TECHNISCHE UNIVERSITÄT MÜNCHEN

Lehrstuhl für Biofunktionalität der Lebensmittel

Bacterial strain specificity of *Enterococcus faecalis* interaction with intestinal epithelial cells in chronic intestinal inflammation

Micha Hoffmann

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzende:
Univ.-Prof. Dr. H. Daniel

Prüfer der Dissertation:
1. Univ.-Prof. Dr. D. Haller
2. Univ.-Prof. Dr. R. F. Vogel

Die Dissertation wurde am 21.09.2009 bei der Technischen Universität München eingereicht und durch die Fakultät für Ernährung, Landnutzung und Umwelt am 11.12.2009 angenommen.

Meinen Eltern

Acknowledgement

Zum Gelingen meiner Dissertation haben viele Leute beigetragen, bei denen ich mich an dieser Stelle ganz herzlich bedanken möchte:

Allen voran bei Prof. Dirk Haller für die immer offene Tür für Diskussionen, für die sehr gute wissenschaftliche Betreuung der Arbeit, für die exzellente Laborausstattung und finanzielle Unterstützung.

Bei Prof. Rudi Vogel, für die Übernahme des Koreferats, für die freundliche Zusammenarbeit und die Nutzung der Laborausstattung für die Virulenzgen-Bestimmung sowie die FPLC.

Bei Prof. Hannelore Daniel für die Übernahme des Vorsitzes in der Prüfungskommission, für die Nutzung der Laboreinrichtungen, vor allem des MALDI-TOF Massenspektrometers und für das entgegengebrachte Vertrauen.

Bei Prof. Balfour Sartor für das Ermöglichen der Arbeit mit den keimfreien Mäusen in North Carolina und für die lehrreichen Vorträge auf Konferenzen und am Institut.

Bei Prof. Gerald Tannock für das Ermöglichen der Studien mit den RLF Mäusen in Neuseeland und die gute Zusammenarbeit.

Bei meinen Freunden und Mitarbeitern Anna Shkoda, Pedro Ruiz, Eva Köstler, Eva Rath, Thomas Clavel, Anja Messlik, Tanja Werner, Gabi Hörmannsperger, Katharina Rank, Marie-Anne von Schillde, Natalie Steck, Emanuel Berger, Katharina Heller, Sigrid Kisling, Ingrid Schmöller, Susan Chang, Anna Zhenchuk, Pia Baur, Job Mapesa, Stefan Wagner, Silvia Pitariu, Nico Gebhardt, Benjamin Tiemann, Theresa Asen, Theresa Habermann, Sonja Böhm und Brita Sturm, sowie bei allen anderen Mitarbeitern am Institut für die Hilfe und Unterstützung bei Problemen aller Art und für die fröhlichen Kaffee-Pausen und gemeinsamen Abende.

Bei meinen Praktikantinnen und Master Studentinnen Gabi, Solveig, Eva, Natalie und Tina für Ihre Leistungen und wertvollen Beiträge zu dieser Arbeit.

Bei Carrie und Peter für die herzliche Zusammenarbeit, und die fröhlichen Abende zusammen.

Bei Georg Lutterschmid für die Einführung in die FPLC.

Bei den Mitarbeiter in Neuseeland - Jens, Diane, Karen, Marco, Magen, Chris, Clarissa und Salina – und in North Carolina – Sandy, Lisa, Thomas, Frank und James und bei alle anderen die mir halfen diese Auslandsreisen zu unvergesslichen Erlebnissen werden zu lassen. Ganz besonders möchte ich mich bei Frank & Christine, sowie Thomas bedanken für die Wochen in denen ich bei Euch wohnen durfte und mich zu Hause gefühlt habe.

Bei Lise Baller für Ihre treue Arbeit in der Spülküche.

Bei Gernot und Marlies Lysek für die angenehmen, guten und fröhlichen Jahre in der Heckenstaller Straße. und besonders für das Korrekturlesen.

Von Herzen danke ich ganz besonders meiner Familie und meiner Frau Jelena für Ihre Unterstützung in jeglicher Hinsicht, für die Kraft die sie mir gaben und für Ihre Liebe.

Publications:

1. Ruiz PA, **Hoffmann M**, Szcesny S, Blaut M, Haller D. Innate mechanisms for *Bifidobacterium lactis* to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats. ***Immunology*** 2005; **115**, 441.

2. **Hoffmann M***, Rath E*, Holzlwimmer G, Quintanilla-Martinez L, Loach D, Tannock G, Haller D. *Lactobacillus reuteri* 100-23 transiently activates intestinal epithelial cells of mice that have a complex microbiota during early stages of colonization. ***J Nutr*** 2008; **138**, 1684.

   *** shared first authorship**

3. **Hoffmann M**, Kim SC, Sartor RB, Haller D. *Enterococcus faecalis* Strains Differentially Regulate Alix/AIP1 Protein Expression and ERK1/2 Activation in Intestinal Epithelial Cells in the Context of Chronic Experimental Colitis. ***J Proteome Res*** 2009; **8**, 1183.

4. Hormannsperger G, Clavel T, **Hoffmann M**, Reiff C, Kelly D, Loh G, Blaut M, Holzlwimmer G, Laschinger M, Haller D. Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. ***PLoS ONE*** 2009; **4**, e4365.

Published Abstracts:

Hoffmann M*, Steck N*, Kim SC, Sartor RB, Haller D. *Enterococcus faecalis* metalloproteinase contributes to the development of chronic intestinal inflammation through impairment of epithelial barrier integrity, ***Int J Med Microbiol*** 2008; 45(298): p. 96

*** shared first authorship**

Poster Presentations:

1. **Hoffmann M**, Ruiz PA, Szcesny S, Blaut M, and Haller D. Innate mechanism for *Bifidobacterium lactis* to activate pro-inflammatory host responses in intestinal epithelial cells after the colonization of germfree rats with probiotic bacteria. Inflammatory Bowel Disease: Research Drives Clinics, Münster, Germany, 2-3. September 2005.

2. **Hoffmann M**, Ruiz PA, Szcesny S, Blaut M, and Haller D. Innate mechanism for *Bifidobacterium lactis* to activate pro-inflammatory host responses in intestinal epithelial cells after the colonization of germfree rats with probiotic bacteria. Innate immunity and its modulation in Inflammatory Bowel Disease, Stuttgart, Germany, 4-5. November 2005.

3. **Hoffmann M**, Kim SC, Sartor BR, and Haller D. Functional epithelial cell proteomics identified Alix/Alg2-Interacting Protein 1 as a target for *Enterococcus faecalis* strain-specific effects on chronic intestinal inflammation in IL-10 deficient mice. 5th meeting of the European Mucosal Immunology Group, Prague, Czech Republic, 5-7. October 2006.

4. **Hoffmann M**, Kim SC, Sartor BR, and Haller D. Functional epithelial cell proteomics identified Alix/Alg2-Interacting Protein 1 as a target for *Enterococcus faecalis* strain-specific effects on chronic intestinal inflammation in IL-10 deficient mice. Fourth International Symposium of the Austrian Proteomics Platform; Seefeld, Austria, 28-31. January 2007.

5. **Hoffmann M**, Kim SC, Sartor BR, and Haller D. Functional epithelial cell proteomics identified Alix/Alg2-Interacting Protein 1 as a target for *Enterococcus faecalis* strain-specific effects on chronic intestinal inflammation in IL-10 deficient mice. 44. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung, Halle-Wittenberg, 8-9. March 2007.

6. **Hoffmann M**, Hew CM, Kim SC, Vogel RF, Sartor RB and Haller D. *Enterococcus faecalis* metalloproteinase contributes to the development of chronic intestinal inflammation in IL-10 deficient mice. 13th International Congress of Mucosal Immunology, Tokyo, Japan, 9-12. July 2007.

7. **Hoffmann M**, Steck N, Hew CM, Kim SC, Vogel RF, Sartor RB and Haller D. *Enterococcus faecalis* metalloproteinase contributes to the development of chronic intestinal inflammation through impairment of epithelial barrier integrity. 45. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung, Bonn, Germany, 13-14. March 2008.

8. **Hoffmann M**, Steck N, Hew CM, Kim SC, Vogel RF, Sartor RB and Haller D. *Enterococcus faecalis* metalloproteinase contributes to the development of chronic intestinal inflammation through impairment of epithelial barrier integrity. 60. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Dresden, Germany, 21-24. September 2008.

Oral Presentation

Hoffmann M, Shkoda A, Kim SC, Sartor BR, and Haller D. Untersuchung stammspezifischer Effekte von *Enterococcus faecalis* auf intestinale Epithelzellen im IL-10-/- Mausmodell für chronisch entzündliche Darmerkrankungen. 43. Wissenschaftlicher Kongress der Deutschen Gesellschaft für Ernährung, Hohenheim, Germany, 9-10. March 2006.

Hoffmann M, Rath E, Holzlwimmer G, Quintanilla-Martinez L, Loach D, Tannock G, Haller D. *Lactobacillus reuteri* 100-23 transiently activates intestinal epithelial cells of mice that have a complex microbiota during early stages of colonization. 61. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie, Goettingen, Germany 20-23. September 2009.

Grants

Research fellowship of the German Academic Exchange Service (DAAD), Dunedin, New Zealand, April 2005

Travel Grant from Symbio Pharm GmbH for the 13th International Congress of Mucosal Immunology, Tokyo, Japan, 9-12. July, 2007.

Content:

List of Abbreviations

Alix	ALG-2-interacting protein X
AS	Aggregation Subastance
ATF	Activating transcription factor
APS	Ammoniumpersulfat
arg	Accessory gene regulator
CD	Crohn's disease
cDNA	Complementary DNA
CFU	Colony forming unit
CARD15	Caspase recruitment domain family, member 15
c-jun	Subunit of the heterodimeric transcription factor AP1
CpG	Cytosine-phosphate-guanosine
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Desoxyribonucleic acid
DSS	Dextran sulfate sodium
ECL	Enhanced chemiluminescence light
ER	Endoplasmatic reticulum
ERK	Extracellular signal-regulated kinase
ESP	Enterococcal Surface Protein
FBS	Fetal bovine serum
F	Frequency
fsr	*E. faecalis* regulator
GALT	Gut-associated lymphoid tissue
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GBAP	Gelatinase biosynthesis-activating pheromone
GF	Germfree
grp78	Glucose regulated protein 78
H&E	Hematoxylin and eosin
HCCA	Hydroxy-cyano-cynamic acid
IBD	Inflammatory bowel disease

IEC	Intestinal epithelial cell
IEF	Iso electric focusing
IFN	Interferon
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
IL-10-/-	IL-10 gene deficient
IP-10	Interferon-gamma-inducible protein-10
IPG	Immobilized pH gradient
JNK	c-Jun NH(2)-terminal kinase
LLR	Leucine-rich repeat
LPS	Lipopolysaccharide
LTA	Lipoteichioic acid
MALDI-TOF	Matrix assisted laser desorption ionisation time-of-flight
MAP kinase	Mitogen-activated protein kinase
MC	Morbus Crohn
MEF	Myoembryogenic fibroblasts
MHC	Major histocompatibility complex
MIP1	Macrophage inflammatory protein 1
MMP	Matrix metalloproteinase
moi	Multiplicity of infection
mRNA	Messenger RNA
MS	Mass spectrometry
MSCRAMM	Microbial Surface Components Recognising Adhesive Matrix Molecules
ms2-mice	*E. faecalis* ms2 14 weeks monoassociated IL-10-/- mice
NaCl	Sodium Chloride
NCBI	National Center for Biotechnology Information
NF	Nuclear factor
NOD2	Nucleotide-binding Oligomerization Domain containing 2
OG1-mice	*E. faecalis* OG1RF 14 weeks monoassociated IL-10-/- mice
PAMPs	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
PMN	polymorphonuclear leucocytes
PRR	patter recognition receptor

RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCID	severe combined immunodeficiency
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Short interfering RNA
TEMED	N,N,N',N'-Tetramethylethylendiamin
TFA	Triflouracetic acid
TIR	Toll-interleukin-1 receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
T_H	T helper cell
TOLLIP	Toll interacting protein
Tris	Tris-(hydroxymethyl)-aminomethan
UC	Ulcerative Colitis
UDP	Uridine diphosphate glucose
WT	Wild type

Summary

Background/Aim. *Enterococcus faecalis* is a commensal intestinal bacterium used as a probiotic and for production of Mediterranean cheese and meat products. However, *E. faecalis* emerged as a nosocomial pathogen playing a role in urinary tract infections, endocarditis and bacteremia. The use of animal models of inflammatory bowel disease (IBD) has shown that commensal intestinal microbes contribute to the initiation and perpetuation of inflammation. However, the role of *E. faecalis* strains in IBD is largely unknown. Therefore, we investigated the interaction between three different *E. faecalis* strains and intestinal epithelial cells (IEC) in the context of IBD, both *in vitro* and *in vivo*. The *E. faecalis* strains, selected on the basis of origin and virulence genes, were: the medical isolate *E. faecalis* strain OG1RF, the endogenous IL-10-/- mouse strain ms2 and the probiotic strain hs3.

Results. *In vitro*, all *E. faecalis* strains were capable of activating the transcription factors NFκB and c-jun and the MAP kinases p-38 and ERK1/2. Furthermore, all strains induced secretion of the cytokine IL-6 and the T-cell attracting chemokine IP-10 in similar amounts. *E. faecalis*-induced IL-6 secretion was TLR-2-dependent and mediated by the NFκB, the ERK1/2 and the p38 MAP kinase pathway, whereas IP-10 secretion was mediated by NFκB and p38. Except for differences in early activation of ERK1/2, no strain-specific differences were found *in vitro*, suggesting similar mechanisms in recognition and signal-transduction at the level of IEC.

Using gnotobiotic IL-10-/- mice, we found differences between the three *E. faecalis* strains. Histopathological analysis revealed moderate colonic inflammation after a 14-week-monoassociation with strain ms2 and severe inflammation with strain OG1RF. The severity of inflammation was associated with an up-regulated IP-10 gene expression in primary IEC. In wild type mice, all *E. faecalis* strains activated the MAP kinases p38 and ERK1/2. However, activation was more pronounced for the probiotic strain hs3. In IL-10-/- mice, p-38 activation was completely lost for all strains after 14 weeks whereas ERK1/2 activation was only lost in severe *E. faecalis* OG1RF-mediated colitis. These results highlight differential roles of *E. faecalis* strains

in the induction of experimental colitis and suggest protective functions for ERK1/2 and p-38.

We used 2D-gel electrophoresis and MALDI-TOF mass spectrometry to compare protein expression in primary IEC under moderate and severe *E. faecalis*-mediated inflammation. Alix, Ezrin and F-actin capping protein alpha-2 subunit were differentially regulated, suggesting changes in cytoskeleton assembly in severe colitis. Alix regulation was confirmed by Western Blot. Knock-down of Alix protein expression in IEC using siRNA did not impair ERK1/2 activation and IP-10 secretion *in vitro*. Therefore, Alix regulation has most likely no function on the observed regulation of ERK1/2 and IP-10 in primary IEC.

Finally, IL10-/- mice were monoassociated with isogenic mutants of *E. faecalis* OG1RF to investigate the role of *E. faecalis* virulence genes in experimental colitis. Mice colonized with a gelatinase-mutant were characterized by significantly lower histopathological scores. Moreover, the deletion of the *fsrB* gene, which is crucial for the expression of gelatinase together with a serine protease, led to significantly attenuated experimental colitis. Hence, *E. faecalis* gelatinase and the *fsr* quorum sensing system contribute to experimental colitis.

Conclusion. Our experiments clearly demonstrate *E. faecalis* strain-specific differences in the initiation of experimental colitis and identified *E. faecalis* gelatinase and the *fsr* quorum sensing system as contributing factors. Therefore, the use of specific protease inhibitors targeting commensal bacterial proteases might be a promising approach to treat IBD.

Zusammenfassung

Hintergrund/Ziel: *Enterococcus faecalis* ist ein kommensales Darmbakterium, das auch in mediterranen Käse- und Wurst-Produkten vorkommt und als Probiotikum verwendet wird. Andererseits ist *E. faecalis* auch ein Krankenhauskeim, der bei Harnwegsentzündungen, Bakterämie und Endokarditis eine Rolle spielt. Tiermodelle für chronisch entzündliche Darmerkrankungen (CED) haben gezeigt, dass kommensale Mikroorganismen zur Entstehung und Aufrechterhaltung von CED beitragen. Die Rolle von *E. faecalis* für CED ist weitgehend unbekannt. Daher haben wir die Wechselwirkung zwischen drei unterschiedlichen *E. faecalis* Stämmen und intestinalen Epithelzellen (IEC) im Kontext von CED untersucht. Für die *in vitro* und *in vivo* Experimente wurden folgende drei Stämme auf der Basis von Virulenzgen-Präsenz und Ursprung ausgewählt: Das medizinische Isolat *E. faecalis* OG1RF, das endogene IL-10-/- Maus-Isolat ms2 und der probiotische Stamm hs3.

Ergebnisse: *In vitro* aktivierten alle *E. faecalis* Stämme die MAP Kinasen p-38, und ERK1/2 sowie die Transkriptionsfaktoren NFκB und c-jun in IEC. Außerdem induzierten alle Stämme die Sekretion des Cytokins IL-6 und des T-Zell Chemokins IP-10 in ähnlichen Mengen. Die *E. faecalis* induzierte IL-6 Sekretion war TLR-2 abhängig und wurde durch die Signalwege NFκB, p38 und ERK1/2 vermittelt während die IP-10 Sekretion NFκB und p38 vermittelt war. Abgesehen von Unterschieden in der frühen Aktivierung des ERK1/2 Signalweges konnten keine stammspezifischen Unterschiede gefunden werden. Daher scheinen die Mechanismen für die Erkennung von verschiedenen *E. faecalis* Stämmen und für die Signalweiterleitung auf der Ebene von IEC ähnlich zu sein.
Wir konnten stammspezifische Effekte in Bezug auf intestinale Entzündung in monokolonisierten IL-10-/- Mäusen, ein Modell für chronische Colitis, nachweisen. Die histopathologische Untersuchung zeigte eine moderate Entzündung des Colons von *E. faecalis* ms2 monoassoziierten Mäusen nach 14 Wochen im Vergleich zu einer starken Entzündung für *E. faecalis* OG1RF. Im Einklang mit den Unterschieden in der Histopathologie war eine signifikant verstärkte Gen-Expression von IP-10 in *E. faecalis* OG1RF monokolonisierten Mäusen. Alle *E. faecalis* Stämme aktivierten die MAP Kinasen p38 und ERK1/2 in primären IEC. Die Aktivierung war jedoch stärker

ausgeprägt für den probiotischen Stamm hs3. In IL-10-/- Mäusen war nach 14 Wochen für alle Stämme keine p-38-Aktivierung mehr nachweisbar. Jedoch war die ERK1/2-Aktivierung noch präsent und nur in starker *E. faecalis* OG1RF vermittelter Colitis deutlich schwächer. Diese Ergebnisse lassen klare *E. faecalis*-stammspezifische Unterschiede in der Induktion von experimenteller Colitis erkennen und deuten protektive Funktionen der MAP Kinasen ERK1/2 und p38 an.

Wir haben 2D-Gelelektrophorese und MALDI-TOF Massenspektroskopie verwendet um die starke und die moderate *E. faecalis* induzierte Entzündung auf der Ebene von primären IEC zu vergleichen. Die Proteine Alix, Ezrin und F-actin capping protein wurden als differentiell reguliert identifiziert, was einen unterschiedlichen Cytoskelett Aufbau im Zustand von starker Entzündung andeutet. Die Regulation von Alix konnten wir mit Western Blot verifizieren. Das gezielte Ausschalten der Alix Protein Expression mit Hilfe von siRNA hatte keinen Einfluss auf die Aktivierung von ERK1/2 und auf die Sekretion von IP-10 in IEC *in vitro*. Daher hat die differentielle Alix Expression in primären IEC höchstwahrscheinlich keine Bedeutung für die beobachtete Regulation von ERK1/2 und IP-10.

Um die *E. faecalis* vermittelte Colitis auf der Ebene der Virulenzgene zu untersuchen haben wir IL-10-/- Mäuse mit isogenen Mutanten von *E. faecalis* OG1RF monokolonisiert. Eine Mutante für die *E. faecalis* Gelatinase zeigte signifikant weniger Entzündung im Vergleich zu *E. faecalis* OG1RF. Zudem führte die Deletion des *E. faecalis fsrB*-Gens, das für die Expression von Gelatinase und einer Serin-Protease notwendig ist, zu einer signifikant schwächeren Colitis. Daher tragen sowohl die Gelatinase als auch das *fsr* Quorum Sensing System von *E. faecalis* zur Colitis bei.

Schlussfolgerung: Eine differentielle Induktion von Colitis durch verschiedene *E. faecalis* Stämme sowie eine Auswirkung der *E. faecalis* Gelatinase auf intestinale Entzündung konnte somit klar gezeigt werden. Daher könnte der Einsatz von Inhibitoren gegen bakterielle Proteasen in der Behandlung von CED erfolgreich sein.

1. Introduction

1.1. Enterococci

Enterococci are commensal lactic acid bacteria in the mammalian and human intestinal tract[1]. Some strains are used as probiotics to enhance the host immune response[2]. Enterococci are also found in different mainly Mediterranean cheese and meet products in which they are important for flavour and ripening[3, 4]. They also serve as starter cultures.

On the other hand, strains of enterococci possess multiple antibiotic resistances and they are a leading cause of hospital-acquired infections. Glycopeptide resistance, especially vancomycin resistance, is a great concern, since these are the antibiotics of the last choice in serious nosocomial infections due to enterococci[5, 6]. In addition, enterococci, especially *Enterococcus faecalis,* play a role in several other infections, e.g. edocarditis[7], bacteremia[8], root canal[9] and urinary tract infections[10]. The interest in enterococci is rising since *E. faecalis* and *Enterococcus faecium* are frequently isolated from these infections, whereas the mechanisms of virulence are still unknown. By now it is almost impossible to discriminate potentially harmful strains from other commensal enterococci, a priori.

1.1.1. Reservoirs and characteristics of enterococci

Enterococci are ubiquitous organisms. They can be isolated from water, food, soil and plants[11, 12,13]. Enterococci can also be found as a natural part of the intestinal microbiota in most mammals and birds. Therefore, enterococci, especially the species *E. faecalis* and *E. faecium,* are often regarded as indicators for fecal contaminations of water. The discrimination of enterococcal strains from fecal origins and from plant origins is important for the control of water quality[12].

In 1938 Sherman described the “Enterococcal Group” of streptococci as bacteria that are able to grow at 10° C, 45° C and at up to pH 9.6, in 6.5% NaCl and that survive heating at 60° C for 30 min[14]. More than 45 years later, in 1984, the genus *Enterococcus* was transferred from the genus *Streptococcus* together with the two species *Enterococcus faecalis* and *Enterococcus faecium* by Schleifer and Kilpper-

Bälz[15]. In 2002 at least 17 species were added to this genus[1]. By now, according to the NCBI taxonomy browser (http://www.ncbi.nlm.nih.gov/sites/entrez), 42 different species are known.

Enterococci are gram positive, catalase negative, non spore forming, non motile, facultative anaerobic cocci. As already mentioned, enterococci can grow under unfavourable growth conditions. They occur singly, in pairs, or in short chains. Enterococci are also able to degrade a wide variety of different carbohydrates. At least 13 sugars are metabolized by all species and 31 additional sugars can be metabolized by at least one or more species[16,17]. The ability to utilize such a broad spectrum of sugars likely enhances the survival of enterococci in competitive environments with limited energy sources, such as the intestine. Interestingly, some *E. faecalis* strains express enzymes capable of degrading human intestinal mucins. Normally, human mucins are poly-O-acetylated or -sulfated at sialic acids on outer non reducing ends of the carbohydrate chain and therefore protected from bacterial degradation. *E. faecalis* can overcome this by expressing extracellular sialate O-acetylesterase and arylesterase[18].

In 2003, the genome of the vancomycin-resistant strain *Enterococcus faecalis* V583 was sequenced[19]. Its main chromosome is 3 218 031-bp-long with 3182 open-reading frames. The three plasmids were found to be 66 320-bp, 57 660-bp and 17 963-bp-long with 72, 64, and 19 open-reading frames respectively. The main chromosome has a G+C content of 37.5% and the three plasmids around 34%. One of the main findings of this genome project was that more than 25% of the genome consists of probably mobile or foreign DNA.

1.1.2. Enterococci in food

As already mentioned, enterococci belong to the commensal intestinal microbiota of mammals and birds. The species *E. faecium, E. faecalis, E. durans* and *E. hirae* are the most often encountered species in the intestine of farm animals like calves, cows, pigs and horses[20]. Therefore, they often contaminate milk- and meat-products during processing. Especially traditionally produced fermented foods made from meat or milk contain enterococci e.g. the Spanish cebreiro cheese[3]. The numbers of enterococci in Mediterranean-type fully ripened cheeses range from 10^5 to 10^7 CFU/g[21]. Since enterococci belong to the “traditional” microbiota of fermented milk products and contribute to ripening and flavour of these products, food scientists use enterococci as starter cultures to accelerate maturation and to improve the flavour of

cheeses and other fermented milk products [22,11]. Furthermore, different capabilities of enterococci, like proteolytic activity or antibiotic production, e.g. the cyclic bacteriocin AS-48, were investigated with the aim to enhance texture and taste or to prevent growth of unwanted bacteria like listeria or bacilli in foods[23,24].

1.1.3. Enterococci as a part of the intestinal microbiota

The intestine harbours a complex microbial ecosystem with a wide diversity of organisms and a high population density. Bacterial cells in the intestine outnumber mammalian host cells by a factor of approximately ten[25]. According to recent studies using molecular techniques based on 16s ribosomal RNA, the intestine contains about 1800 genera, between 15 000 and 36 000 different bacterial species[26] and about 10^{10}-10^{12} bacteria/g intestinal content[27]. Anaerobes outnumber aerobes and facultative species by a factor of about 100 [28]. Although the intestinal microbiota has been studied in great detail, microscopic counts on human feces suggest that only 40% of these bacteria can be cultivated with state-of-the-art bacteriological methodologies on non-selective agar medium [29]. The species composition between different individuals changes. Figure 1 shows a current view of the human intestinal microbial composition.

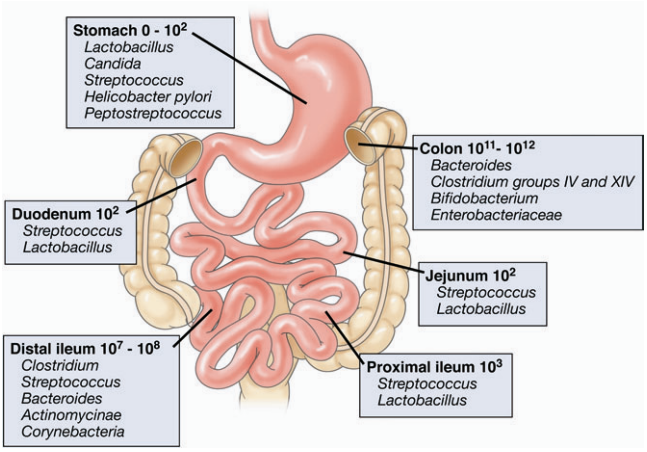


Figure 1: Human intestinal microbial composition (from Sartor 2008[30]).

Also enterococci belong to the „normal“, commensal inhabitants of this complex human intestinal microbiota. However, it has been shown by molecular analysis that the percentage of enterococci in the intestinal microbiota of an adult is not higher than 1%[31] – probably by far below. Therefore, enterococci can be regarded as a minority of the commensal intestinal bacteria. The species *E. faecalis* and *E. faecium*

appear to be detected most often in human feces[32, 33] and *E. faecalis* is considered to be the most common *Enterococcus* species in human feces[12] occurring between 3.5 and 11.5 (log10 CFU/g dry weight) (mean 7.7)[32]. Among the remaining species *Enterococcus durans*, *Enterococcus avium* and *Enterococcus hirae* were reported to be present in human feces[32] and *Enterococcus gallinarum* and *Enterococcus casseliflavus* in the feces of animals[34].

Enterococci are also present in the intestine of infants early after birth. Interestingly, enterococci are less frequent in breast fed infants compared to formula fed infants during the first weeks of life[35].

1.1.4. Enterococci as probiotics

Probiotic bacteria are defined as "*Live microorganisms which when administered in adequate amounts confer a health benefit on the host"* [36]. Most probiotic bacteria belong to the genera *Lactobacillus* or *Bifidobacterium*. However, some enterococci are also used as probiotics. For example, the strain *E. faecium* SF68 (bioflorin) has been successfully used to treat enteritis by decreasing the time for normalisation of patients stool and the duration of diarrhoeal symptoms[37]. Recently it was also shown that *E. faecium* SF68 enhances the immune response to *Giardia intestinalis* in mice[38]. Furthermore, the Causido® culture is a probiotic mixture that consists of two strains of *S. thermophilus* and one strain of *E. faecium*[39]. The hypo-cholesterolaemic effect of this mixture is controverse[40]. Additionally, Symbioflor® is a probiotic preparation sold in pharmacies which contains *E. faecalis*. It was shown to reduce the titres of circulating IgG directed against *E. faecalis* in a group of ten healthy volunteers after the oral intake of 10^7 viable cells for 3 weeks[41]. Finally, enterococci are also widely used as veterinary feed supplements. Since February 2004, 10 preparations containing 9 different strains of *E. faecium* are authorized as additives in the European Union[42].

Since some enterococci, especially *E. faecium,* have emerged as highly antibiotic resistant pathogens, and because of the possibility that the strains transfer antibiotic resistance and virulence genes the use of enterococci as probiotics has raised concerns. Therefore, additional studies investigating the probiotic and pathogenic mechanisms of enterococci are necessary.

1.1.5. Enterococci and potential virulence factors

E. faecium and *E. faecalis* are regarded as opportunistic pathogens and usually cause infections in patients that have severe underlying diseases or that are immunocompromised[43]. *E. faecalis* plays a role in several infections e.g. edocarditis[7], bacteremia[8], root canal[9] and urinary tract infections[10]. Normally, the pathogenesis of bacterial infections follows a sequence of events: colonisation, adhesion to host tissues, invasion of the tissue and resistance to host defence mechanisms. The search for mechanisms in enterococci to fulfil these steps led to a list of genes with potential relation to the virulence of enterococci. Some of these genes and their functions are described below. Since *E. faecalis* is the best investigated and described enterococcal species in the literature, most virulence traits are only described for *E. faecalis*.

Gelatinase and serine protease

Already in 1969, Somkuti and Babel purified an *E. faecalis* proteinase which was able to hydrolytically degrade casein[44]. Later Jensen et al. observed that Cheddar cheese manufactured with *E. faecalis* exhibited more protein breakdown than did cheese manufactured with *E. durans* or control cheese. The finding that *E. faecalis* strains are proteolytically more active than *E. durans* strains was confirmed by a different group[45] . In 1989 Mäkinen et al. purified and extensively characterized an *E. faecalis* metalloproteinase which was termed gelatinase because of its ability to digest gelatine. According to their study, *E. faecalis* gelatinase is a 31.5 kDa strongly hydrophobic metalloproteinase, with an isoelectric point of 4.6 and a broad pH optimum from 6 to 8. Its substrate specificity was found to be similar to that of the mammalian membrane endopeptidase-24.11 and to that of *Streptococcus thermophilus* thermolysin (EC 3.4.24.4)[46]. By now, gelatinase has already been shown to play a pathological role in 3 different animal models: A *Caenorhabditis elegans* life span model[47], a mouse peritonitis model[48] and a rabbit endophthalmitis model[49]. The protein expression of gelatinase is regulated by the *fsr* quorum sensing system which is described in the following section.

The fsr quorum sensing system

The *fsr* (for *E. faecalis* regulator) quorum sensing system senses high cell densities of *E. faecalis* due to high concentrations of an autoinducer, the cyclic peptide

gelatinase biosynthesis-activating pheromone (GBAP)[50]. In turn it regulates expression of different genes, especially expression of two proteases: gelatinase and serine protease.

Qin et al. first described the *fsr* locus composed of three genes, *fsrA, fsrB* and *fsrC*, which have high similarity to the *Staphylococcus aureus arg* quorum sensing system, followed by the two genes *gelE* and *sprE* which encode the two proteases[48].

The *fsrC* gene encodes a membrane associated histidin kinase (Figure 2). This kinase phosphorylates the response regulator (FsrA) (a transcription factor) in case that extracellular GBAP reaches a threshold level which is around 1 nM. Upon activation of FsrA the *fsr* system regulates gene and expression of gelatinase and serine protease. Recent studies suggest, that the *fsr* system regulates by far more than these two genes[51].

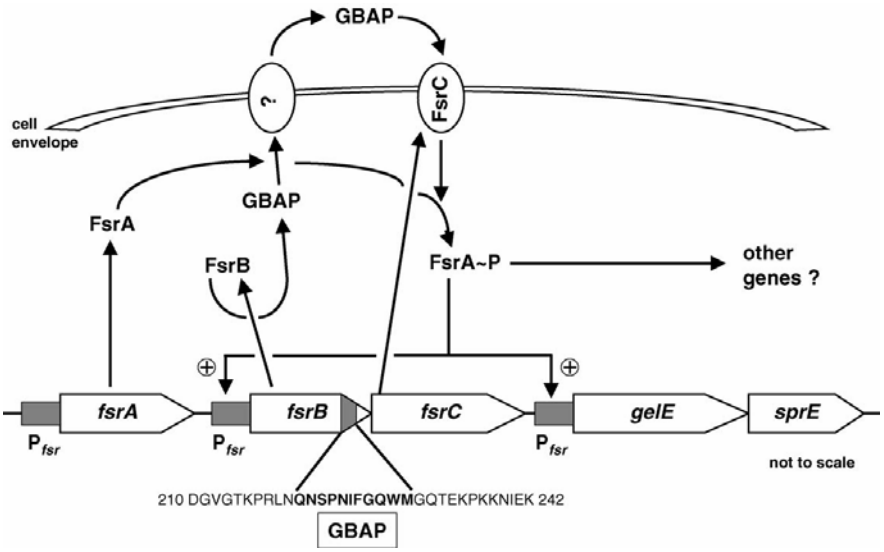


Figure 2: General pathway of the Fsr-driven quorum-sensing regulation in enterococci (from Podbielski 2004[52]).

Recently an additional small open reading frame (*fsrD*, not shown in Figure 2) was found to encode the propeptide for GBAP. *fsrD* is in frame with *fsrB* but translated independently[53]. The *fsrB* gene seems to play a role in processing the GBAP propeptide.

Cytolysin

Cytolysin is a β-haemolyic toxin expressed by some strains of *E. faecalis* with activity against both, eukaryotic and prokaryotic cells[54]. Bacteraemia with strains expressing Cytolysin have been associated with a higher patient mortality compared to non β-

haemolyic strains[55]. Cytolysin activities enhance enterococcal virulence in different infection models[56-58].

Active Cytolysin consists of two peptides which are posttranslationally modified, secreted and proteolytically cleaved by the gene products of *cylM, cylB* and *cylA* respectively as shown in Figure 3. Furthermore, one of the two peptides also plays a role in a quorum sensing autoinduction of the cytolysin operon.

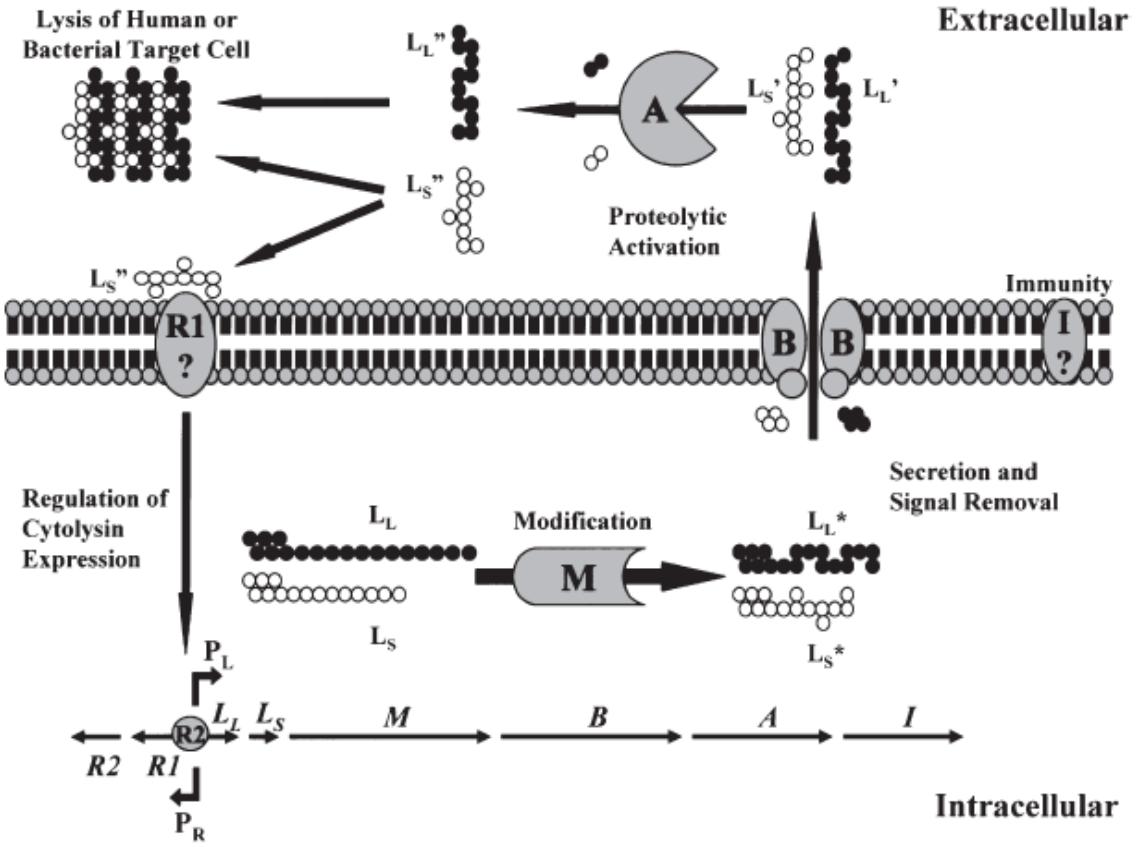


Figure 3: **Model of Cytolysin maturation and regulation.**

$CylL_L$ and $CylL_S$ are synthesized as 68- and 63-amino-acid precursors, respectively, and then post-translationally modified by the product of the cylM gene, generating $CylL_L$* and $CylL_S$*. After modification, both subunits are secreted and processed by CylB, generating $CylL_L$' and $CylL_S$'. Once both cytolysin subunits have been externalized, an identical six-amino-acid sequence is removed from the N-terminus of each subunit by CylA, generating the active toxin subunits, $CylL_L$'' and $CylL_S$''. The cylI gene confers selfprotection. Regulation of cytolysin expression is dependent on the products of the cylR1 and cylR2 genes. $CylL_S$'' has been shown to possess signalling activity that results in the autoinduction of the cytolysin operon by a novel quorum-sensing mechanism (from Coburn 2003[54]).

Adhesion proteins

To survive in a host enterococci have evolved different mechanisms to adhere to host tissues. One protein which facilitates adhesion is the enterococcal surface protein (Esp). Using an isogenic Esp mutant in a mouse model of urinary tract infection Shankar et al. showed that Esp contributes to colonization and persistence of

E. faecalis at this site, whereas histopathology was not changed by the Esp$^+$ strain[59]. Esp also seems to play a role in binding of *E. faecalis* to extracellular matrix[60].

Another protein involved in adhesion of *E. faecalis* is aggregation substance (AS). It is encoded on pheromone-responsive plasmids and was shown to interact with fibronectin to adhere to human colon[61]. AS mediates the formation of cell clumps by binding to a complementary receptor on nearby bacteria[62]. AS can also mediate *E. faecalis* binding to a variety of cells via β-type integrins, including human macrophages and intestinal epithelial cells[63,64].

A further molecule which mediates adhesion of *E. faecalis* to host tissues is ace (adhesin of collagen) which is a collagen binding MSCRAMM of *E. faecalis*[65]. MSCRAMM is an abbreviation for "Microbial Surface Components Recognizing Adhesive Matrix Molecule". The *E. faecalis* MSCRAMM Ace is structurally and functionally related to the staphylococcal Cna protein.

EfaAfs is an *E. faecalis* antigen. Its amino acid sequence shows 55 to 60% homology to a group of streptococcal proteins. These proteins have been shown to be adhesins[66]. Therefore, EfaAfs may function as an adhesin in endocarditis or other diseases. Furthermore, mice infected with an EfaAfs mutant showed more prolonged survival in a mouse peritonitis model than mice infected with the wild type strain[67]. These data clearly indicate a role of EfaAfs in *E. faecalis* virulence.

Antibiotic resistance

A major concern, especially in hospitals, is the emergence of multiple antibiotic resistant enterococci. Enterococci became the third most common cause of hospital acquired infections[68]. Especially resistance to glycopeptides like vancomycin is a great concern, since these are the antibiotics of the last choice in serious nosocomial infections with enterococci[5, 6]. A recent study using 411 vancomycin resistant and vancomycin-susceptible *E. faecium* isolates, "recovered from human and nonhuman sources and community and hospital reservoirs in 5 continents, identified a genetic lineage of *E. faecium* (complex-17) that has spread globally. This lineage is characterized by 1) ampicillin resistance, 2) a pathogenicity island, and 3) an association with hospital outbreaks." [69] Frequent horizontal gene transfer in enterococci enables these bacteria to rapidly respond to changing environmental conditions and may have contributed to the worldwide emergence. The genetic subspecies *E. faecium* called clonal complex 17, is responsible for the majority of

glycopeptide-resistant enterococci-related hospital burden. Preliminary data also suggest that similar high-risk enterococcal clonal complexes may exist within *E. faecalis*[5]. Enterococci are potentially the most important transmitters of antibiotic resistance genes[70].

In summary, enterococci are commensal intestinal bacteria, are used as probiotics but are also often isolated from human infections. Except the screening for antibiotic resistances and for the above mentioned clonal complex 17 there are no well accepted means of distinguishing “safe” from non safe strains. Therefore, further studies on enterococcal virulence are necessary.

1.2. Bacteria host interaction in the intestine

1.2.1. Bacteria host interaction and intestinal homeostasis

Bacteria host interaction in the intestine is a complex interplay between bacteria, bacterial compounds and metabolites, epithelial cells and immune cells and their products. In general bacteria host interaction in the intestine is thought to be beneficial for the host [71]. Bacteria are able to degrade indigestible plant materials like pectin and xylan and secrete products of benefit. Biosynthesis of vitamins and isoprenoids as well as short chain fatty acids (SCFA) like butyrate are examples[72]. Bacteria produced SCFA are estimated to account for up to 10% of the human energy source[73]. However, next to this symbiotic relationship the growth of pathogenic bacteria in the intestinal lumen has to be limited.

Pathogenic bacteria but also commensal bacteria may trigger responses of the intestinal immune system. These immune responses are divided in two categories: The innate and the adaptive immune response. The major difference between these two types of immune responses is the timing and the specificity of the immune reactions. The innate immune response is a very fast response activated by general unspecific features or compounds of bacteria or pathogens, whereas the adaptive immune response is slow and requires the proliferation of B-cells to plasmacytes. B-cells subsequently produce target specific antibodies. In the intestine a specialized mucosal tissue, named the Gut Associated Lymphoid Tissue (GALT), fulfils several tasks of both, the innate and the adaptive immune response. A current view of the intestinal epithelium and the GALT is shown in Figure 4. Peyer´s patches and lymphoid follicles are thought to be the primary inductor sites for intestinal adaptive immune responses and are enriched in antigen presenting dendritic cells, antigen recognizing and immune response regulating T-cells and antigen recognizing B-cells with the capability to proliferate into antibody secreting plasmacytes. M-cells, specialized cells for luminal antigen uptake are present in the epithelial monolayer right above the subepithelial dome of the peyer´s patches. Taking into account the length of the intestine and the number of immune cells present at this site, the intestine is often regarded as the biggest “immune organ” of the human body.

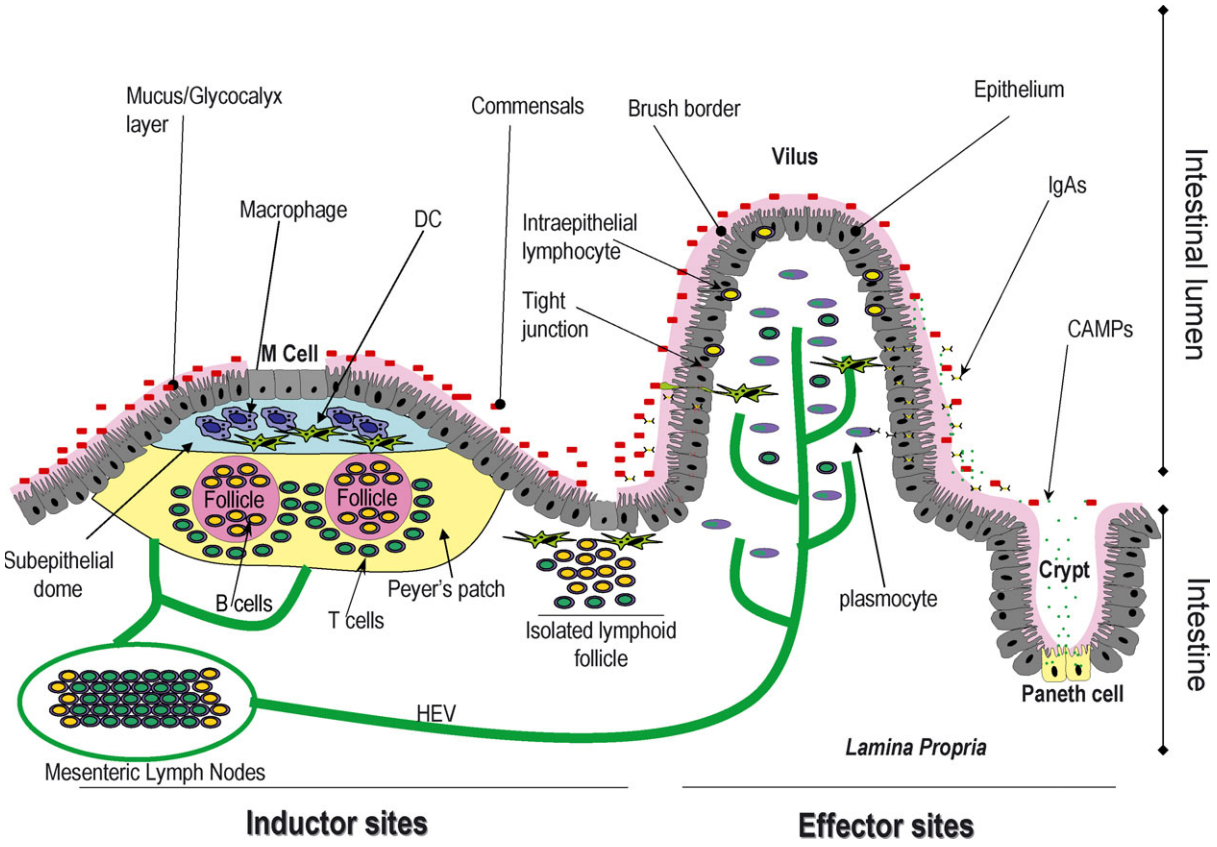


Figure 4: The intestinal immune system (from Magalhaes 2007[74]).

Next to defending pathogens, keeping homeostasis is especially important in the intestine. An inflamed mucosa or a destroyed epithelial layer would interfere with appropriate nutrient uptake and would allow entrance of bacteria into the lamina propria. Therefore, the single layer of Intestinal Epithelial Cells (IEC) and the associated glycocalyx/mucus layer play a prominent role in the intestine. Not only absorption of nutrients and building a barrier for bacteria by the formation of tight junctions but also recognition of antigens and bacteria as well as secretion of immune cell recruiting chemokines, immune response regulating cytokines and antimicrobial compounds are tasks of IEC. The primary focus of this thesis are IEC, therefore they are described in detail.

IEC have a columnar cell shape and are not ciliated in contrast to the epithelium of the respiratory tract. Since the primary function of IEC is the absorption of nutrients, the luminal surface area of the small bowel epithelium is enlarged by prolongations and emarginations of the IEC layer named villi and crypts, respectively. Furthermore, single IEC are vested by microvilli on their apical side which again leads to an

enlargement of the luminal surface. The total surface of the human intestine is estimated to 300m^2.

When looking on specialized IEC located in the IEC layer the already mentioned active regulating and protecting effect of this cell-layer can further be described: Paneth cells are IEC located to the base of the crypts (Figure 5). In order to prevent bacterial colonization of the crypts these cells are able to secrete different antimicrobial compounds, for instance α-defensins[75].

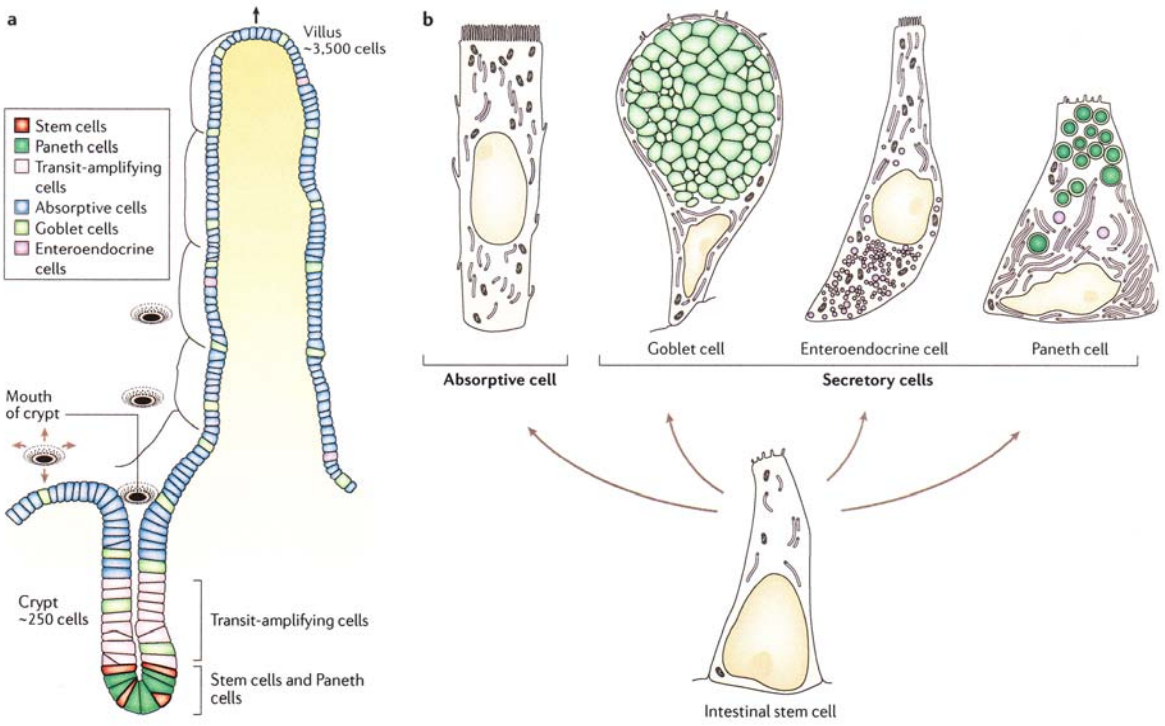


Figure 5: The distribution of epithelial cell types in the mammalian small intestine (from Crosnier 2006[75]).

Goblet Cells are specialized IEC for the secretion of mucins to the intestinal lumen. Theses mucins have a crucial role in protecting the intestine from pathogens, since mucin Muc2 deficient mice develop colitis and later colorectal cancer[76, 77]. Another cell type which can be found in the IEC monolayer is the enteroendocrine cell. Enteroendocrine cells help to keep homeostasis by secreting different mediators like intestinal peptide hormones. Furthermore, the already mentioned M-cells are specialized epithelial cells without microvilli on their luminal side. They are able to transport luminal antigens and bacteria across the epithelial monolayer. After endocytosis of the antigens or phagocytosis of the bacteria by dendritic cells and macrophage the foreign antigens can be presented to other immune cells on MHC

molecules. All theses cells, M-cells, paneth cells, goblet cells and enteroendocrine cells derive from intestinal stem cells located to the base of intestinal crypts (Figure 5). Except for the paneth cells which stay at the base of the crypts, stem cells divide and differentiate into absorptive IEC, goblet cells or enteroendocrine cells which migrate from the crypt towards the villus during differentiation. Therefore, the epithelial cell layer is subject to continuous renewal and the oldest IEC can be found at the tip of the villi. The turnover time for the entire epithelial cell population in mice averages 60h[78, 79].

In the last decade, considerable progress was made in the identification and characterization of pattern recognition receptors. These receptors are able to distinguish frequent compounds/patterns of commensals, pathogens and viruses and subsequently can induce signal transduction, protein synthesis and secretion. Although present on various immune cells, many of these receptors can also be found on IEC.

Toll like receptors (TLRs), the biggest subgroup consisting of at least 14 members[80], play a crucial role in the recognition of commensal and pathogenic bacteria. TLRs are single pass transmembrane receptors. Their structure contains luminal N-terminal extracellular leucine rich repeats which are responsible for the recognition of the bacteria or pathogen associated molecular patterns (PAMPs). On the cytoplasmic side an IL-1 similar domain which is designated Toll/IL-1 receptor (TIR) domain is required for downstream signalling. Upon recognition of their specific compounds, TLRs can induce signal transduction pathways like the NFκB pathway or the MAP kinase pathways[81] which lead to the recruitment of transcription and the activation of gene expression and protein secretion. For example bacterial surface patterns like lipopolysaccharides (LPS) from gram negative bacteria or lipoteichoic acids from gram positive bacteria induce the secretion of inflammatory mediators via TLR-4, TLR-2, or dimers of TLR-2 and TLR-1 or TLR-6 (see Figure 6). Other TLRs recognize single stranded RNA (TLR-7, TLR-8) double stranded RNA (TLR-3),Cytosine-phosphate-guanosine(CpG)-DNA-motifs (TLR-9), or flagellin (TLR-5)[80]. A recent summary of TLRs and their ligands is shown in Figure 6. A combination of a wide variety of cytokines and chemokines like IL-6, TNFα, IL-1, IP-10 or MIP1 may be secreted in response to TLR-activation leading to suppression or activation of targeted immune responses to pathogens. For example the secretion of the

chemokine interferon γ inducible protein-10 (IP-10) recruits T-cells to the site of inflammation.

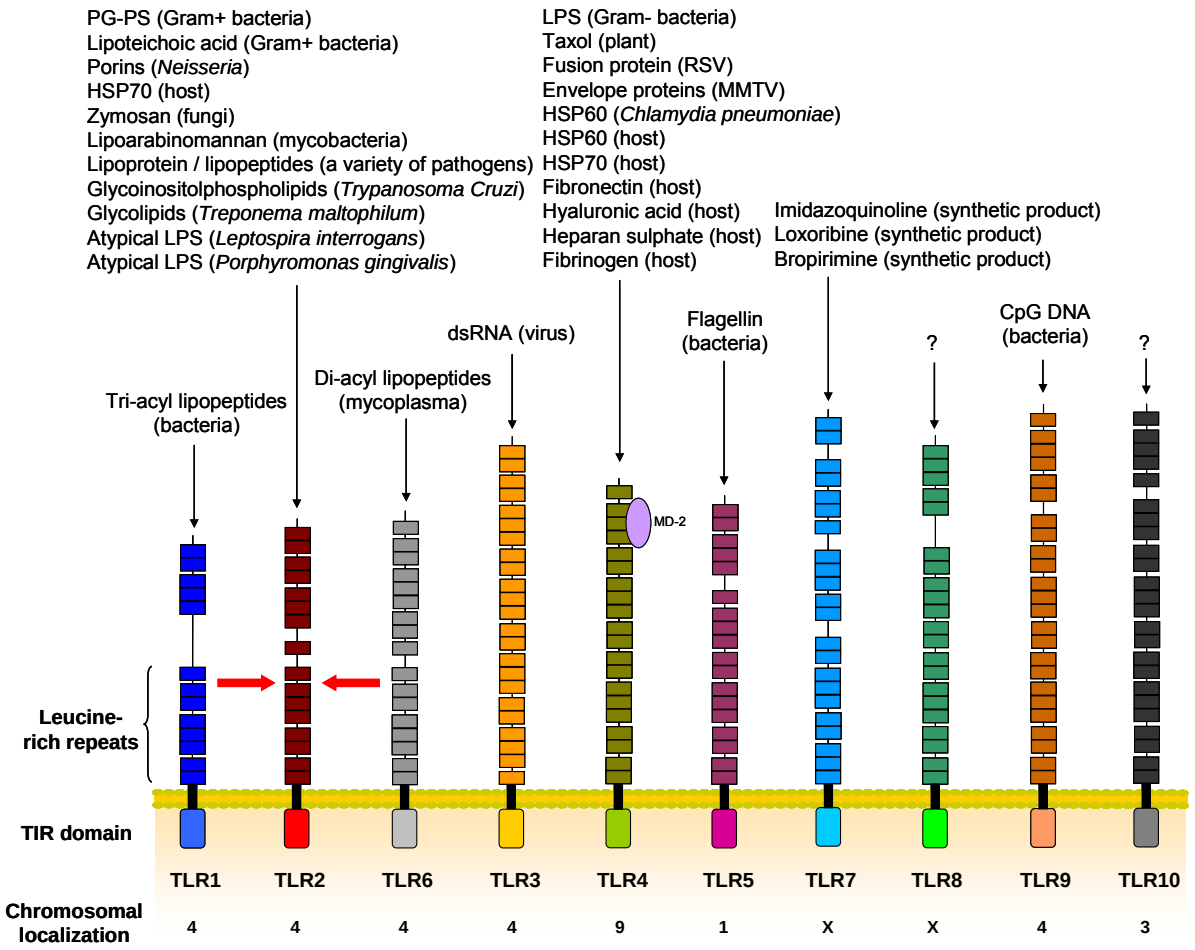


Figure 6: Human TLR protein family and their ligands (from Ruiz 2007[82]).

B-cells in the intestine highly contribute to the repression of inflammatory responses in response to commensal and pathogenic bacteria. B-cells, in general, are cells capable of producing high amounts of target specific designed antibodies after they encounter an antigen which binds to their specific B-cell receptor. The antibodies then bind to their targets and facilitate easy recognition and elimination of the invader mediated by the constant Fc domain, for example uptake and destruction by phagocytes. In the intestine B-cells tend to produce high amounts of a special poly- or dimeric form of the immunoglobulin A (IgA) which can be transported across the epithelium into the lumen using the polymeric immunglobulin receptor (pIgR) on IEC. Therefore, it is also designated secretory IgA. Secretory IgA is of crucial importance for intestinal homeostasis because its constant Fc-domain does not induce the

secretion of inflammatory mediators by immune cells[83]. Furthermore, sIgA can associate with the apical membrane of M-cells and therefore facilitate antigen uptake from the lumen[84]. Other homeostatic functions include (i) prevention of adhesion or entry of luminal antigen into the epithelium[85], (ii) binding of antigen in the lamina propria and secretion to the lumen[86] and (iii) neutralization of proinflammatory antigens[87].

Dendritic Cells (DCs) in the intestine also help to keep homeostasis, for instance by phagocytosis of bacteria. Subsequently antigens may be presented on their cell surface. DCs are enriched in the sub epithelial dome of peyer´s patches. Here, DCs are capable of taking up and presenting antigen after trans-epithelial transport mediated by M-cells (see Figure 7). In the lamina propria DCs are also present. Recently it was discovered, that lamina propria dendritic cells are able to form transepithelial dendrites[88] and that expression of the chemokine receptor CX_3CR1 is necessary for the formation[89]. Using these dendrites DCs are able to directly sample the luminal content for antigen (see Figure 7).

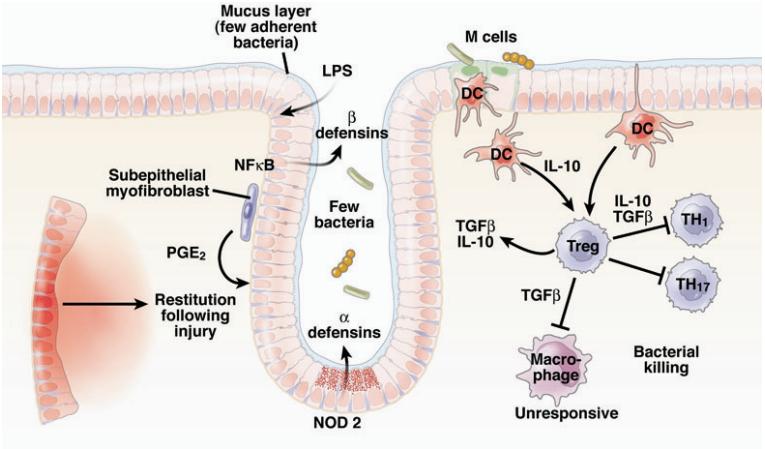


Figure 7: **Bacterial/epithelial interactions in mucosal homeostasis.**

The normal intestine has limited, controlled uptake of microbial antigens; exclusion of viable organisms; a state of nonresponsiveness of epithelial, innate, and adaptive immune cells; and secretion of IL-10 and TGF-β (adapted from Sartor 2008[30]).

1.2.2. Inflammatory Bowel Diseases (IBD)

Inflammatory bowel diseases (IBD) including Crohn´s disease (CD) and ulcerative colitis (UC) are idiopathic, spontaneously relapsing or chronic inflammatory disorders

of the gastrointestinal tract. In UC only the rectum and the colon is affected. The inflammatory process is limited to the mucosa and is histologically characterized by the presence of crypt abscesses and ulcerations. CD may affect any region of the gastrointestinal tract, from the mouth to the rectum. The inflammatory process can extend throughout the intestinal wall (transmural). Histologically, CD is characterized by the formation of granulomas and fibrosis.

The etiologies of CD and UC are still unknown. However, the hypothesis that both, genetic and environmental factors contribute to disease development is generally accepted[90-92]. Genetic predisposition leading to dysregulated mucosal immune responses and the concurrent prevalence of certain environmental triggers in developed countries are strong etiologic factors for disease pathogenesis [93-95]. Genetic predisposition and environmental factors may lead to changes in the intestinal microbiota and metabolite composition and therefore to an overly aggressive, dysregulated immune response and to disease development (Figure 8).

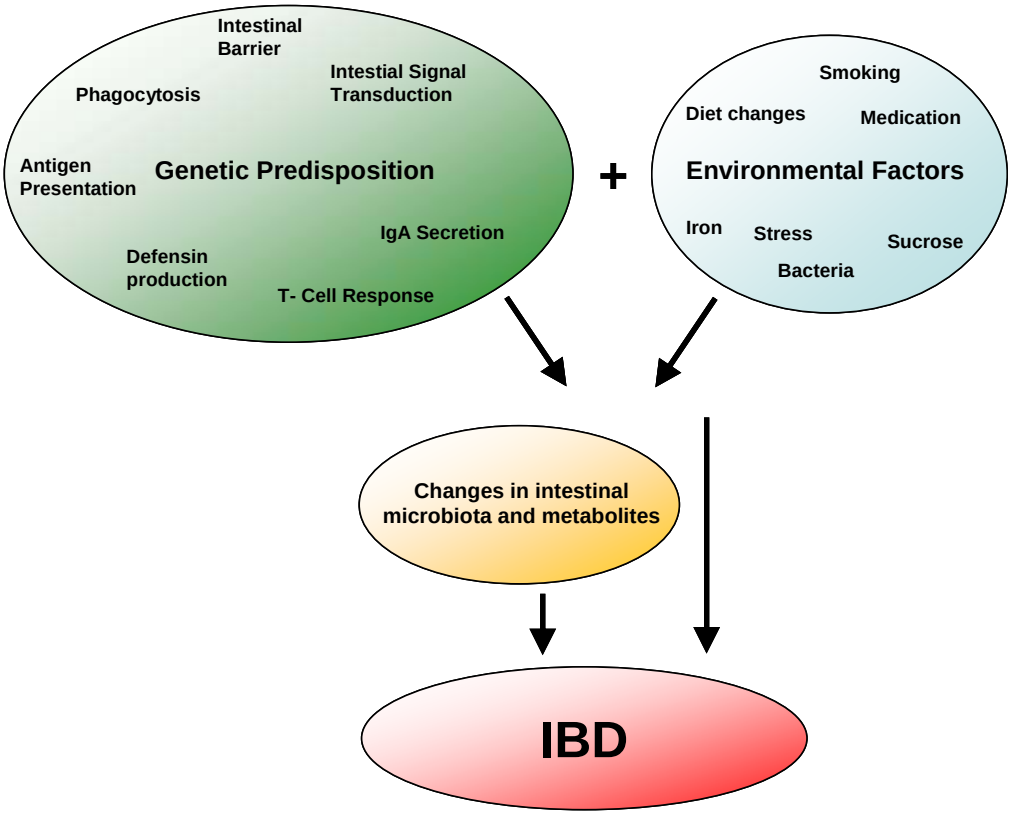


Figure 8: Etiology of Inflammatory Bowel Diseases

1.2.3. Animal models of IBD

The strongest evidence for a role of the commensal microbiota in inducing IBD development comes from animal models which share features with human IBD. The presence of microbiota is a prerequisite for the development of intestinal inflammation in most of these models. In germfree models disease is absent or inflammation is less severe. In Table 1, a selection of microbiota driven animal models and the respective references is presented.

In the years from 1995 to 2001 the number of IBD animal models increased from 18 to at least 63 [96]. According to this development, probably more than 100 animal models exist at the moment. One possible classification of these models is by the mechanism of induction of inflammation: Some models lead to spontaneous development of inflammation, in other models inflammation can be induced by administration of chemicals and in further models inflammation is a result of genetic engineering.

Table 1: Selection of microbiota driven animal models of IBD

Animal model	References
IL-10 deficient	97
C3H/HeJBir	98
SAMP1/Yit	99
CD4+, CD45RBhi transfer	100
IL-2 deficient	101, 102
TCRa deficient	103
HLA-B27 transgenic rats	104
Carrageenan	105

IBD animal models have provided valuable insights into IBD pathogenesis and are frequently reviewed in the literature [91, 106, 107]. For example IBD animal models highlighted the importance of T-cells and T-cell responses for IBD development. T-cells are cells which bear an antigen recognizing T-cell receptor and can be divided in cytotoxic T-cells, T-helper-cells, regulatory T-cells, memory T-cells and NK-T-cells. T-cells can, for instance, differentiate into the T-cell subsets designated T-helper-cells 1 (T_{H1}), T-helper-cells 2 (T_{H2}) and in the recently described T-helper-cells 17 (T_{H17}) [108, 109]. T_{H1} cells produce high amounts of IFNγ and therefore lead to a cell-

mediated immune response which strongly increases phagocytosis by macrophage. In contrast T_{H2} cells produce IL-4, IL-5 and IL-6 and therefore lead to a humoral immune response mediated by immune globulin secreting B-cells. T_{H17} cells were shown to secrete IL-17A, IL-17F and IL-22 and seem to be potent inflammation effectors. Inflammation in IBD animal models is mostly characterized by a overwhelming T_{H1}-response, however the newly identified T_{H17} subset also plays a major role in disease development[110]. Furthermore, the purification of different T-cell subsets and the transfer of theses to an immuno-deficient host with non functional T-cells is sufficient for the onset of intestinal inflammation[111]. These examples show that specific T-cell subsets are able to direct the immune response, and therefore direct chronic intestinal disease.

IL-10 deficient mice and transgenic HLA-B27/human β2 microglobulin rats are IBD animal models characterized by a T_{H1} cytokine response[107]. Both of these animal models have been described with respect to single bacterial species inducing inflammation in ex-germfree mice and are therefore introduced. Additionally, the induction of inflammation using the chemical dextran sulphate sodium (DSS) [112], is mentioned because of its frequent appearance in the literature.

In the DSS animal model 2,5% DSS administered for 5-7 days in the drinking water leads to a mild colitis, 10% results in a pancolitis and 3-4 repeated cycles of DSS administration for 7 days leads to chronic inflammation[113]. T_{H1} and T_{H2} cytokines are induced in chronic DSS-induced colitis and the presence of T-cells or B cells is not required for colitis development[114]. DSS-induced disease is attenuated in mice deficient in the host gelatinase designated matrix metalloproteinase 9 (MMP9)[115]. These data suggests that DSS-induced intestinal injury is mainly mediated by cells of the innate immune system, for instance by polymorphonuclear leukocytes (PMN).
The DSS colitis model seems to be not bacteria-induced since germfree mice also develop colitis [116, 117]. Of note, administration of only 1% DSS-induced death in germfree IQI/Jic mice, an immunologically impaired mice strain, and the commensal intestinal flora was even shown to protect these mice from DSS-induced death[116]. In contrast germfree mice with a BALB/c genetic background only develop mild inflammatory changes after administration of 2.5% DSS in the drinking water and monocolonization with the probiotic E. coli Nissle 1917 did not induce inflammatory

changes[118]. DSS colitis combined with monocolonization using an uropathogenic strain of E. coli was able to increase inflammation[118], indicating that uropathogenic bacteria can aggravate intestinal DSS-induced injury. In summary, the study of bacterial factors on intestinal injury is possible in this model. However, when comparing effects of commensal bacteria on intestinal injury in this model, close attention has to be paid on the genetic background of the mice used, because of different disease susceptibility[119].

Interleukin 10 (IL-10) deficient mice are a genetically engineered T_{H1} model of chronic intestinal inflammation. IL-10 is an important anti-inflammatory cytokine which is produced by T-cells and macrophage. IL-10 gene deficient mice (129/SvEv TAC) develop aggressive chronic colitis as well as ileitis beginning after 2 month of conventional breeding[120]. Specific pathogen free conditions result in inflammation limited to the colon. Crypt abscesses, focal mucosal ulcers and transmural inflammation are common in the late phase. Goblet cell depletion is often mentioned to be present in IL-10-/- mice colitis, however at least one contradictory study exists[121]. The genetic background of IL-$10^{-/-}$ mice highly influences the severity of disease[122]. The mice strain used in this study, 129/SvEv mice, is very susceptible compared to C57Bl/6J mice which are almost resistant to colitis development.
Germfree IL-10 -/- mice stay disease-free[97]. Germfree IL-10-/- mice were frequently used to study the impact of single bacterial species on experimental colitis. Monoassociation of IL-10-/- mice with non-pathogenic commensals, *E. faecalis* or *E. coli* , revealed two variable phenotypes of colitis: *Escherichia coli*-induced mild cecal inflammation after 3 weeks of monoassociation whereas *Enterococcus faecalis*–monoassociated IL-10-/- mice developed distal colitis at 10–12 weeks of age[123]. Dual association with the same *E. coli* and *E. faecalis* strains resulted in aggressive distal colonic-predominant pancolitis with duodenal inflammation by 7 weeks[124]. Monoassociation with other bacterial species including *Bacteroides vulgatus*, *viridans* group *Streptococcus*, *Clostridium sordelli*[125] and *Lactobacillus plantarum*[126] did not lead to the onset of colitis. These data implicate selected commensal bacteria in the development of chronic inflammation in IL-10-/- mice.

Transgenic HLA-B27/human β2 microglobulin rats express two human genes involved in antigen presentation: the human class I major histocompatibility allele

HLA-B27 and human β2 microglobulin. HLA-B27 transgenic rats spontaneously developed inflammatory disease involving the gastrointestinal tract, but also peripheral and vertebral joints, male genital tract, skin, nails, and heart[127]. However, transfection and expression of other human class 1 HLA molecules and human β2 microglobulin failed to induce disease[127]. As already mentioned in Table 1, germfree HLA-B27 transgenic rats do not develop intestinal inflammation[104]. Monoassociation of these rats with a strain of the gram negative anaerobe species *Bacteroides vulgatus* leads to significant increased colitis compared to an *E. coli* strain in which colitis was found to be absent by histological testing[128]. Furthermore, colonization of germfree HLA-B27 transgenic rats with a mixture of 5 different common enteric bacteria isolated from IBD patients (*Enterococcus faecium, Escherichia coli, Peptostreptococcus productus, Eubacterium contortium, and Enterococcus avium*) did not result in cecal colitis whereas the addition of *B. vulgatus* to this mixture resulted in a significantly increased histopathological score in cecum after 1 month. This model therefore also highlights that different bacterial species differ in their potential to induce colitis.

Remarkable differences appear when comparing the bacterial species responsible for colitis induction in monocolonized IL-10-/- mice and HLA-B27 TG rats. *B. vulgatus* is capable of inducing colitis in HLA-B27 TG rats but not in IL-10-/- mice. In IL-10-/- mice *E. faecalis* induces colitis however two enterococcal species did not in HLA-B27 TG rats (Figure 9). These differences could be explained by different genetic susceptibilities of these animal models to specific bacterial properties, enzymes, antigens or metabolites. Furthermore, the use of different *B. vulgatus* strains or different *Enterococci* might have caused the discrepancy.

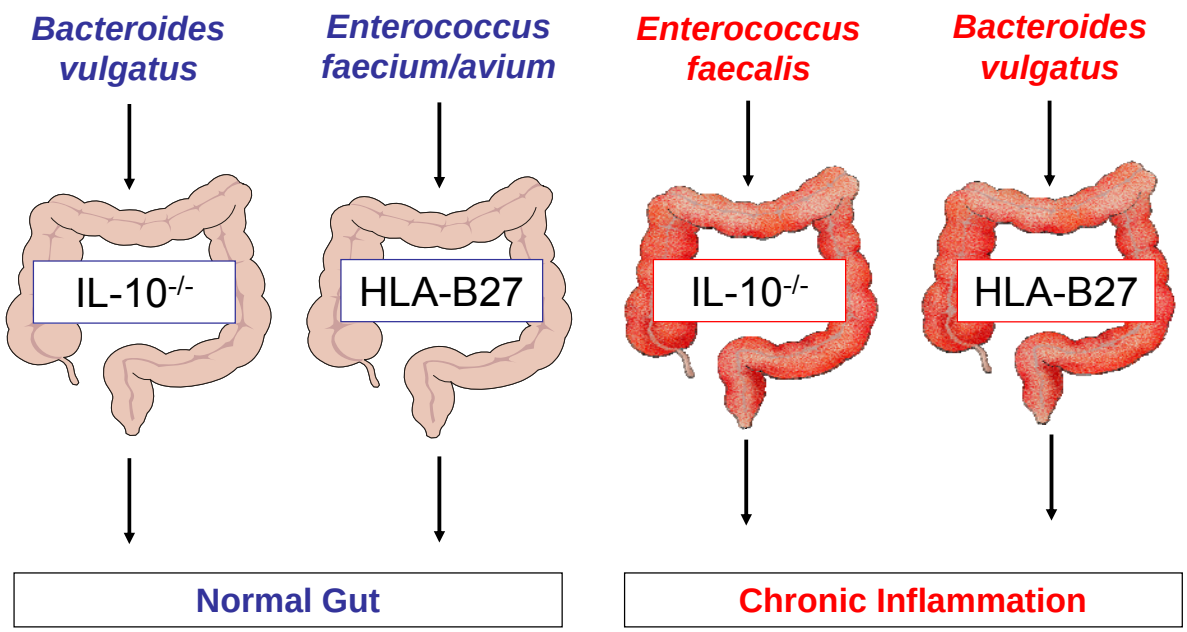


Figure 9: Colitis induction by commensal bacterial species in gnotobiotic animal models reveals remarkable differences (summarized from Kim 2005, Rath 1999, Sellon 1998[97, 123, 128]).

For the luminal containment of commensal and pathogenic bacteria the barrier function of the epithelium is crucial. Some animal models demonstrate the impact of a defective mucosal barrier on the development of IBD: N-cadherin and Cytokeratin 8 are proteins mediating cell-cell adhesion in IEC. Expression of a dominant negative N-cadherin in the epithelium leads to focal areas of intestinal transmural inflammation with defective proliferation, migration and apoptosis in the crypts[129]. Deletion of the Cytokeratin 8 gene is lethal on most mouse genetic backgrounds. However, on a FVB/N background some mice survive and develop colorectal hyperplasia as well as mucosal and submucosal inflammation[130]. Recently also a Mucin 2 deficient mouse model was generated. These mice develop spontaneous colitis[131] underlining the essential role of the mucus layer for intestinal homeostasis.

In summary, IBD animal models have contributed to the discovery of valuable information about IBD pathogenesis. Intact IEC barrier function, mucin production and especially a well regulated mucosal immune response are important to prevent disease. With respect to bacterial colonization, IBD animal models clearly demonstrate the importance of the commensal bacterial microbiota for the initiation and perpetuation of disease. The selective colonization of germfree rodent models of chronic intestinal inflammation like IL-10 deficient mice (IL-10-/-), and HLA-B27

transgenic rats with single bacterial strains implicate *Enterococcus faecalis, Escherichia coli* and *Bacteroides vulgatus* as particularly important to the induction of colitis in these models [123, 128, 132].

1.2.4. Bacteria host interaction in IBD

Role of bacteria for IBD pathogenesis

Next to IBD animal models several findings emphasize that the pathogen-free enteric microbiota plays a major role in the initiation and perpetuation of IBD: (i) Diseases occur mainly at areas of the highest anaerobic bacterial population, either the colon (UC) or the terminal ileum (CD), (ii) probiotic bacteria help to prevent relapses and (iii) some of the disease are successfully treatable with antibiotics [133, 134]. In addition, abnormal bacterial composition was found in UC and CD[26].

Although all these reasons suggest that gastrointestinal microbes influence IBD pathogenesis in susceptible hosts, the types of microbes involved have not been adequately described. It is still controversial whether the entire commensal microbiota, specific bacterial metabolites, enzymes or individual opportunistic pathogens are primarily responsible for the induction of inflammation. Different hypotheses by which bacteria and fungi might induce chronic immune-mediated intestinal injury are summarized in Figure 10 and described below.

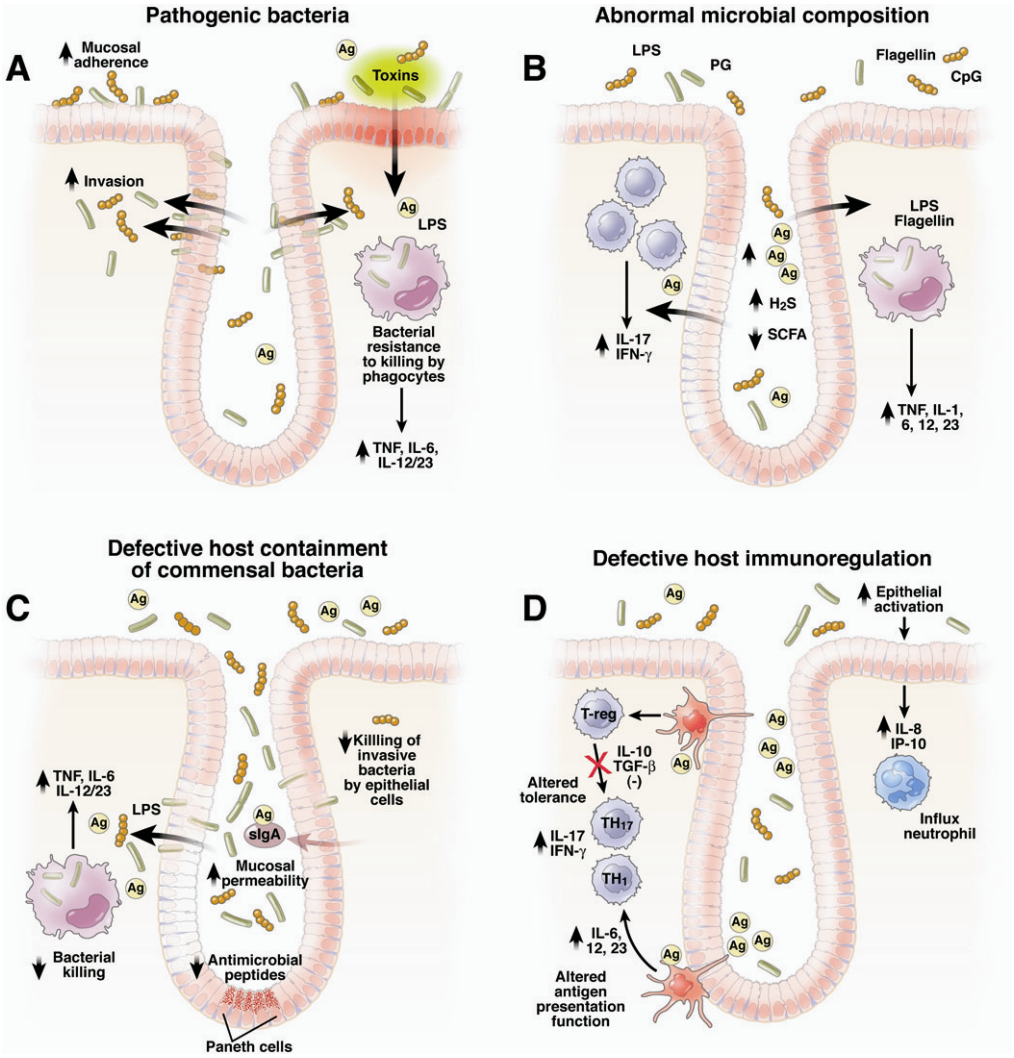


Figure 10: Hypotheses by which the microbiota may induce chronic immune-mediated intestinal injury.

A: Changes in bacterial microbiota could lead to increased numbers of pathogenic or "facultative pathogenic" bacteria, leading to increased adherence of these bacteria to mucosal surfaces, increased toxin production and invasion of host tissue. B: Abnormal bacterial composition might change the metabolic profile of the intestine. Decreased Short Chain Fatty Acid (SCFA) production or increased toxic metabolites like H_2S may increase mucosal permeability and block butyrate metabolism. C: Defective production of secrtory IgA or antimicrobial peptides may lead to defective host containment of commensal bacteria and mucosal overgrowth. Defective bacterial killing in phagocytes can lead to persistent intracellular bacteria. D: Defects in immuno-regulation or ineffective down-regulation of innate immune responses to bacterial antigen can lead to an overly aggressive response to commensal bacteria and their antigens. Dysfunction of regulatory T-cells or antigen presenting cells might be a reason (adapted from Sartor 2008[30]).

Pathogenic bacteria and abnormal bacterial composition

The hypothesis that a single bacterial pathogen is responsible for UC or CD in humans is most likely not true. *Mycobacterium avium* subspecies *paratuberculosis* was subject of numerous studies with respect to CD. However, recent data suggest that this pathogen is not the causative agent of most CD patients[30]. It might play a

role in a small subset of patients with the same susceptibility. Since CD is a heterogeneous group of disorders, it is likely that different genetic susceptibilities are associated with different groups of pathogenic mucosa associated bacteria.

One group of bacteria which has gained interest with respect to CD are adherent invasive *E. coli* (AIEC). The prevalence of AIEC was found to be significantly higher in ileal mucosa from CD patients as in control patients[135]. Strains isolated from CD patients were shown to survive and replicate within macrophages[136]. Expression of Carcinoembryonic antigen-related cell adhesion molecule 6 (CEACAM6) on the apical surface of ileal IEC was reported to act as a receptor for AIEC adhesion[137]. In the DSS colitis model AIEC flaggelin was shown to aggravate colitis, compared to nonflagellated mutants, and TLR-5 mRNA expression was highly increased in colonic tissue[138]. These data suggest AIEC as a prominent example for a possible causative agent for many CD patients.

Several findings emphasize that the balance between beneficial and detrimental bacteria is altered in IBD patients. Mucosa-associated bacteria, particularly *Bacteroides* species and *Enterobacteriaceae* were found to be significantly increased in IBD patients[139]. The total concentration of mucosal bacteria was found to be by far higher in IBD patients compared to controls and increased with the severity of disease[139]. Comparison between CD patients and controls revealed greater numbers of *Escherichia coli* and enterococci and lower numbers of bifidobacteria and ruminococci in the neoterminal ileum after ileocolonic resection for CD[140]. Also other studies confirmed lower bifidobacteria and associated beta-D-galactosidase activity in CD[141]. All these changes in mucosal bacteria and bacterial metabolites could contribute to, or even trigger IBD development. However, by now it is not clear whether the changes are the cause or a consequence of disease.

Defective elimination of pathogenic bacteria and defective immuno-regulation

Defective bacterial recognition is a risk factor for IBD. The pattern recognition receptor NOD2 (**n**ucleotide-binding **o**ligomerization **d**omain containing **2**) also known as CARD15 (**ca**spase **r**ecruitment **d**omain family, member **15)** is able to recognize muramyl dipeptide from bacterial peptidoglycan by a leucine rich repeat domain leading to the activation of NFκB signalling. Mutations in NOD2 were found to be associated with IBD[142]. Crohn´s disease patients with NOD2 polymorphisms show

decreased α-defensin production[143] which might play a functional role for disease development in this subset of patients. Also polymorphisms in the paneth cell defensin A5 were recently suggested to confer susceptibility to IBD[144]. Defensins are antimicrobial peptides produced at a variety of epithelial surfaces. Reduced α-defensin levels are seen in patients with ileal CD whereas reduced β-defensin levels are frequently found in CD patients with colonic disease-involvement[145]. Wehkamp et al. were able to show that the probiotic strain *Escherichia coli* Nissle 1917 is able to induce NFκB-mediated human β-defensin 2 expression in IEC[146].
Furthermore, also mutations in TLRs influence the risk for IBD[147, 148]. These examples highlight the importance of adequate bacterial recognition and bacterial elimination for mucosal homeostasis.

Recently mutations in the autophagy-related 16-like gene (ATG16L1) and in the immunity-related GTPase family M protein (IRGM) which functions in innate immune response, probably through regulation of autophagy, were found to be associated with CD[149, 150]. Autophagy is a process which contributes to killing and processing of intracellular bacteria. ATG16L1 is expressed in IEC and functional knock down was associated with defective autophagy of *Salmonella typhimurium*[151]. Therefore, next to bacterial recognition also defective bacterial killing can contribute to IBD.

Furthermore, defective regulation of homeostatic immune responses to intestinal stimuli often leads to intestinal inflammation. Knock out of the cytokines IL-2 or IL-10 in mice or defective NFκB and TGFβ signalling are examples[90, 97, 101]. Recently it was convincingly demonstrated by Garrett et al. that defective immuno-regulation can even change the composition of the intestinal microbiota to become pathogenic. Loss of T-bet, a T-box transcription factor family member regulating the differentiation and function of immune cells, induced the development of a pathogenic intestinal bacterial population in immuno-deficient T-bet-/- x Rag2-/- mice. Of note, the development of disease in T-bet-/- x Rag2-/- mice was found to be communicable to wild type mice by cohousing compared to cohousing of immuno-deficient Rag2-/- mice with wild type mice[152].

In summary, the evidence that the commensal intestinal microbiota drives inflammation in immunologically susceptible hosts is convincing whereas the majority of mechanisms by which bacteria drive or trigger IBD are still poorly understood.

1.2.5. Enterococci and IBD

Already 42 years ago the relation between gut microbiota and IBD was of particular interest: The faecal microbiota in UC was compared to “normal persons” by Cooke at al. with a short conclusion: No significant differences[153]. One year later Gorbach et al. noticed a slight increase in coliforms in UC patients[154]. In 1975 an observation by Cooke, an increase of *Streptococcus faecalis* in UC patients, was further investigated with the finding, that group-D streptococci (including enterococci) were strongly increased in the stool of UC patients[155]. Because of different microbiological standards and techniques at that time all mentioned studies have to be considered with caution.

An important finding regarding the association of enterococci with IBD was discovered by Balish and Warner[156] in the year 2002 and is summarized in Figure 11. As already mentioned, conventional housed IL-10-/- mice develop aggressive chronic colitis as well as ileitis[120]. Therefore, the role of different bacterial genera for the induction of colitis was discovered in a newly generated germfree model of IL-10-/- mice. Germfree IL-10-/- mice did not develop inflammation whereas colonization of germfree mice with a single strain of *E. faecalis* results in severe chronic colitis after 14 weeks of monoassociation (Figure 11).

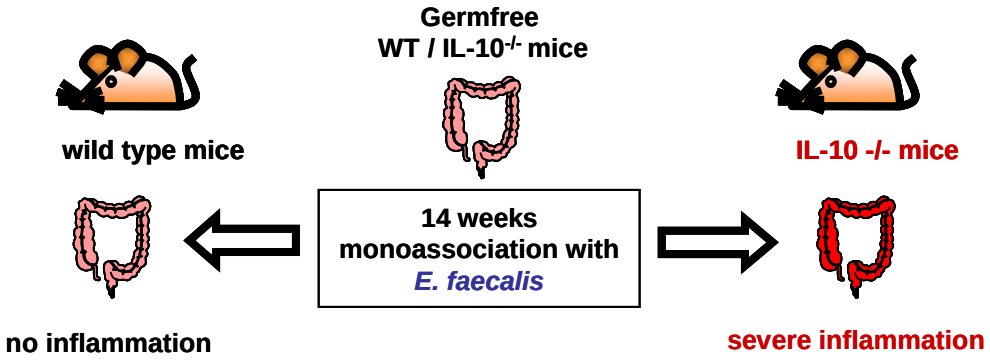


Figure 11: *E. faecalis* monoassociation induces colitis in IL-10-/- mice.

Furthermore, pure cultures of *Candida albicans, Escherichia coli, Lactobacillus casei, L. reuteri, L. acidophilus*, and *Lactococcus lactis*, a *Bifidobacterium* sp. or a *Bacillus* sp. did not lead to colitis development during the 25- to 30-week study. *E. faecalis*-induced colitis in IL-10-/- mice was verified and further investigated on the level of IEC stress mechanisms in our lab[157]. The ability of *E. faecalis* to survive in macrophages[158] could also contribute to intestinal inflammation in IL-10-/- mice.

A further hint for a role of *E. faecalis* in IBD was published by Furrie et al. in 2004: They investigated modulation of the polymorphonuclear leucocyte (PMNs) respiratory bursts by opsonization of bacteria isolated from the rectal mucosa using sera obtained from UC and control patients. In their study they observed a significant difference in the response of PMNs (from healthy donors) to *E. faecalis* opsonized with UC patient serum compared to *E. faecalis* opsonized with serum from healthy control patients. The *E. faecalis* strain they used originated from a UC patient[159]. These results demonstrate the increased presence of *E. faecalis* specific antibodies in serum from UC patients compared to control patients and indicate a role of *E. faecalis* for UC.

Finally, enterococci were also implicated in CD. CD is associated with different extraintestinal manifestations like abscesses, fistulae, and systemic complications like sepsis and endocarditis. A study investigating intraabdominal abscesses revealed *E.coli*, *Bacteroides fragilis*, *Enterococcus* species and *Streptococcus viridans* as the most common organisms recovered from these abscesses[160].

In summary, several different findings suggest a role for enterococci in IBD pathogenesis.

2. The aim of the present study

E. faecalis strains are both commensal intestinal bacteria and opportunistic pathogens. By now, different putative virulence factors of *E. faecalis* have been described. The influences of these *E. faecalis* virulence factors, for instance cytolysin, gelatinase and *efaAfs,* on human diseases have been shown in different animal models. Different studies implicate enterococci in IBD pathogenesis. However, the influence of different *E. faecalis* strains on IBD has not been investigated, yet.

The primary aim of our study was to discover strain-specific differences in *E. faecalis* interaction with IEC in chronic intestinal inflammation. *E. faecalis*-mediated cytokine secretion and signal transduction were investigated at the level of strain-specificity *in vitro* and *in vivo*. The question, whether different *E. faecalis* strains with diverse sets of virulence factors differ in their ability to induce colitis in IL-10-/- mice were addressed with a special focus on *E. faecalis*-induced protein expression in IEC *in vivo*. Finally, the influence of specific *E. faecalis* virulence factors for experimental inflammation should be further evaluated.

To address this aim, three *E. faecalis* strains were selected on the basis of origin and virulence factor presence. The mouse IEC line Mode K was used to measure *E. faecalis* strain-specific IL-6 and IP-10 profiles as well as NFκB and MAP kinase activation by ELISA technique and phosphor-specific immuno-blotting, respectively. The effect of single strains on chronic intestinal inflammation was studied by monoassociation of germfree IL-10-/- mice and by comparing histopathology of formalin fixed paraffin embedded sections by scoring. IEC of theses mice were isolated to monitor differential gene expression and transcription factor activation. 2D-SDS-PAGE and subsequent MALDI-TOF MS was used as a technique to compare *E. faecalis* strain-specific protein expression in IEC and to discover differentially regulated proteins. The *in vitro* and *in vivo* results were compared for similarities in IEC. Potential strain-specific differences were discussed, verified and further investigated to elucidate the underlying molecular mechanisms.

3. Materials and Methods

Table 2: Chemicals and Reagents	
Product	**Supplier**
Acetonitrile	Roth, Karlsruhe
Acrylamide/Bisacrylamide (30%/0.8%)	Roth, Karlsruhe
Acetic acid	Roth, Karlsruhe
Agar Agar	Roth, Karlsruhe
Agarose	Roth, Karlsruhe
Ammonium sulphate ($(NH_4)_2SO_4$)	Roth, Karlsruhe
Ammoniumpersulfat (APS)	Roth, Karlsruhe
Ammoniumhydrogencarbonat (NH_4HCO_3)	Roth, Karlsruhe
Antibiotic Antimycotic solution	Invitrogen, Karlsruhe
Bromophenol Blue	Roth, Karlsruhe
Bio-Rad Protein assay Kit	Bio-Rad, Munich
Calciumchloride	Roth, Karlsruhe
CHAPS	Roth, Karlsruhe
Chloroform	Merck, Darmstadt
Coomassie brilliant Blue (G250)	Serva, Heidelberg
dNTP mixture (RT PCR)	Invitrogen, Karlsruhe
dNTP mix (virulence gene PCR)	Qbiogene, Heidelberg
DMEM: high glucose, with pyridoxine chloride, without sodium pyruvate	Invitrogen, Karlsruhe
DMEM F12	Sigma, Steinheim
Dithiothreitol (DTT)	Roth, Karlsruhe
DEPC (diethylpyrocarbonat)	Roth, Karlsruhe
D-JNKL1 (JNK Inhibitor)	Alexis Biochemicals, San Diego, USA
ECL Detection Kit	GE Healthcare, Freiburg
EDTA	Merck, Darmstadt
Ethanol	Roth, Karlsruhe
EGF	R&D Systems, Minneapolis, USA
First-strand buffer	Invitrogen, Karlsruhe

Foetal bovine serum (FBS)	Invitrogen, Karlsruhe
High Perfect transfection reagent	Qiagen, Hilden
L-Glutamin	Invitrogen, Karsruhe
Light Cycler-Fast Start DNA Master SYBR Green I kit	Roche Diagnostics, Mannheim
Glycerol	Roth, Karlsruhe
Glycin	Roth, Karlsruhe
IFNγ	R&D Systems, Minneapolis, USA
IL-1β	R&D Systems, Minneapolis, USA
Incubation mix (virulence gene PCR)	Qbiogene, Heidelberg
2-Iodacetamide	Merck, Darmstadt
IPG drystrips (pH 3-10)	GE Healthcare, Freiburg,
Isopropanol	Roth, Karlsruhe
IP-10 ELISA Kit	R&D Systems, Minneapolis, USA
IL-6 ELISA Kit	R&D Systems, Minneapolis, USA
Methanol	Roth, Karlsruhe
Milk powder, blotting grade	Roth, Karlsruhe
MMLV-reverse transcriptase	Invitrogen, Karlsruhe
PBS	Invitrogen, Karlsruhe
PD98059 (ERK Inhibitor)	Calbiochem, Merk, Darmstadt
Percoll	Sigma, Steinheim
Pharmalyte pH 3-10	GE Healthcare, Freiburg,
Phosphoric acid	Roth, Karlsruhe
Ponceau S	Roth, Karlsruhe
Protease Inhibitor Mix Complete Mini	Roche Diagnostics, Mannheim
Precision Plus Protein Dual Color Standards	Bio Rad, München
RNase Out	Invitrogen, Karlsruhe
Random Hexamers	Invitrogen, Karlsruhe
SB203589 (p38 Inhibitor)	Calbiochem, Merck, Darmstadt
Sodium chloride	Roth, Karlsruhe
Sodiumdodecylsulfat	Merck, Darmstadt
Sucrose D(+)	Roth, Karlsruhe
Silicon Oil	Serva, Heidelberg
Taq-Polymerase	Qbiogene, Heidelberg

TEMED	Roth, Karlsruhe
Thiourea	Roth, Karlsruhe
TNFα	R&D Systems, Minneapolis, USA
Trifluoracetic acid	Roth, Karlsruhe
Tris-HCL	Roth, Karlsruhe
Trizol	Invitrogen, Karsruhe
Trypsin	Promega, Madison, WI USA
Trypsin-EDTA	Invitrogen, Karsruhe
Trypan Blue Stain 0.4%	Invitrogen, Karsruhe
Tryptone	Becton Dickinson, Sparks, USA
Tween 20	Sigma, Steinheim
Urea	Roth, Karlsruhe
Yeast Extract	Becton Dickinson, Sparks, USA

Table 3: Antibodies and applied dilutions

Antibody	**Dilution**	**Supplier**
Anti-Alix	1:250	Becton Dickinson GmbH, Heidelberg
Anti-Alix	1:1000	Santa Cruz, Europe
anti-β-Actin	1:5000	MP Biomedicals, Solon, OH, USA
anti-RelA	1:1000	Cell Signalling Technolog, Boston, USA
anti-phospho-RelA (Ser536)	1:1000	Cell Signalling Technolog, Boston, USA
anti p38	1:1000	Cell Signalling Technolog, Boston, USA
anti-phospho-p38 (Thr180 / Tyr182)	1:1000	Cell Signalling Technolog, Boston, USA
anti-c-jun	1:1000	Cell Signalling Technolog, Boston, USA
anti-phospho-c-jun (Ser73)	1:1000	Cell Signalling Technolog, Boston, USA
anti-phospho-ERK1/2 (Thr202 / Tyr204)	1:1000	Cell Signalling Technolog, Boston, USA
anti-ERK1/2	1:1000	Cell Signalling Technolog, Boston, USA
anti-Cleaved Caspase 3	1:1000	Cell Signalling Technolog, Boston, USA
ECL anti-Mouse IgG HRP	1:1000	GE Healthcare, Freiburg
ECL anti-Rabbit IgG HRP	1:1000	GE Healthcare, Freiburg
ECL anti-Goat IgG HRP	1:1000	Santa Cruz, Europe

Table 4: Primer sequences for *E. faecalis* virulence gene PCR			
Gene	**Forward Sequence**	**Reverse Sequence**	**size (bp)**
cylM	5´-ctgatggaaagaagatagtat-3´	5´-tgagttggtctgattacattt-3´	742
cylB	5´-attcctacctatgttctgtta-3´	5´-aataaactcttcttttccaac-3´	843
cylA	5´-tggatgatagtgataggaagt-3´	5´-tctacagtaaatctttcgtca-3´	517
gelE	5´-accccgtatcattggttt-3´	5´-acgcattgcttttccatc-3´	419
esp	5´-ttgctaatgctagtccacgacc-3´	5´-gcgtcaacacttgcattgccgaa-3´	933
efaAfs	5´-gacagaccctcacgaata-3´	5´-agttcatcatgctgtagta-3´	705
agg	5´-aagaaaaagaagtagaccaac-3´	5´-aaacggcaagacaagtaaata-3´	1553
cpd	5´-tggtgggttatttttcaattc-3´	5´-tacggctctggcttacta-3´	782
ccf	5´-gggaattgagtagtgaagaag-3´	5´-agccgctaaaatcggtaaaat-3´	543
cob	5´-aacattcagcaaacaaagc-3´	5´-ttgtcataaagagtggtcat-3´	1405

Table 5: Primer sequences for qPCR			
Gene	**Forward Sequence**	**Reverse Sequence**	**size (bp)**
18S	5`-ccaaagtctttgggttccgg-3	5`-aacaactaagaacggccatg-3`	204
GAPDH	5`-atcccagagctgaacg-3`	5`-gaagtcgcaggagaca-3`	198
IP-10	5`-tccctctcgcaaggac-3`	5`-ttggctaaacgctttcat-3`	209
Alix	5´-aggagtcgctaagattgt-3´	5´-tggtgtctcgatggga-3´	204

Table 6: siRNA sequences			
Target gene	**Target sequence**	**double stranded siRNA sequence:**	**Supplier/Product**
Alix	5'-gaggctaacttagagaagaa-3'	sense: r(ggcuaacuugagagaagaa)dTdT antisense: r(uucuucucucaaguuagcc)dTdC	Qiagen/Mm_Pdcd6ip_4_HP
Alix	5'-cagaacctggataatgatgaa-3'	sense: r(gaaccuggauaaugaugaa)dTdT antisense: r(uucaucauuauccagguuc)dTdG	Qiagen/Mm_Pdcd6ip_5_HP)

Table 7:Technical Equipment	
Autoflex 1 MALDI-TOF-MS	Bruker Daltonik GmbH, Bremen, Germany
BiblioSphere Software	Genomatix Software GmbH, München, Germany
Biophotometer	Eppendorf AG, Hamburg, Germany
Centrifuges	SIGMA Laborzentrifugen GmbH, Osterode, Germany
CO_2-Incubator	Binder GmbH, Tuttlingen, Germany
Ettan Dalt II System	GE Healthcare, Freiburg, Germany
Flex Analysis software	Bruker Daltonik GmbH, Bremen, Germany
Flex control software	Bruker Daltonik GmbH, Bremen, Germany
Horizontal Shaker	UniEquip GmbH, München, Germany
Image Scanner	GE Healthcare, Freiburg, Germany
INTAS Gel Imager	Intas Science Imaging Instruments GmbH, Göttingen, Germany
IPGphor 2	GE Healthcare, Freiburg, Germany
Laboratory scales	Gottl. Kern & Sohn GmbH, Balingen, Germany
Laminar flow	Kojair Tech Oy, Vilppula, Finland
Light Cycler	Roche Diagnostics GmbH, Mannheim, Germany
Light microscope	Leica DMIL Leica Microsystems GmbH, Wetzlar, Germany
Protean Mini cell	Bio-Rad Laboratories GmbH, Munich,Germany
Proteom Weaver software	Definiens, Munich, Germany
Spectrophotometer Nanodrop ND-1000	PEQLAB Biotechnologie GmbH, Erlangen, Germany
Thermomixer	Thermo Electron Corporation, Waltham, USA
Thermocycler	Thermo Electron Corporation, Waltham, USA
Trans Blot SD Semi Dry Transfer Cell	Bio-Rad Laboratories GmbH, Munich,Germany
UP200s ultrasonic processor	Hielscher Ultrasonics GmbH, Teltow, Germany
Water bath	Thermo Electron Corporation, Waltham, USA

3.1. Bacteria

For our *in vivo* monoassociation studies as well as for our *in vitro* stimulation of cell lines, we used 5 different *E. faecalis* strains: *E. faecalis* OG1RF (in figures designated as OG1) is a human oral isolate which is well described in the literature. This strain and two isogenic *E. faecalis* OG1RF deletion mutants, the gelatinase mutant *E. faecalis* TX5264 (in figures designated as gelE$^-$), and the fsrB mutant *E. faecalis* TX5266 (in figures designated as fsrB$^-$) were a generous gift from Michael Gilmore (Sheepens Eye Research Institute, Boston, Massachusetts, USA). The strain *E. faecalis* ms2, is an endogenous isolate derived from an IL-10 -/- mouse. Strain *E. faecalis* hs3 is a strain used as a probiotic which is sold as Symbioflor® in pharmacies.

3.2. Growth curves

Luria Broth (LB) Medium containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl was inoculated with 5% (v/v) of an *E. faecalis* overnight culture and grown aerobically at 37°C on a horizontal shaker. 1ml aliquots were aseptically taken and OD_{600nm} was measured.

3.3. Isolation of DNA from *Enterococcus faecalis*

E. faecalis strains were grown overnight in LB and bacteria were lysed using 0.03 g/ml lysozyme for 18 h at 4° C. Subsequently SDS was added to a final concentration of 2% (w/v) and samples were incubated for 10 min at 60°C. NaCl was added to 1 M. An equal volume of a 1:1 (v/v) mixture of phenol:chloroform was added and after 10 min of mixing samples were centrifuged at 5000g for 10 min (RT). The upper phase was mixed with an equal volume of chloroform for 5 min and centrifuged at 5000g for 10 min (RT). The upper phase was again taken and mixed with a 3 M sodium acetate solution 1/9 (v/v). To precipitate the DNA 2.5 total sample volumes of ice cold 99% ethanol were added, the DNA was pelleted by centrifugation at 5000g for 5 min (RT) and resuspended in 200 µl TE Buffer (10 mM Tris-HCl, 1 mM EDTA, pH8).

3.4. Analysis of *Enterococcus faecalis* virulence genes by PCR.

Presence of *E. faecalis* virulence genes in our strains was investigated by polymerase chain reaction (PCR). The specific primer pairs for the virulence genes (*cylM, cylB, cylA, gelE, esp, efaAfs, agg, cpd, ccf and cob*) were designed as described by Eaton and Gasson[161] and are summarized in Table 4.

For PCR a mixture of 0.5 µl DNA, 0.2U Taq polymerase, 40 pmol of forward and reverse primer, 16 µM dNTP mix, 2.5 µl incubation mix (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.2 mg/mL BSA) and water up to 25 µl was used. Samples were amplified in a thermocycler using the following program: 90 sec initial denaturation at 94°C, 29 cycles consisting of 30 sec denaturation at 94°C, 90 sec of annealing at 54°C, and 45 sec elongation at 72°C followed by a final elongation step at 72°C for 5 min. 1.2% (w/v) agarose was diluted in TBE buffer (890 mM Tris-Base, 890 mM boric acid, 20 mM EDTA) to cast gels, samples were loaded and run at 90 V for 1 h. After staining with ethidium bromide the bands were visualized using a gel imager. In the case of presence of a PCR product the size was compared with the expected size in Table 4.

3.5. Mice, bacterial monoassociation, and histology

Germfree 129 SvEv TAC wild type mice and germfree IL-10 gene-deficient (-/-) 129 SvEv TAC mice (derived by Dr. Edward Balish, University of Wisconsin, Madison, WI) were monoassociated at 12-16 weeks of age with one of the *E. faecalis* strains. In collaboration with Prof. R. Balfour Sartor (University of North Carolina, Chapel Hill, NC) the mice were maintained in the National Gnotobiotic Rodent Resource Center at the University of North Carolina, Chapel Hill. Mice were monoassociated by gavage feeding and rectal swabbing with cultured bacteria. The absence of contamination by other bacterial species was evaluated by plating ten-fold dilution series of cecal content of all mice at necropsy. Animal use protocols were approved by the Institutional Animal Care and Use Committee (IACUC), North Carolina State University. Mice were killed 1 or 14-15 weeks after initial bacterial colonization. Germfree mice were used as controls.

Sections of proximal colon, distal colon or from the cecal tip were fixed in 10% (v/v) neutral buffered Formalin. The fixed tissue was embedded in paraffin. Histology scoring (0-4) was analyzed by blindly assessing the degree of lamina propria mononuclear cell infiltration, crypt hyperplasia, goblet cell depletion, and architectural

distortion in hematoxylin and eosin (H&E) stained sections as described by Berg et al.[122]. A score from 0-1 is regarded as no inflammation, 1-2 as mild, 2-3 as moderate and 3-4 as severe inflammation.

3.6. Isolation of primary mouse epithelial cells

Colon and cecum of germfree as well as monoassociated mice were removed, opened longitudinally, washed in calcium/magnesium free PBS, cut into approx. 5 mm pieces and incubated for 30 min at 37°C in 30 ml DMEM containing 5% (v/v) FBS, 1% (v/v) antibiotic antimycotic solution and 1 mM dithiothreitol (DTT). During incubation samples were vortexed 3 times for 60 sec. After incubation samples were filtered and the filtrate was centrifuged for 7 min at 300 g. The pellet was resuspended in 5 ml DMEM containing 5% (v/v) FBS as well as 1% (v/v) antibiotic antimycotic solution. The pieces remaining on the filter were incubated for 10 min at 37°C in 30 ml calcium/magnesium free PBS containing 1.5 mM EDTA, vortexed for 60 sec before and after incubation and filtered. The filtrate was centrifuged for 7 min at 300 g and the pellet was resuspended in the same 5 ml DMEM already mentioned above. This IEC suspension was filled on top of a 20% (v/v) / 40% (v/v) discontinuous Percoll gradient and centrifuged at 600 g for 30 min to purify and collect primary mouse IEC. Finally, one part of the primary IEC was lysed in Trizol reagent for RNA isolation, the other part in proteome lysis buffer for Western Blotting as well as 2D-Gel electrophoresis and stored at -80°C. Absence of T-cell contamination was assessed by Western Blotting for CD3 to confirm purity of the IEC.

3.7. Bacteria culture conditions

Luria broth medium containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.5% (w/v) NaCl was inoculated with 5% (v/v) of an *E. faecalis* overnight culture and grown aerobically at 37°C on a horizontal shaker. After 24 h, OD_{600nm} was determined with a biophotometer, bacteria were harvested in the late stationary growth phase at 4500 g for 10 min and resuspended in Mode K cell culture medium. Mode K cells were stimulated with *E. faecalis* using an moi of 30 (moi= multiplicity of infection = bacteria to epithelial cell ratio).

3.8. Cell culture and cell stimulation

For Cell culture experiments we used the mouse intestinal epithelial cell line Mode K (a generous gift from Ingo Authenrieth, University of Tübingen, Germany). Mode-K cells are adherent non differentiated cells which were initially generated from enterocytes derived from C3H/HeJ mice and were immortalized by SV40 large T gene transfer [162]. Because of a missense mutation in one of the exons of the TLR-4 gene in C3H/HeJ mice[163], Mode-K cells do not respond to LPS and are therefore not sensitive to LPS contaminations. Mode-K cells (passage 15-35) were cultured in 25 or 75 cm^2 flasks, six-well or 12 well culture plates in a humidified 5% CO_2 atmosphere at 37°C. The culture medium consisted of DMEM (without sodium pyruvate, high glucose, with pyridoxine chloride), supplemented with 10% (v/v) FBS, 2 mM L-glutamine and 0.8% (v/v) antibiotic/antimycotic solution and was replaced every 2-3 days. At sub-confluent density cells were passaged with trypsin-EDTA. Cells were washed twice with 10 ml of pre-warmed PBS. 4 ml trypsin-EDTA solution were added and the cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 min to allow detachment of the cells. After collecting and diluting the cells with 10 ml culture medium they were centrifuged at 300 g for 5 min. The pellet was resuspended in fresh culture medium and the cells were seeded at a cell density of approximately $2x10^5$ cells/ml.

For experiments Mode-K cells were stimulated with bacteria (if not indicated moi 30), with cycloheximide (75 µg/ml), or with epidermal growth factor (EGF) (20 ng/ml) as indicated. The pharmacological inhibitors PD98059 and SB203580 were used in 20 µM final concentration, D-JNK-I-1 in 1 µM final concentration. Pharmacological inhibitors were pre-incubated for 1 h before bacterial stimulation.

3.9. Adenoviral infection

Mode K cells were infected overnight with adenoviral dominant-negative (dn)IKKβ (Ad5dnIKKβ) vector (a generous gift from C. Jobin, University of North Carolina, Chapel Hill, NC) in serum-reduced (2% (v/v)) cell culture medium in the absence of antibiotics at an moi of 50. The adenovirus was removed by washing, and fresh cell culture medium was added. Cells were then stimulated with the *E. faecalis* strains (moi30) for 12 h.

3.10. ELISA analysis

For enzyme linked immuno sorbent assay (ELISA) we used an interferon-gamma inducible protein-10 (IP-10) and an interleukin 6 (IL-6) ELISA Kit (R&D Systems, Minneapolis, USA) according to the manufacturer's protocol. The ELISA assay was performed in 96 well polystyrene microplates.

3.11. RNA isolation and quantitative PCR

Primary IEC from wild type as well as IL-10-/- mice were lysed in Trizol reagent. RNA was isolated according to the manufacturer's protocol and dissolved in 20 µl water containing 0.1% (v/v) diethyl-pyrocarbonate (DEPC). RNA concentration and purity was assessed by measuring the absorption at 260 nm and the absorption ratio A260 nm/A280 nm, respectively.

Transcription to cDNA was performed from 1 µg of total RNA. First 1 µg RNA, 8 µl of 5x first-strand buffer, 4 µl of DTT (100 mM) and 6 µl of desoxyribonucleoside triphosphate mixture (300 mM) were incubated for 5 min at 65 °C. After that 10 µl of a solution containing 0.2 µg of random hexamers, 40 U of RNase Out and 200 U of MMLV-reverse transcriptase were added to every sample. For cDNA generation the mixture was incubated for 60 min at 37°C using a thermocycler, followed by heating to 99°C for 1 min.

Primers were designed with the Light Cycler Probe Design software (Roche Diagnostics, Mannheim, Germany). The nucleotide sequences were derived from the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov). Primer-dimer-formation, self-priming and the primer melting temperature were criteria for the selection of optimal primer pairs. Quantitative PCR was performed using glass capillaries in a Light Cycler system. The Light Cycler-Fast Start DNA Master SYBR Green I kit, 1 µg of cDNA and a total sample volume of 10 µl were used. The qPCR program included an initial activation of the hot-start polymerase at 95°C for 10 min, followed by 50 cycles with 15 sec denaturation at 95°C, 10 sec annealing at 60°C and 20 sec extension at 72°C. The crossing point (Ct) of the log-linear portion of the amplification curve was determined. The relative induction of gene mRNA expression was calculated using the following

equation: $E^{\Delta Ct\ (control\ samples - treated\ samples)}$. Values were normalized to the expression of the house-keeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or 18S ribosomal mRNA.

3.12. Protein from primary IEC

Purified IEC were lysed in a buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, and 2% (v/v) pharmalyte pH 3-10. 1% (w/v) DTT and Complete Mini protease inhibitor were freshly added prior to lysis. Complete Mini protease inhibitor was used in a ratio of 1 tablet for every 12.5 ml of extraction volume. Every sample was subjected to 10 ultrasonic impulses on ice (amplitude 35, cycle 0.5) using an UP200s ultrasonic processor followed by 30 min incubation on ice and 30 min centrifugation at 18 000 g and 4°C for 30 min. Supernatants were stored at -80°C. Protein concentrations were determined using the Bio-Rad protein assay as recommended in the manufacturer's protocol.

3.13. Isoelectric focusing and 2D- SDS PAGE

Immobilized pH gradient (IPG) dry strips (pH 3-10, 18 cm) were rehydrated over night using 350 µl buffer containing 8 M urea, 0.5% (w/v) CHAPS, 15 mM DTT and 0.5% (v/v) pharmalyte pH 3-10. Isoelectric focusing (IEF) was performed with an IPGphor 2, using anodic cup loading of 500 µg protein per IPG strip. IEF conditions were as follows: 500 V (1 min, gradient), 4000 V (1.5 h, gradient), 8000 V (28000 Vh, Step-n-hold).

For the second dimension 12.5% (v/v) SDS-PAGE gels were cast using the following gel-solution: Acrylamide/bisacrylamide (30 % (v/v) /0.8 % (v/v)): 333 ml; Tris-HCL pH 8.8: 200 ml; aqua bidest.: 250 ml; SDS 20% (w/v): 4 ml; APS 10% (w/v): 8 ml; TEMED: 200 µl. Prior to electrophoresis, IPG-stripes were incubated for 15 min in an equilibration buffer (6 M urea, 1.5 M Tris-HCL, pH 8.8, 26% (v/v) glycerol, 2% SDS (w/v)) containing 1% DTT (w/v) and further 15 min in the same buffer containing 4% (w/v) iodacetamide instead of the DTT. IPG stripes were then embedded on top of the SDS-polyacrylamide gels using a 0.5% (w/v) agarose gel solution (25 mM Tris-HCL, 192 mM glycin and 0.1% SDS (w/v)). Electrophoresis was carried out at 4 mA per gel for 1 h followed by 12 mA per gel over night. The SDS running buffer contained 25 mM Tris-HCl, 192 mM glycin and 0.1% (w/v) SDS. For protein staining,

gels were fixed in a solution containing 40% (v/v) ethanol and 10% (v/v) acetic acid for 6 h followed by an over-night exposure to a Coomassie solution containing 10% (w/v) $(NH_4)_2SO_4$, 2% (v/v) phosphoric acid, 25% (v/v) methanol, and 0.625% (w/v) Coomassie brilliant blue G-250. Destaining of the gels was performed in aqua bidest. until the background was completely clear. For all comparisons all gels were simultaneously subjected to all steps of 2D-gel electrophoresis including IEF, SDS-PAGE, Coomassie staining and quantitative analysis in order to minimize variability between gels. Only for the comparison of IEC from IL-10-/- mice monoassociated with the *E. faecalis* isogenic mutants this was not possible because 30 gels were necessary for the experiment.

3.14. Image analysis and mass spectrometry

Coomassie-stained gels were scanned and analyzed by Proteome Weaver software including background subtraction and normalization. Except for the experiments using the *E. faecalis* OG1RF isogenic mutants all proteomic experiments were performed in the same way as published by Shkoda et al.[157] to get comparable results. Reference gels from pooled IEC samples of all 5 mice in the control group were generated and compared with 5 single gels from *E. faecalis*-monoassociated mice using Proteome Weaver Software. Spots with at least two fold differences in protein intensity present in at least 3 out of 5 gels were submitted to MALDI-TOF MS and 4 spots were excised. In the experiments using *E. faecalis* OG1RF and its isogenic mutants for all 5 mice in the reference group and in the compared group 2 separate gels were generated. All 10 gels of every group were compared using proteome weaver software. Spots which were at least present in 18 out of 20 gels, which additionally showed significance ($p<0.05$) using the Mann Whitney U Test and which showed at least 1.5 fold regulation were excised and submitted to MALDI-TOF MS. From the 20 compared gels at least 10 spots were excised for subsequent MALDI-TOF MS. Spots were alternately washed using 50 mmol/L NH_4HCO_3 and a 1:1 mixture of acetonitrile and 50 mmol/L NH_4HCO_3 followed by 100% acetonitrile. Spots were digested with 6 µl of 0.02 µg/µl trypsin at 37°C over night. Protein fragments were spotted on a MTP AnchorChip 600/384 target using the thin-layer affinity HCCA AnchorChip preparation by Bruker Daltonics. Peptide mass fingerprints were generated using an Autoflex 1 mass spectrometer and the Flex Control as well as Flex Analysis software. Proteins were identified by using the

Mascot server 1.9 based on mass searches within murine sequences only. The search parameters allowed the carboxyamidomethylation of cystein and 1 missing cleavage. The mass accuracy was set to 100 ppm. As criterion for the positive identification of proteins we used the significant score value of 61 defined by MASCOT. This score value equals a p value of 0.05 and proteins with a higher score value can be regarded as significantly identified. Only proteins which were at least 3 times identified by MALDI-TOF MS using 3 different spots are listed.

In the proteomic result tables the data is abbreviated in the following way: Molecular mass (Mass), isoelectric point (pI), mean Mascot score of at least three identifications (mean score), standard deviation of the mean Mascot score (SD score), mean sequence coverage of at least three identifications (mean SCov), mean regulation factor according to Proteome Weaver software (mean fold change), standard deviation of the fold change (SD fold change) and the "frequency" of the regulated proteins (= number of mice regulated/number of mice investigated).

3.15. Western Blotting

For SDS-PAGE a Protean Mini cell was used according to the manufacturer's instructions and according to the method provided by Laemmli et al.[164]. Cells were harvested in Laemmli buffer and samples were heated (10 min, 95°C) to denature the proteins. In case of primary IEC equal protein amounts of IEC from 5 equally treated mice were pooled and mixed 1:1 with Laemmli Buffer before use. Stimulated Mode-K cells were lysed in Laemmli and stored at -20°C. 50 µg of protein (primary IEC) or 20 µl protein lysates (Mode K cells) were subjected to 10%-15% (v/v)-acrylamide gels depending on the molecular weight of the protein of interest and electrophoresis was performed at 15 mA until the bromophenol blue dye reached the bottom of the gel. Size was confirmed with the Precision Plus protein dual color standards. Proteins were blotted on PVDF membranes using a Trans Blot SD Semi Dry Transfer Cell (0.2 A for 50 min) and semi-dry blotting buffer (Tris 25 mM, glycine 192 mM, methanol 20% (v/v)). Presence of proteins on the membranes was detected by staining with 0.5% (w/v) ponceau S in 1 % (v/v) acetic acid for 1 minute. Thereafter membranes were rinsed with H_2O followed by Tris buffered saline (TBS: 20 mM Tris, 137 mM NaCl) supplemented with 0.1 % (v/v) tween 20 (TBST). Membranes were blocked with a blocking solution containing 5% (w/v) dry milk in TBST for 1 h at RT on a rocking shaker. All antibodies were diluted in this blocking

solution as shown in Table 3. After binding of the antibody (either 1h at RT or over night at 4°C on a rocking shaker) membranes were rinsed 1 time with TBST and then washed 3 times for 10 min with TBST on a rocking shaker. Anti-rabbit IgG, anti-mouse IgG or anti-goat IgG conjugated with horseradish peroxidase were used as secondary antibodies in a 1:1000 blocking buffer dilution for 1 h at RT on a rocking shaker. Subsequently membranes were rinsed and washed as described above. The proteins were detected using an enhanced chemiluminescence light (ECL) detection kit as recommended by the manufacturer.

3.16. Small interference RNA transfection

Mode-K cells were cultured in 24-well cell culture plates using 400 µl DMEM per well containing 10% (v/v) FBS as well as 0.8% (v/v) antibiotic antimycotic solution and 1% (v/v) L-glutamin. After reaching 50-80% confluency cells were transfected using 10 nM synthetic Alix siRNA (Mm_Pdcd6ip_5_HP or Mm_Pdcd6ip_4_HP) or All Star negative control siRNA and 4.5 µl High Perfect transfection reagent according to the manufacturer's protocol. The Mm_Pdcd6ip_5_HP siRNA resulted in better Alix protein knock down and was used for all experiments. In Figure 28 "Alix si 1" represents an experiment with 1.5 µl High Perfect transfection reagent and "Alix si 2" an experiment using 4.5 µl. After 72 h of transfection cells were stimulated with recombinant mouse epidermal growth factor (EGF) for 5 min or with the *E. faecalis* strains at moi 30 for 1 h. For ELISA cell culture supernatants were taken after 12 h of stimulation and stored at -20°C. For Western Blot cells were lysed in 70 µl Lämmli buffer, stored at -20°C and analyzed as described above.

3.17. Bibliometric analysis

Bibliometric analysis for cocitation was performed using BiblioSphere Pathway Edition Software supplied by Genomatix Software GmbH. This software connects regulated proteins with potential interactions. BiblioSphere software is based on cocitations of genes in PubMed abstracts. All networks shown are based on articles in which the genes/proteins were cocitated in one sentence of an abstract. The same sentence also contained a function word (e.g. regulates, inhibition). Colours represent whether proteins were up-regulated (red colours) or down-regulated (blue

colours) compared to control (either germfree mice or *E. faecalis* OG1RF colonized mice).

3.18. Statistical analyses

Data are expressed as the mean ± SD. Statistical analysis was performed by the two-tailed student's t test. Significance was depicted in the following way: (*) for $p<0.05$; (**) for $p<0.01$.

4. Results

4.1. Interaction of three *Enterococcus faecalis* strains with IEC *in vitro*.

4.1.1. Characterization of the *Enterococcus faecalis* strains

Three *E. faecalis* strains were selected for this work including *E. faecalis* OG1RF (in Figures abbreviated as "OG1") which is a well known clinical isolate widely used and described in the literature. *E. faecalis* ms2 is an endogenous isolate derived from an IL-10 deficient mouse and *E. faecalis* hs3 is a human isolate which is used as a probiotic.

To characterize the selected strains growth curves were recorded and ten fold dilution series of the bacteria were plated on LB agar to determine the CFU/ml culture in the stationary growth phase after 24 h. As shown in Figure 12 all three *E. faecalis* strains showed a logarithmic growth phase lasting for about 3 h followed by a stationary phase with a constant OD_{600nm} value up to 24 h. The logarithmic growth phase of strain OG1RF was slightly delayed. Cell counts of the strains in the stationary growth phase after 24 h were similarly around 8.5 (log10 CFU/ml) (Table 8).

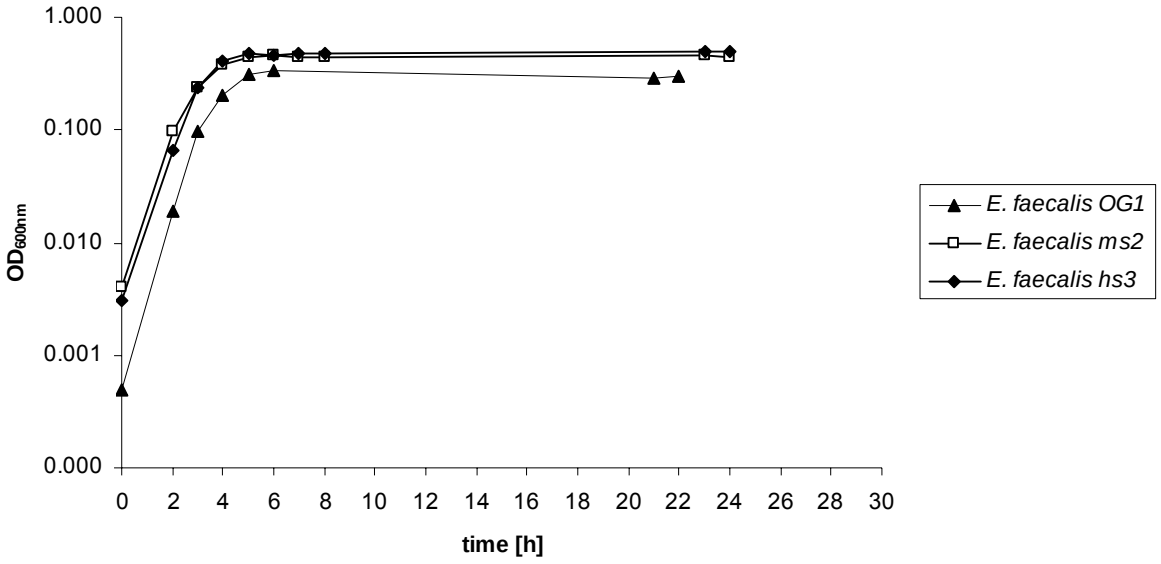


Figure 12: **Growth curves of the selected *Enterococcus faecalis* strains.**

LB Medium was inoculated with 5% of *E. faecalis* overnight cultures and grown aerobically at 37°C on a horizontal shaker. 1 ml aliquots were aseptically taken and OD_{600nm} was measured using a biophotometer. The growth curves are representative for 3 independent experiments.

Table 8: Mean bacterial cell densities after 24h of growth and plating of serial dilutions on LB (n=3).	
E. faecalis strain	log10(CFU/ml)(±SD)
OG1RF	8.51±0.07
ms2	8.54±0.02
hs3	8.41±0.04

Next we investigated the presence of putative virulence genes in our *E. faecalis* strains. Chromosomal DNA was isolated from the *E. faecalis* strains and screened for virulence genes using PCR and specific primers as described by Eaton at al.[161]. In Table 9 the virulence genes of the selected strains are listed. Differences were found for the genes coding for cytolysin (*cyl*ABM), gelatinase (*gelE*) as well as aggregation factor (*agg*). As shown in Table 9 all strains had the genes *cob*, *cpd* and *ccf* which are necessary for pheromone responsive plasmid exchange in *E. faecalis*. None of the strains had the gene *esp*.

Table 9: Presence of putative virulence genes in the selected strains analysed by PCR and specific primers										
E. faecalis strain	Putative virulence genes									
	cylA	*cylB*	*cylM*	*gelE*	*esp*	*efaAfs*	*agg*	*cpd*	*ccf*	*cob*
OG1RF	0	0	0	1	0	1	0	1	1	1
ms2	1	1	1	1	0	1	1	1	1	1
hs3	0	0	0	0	0	1	1	1	1	1

4.1.2. Mode K cells secrete IL-6 and IP-10 and activate different signal transduction pathways in response to the *Enterococcus faecalis* strains.

We used the intestinal epithelial cell line Mode K to investigate strain-specific differences in the interaction of the *E. faecalis* strains with IEC and to characterize the response of IEC to stimulation with the *E. faecalis* strains. Mode K cells are a non carcinoma cell line which originates from C3H/HeJ mice. Since C3H/HeJ mice have a missense mutation in the TLR-4 gene[165], Mode K cells are not sensitive to LPS which is advantageous because LPS frequently contaminates stimulation agents. Furthermore, we knew from our previous study[166] that Mode K cells and also primary IEC derived from germfree Fisher F344 rats secrete the acute phase cytokine IL-6 in

response to the gram positive bacterium *Bifidobacterium lactis.* Therefore, we first investigated whether Mode K cells also secrete IL-6 in response to gram positive *E. faecalis* strains and whether there are any differences between the strains.

In preliminary experiments (data not shown) we used different bacteria to epithelial cell ratios: moi 30, 100 and 300. These experiments demonstrated that Mode K cells secrete IL-6 also in response to *E. faecalis* strains and that a further inflammatory mediator, the T-cell attracting chemokine Interferon γ inducible protein-10 (IP-10), is also secreted (data not shown). However, both, moi 100 and moi 300 led to a pH change in the cell culture medium after 24 h of stimulation, indicating excessive metabolic activity. Therefore, we used moi 30 and moi 3 in our next experiment and also investigated the time dependency of *E. faecalis*-induced IL-6 and IP-10 secretion as well as possible *E. faecalis* strain dependent differences in IL-6 and IP-10 secretion. Figure 13 shows that all *E. faecalis* strains induced a time and dose dependent secretion of IL-6 and IP-10. Cell culture medium did not indicate pH changes for both moi. Therefore, we used moi 30 in all further experiments. Whereas the amount of IL-6 in the culture supernatant did not further increase or only increased slightly after 12 h and 24 h of stimulation, the IP-10 secretion of Mode K cells in response to *E. faecalis* continued for 24 h. The comparison of *E. faecalis* strains showed slight to moderate differences in *E. faecalis*-induced induction of IL-6 and IP-10, especially for the IL-6 secretion induced by *E. faecalis* OG1 compared to the other strains using moi 30 after 6 h and 24 h. However, these differences turned out to be not significant in further experiments (compare with Figure 15).

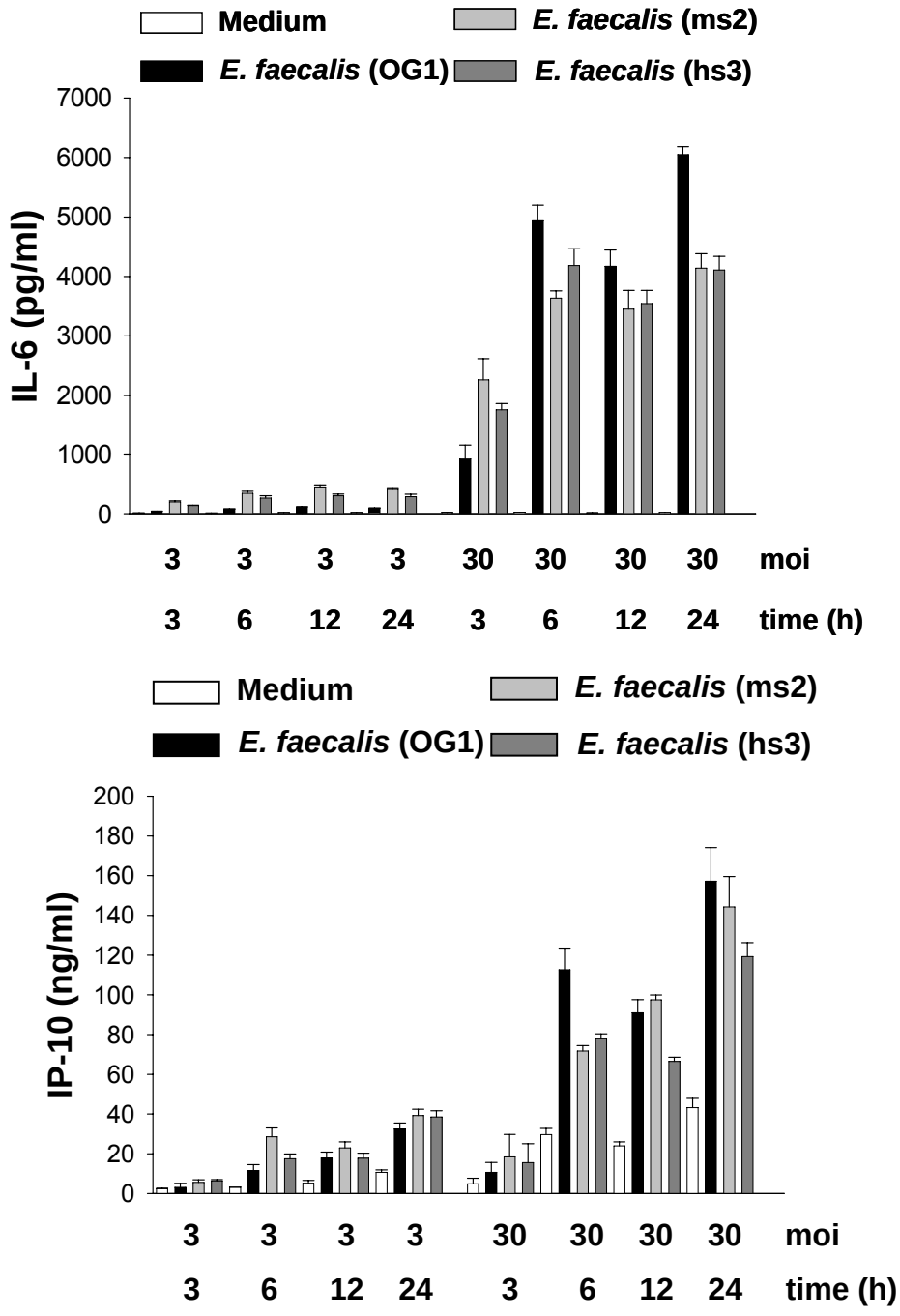


Figure 13: ***Enterococcus faecalis*** **strains induce IL-6 and IP-10 secretion in IEC.**

Mode K cells were stimulated with the *E. faecalis* strains (moi 3 or 30) for 3 h, 6 h, 12 h, or 24 h as indicated. Culture supernatants were analysed by ELISA technique with specific antibodies for IL-6 and IP-10. Results represent the mean ±SD of triplicate samples.

IL-6 and IP-10 are inflammatory mediators which can be regulated by the NFκB and the MAP kinase signal transduction pathways. The MAP kinase pathways are complex signalling pathways which have diverse cellular functions and are capable of activating transcription factors as well as various kinases. Briefly, p38 is implicated in angiogenesis, cell proliferation, inflammation and cytokine production, JNK controls

apoptosis and the development of multiple cell types in the immune system whereas the predominant role of ERK1/2 is proliferation and differentiation[167]. For further reading the reviews by Brown et al. [167], Huang et al. [168] and Katz et al.[169] are suggested. To investigate the activation of these signal transduction pathways in response to the *E. faecalis* strains we stimulated Mode K cells with the *E. faecalis* strains for 1h and 2h and harvested the cells in Lämmli Buffer for Western Blot analysis. The Western Blots showed, for all strains, phosphorylation of the MAP kinases p38 and ERK1/2, of c-jun, which is a subunit of the heterodimeric transcription factor AP1 and is phosphorylated by the MAP kinases JNK, and of RelA, a subunit of the NFκB heterodimer (Figure 14). Of note, stimulation with strain ms2 led to strong ERK1/2 phosphorylation after 1 h whereas stimulation with the other strains did not.

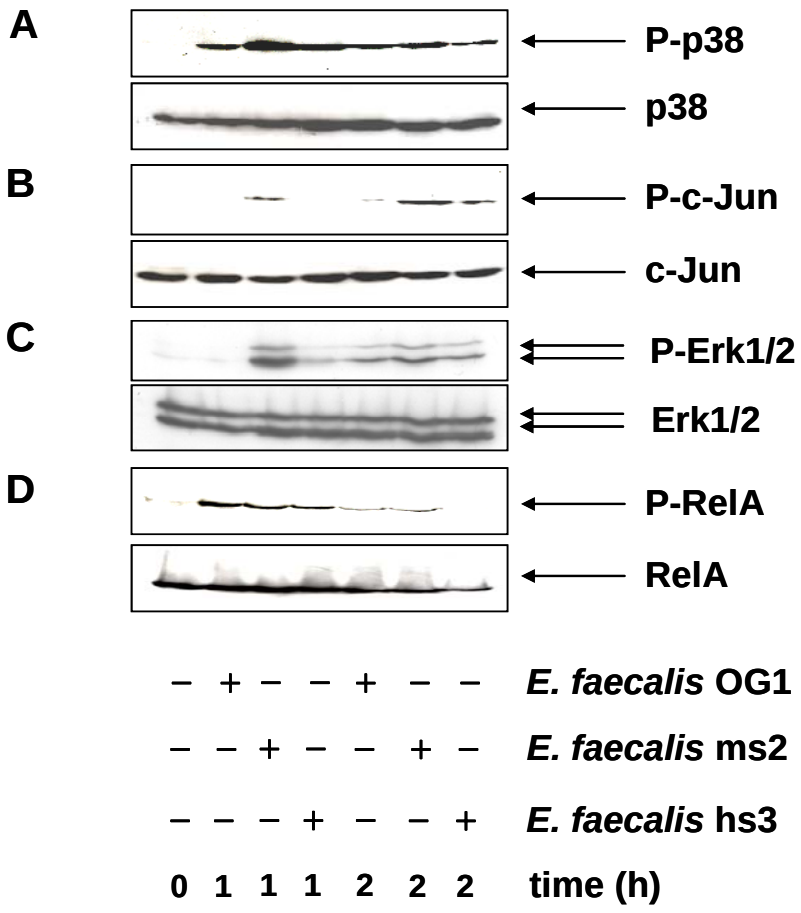


Figure 14: ***Enterococcus faecalis*** **induces activation of MAP-kinases and NFκB (RelA-subunit) in IEC.**

Mode K cells were stimulated with the *E. faecalis* strains (moi 30) for 1 h or 2 h as indicated. Cells were lysed in Lämmli Buffer and analysed by Western Blot with phospho-specific antibodies as described in Materials and Methods.

4.1.3. Impact of NFκB and MAP kinase inhibitors on *Enterococcus faecalis*-induced IL-6 and IP-10 secretion in IEC.

Since *E. faecalis* strains are capable of inducing MAP kinase and NFκB activation, we further used specific inhibitors for the MAP kinase pathways and for the NFκB pathway to evaluate the influence of these pathways for *E. faecalis*-induced IL-6 and IP-10 expression. *E. faecalis*-induced IL-6 and IP-10 expression was found to be dependent on the NFκB and the p38 MAP kinase, but not the JNK MAP kinase pathway (Figure 15). No differences were found comparing the 3 *E. faecalis* strains. Inhibition of the ERK1/2 cascade using the pharmacological inhibitor PD98059 revealed that only *E. faecalis*-induced IL-6 expression but not *E. faecalis*-induced IP-10 expression was strongly attenuated by this inhibitor.

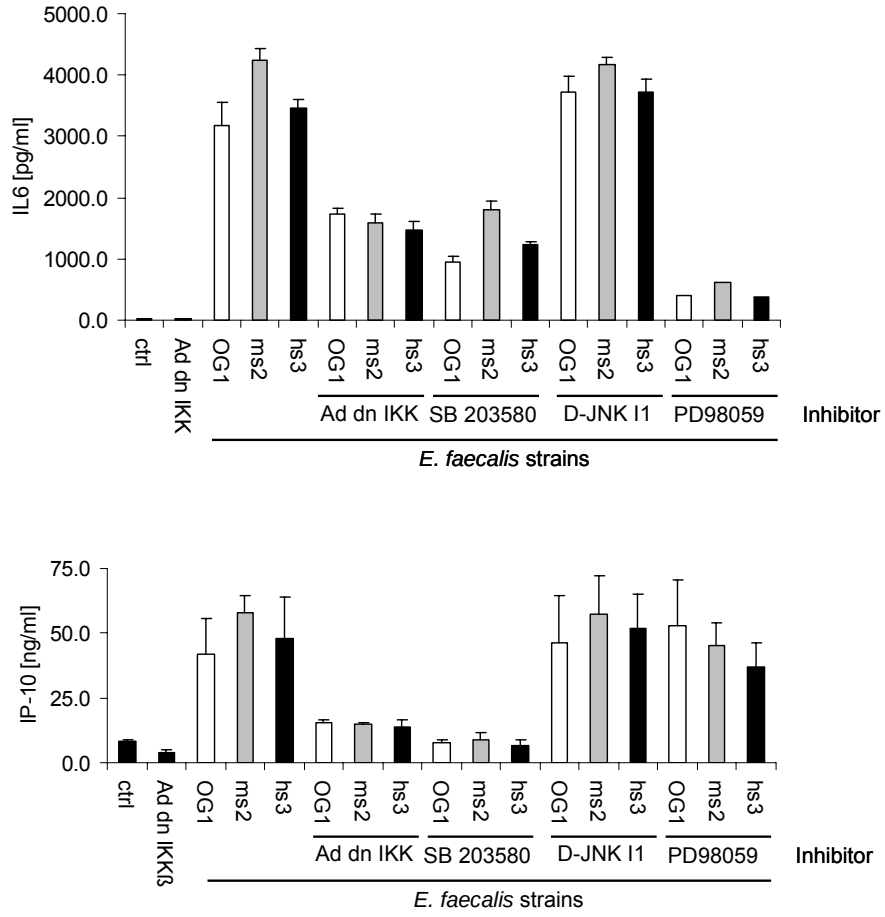


Figure 15: **Impact of NFκB and MAP kinase inhibitors on *Enterococcus faecalis*-induced IL-6 and IP-10 secretion in IEC.**

Mode K cells were infected with Ad dn IKK: Adenovirus dominant negative for IKKβ or pre-treated with the following inhibitors: SB203580: p38 Inhibitor; D-JNKI1: JNK inhibitor; PD98059: Mek1 inhibitor (Erk1/2-cascade). Cells were then stimulated with the *E. faecalis* strains (moi30) for 24 h as indicated. Culture supernatants were analysed by ELISA technique with specific antibodies for IL-6 and IP-10. Results shown represent the mean ±SD of triplicate samples.

4.1.4. All three *Enterococcus faecalis* strains induce IL-6 secretion in myoembryonic fibroblasts cells via toll like receptor-2

Many bacteria have been shown to activate NFκB signalling via toll like receptors (TLR), a group of pattern recognition receptors. Gram positive bacteria are likely to mediate signals via TLR-2. Therefore, we used wild type and TLR-2-/- myoembryonic fibroblasts (MEF) to investigate the role of TLR-2 in mediating *E. faecalis*-induced signals. As shown in Figure 16, IL-6 expression in response to *E. faecalis* in TLR-2-/- MEF cells was completely abrogated compared to wild type MEF demonstrating that the pattern recognition receptor TLR-2 is necessary for *E. faecalis*-induced IL-6 expression. IL-1β-induced IL-6 expression was intact in TLR-2-/- MEF which confirmed responsiveness of the cells.

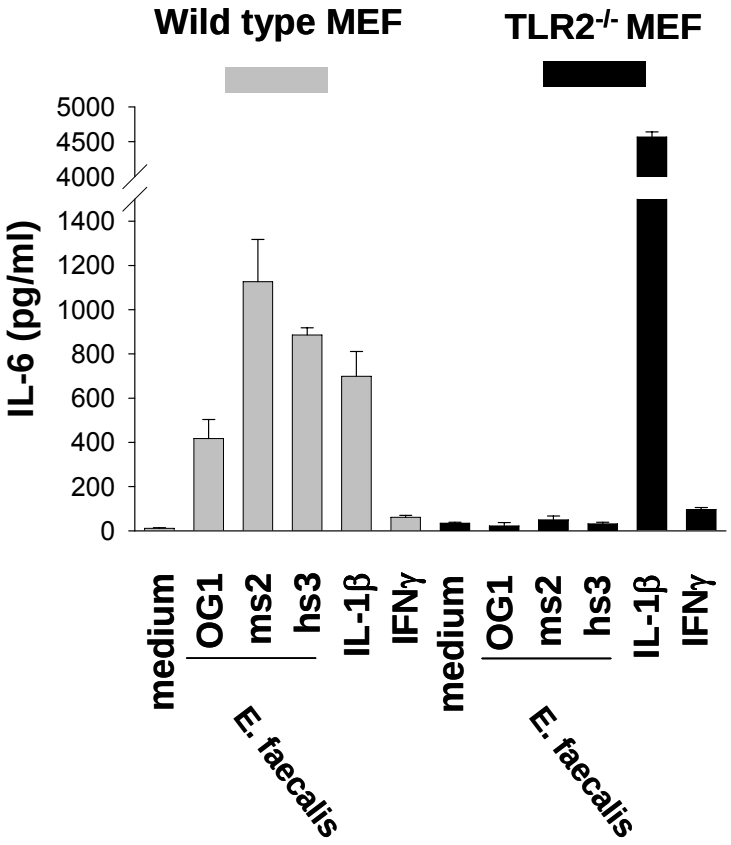


Figure 16: ***Enterococcus faecalis*-induced IL-6 secretion is TLR-2 dependent.**

Wild type MEF cells and TLR-2-/- MEF cells were stimulated with the *E. faecalis* strains (moi 30) or with the cytokines IL-1β or IFNγ (as controls) for 24 h as indicated. Culture supernatants were analysed by ELISA technique for secreted IL-6. Results shown represent the mean ±SD of triplicate samples.

4.2. Interaction of the three *Enterococcus faecalis* strains with IEC *in vivo*.

To investigate the influence of different *E. faecalis* strains on chronic intestinal inflammation and to better understand *E. faecalis* – IEC interaction *in vivo* the germfree IL-10-/- mouse model of chronic experimental colitis was selected. One reason for this was that the complex interaction between a "normal" commensal microbiota or a specific pathogen free (SPF) microbiota and IEC of the host is by far not understood by scientists at the moment - especially not on the level of strain-specific interaction. The big advantage of the germfree approach using monoassociation with single *E. faecalis* strains is that changes in the histopathology of the mice can directly be related to the *E. faecalis* strains used. Therefore, germfree wild type and IL-10-/- mice were monocolonized with the same *E. faecalis* strains which were already characterized *in vitro.* The primary aim of this experiment was to investigate whether different *E. faecalis* strains differ in their ability to induce colitis in IL-10-/- mice (Figure 17).

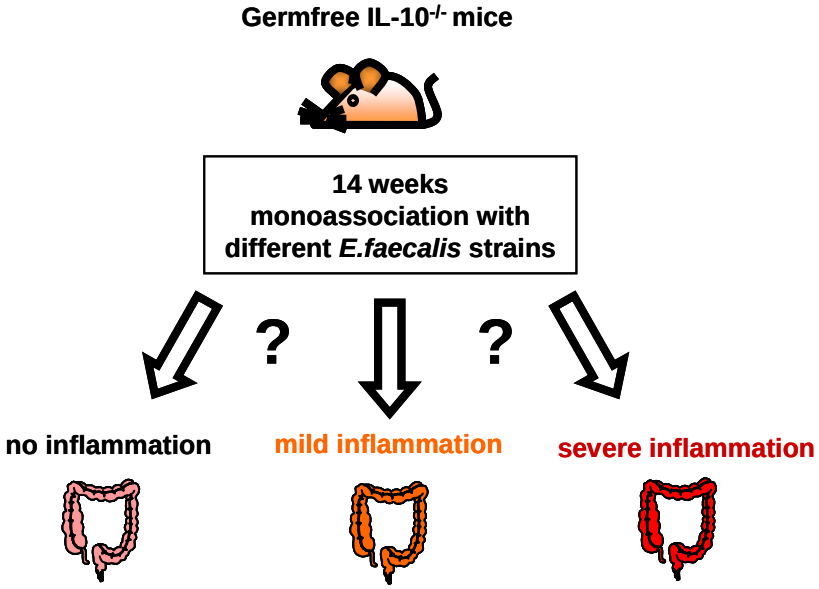


Figure 17: Do all *E. faecalis* strains induce colitis in IL-10-/- mice?

Furthermore, primary epithelial cells were isolated from these mice and RNA as well as protein samples were generated for every mouse as described in Materials and Methods (Figure 18). The luminal bacteria were frozen and bacterial RNA was

isolated and sampled for virulence gene expression by our project collaborators Carrie Hew Ferstl and Rudi Vogel (for results see Table 17).

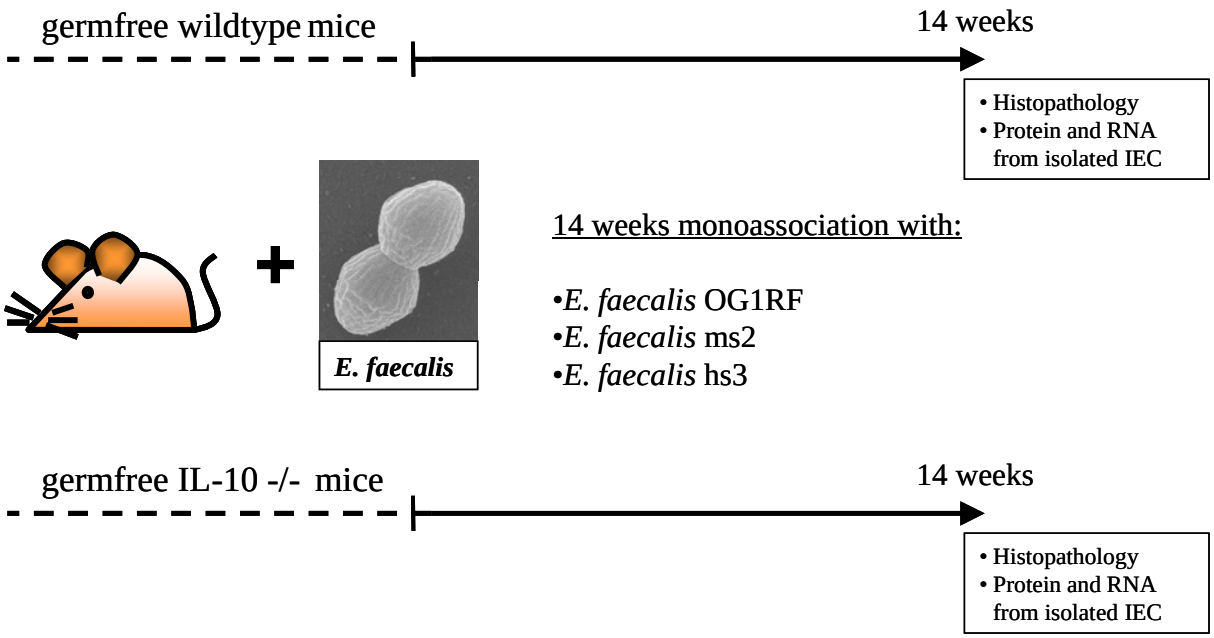


Figure 18: Experimental design.

Wild type and IL-10-/- mice were monoassociated at 12–16 weeks of age (5 mice in each group). Mice were killed 14 weeks after bacterial monoassociation. Blinded histopathological analysis (score 0-4) was performed in formalin-fixed paraffin-embedded tissue sections of the distal colon and the cecum. IEC were isolated for subsequent protein and RNA extraction.

4.2.1. Histopathological analysis revealed significantly different colitis between *Enterococcus faecalis* OG1RF and *E. faecalis* ms2-monoassociated IL-10-/- mice.

To characterize the colitogenic nature of *E. faecalis*, we monoassociated germfree IL-10-/- and wild type mice with the three *E. faecalis* strains. Histopathological analysis of formalin-fixed paraffin-embedded tissue sections from the colon of IL-10-/- mice monoassociated for 14 weeks with *E. faecalis* strain OG1RF (OG1-mice) or with *E. faecalis* strain ms2 (ms2-mice) revealed significant differences in the severity of colonic inflammation (Figure 18). The level of tissue pathology was moderate in ms2-mice (Histology Score: 2.4 ± 0.6; n = 5) compared to severely inflamed OG1-mice (Histology Score: 3.6 ± 0.2; n = 5). Wild type mice were not inflamed demonstrating the non pathogenic nature of the *E. faecalis* strains. Equal colonization was confirmed by plating ten-fold dilution series of cecal contents of all mice (Table 10).

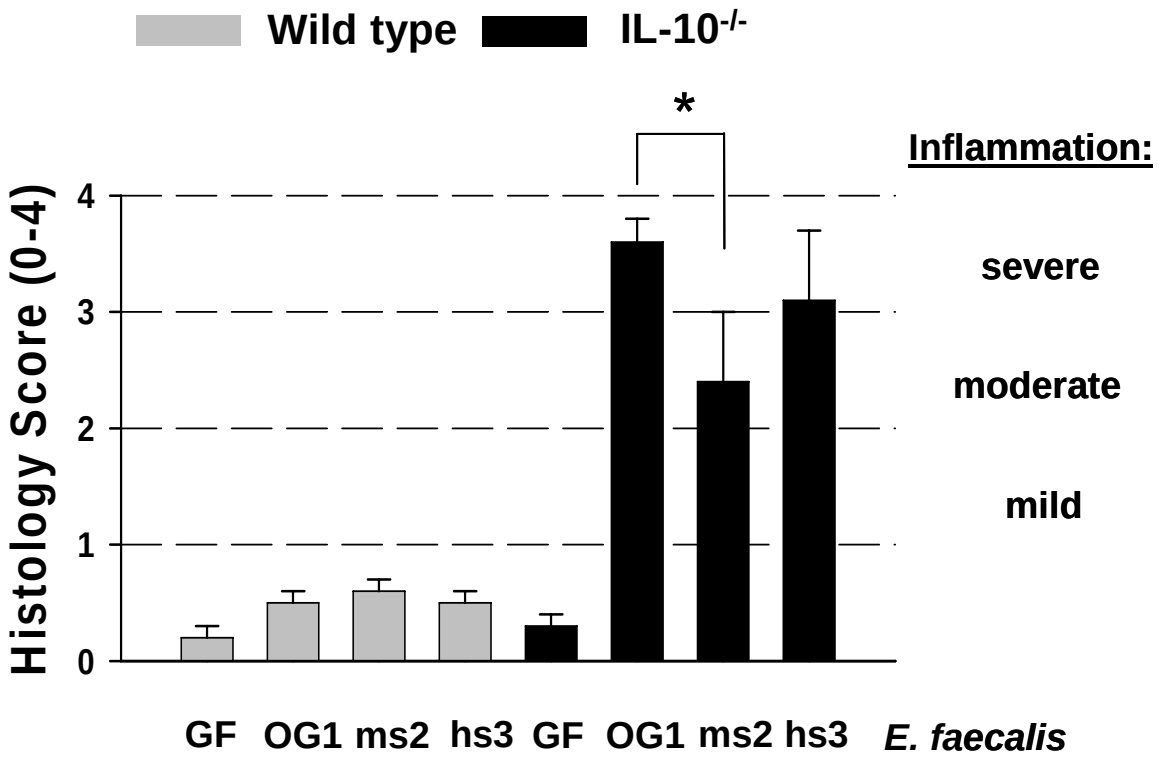


Figure 19 Endogenous *Enterococcus faecalis* ms2 induces significantly less colitis in IL-10-/- mice compared to strain OG1RF.

Germfree wild type and IL-10-/- mice were monoassociated with the *E. faecalis* strains at 12–16 weeks of age (5 mice in each group). Mice were killed 14 weeks after bacterial monoassociation. Blinded histopathological analysis (score 0-4) was performed in formalin-fixed paraffin-embedded tissue sections of the distal colon, as described in Materials and Methods. *p<0.05.

Table 10: Mean bacterial cell densities after plating serial dilutions of cecum content.		
Mice	*E. faecalis* strain	log10(CFU/g)(±SD)
Wild type (n=5)	germfree	-
	OG1RF	9.62±0.05
	ms2	9.67±0.17
IL-10-/- (n=5)	germfree	-
	OG1RF	9.69±0.15
	ms2	9.53±0.22

4.2.2. Differential gene expression of IP-10 in primary IEC from *Enterococcus faecalis*-monoassociated IL-10-/- mice.

The inflammatory chemokine interferon γ inducible protein-10 (IP-10; also referred as CXCL10) is responsible for chemotaxis of activated T-cells to the site of inflammation. Experimental studies in IL10-/- mice demonstrated that the blockade of IP-10 by an anti-IP-10 antibody reduced colitis disease severity[170], supporting the pathological relevance of IP-10 expression for chronic intestinal inflammation. To better understand the role of bacteria-host interactions in *E. faecalis*-mediated experimental colitis, primary IEC were isolated from *E. faecalis*-monoassociated IL-10-/- and wild type mice. Consistent with the level of tissue pathology, IP-10 mRNA expression in IEC from OG1-mice was strongly increased compared to ms2-mice (Figure 20, black bars). Also *E. faecalis* hs3-monoassociated IL-10 -/- mice showed significantly reduced IP-10 mRNA expression. *E. faecalis*-monoassociated wild type mice did not induce high levels of IP-10 mRNA expression in primary IEC (Figure 20, grey bars).

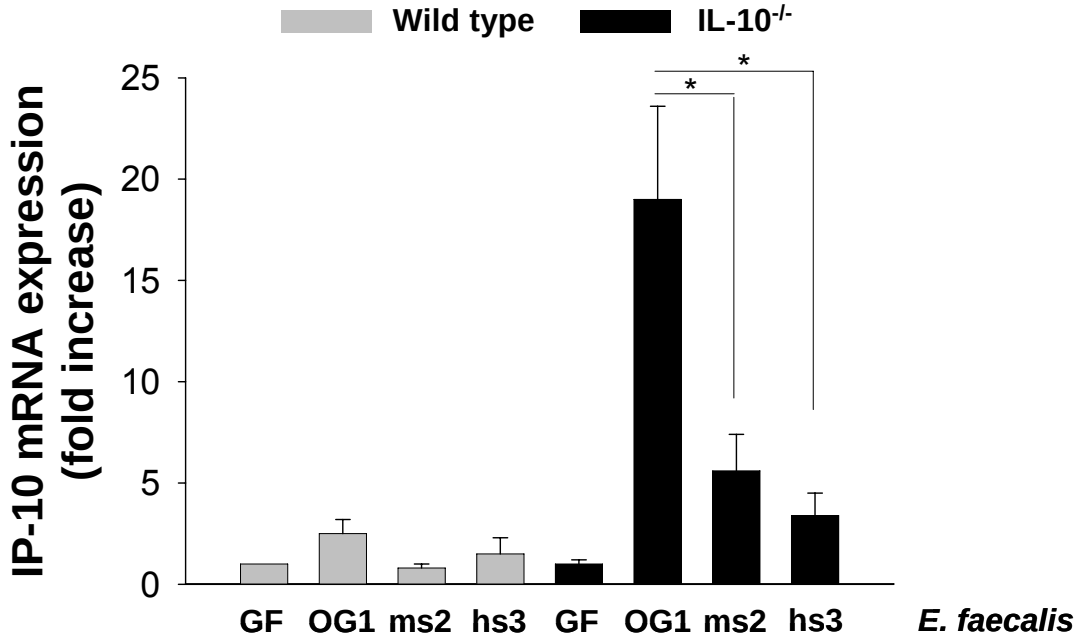


Figure 20: **Epithelial IP-10 mRNA expression is significantly higher in severely inflamed OG1-mice.**

Germfree wild type and IL-10-/- mice were monoassociated with the *E. faecalis* strains at 12–16 weeks of age. Mice (n = 5 in each group) were killed 14 weeks after bacterial monoassociation and primary IEC from large intestine (cecum and colon) were isolated, as described in Materials and Methods. Total RNA was extracted and reverse transcribed into cDNA. Real time PCR was performed using the Light Cycler system with specific primers for murine IP-10 and GAPDH. The induction of IP-10 mRNA was calculated relative to germfree WT controls (mean fold increase ± SD) using the crossing point of the log-linear portion of the amplification curve after normalization with GAPDH. *p<0.05.

4.2.3. MAP kinase activation in primary IEC derived from *Enterococcus faecalis*-monoassociated IL-10-/- mice.

Since *E. faecalis* was able to induce NFκB and MAP kinases *in vitro* in Mode K cells we further investigated the activation of these signal transduction cascades in primary IEC derived from wild type and IL-10 -/- mice. Equal amounts of IEC protein from 5 equally treated mice were pooled and subjected to SDS-PAGE gels. As shown in Figure 21 strain *E. faecalis* hs3 led to strong p38 phosphorylation in wild type mice after 14 weeks of monoassociation. Also c-jun was only significantly phosphorylated in response to *E. faecalis* hs3, but not in response to the other strains.

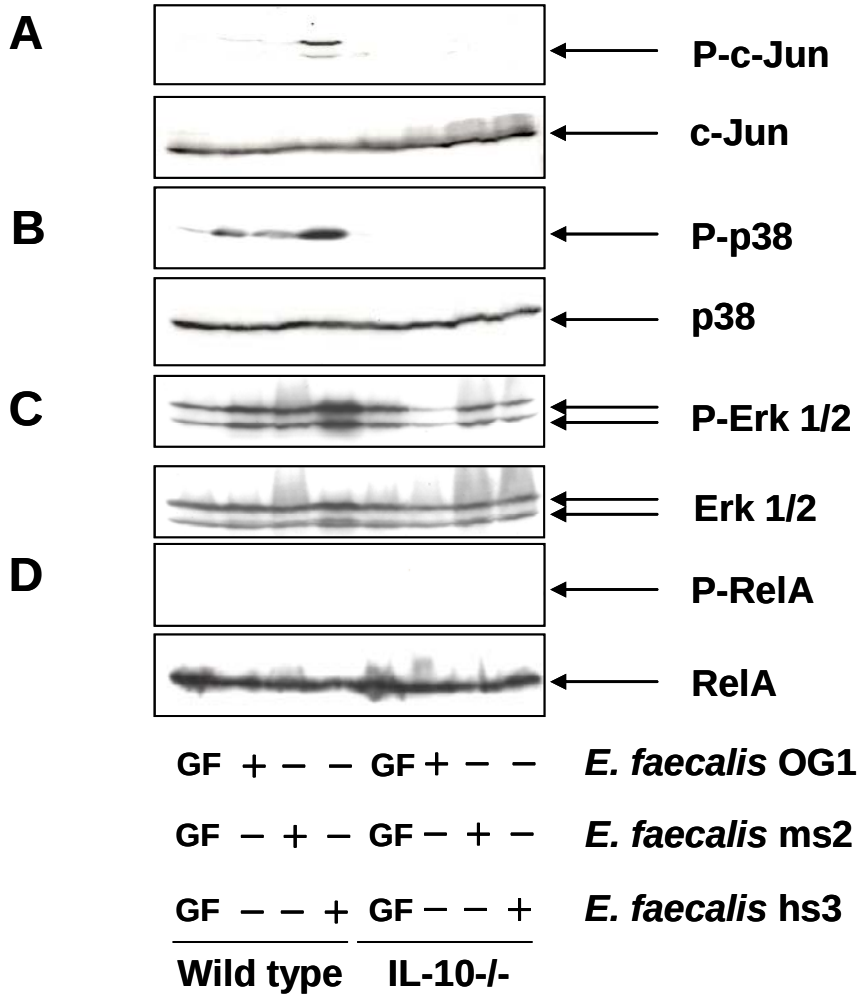


Figure 21: Signal transduction cascades activated by *Enterococcus faecalis* monoassociation of wild type and IL-10-/- mice.

Germfree wild type and IL-10-/- mice were monoassociated with the *E. faecalis* strains at 12–16 weeks of age. Mice (n = 5) were killed 14 weeks after bacterial monoassociation and primary IEC from the large intestine were isolated, as described in Materials and Methods. Protein was isolated from primary IEC and equal amounts of protein from 5 equally treated mice were pooled and subjected to Western Blot analysis with phospho-specific antibodies as described in Materials and Methods.

We were not able to detect phosphorylated c-jun and p38 in IL-10-/- mice and phosphorylated RelA (a NFκB subunit) in wild type and IL-10-/- mice after 14 weeks of monoassociation. In IL-10-/- mice ERK1/2 was found to be phosphorylated after 14 weeks. Of note, phosphorylation of ERK1/2 was lost in the severely inflamed OG1-monoassociated IL-10-/- mice compared to *E. faecalis* ms2 and hs3.

4.2.4. Protein profiling in primary IEC derived from IL-10-/- mice monoassociated with *Enterococcus faecalis* ms2 and *Enterococcus faecalis* hs3.

Previously the effect of *E. faecalis* OG1RF monoassociation on IEC in germfree IL-10-/- mice was investigated in our lab by Anna Shkoda et al. using a proteomic approach [157]. This study revealed increased expression levels of the glucose-regulated ER stress protein (grp)-78 under conditions of chronic inflammation, demonstrating an activated ER stress response in the inflamed epithelium. The results of this proteomic comparison are shown in Table 11.

We therefore investigated IEC derived from *E. faecalis* ms2- and *E. faecalis* hs3-monoassociated IL-10-/- mice in a similar proteomic approach to get comparable results. 2D-PAGE was conducted according to methods developed and provided by Görg et al. and Berkelman et al. [171-173] with minor modifications as described in Material and Methods. Pooled IEC samples from 5 germfree IL-10-/- mice were compared with 5 single *E. faecalis*-monoassociated mice. Isoelectric focusing was performed by using IPG strips with a broad pH gradient from 3 to 10, followed by SDS-PAGE separation of the proteins using 12.5% SDS PAGE gels. Coomassie stained gels were analysed for differentially regulated proteins using the Proteome Weaver software. The fold changes of these proteins were at least two fold different to control intensities (germfree-mice) with a frequency of at least 3 out of 5 mice (referred as frequency in all tables below). Regulated spots were excised from the gels and proteins were identified by matrix assisted laser desorption ionisation – time of flight mass spectrometry (MALDI-TOF MS) as described in Materials and Methods. We identified 10 and 21 differentially regulated proteins in *E. faecalis* ms2- and *E. faecalis* hs3-monoassociated IL-10-/- mice, respectively. Table 12, Table 13,Table 14 and Table 15 summarize molecular mass (Mass), isoelectric point (pI), mean Mascot score (Mean Score), standard deviation of the mean Mascot score (SD Score), mean

sequence coverage (Mean SCov), mean fold change, standard deviation of the fold change (SD Fold Change) and the frequency (number of mice in which the protein was found to be regulated/number of mice investigated) of the regulated proteins. Figure 22, Figure 23 and Figure 24 show the localization of the regulated spots and their regulation.

Table 11: Results published by Shkoda et al.[157]: Differentially regulated proteins in IEC of IL-10-/- mice after 14 weeks of colonization with *Enterococcus faecalis* OG1RF compared to germfree controls.

N°	Gene Name	Accession Number	Name of Protein	Mass [Da] [a]	pI [b]	Mean SCov [%] [c]	Mean Fold Change [d]	SD Fold Change [e]	Frequency [f]
1	Hspa5	A37048	DnaK type molecular chaperone grp78	72491	5.12	27	2.42	0.50	5/5
2	Pcca	Q922N3	Propionyl-CoA-carboxylase, alpha chain	80517	7.00	15	2.73	0.28	3/5
3	Gins1	Q6ZQH1	MKIAA0186 protein	19922	8.65	41	2.74	0.41	3/5
4	Ywhae	S31975	14-3-3 protein epsilon	29326	4.63	24	2.85	0.76	3/5
5	Tubb5	BAB27292	Tubulin beta 5	50064	4.78	28	3.43	0.50	3/5
6	Pkm2	CAA65761	Pyruvate kinase M2	58448	7.18	38	0.34	0.08	5/5
7	Hmgcs2	B55729	Hydroxymethyl-glutaryl-CoA-synthase mitochondrial	53115	7.46	14	0.28	0.08	5/5
8	Ugdh	AAH06749	UDP-glucose dehydrogenase	55482	7.49	37	0.35	0.06	5/5
9	Ckmt1	S24612	Creatine kinase, mitochondrial	47373	8.39	43	0.40	0.06	5/5
10	Serpinb1a	Q9D154	Serin protease inhibitor clade B	42719	5.85	16	0.37	0.08	4/5
11	Mpst	Q99J99	3-mercapto-pyruvate sulfurtransferase	33231	6.11	21	0.40	0.08	4/5
12	Aldh1a1	JQ1004	Aldehyde dehydrogenase NAD precursor	55131	7.89	41	0.30	0.07	3/5
13	Lgals3	A45983	Lactose-binding lectin Mac-2, Galectin-3	27455	8.57	20	0.39	0.01	3/5

[a] Theoretical protein mass according to the Mascot search result. [b] Theoretical isoelectric point of the proteins according to the Mascot search result. [c] Mean sequence coverage of the proteins in % derived from the Mascot search result. [d] Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison. [e] Standard deviation of the mean fold change. [f] Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.

Table 12: Differentially up-regulated proteins comparing IEC derived from germfree IL-10-/- mice (reference) vs. *Enterococcus faecalis* ms2-monoassociated.

N°	Gene Name	Accession Number	Name of Protein	Mass [a] [Da]	pI [b]	Mean Score [c]	SD Score [d]	Mean SCov [%] [e]	Mean Fold Change [f]	SD Fold Change [g]	Fre-quency [h]
1	Psma2	P49722	Proteasome subunit alpha type 2 (EC 3.4.25.1) (Proteasome component C3) (Macropain subunit C3)	25892	8.42	82.8	11.8	41.5	2.39	0.23	5/5
2	Nme1	A46557	nucleoside-diphosphate kinase (EC 2.7.4.6)(fragment)	18843	8.44	88.0	14.4	40.6	2.73	0.57	4/5
3	Hspa5	A37048	dnaK-type molecular chaperone grp78 precursor	72491	5.12	174.8	84.3	34.8	2.50	0.24	5/5
4	Hspd1	Q8C2C7 (S13084)	heat shock protein, 60 kDa (chaperonin groEL precursor) mitochondrial	61089	5.67	123.0	77.8	24.5	3.27	0.70	4/5

[a] Theoretical protein mass according to the Mascot search result. [b] Theoretical isoelectric point of the proteins according to the Mascot search result. [c] Mean Mascot Mowse score derived from the Mascot search result. [d] Standard deviation of the mean Mascot Mowse score. [e] Mean sequence coverage of the proteins in % derived from the Mascot search result. [f] Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison. [g] Standard deviation of the mean fold change. [h] Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.

Table 13: Differentially down-regulated proteins comparing IEC derived from germfree IL-10-/- mice (reference) vs. *Enterococcus faecalis* ms2-monoassociated.

N°	Gene Name	Accession Number	Name of Protein	Mass [a] [Da]	pI [b]	Mean Score [c]	SD Score [d]	Mean SCov [%] [e]	Mean Fold Change [f]	SD Fold Change [g]	Fre-quency [h]
1	Tkt	Q9ESA0	Transketolase (Fragment)	61115	6.54	127.0	31.0	32.0	0.39	0.14	5/5
2	Ugdh	AF061017	UDP-glucose dehydrogenase (Ugdh)	55482	7.49	197.0	45.1	47.8	0.40	0.13	3/5
3	Gpd2	Q8VDT0	Glycerol-3-phosphate dehydrogenase 2	81446	6.26	139.8	17.2	28.0	0.33	0.09	3/5
4	Hmgcs2	Q8N7N8	hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5), mitochondrial	57334	8.53	120	36.0	32.3	0.41	0.06	4/5
5	Eef2	P58252	Elongation factor 2 (EF-2)	96091	6.42	181.5	78.8	24.5	0.35	0.15	3/5
6	Aco2	BC004645	aconitase 2, mitochondrial	86151	8.08	264.5	25.6	41.5	0.36	0.05	3/5

[a] Theoretical protein mass according to the Mascot search result. [b] Theoretical isoelectric point of the proteins according to the Mascot search result. [c] Mean Mascot Mowse score derived from the Mascot search result. [d] Standard deviation of the mean Mascot Mowse score. [e] Mean sequence coverage of the proteins in % derived from the Mascot search result. [f] Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison. [g] Standard deviation of the mean fold change. [h] Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.

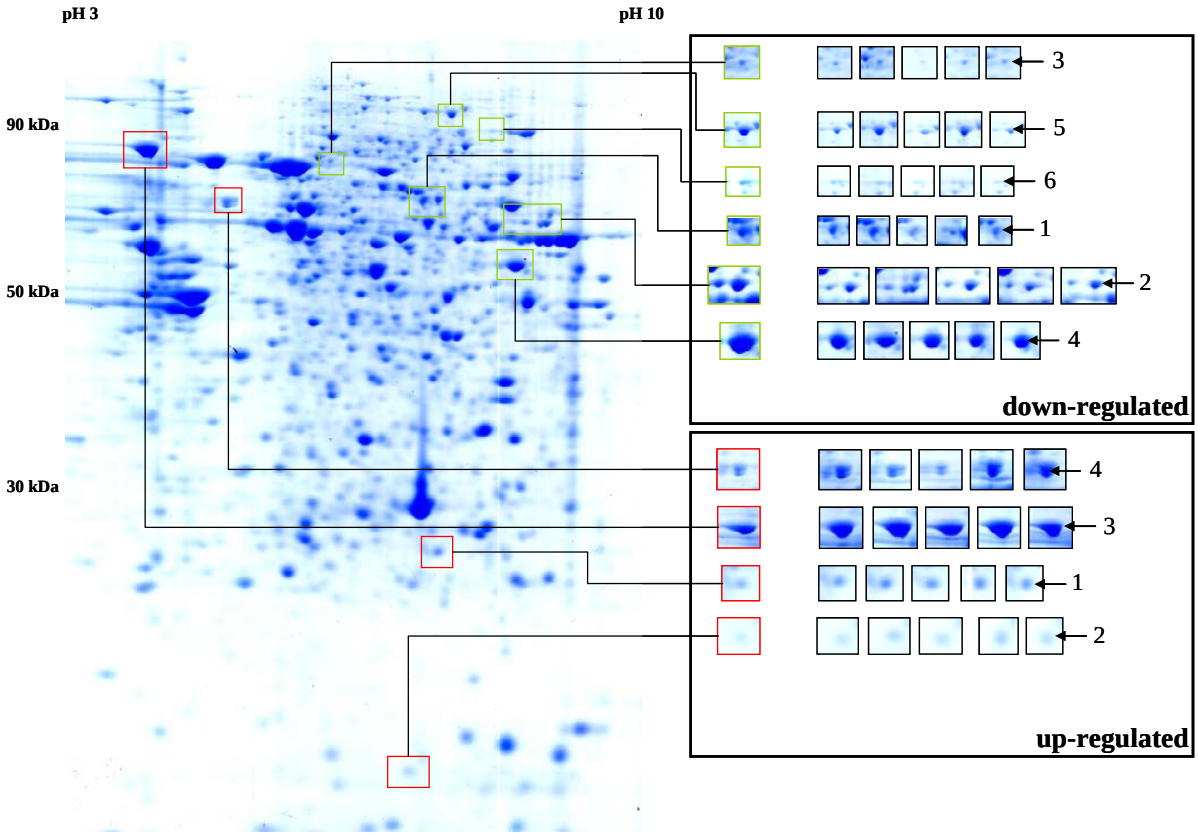


Figure 22: Proteome analysis of primary IEC derived from germfree vs. *Enterococcus faecalis* ms2-monoassociated IL-10-/- mice.
Germfree IL-10-/- mice were monoassociated with *E. faecalis* hs3 for 14 weeks. Primary IEC were purified from germfree and *E. faecalis* hs3-monoassociated animals. 2D SDS-PAGE and proteome analysis was performed as described in Materials and Methods. The reference gel (left side) was obtained with pooled IEC samples from germfree IL-10-/- mice (n=5) and compared with each of the 5 IEC samples from *E. faecalis* ms2-monoassociated mice using the Proteome Weaver software. Localization and regulation of the numbered differentially regulated spots is shown. Identified proteins with at least 2-fold changes in expression level confirmed in at least 3 out of the 5 mice were considered as differentially regulated.

Table 14: Differentially up-regulated proteins comparing IEC derived from germfree IL-10-/- mice (reference) vs. *Enterococcus faecalis* hs3-monoassociated.

N°	Gene Name	Accession Number	Name of Protein	Mass [a] [Da]	pI [b]	Mean Score [c]	SD Score [d]	Mean SCov [%] [e]	Mean Fold Change [f]	SD Fold Change [g]	Fre-quency [h]
Up-regulated Proteins:											
1	Calml4	Q8R1P1	Calmodulin-like 4	17783	7.7	76.5	29.2	37.5	3.21	0.64	5/5
2	Hspa5, Grp78	A37048	dnaK-type molecular chaperone grp78 precursor	72491	5.1	330.7	85.1	47.0	3.41	0.31	5/5
3	Tpt1, Trt	S00775	Translationally controlled tumor protein (TCTP), also known as IgE-dependent histamine-releasing factor (HRF)	19564	4.8	73.0	10.8	26.0	3.06	0.65	5/5
4	Aco2	BC004645	Aconitate hydratase, mitochondrial precursor (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase)	86151	8.1	125.3	50.8	25.0	2.51	0.78	3/5
5	Mrps22	AK013925	mitochondrial ribosomal protein S22	41281	8.6	91.0	36.0	33.9	2.40	0.82	3/5
6	Bcat2	BC017688	branched chain amino-transferase 2, mitochondrial	44669	8.6	80.3	23.7	25.8	2.06	0.52	3/5
7	Nucb1	AK004886	nucleobindin	53376	5	129.3	46.3	42.0	3.63	1.21	3/5
8	Pdia3, Grp58	Q8C2F4; M73329	glucose regulated protein, 58 kDa, Protein disulfide-isomerase A3 precursor (EC 5.3.4.1)	57103	5.8	218.6	65.4	47.8	2.35	0.57	4/5
9	Golph3l	Q8R088	Golgi phosphoprotein 3-like	32999	5.7	104.0	27.0	35.2	2.58	0.65	4/5
10	G6pdx	A56686; S17293; S31406	glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)	59681	6.0	212.3	54.4	42.5	4.16	1.24	5/5

[a] Theoretical protein mass according to the Mascot search result. [b] Theoretical isoelectric point of the proteins according to the Mascot search result. [c] Mean Mascot Mowse score derived from the Mascot search result. [d] Standard deviation of the mean Mascot Mowse score. [e] Mean sequence coverage of the proteins in % derived from the Mascot search result. [f] Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison. [g] Standard deviation of the mean fold change. [h] Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.

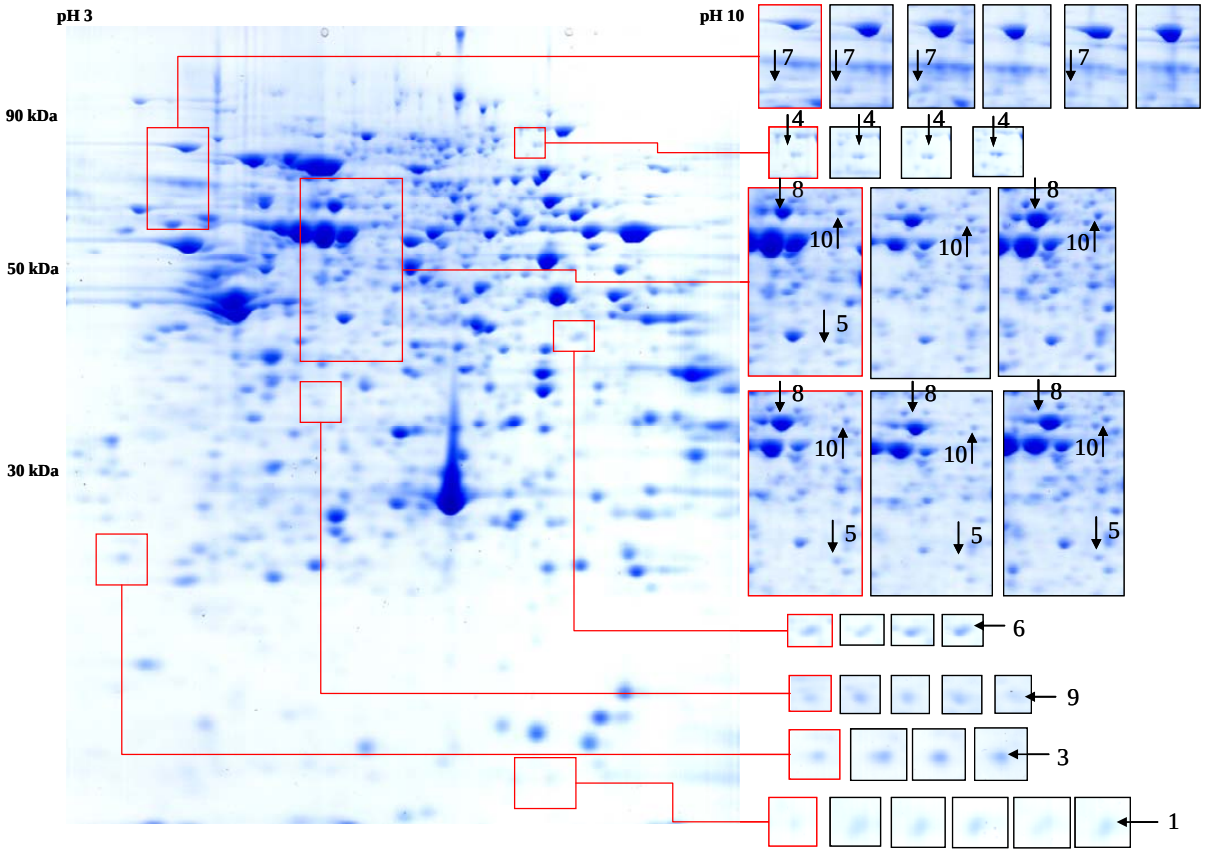


Figure 23: Proteome analysis of primary IEC derived from germfree vs. *Enterococcus faecalis* hs3-monoassociated IL-10-/- mice – up-regulated proteins.
Germfree IL-10-/- mice were monoassociated with *E. faecalis* hs3 for 14 weeks. Primary IEC were purified from germfree and *E. faecalis* hs3-monoassociated animals. 2D SDS-PAGE and proteome analysis was performed as described in Materials and Methods. The reference gel (left side) was obtained with pooled IEC samples from germfree IL-10-/- mice (n=5) and compared with each of the 5 IEC samples from *E. faecalis* hs3-monoassociated mice using the Proteome Weaver software. Localization and regulation of the numbered differentially up-regulated spots is shown. Identified proteins with at least 2-fold changes in expression level confirmed in at least 3 out of the 5 mice were considered as differentially regulated.

Table 15: Differentially down-regulated proteins comparing IEC derived from germfree IL-10-/- mice (reference) vs. *Enterococcus faecalis* hs3-monoassociated.

N°	Gene Name	Accession Number	Name of Protein	Mass [a] [Da]	pI [b]	Mean Score [c]	SD Score [d]	Mean SCov [%] [e]	Mean Fold Change [f]	SD Fold Change [g]	Fre-quency [h]
Down-regulated Proteins:											
1	Glod4	Q9CPV4	Glyoxalase domain-containing protein 4	33581	5.28	165.67	48.63	52.3	0.33	0.07	5/5
2	Hmgcs2	B55729	hydroxymethylglutaryl-CoA synthase (EC 4.1.3.5), mitochondrial	53115	7.46	201.25	42.45	47.25	0.49	0.13	3/5
3	Anxa4	Q7TMN7	Annexin A4	36192	5.4	180.50	63.92	50.0	0.56	0.33	3/5
4	Ak2	Q9WTP6; Q9CY37;	Adenylate kinase isoenzyme 2, mitochondrial (EC 2.7.4.3) (ATP-AMP transphosphorylase)	25686	7.2	75.00	16.46	37.2	0.39	0.05	5/5
5	Prdx6	O08709; Q91WT2; Q9QWP4; Q9QWW0	Peroxiredoxin 6 (EC 1.11.1.15) (Antioxidant protein 2) (1-Cys peroxiredoxin) (1-Cys PRX) (Acidic calcium-independent phospholipase A2) (EC 3.1.1.-) (aiPLA2)(Non-selenium glutathione peroxidase) (EC 1.11.1.7) (NSGPx)	24838	5.7	150.67	34.41	60.4	0.45	0.05	5/5
6	Hao3	Q8JZR9	Hydroxyacid oxidase (Glycolate oxidase) 3	39145	7.6	66.60	13.28	27.0	0.36	0.11	4/5
7	Ugdh	AF061017	UDP-glucose dehydrogenase (Ugdh)	55482	7.49	148.17	63.70	37.5	0.40	0.07	5/5
8	Aldh1a1	JQ1004	Retinal dehydrogenase 1 (EC 1.2.1.36)	55131	7.89	185.00	55.13	42.0	0.46	0.09	3/5
9	Mpst	Q99J99	3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) (MST)	33100	6.12	159.00	20.66	51.3	0.46	0.09	3/5
10	Aldh2	I48966	aldehyde dehydrogenase (NAD) (EC 1.2.1.3) 2 precursor, mitochondrial	57015	7.53	130.75	47.54	33.0	0.43	0.09	4/5
11	Eef2	P58252	Elongation factor 2 (EF-2)	96091	6.42	148.25	34.54	21.8	0.77	0.43	3/5

[a] Theoretical protein mass according to the Mascot search result. [b] Theoretical isoelectric point of the proteins according to the Mascot search result. [c] Mean Mascot Mowse score derived from the Mascot search result. [d] Standard deviation of the mean Mascot Mowse score. [e] Mean sequence coverage of the proteins in % derived from the Mascot search result. [f] Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison. [g] Standard deviation of the mean fold change. [h] Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.

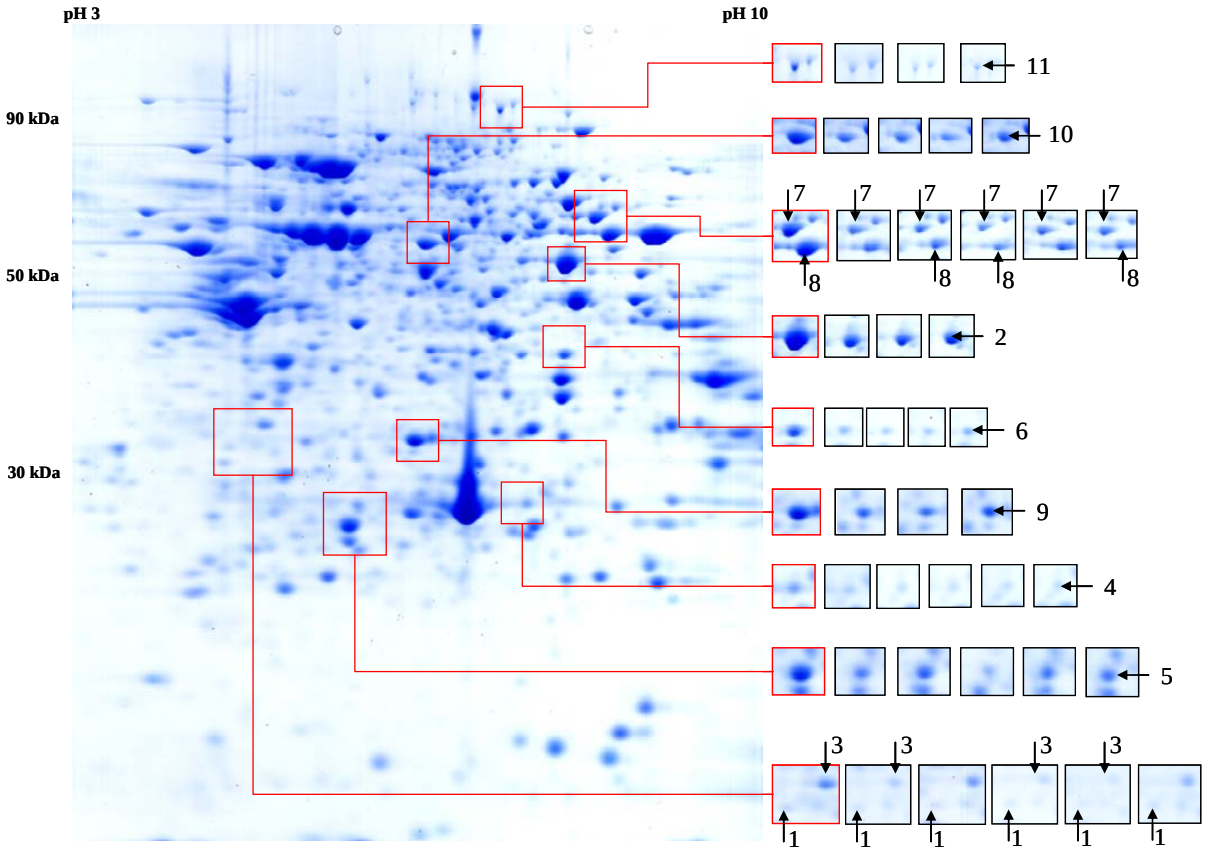


Figure 24: Proteome analysis of primary IEC derived from germfree vs. *Enterococcus faecalis* hs3-monoassociated IL-10-/- mice – down-regulated proteins.
Germfree IL-10-/- mice were monoassociated with *E. faecalis* hs3 for 14 weeks. Primary IEC were purified from germfree and *E. faecalis* hs3-monoassociated animals. 2D SDS-PAGE and proteome analysis was performed as described in Materials and Methods. The reference gel (left side) was obtained with pooled IEC samples from germfree IL-10-/- mice (n=5) and compared with each of the 5 IEC samples from *E. faecalis* hs3-monoassociated mice using the Proteome Weaver software. Localization and regulation of the numbered differentially down-regulated spots is shown. Identified proteins with at least 2-fold changes in expression level confirmed in at least 3 out of the 5 mice were considered as differentially regulated.

Interestingly, we were able to identify the glucose-regulated ER stress protein grp78 to be up-regulated also in *E. faecalis* ms2- and *E. faecalis* hs3-monoassociated IL-10-/- mice compared to germfree animals. Furthermore, the proteins hydroxymethylglutaryl-CoA synthase and UDP-glucose dehydrogenase were found to be down-regulated for all three *E. faecalis* strains, indicating general *E. faecalis*-induced mechanisms in monoassociated IL-10-/- mice.

4.2.5. Bibliometric analysis

BiblioSphere software was used to connect regulated proteins with potential interactions. BiblioSphere software is based on cocitations of genes in PubMed abstracts. For the network tree shown in Figure 25 only genes cocitated in the same sentence of abstracts which also contained a function word (e.g. regulates, inhibition) are shown. Red colours represent up-regulation and blue colours down-regulation compared to control (germfree mice in this case).

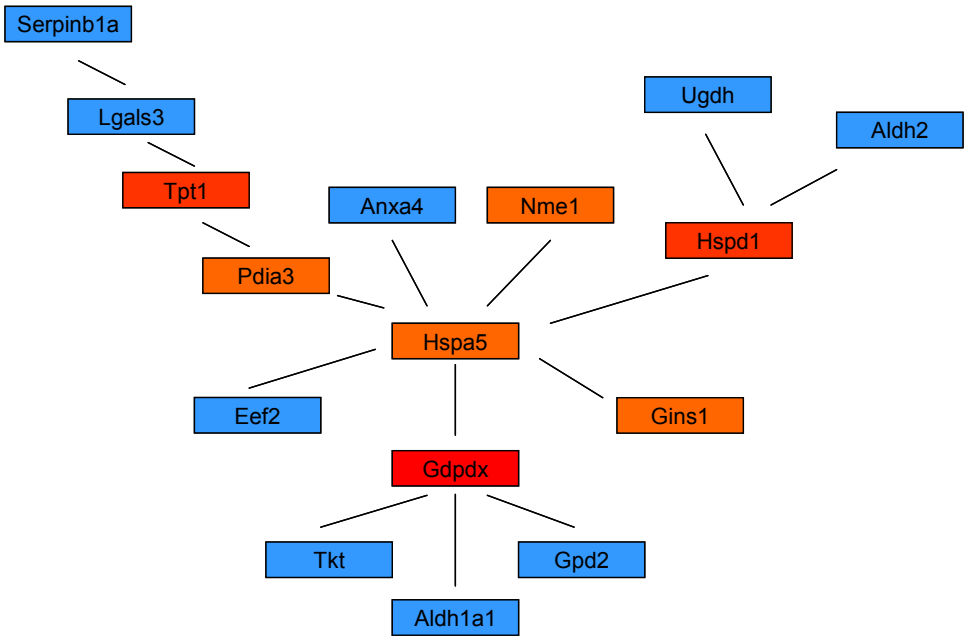


Figure 25: Gene-gene network tree based on regulated proteins between germfree and *Enterococcus faecalis*-monoassociated IL-10-/- mice and bibliometric analysis.
Connections represent cocitations in PubMed abstracts in the same sentence including a function word (e.g. regulates, interacts etc.). Red colour indicate up-regulation, blue colour down-regulation compared to germfree mice. Aldh1a1: Retinal dehydrogenase 1; Aldh2: Aldehyde dehydrogenase, mitochondrial; Anxa4: Annexin A4; Eef2: Elongation factor 2; G6pdx: Glucose-6-phosphate 1-dehydrogenase x; Gins1: MKIAA0186 protein; Gpd2: Glycerol-3-phosphate dehydrogenase, mitochondrial; Hspa5: 78 kDa glucose-regulated protein (grp78); Hspd1: 60 kDa heat shock protein, mitochondrial; Nme1: Nucleoside diphosphate kinase A; Pdia3: Protein disulfide-isomerase A3; Serpinb1a: Serin protease inhibitor clade B = Leukocyte elastase inhibitor A; Tkt: Transketolase; Tpt1: Translationally-controlled tumor protein; Lgals3: Lectin, galactose binding, soluble 3; Ugdh: UDP-glucose 6-dehydrogenase;

4.2.6. Comparison of IL-10 -/- mice monoassociated with the differential colitis inducing *Enterococcus faecalis* strains OG1RF and ms2.

In a further analysis we compared the differential colitis inducing *E. faecalis* strains OG1RF and ms2 in order to investigate mechanisms induced in IEC. Again

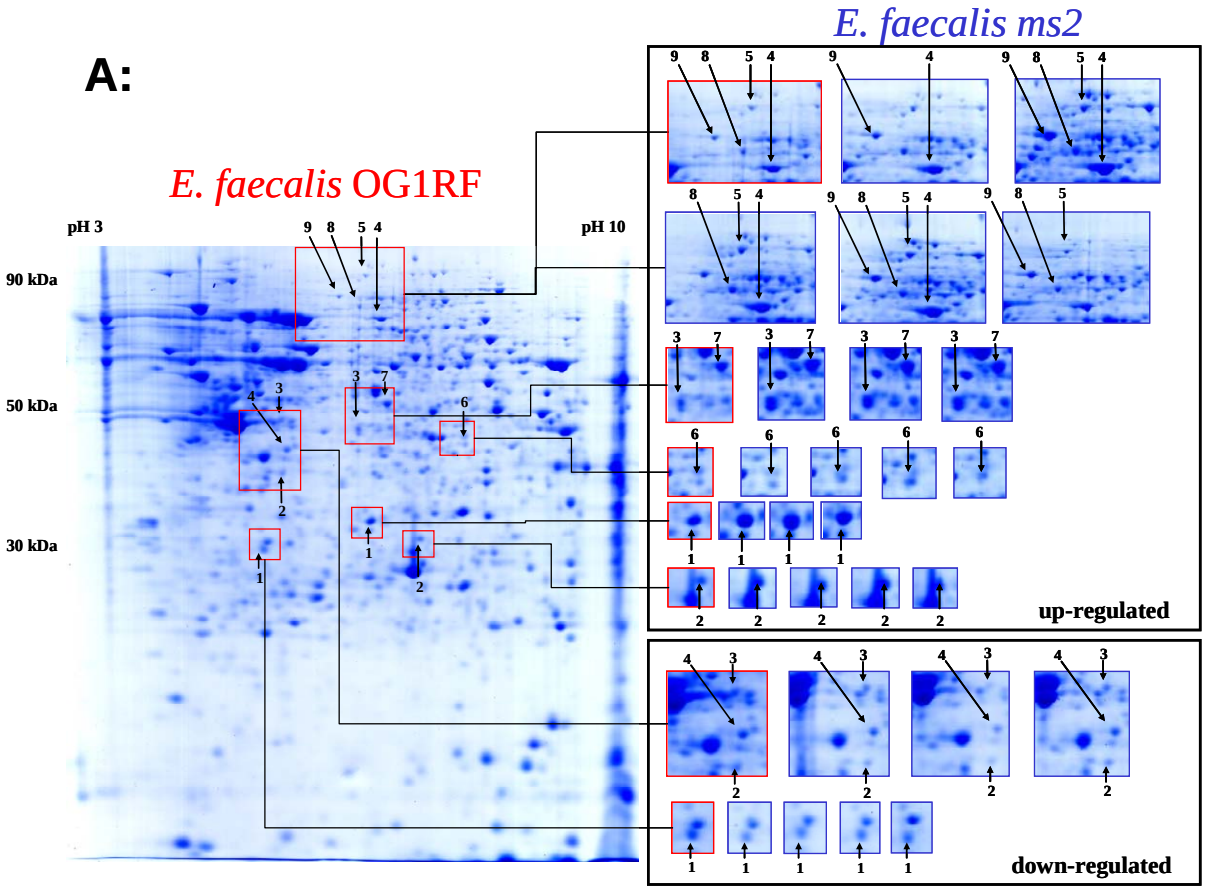
proteomic technique was used to identify novel protein targets in the intestinal epithelium associated with the disease progression from moderately to severely inflamed conditions. To compare protein expression profiles in IEC from OG1-mice and ms2-mice we performed 2D SDS-PAGE gel electrophoresis, Coomassie staining, Proteome Weaver analysis and MALDI-TOF mass spectrometry as described in Materials and Methods. Reference gels from pooled IEC samples of all 5 OG1-mice were generated and compared with 5 gels from single ms2-mice using Proteome Weaver Software. Between 480 and 550 spots were detected in the single gels. We were able to identify 13 differentially regulated proteins (Table 16, Figure 26). The fold change of these proteins was at least two fold different to control intensities (OG1-mice). None of these 13 proteins was found to be regulated in *E. faecalis*-monoassociated wild type mice (Figure 26B).

Table 16: Differentially regulated proteins for the primary IEC proteomic comparison of *Enterococcus faecalis* OG1RF (Reference) vs. *Enterococcus faecalis* ms2

	Gene Name	Accession Number	Name of Protein	Mass [a] [Da]	pI [b]	Mean Score [c]	SD Score [d]	Mean SCov [%] [e]	Mean Fold Change [f]	SD Fold Change [g]	Fre-quency [h]
Up-regulated Proteins:											
1	Mpst	Q99J99	3-mercaptopyruvate sulfurtransferase (EC 2.8.1.2) (MST)	33100	6.12	120.0	17.8	41.8	2.53	0.38	3/5
2	Gsto1	AK027922	Glutathione S-transferase omega 1	27708	6.92	115.3	12.1	37.5	2.92	0.54	4/5
3	Dnajb11	Q99KV1	DNAJ homolog subfamily B member 11 (Erdj3)	40853	5.81	103.0	24.6	31.7	2.29	0.21	3/5
4	Ugdh	AF061017	UDP-glucose dehydrogenase (Ugdh)	55482	7.49	134.2	30.9	41.25	4.07	1.71	4/5
5	Pdcd6ip	Q80Y09	Alix (ALG-2 interacting protein (AIP-1)) (Pdcd6ip-protein)	96822	6.15	229.5	64.0	31.5	3.02	0.33	4/5
6	Ppid	Q9CR16	40 kDa peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8) (PPIase) (Rotamase) (Cyclophilin-40) (CYP-40)	40985	7.05	117.8	21.1	38.0	3.94	0.78	4/5
7	Tufm	Q8BFR5	Elongation factor TU, mitochondrial (P43) homolog	49876	7.23	124.5	7.7	36.0	4.92	1.93	3/5
8	Pcca	Q922N3	Propionyl-Coenzyme A carboxylase, alpha polypeptide	80517	7.00	150.0	25.6	33.0	4.62	1.69	4/5
9	Vil2	P26040	Ezrin (p81) (Cytovillin) (Villin 2)	69407	5,83	87,7	18,7	20,0	2,85	0,91	3/5
Down-regulated Proteins:											
1	Phb	A39682	Prohibitin	29859	5.57	116.3	4.7	53.8	0.344	0.053	4/5

2	Capza2	P47754	F-actin capping protein alpha-2 subunit (CapZ alpha-2)	32987	5.58	113.7	17.0	57.3	0.283	0.145	3/5
3	Suclg2	Q66JT3	Succinate-Coenzyme A ligase, GDP-forming, beta subunit mitochondrial [precursor]	47080	6.58	80.3	18.1	32.0	0.261	0.119	3/5
4	Idh3a	Q8C8A1	Isocitrate dehydrogenase [NAD] subunit alpha, mitochondrial [Precursor]	40099	6.27	80.5	17.0	32.8	0.356	0.126	3/5

[a] Theoretical protein mass according to the Mascot search result. [b] Theoretical isoelectric point of the proteins according to the Mascot search result. [c] Mean Mascot Mowse score derived from the Mascot search result. [d] Standard deviation of the mean Mascot Mowse score. [e] Mean sequence coverage of the proteins in % derived from the Mascot search result. [f] Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison. [g] Standard deviation of the mean fold change. [h] Frequency: number of mice in which the protein was differentially regulated/number of mice investigated.



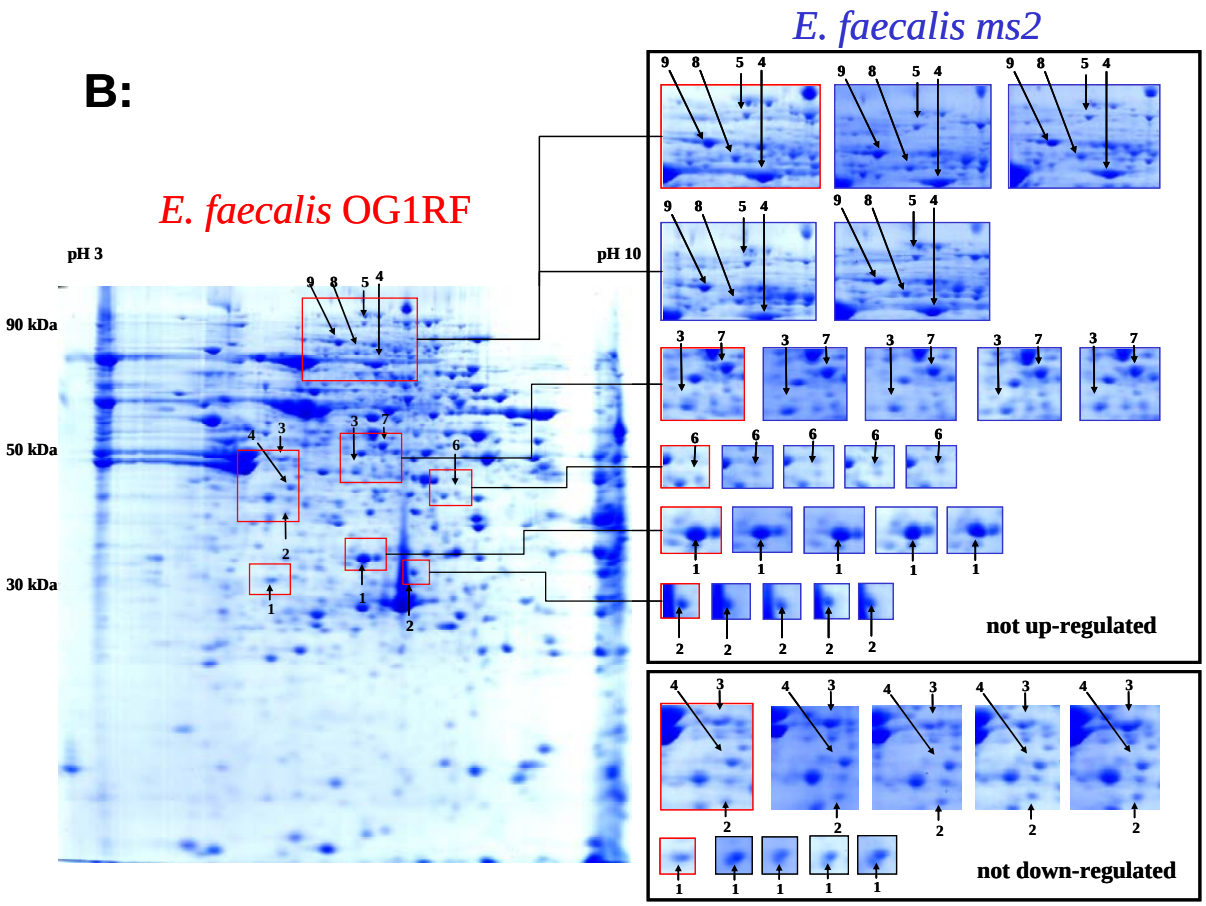


Figure 26: Proteome analysis of IEC derived from *Enterococcus faecalis* OG1RF vs. *Enterococcus faecalis* ms2-monoassociated wild type and IL-10-/- mice.

Germfree IL-10-/- mice (A) and wild type mice (B) were monoassociated either with *E. faecalis* OG1RF (reference) or with *E. faecalis* ms2 for 14 weeks. Primary IEC were purified for 2D SDS-PAGE and proteome analysis was performed as described in Materials and Methods. The reference gel (left side) was obtained with pooled IEC samples from *E. faecalis* OG1RF-monoassociated mice (n=5) and compared with each of the 5 IEC samples from *E. faecalis* ms2-monoassociated mice using the Proteome Weaver software. Localization and regulation of the numbered differentially regulated spots is shown. Identified proteins with at least 2-fold changes in expression level confirmed in at least 3 out of 5 mice were considered as differentially regulated.

4.3. Validation of Alix down-regulation and characterization of Alix function in IEC.

Proteomic analysis revealed reduced Alix expression levels under conditions of severe but not moderate inflammation and Alix was found to be regulated in 4 out of 5 investigated mice. Alix is a multifunctional adaptor protein which can interact with various cytoskeletal elements like focal adhesion kinase [174], F-actin, cortactin and α-actinin [175]. Since also other cytoskeleton proteins were found to be regulated and Alix

therefore might be important in cellular restitution and wound healing processes we further investigated Alix regulation in primary IEC and in IEC lines.
In Figure 27A individual representative Alix 2D-gel spots are shown. Alix 2D-gel spot intensity was only reduced in *E. faecalis* OG1RF-monoassociated IL-10-/- mice but not in *E. faecalis* OG1RF-monoassociated wild type mice, suggesting a role of Alix in the development of chronic intestinal inflammation. Therefore, we next investigated the differential regulation of Alix protein by Western Blot analysis and characterized Alix function in IEC using siRNA knock down.

4.3.1. Validation of epithelial Alix protein down-regulation in severe *Enterococcus faecalis* OG1RF-induced colitis.

In order to validate the down-regulation of Alix in primary IEC from *E. faecalis*-monoassociated wild type and IL-10-/- mice Alix protein expression was measured by Western Blot analysis. Equal amounts of protein derived from 5 single equally treated mice were pooled and subjected to 10% SDS-PAGE gels followed by immuno-blotting for Alix using specific antibodies.
As shown in Figure 27B Alix protein expression was confirmed to be down-regulated in severely but not in moderately inflamed IL-10-/- mice after 14 weeks of monoassociation with *E. faecalis* OG1RF or *E. faecalis* ms2, respectively. Alix protein levels in *E. faecalis* ms2-monoassociated IL-10-/- mice were comparable or slightly higher than in WT- mice. In addition, and most important to understand the kinetics of protein expression changes we demonstrated that Alix protein was down-regulated after 14 but not after 1 week of bacterial monoassociation, suggesting a functional role for Alix in the pathogenesis of experimental colitis (Figure 27C). Equal loading was confirmed using β-actin antibodies.

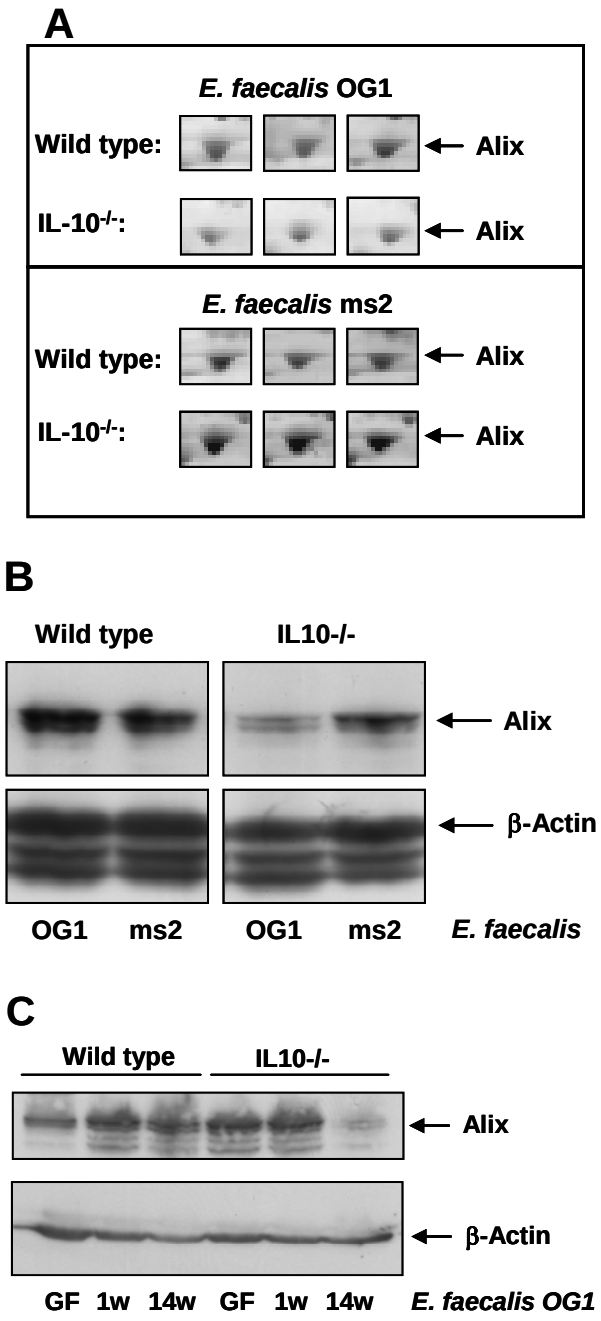


Figure 27: Severely inflamed OG1-mice express less Alix.

Germfree IL-10-/- mice and wild type mice were monoassociated either with *E. faecalis* OG1RF or with *E. faecalis* ms2 for 14 weeks (Figure 27C: 1 and 14weeks). (A) Primary IEC were purified from the large intestine as described in Materials and Methods and 2D SDS-PAGE was performed. Gels were analyzed by Proteome Weaver Software, spots were excised and washed and Alix was detected by MALDI-TOF MS as described in Materials and Methods. 3 representative Alix spots are shown for all groups. (B, C) Protein was extracted from primary IEC and 50 µg of protein sample was subjected to SDS–PAGE followed by Alix and β-actin immuno-blotting using the ECL technique. Bands represent combined equal protein amounts from 5 mice for each group. In Figure 4C germ free (GF) mice were used as controls. Blots shown are representative for 2 different Western Blots using separately pooled samples.

4.3.2. EGF and *Enterococcus faecalis*-induced ERK1/2 phosphorylation was not affected by Alix siRNA knock down.

Alix can directly or indirectly interact with different cell surface receptors, including the Epidermal Growth Factor (EGF) Receptor EGFR [176-178]. EGFR-mediated ERK1/2 activation is probably involved in cell proliferation, cell migration and cell differentiation[169]. As already shown in Figure 21 ERK1/2 phosphorylation levels were reduced in IEC from severely inflamed OG1-mice compared to the moderately inflamed ms2-mice. We therefore asked the question whether ERK1/2 phosphorylation is affected by reduced Alix protein expression. The mouse intestinal epithelial cell line Mode K was used to knock down Alix protein expression by applying siRNA transfection techniques as described in Materials and Methods. As shown in Figure 28, two different siRNA/transfection reagent ratios ("Alix si 1" and "Alix si 2") resulted in a knock down of Alix/AIP 1 protein expression in Mode K cells. We stimulated these cells with EGF for 5 min to investigate the effect of Alix siRNA knock down on EGF-mediated ERK1/2 signalling. However, the siRNA-mediated knock down of Alix protein expression did not reduce EGF-induced ERK1/2 phosphorylation in Mode-K cells.

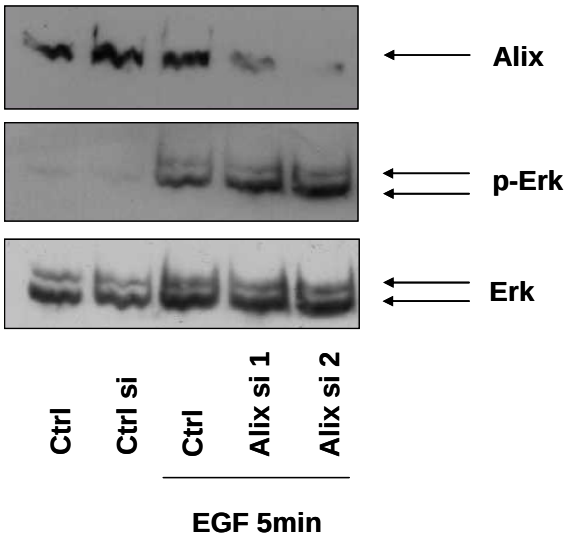


Figure 28: Alix siRNA knock down did not reduce EGF-induced ERK1/2 phosphorylation in Mode K cells.

Mode K cells were transfected with Alix siRNA or control siRNA as described in Materials and Methods and stimulated with 20 ng/ml EGF as indicated. Cells were lysed in Lämmli Buffer and 20 µl of the lysates were subjected to SDS–PAGE followed by Alix, phospho-ERK1/2, total ERK1/2 and β-actin immuno-blotting using the ECL technique.

As already shown, *E. faecalis* ms2 but not *E. faecalis* OG1RF triggered strong ERK1/2 phosphorylation in Mode K cells after 1 h of stimulation (see Figure 14). We therefore next used the capability of *E. faecalis* ms2 to induce early ERK1/2 phosphorylation to further investigate the role of Alix in bacteria-mediated ERK1/2 signalling. Mode K cells were transfected with Alix siRNA or control siRNA and stimulated with the two *E. faecalis* strains for 1 h. As shown in Figure 29 *E. faecalis* ms2-induced ERK1/2 phosphorylation was not reduced by siRNA-mediated Alix knock down.

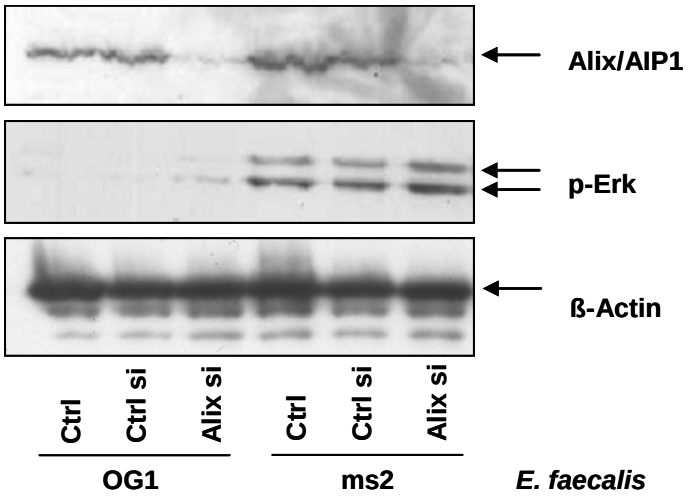


Figure 29: Alix siRNA knock down did not reduce *Enterococcus faecalis*-induced ERK1/2 activation in Mode K cells.

Mode K cells were transfected with Alix siRNA or control siRNA as described in Materials and Methods and stimulated with the *E. faecalis* strains (moi 30) as indicated. Cells were lysed in Lämmli Buffer and 20 µl of the lysates were subjected to SDS–PAGE followed by Alix, phospho-ERK1/2 and β-actin immuno-blotting (as loading control) using the ECL technique.

4.3.3. *Enterococcus faecalis*-induced IP-10 secretion in Mode K cells is not affected by Alix siRNA knock down.

Since *E. faecalis* OG1RF and ms2 differentially induced IP-10 mRNA expression in the intestinal IL-10-/- mouse epithelium (see Figure 20), the role of Alix expression for *E. faecalis*-mediated IP-10 responses was characterized. Mode K cells were transfected with Alix siRNA or with control siRNA, and stimulated with the *E. faecalis* strains alone or in combination with EGF for 12 h. Cell culture supernatants were subjected to ELISA analysis and secreted IP-10 was measured. As shown in Figure

30, Alix knock down did not affect *E. faecalis*-induced IP-10 secretion in the presence or absence of EGF.

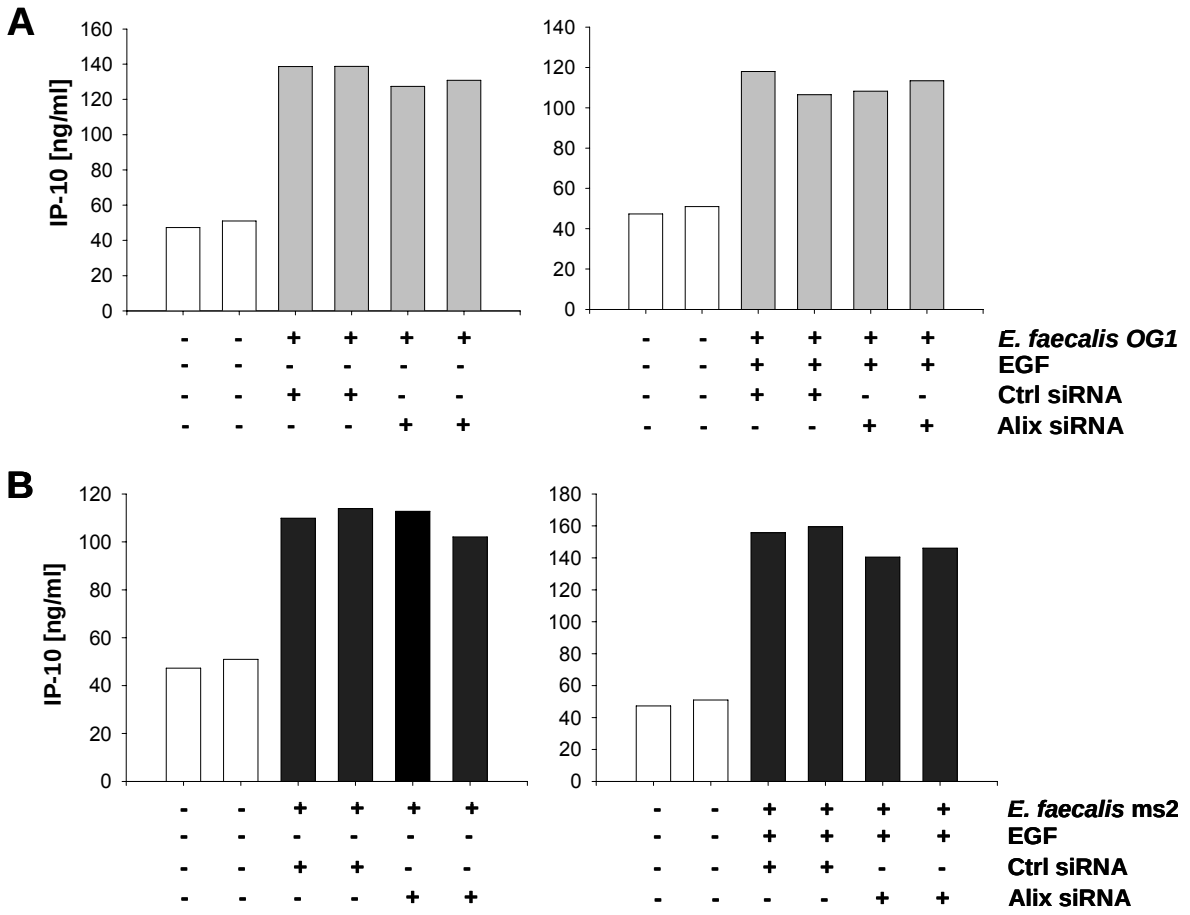


Figure 30: Alix siRNA knock down did not affect IP-10 secretion in Mode K cells.

Mode K cells were transfected with Alix siRNA or control siRNA as described in Materials and Methods and stimulated with *E. faecalis* OG1RF (A), *E. faecalis* ms2 (B) (both moi 30) and with EGF (20ng/ml) for 12 h as indicated. Cell culture supernatants were analyzed for secreted IP-10 Protein using ELISA technique. Bars represent different experiments.

4.4. *Enterococcus faecalis* virulence factor mRNA induction in IL-10-/- mice.

Carrie Hew Ferstl, (Technische Universität München, Institut für Technische Mikrobiologie, Prof. Vogel, Freising) analysed isolated bacterial RNA derived from the gut contents of our *E. faecalis* OG1RF-monoassociated IL-10-/- and wild type mice. The bacterial mRNA was reverse transcribed into cDNA and *E. faecalis* virulence gene expression was determined on the basis of the presence or absence of amplified products following normal PCR with specific primers for *E. faecalis* virulence genes.

As shown in Table 17 virulence gene expression of the *gelE* gene coding for the *E. faecalis* zinc metalloprotease termed gelatinase was induced after 14 weeks of monoassociation in IL-10-/- mice, but not in wild type mice and not in 1 or 2 weeks old IL-10-/- mice. These findings suggested a functional role for gelatinase expression in the pathology of chronic *E. faecalis*-induced colitis. Further analysis using quantitative PCR in a Light Cycler system confirmed up-regulation of the *gelE* gene in IL-10-/- mice compared to wild type mice (data not shown)[179].

Table 17: Virulence gene expression in *Enterococcus faecalis* OG1RF, derived from monoassociated IL-10-/- or wild type mice. These results were conducted by Carrie Hew Ferstl and Rudi F. Vogel[179], Technische Universität München, Institut für Technische Mikrobiologie, Freising.

Bacterium	Mouse genotype	Sampling time	Virulence gene expression			
			efaAfs	*gelE*	*agg*	*esp*
E. faecalis OG1RF	IL-10-/-	1 week	+	-	-	-
		2 weeks	+	-	-	-
		14 weeks	+	+	-	-
E. faecalis OG1RF	Wild type	1 week	+	-	-	-
		2 weeks	+	-	-	-
		14 weeks	+	-	-	-

4.5. Comparison of *Enterococcus faecalis* OG1RF with an isogenic *gelE*-mutant and an isogenic *fsrB*–mutant using monassociation of IL-10-/-mice.

Based on the findings by Carrie Hew Ferstl et al. (see above) we further investigated the function of bacterial proteases on experimental colitis in IL-10-/- mice. Therefore, IL-10-/- mice were monoassociated with *E. faecalis* OG1RF or its isogenic mutant with a deletion in the *gelE* gene (strain TX5264) (Figure 31). We additionally colonized IL-10-/- mice with an *E. faecalis* OG1RF isogenic mutant for *fsrB* (strain TX5266) which is a gene of the *fsr* quorum sensing system. This quorum sensing system regulates the expression of gelatinase and a serine protease in *E. faecalis*. For this reason the *fsrB* mutant is not able to induce gelatinase expression.

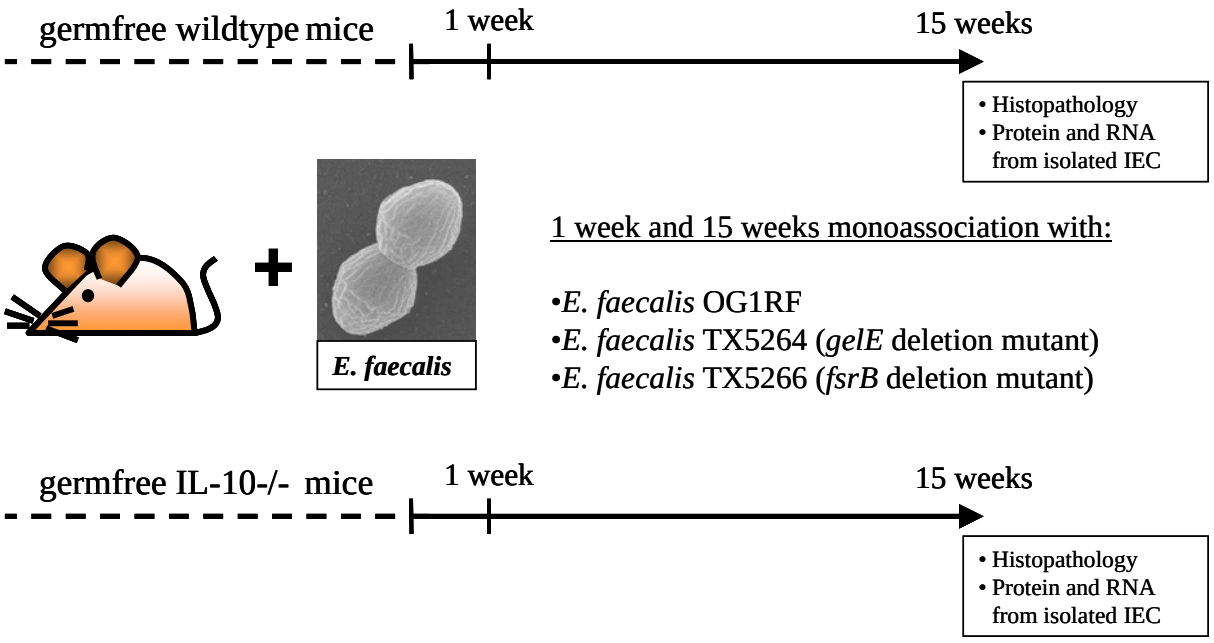
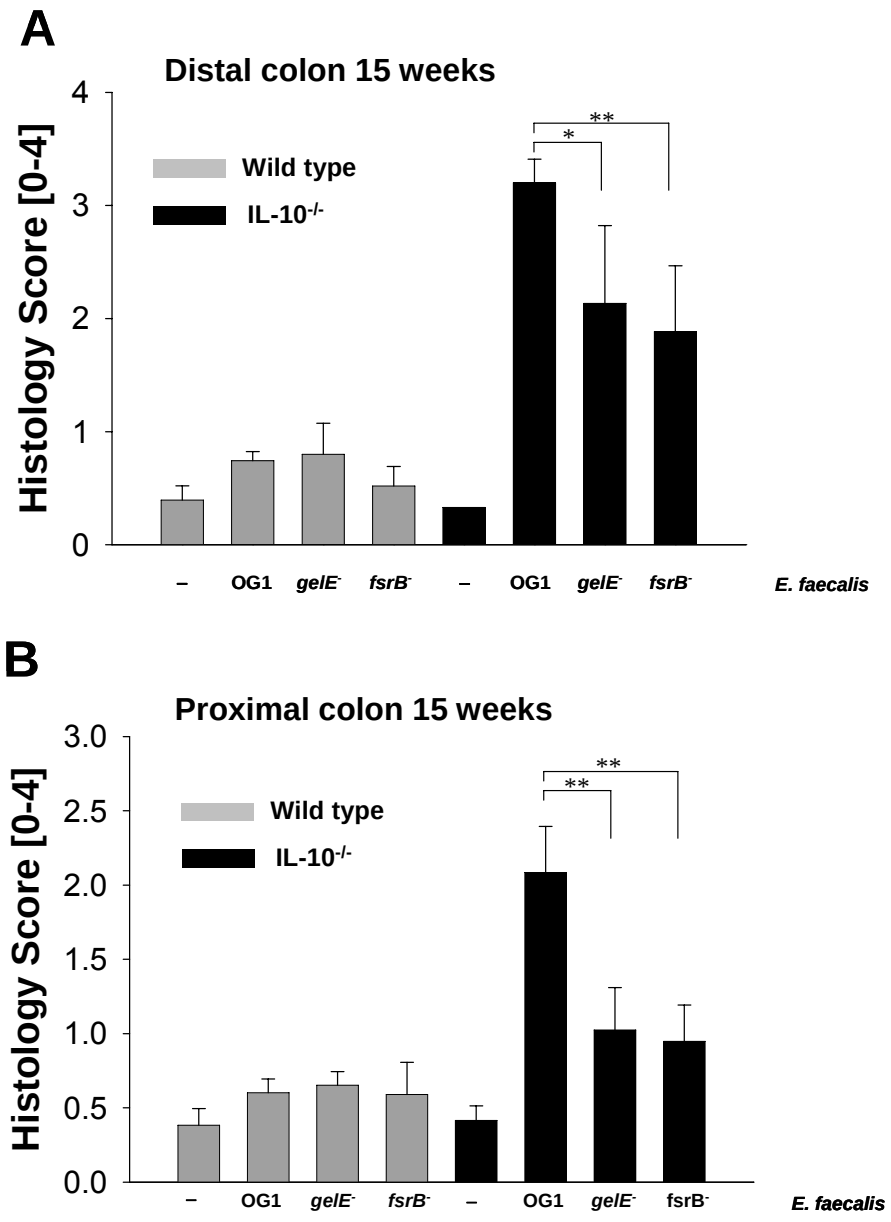


Figure 31: Experimental design.

Wild type and IL-10-/- mice were monoassociated at 12–16 weeks of age (5 mice in each group). Mice were killed 1 or 15 weeks after bacterial monoassociation. Blinded histopathological analysis (score 0-4) was performed in formalin-fixed paraffin-embedded tissue sections of the distal colon and the cecum. IEC were isolated for subsequent protein and RNA extraction.

4.5.1. Histopathological comparison of *Enterococcus faecalis* OG1RF-monoassociated IL-10 deficient mice with *gelE* mutant- and *fsrB* mutant-monoassociated mice.

Histopathological analysis after 15 weeks of monoassociation clearly demonstrates an effect of the *E. faecalis* gelatinase on experimental colitis. IL-10-/- mice monoassociated with the *gelE* mutant showed significantly less colitis in proximal and distal colon when compared to the *E. faecalis* OG1RF (Figure 32A-B). The fsrB mutant induced even more significantly reduced inflammation in the proximal and distal colon as well as in the cecum of IL-10-/- mice, confirming the finding (Figure 32A-C).



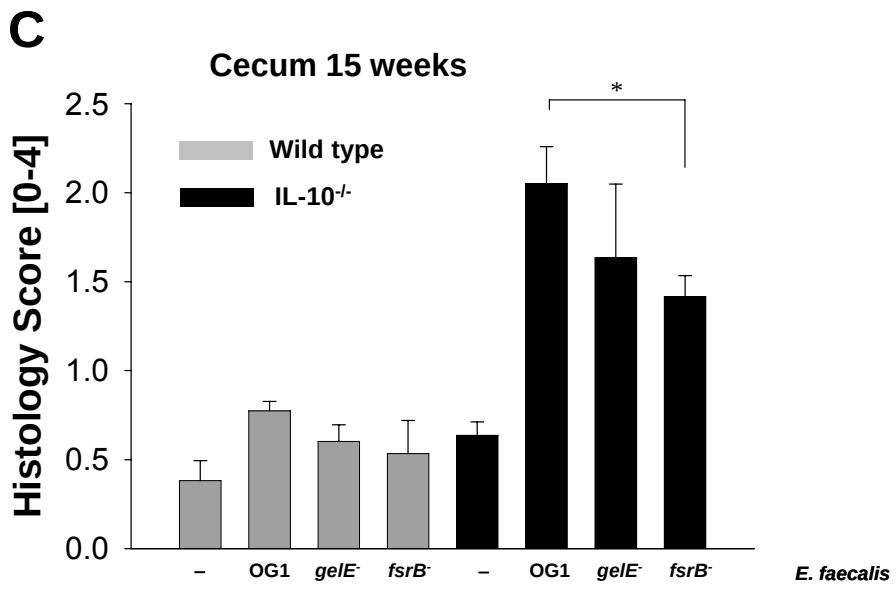


Figure 32: Histological analysis. Both, the *E. faecalis* gelatinase mutant and the *Enterococcus faecalis* fsrB mutant induce significantly less colitis in IL-10-/- mice compared to *Enterococcus faecalis* OG1RF.

Germfree wild type and IL-10-/- mice were monoassociated with *E. faecalis* strain OG1RF or with its isogenic mutants for the gelatinase gene (*gelE*$^-$) and for the fsrB gene (*fsrB*$^-$) at 12–16 weeks of age (n = 5 in each group). Mice were killed 15 weeks after bacterial monoassociation. Blinded histopathological analysis (score 0-4) were performed in formalin-fixed paraffin-embedded tissue sections of the proximal and distal colon and the cecum, as described in Materials and Methods. *p<0.05, **p<0.01.

In Figure 33 representative H&E stained, formalin-fixed paraffin-embedded tissue sections from germfree and *E. faecalis*-monoassociated IL-10-/- mice are shown. Comparing sections from *E. faecalis* OG1RF with the isogenic mutants revealed considerably increased mucosal thickening, goblet cell depletion, and lamina propria cellular infiltration in the *E. faecalis* OG1RF-monoassociated IL-10-/-mice. Furthermore, inflammation seems to progress from the distal colon to the proximal colon, since histological sections and scores from the cecum and the proximal colon show markedly lower inflammation than those from the distal colon.

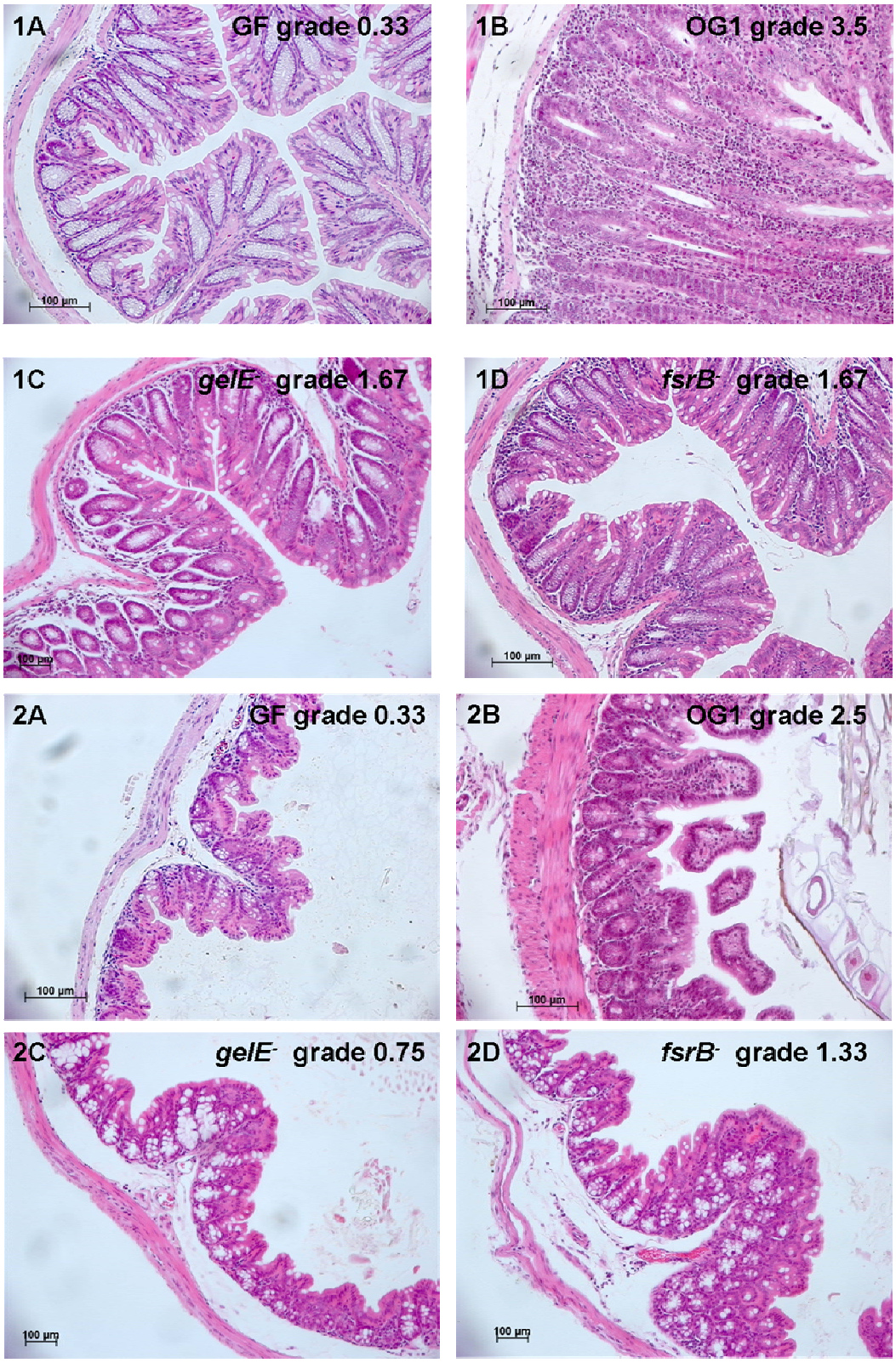


Figure 33: Intestinal inflammation in germfree and *Enterococcus faecalis*-monoassociated IL-10-/- mice.

Germfree (GF) IL-10-/- mice (1A, 2A) were monoassociated with *E. faecalis* OG1RF (1B, 2B) or with its isogenic mutants TX5264 (*gelE*$^-$) (1C, 2C) and TX5266 (*fsrB*$^-$) (1D, 2D) for 15 weeks. Blinded histopathological analyses (grade 0-4) were performed in formalin-fixed paraffin-embedded tissue sections of the distal (1A-D) and proximal (2A-D) colon as described in Materials and Methods. Representative sections are shown. The grade of inflammation is mentioned in the individual sections.

4.5.2. IEC Proteome of IL-10-/- mice monoassociated with *Enterococcus faecalis* OG1RF compared with the isogenic *gelE* or *fsrB* mutant.

To further investigate the function of gelatinase in *E. faecalis*-induced experimental colitis on the level of epithelial cells we compared IEC derived from *E. faecalis* OG1RF and from *E. faecalis* TX5264-monoassociated IL-10-/- mice by a proteomic approach (OG1RF = reference). In a further experiment IEC from *E. faecalis* OG1RF and *E. faecalis* TX5266-monoassociated IL-10-/- mice were compared (OG1RF = reference). For every compared group 10 gels were cast: samples from all 5 biological replicates where run in duplicates. Proteins selected showed significance in the Mann Whitney U Test ($p<0.05$) and were additionally at least 1.5 fold regulated in spot intensity. Table 18 summarizes the regulated proteins. In Figure 34A the location of the regulated proteins is shown. Individual regulated spots are shown in Figure 34B and Figure 34C. Of note, in both comparisons cytokeratin 8 and glyoxalase domain containing protein 4 were found to be down-regulated in the less inflamed mice suggesting a function of these proteins in the pathogenesis of experimental *E. faecalis*-induced colitis.

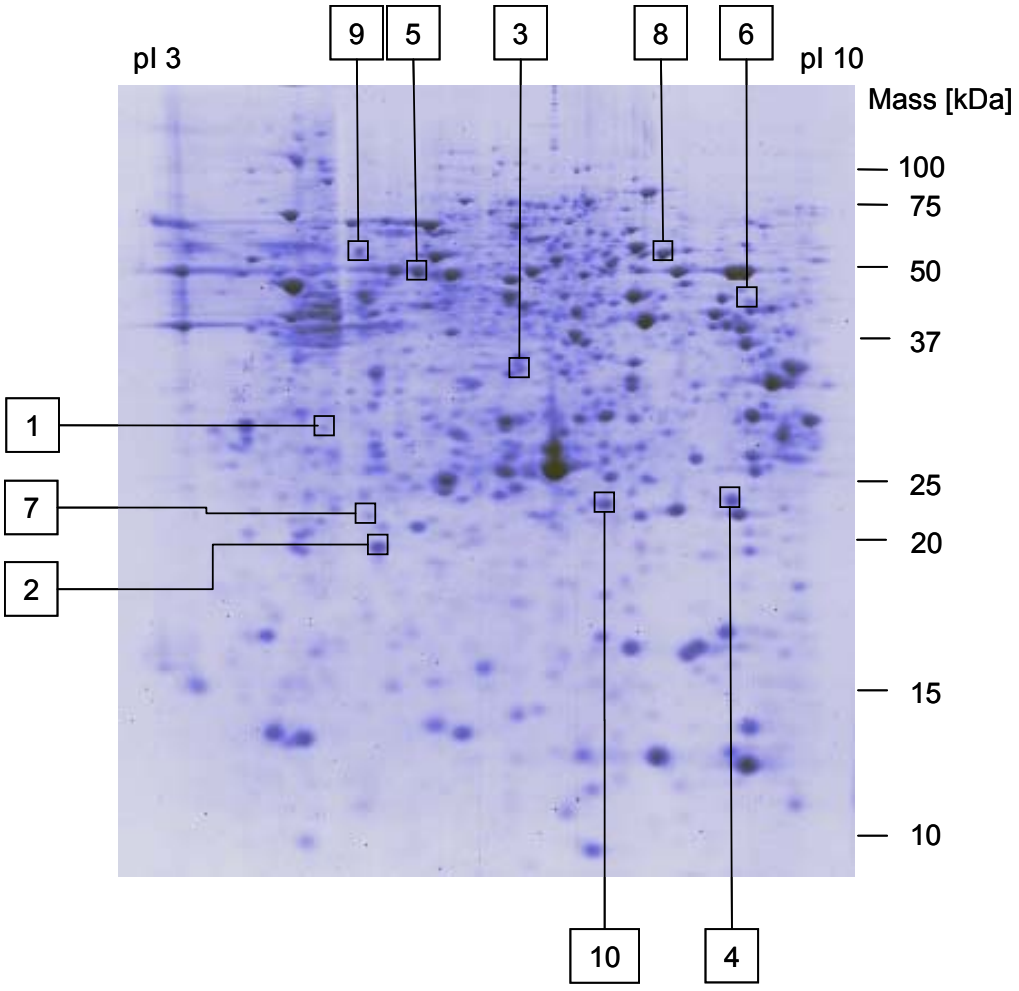
Table 18: Differentially regulated proteins comparing IEC derived from IL-10-/- mice monoassociated with *Enterococcus faecalis* OG1RF (Reference) vs. isogenic mutants.

N°	Gene Name	Accession Number	Name of Protein	Mass [Da][a]	pI [b]	Mean Score [c]	SD Score [d]	Mean SCov [%] [e]	Mean Fold Change [f]
Proteins down-regulated in IL-10 -/- mice monoassociated with *Enterococcus faecalis* gelE mutant TX5264; when compared with *Enterococcus faecalis* OG1RF monocolonized mice (Reference)									
1	Glod4	Q9CPV4	Glyoxalase domain-containing protein 4	33581	5.16	126.25	37.74	44.25	0.55
2	Atp5h	ATP5H	ATP synthase D chain, mitochondrial	18664	5.41	121.29	24.10	69.71	0.55
3	Tufm	Q497E7	Tu translation elongation factor, mitochondrial	49876	7.91	136.33	7.57	40.67	0.57
4	Gstp1	GSTP1_MOUSE	Glutathione S-transferase P 1	23634	8.92	78.63	22.76	45.38	0.56
5	Krt8	P11679	Keratin 8, Cytokeratin 8	53210	5.28	167.14	55.45	35.71	0.41
6	Ola1	BAB28624	Obg-like ATPase 1 (EC 3.6.3.-) (GTP-binding protein 9)	44929	8.59	115.00	50.29	38.75	0.53
7	PyCard	Q9EPB4	Apoptosis-associated speck-like protein containing CARD	21502	5.14	103.00	25.79	70.83	0.54
8	Ugdh	AAC36096	UDP-glucose 6-dehydrogenase	55482	8.57	136.20	59.34	39.80	0.55

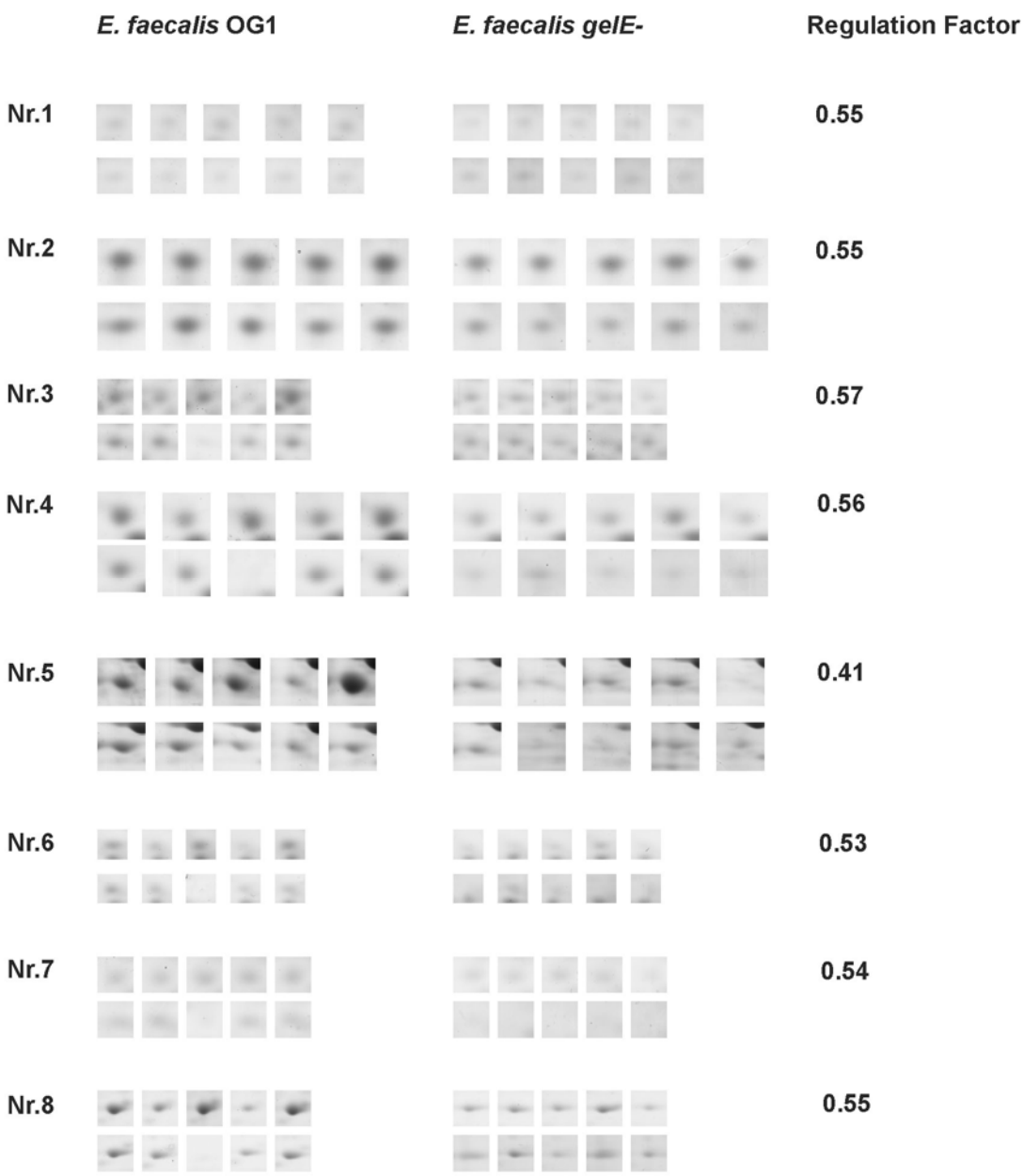
Proteins down-regulated in IL-10 -/- mice monoassociated with *Enterococcus faecalis* fsrB mutant TX5266; when compared with *Enterococcus faecalis* OG1RF monocolonized mice (Reference)									
1	Glod4	Q9CPV4	Glyoxalase domain-containing protein 4	33581	5.16	102.00	55.15	42.50	0.59
5	Krt8	P11679	Keratin 8, Cytokeratin 8	53210	5.28	151.00	56.87	43.00	0.57
9	Hspd1	Q8C2C7	60 kDa heat shock protein, mitochondrial [Precursor]	61089	5.56	119.63	46.63	38.25	0.45
10	Uqcrfs1	Q5SVV1	Ubiquinol-cytochrome c reductase iron-sulfur subunit, mitochondrial [Precursor]	29634	9.71	111.67	17.01	41.33	0.56

[a] Theoretical protein mass according to the Mascot search result. [b] Theoretical isoelectric point of the proteins according to the Mascot search result. [c] Mean Mascot Mowse score derived from the Mascot search result. [d] Standard deviation of the mean Mascot Mowse score. [e] Mean sequence coverage of the proteins in % derived from the Mascot search result. [f] Mean fold change in spot intensity of the regulated proteins derived from the Proteome Weaver software comparison.

A



B


E. faecalis OG1
E. faecalis gelE-
Regulation Factor
Nr.1
0.55
Nr.2
0.55
Nr.3
0.57
Nr.4
0.56
Nr.5
0.41
Nr.6
0.53
Nr.7
0.54
Nr.8
0.55

C

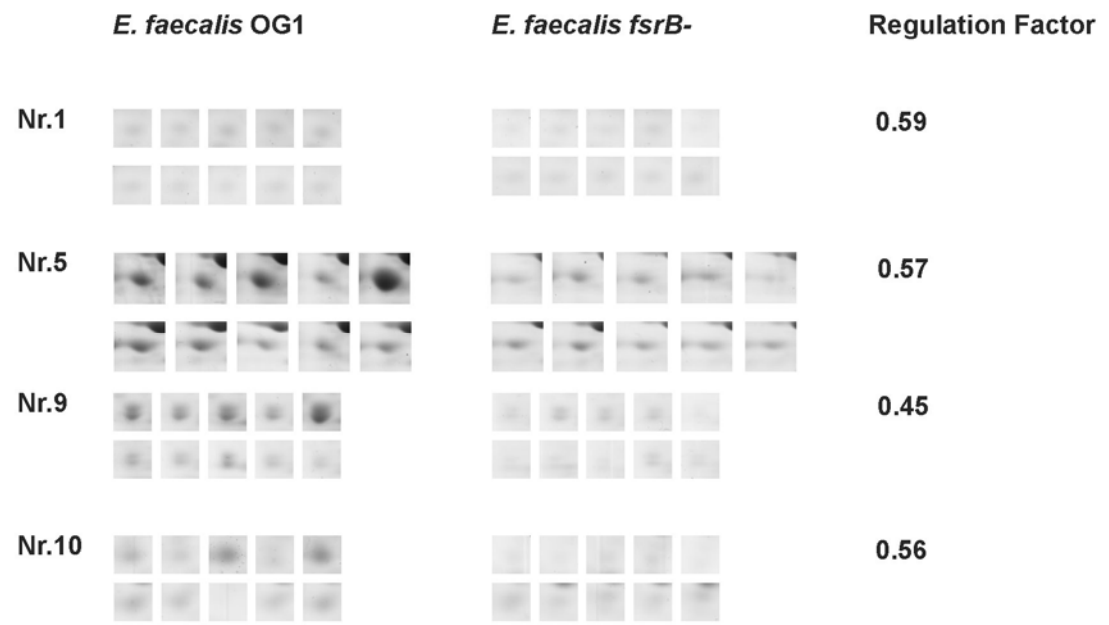


Figure 34: Proteome analysis of primary IEC derived from *Enterococcus faecalis* OG1RF- vs. *Enterococcus faecalis* TX5264 (*gelE*$^-$)- and vs. *Enterococcus faecalis* TX5266 (*fsrB*$^-$)-monoassociated IL-10-/- mice.

Germfree IL-10-/- mice were monoassociated with the *E. faecalis* strains for 15 weeks. Primary IEC were purified from monoassociated animals. 2D SDS-PAGE from n=5 IL-10-/- mice were run in duplicate for every group. Proteome Weaver comparison of the gels and identification of the excised spots by MALDI-TOF MS was performed as described in Materials and Methods. Localization and regulation of the numbered differentially down-regulated spots is shown (A). Regulated spots are shown for (B) *E. faecalis* OG1RF (left side) and *E. faecalis gelE*$^-$ (right side) *or* (C) *E. faecalis* OG1RF (left side) and *E. faecalis fsrB*$^-$ (right side). Identified proteins that showed significant regulation (Mann Whitney Test $p<0.05$) and at least 1.5 mean fold change in expression were considered as differentially regulated.

4.5.3. Bibliometric analysis

Finally, BiblioSphere software was used to find cocitated proteins. For the network tree shown in Figure 35 only proteins cocitated in the same sentence of abstracts which also contained a function word (e.g. regulates, inhibits) are shown. The analysis only includes comparisons of highly inflamed mice (*E. faecalis* OG1RF, control group) with mild to moderate inflamed mice (*E. faecalis* m2, *E. faecalis* TX5264, *E. faecalis* TX5266).

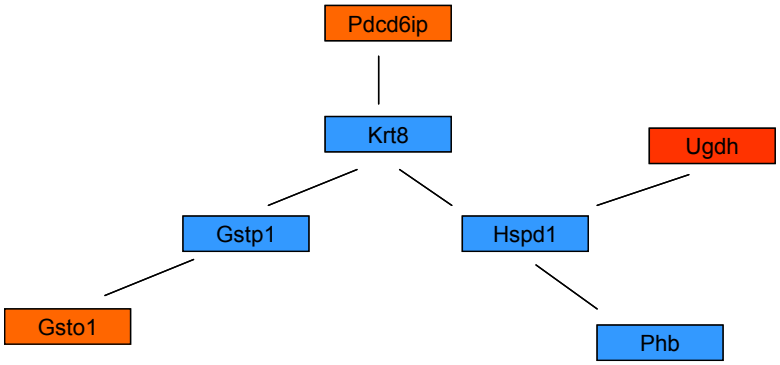


Figure 35: Gene-gene network tree based on regulated proteins between severe and moderately inflamed IL-10-/- mice and bibliometric analysis.

The gene-gene network tree was genrated using BiblioSphere bibliometric analysis. Connections represent a least one cocitation a in PubMed abstract in the same sentence including a function word (e.g. regulates, interacts etc.). Red colour indicates up-regulation, blue colour down-regulation. Gsto1: glutathion S-transferase omega 1; Gstp1: glutathione S-transferase P 1; Hspd1: 60 kDa heat shock protein, mitochondrial; Krt8: Keratin 8; Pdcd6ip: Programmed cell death 6 interacting protein = Alix; Phb: Prohibitin; Ugdh: UDP-glucose 6-dehydrogenase.

5. Discussion

5.1. Interaction of *Enterococcus faecalis* with IEC induces NFκB- and MAP kinase-mediated IL-6 and IP-10 secretion

E. faecalis strains are frequently isolated from traditional Mediterranean food products and some strains are used as probiotics. On the other hand, *E. faecalis* strains can acquire various antibiotic resistances, are regarded as nosocomial pathogens and are also a common cause of endocarditis, urinary tract infections and bacteremia. The role of *E. faecalis* strains in the pathogenesis of IBD is unknown. Therefore, we compared three *E. faecalis* strains of different origins on their mechanisms to induce proinflammatory cytokine and chemokine expression in IEC: A medical human oral isolate, *E. faecalis* OG1RF, which is well known and described in the literature and is known to be colitogenic in IL-10-/- mice[156, 157], the gastrointestinal endogenous isolate *E. faecalis* ms2, derived from an IL-10-/- mouse, and the probiotic isolate *E. faecalis* hs3.
In the beginning of the study we also used two further strains, *E. faecalis* 34.5, a food isolate, and an endogenous *E. faecalis* isolate derived from a wild type mouse (genetic background 129 SvEV TAC). However, since these two strains did not show striking differences in our studies regarding IL-6 and IP-10 secretion in IEC (data not shown), and, since we were only able to use 3 strains for the cost intensive *in vivo* studies in IL-10-/- and wild type mice, we concentrated on the strains *E. faecalis* hs1, ms2, and hs3. Another reason for our decision to choose these 3 strains was the genotypic characterization of the strains. According to the studies of Hew et al., Eaton et al. and our studies the genes for pheromone responsive plasmid exchange (*cpd, ccf, cob*) and the *efaAfs* gene can be found in almost every *E. faecalis* strain [161, 180]. Therefore, the differences in the more sporadic appearing genes *agg, gelE, esp*, and *cylMBA,* were regarded as more interesting. Since *E. faecalis* hs1, ms2 and hs3 showed differences in the presence of the virulence genes *agg*, *gelE* and *cylMBA*, we decided to implement these 3 strains in our studies.

All 3 *E. faecalis* strains were capable of inducing IL-6 secretion in IEC. Our results using pharmacologic inhibitors suggested that *E. faecalis*-induced IL-6 secretion in

IEC is mediated by the NFκB, the ERK1/2 MAP kinase and the p38 MAP kinase pathway. The inhibition of ERK1/2 led to an about 8-fold decrease in IL-6 secretion. However, no significant differences were found between the strains, suggesting that the strains share similar signalling mechanisms to trigger IL-6 secretion.

IL-6 is a cytokine with multiple functions in the intestinal mucosa: IL-6 stimulates acute phase protein synthesis in enterocytes[181], it can induce IgA secretion in B-cells of intestinal Peyer´s Patches[182], and it was reported that high levels of IL-6 were associated with a higher mucosal permeability[183]. Of note, IL-6 was reported to enhance T-cell survival and apoptosis resistance in the lamina propria at inflamed sites[184]. In a recent phase II study IL-6 antibodies were shown to reduce disease activity in CD patients[185]. Hence, our results clearly show the capability of both, a probiotic and a medical *E. faecalis* strain equipped with different virulence factor subsets to induce an inflammatory response in IEC with potential functions on B-cells and/or T-cells. Further the results suggest that the epithelium is also responsive to commensal or probiotic bacteria and not only to infectious pathogens.

We confirmed this finding by different experiments. Using probiotic Lactobacilli, Bifidobacteria and a *Streptococcus* species derived from the probiotic preparation VSL#3 we found that all these species were capable of inducing IL-6 secretion in Mode K cells (unpublished data). *Lactobacillus acidophilus*, *Lactobacillus plantarum* and *Streptococcus thermophilus* induced only low levels of IL-6 compared to *Lactobacillus casei* and the Bifidobacteria. *In vivo* we were able to induce transient IL-6 expression by monoassociation of gnotobiotic Fisher rats with the probiotic *Bifidobacterium lactis* BB12[82]. Finally, we used reconstituted *Lactobacillus* free (RLF) mice to study the effect of a non infectious commensal *Lactobacillus* strain on the activation of signal transduction pathways in IEC in mice that have a complex microbiota (recently published in the Journal of Nutrition; Hoffmann M., Rath E. et al.[186]). RLF mice do not harbour lactobacilli in their intestine, however they harbour a complex microbiota which is functionally similar to that of conventional animals[187, 188]. These mice clearly responded to the first exposure to *Lactobacillus reuteri* by inducing a transient inflammatory reaction with increased levels of IL-6 mRNA and decreased levels of the negative NFκB-regulators A20 and Toll interacting protein (TOLLIP) after 6 days of colonization[186]. These finding demonstrate that the intestinal epithelium is able to regulate the NFκB pathway *in vivo* in response to *Lactobacillus reuteri* even in the presence of an already-established microbiota. Therefore, IL-6

and several other NFκB-induced cytokines and negative regulators of NFκB like A20 or TOLLIP might play a role in the induction of immune tolerance to commensal bacteria in the intestine. Interestingly, van Baarlen et al. [189] very recently published a study showing differential NFκB pathway induction by *Lactobacillus plantarum* in duodenal biopsies of healthy humans. The differential NFκB pathway induction correlated with immune tolerance and therefore supported the hypothesis that NFκB-induced pathways and negative regulators of NFκB play a role in the induction of immune tolerance in the intestine.

The role of IP-10, a chemokine to attract activated T_{H1} lymphocytes and phagocytes, is not elucidated in the pathophysiology of IBD. IP-10 is secreted by endothelial cells, epithelial cells, fibroblasts, keratinocytes, and monocytes[190]. We were able to measure high levels of IP-10 secretion in response to all *E. faecalis* strains. Compared to IL-6 secretion, *E. faecalis*-induced IP-10 secretion in IEC was mediated by the NFκB- and the p38 MAP kinase- but not by the ERK1/2-pathway. Since no differences were found between the individual *E. faecalis* strains the mechanisms of IP-10 secretion in IEC are probably the same for all *E. faecalis* strains. In contrast *Lactobacillus casei* and *Streptococcus thermophilus* were not able to induce IP-10 secretion in Mode K cells (data not shown). We were even able to show, that *Lactobacillus casei* is capable of inhibiting TNFα-induced IP-10 secretion by post-translational degradation[191]. These findings show that probiotic bacteria have different mechanisms to influence epithelial IP-10 secretion.

5.2. Monoassociation of IL-10-/- mice with *Enterococcus faecalis* strains results in different severity of experimental colitis.

A widely accepted hypothesis suggests that the development of IBD is influenced by host susceptibility genes, by immunological and by environmental factors like the commensal intestinal flora[90, 92]. Various rodent models of IBD, for example IL-10-/-, IL-2-/- mice or HLA-B27 transgenic rats have demonstrated the importance of commensal bacteria as a trigger of inflammation[107,192]. Using these models in germfree conditions colonized by single bacterial species yields tools to analyze the individual impact of different bacterial species on intestinal inflammation.

We used germfree IL10-/- mice and demonstrated that monoassociation of these mice with different strains of *E. faecalis* for 14 weeks can lead to significant differences in colonic inflammation. Monoassociation of IL-10-/- mice with the two commensal bacteria *E. faecalis* or *E. coli* resulted in intestinal inflammation with distinctly different kinetics and anatomic distribution in the same host[123]. Furthermore, dual association with both non-pathogenic commensal bacteria led to an aggressive pancolitis with duodenal inflammation after 7 weeks[124]. Hence, *E. coli* and *E. faecalis* seem to have additive effects in IL-10-/- mice.

Our results show that germfree IL-10-/- 129SvEv mice not only allow discriminating colitogenic bacterial species but also discriminating between more or less colitogenic strains and that *E. faecalis* OG1RF strain-specific features initiate a significantly intensified colitis compared to *E. faecalis* ms2. There are different possible reasons to explain this finding for example (i) different expression of *E. faecalis* virulence genes (ii) different abilities of *E. faecalis* to produce extracellular superoxide leading to DNA damage of IEC [193], (iii) different abilities in *E. faecalis* mucin degradation[18], or (iv) changes of the intestinal cytokine profile induced by *E. faecalis* strains [194].

To explain the differential colitis inducing effects we focussed on the induction of host responses in IEC like activated signal-transduction pathways or cytokine mRNA induction and on the analysis of virulence genes present in our strains. Paradoxically analysis of virulence genes of our strains revealed more virulence genes for the less colitis inducing strain *E. faecalis* ms2 (*cylMAB*$^+$, *agg*$^+$, additional to *gelE*$^+$, *efaAfs*$^+$, *cpd*$^+$, *ccf*$^+$, *cob*$^+$, *esp*$^-$) than for *E. faecalis* OG1RF (only *gelE*$^+$, *efaAfs*$^+$, *cpd*$^+$, *ccf*$^+$, *cob*$^+$, *esp*$^-$). These results suggest that the *E. faecalis* ms2 genes for cytolysin and aggregation factor are either not functional or that they are not expressed in the intestinal environment. For both genes it was shown that expression is dependent on the environmental conditions like pH or nutrient supply[180]. Interestingly, expression of the *E. faecalis cylB* gene was significantly reduced in stationary phase bacteria grown in LB media at pH5 compared to pH7[180], suggesting down-regulation of *cylB* in the acidic environment of the intestine. Furthermore, the presence of the additional virlulence genes could be explained by a lack of impact of these genes on chronic intestinal inflammation. However, clear conclusions on the impact of these genes on colitis can only be drawn by using isogenic mutants of a colitiogenic strain like *E. faecalis* OG1RF in a animal model of IBD.

To investigate *E. faecalis*-induced signal transduction pathways and possible strain-specific differences in *E. faecalis* activated pathways, we compared *E. faecalis*-mediated phosphorylation of MAP kinases and NFκB in IEC *in vitro* and *in vivo*.

In vitro all *E. faecalis* strains were capable of inducing activation of c-jun, ERK1/2 and RelA. This is consistent with our finding that inhibitors targeting these pathways resulted in decreased IL-6 secretion in IEC. RelA is a subunit of the NFκB hetero-dimer and phosphorylation leads to nuclear translocation and activation of NFκB-dependent gene-transcription. Activation of all TLRs with their respective ligands can lead to NFκB activation, and peptidoglycan as well as lipoteichoic acid from gram positive bacteria are ligands for TLR-2[81]. Since we also show that TLR-2 deficient MEF cells do not secrete IL-6 in response to *E. faecalis* stimulation compared to TLR-2 competent MEF cells, it is most likely that *E. faecalis*-induced NFκB activation in Mode K cells is also mediated by TLR-2 leading to IL-6 and IP-10 secretion.

In IEC, ERK1/2, p38 and c-jun were activated by all *E. faecalis* strains. c-jun is a subunit of the heterodimeric transcription factor AP1 which is phosphorylated by the MAP kinase JNK. p38, JNK and ERK1/2 activate transcription factors as well as various kinases and play a role in diverse cellular functions. p38 is implicated in angiogenesis, cell proliferation, inflammation and cytokine production, JNK controls apoptosis and the development of multiple cell types in the immune system whereas the predominant role of ERK1/2 is induction of proliferation and differentiation[167]. Furthermore, all MAP kinases play different roles in cell migration events[168]. Strong ERK1/2 phosphorylation was observed after stimulation with *E. faecalis* ms2 for 1h but not for the other strains. This finding was confirmed to be an *E. faecalis* strain-specific effect in several experiments (also compare with Figure 29) suggesting proliferation or differentiation events being early induced by this strain. Strong early ERK1/2 activation points to a different mechanism of signal-induction, which may be mediated by a different receptor.

In vitro E. faecalis-induced IL-6 secretion in IEC was dependent on activated NFκB, p-38, and ERK1/2 whereas IP-10 secretion was only dependent on NFκB and p-38.

In vivo all MAP kinases were activated in the epithelium of wild type mice but only ERK1/2 activation was detected in *E. faecalis* monocolonized IL-10-/- mice. Since p-38 and c-jun activation was lost in IL-10-/- mice these kinases seem to regulate protective mechanisms in the epithelium. We further found that p-38 was present in 1 week but not in 14 weeks *E. faecalis* monocolonized IL-10-/- mice[195] and persistent

TLR-2 protein expression was observed in the epithelium of *E. faecalis* monocolonized Il-10-/- mice up to 28 days, whereas wild type mice were able to downregulate TLR-2 protein expression[196]. Therefore, c-jun or p-38 might also be involved in the inhibition of TLR-2 protein expression in wild type mice which is lost due to a lack of protective TGFβ/SMAD signalling in the epithelium of IL-10-/- mice[196]. High TLR-2 expression probably explains the significantly elevated IP-10 mRNA expression in severe *E. faecalis* OG1RF-mediated colitis, since IP-10 expression may be induced by TLR-2-mediated NFκB signalling.

ERK1/2 phosphorylation was absent in severe *E. faecalis* OG1RF-mediated colitis but not in moderate *E. faecalis* ms2-mediated colitis. Since p38 and c-jun activation was absent in all inflamed *E. faecalis* monocolonized IL-10-/- mice also ERK1/2 phosphorylation might play a protective role in moderate colitis under conditions when p38 and c-jun phosphorylation is absent. Wound healing-associated cell migration and wound closure are protective functions of ERK1/2 signalling in IBD[197]. Recent studies also implicate ERK1/2 in intestinal wound closure[198] and treatment of colitis by growth factors like EGF, which induce ERK1/2 signalling, has been suggested[199, 200].

Experimental studies demonstrated that IP-10 plays an important role in colitis since treatment with an anti-IP-10 antibody reduced the number of colon infiltrating T_{H1} effector cells in a newly established model of colitis[201]. When comparing *E. faecalis* OG1RF and *E. faecalis* ms2 in IL-10-/- mice, we measured increased epithelial IP-10 mRNA expression. This was consistent with the histopathology seen in IL-10-/- mice. Therefore, we confirmed the finding by Noguchi et al.[202] that increased IP-10 expression is associated with higher colitis diseases activity.

Interestingly, *E. faecalis* hs3 induced lower IP-10 mRNA expression in the epithelium of IL-10-/- mice compared to *E. faecalis* OG1RF whereas the grade of colitis induced by *E. faecalis* hs3 was still severe. This might point to a different less IP-10-mediated mechanism of colitis in *E. faecalis* hs3 monocolonized IL-10-/-mice. This seems to be possible, since *E. faecalis* hs3 also led to strong p38 phosphorylation in wild type mice after 14 weeks of monoassociation compared to the other strains, suggesting different p-38 signalling mechanisms. Hence, *E. faecalis* hs3 has the potential of inducing differential signal transduction pathways, at least in wild type mice. Therefore, these results present an attractive starting point for further studies on the signal transduction mechanisms induced by *E. faecalis* hs3 in IEC.

5.3. Proteins regulated by *Enterococcus faecalis* strains in monoassociated IL-10-/- mice suggest induction of stress, detoxification and cytoskeleton remodelling mechanisms in severe colitis

For all three *E. faecalis* strains UDP-glucose dehydrogenase and hydroxymethylglutaryl-CoA synthase were found to be down-regulated in IL-10-/- mice compared to germfree mice. Of note, Shkoda et al. found both enzymes to be down-regulated in IL-10-/- mice already after 2 weeks of monoassociation with *E. faecalis* OG1RF.[195] Therefore, the regulation of these enzymes seems to be an early *E. faecalis*-induced effect on IEC which occurs before the onset of colitis. Hydroxylmethylglutaryl-CoA-synthase is a protein localized in mitochondria and is involved in the synthesis of the ketone body acetoacetic acid. Ketone bodies are mainly synthesized in energy deficiency (catabolism) and are used as a source of energy in heart and brain. A recent study demonstrated effects of the gut microbiota on myocardial ketone body metabolism during nutrient deprivation[203]. In contrast to germfree mice, the commensal microbiota was able to supply energy for the heart, probably by the secretion of short chain fatty acids. Taken together, down-regulation of hydroxylmethylglutaryl-CoA-synthase-mediated ketone body synthesis in the epithelium induced by *E. faecalis* would suggest that this beneficial effect of the microbiota is decreased or not present in *E. faecalis* monoassociated mice.

UDP-glucose dehydrogenase is an enzyme involved in the biosynthesis of glycosaminoglycans, hyaluronan, chondroitin sulfate, and heparan sulfate [204]. These substrates might serve as nutrients for *E. faecalis*. Interestingly, it was shown by Baldassarri et al. that glycosaminoglycans mediate invasion and survival of *Enterococcus faecalis* into macrophages[158]. Furthermore, it was shown that glycosaminoglycans, especially heparin and/or heparan sulfate play an important role in the binding of enterococci to human colon cells [205]. Therefore, the down regulation of UDP-glucose dehydrogenase might be an early counter regulation of IEC against bacterial adhesion.

Grp78 was found to be up-regulated for all *E. faecalis* strains in IL-10-/- mice compared to germfree mice. This protein is a chaperone and the major inducer of the unfolded protein response, which is a result of mis- or unfolded protein accumulation in the endoplasmic reticulum (ER). Induced by dissociation of grp-78 from IRE1,

PERK, and AFT6, these proteins then mediate an ER stress response leading to up-regulated transcription of genes that increase ER protein folding and secretory capacities. We recently published that primary IEC from IL-10-/- mice as well as IBD patients revealed increased expression levels of grp78 under conditions of chronic inflammation. As a mechanism, we show that IL-10 modulates the recruitment of ATF6 to the grp78 promoter in IEC [157]. The up-regulation of grp78 in all monoassociated IL-10-/- mice reveals that this response is not specific for the medical isolate *E. faecalis* OG1RF and therefore increases the probability that inadequate ER stress induction is a mechanism which can lead to colitis. Very recently an association of a polymorphism in the XBP1 gene with IBD patients was found. XBP1 is spliced by IRE1 after ER stress induction to induce protective gene transcription[206]. This finding underlines that dysregulated ER stress responses might be a cause for IBD.

Comparing different grades of inflammation induced by different *E. faecalis* strains in IEC might reveal protective strain-specific-induced proteins. Therefore, we directly compared *E. faecalis* OG1RF and ms2. Monoassociation of germfree IL-10-/- mice coupled with 2D gel electrophoresis and MALDI-TOF mass spectrometry resulted in the identification of 13 proteins with differentially regulated steady state protein expression in the epithelium of severe inflamed OG1-mice compared to moderately inflamed ms2-mice. Parallel proteomic analysis of wild type mice and IL10-/- mice made it possible to show that these proteins were exclusively regulated in IL-10-/- mice. Since OG1-mice were significantly more inflamed than ms2-mice, the differences found by proteomic comparison are likely to be related to the different histopathology induced by the two strains, rather than to changes directly triggered by these strains. This hypothesis is supported by the fact that 3 of the 13 regulated proteins - F-actin capping protein alpha-2 subunit, Ezrin, and Alix - are associated with cytoskeleton functions and cytoskeleton interactions, suggesting morphological rearrangements during development of severe colitis.

Another protein found to be regulated in severe colitis in OG1-mice is prohibitin. Theiss et al.[207] already showed that prohibitin is down-regulated in biopsies from Crohn´s disease patients and in animal models of experimental colitis including DSS-induced colitis and piroxicam-induced colitis in IL-10-/- mice. It was recently suggested that prohibitin expression is modulated by the acute phase cytokine IL-6 in

IEC [208]. We revealed increased expression levels of prohibitin in primary IEC from severely inflamed OG1-mice compared to moderately inflamed ms2-mice. Since prohibitin protects against oxidative stress in intestinal epithelial cells [207], the elevated prohibitin protein expression in the intestinal epithelium of OG1-mice might reflect a response against the accumulation of reactive oxygen species produced by macrophage and neutrophils under conditions of severe inflammation.

DNAJ homolog subfamily B member 11, also designated Erdj3, is a soluble ER-glycoprotein which is up-regulated during ER stress[209]. Erdj3 was found to be up-regulated in OG1-mice. ERdj3 binds directly to unfolded proteins [210] and the release of Erdj3 from its substrate is regulated by grp78 [211]. Hence, up-regulation of Erdj3 further confirms the induction of an ER-stress response in primary IEC from IL-10-/- mice and might lead to a better understanding of the intensity of an ER stress response, since it was less expressed in less inflamed mice.

Shkoda et al.[195] found 3-mercaptopyruvate-sulfurtransferase (MST) to be down-regulated (2.5 fold) in *E. faecalis* OG1RF-induced colitis. Consistently we found this protein to be down-regulated in severe *E. faecalis* hs3-induced colitis (reference: germfree mice) and up-regulated when comparing moderate colitis in ms2-mice with severe colitis in OG1-mice (reference). These results suggest that inhibition of epithelial MST expression is a characteristic of severe IBD. MST is an enzyme which functions in cyanide detoxification[212] and in cysteine catabolism to form pyruvate, suggesting lower availability of cysteine in IEC to form pyruvate. This would support the hypothesis that under conditions of chronic inflammation, IEC are in a state of energy deficiency in which cysteine is not available to be degraded to provide energy in the form of pyruvate. Ramasamy et al.[213] showed in UC patients that MST expression in the colonic mucosa is lost under conditions of chronic inflammation. According to this finding, it is more likely that decreased MST expression is a general feature of severe IBD and not an *E. faecalis* specific effect. The intensity of the MST-spot in our 2D-gels suggests that MST is expressed in high amounts in IEC and therefore probably has an important physiological role, for example energy supply by providing pyruvate.

Using a proteomic approach the colitogenic effect of the *E. faecalis* gelatinase mutant and the *fsrB* mutant strains was compared with the wild type strain OG1RF. Interestingly, for both comparisons the two proteins glyoxylase domain-containing protein 4 and cytokeratin 8 were found to be down-regulated in conditions of

moderate colitis, suggesting general mechanisms of gelatinase and the *E. faecalis fsr* quorum sensing system. Vice versa this means that these two proteins are up-regulated in severe colitis induced by *E. faecalis* OG1RF.

No references were found for glyoxylase domain-containing protein 4. According to the Uniprot proteinknowledgebase (http://www.uniprot.org/uniprot/Q9CPV4), this protein is probably localized in mitochondria and interacts with a member of the Nudix hydrolase familiy designated NUDT9 (This information is based on protein similarities). NUDT9 was shown to be an ADP-ribose (ADPR) pyrophosphatase, which plays a role in the removal of the potentially toxic ADPR[214, 215]. Interestingly, also glyoxalases have a function in the detoxification of the cytotoxic methylglyoxal, a normal byproduct of carbohydrate metabolism[216]. Therefore, glyoxylase domain-containing protein 4 might also be involved in detoxification processes in the intestinal epithelium under conditions of chronic inflammation. This conclusion is supported by our finding that up-regulated grp78 indicates induced stress mechanisms in the epithelium of *E. faecalis* monoassociated IL-10-/- mice which are probably associated with higher energy supply and toxic by-products.

Cytokeratin 8, also designated Keratin 8 or Cytokeratin EndoA, is a type II intermediate filament protein expressed in the intestinal epithelium[217]. The spot intensity in our 2D gels suggests that expression of Keratin 8 in colonic IEC is high, especially in severe colitis. The function of Keratin 8 is essential in the intestinal epithelium because the intermediate filament Keratin 7, which is able to substitute Keratin 8 functions in other epithelia, is not expressed in IEC[217]. This explains why keratin 8-deficient mice develop hyperplasia and inflammation in the colon [130] but not in many other epithelia. Although colonic inflammation in keratin 8-deficient mice was already discovered in 1994[130], its relevance in the pathogenesis of chronic intestinal inflammation is just starting to be discovered. In 2004, mutations in the Keratin 8 gene were linked with IBD in patients[218]. Recently a novel Keratin 8 mutation was correlated with extensive UC[219].

We found Keratin 8 up-regulated in severe *E. faecalis*-mediated colitis compared to mild disease mediated by the *E. faecalis* OG1RF mutants, suggesting that dysregulated Keratin 8 expression is an attribute of colitis. Interestingly, the stress-induced ER-chaperone grp78 can associate with cytosolic Keratin 8 *in vitro* under conditions that induce grp78 expressions, for instance glucose starvation or tunicamycin treatment.[220] In addition, simple epithelial Keratins like Keratin 8 were

shown to protect cells from TNFα- and Fas-mediated apoptosis[221, 222]. Since high TNFα expression is a characteristic of CD, increased Keratin 8 might allow preserving a low level of epithelial barrier function by the inhibition of IEC apoptosis under condition of severe inflammation. This is consistent with a recent publication implicating IL-6-induced Keratin 8 expression in barrier function alterations[223]. Cytosolic grp78 might protect Keratin 8 from degradation.

In summary, and additional to the already discussed regulated proteins, also elevated levels of Keratin 8 and glyoxylase domain-containing protein 4 suggest mechanisms of cytoskeleton remodeling, stress induction, and detoxification under conditions of severe colitis.

5.4. Role of Alix in *Enterococcus faecalis* stimulated IEC.

We showed for the first time that Alix is down-regulated in severe but not moderate intestinal inflammation, suggesting a protective role of Alix in the process of disease progression. Alix is homologous to two different yeast proteins, Bro1 and Rim20, and to the *Aspergillus nidulans* PalA Protein. PalA/Rim20 have a function in pH regulation by binding the transcription factor PacC/Rim101 and allowing its cleavage.[224] In higher eukaryotes, no PacC/Rim101 homologue has been identified.[225] The second Saccharomyces cerevisiae Alix homologue, Bro1, can be recruited to endosomes by binding to Vps32[226] which is part of the ESCRT-III complex (endosomal sorting complex required for transport III). Bro1 is conserved in evolution and mammalian Alix Protein also includes an N-terminal Bro1 domain. Additional to that domain, Alix structure contains a middle domain with a recently solved V-structure[227] and a proline-rich domain (PRD) which can bind to endophilins, SETA/CIN8530 and apoptosis linked gene 2 (ALG2).[228] The N-terminal Bro1 domain, as well as the PRD binds filamentous actin. The middle domain can recruit alpha actinin which bundles F-actin in stress fibers and cortactin which has a function in clathrin-mediated endocytosis.[175] Interestingly, Alix directly or indirectly interacts with different cell surface receptors like the transferrin receptor[176], the vasopressin receptor,[177] the platelet-derived growth factor beta-receptor[229], and the EGFR.[178] In this context, Alix has been suggested to play a role in down-regulation and sorting of cell surface receptors through the endosomal compartments toward the lysosome.[229] In addition, Alix can associate with different cytoskeleton elements. For example, Schmidt et

al.[174] demonstrated the interaction of Alix with the focal adhesion kinase and other cytoskeletal elements. Focal adhesion kinase is a protein involved in integrin-mediated cell migration.[230] Consistent with these findings, siRNA-mediated knock down of Alix inhibited cytoskeleton assembly in WI38 cells.[175] In addition to Alix, we found F-actin capping protein alpha-2 subunit and Ezrin to be regulated under conditions of severe inflammation. Both proteins are associated with cytoskeleton functions and cytoskeleton interactions, suggesting morphological rearrangements during development of severe colitis. Therefore, one possible function of Alix might be associated with mechanisms of cellular restitution and orchestrating the re-epithelialization process of intestinal wounds.

Stimulation of the EGFR may lead to phosphorylation of the MAP kinase ERK1/2. Since Alix may associate with the EGF Receptor and Alix expression in 293T cells inhibited insulin like growth factor-induced MAP kinase activation,[231] we postulated an inhibiting function of Alix on ERK1/2 phosphorylation. The Ras/Raf/MEK/ERK1/2 signalling cascade is important for cell proliferation and differentiation and is implicated in cell cycle regulation.[232] Nakamura et al.[233] showed that 5-hydroxy-l-tryptophan, a serotonin precursor, decreased phosphorylation of ERK1/2 and specifically induced actin remodelling in gut microvilli. Tarnawski et al.[197] demonstrated that gastric ulceration triggers overexpression of EGF and EGFR in epithelial cells of the ulcer margins. Interestingly, healing of the epithelial cell compartment of gastric ulcers involved activation of the EGFR-ERK1/2 signal transduction pathway, including a dramatic increase in ERK1/2 activity. Since we showed that ERK1/2 phosphorylation was dramatically reduced in severely inflamed mice, ERK1/2 activation might have a protective role for the epithelium by modulating IEC migration and cytoskeletal remodelling under conditions of moderate intestinal inflammation. Although Alix has been associated with EGFR signalling,[178] we showed that Alix siRNA knock down did not impact EGF-mediated ERK1/2 phosphorylation in epithelial cell cultures.

5.5. *Enterococcus faecalis* gelatinase and the *fsr* quorum sensing system contribute to experimental colitis – what is the role of bacterial proteases in IBD?

Inspired by the findings from Carrie Hew Ferstl et al.[179] who demonstrated that *E. faecalis* gelatinase gene expression was induced in our OG1-mice at 14 weeks but not at earlier time points (see Table 17), we further investigated the effect of this protease on colitis in gnotobiotic IL-10-/- mice. Therefore, we used an *E. faecalis* OG1RF isogenic deletion mutant for the *gelE* gene. In an additional approach, and to confirm results we monoassociated IL-10-/- mice with an *E. faecalis fsrB* deletion mutant, for it is known that the *fsr* quorum sensing system regulates gelatinase protein expression.

We were able to show that intestinal inflammation in monoassociated IL-10-/- mice was significantly reduced using the *gelE* mutant compared to the wild type strain OG1RF. This finding was significant for distal colon and proximal colon. Therefore, our results clearly demonstrate that *E. faecalis* gelatinase contributes to the development of experimental colitis. The effect of gelatinase might either be mediated by gelatinase itself or by a gelatinase-cleaved substrate.

As seen in Figure 32A-C inflammation was even more reduced in *E. faecalis* fsrB$^-$-monoassociated IL-10-/- mice. In contrast to the *E. faecalis* gelE$^-$ mutant significance was not only reached for the distal and proximal colon, but also for the cecum. These results verify the colitogenic effect of *E. faecalis* gelatinase. However, it is not surprising that deletion of the *fsrB* gene leads to a stronger effect, when keeping in mind that the *fsr* quorum sensing system not only regulates expression of gelatinase but also of serine protease and of numerous other proteins[51]. Therefore, this result also raises several new questions, for instance: Which other *fsrB*-induced proteins orchestrate with gelatinase? Is gelatinase itself the colitis increasing component or does it only activate the serine protease? The latter question will be discussed below.

E. faecalis gelatinase belongs to the thermolysin family (M4) of zinc containing metalloproteases [46]. *Bacillus thermoproteolyticus* thermolysin is the type example for this group. Interestingly, these zinc metalloproteases are frequently found in prokaryotic organisms and they are often implicated in bacteria-mediated diseases.

Examples are *Clostridium perfringens* λ-toxin[234], *Helicobacter pylori* hemagglutinin/proteinase[235, 236] or *Pseudomonas aeruginosa* pseudolysin[237]. Therefore, and to investigate whether our observed colitis contributing effects are specific for *E. faecalis* gelatinase, it would be interesting whether monoassociation of IL-10-/- mice with other commensal bacteria expressing similar M4-famlily members are also capable of inducing colitis and whether the colitis is also aggravated by these enzymes. These experiments would shed light on the question whether bacterial metalloproteases in general contribute to the development of colitis and whether the mechanisms of action are similar.

Our results suggest that treatment of colitis using a general protease inhibitor against commensal bacterial zinc metalloproteases might be possible. By now, treatment of IBD using protease inhibitors was mainly focused on the inhibition of host proteases. Examples for targets are the aggressive human matrix metalloproteinase MMP9 [238], which has been implicated in IBD as a tissue degrading mediator of disease[239] or the T-cell proteases dipeptidyl peptidase IV (DPIV/CD26) and aminopeptidase N (APN/CD13)[240], which play a role in immune activation by hydrolyzing bioactive polypeptides. However, the inhibition of commensal bacterial zinc metalloproteases by specific protease inhibitors might be a new treatment of IBD.

Another example for a thermolysin family member is the *Staphylococcus aureus* metalloproteinase aureolysin[241]. Interestingly, aureolysin is also regulated by a quorum sensing system, the *arg* system (accessory gene regulator)[242]. The *arg* system shows intriguing similarities to the *fsr* system. The deduced amino acid sequence of FsrA shows 26% identity and 38% similarity to *Staphylococcus aureus* AgrA (the transcription factor in the agr locus), FsrB shows 23% identity and 41% similarity to *S. aureus* AgrB, and FsrC shows 23% identity and 36% similarity to *S. aureus* AgrC[48]. Therefore, mechanisms in the better described *arg* system might be transferable to the *fsr* system. Furthermore, aureolysin is able to cleave and activate another *Staphylococcus aureus* protease, the serine protease V8[243]. This enzyme was shown to efficiently inactivate α1-proteinase inhibitor (α1-PI), a potent neutrophil elastase inhibitor of human plasma, by specific cleavage at the exposed reactive site. This cleavage leads to a molecule with potent chemotactic activity toward neutrophils[244]. Therefore, it is tempting to speculate that *E. faecalis* gelatinase is responsible for the activation of another protease, may be the co-transcribed *E.*

faecalis serine protease. Furthermore, active *E. faecalis* serine protease might induce chemotactic activity by cleavage of inhibitors in a similar manner.

To investigate mechanisms of a protease, its substrate specificity is valuable information. An early study by Shockman et al.[245] suggested casein, gelatin, hemoglobin, and a streptococcal lysine as substrates of *E. faecalis* gelatinase. Makinen et. al.[46] confirmed gelatin and casein as substrates, and expanded the list by adding insulin β-chain, collagen, fibrinogen, and several sex pheromone-related peptides of *E. faecalis*. Five years later they also identified human endothelin as an *E. faecalis* gelatinase substrate[246]. This should be investigated further, since endothelin-3 is a peptide hormone, which was recently found to stimulate human colonic epithelial cell proliferation and survival of goblet cells using an organotypic culture system[247]. The substrates of *E. faecalis* serine protease are poorly described. Hence, further investigation of the substrates of *E. faecalis* serine protease and *E. faecalis* gelatinase might pave the way for understanding pathologic mechanisms of *E. faecalis* - not only with respect to experimental colitis, but also - probably even more important - to the mechanisms of bacteremia and endocarditis.

One additional mode of action of *E. faecalis* gelatinase might be the activation of protease-activated receptor 2 (PAR-2). PARs are G-protein coupled receptors and are activated by the proteolytic cleavage of their N-terminal domain. Interestingly, these receptors were linked to irritable bowel syndrome (IBS) and were recently suggested as a target for the treatment of IBS[248]. In addition, Serralysin, a bacterial zinc metalloprotease secreted by *Serratia marcescens*, a gram negative opportunistic pathogen causing nosocomial infections, activated NFκB via PAR-2 in epithelial cells[249], suggesting a similar mechanism for the effect of *E. faecalis* gelatinase.

The already mentioned human MMP9 and MMP2 are also zinc metalloproteases and gelatinases. MMP2 and MMP9 are parts of complex protease cleavage cascades which are in general activated by the cleavage of an inactive zymogen of one of the cascade members. After activation, MMP9 cleaves and destroys extracellular matrix tissue, which is necessary in tissue remodeling, repair, and for the movement of immune cells. Interestingly, MMP9 and MMP2 are up-regulated in colitis[238]. Therefore, it might be possible that *E. faecalis* gelatinase activates a human protease

cascade by cleavage of a human inactive MMP zymogen or by cleavage of its inhibitors. Since balance between MMPs and their tissue inhibitors is essential for homeostasis in the gut, and imbalances are implicated in IBD[239], this might represent a second possible mode of action of *E. faecalis* gelatinase or serine protease.

6. List of Tables

7. List of Figures

8. References

1. Tannock G.W. (2002) Enterococci as members of the intestinal microflora of humans. In: *The enterococci: pathogenesis, molecular biology and antibiotic resistance* (ed. M. S. Gilmore), p. 101. American Society for Microbiology, Washington D.C.
2. Kropec A., Hufnagel M., Zimmermann K. & Huebner J. (2005) In vitro assessment of the host response against Enterococcus faecalis used in probiotic preparations. *Infection*, **33,** 377.
3. Centeno J.A., Menendez S., Hermida M. & Rodriguez-Otero J.L. (1999) Effects of the addition of Enterococcus faecalis in Cebreiro cheese manufacture. *Int J Food Microbiol*, **48,** 97.
4. Hugas M., Garriga M. & Aymerich M.T. (2003) Functionality of enterococci in meat products. *International Journal of Food Microbiology*, **88,** 223.
5. Willems R.J. & Bonten M.J. (2007) Glycopeptide-resistant enterococci: deciphering virulence, resistance and epidemicity. *Curr Opin Infect Dis*, **20,** 384.
6. Klein G. (2003) Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *Int J Food Microbiol*, **88,** 123.
7. Nallapareddy S.R., Singh K.V., Sillanpaa J., Garsin D.A., Hook M., Erlandsen S.L. & Murray B.E. (2006) Endocarditis and biofilm-associated pili of Enterococcus faecalis. *J Clin Invest*, **116,** 2799.
8. Graninger W. & Ragette R. (1992) Nosocomial bacteremia due to Enterococcus faecalis without endocarditis. *Clin Infect Dis*, **15,** 49.
9. Stuart C.H., Schwartz S.A., Beeson T.J. & Owatz C.B. (2006) Enterococcus faecalis: its role in root canal treatment failure and current concepts in retreatment. *J Endod*, **32,** 93.
10. Guzman C.A., Pruzzo C., LiPira G. & Calegari L. (1989) Role of adherence in pathogenesis of Enterococcus faecalis urinary tract infection and endocarditis. *Infect Immun*, **57,** 1834.
11. Villani F. & Coppola S. (1994) Selection of enterococcal strains for water buffalo Mozzarella cheese manufacture. *Annali di Microbiologia ed Enzimologia*, **44,** 97.
12. Leclerc H., Devriese L.A. & Mossel D.A. (1996) Taxonomical changes in intestinal (faecal) enterococci and streptococci: consequences on their use as indicators of faecal contamination in drinking water. *J Appl Bacteriol*, **81,** 459.
13. Ulrich A. & Muller T. (1998) Heterogeneity of plant-associated streptococci as characterized by phenotypic features and restriction analysis of PCR-amplified 16S rDNA. *J Appl Microbiol*, **84,** 293.
14. Sherman J.M. (1938) The Enterococci and Related Streptococci. *J Bacteriol*, **35,** 81.
15. Schleifer K.H. & Kilpper-Balz R. (1984) Transfer of Streptococcus faecalis and Streptococcus faecium to the genus Enterococcus nom. rev. as Enterococcus faecalis comb. nov. and Enterococcus faecium comb. nov. *Int. J. Syst. Bacteriol.*, **34,** 31.
16. Devriese L.A., Pot B. & Collins M.D. (1993) Phenotypic identification of the genus Enterococcus and differentiation of phylogenetically distinct enterococcal species and species groups. *J Appl Bacteriol*, **75,** 399.

17. Farrow J.A., Jones D., Phillips B.A. & Collins M.D. (1983) Taxonomic studies on some group D streptococci. *J Gen Microbiol*, **129,** 1423.
18. Corfield A.P., Wagner S.A., Clamp J.R., Kriaris M.S. & Hoskins L.C. (1992) Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. *Infect Immun*, **60,** 3971.
19. Paulsen I.T., Banerjei L., Myers G.S., Nelson K.E., Seshadri R., Read T.D., Fouts D.E., Eisen J.A., Gill S.R., Heidelberg J.F., Tettelin H., Dodson R.J., Umayam L., Brinkac L., Beanan M., Daugherty S., DeBoy R.T., Durkin S., Kolonay J., Madupu R., Nelson W., Vamathevan J., Tran B., Upton J., Hansen T., Shetty J., Khouri H., Utterback T., Radune D., Ketchum K.A., Dougherty B.A. & Fraser C.M. (2003) Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. *Science*, **299,** 2071.
20. Devriese L.A., Van de Kerckhove A., Kilpper-Balz R. & Schleifer K.H. (1987) Characterization and identification of Enterococcus species isolated from the intestines of animals. *Int. J. Syst. Bacteriol.*, **37,** 257.
21. Franz C.M., Holzapfel W.H. & Stiles M.E. (1999) Enterococci at the crossroads of food safety? *Int J Food Microbiol*, **47,** 1.
22. Tzanetakis N., Vafopoulou-Mastrojiannaki A. & Litopoulou-Tzanetaki E. (1995) The quality of white-brined cheese from goat´s milk made with different starters. *Food Microbiology*, **12,** 55.
23. Grande M.J., Abriouel H., Lucas Lopez R., Valdivia E., Ben Omar N., Martinez-Canamero M. & Galvez A. (2007) Efficacy of enterocin AS-48 against bacilli in ready-to-eat vegetable soups and purees. *J Food Prot*, **70,** 2339.
24. Molinos A.C., Abriouel H., Ben Omar N., Valdivia E., Lopez R.L., Maqueda M., Canamero M.M. & Galvez A. (2005) Effect of immersion solutions containing enterocin AS-48 on Listeria monocytogenes in vegetable foods. *Appl Environ Microbiol*, **71,** 7781.
25. Savage D.C. (1977) Microbial ecology of the gastrointestinal tract. *Annu Rev Microbiol*, **31,** 107.
26. Frank D.N., St Amand A.L., Feldman R.A., Boedeker E.C., Harpaz N. & Pace N.R. (2007) Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci U S A*, **104,** 13780.
27. Zoetendal E.G., Collier C.T., Koike S., Mackie R.I. & Gaskins H.R. (2004) Molecular ecological analysis of the gastrointestinal microbiota: a review. *J Nutr*, **134,** 465.
28. Moore W.E. & Holdeman L.V. (1974) Human fecal flora: the normal flora of 20 Japanese-Hawaiians. *Appl Microbiol*, **27,** 961.
29. Tannock G.W., Munro K., Harmsen H.J., Welling G.W., Smart J. & Gopal P.K. (2000) Analysis of the fecal microflora of human subjects consuming a probiotic product containing Lactobacillus rhamnosus DR20. *Appl Environ Microbiol*, **66,** 2578.
30. Sartor R.B. (2008) Microbial influences in inflammatory bowel diseases. *Gastroenterology*, **134,** 577.
31. Sghir A., Gramet G., Suau A., Rochet V., Pochart P. & Dore J. (2000) Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridization. *Appl Environ Microbiol*, **66,** 2263.
32. Finegold S.M., Sutter V.L. & Mathisen G.E. (1983) Normal indigenous intestinal flora. In: *Human Intestinal Microflora in Health and Disease* (ed. D. J. Hentges), p. 3. Academic Press, New York.

33. Devriese L.A. & Pot B. (1995) The genus *Enterococcus*. In: *The lactic acid bacteria. The genera of lactid acid bacteria.* (ed. B. J. B. Wood & W. H. Holzapfel), Vol. 2, p. 327. Blackie Academic and Professional, London.
34. Poeta P., Radhouani H., Sargo R. & Igrejas G. (2008) In vitro activity of dalbavancin against enterococci isolates from wild animals, pets, poultry and humans in Portugal. *J Basic Microbiol*, **48,** 526.
35. Stark P.L. & Lee A. (1982) The microbial ecology of the large bowel of breast-fed and formula-fed infants during the first year of life. *J Med Microbiol*, **15,** 189.
36. FAO/WHO (2001) Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria.
37. Bellomo G., Mangiagle A., Nicastro L. & Frigerio G. (1980) A controlled double-blind study of SF68 strain as a new biological preparation for the treatment of diarrhoea in pediatrics. *Curr. Ther. Res.*, **28,** 627.
38. Benyacoub J., Perez P.F., Rochat F., Saudan K.Y., Reuteler G., Antille N., Humen M., De Antoni G.L., Cavadini C., Blum S. & Schiffrin E.J. (2005) Enterococcus faecium SF68 enhances the immune response to Giardia intestinalis in mice. *J Nutr*, **135,** 1171.
39. Agerholm-Larsen L., Bell M.L., Grunwald G.K. & Astrup A. (2000) The effect of a probiotic milk product on plasma cholesterol: a meta-analysis of short-term intervention studies. *Eur J Clin Nutr*, **54,** 856.
40. Lund B., Adamsson I. & Edlund C. (2002) Gastrointestinal transit survival of an Enterococcus faecium probiotic strain administered with or without vancomycin. *Int J Food Microbiol*, **77,** 109.
41. Jansen G., Deddens B., Wilkinson M. & van der Waaij D. (1993) Significant decrease of titres of circulating IgG after oral intake of a preparation of Enterococcus faecalis in a group of ten healthy volunteers. *Infection*, **21,** 193.
42. Foulquie Moreno M.R., Sarantinopoulos P., Tsakalidou E. & De Vuyst L. (2006) The role and application of enterococci in food and health. *Int J Food Microbiol*, **106,** 1.
43. Morrison D., Woodford N. & Cookson B. (1997) Enterococci as emerging pathogens of humans. *Soc Appl Bacteriol Symp Ser*, **26,** 89S.
44. Somkuti G.A. & Babel F.J. (1969) Hydrolytic break down of casein by a proteinase of *Streptococcus faecalis var. liquefaciens*. *Journal of Dairy Science*, **52,** 1186.
45. Sarantinopoulos P., Andrighetto C., Georgalaki M.D., Rea M.C., Lombardi A., Cogan T.M., Kalantzopoulos G. & Tsakalidou E. (2001) Biochemical properties of *enterococci* relevant to their technological performance. *International Dairy Journal*, **11,** 621.
46. Makinen P.L., Clewell D.B., An F. & Makinen K.K. (1989) Purification and substrate specificity of a strongly hydrophobic extracellular metalloendopeptidase ("gelatinase") from Streptococcus faecalis (strain 0G1-10). *J Biol Chem*, **264,** 3325.
47. Sifri C.D., Mylonakis E., Singh K.V., Qin X., Garsin D.A., Murray B.E., Ausubel F.M. & Calderwood S.B. (2002) Virulence Effect of Enterococcus faecalis Protease Genes and the Quorum-Sensing Locus fsr in Caenorhabditis elegans and Mice. *Infect Immun*, **70,** 5647.

48. Qin X., Singh K.V., Weinstock G.M. & Murray B.E. (2000) Effects of Enterococcus faecalis fsr genes on production of gelatinase and a serine protease and virulence. *Infect Immun*, **68,** 2579.
49. Mylonakis E., Engelbert M., Qin X., Sifri C.D., Murray B.E., Ausubel F.M., Gilmore M.S. & Calderwood S.B. (2002) The Enterococcus faecalis fsrB gene, a key component of the fsr quorum-sensing system, is associated with virulence in the rabbit endophthalmitis model. *Infect Immun*, **70,** 4678.
50. Nakayama J., Cao Y., Horii T., Sakuda S., Akkermans A.D., de Vos W.M. & Nagasawa H. (2001) Gelatinase biosynthesis-activating pheromone: a peptide lactone that mediates a quorum sensing in Enterococcus faecalis. *Mol Microbiol*, **41,** 145.
51. Bourgogne A., Hilsenbeck S.G., Dunny G.M. & Murray B.E. (2006) Comparison of OG1RF and an isogenic fsrB deletion mutant by transcriptional analysis: the Fsr system of Enterococcus faecalis is more than the activator of gelatinase and serine protease. *J Bacteriol*, **188,** 2875.
52. Podbielski A. & Kreikemeyer B. (2004) Cell density--dependent regulation: basic principles and effects on the virulence of Gram-positive cocci. *Int J Infect Dis*, **8,** 81.
53. Nakayama J., Chen S., Oyama N., Nishiguchi K., Azab E.A., Tanaka E., Kariyama R. & Sonomoto K. (2006) Revised model for Enterococcus faecalis fsr quorum-sensing system: the small open reading frame fsrD encodes the gelatinase biosynthesis-activating pheromone propeptide corresponding to staphylococcal agrd. *J Bacteriol*, **188,** 8321.
54. Coburn P.S. & Gilmore M.S. (2003) The Enterococcus faecalis cytolysin: a novel toxin active against eukaryotic and prokaryotic cells. *Cell Microbiol*, **5,** 661.
55. Huycke M.M., Spiegel C.A. & Gilmore M.S. (1991) Bacteremia caused by hemolytic, high-level gentamicin-resistant Enterococcus faecalis. *Antimicrob Agents Chemother*, **35,** 1626.
56. Ike Y., Hashimoto H. & Clewell D.B. (1984) Hemolysin of Streptococcus faecalis subspecies zymogenes contributes to virulence in mice. *Infect Immun*, **45,** 528.
57. Singh K.V., Qin X., Weinstock G.M. & Murray B.E. (1998) Generation and testing of mutants of Enterococcus faecalis in a mouse peritonitis model. *J Infect Dis*, **178,** 1416.
58. Garsin D.A., Sifri C.D., Mylonakis E., Qin X., Singh K.V., Murray B.E., Calderwood S.B. & Ausubel F.M. (2001) A simple model host for identifying Gram-positive virulence factors. *Proc Natl Acad Sci U S A*, **98,** 10892.
59. Shankar N., Lockatell C.V., Baghdayan A.S., Drachenberg C., Gilmore M.S. & Johnson D.E. (2001) Role of Enterococcus faecalis surface protein Esp in the pathogenesis of ascending urinary tract infection. *Infect Immun*, **69,** 4366.
60. Toledo-Arana A., Valle J., Solano C., Arrizubieta M.J., Cucarella C., Lamata M., Amorena B., Leiva J., Penades J.R. & Lasa I. (2001) The enterococcal surface protein, Esp, is involved in Enterococcus faecalis biofilm formation. *Appl Environ Microbiol*, **67,** 4538.
61. Isenmann R., Schwarz M., Rozdzinski E., Christ C., Schmidt E., Augat P., Marre R. & Beger H.G. (2002) Interaction of fibronectin and aggregation substance promotes adherence of Enterococcus faecalis to human colon. *Dig Dis Sci*, **47,** 462.

62. Dunny G.M., Leonard B.A. & Hedberg P.J. (1995) Pheromone-inducible conjugation in Enterococcus faecalis: interbacterial and host-parasite chemical communication. *J Bacteriol*, **177,** 871.
63. Sartingen S., Rozdzinski E., Muscholl-Silberhorn A. & Marre R. (2000) Aggregation substance increases adherence and internalization, but not translocation, of Enterococcus faecalis through different intestinal epithelial cells in vitro. *Infect Immun*, **68,** 6044.
64. Sussmuth S.D., Muscholl-Silberhorn A., Wirth R., Susa M., Marre R. & Rozdzinski E. (2000) Aggregation substance promotes adherence, phagocytosis, and intracellular survival of Enterococcus faecalis within human macrophages and suppresses respiratory burst. *Infect Immun*, **68,** 4900.
65. Rich R.L., Kreikemeyer B., Owens R.T., LaBrenz S., Narayana S.V., Weinstock G.M., Murray B.E. & Hook M. (1999) Ace is a collagen-binding MSCRAMM from Enterococcus faecalis. *J Biol Chem*, **274,** 26939.
66. Lowe A.M., Lambert P.A. & Smith A.W. (1995) Cloning of an Enterococcus faecalis endocarditis antigen: homology with adhesins from some oral streptococci. *Infect Immun*, **63,** 703.
67. Singh K.V., Coque T.M., Weinstock G.M. & Murray B.E. (1998) In vivo testing of an Enterococcus faecalis efaA mutant and use of efaA homologs for species identification. *FEMS Immunol Med Microbiol*, **21,** 323.
68. Giraffa G. (2002) Enterococci from foods. *FEMS Microbiol Rev*, **26,** 163.
69. Willems R.J., Top J., van Santen M., Robinson D.A., Coque T.M., Baquero F., Grundmann H. & Bonten M.J. (2005) Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex. *Emerg Infect Dis*, **11,** 821.
70. Mundy L.M., Sahm D.F. & Gilmore M. (2000) Relationships between enterococcal virulence and antimicrobial resistance. *Clin Microbiol Rev*, **13,** 513.
71. Ley R.E., Peterson D.A. & Gordon J.I. (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, **124,** 837.
72. Gill S.R., Pop M., Deboy R.T., Eckburg P.B., Turnbaugh P.J., Samuel B.S., Gordon J.I., Relman D.A., Fraser-Liggett C.M. & Nelson K.E. (2006) Metagenomic analysis of the human distal gut microbiome. *Science*, **312,** 1355.
73. McNeil N.I. (1984) The contribution of the large intestine to energy supplies in man. *Am J Clin Nutr*, **39,** 338.
74. Magalhaes J.G., Tattoli I. & Girardin S.E. (2007) The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. *Semin Immunol*, **19,** 106.
75. Crosnier C., Stamataki D. & Lewis J. (2006) Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet*, **7,** 349.
76. van der Sluis M., Bouma J., Vincent A., Velcich A., Carraway K.L., Buller H.A., Einerhand A.W., van Goudoever J.B., Van Seuningen I. & Renes I.B. (2008) Combined defects in epithelial and immunoregulatory factors exacerbate the pathogenesis of inflammation: mucin 2-interleukin 10-deficient mice. *Lab Invest*, **88,** 634.
77. Velcich A., Yang W., Heyer J., Fragale A., Nicholas C., Viani S., Kucherlapati R., Lipkin M., Yang K. & Augenlicht L. (2002) Colorectal cancer in mice genetically deficient in the mucin Muc2. *Science*, **295,** 1726.

78. Cheng H. & Bjerknes M. (1983) Cell production in mouse intestinal epithelium measured by stathmokinetic flow cytometry and Coulter particle counting. *Anat Rec*, **207,** 427.
79. Cheng H. & Bjerknes M. (1982) Whole population cell kinetics of mouse duodenal, jejunal, ileal, and colonic epithelia as determined by radioautography and flow cytometry. *Anat Rec*, **203,** 251.
80. Akira S., Uematsu S. & Takeuchi O. (2006) Pathogen recognition and innate immunity. *Cell*, **124,** 783.
81. Kawai T. & Akira S. (2007) Signaling to NF-kappaB by Toll-like receptors. *Trends Mol Med*, **13,** 460.
82. Ruiz P. (2007) Molecular mechanisms for the inhibition of the nuclear factor kappa B signal transduction in intestinal epithelial cells under conditions of chronic inflammation. In: *PhD Thesis, Fachgebiet für Experimentelle Ernährungsmedizin*. Technische Universität München, Freising-Weihenstephan.
83. Russell M.W., Sibley D.A., Nikolova E.B., Tomana M. & Mestecky J. (1997) IgA antibody as a non-inflammatory regulator of immunity. *Biochem Soc Trans*, **25,** 466.
84. Roy M.J. & Varvayanis M. (1987) Development of dome epithelium in gut-associated lymphoid tissues: association of IgA with M cells. *Cell Tissue Res*, **248,** 645.
85. Nagler-Anderson C. (2001) Man the barrier! Strategic defences in the intestinal mucosa. *Nat Rev Immunol*, **1,** 59.
86. Robinson J.K., Blanchard T.G., Levine A.D., Emancipator S.N. & Lamm M.E. (2001) A mucosal IgA-mediated excretory immune system in vivo. *J Immunol*, **166,** 3688.
87. Fernandez M.I., Pedron T., Tournebize R., Olivo-Marin J.C., Sansonetti P.J. & Phalipon A. (2003) Anti-inflammatory role for intracellular dimeric immunoglobulin a by neutralization of lipopolysaccharide in epithelial cells. *Immunity*, **18,** 739.
88. Rescigno M., Urbano M., Valzasina B., Francolini M., Rotta G., Bonasio R., Granucci F., Kraehenbuhl J.P. & Ricciardi-Castagnoli P. (2001) Dendritic cells express tight junction proteins and penetrate gut epithelial monolayers to sample bacteria. *Nat Immunol*, **2,** 361.
89. Niess J.H., Brand S., Gu X., Landsman L., Jung S., McCormick B.A., Vyas J.M., Boes M., Ploegh H.L., Fox J.G., Littman D.R. & Reinecker H.C. (2005) CX3CR1-mediated dendritic cell access to the intestinal lumen and bacterial clearance. *Science*, **307,** 254.
90. Xavier R.J. & Podolsky D.K. (2007) Unravelling the pathogenesis of inflammatory bowel disease. *Nature*, **448,** 427.
91. Wirtz S. & Neurath M.F. (2007) Mouse models of inflammatory bowel disease. *Adv Drug Deliv Rev*, **59,** 1073.
92. Brown S.J. & Mayer L. (2007) The immune response in inflammatory bowel disease. *Am J Gastroenterol*, **102,** 2058.
93. Loftus E.V., Jr. (2004) Clinical epidemiology of inflammatory bowel disease: Incidence, prevalence, and environmental influences. *Gastroenterology*, **126,** 1504.
94. Ahmad T., Tamboli C.P., Jewell D. & Colombel J.F. (2004) Clinical relevance of advances in genetics and pharmacogenetics of IBD. *Gastroenterology*, **126,** 1533.

95. Bouma G. & Strober W. (2003) The immunological and genetic basis of inflammatory bowel disease. *Nat Rev Immunol*, **3,** 521.
96. Hoffmann J.C., Pawlowski N.N., Kuhl A.A., Hohne W. & Zeitz M. (2002) Animal models of inflammatory bowel disease: an overview. *Pathobiology*, **70,** 121.
97. Sellon R.K., Tonkonogy S., Schultz M., Dieleman L.A., Grenther W., Balish E., Rennick D.M. & Sartor R.B. (1998) Resident enteric bacteria are necessary for development of spontaneous colitis and immune system activation in interleukin-10-deficient mice. *Infect Immun*, **66,** 5224.
98. Brandwein S.L., McCabe R.P., Cong Y., Waites K.B., Ridwan B.U., Dean P.A., Ohkusa T., Birkenmeier E.H., Sundberg J.P. & Elson C.O. (1997) Spontaneously colitic C3H/HeJBir mice demonstrate selective antibody reactivity to antigens of the enteric bacterial flora. *J Immunol*, **159,** 44.
99. Matsumoto S., Okabe Y., Setoyama H., Takayama K., Ohtsuka J., Funahashi H., Imaoka A., Okada Y. & Umesaki Y. (1998) Inflammatory bowel disease-like enteritis and caecitis in a senescence accelerated mouse P1/Yit strain. *Gut*, **43,** 71.
100. Aranda R., Sydora B.C., McAllister P.L., Binder S.W., Yang H.Y., Targan S.R. & Kronenberg M. (1997) Analysis of intestinal lymphocytes in mouse colitis mediated by transfer of CD4+, CD45RBhigh T cells to SCID recipients. *J Immunol*, **158,** 3464.
101. Ehrhardt R.O., Ludviksson B.R., Gray B., Neurath M. & Strober W. (1997) Induction and prevention of colonic inflammation in IL-2-deficient mice. *J Immunol*, **158,** 566.
102. Schultz M., Tonkonogy S.L., Sellon R.K., Veltkamp C., Godfrey V.L., Kwon J., Grenther W.B., Balish E., Horak I. & Sartor R.B. (1999) IL-2-deficient mice raised under germfree conditions develop delayed mild focal intestinal inflammation. *Am J Physiol*, **276,** G1461.
103. Dianda L., Hanby A.M., Wright N.A., Sebesteny A., Hayday A.C. & Owen M.J. (1997) T cell receptor-alpha beta-deficient mice fail to develop colitis in the absence of a microbial environment. *Am J Pathol*, **150,** 91.
104. Taurog J.D., Richardson J.A., Croft J.T., Simmons W.A., Zhou M., Fernandez-Sueiro J.L., Balish E. & Hammer R.E. (1994) The germfree state prevents development of gut and joint inflammatory disease in HLA-B27 transgenic rats. *J Exp Med*, **180,** 2359.
105. Onderdonk A.B., Franklin M.L. & Cisneros R.L. (1981) Production of experimental ulcerative colitis in gnotobiotic guinea pigs with simplified microflora. *Infect Immun*, **32,** 225.
106. Mizoguchi A. & Mizoguchi E. (2008) Inflammatory bowel disease, past, present and future: lessons from animal models. *J Gastroenterol*, **43,** 1.
107. Strober W., Fuss I.J. & Blumberg R.S. (2002) The immunology of mucosal models of inflammation. *Annu Rev Immunol*, **20,** 495.
108. Park H., Li Z., Yang X.O., Chang S.H., Nurieva R., Wang Y.H., Wang Y., Hood L., Zhu Z., Tian Q. & Dong C. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol*, **6,** 1133.
109. Harrington L.E., Hatton R.D., Mangan P.R., Turner H., Murphy T.L., Murphy K.M. & Weaver C.T. (2005) Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol*, **6,** 1123.

110. Monteleone I., Pallone F. & Monteleone G. (2009) Interleukin-23 and Th17 cells in the control of gut inflammation. *Mediators Inflamm*, **2009,** 297645.
111. Powrie F., Leach M.W., Mauze S., Caddle L.B. & Coffman R.L. (1993) Phenotypically distinct subsets of CD4+ T cells induce or protect from chronic intestinal inflammation in C. B-17 scid mice. *Int Immunol*, **5,** 1461.
112. Okayasu I., Hatakeyama S., Yamada M., Ohkusa T., Inagaki Y. & Nakaya R. (1990) A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice. *Gastroenterology*, **98,** 694.
113. Cooper H.S., Murthy S.N., Shah R.S. & Sedergran D.J. (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest*, **69,** 238.
114. Dieleman L.A., Ridwan B.U., Tennyson G.S., Beagley K.W., Bucy R.P. & Elson C.O. (1994) Dextran sulfate sodium-induced colitis occurs in severe combined immunodeficient mice. *Gastroenterology*, **107,** 1643.
115. Santana A., Medina C., Paz-Cabrera M.C., Diaz-Gonzalez F., Farre E., Salas A., Radomski M.W. & Quintero E. (2006) Attenuation of dextran sodium sulphate induced colitis in matrix metalloproteinase-9 deficient mice. *World J Gastroenterol*, **12,** 6464.
116. Kitajima S., Morimoto M., Sagara E., Shimizu C. & Ikeda Y. (2001) Dextran sodium sulfate-induced colitis in germ-free IQI/Jic mice. *Exp Anim*, **50,** 387.
117. Hudcovic T., Stepankova R., Cebra J. & Tlaskalova-Hogenova H. (2001) The role of microflora in the development of intestinal inflammation: acute and chronic colitis induced by dextran sulfate in germ-free and conventionally reared immunocompetent and immunodeficient mice. *Folia Microbiol (Praha)*, **46,** 565.
118. Hudcovic T., Stepankova R., Kozakova H., Hrncir T. & Tlaskalova-Hogenova H. (2007) Effects of monocolonization with Escherichia coli strains O6K13 and Nissle 1917 on the development of experimentally induced acute and chronic intestinal inflammation in germ-free immunocompetent and immunodeficient mice. *Folia Microbiol (Praha)*, **52,** 618.
119. Mahler M., Bristol I.J., Leiter E.H., Workman A.E., Birkenmeier E.H., Elson C.O. & Sundberg J.P. (1998) Differential susceptibility of inbred mouse strains to dextran sulfate sodium-induced colitis. *Am J Physiol*, **274,** G544.
120. Kuhn R., Lohler J., Rennick D., Rajewsky K. & Muller W. (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*, **75,** 263.
121. Makkink M.K., Schwerbrock N.M., Mahler M., Boshuizen J.A., Renes I.B., Cornberg M., Hedrich H.J., Einerhand A.W., Buller H.A., Wagner S., Enss M.L. & Dekker J. (2002) Fate of goblet cells in experimental colitis. *Dig Dis Sci*, **47,** 2286.
122. Berg D.J., Davidson N., Kuhn R., Muller W., Menon S., Holland G., Thompson-Snipes L., Leach M.W. & Rennick D. (1996) Enterocolitis and colon cancer in interleukin-10-deficient mice are associated with aberrant cytokine production and CD4(+) TH1-like responses. *J Clin Invest*, **98,** 1010.
123. Kim S.C., Tonkonogy S.L., Albright C.A., Tsang J., Balish E.J., Braun J., Huycke M.M. & Sartor R.B. (2005) Variable phenotypes of enterocolitis in interleukin 10-deficient mice monoassociated with two different commensal bacteria. *Gastroenterology*, **128,** 891.
124. Kim S.C., Tonkonogy S.L., Karrasch T., Jobin C. & Sartor R.B. (2007) Dual-association of gnotobiotic IL-10-/- mice with 2 nonpathogenic commensal bacteria induces aggressive pancolitis. *Inflamm Bowel Dis*, **13,** 1457.

125. Sydora B.C., Tavernini M.M., Doyle J.S. & Fedorak R.N. (2005) Association with selected bacteria does not cause enterocolitis in IL-10 gene-deficient mice despite a systemic immune response. *Dig Dis Sci*, **50,** 905.
126. Schultz M., Veltkamp C., Dieleman L.A., Grenther W.B., Wyrick P.B., Tonkonogy S.L. & Sartor R.B. (2002) Lactobacillus plantarum 299V in the treatment and prevention of spontaneous colitis in interleukin-10-deficient mice. *Inflamm Bowel Dis*, **8,** 71.
127. Hammer R.E., Maika S.D., Richardson J.A., Tang J.P. & Taurog J.D. (1990) Spontaneous inflammatory disease in transgenic rats expressing HLA-B27 and human beta 2m: an animal model of HLA-B27-associated human disorders. *Cell*, **63,** 1099.
128. Rath H.C., Wilson K.H. & Sartor R.B. (1999) Differential induction of colitis and gastritis in HLA-B27 transgenic rats selectively colonized with Bacteroides vulgatus or Escherichia coli. *Infect Immun*, **67,** 2969.
129. Hermiston M.L. & Gordon J.I. (1995) Inflammatory bowel disease and adenomas in mice expressing a dominant negative N-cadherin. *Science*, **270,** 1203.
130. Baribault H., Penner J., Iozzo R.V. & Wilson-Heiner M. (1994) Colorectal hyperplasia and inflammation in keratin 8-deficient FVB/N mice. *Genes & Dev.*, **8,** 2964.
131. Van der Sluis M., De Koning B.A., De Bruijn A.C., Velcich A., Meijerink J.P., Van Goudoever J.B., Buller H.A., Dekker J., Van Seuningen I., Renes I.B. & Einerhand A.W. (2006) Muc2-deficient mice spontaneously develop colitis, indicating that MUC2 is critical for colonic protection. *Gastroenterology*, **131,** 117.
132. Waidmann M B.O., Frick JS, Lehr HA, Schubert S, Dobrindt U, Loeffler J, Bohn E, Autenrieth IB (2003) Bacteroides vulgatus protects against Escherichia coli-induced colitis in gnotobiotic interleukin-2-deficient mice. *Gastroenterology*.
133. Sartor R.B. (2004) Therapeutic manipulation of the enteric microflora in inflammatory bowel diseases: antibiotics, probiotics, and prebiotics. *Gastroenterology*, **126,** 1620.
134. Tamboli C.P., Caucheteux C., Cortot A., Colombel J.F. & Desreumaux P. (2003) Probiotics in inflammatory bowel disease: a critical review. *Best Pract Res Clin Gastroenterol*, **17,** 805.
135. Darfeuille-Michaud A., Boudeau J., Bulois P., Neut C., Glasser A.L., Barnich N., Bringer M.A., Swidsinski A., Beaugerie L. & Colombel J.F. (2004) High prevalence of adherent-invasive Escherichia coli associated with ileal mucosa in Crohn's disease. *Gastroenterology*, **127,** 412.
136. Glasser A.L., Boudeau J., Barnich N., Perruchot M.H., Colombel J.F. & Darfeuille-Michaud A. (2001) Adherent invasive Escherichia coli strains from patients with Crohn's disease survive and replicate within macrophages without inducing host cell death. *Infect Immun*, **69,** 5529.
137. Barnich N., Carvalho F.A., Glasser A.L., Darcha C., Jantscheff P., Allez M., Peeters H., Bommelaer G., Desreumaux P., Colombel J.F. & Darfeuille-Michaud A. (2007) CEACAM6 acts as a receptor for adherent-invasive E. coli, supporting ileal mucosa colonization in Crohn disease. *J Clin Invest*, **117,** 1566.
138. Carvalho F.A., Barnich N., Sauvanet P., Darcha C., Gelot A. & Darfeuille-Michaud A. (2008) Crohn's disease-associated Escherichia coli LF82

aggravates colitis in injured mouse colon via signaling by flagellin. *Inflamm Bowel Dis*, **14,** 1051.
139. Swidsinski A., Ladhoff A., Pernthaler A., Swidsinski S., Loening-Baucke V., Ortner M., Weber J., Hoffmann U., Schreiber S., Dietel M. & Lochs H. (2002) Mucosal flora in inflammatory bowel disease. *Gastroenterology*, **122,** 44.
140. Neut C., Bulois P., Desreumaux P., Membre J.M., Lederman E., Gambiez L., Cortot A., Quandalle P., van Kruiningen H. & Colombel J.F. (2002) Changes in the bacterial flora of the neoterminal ileum after ileocolonic resection for Crohn's disease. *Am J Gastroenterol*, **97,** 939.
141. Favier C., Neut C., Mizon C., Cortot A., Colombel J.F. & Mizon J. (1997) Fecal beta-D-galactosidase production and Bifidobacteria are decreased in Crohn's disease. *Dig Dis Sci*, **42,** 817.
142. Hugot J.P., Chamaillard M., Zouali H., Lesage S., Cezard J.P., Belaiche J., Almer S., Tysk C., O'Morain C.A., Gassull M., Binder V., Finkel Y., Cortot A., Modigliani R., Laurent-Puig P., Gower-Rousseau C., Macry J., Colombel J.F., Sahbatou M. & Thomas G. (2001) Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease. *Nature*, **411,** 599.
143. Wehkamp J., Salzman N.H., Porter E., Nuding S., Weichenthal M., Petras R.E., Shen B., Schaeffeler E., Schwab M., Linzmeier R., Feathers R.W., Chu H., Lima H., Jr., Fellermann K., Ganz T., Stange E.F. & Bevins C.L. (2005) Reduced Paneth cell alpha-defensins in ileal Crohn's disease. *Proc Natl Acad Sci U S A*, **102,** 18129.
144. Ferguson L.R., Browning B.L., Huebner C., Petermann I., Shelling A.N., Demmers P., McCulloch A., Gearry R.B., Barclay M.L. & Philpott M. (2008) Single nucleotide polymorphisms in human Paneth cell defensin A5 may confer susceptibility to inflammatory bowel disease in a New Zealand Caucasian population. *Dig Liver Dis*, **40,** 723.
145. Ramasundara M., Leach S.T., Lemberg D.A. & Day A.S. (2009) Defensins and inflammation: the role of defensins in inflammatory bowel disease. *J Gastroenterol Hepatol*, **24,** 202.
146. Wehkamp J., Harder J., Wehkamp K., Wehkamp-von Meissner B., Schlee M., Enders C., Sonnenborn U., Nuding S., Bengmark S., Fellermann K., Schroder J.M. & Stange E.F. (2004) NF-kappaB- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium. *Infect Immun*, **72,** 5750.
147. Franchimont D., Vermeire S., El Housni H., Pierik M., Van Steen K., Gustot T., Quertinmont E., Abramowicz M., Van Gossum A., Deviere J. & Rutgeerts P. (2004) Deficient host-bacteria interactions in inflammatory bowel disease? The toll-like receptor (TLR)-4 Asp299gly polymorphism is associated with Crohn's disease and ulcerative colitis. *Gut*, **53,** 987.
148. Pierik M., Joossens S., Van Steen K., Van Schuerbeek N., Vlietinck R., Rutgeerts P. & Vermeire S. (2006) Toll-like receptor-1, -2, and -6 polymorphisms influence disease extension in inflammatory bowel diseases. *Inflamm Bowel Dis*, **12,** 1.
149. Hampe J., Franke A., Rosenstiel P., Till A., Teuber M., Huse K., Albrecht M., Mayr G., De La Vega F.M., Briggs J., Gunther S., Prescott N.J., Onnie C.M., Hasler R., Sipos B., Folsch U.R., Lengauer T., Platzer M., Mathew C.G., Krawczak M. & Schreiber S. (2007) A genome-wide association scan of nonsynonymous SNPs identifies a susceptibility variant for Crohn disease in ATG16L1. *Nat Genet*, **39,** 207.

150. Parkes M., Barrett J.C., Prescott N.J., Tremelling M., Anderson C.A., Fisher S.A., Roberts R.G., Nimmo E.R., Cummings F.R., Soars D., Drummond H., Lees C.W., Khawaja S.A., Bagnall R., Burke D.A., Todhunter C.E., Ahmad T., Onnie C.M., McArdle W., Strachan D., Bethel G., Bryan C., Lewis C.M., Deloukas P., Forbes A., Sanderson J., Jewell D.P., Satsangi J., Mansfield J.C., Cardon L. & Mathew C.G. (2007) Sequence variants in the autophagy gene IRGM and multiple other replicating loci contribute to Crohn's disease susceptibility. *Nat Genet*, **39,** 830.
151. Rioux J.D., Xavier R.J., Taylor K.D., Silverberg M.S., Goyette P., Huett A., Green T., Kuballa P., Barmada M.M., Datta L.W., Shugart Y.Y., Griffiths A.M., Targan S.R., Ippoliti A.F., Bernard E.J., Mei L., Nicolae D.L., Regueiro M., Schumm L.P., Steinhart A.H., Rotter J.I., Duerr R.H., Cho J.H., Daly M.J. & Brant S.R. (2007) Genome-wide association study identifies new susceptibility loci for Crohn disease and implicates autophagy in disease pathogenesis. *Nat Genet*, **39,** 596.
152. Garrett W.S., Lord G.M., Punit S., Lugo-Villarino G., Mazmanian S.K., Ito S., Glickman J.N. & Glimcher L.H. (2007) Communicable ulcerative colitis induced by T-bet deficiency in the innate immune system. *Cell*, **131,** 33.
153. Cooke E.M. (1967) A quantitative comparison of the faecal flora of patients with ulcerative colitis and that of normal persons. *J Pathol Bacteriol*, **94,** 439.
154. Gorbach S.L., Nahas L., Plaut A.G., Weinstein L., Patterson J.F. & Levitan R. (1968) Studies of intestinal microflora. V. Fecal microbial ecology in ulcerative colitis and regional enteritis: relationship to severity of disease and chemotherapy. *Gastroenterology*, **54,** 575.
155. van der Wiel-Korstanje J.A. & Winkler K.C. (1975) The faecal flora in ulcerative colitis. *J Med Microbiol*, **8,** 491.
156. Balish E. & Warner T. (2002) Enterococcus faecalis induces inflammatory bowel disease in interleukin-10 knockout mice. *Am J Pathol*, **160,** 2253.
157. Shkoda A., Ruiz P.A., Daniel H., Kim S.C., Rogler G., Sartor R.B. & Haller D. (2007) Interleukin-10 blocked endoplasmic reticulum stress in intestinal epithelial cells: impact on chronic inflammation. *Gastroenterology*, **132,** 190.
158. Baldassarri L., Bertuccini L., Creti R., Filippini P., Ammendolia M.G., Koch S., Huebner J. & Orefici G. (2005) Glycosaminoglycans mediate invasion and survival of Enterococcus faecalis into macrophages. *J Infect Dis*, **191,** 1253.
159. Furrie E., Macfarlane S., Cummings J.H. & Macfarlane G.T. (2004) Systemic antibodies towards mucosal bacteria in ulcerative colitis and Crohn's disease differentially activate the innate immune response. *Gut*, **53,** 91.
160. Keighley M.R., Eastwood D., Ambrose N.S., Allan R.N. & Burdon D.W. (1982) Incidence and microbiology of abdominal and pelvic abscess in Crohn's disease. *Gastroenterology*, **83,** 1271.
161. Eaton T.J. & Gasson M.J. (2001) Molecular Screening of Enterococcus Virulence Determinants and Potential for Genetic Exchange between Food and Medical Isolates. *Appl Environ Microbiol*, **67,** 1628.
162. Vidal K., Grosjean I., evillard J.P., Gespach C. & Kaiserlian D. (1993) Immortalization of mouse intestinal epithelial cells by the SV40-large T gene. Phenotypic and immune characterization of the MODE-K cell line. *J Immunol Methods*, **166,** 63.
163. Sultzer B.M., Castagna R., Bandekar J. & Wong P. (1993) Lipopolysaccharide nonresponder cells: the C3H/HeJ defect. *Immunobiology*, **187,** 257.
164. Laemmli U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227,** 680.

165. Poltorak A., He X., Smirnova I., Liu M.Y., Van Huffel C., Du X., Birdwell D., Alejos E., Silva M., Galanos C., Freudenberg M., Ricciardi-Castagnoli P., Layton B. & Beutler B. (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science*, **282,** 2085.
166. Ruiz P.A., Hoffmann M., Szcesny S., Blaut M. & Haller D. (2005) Innate mechanisms for Bifidobacterium lactis to activate transient pro-inflammatory host responses in intestinal epithelial cells after the colonization of germ-free rats. *Immunology*, **115,** 441.
167. Brown M.D. & Sacks D.B. (2009) Protein scaffolds in MAP kinase signalling. *Cell Signal*, **21,** 462.
168. Huang C., Jacobson K. & Schaller M.D. (2004) MAP kinases and cell migration. *J Cell Sci*, **117,** 4619.
169. Katz M., Amit I. & Yarden Y. (2007) Regulation of MAPKs by growth factors and receptor tyrosine kinases. *Biochim Biophys Acta*, **1773,** 1161.
170. Hyun J.G., Lee G., Brown J.B., Grimm G.R., Tang Y., Mittal N., Dirisina R., Zhang Z., Fryer J.P., Weinstock J.V., Luster A.D. & Barrett T.A. (2005) Anti-interferon-inducible chemokine, CXCL10, reduces colitis by impairing T helper-1 induction and recruitment in mice. *Inflamm Bowel Dis*, **11,** 799.
171. Gorg A., Drews O., Klaus A., Luck C., Reil G., Scheibe B., Wildgruber R. & Weiss W. (1998) Two-dimensional electrophoresis with immobilized pH gradients for proteome analysis. A laboratory manual.
172. Gorg A., Weiss W. & Dunn M.J. (2004) Current two-dimensional electrophoresis technology for proteomics. *Proteomics*, **4,** 3665.
173. Berkelman T., Senstedt T., Bjellqvist B., Laird N., Olsson I., Stochaj W. & Westermeier R. (1998) *2-D Electrophoresis using immobilized PH Gradients - Principles and Methods*. Amersham Pharmacia Biotech Inc.
174. Schmidt M.H., Chen B., Randazzo L.M. & Bogler O. (2003) SETA/CIN85/Ruk and its binding partner AIP1 associate with diverse cytoskeletal elements, including FAKs, and modulate cell adhesion. *J Cell Sci*, **116,** 2845.
175. Pan S., Wang R., Zhou X., He G., Koomen J., Kobayashi R., Sun L., Corvera J., Gallick G.E. & Kuang J. (2006) Involvement of the conserved adaptor protein Alix in actin cytoskeleton assembly. *J Biol Chem*, **281,** 34640.
176. Geminard C., De Gassart A., Blanc L. & Vidal M. (2004) Degradation of AP2 during reticulocyte maturation enhances binding of hsc70 and Alix to a common site on TFR for sorting into exosomes. *Traffic*, **5,** 181.
177. Yi X., Bouley R., Lin H.Y., Bechoua S., Sun T.X., Del Re E., Shioda T., Raychowdhury M.K., Lu H.A., Abou-Samra A.B., Brown D. & Ausiello D.A. (2007) Alix (AIP1) is a vasopressin receptor (V2R)-interacting protein that increases lysosomal degradation of the V2R. *Am J Physiol Renal Physiol*, **292,** F1303.
178. Schmidt M.H., Hoeller D., Yu J., Furnari F.B., Cavenee W.K., Dikic I. & Bogler O. (2004) Alix/AIP1 antagonizes epidermal growth factor receptor downregulation by the Cbl-SETA/CIN85 complex. *Mol Cell Biol*, **24,** 8981.
179. Hew Ferstl C. (2008) Impact of food processing and gut transit on *Enterococcus faecalis* virulence gene expression. In: *PhD Thesis, Lehrstuhl für Technische Mikrobiologie*. Technische Universität München, Freising-Weihenstephan.
180. Hew C.M., Korakli M. & Vogel R.F. (2007) Expression of virulence-related genes by Enterococcus faecalis in response to different environments. *Syst Appl Microbiol*, **30,** 257.

181. Molmenti E.P., Ziambaras T. & Perlmutter D.H. (1993) Evidence for an acute phase response in human intestinal epithelial cells. *J Biol Chem*, **268,** 14116.
182. Beagley K.W., Eldridge J.H., Lee F., Kiyono H., Everson M.P., Koopman W.J., Hirano T., Kishimoto T. & McGhee J.R. (1989) Interleukins and IgA synthesis. Human and murine interleukin 6 induce high rate IgA secretion in IgA-committed B cells. *J Exp Med*, **169,** 2133.
183. Wang W., Smail N., Wang P. & Chaudry I.H. (1998) Increased gut permeability after hemorrhage is associated with upregulation of local and systemic IL-6. *J Surg Res*, **79,** 39.
184. Atreya R., Mudter J., Finotto S., Mullberg J., Jostock T., Wirtz S., Schutz M., Bartsch B., Holtmann M., Becker C., Strand D., Czaja J., Schlaak J.F., Lehr H.A., Autschbach F., Schurmann G., Nishimoto N., Yoshizaki K., Ito H., Kishimoto T., Galle P.R., Rose-John S. & Neurath M.F. (2000) Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis in vivo. *Nat Med*, **6,** 583.
185. Ito H. (2005) Treatment of Crohn's disease with anti-IL-6 receptor antibody. *J Gastroenterol*, **40 Suppl 16,** 32.
186. Hoffmann M., Rath E., Holzlwimmer G., Quintanilla-Martinez L., Loach D., Tannock G. & Haller D. (2008) Lactobacillus reuteri 100-23 transiently activates intestinal epithelial cells of mice that have a complex microbiota during early stages of colonization. *J Nutr*, **138,** 1684.
187. Tannock G.W., Crichton C., Welling G.W., Koopman J.P. & Midtvedt T. (1988) Reconstitution of the gastrointestinal microflora of lactobacillus-free mice. *Appl Environ Microbiol*, **54,** 2971.
188. Tannock G.W. & Archibald R.D. (1984) The derivation and use of mice which do not harbour lactobacilli in the gastrointestinal tract. *Can J Microbiol*, **30,** 849.
189. van Baarlen P., Troost F.J., van Hemert S., van der Meer C., de Vos W.M., de Groot P.J., Hooiveld G.J., Brummer R.J. & Kleerebezem M. (2009) Differential NF-kappaB pathways induction by Lactobacillus plantarum in the duodenum of healthy humans correlating with immune tolerance. *Proc Natl Acad Sci U S A*, **106,** 2371.
190. Elson C.O., Cong Y., Lorenz R. & Weaver C.T. (2004) New developments in experimental models of inflammatory bowel disease. *Curr Opin Gastroenterol*, **20,** 360.
191. Hormannsperger G., Clavel T., Hoffmann M., Reiff C., Kelly D., Loh G., Blaut M., Holzlwimmer G., Laschinger M. & Haller D. (2009) Post-translational inhibition of IP-10 secretion in IEC by probiotic bacteria: impact on chronic inflammation. *PLoS ONE*, **4,** e4365.
192. Elson C.O., Cong Y., McCracken V.J., Dimmitt R.A., Lorenz R.G. & Weaver C.T. (2005) Experimental models of inflammatory bowel disease reveal innate, adaptive, and regulatory mechanisms of host dialogue with the microbiota. *Immunol Rev*, **206,** 260.
193. Huycke M.M., Abrams V. & Moore D.R. (2002) Enterococcus faecalis produces extracellular superoxide and hydrogen peroxide that damages colonic epithelial cell DNA. *Carcinogenesis*, **23,** 529.
194. Wang S., Ng L.H., Chow W.L. & Lee Y.K. (2008) Infant intestinal Enterococcus faecalis down-regulates inflammatory responses in human intestinal cell lines. *World J Gastroenterol*, **14,** 1067.

195. Shkoda A. (2006) Functional epithelial cell proteomics under conditions of chronic intestinal inflammation. In: *PhD Thesis, Fachgebiet für Experimentelle Ernährungsmedizin*. Technische Universität München, Freising-Weihenstephan.
196. Ruiz P.A., Shkoda A., Kim S.C., Sartor R.B. & Haller D. (2005) IL-10 gene-deficient mice lack TGF-beta/Smad signaling and fail to inhibit proinflammatory gene expression in intestinal epithelial cells after the colonization with colitogenic Enterococcus faecalis. *J Immunol*, **174,** 2990.
197. Tarnawski A.S. (2005) Cellular and molecular mechanisms of gastrointestinal ulcer healing. *Dig Dis Sci*, **50 Suppl 1,** S24.
198. Suer S., Ampasala D., Walsh M.F. & Basson M.D. (2009) Role of ERK/mTOR signaling in TGFbeta-modulated focal adhesion kinase mRNA stability and protein synthesis in cultured rat IEC-6 intestinal epithelial cells. *Cell Tissue Res*, **336,** 213.
199. Dieckgraefe B.K., Korzenik J.R. & Anant S. (2006) Growth factors as treatment options for intestinal inflammation. *Ann N Y Acad Sci*, **1072,** 300.
200. Beck P.L. & Podolsky D.K. (1999) Growth factors in inflammatory bowel disease. *Inflamm Bowel Dis*, **5,** 44.
201. Suzuki K., Kawauchi Y., Palaniyandi S.S., Veeraveedu P.T., Fujii M., Yamagiwa S., Yoneyama H., Han G.D., Kawachi H., Okada Y., Ajioka Y., Watanabe K., Hosono M., Asakura H., Aoyagi Y. & Narumi S. (2007) Blockade of interferon-gamma-inducible protein-10 attenuates chronic experimental colitis by blocking cellular trafficking and protecting intestinal epithelial cells. *Pathol Int*, **57,** 413.
202. Noguchi A., Watanabe K., Narumi S., Yamagami H., Fujiwara Y., Higuchi K., Oshitani N. & Arakawa T. (2007) The production of interferon-gamma-inducible protein 10 by granulocytes and monocytes is associated with ulcerative colitis disease activity. *J Gastroenterol*, **42,** 947.
203. Crawford P.A., Crowley J.R., Sambandam N., Muegge B.D., Costello E.K., Hamady M., Knight R. & Gordon J.I. (2009) Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. *Proc Natl Acad Sci U S A*, **106,** 11276.
204. Spicer A.P., Kaback L.A., Smith T.J. & Seldin M.F. (1998) Molecular cloning and characterization of the human and mouse UDP-glucose dehydrogenase genes. *J Biol Chem*, **273,** 25117.
205. Sava I.G., Zhang F., Toma I., Theilacker C., Li B., Baumert T.F., Holst O., Linhardt R.J. & Huebner J. (2009) Novel interactions of glycosaminoglycans and bacterial glycolipids mediate binding of enterococci to human cells. *J Biol Chem*, **284,** 18194.
206. Kaser A., Lee A.H., Franke A., Glickman J.N., Zeissig S., Tilg H., Nieuwenhuis E.E., Higgins D.E., Schreiber S., Glimcher L.H. & Blumberg R.S. (2008) XBP1 links ER stress to intestinal inflammation and confers genetic risk for human inflammatory bowel disease. *Cell*, **134,** 743.
207. Theiss A.L., Idell R.D., Srinivasan S., Klapproth J.M., Jones D.P., Merlin D. & Sitaraman S.V. (2007) Prohibitin protects against oxidative stress in intestinal epithelial cells. *Faseb J*, **21,** 197.
208. Theiss A.L., Obertone T.S., Merlin D. & Sitaraman S.V. (2007) Interleukin-6 transcriptionally regulates prohibitin expression in intestinal epithelial cells. *J Biol Chem*, **282,** 12804.
209. Shen Y., Meunier L. & Hendershot L.M. (2002) Identification and characterization of a novel endoplasmic reticulum (ER) DnaJ homologue,

which stimulates ATPase activity of BiP in vitro and is induced by ER stress. *J Biol Chem*, **277,** 15947.
210. Jin Y., Zhuang M. & Hendershot L.M. (2009) ERdj3, a luminal ER DnaJ homologue, binds directly to unfolded proteins in the mammalian ER: identification of critical residues. *Biochemistry*, **48,** 41.
211. Jin Y., Awad W., Petrova K. & Hendershot L.M. (2008) Regulated release of ERdj3 from unfolded proteins by BiP. *Embo J*, **27,** 2873.
212. Nagahara N. & Sawada N. (2006) The mercaptopyruvate pathway in cysteine catabolism: a physiologic role and related disease of the multifunctional 3-mercaptopyruvate sulfurtransferase. *Curr Med Chem*, **13,** 1219.
213. Ramasamy S., Singh S., Taniere P., Langman M.J. & Eggo M.C. (2006) Sulfide-detoxifying enzymes in the human colon are decreased in cancer and upregulated in differentiation. *Am J Physiol Gastrointest Liver Physiol*, **291,** G288.
214. Perraud A.L., Fleig A., Dunn C.A., Bagley L.A., Launay P., Schmitz C., Stokes A.J., Zhu Q., Bessman M.J., Penner R., Kinet J.P. & Scharenberg A.M. (2001) ADP-ribose gating of the calcium-permeable LTRPC2 channel revealed by Nudix motif homology. *Nature*, **411,** 595.
215. Perraud A.L., Shen B., Dunn C.A., Rippe K., Smith M.K., Bessman M.J., Stoddard B.L. & Scharenberg A.M. (2003) NUDT9, a member of the Nudix hydrolase family, is an evolutionarily conserved mitochondrial ADP-ribose pyrophosphatase. *J Biol Chem*, **278,** 1794.
216. Creighton D.J., Zheng Z.B., Holewinski R., Hamilton D.S. & Eiseman J.L. (2003) Glyoxalase I inhibitors in cancer chemotherapy. *Biochem Soc Trans*, **31,** 1378.
217. Owens D.W. & Lane E.B. (2004) Keratin mutations and intestinal pathology. *J Pathol*, **204,** 377.
218. Owens D.W., Wilson N.J., Hill A.J., Rugg E.L., Porter R.M., Hutcheson A.M., Quinlan R.A., van Heel D., Parkes M., Jewell D.P., Campbell S.S., Ghosh S., Satsangi J. & Lane E.B. (2004) Human keratin 8 mutations that disturb filament assembly observed in inflammatory bowel disease patients. *J Cell Sci*, **117,** 1989.
219. Tao G.Z., Strnad P., Zhou Q., Kamal A., Zhang L., Madani N.D., Kugathasan S., Brant S.R., Cho J.H., Omary M.B. & Duerr R.H. (2007) Analysis of keratin polypeptides 8 and 19 variants in inflammatory bowel disease. *Clin Gastroenterol Hepatol*, **5,** 857.
220. Liao J., Price D. & Omary M.B. (1997) Association of glucose-regulated protein (grp78) with human keratin 8. *FEBS Lett*, **417,** 316.
221. Caulin C., Ware C.F., Magin T.M. & Oshima R.G. (2000) Keratin-dependent, epithelial resistance to tumor necrosis factor-induced apoptosis. *J Cell Biol*, **149,** 17.
222. Gilbert S., Loranger A., Daigle N. & Marceau N. (2001) Simple epithelium keratins 8 and 18 provide resistance to Fas-mediated apoptosis. The protection occurs through a receptor-targeting modulation. *J Cell Biol*, **154,** 763.
223. Wang L., Srinivasan S., Theiss A.L., Merlin D. & Sitaraman S.V. (2007) Interleukin-6 induces keratin expression in intestinal epithelial cells: potential role of keratin-8 in interleukin-6-induced barrier function alterations. *J Biol Chem*, **282,** 8219.
224. Arst H.N. & Penalva M.A. (2003) pH regulation in Aspergillus and parallels with higher eukaryotic regulatory systems. *Trends Genet*, **19,** 224.

225. Sadoul R. (2006) Do Alix and ALG-2 really control endosomes for better or for worse? *Biol Cell*, **98,** 69.
226. Vincent O., Rainbow L., Tilburn J., Arst H.N., Jr. & Penalva M.A. (2003) YPXL/I is a protein interaction motif recognized by aspergillus PalA and its human homologue, AIP1/Alix. *Mol Cell Biol*, **23,** 1647.
227. Fisher R.D., Chung H.Y., Zhai Q., Robinson H., Sundquist W.I. & Hill C.P. (2007) Structural and biochemical studies of ALIX/AIP1 and its role in retrovirus budding. *Cell*, **128,** 841.
228. Vito P., Pellegrini L., Guiet C. & D'Adamio L. (1999) Cloning of AIP1, a novel protein that associates with the apoptosis-linked gene ALG-2 in a Ca2+-dependent reaction. *J Biol Chem*, **274,** 1533.
229. Lennartsson J., Wardega P., Engstrom U., Hellman U. & Heldin C.H. (2006) Alix facilitates the interaction between c-Cbl and platelet-derived growth factor beta-receptor and thereby modulates receptor down-regulation. *J Biol Chem*, **281,** 39152.
230. Sieg D.J., Hauck C.R. & Schlaepfer D.D. (1999) Required role of focal adhesion kinase (FAK) for integrin-stimulated cell migration. *J Cell Sci*, **112 (Pt 16),** 2677.
231. Sperandio S., Poksay K., de Belle I., Lafuente M.J., Liu B., Nasir J. & Bredesen D.E. (2004) Paraptosis: mediation by MAP kinases and inhibition by AIP-1/Alix. *Cell Death Differ*, **11,** 1066.
232. Chambard J.C., Lefloch R., Pouyssegur J. & Lenormand P. (2007) ERK implication in cell cycle regulation. *Biochim Biophys Acta*, **1773,** 1299.
233. Nakamura K., Sato T., Ohashi A., Tsurui H. & Hasegawa H. (2008) Role of a serotonin precursor in development of gut microvilli. *Am J Pathol*, **172,** 333.
234. Jin F., Matsushita O., Katayama S., Jin S., Matsushita C., Minami J. & Okabe A. (1996) Purification, characterization, and primary structure of Clostridium perfringens lambda-toxin, a thermolysin-like metalloprotease. *Infect Immun*, **64,** 230.
235. Hurtado A., Chahal B., Owen R.J. & Smith A.W. (1994) Genetic diversity of the Helicobacter pylori haemagglutinin/protease (hap) gene. *FEMS Microbiol Lett*, **123,** 173.
236. Smith A.W., Chahal B. & French G.L. (1994) The human gastric pathogen Helicobacter pylori has a gene encoding an enzyme first classified as a mucinase in Vibrio cholerae. *Mol Microbiol*, **13,** 153.
237. Hersh L.B. & Morihara K. (1986) Comparison of the subsite specificity of the mammalian neutral endopeptidase 24.11 (enkephalinase) to the bacterial neutral endopeptidase thermolysin. *J Biol Chem*, **261,** 6433.
238. Garg P., Vijay-Kumar M., Wang L., Gewirtz A.T., Merlin D. & Sitaraman S.V. (2009) Matrix metalloproteinase-9-mediated tissue injury overrides the protective effect of matrix metalloproteinase-2 during colitis. *Am J Physiol Gastrointest Liver Physiol*, **296,** G175.
239. Medina C. & Radomski M.W. (2006) Role of matrix metalloproteinases in intestinal inflammation. *J Pharmacol Exp Ther*, **318,** 933.
240. Bank U., Bohr U.R., Reinhold D., Lendeckel U., Ansorge S., Malfertheiner P. & Tager M. (2008) Inflammatory bowel diseases: multiple benefits from therapy with dipeptidyl- and alanyl-aminopeptidase inhibitors. *Front Biosci*, **13,** 3699.
241. Banbula A., Potempa J., Travis J., Fernandez-Catalan C., Mann K., Huber R., Bode W. & Medrano F. (1998) Amino-acid sequence and three-dimensional structure of the Staphylococcus aureus metalloproteinase at 1.72 A resolution. *Structure*, **6,** 1185.

242. Shaw L., Golonka E., Potempa J. & Foster S.J. (2004) The role and regulation of the extracellular proteases of Staphylococcus aureus. *Microbiology*, **150,** 217.
243. Drapeau G.R. (1978) Role of metalloprotease in activation of the precursor of staphylococcal protease. *J Bacteriol*, **136,** 607.
244. Potempa J., Fedak D., Dubin A., Mast A. & Travis J. (1991) Proteolytic inactivation of alpha-1-anti-chymotrypsin. Sites of cleavage and generation of chemotactic activity. *J Biol Chem*, **266,** 21482.
245. Shockman G.D. & Cheney M.C. (1969) Autolytic enzyme system of Streptococcus faecalis. V. Nature of the autolysin-cell wall complex and its relationship to properties of the autolytic enzyme of Streptococcus faecalis. *J Bacteriol*, **98,** 1199.
246. Makinen P.L. & Makinen K.K. (1994) The Enterococcus faecalis extracellular metalloendopeptidase (EC 3.4.24.30; coccolysin) inactivates human endothelin at bonds involving hydrophobic amino acid residues. *Biochem Biophys Res Commun*, **200,** 981.
247. Kalabis J., Li G., Fukunaga-Kalabis M., Rustgi A.K. & Herlyn M. (2008) Endothelin-3 stimulates survival of goblet cells in organotypic cultures of fetal human colonic epithelium. *Am J Physiol Gastrointest Liver Physiol*, **295,** G1182.
248. Bueno L. (2008) Protease activated receptor 2: a new target for IBS treatment. *Eur Rev Med Pharmacol Sci*, **12 Suppl 1,** 95.
249. Kida Y., Inoue H., Shimizu T. & Kuwano K. (2007) Serratia marcescens serralysin induces inflammatory responses through protease-activated receptor 2. *Infect Immun*, **75,** 164.

Lebenslauf

Persönliche Daten

Name:	Micha Hoffmann
Geburtsdatum:	24.02.1976
Geburtsort:	Mühlheim an der Ruhr, Deutschland

Schulausbildung

1982-1986	Grundschule Dachau Süd
1986-1995	Ignaz Taschner Gymnasium, Dachau
	Abschluss: Allgemeine Hochschulreife

Zivildienst

07/1995 – 08/1996	Zivildienstleistender, Fahrtendienst, Caritas, Dachau

Praktika

11/1996 – 03/1997	Scheibe-Flugzeugbau GmbH, Dachau
05/1997 – 08/1997	Coca-Cola Erfrischungsgetränke AG, Fürstenfeldbruck
01/2001	Staatliche Molkerei Weihenstephan GmbH & Co. KG

Hochschulausbildung

11/1997 – 01/2003	Studium: Technologie und Biotechnologie der Lebensmittel, Technischen Universität München
	Abschluss: Dipl.-ing. univ. Technologie und Biotechnologie der Lebensmittel
seit 02/2004	Promotion, Technischen Universität München Lehrstuhl für Biofunktionalität der Lebensmittel

Auslandspraktika und Nebentätigkeiten:

04/2000	Lehrstuhl für Technische Mikrobiologie, Technische Universität München; wissenschaftliche Hilfskraft
08/2000 – 09/2000	Margarine und Mayonnaise produzierenden Firma Vista Ltd., Kiew, Ukraine; Praktikum
09/1999 – 06/2003	Informationstechnologie, Technischen Universität München; studentische/wissenschaftliche Hilfskraft sowie wissenschaftlicher Mitarbeiter
06/2003 – 12/2003	Lehrstuhl für Mikrobiologie, Universität von Otago, Dunedin, Neuseeland; Dreimonatiges Forschungsprojekt sowie drei Monate Sprachurlaub
04/2005	Lehrstuhl für Mikrobiologie, Universität von Otago, Dunedin, Neuseeland; DAAD Kurzstipendium