Host – Microbe Interactions In The Inflamed Gut

Doctoral Thesis

by

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Host – Microbe Interactions In The Inflamed Gut

Dissertation

zur Erlangung des akademischen Grades

"doctor rerum naturalium"

(**Dr. rer. nat.**)

in der Wissenschaftsdisziplin " Gastrointestinale Mikrobiologie "

eingereicht an der

Mathematisch-Naturwissenschaftlichen Fakultät

der Universität Potsdam

von

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aus Tamil Nadu, Indien

Potsdam, den 23rd October, 2013

Published online at the
Institutional Repository of the University of Potsdam:
URL http://opus.kobv.de/ubp/volltexte/2014/6955/
URN urn:nbn:de:kobv:517-opus-69558
http://nbn-resolving.de/urn:nbn:de:kobv:517-opus-69558

For my family

Abstract

Initiation and perpetuation of inflammatory bowel diseases (IBD) may result from an exaggerated mucosal immune response to the luminal microbiota in a susceptible host. We proposed that this may be caused either 1) by an abnormal microbial composition or 2) by weakening of the protective mucus layer due to excessive mucus degradation, which may lead to an easy access of luminal antigens to the host mucosa triggering inflammation.

We tested whether the probiotic *Enterococcus faecium* NCIMB 10415 (NCIMB) is capable of reducing chronic gut inflammation by changing the existing gut microbiota composition and aimed to identify mechanisms that are involved in possible beneficial effects of the probiotic. To identify health-promoting mechanisms of the strain, we used interleukin (IL)-10 deficient mice that spontaneously develop gut inflammation and fed these mice a diet containing NCIMB (10^6 cells g^{-1}) for 3, 8 and 24 weeks, respectively. Control mice were fed an identically composed diet but without the probiotic strain. No clear-cut differences between the animals were observed in pro-inflammatory cytokine gene expression and in intestinal microbiota composition after probiotic supplementation. However, we observed a low abundance of the mucin-degrading bacterium *Akkermansia muciniphila* in the mice that were fed NCIMB for 8 weeks. These low cell numbers were associated with significantly lower interferon gamma (IFN-γ) and IFN-γ-inducible protein (IP-10) mRNA levels as compared to the NCIMB-treated mice that were killed after 3 and 24 weeks of intervention. In conclusion, NCIMB was not capable of reducing gut inflammation in the IL-$10^{-/-}$ mouse model.

To further identify the exact role of *A. muciniphila* and uncover a possible interaction between this bacterium, NCIMB and the host in relation to inflammation, we performed *in vitro* studies using HT-29 colon cancer cells. The HT-29 cells were treated with bacterial conditioned media obtained by growing either *A. muciniphila* (AM-CM) or NCIMB (NCIMB-CM) or both together (COMB-CM) in Dulbecco's Modified Eagle Medium (DMEM) for 2 h at 37 °C followed by bacterial cell removal. HT-29 cells treated with COMB-CM displayed reduced cell viability after 18 h ($p<0.01$) and no viable cells were detected after 24 h of treatment, in contrast to the other groups or heated COMB-CM. Detection of activated caspase-3 in COMB-CM treated groups indicated that death of the HT-29 cells was brought about by apoptosis. It was concluded that either NCIMB or *A. muciniphila* produce a soluble and heat-sensitive factor during their concomitant presence that influences cell viability in an *in vitro* system. We currently hypothesize that this factor is a protein, which has not yet been identified.

Based on the potential effect of *A. muciniphila* on inflammation (*in vivo*) and cell-viability (*in vitro*) in the presence of NCIMB, we investigated how the presence of *A. muciniphila* affects the severity of an intestinal *Salmonella enterica* Typhimurium (*S*Tm)-

induced gut inflammation using gnotobiotic C3H mice with a background microbiota of eight bacterial species (SIHUMI, referred to as simplified human intestinal microbiota). Presence of *A. muciniphila* in *S*Tm-infected SIHUMI (SIHUMI-AS) mice caused significantly increased histopathology scores and elevated mRNA levels of IFN-γ, IP-10, tumor necrosis factor alpha (TNF-α), IL-12, IL-17 and IL-6 in cecal and colonic tissue. The number of mucin filled goblet cells was 2- to 3- fold lower in cecal tissue of SIHUMI-AS mice compared to SIHUMI mice associated with *S*Tm (SIHUMI-S) or *A. muciniphila* (SIHUMI-A) or SIHUMI mice. Reduced goblet cell numbers significantly correlated with increased IFN-γ (r^2 = -0.86, ****P<0.001*) in all infected mice. In addition, loss of cecal mucin sulphation was observed in SIHUMI-AS mice. Concomitant presence of *A. muciniphila* and *S*Tm resulted in a drastic change in microbiota composition of the SIHUMI consortium. The proportion of *Bacteroides thetaiotaomicron* in SIHUMI, SIHUMI-A and SIHUMI-S mice made up to 80-90% but was completely taken over by *S*Tm in SIHUMI-AS mice contributing 94% to total bacteria. These results suggest that *A. muciniphila* exacerbates *S*Tm-induced intestinal inflammation by its ability to disturb host mucus homeostasis.

In conclusion, abnormal microbiota composition together with excessive mucus degradation contributes to severe intestinal inflammation in a susceptible host.

Keywords: *IBD, Probiotics, Immune response, Mucus, Goblet cells, Chronic and acute inflammation, Apoptosis, Commensal, Cytokines, Pathogen, Infection*

Zusammenfassung

Die Initiation and die Manifestation von entzündlichen Darmerkrankungen (inflammatory bowel diseases - IBD) können aus einer übersteigerten mukosalen Immunreaktion auf die luminale Mikrobiota in einem empfänglichen Wirt resultieren. Wir schlagen vor, dass dies entweder durch 1) eine abnormale mikrobielle Zusammensetzung oder 2) die Abschwächung der schützenden Schleimschicht, eingeleitet durch deren fortgeschrittenen Abbau, verursacht werden kann. Diese Entwicklung ermöglicht einen erleichterten Zugang des luminalen Antigens zu der Mukosa des Wirts und somit die Auslösung der Entzündung.

Wir haben getestet, ob das probiotische Bakterium *Enterococcus faecium* NCIMB 10415 (NCIMB) in der Lage ist, der chronischen Darmentzündung durch Veränderung der Zusammensetzung der Darmmikrobiota entgegenzuwirken und strebten an, die zugrunde liegenden Mechanismen der probiotischen Wirkungsweise zu identifizieren.

Für die Aufklärung der gesundheitsfördernden Mechanismen dieses Bakterienstammes wurden Interleukin-10 defiziente Mäuse verwendet, die spontan eine Darmentzündung entwickeln. Den Mäusen wurde für 3, 8 und 24 Wochen eine NCIMB enthaltende Diät verabreicht. Die Kontrollgruppe erhielt eine identisch zusammengesetzte Diät ohne die probiotischen Bakterien. Nach der Fütterung waren keine eindeutigen Unterschiede zwischen den Gruppen hinsichtlich der Genexpression von pro-inflammatorischen Zytokinen und der Zusammensetzung der Darmmikrobiota zu beobachten, obwohl eine geringere Zellzahl des schleimabbauenden Bakteriums *Akkermansia muciniphila* in den mit NCIMB gefütterten Mäusen nach 8 Wochen festgestellt wurde. Diese geringere Häufigkeit war mit einer signifikante Abnahme der mRNA von IFN-γ and IP-10 im Vergleich zu den NCIMB-gefütterten Mäusen nach 3 und 24 Wochen der Intervention verbunden. Daraus folgt, dass NCIMB nicht in der Lage ist, dem Verlauf der Darmentzündung im IL-$10^{-/-}$-Mausmodell entgegenzuwirken.

Um die Rolle von *A. muciniphila* und eine mögliche Wechselwirkung zwischen dem Bakterium NCIMB und dem Wirt in dem Entzündungsprozess aufzuklären, wurde ein *in vitro*-Experiment unter Nutzung der Dickdarmkrebs-Zelllinie HT-29 durchgeführt. Die HT-29-Zellen wurden mit bakteriell konditionierten Medien von entweder *A. muciniphila* (AM-CM) oder NCIMB (NCIMB-CM) bzw. der Kokultivierung beider Spezies (COMB-CM) inkubiert. Im Gegensatz zu den anderen Gruppen (AM-CM, NCIMB-CM) als auch zu dem erhitzten COMB-CM Ausführung zeigten die COMB-CM behandelten HT-29-Zellen eine reduzierte Lebensfähigkeit nach 18 h ($p<0.01$), und nach 24 h waren keine lebenden Zellen mehr nachweisbar. Der Nachweis der aktivierten Caspase-3 in den mit dem COMB-CM behandelten Zellen bestätigte die Vermutung, dass das Absterben der HT-29-Zellen durch Apoptose eingeleitet wurde. Es gibt einen deutlichen Hinweis dafür, dass entweder NCIMB

oder *A. muciniphila* einen löslichen und hitze-empfindlichen Faktor während ihrer gemeinsamen Kultivierung produziert, welcher die Lebensfähigkeit der Zellen maßgeblich in einem *in-vitro*-System beeinflusst. Wir vermuten, dass es sich hierbei um ein Protein handelt und werden dieses in naher Zukunft genauer charakterisieren.

In der nachfolgenden Studie wurde untersucht, wie die Anwesenheit von *A. muciniphila* den Ausprägungsgrad einer intestinalen *Salmonella enterica* Typhimurium (*S*Tm) induzierten Darmentzündung beeinflusst. Dafür wurden gnobiotische C3H-Mäuse mit einem mikrobiellen Hintergrund von acht Bakterienspezies (SIHUMI) verwendet. Die Anwesenheit von *A. muciniphila* in den *S*Tm-infizierten SIHUMI-AS-Mäusen führte zu einem signifikant erhöhten Auftreten von histopathologischen Befunden und erhöhten mRNA-Mengen für IFN-γ, IP-10, TNF-α, IL-12, IL-17 und IL-6 im Gewebe von Zäkum und Kolon. Die Anzahl der schleimgefüllten Becherzellen im Zäkumgewebe war in den SIHUMI-AS-Mäusen zwei- bis dreifach niedriger als in den SIHUMI-S (SIHUMI-Mäusen mit *S*Tm), SIHUMI-A (SIHUMI-Mäusen mit *A. muciniphila*) oder SIHUMI-Mäusen. Die Abnahme der Becherzellen korrelierte in allen infizierten Mäusen signifikant mit der Zunahme der IFN-γ-mRNA (r^2 = -0.86, ****P<0.001*). Zusätzlich wurde eine Sulfatierung des zäkalen Schleimes in den SIHUMI-AS-Mäusen beobachtet. Die gleichzeitige Anwesenheit von *A. muciniphila* und *S*Tm verursachte eine drastische Veränderung der Mikrobiota-Zusammensetzung des SIHUMI-Konsortiums. *Bacteroides thetaiotaomicron,* dessen Beitrag in den SIHUMI-, SIHUMI-A- und SIHUMI-S-Mäusen 80-90% ausmacht, wurde in SIHUMI-AS-Mäusen vollständig durch *S*Tm mit einem Anteil von 94% verdrängt. Diese Ergebnisse zeigen, dass *A. muciniphila* durch seine Fähigkeit, die Homöostase/Selbstregulation der Schleimbildung zu stören, die *S*Tm-induzierte Darmentzündung verschärft.

Es kann geschlußfolgert werden, dass eine abweichende Zusammensetzung der Mikrobiota in Kombination mit einem massiven Abbau des Mucus zur schweren intestinalen Entzündung im empfänglichen Wirt beiträgt.

Keywords: *IBD, Probiotika, Immunreaktion, Schleim, Becherzellen, chronische und akute Entzündung, Apoptose, kommensal, Zytokine, Pathogen, Infektion*

ACKNOWLEDGEMENT

This thesis would not be possible without the support and encouragement of many great individuals.

I would first like to thank my advisor, *Professor Michael Blaut*. Over the years, his guidance has cultivated my abilities to think like a scientist. As a mentor, he constantly challenged me to think rigorously about science and to carry high standards in all aspects ('no control, no Ph.D.'). I sincerely thank you for pushing me to excel on all fronts.

In addition, I am grateful to my supervisor, *Dr. Gunnar Loh* for his support and insight on my projects who helped me with critical reviews during the publication of the projects.

I certainly would not have made it this far without the help of the awesome *Prof. Blaut* lab crew and the technicians in DIfE, especially *Mrs. Marion Urbich, Mrs. Ines Grüner*, *Mrs. Ute Lehmann, Mrs. Elizabeth Meyer* and *Mrs. Stefanie Deubel* who helped me with histology, animal and cell culture experiments. I could not imagine being in a more supportive lab. Thank you for making the journey so memorable and enjoyable.

A special thanks to all the *members of the Ph.D. committee* for their support and the critical reviewing of my thesis. In addition, a special thanks to *Dr. Delphine Saulnier* for initial reviewing of my thesis.

A special thanks to all the members of SFB 852, had a great and fruitful time with interesting lectures. I also thank the German Research Foundation (DFG) for their financial support.

And finally, at the end of the day, when all is said and done and the last pipette tip has been used, I have my supportive and lovely family (Papa: *Mr. P. Ganesh*, Mom: *Mrs. Vijayalakshmi Ganesh*; Bro: *Mr. Arunkumar Ganesh* and Fiancé: *Mr. Arghya Mondal*) and friends (*Mr. Navaneethakrishnan, Mr. Ananyo Dan*, *Mrs. P. Selvanayaki* and *Mrs. Janiene Steinert*) to thank for encouraging me when the going got tough and for celebrating with me when the hard work paid off.

Even though none of you will probably understand and appreciate all the mice and bacteria used for my experiment, the way I do, you have been my biggest supporters and I am so grateful.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 3

LIST OF TABLES

ABBREVIATION

IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cells
GI	Gastrointestinal tract
UC	Ulcerative colitis
CD	Crohn's disease
NCIMB 10415/ NCIMB	*Enterococcus faecium* NCIMB 10415
***S*Tm**	*Salmonella* Typhimurium DT104
A. muciniphila	*Akkermansia muciniphila*
Amuc	*Akkermansia muciniphila*
VSL#3	Probiotic cocktail of 8 bacterial species
TLR	Toll like receptors
IFN	Interferon
IL	Interleukin
TNF	Tumor necrosis factor
IL-10	Interleukin 10
IEC	Intestinal epithelial cell
UPR	Unfolded protein response
NOD2	Nucleotide-binding oligomerization domain-2
CARD	Caspase recruitment domain
MyD88	Myeloid differentiation primary response gene 88
LPS	Lipopolysaccharide
NF-κB	Nuclear factor kappa - enhancer activated B cell
T_H1/2	T helper cell 1 (or) 2
GWAS	Genome-wide association study
ATG16L1	Autophagy-related protein 16-1
NSAID	Non- steroidal anti-inflammatory drug
SLC22A4/A5	Solute carrier family 22, member 4 (or) 5
WT	Wild Type
DSS	Dextran sodium sulphate
CD4	Cluster of differentiation 4
ICAM1	Intercellular Adhesion Molecule 1
FoxP3	Forkhead box P3
MAPK	Mitogen-activated protein kinase

TPH1	Tryptophan hydrolase 1
MUC	Mucin
PTS Domain	Proline, threonine and serine domain
T3SS	Type 3 Secretion system
SPI	*Salmonella* pathogenic island
ROS	Reactive oxygen species
mLN	Mesenteric lymph node
ZO	Zonula occludin
H_2S	Hydrogen sulfide
$S_2O_3^{2-}$	Thiosulphate
$S_4O_6^{2-}$ / ttr	Tetrathionate
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
PCR	Polymerase chain reaction
FITC	Fluorescein isothiocyanate
PBS	Phosphate buffered saline
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
DGGE	Denaturing gradient gel electrophoresis
DMEM	Dulbecco`s Modified Eagle Medium
CM	Conditioned media
COMB-CM	Combined *A. muciniphila* and NCIMB-CM
AM-CM	*A. muciniphila*-CM
NCIMB-CM	*E. faecium* NCIMB 10415-CM
BHI	Brain-heart infusion broth
FBS	Fetal bovine serum
ELISA	Enzyme-linked immunosorbent assay
DIGE	Differential gel electrophoresis
YCFA	Yeast casitone fatty acid media
SIHUMI	Simplified human intestinal microbiota (8 bacterial species)
SIHUMI-A	SIHUMI with *A. muciniphila*
SIHUMI-S	SIHUMI with *S.* Typhimurium
SIHUMI-AS	SIHUMI with both *A. muciniphila* and *S.* Typhimurium
PMN	Polymorphonuclear leukocyte
NANA	N- acteylneuraminic acid
PAS	Periodic acid-Schiff
AB	Alcian blue
LDH	L-lactic dehydrogenase

NAD^+	Nicotinamide adenine dinucleotide
AU	Arbitrary unit
HID	High iron diamine
nm	Nanometer
p.i.	Post infection
ER	Endoplasmic reticulum
NMRI mice	Naval medical research institute, mice
HLA-B27 gene	Human leukocyte antigen subtype B27
EcN	*E. coli* NISSLE
CRC	Colorectal cancer
MDa	Mega Dalton
mRNA	Messenger RNA
BSA	Bovine serum albumin
h	Hour
H	Heated
α	Alpha
β	Beta
γ	Gamma
µ	Micro
-/-	Knock out

"*S*" behind any abbreviation points out the *plural*

Title: General Introduction

1.1 Intestinal microbiota

The mammalian intestinal mucosal surface harbors a very dense and diverse microbial community, the "intestinal microbiota" (>10^{12} bacteria /g) (**Figure 1**), which has profound effects on the host (**Bäckhed *et al.*, 2005; Stecher *et al.*, 2012; Duerkop *et al.*, 2009; Blaut, 2013**). Most (>90%) of this microbial community belong to two different phyla that account for the majority of gram-negative bacteria (Bacteroidetes) and gram-positive bacteria (Firmicutes); the remainder belong to rarer phyla such as Proteobacteria, Actinobacteria and Verrucomicrobia as well as Archaea and Fungi (**Kaser *et al.*, 2010; Harris *et al.*, 2011; Blaut, 2013**). Homeostatic and symbiotic interactions facilitate the peaceful co-existence between microbiota and host. The microbiota also inhibits colonization by most intestinal incoming pathogens (**Kaiser *et al.*, 2012**). Stable interactions between host and the intestinal microbiota provide various health benefits to the host such as nutrient degradation, improvement of intestinal epithelial barrier function, colonization resistance against pathogens and modulation and maintenance of the immune system contributing to health and well-being (**Segawa *et al.*, 2011; Hemarajata & Versalovic, 2013**). For example, presence of *Lactobacillus acidophilus* causes immune modulation and induction of intracellular lymphocyte expansion in mice (**Roselli *et al.*, 2009**). Similarly, *Bacteroides thetaiotaomicron* affects innate immune capabilities by regulating antimicrobial peptide (e.g., angiogenin) expression within the intestinal epithelium through direct activation of Toll-like receptors (TLR) on Paneth cells (**Kaser *et al.*, 2010**). However these mutual interactions between intestinal microbiota and host are dependent on limiting factors such as preventing bacterial penetration into host tissues.

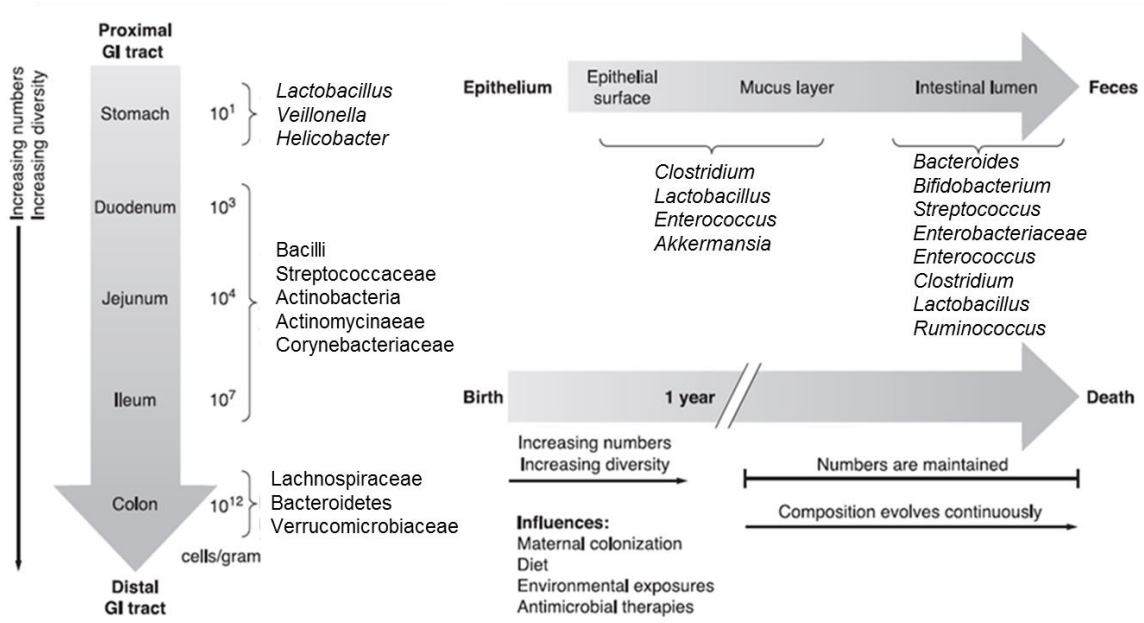


Figure 1. Composition of intestinal microbiota in humans (adapted from **Sekirov *et al.,* 2010; Harris *et al.,* 2011; Derrien *et al.,* 2004; Segata *et al.,* 2012**).

Maintaining, monitoring and controlling bacterial interactions with the intestinal epithelium are an important strategy for minimizing bacterial invasion into the host tissue (**Duerkop *et al.,* 2009**). Indeed, studies on germ-free animals, which by definition are devoid of any bacteria in any parts of their body, showed that they had decreased intestinal epithelial turnover together with higher concentrations of urea in the colon indicating that microorganism play a key role in nitrogen recycling (**Hooper *et al.,* 2002**). They also showed deficiencies in immune development (**Fujimura *et al.,* 2010**) compared to conventional mice. Louis Pasteur already emphasized that the microbiota is an essential and vital element for the host (**Guerrero & Berlanga, 2006**).

Dysregulation of intestinal mucosa homeostasis can lead to a multitude of ailments, including inflammatory bowel diseases ((IBD), Crohn's disease (CD) and Ulcerative colitis (UC) (**Figure 2**), two major forms of IBD) and chronic human immunodeficiency disorders.

The intestinal microbiota is key for mucosal homeostasis and is therefore implicated in the progression of these disorders (**Sekirov *et al.*, 2010**).

Until now, most of the biological functions mediated by resident bacteria and their multiple interactions with the host are still poorly understood. The present thesis sought to better understand the molecular mechanisms underlying host-microbe interactions and their implications in the development and progression of IBD. Various mouse models with chronic or acute intestinal inflammation were used to investigate the impact of intestinal bacteria on IBD.

1.2 Inflammatory bowel diseases

CD and UC, two major forms of IBD are chronic, immunologically mediated diseases characterized by overly aggressive T-cell mediated immune responses to a subset of commensal enteric bacteria in susceptible individuals (**Backhed *et al.*, 2004; Turnbaugh *et al.*, 2006; Sartor, 2006**). The onset and reactivation of disease are triggered by environmental factors that transiently break the mucosal barrier, stimulate immune responses or alter the balance between beneficial and pathogenic enteric bacteria (**Sartor, 2006**).

1.2.1 Clinical manifestation

UC is a continuous inflammation that starts in the rectum and is restricted to the colon while the inflammation in CD can occur anywhere in the gastrointestinal tract, often with lesions (**Figure 2**). Inflammation in CD preferentially occurs in the terminal ileum and affects all layers of the bowel, whereas inflammation in UC is confined to the mucosa and develops into ulcer (**Lakatos *et al.*, 2006**). Clinical symptoms of IBD comprise fever, abdominal pain, diarrhea and rectal bleeding. In addition patients very often experience loss of appetite, malnutrition, weight loss and fatigue (**Podolsky, 2002**).

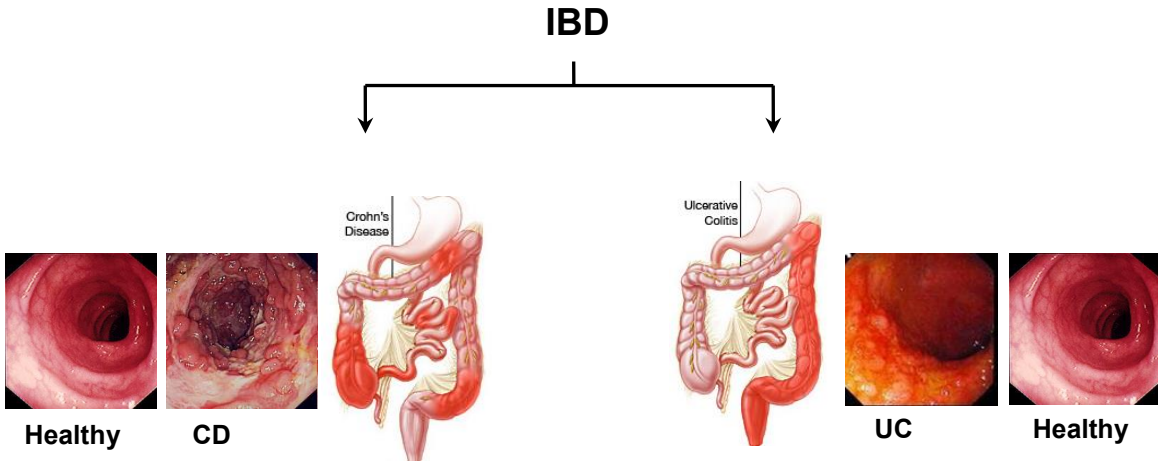


Figure 2. Clinical forms of inflammatory bowel diseases (IBD), CD and UC, compared to the healthy colon of the gastrointestinal tract (Red color patches in the graphic diagram represents severe inflammation). (http://www.studyblue.com/notes/note/n/4-ibd/deck/5054161 and http://www.intechopen.com/books/colonoscopy/endoscopic-approach-in-ulcerative-colitis)

1.2.2 Epidemiology

A recent epidemiological study reports an increasing incidence and prevalence of IBD. The highest annual incidence of UC per 100,000 person-years was 24.3 in Europe; 6.3 in Asia and the Middle East and 19.2 in North America. The highest annual incidence of CD per 100,000 person-years was 12.7 in Europe, 5.0 in Asia and the Middle East, and 20.2 in North America. The highest prevalence values for IBD per 100,000 persons were reported for Europe (UC 505; CD 322) and North America (UC 249; CD 319) (**Molodecky, *et al.,* 2012**).

1.2.3 Immune dysregulation

Dysregulated mucosal immune response of both CD and UC displays activated innate (macrophages, dendritic cells) and acquired (T- and B- cell) immune responses as well as loss of tolerance to enteric commensal bacteria (**Bach, 2002**). The latter is considered to be the main reasons for IBD (**Lidar *et al.*, 2009; Mladenova & Kohonen-Corish, 2012; Iapichino *et al.*, 2008**). As an example, transgenic rats expressing human leukocyte antigen subtype-B27

(HLA-B27) and human β2-microglobulin spontaneously develop chronic colitis that resembles human IBD. However, IBD does not develop under germ-free conditions, further suggesting a relation between IBD and intestinal microbiota (**Yan & Polk, 2004**).

Bacterial antigens (lipopolysaccharides (LPS) or peptidoglycan) are recognized by pattern recognition receptors, such as nucleotide-binding oligomerization domain-containing 2 protein also called caspase recruitment domain containing protein 15 (NOD2/CARD15) and toll-like receptors (TLR). The recognition of these foreign molecules leads subsequently to activation of nuclear factor kappa B (NF-$_K$B). Activation of NF-$_K$B stimulates the expression of various molecules relevant to the pathogenesis of IBD. These include pro-inflammatory molecules such as interleukin (IL) -1β, interferon (IFN), tumor necrosis factor (TNF), IL-6, IL-8, IL-23, IL-17, IL-12 or the intercellular cell adhesion molecule 1 (ICAM1), which is necessary for the infiltration of activated immune cells into the inflammatory sites (**Sartor, 2006**). The activation of innate immune responses is similar in both forms of IBD except for defensin production. Defensins are antimicrobial peptides produced by immune cells as a part of innate immune response (**Ganz, 2003**). Evidence for the altered defensins levels were shown in CD, where reduced α-defensin levels are seen in patients with ileal disease and reduced β-defensin levels in those with colonic involvement (**Ramasundara *et al.*, 2008**). However, acquired immune related T-cell expression profiles differ between CD and UC. The T_H1 cytokine profile, which includes IFN-γ and IL-12 stimulation, are higher in patients with CD whereas T_H2 cytokines, IL-4 and IL-13 are higher in UC patients (**Sartor, 2006**).

1.2.4 Genetic susceptibility

Advanced studies based on single nucleotide polymorphisms and candidate gene approaches revealed associations of genetic alterations with IBD aetiology. IBD associated genes regulate several important biological functions, including bacterial sensing, mucosal barrier function and immunoregulation. However, only the combination of such genetic

alterations leads to the development of a disease phenotype (**Zhernakova *et al.*, 2009; Jostins *et al.*, 2012**).

The key immune mediators involved in the maintenance of the microbiota in the intestine include NOD2/CARD15, TLR, MyD88 (myeloid differentiation primary response gene 88), autophagy-related protein 16-1 (ATG16L1), solute-carrier family 22 member-5 (SLC22A4/5), peroxisome proliferative-activated receptor γ (PPARG) and multidrug resistance gene (MDR1) (**De Cruz *et al.*, 2012; Sartor, 2006**).

NOD2/CARD15 is an intracellular receptor expressed in dendritic cells, Paneth cells and intestinal epithelial cells, responsible for the recognition of bacterial peptidoglycan through the binding of muramyl dipeptide (**Cario, 2005**). Deletion of NOC2/CARD15 in mice decreased intestinal α-defensin production and enhanced the susceptibility towards infections by bacterial pathogens (**Kobayashi *et al.*, 2005**). It was also demonstrated that NOD2/CARD15-defective cells were unable to down regulate NF-$_K$B activation, which may result in excessive pro-inflammatory reactions (**Watanabe *et al.*, 2004**). MyD88-dependent activation of TLRs in Paneth cells and other intestinal epithelial cells sense enteric bacteria and regulate the production of antimicrobial peptides, thereby limiting bacterial penetration of host tissue (**De Cruz *et al.*, 2012**). Therefore, defect in MyD88 region leads to inflammation. In addition, defects in ATG16L1 have been linked to CD. ATG16L1 is a protein complex essential for autophagy, the major process by which intracellular components are targeted to lysosomes for degradation (**Rioux *et al.*, 2007**). Similarly, functional variants of SLC22A4/A5 reduce the carnitine-mediated transport of long-chain fatty acids across the mitochondrial membrane resulting in the inhibition of fatty acid oxidation. Since fatty oxidation is likely essential for a normal gut function, its disturbance may cause a metabolic induction of colitis (**Shekhawat *et al.*, 2007**). PPARG is nuclear receptor that inhibits NF-$_K$B translocation. Polymorphism in PPARG was associated with the development of CD (**Sartor, 2006**). MDR1 encodes the P-glycoprotein 170, a transporter that governs efflux of drugs and possibly xenobiotic

compounds from cells. It has been shown that the deletion of MDR1 gene in mice leads to the development of colitis (**Panwala *et al.*, 1998**).

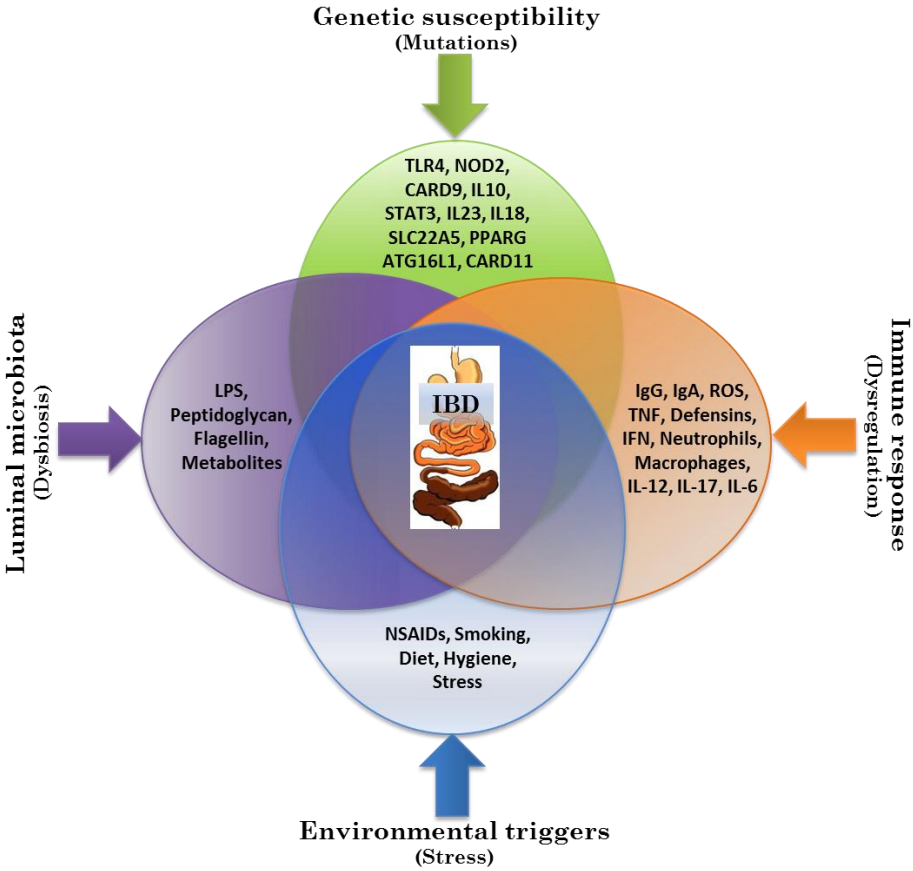


Figure 3. IBD as a multifactorial disorder. The development and course of IBD are affected by several factors including genetic susceptibility of the host, the intestinal microbiota, other environmental factors and the host immune system. These factors cross-regulate each other in multiple ways with several sub-factors as shown. (Adapted from **Sartor, 2006; Kaser *et al.*, 2010; Jostins *et al.*, 2012**).

1.2.5 Environmental triggers

Environmental factors including smoking (which is protective in ulcerative colitis but detrimental in CD), diet, the use of antibiotics and non- steroidal anti-inflammatory drugs (NSAIDs), stress, infection and dysbiosis (changes from beneficial to aggressive microbiota composition) also contribute to the development and exacerbation of IBD (**Sartor, 2006**). Recent studies revealed that commensal bacteria are depleted in IBD and that both major

classes of commensal phyla, Firmicutes and Bacteroidetes are affected (**Kaser *et al.*, 2010**). *E. coli*, the predominant aerobic gram-negative species of the normal intestinal microbiota, colonized the terminal ileum of CD patients being abundant in both acute and chronic ileal lesions in these patients (**Lidar *et al.*, 2009**). In addition, the butyrate producing bacterium *Fecalibacterium prausnitzii* is reduced in patients with CD (**Sokol *et al.*, 2008**). Butyrate has been proposed to decrease the pro-inflammatory cytokines in lamina propria mononuclear cells from patients with CD by inhibiting the activation of NF-$_K$B (**Blaut & Clavel, 2007**).

1.3 Probiotics

Probiotics are defined as "beneficial live micro-organisms which when administrated in adequate amounts confer beneficial effects on the host health" (**Morelli, 2013**). Most known probiotics until now are either lactobacilli or bifidobacteria representatives of which are normal inhabitants of the gastro-intestinal (GI) tract (**Wohlgemuth *et al.*, 2010; Blum *et al.*, 2002**). Recently, animal experiments and human studies suggest that therapeutic manipulation of the balance between beneficial and detrimental intestinal bacterial species can influence health and disease (**Sartor, 2004; Fitzpatrick, 2013**). The known mechanisms of probiosis include manipulation of intestinal microbial communities, suppression of pathogens, immunomodulation, activation of anti-apoptotic genes in human or mouse intestinal epithelial cells from cytokine induced apoptosis, differentiation and fortification of the intestinal barrier (**Thomas and Versalovic, 2010**). For example, simultaneous treatment with probiotic *Streptococcus thermophilus* ATCC19258 and *Lactobacillus acidophilus* ATCC4356, prevent invasion of entero-invasive *E. coli* and enhance the intestinal epithelial barrier function by amplifying the phosphorylation of occludin and ZO-1 together with a reduction of pro-inflammatory responses in vitro (**Resta-Lenert & Barret, 2003**) . Another similar study also demonstrated that application of probiotic *E. coli* NISSLE (EcN) is able to mediate up-

regulation of ZO-1 expression in murine IECs and confer protection from the DSS colitis-associated increase in mucosal permeability to mice luminal substances (**Ukena *et al.*, 2007**).

Loss of tolerance to the patient's own commensal microbiota has been implicated in the development of IBD (**Borchers *et al.*, 2009**). Use of probiotics, to shift the existing microbiota balance in favor of protective microbial species and to treat IBD, has been extensively reviewed (**Packey & Sartor, 2009**). The ability of some probiotics to synthesize bacteriocins (**Awaisheh *et al.*, 2013**) or to induce the secretion of antibacterial cryptidins by Paneth cells (**Ayabe *et al.*, 2004; Hooper *et al.*, 2003**) could account for such changes in microbiota composition or even for the protection against pathogenic bacteria. In addition to the effects mediated by bacteria-bacteria interactions, probiotics may have a direct effect on host physiology. In the inflamed gut, the down-regulation of pro-inflammatory cytokines by probiotics may be an important factor for the observed improvement of symptoms (**Ma *et al.*, 2004**). For example, *Lactobacillus casei* DN-114001 treatment increases the number of CD4(+)FoxP3(+) regulatory T cells in mesenteric lymph nodes (mLN) decreases the production of the pro-inflammatory cytokines TNF-α and IFN-γ, changes the gut microbiota composition and prevents DSS induced colitis in BALB/c mice (**Zakostelska *et al.*, 2011**). However, only few molecular mechanisms underlying probiotic action have so far been identified. Activation of TLR9 by bacterial DNA has been proposed as one possible mechanism of a probiotic-mediated amelioration of experimental colitis (**Rachmilewitz *et al.*, 2004**). TLRs belong to highly conserved receptors of the innate immune system. TLR activation results in the translocation of the nuclear factor $NF_{\kappa}B$ into the cell nucleus triggering transcription of immunorelevant genes (**Cario and Podolsky, 2005**). In addition, *L. casei* inhibits post-transcription of pro-inflammatory interferon γ-induced protein 10 (IP-10) in intestinal epithelial cells of colitic IL-10 knock-out mice (**Hormannsperger *et al.*, 2009**).

An intact intestinal epithelial cell layer is of utmost importance for preventing the uncontrolled intrusion of pathogenic bacteria. However, pathogenic bacteria are capable of

compromising the integrity of the epithelium by disrupting the tight junctions between epithelial cells (**Berkes *et al.,* 2003**). Bacterial factors improving epithelial integrity have been identified for the probiotic *Lactobacillus* GG. This strain produces two proteins which protect epithelial cells from apoptosis and thereby increase mucosal integrity. The secreted proteins activate anti-apoptotic protein kinase B (PKB/Akt) in a phosphatidylinositol-3'-kinase (PI3K)-dependent pathway and inhibit the pro-apoptotic p38/mitogen-activated protein kinase (MAPK) (**Yan *et al.,* 2007**). These findings clearly demonstrate that bacterial interactions directly or indirectly have an impact on host physiology.

However, treatment of CD and UC with probiotics has not replaced standard medications. This is in part due to the fact that human trials only support a use of probiotics for the maintenance of remission in UC. Similarly, the strain *Enterococcus faecium* NCIMB 10415 (NCIMB) which was isolated from a healthy Swedish baby (**Benyacoub *et al.,* 2005**), is used as a probiotic extensively in animal nutrition mainly in swine. However, the mechanisms underlying probiotic action on chronic inflammatory disorders have not been fully elucidated (for detailed explanations, experimental evidences with discussion, see **Chapter 3**).

1.4 Role of the intestinal mucus layer

Human organs such as respiratory, digestive and urinary tracts that are exposed to the external environment are protectively coated with a continuous layer of mucus. Mucus is a highly hydrated gel (± 95% water) mainly composed of mucins (family of heavily glycosylated proteins) together with salts, lipids (e.g., fatty acids, phospholipids and cholesterol), proteins (e.g., lysozyme that cleaves peptidoglycan), immunoglobulin, defensins, growth factors and desquamated epithelial cells. They possess various major roles and can act as: 1) a lubricant facilitating the passage of food components; 2) a selective barrier by allowing passage of low molecular weight components, nutrients, gases, waste through the

cells; and 3) a thick protective layer for the underlying host tissues against penetration of harmful substances such as drugs, toxins, heavy metals, acid (**Allen & G Flemstrom, 2005**), resident or pathogenic bacteria and viruses or parasites (**Miyake *et al.,* 2006; Deplancke & Gaskins, 2001**).

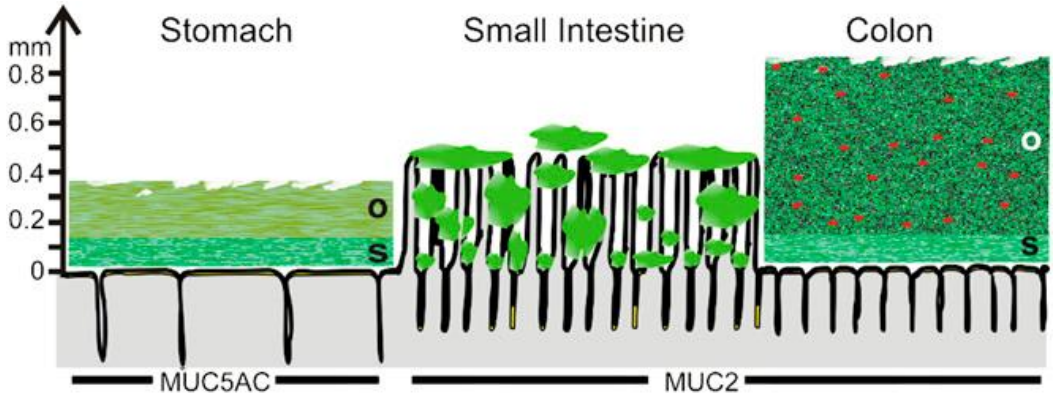


Figure 4. Scheme depicting the mucus organization in the gut. The mucus thicknesses given are from rat. The red dots in the outer mucus layer of colon illustrate bacteria. The genes encoding the gel-forming mucins (green) expressed by the surface goblet cells in the different parts of the intestine are marked by name. o- outer loose mucus layer; s- inner firmly attached mucus layer. Mucus thickness and length of the villi vary along the gut (Obtained from **Johansson *et al.,* 2011**)

Mucus is constantly secreted, shed, produced and its life time is short, varying from a few minutes to several hours (**Bell, 1985**). Two different mucus layers have been observed in the GI tract (**Johansson *et al.,* 2011**). The external layer, also named mobile or non-adherent layer, is largely soluble and constantly removed. It acts as a lubricant by expulsing aggressive agents (bacteria, viruses) trapped in this layer. The inner layer, also known as adherent layer, is firmly attached to the epithelial surfaces and acts as a selective barrier (**Allen & Flemstrom, 2005**). The thickness of the mucus layer follows a gradient from the small intestine to the colon depending on the microbial load (**Figure 4**).

Mucins are responsible for the viscosity and elastic gel-like properties of mucus and are highly glycosylated proteins that have numerous O-glycans attached to domains rich in

proline, threonine and serine (PTS domain) (**Pelaseyed & Hannson, 2010**). The decorated carbohydrate chains are linked to the core protein via N-acetylglucosamine (GlcNac) that is coupled to the hydroxyl group of serine or threonine amino acid via a-O-glycosidic linkage. The backbone region consists of successive galactose and GlcNac residues. The peripheral region (with a size of 2-20 monosaccharides) is comprised of one or more of the major oligosaccharides N- acetylgalactosamine (GalNac), fucose, galactose, and GlcNac (**Johansson & Hansson, 2008; Carraway & Hull, 1991; Hannson, 2012; Bergstrom *et al.,* 2010**). Furthermore, the mucin structures may be modified due to the addition of sialic acid or sulphate residues that together contribute to the specific function of mucin in the GI tract (**Cabotaje *et al.,* 1994**). The two PTS domains are interrupted by two small cysteine domains (CysD) and the whole molecule has large cysteine-rich N- and C-termini resulting in a high molecular weight (up to 40 MDa) (**Specian & Oliver, 1991; Johansson & Hansson, 2010; van der post *et al.,* 2010**) (**Figure 5**). The human genome contains a family of mucin genes, designated as MUC followed by a number that indicates their order of discovery (**Dekker, 2002**). The MUC family has at least 19 members that differ considerably in size. MUC2 represents the main secreted mucin by goblet cells into the intestine (**Johansson *et al.,* 2011; Ambort *et al.,* 2010**). The separation of intestinal bacteria and epithelium by intestinal mucus has been proposed to be essential for the maintenance of homeostasis in the small intestine and colon (**Johansson & Hansson, 2011**).

The mucus layer in the intestine of CD or UC patients is disturbed. In case of UC, the mucus layer was found to be thinner or absent in the inflamed part, while in the case of CD the mucus thickness was significantly higher compared to uninflamed controls (**Pullan, 1994**). Thus, the integrity of the mucus layer is crucial for preventing intestinal bacteria from invading host tissues. Intestinal mucus has a dual role as it protects the mucosa from certain microorganisms and provides an initial binding site. Therefore, mucins offer binding sites for bacterial adhesion. For instance, mucins from human breast milk, in which MUC1 is predominant, bind to certain pathogenic microorganisms such as *Campylobacter* (**Ruiz-Palacios**

et al., 2003), *Escherichia coli* (Martin-Sosa *et al.*, 2002), and thus interfere with their colonization in the infant GI tract. Besides providing attachment sites to bacteria, mucus can provide energy to intestinal bacteria. Mucus is an important source of carbon for bacteria,

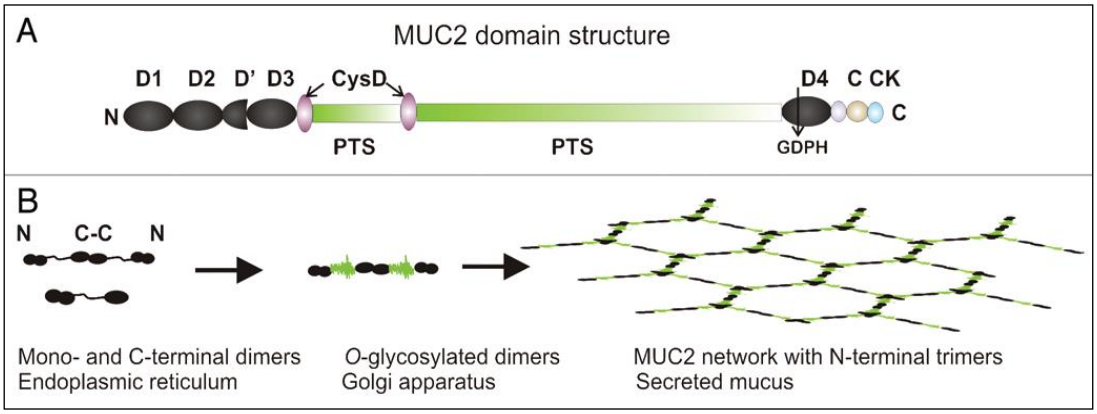


Figure 5. The MUC 2 mucin forms a network in mucus. (A) The domain organization of the MUC 2 mucin. **(B)** The formation of MUC 2 mucin in the endoplasmic reticulum, in the Golgi apparatus, and structure as its release from the goblet cells (Johansson *et al.*, 2008).

mainly in the distal colon where the availability of carbohydrates is limited. Mucin degradation is achieved by a combination of saccharolytic and proteolytic enzymes from the host and from bacteria (Corfield *et al.*, 1992). However, mucin degradation is often regarded as an initial stage in pathogenesis, since it disturbs the first protection of the host mucosal surfaces and changes its composition (Corfield & Myerscough, 2000). Furthermore, loss of the protective mucus layer may expose GI tract cells to luminal antigens (Derrien *et al.*, 2004). In addition, defects in the mucus layer allow bacteria to reach the epithelia, which triggers intestinal inflammation because the luminal antigens will be exposed to TLRs (Johansson & Hansson, 2010; Johansson & Hansson, 2011; Haridass *et al.*, 2010; Johansson *et al.*, 2013). It is known that mucus degradation causes defects in mucus layer integrity. However, changes in the intestinal mucus layer integrity due to a mucin degrading bacterium and its effects on the host health has so far hardly been studied (refer **Chapter 5** for detailed explanation).

1.5 Mucus degrading bacterium *Akkermansia muciniphila*

Akkermansia muciniphila is capable of using mucin as energy, carbon and nitrogen source. Cells are oval-shaped, non-motile and stain Gram-negative. The organism is a strictly anaerobic, chemo-organotrophic bacterium and mucolytic in pure culture. *Akkermansia muciniphila* belongs to the phylum Verrucomicrobia. The bacterium colonizes the human gut early in life and is able to grow on gastric mucin, BHI and Columbia media, and on N-acetylglucosamine, N-acetylgalactosamine and glucose (**Derrien *et al.,* 2004**). Fermentation of sulphated mucins by *A. muciniphila* leads to the release of sulphate. Electron microscopy revealed the presence of filamentous structures on cells that were grown in mucin medium (**Figure 6**). In 2004, Derrien and co-workers assumed that these filaments are capsular polymers that connect the cells. Since this aggregation is mainly observed in mucin medium, it may be speculated that this capsule may aid in adhesion and colonization of mucin-secreting epithelia in the GI-tract (refer **Chapter 4** and **Chapter 5** for detailed information).

A B

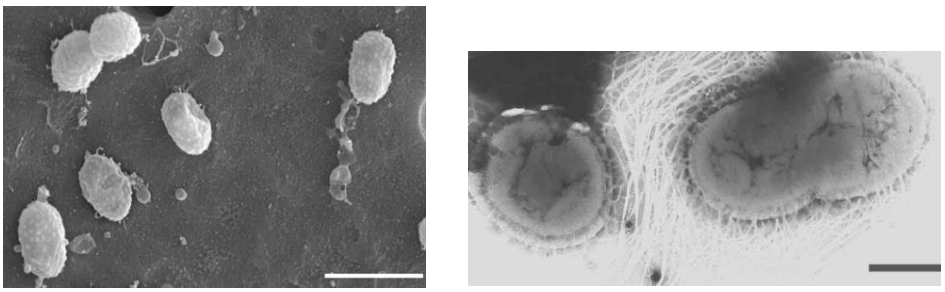


Figure 6. Electron microscopic images of *A. muciniphila*. (A) Bar, 1 μm. (B) Thickened but extensive capsule fibers of the cells. Bar, 0.5 μm. (**Derrien *et al.,* 2004**)

1.6 Impact of *Salmonella enterica* Typhimurium on the host

One of the most important functions of the microbiota in the mammalian intestine is to promote resistance to colonization by pathogens (**Sekirov & Finlay, 2009; Stecher & Hardt, 2008; Bailey, 2012**). Recent studies show that enteric bacterial pathogens induce inflammation to overcome resistance to colonization (**Stecher *et al.*, 2007**). Nutrients and cofactors produced during inflammation can be selectively utilized by enteric pathogens to grow in the intestinal lumen. For example, one of the fermentation end products generated by the commensal microbiota is hydrogen sulphide (H_2S), a cytotoxic compound that is converted to thiosulphate ($S_2O_3^{2-}$) by quinone oxidoreductase, persulfide transferase or rhodanese in the colonic mucosa (**Hildebrandt & Grieshaber, 2008**). During inflammation, neutrophils that transmigrate into the intestinal lumen release reactive oxygen species (ROS) in an attempt to kill enteropathogens (**Thiennimitr *et al.,* 2012**). A by-product of the release of ROS (mainly by NADPH oxidase) is the oxidation of thiosulphate ($S_2O_3^{2-}$) to tetrathionate ($S_4O_6^{2-}$) (**Winter *et al.,* 2010**) in the lumen. In contrast to the commensal fermenting microbiota, *Salmonella enetrica* Serotype Typhimurium (a murine pathogen) can use tetrathionate as a terminal electron acceptor to support its growth by anaerobic respiration, which is more efficient for energy production than fermentation (**Thiennimitr *et al.,* 2012**). These new findings highlight the concept that pathogens evolved virulence mechanisms to allow access to host nutrients. Invasion, colonization and induction of inflammation by *S.* Typhimurium require the function of two type III secretion systems, T3SS-1 and T3SS-2, encoded by genes in the *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2), respectively (**Swart & Hensel, 2012; Loetscher *et al.*, 2012**). These systems deliver effector proteins (namely SopE, SipA, SopA, SopB and SopD) into host cells to trigger specific responses in favor of the pathogen. The resulting inflammatory responses, including the epithelial transmigration of neutrophils, cause changes in the availability of cofactors (iron-sulfur clusters) and nutrients (sugars, amino acids,

ammonia and urea) that allow the pathogenic bacteria in the intestinal lumen to out-compete the commensal microbiota (**Rohmer *et al.*, 2011**). "Blooms" of *S.* Typhimurium in the intestine facilitate translocation into host tissues and are thus critical for the organism's success as a pathogen (**Bliska & Velden, 2012; Stecher *et al.*, 2012**). Previous studies demonstrated that a *Salmonella* Typhimurium-infection induces an acute inflammatory response in the host and is therefore an excellent model for studying the immune responses that are relevant to human diseases, especially inflammatory disorders (**Zirk, 1999**). Furthermore, *S.* Typhimurium has to successfully penetrate the protective external mucus barrier for successful translocation and induction of severe inflammation in the intestine (**Deplancke & Gaskins, 2001**). This prompted us to investigate the inflammatory responses by the host to *S.* Typhimurium-infection in the presence of a mucus degrading commensal (Detailed explanation with experiments refer **Chapter 5**).

1.7 Mouse model of gut inflammation

Animal models of intestinal inflammation are indispensable for understanding the pathogenesis of CD and UC, the idiopathic forms of inflammatory bowel disease in humans. The clinical appearance of human IBD is heterogeneous, a fact that is also reflected by the steadily increasing number of mouse strains displaying IBD-like intestinal alterations. The analysis of these models together with genetic studies in humans greatly enhanced insights into immunoregulatory processes in the gut and led to the generally accepted hypothesis that a dysregulated immune response against components of the commensal microbiota and pathogens is critically involved in IBD pathophysiology (**Maharshak *et al.*, 2013; Hapfelmeier *et al.*, 2005**). In animals inflammation can be induced by chemical agents (dextran sodium sulphate (DSS) or 2, 4, 6-Trinitrobenzenesulfonic acid (TNBS)), by certain bacteria (commensal or pathogenic) or occur spontaneously in genetically susceptible mice.

Accordingly, IBD mouse models can be divided into six categories 1) Gene knock-out models; 2) Transgenic models; 3) Spontaneous colitis models; 4) Chemically induced colitis models; 5) Adoptive transfer models and 6) Pathogenic infection models.

A gene knock-out mouse model which lacks the anti-inflammatory gene Interleukin-10 develops chronic intestinal inflammation under conventional conditions. Mice with a targeted disruption in the IL-10 gene spontaneously develop chronic intestinal inflammation since IL-10 is an anti-inflammatory cytokine that suppresses effector functions of $CD4^+$ T_H1 immune cells and macrophages (**Wirtz *et al.,* 2007; Cohen *et al.,* 2004; Blumberg *et al.,* 1999**). The onset and severity of colitis is strongly influenced by the husbandry conditions of the mice, especially the commensal microbiota and the genetic background of the mouse strain, since animals raised under germ-free conditions are disease-free (**Kim *et al.*, 2005**). The spontaneous onset of gastrointestinal inflammation becomes evident between 6 weeks and 6 months of age (**Scheinin *et al.,* 2003**).

Wild-type mice infected with *S.* Typhimurium are characterized by rapid crypt loss, goblet cell loss and epithelial erosions, mucosal and submucosal infiltration by acute inflammatory cells in particular neutrophils and with a marked edema in the cecum and to a lesser degree in the colon (**Eckmann, 2006; Mizoguchi, 2012**). Furthermore, SPI-2-dependent intracellular proliferation of *Salmonella* triggers MyD88-dependent innate immune responses in the intestinal tract (**Hapfelmeier *et al.,* 2005**). Since, *S.* Typhimurium triggers an acute intestinal inflammation (**Hapfelmeier *et al.,* 2005**), mice infected with this pathogen represent a highly suitable model for investigating immune disorders (**Zirk *et al.,* 1999**).

Objectives

IBD can limit the quality of life because of severe abdominal pain, vomiting, diarrhea and it is rarely fatal on its own. However, patients with IBD have a higher risk of developing colorectal cancer (CRC), which is most probably one of the main reasons for death (**Bansal & Sonnenberg, 1996**). A recent meta-analysis has demonstrated that 1 in 5 patients with UC will develop CRC over a period of 30 years (**Staa *et al.*, 2005**). The intestinal microbiota plays an important role in the onset and perpetuation of different IBD phenotypes (**Loh & Blaut, 2012; Pflughoeft & Versalovic, 2012**). This could be due to an abnormal microbial composition (**Sartor, 2004**) or to an excessive degradation of the protective mucus layer by commensals (**Embden *et al.*, 1989**) weakening mucosal integrity (**Pullan *et al.*, 1994**). However the role of the intestinal microbiota in the development of IBD has so far not been clearly understood. Therefore the aim of the presented thesis was to characterize host-microbe interactions playing a role in IBD. For the experiments presented in this thesis we took the advantage of various mouse models to investigate the following questions:

(1) Does the probiotic *E. faecium* NCIMB 10415 attenuate intestinal inflammation in conventional IL-$10^{-/-}$ mice? **(Chapter 3)**

- Does the supplementation of NCIMB 10415 lead to changes in gut microbiota composition?
- Do changes in bacterial composition in turn enhance the gut barrier function and improves inflammatory symptoms?

(2) Does the interaction between *E. faecium* NCIMB 10415 and *A. muciniphila* cause inflammatory responses in HT-29 cell lines? **(Chapter 4)**

- Do these inflammatory responses influence the HT-29 cell growth?
- Do these interactions lead to the production of inflammatory molecules?

(3) Does the presence of mucin-degrading bacterium *A. muciniphila* play an important role in the development and progression of gut inflammation in *S.* Typhimurium-infected gnotobiotic mice? **(Chapter 5)**

- Does the presence of *A. muciniphila* have an impact on host mucus composition and production?
- Do the changes in mucus composition contribute to an exacerbation of *S.* Typhimurium-induced inflammation?

Title: *Enterococcus faecium* NCIMB 10415 does not protect interleukin-10 knock-out mice from chronic gut inflammation

(Majority of the text used to explain this chapter has been taken from the literature with the aforementioned title published in Beneficial Microbes, 2012. The Authors contributed were **Ganesh BP, Richter J, Blaut M** and **Loh G**)

3.1 Introduction

Probiotics are defined as live micro-organisms that confer a health benefit on the host (Sanders, 2008) and are frequently used in human and animal nutrition to prevent gastrointestinal disorders (**Chaucheyras-Durand & Durand, 2010; Marteau *et al.,* 2001**). *Enterococcus faecium* NCIMB 10415 (NCIMB 10415), also referred to as SF68, reduces the duration of acute diarrhea in humans (**Buydens & Debeuckelaere, 1996; Wunderlich et al., 1989**) and diarrhea incidence in piglets in the post-weaning period (**Taras *et al.,* 2006; Zeyner & Boldt, 2006**). Possible effects of the strain on chronic gut inflammation have not been investigated so far.

Widely accepted probiotic mechanisms that may also be responsible for the observed effects of NCIMB 10415 include the influence on gut microbiota composition and intestinal pathogens, the induction of innate and adaptive immune functions and beneficial effects on the intestinal epithelial barrier function (**Wohlgemuth *et al.*, 2010**). However, the use of NCIMB 10415 in piglets did not alter intestinal microbiota composition (**Broom *et al.*, 2006**). Its application failed to reduce the occurrence of pathogenic *Escherichia coli* strains and even increased the cell numbers of *Salmonella enterica* Typhimurium in feces and internal organs of experimentally infected pigs (**Szabo *et al.*, 2009; Taras *et al.*, 2006**). Feeding NCIMB 10415 decreases fecal immunoglobulin (Ig) A and circulating Ig G concentrations as well as levels of epithelial cytotoxic $CD8^+$ T-cells in piglets indicating that it does not enhance the immune response towards potential pathogens (**Scharek *et al.*, 2007; Scharek *et al.*, 2005**). Moreover,

NCIMB 10415 did not improve the epithelial barrier function in piglets as concluded from unchanged paracellular permeability of jejunal tissues in an *ex vivo* system (**Lodemann *et al.*, 2006**).

We hypothesized that NCIMB 10415 is not only effective against acute diarrhea but also alleviates chronic gut inflammation and tested if the strain improves gut health in interleukin-10 deficient (IL-10$^{-/-}$) mice. These mice develop intestinal inflammation without further intervention under our housing conditions (**Wohlgemuth *et al.*, 2009**). In addition to investigating possible effects of NCIMB 10415 on gut histopathology in these animals, we compared mucosal cytokine expression, gut barrier function and intestinal microbiota composition in NCIMB 10415-treated and control mice.

3.2 Materials and Methods

3.2.1 Animal experiment

The experiments were conducted in specific pathogen-free 129(B6)-IL10^{tm1Cgn}/J (IL-10$^{-/-}$) mice. IL-10 knock-out was cross verified by genotyping the DNA of the mice. Animals were housed in individually ventilated cages under controlled housing conditions (22 °C room temperature, 55 % air humidity, 12 h light/dark cycle) with free access to feed and drinking water. The mice were weaned at 28 days of age and fed a diet with (NCIMB) or without (Control) $\log_{10}$ 6.5 to $\log_{10}$ 6.7 colony forming units of *E. faecium* NCIMB 10415 per gram (dry weight) of diet (n=36, each) (**Figure 1**). The diet was composed of wheat (45 %), soybean meal (26 %), skim milk powder (12 %), oat (10 %), corn meal (1 %) and soybean oil (1 %). Minerals and vitamins were added to meet the requirements of the mice. For quantification of viable NCIMB 10415 cells, dietary material was diluted 1:10 and homogenized in sterile phosphate buffered saline (PBS; 37 mM NaCl, 2.7 mM KCl, 4.3 mM

Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). After plating 10 fold dilution series on bile-esculin agar (Oxoid, Basingstoke, UK) and subsequent aerobic incubation at 37 °C for 24 hours, bacterial colonies were counted and the purity of the colonies was checked by Gram-staining according to standard procedures (http://www.sdstate.edu/sdces/fcs/upload/GramStaining PPT.pdf).

Twelve animals per group were killed after an intervention period of 3, 8, and 24 weeks, respectively. One week prior to killing, the intestinal permeability was measured with fluorescein isothiocyanate-labelled dextran (see below). After killing the mice by cervical dislocation, colonic and cecal contents were collected for microbiota analysis (see below). Dry weight of intestinal contents was determined by overnight freeze-drying of the contents using Gamma 1a apparatus (Christ, Osterode, Germany) followed with weighing. Tissue sections from colon and cecum were fixed in 10 % neutral buffered formalin for

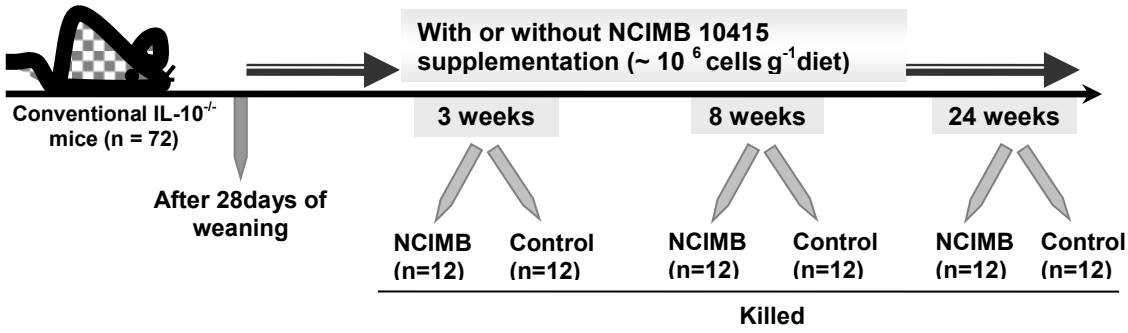


Figure 1. Design of the animal experiment. Seventy two C3H conventional IL-10$^{-/-}$ mice were allocated to 2 groups. These groups differ from each other by the presence (experimental) or absence (control) of probiotic *E. faecium* NCIMB 10415. The groups were further divided into 3 groups depending on the time of probiotic feeding. After 28 days of weaning, NCIMB was supplemented with the diet. Each group was composed of 12 mice (6 males and 6 females).

histopathology scoring (see below) and the remaining mucosa was carefully scraped off, frozen in liquid nitrogen and stored at -80 °C until further analysis. To study the intestinal barrier function in more detail, colonic tissue from mice fed the experimental or the control

diet for 24 weeks was subjected to electrophysiological measurements (see below). The experimental procedures were approved by the local Animal Welfare Committee and the local authority under the permission number 23-2347-8ä42008.

3.2.2 Evaluation of intestinal inflammation

3.2.2.1 Histopathology scores

Cecal and colonic sample material was embedded in paraffin and sectioned at 4 µm. After staining with haematoxylin and eosin (H&E), gut inflammation was evaluated based on immune cell infiltration, mucosal hyperplasia and the presence of edema. The occurrence of abscesses and ulcerations as well as the loss of differentiation were taken into account (**Burich *et al.*, 2001**). Inflammatory cell infiltration scores were as follows: No inflammation- 0; low grade inflammation of Mucosa- 1; moderate inflammation in Mucosa and Submucosa- 2; high degree inflammation in Mucosa and Submucosa- 3, Crypt abscesses- +1, Activation of lymphatic facilities- +1, Erosion and/or Ulceration of crypts- +1, Mucosa: Intact- 0; Mild epithelial Hyperplasia- 1; Moderate epithelial Hyperplasia- 2; Branching- 3; Goblet cell loss- +1, Edema in Mucosa/ Submucosa: No- 0; Existing- 1. Histopathology scoring was kindly performed at FU Berlin in Prof. Klopfleisch's laboratory.

3.2.2.2 mRNA expression levels of pro-inflammatory cytokines

To measure the relative expression levels of the pro-inflammatory cytokines IFN-γ, TNF-α, interferon gamma-induced protein (IP) 10, IL-6, IL-12a, IL-23, IL-17, and IL-4, RNA was extracted from mucosal samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany). One µg of RNA was reverse-transcribed to single-stranded cDNA with the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Real-time PCR was performed using the 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad,

USA). The RT-PCR reaction mix (25 µl) contained the template DNA (1 µl), the QuantiFast SYBR Green PCR master mix (12.5 µl) (Qiagen, Hilden, Germany), the respective primer pairs (0.5 µl) and water (10.5 µl). The primer pairs used were for IFN-γ, IP-10, IL-12a, IL-17, TNF-α, IL-6, IL-23 and IL-4, the forward and the reverse primer sequences are given in **Table 1**. The expression levels of the target genes were calculated using the relative standard curve method after normalizing the target gene expression to the expression of the house-keeping gene encoding glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The expression of the latter gene was measured with the primers GAPDH-for (CAA GGT CAT CCA TGA CAA CTT TG) and GAPDH-rev (GTC CAC CAC CCT GTT GCT GTA G). Subsequently, the expression of the selected genes in the intestinal mucosa of the NCIMB treated and untreated mice were compared for relative expression with the physiological

Cytokines	Oligonucleotides sequences		Size of PCR Products (bp)	Annealing temp. (°C)
	Forward primer 5'- 3'	Reverse primer 5'- 3'		
IFN-γ	GCCAAGTTTGAGGTCAACAACCC	CCG AAT CAG CAG CGA CTC CT	124	62
IP-10	TGGGACTCAAGGGATCCCTC	TGGCAATGATCTCAACACGTGG	142	62
IL-12a	ATGTGTCAATCACGCTACCTCCTC	GGTCTTCAGCAGGTTTCGGG	128	62
IL-17	ACTCTCCACCGCAATGAAGACA	CCCTCTTCAGGACCAGGATCTC	150	62
TNF-α	CCTCACACTCAGATCATCTTCTC	GTCTTTGAGATCCATGCCGT	141	60
IL-6	CTCTGCAAGAGACTTCCATCCA	TAAGCCTCCGACTTGTGAAGTA	150	60
IL-23	TAGCCTGGAACGCACATGCAC	GCAAGCAGAACTGGCTGTTGTA	146	64
IL-4	TCTCGAATGTACCAGGAGCCA	CTCTGTGGTGTTCTTCGTTGCT	150	62
IL-18	ACTGTACAACCGCAGTAATACGC	AGTGAACATTACAGATTTATCCC	434	58

Table 1. The primer sequences used for mRNA quantification of pro-inflammatory cytokines

mucosal cytokine expression levels in healthy 129SvEv wild-type mice (n=3) which were housed under the very same conditions. The mucosal cytokine expression value obtained in the latter were set to 1.0 and used as an internal calibrator.

3.2.3 Intestinal permeability

Paracellular epithelial permeability was measured with 4 kDa fluorescein isothiocyanate-labelled dextran (DX-4000-FITC) one week before the end of the respective intervention phases. Mice were fasted for 6 hours and subsequently DX-4000-FITC (Sigma-Aldrich) was intragastrically applied at a dose of 8 mg / g body weight. One hour after DX-4000-FITC application, 60 µl blood was collected under anesthesia from the retrobulbar venous plexus. The blood was centrifuged at 2000 x g for 5 min at 4 °C and the plasma was diluted with an equal volume of PBS. DX-4000-FITC concentrations in the plasma were measured with a fluorescence spectrophotometer at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Standard curves were created using plasma from control mice spiked with known amounts of DX-4000-FITC. Additionally, The epithelial and sub-epithelial resistance of the colonic wall in mice after the 24 weeks intervention phase was determined *ex vivo* using one-path impedance spectroscopy as described elsewhere (**Gitter *et al.*, 1997**) and was kindly performed at Charite-Campus Benjamin Franklin with Dr. Richter.

3.2.4 Microbiota composition analysis

3.2.4.1 Denaturing gradient gel electrophoresis (DGGE)

Bacterial DNA from cecal contents was extracted with the PSP Spin stool DNA plus Kit (Invitek, Berlin, Germany). The DNA was amplified by polymerase chain reaction (PCR) with the forward and reverse primers: U968-GC-f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3') and L1401-r (5'-CGG TGT GTA CAA GAC CC-3') to amplify the variable V6-V8 regions of the bacterial 16S rRNA genes (**Wohlgemuth *et al.*, 2009**). PCR was performed in a 25 µl reaction mixture including 0.5 µl of each primer (10 pmol/µl), 2.5 µl of 10 x PCR buffer, 1.5 µl of 50 mM

$MgCl_2$, 2.5 U Taq DNA polymerase (Invitrogen, Karlsruhe, Germany), 0.4 µl of each 12.5 mM dNTP (Invitek, Berlin, Germany) and 1 µl of template DNA. Touch down PCR conditions were the following: denaturation for 5 min at 94 °C; 21 cycles of 30 s at 94 °C, 20 s at 66 °C (with 0.3 °C increase per cycle), and 40 s at 72 °C; 14 cycles of 30 s at 94 °C, 20 s at 59 °C and 40 s at 72 °C followed by a final extension step of 10 min at 72 °C. The size of the PCR products was checked on 1 % agarose gels in comparison to a 100 bp DNA ladder. The products were purified with the High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) and the concentrations of the amplicons were determined spectrophotometrically. For DGGE, 100 ng of amplified DNA was applied to each lane of a gel with a 40 % to 60 % denaturing gradient which was formed by mixing polyacrylamide solutions containing 0 % or 100 % of denaturing agents (40% formamide, 7 M urea) denaturing polyacrylamide solution. Electrophoresis was carried out using a denaturing gradient gel electrophoresis system (C.B.S. Scientific, Del Mar, USA) at 212 V for 10 min followed by a run at 100 V for 22 h. TAE buffer (242 g/l Tris, 57.1 ml acetic acid, 100 ml 500 mM EDTA, pH 8.3) was used at 60 °C as the running buffer. After the run, the gels were silver stained (**Wohlgemuth *et al.*, 2009**) and microbiota similarity between the animals was evaluated using the Dice's similarity coefficient. Bottom-up cluster analysis was performed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) (**Vanhoutte *et al.*, 2004**).

3.2.4.2 16S rRNA gene sequencing

For microbiota analysis based on 16S rRNA gene sequencing, DNA from cecal contents of all animals per group was pooled and subsequently amplified with the reverse primer 1492-r and a mixture of four 27-f primers (Frank *et al*., 2008). PCR products were purified and ligated into the pGEM-T Easy vector (Promega, Mannheim, Germany), using a

vector : insert ratio of 1:3. The required amount of DNA for the correct vector : insert ratio was calculated as follows:

[(ng of vector x size of insert (in kb)) ÷ size of vector (in kb)] x Molar amount of (insert ÷ vector) = ng insert

e.g. [(50 ng vector × 1.46 kb insert) ÷ 3.0 kb vector] × (3 ÷ 1) = 73 ng insert

The DNA resulting from the ligation reactions was subsequently transformed into competent cells of *E. coli* JM-109 (Promega, Mannheim, Germany) by heat shock. Briefly, 50 µl of competent cells, with a density of ~10^8 bacterial cells/ µl were added to 2 µl of the ligation reaction and incubated for 20 min on ice. Cells were then subjected to a heat shock for 45 s at 42 °C in a water bath and immediately returned on ice for 2 min. Finally, 950 µl of SOC medium (Carl Roth, Karlsruhe, Germany) was added and reactions were incubated for 5 h at 37 °C on a shaker. A 100 µl aliquot of transformed cells was plated in duplicate on LB/ampicillin/IPTG/X-Gal plates and incubated overnight at 37 °C. Colonies with an insert were selected by white/blue screening of the colonies. White colonies were cultured in 5 ml LB medium containing 100 µg/ ml ampicillin (Carl Roth, Karlsruhe, Germany). Plasmid DNA from the culture was isolated according to the manufacturer's instruction (Innu PREP Plasmid Mini Kit, Analytik Jena, Jena, Germany). Cultured white colonies were tested for the correct insert size by colony PCR using vector specific T7 (5'- TAA TAC GAC TCA CTA TAGG G-3') and SP6 (5'-GAT TTA GGT GAC ACT ATA G-3') primers. Inserts were sequenced using vector specific primers: T7 and SP6 (Eurofins MWG Operon, Ebersberg, Germany). Roughly one hundred colonies from each group were randomly selected for sequencing (MWG Eurofins, Ebersberg, Germany). The resulting 16S rRNA gene sequences were subjected to genome database (GenBank: www.ncbi.nlm.nih.gov) searches. The BLAST

function was used to identify the most closely related bacterial species. A sequence identity of ≥ 98 % was set as a threshold for species identification. Sequences with a lower sequence identity were assigned to the respective bacterial family.

3.2.5 Quantification of intestinal bacteria

E. faecium NCIMB 10415 was quantified in colonic and cecal contents whereas *A. muciniphila* in cecal material using quantitative real-time PCR. The latter strain was selected because the microbiota analysis indicated a special role of this bacterium in our model (**Figure 7**). Bacterial DNA was extracted from cecum and colon sample material with the PSP Spin Stool DNA plus Kit (Invitek, Berlin, Germany) and used as template. PCR was performed with the QuantiFast SYBR Green PCR master mix (Qiagen, Hilden, Germany) using the Applied Biosystems 7500 Fast Real-time PCR system. The primers Cyl-1 (TCG GAA TTT GCC AGA AGA AC) and Cyl-2 (CTG GTG AAG CAG GGT TTC AT) which are specific for a unique plasmid sequence present in *E. faecium* NCIMB 10415 were used (**Vahjen et al., 2007**). For the quantification of *A. muciniphila,* 16S rRNA gene-targeted primers ACG GGT GGC AGC AGT CGA GA and TGG TTC CGA ACA ACG CTT GAG ACC were applied. Standard curves were generated by spiking fecal material from control mice with known amounts of NCIMB 10415 cells. For *A. muciniphila* fecal material from germ-free mice was used. Bacterial cell numbers were calculated as $\log_{10}$ cells / g of sample material (dry weight).

3.2.6 Statistical analysis

Data distribution was tested with the Kolmogorov–Smirnov test. Differences between groups were analyzed using One-way ANOVA using the Kruskal-Wallis test followed by the Dunnett's Multiple Comparison Test. Differences between the groups in mRNA expression

levels were analyzed using the paired t-test (SPSS 16.0 for Windows, SPPS, Chicago, USA). Data with normal distribution are presented as means with standard deviation. Not normally distributed data are presented as box-whisker plots indicating the medians, the 2.5th and the 97.5th percentiles and data ranges (Prism 4.0 for Windows, Graph Pad Software Inc., La Jolla, USA). Differences were considered significant at $p \leq 0.05$.

3.3 Results

3.3.1 NCIMB 10415 is capable of proliferating in the intestine of IL-10$^{-/-}$ mice

We first tested whether NCIMB 10415 was able to survive in the murine gut. In cecum and colon contents of the experimental mice the strain reached cell numbers of $\log_{10}$ 8.5 to $\log_{10}$ 9.0 cells / g (dry weight) while it was not detected in the intestine of control mice. NCIMB 10415 cell numbers determined after 3, 8 and 24 weeks were in the same range (**Table 2**).

3.3.2 NCIMB 10415 does not consistently influence gut inflammation in IL-10$^{-/-}$ mice

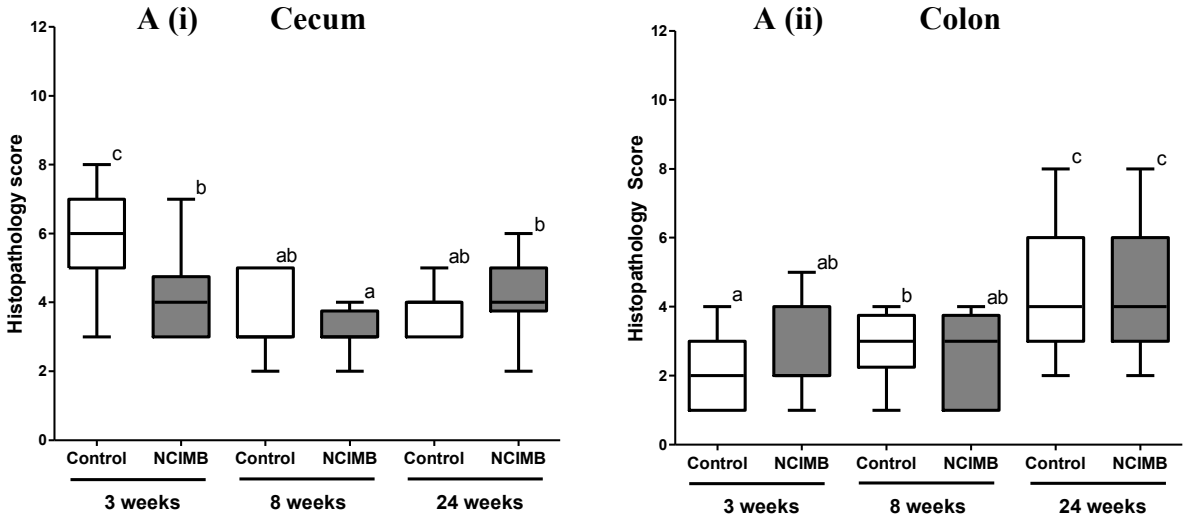
A possible effect of NCIMB 10415 on gut inflammation in the IL-10$^{-/-}$ mouse model was addressed by tissue histopathology scoring and by the measurement of selected cytokines. Most animals developed a mild inflammation in cecum and colon characterized by mucosal hyperplasia, immune cell infiltration and edematous alterations of the mucosa and sub-mucosa. Eight of the 72 animals displayed a stronger immune cell infiltration into the mucosa and sub-mucosa in conjunction with the formation of crypt abscesses, erosions and ulcerations and the activation of the surrounding lymphatic tissue, corresponding to histopathology scores

of more than 6 (moderate inflammation). The inflammation score in the colon but not in the cecum increased with age and became maximal in the animals killed 24

	Probiotic Diet g^{-1}	Cecum g^{-1} (DW)		Colon g^{-1} (DW)	
	CFU $\log_{10}$	Probiotic $\log_{10}$	Control $\log_{10}$	Probiotic $\log_{10}$	Control $\log_{10}$
3 weeks	6.45 ± 0.12	8.70 ±0.20	n.d.	9.07 ± 0.39	n.d.
8 weeks	6.73 ± 0.10	8.57 ± 0.22	n.d.	8.61 ± 0.39	n.d.
24 weeks	6.62 ±0.12	8.53 ± 0.26	n.d.	8.80 ± 0.29	n.d.

Table 2. Absolute quantification of *E. faecium* NCIMB 10415. From the diet, cecum and colon material from the probiotic treated and the control groups after the intervention phases of 3, 8 and 24 weeks. The data's were indicated as mean ± SD, obtained from animal per groups, n=12. Not detected, n.d.

weeks after weaning. We observed a significantly lower cecal inflammation score in the NCIMB-treated than the control mice after three weeks of intervention. At all other time points, no differences in the grade of cecal and colonic inflammation were observed (**Figure 2 A** and **B**). There were also no significant differences between the animals in the relative mucosal mRNA expression levels of TNF-α, IL-6, and IL-23 (**Figure 9**). The expression levels of IL-4 were below the detection limit in all mice (data not shown). In contrast, a significant higher expression of IFN-γ and IP-10 than in the control mice was observed in the mice treated with NCIMB 10415 for 3 and 24 weeks. An increased expression of IL-12a was only observed in those animals that have been treated with NCIMB for 3 weeks. The relative expression of IL-17 was lower in NCIMB-treated mice as compared to control mice, but only at 8 weeks after start of intervention (**Figure 3**).



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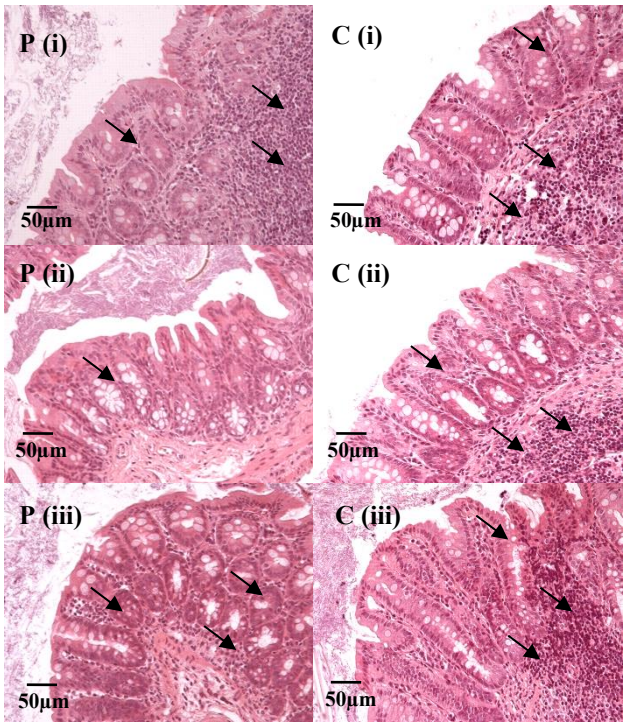

Figure 2. Treatment of IL-10$^{-/-}$ mice with probiotic NCIMB 10415 does not lead to a reduction in intestinal histopathology scores. (A) *E. faecium* NCIMB 10415 does not exert clear-cut anti-inflammatory effects in cecum (i) colon (ii) of IL-10$^{-/-}$ mice. Evaluation of tissue pathology was performed using a scoring system ranging from 0 (no inflammation) to 12 (severe inflammation). Different superscripts indicate statistically significant differences ($p \leq 0.05$). **(B)** Cecal tissue sections stained with H&E. Original magnification 20X. Arrows indicate the severe immune cell infiltration. P- Probiotic, C- Control

i. 3 weeks – severe infiltration of immune response in both treated and control group
ii. 8 weeks – inflammatory infiltration, lower in treated group than in the control group
iii. 24 weeks –severe infiltration observed in both treated and control group.

3.3.3 NCIMB 10415 does not improve gut barrier function in IL-10$^{-/-}$ mice

To test whether NCIMB 10415 influences gut permeability, plasma concentrations of FITC-dextran were measured in all animals one hour after oral administration of the marker. No differences between the animals in the experimental and control groups were observed at any time point. Independent of NCIMB 10415 treatment, the highest plasma FITC-dextran concentrations were measured at 24 weeks after start of the intervention. The lowest concentrations were measured in animals fed NCIMB 10415 for 8 weeks (**Figure 4**).

To test a possible effect of NCIMB 10415 on gut barrier function, one-path impedance spectroscopy was performed at Charite by Dr. Richter and me together. With this approach it is possible to differentiate between the ion conductance of the epithelial cell layer and the underlying tissue and, thus, to locate intestinal permeability changes. However, no differences in the epithelial or the sub-epithelial resistance were observed resulting in an equal total transmural resistance in all animals tested (**Figure 5**).

3.3.4 NCIMB 10415 does not change intestinal microbiota composition in IL- 10$^{-/-}$ mice

To investigate whether the daily uptake of NCIMB 10415 changes microbiota composition in IL-10$^{-/-}$ mice, we compared intestinal colonization patterns of NCIMB-treated and control mice. No obvious differences in the DGGE band profiles of the experimental and the control mice were observed by visual inspection of the gels. This was confirmed by computer-based band profile evaluation and subsequent cluster analysis. However, the mice killed after an intervention phase of 8 weeks clustered separately from all other animals independent of the NCIMB 10415 treatment (**Figure 6**). To identify NCIMB-induced changes in dominant bacterial groups that had possibly been missed in the DGGE approach, we performed 16S rRNA gene sequencing analysis. As expected, the majority of identified sequences represented the Firmicutes (60 – 75 %) and the Bacteroidetes (21 – 34 %).

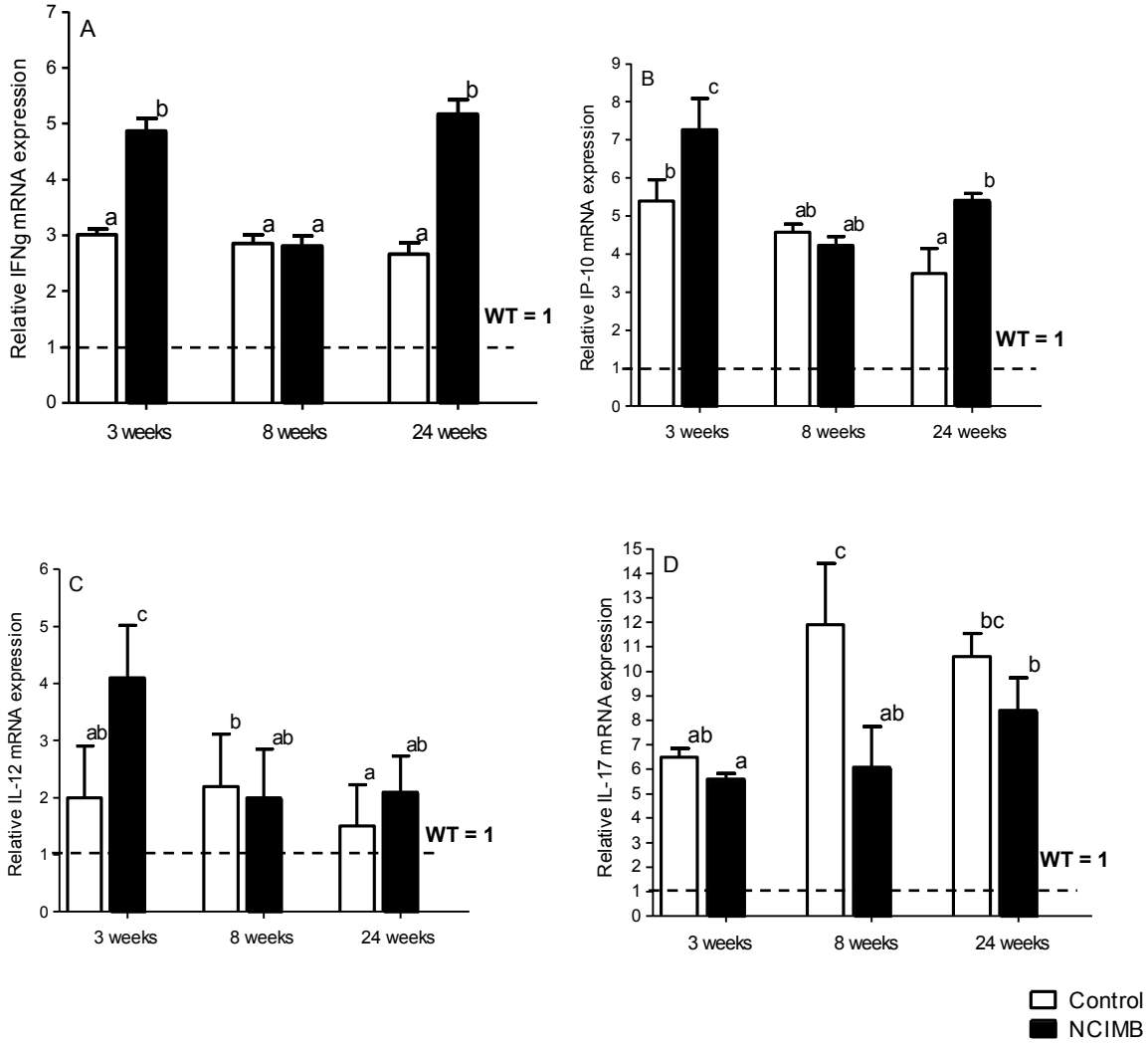

Figure 3. Treatment of IL-10$^{-/-}$ mice with NCIMB 10415 does not affect gene expression of pro-inflammatory cytokines. Effects of *E. faecium* NCIMB 10415 on the mucosal expression of interferon (IFN) γ **(A),** interferon-inducible protein (IP) 10 **(B),** interleukin (IL) 12a **(C),** and IL-17 **(D)** at the mRNA level in IL-10$^{-/-}$ mice. Gene expression of the corresponding genes in wild-type (WT) mice was arbitrarily set to 1. Different superscripts indicate statistically significant differences ($p \leq 0.05$).

Members of the Proteobacteria, Actinobacteria and Verrucomicrobia were either not detected or contributed only a small proportion (≤ 5 %) to the total number of sequences. No clear effect of the treatment with NCIMB10415 on microbiota composition was obvious from 16S rRNA gene sequence analysis (**Figure 7 & Table 3**). However, a closer look at the sequences revealed that the Verrucomicrobia which were exclusively represented by the species

Akkermansia muciniphila (> 99 % sequence identity), were present in all animals killed after 3 (1 to 4 %) and 24 (3 to 5 %) weeks but absent from NCIMB-treated animals killed after 8 weeks. In the control mice that were killed at this time point, this species contributed 1 % to total intestinal bacteria. To corroborate this finding, numbers of *A. muciniphila* were quantified in intestinal contents by quantitative PCR. In essence, the quantitative real-time PCR results confirmed that the cell numbers of *A. muciniphila* were significantly lower in the animals treated with probiotic NCIMB 10415 for 8 weeks (**Figure 8**).

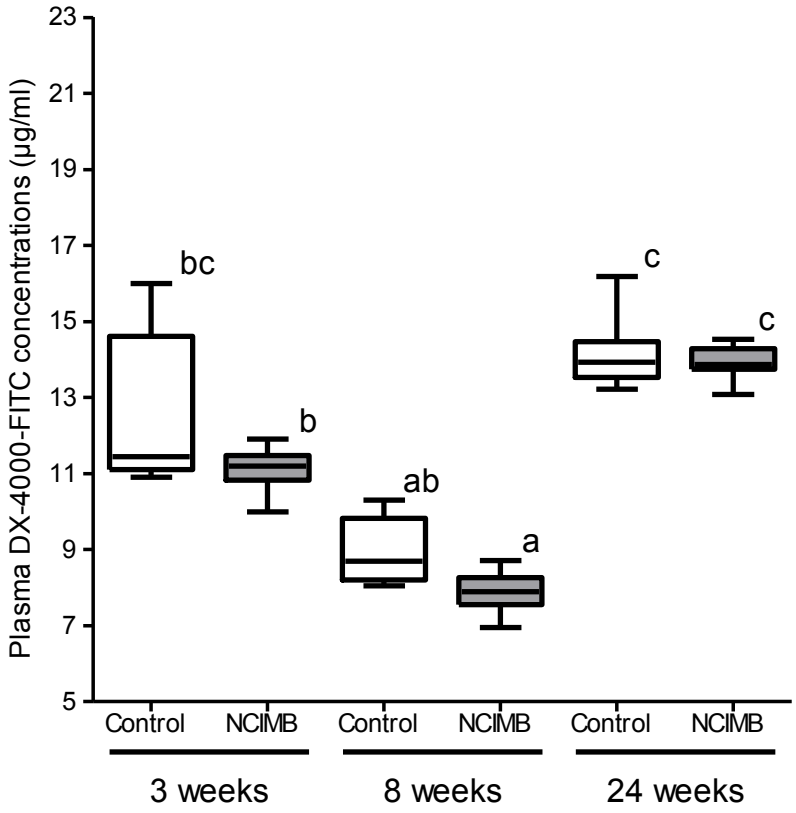

Figure 4. Treatment of IL-10$^{-/-}$ mice with NCIMB 10415 feeding did not affect gut permeability. *E. faecium* NCIMB 10415 given to IL-10$^{-/-}$ mice in their diet does not result in an improved intestinal epithelial barrier function shown are from plasma DX-4000-FITC concentrations 1 h after oral application. Different superscripts indicate statistically significant differences ($p \leq 0.05$).

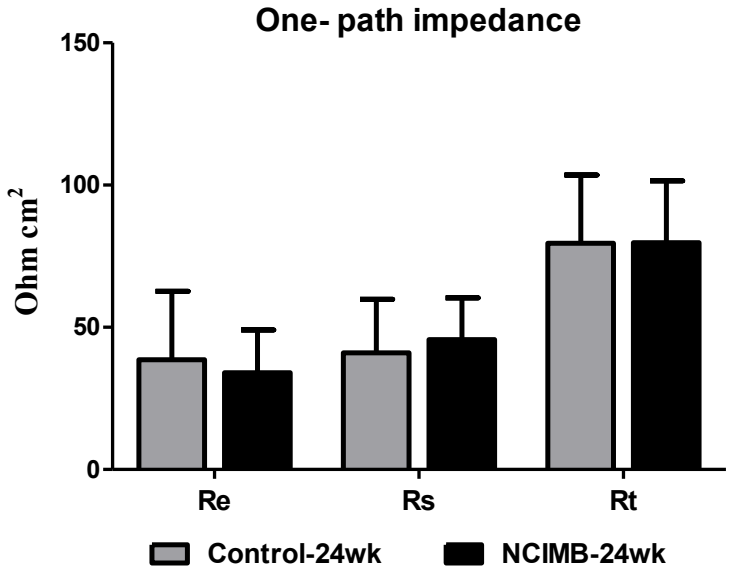

Figure 5. Treatment of IL-10$^{-/-}$ mice with NCIMB 10415 for 24 weeks does not show changes in gut permeability measured with Ussing chamber. Permeability of the distal colon of mice fed with probiotic *E. faecium* does not differ from that of control groups. This was indicated by no changes in the colonic epithelial resistance (Re), the sub-epithelial resistance (Rs) and transmural resistance (Rt) of mice given NCIMB 10415 for 24 weeks compared to the control mice. Data represents mean ± SD.

3.4 Discussion

Enterococcus faecium NCIMB 10415 reduces duration and severity of diarrhea in humans and piglets (**Taras *et al.*, 2006; Zeyner & Boldt, 2006**). We tested whether the strain is also protective against chronic gut inflammation and aimed to identify possible anti-inflammatory mechanisms. We fed NCIMB 10415 to IL-10$^{-/-}$ mice from weaning on over a period of 3, 8 and 24 weeks and investigated if NCIMB 10415 influences intestinal barrier function and gut microbiota composition in this animal model.

Since there were no clear-cut differences in gut pathology between the NCIMB-treated and control animals after 3, 8 and 24 weeks of intervention (**Figure 2**), we do not propose a strong anti-inflammatory effect of NCIMB 10415 in IL-10$^{-/-}$ mice. In addition, effects on the expression of pro-inflammatory cytokines were inconsistent. The relative expression of

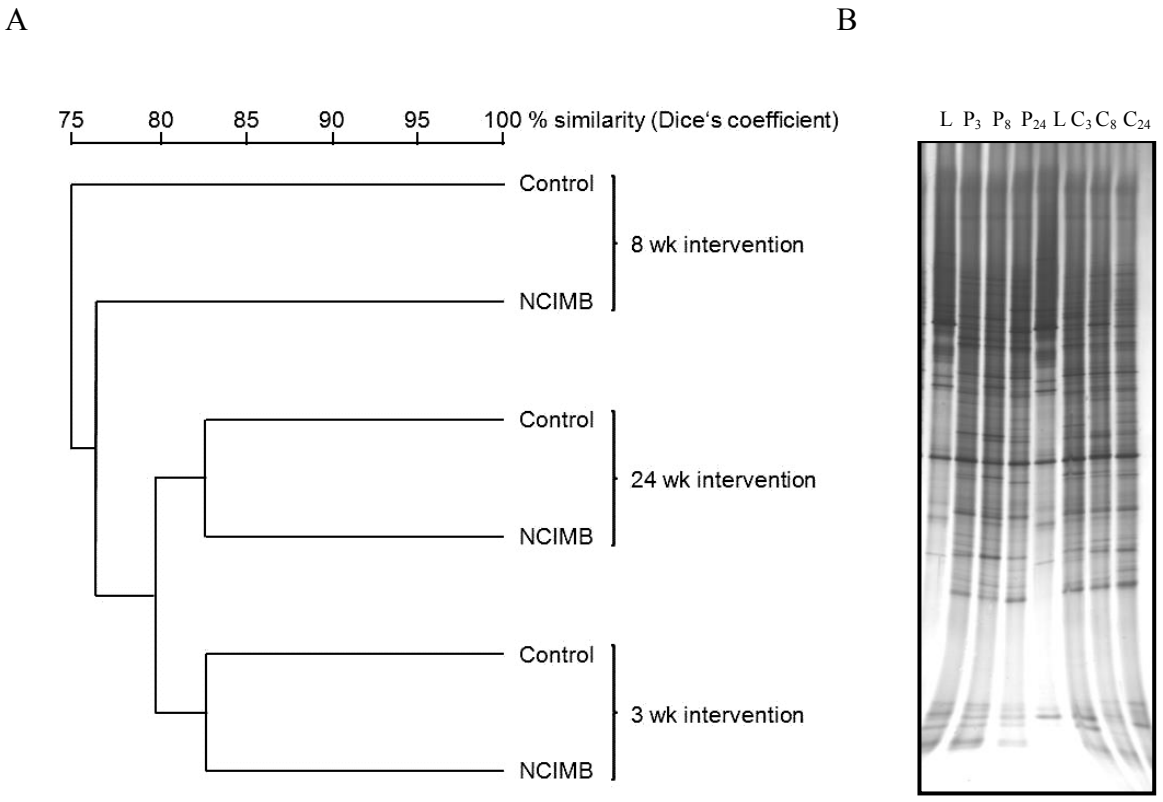

Figure 6. Treatment of IL-10$^{-/-}$ mice with NCIMB 10415 revealed no differences in intestinal microbial community analyzed by DGGE. (A) Intestinal microbiota composition similarity based on DGGE band patterns and cluster analysis of IL-10$^{-/-}$ mice supplemented with NCIMB 10415 showed no differences. The duration of probiotic intervention (3, 8 and 24 weeks) is indicated. Weeks are indicated as wk. **(B)** DGGE gel showing the cecal microbiota band patterns from probiotic (P) and control (C) groups, (L-ladder) of the pooled cecal bacterial DNA samples from mice. 3, 8 and 24 represent the weeks of probiotic supplementation with the respective control groups.

IFN-γ and IP-10 was increased in mice fed NCIMB 10415 for 3 and 24 but not in mice fed the strain for 8 weeks (**Figure 3**). Elevated expression levels of IL-12a mRNA were only observed in mice after 3 weeks of intervention. IL-17 expression was only lower after 8 weeks of feeding NCIMB 10415 to the mice (**Figure 3**). Site specific anti-inflammatory effects in IL-10$^{-/-}$ mice characterized by a reduced grade of cecal inflammation and a similar or even slightly higher grade of colonic inflammation have previously been demonstrated for VSL#3, a probiotic cocktail consisting of 8 bacterial species (**Hoermannsperger *et al.*, 2009; Reiff *et al.*, 2009**). In contrast to NCIMB 10415, VSL#3 reduced the intestinal expression of

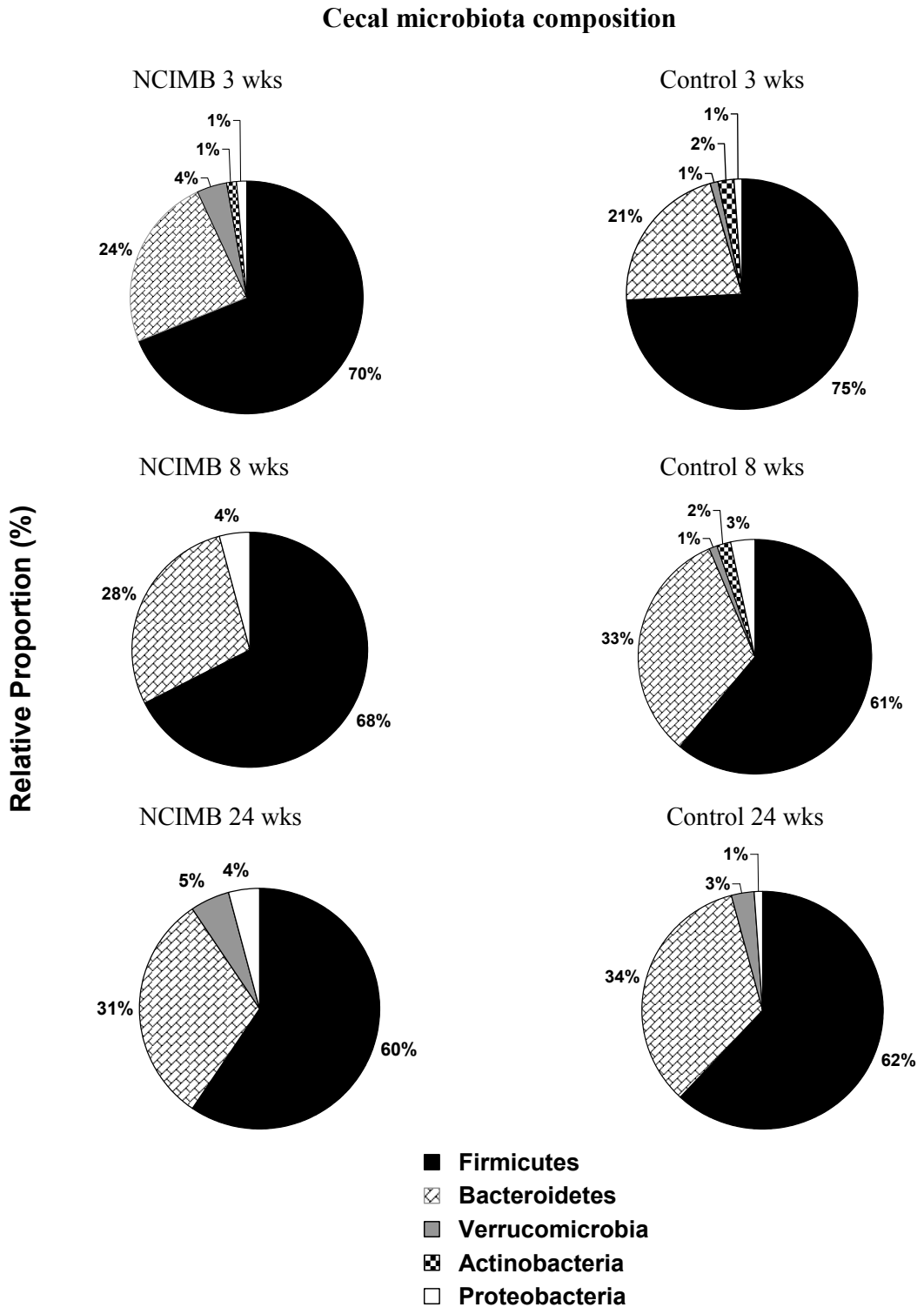

Figure 7. 16S rRNA gene sequencing revealed no differences in cecal microbiota composition in IL-10$^{-/-}$ mice in response to NCIMB 10415 feeding. The pie chart shows cecal microbiota composition at phylum level. Relative contribution of the phyla to the cecal microbiota of mice groups with NCIMB 10415 or not. Only phyla that contribute at least 1% to one of the profiles are indicated. The phylum Verrucomicrobia were completely absent in the probiotic fed groups at 8 wks but not in 3 and 24 weeks and in the respective control groups at all-time points. The number of clones used for sequencing was 3 wks – 170 clones, 8 wks – 194 clones & 24 wks – 193 clones.

genes associated with a T_h1-type immune response such as TNF-α and TNF-α-induced chemokines including CXCL10 (IP-10). The changes observed in the reduction of inflammatory response by VSL#3 administrations might in part explain that different probiotic strains behave differently and treatment with different probiotics show different outcomes on gut inflammation. In rats with antibiotic-associated diarrhea, the treatment with the probiotic *E. faecium* strain L5 improved clinical symptoms in conjunction with higher IL-10 expression in the intestinal mucosa suggesting that IL-10-dependent mechanisms might be involved in protective effects of probiotic *E. faecium* strains (**Tarasova *et al.*, 2010**).

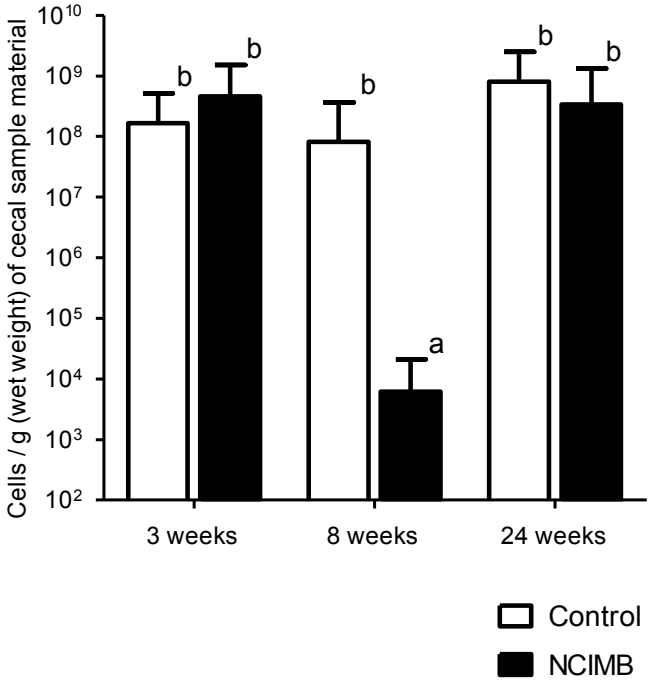

Figure 8. Treatment of IL-10$^{-/-}$ mice with NCIMB 10415 showed differences in cecal *A. muciniphila* cell numbers. Cell numbers of *Akkermansia muciniphila* in cecal contents obtained from NCIMB 10415-treated mice and control mice killed after 3, 8 and 24 weeks of intervention as determined by quantitative real time PCR. Different superscripts indicate statistically significant differences ($p \leq 0.05$).

Probiotic strains such as *E. coli* Nissle 1917 are able to improve gut barrier function by inducing the expression of tight junction proteins and influencing their cellular redistribution

(**Zyrek *et al.*, 2007**). Other mechanisms involved in barrier-improving probiotic effects include the prevention of epithelial injury associated with increased pro-inflammatory cytokine levels. Such an effect has been demonstrated for *Lactobacillus rhamnosus* GG which produces two proteins with anti-apoptotic effects (**Yan *et al.*, 2007**). However, our study does not reveal any effects of NCIMB 10415 on epithelial integrity which is in line with previous observations in piglets (**Lodemann *et al.*, 2006**).

Various probiotics are known to produce antimicrobial substances, to out-compete pathogenic bacteria or to induce host defensin production (**Wohlgemuth *et al.*, 2010**) and thereby change gut microbiota composition (**Gerritsen *et al.*, 2011**). Thus, we speculated that NCIMB 10415 affects the gut microbiota of IL-10$^{-/-}$ mice. However, we did not observe any changes in response to the treatment of the mice with this strain. Similar findings have been reported for piglets (**Broom *et al.*, 2006**). It is not very likely that the numbers of NCIMB 10415 in the intestine of the experimental mice were too low to exert beneficial effects on the above mentioned parameters. We fed approximately $\log_{10}$ 6 cells per gram of diet and detected more than $\log_{10}$ 8 cells per gram of intestinal content (dry weight) in the experimental mice (**Table 2**). This finding suggests that NCIMB 10415 was able to proliferate in the intestine of the mice and that it was metabolically active. In contrast to our findings, cell numbers of NCIMB 10415 have been reported to be highly variable in sow feces and in intestinal contents of their offspring where they do not reach such high numbers when $\log_{10}$ 6 cells per gram of diet were fed (**Vahjen *et al.*, 2007**).

One interesting finding in our study was the reduced occurrence of *A. muciniphila* in the NCIMB-treated animals after 8 week of intervention (**Figure 8**). We do not think that this was a result of the treatment but rather coincidental because *A. muciniphila* cell numbers in all other mice were identically high. However, the mice with lower numbers of this bacterium

displayed a reduced cecal histopathology (**Figure 2**) in conjunction with lower paracellular permeability (**Figure 4**) of the gut wall than the NCIMB-treated animals killed after 3 and 24

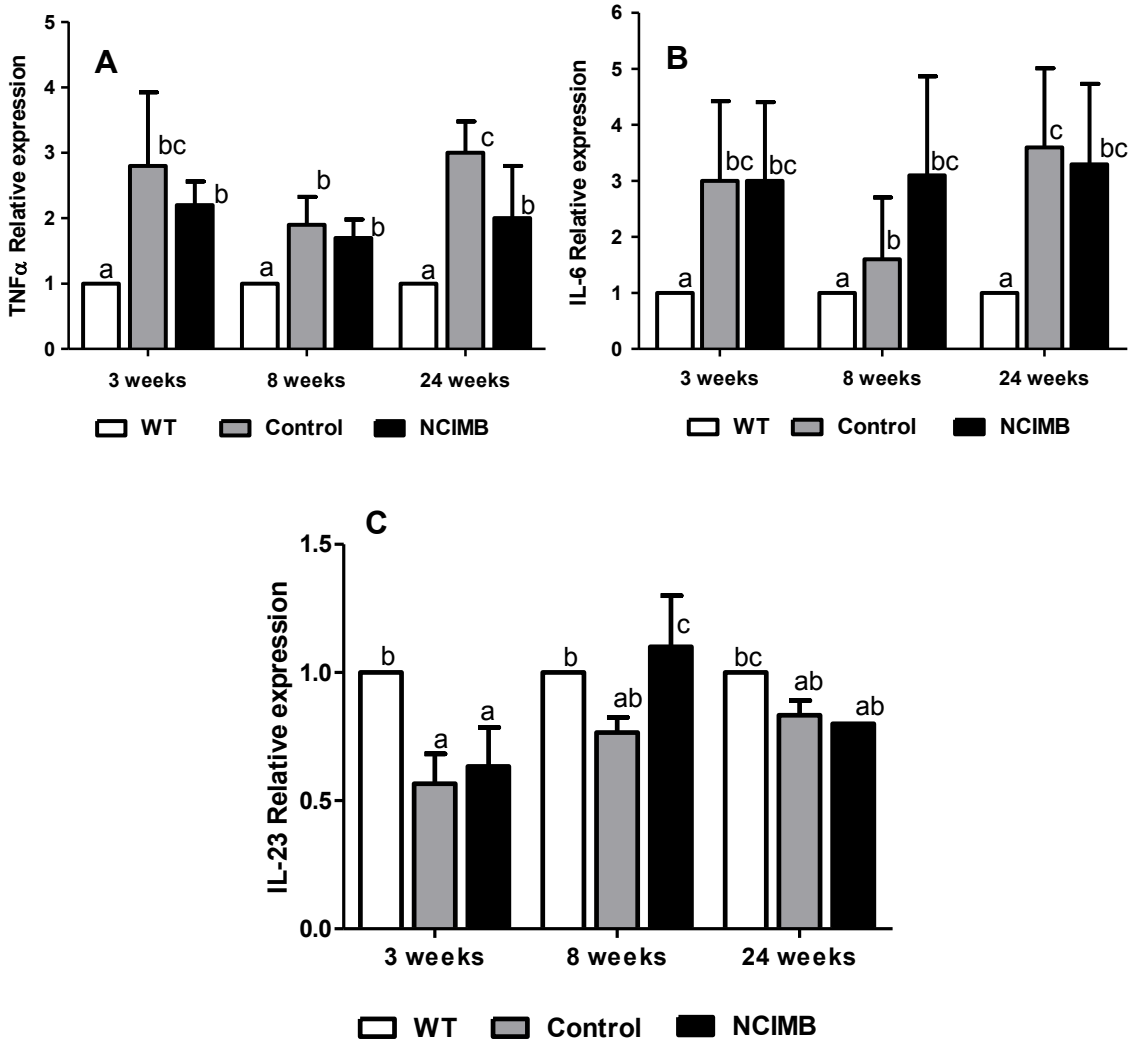

Figure 9. Treatment of IL-10$^{-/-}$ mice with NCIMB 10415 does not exert clear effect on TNF-α, IL-6 and IL-23 mRNA levels. Inconsistent effect of *E. faecium* NCIMB 10415 on the mucosal expression levels of tumor necrosis factor (TNF) α **(A),** Interleukin (IL) 6 **(B)** and IL-23 **(C)** at the mRNA level in IL-10$^{-/-}$ mice. WT- Wild type, cecum mRNA expression was set to be 1 and used as a calibrator. Different superscripts indicate statistically significant differences ($p \leq 0.05$).

weeks. In addition, we found a higher expression of IFN-γ and IP-10 in the NCIMB-treated than in the control mice after 3 and 24 weeks of intervention but not after 8 weeks when *A. muciniphila* was absent. It has been demonstrated very recently that the mucin-degrading *A.*

muciniphila is capable of inducing host genes involved in immune responses and cell differentiation (**Derrien *et al.*, 2011**). It may be speculated that interactions between *A. muciniphila* and the host, between *A. muciniphila* and NCIMB 10415 or a triangular relationship between all three partners influence intestinal inflammation in the IL-10$^{-/-}$ mouse model. However, our experiments do not provide a clear picture. Further studies are needed to verify this assumption.

In summary, we found no indication for a valid and consistent effect of NCIMB 10415 on chronic gut inflammation, intestinal permeability or gut microbiota composition in the IL-10$^{-/-}$ mouse. We cannot rule out that IL-10-dependent mechanisms are involved in the probiotic mode of action of NCIMB 10415. It appears that the IL-10$^{-/-}$ mouse is an inadequate model for investigating these effects. In addition, protective effects of NCIMB 10415 may be restricted to acute intestinal disorders such as infectious diarrhea. Possible interactions between NCIMB 10415, *A. muciniphila* and the host, which might affect host health, will be addressed in further chapters.

Table 3: 16S rRNA gene sequencing data from cecal bacterial DNA

	Probiotic 3 wks	Control 3 wks	Probiotic 8 wks	Control 8 wks	Probiotic 24 wks	Control 24 wks
Bacteria (%)	100	100	100	100	100	100
Firmicutes	69.3	74.4	66.6	61	59.4	61.9
Aerococcaceae	—	6.7	—	2	—	1
Blautia	4	3.3	3	5.1	2.1	8.3
Clostridiaceae	14.6	9	18.2	12.6	14.6	15.5
Enterococcaceae	—	3.3	4	—	1	1
Erysipelotrichaceae	—	1.1	—	—	3.1	—
Eubacteriaceae	4	10	7.1	7.4	8.3	4.1
Paenibacilliaceae	—	1.1	—	—	1	1
Lachnospiraceae	13.3	22.2	14.1	17.2	11.5	19.6
Listeriaceae	—	2.3	—	—	—	—
Ruminococcaceae	33.3	14.4	17.2	13.7	12.5	10.3
Veillonellaceae	—	1.1	—	—	—	—
Peptococcaceae	—	—	1	1.1	2.1	1
Staphylococcus	—	—	—	1.1	—	—
Lactobacillaceae	—	—	1	—	1	—
Bacillaceae	—	—	1	—	1	—
Streptococcaceae	—	—	—	—	1	—
Bacteroidetes	24	21.1	28.3	32.6	31.3	34
Bacteroidaceae	17.6	14.4	18.2	17.9	22.9	20.6
Porphyromonadaceae	6.8	3.3	9.1	11.6	6.3	11.3
Prevotellaceae	—	1.1	—	3.2	2.1	2.1
Rikenellaceae	—	2.2	1	—		
Verrucomicrobia	4	1.1	—	1.1	5.2	3.1
Verrucomicrobiaceae	4	1.1	—	1.1	5.2	3.1
Actinobacteria	1.4	2.3	1	2.1	—	—
Mycobacteriaceae	—	2.3	—	2.1	—	—
Bifidobacteriaceae	1.4	—	1	—	—	—
Proteobacteria	1.3	1.1	4	3.2	4.1	1
Desulfovibrionaceae	1.3	1.1	—	—	—	1
Campylobacteraceae	—	—	1	3.2	—	—
Acetobacteraceae	—	—	3	—	—	—
Enterobacteriaceae	—	—	—	—	3.1	—
Sphingomonadaceae	—	—	—	—	1	—
No. of Clones	75	90	98	95	96	97

Table 3. 3 weeks – 165 clones; 8 weeks – 193 clones; 24 weeks – 193 clones

Title: Co-culture of probiotic *E. faecium* NCIMB 10415 with *Akkermansia muciniphila* results in the production of a cytotoxic factor

4.1 Introduction

Different possible mechanisms of probiotic action have been proposed, including both suppression and stimulation of host immune responses (**Pagnini *et al.,* 2009**). While investigating the effects of probiotic *Enterococcus faecium* NCIMB 10415 (NCIMB) in an IL-10$^{-/-}$ mouse model of chronic gut inflammation, we observed more severe inflammatory symptoms in NCIMB 10415-treated mice when the commensal bacterium *Akkermansia muciniphila* was present at higher cell numbers (**Ganesh *et al.,* 2012**) (**Chapter 3**).

It has recently been reported that *A. muciniphila* is a dedicated mucin degrading commensal (**Collado *et al.,* 2007; Derrien *et al.,* 2008**). Since we observed increased levels of pro-inflammatory cytokines in the presence of higher cell numbers of (9 $\log_{10}$) *A. muciniphila* in IL-10$^{-/-}$ mice (**Ganesh *et al.,* 2012**), we speculated that the presence of mucin degrader together with NCIMB 10415 might contribute to an increase in inflammatory responses. We hypothesized that either NCIMB or *A. muciniphila* produces bacterial factor/s with pro-inflammatory properties. Therefore we investigated the effects of microbe-microbe interactions on the host cells, using *in vitro* models. For this purpose, HT-29 colon cancer cell lines were treated with cell culture medium or cell culture medium conditioned with either NCIMB or *A. muciniphila* or both together. HT-29 colon cancer cell-lines serves as a suitable *in vitro* model because this cell line behaves similar to that of intestinal epithelial cells under inflammatory conditions (**Bruno *et al.,* 2005; Su *et al.,* 1999**).

4.2 Materials and Methods

4.2.1 Conditioned media

The overnight bacterial cultures of probiotic NCIMB grown in BHI medium (Sigma Aldrich) and mucin degrader *A. muciniphila* grown in Columbia broth (DIFCO) were adjusted to approximately 8 $\log_{10}$ cells per ml using a counting chamber. The bacterial cells (~8 $\log_{10}$ cells ml^{-1}) from NCIMB or *A. muciniphila* were washed with sterile PBS and were inoculated either separately or together into sterile Dulbecco's Modified Eagle Medium (DMEM) medium (Invitrogen). The bacterial cells (10^8 cells ml^{-1}) in DMEM were incubated under anaerobic condition with CO_2 / N_2 (80/20; v/v) for 2 hours at 37 °C. After 2 hours incubation the bacterial cells from the respective groups were removed from DMEM medium by centrifugation at 850 x *g* for 15 min at 4 °C. The supernatant also called conditioned media (CM) was passed through a membrane filter (pore size: 0.25 µm) to obtain bacterial cell-free CM. The CM was stored at -20 °C until use. Three different CM media were used: (1) AM-CM: DMEM medium conditioned with *A. muciniphila* only, (2) NCIMB-CM: DMEM medium conditioned with NCIMB only and (3) COMB-CM: DMEM medium conditioned with both *A. muciniphila* and NCIMB. In addition, the aliquots of these media (CM) heated at 80 °C for 20 min to inactivate bacterial proteins or other heat sensitive factors: (4) AM-CM_H, (5) NCIMB-CM_H and (6) COMB-CM_H, used respectively.

4.2.2 HT-29 cell culture

The HT-29 human colon adenocarcinoma cell line was maintained in DMEM medium supplemented with 5% fetal bovine serum (FBS) and antibiotics (100 U /ml penicillin and 100 µg /ml streptomycin). All cell culture reagents were from Invitrogen Life Technologies. HT-29 cells were plated at an initial confluence of 30–40% and sub-cultured by trypsinization twice weekly to maintain continuous cellular proliferation. Cells were re-plated at the same

cell density at each passage. Cells were treated with either DMEM medium or filtered bacterial free CM (AM-CM, NCIMB-CM, COMB-CM, AM-CM_H, NCIMB-CM_H and COMB-CM_H) for a period of 0, 2, 4, 6, 8, 18 and 24 hours (2 ml CM / 10^6 HT-29 cells). Cell-culture facilities were kindly provided by Prof. Brigelius-Flohé's laboratory, DIfE.

4.2.3 Cell-viability measured after treating HT-29 cells with different CM

Sub-cultured HT-29 cells were counted using a haemocytometer and adjusted to approximately 10^6 cells per ml. After transfer of HT-29 cells into a 35 mm sterile dish, containing DMEM supplemented with 10% FBS the cells were incubated for three days at 37 °C with 5 % CO_2 to proliferate and form a monolayer. The HT-29 cell layer was then washed with sterile PBS and treated individually with various CM. Treatments of the HT-29 cells with the different CM were performed for 0, 2, 4, 6, 8, 18 and 24 hours (h). At each time point, HT-29 cells were washed with sterile PBS (1x), trypsinized stained with trypan blue (10:1 ratio) and counted using a haemocytometer. Trypan blue stains dead HT-29 cells blue.

4.2.4 Quantification of cytokines in HT-29 cell-free supernatants using ELISA after treatment with different CM

HT-29 cells were treated with the respective CM for the indicated times. After treatment the supernatants were collected and used to quantify the IFN-γ and TNF-α protein concentrations by enzyme-linked immunosorbent assay (ELISA) using a commercially available ELISA kit (Abcam, Germany). The assay was performed in duplicate and the concentrations were calculated from the standard curves according to the manufacturer's instruction. The detection limits for the IFN-γ was 46.9 pg /ml and for TNF-α 31.3 pg /ml respectively. Samples were measured spectrophotometrically according to the manufacture's instruction.

4.2.5 mRNA levels of pro-inflammatory cytokines in HT-29 cells after treatment with various CM

RNA was extracted from the HT-29 cells after treatment with various CM at specific time points using the miRNeasy® mini kit (Qiagen, Hilden, Germany) to quantify the relative expression levels of the human pro-inflammatory cytokines IFN-γ and TNF-α. One µg of RNA was reverse-transcribed to single-stranded cDNA using the Revert Aid H minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Reverse transcriptase real-time PCR was performed for the samples using the 7500 Fast Real-Time PCR system (Applied Biosystems, Carlsbad, USA) and quantified using the specific standards. The RT-PCR reaction mix per sample (25 µl) contained the template DNA (1 µl), the QuantiFast SYBR Green PCR master mix (12.5 µl) (Qiagen), the respective primer pairs (0.5 µl each) and water (10.5 µl). The forward and reverse primers used were IFN-γ (**Ethuin *et al.,* 2004**) and TNF-α (**Silswal *et al.,* 2005**). Relative mRNA target gene expression levels were normalized using the values obtained for β-actin (**Ethuin *et al.,* 2004**) selected as reference gene (house-keeping gene). Subsequently, mRNA levels of pro-inflammatory cytokines of the control group (HT-29 cells treated with only DMEM) were set to 1.0 and used as the calibrator to identify the relative mRNA fold change between the DMEM treated and the various CM treated groups.

4.2.6 Detection of apoptotic HT-29 cells after treatment with different CM

Apoptosis in human colon carcinoma HT-29 cells treated with different CM for 0, 2, 4, 6, 8 and 24 h was detected using a CaspGLOW™ Fluorescein Active Caspase-3 Staining Kit (Biovision, Research Products, Mountain View, CA, USA) according to the manufacturer's instructions. FITC-DVED-FMK (Biovision) is cell permeable, non-toxic, and irreversibly binds to activated caspase-3 in HT-29 cells undergoing apoptosis. The DEVD-

FMK labeled with FITC allows for direct detection of activated caspase in apoptotic cells by fluorescence microscopy or fluorescence plate reader (Ex / Em = 485 to 535 nm).

4.2.7 Extra-cellular bacterial protein extraction from different CM's

The extra-cellular bacterial proteins were extracted from bacteria-free CM. During the protein extraction steps the CM were always maintained on ice at 4 °C. Eight hundred microliters from each CM were used. After addition of benzonase and protease inhibitor the proteins were precipitated by the 2D-Quant precipitant and co-precipitate kits followed by purification using the 2D-cleanup kit (GE Health care, USA). The proteins were purified and suspended in 50 µl rehydration buffer (1g CHAPS, 3.8 g thiourea and 10.5 g urea in 25 ml with distilled water and store at -20 °C), which were then stored at -80 °C until use. Subsequently, the quantification of bacterial proteins were determined spectrophotometrically using Bradford assay (Ex – 595 nm). Standard curves were obtained for Bradford assay using bovine serum albumin (BSA) dilutions (dilutions (mg /ml): 0.3, 0.25, 0.2, 0.15, 0.1, 0.05 and 0.02) and measured in duplicates.

4.2.8 Two-dimensional gel electrophoresis (2D-DIGE) for protein identification

2D-DIGE is commonly used to analyze proteins. Mixtures of proteins are separated by two properties in two dimensions. The two dimensions that proteins are separated into initially using isoelectric point, protein complex mass in its native state and protein mass. The gels were followed with two different labeling methods for identification of differentially expressed proteins between the groups. First the reference gel was stained with Ruthenium (II) tris (4, 7-diphenyl-1, 10-phenantrolin disulfonate) (RuBPS) and this was followed by analytical gel with the fluorescence 2D-differential gel electrophoresis (DIGE). Briefly, for the detection of differentially expressed proteins on the analytical gel, lysine residues of the proteins were labeled with fluorescent cyanine-based dyes (CyDye) before isoelectric

focusing using a well-defined program (Program: 30V- 10 h, 500V- 1 h, 1000V- 1 h, 10000- 3 h and 10000V- 42500 Vh) performed with IPG-strips (24 cm long and with a pH-range of 4-7) (GE Healthcare, Germany) followed by gel electrophoresis (**Tannu *et al.,* 2006**). This technique allows the labeling of 2 to 3 individual samples with different Cy-dyes at once in a single gel. The CyDye has an *N*-hydroxysuccinimidyl ester reactive group and forms a covalent bond with the epsilon amino group of lysine residues, thus each labeled protein can be visualized as a single protein spot (**Tannu *et al.,* 2006**). Proteins extracted from AM-CM, NCIMB-CM and COMB-CM was labeled with Cy3 or Cy5 dyes (GE Healthcare). A mix of all samples was labeled with Cy2 (GE Healthcare) and served as an internal standard.

Electrophoresed gels were scanned with a pixel size of 100 µm and an intensity of 510 V used for reference gel (RuBPS staining) and 460 V was used for analytic gel (Cy-labeled gels) using Typhoon Scanner TRIO Variable Mode Imager (GE Healthcare, Germany). Gel editing was performed with Image Quant TL Toolbox (GE Healthcare, Germany) and DIGE gel analysis was performed using the DeCyder^{TM} 2D 6.5 software (GE Healthcare, Germany). Furthermore, protein spots with an average ratio of the difference in expression levels of > 2 and < -2 were only taken into consideration for further analysis. 2D-DIGE analysis was kindly performed by master's student Ms. Adler.

4.2.9 Statistical Analysis

Data distribution was tested with the Kolmogorov–Smirnov test. Differences between groups were analyzed using One-way ANOVA for normally distributed data and the Kruskal-Wallis test for non-normally distributed data followed by the Bonferroni/ Dunnett's Multiple Comparison Test. Data with normal distribution are presented as means with standard error and non-normally distributed data are presented as medians with range (Prism 4.0 for Windows, Graph Pad Software Inc., La Jolla, USA). Differences were considered significant

at $p \leq 0.05$. The statistical significance for proteins spots were performed by DeCyder™ 2D 6.5 software and was determined as $p<0.05$ for T-Test (GE Healthcare).

4.3 Results

4.3.1 Treatment of HT-29 cells with COMB-CM caused cell death

Treatment of HT-29 cells with control DMEM, *A. muciniphila* conditioned (AM) -CM and NCIMB conditioned (NCIMB) -CM, did not result in any changes in HT-29 cell viability

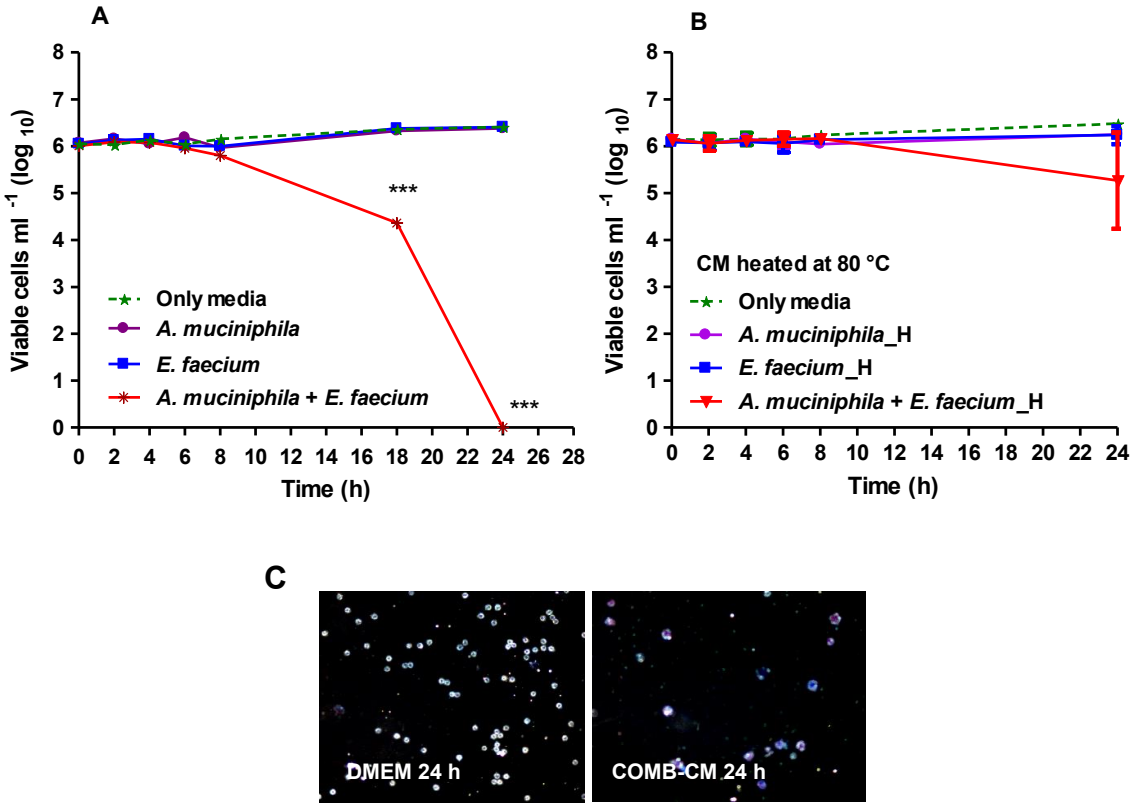

Figure 1. HT-29 cells are killed by treatment with COMB-CM. (A) HT-29 cells were treated with DMEM, AM-CM, EF-CM and COMB-CM. **(B)** HT-29 cells treated with heated DMEM, AM-CM_H, EF-CM_H and COMB-CM_H. After treatment (A & B) for 0, 2, 4, 6, 8, 18 and 24 h, the HT-29 cells stained with trypan blue to count viable cells. Data are expressed as mean ± standard error. n=3 per group at each time point. Stars indicate statistically significant differences (*** $P \leq 0.001$). H-heated, h-hours. **(C)** Image showing HT-29 viable cells in white color without stain and the dead cells in blue color after trypan blue staining and viewed under the microscope with a magnification of 400 x.

In contrast, HT-29 cells treated with the DMEM conditioned with both *A. muciniphila* and NCIMB (COMB-CM) displayed reduced cell viability after 18 h ($p<0.01$). Furthermore, no viable cells were detected after 24 h of treatment with COMB-CM (**Figure 1A & 1C**). This cytotoxic effect was abolished by heat treatment of COMB-CM at 80 °C causing no HT-29 cell death (**Figure 1B**).

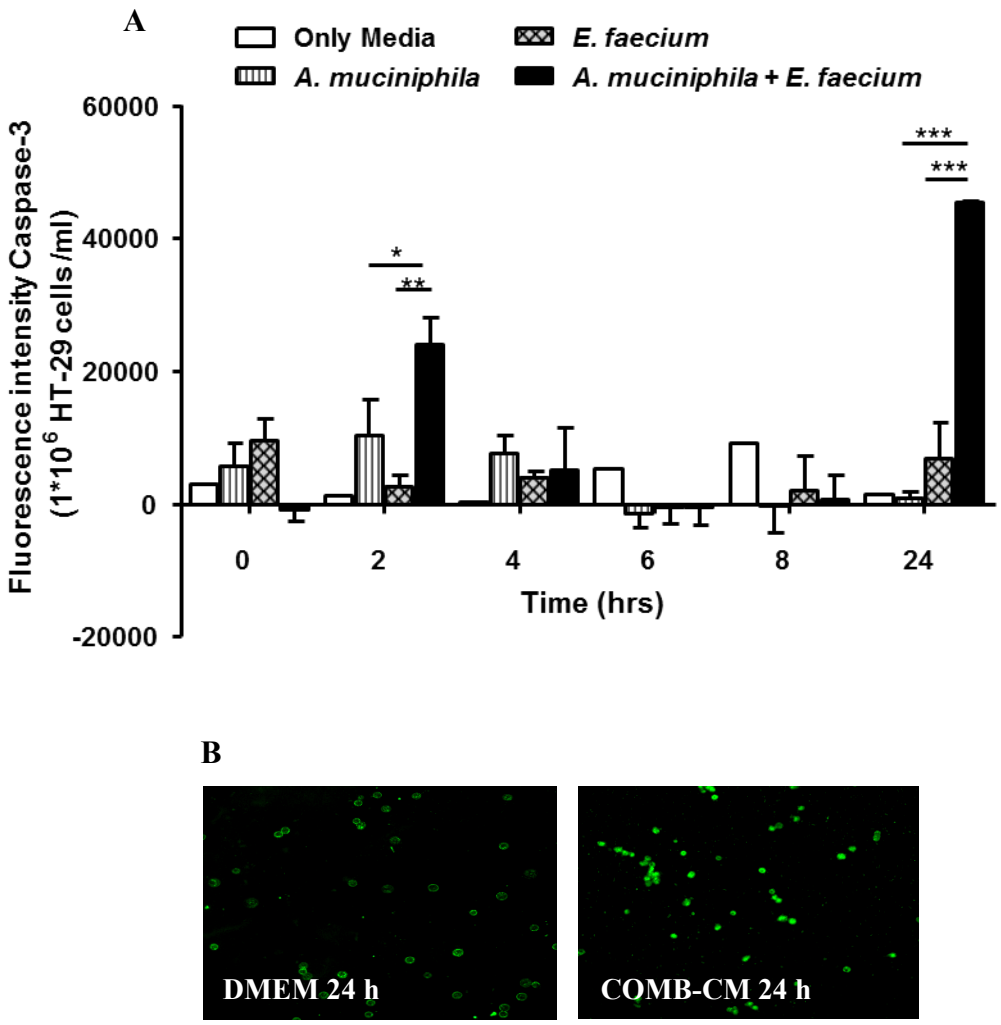

Figure 2. HT-29 cells showed increased Caspase-3 after treatment with COMB-CM. (A) The HT-29 cells were treated with DMEM, AM-CM, EF-CM and COMB-CM, for a period of 0, 2, 4, 6, 8 and 24 h. After treatment HT-29 cells were washed, trypsinized and stained with fluorescence Caspase-3 inhibitor and quantified spectrophotometrically (see materials and methods). Data are expressed as median with range. n=3 per group at each time point. Star indicate statistically significant differences (** *P<0.01*, * *P<0.05*). **(B)** Apoptotic HT-29 cells were stained with caspase-3 fluorescence dye (bright green- apoptotic cells) after treatment with respective CM and viewed under the microscope with a 400-fold magnification.

4.3.2 Treatment with COMB-CM leads to increased Caspase-3 protein levels in HT-29 cells

To investigate whether the cell death was caused by apoptosis or necrosis we quantified Caspase-3 protein expression levels in HT-29 cells which were treated with control DMEM or AM-CM, EF-CM or COMB-CM. We observed a significant increase in active Caspase-3 protein expression in HT-29 cells treated with COMB-CM for 24 h compared to other groups (**Figure 2A**). Detection of activated caspase-3 indicated that cell death was brought about by apoptosis. No or only few apoptotic cells were detected after treatment of HT-29 cells with any other CM/ DMEM (**Figure 2B**).

4.3.3 No clear difference in gene expression levels of pro-inflammatory cytokines after treating HT-29 cells with COMB-CM

Based on our previous observations in mice (**Ganesh *et al.*, 2012**) we speculated that the simultaneous presence of NCIMB and *A. muciniphila* triggers pro-inflammatory responses

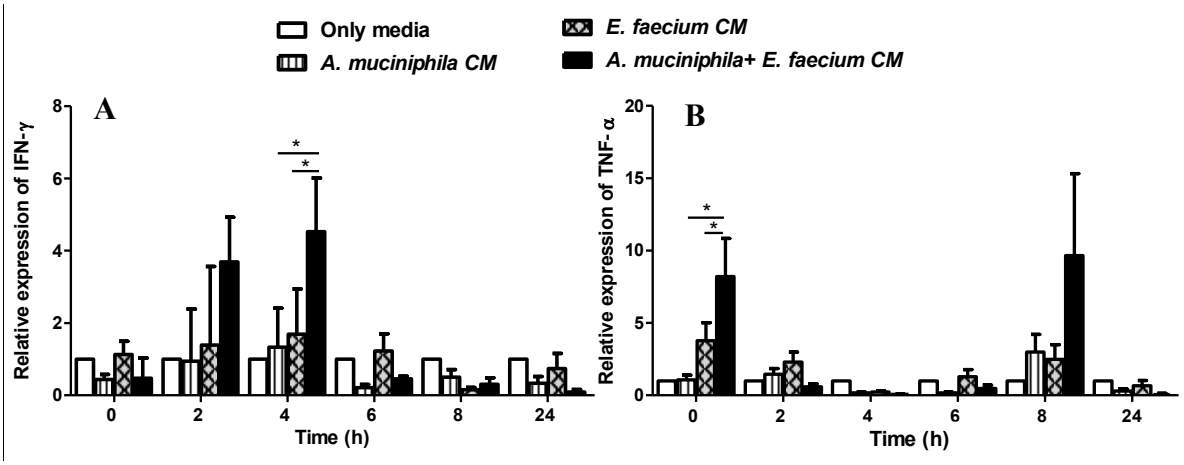

Figure 3. HT-29 cells showed increased mRNA levels of pro-inflammatory cytokines after treatment with COMB-CM. The HT-29 cells are treated with different CM's: DMEM, AM-CM, EF-CM and COMB-CM, respectively, for a period of 0, 2, 4, 6, 8 and 24 h. After treatment HT-29 cells are washed, trypsinized and used to extract mRNA to identify the gene expression levels of IFN-γ **(A)** and TNF-α **(B)** cytokines between the groups at each time points individually. Data are expressed as median with range. n=3 per group at each time point. Star indicate statistically significant difference (* *P<0.05*).

in intestinal epithelial cells *in vitro*. However, mRNA levels of TNF-α and IFN-γ in HT-29 cells after treatment with different CM did not provide a clear picture. Cells treated with COMB-CM showed significantly higher ($p \leq 0.05$) TNF-α mRNA levels than cells treated with DMEM, AM-CM and NCIMB-CM, respectively (**Figure 3B**). However, this was only observed at 0 h and 8 h. TNF- α expression was similar or even slightly lowers at all other time points tested. In addition, mRNA expression of IFN-γ was significantly higher ($p \leq 0.05$) at 0 h, 2 h and 4 h of treatment with COMB-CM than in all other groups treated at various time points (**Figure 3A**). However, this effect was no longer observed at the later time points of the same group.

4.3.4 Differentially expressed extra-cellular bacterial proteins identified in COMB-CM

We observed HT-29 cell death in the treatment with COMB-CM and when the COMB-CM was heated the cytotoxicity of CM_H on HT-29 cells was abolished. This leads to the prediction that the effect observed could be due to a cytotoxic bacterial factor present in COMB-CM. therefore 2D-DIGE analysis was performed for the extra-cellular bacterial proteins. Interestingly we observed very few differentially expressed bacterial proteins (**Figure 4 and 5**) in COMB-CM compared to AM-CM or NCIMB-CM proteins. The proteins were considered to be significantly differentially expressed only when their average ratio was at least above 2.0 fold higher in the experimental group compared to the control group (up-regulated) and vice versa (down-regulated). Further experiments are necessary to find out the potential function of all the differentially expressed proteins (**Figure 4**).

4.4 Discussion

Commensal bacteria are known to influence a number of host processes including nutrition, development and immune responses that are relevant for both health and disease

(**Yan *et al.*, 2004**). Therefore, manipulation of the intestinal microbiota has been used as an approach for disease prevention and treatment (**Sartor, 2004**). Although the defensive response of the intestinal epithelium (IE) against pathogenic bacteria has been extensively explored, less information is available in the interactions of IE with commensal bacteria and their effects on host health. A commonly held belief is that nonpathogenic and probiotic bacteria exert opposing effects to those induced by pathogenic bacteria, including suppression rather than stimulation of pro-inflammatory cytokines in epithelial cells and macrophages (**Jijon *et al.*, 2004**). However, several studies underscore the importance of "physiologic inflammation" induced by the commensal microbiota, both for the development

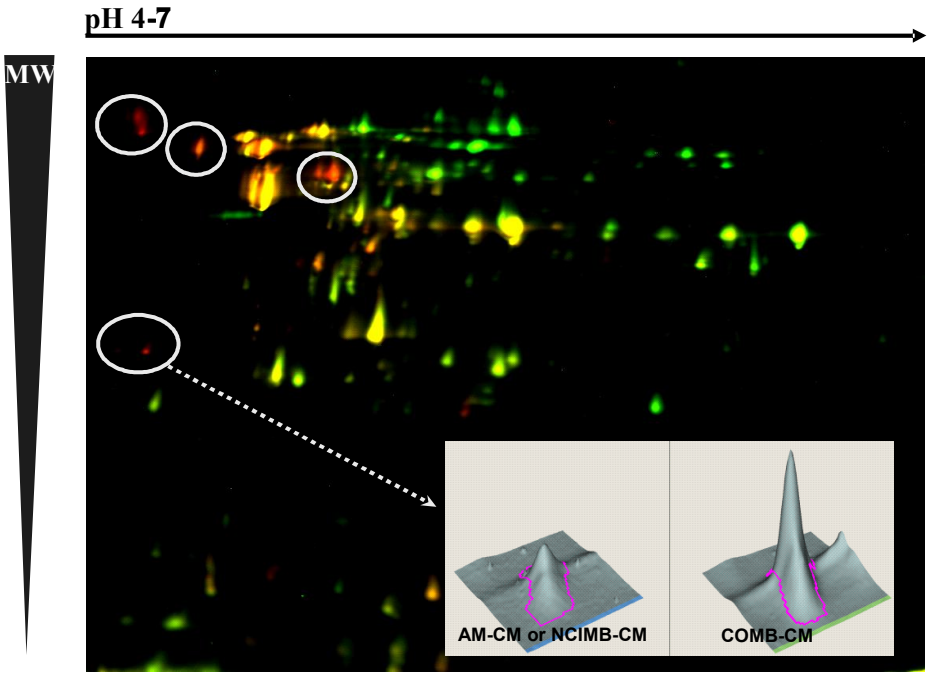

Figure 4. COMB-CM contains proteins whose expression differs from that of AM-CM or NCIMB-CM. Conditioned media were prepared by inoculating *A. muciniphila* (Am-CM) or NCIMB (NCIMB-CM) or both together (COMB-CM). The extra-cellular proteins were extracted from the CM (see materials and methods) and subjected to 2D-DIGE to identify the differentially expressed proteins. Yellow spots indicate the proteins present in all CM (AM-CM, NCIMB-CM and COMB-CM), green spots indicate protein present in AM-CM or NCIMB-CM whereas red spots indicate proteins only present in COMB-CM. (Red-Cy5; Green-Cy3; Yellow-Cy2).

of the immune system and for the response to pathogenic bacteria in the gut. Accordingly, the possibility that probiotic bacteria elicit the same kind of pro-inflammatory response is currently being explored. In addition, probiotics have recently received clinical attention for their potential to prevent and/or treat IBD (**Yan *et al.,* 2007**). However it is very important to verify and screen probiotics for their potential effects on host health with more than one experiment using different models before advertising them as therapeutics. Even though we found no beneficial effects of probiotic NCIMB in conventional IL-$10^{-/-}$ mice for chronic gut inflammation, we found indications that higher cell numbers of *A. muciniphila* were associated with increased inflammatory symptoms in the presence of NCIMB (**Ganesh *et al.,* 2012**).

Our experiments revealed that all the HT-29 colon cancer cells treated with COMB-CM for 24 h killed these cells completely. This was not the case when the HT-29 cells were treated with the AM-CM or NCIMB-CM (**Figure 1**). This suggests that the concomitant presence of NCIMB and *A. muciniphila* led to the production of a soluble and heat-sensitive factor that influences the viability of HT-29 cells. This suggests that either of the two bacterial species induce the formation of this factor in the other one or that two independent factors from the two organisms exerted this effect when combined. We currently hypothesize that the cytotoxic factor in COMB-CM is a protein and possibly one of the differentially expressed proteins were identified in the 2D-DIGE gels (**Figure 4**). However, this hypothesis needs to be tested. As a first step, all differentially expressed proteins found in various CM need to be identified to clarify which ones are produced by NCIMB or by *A. muciniphila* during their concomitant presence. Elucidation of their function will help to better characterize their effects on epithelial cells.

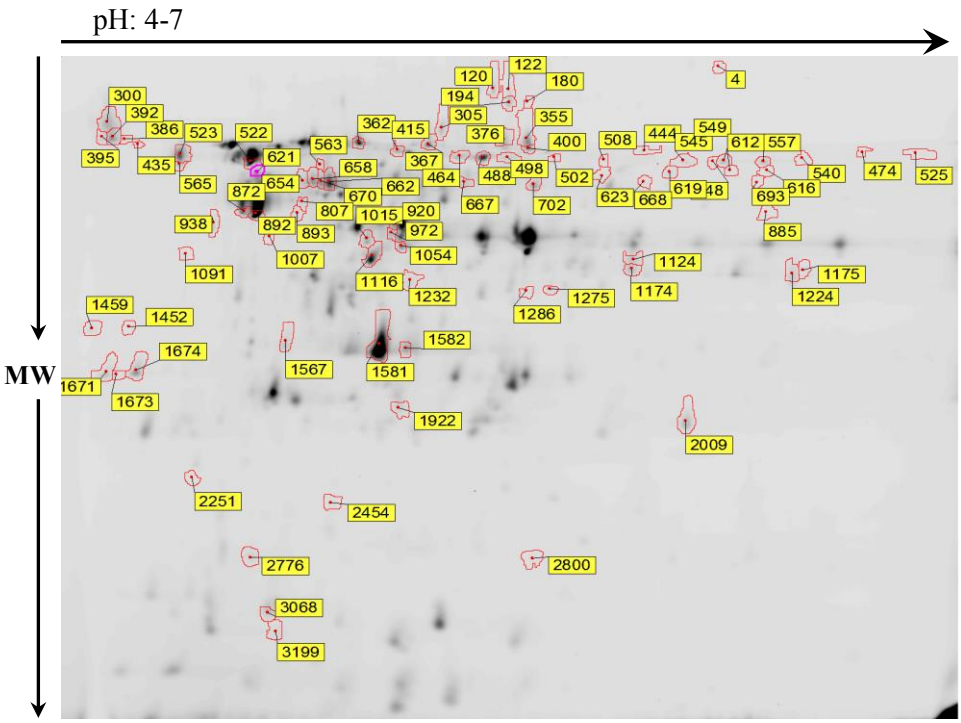

Figure 5: COMB-CM contains proteins spots whose expression is different from that of AM-CM or NCIMB-CM. Proteins were separated (pH=4-7) in the first dimension according to their isoelectric point and according to their molecular weights (MW) using a 12.5% polyacrylamide gels in the second dimension. Spot numbers were provided by DeCyder™ software.

Even though the study has not yet led to the identification of the assumed cytotoxic factor, it is quite clear from the data that interactions between a known probiotic bacterium and a commensal mucin degrading bacterium leads to the production of bacterial factor/s that killed HT-29 cells and led to an increase in IFN-γ and TNF-α mRNA levels at various time-points.

Title: Commensal *A. muciniphila* exacerbates gut inflammation in *Salmonella* Typhimurium-infected gnotobiotic mice

(Majority of the text used to explain this chapter has been taken from the literature with the aforementioned title published in PLoS ONE, 2013. The Authors contributed were **Ganesh BP, Klopfleisch R, Loh G** and **Blaut M**)

5.1 Introduction:

The intestinal mucus layer provides a barrier against invasion of the epithelium by intestinal bacteria. Recent studies suggest that the pathophysiology of ulcerative colitis (UC) involves a disruption of the mucus layer integrity followed by depletion of mucus secretory goblet cells (**Swidsinski *et al.,* 2007; Sartor, 2009; Kim & Ho, 2010; Derrien *et al.,* 2010**). Besides serving as a barrier, mucus also represents a growth substrate and a site of adhesion for intestinal bacteria (**Derrien *et al.,* 2010; Falk *et al.,* 1998**). Excessive mucin degradation by intestinal bacteria may contribute to IBD by facilitating the access of luminal antigens to the intestinal immune system and by changes in the resident gut microbial community (**Corfield & Myerscough, 2000; Wiggins *et al.,* 2001; Campieri & Gionchetti, 2001; Gassler *et al.,* 2001; Sartor, 2006; Sekirov *et al.,* 2010; Duerkop *et al.,* 2009**).

Using IL-$10^{-/-}$ mice as a model of chronic gut inflammation, we previously observed that intestinal inflammation was reduced after 8 weeks of treatment with the probiotic bacterium *Enterococcus faecium* NCIMB 10415 (**Ganesh *et al.,* 2012**) (**Chapter 3**). This reduction in inflammation coincided with a lower abundance of *Akkermansia muciniphila*, a mucin-degrading commensal and a member of the Verrucomicrobia, from 10^8 to 10^4 cells g^{-1}, suggesting that this organism promoted inflammation (**Ganesh *et al.,* 2012**). Moreover, in a T-cell transfer-mediated mouse model of intestinal inflammation the proportion of bacteria belonging to the phylum Verrucomicrobia was fivefold increased compared to control mice (**Stecher *et al.,* 2007**). *A. muciniphila* is the main intestinal representative of this phylum

(**Derrien *et al.,* 2010**), suggesting that *A. muciniphila* numbers increased in response to inflammation (**Stecher *et al.,* 2007**). *A. muciniphila* is a commensal bacterium that colonizes the human gut early in life (**Derrien *et al.,* 2004; Collado *et al.,* 2007; Derrien *et al.,* 2008**). Because of its ability to degrade mucins, we hypothesized that this organism might contribute to intestinal inflammation.

To test this hypothesis we took advantage of a well-defined gnotobiotic mouse model associated with a defined simplified human intestinal microbiota (SIHUMI) of eight bacterial species (**Becker *et al.,* 2011**), complemented with *A. muciniphila* or/and with *Salmonella enterica* Serovar Typhimurium (*S.* Typhimurium). The latter is a murine pathogen (**Zirk *et al.,* 1999**) that triggers acute inflammatory responses (**Bruno *et al.,* 2009**) in TLR11 knock-out mice or streptomycin-treated mice (**Mathur *et al.,* 2012; Winter *et al.,* 2010**) and therefore represents a highly suitable model for investigating immune disorders (**Zirk *et al.,* 1999**). We therefore used *Salmonella* Typhimurium to induce intestinal inflammation in SIHUMI mice to investigate whether *A. muciniphila* influences the infectious and inflammatory symptoms caused by *Salmonella* Typhimurium in these mice. Here we demonstrate that *A. muciniphila* exacerbates *S.* Typhimurium-induced inflammation in the SIHUMI mouse model indicating that the former organism turns into a harmful bacterium under inflammatory conditions. Our experiments suggest that this is at least in part based on *A. muciniphila*'s ability to interfere with host mucus formation and production.

5.2 Materials and methods

5.2.1 Bacterial Strains

The bacterial strains used in this study were: *A. muciniphila* ATCC BAA-835 and *S.* Typhimurium DT104 as well as members of a simplified human intestinal microbiota

(SIHUMI) consisting of eight bacterial species *(Bifidobacterium longum* NCC 2705*, Blautia producta* DSMZ 2950*, Bacteroides thetaiotaomicron* DSMZ 2079*, Clostridium ramosum* DSMZ 1402*, Clostridium butyricum* DSMZ 10702*, Escherichia coli* K-12 MG1655*, Lactobacillus plantarum* DSMZ 20174 and *Anaerostipes caccae* DSMZ 14662*)*. All strains were routinely cultured at 37°C. The SIHUMI members and *S.* Typhimurium were cultured in yeast casitone fatty acid (YCFA) medium (**Becker *et al.,* 2011**) while *A. muciniphila* was cultured in Columbia broth (Difco). All strains were cultured under strictly anoxic conditions using N_2 / CO_2 (80/20; v/v) as the gas phase.

5.2.2 Animal experiments

Germ-free C3H mice were bred in Trexler-type isolators. After weaning, all mice were colonized by gavaging the fecal supernatant of SIHUMI mice (Becker *et al.,* 2011). Forty of these SIHUMI mice were allocated to four groups (10 mice per group). The groups differed in their microbial status: they were subsequently colonized with *A. muciniphila* (SIHUMI-A) or with *S.* Typhimurium (SIHUMI-S) or with both *A. muciniphila* and *S.* Typhimurium (SIHUMI-AS) as indicated in **Figure 1**. The bacteria were grown anaerobically overnight at 37 °C and their cell numbers were determined using a counting chamber. Mice were inoculated with: $5*10^7$ cells of *S.* Typhimurium suspended in 200 µl medium and $1*10^8$ cells of *A. muciniphila* suspended in 200 µl medium by gastric gavage. Mice colonized with only SIHUMI members received 200 µl of sterile medium. Successful bacterial colonization after inoculation was validated in the feces by qPCR (see below).

After anaesthetization the animals were killed by cervical dislocation at the times indicated in Figure 1. Cecal and colonic contents were collected and bacterial cells were enumerated by qPCR. Spleen and mesenteric lymph nodes were collected for enumeration of *S.* Typhimurium. Colon tissue samples were fixed in formalin and Carnoy's solution for histochemical analysis and measurement of mucus thickness. Cecum tissue samples were

fixed with formalin and embedded in paraffin (p) for immunohistochemical analysis (IHC-p), stained with haematoxylin and eosin (H&E) for histopathology scoring and with alcian blue (AB) for enumeration of mucin-filled goblet cells. Bacterial cells were detected by 16S rRNA-targeted fluorescence in-situ hybridization (FISH). Five of the 10 mice per group were used for colon mucosa scrapings while colon tissue of the remaining 5 mice was subjected to Carnoy's fixation. In addition, cecal tissue from all 10 mice per group was used in part for mucosa scrapings and in part subjected to formalin fixation. Scraped mucosa samples were flash frozen in liquid nitrogen and stored at -80 °C until use. Approximately 25 mg of the frozen intestinal mucosa scrapings were homogenized for RNA extraction. Blood was collected for measuring serum inflammatory biomarkers using ELISA. All samples were frozen at -80 °C until use.

5.2.3 Ethics statement

Approval for the animal experiments was granted by the animal welfare authority (State of Brandenburg) under the number V3-2347-42-2011.

5.2.4 Quantification of bacterial cell numbers

Bacterial DNA was extracted from cecal, colonic and fecal sample material using the PSP® Spin Stool DNA plus Kit (Invitek, Berlin, Germany) and used for the quantification of *A. muciniphila*, *S.* Typhimurium and the members of the SIHUMI consortium present in the gut contents. Bacteria were quantified using quantitative Real-Time PCR targeting the 16S rRNA gene in the case of *A. muciniphila* (**Ganesh *et al.*, 2012**), the ttr (tetrathionate respiration) - region in the case of *S.* Typhimurium, as described previously (**Malorny *et al.*, 2007**) and the HSP-60 gene for each member of the SIHUMI consortium (**Slezak *et al.*, 2013**). All primers were purchased from MWG Eurofins (Germany). Standard curves were obtained by spiking feces from germfree mice with known cell numbers of *A. muciniphila*, *S.* Typhimurium or

individual SIHUMI bacteria. The Applied Biosystems 7500 FAST Real-Time PCR (Life Technologies GmbH, Darmstadt, Germany) was used for amplification and fluorescent data collection. The supplied software was used to calculate absolute cell numbers according to the calibration curves. The master mix consisted of 12.5 µl QuantiFast SYBR Green 2000 (Qiagen, Hilden, Germany), 0.5 µl of each primer (10 µM), 1 µl of sample and adjusted with water to a final volume of 25 µl per well. After PCR amplification, the specificity of the primers was checked by inspecting the melting curve and determining the size of the amplicon by agarose gel electrophoresis (1 %). Bacterial DNA from mesenteric lymph nodes (mLN) and spleen was extracted with the Blood and Tissue DNA extraction kit (Qiagen) and used for the quantification of *S.* Typhimurium. Standards were obtained by spiking homogenized mLN or spleen of SIHUMI mice with known cell numbers of *S.* Typhimurium.

5.2.5 Evaluation of intestinal inflammation

Formalin fixed cecal and colonic sample material was embedded in paraffin and sectioned at 4 µm. After staining with haematoxylin and eosin, gut inflammation was evaluated by an experienced pathologist in a blinded fashion. The histopathology scores was based on the following parameters: villous atrophy and fusion: 1= scant (ca. 10); 2= moderate; 3= dense, lymphocytes: 0= one small; 1= some (2-4); 3= numerous (>5) or 1 large, necrotic epithelial cells: 1= scant (ca. 10); 2= moderate; 3= dense, PMN: 0= none; 1= few extravascular PMNs; 2= many polymorph nuclear cells (neutrophils), neutrophils: 1= Scant (1); 2= moderate, 3= dense, infiltration: 0= none; 1=rare (<15 %); 2= moderate; 3= abundant (>50 %), desquamation: 1= Patchy (<30 %); 2= diffuse (> 30 %), edema: 0= none to mild (<10 of the mucosa); 1= moderate; 2= severe, ulceration: 0= no; 1= present, Crypt abscesses: 0= none; 1= rare; 2= moderate; 3= abundant, Peyer patch hyperplasia: 0= none; 1= present and epithelial hyperplasia: 0= none; 1: present.

5.2.6 mRNA levels of pro-inflammatory cytokines from intestinal mucosa samples

To quantify the relative mRNA expression levels of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interferon gamma-inducible protein (IP)-10, interleukin (IL)-6, IL-12, IL-23, IL-17, IL-18 and IL-4, RNA was extracted from intestinal mucosa samples using the miRNeasy® mini kit (Qiagen, Hilden, Germany). One µg of RNA was reverse-transcribed to single-stranded cDNA using the RevertAid H minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany). Reverse transcriptase real-time (RT) PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system (Life Technologies GmbH). The RT-PCR reaction mix (adjusted with H_2O to a total volume of 25 µl) contained 1 µl template DNA, 12.5 µl QuantiFast SYBR Green PCR master mix (Qiagen), 0.5 µl of the respective primers (10 µM each). The forward and reverse primers used for IFN-γ, IP-10, IL-12, IL-17, TNF-α, IL-6, IL-23, IL-4 and IL-18 quantification were described previously (**Ganesh *et al.*, 2012**) (**Page No: 24**). MUC2 forward (5'-GTGGCTGCGTGCCT AGTCCT-3') and reverse primers (5'-AGGCCGGCCCGAGAGTAGAC-3') were designed using Primer BLAST (NCBI). Relative mRNA target gene expression levels (Ratio = $[(E_{target})^{dCP_{target}\ (control\text{-}sample)}] / [(E_{ref.})^{dCP_{ref.}\ (control\text{-}sample)}]$) were normalized to the house keeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and used as a reference. Subsequently, intestinal mucosal cytokine and MUC2 gene expression values of the SIHUMI group were set to 1.0 and used as the calibrator to identify the relative mRNA fold difference between the SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS groups.

5.2.7 Measurement of cytokines in blood plasma using ELISA

Serum levels of IFN-γ, TNF-α and IL-6 concentrations were measured in duplicate by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Abcam, Cambridge, UK). The concentrations were calculated from standard curves according to the

manufacturer's instruction. The detection limits for the aforementioned cytokines were 46.9 pg/ ml, 31.3 pg/ ml and 8.06 pg/ ml, respectively.

5.2.8 Immunohistochemical analysis

Formalin-fixed, paraffin-embedded cecal sections (2 µm) were incubated overnight at 4 °C with a primary antibody targeting the mouse macrophage-specific receptor F4/80 (Abcam, Cambridge, UK) after antigen retrieval according to the manufacturer's instructions. Samples were washed and subsequently incubated with Histofine (anti-rat secondary antibody fab' fragment from Nichirei, Tokyo, Japan) for 30 min at RT. Immunoperoxidase staining was performed with the diaminobenzidine substrate kit (Sigma-Aldrich, Munich, Germany). Sections were counterstained with haematoxylin and examined by light microscopy in a blinded fashion. Approximately, 50 µm of cecal lamina muscularis corresponding to approximately 30 crypts per mouse and per section were scored. The scores represent positively stained cells in lamina propria and sub-mucosa as follows, 0= none (between 0 and 4), 1= normal (between 5 and 8), 2= moderate (between 9 and 12) and 3= severe (between 13 and above). The scores are shown individually for lamina propria and sub-mucosa. Similarly, 2 µm cecal tissue sections were incubated overnight at 4 °C with MUC2 primary antibody (Santa Cruz) and followed with Alexa fluor 488 labeled IgG secondary antibody for 60 min. The sections were examined with laser scanning confocal microscopy (Leica, Germany) with an appropriate wavelength of 488 nm. The access to confocal microscopy was kindly provided by the laboratory of Prof. Meyerhof from DIfE.

5.2.9 Alcian blue staining of cecal tissue samples

The formalin-fixed cecal tissue was sectioned at 4 µm and stained with alcian blue (AB) at pH-2.5, which stains acidic mucins blue. Goblet cells were enumerated in a 50 µm stretch of the lamina muscularis corresponding to approximately 30 crypts per section and per

mouse using an Eclipse E600 microscope (NIKON, Germany) and inspecting the images captured with a MV-1500 digital camera and Lucia G software version 4.51 (Laboratory imaging s.r.o.) for Windows 7 (Microsoft, Munich, Germany) was used. To distinguish different mucins, colonic and cecal tissue sections were stained with periodic acid Schiff (PAS)/ AB. Cecal tissue sections were additionally stained with high iron diamine (HID)/ AB at pH-2.5, which stains sulphated mucins (sulphomucin) brown and sialylated mucins (sialomucin) blue. Images were analyzed using an Eclipse E600 microscope and captured with a MV-1500 digital camera (NIKON, Düsseldorf, Germany).

5.2.10 FISH analysis for bacterial detection in cecal tissue sections

Formalin-fixed paraffin-embedded cecal tissue sections (4 µm) were initially treated with Roti-Histol (Carl Roth, Karlsruhe, Germany) and then hybridized at 50°C with the 5'-Cy3-labeled *A. muciniphila*-specific probe S-S-MUC-1437-a-A-20 (5'-CCTTGCGGTTGGC TTCAGAT-3') (**Derrien *et al.*, 2011**) and at 45°C with the 5′-Cy3 labeled *Salmonella*-specific probe L-S-Sal-1713-a-A-18 (5'-AATCACTTCACCTACGTG-3') (MWG Eurofins, Germany) as described previously (**Nordentoft *et al.*, 1997**). After overnight hybridization sections were counter-stained with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for visualization of cell nuclei and the slides were analyzed using a Nikon E600 epifluorescence microscope equipped with appropriate filter set, wavelength ranging from 330 to 700 nm (NIKON, Germany).

5.2.11 PAS/AB staining of colonic tissue samples

Carnoy's-fixed colonic tissue (Swiss-rolls) was sectioned at 4 µm and stained with periodic acid Schiff/ Alcian blue (PAS/AB) at both pH 2.5 and 1 (at pH 2.5 acidic mucins are stained blue and neutral mucins are stained magenta, at pH 1: highly sulphated mucins are

stained blue). Mucus layer thickness was measured on the colonic tissue sections. Approximately 30 crypts per section were analyzed using an Eclipse E600 microscope (NIKON, Germany) with Lucia G version 4.51 software for Windows 7 (Laboratory imaging s.r.o.) (Microsoft, Munich, Germany). Images were captured with a MV-1500 digital camera (NIKON, Düsseldorf Germany).

5.2.12 Sialic acid quantification in intestinal mucosal scrapings

The cecal or colonic mucosal samples were homogenized and a portion of 5 mg was used to quantify N-acetylneuraminic acid (NANA). For that purpose the sialic acid quantification kit for whole cells (Sigma-Aldrich, Germany) was modified as follows: The homogenized tissue samples were suspended in sialidase buffer and subjected to an overnight treatment with α→ (2, 3, 6, 8, and 9)-neuraminidase at 37 °C. Subsequently samples were incubated with N-acetylneuraminic acid aldolase which catalyzed the conversion of NANA to pyruvate and the initial absorbance at 340 nm (A_{340}) was recorded after addition of NADH. Following the addition of L-lactic dehydrogenase (LDH) and sample incubation for 10 min at 37 °C, reduction of pyruvate to lactate and the equimolar formation of NAD^+ was recorded at final A_{340}. The decrease in absorbance at A_{340} was measured and used to calculate the total nmoles of NANA released as follows ([nmoles NANA = {[($A_{340\ Initial}$ - $A_{340\ Final}$)] *1000} /6.22]; 6.22, extinction coefficient of NADH at 340 nm) (**Dwek *et al.,* 1993**).

5.2.13 Statistical analysis

Data were tested for normal distribution using the Kolmogorov–Smirnov test. Normally distributed data are presented as means with standard error while the medians with their range are given for non-normally distributed data. Significance of differences between SIHUMI, SIHUMI-S, SIHUMI-A and SIHUMI-AS mice were analyzed using the One-way

analysis of variance test for normally distributed data (or) the Kruskal-Wallis test for non-normally distributed data, followed by either Bonferroni /Tukey or Dunn's comparison post-hoc tests. Differences between SIHUMI-S and SIHUMI-AS mice were analyzed using students t-test followed by the Mann-Whitney test for non-normally distributed data. The correlation between IFN-γ expression levels and number of mucin filled goblet cells in the mice were analyzed by the Pearson correlation coefficient test. Differences between the groups were considered significant at *$P < 0{\cdot}05$, **$P<0.01$, ***$p<0.001$. SPSS 16.0 (IBM, Munich, Germany) for Windows 7 was used for data analysis. Prism 5.0 software (Graph Pad Software, Inc., La Jolla, CA, USA) for Windows, was used for data presentation.

5.3 Results

5.3.1 *A. muciniphila* profoundly affects microbial community composition of SIHUMI mice associated with S. Typhimurium

To induce intestinal inflammation, mice associated with a simplified intestinal microbiota (SIHUMI) were additionally colonized with *A. muciniphila* and subsequently infected with *S.* Typhimurium (SIHUMI-AS). SIHUMI mice and SIHUMI mice associated with either *A. muciniphila* (SIHUMI-A) or *S.* Typhimurium (SIHUMI-S) served as controls (**Figure 1**). Bacterial cell numbers in the intestinal contents were quantified using qPCR.

Five days post infection (p.i.) *S.* Typhimurium became the predominant species representing 94 % of total bacteria in the cecum of SIHUMI-AS mice (**Figure 2D**). In contrast, in SIHUMI-S mice *S.* Typhimurium made up merely 2.2 % of total bacteria. *A. muciniphila* accounted for 8.4 % of total bacterial cells in the SIHUMI-A group, but was as low as 1.3 % in the SIHUMI-AS group. *B. thetaiotaomicron* was dominant in SIHUMI, SIHUMI-A and SIHUMI-S mice making up 80-90 % of total bacteria but was reduced to 0.02 % in the SIHUMI-AS mice. The proportion of other community members was also lower in

the SIHUMI-AS group compared to the other mouse groups. For example, *E. coli* became undetectable in the SIHUMI-AS mice whereas this organism made up 0.14 % of total bacteria in the SIHUMI-S animals (**Figure 2C, 2D**). This was less than the initial *E. coli* proportion of 0.52 % and 0.88 % in the uninfected control groups SIHUMI and SIHUMI-A, respectively (**Figure 2A, 2B**).

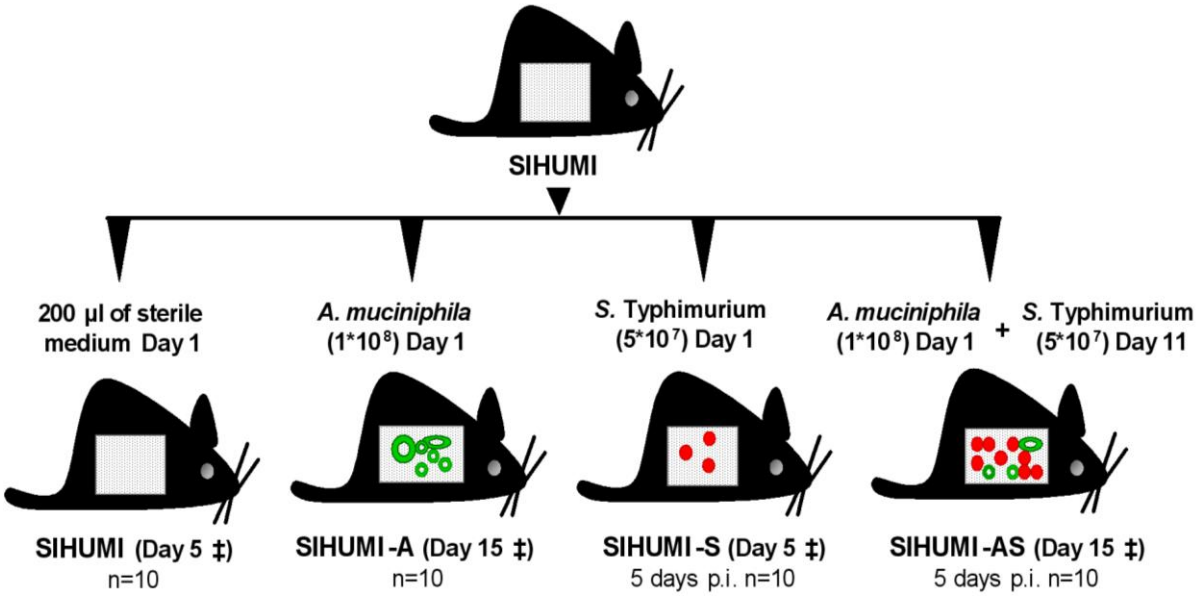

Figure 1. Design of the animal experiment. Forty C3H mice associated with a defined microbial community of 8 bacterial species (SIHUMI) was allocated to four different groups (10 mice per group). Each mouse was associated with 8 bacterial species (SIHUMI). Twelve weeks-old SIHUMI mice were subsequently associated with *A. muciniphila* (SIHUMI-A) or *S.* Typhimurium (SIHUMI-S) or with both *A. muciniphila* and *S.* Typhimurium (SIHUMI-AS). SIHUMI mice received only sterile medium. Times of association, infection and killing are as indicated. ‡ - killed.

Interestingly, there was no significant difference in the absolute *S.* Typhimurium cell numbers between SIHUMI-S and SIHUMI-AS mice but all other members of the community were 1 to 5 logs lower when both S. Typhimurium and A. *muciniphila* were present (SIHUMI-AS) suggesting that the latter organism (*A. muciniphila*) caused a decrease of all other community members except *S.* Typhimurium (**Table 1**). Bacterial cell numbers in the colon revealed a pattern similar to that observed for cecum (**Figure 12 and Table 3**).

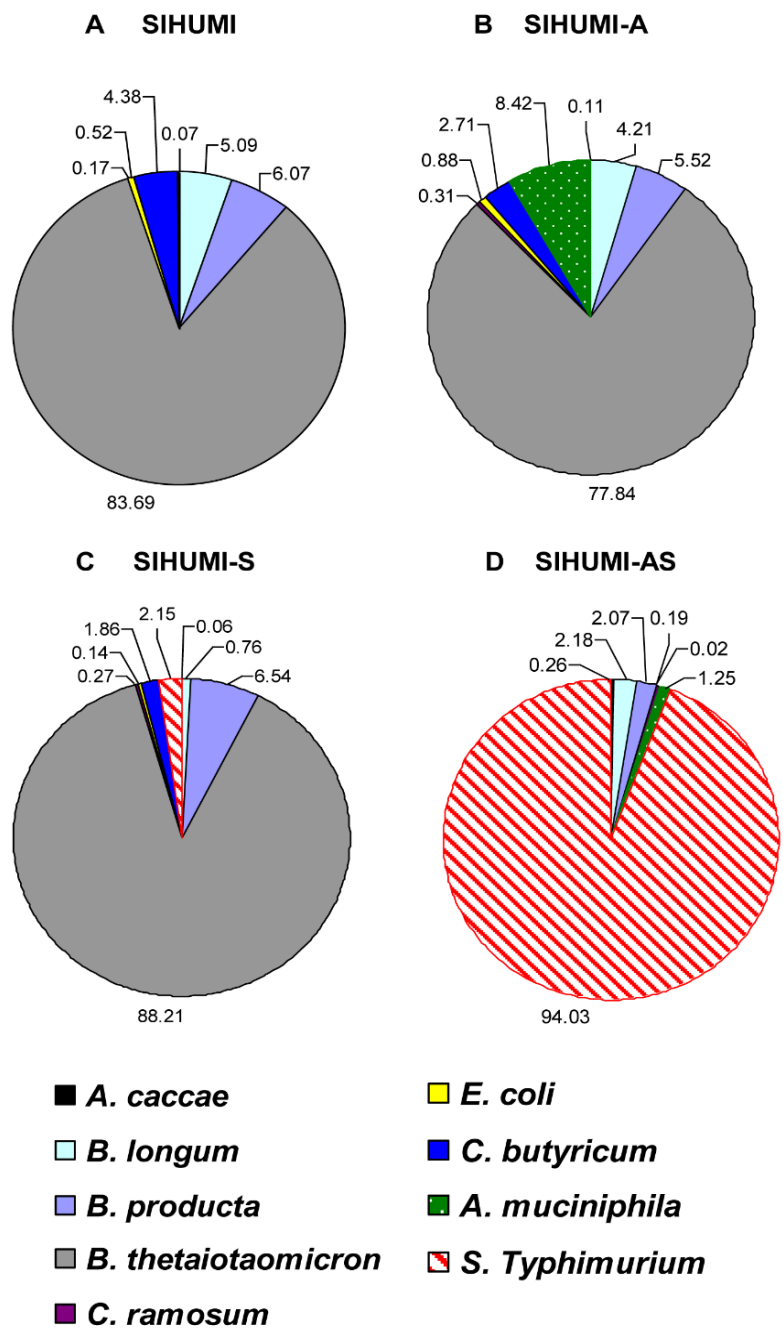

Figure 2. Presence of *A. muciniphila* renders *S.* Typhimurium the dominant species in gnotobiotic SIHUMI mice. Cecal contents were collected from gnotobiotic C3H mice, differing in their microbial status: (A) Mice with a defined microbial community of eight bacterial species (SIHUMI), (B) SIHUMI mice additionally colonized with *A. muciniphila* (SIHUMI-A), (C) SIHUMI mice infected with *S.* Typhimurium (SIHUMI-S) and (D) SIHUMI mice colonized with *A. muciniphila* and 10 days later infected with *S.* Typhimurium (SIHUMI-AS) (see Figure 1). Total DNA was extracted and bacterial cell numbers were quantified by qPCR with primers targeting the HSP60 gene of the SIHUMI members, the 16S rRNA gene of *A. muciniphila* and the ttr-region of *S.* Typhimurium. Calculation of the cell numbers was based on DNA obtained from cell suspensions containing known cell numbers of the targeted bacterial species (see materials and methods). Presence of *A. muciniphila* in SIHUMI-AS mice is attributed to an increase in the proportion of *S.* Typhimurium cells at the expense of other community members showing reduced proportion of SIHUMI members. Ten animals per group were used. The exact bacterial cell numbers and *P-values* for the differences between the groups are provided in Table 1.

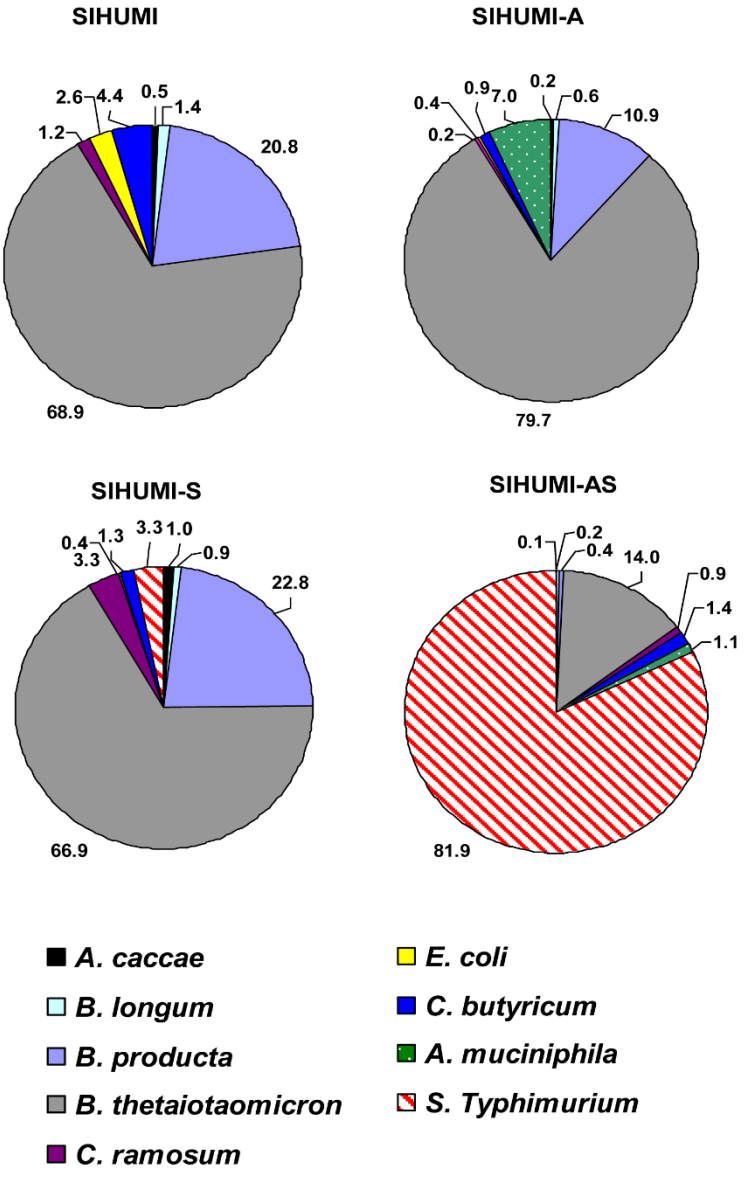

Figure 12. Presence of *A. muciniphila* renders *S.* Typhimurium the dominant species in colon of gnotobiotic SIHUMI mice. Colonic contents were recovered from gnotobiotic C3H mice assigned to 4 groups, differing in their microbial status: (A) Mice with a defined microbial community of eight bacterial species (SIHUMI), (B) SIHUMI mice colonized additionally with *A. muciniphila* (SIHUMI-A), (C) SIHUMI mice infected with *S.* (SIHUMI-S) and (D) SIHUMI mice colonized with *A. muciniphila* and 10 days later infected with *S.* Typhimurium (SIHUMI-AS). Total DNA was extracted and bacterial cell numbers were quantified by primers targeting the HSP60 gene of the SIHUMI members, the 16S rRNA gene of *A. muciniphila* and the ttr-region of *S.* Typhimurium using quantitative PCR. Calculation of the cell numbers was based on DNA obtained from cell suspensions containing known cell numbers of the targeted bacterial species (see materials and methods). Reduced proportion of SIHUMI members in SIHUMI-AS mice is attributed to an increase in the proportion of *S.* Typhimurium cells. Ten animals per group were used. The exact bacterial cell numbers and *P-values* for the differences between the groups are provided in Table 3.

5.3.2 Presence of *A. muciniphila* aggravates inflammatory symptoms caused by *S.* Typhimurium in SIHUMI mice

Histopathological analysis revealed that 5 days p.i. SIHUMI-AS mice showed a 24 % higher cecal histopathology score compared to the SIHUMI-S mice and more than 4.5- to 5-fold higher scores compared to the SIHUMI and the SIHUMI-A mice (no inflammation: 4.0 ± 1.6 and 3.9 ± 1.8) (**Figure 3A, 3B**). This result indicates that *A. muciniphila* exacerbates the symptoms of cecal inflammation caused by *S.* Typhimurium infection in SIHUMI-AS mice. The colon of the infected mice did not display histopathological signs of inflammation (data not shown).

	SIHUMI (DW)	SIHUMI-A (DW)	SIHUMI-S (DW)	SIHUMI-AS(DW)
	log $_{10}$ (g^{-1})	log $_{10}$ (g^{-1})	log $_{10}$ (g^{-1})	log $_{10}$ (g^{-1})
A. caccae	8.20±0.26 ^{ab}	8.52±0.21 ^{b}	8.15±0.31 ^{ab}	7.45±1.26 ^{a}
B. longum	10.03±0.44 ^{b}	10.11±0.27 ^{b}	9.22±0.55 ^{ab}	8.37±2.10 ^{a}
B. producta	10.11±0.35 ^{b}	10.23±0.29 ^{b}	10.16±0.26 ^{b}	8.34±1.34 ^{a}
B. thetaiotaomicron	11.25±0.20 ^{b}	11.38±0.22 ^{b}	11.29±0.30 ^{b}	6.29±0.75 ^{a}
C. ramosum	8.55±0.27 ^{a}	8.98±0.37 ^{a}	8.76±0.28 ^{a}	7.31±0.98 ^{a}
E. coli	9.05±0.19 ^{bc}	9.43±0.98 ^{c}	8.48±0.28 ^{bc}	4.90±0.41 ^{a}
C. butyricum	9.97±0.25 ^{b}	9.92±0.34 ^{b}	9.61±0.35 ^{b}	4.15±0.50 ^{a}
A. muciniphila	n.d.	10.41±0.20 ^{b}	n.d.	8.12±0.60 ^{a}
***S.* Typhimurium**	n.d.	n.d.	9.67±0.16 ^{a}	9.99±0.45 ^{a}
Total bacteria(HSP60)	11.99±0.54 ^{bc}	11.65±0.62 ^{b}	11.96±0.43 ^{bc}	10.62±1.02 ^{a}

Table 1. *S.* Typhimurium becomes the dominant species in SIHUMI mice when previously associated with *A. muciniphila*. Data are expressed as mean ± standard error. Different superscripts indicate statistically significant differences ($P \leq 0.05$). n = 10 mice per group. DW – dry weight.

In line with the histopathology scores, mRNA levels of selected pro-inflammatory cytokines in cecal mucosa were up-regulated in the SIHUMI-AS mice 5 days p.i. compared to all other groups. Interferon-gamma (IFN-γ) expression was approximately 2.5-fold higher in SIHUMI-AS mice compared to SIHUMI-S mice and approximately 40-fold higher compared to SIHUMI or SIHUMI-A mice (**Figure 4A**).

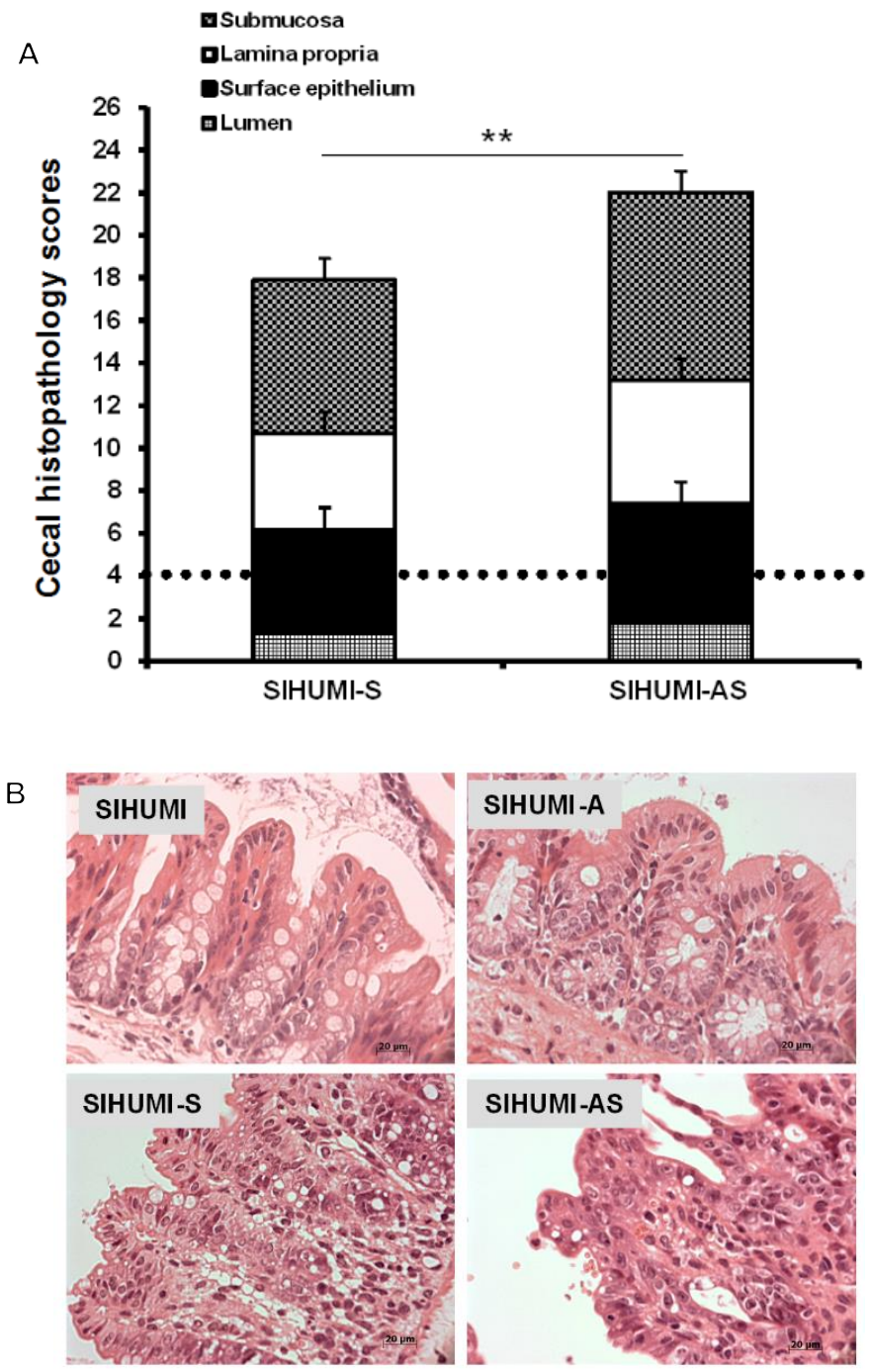

Figure 3. Concomitant presence of *A. muciniphila* and *S.* Typhimurium results in increased histopathology scores in SIHUMI mice. (A) Gnotobiotic C3H mice containing 8 defined microbial species (SIHUMI) were subsequently inoculated with *A. muciniphila* or *S.* Typhimurium or consecutively with both organisms (see Figure 1). SIHUMI and SIHUMI-A mice had the lowest histopathology scores (≤4.0) with no signs of inflammation and were therefore taken as baseline (dotted line). Data are expressed as median with range. **P<0.05, **P<0.01, ***P<0.001*. n = 10 mice per group. (B) Representative microscopy images of pathological changes observed in cecum tissue sections fixed with formalin and stained with hematoxylin and eosin (4 µm) of the four mouse groups. n = 10 mice per group; Magnification 1000-fold.

Essentially similar patterns were observed for IFN-γ-induced protein 10 (IP-10), tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-17. The pattern for IL-12 differed from that of the other cytokines investigated, with 1.5- and 2- fold higher IL-12 mRNA levels in the SIHUMI-A and SIHUMI-AS groups compared to the other two groups (**Figure 4A**). In spite of these minor differences, the presence of *A. muciniphila* in the *S.* Typhimurium-infected SIHUMI mice coincided with significantly higher mRNA expression levels of the pro-inflammatory cytokines except IL-18, which was significantly down-regulated (**Figure 4A**). The mRNA expression patterns of IFN-γ, IL-17, IL-6, TNF-α, IL-12 and IP-10 in colonic tissue were very similar to those observed in cecal tissue (**Figure 13**).

To check for systemic effects of infection, we also quantified the protein levels of pro-inflammatory cytokines in serum. Five days p.i. SIHUMI-AS mice had 1.5- to 3- fold higher serum levels of IFN-γ compared to SIHUMI-S mice, SIHUMI mice or SIHUMI-A mice (**Figure 4B**). However, TNF-α and IL-6 protein levels in serum were below the detection limit.

The increased intestinal inflammation in the SIHUMI-AS mice compared to the SIHUMI-S mice coincided with a predominance of *S.* Typhimurium cells in the SIHUMI-AS mice suggesting that *A. muciniphila* exacerbated the pathogen-induced inflammation. To investigate whether the increased inflammation was accompanied by an enhanced translocation of *S.* Typhimurium into host tissue, *S.* Typhimurium was enumerated in mesenteric lymph nodes (mLN) and spleen. Five days p.i. the cell number of *S.* Typhimurium in the mLN of SIHUMI-AS mice was 10-fold higher compared to that observed for SIHUMI-S mice (**Figure 5A, 5B**). However, *S.* Typhimurium was not detectable in the spleens of the mice infected with the pathogen.

Infection by *S.* Typhimurium involves its survival within host macrophages (**Monack *et al.,* 2004**) and promotes macrophage recruitment (**Nix *et al.,* 2007; Thiennimitr *et al.,* 2012**). To investigate whether the presence of *A. muciniphila* enhanced this process, we scored

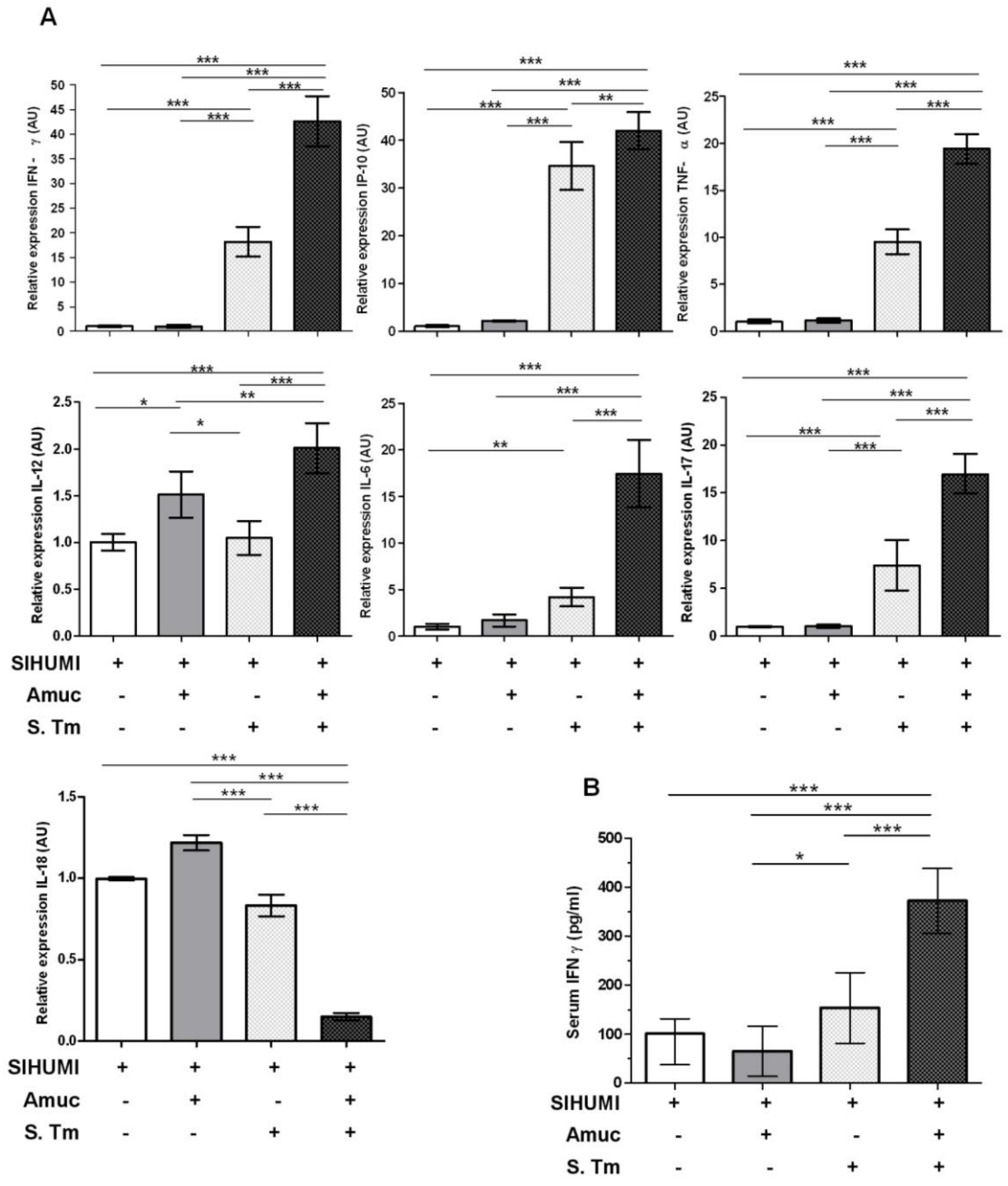

Figure 4. Presence of both *A. muciniphila* and *S.* Typhimurium is accompanied by increased pro-inflammatory cytokines. (A) Cecal mRNA levels of IFN-γ, IP-10, TNF-α, IL-12, IL-6, IL-17 and IL-18 in gnotobiotic SIHUMI mice were measured. mRNA was extracted from cecum mucosa of mice belonging to either one of four groups: SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS (see Figure. 1). The mRNA was converted to cDNA for quantitative real-time PCR measurement (see materials and methods). Inoculation of the gnotobiotic SIHUMI mice with *A. muciniphila* followed by *S.* Typhimurium infection (SIHUMI-AS) caused an increase in mRNA levels of pro-inflammatory cytokines except IL-18. Data are expressed as mean ± standard error. n=6 per group. Star indicates statistically significant differences (**P<0.05, **P<0.01, ***P<0.001)*. AU: Arbitrary units. (Amuc – *A. muciniphila;* S. Tm – *S.* Typhimurium). (B) Serum protein levels of IFN-γ were increased in SIHUMI-AS mice compared to the other mouse groups. Data are expressed as mean ± standard error. n = 10 mice per group. **P<0.05, **P<0.01, ***P<0.001*. (Amuc – *A. muciniphila;* S. Tm – *S.* Typhimurium).

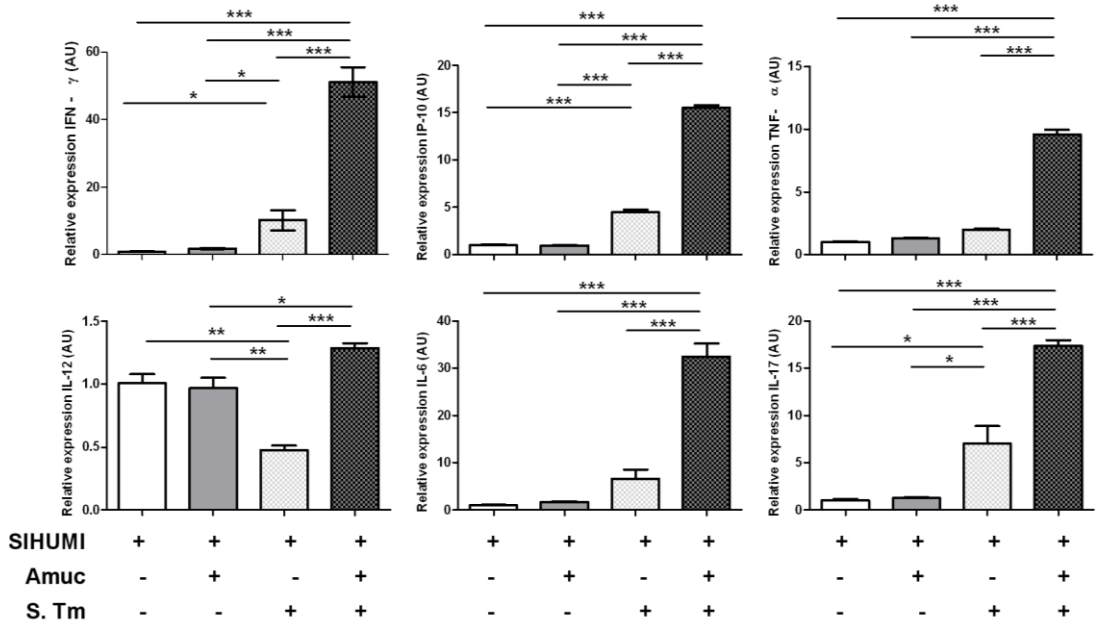

Figure 13. Presence of both *A. muciniphila* and *S.* Typhimurium is accompanied by increased colonic pro-inflammatory cytokine mRNA levels. Colonic mRNA levels of IFN-γ, IP-10, TNF-α, IL-12, IL-6 and IL-17 in gnotobiotic C3H mice were measured. mRNA was extracted from colon mucosa of mice belonging to either one of four groups: SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS (see Figure. 1). The mRNA was converted to cDNA for quantitative real-time PCR measurement (see materials and methods). Inoculation of the gnotobiotic SIHUMI mice with *A. muciniphila* followed by *S.* Typhimurium infection (SIHUMI-AS) caused an increase in mRNA levels of pro-inflammatory cytokines. Data are expressed as mean ± standard error. n=6 per group. Star indicates statistically significant differences (**P<0.05, **P<0.01, ***P<0.001)*. AU: Arbitrary units. (Amuc – *A. muciniphila;* S. Tm – *S.* Typhimurium).

	SIHUMI (DW) $\log_{10}(g^{-1})$	SIHUMI-A (DW) $\log_{10}(g^{-1})$	SIHUMI-S (DW) $\log_{10}(g^{-1})$	SIHUMI-AS (DW) $\log_{10}(g^{-1})$
A. caccae	8.6±0.6 ^{b}	8.9±0.3 ^{b}	9.2± 0.3 ^{b}	6.9±1.2 ^{a}
B. longum	9.0±0.2 ^{b}	9.4±0.2 ^{b}	9.1±0.5 ^{b}	7.4±1.4 ^{a}
B. producta	10.2±0.5 ^{b}	10.6±0.2 ^{b}	10.5±0.2 ^{b}	7.7±1.3 ^{a}
B. thetaiotaomicron	10.7±0.5 ^{b}	11.5±0.4 ^{b}	11.0±0.3 ^{b}	9.2±0.9 ^{a}
C. ramosum	9.0±0.3 ^{b}	8.9±0.5 ^{b}	9.7±0.3 ^{c}	8.0±1.1 ^{a}
E. coli	9.3±0.4 ^{b}	9.2±0.6 ^{b}	8.7±0.3 ^{b}	6.1±0.8 ^{a}
C. butyricum	9.5±0.3 ^{b}	9.5±0.3 ^{b}	9.3±0.4 ^{b}	8.2±1.4 ^{a}
A. muciniphila	n.d.	10.4±0.2 ^{b}	n.d.	8.1±0.6 ^{a}
S. Typhimurium	n.d.	n.d.	9.7±0.2 ^{a}	10.0±0.4 ^{a}
Total bacteria (HSP60)	10.6±0.7 ^{b}	11.1±0.8 ^{b}	10.7±0.6 ^{b}	9.3±0.7 ^{a}

Table 3. *S.* Typhimurium becomes the dominant species in colon of SIHUMI mice when previously associated with *A. muciniphila*. Data are expressed as mean ± standard error. Different superscripts indicate statistically significant differences (*P≤ 0.05).* n = 10 mice per group. DW – dry weight

macrophage infiltration in cecal tissue by immunohistochemical detection of the F4/80 receptor present on mouse macrophages (**Kallis *et al.*, 2010**). The degree of macrophage infiltration into cecal lamina propria and submucosa was evaluated by a score ranging from 0 to 3 (as defined in the methods section). SIHUMI-AS mice displayed a significantly higher infiltration score for both lamina propria and submucosa than the SIHUMI-S, SIHUMI-A or SIHUMI mice (**Figure 6A, 6B**). In addition, FISH analysis revealed that *A. muciniphila* was in close contact with the cecal epithelium (**Figure 9A**) in SIHUMI-A mice. In SIHUMI-S mice *S.* Typhimurium was also detected mostly on the epithelial surface whereas in SIHUMI-AS mice *S.* Typhimurium was detected deep inside the cecal tissues (**Figure 9B, 9C**).

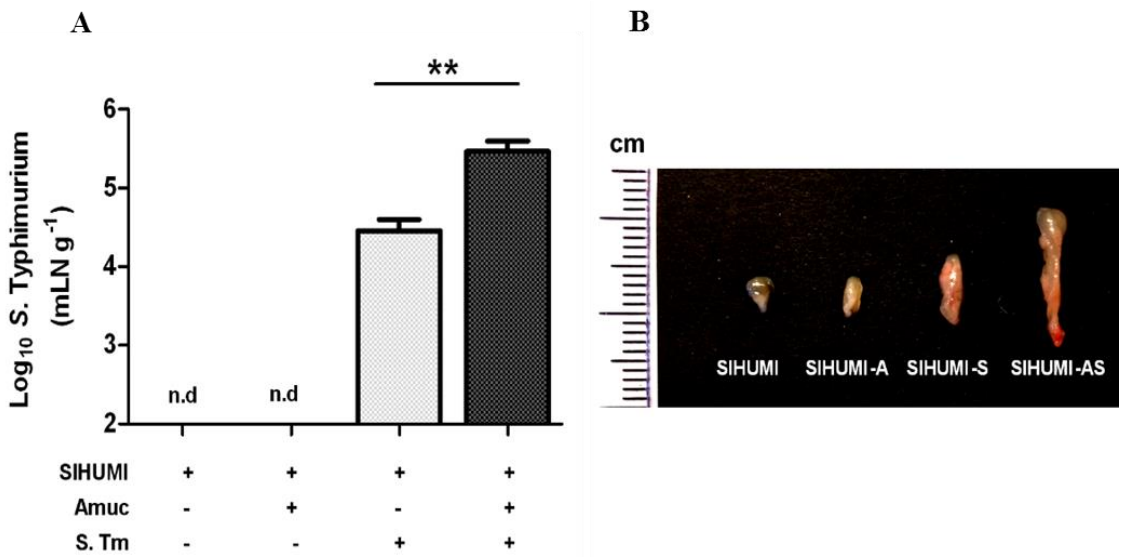

Figure 5. SIHUMI mice colonized with both *A. muciniphila* and *S.* Typhimurium display enlarged mLN and elevated *S.* Typhimurium cell numbers. (A) Mesenteric lymph nodes (mLN) were obtained from four groups of gnotobiotic C3H mice. SIHUMI mice were subsequently inoculated with *A. muciniphila* or *S.* Typhimurium or consecutively with both organisms (see Figure 1). The mLN tissue was homogenized and DNA was isolated to quantify *S.* Typhimurium using quantitative PCR with primers targeting the ttr-region of *S.* Typhimurium. Absolute cell numbers were calculated based on calibration curves with known concentrations of *S.* Typhimurium. The mLN of SIHUMI-AS mice contained 10-fold higher cell numbers of *S.* Typhimurium compared to SIHUMI-S mice. Data are expressed as mean ± standard error. n = 10 mice per group. **P<0.05, **P<0.01, ***P<0.001*. n.d: not detected. (Amuc – *A. muciniphila;* S. Tm – *S.* Typhimurium). (B) The photograph shows four lymph nodes, each representative of one of the four mouse groups and a cm scale. Twelve week old gnotobiotic SIHUMI mice with both *A. muciniphila* and *S.* Typhimurium displayed an increased size of their mesenteric lymph nodes compared to SIHUMI mice infected with *S.* Typhimurium only.

5.3.3 Presence of *A. muciniphila* in *S.* Typhimurium-infected SIHUMI mice facilitates pathogen translocation by interfering with mucus formation

Since *A. muciniphila* is capable of degrading mucins, we hypothesized that this organism modified the mucus layer, which in turn enhanced exposure of the mucosa to *S.* Typhimurium, resulting in enhanced translocation of the pathogen. Stronger inflammatory and infectious symptoms in SIHUMI-AS mice compared to SIHUMI-S mice were characterized by increased cell numbers of *S.* Typhimurium in mLN (**Figure 5A**), suggesting that the presence of *A. muciniphila* facilitated the translocation of the pathogen from the intestinal lumen into host tissue. We therefore investigated how the presence of *A. muciniphila* affected mucin formation, mucus thickness, mucus composition and number of mucin-filled goblet cells. Therefore, mRNA expression levels of cecal MUC2 were determined and cecum tissue sections were stained with alcian blue (AB) for quantification of goblet cells filled with acidic mucin. MUC2 gene expression was twofold higher in mice associated with *A. muciniphila* (SIHUMI-AS and SIHUMI-A mice) compared to SIHUMI-S mice or SIHUMI mice. MUC2 gene expression in the latter two groups was not significantly different (**Figure 7A**).

Higher MUC2 gene expression in the SIHUMI-AS mice suggested that these mice produced more mucin than the mice of the other groups. This was evaluated by staining of cecum tissue sections with AB. Microscopic examination of thin sections from cecum tissue collected 5 days p.i. revealed striking differences in the number of acidic mucin-containing goblet cells between the groups (**Figure 7B**). In spite of showing the highest MUC2 gene expression, SIHUMI-AS mice displayed significantly lower numbers of mucin-filled goblet cells than SIHUMI-S mice or SIHUMI mice. Moreover, the cecal mucosa from SIHUMI-A mice showed the highest number of mucin-filled goblet cells compared to the mice from the other three groups (**Figure 7B&C, 11**). Essentially the same results were obtained for colonic

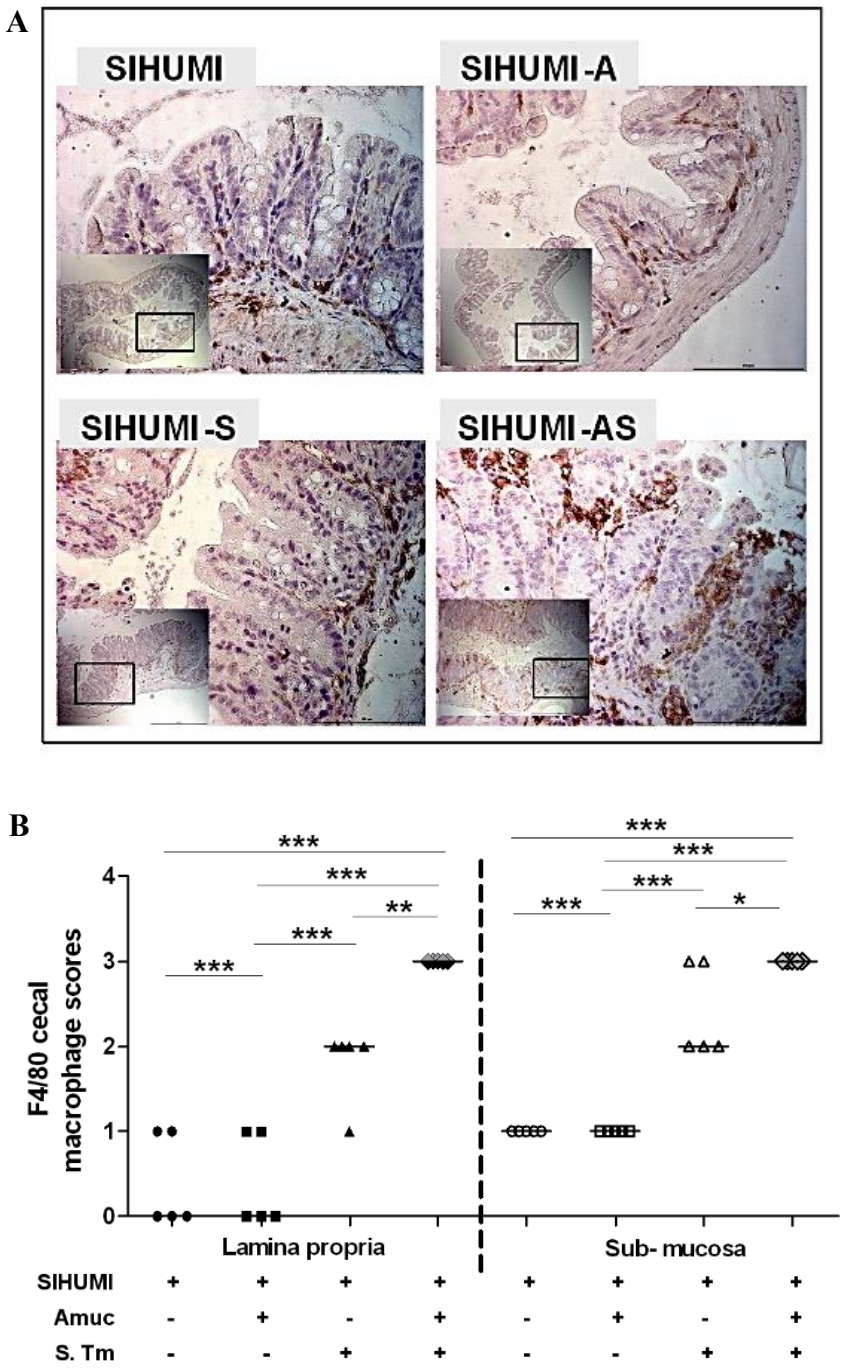

Figure 6. SIHUMI mice colonized with both *A. muciniphila* and *S.* Typhimurium display an increased cecal macrophage infiltration. (A) Formalin fixed paraffin embedded cecum tissue was thin sectioned at 2 µm. Macrophages were stained by targeting the F4/80 receptor expressed on mouse macrophages using immunohistochemistry with specific antibodies. Brown color indicates positively stained macrophages. Magnification 400-fold. Bar = 100 µm. (B) Positively stained macrophages were enumerated along a stretch of 50 µm of lamina muscularis for both lamina propria and sub-mucosa (see materials and methods). SIHUMI mice colonized with both *A. muciniphila* and *S.* Typhimurium had the highest macrophage infiltration scores compared to the other groups (see Figure. 1). Data are expressed as median with range. n = 5 mice per group. **P<0.05, **P<0.01, ***P<0.001*. (Amuc – *A. muciniphila;* S. Tm – *S.* Typhimurium).

tissue where we observed the highest number of mucin-filled goblet cells in the SIHUMI-A group and lowest in the SIHUMI-AS group compared to the other groups (**Figure 14A**). The cytokine patterns observed in cecum (**Figure 4A**) and colon (**Figure 13**) mucosa were very similar. Since only colonic tissue had been fixed with Carnoy's we used colonic tissue sections for investigating the impact of *A. muciniphila* on mucus layer thickness (µm): SIHUMI-A mice (22.3 ± 3.8) had the thickest mucus layer compared to SIHUMI (9.8 ± 1.1), SIHUMI-S (10.7 ± 0.8) and SIHUMI-AS mice (7.5 ± 0.9), whereas SIHUMI-AS mice showed 2-fold reduced mucus thickness compared to SIHUMI-A mice (**Figure 14B**). In addition, thin sections from cecum tissue stained with high iron diamine (HID)/ AB revealed a reduction in sulphated mucins in SIHUMI-AS mice compared to the other mouse groups (**Figure 8**). We therefore quantified NANA in cecal and colonic mucosa of the different mouse groups. NANA concentrations in cecum mucosa were 2- to 2.5- fold higher in SIHUMI-AS mice compared to SIHUMI or SIHUMI-S mice. However, SIHUMI-A mice showed the highest NANA concentration compared to all other mouse groups (**Table 2**). The NANA concentration in colonic mucosa reveals similar differences between the groups as observed for cecum mucosa. It may be concluded that the presence of *A. muciniphila* results in an increased concentration of sialylated mucins, which however was partially reduced when *S.* Typhimurium was simultaneously present.

5.4 Discussion

5.4.1 A commensal intestinal bacterium may turn into a pathobiont and contribute to an aggravation of disease symptoms

The majority of bacteria in the gastrointestinal tract are considered commensals, i.e. they do not harm the host. Our data show that the commensal *A. muciniphila* exacerbates *S.* Typhimurium-induced intestinal inflammation. However, this detrimental effect on the host

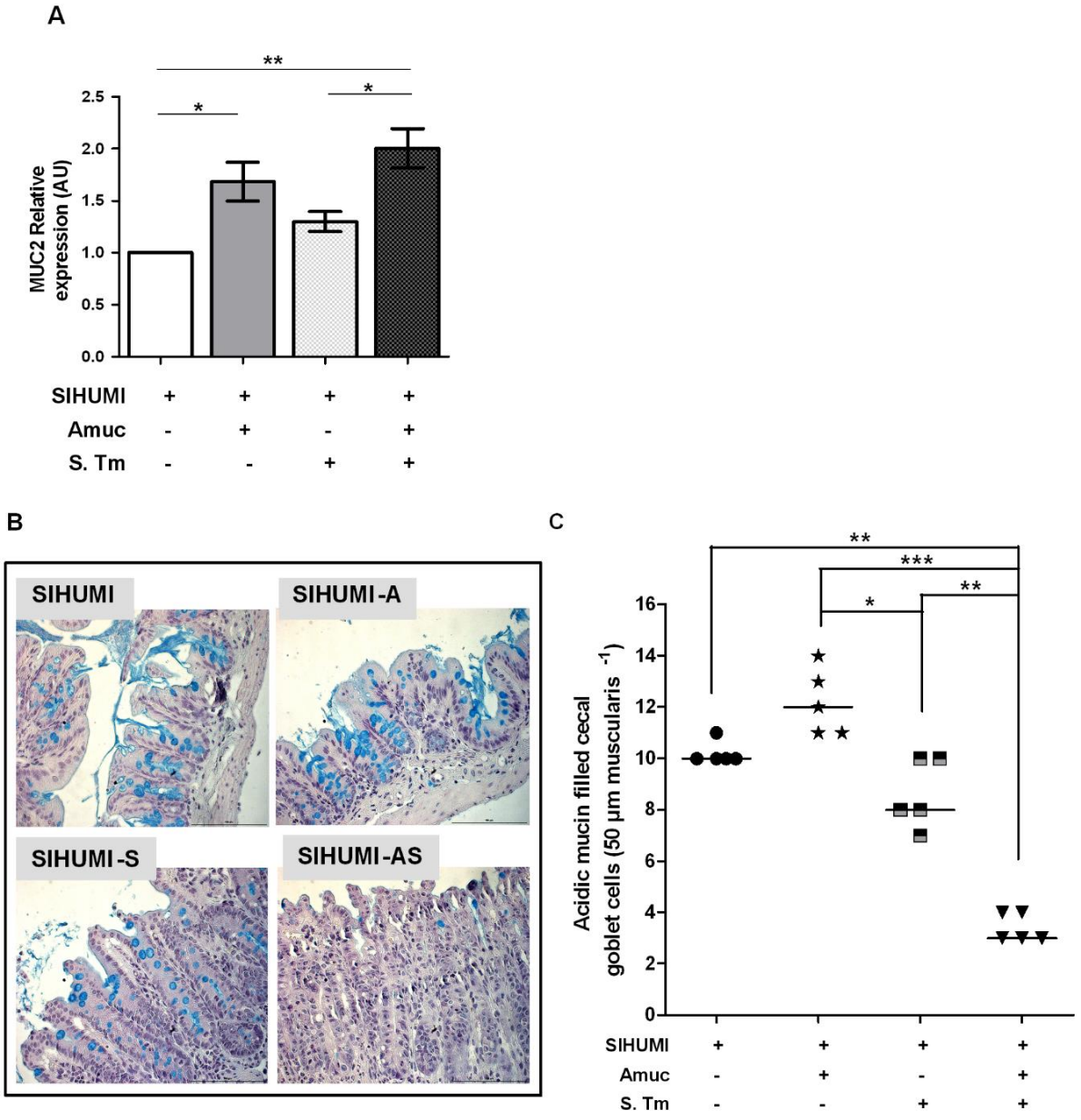

Figure 7. SIHUMI mice with both *A. muciniphila* and *S.* Typhimurium display increased MUC2 mRNA levels (A) and reduced numbers of mucin filled goblet cells (B and C). (A) mRNA was extracted from cecum mucosa of mice belonging to either one of four groups: SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS. MUC2 mRNA from cecum mucosa was converted to cDNA and expression levels were quantified using real-time PCR (see materials and methods). SIHUMI-A and SIHUMI-AS mice showed significantly higher MUC2 gene expression compared to the other two groups, harboring no *A. muciniphila*. Data are expressed as mean ± standard error. n = 6 per group. **P<0.05, **P<0.01, ***P<0.001.* (Amuc – *A. muciniphila;* S. Tm – *S.* Typhimurium). (B) Formalin fixed cecal tissue sections from SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS mice were stained with alcian blue (pH-2.5) and haematoxylin. Images are representative of 5 mice per group. Magnification 400-fold. SIHUMI-AS mice display the lowest number of positively stained mucin-filled goblet cells compared to the other three groups. The bar represents 100 µm. (C) Quantitative analysis of the number of acidic mucin-filled goblet cells (blue) enumerated in cecal tissue sections

from SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS mice for a 50 µm stretch of lamina muscularis corresponding to approximately 30 cecal crypts per section. Two sections per mouse were analyzed. The number of cecal mucin filled goblet cells was elevated when *A. muciniphila* was present (SIHUMI-A) but the concomitant presence of *S.* Typhimurium (SIHUMI-AS) resulted in the lowest number of mucin filled goblet cells of gnotobiotic SIHUMI mice compared to the other mouse groups. Data are expressed as mean ± standard error. n = 5 mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$. (Amuc – *A. muciniphila;* S. Tm – *S.* Typhimurium).

can only arise under certain circumstances, in this case in the presence of both a commensal mucin degrader and a pathogen. In our study, *S.* Typhimurium-triggered inflammation turned *A. muciniphila* into a pathobiont (a resident intestinal bacterium that under certain circumstances causes disease) (**Ayres *et al.*, 2012; Chow & Mazmanian, 2010**). The experiments presented here indicate that the concomitant presence of these two organisms in SIHUMI mice disturbs mucus layer homeostasis, which in turn aggravates infectious and inflammatory symptoms. The molecular interactions between a mucin-degrading commensal bacterium and a pathogenic bacterium on host health have not yet been studied and are not well understood.

The current study was triggered by previous observations in conventional IL-$10^{-/-}$ mice treated with a probiotic *E. faecium* strain (**Ganesh *et al.*, 2012**). In these mice, a significant increase in pro-inflammatory cytokine expression levels was associated with an increase in cell numbers of *A. muciniphila* (**Ganesh *et al.*, 2012**). The results presented herein are in accordance with these observations because the latter organism also affected inflammatory parameters in our present study. For example, mRNA expression levels of IFN-γ, IP-10, TNF-α, IL-6, IL-12 and IL-17 were increased in SIHUMI-AS compared to SIHUMI-S mice (**Figure 4A, 4B**). It may be concluded that *S.* Typhimurium alone leads to a considerably weaker gut inflammation as compared to when *A. muciniphila* is also present.

IL-12 and IL-18 have been described to increase in response to a *S.* Typhimurium infection and in turn to induce the production of IFN-γ which enhances the ability of macrophages to kill intracellular pathogens (**Thiennimitr *et al.*, 2012; Berclaz *et al.*, 2002**).

Interestingly, in our experiment, we only observed an up-regulation of IL-12 in the SIHUMI-AS mice, while IL-18 was significantly down-regulated in these mice compared to the other groups. IL-12 formation by infected macrophages is an important defense against *Salmonella* because it leads to the recruitment of Natural Killer (NK) cells to the infected site, a higher production of IFN-γ, and in turn an enhanced differentiation of monocytes to macrophages (**Lapaque *et al.*, 2009; Car *et al.*, 1995; Munder *et al.*, 1998**). In line with these studies, we

	SIHUMI	SIHUMI-A	SIHUMI-S	SIHUMI-AS
NANA (nmoles /g)				
Cecum mucosa	**546.6 ± 266.2** ^{ab}	**2371.7 ± 834.3** ^{bc}	**333.1 ± 125.3** ^{a}	**1661.1 ± 568.7** ^{b}
Colon mucosa	**750.5 ± 139.6** ^{a}	**1245.0 ± 81.6** ^{b}	**820.6 ± 129.6** ^{a}	**1177.5 ± 50.9** ^{b}
Mucus thickness(µm) (supplementary materials & methods)				
Colon (/crypt)	**9.4 ± 1.1** ^{ab}	**22.3 ± 3.8** ^{c}	**10.7 ± 0.8** ^{b}	**8.1 ± 0.9** ^{a}

Table 2. Colonization of C3H SIHUMI mice with *A. muciniphila* shows an increase in N-acetyl neuraminic acid (NANA) irrespective of the infection. NANA concentration quantified in both cecum and colon mucosa of the SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS mice. Data were represented as mean ± SEM. Different superscripts indicate statistically significant differences of $P \leq 0.05$. n=5

observed significantly higher numbers of cecal macrophages accompanied by higher cecal and colonic mRNA levels of IL-12 and IFN-γ in SIHUMI-AS compared to SIHUMI-S mice. *S.* Typhimurium survives and grows inside macrophages from where the pathogen invades host tissues (**Van der Velden *et al.*, 2000**). In accordance with the elevated IL-12 and IFN-γ mRNA levels *S.* Typhimurium cell numbers were 10 fold higher in mLN of SIHUMI-AS compared to SIHUMI-S mice. IL-18 in conjunction with IL-12 is involved in phagocytosis of intracellular pathogens (**Berclaz *et al.*, 2002**). The observed suppression of IL-18 in SIHUMI-AS compared to SIHUMI-S mice, led us to speculate that the concomitant presence of *A.*

muciniphila and *S.* Typhimurium facilitates growth of the pathogen in the infected macrophages because down-regulation of IL-18 protects *S.* Typhimurium from being killed.

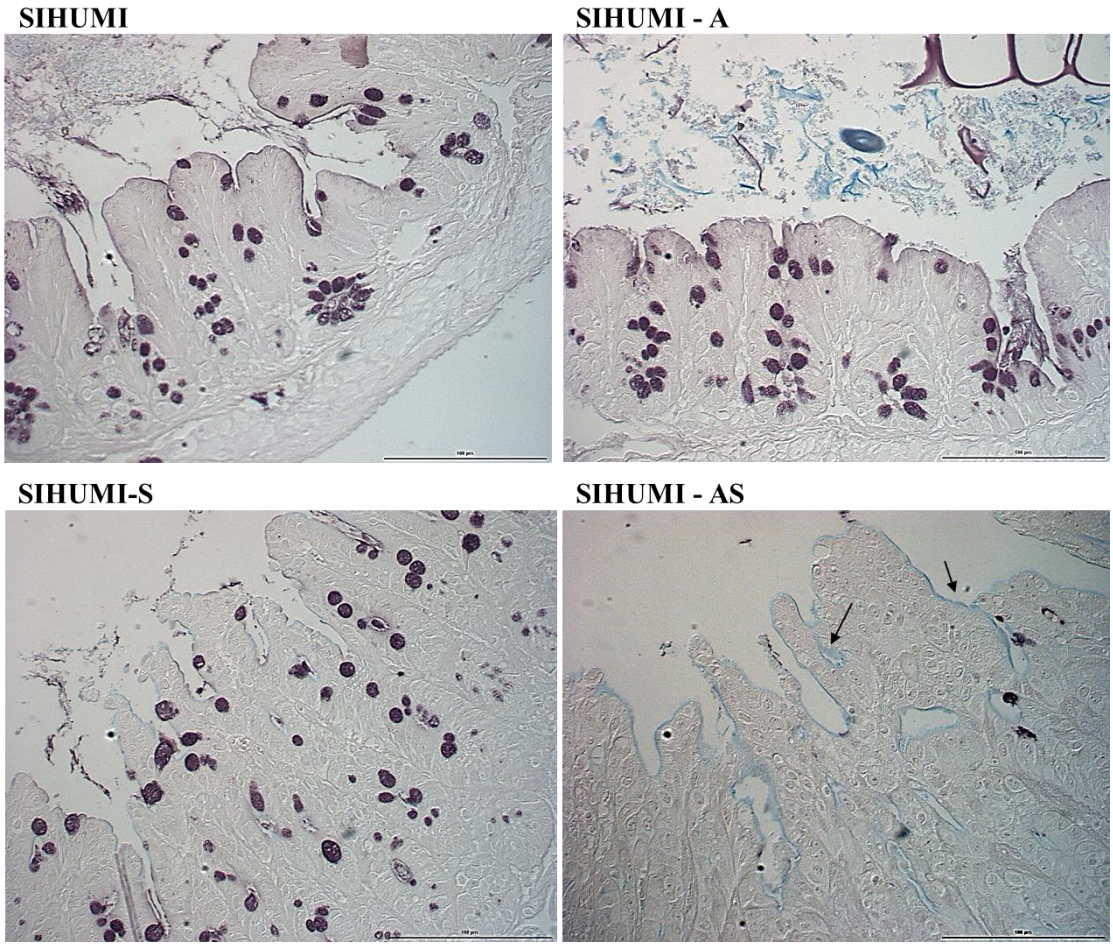

Figure 8. SIHUMI mice colonized with both *A. muciniphila* and *S.* Typhimurium display reduced mucus sulphation. Formalin fixed thin sections (4 µm) of cecal tissue of mice belonging to either one of four groups: SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS (see Figure. 1) were stained with high iron diamine (HID) / AB at pH-2.5 and subsequently analyzed. Brown color indicates sulphated mucins while blue color indicates sialylated mucins. SIHUMI-AS mice display few sulphated mucins compared to the other mouse groups. Magnification 400 x. Bars indicate 100 µm.

In addition, SIHUMI-AS mice showed significantly higher cecal histopathology scores compared to SIHUMI-S infected mice. Contrary to cecum, the colon displayed 5 days p.i. no elevated histopathology scores in SIHUMI-AS mice and SIHUMI-S mice. This may be explained by the fact that it takes several days for the inflammation to spread from cecum to

colon (**Kaiser *et al.,* 2012**). Previously known that the presence of junctional claudin-2 causes the formation of cation-selective channels sufficient to transform a 'tight' tight junction into a leaky one (**Amasheh *et al.,* 2002**). The measurement of claudin-2 mRNA levels in intestinal mucosa revealed no changes in its expression in SIHUMI-AS compared to SIHUMI-A and SIHUMI mice (**Figure 15**). However, SIHUMI-S mice showed the highest mRNA expression levels of claudin-2.

5.4.2 *A. muciniphila's* ability to disturb host mucus-homeostasis appears crucial for its ability to exacerbate infectious and inflammatory symptoms caused by *S.* Typhimurium

Commensal bacterium *A. muciniphila* is known for its ability to degrade mucins (**Derrien *et al.,* 2004; Derrien *et al.,* 2008**). Recent studies propose that excessive mucin degradation facilitates the access of pathogen to the mucosa (**Wiggins *et al.,* 2001; Linden *et al.,* 2008**). The experiments presented in this paper support the view that the presence of the mucin-degrading *A. muciniphila* causes an aggravation of intestinal inflammatory symptoms caused by *S.* Typhimurium infection. Using a consortium of eight bacterial species (**Becker *et al.,* 2011**) as a background microbiota we observed that the concomitant presence of *A. muciniphila* and *S.* Typhimurium resulted in mucus-related differences that were absent or less pronounced if either one of the strains was present. For example, the number of mucin-filled goblet cells in SIHUMI-AS mice was 2.5- to 4- fold lower than in any other of the mouse groups (**Figure 7B, 7C**). Paradoxically, the MUC2 gene expression level in the SIHUMI-AS mice was higher than that in the SIHUMI-S or the SIHUMI mice (**Figure 7A**). Why higher mRNA levels of MUC2 in mucosal tissue did not coincide with higher numbers of mucin-filled goblet cells is not quite clear. Two explanations are conceivable: 1. Previous studies indicate that severe inflammation causes endoplasmic reticulum (ER) stress in intestinal epithelial cells and in goblet cells (**Varki & Varki A, 2007; Shkoda *et al.,* 2007; Bogaert *et***

***al.,* 2011; Heazlewood *et al.,* 2008; Söderholm & Perdue, 2001**). For example, a ribotoxic stress response caused apoptosis of intestinal epithelial cells triggered by Shiga toxin-producing *E. coli* (**Smith *et al.,* 2003**) and of goblet cells (**McGuckin *et al.,* 2011; Kaser & Blumberg, 2010**). Such stress acting on goblet cells might result in increased expression of the MUC2 gene to compensate for the loss of mucin-filled goblet cells. However, owing to cellular stress, decoration of the mucin polypeptide backbone with carbohydrates would remain fragmentary. Since AB does not stain the mucin polypeptide backbone, undecorated mucin would therefore not be detectable with AB in goblet cells. Therefore MUC2 antibody was used to identify the protein expression of MUC2 backbone which also showed reduced MUC2 protein expression in SIHUMI-AS mice (**Figure 11**). 2. Previous findings demonstrated that infection with *S.* Typhimurium enhances mucin excretion from goblet cells by increased expression of IFN-γ (**Songhet *et al.,* 2011**). Therefore, the 2.5- fold higher IFN-γ expression level in SIHUMI-AS mice relative to SIHUMI-S mice may have led to an emptying of goblet cells to restrict the load of pathogens in the host. In support of this assumption we observed a significant inverse correlation (r^2= -0.86, *P<0.001*) between the number of mucin filled goblet cells and IFN-γ gene expression levels (data not shown).

Mucus is constantly secreted into the intestine, where it forms a protective gel-like structure of approximately 150 μm thicknesses on the mucosal surface. Cecum and colon mucosa is covered with a tightly packed inner mucus layer and a less dense outer layer. The inner layer serves as a barrier that prevents bacterial access to the epithelium (**Johansson *et al.,* 2011; Johansson & Hansson, 2010**). Even though the inner mucus layer is usually devoid of bacteria, we detected *A. muciniphila* in close contact with the cecal epithelial surface in the SIHUMI-A mice (**Figure 9A**). We speculate that *A. muciniphila* promotes mucin formation and thereby supports its own growth via mucin degradation similar to what has been observed for *B. thetaiotaomicron* in NMRI mice; utilization of fucose by this organism triggered the synthesis of fucosylated glycoconjugates by the host epithelium (**Falk *et al.,* 1998**).

The reduced brown color observed after HID/ AB (pH-2.5) staining in cecal tissue sections of SIHUMI-AS mice compared to those of the other mouse groups indicate a loss of mucin sulphation in this group (**Figure 8**) together with an increase in NANA concentration of cecal mucosa (**Table 2**). Interestingly, changes in intestinal mucin composition characterized by a lower degree of sulphation (**Corfield & Myerscough, 2000; Raouf *et al.,* 1992**) and a higher degree of sialylation have previously been reported to occur in UC, Crohn's Disease (CD) and gastric ulcer caused by *Helicobacter pylori*-induced inflammation (**Corfield & Myerscough, 2000; Raouf *et al.,* 1992; Mahdavi *et al.,* 2010**). These changes might facilitate access of intraluminal antigens and thereby possibly aggravate inflammatory symptoms in the host intestine.

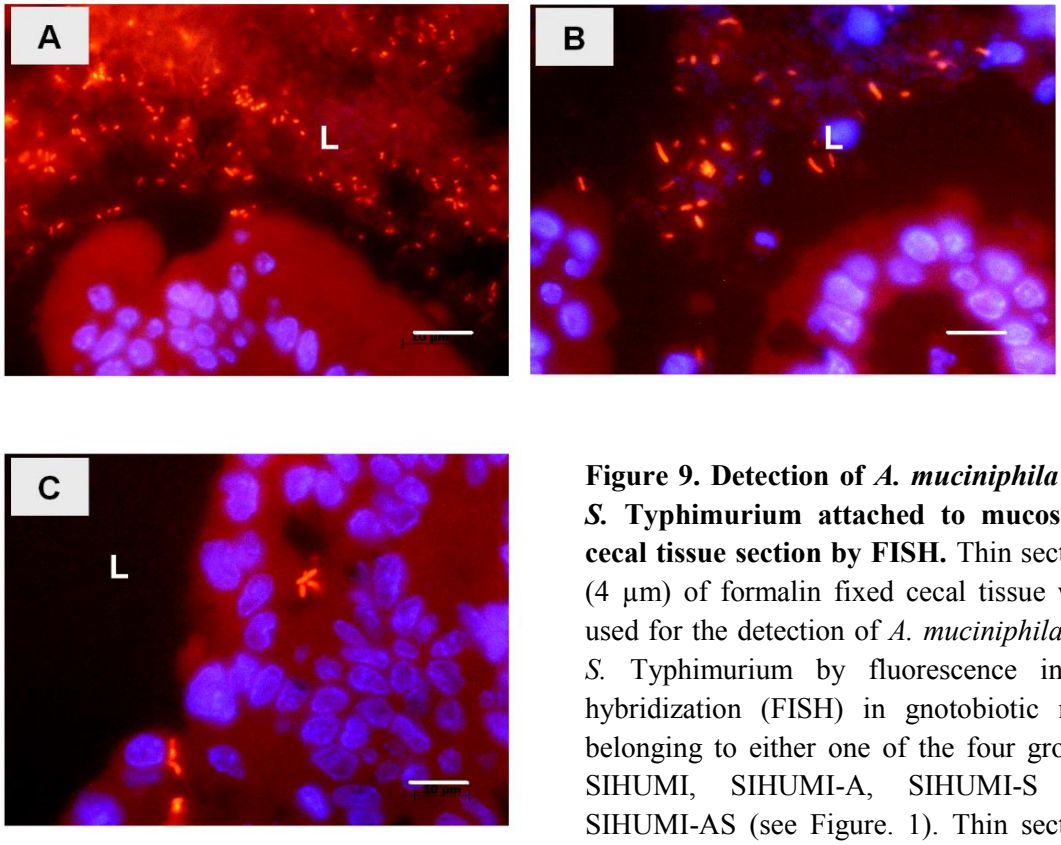

Figure 9. Detection of *A. muciniphila* and *S.* Typhimurium attached to mucosa in cecal tissue section by FISH. Thin sections (4 µm) of formalin fixed cecal tissue were used for the detection of *A. muciniphila* and *S.* Typhimurium by fluorescence in-situ hybridization (FISH) in gnotobiotic mice belonging to either one of the four groups: SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS (see Figure. 1). Thin sections were hybridized with Cy3 labeled oligonucleotide probes (see supplementary materials and methods) targeting *A. muciniphila* (S-S-MUC-1437-a-A-20) *at* 55 °C and *S.* Typhimurium (L-S-Sal-1713-a-A-18) at 45 °C. DNA was counterstained with 4',6-diamidino-2-phenylindole (DAPI). (A) *A. muciniphila* is in close contact to the epithelial surface in SIHUMI-A mice. (B) *S.* Typhimurium cells are found mostly on the epithelial cell surface of SIHUMI-S mice. (C) *S.* Typhimurium is in cecal tissue of SIHUMI-AS mice. Magnification 1000 x. The scales represent 20 µm. L: lumen.

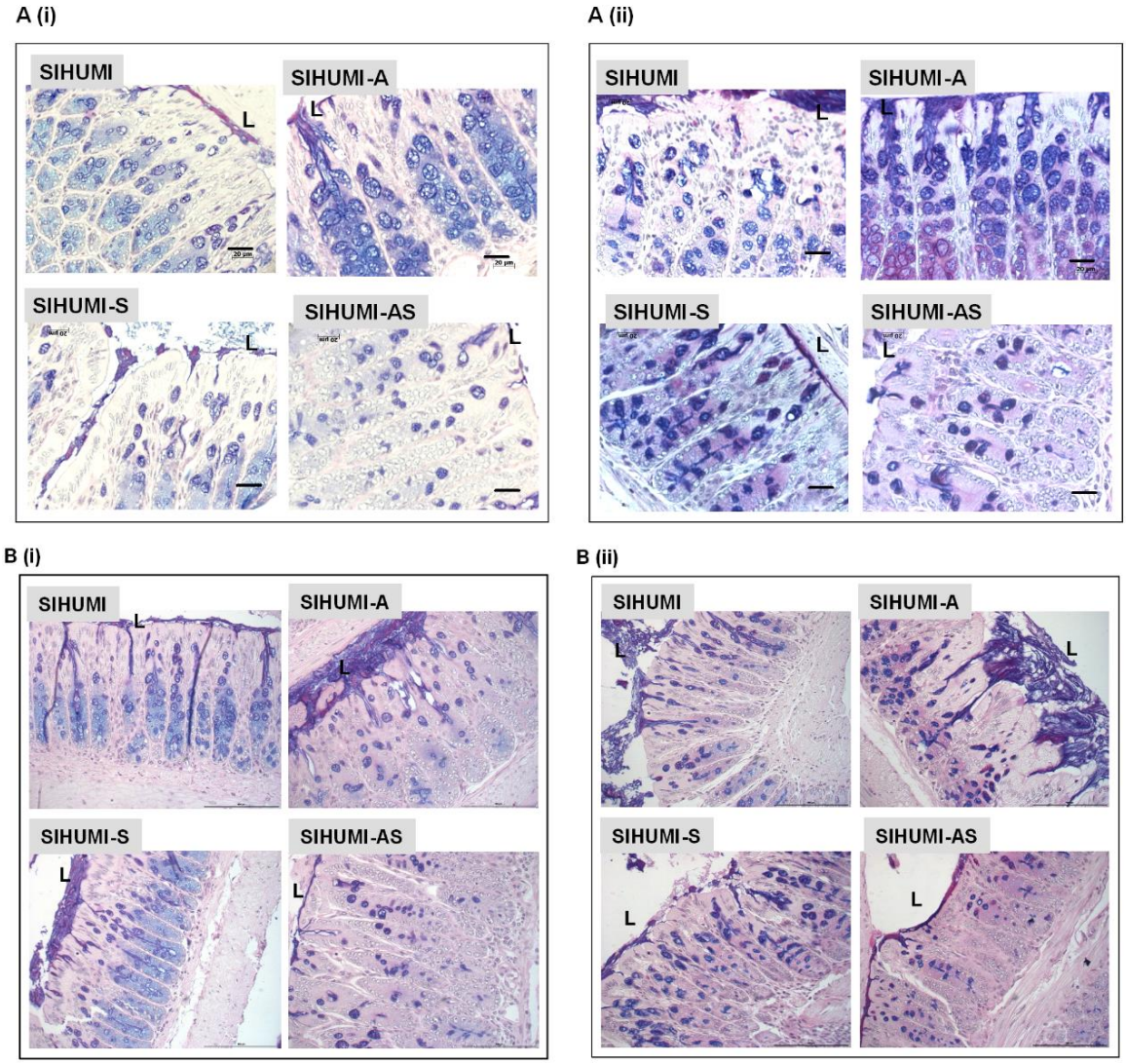

Figure 14. Presence of both *A. muciniphila* and *S.* Typhimurium caused reduction in number of mucin filled goblet cells in colon of SIHUMI mice. Carnoy's-fixed cecal tissue sections (4 µm) from SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS (see Figure. 1) mice were stained with periodic acid Schiff/Alcian blue (PAS/AB) at both pH-2.5 and pH-1. Images are representative of 5 mice per group. (A-i) All acidic mucins are stained blue with AB at pH-2.5 whereas all neutral mucins are stained magenta with PAS; (A-ii) highly sulphated mucins are stained blue with AB at pH-1. All the images from (A-i & A-ii) are obtained with a magnification of 1000-fold. Bars indicate 20 µm. (B-i) colonic tissues stained with PAS/AB at pH-2.5; (B-ii) colonic tissues stained with PAS/AB at pH-1 obtained with a magnification of 400-fold. Bars indicate 100 µm. SIHUMI-AS mice display the lowest number of positively stained colonic mucin-filled goblet cells compared to the other three groups at any given pH. L: lumen.

5.4.3 Presence of both *A. muciniphila* and *S.* Typhimurium is associated with drastic changes in microbiota composition

The above findings indicate that the presence of *A. muciniphila* within the SIHUMI-AS consortium is responsible for the exacerbation of inflammation observed in the corresponding mice. One of the most prominent differences between *S.* Typhimurium-infected SIHUMI mice with or without *A. muciniphila* relates to drastic differences in microbiota composition. The data indicate that *A. muciniphila* promotes the growth of *S.* Typhimurium, which reaches a proportion of 94 % in the presence of *A. muciniphila* compared to 2.2 % in its absence, while other community members including *B. thetaiotaomicron* and *E. coli* decreased dramatically from 88 % to 0.02 % and 0.14 % to 0.01 %, respectively (**Figure 2**). The reasons for this dramatic change are not really known. It may be speculated that this phenomenon is related to *A. muciniphila*'s ability to exacerbate *S.* Typhimurium-induced inflammation (**Figure 2D**) whereas the presence of *A. muciniphila* is without consequence when *S.* Typhimurium is absent. Presence of either *A. muciniphila* or *S.* Typhimurium alone did not lead to such a dramatic shift in the existing microbiota composition as evident from a comparison of SIHUMI, SIHUMI-A and SIHUMI-S mice, which showed no major differences in the relative proportions of the SIHUMI community members between these mouse groups (**Figure 2A, 2B, 2C**). Enteropathogenic bacteria such as *S.* Typhimurium are known to breach colonization resistance and to invade host tissues by exploiting host inflammation (**Stecher *et al.*, 2007; Endt *et al.*, 2010; Loetscher *et al.*, 2012**). Higher numbers of *S.* Typhimurium in mesenteric lymph nodes of the SIHUMI-AS mice compared to SIHUMI-S mice suggest that A. *muciniphila* contributes to an impairment of colonization resistance and enhances intestinal inflammation. In fact, we observed higher mRNA levels of pro-inflammatory markers in SIHUMI-AS compared to SIHUMI-S mice (**Figure 4A**).

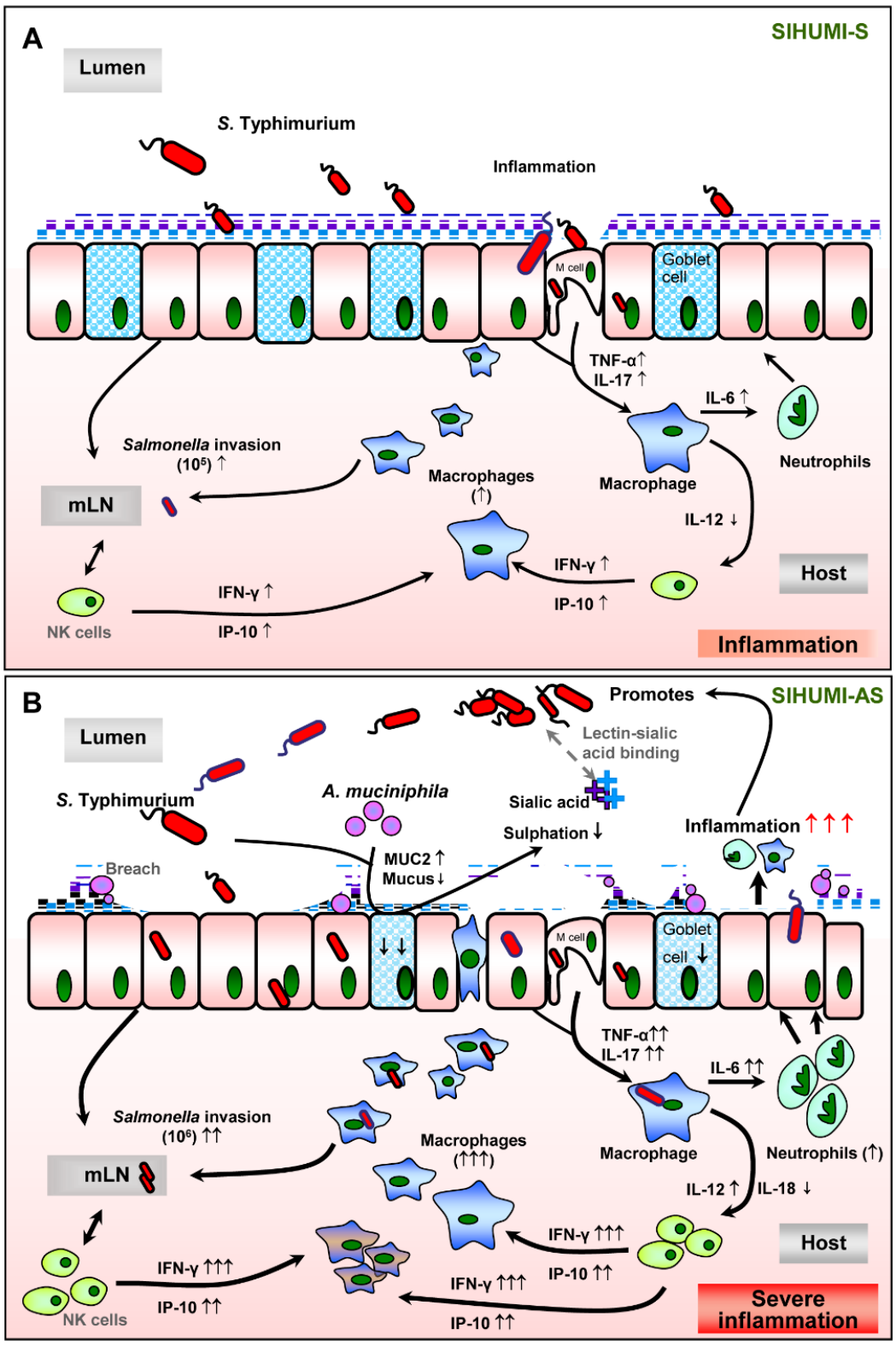

Figure 10. Hypothetical Scheme. The presence of *A. muciniphila,* leads to the exacerbation of *S.* Typhimurium-induced intestinal inflammation. We propose that the presence of *A. muciniphila* causes changes in the mucin composition and production, which in turn facilitates the invasion of *S.* Typhimurium into the host. Increased inflammatory status was characterized by increased pro-inflammatory cytokines, increased macrophage infiltration and invasion of the pathogen into the

lymph nodes, reduced number of mucin-filled goblet cells in SIHUMI-AS mice (B) compared to SIHUMI-S mice (A). Our data suggests that in the presence of both *A. muciniphila* and *S.* Typhimurium, mucus sulphation is diminished and this may facilitate the access of *S.* Typhimurium to sialic acid in mucus. Sialic acid may serve as a substrate and adhesion site for *S.* Typhimurium in the gut (**Giannasca *et al.,* 1996, Severi *et al.,* 2007**). Increased gene expression of IFN-γ and IP-10 indicate an increase in NK-cells. mLN - mesenteric lymph nodes, NK- Natural killer cells. (↑- increased; ↓- decreased; grey dotted line – assumed processes: lectin-sialic acid binding (**Giannasca *et al.,* 1996**), M-cell for pathogen transit (**Foster & Macpherson. 2010; Clark *et al.,* 1996**); black line – supported by data of the present study).

We propose that the enhanced inflammatory host response in the SIHUMI-AS mice was responsible for the dramatic decrease in the *B. thetaiotaomicron* population. We speculate that the decimation of *B. thetaiotaomicron* might be due to the generation of higher concentrations of reactive oxygen and nitrogen species (**Thiennimitr *et al.,* 2012; Winter *et al.,* 2013**) in the more severely inflamed SIHUMI-AS mice compared to SIHUMI-S mice. The reduction of *E. coli* numbers in the SIHUMI-AS versus the SIHUMI-S mice was moderate compared to that of *B. thetaiotaomicron* and is in contradiction to previous studies where an increase *in E. coli* was observed in conventional mice in response to a *S.* Typhimurium-induced gut inflammation (**Stecher *et al.,* 2007; Winter *et al.,* 2013**) or in inflamed IL-10$^{-/-}$ mice (**Wohlgemuth *et al.,* 2009**). One possible explanation for reduced *E. coli* cell numbers in the SIHUMI-AS mice could be due to the fact that we used a non-pathogenic laboratory strain of *E. coli* which lacks fitness genes (**Moulin-Schouleur *et al.,* 2006**) and might therefore be more susceptible to inflammatory conditions.

Composition of the microbiota of IBD patients significantly differs from that in healthy controls (**Kleessen *et al.,* 2002**). Currently, an imbalance in gut microbiota is regarded as one possible factor triggering the inflammation in UC and CD (**Sartor, 2009; Campieri & Gionchetti, 2001; Sartor, 2006**). Our data suggest that the presence of a dedicated mucin-degrading bacterium supports a pathogen-induced inflammation, which in turn leads to alterations in the existing gut microbiota composition.

A possible limitation of our mouse model lies in the use of a simplified human intestinal microbiota, which does not completely reflect the features of a conventional microbiota. Therefore, we cannot directly extrapolate the observed effects from the SIHUMI-AS mice to conventional mice. However, in spite of these limitations this model offers the chance to identify the molecular mechanisms underlying the interactions between a pathogen, a commensal microbiota and the host because each SIHUMI member is known and can be tracked.

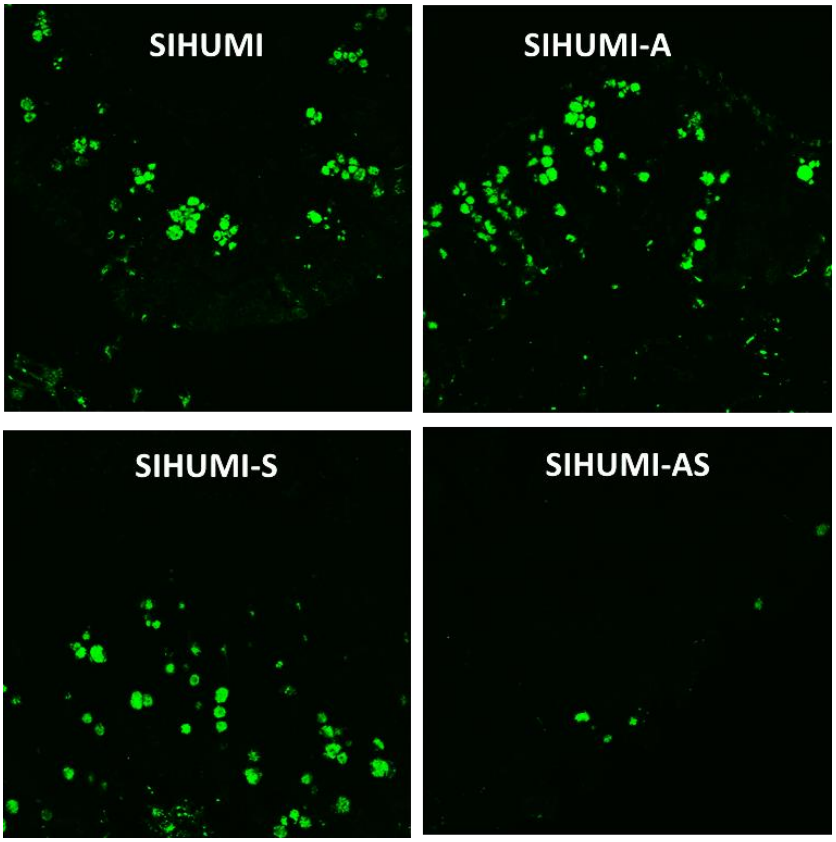

Figure 11. SIHUMI mice colonized with both *A. muciniphila* and *S.* Typhimurium display reduced MUC2 protein formation. Formalin fixed thin sections (2 µm) of cecal tissue of mice belonging to either one of four groups: SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS (see Figure. 1) were stained targeting MUC2 protein expressed in the goblet cells using fluorescence-immunohistochemistry with specific antibodies and subsequently incubated with Alexa Fluor 488 labeled secondary antibody and analyzed using confocal microscopy. Green color indicates the expression of MUC2 protein. Magnification 400 x.

Taken together our experiments indicate that *A. muciniphila* facilitates infection by *S.* Typhimurium in mice colonized with a simplified human intestinal microbiota and thereby exacerbates infectious and inflammatory symptoms (**Figure 10B**). This was not the case in SIHUMI mice colonized with *S.* Typhimurium in the absence of *A. muciniphila* (**Figure 10A**).This is an impressive example on how a community member changes its role in the ecosystem in response to the presence of a pathogen and how it shifts from a commensal to a harmful bacterium (pathobiont).

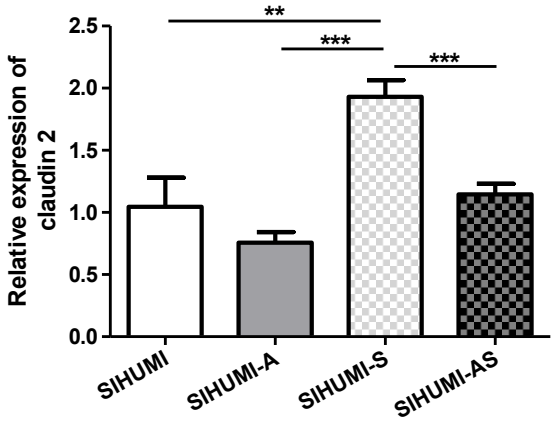

Figure 15. Presence of *A. muciniphila* together with *S.* Typhimurium is accompanied by reduced cecal claudin-2 mRNA levels. Cecal mRNA level of claudin-2 in gnotobiotic C3H mice was measured. mRNA was extracted from cecal mucosa of mice belonging to either one of four groups: SIHUMI, SIHUMI-A, SIHUMI-S and SIHUMI-AS (see Figure. 1). The mRNA was converted to cDNA for quantitative real-time PCR measurement (see materials and methods). Data are expressed as mean ± standard error. n=6 per group. Star indicates statistically significant differences (**P<0.05, **P<0.01, ***P<0.001)*.

Conclusions

Host-bacteria interactions (whether beneficial or harmful) are defined by a dynamic exchange of molecules that mediate various biological outcomes. These interactions by commensal bacteria significantly increase the metabolic capacity of the host by fermenting non-digestible food components (**Savage, 1986**) and are critically involved in the maturation of the host immune system and in epithelial barrier function against gastrointestinal infections (**Berg, 1996**). It has been recently reported that abnormal proportion of intestinal bacteria might contribute to various disease outcomes. Additionally, intestinal mucus is an important interface between the luminal microbiota and the underlying epithelium. The organic mucus component, mucins, can interact with bacteria in various ways and at this location (mucus layer) major interactions are believed to take place between lumen and host intestinal epithelium (**Kleessen & Blaut, 2005**). However, recently it has been found that excessive mucin degradation by commensal bacteria might be involved in the onset and perpetuation of inflammatory bowel diseases (**Sartor, 2006; Corfield & Myerscough, 2000; Johansson & Hansson, 2008**). The experiments described in this thesis drives to find out (**1**) whether the modulation of intestinal microbiota by the use of probiotic *E. faecium* NCIMB 10415 protects the host from adverse effects caused by abnormal bacterial proportion in a mouse model bearing chronic gut inflammation (**2**) whether the presence of mucin degrading commensal bacterium *A. muciniphila* in a *S.* Typhimurium-infected mice causes severe intestinal inflammation.

As to (**1**), we found no indication for a consistent effect of NCIMB on chronic gut inflammation, intestinal permeability or gut microbiota composition in the conventional IL-$10^{-/-}$ mice. The data indicate that NCIMB behaves like any other commensal bacterium under the chosen conditions. We cannot rule out that IL-10-dependent mechanisms are involved in the probiotic mode of action of NCIMB and that the IL-$10^{-/-}$ mouse might therefore be an inadequate model for investigating these effects. Protective effects of NCIMB may be

restricted to severe intestinal disorders such as infectious diarrhea **(Chapter 3)**. Nevertheless, mice treated with NCIMB for 8 weeks showed a significant reduction in *A. muciniphila* cell numbers and this was associated with less severe symptoms of intestinal inflammation (**Ganesh *et al.,* 2012**). Based on these observations the role of *A. muciniphila* and NCIMB was investigated in HT-29 cells. The experiments suggest that a co-culture of NCIMB and *A. muciniphila* produces a soluble and heat-sensitive factor/s that influences cell viability of HT-29 cells. We currently hypothesize that this factor is a protein and aim at identifying this compound in the future. This is the pre-requisite for finding out what triggers its formation and how it affects epithelial cell physiology *in vivo* **(Chapter 4)**.

As to (**2**) we observed that *A. muciniphila* aggravates *S.* Typhimurium-caused intestinal inflammation in a gnotobiotic mouse model. We found indications that the presence of *A. muciniphila* facilitates *S.* Typhimurium-induced infection by disturbing the mucus layer composition and mucin production in gnotobiotic mice colonized with a simplified human intestinal microbiota and thereby exacerbates infectious and inflammatory symptoms. This is an impressive example on how a community member changes its role in the ecosystem in response to the presence of a pathogen and how it shifts from a commensal to a harmful bacterium (pathobiont) (**Ganesh *et al.,* 2013**) **(Chapter 5).** Until now, we have not yet identified the bacterial molecule/s that mediates the observed effects in the host. To move a step forward we have started to perform deep sequencing of intestinal bacterial mRNA from gut contents of infected and uninfected mice to identify bacterial genes that are differentially expressed between mice with and without intestinal inflammation.

Perspectives and recommendations

This thesis provides new insights into one possible role of the mucin-degrading commensal *A. muciniphila* namely in the development and progression of severe intestinal inflammation. This is based on several observations: Higher cell numbers *of A. muciniphila*

were associated with increased inflammatory responses in conventional IL-10$^{-/-}$ mice in a probiotic supplementation trial. This led us to speculate that NCIMB stimulates inflammatory responses resulting in high cell numbers (10^8 /g) of *A. muciniphila*. Mice fed NCIMB for 8 weeks were less inflamed and this coincided with 4 log lower cell numbers of *A. muciniphila*. Even though we found no beneficial effects of NCIMB on inflammation in IL-10$^{-/-}$ mice, we realized for the first time that mucin degradation might lead to an exacerbation of an existing gut inflammation.

Higher cell numbers of *A. muciniphila* were associated with more severe symptoms of intestinal inflammation induced by *S.* Typhimurium in gnotobiotic mice and in conventional IL-10$^{-/-}$ mice. We decided to use *S.* Typhimurium instead of chemical agents to induced gut inflammation because the later might disturb the nature of the protective mucus layer in the intestine before *A. muciniphila* colonization. Therefore, we selected a gnotobiotic mouse model which was first associated with a bacterial consortium consisting of eight species and subsequently with *S.* Typhimurium. In this model presence of *A. muciniphila* exacerbated inflammation and infectious symptoms by the pathogen compared to its absence. In conflict with our observations, *A. muciniphila* cell numbers were reduced in IBD subjects (**Wen-Png *et al.,* 2010**). We cannot exclude that the effect of *A. muciniphila* in *S.* Typhimurium infected gnotobiotic mice differs from that reported for IBD patients because we used a gnotobiotic mouse model. It will therefore be necessary to corroborate the role of *A. muciniphila* in other mouse models.

Our study serves as the first example that *A. muciniphila* exacerbates *S.* Typhimurium-induced inflammation by changing the intestinal mucus composition followed with reduction in MUC2 protein formation. This might be due to *A. muciniphila's* mucin-degrading capabilities. Furthermore, reduced mucin sulphation together with reduced MUC2 formation might facilitate the access of luminal antigens to host tissues leading to severe inflammation.

This is in accordance with the observed correlation between reduced inflammation and low numbers of *A. muciniphila* cells in IL-10$^{-/-}$ mice. Our data shows that presence of *A. muciniphila* in gnotobiotic mice leads to increased number of mucin-filled goblet cells. We speculate that the changes in intestinal mucus composition and production observed in response to *A. muciniphila* facilitates the binding of the pathogen and the use of mucus as an energy source, which might boost the growth of the pathogen and its translocation into the host tissues/ organs. Our data also shows *A. muciniphila* cell numbers decreased significantly in response to inflammation. This might be the reason why IBD patients showed less *A. muciniphila* as described by Wen-Png *et al.* in 2010. It should be checked whether the observations in the *S.* Typhimurium infection model can also be made in other models of inflammation including the use of other pathogens. It is also necessary to investigate the changes of intestinal mucus composition after *A. muciniphila* colonization in more detail. This could be achieved by taking advantage of more simplified mouse models. Such approaches will provide new insights into the molecular interactions between the mucin-degrading bacterium and the host and might also help to better understand the interactions of the pathogen with the modified mucus layer for successful colonization.

Recently researchers stated that they "**strongly believe**" that *A. muciniphila* could one day be used to treat disorders such as obesity, diabetes and colitis in humans since it improves mucus thickness (http://www.nature.com/news/gut-microbe-may-fight-obesity-and-diabetes-1.1297 5). My thesis is most likely the first example to demonstrate that the commensal *A. muciniphila* plays a major role in exacerbation of inflammation by supporting pathogen invasion. This does not exclude that *A. muciniphila* may be is beneficial for some other diseases such as obesity but probably not in IBD. However, more experimental approaches are required to better understand the molecular interaction of *A. muciniphila* with the host under inflammatory conditions.

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LIST OF PUBLICATIONS AND CONFERENCES

PUBLICATIONS:

1. *BP Ganesh, JF Richter, M Blaut and G Loh*. (March 2012). ***Enterococcus faecium* NCIMB 10415 does not protect interleukin-10 knock-out mice from chronic gut inflammation.** Beneficial Microbes.3(1): 43-50

2. *BP Ganesh, R Klopfleisch, G Loh and M Blaut*. (September 2013). **Commensal *Akkermansia muciniphila* exacerbates gut inflammation in *Salmonella* Typhimurium-infected gnotobiotic mice.** PLoS ONE. 8 (9): e74963 (1-15).

CONFERENCES:

Presentation **:** Presentation (talk) on host – microbe interactions on IBD at ***SFB 852 mini-symposiums*** on ***2010, 2011*** and ***2012*** in Berlin, Germany.

Presentation **:** Presentation (talk) on effects of probiotic *E. faecium* on IBD using IL-10 KO mouse model and possible interactions of this bacterium on commensal mucin degrading bacteria using HT-29 colon cancer cell lines in ***IPC 2012*** at Kosice, Slovakia

Poster **:** Poster presentation on effects of probiotic *E. faecium* on chronic gut inflammation in ***NuGO week 2011*** at Wageningen, Netherlands.

Poster **:** Poster presentation on probiotic effects of *E. faecium* in IL $10^{-/-}$ mice at ***66th conference of the nutritional physiology 2012***, Gottingen, Germany.

Poster **:** Poster presentation on *Akkermansia muciniphila* exacerbates *Salmonella* induced gut inflammation in gnotobiotic mice at ***6th Seeon conference 2013, Munich, Germany***

AWARDS:

1. **Best presentation** (talk) award during **SFB 852** Mini-Symposium **2011** at Berlin, Germany.
2. **Best poster** award in **Seeon** conference **2013** at Munich, Germany.

MENTOR:

1. Successful completion of the diploma thesis supervised for 7 months (Ms. Maria Adler)
2. Practical training supervised for 1 to 3 months (Ms. Adler and Mr. Eid Labib)