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Mechanistic impact of *Enterococcus faecalis* metalloprotease on bacterial virulence in the context of chronic intestinal inflammation

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ABSTRACT

Host-derived proteases have been implicated in gastrointestinal disorders including inflammatory bowel diseases (IBD), the etiology of which has not been elucidated yet. The pathogenesis of IBD is characterized by a deregulated immune response towards the intestinal microbiota. *Enterococcus faecalis* (*E. faecalis*) are Gram-positive lactic acid bacteria living as commensal inhabitants in the mammalian gastrointestinal tract and belonging to the core microbiome in the human gut. The secreted zinc-dependent metalloprotease gelatinase (GelE) has been associated with *E. faecalis* virulence in several models. Thus, the aim of the study was to unravel the mechanism how *E. faecalis* GelE, as a protease produced by commensal gut bacteria, contributes to the development of chronic intestinal inflammation.

The characterized GelE expressing *E. faecalis* strain OG1RF, isogenic GelE mutants and GelE reconstituted mutant strains have been used in order to demonstrate protease specificity. Concentrated bacterial culture supernatant from GelE producing strains impaired barrier function of polarized intestinal epithelial cells (IEC). GelE expressing *E. faecalis* strains isolated from faecal samples of IBD patients and a healthy volunteer exhibited the same effect on epithelial barrier impairment suggesting that GelE activity of *E. faecalis* might be relevant for IBD. Additional experiments with purified GelE and Marimastat, an inhibitor for GelE activity, confirmed GelE to be responsible for the loss of barrier function in IEC. The reduction of epithelial barrier was associated with reduced expression levels of the tight junction protein Occludin. Bacterial GelE and pro-inflammatory cytokines exerted potentiating effects with respect to epithelial barrier impairment of IEC. The presence of *E. faecalis* GelE significantly reduced the ectodomain of adherence junction protein E-Cadherin in inflamed interleukin-10 deficient mice, but not in wild type mice. Furthermore, cleavage sites for GelE in the sequence of recombinant murine E-Cadherin could be identified, suggesting the possibility for a direct GelE-mediated degradation. Additionally, mucosal stimulation with purified GelE revealed the loss of epithelial barrier function and extracellular E-Cadherin in the distal colon of mice susceptible to intestinal inflammation. The generation of a *gelE-epaB* double deletion mutant revealed additive effects of GelE and enterococcal polysaccharide (epa) B, a putative glycosyl transferase, on enterococcal biofilm formation and virulence.

In summary, this study demonstrates that *E. faecalis* GelE contributes to the development of chronic intestinal inflammation in the disease susceptible host through the degradation of epithelial junction proteins. Future experiments with isogenic mutants for various structures including the *epa* locus will reveal the colitogenic mechanisms of *E. faecalis* and contribute to the understanding, how commensal bacteria are involved in the development of IBD.

ZUSAMMENFASSUNG

Endogenen Proteasen wird bei einer Vielzahl von Darmerkrankungen eine Rolle zugeschrieben. Dies gilt auch für chronisch entzündliche Darmerkrankungen (CED), deren Ursache bisher noch nicht aufgeklärt werden konnte. Eine überschießende Immunantwort gegen die intestinale Mikrobiota scheint Teil der Pathogenese von CED zu sein. *Enterococcus faecalis* (*E. faecalis*) ist ein kommensaler Bewohner des menschlichen Gastrointestinaltraktes und ist Teil des dort ansässigen humanem Mikrobioms. Das Ziel der vorliegenden Arbeit war es, den Einfluss der Metalloprotease Gelatinase (GelE), einem Virulenzfaktor von *E. faecalis*, auf die Entstehung von CED zu untersuchen.

Um den Effekt der Protease zu untersuchen, wurde der charakterisierte GelE produzierende *E. faecalis* Stamm OG1RF, sowie isogene GelE Mutanten und rekonstituierte Stämme verwendet. Konzentrierter Bakterienüberstand von GelE positiven Stämmen reduzierte die Barrierefunktion von kultivierten intestinalen Epithelzellen (IEZ). Die Isolierung GelE produzierender *E. faecalis* Stämme aus dem Fäzes von CED Patienten und einer gesunden Kontrolle zeigte, dass GelE von diesen Stämmen ebenfalls die Barriere von IEZ reduzierte. Dies verdeutlicht die Relevanz von *E. faecalis* GelE für CED. Weitere Experimente mit gereinigter GelE und Marimastat, einem Inhibitor der proteolytischen Aktivität, bestätigten die Spezifität des GelE-vermittelten Effektes auf die Barrierefunktion von IEZ. Der Verlust der Barrierefunktion war mit einer Reduktion des Tight Junktion Proteins Occludin assoziiert. Pro-inflammatorische Zytokine potenzierten den GelE-vermittelten Verlust der Barrierfunktion in IEZ. Im Gegensatz zu Wildtyp Mäusen, konnte die extrazelluläre Domäne des Adherence Junktion Proteins E-Cadherin nicht mehr in Interleukin-10 defizienten Mäusen nach Monokolonisierung mit OG1RF nachgewiesen werden. Außerdem konnten Spaltungsstellen in der Aminosäure Sequenz von rekombinantem murinem E-Cadherin nachgewiesen werden. Dies deutet auf die Möglichkeit eines direkten, durch GelE-vermittelten Abbaus hin. Zudem führte die Stimulation von distalem Kolongewebe mit gereinigter GelE zu dem Verlust der mukosalen Barrierefunktion und der Reduktion von extrazellulärem E-Cadherin im sukzessiblen Wirt. Die additiven Effekte von GelE und dem enterococcal polysaccharide antigen (epa) B, einer putativen Glykosyltransferase, auf Biofilm Bildung und Virulenz von *E. faecalis* konnte durch die Generierung einer Doppelmutante gezeigt werden.

Die vorliegende Arbeit zeigte, dass *E. faecalis* GelE zu der Entstehung von CED im sukzessiblen Wirt beitragen kann. Zukünftige Experimente mit Mutanten für verschiedene Strukturen wie dem *epa* Lokus werden die kolitogenen Mechanismen von *E. faecalis* aufklären und einen Beitrag dazu leisten, die Rolle der kommensalen Darmbakterien bei der Entstehung von CED zu verstehen.

1 Introduction

1.1 The importance of proteases

1.1.1 General characteristics and functions of proteases

In comparison to proteolytic enzymes catalyzing the cleavage of peptide bonds in other proteins, proteases are degradative enzymes mediating the total hydrolysis of proteins. Due to the huge diversity of action and structure, proteases have been divided on the basis of three major criteria: 1) type of reaction catalyzed (basically endo- and exopeptidases) 2) chemical nature of their catalytic site and 3) evolutionary relationship with reference to structure. Based on the functional group present at the active site, proteases have been further classified into serine, aspartate, cysteine, threonine, glutamic acid and metalloproteases [1]. Protease synthesis and storage requires tight control mechanisms in order to prevent excessive proteolytic activity and subsequent tissue damage. Therefore most proteases are synthesized and stored in the zymogen form. This inactive form serves to protect intracellular organelles which are responsible for protein synthesis and conversion from degradation. Proteolytic zymogen activation through conformational changes and the formation of an intact active site is either mediated by mature proteases or by autocatalytic domains. This process is often carefully regulated by the contribution of several mechanisms involved in controlling protease maturation and termination of the proteolytic action [2].

Proteases stand at the beginning of catabolism in almost every organ in every species. They participate and control a tremendous variety of different physiological events in the body such as the digestion of food compounds, the activation and procedure of the blood-clotting cascades or apoptotic events. Bacteria and other organisms such as fungi or arachnids produce and secrete proteases which can be part of their virulence factors. Furthermore, proteases are responsible for the digestion of complex extracellular matrix (ECM) compounds into monomers that can be absorbed providing energy and nutrient supply for the respective organism. Tight regulation of proteolytic activity in the body plays a pivotal role for homeostasis, especially in symbiotic communities such as the gut lumen. Proteolytic action of proteases can be inhibited by specific protease inhibitors, but also through environmental conditions including temperature, acidification or osmotic changes. The complex balance between proteases and anti-proteases or inhibitors maintains homeostasis, but is disturbed under pathophysiological conditions and therefore provides possibilities for therapeutic intervention. Proteolytic action can be disturbed in two directions, either there is too much proteolytic degradation or too few, which can be both contributing or leading to

disease development. Both situations can result from either a deregulation of the respective proteases or their inhibitors.

1.1.2 Host-derived and bacterial proteases in the gut – an unexplored world of complexity

The proteolytic capacity of the gut and the intestinal lumen consist of host- and bacteria-derived proteases in an unknown composition and proportion which depends on the intestinal region, the health status of the host, the diet and presumably other unknown factors. Digestive proteases including pepsin, trypsin, chemotrypsin, elastases and cathepsins are basically released in the small intestine in order to disintegrate and hydrolyse protein sources in food. Another remarkable group of host-derived proteases are matrix metalloproteinases (MMPs) which fulfil various functions including the degradation and turnover of ECM and the activation of signalling molecules including cytokines, chemokines, growth factors and junction proteins. The awareness of the intestinal bacterial proteolytic activity has already been described by MacFarlane and colleagues in the 1980s. The authors describe the bacterial proteolytic activity in faecal samples by using different p-nitroaniline substrates and various chemical protease inhibitors [3]. They identified *Bacteroidetes* and *Propionibacterium* spp. as the major sources for bacterial proteolytic activity [4]. Figure 1 summarizes the mucosal protease pools in the intestine. Intestinal epithelial cells (IEC) are located at the centre, dividing the luminal proteases which can be host- and bacteria-derived proteases from the proteases that have been produced by immune cells or other cell types in the circulation. IEC themselves express a variety of secreted and membrane-associated proteases such as MMPs or peptidases that are responsible for the hydrolysis of proteins and play an essential role in nutrient uptake. Although the intestinal proteolytic balance has not been characterized yet, the host has evolved protective mechanisms to antagonize an imbalance or the overreaction of bacterial molecules.

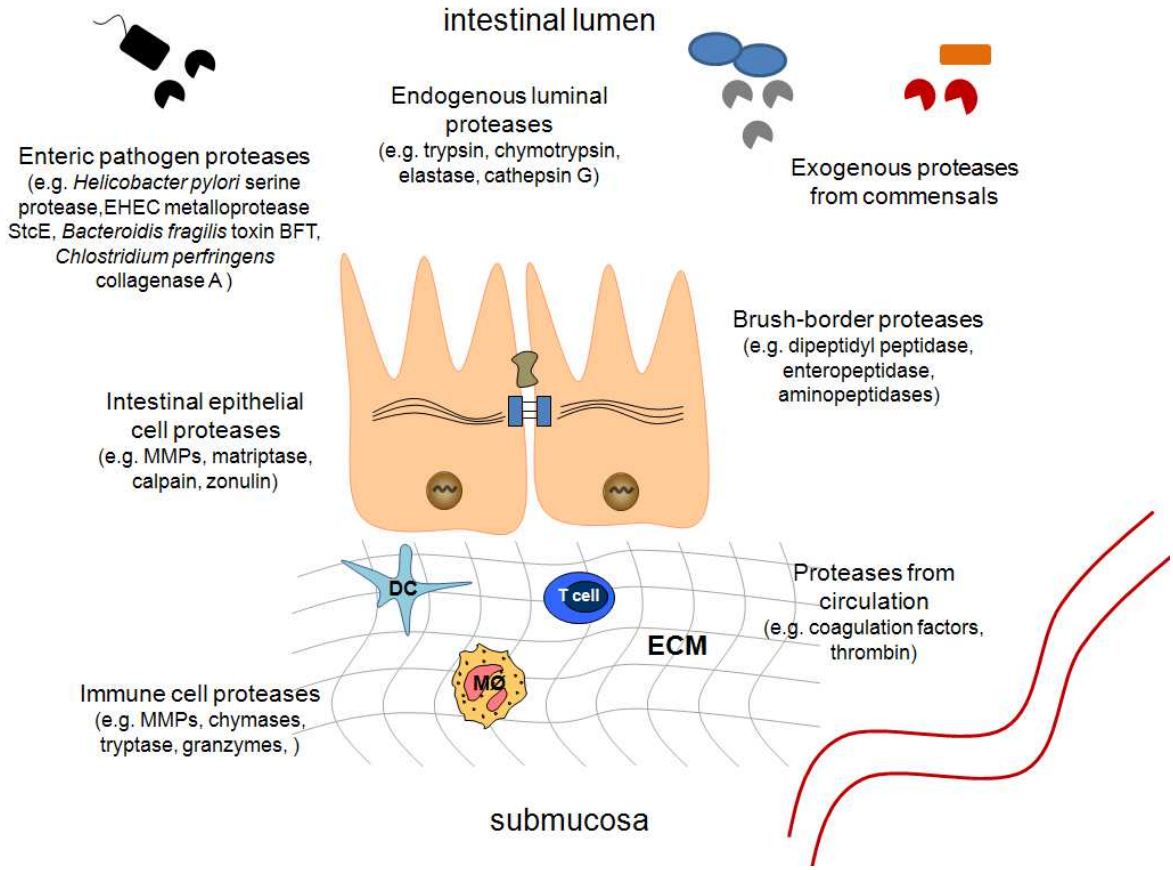


Figure 1: Endogenous and exogenous proteases in the gut. The different protease pools (intestinal, epithelial and submucosal) consist of a variety of different proteases produced by various cell types. The maintenance of mucosal homeostasis is dependent on the proteolytic balance. Luminal proteases are of endogenous and exogenous origin in an unknown composition and proportion.

1.2 Inflammatory bowel diseases

Inflammatory bowel diseases (IBD), comprising the two main idiopathic pathologies ulcerative colitis (UC) and Crohn's disease (CD), are spontaneously relapsing immune-mediated inflammatory disorders of the gastrointestinal tract. The pathogenesis of IBD is characterized by an aggressive and deregulated immune response towards the intestinal commensal microbiota [5]. In the last years, IBD research focused on gene-environment interactions in order to elucidate the initial triggers for disease onset and to improve the understanding of the complex interplay between different aspects of the disease [6]. Environmental factors including diet, life style or frequent medication such as non-steroidal anti-inflammatory drugs or antibiotics do not only influence the host, but also the composition and functionality of the gut microbiota. The already existing intra-individual differences in the intestinal microbiota pose an additional challenge for the investigation of microbe-host

interactions in the context of IBD. Despite the substantial advances in understanding the pathogenesis of IBD, the therapeutic options remain limited targeting symptoms instead of causes. The common goal is the suppression of the abnormal immune response using aminosalicylates, corticosteroids and TNF-inhibitors. Antibiotics and probiotics are utilized to modulate the intestinal microbiota and have been shown to be effective, but not generally in all patients.

1.2.1 Proteases in chronic intestinal inflammation

Excessive concentrations of proteases have been found in faeces of UC patients [7]. Consistent with these findings, secreted factors of colonic biopsy samples from IBD and irritable bowel syndrome patients revealed increased proteolytic activity [8]. Although the origin of the proteolytic activity has not been elucidated, it is conceivable that proteases released from biopsy specimen are derived from the host. However, elevated faecal proteolytic activity might originate from colonic luminal bacteria which release serine, cysteine and metalloproteases [3]. Oral antibiotic treatment of mice resulted in reduced numbers of colonic bacteria and reduced colonic luminal serine protease activity which provides further evidence for the bacterial source of the proteases [9]. Bacterial proteolytic activity could be demonstrated even in the absence of inflammation hypothesizing that bacterial proteases are ubiquitously present in the gut lumen but might impact the development of IBD in a susceptible situation [10].

The deregulated expression and/or activity of host-derived MMPs has been implicated in several diseases including arthritis, atherosclerosis and colon cancer [11,12]. Increasing evidence suggests that MMPs are the predominant endogenous proteases involved in the pathogenesis of IBD [13,14]. MMPs influence disease progression in multiple ways involving the function and migration of inflammatory cells as well as matrix deposition and degradation. The expression and activity of certain MMPs is increased during acute inflammation [15], but also an imbalance between MMPs and their natural tissue inhibitors (TIMP) has been reported for IBD [16]. Beside MMPs, the expression and/or activity of other host-derived proteases including trypsin, neutrophil elastase [17], mast cell tryptase [18], cathepsins [19] and thrombin [20] has been associated with intestinal inflammation.

1.2.2 Intestinal mucosal barrier dysfunction as a central key player in the pathogenesis of IBD

Intestinal homeostasis is provided and maintained by the mucosal barrier which separates the intestinal lumen with all its components (food, digestive enzymes, and microbes) from the host and its underlying organs and tissues. The mucosal barrier in the gut can be divided into three different authorities: the secreted mucus layer, the intestinal epithelial cells (IEC) and the immune cells of the gut associated lymphoid tissue (figure 2) [21]. Especially IEC form a tight line of defense against harmful molecules and microorganisms, in parallel with their task of allowing transport and permeation of nutrients and electrolytes at the same time [22]. Intercellular junctional complexes composed of tight junctions (TJ) and adherence junctions (AJ) maintain physical barrier and provide cell-cell contact between adjacent IEC. TJ are organized in 1) transmembrane proteins that bridge the intercellular space (junction adhesion molecules, claudins, occludin), 2) adapter molecules that link integral TJ proteins to the actin skeleton and other cytosolic cell signalling molecules (ZO-1, ZO-2, ZO-3) and 3) cytosolic and nuclear proteins that regulate various cell functions including differentiation, polarity or tumor suppression on transcriptional or post-transcriptional level [23]. E-Cadherin is the best described member of the calcium-dependent cell-cell adhesion molecules and the integral protein of the AJ in IEC. It consists of a single transmembrane domain and five tandemly extracellular repeated domains, the so called extracellular Cadherin repeats (EC1-5). The cytosolic domain consists of two subdomains: the membrane proximal conserved domain which includes the binding site for the intracellular adapter protein $p120^{ctn}$ and the β-catenin binding domain. E-cadherin plays a crucial role in epithelial cell differentiation by controlling proliferation and providing and maintaining cell polarity (“apicobasal”) [24,25]. Furthermore, the loss of E-Cadherin progress towards malignancy in epithelial tumors, suggesting E-Cadherin as tumor suppression molecule [26,27].

Impaired intestinal epithelial barrier function has been associated with IBD [21,28,29], although it is still unresolved whether the loss of barrier integrity is cause or consequence of chronic inflammation. Epithelial barrier dysfunction is primarily characterized by an enhanced paracellular permeability resulting from an increased flux across the paracellular space [30]. Epithelial barrier damage as a consequence of tissue damage resulting from apoptosis, necroptosis, erosion and ulceration, or TJ regulation both lead to the loss of epithelial barrier function both occurring in IBD [31,32].

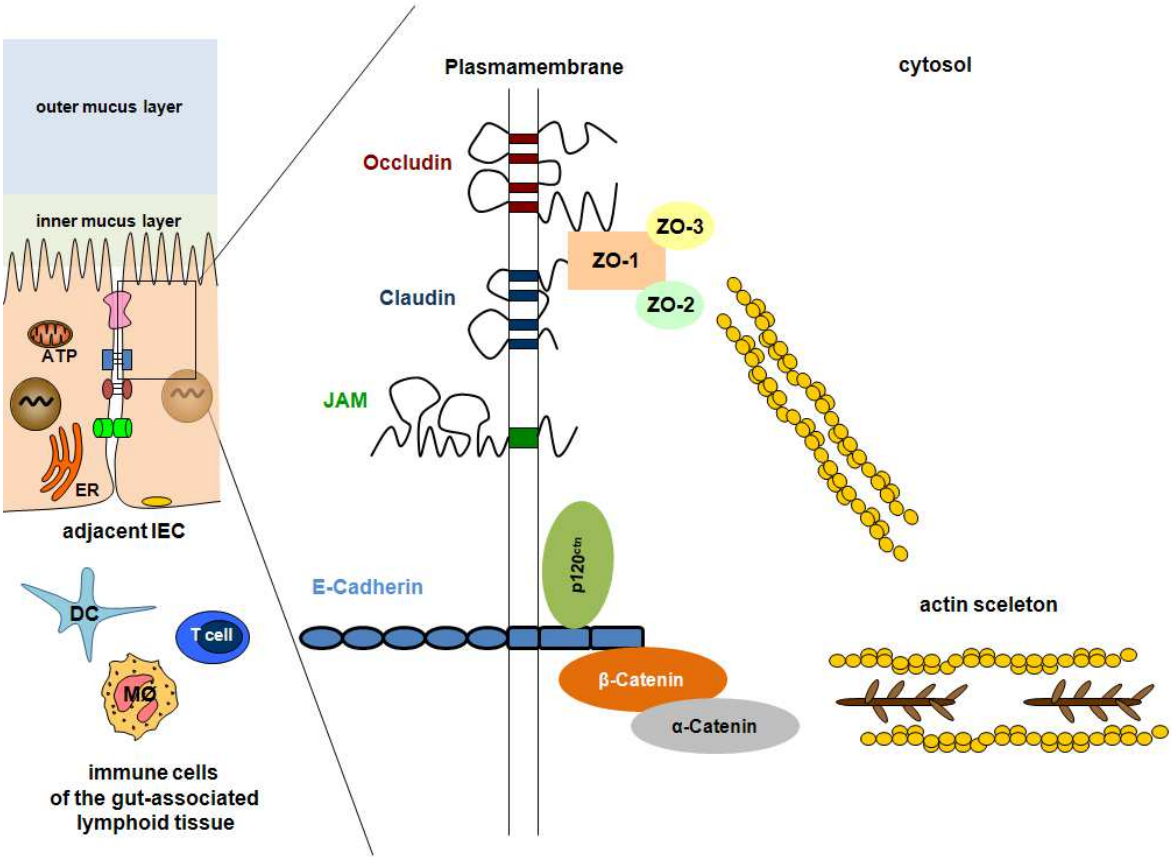


Figure 2: Intestinal mucosal barrier and organisation of the junction complexes in IEC. The mucosal barrier has different authorities: the inner and outer mucus layer, IEC and underlying immune cells provide barrier function and maintain intestinal homeostasis. Adjacent IEC are connected with TJ and AJ which regulate the paracellular flux of molecules and provide barrier integrity of the IEC monolayer. Structurally transmembrane proteins control selective paracellular permeability with their extracellular domains. They are connected with intracellular adapter proteins which communicate with the actin skeleton and many other pathways involved in barrier regulation and cell signalling.

There are numerous studies demonstrating that the expression of TJ proteins is diminished or deregulated in tissue of IBD patients and IEC from IBD animal models. Whereas occludin and junction adhesion molecule A (JAM-A) expression is reduced in intestinal inflammation [33], claudin-2, which increases paracellular flux of sodium ions and small uncharged molecules, is enhanced in TJ in IBD [34,35]. Other members of the claudin family, claudin-3, -4, -5 and -8 are removed from the TJ suggesting that the regulation of paracellular permeability by TJ proteins is complex but selective [36,37]. In contrast to TJ, the functions of AJ are more general, as they mediate cell-cell contact and are responsible for appropriate epithelial cell differentiation. In the context of IBD, AJ dysfunction could be demonstrated by reduced expression levels of E-Cadherin in the inflamed intestinal epithelium [38-40].

Furthermore, polymorphisms in the E-Cadherin encoding gene *CDH1* lead to a cytosolic mis-localization of E-Cadherin and are associated with CD demonstrating that AJ functionality is critical for IBD susceptibility [41].

Cytokine production, especially studied for tumor necrosis factor α (TNF) and interferon-γ (IFN-γ), has been shown to be critical for epithelial barrier function. Various studies demonstrated that the pro-inflammatory cytokines TNF and IFN-γ alter barrier function of cultivated epithelial cells [42,43], but less data are available for primary tissue. Schulzke and colleagues described changes of mucosal morphology, the impairment of barrier function and alterations in TJ profile in rectal explants from a rat model after exposure to TNF and IFN-γ [44] supporting *in vivo* relevance. Mechanistically, pro-inflammatory cytokines modulate epithelial barrier function through the transcriptional inhibition of TJ proteins [45] and redistribution of TJ proteins by myosin light chain kinase (MLCK) dependent contraction of the actin filaments [46,47]. Few studies have been conducted to investigate the impact of pro-inflammatory cytokines on AJ proteins. Yi *et al.* demonstrated a loss of E-Cadherin protein expression suggesting that not only TJ, but also AJ proteins are affected by a pro-inflammatory milieu and are involved in epithelial barrier disruption in IBD [48].

1.2.3 Bacteria-Host interaction: Commensal gut microbiota in IBD

The microbial ecosystem of humans consists of between 15,000 and 36,000 species belonging to ~1800 genera [49]. In total the estimated number of microbes range between 10^2 in the duodenum and 10^{12} in the colon [50]. Culture-independent methods such as large scale analysis of the 16s ribosomal DNA and metagenomics allowed the investigation of the gut microbiome in humans and animals and revolutionized the understanding of the microbial composition and functionality of the largely not-yet-cultivated organisms [51]. The intestinal microbiota is crucial for the development and maintenance of mucosal homeostasis. Microbe-host interactions can have a mutualistic (benefit for both species), symbiotic (at least one partner benefits from the relationship without harming the other) or commensal (coexistence without any obvious benefit or disadvantage) character [52]. The host benefits from intestinal microbes by the supply of essential nutrients through the digestion of indigestible food, the maintenance of IEC homeostasis and the regulation and development of the intestinal immune system. Furthermore, the so called “colonization resistance” is based on the hypothesis that commensal microbes compete with pathogenic bacteria for nutrients, binding sites and produce bactericidal compounds or inhibitory molecules leading to the prevention of a pathogenic infection [53]. The host immune system has to be tolerant against commensal microbial communities and effective in recognition and defence against

pathogens. Therefore the gut has evolved several strategies to live and cross-talk with commensal bacteria [52]. Some of those mechanisms are described in the following paragraph and illustrated in figure 3.

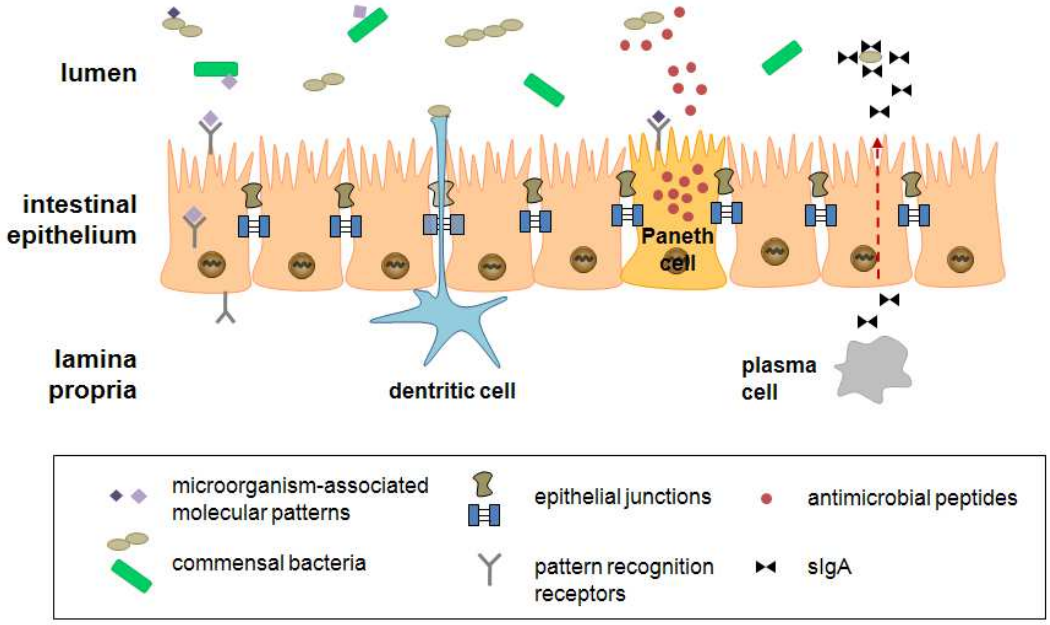


Figure 3: Cross-talk between commensal gut bacteria and the intestinal epithelium. Pattern recognition receptors and dentritic cells sense microorganism-associated molecular patterns and mediate the tolerance against commensal gut bacteria. Antimicrobial peptides prevent direct contact between luminal bacteria and the intestinal epithelium. Secretory IgA (sIgA) bind to bacterial antigens and anticipate the induction of a pro-inflammatory immune response.

The production and secretion of antimicrobial peptides (defensins) by paneth cells protects the epithelium from bacterial colonization. This enables the appropriate development and differentiation of various IEC subtypes from intestinal stem cells located at the bottom of the crypts. Secretory immunoglobulin A (sIgA) produced by plasma cells fulfil an important role in intestinal homeostasis by the inhibition of pro-inflammatory signals through the binding of bacterial antigens. The interplay between dentritic cells and regulatory T cells involving inhibitory molecules such as interleukin (IL) -10 and transforming growth factor (TGF) -β promote nonresponsiveness to the commensal microbiota [54,55]. Pattern recognition receptors (PRRs) including toll-like receptors (TLR) and intracellular nucleotide-binding oligomerization domain (NOD) -like receptors sense microorganism-associated molecular patterns (MAMPs) from commensal and pathogenic bacteria. Receptor activation induces

signalling cascades including nuclear factor-κB (NF-κB) and mitogen-activated protein (MAP) kinases resulting in the subsequent transcription of pro- and anti-inflammatory proteins [56].

The microbial composition varies between individuals and depends on environmental factors such as the anatomical location, diet, the intake of drugs and antibiotics or the health status of the host. The predominant species within the gut are Firmicutes, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* [57,58]. Several studies in experimental rodent models as well as clinical trials suggest the crucial role of the gut microbiota in the development of IBD [59,60]. It has been suggested that alterations in microbial diversity and composition in disease susceptible populations may alter innate defence mechanisms leading to chronic activation of the mucosal immune system [61,62]. The study conducted by Frank *et al*. described the intestinal dysbiosis in 190 resected tissue specimen from IBD patients and revealed increased numbers of *Proteobacteria* and *Actinobacteria* and a decrease in Firmicutes and *Bacteroidetes* [58]. Furthermore, a general reduction in biodiversity has been demonstrated in this study suggesting, that the compositional changes are more related to the inflamed status of the host, as the pathology and immunology of CD and UC are distinctly different [63]. However, there is no IBD-associated specific microbiota. In fact, it has to be considered that commensalism change into a harmful situation for the host which then becomes a continuous inflammatory trigger and leads to the development of chronic intestinal inflammation.

1.2.4 Gastrointestinal infections as critical trigger for IBD

Pathogens are hypothesized to be the initiation step for the pathogenesis of IBD and are discussed to potentiate disease progression [59,64,65]. Pathogens have evolved several strategies to undermine the hosts defence mechanisms. Recent work by McGuckin and colleagues discuss pathogen-mucin interactions and summarize mechanisms how pathogens overcome intestinal mucus layers. In principle there are three strategies: 1) enzymatic digestion of mucus 2) avoidance of mucus by entering via M cells and 3) diffusion of secreted toxins through the mucus and induce barrier impairment by regulating TJ proteins and mucin expression [66]. Beside the direct invasion of epithelial cells and the usage of type specific secretion systems in order to colonize the epithelial layer, the production of toxins and proteases is a very common mechanism to disrupt epithelial barrier integrity that allow subsequent penetration and translocation of the respective organism [65].

A number of microorganisms including *Helicobacter pylori*, *Salmonella*, *Listeria*, *Chlostridium* species, *Yersinia* and *Shigella* have been discussed as etiologic factors in IBD, but there is

no conclusive evidence showing that there is one single pathogen causing disease [59,63,65]. *Mycobacterium avium* spp *paratuberculosis* (MAP) has been associated with CD for several reasons. MAP causes spontaneous granulomatous enterocolitis in ruminants which is similar to transmural inflammation in human CD. Indeed MAP has been identified in biopsy specimen from CD patients and anti-MAP antibodies could be detected in the serum [67,68]. Nevertheless, it remains unresolved whether the chronic state of inflammation is caused by MAP or whether MAP potentiates or sustains the already existing pathology. One of the first organisms implicated in IBD was *Escherichia coli* (*E. coli*). It has been demonstrated that ileal mucosal lesions in CD patients are predominantly colonized by adherent and invasive *E. coli* (AIEC). AIEC-infected macrophages produce TNF which is responsible for granuloma formation occurring in 37-55% of CD patients [69,70]. Similar to MAP, evidence for AIEC as initial trigger for CD is missing. Additionally, other facultative pathogens such as *Bacteroidis fragilis* [71] and *Staphylococcus aureus* [72] have been discussed to play a role in the pathogenesis of IBD. In general it seems obvious that IBD patients have a higher risk for enteric pathogenic infections, but *vice versa* there is a risk of chronicity after pathogen-induced intestinal inflammation. Further studies are needed to elucidate the role of pathogenic infections in the etiology of IBD. One proof for the induction of IBD by pathogenic bacteria are mouse models that develop chronic intestinal inflammation after infection with *Citrobacter rodentium* [73], the rodent equivalent to human enteropathogenic *E. coli*, and *Salmonella enterica* spp *enterica* serovar Typhimurium[74].

1.2.5 Bacterial proteases in IBD

Many endeavours have been made to identify the bacterial structures or molecules responsible for the pathogenicity of microbes. Beside the mechanism of type-specific secretion systems allowing the infiltration of bacterial material or whole bacteria into host cells, proteases have been described to be involved in the infectious process of pathogens. However, the pattern of different types of proteases and their expression, regulation, activation and substrate specificity is very diverse. Host tissue provides different target points for bacterial proteases. In addition to the activation of specific types of host receptors and the degradation of extracellular matrix, the disruption of epithelial barrier function exhibits the most frequently described consequence of bacterial proteases. In consideration of the complex pathogenesis of IBD, targeting host epithelial barrier function is a central mechanism. Table 1 itemizes proteases from pathogens targeting epithelial or endothelial barrier function in different organs summarizing general mechanisms attributed to bacterial proteases and therefore might be relevant in IBD.

Table 1: Bacterial proteases target epithelial cell barrier function [75].

Species	Classification	Protease	Host target structure/ proposed mechanism	Reference
Bacillus anthracis	Pathogen	Metalloprotease lethal toxin (LT) M4 metalloprotease neutral protease (Npr599) M6 metalloprotease immune inhibitor A metalloprotease (InhA)	LT impairs barrier function in primary human endothelial cells (altered VE-Cadherin distribution) Npr599 and InhA reduce endothelial barrier function through increased syndecan-1 ectodomain shedding in cultivated murine mammary gland cells	[76]
Citrobacter rodentium	Pathogen	Lymphostatin: virulence factor consisting of a glycosyltransferase, a protease and an aminotransferase	Disruption of epithelial barrier function via modulation of the small GTPase Rho and Cdc42	[77]
Clostridium difficile, Clostridium sordellii, Clostridium novyi	Pathogen	Large clostridial toxins (glycosy-ltransferases)	Inactivation of GTPases Rho, Rac and Cdc42 in intestinal epithelial cells	[78]
Clostridium perfringens	Opportunistic pathogen	Collagenase A	Intestinal barrier function, basal type-IV-collagen, mucus	[10]
Enterohemorrhagic *Escherichia coli*	Pathogen	Metalloprotease StcE	Cleavage of mucin 7 and glycoprotein 340, facilitation of adherence to epithelial like HEp-2 cells	[79]
Enterotoxigenic *Bacteroidis fragilis*	Opporstunistic pathogen	Metalloprotease fragylisin or *B. fragilis* toxin (BFT)	Induction of γ-secretase dependent shedding if E-Cadherin ectodomain in HT29 cells	[80]
Helicobacter pylori	Pathogen	Serine protease *Helicobacter pylori* high temperature requirement A (HpHtrA)	Reduction of epithelial barrier integrity through targeting E-Cadherin	[81]
Pseudomonas aeroginosa	Pathogen	Pseudomonas elastase	Reduction of barrier function in MDCK cells, altered ZO-1 expression and disturbed microfilaments	[82]
Staphylococcus aureus	Opportunistic pathogen	Serine proteases	Modulation of chemokine expression through NF-κB activation	[83]
Vibrio cholerae	Pathogen	Metalloprotease heamagglutinin/ protease	Reduction of barrier integrity through actin and tight junction rearrangement	[84]

The question of how and when commensal-derived proteases are involved in IBD is of clinical importance. IBD patients have an increased number of mucosa-associated bacteria, and the thickness of the intestinal mucus layer is diminished [85,86]. Mucolytic activity allows bacteria to use mucus as carbohydrate source and enable them to survive in the niche of the intestinal outer mucus layer. Over 20 years ago Rhodes and colleagues hypothesized that bacterial mucus degradation could be associated with IBD, but they could not correlate bacterial glycosidase activity with disease severity in IBD patients [87]. However, a recent study demonstrated a shift in the mucolytic consortium of bacteria in IBD patients. The most abundant mucolytic species in healthy controls, *Akkermansia muciniphila*, is reduced in IBD, whereas *Ruminococcus* species are disproportionally increased under conditions of chronic inflammation. *Ruminococcus* α- and β-glycosidases remove terminal sugars from the mucus matrix that subsequently become accessible for other bacteria providing a possible explanation for the increase in total mucosa-associated bacteria in IBD [88]. As already mentioned, pathogens have evolved several strategies to disrupt and avoid mucosal barriers. Thereby, the enzymatic degradation of mucins is one of the most common mechanisms [66]. The substrate specificity of bacterial proteases is often not restricted to one molecule. Proteases, that have been demonstrated to digest mucins, have also been shown to facilitate adherence to host cells [79] or to disrupt epithelial barrier function [10]. Alterations in microbial composition and mucus structure under disease susceptible conditions might allow commensal-derived proteases to gain access to the epithelium targeting protein substrates that would not be accessible to them under physiological conditions. As illustrated in figure 4, one could think of four critical scenarios that might allow access of commensal proteases to IEC: 1) commensals possess a mucolytic activity 2) attenuated mucosal barrier function during or after a pathogenic infection 3) diminished mucosal barrier function due to a genetic predisposition and 4) the degradation of antimicrobial peptides.

The impact of commensal-derived proteases in the development of chronic intestinal inflammation might be depending on additional barrier attenuating factors and certain disease susceptibility. Although this hypothesis fits into the suggested multi-factorial pathogenesis of IBD, further evaluation is required in order to assess the overall impact in disease development.

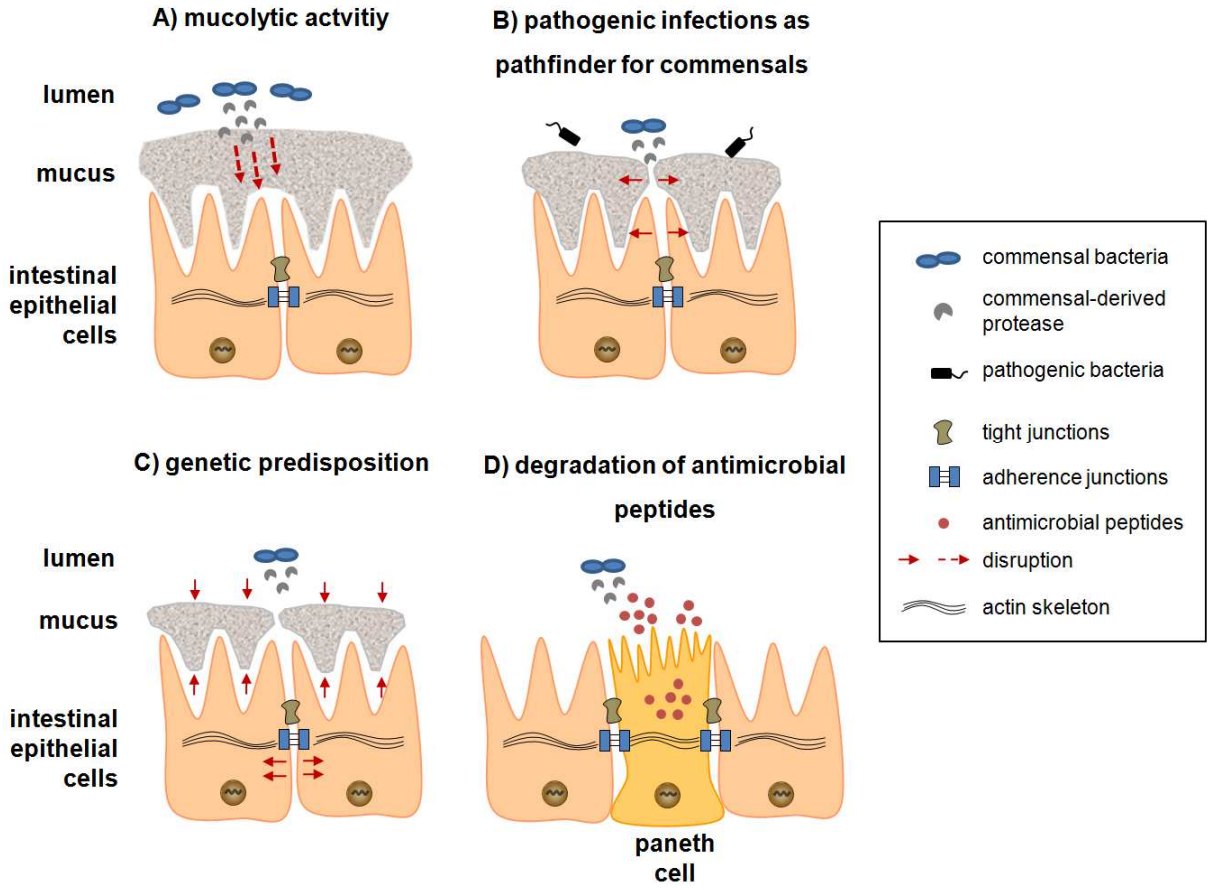


Figure 4: Scenarios how commensal proteases gain access to IEC. A) Some commensals live in the outer mucus layer and use intestinal mucus as carbohydrate source. B) During or after pathogenic infections, mucus and barrier function of IEC are diminished. C) A genetic predisposition leads to reduced production and stability of mucus or an impaired barrier function of IEC. D) Bacterial proteases hydrolyse antimicrobial peptides.

1.2.6 Receptors for bacterial proteases in the gut

Protease-activated receptors

Protease-activated receptors (PARs) are G-protein coupled receptors which are activated by proteolytic cleavage of the N-terminal tethered ligand. PARs are expressed on various cell types including endothelial and epithelial cells, smooth muscle cells, fibroblasts, platelets, neurons and immune cells [89]. The activation of PARs by proteases that have been released during inflammation and injury contributes to inflammatory mechanisms, cytokine production, nociception and repair mechanisms. Serine proteases such as trypsin, thrombin and cathepsin G are the best studied PAR activating proteases [90]. The potential of

bacterial proteases to target PARs has been shown for respiratory and oral epithelial cells as well as for platelets and neutrophils. The activation of PAR receptors has been shown for the cysteine protease gingipain-R from *Porphyromonas gingivalis* [91,92], the metalloprotease *P. aeroginosa* elastase from *Pseudomonas aeroginosa* [93] and the metalloprotease serralysin from *Serratia marcescens* [94]. The expression of PARs on the apical surface of IEC suggests a proteolytic activation from the luminal side of the gut which is most likely not only mediated by host proteases, but also by bacterial proteases.

Pattern recognition receptors

Toll-like receptors (TLR) are part of the innate immune system and belong to the pattern recognition receptor family that identifies microbial pathogens through the recognition of pathogen- or microbial-associated molecular patterns (carbohydrates, nucleic acids, peptidoglycans, lipoteichoic acids, lipoproteins). Direct proteolytic activation of a full-length TLR has been recently described for the avian TLR15. Virulence-associated microbial-derived proteases from fungi *Candida guilliermondii*, *Trichosporon* spp., *Penecillium* spp., *Mucor* spp., and gram-negative opportunistic pathogen *P. aeruginosa* have been indentified to activate TLR15, which is an unique type of receptor that combines TLR characteristics with an activation mechanism typical for the evolutionary distinct PARs [95]. Although it remains unanswered which proteases activate TLR15 one could speculate that these are serine proteases as the receptor activation could be inhibited by the serine protease inhibitor phenylmethanesulfonylfluoride (PMSF). Mammalians seem to lack a TLR that is able to sense proteolytic activity, although mammalian TLR4 can be activated by elastase-activated compounds from the extracellular matrix [96]. Furthermore lipopolysaccharide (LPS) recognition by TLR4 was indirectly influenced by trypsin which is augmented in ileal inflammation and cleaved MD-2, an accessory glycoprotein essential for TLR4 signalling. The proteolysis of MD-2 provided a mechanism for intestinal epithelial LPS tolerance that helped to regulate immune responses to commensal bacteria-derived ligands [97].

E-Cadherin

The adherence junction protein E-Cadherin is a calcium-dependent single-pass transmembrane protein generally expressed in the lateral plasma membrane of epithelial cells. E-Cadherin provides contact to adjacent cells and plays a major role in epithelial cell differentiation. The intracellular domain is highly conserved and signals through cytoplasmic proteins from the catenin family [24]. The extracellular domain could act as a receptor for bacterial or fungal entry into epithelial cells, which so far has only been demonstrated for

surface proteins. Internalin A from *Listeria monocytogenes* [98] and invasion Als3 from *Candida albicans* [99,100] interact with E-Cadherin and mediate the internalization of the respective microorganisms. E-Cadherin is also targeted by bacterial proteases, the metalloprotease toxin BFT from *Bacteroidis fragilis* was shown to induce the shedding of the E-Cadherin ectodomain through an unknown IEC receptor-mediated induction of γ-secretase [80]. A direct E-Cadherin cleavage could be demonstrated for the trypsin-like serine protease HpHtrA from *Helicobacter pylori* [81]. The proteolytic cleavage of E-Cadherin, either direct or indirect, reflects an important mechanism for bacteria, especially pathogens for reaching the intercellular space and to translocate across the epithelium.

Protease-dependent receptor activation

The release of tumor necrosis factor α (TNF) is associated with an increased permeability of the gut epithelial barrier. Blockade of TNF with anti-TNF antibodies is an established strategy in the treatment of IBD. The increase of soluble biologically active TNF arises from the conversion of membrane-bound TNF by TNF-converting enzyme (TACE) [101,102]. TACE or ADAM17 belong to the ADAM (a disintegrin and a metalloprotease) family of metal-dependent proteases and has been additionally implicated in the shedding of other membrane-bound precursors of cytokines and growth factors [103]. One of these factors is the transforming growth factor-α which in turn activates the epidermal growth factor receptor (EGFR). Phosphorylated EGFR induces the activation of mitogen activated protein kinases and mediate changes in intestinal permeability [104]. Many of these processes and receptor activation mechanisms are described in the context of carcinogenesis [105], a frequent IBD-associated complication. Especially the fact, that numerous cytokines and growth factors are membrane-associated as precursors and require proteolytic conversion may represent a novel mechanism for bacterial-derived proteases.

1.3 Enterococci: a narrow path between commensalism and pathogenicity

Enterococci are Gram-positive lactic acid bacteria that are found ubiquitously in the environment (water, soil, plants). Furthermore they are commensal inhabitants of the gastrointestinal tract of mammals, vertebrates and insects. They play an important beneficial role in food and feed applications, some strains even exert a probiotic function. On the other hand, there have been emerging concerns about enterococci because of their resistances to antimicrobial agents and their involvement in hospital-acquired infections.

1.3.1 Characteristics, distribution and association with disease

The attention and awareness of microorganisms and bacteria started particularly in the 19th century with the main considered founders of microbiology Louis Pasteur, Ferdinand Cohn and Robert Koch. In 1899 the term “entérocoque” appeared for the first time in literature [106]. This name was given to a Gram-positive organism in order to emphasize its intestinal origin. A few years later, in 1906, Andrewes and Horder isolated a strain from an endocarditis patient which exerted the same characteristics of the human intestinal *Streptococcus* strain [107]. Accordingly, the authors called their new isolate *Streptococcus faecalis*. After the first description of the “enterococcal group” of streptococci in 1938 [108] they have been classified as “faecal streptococci or Lancefield’s group D streptococci”, until 1984 when this group has been divided into three separate genera: *Streptococcus*, *Lactococcus* and *Enterococcus*. Enterococci are catalase negative, non spore forming, facultative anaerobic cocci that are arranged in pairs or short chains. They can be separated from other cocci through their ability to grow under harsh conditions including a pH range from 4 to 10, a temperature range from 10°C up to 45°C or the prese nce of 40% bile acids [109]. The most prominent species among enterococci are *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*), but at least 20 species have been identified in the genus *Enterococcus* [110]. Today over 70 different species of *E. faecalis* are listed in the taxonomy browser of the NCBI database. However, due to the development of new techniques for cultivation and identification of strains, this number is increasing continuously. Recently it has been demonstrated that *E. faecalis* belongs to the human core gut microbiome and therefore represents one of the most common commensal species in the human intestine [61]. The numbers range from 10^5 to 10^7 cfu/g stool [111].

Enterococci in food

Enterococci have been used in food fermentations for a long time. Despite the fact that faecal strains from the intestine of farm animals contaminate especially milk and meat products which can cause human disease, people have used enterococci strains as starter or ripening cultures in traditionally fermented foods made of meat and milk. Especially the traditional Mediterranean style production of cheese and sausages uses the ripening and aroma development properties of an enterococcal fermentation [109,112]. Another benefit of enterococci in food is the production of bacteriocins which have been shown to exert an antimicrobial activity against harmful foodborne pathogens such as *Listeria monocytogenes* [112,113]. The usage of enterococci as probiotics is more occasionally compared to the commonly used genera *Bifidobacterium* or *Lactobacillus*. Probiotics are defined according to

the WHO/FAO in 2001 as living microorganisms that confer a health benefit for the host when administered in adequate amounts. Currently, some products such as Symbioflor 1 (SymbioPharm, Herborn, Germany), Cylactin (Hoffman-LaRoche, Basel, Switzerland), ECOFLOR (Walthers Health Care, Den Haag, The Netherlands) and Causido (Arla Foods, Viby, Denmark) contain enterococci as probiotic preparation for humans or as veterinary feed supplements [112]. The best described probiotic strain is *E. faecium* SF68 which was effective in the treatment of diarrhea [114], the prevention of antibiotic-associated diarrhea [115] and in a short-term reduction of blood cholesterol in humans [116]. Other mechanisms suggest the activation of the mucosal immune system, the growth-limitation of pathogenic bacteria or the promotion of intestinal epithelial homeostasis. In general enterococci can fulfil some of the essential characteristics of probiotics such as the adherence to host cells or the ability to survive gastric acid and bile salts [117]. Usually probiotic strains have been naturally present in the human intestine which assures their safety of usage, thus new isolates are often tested for their probiotic potential [118]. Although the probiotic benefits of some strains are well established, the application of enterococci as probiotics remains controversial because of their association with human disease.

Enterococci as infectious organisms

Enterococci are opportunistic pathogens meaning that they remain harmless in healthy individuals but can cause severe problems in hospitalized patients who have received multiple courses of antibiotics or are immunocompromised. This is not surprising as enterococci have been described as the main cause for endocarditis and urinary tract infections since the early 1900s [119]. Furthermore, they have been identified to cause bacteremia, infections of the blood stream and central nervous system, surgical wound infections and neonatal as well as hepatobiliary sepsis [119-121]. Nowadays enterococci, mainly *E. faecalis* and *E. faecium*, are regarded to be the third common cause for nosocomial infections in the US [122] and the incidence is increasing in Europe [123]. The major problem in the treatment of enterococcal-mediated infections is the rapid acquisition of antimicrobial resistances through the accumulation and transfer of genetic elements. Attention has been focused on enterococci because of their ability to acquire genetic determinants conferring resistances to many classes of antibiotics including chloramphenicol, erythromycin, tetracycline, penicillin and vancomycin [119,124]. Especially the resistance to vancomycin, which was described in 1986 in isolates from patients in France and England for the first time [125], gave cause to serious concern as this therapy has been used at last resort treatment for multiple antibiotic resistant enterococci [126]. Although antibiotic resistant enterococci have been isolated from different sources (human, animals and food), clinically

relevant resistances against penicillin or vancomycin are very rare in food isolates suggesting that clinical enterococci strains have rather adapted to the environmental pressure of antibiotic therapies in hospitals [124]. The existence of transfer mechanisms for resistance genes through conjugative plasmids or transposons is another serious problem with enterococci. Thereby enterococci can transpose genes between each other [127], but they can also transfer genes to other species as it has been reported for *Staphylococcus aureus* [128] and *Listeria* species [129]. Due to the major concerns about the treatment problems with multiresistances, it might be reasonable to assess the virulent potential and innocuousness of enterococci in a case-by-case evaluation of each strain, especially with the focus on the usage of enterococci in food [124].

Enterococci in IBD

As part of the commensal gut community, enterococci have been carefully discussed to play a role in the pathogenesis of IBD. An increased risk for bacterial endocarditis in IBD has already been demonstrated in 1993 [130]. In addition, hospitalized IBD patients have an increased risk for vancomycin resistant enterococcal infection which was in turn associated with the length of stay in hospital [131]. Increased numbers of enterococci have been detected in biopsies of children with UC and CD compared to healthy controls [86]. The microscopic analysis of colonic biofilms demonstrated that enterococci growing in microcolonies occurred on mucosal surfaces of UC patients, but not in healthy people suggesting that spatial distribution and mucosal proximity of the microorganisms might be linked to the disease process rather than their absolute numbers [132]. Furthermore, these authors revealed higher titres of *E. faecalis* specific antibodies in the serum of UC patients compared to healthy controls, demonstrating an immune-mediated recognition of *E. faecalis* under conditions of chronic inflammation. So far, the direct association of enterococci and the development of chronic intestinal inflammation could only be demonstrated under experimental conditions in *E. faecalis* monoassociated interleukin 10 deficient (IL-$10^{-/-}$) mice [133]. Kept under germ-free conditions IL-$10^{-/-}$ mice remain free of intestinal pathology, but if they have been colonized with bacteria the pathological outcome of disease was strain dependent [134,135]. Wild type (Wt) counterparts did not develop colitis when colonized with *E. faecalis* demonstrating the commensal nature in a healthy host. This model clearly showed that there are two critical aspects playing a role in the pathogenesis of IBD, the genetic susceptibility of the host and the characteristics of a certain microorganism. Hoffmann *et al* have demonstrated that pathology in monoassociated IL-$10^{-/-}$ mice is also dependent on the *E. faecalis* strain suggesting that not only the species but also strain-specific properties are crucial for disease development [136].

1.3.2 Putative virulence factors in enterococci

In general, microorganisms express and secrete virulence factors that enable them to survive within a host. Usually these proteins, toxins, carbohydrates or glycoconjugates are produced in order to get access to nutrients, to adhere and invade host cells or to evade host defence mechanisms. The expression of virulence factors is attributed to pathogens that invade the host with active mechanisms. The pathogenicity traits of enterococci have been extensively studied with respect to the expression of putative virulence factors. As a result of limitations in antimicrobial therapy against enterococcal infections, this is of considerable interest. Enterococci express a variety of putative virulence factors including proteins, carbohydrates and glycoconjugates. Most of the studies investigating virulence-associated genes of enterococci have been conducted for *E. faecalis* and *E. faecium*. Table 2 summarizes the most often characterized putative virulence factors of *E. faecalis* that have been tested in several infection models including different host species (rat, rabbit, mouse, insects and nematodes). Most of the virulence-associated genes in *E. faecalis* have been identified to be involved in biofilm formation. Biofilms are populations of cells irreversibly accumulated at various biotic and abiotic surfaces. Bacteria cells are embedded in a self-produced hydrated matrix of exopolymeric substances [137]. The tolerance against antibiotic treatment, phagocytosis and other components of the innate and adaptive host defence mechanisms emphasizes the clinical relevance of bacterial biofilms [138]. The genetic determinants and metabolic pathways regulating biofilm formation in enterococci have been studied extensively, but remain not fully understood [139]. The communication of bacteria in biofilms is provided by quorum sensing systems which sense cell density and react with the induction of corresponding target genes [138].

The *fsr* quorum sensing system

The *E. faecalis* regulator (*fsr*) locus, consisting of *fsrA*, *fsrB*, *fsrC* and *fsrD*, is involved in *E. faecalis* biofilm formation [140] and virulence [141-143]. As homologue to the accessory gene regulator (*agr*) system in *Staphylococcus aureus*, the *fsr* genes encode for a two component quorum sensing system [144]. The cell wall-associated histidine kinase FsrC senses the extracellular accumulation of the gelatinase biosynthesis activating pheromone (GBAP), a peptide lacton encoded by *fsrD* [145]. After reaching the threshold concentration of GBAP (1nM), FsrC phosphorylates the response regulator and transcription factor FsrA which activates in turn the expression of the co-transcribed proteases gelatinase (*gelE*) and serine protease (*sprE*) [140,146].

Table 2: Putative virulence factors of *E. faecalis*. (Modified by Sava *et al,* 2009) [147]

virulence gene/ gene locus	virulence factor/ encoding molecule	pathophysiology/ virulence	reference
adhesion of collagen from *E. faecalis* (*ace*)	microbial surface components recognizing adhesive matrix molecules (MSCRAMM)	– binding to collagen type I and IV, laminin and dentin – pathogenesis in endocarditis	[148-150]
aggregation substance (*agg*)	group of surface proteins	– promotes conjugation by directing bacterial aggregation – facilitates bacterial internalization into intestinal epithelial cells – binding to host cells (tubular cells, macrophages) and ECM – pathogenesis in endocarditis	[151-156]
cytolysin operon (*cyl*)	hemolysin/ bacteriocin class I lantibiotic	– lysis of gram-positive bacteria and eukaryotic cells – pathogenesis in peritonitis and endophthalmitis – *C. elegans* and *Drosophila melanogaster* infection	[157-161]
***E. faecalis* antigen A (*efaA*)**	lipoprotein adhesin	– pathogenesis of peritonitis	[162]
endocarditis and biofilm associated pili (*ebp*) locus	pili	– biofilm formation – pathogenesis in endocarditis and urinay tract infection	[163,164]
enterococcal polysaccharide antigen (*epa*) locus	proteins involved in carbohydrate metabolism putative glycosyl transfereases	– biofilm formation – pathogenesis in endocarditis and urinary tract infection – resistance to killing by PMNs and infection by phages – translocation across intestinal epithelial cells	[165-169]
enterococcal surface protein (*esp*)	surface protein	– biofilm formation – pathogenesis of urinary tract infection	[170,171]
gelatinase (*gelE*) *E. faecalis* regulator (*fsr*) locus	Zn-dependent metalloprotease fsr proteins of a two-component quorum sensing system	– biofilm formation – pathogenesis in endocarditis and endophthalmitis – *C. elegans* and *Galleria mellonella* infection – translocation across intestinal epithelial cells	[141-144,158,172,173]
general stress protein (*gls*) 24	Stress protein	– pathogenesis in endocarditis and peritonitis	[174,175]
cell wall and capsular polysaccharides	lipoteichoic acid (LTA)	– target of opsonic antibodies	[176]
glycolipids		– biofilm formation – binding to intestinal epithelial cells – pathogenesis of mouse sepsis	[177,178]

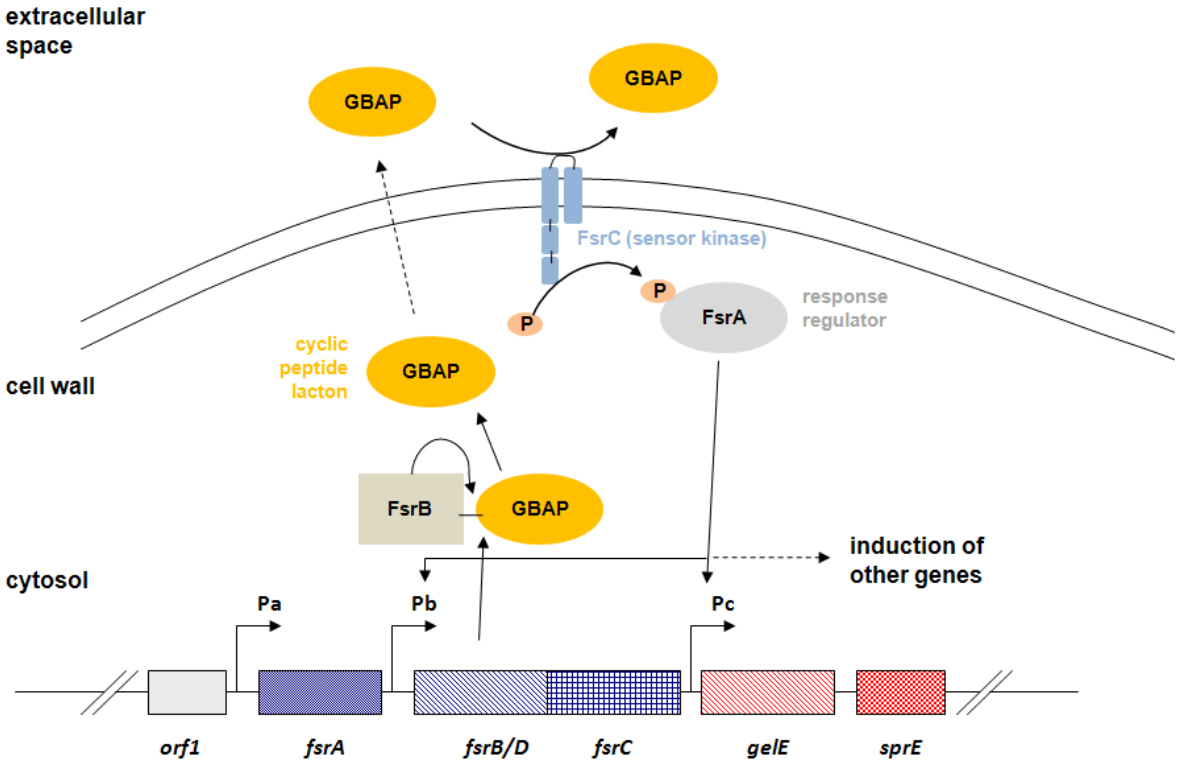


Figure 5: The *fsr* quorum sensing sytem of *E. faecalis*. After the recognition of a critical concentration of GBAP, the sensor kinase FsrC phospholylates the response regulator and transcription factor FsrA. FsrA induces expression of the fsr system, gelatinase and serine protease and other target genes.

The metalloprotease gelatinase

Gelatinase (GelE) is a zinc dependent secreted metalloprotease, capable of degrading a number of substrates including fibrin, fibrinogen, collagen, laminin, haemoglobin, endothelin and casein. It displays a similar substrate specificity as the mammalian endopeptidase-24.11 and *Streptococcus thermophilus* thermolysin (EC3.4.24.4.) [179]. As member of the M4 family of bacterial zinc metallo-endopeptidases, GelE is synthesized as a 509 amino acid prepropolypeptide which ends up in the mature protease after proteolytic cleavage of 192 amino acids at the amino terminal end. It is supposed that GelE maturation is preceded by autocatalytic processing and transient association of the propeptide with the active form. A more recent work additionally suggests a C-terminal processing for full protease activity [180]. GelE controls bacterial survival and dissemination through the regulation of coccal chain length and the degradation of misfolded proteins on the bacterial surface [181,182]. Several studies with genetically modified strains lacking *gelE* or *fsrB* creating a GelE negative phenotype demonstrated that GelE is involved in biofilm formation [139,183].

Furthermore proteolytic activity of GelE seems to be essential in this process [184]. As demonstrated in table 2, GelE is one of the best studied virulence factors in *E. faecalis.* Virulence of GelE has been demonstrated utilizing various models for enterococcal infections suggesting GelE as one interesting candidate to play a role in *E. faecalis*-mediated development of chronic intestinal inflammation.

2 Study objective

Although there has been much effort and progress in understanding the complex pathogenesis of IBD, the initial onset for disease development remains to be elucidated. Host proteases are involved in this process by inducing signalling pathways, proteolytic activation of inflammatory mediators, degradation of ECM and subsequent tissue destruction. The current knowledge about the function of bacterial proteases in the context of bacterial-associated virulence is limited to proteases from pathogens. The investigation of the gut microbiota in terms of composition and functionality gives novel insights with regard to the commensal microbiota which is different in an inflamed host compared to a healthy environment. Commensal microorganisms get access to the host and might change their commensal character towards an invasive and aggressive phenotype. This scenario has already been demonstrated for *E. faecalis* in IL-10$^{-/-}$ mice where a commensal strain induces severe colitis in the genetic susceptible host. Furthermore not all *E. faecalis* strains induced colitis to the same extent in IL-10$^{-/-}$ mice, suggesting that distinct bacterial features are responsible for disease development and/or progression [136]. Similar to pathogens, virulence-associated genes are hypothesized to mediate the invasive and pathogenic character of *E. faecalis*. Previous work demonstrated that IL-10$^{-/-}$ mice monoassociated with GelE deficient *E. faecalis* mutants developed significantly less inflammation in the colon after 15 weeks [185]. This experiment showed for the first time the involvement of a bacterial protease in the development of chronic intestinal inflammation.

The aim of the present work was to investigate mechanistically how *E. faecalis* GelE influence and contribute to intestinal inflammation. Similar to mechanisms of proteases from pathogens, the interaction between GelE and the intestinal mucosal barrier function was determined. Furthermore, the special focus of this work was based on the identification of host target proteins which can be proteolytically degraded by GelE.

3 Material and Methods

3.1 Bacteria

The impact of *E. faecalis* GelE on the development of intestinal inflammation was investigated by using different GelE expressing strains and isogenic mutants listed in table 3. The well characterized *E. faecalis* strain OG1RF was used as *gelE* expressing bacterial Wt strain [186,187]. The isogenic mutants, TX5264 (Δ*gelE*), TX5243 (Δ*sprE*) and TX5266 (Δ*fsrB*), were kindly provided by M. Gilmore (Sheepens Eye Research Institute, Boston, USA). The strains TX5439 [173] (Δ*gelE* reconstitution) and TX5266.01 [141] (Δ*fsrB* reconstitution) were provided by B. Murray (University of Texas Medical School, Houston, USA). The reconstitutions were accomplished by introducing stable plasmids (pTEX5438 and pTEX5249) which are not incorporated into the genome of the bacteria and therefore require the presence of erythromycin (10 µg/mL). The non-characterized *E. faecalis* strains CD11, CD28.1, UC7, UC18.1 and AH114 were isolated from faecal samples of IBD patients and healthy controls according to their ability to degrade gelatine [188]. Cultures were spotted onto tryptic soy agar supplemented with 0.5 g/L L-cysteine and 1.6% Difco gelatine and displayed zonal changes around them. Bacteria were considered GelE positive and were identified by 16S-23S ribosomal DNA intergenic spacer region sequencing.

Table 3. *E. faecalis* OG1RF and isogenic mutants used in this study.

strain	characteristics[α]	reference
OG1RF	Wild type Strain; $GelE^+$ $SprE^+$ Rif^r Fus^r	[186,187]
TX5264	OG1RF *gelE* in-frame deletion mutant; $GelE^-$ $SprE^+$ Rif^r Fus^r	[141]
TX5243	OG1RF *sprE* insertion mutant; $GelE^+$ $SprE^-$ Rif^r Fus^r Em^r	[144]
TX5266	OG1RF *fsrB* in-frame deletion mutant; $GelE^-$ $SprE^-$ Rif^r Fus^r	[146]
TX5439	TX5264 harboring pTEX5249; $GelE^-$ $SprE^+Rif^r$ Fus^r Em^r	[173]
TX5266.01	TX5266 harboring pTEX5438; $GelE^-$ $SprE^-$ Rif^r Fus^r Em^r	[141]
TX5692	OG1RF *epaB* deletion mutant; $GelE^+$ $SprE^+$ Rif^r Fus^r	This study
TX5693	TX5264 *epaB* deletion mutant; $GelE^-$ $SprE^+$ Rif^r Fus^r	This study
TX5694	TX5266 *epaB* deletion mutant; $GelE^-$ $SprE^+$ Rif^r Fus^r	This study

[α]Abbreviations: GelE, gelatinase; SprE, serine protease; Rif, rifampicin; Fus, fusidic acid; r, resistance.

3.1.1 Generation of the *epaB* deletion mutants

The *epaB* deletion mutant was created using the pHOU1 plasmid [189]. DNA fragments upstream (902 bp) and downstream (1,112 bp) of the *epaB* gene were amplified with primer pairs of 1F (TGC TGG AAT TCG GAT AGA TTT TGT GAC GTT) + 1Ra (TCT AAA ATT TAA GAG GAA TGA TGA CTT TGT AGC A) and 2Fa (GTA AGG AGA ATT TAA AAT CTT TAT GCA ATC AAT G) + 2Ra (CGC GGA TCC AAA TGC AAA ATT AGC AAT CACTC), respectively. Amplified fragments were connected by cross-over PCR, digested with *Bam*HI and *Eco*RI and then ligated into pHOU1 digested with the same restriction enzymes. The construct, designated pJH132, was electrophorated into *E. faecalis* CK111 [190] which was then conjugated as described previously [189] with *E. faecalis* OG1RF and Δ*gelE*. The first recombination was selected on Brain Heart Infusion (BHI) (BD, Sparks, USA) agar plates containing gentamicin (200 µg/mL), fusidic acid (25 µg/mL) and X-gal (200 µg/mL). Blue colonies resistant to gentamicin and fusidic acid were further characterized to verify recombination into the *epaB* region using outside primer pairs of upF (AAT CGG TAT TTT GTT AGC AGC ATT) + 2Ra and 1F + DnR (CAA ATG CAA AAT TAG CAA TCA CTC). The second cross-over event was obtained by spreading a diluted culture of first cross-over cells onto BHI containing X-gal (200 µg/ml) agar. White colonies tested sensitive to gentamicin were isolated and further confirmed by DNA sequencing after PCR amplification (upF + DnR). The open reading frame of *epaB* is composed of 789 bp (encoding 262 amino acids); 726 bp starting from the start codon were deleted from the *epaB* mutant. The previously used counter selection medium MM9YEG supplemented with 10 mM p-Cl-Phe was not successful in selecting for excision of pJH132 which we later found was due to severe inhibition of growth of the *epaB* deletion mutant by 10 mM p-Cl-Phe.

3.1.2 Cultivation and preparation of concentrated conditioned media

Bacteria were cultivated in BHI (BD, Sparks, USA) at 37℃ under aerobic conditions (5% CO_2). For preparation of concentrated conditioned media (conc CM) bacterial culture supernatant from an overnight culture was concentrated (factor 50) by using an Amicon Filter System (Millipore, Bellerica, USA) with an exclusion size of 10 kDa.

3.1.3 Assessment of the gelatinolytic activity by cultivation

Bacterial cultures grown for 24 h in BHI medium were transferred (5 µL) onto BHI supplemented with 3% (w/v) gelatine. Plates were incubated for 24 h at 37°C (5% CO_2) followed by 6 h at 4°C. GelE production was indicated by zonal changes around the colonies.

3.1.4 Assessment of GelE activity with an Azocasein assay

The GelE activity was assessed using an Azo-dye labeled Casein (Azocasein, Sigma Aldrich, Saint Louis, USA). Samples were incubated with 200 µL Azocasein (3 mg/mL) in 50 mM Tris HCl, 150 mM sodium chloride and 1 mM calcium chloride in a 96 well plate at 37°C for one hour. 15% trichloroacetic acid (100 µL per sample) precipitated unconverted Azocasein which sticks to the bottom of the well plate after centrifugation (3000xg, 10 min). pH of supernatants (100 µL) was adjusted with 0.5 M NaOH (100 µL) to neutral, colour reaction was quantified by measuring the optical density at 440 nm. Subtilisin A from *Bacillus licheniformis* (Sigma Aldrich, Saint Louis, USA) was used as reference protease to calculate the protease activity in units/mL.

3.1.5 Determination of gelatinolytic activity with zymography

MMP-2 and MMP-9 activity was determined by using gelatine zymography, a gel-based activity assay. Recombinant murine pro-MMP2 and pro-MMP-9 (R&D systems, Wiesbaden-Nordenstadt, Germany) was incubated with purified GelE or activated by pre-incubation with p-Aminophenylmercuric acetate (APMA) (Sigma Aldrich, Saint Louis, USA) for 1.5 h at 37°C. Samples were mixed with Laemmli without dithiothreitol and run through gelatine containing SDS gel (0.1% w/v gelatine). SDS was removed by shaking in 2.5% Triton X-100 (Roth, Karlsruhe, Germany) and protease activity was visualized after incubation overnight at 37°C with Coomassie staining. The activated form of MMP-9 could be further detected by silver staining which was purchased from Amersham Biosciences (Arlington Heights, USA) and performed according the manufacturer's instructions.

3.1.6 Biofilm formation

Biofilm formation was assessed according to Baldassarri *et al* [191]. 180 µL tryptic soy broth (TSB) supplemented with 1% glucose were inoculated with 20 µL of stationary phase cultures in polystyrene tissue culture plates and incubated overnight. Growth rates were

assessed by measuring the optical density (OD) at 600 nm. After three washing steps with phosphate buffered saline (PBS), plates were dried at 60°C for one hour and then stained with 2% Hucker's crystal violet (Sigma Aldrich, Taufkirchen, Germany) for 2 minutes. Excess stain was removed by rinsing the plates thoroughly under tap water. Plates were dried for 10-20 minutes at 60°C. Crystal violet staining was measured at 630 nm and biofilm formation was normalized to growth with the biofilm index ($OD_{biofilm}$ x ($0.5/OD_{growth}$))[192].

To determine biofilm thickness, E. faecalis cells were cultivated overnight in TSB supplemented with 1% glucose in collagen-coated chambers at 37°C with gentle shaking. Bacteria and liquid broth were removed carefully and carbohydrate structures in the biofilm were stained for 1 h using a 1:100 dilution of Alexa Fluor 488 labelled concanavalin A (con A) (Invitrogen, Carlsbad, USA) in PBS. The staining solution was replaced by PBS which was removed immediately before microscopic acquisition of biofilm images using an Olympus BX61 confocal microscope (Olympus, Hamburg, Germany). Biofilm thickness was calculated according to the number of acquired images and 3D illustrations were prepared using Volocity 5.4.1 software (PerkinElmar).

3.1.7 Assessment of gene expression on transcriptional level using quantitative real time PCR (qRT-PCR)

Bacteria were cultivated in BHI (BD, Sparks, USA) at 37°C under aerobic conditions. Expression of mRNA was determined after 4 and 6 h as indicated in the results section. Bacteria were lysed with Lysozym (40 mg/mL) and RNA was extracted by RNeasy Mini columns (Quiagen, Hilden, Germany) according the manufacturer's instructions. Transcription to cDNA was performed with 100 ng of total RNA using a standard protocol for reverse transcription [193]. qRT-PCR was performed with 1 µL cDNA in a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). For detection and quantification of genes of interest, we used SYBR green (Roche Diagnostics, Mannheim, Germany) to detect copies of cDNA and gene specific primers listed in table 4. Crossing points were determined and relative induction of gene mRNA expression was calculated according to the $2^{-\Delta\Delta Cp}$ method as previously described [194]. Values were normalized to PyrC and GDH expression. To determine PCR products and primer specificity PCR amplicons were subjected to electrophoresis on agarose gels (2%).

Table 4. qPCR primer sequences for the detection of virulence associated genes in *E. faecalis.*

Gene	Forward sequence	Reverse sequence
gelE	5´-ACCCCGTATCATTGGTTT-3´	5´-ACGCATTGCTTTTCCATC-3´
sprE	5´-CCTGTCTGCAAATGCAGAAG-3´	5´-CTGCCACTTCTTGTCTTCTG-3´
fsrB	5´-TGCTCAAAAAGCAAAGCCTTATAA-3´	5´-GATGACGAGACCGTAGAGTATTACTGAA-3´
epaB	5´-GGGCGCCCTTTTCATACATGTGA-3´	5´-GGTGGGTTCGATGAACGTTTCTTCA-3´
epaC	5´-CTGCCACAGTCGGATCTAACGGC-3´	5´-AATCCAGGCCTAGCGACCGC-3´
epaD	5´-CGAAAGGCCATACCTGCCCCA-3´	5´-CGCACCCTGACATCCAAGTGGT-3´
epaG	5´-TCTGCACGCCACCAGTCTTCAT-3´	5´-GCATGTCAACGATCGTGCAGGA-3´
epaN	5´-TGCCTGCCATTTGTGCATCGG-3´	5´-AGTCCCAGTCGCAGCCACGA-3´
pyrC	5´- TTCAGCCGTATCTGGTAC-3´	5´- AGAGAACCAGGCTTTACG-3´
gdh	5´- CAAGCATGGTTCCTATGG -3´	5´- AGTGTTGTTCCGGTTACG -3´

3.1.8 Infection model *Galleria mellonella* (*G. mellonella*)

G. mellonella larvae (HW-Terra KG, Germany) were infected with *E. faecalis* OG1RF and the mutant strains in order to assess enterococcal virulence. *E faecalis* strains were grown in BHI medium for 6 h at 37℃ under aerobic conditions . Cells were, collected by centrifugation and washed once with PBS. Bacterial cell number was adjusted to $0.4x10^6$ cfu/µL in PBS and aliquots were frozen at -80℃. Concurrent to bacter ial inoculation, serial dilutions of each aliquot were plated on BHI plates to confirm the adjusted cell number. *G. mellonella* larvae were sorted by weight (0.22-0.29 grams). Each larvae was infected at the second to last proleg with 5 µL of PBS containing $2x10^6$ cfu or 5 µL PBS as control using a 20 µL syringe (Hamilton Bonaduz AG, Switzerland) and 0.210 x 51 mm needles (Hamilton Bonaduz AG, Switzerland). Per group 10 larvae were incubated at 37℃ in a petri dish containing litter. The insect mortality was then monitored at 6-h intervals for 3 days post infection. Insect mortality was monitored at 6-h intervals for 3 days post infection. Experiments were repeated three times and resulting survival curves were statistically analyzed by log rank test (GraphPad Prism).

3.1.9 Infection model *Caenorhabditis elegans* (*C. elegans*)

C. elegans N2 was maintained and cultivated as previously described [195]. For nematode infection, *E. faecalis* strains were cultivated under routine conditions overnight. Bacteria cultures were concentrated (10-fold) and spread on nematode growth medium (NGM) agar plates which were incubated at 37°C overnight. Plates were equilibrated at room temperature before *C. elegans* L4 larvae were transferred individually onto the bacterial lawn of the respective *E. faecalis* strain or *E. coli* OP50 as control. Nematodes were transferred to fresh NGM plates with the corresponding feeding strain every second day. Worms were considered dead if they failed to respond to touch. The number of viable and killed worms was determined daily. Data are based on one experiment with 3 individual NGM plates per bacteria strain and 30-40 nematodes per plate. They were combined in survival curves which were statistically analyzed by log rank test using Prism 4.03 software (GraphPad software).

3.2 Protein purification

3.2.1 GelE purification from the culture supernatant of enterococci strains

GelE from *E. faecalis* was purified after a modified protocol from Mäkkinen *et al* [179]. Bacteria were cultivated in BHI medium (37°C, aerobic condition) overnight and GelE containing supernatants were achieved by centrifugation (4500 rpm, 20 min). In order to remove some of the disturbing proteins from the supernatant 30% ammonium sulphate saturation (176 g/L) was implemented. The precipitates were removed by centrifugation (5500 rpm, 30 min) and GelE was precipitated by applying 60% ammonium sulphate saturation (addition 198 g/L). After centrifugation (5500 rpm, 30 min) precipitates were dissolved in 2 mL deionized H_2O and then dialyzed against deionized H_2O and 20 mM L-Histidine (Roth, Karlsruhe, Germany), pH 6,0 for 24 h respectively. GelE was purified by Anion Exchange Chromatography using a Resource Q 4 mL column (Amershem Biosciences, Arlington Heights, USA). The binding buffer contained 20mM L-Histidine, the elution buffer included additionally 1 M NaCl, the flow rate was 4 mL/min. GelE containing fractions (tested with Azocasein) were pooled and concentrated using an Amicon Filter System (Millipore, Bellerica, USA) with an exclusion size of 10 kDa. The protein concentration was assessed with RotiQuant protein assay (Roth, Karlsruhe, Germany) according to the manufacturer's instructions. The purity of GelE was determined by SDS-PAGE (10 % acrylamide gel) followed by coomassie or silver staining.

3.2.2 Purification of the *E. faecalis* lipoprotein EF1362

E. faecalis EF1362 was used as an unrelated protein control (UPC). The recombinant protein was overexpressed and purified as previously described [196] using a His6 tag fused to its N terminus. The *ef1362* gene was amplified by PCR and cloned into the pQE30 vector (Qiagen, Hilden, Germany). The protein was expressed in the *E. coli* M15 (pREP4) strain carrying pQE30 vector containing the *ef1362* gene. The production of His6-EF1362 was induced by the addition of 1 mM of isopropyl-D-thiogalactopyranoside. Cells were harvested by centrifugation, disrupted and the cell lysate was centrifuged in order to recover the soluble fraction of proteins. His-tagged EF1362 was purified using Ni-nitrilotriacetic acid columns from the Protino kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

3.3 Cell culture

3.3.1 Transwell experiments: measurement of the transepithelial electrical resistance and translocation across polarized intestinal epithelial cells

Ptk6 cells were kindly provided by R. Whitehead (Vanderbilt University, Nashville, Tennessee) and cultivated as described previously [197]. Cells grew at 33℃ in RPMI-1640 medium (Invitrogen, Carlsbad, USA) containing 5% fetal calf serum (FCS) (Biochrom, Berlin, Germany), 1 µg/mL Insulin-Transferrin-Selenium A (Invitrogen, Carlsbad, USA), 10 Units/ml murine IFN-γ and Antibiotic-Antimycotic (Invitrogen, Carlsbad, USA). For transwell experiments Ptk6 cells ($3x10^5$ cells/mL) were grown on 0.4 µm polyester Transwell filters (Costar Corning, Corning, USA) in cell culture medium without IFN-γ at 37℃. The colon carcinoma cell line T84 was used to validate the observed effects in Ptk6. T84 cells were cultivated in DMEM-F12 medium (Sigma Aldrich, Taufkirchen, Germany) and seeded for transwell experiments in density of $5x10^5$ cells/mL. Cells were apically stimulated as indicated in the results section. The transepithelial electrical resistance (TER) was measured using a Volt-Ohm-meter by Millipore (Millipore, Schwalbach/TS, Germany). TER development was examined and cells were stimulated as indicated in the results section when TER values have reached a constant level. To measure translocation medium was replaced by Krebs solution containing 500 µg/mL sodium fluorescein or 1 µg/mL FITC-Dextran 4000 (FD-4) or FITC-Dextran 10.000 (FD-10) (Sigma Aldrich, Taufkirchen, Germany) in the apical compartment. Basolateral medium was replaced by plain Krebs solution. The transwell plate was incubated at 37℃ under gentle shaking (100 rpm). After 1 h

translocated amounts were quantified in the basolateral compartment using a fluorimeter (Thermo Scientific, Waltham, USA).

3.3.2 Translocation experiments with *E. faecalis*

In order to test translocation of *E. faecalis* across polarized epithelial cells, the human colon carcinoma cell line T84 was used according to a previously published protocol [166,173]. Briefly, cells grew in DMEM/F12 medium (Sigma Aldrich, Taufkirchen, Germany) including 10% FCS (Biochrom, Berlin, Germany) on 3 µm polyester Transwell filters (Costar Corning, Corning, USA) until they have reached maximal polarity and transepithelial electrical resistance remained constant. Cells were apically stimulated with $8x10^7$ cfu/well and kept at 37℃. Basolateral aliquots were plated on BHI agar after 6 h in order to assess number of translocated bacteria.

3.4 Ussing chamber experiments

In order to investigate the influence of *E. faecalis* GelE on distal colonic tissue resistance and translocation of sodium fluorescein from the luminal to the basolateral side we used Ussing chamber systems (Easy mount chambers, Physiologic instruments, San Diego, USA). The technical details and the principle of the measurement have been described previously [198]. We used colon segments from specific pathogen free (SPF) housed IL-$10^{-/-}$, Rag$2^{-/-}$ mice (with and without transferred $CD4^+$ T cells), NOD2 deficient (NOD$2^{-/-}$) mice and conventional housed heterozygous $TNF^{\Delta ARE/Wt}$, a mouse model for experimental ileitis [199],. Distal colon from susceptibility models for intestinal inflammation and their adequate Wt counterparts (129Sv/Ev or C57BL/6) was prepared as whole mount into slider with a recording area of 0.25 cm^2. Human colonic tissue samples were obtained from 6 bowel cancer patients undergoing surgery at the departments of surgery at the University Hospital of the Technical University Munich and the Clinical centre in Freising. Human tissue was dissected and the mucosa was mounted into slider with a recording area of 0.5 cm^2. Apical and basolateral sides were bathed separately in 3 mL Krebs solution. The bath was maintained at 37℃ and aerated continuously with Carbogen (95% O_2 and 5% CO_2). After an equilibration period of 45 min, the Krebs solution in both compartments was replaced by 3 ml fresh Krebs solution containing sodium fluorescein (500 µg/mL) in the mucosal compartment and plain Krebs solution in the basolateral compartment. Purified GelE or UPC (10 µg/mL) were added to the apical part of the chamber system. TER was calculated from the short circuit current and the resulting voltage difference. After 5 h the tissue was disintegrated and incorporated in lysis

buffer for subsequent Western blot analysis. Translocated sodium fluorescein was measured in 100 µL aliquots from basolateral compartments using a fluorimeter (Thermo Scientific, Waltham, USA).

3.5 Histological and immunohistochemical methods

3.5.1 H&E staining and histological scoring

Paraffin embedded tissue sections (5 µm) from distal colon were deparaffinised and stained with hematoxylin and eosin. Histological scoring was done in a blinded fashion by assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion and architectural distortion as previously described [200]. Scores ranging between 0 (non-inflamed) to 12 (severe inflammation) indicate the inflammatory state.

3.5.2 Alcian blue staining

Paraffin embedded tissue sections (5 µm) from distal colon were deparaffinised (two changes in xylene for 3 min each, 2 min in 100% ethanol, 2 min in 96% ethanol, 1 min in 70% ethanol). Specimen were washed with H_2O_{dest} for 2 min and then incubated for 5 min in 1% Alcian Blue solution (Neolab, Heidelberg, Germany). After a washing step with H_2O_{dest} for 3 min, nuclei were stained using Nuclear Fast Red (Roth, Karlsruhe, Germany). Specimen were re-hydrated and covered with mounting medium (Roth, Karlsruhe, Germany).

3.5.3 Immunofluorescence staining *in vivo* and *in vitro*

Standard immunohistochemical procedures were performed to stain gram positive bacteria (anit-lipoteichoic acid antibody), β-Catenin (Anti-β-Catenin from Invitrogen, Carlsbad, USA), extracellular E-Cadherin (Anti-E-Cadherin from SantaCruz sc-8426, Heidelberg, Germany) and intracellular E-Cadherin (Abcam ab76055, Cambridge, UK) in paraffin embedded tissue sections. Paraffin embedded tissue sections (5 µm) from distal colon were deparaffinised (two changes in xylene for 3 min each, 2 min in 100% ethanol, 2 min in 96% ethanol, 1 min in 70% ethanol). After 2 min in deionized H_2O antigen unmasking was achieved through heat treatment in 10 mM sodium citrate (pH 6,0). Therefore the specimens were cooked in a tender cooker for 23 min in the microwave. Specimens were cooled at room temperature for 30 min. To block unspecific binding specimen were incubated in 5% normal goat serum in

PBS including 0.3% Triton-X 100 for 30 min. Primary antibodies were diluted (1:100) in antibody diluent buffer containing 1% BSA and 0.3% Triton-X 100 in deionized H_2O. Specimens were incubated with the antibodies at 4℃ over night. The slides were washed in 3 changes of PBS over 15 min. Alexa Fluor 488 goat anti rabbit IgG (Invitrogen Molecular Probes, Carlsbad, USA) and Alexa Fluor 546 goat anti mouse IgG (Invitrogen Molecular Probes, Carlsbad, USA) were applied to the specimen in a 1:200 dilution in antibody diluent buffer also containing DAPI (Invitrogen, Carlsbad, USA) as counterstaining for the nuclei for one hour at room temperature. Slides were washed three times with PBS, 3 min each, and mounted in vectashield (Vector laboratories).

Ptk6 and T84 cells were fixed with 4% formalin on transwell inserts and stained with anti-E-Cadherin (SantaCruz sc-7870, Heidelberg, Germany) describing the extracellular domain of the protein, anti-E-Cadherin (Abcam ab76055, Cambridge, UK) describing the intracellular domain of the protein, anti-Occludin (Invitrogen, Carlsbad, USA) and anti-Zonula occludens 1 (ZO-1) (Invitrogen, Carlsbad, USA) after stimulation as indicated in the results section. Bacteria were stained with anit-LTA antibody. Alexa Fluor 488 goat anti rabbit IgG and Alexa Fluor 546 goat anti mouse IgG (Invitrogen, Carlsbad, USA) were used to detect the proteins. Nuclei were stained with DAPI (Invitrogen, Carlsbad, USA). Confocal microscopy was performed using a Leica SP5 confocal microscope.

3.6 Mice, bacterial monoassociation and isolation of primary IEC

The experiments with IL-$10^{-/-}$ and Wt mice were performed in collaboration with RB Sartor in the gnotobiotic facility of the University of North Carolina, Chapel Hill. Germ-free IL-$10^{-/-}$ and Wt mice on the 129S6/SvEv background were monoassociated at 15-18 weeks of age with *E. faecalis* OG1RF and the mutant strains Δ*gelE* and Δ*fsrB* by oral gavage and rectal swab. Animal use protocols were approved by the Institutional Animal Care and Use Committees of the University of North Carolina at Chapel Hill. Primary colonic IEC were isolated and purified as described previously [193]. Briefly, colonic tissue was disintegrated in little pieces and incubated in DMEM (Invitrogen, Carlsbad, USA) containing 10% FCS (Invitrogen, Carlsbad, USA) and 1 mM dithiothreitol at 37℃ for 30 min. The suspension was filtered and the remaining tissue was incubated in 30 mL phosphate buffered saline (PBS) containing 1.5 mM EDTA for 10 min at 37℃. After another filtration and centrifugation step (400 g, 5 min) the cell pellet was suspended in DMEM with 5% FCS. Primary IEC were purified by centrifugation through a 20%/40% discontinuous Percoll gradient at 600 g for 30 min. Finally primary IEC were collected in lysis buffer or in Trizol (Invitrogen, Carlsbad, USA) for subsequent Western Blot analysis and RNA isolation respectively.

3.7 Western Blot analysis

Primary isolated IEC were mixed 1:1 with Laemmli buffer, whereas Ptk6 cells were directly harvested with Laemmli. After heat denaturation (95℃, 10 min) equal amounts (30 µg in the case of cells, 50 µg of IEC) samples were subjected to 7, 10 or 15% acrylamid gels depending on the molecular weight of the protein of interest. After electrophoresis proteins were transferred on PVDF membranes (Millipore, Schwalbach, Germany) using a Trans Blot SD Semi Dry Transfer Cell System (peQLab, Erlangen, Germany). Membranes were blocked in 5% milk to prevent unspecific binding of the antibodies. Anti-E-Cadherin (Santa Cruz sc-7870, Heidelberg, Germany) which is raised against amino acids 600-707 mapping the extracellular domain of the protein, anti-E-Cadherin (Abcam ab76055, Cambridge, UK) which binds to the intracellular domain of the protein, anti-occludin, anti-Claudin 1, anti-Claudin 4 (Invitrogen, Carlsbad, USA), anti-JAM-A (SantaCruz, Heidelberg, Germany), anti-β-Catenin (Cell signalling, Boston, USA) and anti-β-actin (MP Biomedicals, Solon, USA) were used with the respective HRP-conjugated secondary antibody (Dianova, Hamburg, Germany) to detect immunoreactive proteins with an enhanced chemiluminescence light-detection kit (GE Healthcare, Buckinghamshire, UK).

Table 5. Primer sequences for qPCR.

Protein	Gene	Forward sequence	Reverse sequence
E-Cadherin	*Cdh-1*	5'-ATCCTCGCCCTGCTGATT-3'	5'-ACCACCGTTCTCCTCCGTA-3'
JAM-A	*F11r*	5'-AGAACAAAGAAAGGGACTGCAC-3'	5'-ACCAGGAACGACGAGGTCT-3'
Occludin	*ocln*	5'-CACGACAGGTGGGGAGTC-3'	5'-TTGATCTGAAGTGATAGGTGGATATT-3'
Claudin-1	*cldn1*	5´-CCCTTGACCCCCATCAAT-3´	5´-ACACCTCCCAGAAGGCAGA-3´
Claudin-2	*cldn2*	5´-CCAGTGCGATATCTACAGTACCC-3´	5´-TGCACTGGACGTCACCAT-3´
Claudin-4	*cldn4*	5´-CGGGCAGAAGAGGGAAAT-3´	5´-TGCTACGAGGTGGGCAAC-3´
ZO-1	*Tjp-1*	5´-AGGCAGCTCACGTAGGTCTC-3´	5´-GGTTTTGTCTCATTTCTTCAG-3´
TNF	*tnfa*	5´-TGCCTATGTCTCAGCCTC-3´	5´- GAGGCCATTTGGGAACTTCT-3´
IFN-γ	*ifng*	5´-GGAGGAACTGGCAAAAGGAT-3´	5´-TTCAAGACTTCAAAGAGTCTGAGG-3´
Mucin 2	*muc2*	5´- GGCAGTACAAGAACCGGAGT-3´	5´-GGTCTGGCATGCCTCGAA-3´
MMP-2	*mmp2*	5'-CAGGAGGAGAAGGCTGTGTT-3'	5'-GGTCAGTGGCTTGGGGTAT-3'
MMP-9	*mmp9*	5'-ACGACATAGACGGCATCCA-3'	5'-GCTGTGGTTCAGTTGTGGTG-3'
GAPDH	*gapdh*	5'-TCCACTCATGGCAAATTCAA-3'	5'-TTTGATGTTAGTGGGGTCTCG-3'

3.8 RNA isolation, reverse transcription and qRT-PCR

RNA was extracted using Trizol Reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. Transcription to cDNA was performed with 1 µg of total RNA

using a standard protocol for reverse transcription [193]. Real time PCR was performed with 1 µL cDNA in a LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). For detection and quantification of genes of interest we used the Universal Probe Library system (Roche Diagnostics, Mannheim, Germany) with specific primers listed table 5. Crossing points were determined and relative induction of gene mRNA expression was calculated according to the $2^{-\Delta\Delta Cp}$ method as previously described [194]. Values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression.

3.9 Degradation of recombinant E-Cadherin

Recombinant murine E-Cadherin (Leinco, St. Louis, USA) (500 ng) was incubated with purified GelE (100 and 250 ng) or APMA-activated MMP-9 (500 ng) at 37°C. After SDS-Page on a 7% or 5% polyacrylamide gel proteins were stained with Flamingo (Biorad, Hercules, USA) and visualized with a typhoon TRIO+ variable mode imager (GE Healthcare, Buckinghamshire, UK).

3.10 Identification of proteins and cleavage sites using LC-MS/MS analysis

To determine possible impurities in our purified protein samples of GelE from *E. faecalis* OG1RF, CD11 and UC7, we subjected the samples to LC-MS/MS analysis. Furthermore, in order to identify possible GelE cleavage sites in murine recombinant E-Cadherin, E-Cadherin (1 µg) was incubated with GelE (100 ng and 250 ng) for 5 seconds prior to heat-inactivation. Proteins from purified GelE samples as well as from GelE-treated E-Cadherin samples were further denaturated as previously described [201] by adding urea in 50 mM triethylammonium bicarbonate (TEAB) to a final concentration of 8 M. Cystein residues were reduced with 10 mM dithiothreitol for 30 minutes at 56°C prior to alkylation with 100 mM iodoacetamide for 60 minutes. The samples were diluted with 50 mM TEAB to a final urea concentration of 2 M. After addition of 20 ng Trypsin (Promega), the proteins were digested over night. Desalting and concentration of peptides prior to LC-MS/MS was performed with StageTips[202]. LC-MS/MS measurements were performed on an amaZon ETD mass spectrometer (Bruker Daltonik, Bremen, Germany) coupled to an easy-nLC (Proxeon, DK). Peptides were separated on a self packed 0.075x15 cm reversed-phase column (Reprosil, Dr. Maisch, Ammerbuch, Germany) using a 30 minutes linear gradient (0-35% acetonitrile in 0.1% formic acid, flow rate 300 nL/min). Intact masses of eluting peptides were determined in enhanced scan mode and the ten most intense peaks were selected for further fragmentation by

collision-induced dissociation (CID) and acquisition of fragment spectra in ultra scan mode. Singly charged ions as well as ions with unknown charge state were rejected. Dynamic exclusion was enabled and dynamic exclusion duration was set to 10 seconds. Peaklist files were generated using DataAnalysis 4.0 (Bruker Daltonik, Bremen, Germany) and database searches were performed using the Mascot search engine version 2.3 (Matrix Science, London, UK) with a parent ion tolerance of 0.3 Da and a fragment ion tolerance of 0.5 Da against the NCBInr database (07/20/2010) considering oxidation of methionine (15.99 Da), carbamidomethylation of cystein residues (57.01 Da) and semi-tryptic peptides. Search result files were imported into Scaffold 3.0 (Proteome Software, Portland, Oregon) and all peptide-spectrum matches with a Mascot ion score greater than 40 were accepted. Normalized spectrum abundance factors were calculated according to Zybailov *et al* [203].

3.11 Determination of GelE antigenicity

To investigate whether GelE was able to stimulate an immune response we used a protocol which has been already described in detail [204]. Briefly, T cell depleted splenocytes (antigen presenting cells, APC) from SPF 129SvEv mice were either pulsed with bacterial lysates from *E. faecalis* OG1RF or Δ*gelE* at a concentration of 10 µg/mL or with purified GelE (10 µg/mL) over night. Keyhole limpet hemocyanin (KLH) was used as an unrelated antigen control. APC were collected and co-cultured with $CD4^+$ T cell which were isolated from mesenteric lymph nodes of *E. faecalis* OG1RF monoassociated IL-$10^{-/-}$ mice. The IFN-γ response in the supernatants was evaluated using an enzyme-linked immunosorbent assay (BD, Sparks, USA) after 3 days of cultivation.

3.12 DNA laddering

The DNA laddering kit from BioVision (Apoptotic DNA Ladder Extraction Kit, BioVision, Mountain View, USA) was used to investigate the apoptotic potential of *E. faecalis* GelE. Ptk6 cells were stimulated in 6 well plates with conc CM from *E. faecalis* OG1RF and Δ*gelE*, purified GelE and a combination of Breveldin A and Staurosporin (0.5 µM each). After 24 h cells were harvested (including the already detached cells in the supernatant) and fragmented DNA was extracted according to the manufacturer's instructions.

3.13 Wnt reporter assay

The human embryonic kidney cell line HEK293 has been used to study the effect of purified GelE on the induction of the Wnt pathway. Cells grew in DMEM medium (Invitrogen, Carlsbad, USA) supplemented with 10% FCS (Biochrom, Berlin, Germany) and 5% non essential amino acids (Invitrogen, Carlsbad, USA). Cells were incubated with the multi transfection mix including vectors for TOP/FOP, Renilla, Wnt, pcDNA 3 and Lipofectamin LTX reagent (Invitrogen, Carlsbad, USA) for 4 h. The plasmids have been provided by Korinek and Clevers. Afterwards cells were stimulated with purified GelE in different concentrations for 20 h. Chemoluminescence was determined by using a luminometer. The ratio between TOP and FOP values represents the induction of Wnt pathway.

3.14 Statistical analysis

Data are expressed as mean values ± standard deviation (SD). Statistical examinations were performed by using Sigma Plot 11.0 software. Data comparing different groups or treatments were analyzed with one way ANOVA. If normality test failed, data were evaluated using ANOVA on ranks and all pair wise multiple comparison procedures were performed according to Tukey or Student-Newman-Keuls Method. Differences were considered significant if p-values are ≤ 0.05 (*) or ≤ 0.001 (**).

4 Results

4.1 Characterization of the gelatinolytic ability of *E. faecalis*

4.1.1 GelE expression in *E. faecalis* OG1RF, its isogenic mutant strains and non-characterized isolates from IBD patients

The impact of *E. faecalis* GelE on the development of intestinal inflammation *in vivo* has been investigated by the work of Micha Hoffmann [185]. The monoassociation of IL-10$^{-/-}$ mice with the GelE expressing *E. faecalis* strain OG1RF and isogenic mutant strains lacking GelE expression revealed a significant reduction of pathology in the absence of GelE [205]. To elucidate the pro-inflammatory mechanism of GelE, the proteolytic activity was evaluated and characterized in the GelE expressing *E. faecalis* strain OG1RF and the isogenic mutant strains TX5364 (Δ*gelE*) and TX5266 (Δ*fsrB*) both lacking *gelE* expression. To show specificity for the GelE mediated-effect, the strains TX5439 (Δ*gelE* reconstituted strain) and TX5266.01 (Δ*fsrB* reconstituted strain) were used. Protease expression in the reconstituted mutants was induced by an erythromycin sensitive *gelE* or *fsrB* containing vector. All strains grew equally well in BHI medium under aerobic conditions at 37℃ (figure 6).

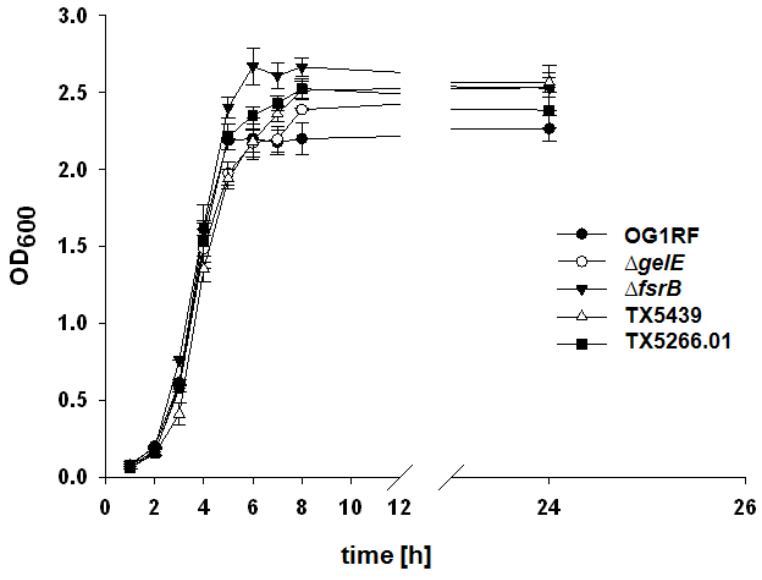


Figure 6: Growth curves of *E. faecalis* strains in BHI medium. OD was measured at 600 nm over time in bacterial cultures which were aseptically inoculated with 1% from an overnight culture. Bacteria grew under aerobic conditions in BHI medium at 37℃. The data represent biological triplicates from one of three independent experiments.

The gelatinolytic activity of the strains was tested with gelatine containing BHI agar. The results show that all strains produced an enzyme able to degrade gelatine as demonstrated as halos around the colonies (figure 7A). An Azocasein activity assay revealed the proteolytic activity in concentrated conditioned media (conc CM), which was prepared as a 50 fold concentration of supernatants from 24 h bacteria cultures of the different strains. As expected the isogenic mutants Δ*gelE* and Δ*fsrB* did not produce GelE in comparison to OG1RF and the reconstituted strains TX5439 and TX5266.01 (figure 7B).

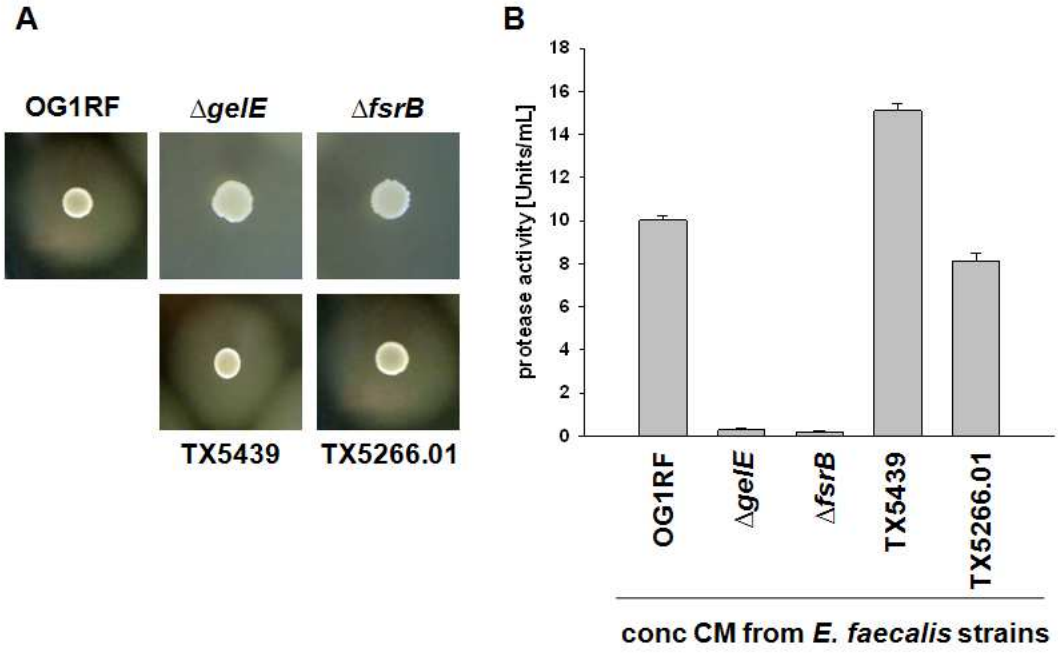


Figure 7: Determination of proteolytic activity in *E. faecalis* OG1RF and the isogenic mutant strains. Bacteria grew on gelatine containing BHI agar overnight at 37℃. Colony surrounding halos represent degradation of gelatine (A). The proteolytic activity in the conc CM was determined with an Azocasein activity assay. Subtilisin A from *Bacillus licheniformis* was used as reference protease to calculate the protease activity in Units/mL. Values represent triplicates from one of three independent experiments (B).

In order to elucidate whether GelE production is relevant in human IBD, faecal samples from IBD patients and one healthy volunteer were screened for GelE producing *E. faecalis* strains. 16S-23S ribosomal DNA intergenic spacer region sequencing identified the species possessing a gelatinolytic activity. With this method, GelE producing *E. faecalis* stains CD11 and CD18.1 could be isolated from the faeces of CD patients, UC7 and UC28.1 from the faeces of UC patients and AH114 from the faeces of a healthy volunteer. GelE activity of the

different strains was confirmed with gelatine containing BHI agar (figure 8A). Growth curves in BHI medium revealed that CD isolate CD28.1 and AH114 from the healthy control did not reach such high density cultures as the other strains (figure 8B). Furthermore *gelE* mRNA expression was determined in the bacteria after 6 h growth in BHI medium at 37℃ under aerobic conditions. Proteolytic activity of GelE was assessed in conc CM from 24 h bacteria cultures. The results demonstrate that GelE expression and activity was approximately half compared to GelE from OG1RF under the conditions that have been tested suggesting that all isolated strains possess the ability to produce functional GelE, but the amount or activity might be strain-specific or dependent on environmental influences (figures 8C and D).

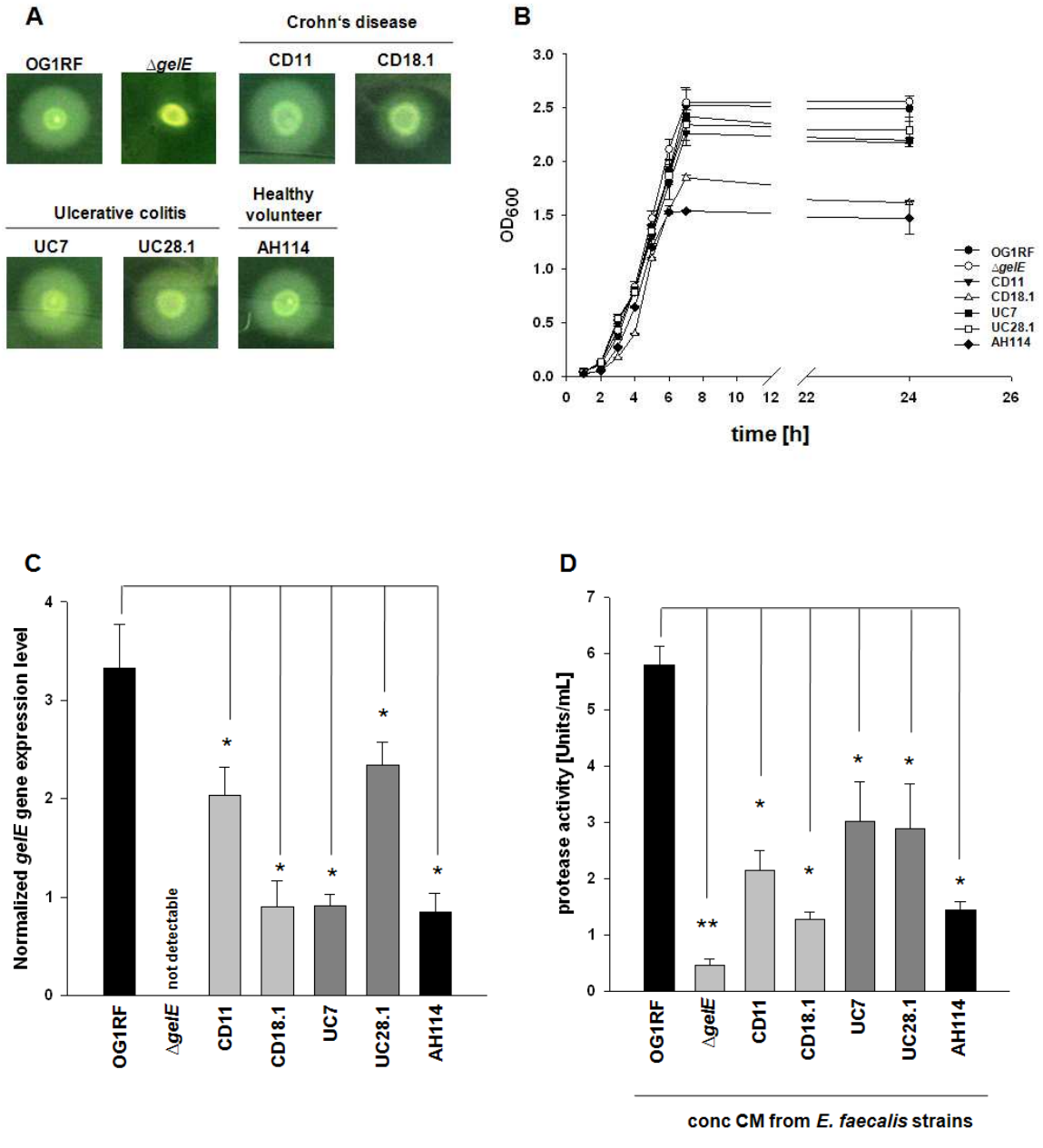


Figure 8: Growth and determination of GelE expression and activity in *E. faecalis* isolates from IBD patients. *E. faecalis* strains possessing a gelatinolytic activity on gelatine containing BHI agar were isolated from faecal samples (A). Growth curves were calculated by measuring the OD at 600 nm over time in bacterial cultures which were aseptically inoculated with 1% from an overnight culture. Bacteria grew under aerobic conditions in BHI medium at 37°C. The data represent biological duplicates from one of three independent experiments (B). GelE production was assessed on transcript-level normalized to *gdh* and *pyrC* expression in the bacteria after 6 h growth in BHI medium (N=3) (C). Proteolytic activity in the concentrated conditioned media (conc CM) from *E. faecalis* strains was determined using an Azocasein activity assay (N=3) (D). Statistically significant differences are calculated by one way ANOVA and marked as follows: *p≤0.05, **p≤0.001.

4.1.2 GelE purification using anion exchange chromatography

In order to provide final evidence for the *E. faecalis* GelE-mediated effects, the protease was purified from the culture supernatant by using anion exchange chromatography. Before chromatographic fractionation, GelE was precipitated with 60% ammonium sulphate saturation. The appropriate concentration of ammonium sulphate was determined with an Azocasein assay which demonstrated that GelE fully precipitated at 55% ammonium sulphate saturation (figure 9A). All fractions from the chromatographic separation were tested for proteolytic activity with an Azocasein assay which revealed that GelE eluted in fractions A4 till A10 (figure 9B).

The protease positive fractions were unified and tested for purity with SDS-PAGE followed by Coomassie staining (figure 10A). The distinct protein with the size of about 32-34 kDa could be significantly identified as *E. faecalis* GelE with MALDI-TOF MS analysis. The *E. faecalis* lipoprotein EF1362 was purified with affinity chromatography and used as *E. faecalis* unrelated protein control in the experiments of the study. GelE was purified from the culture supernatant of *E. faecalis* OG1RF and TX5439 (figure 10A). Purified GelE from OG1RF and TX5439 exhibited a comparable proteolytic activity in the conc CM of the respective strains (figure 10B). Furthermore GelE from IBD isolates CD11 and UC7 was purified according to the same protocol. However, the activity of purified GelE from CD11 and UC7 was significantly lower to GelE from OG1RF. Silver staining revealed additional proteins which were more abundant in the GelE samples from IBD isolates (figure 3C). Despite the fact that GelE was the most abundant protein, LC-MS/MS analysis identified a set of other proteins in those samples listed in table 6 which might be responsible for the lower proteolytic activity in those samples.

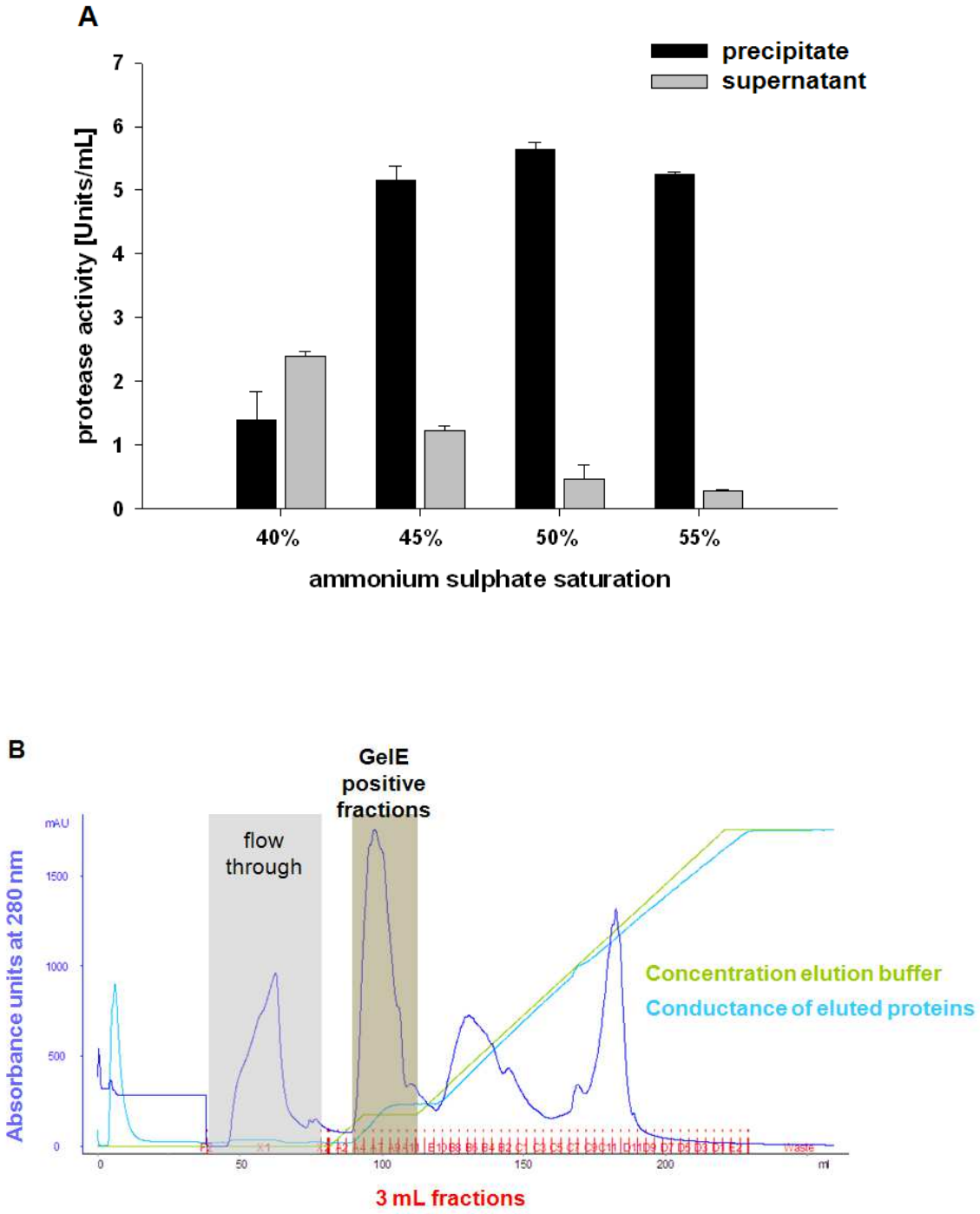


Figure 9: GelE purification from the culture supernatant of *E. faecalis* OG1RF. GelE activity was assessed with Azocasein assay in the bacterial culture supernatant of OG1RF after Ammonium sulphate precipitation at different saturation concentrations (A). GelE was precipitated with 60% ammonium sulphate saturation from 1 litre OG1RF culture, resuspend in 4mL starting buffer and applied to anion exchange chromatography. Measurement of the absorbance units reflects the elution of proteins (B).

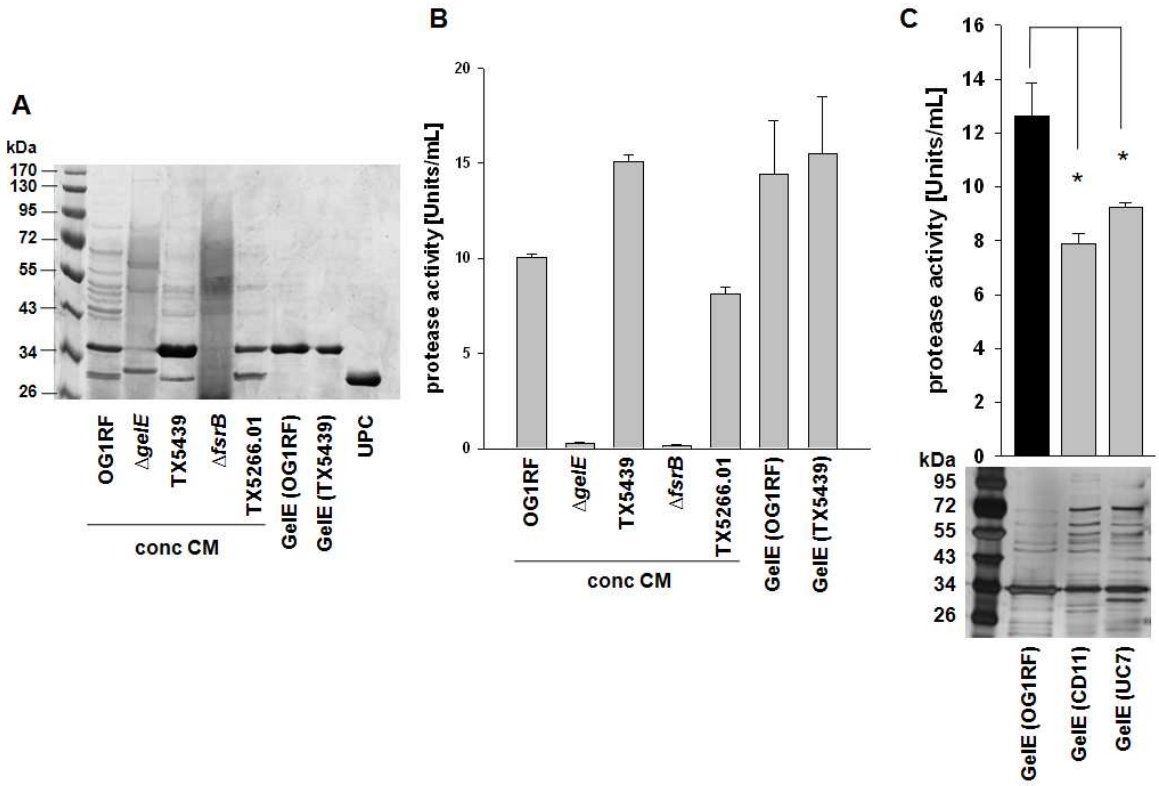


Figure 10: Characterization of purified GelE from *E. faecalis*. SDS PAGE followed by Coomassie staining demonstrates purity of purified GelE from *E. faecalis* strains OG1RF and TX5439 (A). Purified GelE was proteolytically active in an Azocasein activity assay (N=3) (B). Proteolytic activity from the purified GelE samples was significantly lower to GelE from OG1RF. Silver stain revealed more additional proteins in those samples (N=3) (C). Statistically significant differences are calculated by one way ANOVA and marked as follows: *p≤0.05.

4.1.3 Inhibition of GelE activity by EDTA, heat treatment and Marimastat

For further characterization of GelE specificity, various protease inhibitors have been tested to inhibit proteolytic activity. Finally, GelE activity could be inhibited by EDTA, heat treatment and the broad spectrum MMP inhibitor Marimastat (figure 11A). The inhibitory kinetic of Marimastat differs between bacterial GelE and host-derived MMPs which might be due to the comparatively high concentration of GelE necessary for the Azocasein Assay. The half maximal inhibitory concentration (IC_{50}) of Marimastat was calculated to be 183.12 µM for GelE inhibition (figure 11B).

Table 6. Abundance estimation and enumeration of identified proteins in purified GelE samples from *E. faecalis* OG1RF, CD11 and UC7

			Normalized spectrum abundance factor (NSAF)		
GI number	Protein name	MW /kDa	OG1RF	CD	UC
gi|148311	gelatinase [Enterococcus faecalis]	55 kDa	0.5647	0.1407	0.3143
gi|194271937	secreted antigen	48 kDa	0	0.0283	0.0374
gi|116494821	elongation factor Tu	44 kDa	0	0.0177	0.0147
gi|227519758	succinate dehydrogenase	54 kDa	0	0.029	0.0125
gi|257079279	streptococcin II/1	34 kDa	0	0.0139	0.0703
gi|255970739	rod shape-determining protein MreC	26 kDa	0	0.0231	0.0067
gi|238858583	conserved hypothetical protein	138 kDa	0	0.0031	0.0008
gi|229546049	translation initiation factor IF-2	88 kDa	0	0.0031	0.0033
gi|256958560	GAF domain-containing protein	17 kDa	0	0.0211	0.01
gi|229546605	thioredoxin superfamily protein	17 kDa	0	0.0184	0.0106
gi|227517960	nucleoside-diphosphate kinase	15 kDa	0	0.0133	0.0077
gi|227519967	ABC superfamily ATP binding cassette transporter, binding protein	38 kDa	0	0.0043	0.003
gi|229545471	membrane-oligosaccharide glycerophosphotransferase	71 kDa	0	0.002	0.0017
gi|227518007	carbamate kinase	33 kDa	0	0.001	0.0051
gi|229546902	lysyl-tRNA synthetase	57 kDa	0	0.0012	0.0011
gi|29375185	hypothetical protein EF0573 [	58 kDa	0	0.0438	0
gi|29377904	surface exclusion protein PrgA	98 kDa	0	0.0133	0
gi|229546655	autolysin	77 kDa	0	0.0112	0
gi|227517533	ABC superfamily ATP binding cassette transporter, binding protein	36 kDa	0	0.026	0
gi|227517158	pheromone cAD1 precursor lipoprotein	33 kDa	0	0.0187	0
gi|229545341	ABC superfamily ATP binding cassette transporter, binding protein	35 kDa	0	0.0168	0
gi|256958217	peptidase	35 kDa	0	0.0084	0
gi|217388438	aggregation substance	143 kDa	0	0.0028	0
gi|21693365	EF0109	207 kDa	0	0.0019	0
gi|227519416	ErfK/YbiS/YcfS/YnhG family protein	54 kDa	0	0.0051	0
gi|10863218	collagen adhesin precursor	64 kDa	0	0.0042	0
gi|257087356	cell surface protein	164 kDa	0	0.0014	0
gi|256963137	chitin-binding	21 kDa	0	0.011	0
gi|227518733	conserved hypothetical protein	51 kDa	0	0.0048	0
gi|256959387	predicted protein	19 kDa	0	0.0104	0
gi|256617460	surface protein	108 kDa	0	0	0.0482
gi|29374872	adenylate kinase	24 kDa	0	0	0.0318
gi|257078462	lysozyme M1	30 kDa	0	0	0.0139
gi|229545818	3-phosphoshikimate 1-carboxyvinyltransferase	46 kDa	0	0	0.0086
gi|229545261	ABC superfamily ATP binding cassette transporter, binding protein	54 kDa	0	0	0.0054
gi|255972162	N-acetylglucosaminidase	24 kDa	0	0	0.0077

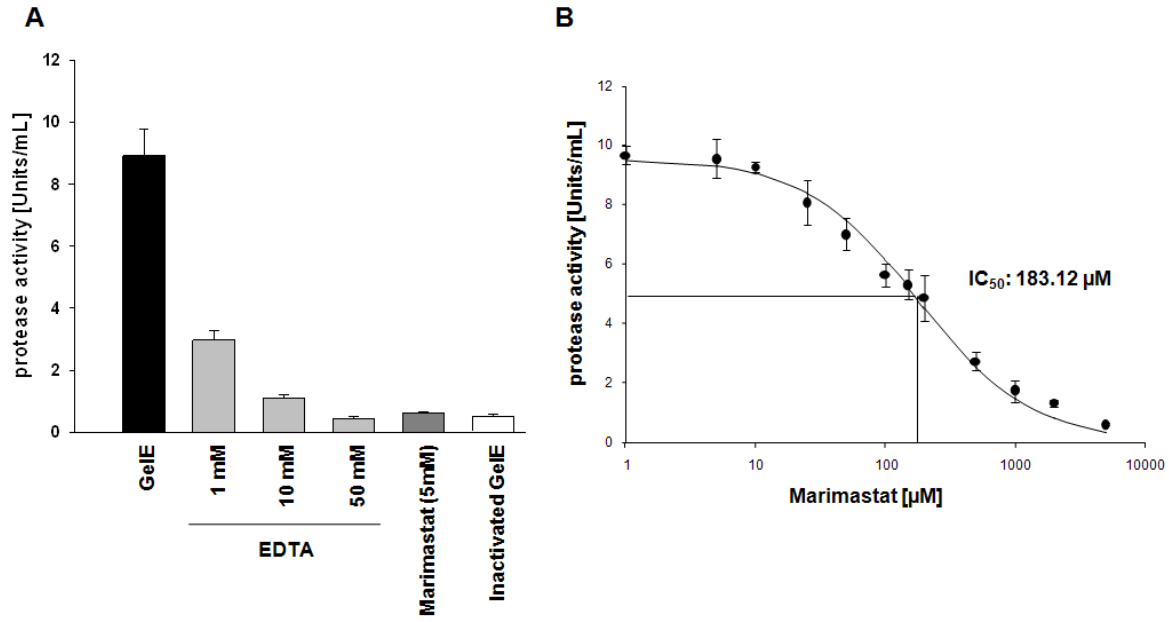


Figure 11: Inhibitors for *E. faecalis* GelE. GelE activity can be inhibited by EDTA, Marimastat and heat treatment (inactivated) (A). The IC_{50} for the inhibition of GelE activity could be calculated for Marimastat to be 183.12 µM (B).

4.1.4 *E. faecalis* GelE is not inducing apoptosis and is not an antigenic structure

One of the first proposed mechanisms for the pro-inflammatory effect that has been observed for *E. faecalis* GelE *in vivo* was the induction of apoptotic pathways. In order to investigate whether GelE exhibits pro-apoptotic features, Ptk6 cells were stimulated with conc CM of *E. faecalis* OG1RF, Δ*gelE* and purified GelE for 24 h and apoptosis was determined by DNA laddering. This analysis revealed that GelE is not inducing apoptosis in contrast to Breveldin A and Staurosporin, a combination that has been used as pro-apoptotic stimulus (figure 12A). To determine whether GelE evokes an antigen-specific T cell response, MLN-derived $CD4^+$ T cells from *E. faecalis* OG1RF monoassociated IL-$10^{-/-}$ mice were co-cultured with antigen presenting cells (APC) that had been pulsed with bacterial lysates from *E. faecalis* OG1RF and Δ*gelE*, as well as with purified GelE and KLH as negative control. Interestingly, the antigenic capacity of GelE seemed not to be associated with the development of tissue pathology in OG1RF monoassociated IL-$10^{-/-}$ mice, as purified GelE did not trigger interferon-γ responses in the APC-T cell co-culture system (figure 12B). In addition, lysates from Wt *E. faecalis* and Δ*gelE* triggered comparable IFN-γ responses in activated T cells, clearly supporting the hypothesis that antigen-independent mechanisms are important for the disease promoting effects of GelE.

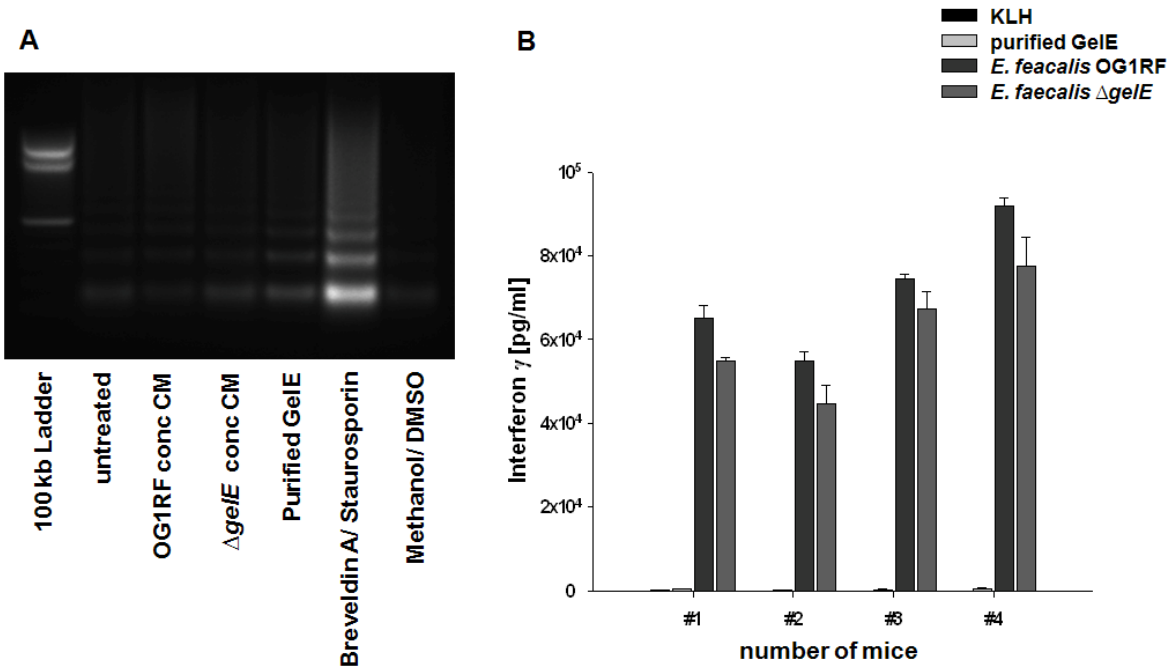


Figure 12: Impact of *E. faecalis* GelE on apoptosis and its antigenic potential. In contrast to Bereveldin A and Staurosporin, GelE did not induce apoptosis in Ptk6 cell which have been stimulated with conc CM of *E. faecalis* OG1RF and Δ*gelE* or purified GelE for 24 h. Apoptosis was determined by DNA laddering (A). To test antigenicity of GelE, splenic APC from Wt mice were pulsed with KLH (negative control), purified GelE, or bacterial lysates from *E. faecalis* OG1RF and Δ*gelE* (10 µg/mL) for 18 hr. APC were co-cultured with $CD4^+$ T cells from *E. faecalis* OG1RF monoassociated mice. Supernatants were collected after 72 hr and IFN-γ was measured by immunosorbent assay. Values represent mean ± SD of IFN-γ concentration in triplicate co-culture supernatants from 4 animals (B).

4.1.5 *E. faecalis* GelE does not possess a mucolytic activity

One possible mechanism for bacterial proteases to be involved in the development of intestinal inflammation is the degradation of glycoproteins in the intestinal outer mucus layer. This feature can be beneficial or harmless for the host, depending on the protease-producing microorganism. However, mucolytic activity enables the microorganism itself or allows other bacteria to get closer to the intestinal epithelial cell layer. Despite the fact that enterococci are members of the intestinal mucosa-associated microbiota, possibly due to the fact that they are able to form biofilms, there is only weak evidence for the ability to digest mucus or mucin glycoproteins. Staining of acidic mucus substances using Alcian blue revealed a strong reduction of mucus in IL-$10^{-/-}$ mice monoassociated with the GelE producing *E. faecalis* strain OG1RF. The animals colonized with the GelE lacking strains Δ*gelE* and Δ*fsrB* showed similar mucus production compared to GF controls (figure 13A). Accordingly,

transcript level of MUC2, the most prominent mucin expressed in the colon, is reduced in isolated IEC of OG1RF monoassociated IL-10$^{-/-}$ mice (figure 13B).

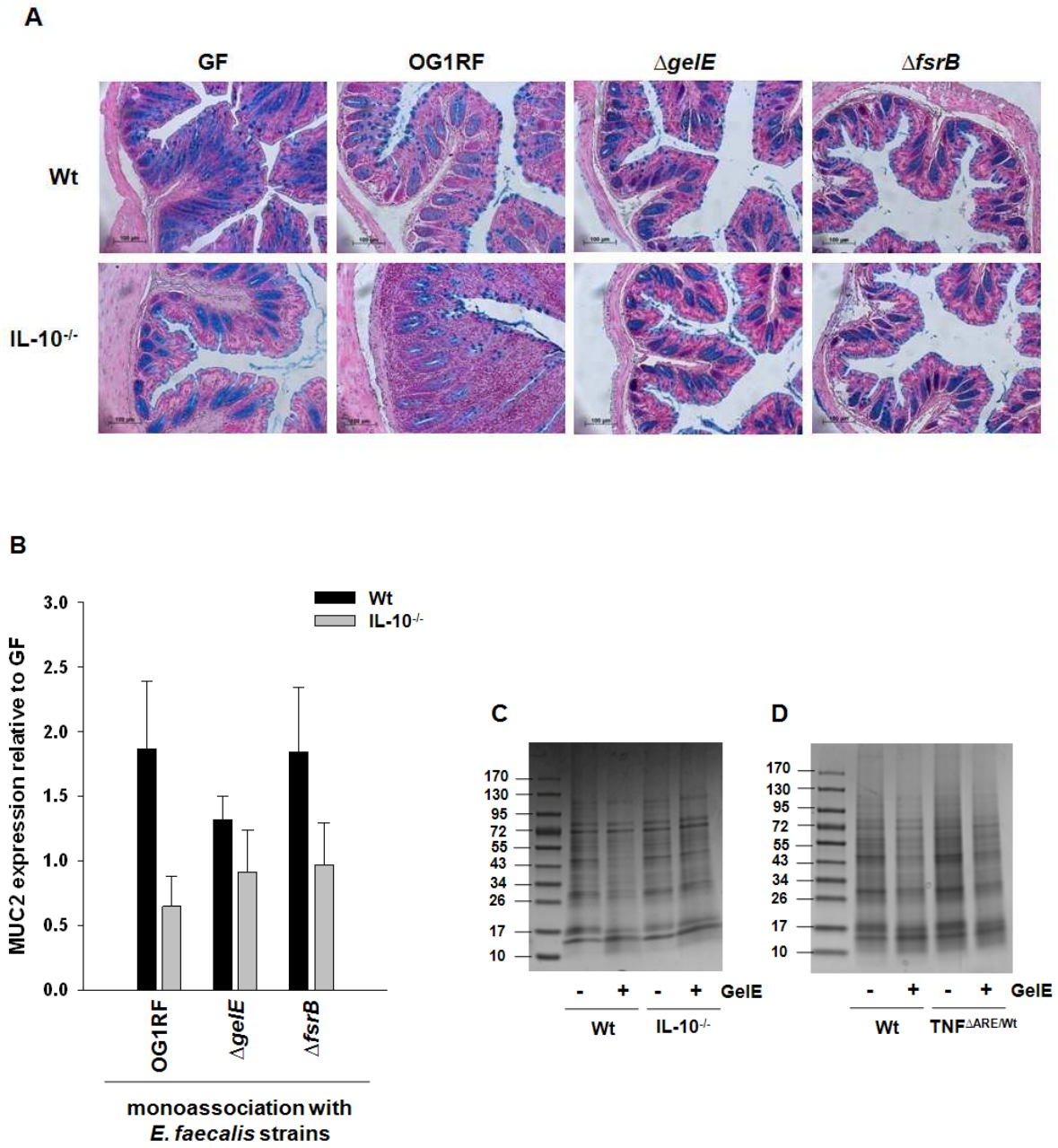


Figure 13: Impact of *E. faecalis* GelE on intestinal mucus production and degradation. Acidic mucus in the distal colon was stained with Alcian blue in paraffin embedded tissue sections of *E. faecalis* monoassociated mice (A). MUC2 mRNA expression revealed no differences neither between the different colonized *E. faecalis* strains nor between wt and IL-10-/- mice (B). Purified GelE was incubated for 1 h at 37℃ with scratched mucus from the colon of IL-10$^{-/-}$, TNF$^{\Delta ARE/Wt}$ and their respective Wt mice. Mucus proteins were separated with SDS PAGE and visualized with coomassie (C and D).

Mucins are produced and secreted by goblet cells whose number is decreased under inflamed conditions. The reduction in mucus staining and the lower transcript level of MUC2 is a result of a decreased number of goblet cells in the OG1RF monoassociated IL-10$^{-/-}$ mice. GelE producing *E. faecalis* strains from IBD patients (UC7, UC28.1 and CD11) and from a healthy volunteer (AH114) did not degrade type III porcine gastric mucin in an *in vitro* assay [10]. Furthermore, purified GelE from *E. faecalis* OG1RF did not alter the protein pattern of scratched mucus from IL-10$^{-/-}$ and TNF$^{\Delta ARE/Wt}$ mice or their respective Wt counterparts providing concurrent evidence that *E. faecalis* GelE is not able to digest intestinal mucus (figure 13C and D).

4.2 *E. faecalis* GelE impairs barrier integrity of intestinal epithelial cells

4.2.1 GelE disrupt barrier function of IEC

Proteases from pathogenic bacteria often target barrier function of IEC in order to infect the host. The impact of *E. faecalis* GelE on barrier function of IEC was addressed with transwell experiments in Ptk6 cells, a murine colonic epithelial cell line that forms polarized monolayers. The conc CM of GelE producing strains OG1RF, TX5439 and TX5266.01 significantly decreased the transepithelial electrical resistance (TER) of cells after 24 h apical stimulation (figure 14A). The GelE lacking strains Δ*gelE* and Δ*fsrB* had no impact on TER values. The functional disruption of barrier integrity mediated by GelE could be confirmed with a significantly higher translocation rate of sodium fluorescein from the apical to the basolateral side of the chamber system (figure 14B).

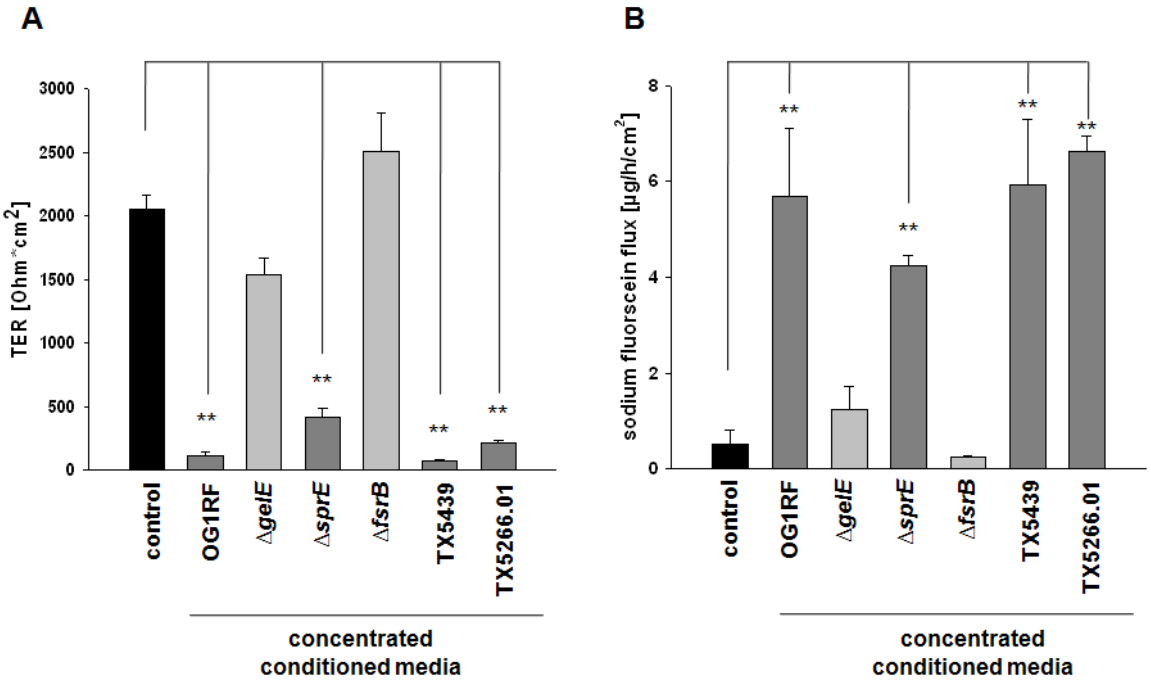


Figure 14: Production of GelE in *E. faecalis* mediates loss of barrier function in cultivated intestinal epithelial cells. Ptk6 cells were apically stimulated with 50 µL conc CM of *E. faecalis* OG1RF, Δ*gelE*, Δ*sprE*, Δ*fsrB* and TX5439 (Δ*gelE* reconstituted strain), TX5266.01 (Δ*fsrB* reconstituted strain). Barrier function was assessed after 24 h by measuring the transepithelial electrical resistance (TER) (A) and translocation of sodium fluorescein (B). Values represent triplicates from one of three independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: **p≤0.001.

The physiological relevance of the so far described results is based on the fact that enterococci belong to the commensal inhabitants of the human gastrointestinal tract. In order to proof that GelE from faecal *E. faecalis* isolates of IBD patients and a healthy volunteer exhibit the same effect, Ptk6 cells were apically stimulated with conc CM from *E. faecalis* strains CD11, CD28, UC7, UC18 and AH112. Although the proteolytic activity was adjusted to the same amount, GelE from the isolated strains had not the same potential to break epithelial integrity as GelE from OG1RF (figure 15A). This is especially obvious with respect to the translocation data of sodium fluorescein (figure 15B).

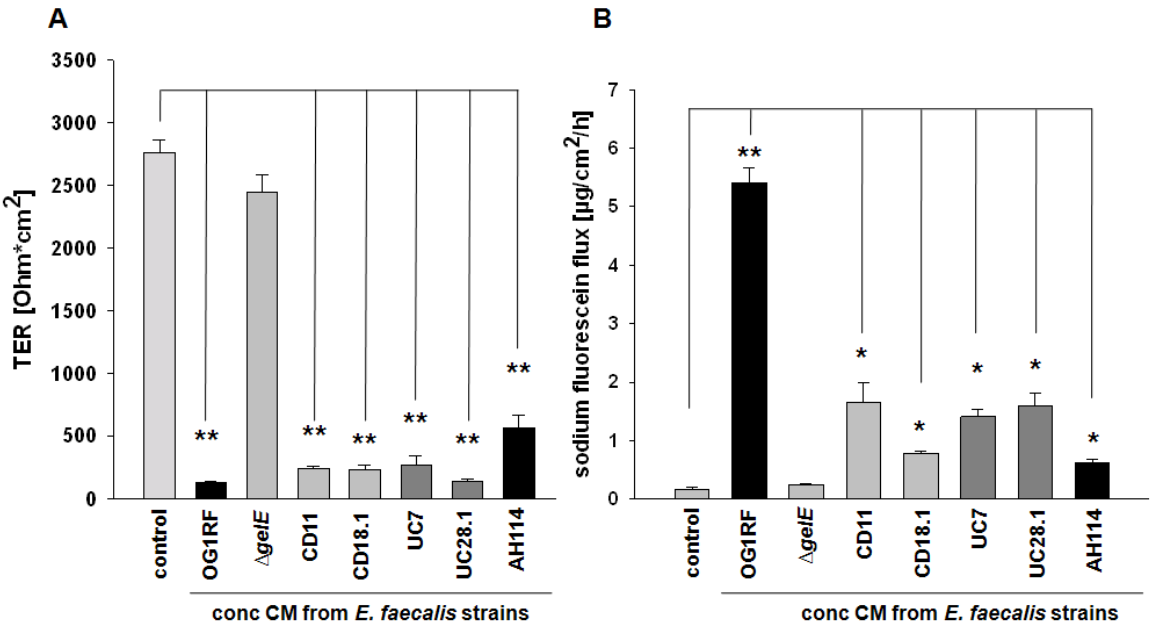


Figure 15: GelE-mediated effect of *E. faecalis* isolates from IBD patients on barrier function of Ptk6 cells. Equal proteolytic activity of conc CM from *E. faecalis* strains OG1RF, AH114 and the IBD isolates CD11, CD18.1, UC7 and UC28.1was used for apical stimulation of Ptk6 cells for 24 h. TER (A) and translocation of sodium fluorescein (B) was evaluated to determine the GelE effect on barrier function. Values represent triplicates from one of three independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: *p≤0.05, **p≤0.001.

Additional experiments with purified proteolytically active GelE from OG1RF, TX5439 and TX5243, the Δ*sprE* mutant, and the IBD isolates CD11 and UC7 revealed that GelE, derived from the OG1RF background, exhibited the same barrier breaking effect. In contrast, GelE from the faecal isolates CD11 and UC7 had not such a strong impact (figure 16A and B). However, these results clearly show that GelE is the mediator of action and not another secreted component of the bacteria. The differences between GelE from different *E. faecalis* strains might originate from impurities in the GelE samples from IBD isolates containing other *E. faecalis*-derived proteins. Further evaluation of the GelE effect on barrier function could be demonstrated with purified GelE, heat-inactivated GelE and inhibited GelE with the broad spectrum MMP inhibitors Marimastat. Inhibited or inactive GelE had no impact on TER values or increased translocation of sodium fluorescein (figure 16C and D). Accordingly, proteolytic activity is required to disrupt epithelial barrier integrity, suggesting that GelE regulate or degrade barrier promoting proteins.

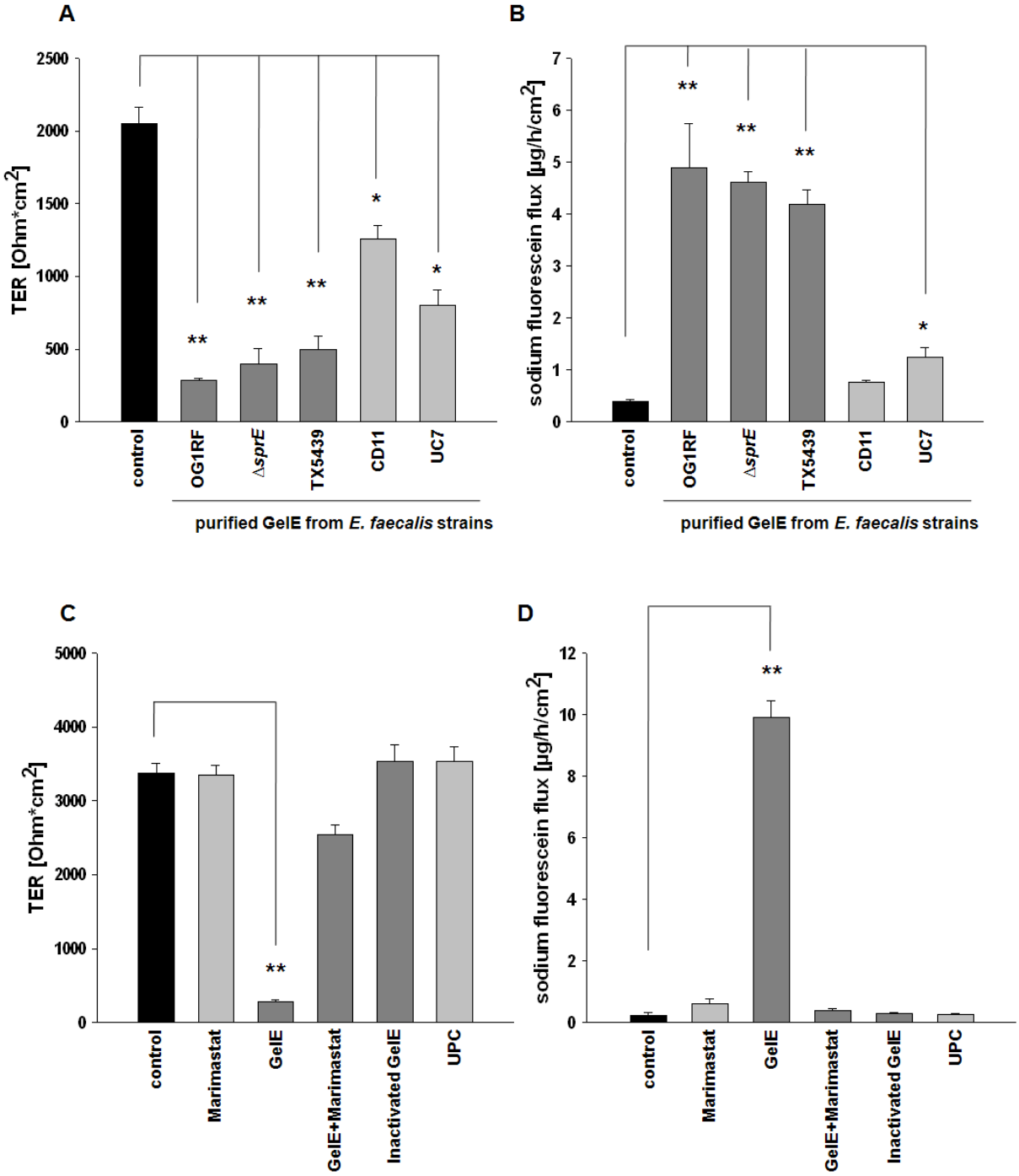
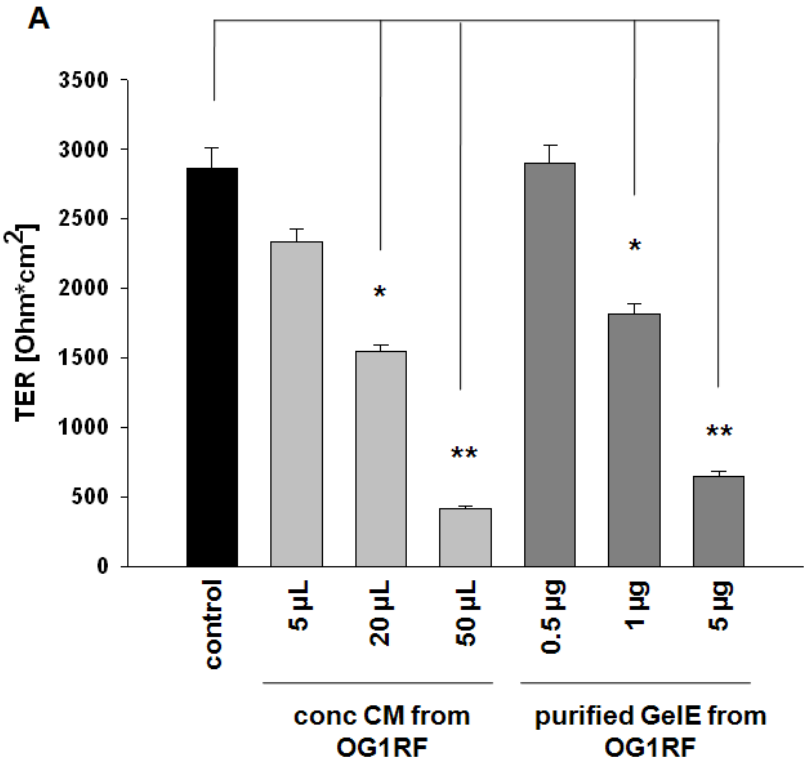


Figure 16: Evaluation of the GelE-mediated barrier disruption using purified GelE and the inhibitor Marimastat. Equal proteolytic activity of GelE from *E. faecalis* OG1RF, Δ*sprE*, TX5439 and the IBD isolates CD11 and UC7 was used to apically stimulate Ptk6 cells for 24 h. GelE from CD11 and UC7 did not reduce barrier function to the same extent as GelE from the OG1RF background (A and B). After 15 min pre-incubation with GelE Marimastat inhibited the GelE mediated disruption of barrier function. Further specificity could be demonstrated by using heat-inactivated GelE and the *E. faecalis* lipoprotein 1362 as unrelated protein control (UPC) (C and D). Values represent triplicates from one of three independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: *p≤0.05, **p≤0.001.

The GelE-mediated effect is concentration dependent providing further evidence for an unspecific degradation mechanism that requires a certain concentration of the protease (figure 17A). The GelE-mediated disruption of barrier integrity could be further characterized by using different permeability markers with different sizes. The bigger the molecule the lower the translocation rate after GelE treatment. Of note, even the biggest permeability marker, FITC-Dextran 10 kDa, showed a significantly higher translocation compared to untreated controls suggesting that the GelE-mediated disruption is more than a physiological temporary opening of the paracellular pathway (figure 17B). Furthermore the GelE-mediated effect could be validated in T84 cells, a human colon carcinoma cell line, supporting the hypothesis for an unspecific, species independent mechanism for *E. faecalis* GelE (figure 17C).



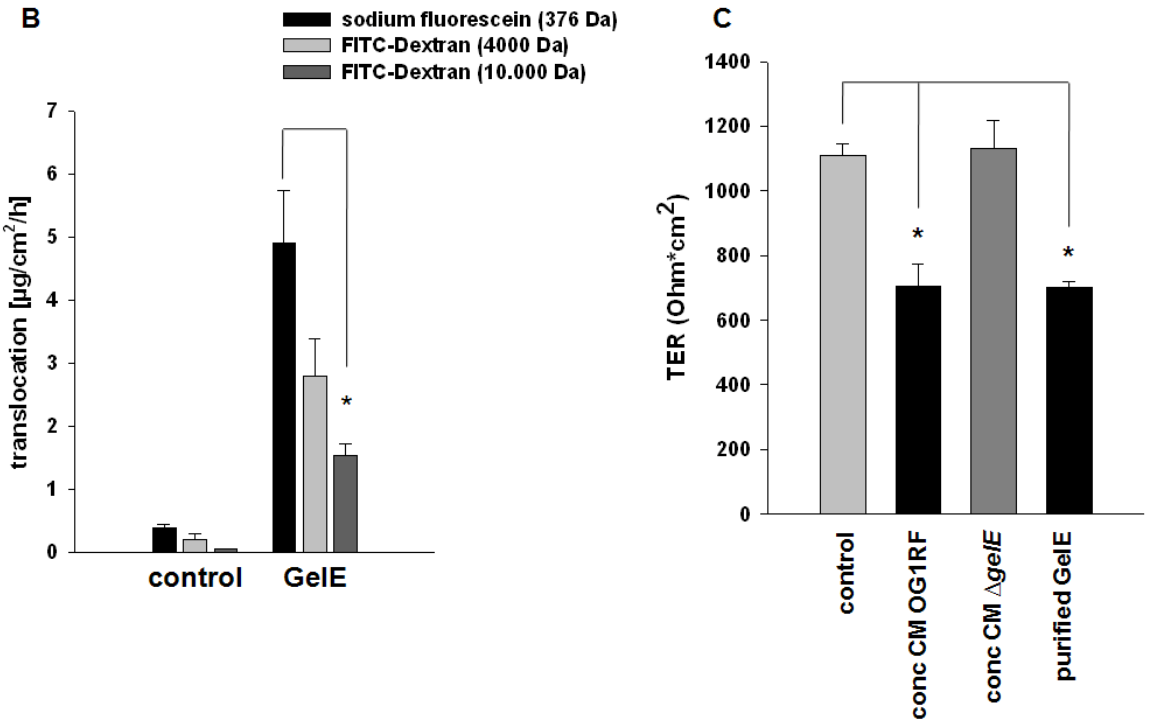


Figure 17: Characterization of the GelE effect on intestinal epithelial barrier function. The GelE mediated disruption of barrier function in Ptk6 cells is concentration dependent (A). After apical stimulation with GelE from *E. faecalis* OG1RF the different permeability markers sodium fluorescein, FITC-Dextran 4000 and FITC-dextran 10000 described barrier disruption of Ptk6 cells (B). *E. faecalis* GelE significantly reduce TER values of T84 cells (C). Values represent triplicates from one of two independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: *p≤0.05, **p≤0.001.

4.2.2 The GelE-mediated loss of barrier function in IEC is associated with a reduction of tight junction protein Occludin

The epithelial barrier integrity is provided by proteins of the junctional complexes, namely the tight and adherence junctions. Western Blot analysis and immunofluorescence staining of the most important or frequently described proteins of the junctional complexes revealed a significant reduction in TJ protein Occludin after apical stimulation of Ptk6 cells with purified GelE for 24 h (figure 18A and B).

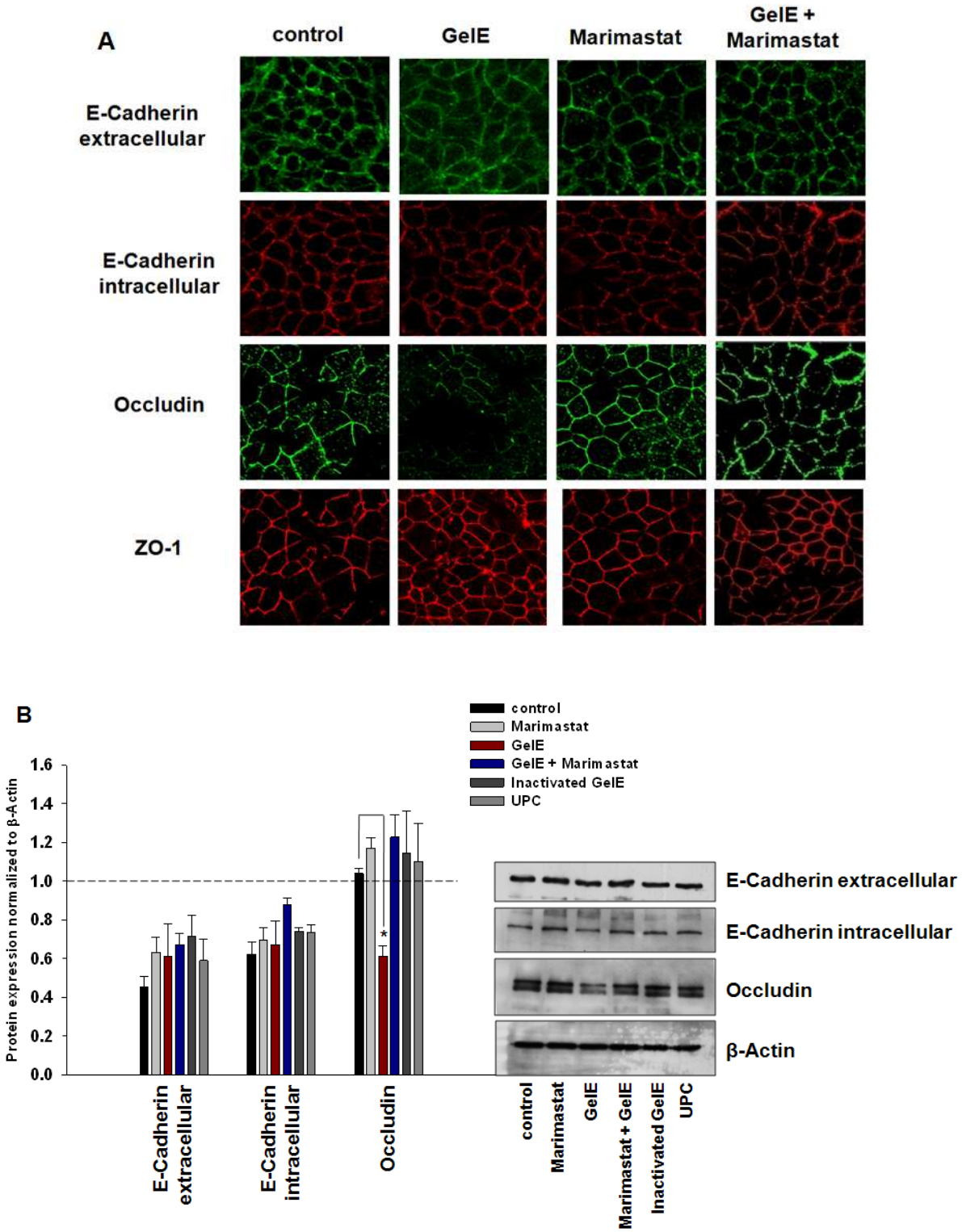


Figure 18: Impact of *E. faecalis* GelE on barrier comprising proteins of TJ and AJ. Immunofluorescence staining of TJ and AJ proteins in Ptk6 cells after GelE stimulation for 24 h revealed a visible reduction of TJ protein Occludin (A). Further evaluation using Western Blot and subsequent densitometric analysis from 3 triplicates demonstrated a significant ($p \leq 0.05$) reduction of Occludin expression after GelE treatment (B).

4.2.3 Synergistic effects of *E. faecalis* GelE and pro-inflammatory cytokines

It has been shown that pro-inflammatory cytokines TNF and IFN-γ disrupt epithelial barrier function through the modulation of cytoskeletal filaments and the activation of MAP kinases [42,43]. To mimic the inflammatory environment, Ptk6 cells were basolaterally pre-stimulated with TNF and IFN-γ for 12 h followed by 24 h stimulation with GelE from the apical side. GelE as well as the combination of the pro-inflammatory cytokines significantly decreased TER values associated with an enhanced translocation of sodium fluorescein (figure 19A and B). Of note, the treatment of Ptk6 monolayers with TNF and IFN-γ, in combination with GelE, potentiated the sodium fluorescein flux, suggesting a synergistic effect of host-derived cytokines and *E. faecalis* GelE in triggering the functional loss of intestinal epithelial barrier integrity.

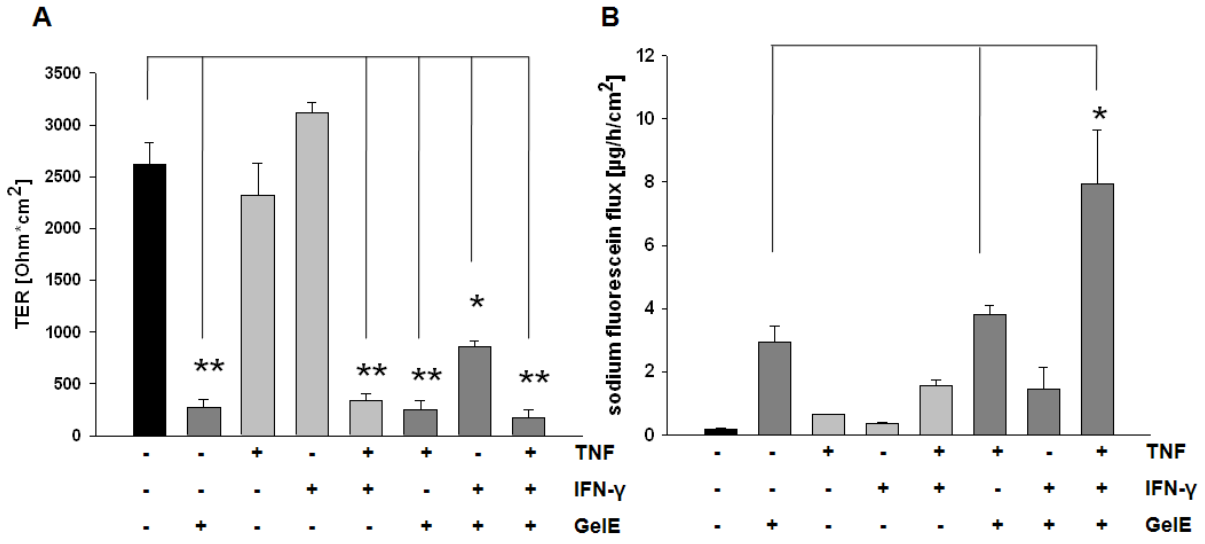
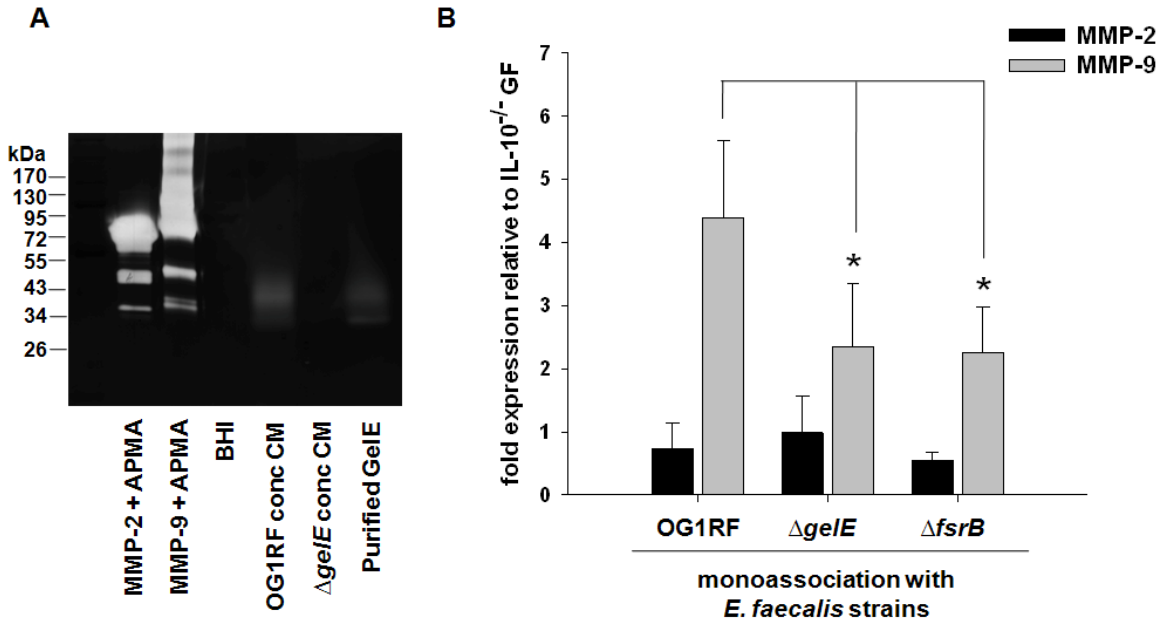


Figure 19: Synergistic effects of *E. faecalis* GelE and pro-inflammatory cytokines TNF and IFN-γ on barrier impairment of epithelial cells. To show synergistic effects of GelE and pro-inflammatory cytokines on epithelial permeability, cells were pre-incubated basolateral with TNF (20 ng/mL) and IFN-γ (50 ng/mL) for 12 h followed by apical GelE stimulation for 24 h. Barrier function was assessed by measuring the TER (A) and translocation of sodium fluorescein (B). Values represent triplicates from one of two independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: **p≤0.001.

4.3 Interaction between *E. faecalis* GelE and host-derived gelatinases

The synthesis as zymogen is one of the similarities between host-derived gelatinases, MMP-2 and -9, and *E. faecalis* GelE. Although gelatinases hydrolyse gelatine, the stoichiometry is very different between endogenous gelatinases and *E. faecalis* GelE. In contrast to APMA activated MMP-2 and -9 (50 ng), a 100 times higher concentration of *E. faecalis* GelE (5 µg) is necessary to detect gelatine degradation in a gelatine zymography (figure 20A). MMP-2 mRNA expression is not different in IEC of *E. faecalis* monoassociated IL-10$^{-/-}$ mice. In contrast, MMP-9 expression is significantly increased in the group of *E. faecalis* OG1RF colonized IL-10$^{-/-}$ mice compared to the animals colonized with the GelE lacking strains Δ*gelE* and Δ*fsrB* (figure 20B). MMP-9 expression was not detectable in Wt mice providing evidence that MMP-9 was induced in the development of colonic inflammation and not by the monoassociation with *E. faecalis*. In order to answer the question of whether *E. faecalis* GelE directly activates MMP-9, recombinant murine pro-MMP-9 was incubated with purified GelE. Active MMP-9 was determined with SDS-Page followed by silver staining and zymography. Compared to APMA that was used as positive control, *E. faecalis* GelE was not able to activate recombinant pro-MMP-9 (figure 20C). In addition purified GelE as well as conc CM of *E. faecalis* OG1RF could not induce MMP-9 mRNA expression in Ptk6 cells after 24 h stimulation supporting the hypothesis that the induction of MMP-9 expression in the intestinal epithelium of the monoassociated IL-10$^{-/-}$ mice was a consequence of the ongoing inflammation and not directly associated with the presence of bacterial GelE (figure 20D).



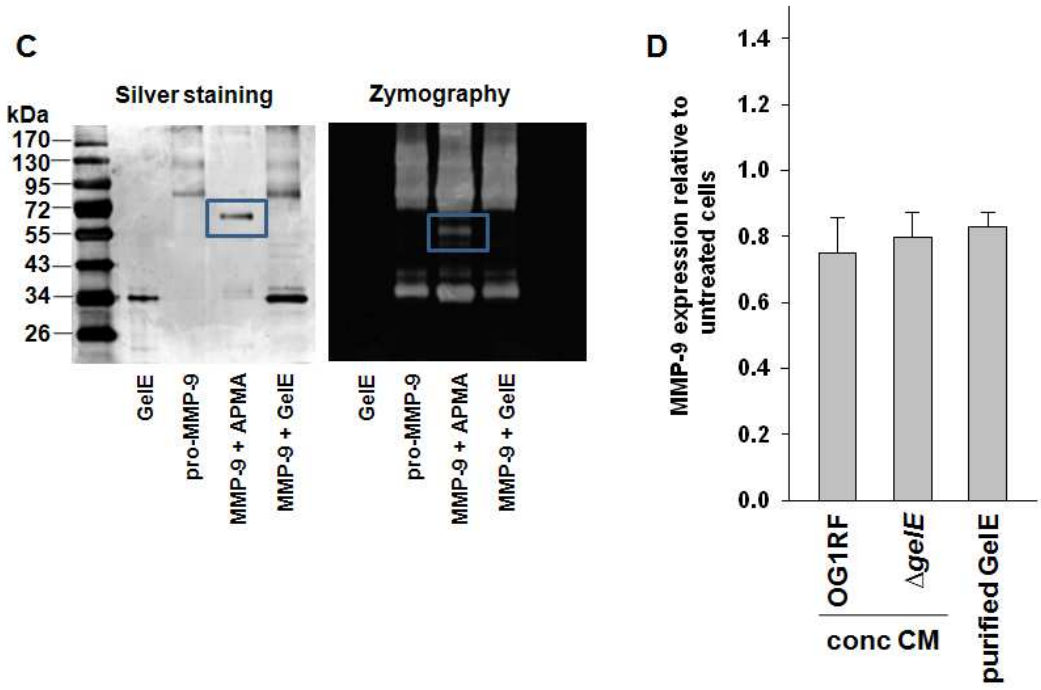


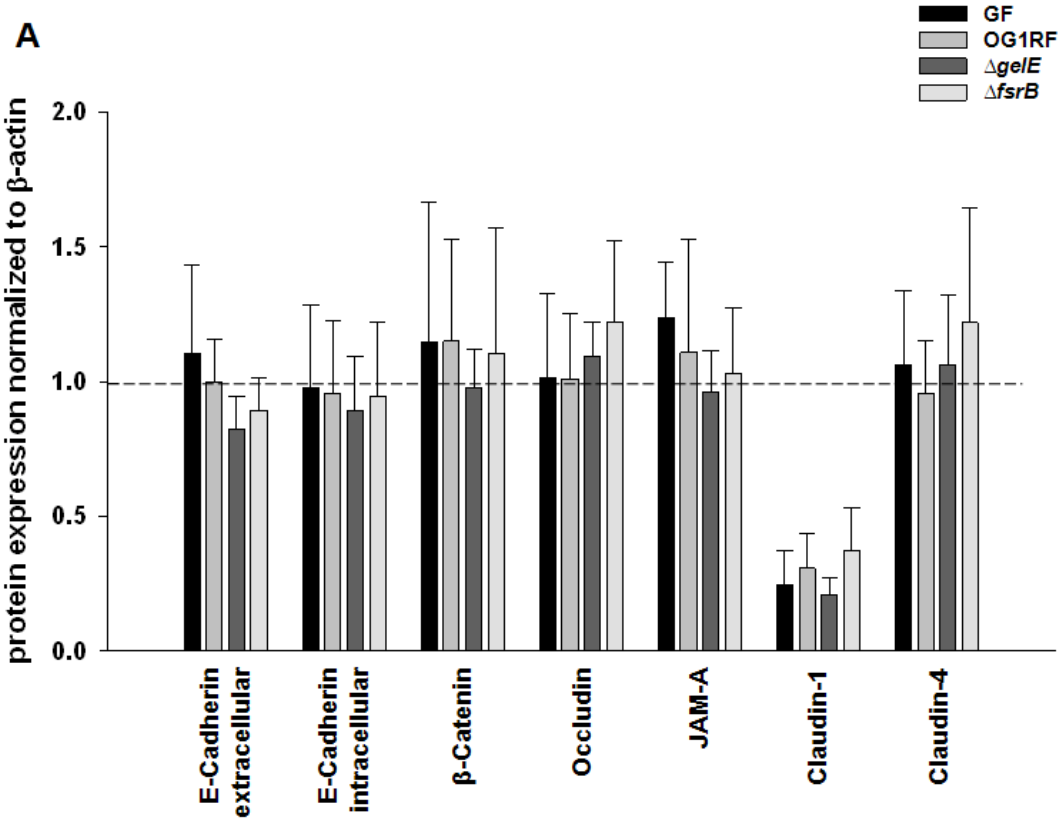
Figure 20: Induction and activation of host-derived gelatinases MMP-2 and -9 by *E. faecalis* GelE. Transcript levels of MMP-2 and MMP-9 were determined with qPCR normalized to GAPDH (house keeper) and calculated as fold expression compared to GF (A and B). Pro- MMP-9 could be activated by APMA (blue box), but not through *E. faecalis* GelE demonstrated on protein level (Silver stain) and by determining the proteolytic activity (Zymography) (C). *E. faecalis* GelE could not induce MMP-9 mRNA expression in Ptk6 cells which was assessed by qPCR normalized to GAPDH and calculated as fold expression relative to untreated cells (D). qPCR values represent douplicates from one of two independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: *p≤0.05.

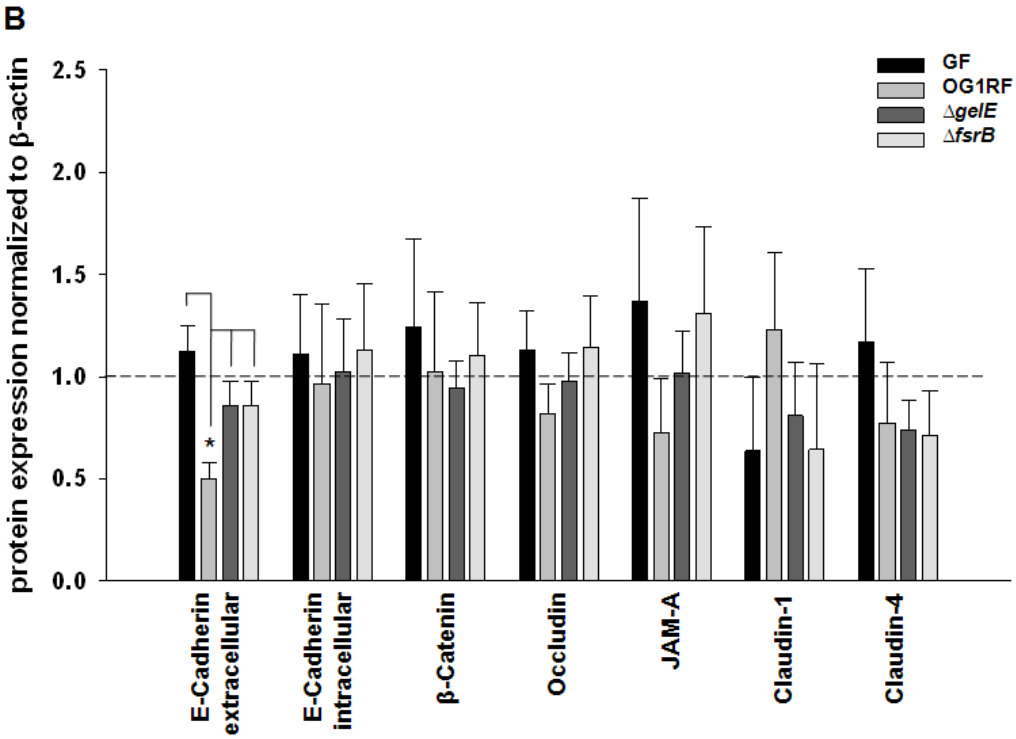
4.4 Impact of *E. faecalis* GelE on junction proteins *in vivo*

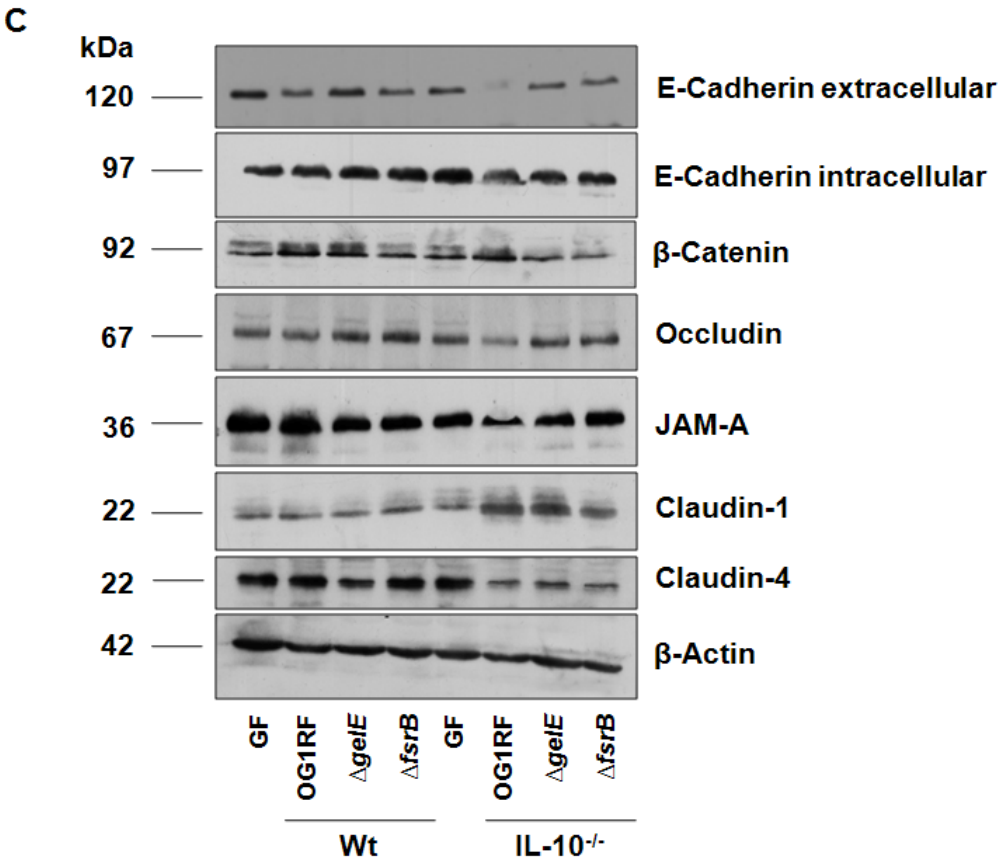
4.4.1 *E. faecalis* GelE mediates loss of extracellular domain of AJ protein E-Cadherin

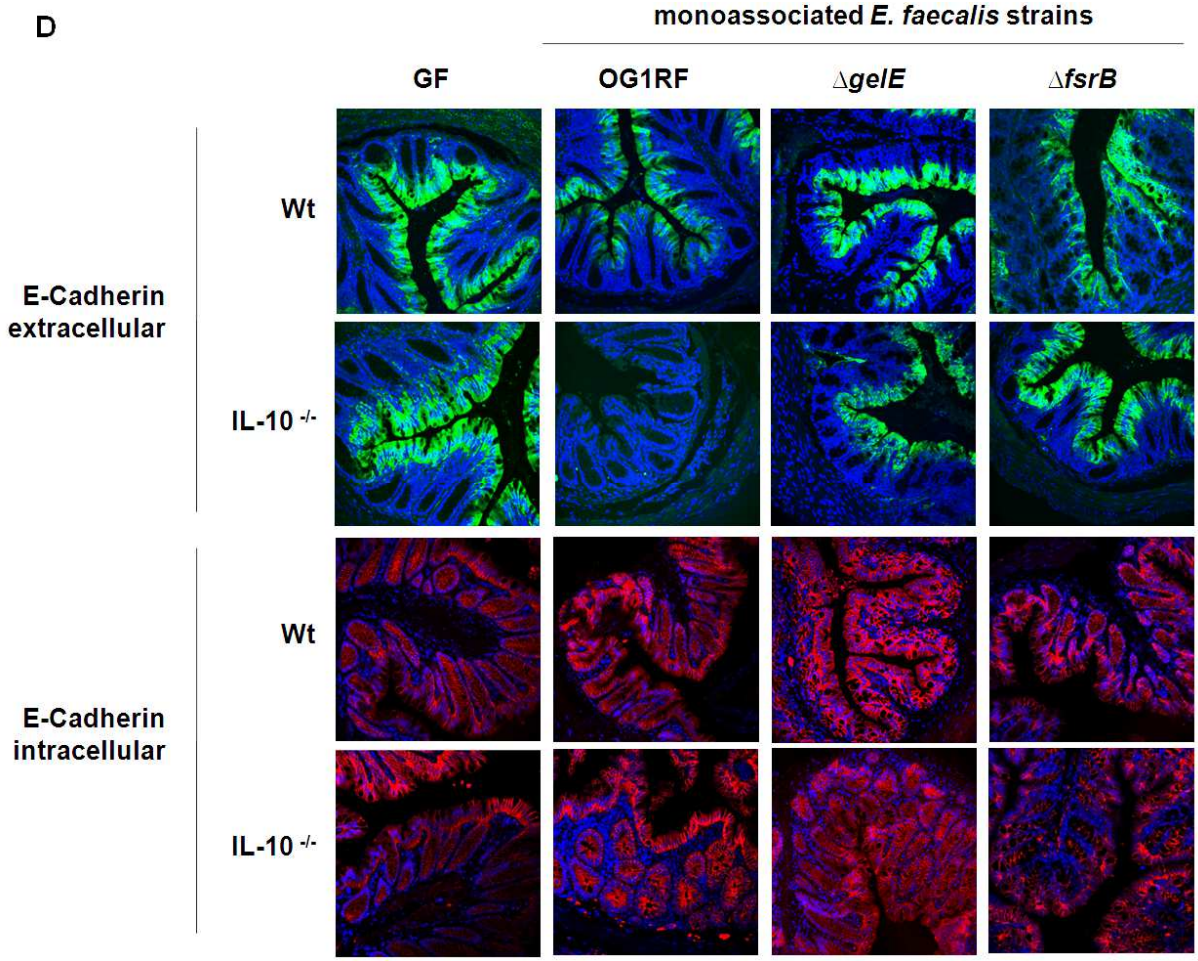
Cell culture experiments have demonstrated that *E. faecalis* GelE reduces barrier integrity of intestinal epithelial cells which is partly mediated through the loss of TJ protein Occludin. The relevance for the loss of Occludin could not be validated *in vivo*. However, Western Blot analysis of IEC revealed a non-significant reduction of TJ proteins Occludin and JAM-A in the *E. faecalis* OG1RF monoassociated IL-10$^{-/-}$ mice. The most remarkable reduction could be observed for the extracellular domain of the AJ protein E-Cadherin in the presence of GelE. An antibody raised against the intracellular domain did not reveal any differences between the colonization with the different *E. faecalis* strains (figures 21A-C). Most importantly, the

loss of extracellular domain of E-Cadherin in *E. faecalis* OG1RF-monoassociated IL-10$^{-/-}$ mice was confirmed by immunofluorescence staining of paraffin embedded distal colon sections. Expression levels of the intracellular domain of E-Cadherin remained unaffected (figure 21D). Additionally, there were no differences detectable at the gene transcript level between the different monoassociated IL-10$^{-/-}$ mice, neither for E-Cadherin mRNA nor for any tested TJ protein (figure 21E).




B
protein expression normalized to β-actin
2.5
2.0
1.5
1.0
0.5
0.0
GF
OG1RF
ΔgelE
ΔfsrB
*
E-Cadherin extracellular
E-Cadherin intracellular
β-Catenin
Occludin
JAM-A
Claudin-1
Claudin-4


C
kDa
120
97
92
67
36
22
22
42
E-Cadherin extracellular
E-Cadherin intracellular
β-Catenin
Occludin
JAM-A
Claudin-1
Claudin-4
β-Actin
GF
OG1RF
ΔgelE
ΔfsrB
GF
OG1RF
ΔgelE
ΔfsrB
Wt
IL-10-/-


D
monoassociated E. faecalis strains
GF
OG1RF
ΔgelE
ΔfsrB
E-Cadherin extracellular
Wt
IL-10 -/-
E-Cadherin intracellular
Wt
IL-10 -/-

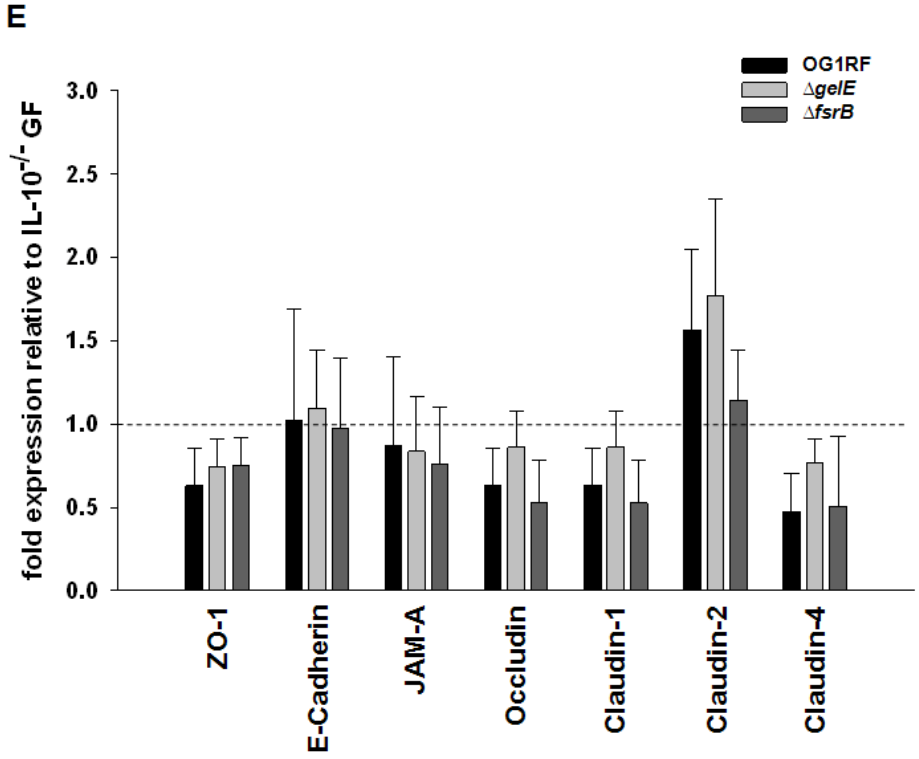
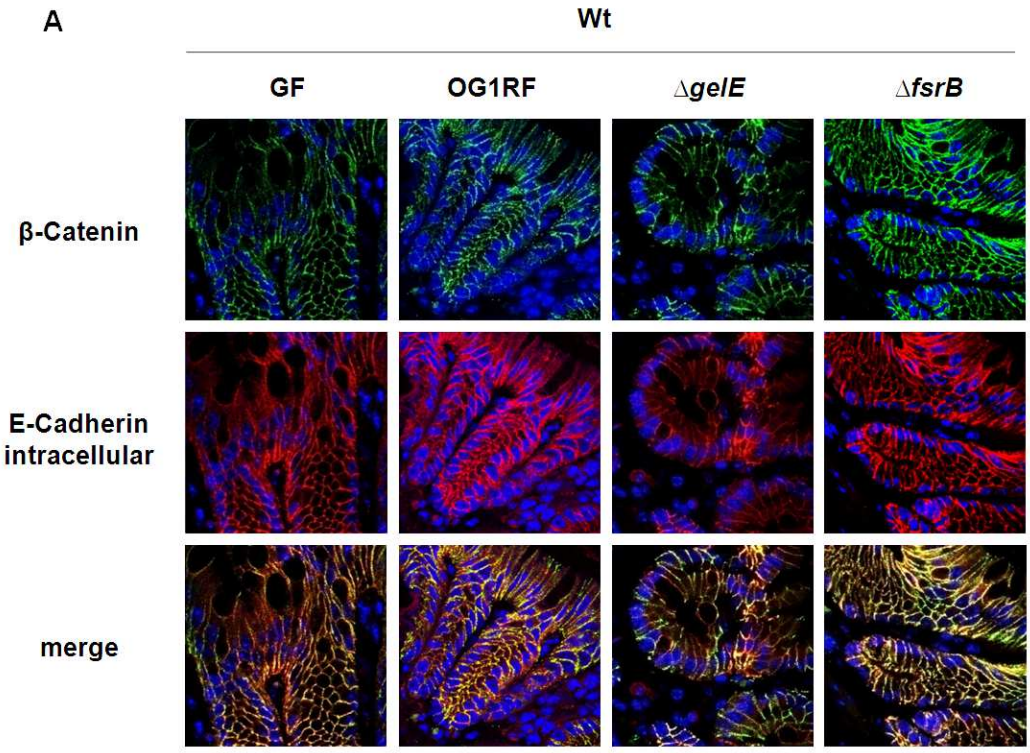

E
OG1RF
ΔgelE
ΔfsrB
fold expression relative to IL-10-/- GF
3.0
2.5
2.0
1.5
1.0
0.5
0.0
ZO-1
E-Cadherin
JAM-A
Occludin
Claudin-1
Claudin-2
Claudin-4

Figure 21: Impact of *E. faecalis* GelE on junction proteins in the intestinal epithelium of monoassociated Wt and IL-10$^{-/-}$ mice. Western Blot and denistometric analysis of isolated IEC revealed a significant reduction in the extracellular domain of AJ protein E-Cadherin in the *E. faecalis* OG1RF monoassociated IL-10$^{-/-}$ mice. Denistometric analysis has been calculated from 5 animals per group (mean values ± SD) from *E. faecalis* monoassociated Wt (A) and IL-10$^{-/-}$ (B) mice. Figure C shows representative Western Blots from all tested junction proteins. Immunofluorescence staining of paraffin embedded distal colon sections confirmed the loss of extracellular E-Cadherin while the intracellular domain remained intact (D). Transcript levels of junction proteins were determined with qPCR. Values (n=5) were normalized to GAPDH and expressed as fold expression compared to GF.

4.4.2 Induction of signalling pathways by *E. faecalis* GelE

The induction of pro-inflammatory signalling pathways including the activation of NF-κB or MAP kinases specifically mediated by *E. faecalis* GelE could not be demonstrated. Immunofluorescence staining of intracellular E-Cadherin and the intracellular adapter protein β-Catenin indicated a cytosolic and not membrane associated distribution of β-Catenin in *E. faecalis* OG1RF monoassociated IL-10$^{-/-}$ mice (figure 22A and B). β-Catenin also acts as transcription factor in Wnt signalling, suggesting that the GelE-mediated induction of Wnt signalling in the susceptible host might be of physiological relevance. Interestingly, a Wnt reporter assay in HEK293 cells showed that Wnt signalling was significantly induced by *E. faecalis* GelE (figure 22C).



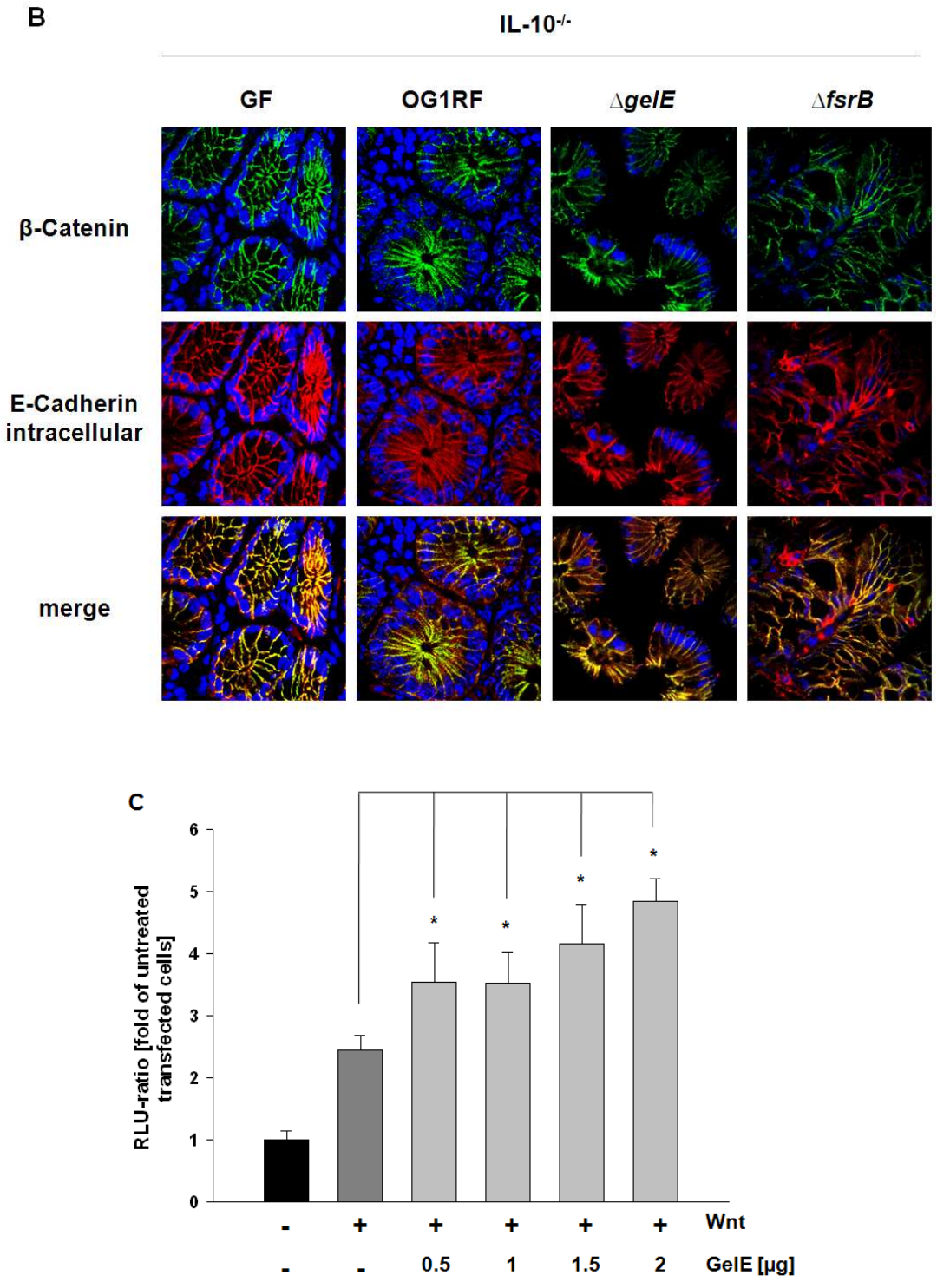
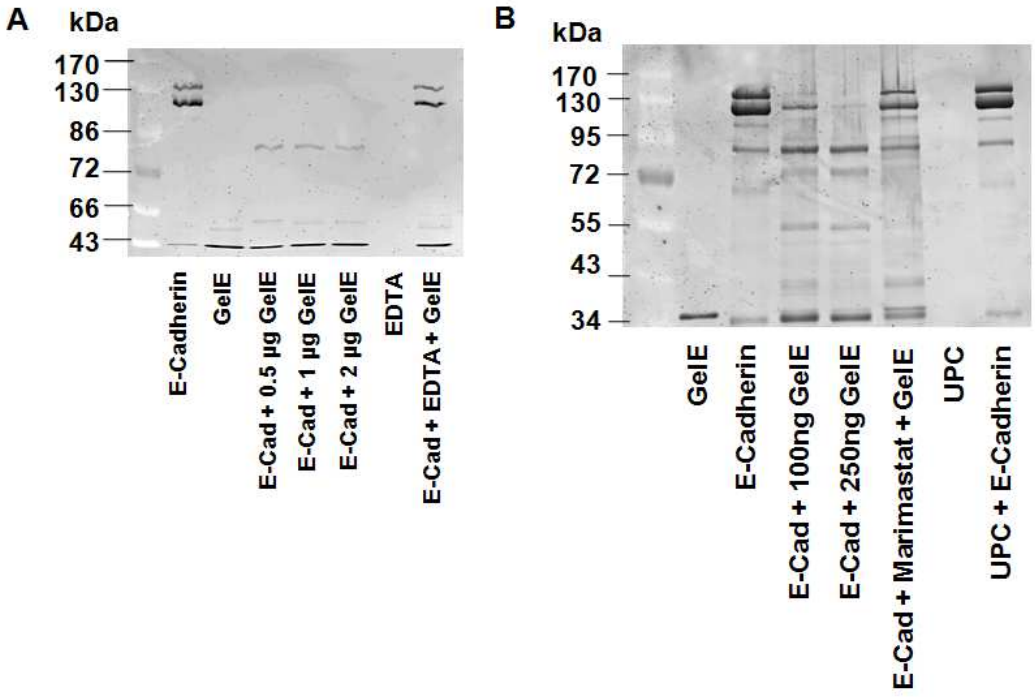


Figure 22: Induction of Wnt signalling by *E. faecalis* GelE. Immunofluorescence staining of paraffin embedded distal colon sections demonstrated an intracellular localization of β-Catenin (Alexa Fluor 488, green), the intracellular adapter protein of E-Cadherin (Alexa Fluor 546, red) in *E. faecalis* OG1RF monoassociated IL-10$^{-/-}$ mice. Representative images are shown for monoassociated Wt (A) and IL-10$^{-/-}$ mice (B). *E. faecalis* GelE induced Wnt signalling in a Wnt reporter assay in HEK293 cells (C).

4.4.3 E-Cadherin can be directly degraded by *E. faecalis* GelE

E. faecalis GelE degrades recombinant murine E-Cadherin in a concentration and time dependent manner. Degradation products could be detected with SDS PAGE followed by Flamingo staining at lower concentration ranges of GelE (100 and 250 ng). EDTA and Marimastat could prevent the GelE-mediated degradation of E-Cadherin demonstrating that the proteolytic activity of GelE is required (figure 23A and B). GelE from the IBD isolates CD11 and UC7 also degraded recombinant E-Cadherin, suggesting that this protease substrate combination is independent of the *E. faecalis* strain and could be mediated by any *E. faecalis* GelE (figure 23C). In contrast, active MMP-9 did not degrade recombinant E-Cadherin, supporting the hypothesis that GelE from *E. faecalis* degrades E-Cadherin, but not host-derived gelatinases (figure 23D). Furthermore LC-MS/MS analysis identified various additional cleavage sites in the extracellular domain of recombinant E-Cadherin after GelE incubation (appendix table 8 and 9), supporting the possibility for a direct GelE-mediated degradation *in vivo* (figure 24).



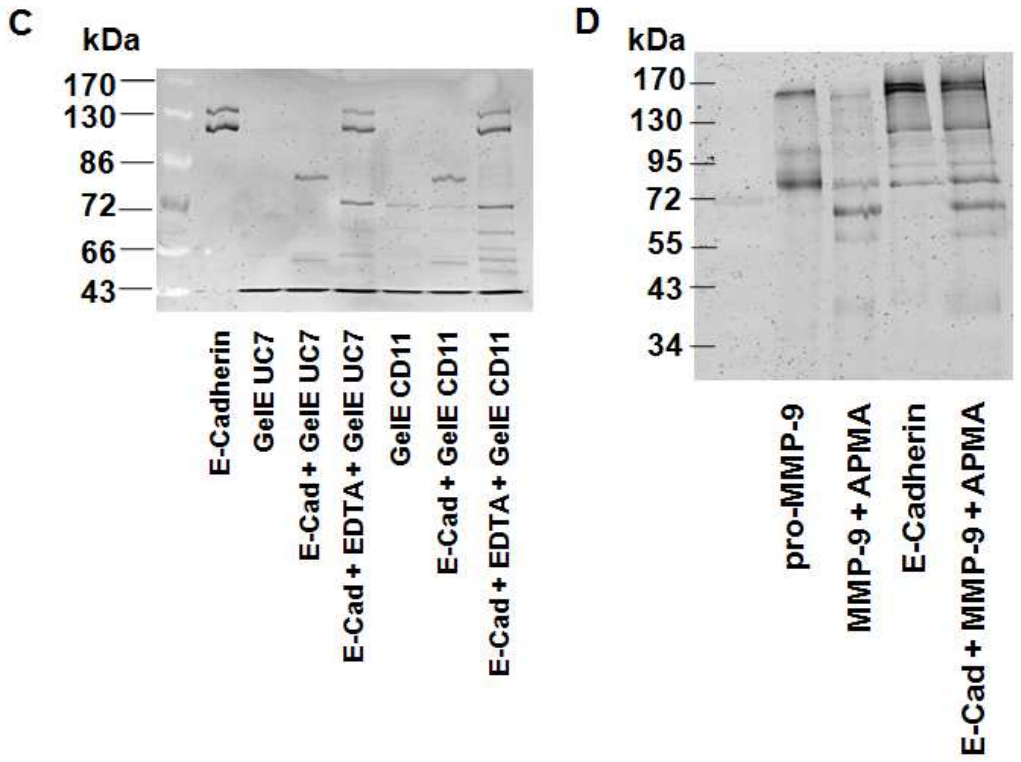


Figure 23: Purified *E. faecalis* GelE directly degrades recombinant murine E-Cadherin. Various concentrations of purified GelE have been tested to degrade recombinant murine E-Cadherin and degradation products have been visualized by SDS PAGE with subsequent flamingo staining. 0.5, 1 and 2 µg GelE and EDTA-inhibited GelE were incubated with E-Cadherin for 5 min at 37℃. All concentrations degr aded E-Cadherin with one prominent degradation product visible at ~ 80 kDa (A). Lower amounts of GelE (100 and 250 ng) and a shorter incubation time (~5 sec) revealed more degradation products for the GelE-mediated degradation of E-Cadherin (B). Purified GelE from UC7 and CD11 also degraded recombinant murine E-Cadherin which could be inhibited by EDTA (C). APMA-activated MMP-9 did not degrade recombinant murine E-Cadherin (D).

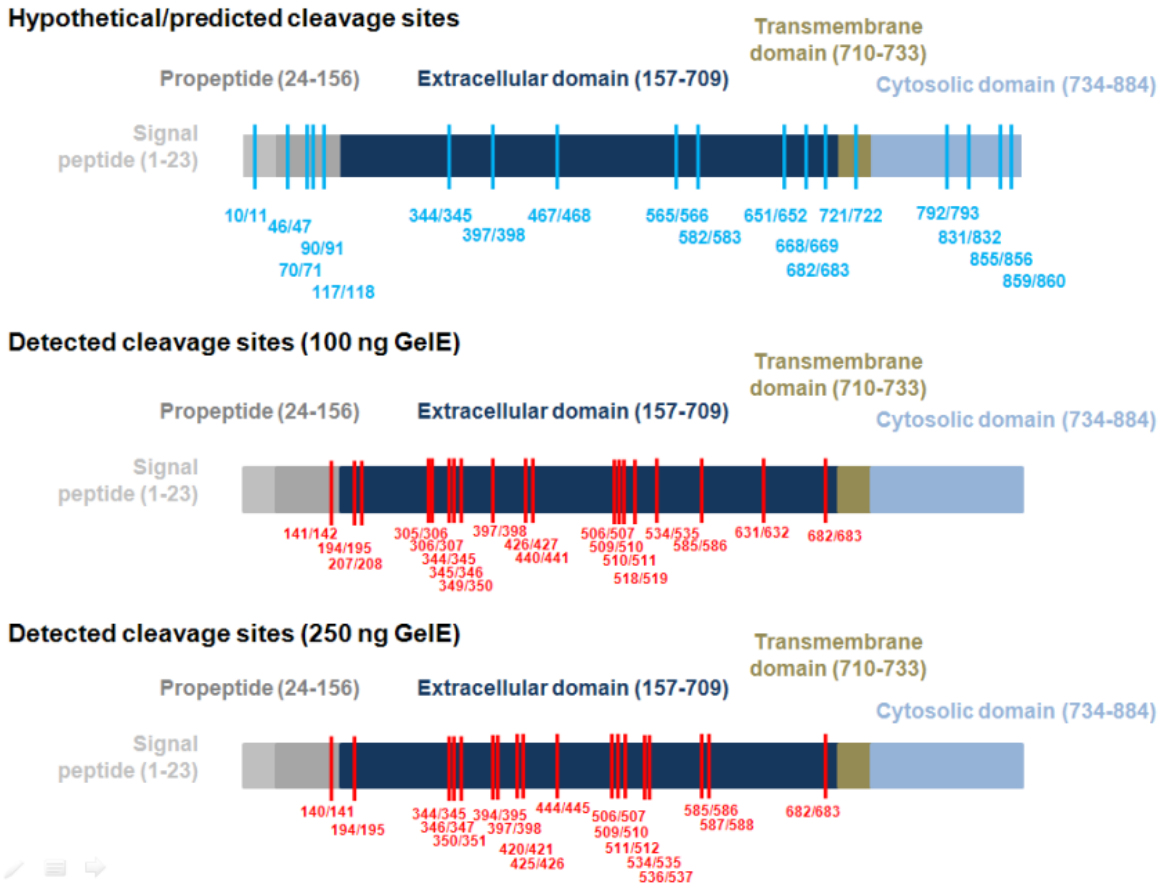


Figure 24: Identified cleavage sites for *E. faecalis* GelE in the AA sequence of murine E-Cadherin. LC-MS/MS analysis revealed cleavage sites for the GelE-mediated degradation of recombinant murine E-Cadherin. The hypothetical cleavage sites have been extracted by Mäkkinen *et al* [179].

4.5 *E. faecalis* GelE reduce barrier function in IBD mouse models

In order to investigate the relevance of the *E. faecalis* GelE-mediated epithelial barrier impairment *in vivo*, the impact of mucosal GelE stimulation on barrier function of distal colon segments from mice has been studied by using Ussing chamber systems. Wt mice did not develop inflammation after monoassociation with *E. faecalis*, demonstrating the commensal character of the bacteria and suggesting that *E. faecalis* contribute to chronic intestinal inflammation only in the susceptible host. In line with the observations in the monoassociation experiment, susceptibility models for intestinal inflammation have been used to study the impact of *E. faecalis* GelE on barrier function. Another intention was to use 8 weeks old mice that have not developed tissue pathology at this time point to evaluate whether *E. faecalis* GelE could act as trigger in those susceptible but disease-free mice. One model which was used was the $TNF^{\Delta ARE/Wt}$ model. These mice develop spontaneous Crohn's

disease-like ileitis due to a genetic modification that results in an increased mRNA stability of the pro-inflammatory cytokine TNF [199]. So far, there is nothing known about a predisposition in terms of barrier function. In contrast to the terminal ileum there is no inflammation in the distal colon of $TNF^{\Delta ARE/Wt}$ and the production of acidic mucus seems to be comparable to the Wt situation (figure 25A). However, they have an increase in TNF mRNA production which implies that the mice have a predisposition in barrier function as it has been demonstrated that pro-inflammatory cytokines including TNF impair intestinal epithelial barrier function (figure 25B) [42,43]. An impaired mucosal barrier function has already been reported for IL-$10^{-/-}$ mice [206,207]. The staining of acidic mucus revealed an already existing reduction of mucus in the distal colon of 8 weeks old IL-$10^{-/-}$ mice which was associated with a slight increase in TNF production but in the absence of tissue pathology (figure 25B and C).

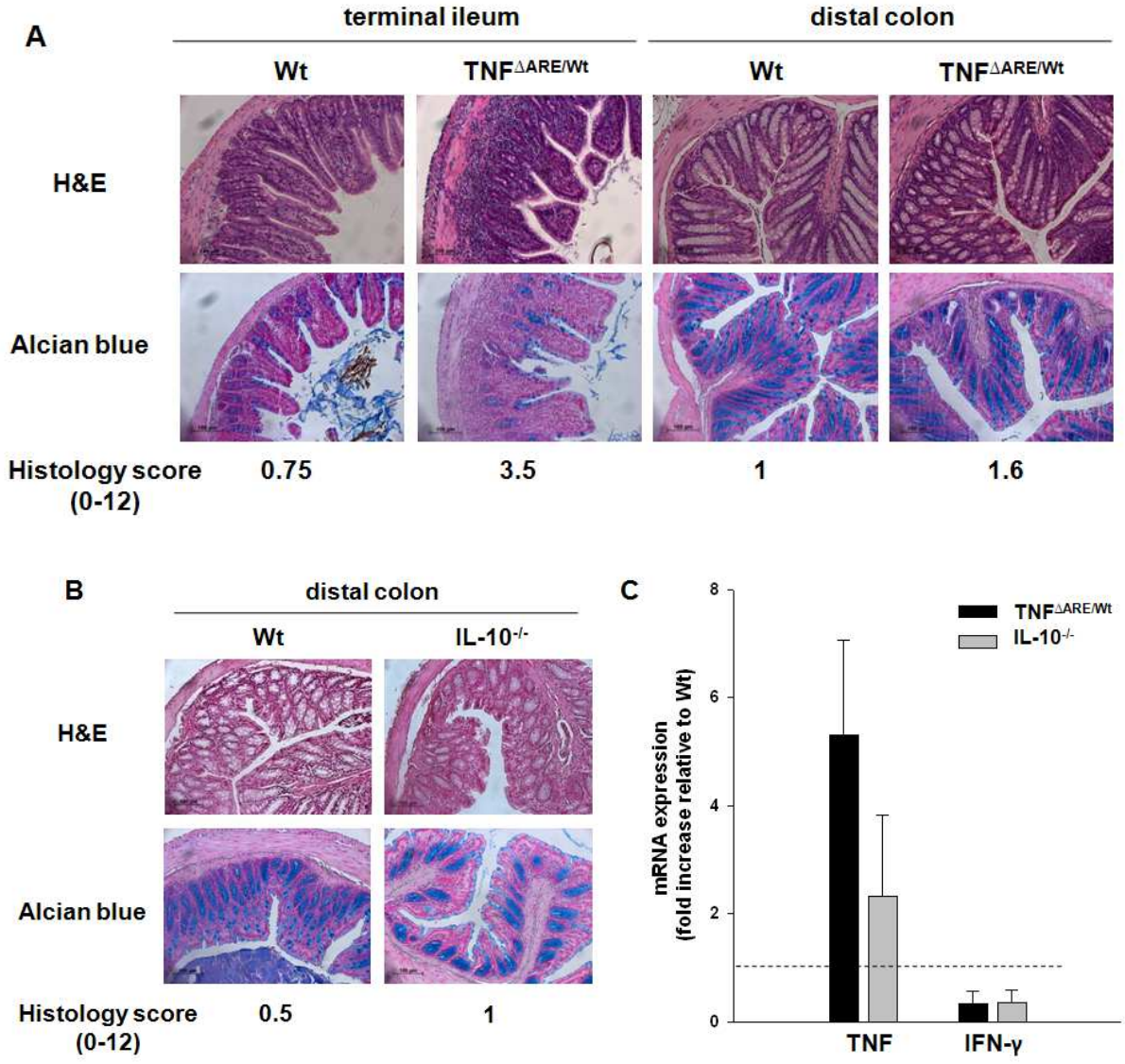
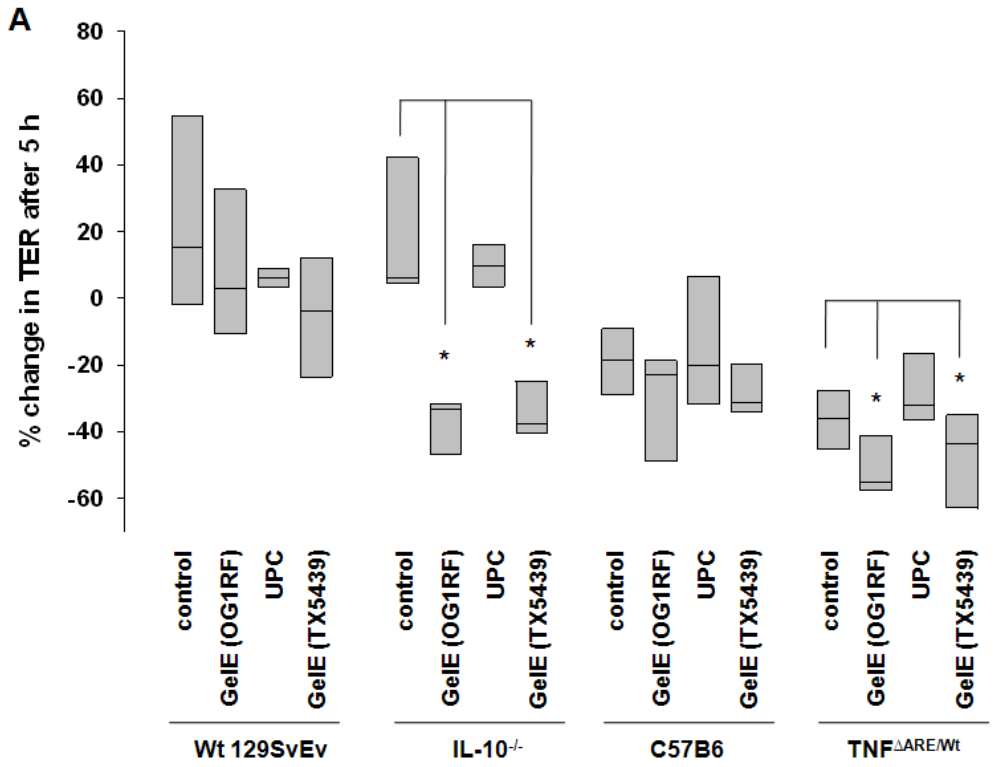


Figure 25: Acidic mucus production and cytokine expression in the colon of IL-10$^{-/-}$ and TNF$^{\Delta ARE/Wt}$ mice. H&E staining of paraffin embedded tissue sections demonstrate that TNF$^{\Delta ARE/Wt}$ mice developed chronic inflammation in the terminal ileum at the age of 8 weeks, but not in the colon (Histology score 0-12). Inflammation was associated with a reduction of acidic mucus substances as demonstrated by the Alcian blue staining (A). IL-10$^{-/-}$ mice show no colonic pathology and reduced acidic mucus production at the age of 8 weeks (B). qPCR analysis of colonic tissue from TNF$^{\Delta ARE/Wt}$ and IL-10$^{-/-}$ mice revealed increased levels of the pro-inflammatory cytokine TNF (C).

Mucosal stimulation of distal colon segments from TNF$^{\Delta ARE/Wt}$ and IL-10$^{-/-}$ mice with purified GelE from *E. faecalis* OG1RF and TX5439 resulted in a significant decrease of TER values after 5 h (figure 26A). Wt mice were not affected supporting the hypothesis that *E. faecalis* GelE has an impact under disease susceptible but pathology-free conditions. The loss of barrier function could be validated with a significant reduction of the extracellular domain of AJ protein E-Cadherin after GelE stimulation in the susceptible mice (figure 26B). Despite the fact that other mechanisms could not be excluded, these results give further evidence for a direct GelE-mediated degradation of E-Cadherin.



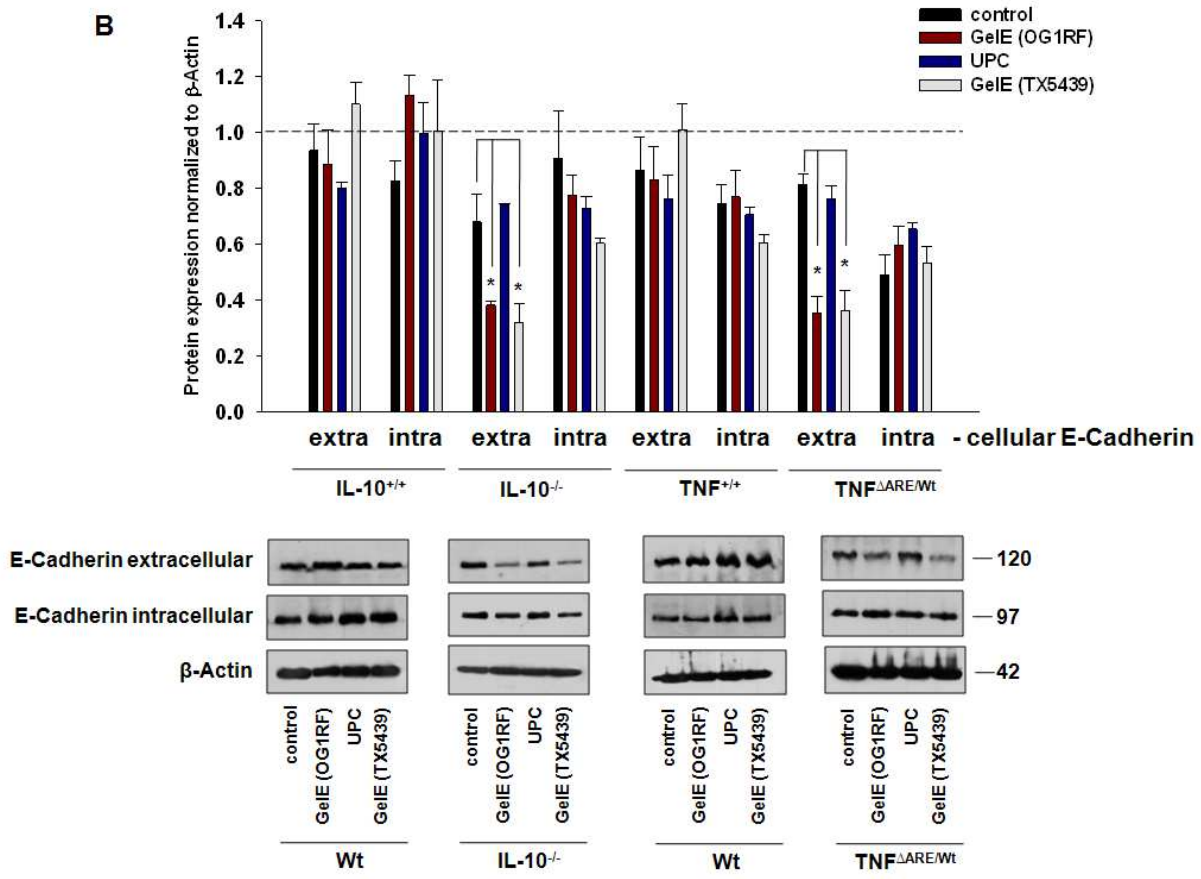


Figure 26: Impact of *E. faecalis* GelE on barrier function of IL-10$^{-/-}$ and TNF$^{\Delta ARE/Wt}$. Distal colon segments from 8 week old IL-10$^{-/-}$ and TNF$^{\Delta ARE/Wt}$ mice and their Wt counterparts were apically stimulated with purified GelE from *E. faecalis* OG1RF and TX5439 (10 µg/mL) for 5 h in Ussing chamber systems. The *E. faecalis* lipoprotein EF1362 was used as unrelated protein control (UPC). Results are expressed as % change of TER values compared to the initial value of the tissue (A). Data represent median with the 25th and 75th percentile from 5 animals per group, statistical significance was calculated by one way ANOVA (*p≤0,05). E-Cadherin protein expression was assessed by Western blot and densitometric analysis of 3 animals per group and treatment (B).

Another experimental colitis model that has been tested for GelE sensitivity were Rag2 deficient (Rag2$^{-/-}$) mice who lack B and T cells. After the transfer of colitogenic CD4^{+} T cells isolated from IL-10$^{-/-}$ mice, Rag2$^{-/-}$ mice developed colitis after 8 weeks. Besides, NOD2$^{-/-}$ mice who lack the intracellular pattern recognition receptor NOD2 and are susceptible for certain bacterial infections [208,209], have been studied as well for the susceptibility for the GelE-mediated disruption of epithelial barrier integrity. Appropriate Wt counterparts, 129SvEv and C57B6, were used as controls. Mucosal stimulation with *E. faecalis* GelE resulted in a significant loss of barrier function after 5 h in the distal colon segments from inflamed CD4^{+} T

cell transferred Rag2$^{-/-}$ mice and NOD2$^{-/-}$ mice (figure 27). In summary *E. faecalis* GelE impaired mucosal barrier function in all tested disease susceptibility models, but not in Wt counterparts, supporting the hypothesis that commensal-derived proteases remain harmless in the healthy host. Colon resections from carcinoma patients were used in Ussing chamber systems to assess the impact of *E. faecalis* GelE on human tissue. Despite GelE reduced barrier function in the human tissue, the results were not significant which might be a result of the heterogeneity of the tissue and the patients.

Beside the measurement of the TER, the translocation of sodium fluorescein was determined in the Ussing chamber experiments as marker for paracellular permeability and barrier function. All animals on 129SvEv background displayed increased translocation of sodium fluorescein after mucosal stimulation with GelE for 5 h. In contrast, only the disease susceptible mice on C57B6 background reacted with an enhanced paracellular permeability, suggesting that the genetic background sets the stage for certain susceptibility or the permeability marker is too small to demonstrate a specific effect (figure 28).

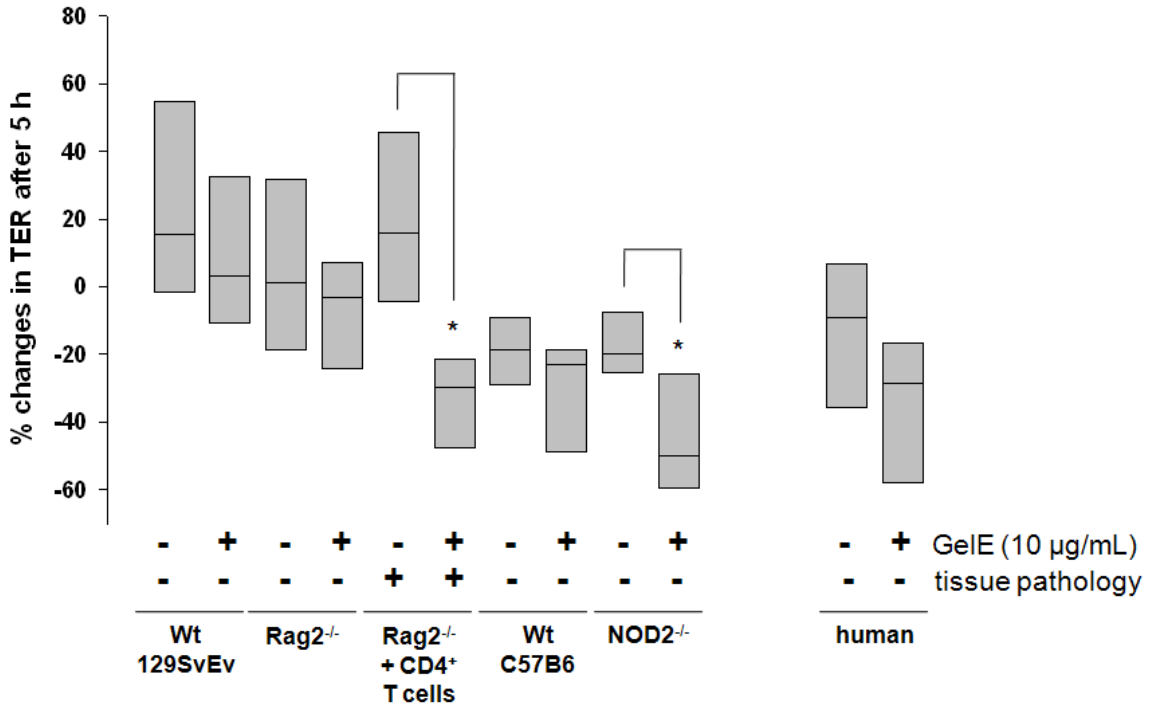


Figure 27: *E. faecalis* GelE impairs barrier function in the susceptible host. Further susceptibility models have been tested for the impact of *E. faecalis* GelE on colonic barrier function. $Rag2^{-/-}$ mice lack B and T cells and develop colitis after transfer of $CD4^{+}$ T cells. $NOD2^{-/-}$ mice lack the intracellular pattern recognition receptor NOD2 and are susceptible for certain bacterial infections. Appropriate wild type (Wt) counterparts 129SvEv and C57B6 were used as controls. Except for T cell transferred $Rag2^{-/-}$ mice, all other models have not developed histological changes with respect to inflammation in the colon after 8 weeks. Distal colon segments from mice and human tissue from colonic surgeries of carcinoma patients were apically stimulated with purified GelE from *E. faecalis* OG1RF (10 µg/mL) for 5 h in Ussing chamber systems. Results are expressed as % change of TER values compared to the initial value of the tissue. Data represent median with the 25th and 75th percentile from 5 animals per group, significant differences were indicated with*p≤0.05.

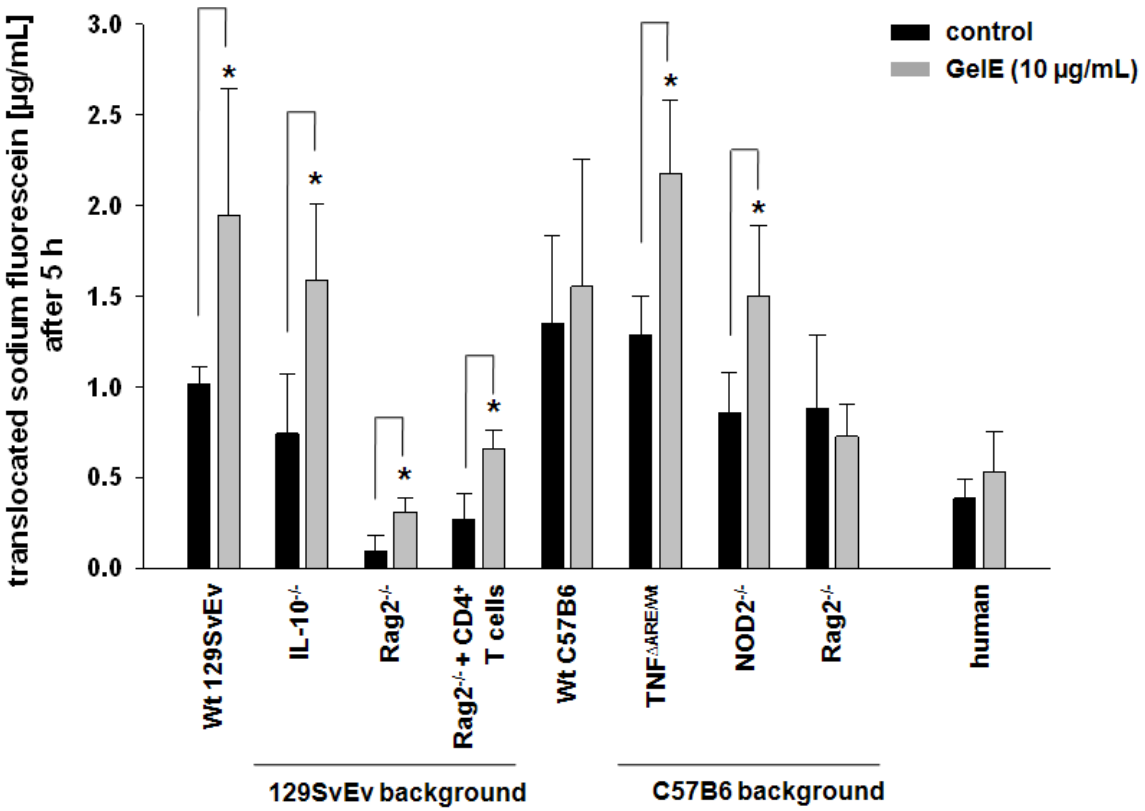


Figure 28: *E. faecalis* GelE increase translocation of sodium fluorescein across the mucosal barrier. The translocation of sodium fluorescein as permability marker was assessed in Ussing chamber experiments in order to investigate the functional epithelial barrier impairment after *E. faecalis* GelE stimulation. The translocated amount of sodium fluorescein was measured after 5 h apical stimulation with purified GelE (10 µg/mL).

4.6 Additive effects of GelE and EpaB on *E. faecalis* virulence

So far, the results have demonstrated the role of *E. faecalis* GelE in the development of chronic intestinal inflammation. GelE contributes to *E. faecalis*-mediated inflammation by impairing epithelial barrier function and thus facilitating the translocation of antigens or possibly of bacteria themselves. The experiments further demonstrated that GelE was not the initial trigger for inflammation and did not induce a pro-inflammatory T cell response. *E. faecalis* express a variety of different virulence-associated molecules which can be implicated to act as antigenic structure. The enterococcal polysaccharide antigen (epa) locus has been reported to play a role in bacterial virulence and is one important mediator for bacterial biofilm formation [165-168]. Especially the *epaB* gene which encodes for a putative glycosyl transferase was involved in virulence in several models for enterococcal-mediated infection. The next purpose of the present work was the generation and characterization of an *epaB* deletion and a *gelE-epaB* double deletion mutant which should be tested for their antigenic ability in order to unravel the colitogenic mechanisms of *E. faecalis*.

4.6.1 Generation of *epaB*, *gelE-epaB* and *fsrB-epaB* deletion mutants

In order to analyze possible additive effects of GelE and EpaB on *E. faecalis*-mediated intestinal inflammation, deletion mutants TX5692 (Δ*epaB*), TX5693 (Δ*gelE-epaB*) and TX5694 (Δ*fsrB-epaB*) were constructed by deletion of *epaB* gene from OG1RF, Δ*gelE* and Δ*fsrB* (figure 29A). Colony PCR using primers flanking the *epaB* region indicated deletion of *epaB* (figure 29B) which was further confirmed by DNA sequencing. Growth of Δ*epaB*, Δ*gelE-epaB* and Δ*fsrB-epaB* mutants was comparable to OG1RF, Δ*gelE* and Δ*fsrB* strains in BHI broth (figure 29C).

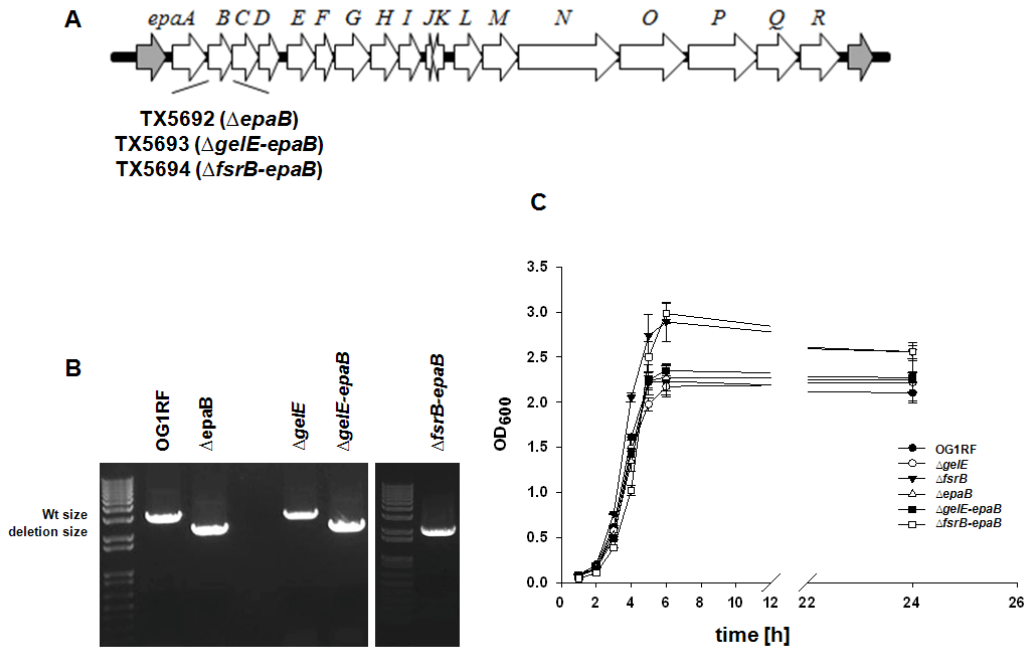


Figure 29: Generation and growth of *epaB* deletion mutants. Deletion mutants of *epaB* were generated in *E. faecalis* OG1RF, Δ*gelE* and Δ*fsrB* (A). Amplification of the *epaB* region by using outside primers confirmed the double cross-over and therefore the deletion of *epa* (B). Growth curves were calculated by measuring the OD at 600 nm over time in bacterial cultures which were aseptically inoculated with 1% from an overnight culture. Bacteria grew under aerobic conditions in BHI medium at 37℃. The data represent biological duplicates from one of three independent experiments (C).

The deletion of *epaB* could be further validated on transcriptional level by using qRT-PCR analysis with *epaB* specific primers. In contrast to OG1RF, Δ*gelE* and Δ*fsrB*, the *epaB* deletion mutants lack EpaB mRNA expression (figure 30A). Agarose gel electrophoresis finally confirmed the absence of epaB transcript (figure 30B).

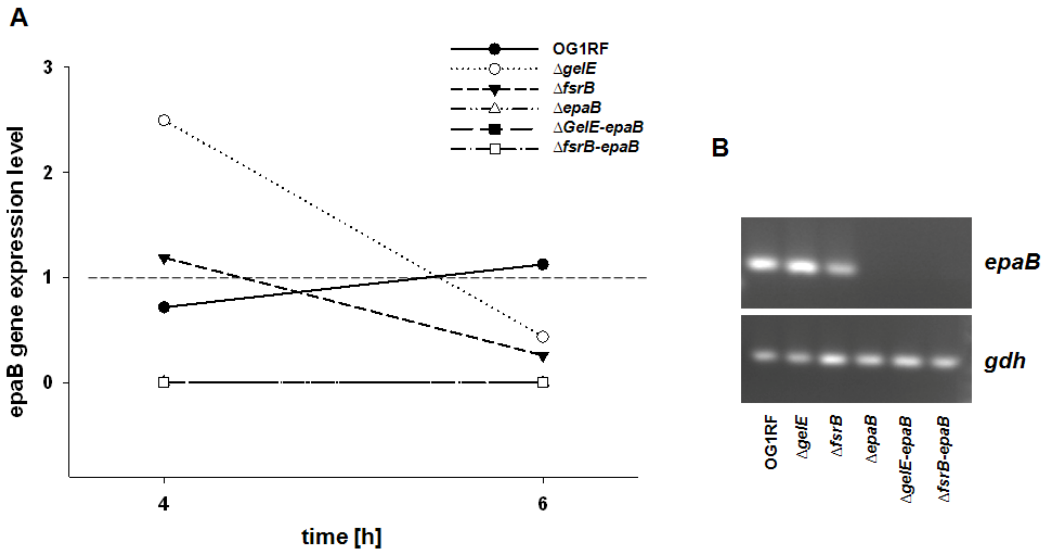


Figure 30: Confirmation of *epaB* deletion on transcript level. Bacterial epaB expression was assessed on transcript level after 4 (mid-log phase) and 6 h (stationary phase) of growth in BHI medium at 37℃ under aerobic conditions (A). Data were normalized to *gdh* as housekeeping gene and represent one out of 3 independent repetitions. Amplicons were validated by using agarose gel electrophoresis (B).

4.6.2 Characterization of Δ*epaB*, Δ*gelE-epaB* and Δ*fsrB-epaB* mutants

The majority of *epa* genes encode for putative glycosyl transferases, but their function, regulation or possible interactions during growth are not well understood. In order to investigate whether the deletion of *epaB* affects expression of other *epa* genes, qRT-PCR analysis was performed after 4 h (mid-log phase) and 6 h of growth (stationary phase). Expression of *epaC* and *epaD,* located downstream of *epaB* was not reduced by the *epaB* deletion (figure 31A and B). Furthermore, expression of the further downstream located genes *epaG* (putative gylcosyl transferase) and *epaN* (putative dTDP-glucose 4,6-dehydratase) was not diminished by *epaB* deletion (figure 31C and D).

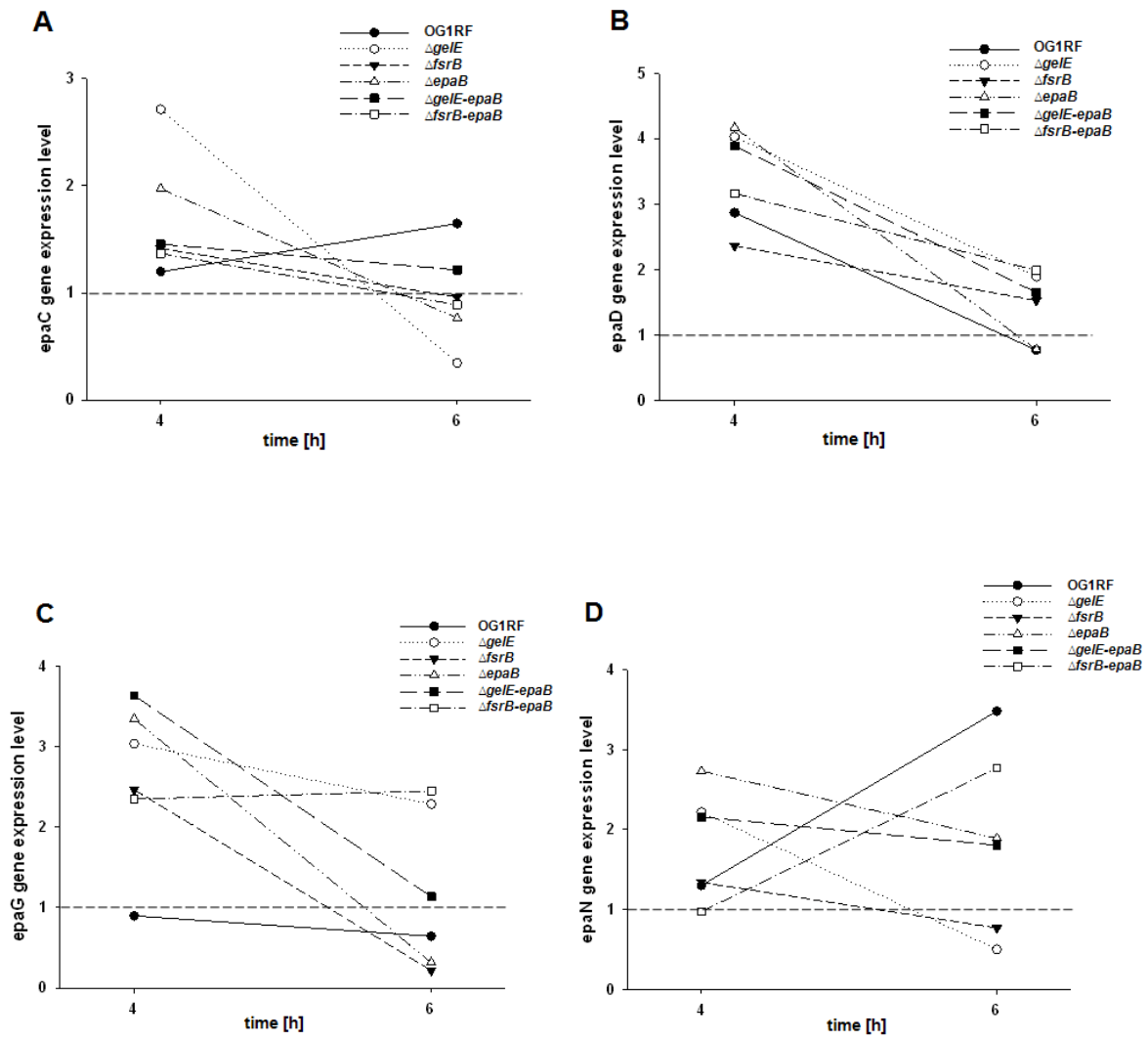
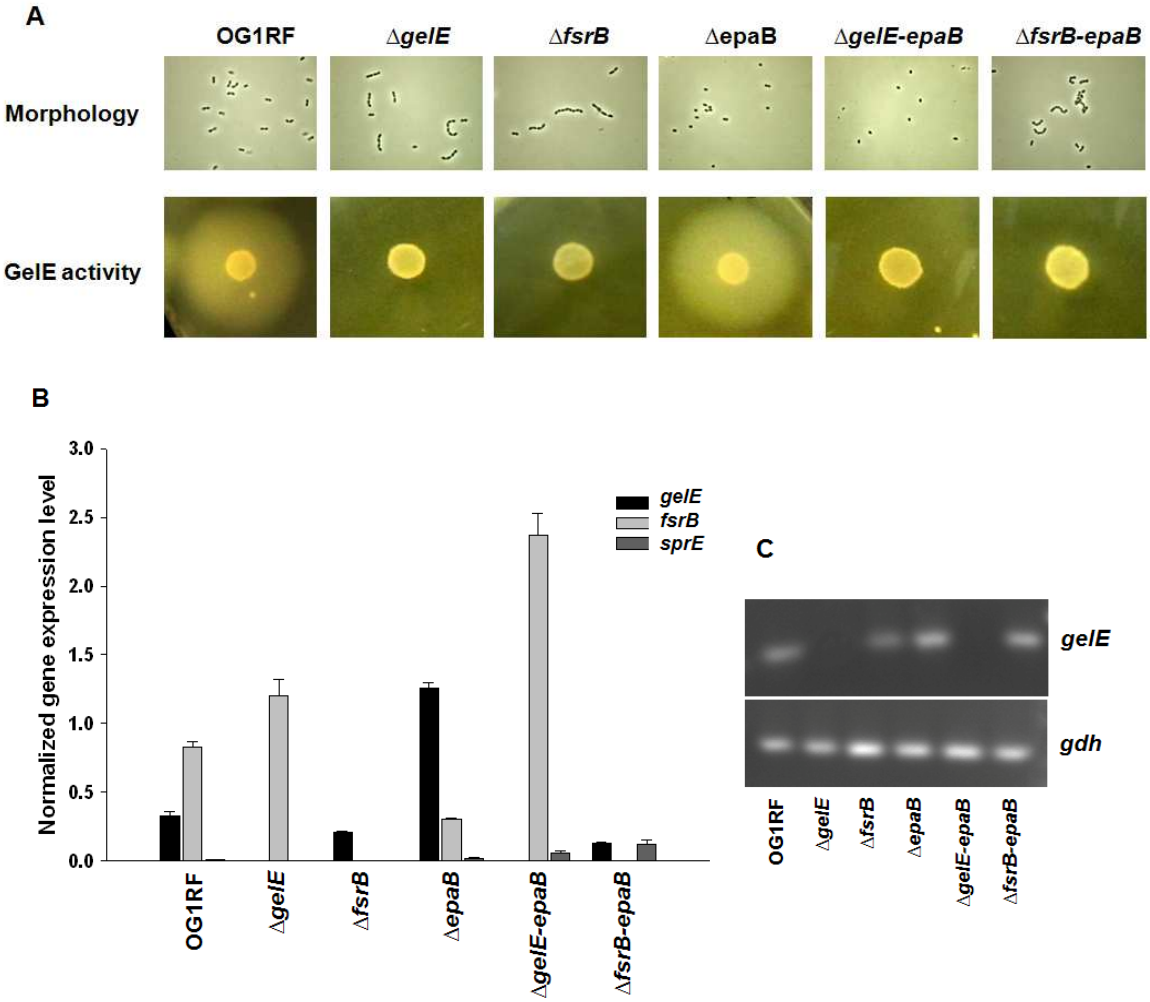


Figure 31: Effect of *epaB* deletion on transcription of downstream epa genes. mRNA expression of epaC. epaD, epaG and epaN was determined after 4 (mid-log phase) and 6 h (stationary phase) of growth in BHI medium at 37℃ under aerobic conditions (A-D). Data were normalized to GDH as housekeeping gene and represent one out of 2 independent repetitions.

Similar to the previously described *epaB* disruption mutant [168], Δ*epaB* and Δ*gelE-epaB* displayed an altered morphological phenotype compared to OG1RF as demonstrated in figure 32A. Both, Δ*epaB* and Δ*gelE-epaB* mutants, had a rounded shape and occurred mainly as single cells. In contrast, the phenotype of Δ*fsr* was maintained in the Δ*fsr-epaB* mutant. The previously described medium chain length phenotype of OG1RF and the longer chain length of Δ*gelE* [181] were abrogated by the deletion of *epaB,* suggesting a pivotal role of EpaB in synthesizing the Epa polysaccharide which is involved in generation and maintainence of appropriate cell wall dynamics. Although *gelE* expression was induced on

transcript level in Δ*fsrB* and the Δ*fsrB-epaB* mutants, there was no gelatinolytic activity visible on gelatine containing BHI agar (figure 32A-C). In contrast, the Azocasein assay revealed an enhanced proteolytic activity in the conc CM of Δ*fsrB-epaB* mutant which might originate from the increased expression of the *E. faecalis* serine protease (*sprE*) detected by qPCR analysis (figure 32B-D). In general the deletion of *epaB* affects membrane carbohydrate metabolism which might lead to the weakening of membrane stability and the secretion of numerous proteins (figure 32E).

In order to further verify equivalent function of GelE from the *epa* deletion mutants, polarized intestinal epithelial cells Ptk6 were apically exposed to conc CM which had been adjusted to the same amounts of GelE activity (1 Unit/mL). The conc CM of Δ*epaB* decreased barrier function of the cells comparable to the conc CM of OG1RF (figure 32F). Furthermore, a comparable functional disruption of epithelial barrier integrity was demonstrated by enhanced translocation of sodium fluorescein, which was used as permability marker (figure 32G). The *fsrB-epaB* deletion mutant did not impair barrier function of Ptk6 cells providing further evidence that the proteolytic activity result from the serine protease which has no impact on IEC barrier integrity.



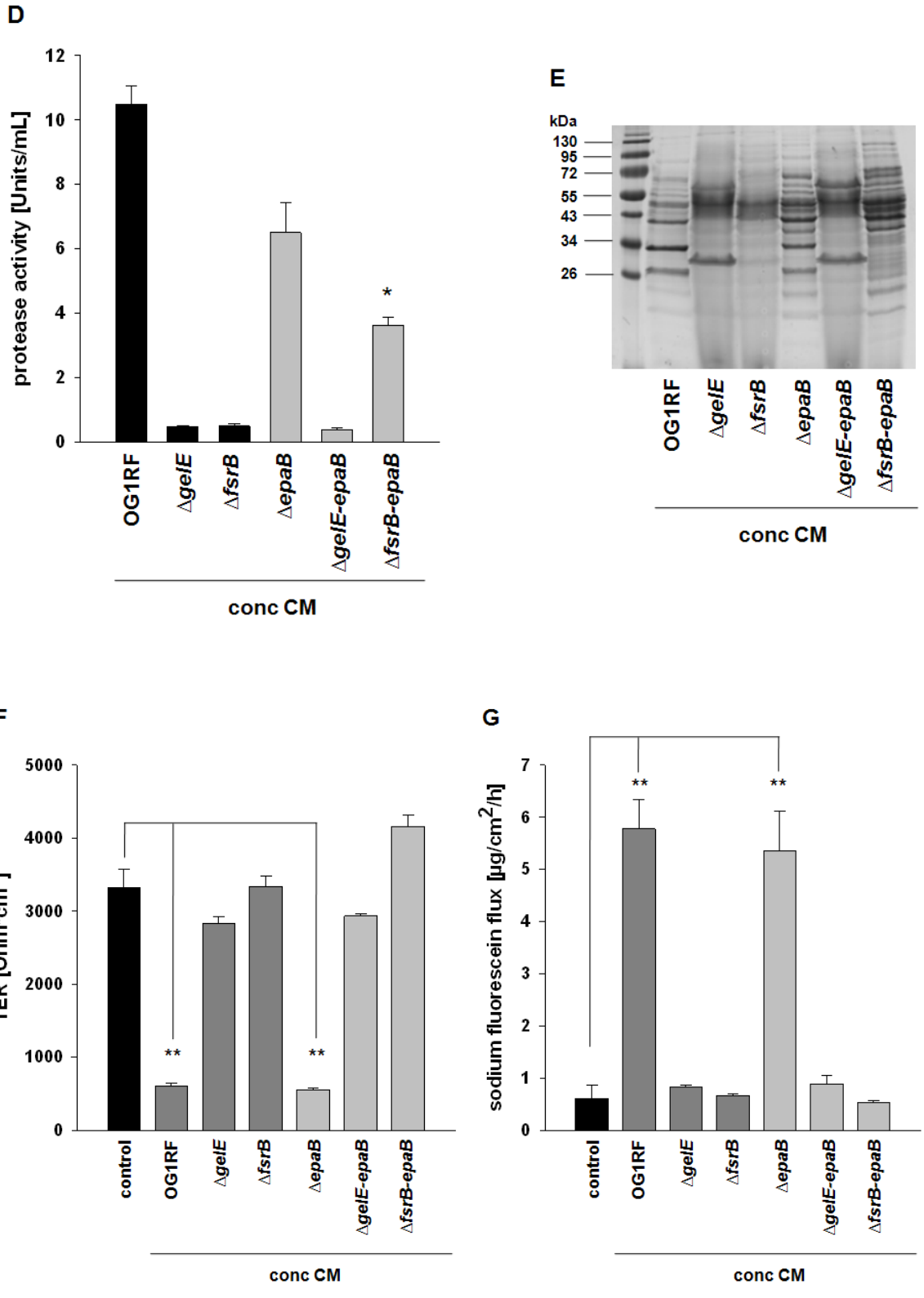
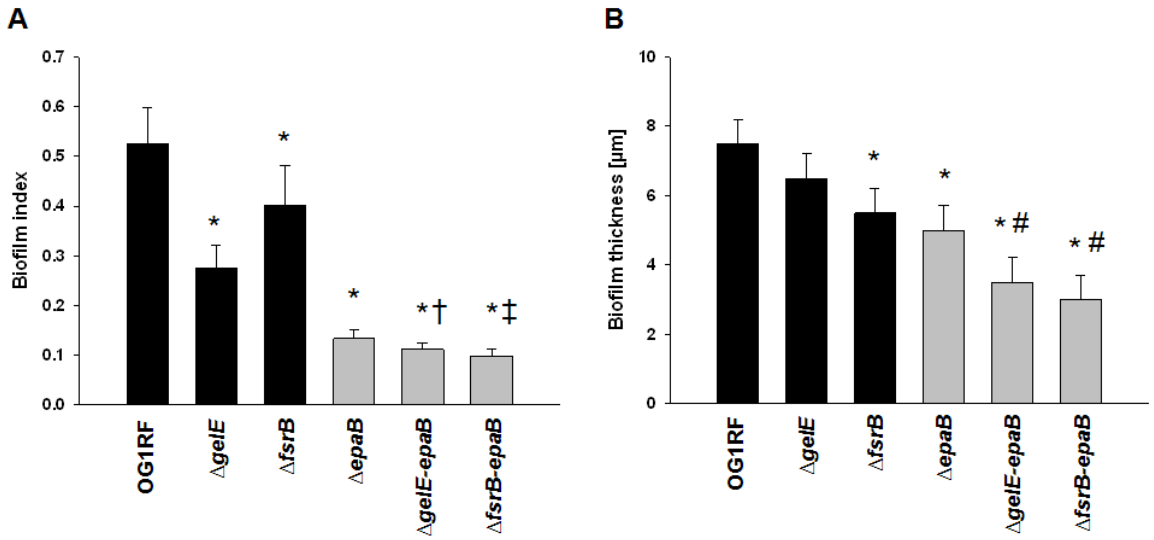

D
protease activity [Units/mL]
12
10
8
6
4
2
0
*
OG1RF
ΔgelE
ΔfsrB
ΔepaB
ΔgelE-epaB
ΔfsrB-epaB
conc CM
E
kDa
130
95
72
55
43
34
26
OG1RF
ΔgelE
ΔfsrB
ΔepaB
ΔgelE-epaB
ΔfsrB-epaB
conc CM
F
TER [Ohm*cm2]
5000
4000
3000
2000
1000
0
**
**
control
OG1RF
ΔgelE
ΔfsrB
ΔepaB
ΔgelE-epaB
ΔfsrB-epaB
conc CM
G
sodium fluorescein flux [µg/cm2/h]
7
6
5
4
3
2
1
0
**
**
control
OG1RF
ΔgelE
ΔfsrB
ΔepaB
ΔgelE-epaB
ΔfsrB-epaB
conc CM

Figure 32: Impact of *epaB* deletion on GelE expression and activity. Morphologically the *gelE-epaB* mutant displayed a comparable phenotype as the single *epaB* deletion; however the *fsrB-epaB* mutant was more comparable to the *fsrB* phenotype (A). Bacterial gelatinolytic activity was determined on gelatine containing BHI agar (B). GelE expression and activity was determined on transcriptional level (B and C) and by using an Azocasein assay with the concentrated CM (D). SDS-PAGE and Coomassie staining revealed that the conc CM of the *epaB* deletion mutants contain more proteins compared to Δ*gelE*, Δ*fsrB* and the Wt OG1RF (E). Equal proteolytic activity of conc CM was used for apical stimulation of Ptk6 cells for 24 h. TER (F) and translocation of sodium fluorescein (G) was evaluated to determine the effect of GelE on barrier function. Values represent triplicates from one of two independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: *p≤0.05, **p≤0.001.

4.6.3 Synergistic effects of EpaB and GelE in the context of enterococcal virulence

The purpose to generate the Δ*gelE-epaB* mutant was to unravel the colitogenic mechanisms of *E. faecalis* on the basis of structure-function analysis. Future studies will address this issue by monocolonization studies with IBD mouse models. The present study further characterized the *epaB* deletion mutants in terms of possible synergistic and/or additive effects of GelE and EpaB in enterococcal virulence.



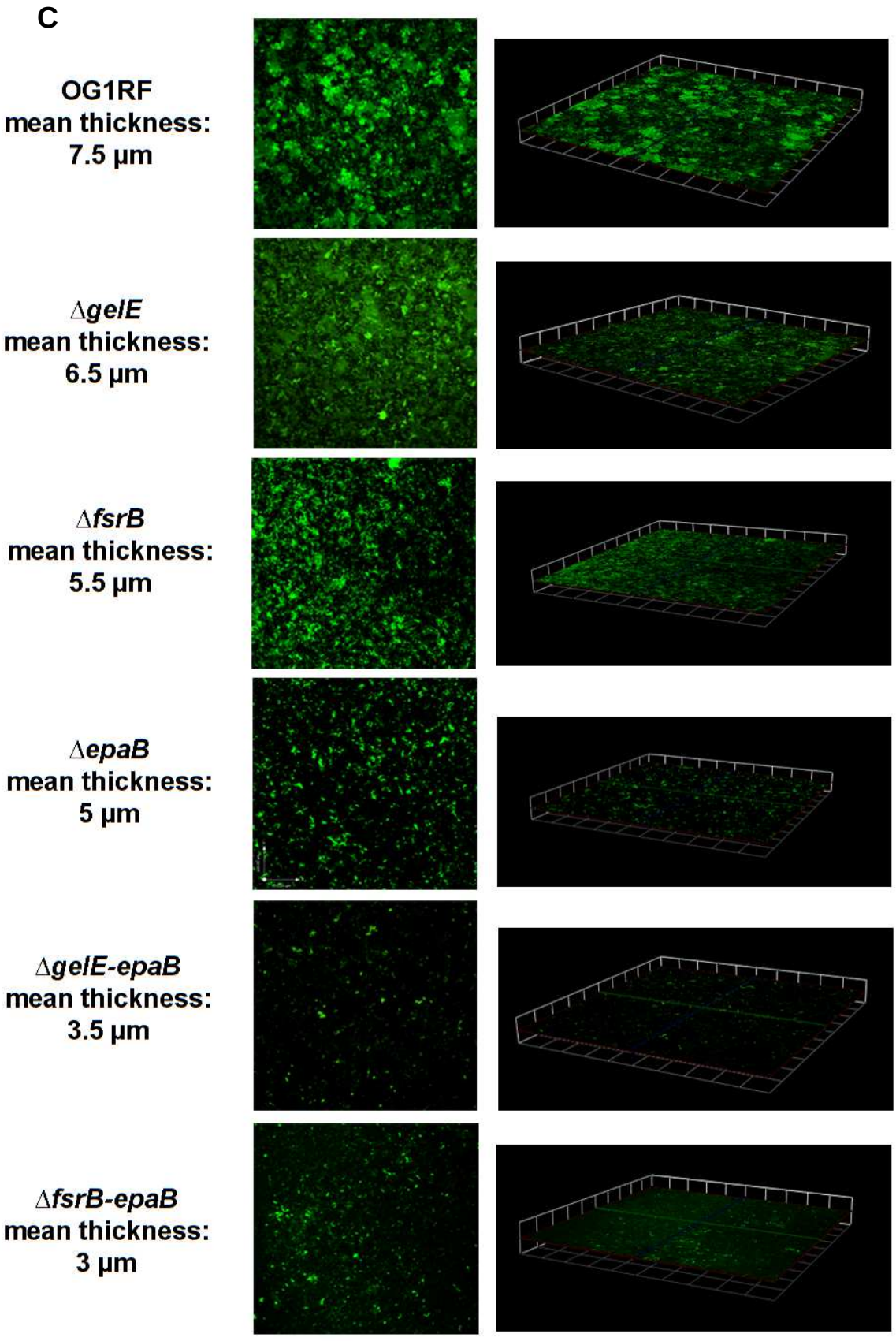

C
OG1RF
mean thickness:
7.5 µm
ΔgelE
mean thickness:
6.5 µm
ΔfsrB
mean thickness:
5.5 µm
ΔepaB
mean thickness:
5 µm
ΔgelE-epaB
mean thickness:
3.5 µm
ΔfsrB-epaB
mean thickness:
3 µm

Figure 33: Assessment of biofilm formation. Biofilm formation was assessed by crystal violet staining and normalized to growth in order to calculate the biofilm index (A). Staining of carbohydrate structures (Alexa Fluor 488) revealed constitution and thickness of biofilm formation. Data represent duplicates from two independent experiments. Statistically significant differences are calculated by one way ANOVA and marked as follows: * significant difference compared to OG1RF, † significant difference to Δ*gelE*, ‡ significant difference to Δ*fsrB* and # significant difference to the respective single mutant, Δ*gelE*, Δ*fsrB*, and Δ*epaB*.

Determination of the biofilm index revealed a significant reduction of biofilm formation for all tested mutants compared to OG1RF (*). However, additive effects of GelE and EpaB or FsrB and EpaB could not be detected. The double mutants displayed only a significant difference compared to Δ*gelE* (†) and Δ*fsrB* (‡) mutants, but not compared to *epaB* deletion (figure 33A). In contrast, additional evaluation of biofilm formation by confocal images of immunofluorescence stained extracellular glycoproteins indicated additive effects of GelE and EpaB as well as FsrB and EpaB on biofilm formation. This showed a significant reduction in biofilm thickness of the Δ*gelE-epaB* and ΔfsrB-epaB double mutants when compared to OG1RF and the respective single mutants Δ*epaB* (#), Δ*gelE* and Δ*fsrB* (figure 33B and C). Due to the fact that the phenotype of the Δ*fsrB-epaB* mutant needs further evaluation with respect to the non-distinctive expression and activity of GelE, additional experiments have been performed to elucidate attenuated virulence only of Δ*gelE-epaB* mutant.

Experiments using the human colon carcinoma cell line T84 demonstrated that the deletion of *gelE* and *epaB* significantly diminished translocation across polarized cell monolayers compared to OG1RF and the single mutants Δ*gelE* and Δ*epaB* (figure 34A). Additionally, immunofluorescence staining of *E. faecalis* and AJ protein E-Cadherin indicate that Δ*gelE-epaB* mutant was not able to access the surface of cells in comparison to the other strains. Of note, the expression of GelE seems to be important for bacterial internalization which can only be observed in OG1RF and Δ*epaB* infected cells (figure 34B). However, there are no convincing data in literature demonstrating *E. faecalis* internalization into epithelial cells. Furthermore, the paracellular route cannot be excluded for *E. faecalis* to overcome IEC barrier as translocation could also be demonstrated for the Δ*gelE* mutant. Additional experiments are needed to validate the observation of *E. faecalis* internalization into epithelial cells by a higher magnification and staining of additional IEC marker and the actin skeleton to resolve bacterial intracellular localization.

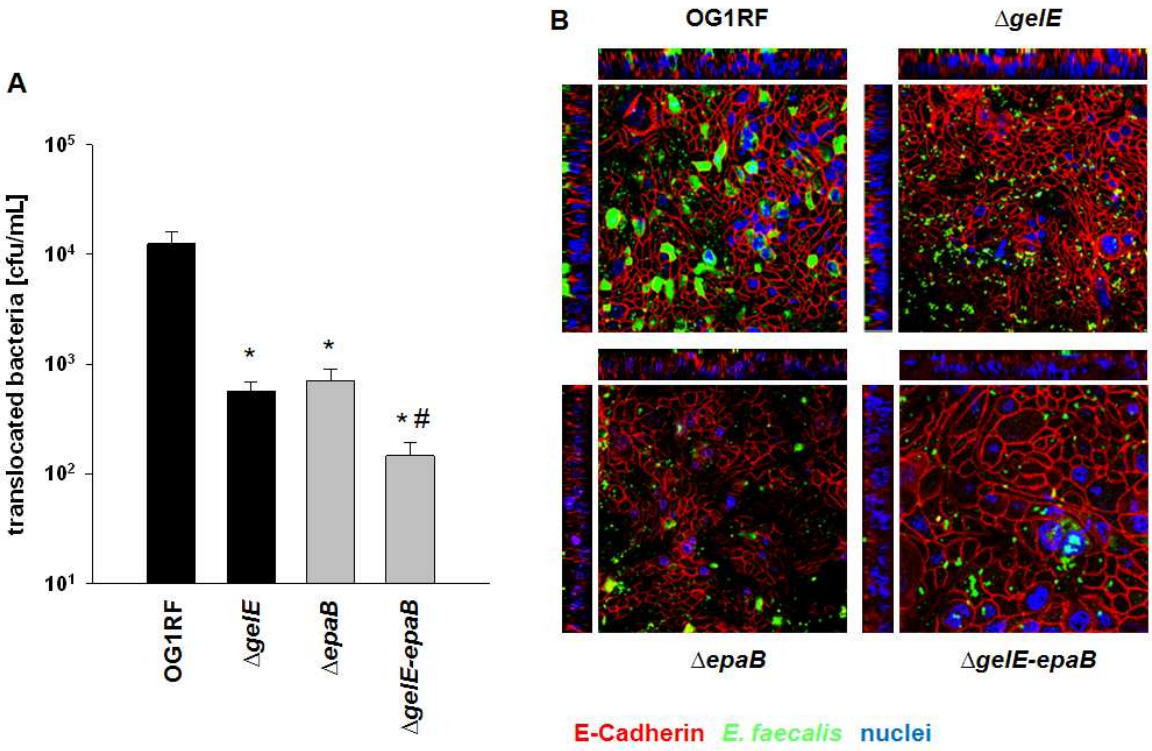


Figure 34: Deletion of GelE and EpaB attenuated translocation across polarized IEC. Polarized T84 cells were infected with mid-log cultures of *E. faecalis* ($8x10^7$ cfu/well). Translocated bacteria in the basolateral compartment were determined by plating on BHI agar 6 h post-infection (A). Statistically significant differences are calculated by one way ANOVA and marked as follows: * significant difference compared to OG1RF, # significant difference to Δ*gelE* and Δ*epaB* single mutants. Immunofluorescence staining of *E. faecalis* (Alexa Fluor 488) and E-Cadherin (Alexa Fluor 546) suggest a GelE-dependent intracellular localization of the bacteria. IEC surface adherence was attenuated in the Δ*gelE-epaB* mutant.

Further evidence for additive effects for GelE and EpaB was provided by *G. mellonella* and *C. elegans* models of enterococcal infection. Deletion of *epaB* attenuated insect mortality compared to OG1RF and Δ*gelE* mutant. Furthermore, the deletion of *gelE* and *epaB* almost completely attenuated enterococcal virulence in *G. mellonella*. Generally the differential outcome of insect mortality was significantly different between all strains (log rank test, p=0.0084) (figure 35). Enterococcal infection of *C. elegans* was accomplished by the oral route and nematode survival was monitored daily. Nematode mortality was significantly reduced when worms were fed with the Δ*gelE-epaB* mutant compared to each of the single mutants and compared to OG1RF (log rank test, p<0.0001) (figure 36).

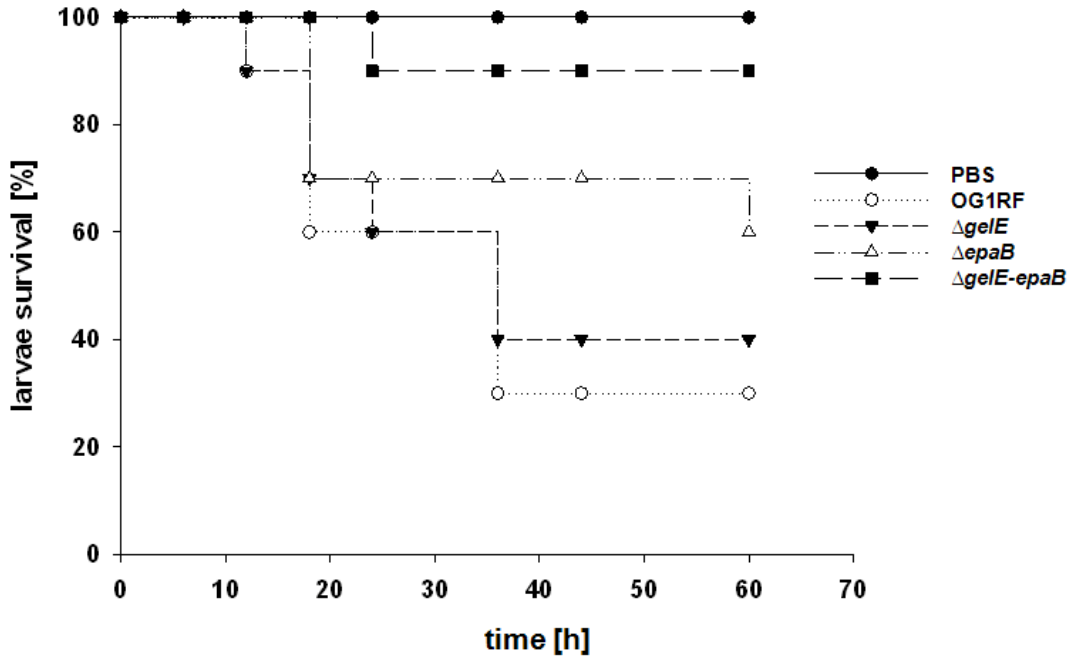


Figure 35: Survival of *G. mellonella* upon *E. faecalis* infection. Larvae survival was frequently monitored after injection of $2x10^6$ cfu *E. faecalis* per insect (N=10 larvae per treatment). The control group received 5 µL PBS per larva. The Kaplan-Meier plot is based on one representative out of three independent experiments and demonstrates that deletion of *gelE* and *epaB* significantly attenuated enterococcal virulence (p=0.0084).

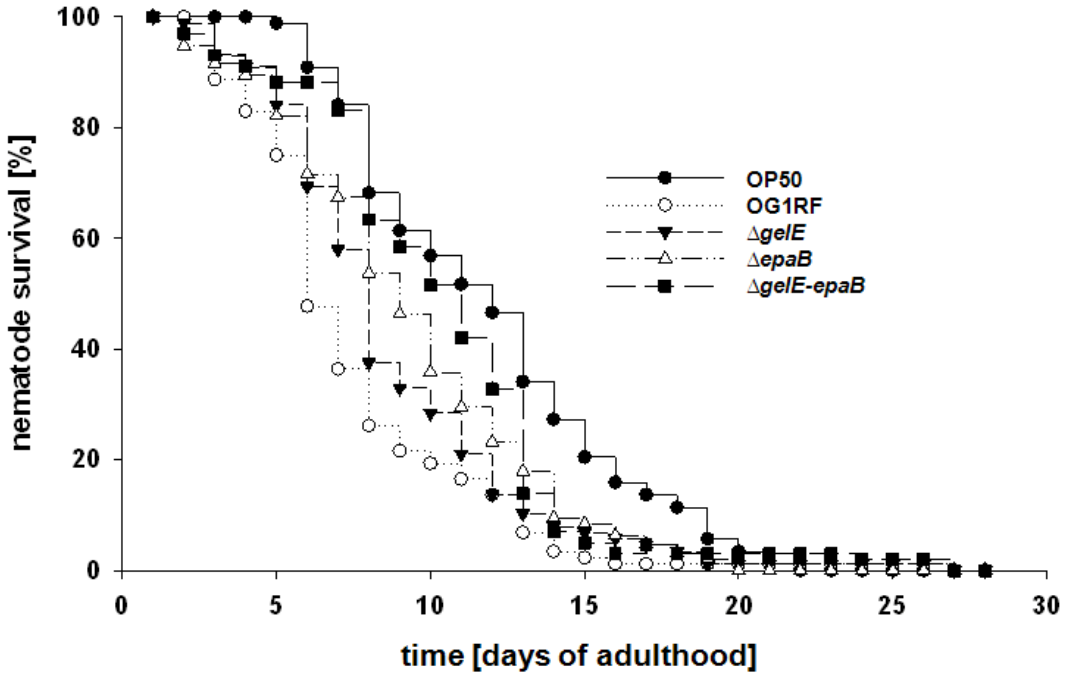


Figure 36: Survival of C. elegans upon *E. faecalis* infection. Nematodes were fed with E. faecalis strains and E. coli OP50 as control (N≥90 per group representing the sum of three triplicates from one experiment). Survival was monitored daily and plotted in a Kaplan-Meier graph. The survival curves were compared using a log rank test (p<0.0001).

5 Discussion

The present study demonstrates for the first time how a protease from commensal gut bacteria contributes to the development of chronic intestinal inflammation (figure 37). The zinc dependent metalloprotease GelE from *E. faecalis* disrupted intestinal epithelial barrier integrity through targeting the junction proteins Occludin and E-Cadherin. The presence of disease susceptibility, but not tissue inflammation, is required for *E. faecalis* GelE-mediated disruption of mucosal barrier function supporting the hypothesis that commensal bacteria-derived proteases remain harmless in the healthy host.

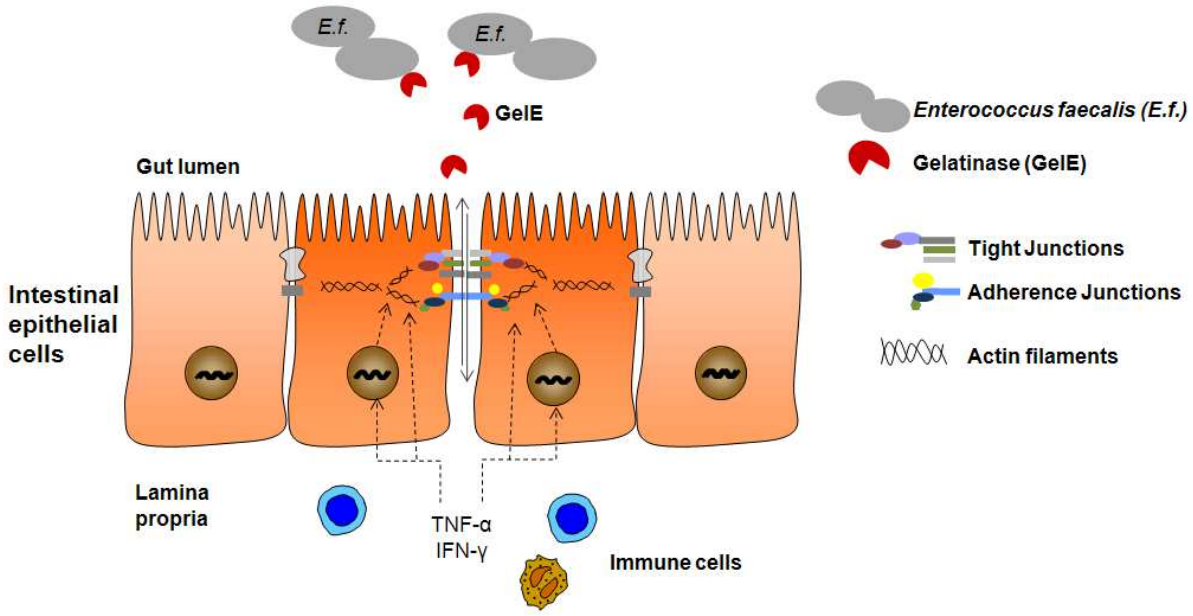


Figure 37: Proposed mechanism for the *E. faecalis* GelE-mediated loss of epithelial barrier function in the susceptible host. *E. faecalis* GelE gets access to IEC under disease susceptible conditions e.g. the presence of pro-inflammatory cytokines such as TNF and IFN-γ. The commensal-derived protease target junction proteins including Occludin and E-Cadherin in the pre-conditioned tissue and opens the paracellular pathway for bacterial or antigen translocation.

5.1 *E. faecalis* GelE impairs intestinal barrier function through degradation of junction proteins

The AJ protein E-Cadherin is involved in epithelial cell differentiation and provides barrier function between adjacent cells. It is targeted and processed by various host-derived proteases including stromelysin-1, matrilysin [210], ADAM-10 [211] and meprin-β [212]. One

of the most frequently described infectious mechanisms of pathogenic bacteria is the disruption of intestinal epithelial barrier function. The bacterial molecules are often proteases or toxins possessing a proteolytic function. Although E-Cadherin is expressed at the lateral side of epithelial cells it is frequently targeted directly or passively by pathogenic molecules and proteases. The direct cleavage of the E-Cadherin ectodomain has been demonstrated for the serine protease HtrA from *Helicobacter pylori* [81]. In consistence to the present study with *E. faecalis* GelE, the authors have used recombinant E-Cadherin and purified HtrA, as well as a HtrA inhibitor, to show the HtrA-mediated cleavage of E-Cadherin. However, it remains unresolved whether this direct cleavage is physiologically relevant *in vivo*. Other pathogenic structures, including the surface protein Internalin A from *Listeria monocytogenes* and the invasin Als3 from *Candida albicans,* use the extracellular domain of E-Cadherin as receptor for bacterial and fungal internalization [98,100,213]. In addition, the metalloprotease toxin BFT from *Bacteroides fragilis* was shown to induce the shedding of the E-Cadherin ectodomain through an unknown IEC receptor-mediated induction of γ-secretase, suggesting the possibility that indirect bacterial protease-mediated effects might be implicated in the loss of E-Cadherin [80]. The study conducted by Sumitomo *et al* reported that the bacteriocin streptolysin S from *Streptococcus pyogenes* acts in concert with the host cysteine protease calpain. The interplay of the proteases led to an impairment of epithelial barrier function by degradation of junction proteins Occludin and E-Cadherin and thus facilitated bacterial translocation across epithelial cells [214]. Although the E-Cadherin ectodomain was directly degraded by purified GelE, it remains to be answered whether this is the mechanism *in vivo* or whether GelE induces signalling pathways or other host-derived proteases which are in turn responsible for the loss of extracellular E-Cadherin.

The genetic susceptibility of the host was crucial for the *E. faecalis* GelE-mediated disruption of epithelial barrier function. In contrast to C57B6 Wt mice, Wt mice with 129SvEv background displayed an enhanced translocation of sodium fluorescein after mucosal GelE exposure (figure 28). This indicates that the 129SvEv background was more susceptible for the GelE treatment. Alongside, the genetic outbred of IL-$10^{-/-}$ mice dramatically influence severity of disease. Compared to C57B6, BALB/c and C3H/HeJBir backgrounds, IL-$10^{-/-}$/129SvEv mice develop most severe colitis [215,216]. The cytokine deficiency-induced colitis susceptibility locus (*cdcs1*) on chromosome 3 was identified to exacerbate chronic intestinal inflammation in combination with the experimental crossing of IL-10 deficiency in C3H/HeJBir mice [217,218]. The differences in disease susceptibility of IL-$10^{-/-}$ mice, depending on the genetic background, resembles the complexity of genetics in human IBD and addresses the need for individual causal research.

5.2 Comparison between endogenous gelatinases and *E. faecalis* GelE

E. faecalis GelE is a zinc dependent secreted metalloprotease and member of the MEROPS M4 family with thermolysin from *Bacillus thermoproteolyticus* as the representative protease [219]. The MEROPS database classifies proteases into families based on sequence homology [220]. Formerly termed coccolysin, *E. faecalis* GelE was named due to the ability to degrade gelatine similar to the host-derived gelatinases A (MMP-2) and B (MMP-9). MMP-2 and -9 share their main matrix substrates (type IV collagen, gelatine) and differ from other MMPs in terms of their structure [221]. However, the effects of endogenous gelatinases are rather diverse. While MMP-2 helps in maintaining barrier function in the intestine, MMP-9 expression and activity is increased in several animal models for colitis indicating that MMP-9 is responsible for tissue damage, one of the most serious consequences in IBD [222-224]. Beside the analogy in terms of synthesis as zymogens and the affinity for certain substrates, *E. faecalis* GelE and host gelatinases are not homologues and do not share much similarity as demonstrated in table 7. The major differences are based in the enzyme kinetics, stoichiometry and substrate specificities. Bacterial and endogenous gelatinases can be inhibited by Marimastat, a known broad spectrum MMP inhibitor which blocks the active site of the proteases. However the IC_{50} values differ dramatically between MMP-2 and -9 and for *E. faecalis* GelE ranging from nM to µM concentrations. This difference might be due to differences in substrate binding affinity which is much higher for the endogenous gelatinases. A much lower amount of endogenous MMPs is necessary to convert a certain amount of substrate compared to *E. faecalis* GelE. Zymography with gelatine as substrate revealed that much lower amounts of MMP-2 and -9 compared to *E. faecalis* GelE are necessary to detect gelatine degradation (figure 20A). These differences are physiologically relevant as excessive endogenous gelatinase activity would lead to severe tissue destruction and a tight regulation is pivotal for intestinal homeostasis. In contrast GelE is synthesized by the bacteria during their reproduction under the control of the fsr quorum sensing system, although there is some evidence for a fsr-independent GelE production [225]. GelE is secreted into the immediate bacterial environment in order to remove misfolded proteins on the bacterial surface and control bacterial dissemination through the restriction of coccal chain length [182]. More recently, it has been demonstrated that GelE contributes to cell death and extracellular DNA release through fratricide, the lysis of sibling cells mediated by isogenic cells within the same population of bacteria growing in biofilm [226]. In comparison to the endogenous gelatinases, GelE activity is controlled within the cells through the expression level and presumably through unknown mechanisms during protease release. So far, there are no extracellular control mechanisms or bacterial inhibitors described.

Table 7. Comparison between endogenous gelatinases and *E. faecalis* GelE

species	protease	molecular weight latent/active	synthesis	substrates	marimastat inhibition IC_{50}
endogenous	Gelatinase A (MMP-2) Gelatinase B (MMP-9)	72/66 kDa 92/86 kDa	zymogen	Non-fibrillar collagens, gelatine, fibronectin, elastin	MMP-2: 6 nM MMP-9: 3 nM
bacterial	*E. faecalis* gelatinase (GelE)	55.6/ 33-34.5 kDa	zymogen	casein, gelatine, collagen, fibrin, fibrinogen, hemoglobin, endothelin	GelE: 183.12 µM

5.3 Receptor activation by *E. faecalis* GelE

Activation of PARs

The most prominent receptors for endogenous and bacterial proteases are the G-protein coupled PARs, 4 of which have been described so far. PARs are expressed on a variety of different cell types including epithelial cells, immune cells and neurons and are involved in mechanisms of inflammation and pain. Therefore they and their activating proteases have been considered as targets for the development of therapeutic options in the treatment of inflammation and pain associated diseases [90]. It is known that proteases from pathogenic bacteria including the cysteine protease gingipain-R from *Porphyromonas gingivalis* [91,92], the metalloprotease *P. aeroginosa* elastase from *Pseudomonas aeroginosa* [93], the metalloprotease serralysin from *Serratia marcescens* [94] and proteases from host dust mites [227,228] activate PARs. The activation is mediated by the proteolytic cleavage of the tethered ligand that binds to the receptor and induces intracellular signalling. Released peptidases from the peridontitis pathogen *Treponema denticola* exert an inhibitory effect on proteolysis mediated PAR2 activation, indicating that some proteases are able to disarm and therefore silent PARs by proteolytic removal of the activation site [90,229]. Despite there is increasing evidence for a physiologically relevant PAR activation, mediated by bacterial proteases, the current knowledge about bacterial proteases relevant to the intestinal

environment is limited. Experiments with IEC failed to demonstrate *E. faecalis* GelE-mediated activation of PARs. However, purified GelE negatively influenced the ability of a selective PAR2-activating peptide to excite neurons suggesting that GelE may signal through PAR2 dependent pathways [75]. Although GelE partially activated guinea-pig enteric neurons directly, it could be speculated that GelE might disarm the receptor through stepwise proteolytic degradation of whole parts of the extracellular domain. As the cleavage sites of *E. faecalis* GelE seem to be non-specific before hydrophobic amino acids, it is more likely that GelE activates IEC receptor signalling through proteolytic cleavage of the receptors.

Wnt signalling

E. faecalis GelE-mediated induction of Wnt signalling could be demonstrated by using a Wnt specific reporter assay *in vitro* (figure 22A). Wnt signals are involved in stem cell differentiation, cell cycle control and adult tissue maintenance. Dysfunction of Wnt signalling has been associated with human degenerative diseases and cancer [230]. The so far for the intestine described canonical Wnt pathway involves the nuclear translocation of β-Catenin and the subsequent activation of transcription factor T cell factor [231,232]. Despite the fact that β-Catenin also acts as intracellular adapter protein to AJ protein E-Cadherin, the mechanisms in Wnt signalling are different. Nevertheless, β-Catenin somehow links epithelial cell polarity with Wnt signals [233]. The present study could not resolve the question of whether GelE acts as a Wnt signal and therefore disturbs epithelial cell differentiation. Immunofluorescence staining of β-Catenin revealed an intracellular localization in IL-10$^{-/-}$ mice monoassociated with the GelE expressing *E. faecalis* strain OG1RF (figure 22C), but it remains unclear whether this is a consequence of inflammation or a direct GelE-mediated effect. Enhanced cell proliferation and modulation of the β-Catenin subcellular localization could be demonstrated for the ADAM 10 mediated shedding of the E-Cadherin ectodomain [211] demonstrating a possible mechanism for a metalloprotease to be associated with disturbed β-Catenin signalling. The data of this study provide preliminary evidence that there might be direct receptor activation by *E. faecalis* GelE, but additional experiments with endogenous protease inhibitors or transgenic mouse models need to be performed in order to dissect the effect of the bacterial protease from that of the endogenous proteases.

5.4 Clinical implications for bacteria-derived proteases

The inhibition of host-derived proteases has been discussed as therapeutic option in IBD, but there are only limited reports with inconsistent results. Approaches in animal models

revealed that inhibition of cathepsins [19] and tryptase [234] ameliorates chemical induced colitis. Furthermore, the serine protease inhibitor camostat has been reported to induce and maintain remission in UC patients [235]. Various MMP inhibitors have been shown to improve inflammation particularly in rat models for IBD [236-238], but also in dextran sodiumsulphate (DSS)-induced colitis in mice. Despite the fact that MMP inhibitors exhibited beneficial therapeutic effects in experimental colitis models, a clinical trial investigating the beneficial potential of the MMP inhibitor prinomastat in lung cancer failed due to unacceptable adverse effects [239]. To date, studies that use inhibitors of host-derived proteases showed rather poor efficacy in the treatment of human IBD. Adverse side effects presumably resulting from non-specific actions and/or broad-spectrum inhibition of host proteases need to be addressed before protease inhibitors become a therapeutic option. Therefore, the usage of endogenous protease inhibitors could be considered as treatment option in IBD to avoid adverse side effects and to reconstitute the proteolytic balance in the gut. One example is elafin, a serine protease inhibitor, the expression of which is reduced in the gut mucosa of IBD patients [240]. Furthermore, it has been demonstrated that elafin protects against DSS colitis through the inhibition of pro-inflammatory mediators and the strengthening of epithelial barrier function [241]. The interaction between bacterial proteases and endogenous inhibitors and *vice versa* between bacterial protease inhibitors and endogenous proteases should be considered as important mechanisms in bacteria-host interactions. The current knowledge about such interactions is very limited. The work from Kantyka *et al.* described that *Staphylococcus aureus* cysteine proteases can be inhibited by epithelial-derived serpin Squamous Cell Carcinoma Antigen 1 [242]. Usually the group of serpins (serine protease inhibitors) inhibit endogenous cysteine proteases including papain, cathepsins or mast cell chymase and are involved in many physiological processes. Therefore the inhibition of bacterial proteases exhibits a novel mechanism how bacterial virulence can be attenuated by the host. Furthermore, it has been demonstrated that *Bifidobacterium longum* serpin inhibits human neutrophil elastase, an inflammation associated protease produced by neutrophils, suggesting that the bacterial contribution to intestinal proteolysis might be also beneficial for the host.

A few clinical trials have shown that the administration of probiotics, such as the probiotic mixture VSL#3 induce and maintain remission in UC patients [243,244]. Although probiotic efficacy could be demonstrated in IBD, the active functional components/structures, the molecular mechanisms or the primary probiotic target still remain to be clarified. It may be speculated that the protective role of probiotics may be related to the release of proteases and/or protease inhibitors. Such a mechanism has been recently reported for the serine protease Lactocepin from *Lactobacillus paracasei* which inhibited the recruitment of pro-

inflammatory cells to the side of inflammation through degradation of chemokines [245]. In general the inhibition of harmful and excessive proteolytic activity in the gut represents a strategy for the development of novel therapeutic options in the treatment of IBD and other inflammatory intestinal disorders.

5.5 Possible role for EpaB in *E. faecalis*-mediated chronic intestinal inflammation

The idea that *E. faecalis* GelE might be implicated in the development of chronic intestinal inflammation arose from the knowledge about the role of microbial metalloproteases for the virulence traits of pathogens and the fact that endogenous MMPs are involved in the pathogenesis of IBD. The presence of GelE was shown to potentiate the development of experimental colitis in *E. faecalis* monoassociated IL-10$^{-/-}$ mice, independent of antigen-specific activation of colitogenic CD4^{+} T cells (figure 12B). Previous studies demonstrated the colitogenic character of the well described *E. faecalis* strain OG1RF in IL-10$^{-/-}$ mice [133,135], but the structural characteristics of this commensal strain responsible for disease initiation remained unclear. *E. faecalis* strains are part of the human core gut microbiome [246] but are also regarded as nosocomial pathogens playing a role in several infectious disease such as endocarditis [163], bacteremia [247] and urinary tract infections [248]. The role of *E. faecalis* GelE seems to be restricted to its ability of epithelial barrier impairment and the proteolytic modulation of the immune response. Zeng *et al* already postulated that GelE is important for bacterial translocation across T84 cells [173]. The present work could unravel the mechanism by demonstrating that GelE impairs epithelial barrier function through the degradation of junction proteins. Of note, the GelE-mediated degradation of proteins of the complement system inhibits the opsonization of bacterial cells [172] and decreases neutrophil migration to the side of infection [249], describing defence mechanisms of how *E. faecalis* GelE contributes to bacterial escape from the immune system. Future studies considering other bacterial molecules as initial trigger for the development of chronic inflammation have to be performed in order to elucidate the colitogenic mechanisms of *E. faecalis*.

Enterococcal polysaccharide antigen (epa) was detected in the sera of patients with systemic enterococcal infections suggesting that cell wall polysaccharides have been recognized by the adaptive immune system [165,250,251]. Polysaccharides are involved in bacterial pathogenesis by mediating adherence and invasion of host cells [252,253], resistance to host defence mechanisms [254-256] or the induction of pro-inflammatory responses [257,258]. The *E. faecalis epa* gene cluster consists of 18 genes coding for enzymes and transporters

that are involved in cell wall polysaccharide metabolism. The disruption of *epaB* encoding for a putative glycosyl transferase, resulted in attenuated *E. faecalis* virulence [165-169]. Carbohydrate preparation from OG1RF and *epaB* disruption mutant revealed that EpaB is mediating the transfer of rhamnose, a 6-deoxy-hexose sugar, to cell wall polysaccharides [259]. Although various studies have addressed the impact of EpaB on enterococcal virulence, structure-function mechanisms have not been described yet. Especially the potential of enterococcal cell wall polysaccharides to serve as an antigen support the hypothesis that EpaB may contribute to chronic intestinal inflammation. In order to investigate the colitogenic or antigenic effect of EpaB *in vivo* it was necessary to generate deletion mutants. The characterization of the Δ*epaB* and Δ*gelE-epaB* mutants demonstrated that there are additive effects of the two factors in terms of biofilm formation (figure 33), bacterial translocation (figure 34) and enterococcal virulence (figure 35 and 36). However, it has to be considered that the deletion of *gelE* or *epaB* result in different bacterial morphology and decrease the ability to grow as biofilm compared to the Wt *E. faecalis* strain.

5.6 Conclusion and perspective

Enterococci live as harmless bacteria in a healthy environment, but can turn into infectious organisms under certain circumstances. The “commensal-to-pathogen switch” of *E. faecalis* has been demonstrated in an insect model of infection and sepsis [260]. The authors demonstrated that *E. faecalis*, also a commensal inhabitant of the lepidopteran model *Manduca sexta* itself, is only able to translocate and infect the hemocoel of the insect after *Bacillus thuringiensis* toxin-mediated disruption of the intestinal barrier function. Furthermore, *E. faecalis* injection resulted in a robust immune response and rapid death, suggesting that *E. faecalis* turns into a pathogen when it gets access to the host and its organism. This concept can be applied to the involvement of *E. faecalis* in IBD as well, where this commensal strain plays a role only in the susceptible host.

The present study demonstrates that *E. faecalis* metalloprotease GelE contributes to the development of chronic intestinal inflammation through the impairment of mucosal barrier function and the degradation of epithelial junction proteins. Although GelE facilitates antigen translocation, the protease itself was not the pro-inflammatory trigger recognized by the immune system. Thus, other *E. faecalis* structures will be investigated for their inflammatory potential in order to unravel the colitogenic mechanisms of *E. faecalis*. The approach, which has been already addressed in the present work, considers cell wall polysaccharides as they have been shown to be recognized by the adaptive immune system. Besides, bacterial lipoproteins can be recognized by the innate immune system [261]. In literature, it has been

reported that lipoproteins from group B streptococci and *Listeria monocytogenes* activate the pattern recognition receptor TLR2 and induce an inflammatory response [262,263]. Therefore *E. faecalis* lipoproteins can be regarded to play a role in IBD-associated enterococcal pathogenesis through specific receptor activation of TLR2. To dissect the impact of certain bacterial structures on the development of chronic intestinal inflammation, it is crucial to colonize germfree animals with isogenic *E. faecalis* mutants lacking the respective molecule or structure. The identification of inflammatory triggers and structure-function mechanisms of *E. faecalis* will help to understand the role of commensal gut bacteria in the complex pathogenesis of IBD and will possibly provide novel therapy options for patients.

6 APPENDIX

Table 8. Identification of cleavage sites in recombinant murine E-Cadherin for *E. faecalis* GelE-mediated degradation (100 ng GelE)

		Best Mascot	Position in Protein		Cleavage site	
Sequence	# Spectra	Ion Score	Start	Stop	Before peptide	After peptide
(R)HHHRDPASESNPEL(L)	3	52.56	128	141	R|H	L|L
(S)ITGQGADKPPVGVFIIER(E)	2	71.21	194	211	S|I	R|E
(S)ITGQGADKPPVGVF(I)	3	61.58	194	207	S|I	F|I
(Y)TIVSQDPELPHK(N)	2	52.96	305	316	Y|T	K|N
(T)IVSQDPELPHK(N)	1	66.96	306	316	T|I	K|N
(T)LVVQAADLQGEGLSTTAK(A)	3	116.72	345	362	T|L	K|A
(L)VVQAADLQGEGLSTTAK(A)	1	98.43	346	362	L|V	K|A
(A)ADLQGEGLSTTAK(A)	1	56.53	350	362	A|A	K|A
(T)LKVTDDDAPNTPAWK(A)	2	77.09	398	412	T|L	K|A
(F)VVVTDPTTNDGILKT(A)	1	79.97	426	440	F|V	T|A
(R)RVEVPEDFGVGQEITSYT(A)	2	90.95	494	511	R|R	T|A
(R)RVEVPEDFGVGQEITS(Y)	3	89.22	494	509	R|R	S|Y
(R)RVEVPEDFGVGQE(I)	4	85.14	494	506	R|R	E|I
(E)ITSYTAREPDTF(M)	1	50.1	507	518	E|I	F|M
(W)LEINPETGAIFTR(A)	1	69.4	535	547	W|L	R|A
(L)VLLDVNDNAPIPEPR(N)	1	51.31	586	600	L|V	R|N
(R)NPQPHIITILDPDLPPNTSPFTAE(I)	1	46.72	608	631	R|N	E|L
(K)LADNQNKDQVTT(L)	2	52.56	671	682	K|L	T|L

Table 9. Identification of cleavage sites in recombinant murine E-Cadherin for *E. faecalis* GelE-mediated degradation (250 ng GelE)

		Best Mascot	Position in Protein		Cleavage site	
Sequence	# Spectra	Ion Score	Start	Stop	Before peptide	After peptide
(R)HHHRDPASESNPEL(L)	1	46.63	128	141	R|H	L|L
(S)ITGQGADKPPVGVFIIER(E)	2	88.97	194	211	S|I	R|E
(T)LVVQAADLQGEGLSTTAK(A)	3	119.87	345	362	T|L	K|A
(L)VVQAADLQGEGLSTTAK(A)	1	61.71	346	362	L|V	K|A
(A)ADLQGEGLSTTAK(A)	1	65.34	350	362	A|A	K|A
(K)DINDNAPVFNPSTYQGQVPENEVNAR(I)	1	43.58	369	394	K|D	R|I
(T)LKVTDDDAPNTPAWK(A)	2	64.68	398	412	T|L	K|A
(D)PDQQFVVVTDPTTNDGILK(T)	1	75.68	421	439	D|P	K|T
(F)VVVTDPTTNDGILK(T)	1	42.95	426	439	F|V	K|T
(L)DFEAKQQYILHVR(V)	1	42.08	445	457	L|D	R|V
(R)RVEVPEDFGVGQEITS(Y)	2	81.28	494	509	R|R	S|Y
(R)RVEVPEDFGVGQE(I)	5	81.17	494	506	R|R	E|I
(R)RVEVPEDFGVGQEITSYT(A)	2	70.82	494	511	R|R	T|A
(W)LEINPETGAIFTR(A)	1	61.1	535	547	W|L	R|A
(E)INPETGAIFTR(A)	1	54.25	537	547	E|I	R|A
(L)VLLDVNDNAPIPEPR(N)	1	47.41	586	600	L|V	R|N
(L)LDVNDNAPIPEPR(N)	2	80.3	588	600	L|L	R|N
(K)LADNQNKDQVTT(L)	2	52.73	671	682	K|L	T|L

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ABBREVIATIONS

ADAM	a disintegrin and a metalloprotease
AIEC	adherent invasive *Escherichia coli*
AJ	adherence junction
APC	antigen presenting cells
APMA	p-aminophenylmercuric acetate
BHI	brain heart infusion
CD	Crohn's disease
cDNA	complementary deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
DNA	deoxyribonucleic acid
DSS	dextransodiumsulphate
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
C. elegans	*Caenorhabditis elegans*
conc CM	concentrated conditioned media
E. coli	*Escherichia coli*
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
EGFR	epidermal growth factor receptor
Epa	enterococcal polysaccharide antigen
FCS	fetal calf serum
Fsr	*E. faecalis* regulator
GAPDH	glyceraldehyd 3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GelE	Gelatinase from *E. faecalis*
GF	germ-free

G. mellonella	*Galleria mellonella*
H&E	hematoxylin & eosin
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
IFN-γ	interferon-γ
IL	interleukin
IL-$10^{-/-}$	interleukin-10 deficient
JAM-A	junction adhesion molecule A
KLH	keyhole limpet hemocyanin
LC	liquid chromatography
LPS	lipopolysaccharide
MAP	mitogen-activated protein
MLCK	myosin light chain kinase
MLN	mesenteric lymph node
MMP	matrix metalloprotease
mRNA	messenger ribonucleic acid
MS	mass spectrometry
MAP	*Mycobacterium avium* spp *paratuberculosis*
NF-κB	nuclear factor-κB
NOD	nucleotide binding oligomerization domain
OD	optical density
PAR	protease-activated receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonylfluoride
qRT-PCR	quantitative real-time polymerase chain reaction
RNA	ribonucleic acid
SDS-PAGE	sodium dodecylsulfate polyacrylamid gel electrophoresis
spp	subspecies

SprE	*E. faecalis* serine protease
TACE	tumor necrose factor-converting enzyme
TER	transepithelial electrical resistance
TGF	transforming growth factor
TIMP	tissue inhibitor of matrix metalloproteinase
TJ	tight junction
TLR	toll-like receptor
TNF	tumor necrose factor α
TSB	tryptic soy broth
UC	ulcerative colitis
UPC	unrelated protein control
Wt	wild type
ZO	zonula occludens

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ERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Doktorarbeit selbstständig angefertigt habe. Es wurden nur die in der Arbeit ausdrücklich benannten Quellen und Hilfsmittel benutzt. Wörtlich oder sinngemäß übernommenes Gedankengut habe ich als solches kenntlich gemacht.

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