.4.2 Effects of supplementation with *E. faecium* on the expression of inflammasome components in the porcine intestine

In the experiments of the present thesis, mother sows were supplemented with *E. faecium* 28 days before farrowing. Thus, piglets came into contact with the probiotic on their first day of life through their mothers (birth, feces, straw bedding). In addition, the probiotic was administered to the piglets with their pre-starter diet beginning at day 12. At the age of 29 days (one day before weaning), the piglets were killed, and the expression of inflammasome components in the jejunum, ileum, and colon was compared with that of control animals. With regard to ASC expression, significant effects could be detected following supplementation of piglets with *E. faecium*. Other inflammasome components tested were not affected by supplementation with the probiotic in the present study.

The adaptor ASC is a component of various inflammasomes such as NLRP1, NLRP3, NLRP6, NLRC4, and AIM2 (Bauernfeind et al. 2013). It is required by all inflammasomes for the activation of caspase-1 (Stutz et al. 2013). The mRNA expression of ASC was higher in the jejunum and ileum of probiotic-treated piglets than in control piglets.

The previously mentioned oral-aboral expression pattern for NLRP6, ASC, and caspase-1 in 29-day-old control piglets could also be found in 29-day-old piglets supplemented with *E. faecium*. Hence, the characteristic expression pattern effect was confirmed and shown to occur independently of supplementation with the probiotic.

The expression of NLRP3, caspase-1, and IL-18 was not changed in the present study in piglets supplemented with *E. faecium* compared to control pigs. Similar observations have also been described in the study of Schmitz et al. (2015), showing no impact of *E. faecium* on NLRP3, caspase-1, and IL-18 expression levels in the duodenum of dogs. The results suggest that *E. faecium* does not mediate its effects *via* an alteration in the expression of the inflammasome components NLRP3, caspase-1, and IL-18. However, the expression of NLRP6 in the ileum and of IL-1β in the jejunum was numerically upregulated by the probiotic in the present study.

In the literature, almost no studies have been conducted in pigs in order to analyze the influence of *E. faecium* on the expression of the inflammasome components that were examined in the present work. However, with respect to IL-1β protein release, the study of Huang et al. (2012) revealed interesting findings. *E. faecium* EF1 reduced IL-1β protein expression in the jejunum and ileum of 25-day-old piglets. The piglets were supplemented with 5-6×10^8 colony-forming units (CFU) *E. faecium* EF1 per ml in their milk at days 1, 3, and 5 post-partum (Huang et al. 2012). In the present study, such effect could not be found. However, the study design used here was not identical to that of Huang et al. (2012). In the present investigation, the piglets were 29 days old and received only 4×10^6 CFU *E. faecium* per gram

of feed in their pre-starter diet. Mother sows were also fed with 4×10^6 CFU *E. faecium* per gram of feed at 28 days before farrowing and after parturition. These differences in the design of the studies might have led to the different outcomes. However, in agreement with the results in the present work, IL-1β mRNA expression was not altered in intestinal tissue of dogs that was incubated with *E. faecium ex vivo* for 5 h (Schmitz et al. 2015). Notably, the study of Huang et al. (2012) examined protein levels, whereas the present study and the study of Schmitz et al. (2015) analyzed the mRNA expression of IL-1β. Probably, the protein release of IL-1β is modulated functionally by supplementation with *E. faecium* without affecting the mRNA expression.

Indeed, the present study provides evidence for the functional activation of IL-1β protein release without corresponding effects on the mRNA level during ETEC infection. This is discussed in more detail in section 7.4.3 ‘Effects of pre-incubation with *E. faecium* on the expression of inflammasome components in the jejunum during ETEC infection’.

7.4.3 Effects of pre-incubation with *E. faecium* on the expression of inflammasome components in the jejunum during ETEC infection

Few studies have investigated probiotic effects on the expression of inflammasome components during infection with noxes or pathogens (Schmitz et al. 2015, Vilahur et al. 2015, Wu et al. 2015, Liu et al. 2016, Loss et al. 2018, Loss et al. 2018). On the assumption that the probiotic *E. faecium* mediates protective effects *via* the modulation of inflammasome components more intensively during a pathogenic challenge, an *ex vivo* challenge trial was conducted within the frame of the present thesis. As previously mentioned, inflammasome components are often described as being present in immune cells (Mariathasan et al. 2004, Laudisi et al. 2013). Conventionally fed pigs were sacrificed at the age of 80 days, and isolated jejunal epithelium was mounted in Ussing chambers. The jejunum was incubated with *E. faecium*, ETEC, both bacterial strains, or without bacteria. Intriguingly, no effects could be detected on the mRNA expression of NLRP3, NLRP6, ASC, caspase-1, IL-1β, or IL-18, on incubation with neither ETEC nor *E. faecium*. In contrast, a former study identified upregulated NLRP3 expression in the jejunum of 29- to 30-day-old German Landrace piglets incubated with ETEC in Ussing chambers at a final concentration of ~10^8 CFU/ml at the mucosal side for 3 h (Lodemann et al. 2017). The concentration of ETEC in the present study was the same. However, the age and breed of the pigs was different as 80-day-old Danbred x Pietrain growers were used in the present study compared with the 29- to 30-day-old German Landrace piglets in the study of Lodemann et al. (2017). The growers in the present study possibly responded differently to ETEC infection because of breed- and age-dependent modulations in the jejunum. Evidence for breed-dependent differences in the susceptibility to ETEC infection

comes from Gao et al. (2013). European Landrace pigs showed a significantly higher incidence of ETEC-induced diarrhea, shed more *E. coli*, and were inhabited by a greater microbial population of *E. coli* in their colon than challenged Jinhua pigs (Gao et al. 2013). Age-dependent effects during ETEC infection were examined in the study of Runnels et al. (1980). $K99^+$ ETEC adhered less to the intestinal epithelial cells of 6-week-old pigs than to intestinal cells of 21-day-old piglets (Runnels et al. 1980). The reason for this phenomenon is still not fully understood. The resistance to ETEC infection in older pigs is suggested not to be dependent on the decreased appearance of $K88^+$ and F6 receptors, as these receptors are also expressed abundantly in older pigs (Dean 1990, Erickson et al. 1992). An aquired immunity by natural exposure to $K88^+$ and F6 ETEC seems also not responsible for the decreased susceptibility to ETEC infection in adult pigs (Francis 2002). Francis (2002) suggested that modulations in the intestinal brush border glycocalyx are associated with decreased susceptibility to ETEC infection in adult pigs, since a modified glycocalyx might render receptors less available for the adhesion of ETEC fimbriae. Thus, age- and breed-dependent changes might explain the absence of ETEC-induced effects on the NLRP3 mRNA expression in the porcine jejunum in the present study.

As a readout of NLRP3 activity, IL-1β was measured in the jejunum and in the incubation medium of the jejunum during the infection trial with ETEC. As previously mentioned, the mRNA expression of IL-1β was not affected by bacterial incubation in the jejunum. Contrary to our results, IL-1β mRNA expression was upregulated in ETEC-infected Caco-2 cells at 2 h post infection, which could be counteracted by the probiotic bacteria *Bifidobacterium animalis* and *Lactobacillus rhamnosus* (Roselli et al. 2006). However, these different observations in IL-1β expression during ETEC infection might be explained by the facts that the study of Roselli at al. (2006) was conducted *in vitro* with different probiotic strains and that complex jejunal tissue was examined in the present study.

Despite the lack of effects on IL-1β mRNA levels by bacterial incubation of the jejunum in the present work, a clear effect was seen at the protein level. The release of IL-1β protein into the incubation medium of the jejunum was increased by ETEC. This effect was not detected in the incubation medium of ETEC-infected tissue pre-treated with *E. faecium*. In previous studies, the release of IL-1β was also elevated by ETEC in Caco-2/TC7 cells and could be abolished by the probiotic *Lactobacillus amylovorus* (Finamore et al. 2014). The mRNA levels of IL-1β are probably upregulated in the jejunum earlier than 3 h after infection, which was the time point of measurement in the present study. Another explanation might be that the release of IL-1β in the jejunum is mediated *via* the functional activation of the inflammasome pathway, an event that is not dependent on IL-1β mRNA expression. After the first step of inflammasome activation (signal 1), the translated precursors are already present within the cell (see section 2.5 ‘Inflammasomes’). Thus, the assembly of the inflammasome complex and the activation of

caspase-1, which cleaves pro-IL-1β to IL-1β, might occur without the upregulation of IL-1β mRNA when sufficient pro-IL-1β is contained within the cell. One can also assume that IL-1β protein is released by the activation of other inflammasomes or non-canonical inflammasomes that have not been included in the setup of the present work.

The work presented here thus provides evidence for a protective effect of *E. faecium* mediated *via* an inflammasome pathway. A more detailed analysis of these pathways should provide an interesting topic for further research.

7.5 Summary and Conclusion

In the present study, protective effects of the probiotic *E. faecium* have been revealed during infection with ETEC. Protective effects on intestinal barrier properties included the prevention of the ETEC-induced effects like a drop in R_t, destruction of the physiological epithelial cell structure, and cytotoxicity in IPEC-J2 cells. Furthermore, the expression and release of inflammatory cytokines during ETEC infection were reduced by *E. faecium* in IPEC-J2 cells. The concentration of the second messenger cAMP was elevated in ETEC-infected cells but not in *E. faecium* pre-incubated cells. This collectively indicates that the probiotic mediates its protective effects at an early stage of ETEC infection, since the release of cAMP is one of the early effects in ETEC pathogenesis.

The results presented here also suggest that the porcine inflammasome is a possible mediator of probiotic effects during ETEC infection. Characteristics of the expression of inflammasome components have been revealed in porcine intestinal tissue. For the first time, a characteristic oral-aboral expression pattern of the inflammasome NLRP6 and the inflammasome components ASC and caspase-1 has been detected in the intestine of piglets. In humans, these components are expressed in a similar way suggesting that the pig when used for preclinical and translational inflammasome research, is a good and representative animal model in human medicine. Pre-incubation with *E. faecium* decreased the ETEC-induced release of IL-1β protein in the supernatant of the jejunum of pigs incubated with the bacterial strains *ex vivo*. Since IL-1β is released by inflammasome activation, the porcine inflammasome is probably a mediator of protective probiotic effects at the functional level.

Based on the revealed cellular effects of *E. faecium* during ETEC infection, the use of *E. faecium* for the prevention of ETEC-induced diarrhea in piglets seems to be a promising antibiotic-free strategy for pig farming.

Chapter 8: Summary/Zusammenfassung

Summary of the thesis:

Probiotic effects on epithelial barrier properties and inflammatory response in an infection model of the porcine intestine

Martina Kern

The probiotic *Enterococcus faecium* NCIMB 10415 (hereafter called *E. faecium*) is a licensed feed additive for pigs in the EU. Piglets supplemented with *E. faecium* showed a decreased incidence and severity of post-weaning diarrhea in previous studies. The main cause of post-weaning diarrhea in piglets is infection with enterotoxigenic *Escherichia coli* (ETEC). However, the underlying cellular mechanisms of the protective effects of *E. faecium* against ETEC infection have only rarely been analyzed.

Therefore, the present study investigated the effects of the probiotic *E. faecium* on epithelial barrier properties and cytokine expression in the intestinal porcine epithelial cell line J2 (IPEC-J2) during an infection with ETEC *in vitro*. A decrease of the transepithelial electrical resistance (R_t) in ETEC-infected cells was detected that was not seen in cells pre-incubated with *E. faecium*. These observations confirmed the results of a former study conducted at our institute. The expression of tight junction (TJ) proteins analyzed in the present study was not influenced by incubation with the bacterial strains. However, a modulation of the cell structure was seen in ETEC-infected cells, i.e. cells arranged on top of each other, indicating increased cell shedding. This was not seen in ETEC-infected cells pre-incubated with *E. faecium*. The rate of apoptosis was not modulated by ETEC. However, the cytotoxicity that significantly increased in ETEC-infected cells, was ameliorated by pre-incubation with *E. faecium*. At the mRNA level, ETEC upregulated the proinflammatory cytokine tumor necrosis factor (TNF)-α and increased the release of the cytokines TNF-α, interleukin (IL)-1α, and IL-6 at the protein level. A protective effect of the probiotic could be detected for the ETEC-induced increased TNF-α mRNA expression and IL-6 protein release. Both were decreased by pre-incubation of cells with *E. faecium*. The second messenger 3′,5′-cyclic adenosine monophosphate (cAMP) was also elevated by ETEC, but this was counteracted in cells pre-incubated with *E. faecium*.

In a further part of the present study, the inflammasome was analyzed as a potential mediator of probiotic effects. First, the basal expression of inflammasome components was analyzed at the mRNA level in the intestine of piglets and fattening pigs. The expression of nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3), NLRP6, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), caspase-1, IL-1β, and IL-18 was detected in the jejunum, ileum, and colon of pigs. A characteristic expression pattern

was revealed for NLRP6, ASC, and caspase-1 in 29-day-old piglets that showed a decreasing expression of these components in an oral-aboral direction. Such expression pattern could not be detected in 70-day-old fattening pigs. Furthermore, the effects of the probiotic *E. faecium* on the mRNA expression of inflammasomes and inflammasome components were analyzed in a feeding trial. Piglets supplemented with *E. faecium* showed increased mRNA expression of ASC in the jejunum. The mRNA expression of NLRP3, NLRP6, caspase-1, IL-1β, and IL-18 was not modulated by the probiotic.

In order to analyze probiotic effects during an infection with a pathogen, the jejunum of 80-day-old pigs was incubated with *E. faecium*, ETEC, *E. faecium* and ETEC, or without bacteria in an Ussing chamber experiment *ex vivo*. The mRNA expression of the inflammasome components was not modulated by the bacterial strains. However, at the protein level, the release of IL-1β was significantly increased by ETEC. A protective effect was found for the probiotic *E. faecium*. ETEC-induced increased release of IL-1β protein was significantly reduced by pre-incubation with *E. faecium*.

In conclusion, several protective effects of the probiotic *E. faecium* have been detected at the cellular level during ETEC infection in the present work. The probiotic effects were mediated *via* the reduced decrease in R_t, the downregulated expression and release of inflammatory cytokines, the decreased intracellular concentrations of cAMP, the maintenance of the physiological epithelial cell structure, and the lessening of cytotoxic effects during ETEC infection. A characteristic oral-aboral expression pattern was revealed for the inflammasome NLRP6 and the inflammasome component ASC and caspase-1 in the intestine of piglets. The detected expression pattern was similar to the expression pattern in humans thereby reinforcing the use of pigs as an animal model for preclinical and translational inflammasome research. Pre-incubation with *E. faecium* reduced the ETEC-induced release of IL-1β protein. IL-1β release is associated with inflammasome activation. Hence, the present study provides evidence for the inflammasome as a mediator of protective probiotic effects at the functional level during infection with ETEC *ex vivo*.

The protective effects of *E. faecium* demonstrated in this study represent important findings concerning the cellular mode of action of the probiotic during ETEC infection. Based on these findings, the prophylactic use of *E. faecium* can be considered for the reduction of the incidence and severity of ETEC infections in piglets as a very promising strategy. The latter has the potential to decrease the necessity of the therapeutic use of antibiotics in pig farming.

Zusammenfassung der Dissertation zum Thema:

Probiotische Effekte auf epitheliale Integrität und entzündliche Reaktionen in einem Infektionsmodell des Schweinedarmes

Martina Kern

Das Probiotikum *Enterococcus faecium* NCIMB 10415 (im Folgenden *E. faecium* genannt) ist als Futterzusatzstoff für Schweine in der EU zugelassen. Ferkel, welche ein mit *E. faecium* supplementiertes Futter erhielten, zeigten in früheren Studien ein geringeres Auftreten und einen weniger schwerwiegenden Verlauf von Ferkeldurchfällen. Hauptverursacher von Durchfällen bei Absetzferkeln sind enterotoxische *Escherichia coli* (ETEC). Die zugrundeliegenden zellulären Effekte, über welche *E. faecium* protektive Effekte bei einer ETEC-Infektion vermittelt, wurden bisher kaum erforscht.

Die vorliegende Dissertation untersuchte zunächst den Einfluss des Probiotikums *E. faecium* auf die epitheliale Integrität und Zytokinfreisetzung während einer *in vitro* Infektion mit ETEC in der porzinen intestinalen epithelialen Zelllinie J2 (IPEC-J2). ETEC verursachte einen Abfall des transepithelialen Widerstandes (R_t). Dieser Abfall wurde durch Präinkubation mit *E. faecium* vermindert. Dies bestätigte das Ergebnis einer vorherigen Studie an unserem Institut. Die in der vorliegenden Dissertation untersuchten Tight-Junction (TJ)-Proteine wurden durch die Inkubation mit den verschiedenen Bakterienstämmen nicht beeinflusst. Jedoch zeigten die mit ETEC infizierten Zellen eine Veränderung ihrer Struktur im Zellverband. Einzelne Zellen ragten aus dem Zellverband heraus, nachdem sie mit ETEC inkubiert wurden. Dies weist auf eine erhöhte Abschilferung dieser Zellen hin. Die Veränderung war nicht zu erkennen, wenn die Zellen zuvor mit *E. faecium* präinkubiert wurden. Die Apoptose-Rate der Zellen wurde durch ETEC nicht beeinflusst. Jedoch bewirkte die Inkubation mit ETEC eine erhöhte Zytotoxizität in den Zellen, welche durch Präinkubation mit *E. faecium* vermindert werden konnte. Des Weiteren wurden durch die Inkubation der IPEC-J2 Zellen mit ETEC auf mRNA-Ebene das proinflammatorische Zytokin Tumornekrosefaktor (TNF)-α vermehrt exprimiert und auf Protein-Ebene die Zytokine TNF-α, Interleukin (IL)-1α und IL-6 im höheren Maße ausgeschüttet. Hierbei konnte ein protektiver Effekt des Probiotikums *E. faecium* festgestellt werden, indem die ETEC-induzierte erhöhte Expression von TNF-α mRNA sowie die gesteigerte Ausschüttung von IL-6 Protein unterbunden wurde. Zudem wurde durch ETEC die Konzentration des sekundären Messengers cyklisches Adenosinmonophosphat (cAMP) in den Zellen erhöht, welche in Zellen, die mit *E. faecium* präinkubiert wurden, nicht anstieg.

Um das Inflammasom als potentiellen Vermittler probiotischer Effekte zu untersuchen, wurde zunächst das Vorkommen von Komponenten des Inflammasoms im Darm von Absetzferkeln und Mastschweinen auf mRNA-Ebene untersucht. Die mRNA Expression von NLRP3, NLRP6, ASC, Caspase-1, IL-1β und IL-18 konnte im Jejunum, Ileum und Colon von Schweinen

nachgewiesen werden. Ein charakteristisches Expressionsmuster, welches eine Abnahme der Expression von oral nach aboral zeigte, wurde für NLRP6, ASC und Caspase-1 bei 29 Tage alten Absetzferkeln entdeckt. Bei 70 Tage alten Mastschweinen war dieses Expressionsmuster nicht mehr zu erkennen. In einem weiteren Versuch wurde der Einfluss des Probiotikums *E. faecium* auf die mRNA Expression von Komponenten des Inflammasoms in einem Fütterungsversuch mit *E. faecium* erforscht. Mit *E. faecium* gefütterte Ferkel zeigten eine erhöhte mRNA Expression von ASC im Jejunum. Die mRNA Expression von NLRP3, NLRP6, Caspase-1, IL-1β und IL-18 wurde durch die Fütterung mit dem Probiotikum nicht beeinflusst. Um Effekte des Probiotikums während einer Infektion mit einem Pathogen zu analysieren, wurde in einem Ussingkammer-Versuch Jejunum von 80 Tage alten Schweinen *ex vivo* mit *E. faecium*, ETEC, *E. faecium* und ETEC sowie ohne Bakterien inkubiert. Die Expression der untersuchten Komponenten des Inflammasoms wurde durch die Inkubation mit den verschiedenen Bakterien nicht beeinflusst. Jedoch wurde auf Protein-Ebene die Ausschüttung von IL-1β durch ETEC signifikant erhöht. Das Probiotikum *E. faecium* zeigte hierbei einen protektiven Effekt, indem es die ETEC-induzierte erhöhte Expression von IL-1β reduzierte.

Zusammenfassend konnten in der vorliegenden Arbeit mehrere protektive Effekte des Probiotikums *E. faecium* während einer Infektion mit ETEC auf zellulärer Ebene nachgewiesen werden. Diese protektiven probiotischen Effekte wurden über einen verminderten Abfall des R_t, eine reduzierte Expression und Ausschüttung inflammatorischer Zytokine, eine geringere Konzentration von cAMP in den Zellen, die Erhaltung der physiologischen epithelialen Zellstruktur und verminderte zytotoxische Effekte während einer Infektion mit ETEC vermittelt. Für das Inflammasom NLRP6 und die Inflammasom-Komponenten ASC und Caspase-1 wurde ein charakteristisches oral-aborales Expressionsmuster im Darm von Absetzferkeln entdeckt, welches mit dem Expressionsmuster des Menschen übereinstimmt und das Schwein als Versuchstier für die präklinische und translationale Inflammasom-Forschung prädestiniert. Durch Präinkubation mit *E. faecium* wurde die Ausschüttung des proinflammatorischen Zytokins IL-1β, welches durch Aktivierung des Inflammasoms freigesetzt wird, im ETEC-infizierten Gewebe reduziert. Das Inflammasom als Vermittler probiotischer Effekte konnte somit auf funktioneller Ebene während einer *ex vivo* Infektion mit ETEC nachgewiesen werden. Die in dieser Dissertation entdeckten protektiven Effekte des Probiotikums *E. faecium* liefern wichtige Erkenntnisse über die Wirkungsweise des Probiotikums während einer Infektion mit ETEC auf zellulärer Ebene. Der prophylaktische Einsatz von *E. faecium* zur Reduzierung des Auftretens und Minderung des Schweregrades einer ETEC-Infektion bei Ferkeln kann auf Grundlage der in dieser Arbeit beschriebenen protektiven Effekte als sehr vielversprechende Methode angesehen werden, die das Potential hat die Notwendigkeit eines therapeutischen Einsatzes von Antibiotika in der Schweinehaltung bei dieser Erkrankung zu reduzieren.

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2016

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In: 70th conference 8th-10th March 2016 in Hannover: review, abstracts, workshop – Gesellschaft für Ernährungsphysiologie (Hrsg.)

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