Probiotic Bacteria Enhance the Antibacterial Barrier of Enterocytes: Insights into Their Mechanism of Action

Dissertation zur Erlangung des Doktorgrades
der Naturwissenschaften (Dr. rer. nat.)

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aus München
2006

The studies underlying this work were carried out at the Dr. Margarete Fischer-Bosch Institute of Clinical Pharmacology in Stuttgart in the laboratory group of Prof. E. F. Stange and supported by the Robert Bosch Foundation.

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Eingereicht am:	20.11.06
Mündliche Prüfung am:	23.02.07

Die vorliegende Arbeit wurde am 23.02.07 von der Fakultät Naturwissenschaften der Universität Hohenheim als „Dissertation zur Erlangung des Doktorgrades der Naturwissenschaften“ angenommen.

Great works are performed, not by strength, but by perseverance. He that shall walk, with vigor, three hours a day, will pass, in seven years, a space equal to the circumference of the globe.

Samuel Johnson (1709-84), English poet

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LIST OF ABBREVIATIONS

AIDA	Advanced Image Data Analyser
AMP	Antimicrobial peptide
AP-1	Activator protein 1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
bp	Base pairs
BSA	Bovine serum albumine
Caco-2	Colon adenocarcinoma cells
CD	Crohn´s Disease
CD8	Coreceptor of cytotoxic T cell receptor
cDNA	Complementary desoxyribonucleic acid
CF	Cystic fibrosis
CFU	Colony forming units
CpG	Deoxycytidylate-phosphate-deoxyguanylate
DEPC	Diethylpyrocarbonate
DMEM	Dulbecco Modified Eagle Medium
DMF	Dimethylformamide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
ERK	Extracellular signal-regulated kinase
Fig.	Figure
GAPDH	Glycerylaldehyde-phospho-dihydrogenase
GIT	Gastrointestinal tract
GM-CSF	Granulocyte macrophage-colony-stimulating factor
hBD	Human beta defensin
HBSS-CMF	Hanks balanced salt solution – Ca^{2+} and Mg^{2+} free
HD	Human defensin
HEPES	Hydroxyethylpiperazine-N`-ethanesulfonic acid
HNP	Human neutrophil peptide
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN	Interferon
IgA	Immunglobulin A
IL	Interleukin

IRAK	Interleukin-1 receptor-associated kinase
JNK	c-Jun N-terminal kinase
kD	Kilo Dalton
LDL	Low density lipoprotein
LPS	Lipopolysaccharide
M	Mol/l
MAPK	Mapkinase
MOPS	3-(N-Morpholino)-propanesulfonic acid
mRNA	Messenger ribonucleic acid
MRSA	Methicillin-resistent *Staphylococcus aureus*
MTT	3-[4,5-dimethylthiazolyl-2]-2,5-diphenyl tetrazolium bromide)
MWCO	Molecular weight cut off
NF-IL-6	Nuclear factor of interlukin 6
NF-κB	Nuclear factor kappa B
ODN	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PMA	Phorbolmuramylacetate
RNA	Ribonucleic acid
rpm	Rounds per minute
RPMI	Roswell Park Memorial Institute
RT-PCR	Reverse transcription-polymerase chain reaction
sp.	Subspecies (bacteria)
STAT	Signal Transducer and Activator of Transcription
TAE	Tris/Acetic acid/EDTA
TEMED	Tetramethylethylenediamin
TGF	Transforming growth factor
Th1	Inflammatory T cells type I
Th2	T-helper cells type II
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TRAF6	TBF-receptor-associated factor 6
TRIS	Tris-(hydroxymethyl-)methylamine
UC	Ulcerative colitis
v/v	Volume per volume
w/v	Weight per volume
X-Gal	5-brom-4-chlor-indolyl-ß-galactopyranoside

SUMMARY

In the healthy intestine there is a stable balance of luminal bacteria and host factors to prevent infections or inflammatory bowel diseases (IBD). A complex network of environmental, genetic, and immunoregulatory factors may precipitate the onset of ulcerative colitis (UC) and Crohn's disease (CD), the primary manifestations of inflammatory bowel disease (IBD). It is currently believed that IBD results from an aberrant immune response of the intestinal mucosa towards the normal commensal bacterial flora. Alternatively, a primary defect in the mucosal barrier might permit bacterial invasion and trigger inflammation. In our research group the hypothesis was proposed that the defective barrier in Crohn´s disease may be due to a lack of defensins which form a chemical barrier against luminal bacteria.

A major gut defensin is the human beta defensin-2 (hBD-2) which is an inducible antimicrobial peptide synthesized and secreted by the epithelium to counteract bacterial adherence and invasion. Proinflammatory cytokines, as well as certain bacterial strains, have been identified as potent endogenous inducers. In recent studies, Fellermann *et al* demonstrated that the defective expression of hBD-2 which was measured in the gut mucosa of patients with Crohn´s disease was due to a reduced copy number of the hBD-2 gene. In patients with ulcerative colitis β-defensin expression is low in the colon during remission, but readily inducible during inflammation. Probiotic bacteria might act beneficially in the human gut by inducing the expression of defensins and thereby reinforcing the mucosal barrier. Recently, our group has been the first to describe hBD-2 induction by the probiotic strain *E. coli* Nissle (Mutaflor®) which is an effective treatment for ulcerative colitis during remission.

The aim of the present work was to determine the underlying molecular mechanisms. We determined a time- and dose-dependent expression pattern of hBD-2 in Caco-2 cells upon stimulation with IL-1β, *E. coli* Nissle culture supernatant and diverse other probiotic strains. We further investigated the transcriptional regulation of hBD-2 expression mediated by probiotics. The hBD-2 promoter contains several elements known to be involved in transcriptional upregulation such as the NF-κB element, which is believed to be one of the main regulators of the hBD-2 gene expression. However, for certain signals, the expression

of the hBD-2 gene has been reported to depend on the activation of a second transcription factor, such as AP-1. Most importantly, *E. coli* Nissle was shown to shed or secrete factors, contained in the bacterial supernatant, which were sufficient to trigger activation of NF-κB and AP-1 and to induce hBD-2. Our results indicated further that the supernatant-induced activation of the MAP kinase pathways ERK½, JNK, and p38 may be directly responsible for the probiotic supernatant-induced activation of the transcription factors AP-1 and NF-κB and subsequent synthesis of hBD-2.

A further aim of the present study was to identify and isolate the bacterial components which are responsible for *E. coli* Nissle mediated hBD-2 induction. As *E. coli* Nissle culture supernatant was found to be a more potent stimulant than the bacterial pellet, we investigated the characteristics of the unknown soluble or shed molecules in the bacterial culture media. The first analysis revealed the factor as a heat resistant and proteinase sensitive molecule. Both, *E. coli* Nissle specific lipopolysaccharide (LPS) and bacterial DNA, which might contain immunostimulatory DNA motifs, failed to trigger hBD-2 expression.

Based on the knowledge of the surface composition several *E. coli* Nissle deletion mutants were constructed and tested for their ability to induce hBD-2 expression in Caco-2 cells. Deletion mutants for flagellin, the flagella filament protein, specifically exhibited an impaired immunostimulatory capacity. Reinsertion of the flagellin gene restored the induction capacity to normal levels. Next, we isolated flagellins from different bacteria strains (*Salmonella enterica* serovar Enteritidis, *E. coli* ATCC 25922, *E. coli* Nissle and the uropathogenic *E. coli* strain CFT073Δ*hly,* whose genome structure resembles closely that of *E. coli* Nissle). In the Western blot anti-H1 flagellin displayed immunoreactivity against the different types of flagellins, due to the highly conserved central region of the flagellin filament structure.

Incubation of Caco-2 cells with isolated *E. coli* Nissle flagellin (molecular size 60.81 kDa) induced hBD-2 promoter activation in a dose-dependent manner. The induction of hBD-2 expression by flagellin was confirmed with a positive control (*Salmonella* flagellin). Interestingly, the serotype-identical CFT073Δ*hly* flagellin expressed only moderate hBD-2 inducing ability compared to *E. coli* Nissle flagellin. Thus, differences in extracellular matrix

e.g. the glycosilation degree might underlie the differentially modulated hBD-2 response of Caco-2 cells by the two flagellins. H1 flagellin antiserum abrogated hBD-2 expression induced by flagellin as well as *E. coli* Nissle supernatant, confirming that flagellin is the major stimulatory factor of *E. coli* Nissle.

In conclusion, flagellin of *E. coli* Nissle provides reinforcement of mucosal antimicrobial function, apparently without inducing inflammation. This might explain the beneficial effects of *E. coli* Nissle on remission maintenance in ulcerative colitis. In patients with Crohn´s disease there is evidence against a therapeutic effect of probiotics and this may be explained by a defective defensin system. Future investigations about strain-specific beneficial functions might contribute to the therapeutic application of science-based probiotic products.

ZUSAMMENFASSUNG

Im gesunden Darm herrscht ein stabiles Gleichgewicht zwischen der luminalen Flora und körpereigenen Abwehrkräften, das Infektionen oder entzündlichen Erkrankungen im Darm vorbeugt. Ein komplexes Netzwerk aus Umwelteinflüssen, genetischen und immunregulatorischen Faktoren kann den Ausbruch von Colitis ulcerosa oder Morbus Crohn, den Haupterscheinungsformen der chronisch entzündlichen Darmerkrankungen (CED) herbeiführen. Eine überschießende Immunantwort der intestinalen Epithelzellen gegenüber der normalen Darmflora wird als auslösender Faktor für die intestinale Entzündung postuliert. Alternativ, könnte auch ein Primärdefekt der mukosalen Barriere verantwortlich für eine bakterielle Invasion und die darauffolgende Entzündung sein. In unserer Arbeitsgruppe wurde vor kurzem die Hypothese entwickelt, dass die gestörte Darmbarrierefunktion bei Morbus Crohn in einem Mangel an Defensinen begründet sein könnte, welche eine Art chemische Barriere gegen luminale Bakterien darstellen.

Eines der wichtigsten Defensine des Darms ist das humane beta Defensin-2 (hBD-2) welches ein, durch proinflammatorische Zytokine sowie spezielle Bakterienstämme induzierbares, antimikrobiell wirksames Peptid ist. Es wird von Epithelzellen synthetisiert und sezerniert zur Abwehr einer bakteriellen Adhärenz und Invasion. Fellermann *et al* wies vor kurzem nach, dass die in der Darmschleimhaut des Kolons von Morbus Crohn Patienten gemessene defekte hBD-2 Expression durch eine verringerte Genkopienzahl des humanen beta-Defensins-2 bedingt ist. Bei Patienten mit Colitis ulcerosa ist die β-Defensin Expression im Kolon in der beschwerdefreien Phase (=Remission) niedrig, in der Entzündung wird diese jedoch umgehend induziert. Probiotika könnten durch eine Induktion der Defensinexpression im Darm einen günstigen Einfluss ausüben und somit die Darmbarriere stärken.

Unsere Arbeitsgruppe hat vor kurzem erstmals eine hBD-2 Induktion durch den probiotischen Keim *E. coli* Nissle (Mutaflor®) nachgewiesen, der mit gutem Behandlungserfolg in der Remission der Colitis ulcerosa Einsatz findet. Das Ziel der vorliegenden Arbeit war, die zugrunde liegenden molekularen Mechanismen zu untersuchen. Wir bestätigten ein zeit- und dosisabhängiges hBD-2 Expressionsmuster in Caco-2 Zellen durch Stimulation mit IL-1β, *E. coli* Nissle Kulturüberstand und diversen anderen probiotischen Keimen.

Des Weiteren untersuchten wir die transkriptionelle Regulation der probiotika-vermittelten hBD-2 Expression. Der hBD-2 Promotor enthält verschiedene, an der Aktivierung der Transkription beteiligte Elemente, wie z.B. dem nukleären Faktor NF-κB welches ein Hauptregulator der hBD-2 Expression zu sein scheint. Es gibt jedoch Hinweise darauf, dass die Expression des hBD-2 Gens durch gewisse Stimulanzien noch von einem weiteren Transkriptionsfaktor abhängt, wie z.B. AP-1. Sezernierte oder abgelöste Faktoren des *E. coli* Nissle, welche sich im Kulturüberstand befinden, waren ausreichend in der Lage hBD-2 durch die Aktivierung von NF-κB und AP-1 zu induzieren. Die überstandsinduzierte Aktivierung der MAP-Kinase-Signalwege über ERK½, JNK und p38 scheint hierbei direkt verantwortlich zu sein für die über AP-1 und NF-κB aktivierte Synthese von hBD-2.

Ein weiteres Ziel dieser Arbeit war die Identifizierung und Isolation der bakteriellen Bestandteile des *E. coli* Nissle welche für die hBD-2 Induktion verantwortlich sind. Da der Bakterienkulturüberstand ein stärkerer Stimulus als das Bakterienpellet war, untersuchten wir die Charakteristiken der unbekannten löslichen oder abgegebenen Moleküle im bakteriellen Kulturmedium. Erste Untersuchungen ergaben, dass der gesuchte Faktor eine hitzeresistente, durch Proteinase verdaubare Komponente des Bakteriums darstellt. Das für *E. coli* Nissle spezifische Lipopolysaccharid (LPS) sowie dessen bakterielle DNA, welche immunstimulatorische DNA-Motive enthalten könnte, hatten keinen hBD-2 induzierenden Einfluss.

Auf der Grundlage bekannter oberflächlich exprimierter Moleküle des *E. coli* Nissle wurden mehrere Deletionsmutanten konstruiert und auf ihre Fähigkeit, die hBD-2 Expression in Caco-2 Zellen auszulösen, untersucht. Flagellin-Deletionsmutanten wiesen eine stark verminderte immunstimulatorische Kapazität auf. Das erneute Einfügen des deletierten Flagellingens regenerierte die Fähigkeit zur hBD-2 Induktion vollständig. Des Weiteren isolierten wir Flagellen aus verschiedenen Bakterienstämmen (*Salmonella enterica* serovar Enteritidis, *E. coli* ATCC 25922, *E. coli* Nissle und dem uropathogenen *E. coli* Stamm CFT073Δ*hly*, dessen Genom dem des *E. coli* Nissle sehr ähnlich ist). Im Western Blot wurde eine Immunreaktivität von Anti-H1 Flagellin gegen die verschiedenen Flagellintypen nachgewiesen, was sich durch die hoch konservierte Zentralregion der Flagellinfilamentstruktur erklären lässt.

Die Inkubation von Caco-2 Zellen mit dem isolierten *E. coli* Nissle Flagellin (Molekulargewicht: 60.81 kDa) induzierte dosisabhängig die hBD-2 Promotoraktivierung. Die Induktion der hBD-2 Expression durch Flagellin wurde mit einer Positivkontrolle (*Salmonella* Flagellin) bestätigt. Interessanterweise war die Induzierbarkeit durch das serotypidentische CFT073Δ*hly* Flagellin, verglichen mit der des *E. coli* Nissle Flagellins reduziert. Unterschiede in der Extrazellulärmatrix, z.B. dem Glykosilierungsgrad, könnten die verschieden ausgeprägte hBD-2 Antwort der Caco-2 Zellen auf die beiden Flagelline erklären. Zusätzlich wurde die Flagellin- und *E. coli* Nissle Überstand-induzierte hBD-2 Expression durch H1-Flagellin Antiserum gehemmt. Diese Befunde lassen schließen, dass Flagellin der hauptverantwortliche Faktor für die Stimulation der hBD-2 Expression durch *E. coli* Nissle ist.

Das Flagellin des *E. coli* Nissle stärkt somit die mukosale antimikrobielle Funktion des Darmepithels ohne eine Entzündung auszulösen. Dies könnte die positive Wirkung des *E. coli* Nissle auf die Remissionserhaltung der Colitis ulcerosa erklären. Bei Patienten mit Morbus Crohn gibt es Hinweise gegen einen therapeutischen Nutzen von Probiotika, was durch ein defektes Defensinsystem erklärt werden könnte. Die Charakterisierung positiver Eigenschaften relevanter Keime könnte einen Beitrag zur evidenz-basierten therapeutischen Verwendung probiotischer Produkte liefern.

1 INTRODUCTION

1.1 The composition and benefits of the normal flora

The human gastrointestinal tract (GIT) is colonized by a remarkable community of symbionts and commensals performing an essential role in nutrient processing as well as innate and adaptive immunity. Approximately 500 different species of microorganisms exert a metabolic activity comparable to that of the liver (Berg 1996; Bocci 1992). Differences in the composition of the flora are influenced by age, diet, cultural conditions, and the use of antibiotics. In the upper gastrointestinal tract of adults, the oesophagus contains only the bacteria swallowed with saliva and food (Fig. 1).

Due to the high acidity of the gastric juice, very few types of bacteria (mainly acid-tolerant lactobacilli) can be cultured from the normal stomach. However, the infestation of the stomach with the pathogenic bacterium *Helicobacter pylori* is one of the most common infectious diseases worldwide known to cause peptic ulcer disease, and probably also gastric cancer (Stolte 1998; Todar 2002).

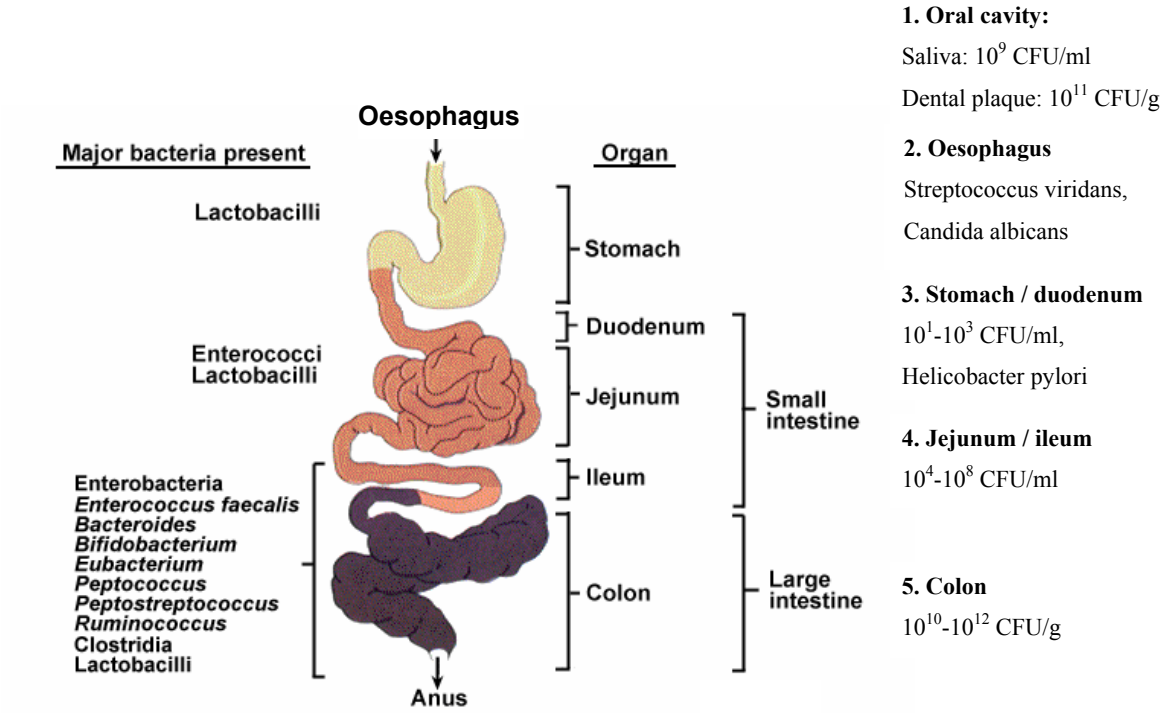


Figure 1: Bacteria distribution in the gastrointestinal tract. CFU, colony forming units (Source: www.agen.ufl.edu/~chyn/age2062/lect/lect, right panel adapted from Otte 2003)

The proximal small intestine is populated by a relatively sparse, gram-positive flora consisting mainly of lactobacilli and *Enterococcus faecalis.* The distal part of the small intestine contains higher concentrations of bacteria (10^8/ml) and additional species including coli forms and *Bacteroides,* next to lactobacilli and enterococci. The flora of the large intestine (colon) is qualitatively similar to that found in faeces. In the colon, with 10^{11} bacteria/ml faeces, coliforms become more prominent and enterococci, clostridia and lactobacilli can be regularly found, but the predominant species are anaerobic *Bacteroides* and anaerobic lactic acid bacteria of the genus *Bifidobacterium* (Todar 2002).

At birth, the entire intestinal tract is sterile, but following delivery, multiple different antigens challenge the intestine of the newborn and the maternal intestinal flora is a major source of bacteria for the neonatal gut. After the first week of life, a stable bacterial flora is usually established (Fanaro 2003). In breast-fed infants, bifidobacteria account for more than 90 % of the total intestinal bacteria as opposed to the bacterial distribution found in bottle-fed infants where other bacteria such as bacteroides, enterococci or clostridia prevail. Apparently, human milk contains a growth factor that enhances the growth of bifidobacteria which play an important role in preventing colonization of the infant intestinal tract by non-indigenous or pathogenic species.

The indigenous bacteria of the gastrointestinal tract, mainly because of their enormous number, seem to have the greatest overall impact on their host. To understand the consequences of a disturbed flora it is important to know more about the beneficial effects of the normal flora.

Benefits of the host's indigenous flora:

- Colonization with pathogens is prevented by competition for attachment sites or for essential nutrients (Goldin 1998)
- Bacteria are antagonized. This may occur through the production of highly specific bacteriocins, which inhibit or kill nonindigenous species (Riley 2002)
- Bacteria synthesize and excrete vitamins in excess of their own needs, which can be absorbed as nutrients by the host, e.g. enteric bacteria secrete Vitamin K and several B vitamins such as Vit. B_{12} (Hill 1997)

- Development of the gut-associated lymphoid tissues is stimulated (Rhee 2004)
- Production of cross-reactive antibodies is stimulated. Low levels of antibodies produced by the normal flora are known to crossreact with certain pathogens and thereby prevent infection or invasion by pathogenic strains (Wijburg 2006).

1.2 Etiology, pathophysiology and the role of the luminal flora in IBD

There is increasing evidence that the balance of the luminal flora also plays a critical role in the initiation and perpetuation of colitis. The incidence of "non-specific" inflammatory bowel diseases (IBD), which include ulcerative colitis (UC) and Crohn´s disease (CD), once regarded as medical curiosities, has increased sharply in the late 1900s and now affects millions of people worldwide. The specific type of IBD can be difficult to distinguish because the symptoms are often similar. The acute clinical characteristics are diarrhoea, abdominal pain, fever and weight loss; the acute pathologic features include a constant flux of neutrophils into inflamed mucosa, eventually penetrating the epithelium into the intestinal lumen. Crohn´s disease may affect the entire length of the gastrointestinal tract but preferentially manifests in the terminal ileum, as was originally described by Crohn *et al* (1932). Ulcerative colitis is usually restricted to the colon where inflammation appears to be continuous.

Since the etiology of ulcerative colitis and of Crohn´s disease remains elusive, many unresolved aspects of the pathogenesis of IBD are matters of investigation including: environmental factors, emerging new types of bacteria, the body's defences against bacteria and viruses, psychoneuro-immunological, genetic and neurohormonal mechanisms mediating stress and emotional disturbances (Kirsner 2001).

Genetic factors

Familial distributions of IBD involved first-degree relatives more often than second- or third-degree relatives (Orholm 1991). Sartor (1998) suggested that a genetically determined, overly aggressive immune response to ubiquitous resident luminal bacterial constituents might be the cause for chronic intestinal inflammation. This dysfunctional response could be mediated by either defective immunoregulatory events and/or abnormal epithelial barrier function.

Genetic research programs have identified single nucleotide polymorphisms in the coding region of the bacterial sensing gene NOD2 (IBD1 locus on chromosome 16) with a susceptibility to ileal Crohn´s disease (Hampe 2001; Ogura 2001; Hugot 2001). Between 17-25 % of Crohn´s disease patients carry mutations in the NOD2/CARD15 gene resulting in decreased NF-κB expression, which might alter the host's immune response to the bacterial flora (Hampe 2001). Several other loci are associated with either UC or CD while others are involved in the pathogenesis of both IBD forms (Ahmad 2004). Genetic variation in other innate immune receptors, notably the Toll-like receptor (TLR) 4, has been defined and may also play a role in CD pathogenesis (Brand 2005).

Furthermore, Fellermann *et al* (2006) determined a reduced gene copy number of the human beta defensin-2 (hBD-2) in colonic CD but not in ileal CD, UC or controls. Thus a low hBD-2 gene copy number creates a predisposition to colonic CD. The human leukocyte antigen (HLA) class II genes are also candidates in the pathogenesis of IBD as their products play a central role in the immune response. However, they may play a greater role as disease phenotype modifiers than in determining overall disease susceptibility (Satsangi 1996).

Immunological factors

Both ulcerative colitis and Crohn´s disease are characterized by an increased expression of "general" proinflammatory cytokines (TNF-α, IL-1, IL-6), which suggests an abnormally intense inflammatory response to commensal bacteria. This loss of immune tolerance to non-pathogenic microbes results in pathologic immune reactivity and a self-supporting inflammatory cascade. A role for cytokines is also indicated by favourable clinical responses to blockage of them, for instance by anti-IL-12 thereby enhancing the epithelial barrier and inhibiting proinflammatory cytokines.

Microbiological factors

Several studies provide substantial evidence that the normal enteric flora plays a key role in the development of IBD. In the majority of IBD animal models, intestinal inflammation fails to develop when the animals are raised in a germfree environment. For example, interleukin-2 (IL-2) - or interleukin-10 (IL-10) -deficient mice spontaneously develop colitis

under conventional housing conditions, which is highly attenuated under germfree conditions (Ehrhardt 1997; Sellon 1998). Similarly, T-cell receptor knock-out mice fail to develop colitis in the absence of normal bacteria (Dianda 1997).

Changes in the normal flora of IBD patients, more precisely high numbers of unusual bacteria, reduced biodiversity and a generalized or localized dysbiosis have been reported frequently (Ott 2004; Seksik 2006). More than 50 years ago Seneca *et al* (1950) determined already an 85-fold increase compared to healthy individuals in the total number of microorganisms and a fifty-fold increase in the numbers of coliforms in the stools of patients with severe ulcerative colitis. They postulated that the large number of bacteria created damaging proteolytic enzymatic activity, which injured the intestinal mucosa and was followed by secondary bacterial infection.

Several studies were carried out to find possible pathogenic characteristics of *E. coli* organisms in ulcerative colitis. The isolated coliforms from patients with ulcerative colitis had a higher incidence of *in vitro* adhesive and invasive properties compared to controls (Dickinson 1980). The UC strains also included a higher proportion of coliforms that produced hemolysin and necrotoxin and caused a severe tissue reaction in segments of rabbit ileum (Cooke 1974; Cooke 1968). In jejunum, ileum and colon resectates from Crohn´s disease patients an abnormal ileal flora was inferred by the detection of increased amounts in *E. coli* and *Bacteroides fragilis* in the ileum (Keighley 1978). The latter strain was found to be responsible for >60% of the mucosal biofilm mass in IBD patients but only for 30% in patients with self-limiting colitis and <15% of the biofilm mass in patients with inflammatory bowel syndrome (Swidsinski 2005).

Swidsinski *et al* reported that mucosal bacteria concentrations progressively increase from self-limiting colitis to indeterminate colitis with the highest amounts of bacteria found in Crohn´s disease patients compared to the virtually sterile mucosal surface of healthy controls. However, he did not observe any difference in the composition of the mucosal flora in patients with inflammatory bowel disease or in controls (Swidsinski 2002).

At sites of inflammation, bacteria may encounter dysfunctional or absent epithelium, with gross changes to the barriers that normally attenuate invasion and exposure of stromal sites for bacterial adhesion (Braun 2002). Darfeuille-Michaud *et al* (1998) hypothesized that entero-adherent *E. coli* strains in ileal mucosa might disrupt the intestinal barrier by synthesizing alpha-hemolysin. Swidsinski *et al* (2005) however uncovered little evidence for these modes of bacterial invasion and hypothesized that crossing of mucus barriers and bacterial adhesion would occur in advance of mucosal destruction.

Research focussed for some time on the identification of possible agents responsible for IBD such as mycobacteria (*Mycobacterium avium paratuberculosis* (Chiodini 1984)), bacterial components (e.g., lipopolysaccharides, peptidoglycans), metabolic products (endotoxins) and the host intestinal bacteria, but none of the investigations confirmed a role for specific factors related to CD or UC. The apparent rarity of IBD in third world countries with a high incidence of specific intestinal infections and parasitic infestations is of particular interest. Interestingly, mice previously exposed to helminthic infestations (a worm classified as a parasite) apparently are protected against experimentally-induced intestinal inflammation (Elliott 2003) via a helminth-induced mucosal Th_2 response (Shi 1998). Recently, disease activity was also shown to be diminished in IBD patients after exposure to the helminth *Trichuris suis* (Elliott 2005; Summers 2005).

An attempt to correlate inflammatory bowel disease and domestic hygiene in infancy (Gent 1994) was made in England including 364 IBD patients with matched controls (133 CD and 231 UC patients). “Evidence” of a “sanitized” environment was taken as the availability of a hot water tap and bathroom in the childhood home. While the results supported the hypothesis of delayed early exposure to enteric infections as a contributing factor in the development of Crohn´s disease (not ulcerative colitis), the limitations of this study are apparent.

The frequent onset of IBD following an acute enterocolonic infection (e.g., traveller’s diarrhoea) might suggest the implication of a food- or waterborne microbial agent despite the lack of evidence that either CD or UC are classical infectious diseases i.e. the aquisition of a specific pathogen by direct contact with a patient. Multiple bacterial species are appar-

ently involved in the onset of IBD, but rather as precipitants of the tissue reaction, modified by genetic and immune influences, than as direct causes of inflammation. This hypothesis is supported by clinical observations that a reduction of intestinal bacterial concentrations by various techniques could lead to clinical improvement and diminished intestinal inflammation (Sellon 1998). Rutgeerts *et al* (1991) reported that the recurrence of an inflammation in the neoterminal ileum of Crohn´s disease patients after resection of the distal ileum correlated with the contact of bacteria in the faeces. If the fecal stream was diverted via terminal ileostomy, biopsies from the patients showed no inflammatory changes characteristic to CD.

Epithelial permeability

Finally, the role of bacteria in the pathogenesis of IBD is further confirmed by an increased intestinal permeability in CD patients, suggesting an enhanced entry of bacteria and bacterial products into the intestinal wall, initiating or aggravating an inflammatory response (Hollander 1986). An association between IBD and genes which are involved in mucosal transport and integrity (e.g., the novel organic cation transporter OCTN or DGL5) has been reported recently (Vermeire 2006), supporting the assumption of an increased permeability preceding the onset of symptomatic disease (Wyatt 1993).

1.3 Probiotics – Definition and characteristic traits

The microbiological aspects of IBD have also renewed interest in the potential therapeutic benefits of stabilising the normal intestinal microflora by probiotics (e.g., *Lactobacillus sp*., *Bifidobacterium sp*., *Streptococcus sp*., and the yeast *Saccharomyces boulardii*) as adjuncts to conventional treatment (Campieri 1999).

Probiotics have been defined after several revisions as follows: “A preparation of or a product containing viable, defined microorganisms in sufficient numbers, which alter the microflora (by implantation or colonization) in a compartment of the host and by that exert beneficial health effects in this host” (Schrezenmeir 2001). The consumption of “probiotics” originates centuries before any theoretical understanding of possible mechanisms of action. A Persian version of the Old Testament (Genesis 18:8), states, that “Abraham owed his longevity to the consumption of sour milk” (Schrezenmeir 2001).

The Roman historian Plinius recommended in 76 BC the administration of fermented milk products for the treatment of gastroenteritis (Bottazzi 1983).

The theoretical concept of probiotics was first described by the Russian Nobel-prize-holder E. Metchnikoff in the early 20th century. He noted that people in Bulgaria, considered as "underdeveloped", had a higher life expectancy than people in other countries. Metchnikoff (1907) claimed that the intake of yogurt containing lactobacilli (e.g., *Lactobacillus bulgaricus*) created a hostile environment for pathogens by the production of acid, leading to a reduction of toxin-producing bacteria in the gut. The euphoria surrounding this claim soon decreased after scientists proved the lack of resistance to gastric acid and bile salts of the classic strains *Lactobacillus bulgaricus* and *Streptococcus thermophilus* found in yoghurt. Probiotics regained interest with the observation that *lactobacilli* or *bifidobacteria* strains survive the transport through the gastrointestinal tract (Rettger 1935).

Microorganisms need to meet the following criteria to be considered probiotic:

- Safety: no pathogenic effect may result from the use of specific bacterial strains
- Survival of the gastrointestinal passage (bile and acid resistance)
- Capacity to adhere to the mucosal epithelium of the GIT
- Metabolic activity in the gastrointestinal tract (GIT), implies colonization
- Antimicrobial activity against pathogenic bacteria
- Sufficient germ density after long time storage

Both, the unspecific (increased leukocyte, macrophage or lymphocyte activity (Schiffrin 1995; Goldin 1998)) as well as the specific immune response (increased intestinal IgA response) can be influenced by probiotics. Allergies seem to be delayed or reduced, and the incidence of infections was shown to be markedly decreased after newborn or premature infants had received the probiotic *E. coli* strain PZ720 (Lodinová-Zádníková 2003).

A summary of some molecular mechanisms of probiotic action which underlie the beneficial effects is provided by Table 1 (according to Marco 2006).

Table 1: Molecular mechanisms of action by which probiotics modulate human health.

Effect	Molecular mechanisms	Reference
Modulation of the immune response	Inactivation of NF-κB (e.g. through proteasome inhibition or inhibition of NF-κB translocation to the nuclei) → e.g. reduction of TNFα-induced IL-8 expression	Petrof 2004 Ma 2004
	Induction of IL-10 producing, regulatory T cells *in vitro* through the interaction of *Lactobacillus* strains with a specific dendritic cell receptor (DC-SIGN)	Smits 2005
Strengthening of the intestinal barrier	Induction of heat shock proteins by VSL#3 *in vitro* and *in vivo* → stabilization of the actin cytoskeleton	Petrof 2004
	Induction of antimicrobial peptides (hBD-2)	Wehkamp 2004
Antagonizing of pathogens by:		
Production of antimicrobial products	*L. rhamnosus* GG exerts antimicrobial activity against *Salmonella typhimurium* by secreting bactericidal substances + lactic acid (↓ pH)	De Keersmaecker 2006
Competition for mucosal adhesion sites	Exhibition of adhesins similar to those of pathogens → inhibit pathogen attachment (e.g. *L. plantarum* binds to mannose receptor likewise as enteropathogenic *E. coli*)	Pretzer 2005

1.3.1 Prophylactic or therapeutic application of probiotics

Prescription of probiotics or "biotherapeutic agents" is generally considered to improve constitutional health or to overcome disease as an adjunct to medical therapy. In the past decades, several *in vitro* and *in vivo* trials have been conducted in search of more solid evidence of the benefits of probiotics.

Gastrointestinal infections

The main prerequisite for the protective effect of probiotics in the gut is the adhesion to and colonization of the mucosa. It is hypothesized that masking of cell wall receptors or direct inactivation via aggregation, suppresses the negative potential of pathogens. Bernet-Camard *et al* (1997) reported that the *L. acidophilus (johnsonii)* strain La1 remarkably inhibited the adhesion and invasion of pathogens such as *Salmonella, Listeria* or *Shigella* to enterocytes. The reduced invasion of pathogens was thereby partially due to antimicrobial substances secreted by La1. Another study has reported a reduced incidence of acute diarrhoea and rotavirus shedding in hospitalized infants under 2 years of age (39 % control formula versus 10 % supplemented formula) (Saavedra 1994) receiving a formula supplemented with *Bifidobacterium bifidum* or *Streptococcus thermophilus*. Several other studies

confirm, especially in infants, that the duration and extent of diarrhoea can be cut by the intake of probiotics (Gaon 2003; Rosenfeldt 2002). In adults, the efficacy of probiotics is presented by shorter durations of traveller's diarrhoea and other self-limited gastrointestinal infections (Black 1989; Vanderhoof 2001; Marteau 2001).

Anticancerogenic effects

Evidence for cancer-preventing properties of pro- and prebiotics is found in studies on faecal enzyme activities in animals and humans, detoxification of genotoxins in the gut *in vitro* and *in vivo* and suppression of carcinogen-induced preneoplastic lesions and tumours in laboratory animals (Goldin 1996). Some of these studies indicate that combinations of pro- and prebiotics ('synbiotics') are more effective. Epidemiological and intervention studies provide some, albeit limited, evidence for protective effects of products containing probiotics in humans. Scientifically evident is the reduction of key enzyme activities in cancerogenesis as well as cell proliferation (Ballongue 1997).

Use of probiotics in inflammatory bowel disease

Therapy of IBD often involves induction of remission with corticosteroids and maintenance therapy with a combination of aminosalicylates and immunomodulators. Patients treated with long-term corticosteroids may suffer from complications including growth failure or osteopenia. Aminosalicylates (e.g., sulfasalazine, mesalamine) are also used to maintain remission and have a modest effect with more than 50 % relapses after 1 year.

Alternative and complementary therapies such as probiotics might induce or contribute to remission maintenance in IBD by modifying the bacterial environment without severe adverse effects. Favourable investigations in experimental murine colitis have been conducted with *E. coli* Nissle leading to an amelioration of disease (Schultz 2004) next to anti-inflammatory influences on T cell proliferation and function (Sturm 2005). However, *Lactobacillus rhamnosus* strain GG (LGG) failed to extend remission time in patients with CD. The time to relapse was identical between the probiotic and placebo group (Bousvaros 2005).

Despite the failure of most probiotics in Crohn´s disease, *Saccharomyces boulardii*, a non-pathogenic yeast exerted beneficial effects in the maintenance treatment of Crohn's dis-

ease when combined with mesalamine (Guslandi 2000). Studies on the impact of probiotics on remission maintenance in ulcerative colitis and prophylaxis of pouchitis are very convincing. LGG was capable to prolonge the relapse-free time in UC (Zocco 2006).
Three independent studies have found an equal efficacy for *E. coli* Nissle compared to aminosalicylates, the standard treatment for remission maintenance in ulcerative colitis (Kruis 1997; Rembacken 1999; Kruis 2004). The probiotic mixture VSL#3, composed of eight different bacterial species, is effective in primary and secondary prevention of pouchitis after proctocolectomy in ulcerative colitis and improved the quality-of-life scores of the patients (Gionchetti 2000; Gionchetti 2003; Mimura 2004). Further, VSL#3-treatment of patients with mild to moderate UC, not responding to conventional therapy, resulted in a combined induction of remission/response rate of 77 % with no adverse events (Bibiloni 2005).

1.4 Defensins – genetics, expression and regulation

We have recently provided a possible explanation for the probiotic effect of *E. coli* Nissle as it provokes inducible human beta defensin-2 (hBD-2) expression in an intestinal epithelial cell line (Wehkamp 2004). Defensins, such as hBD-2, are components of the armoury of endogenous antimicrobials, which are part of the innate immune system. They are highly conserved molecules in terms of genomics, proteomics regulation and function from *Drosophila* to higher mammals (Bevins 1996; Boman 1996, 1998; Lehrer and Ganz 1999; Schroeder 1999a). Defensins are small cationic peptides with a characteristic β-sheet-rich fold and a framework of six disulphide-linked cysteines (Fig. 2).

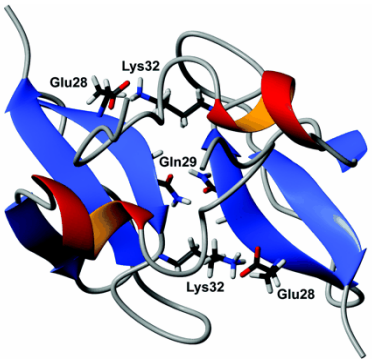


Figure 2: β-sheet structure of the human β-defensin 2
(Source: http://www.phoenixpeptide.com/Catalog%20Files/BD/bc0727004011.gif)

Defensins can be classified into two main subfamilies, α- and β-defensins, based on their tertiary structure (another subfamily, the circular θ-defensins does not exist in humans but in rhesus macaque leukocytes (Tang 1999)). Defensins are either constitutively expressed (e.g., hBD-1) or inducible by bacteria or cytokines (hBD-2, -3 and -4) (Table 2).

To date, six human alpha-defensins have been identified and are further subdivided into two groups, the human neutrophil peptides (HNPs 1-4) and the human defensins (HDs 5-6). The human α-defensin peptides (3.5 - 4 kDa) are abundant within neutrophils and Paneth cells of the small intestine (Boman 1998, Lehrer and Ganz 1999, Schroeder 1999b) whereas the distribution of β-defensins (4 - 6 kDa) is strictly limited to epithelial cells.

Table 2: Overview of representative human antimicrobial peptides. The table shows the expression pattern in different cell types (adapted from Oppenheim 2003).

Peptide		**Constitutive expression**	**Induced by proinflammatory cytokines and endotoxin (LPS)**
α-defensins	HNP 1-4	Neutrophil granules	Monocytes
	HD 5-6	Paneth cell granules (small intestine)	CD8 T lymphocytes (CTL)
β-defensins	HBD 1	Keratinocytes	Keratinocytes
		Barrier epithelial cells	Monocytes and dendritic cells
	HBD 2-4		Barrier epithelial cells, Keratinocytes, Mast cells
	HBD 5-6	Epididymis	Monocytes and dendritic cells

Human β-defensins provide a first line of defence against potentially pathogenic microbes at the body's mucosal frontiers (Boman 1998, Lehrer and Ganz 1999, Schroeder 1999b). Evaluation of the human genome suggests the existence of an additional 25 β-defensins that have not yet been identified (Schutte 2002).

The role of β-defensins will be further considered in more detail. Single genes, clustered amongst the defensin locus within chromosome 8 p22-23.1 encode inactive pre-pro-peptide forms of the β-defensin gene products (Liu 1997, 1998; Harder 1997b). They are subsequently processed by proteolytic factors to form mature, bioactive peptides of 28 to 44 amino acids in length (Bals 1998, Valore 1998). In epidermal keratinocytes, human β-

defensin 2 (hBD-2) is secreted in lamellar bodies (Oren 2003), which are lipid-containing vesicles that are secreted into the intercellular space and impermeabilize the skin to water. Apparently, these vesicles also generate the antimicrobial barrier in the epidermis.

In addition to defensins, other human antimicrobial peptides are cathelicidins ((LL-37/hCAP-18) (in neutrophil granules, barrier epithelial cells, keratinocytes, monocytes and T-lymphocytes), hepcidins (in blood ultrafiltrate and urine, acting as an iron-regulatory hormone (Kluver 2002; Krause 2003, Ganz 2006)), dermicidins (eccrine sweat glands (Rieg 2004; Schittek 2001)), histatins found in saliva (Oppenheim 1988; Sabatini 1989) and thrombocidins (or platelet microbicidal proteins) which are released upon contact with pathogens or stimulation with thrombin (Krijgsveld 2000; Yeaman 1999).

1.4.1 Mechanisms of activity of defensins

Defensins, or so-called arginine-rich cationic peptides, are defined by their high cathodal electrophoretic mobility, which influences their antimicrobial activity against gram-negative and -positive bacteria, fungi and viruses (Table 3).

Table 3: Antimicrobial spectrum of ß-defensins

hBD-1	E. coli , Haemophilus influenzae
hBD-2	Gram-negative bacteria, Candida albicans, H. pylori, S. typhimurium, Haemophilus influenzae, Pseudomonas aeruginosa, HIV, rhinovirus
hBD-3	Gram-negative (P. aeruginosa, E.coli, H. pylori) and gram-positive bacteria (Streptococcus pyogenes, Staphylococcus aureus, MRSA, Enterococcus faecium), S. cerecisia, Cand. albicans
hBD-4	Pseudomonas aeruginosa, Staphylococcus carnosus

Membranes were recognized early on as the targets for many antibacterial peptides (Hultmark 1980; Steiner 1982) and permeabilization of target membranes is the crucial step in defensin-mediated antimicrobial activity and cytotoxicity.

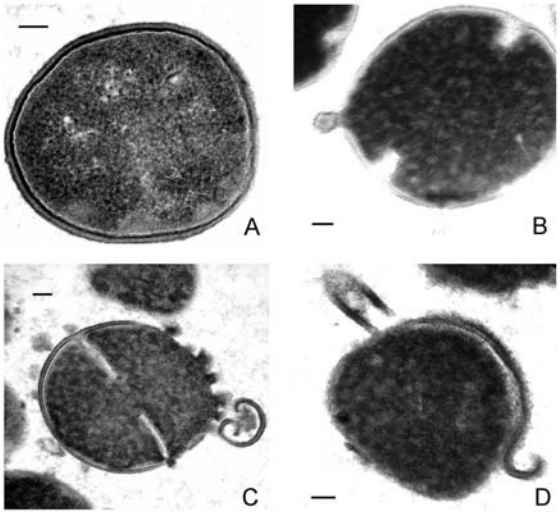


Figure 3: Staphylococcus aureus killing by defensins. Transmission electron micrographs of *S. aureus* (10^8 cells/ml) incubated in 10 mM phosphate buffer for 2 h (*A*) or treated with synthetic hBD-3 (500 µg/ml) for 30 min (*B*) or 2 h (*C* and *D*) are shown. *Bars* represent 0.1 µm. (adapted from Harder 2001)

Harder *et al* presented the antimicrobial activity of isolated hBD-3 (Fig. 3). When *S. aureus* was incubated with hBD-3, it showed perforations of the peripheral cell wall with an explosion-like liberation of the plasma membrane within 30 min. After 2 h most bacteria were lysed with different degrees of cellular disintegration.

Artificial membranes have been used to demonstrate voltage-dependent channel formation by defensins (Kagan 1990). When a negative potential is applied to the membrane side opposite to the defensin-containing solvent, the defensins are electrophoretically driven into the membrane, they aggregate to form complexes based on their amphipathic structure, which results in a loss of membrane integrity, ATP production, potassium pump function, respiration and eventually cell death (Fig. 4).

Necessary conditions for sufficient electrostatic forces between anionic phospholipid headgroups and cationic defensins are low concentrations of salts and plasma proteins, which would otherwise competitively inhibit the defensin antimicrobial activity (Bals 1998; Lehrer 1988).

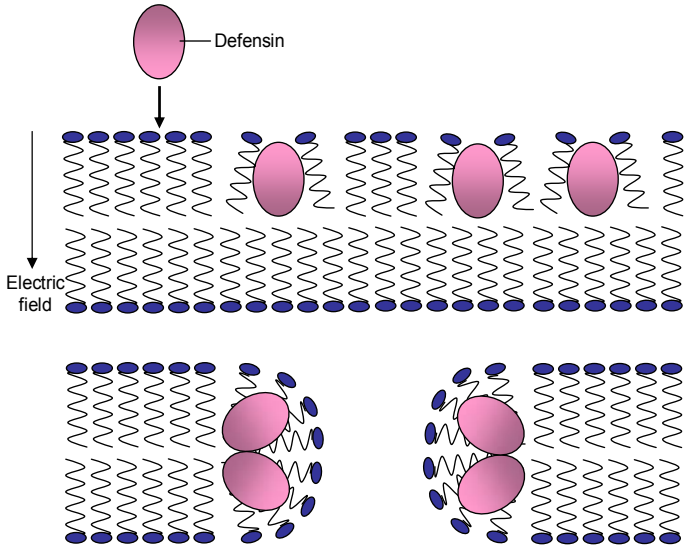


Figure 4: Model of antimicrobial activity of defensins. Defensins as amphipathic molecules have clusters of positively charged amino-acid side chains (pink) and hydrophobic amino-acid side chains (grey). Electrostatic attraction (by the negatively charged microbial phospholipid membrane) and the transmembrane bioelectric field draw the peptide molecules towards and into the membrane. The accumulation of the peptides leads to straining of the membrane and after a new arrangement to the formation of pores in the membrane (adapted from Ganz 2003).

When viruses attack cells, glycoproteins on both, the cell surface and on the virus, are spread apart which results in membrane fusion.

It was recently reported that the θ-defensins retrocyclin-1 and -2 as well as hBD-3 inhibited membrane fusion mediated by the viral hemagglutinin by erecting a protective barricade of immobilized surface glycoproteins (Leikina 2005; Owen 2004). While this does not necessarily kill the virus, cell attack is stopped and the viruses can be destroyed by other immune responses.

Additional activity spectrum of defensins

It is increasingly evident that defensins do not only act as endogenous antibiotics, but display also a range of other functions, including activities that are involved in regulating immune responses and inflammation (e.g., interaction with Toll-like receptors, chemotactic effects), wound repair, and sperm function (Hiemstra 2006; Kluver 2006).

Activities of β-defensins include:

- Induction of prostaglandin D_2 production in mast cells (Niyonsaba 2001)
- Degranulation of mast cells → histamine release (Niyonsaba 2001)
- Chemotactic activity regarding cells expressing the receptor CCR6 (e.g., immature dendritic cells and some CD8 T lymphocytes) (Yang 1999)
- Immunoadjuvant effects in mice (Biragyn 2001):
  Enhanced antitumour adaptive immune responses
  Murine β-defensin (mBD-2) induces cytokines and chemokines

Recently, Kougias *et al* (2005) detected neutrophil defensins and cathelicidins in high concentrations in atherosclerotic plaques in humans, which may imply a role as potential mediators of vascular disease. Preliminary results from *in vitro* and animal studies revealed that defensins are involved in lipoprotein metabolism in the vessel wall, favouring LDL and lipoprotein (a) accumulation and its modification in the endothelium. They also exhibit prothrombotic activity and inhibitory effects in angiogenesis. Cathelicidins on the other hand enhance endothelial proliferation and induce angiogenesis in animal models; further, they regulate endothelial cell apoptosis. These observations suggest an important role of defensins as a link between innate and adaptive immune responses.

1.4.2 Association of a dysregulated defensin expression with diseases

Recent studies describing clinical associations between diseases and defensin production suggest that these peptides have important and diverse functions.

Cystic fibrosis

The main cause of morbidity and mortality in cystic fibrosis (CF) patients is respiratory failure due to progressive destruction of the airways and lungs by recurrent infections and inflammation. In early childhood, the epithelial surfaces of the respiratory tract can be colonized by *S. aureus* and/or *Hemophilus influenzae*, which are eventually succeeded by *Pseudomonas aeruginosa* supporting the onset of progressive lung destruction. These events indicate a local defect in epithelial host defence, as the infection usually remains limited to the lung. Smith *et al* (1996) suggested that the local host defence impairment is caused by inhibited defensin activity through abnormally elevated salt concentrations in

epithelial cells, due to the defective transepithelial chloride transport. In a human bronchial xenograft model hBD-1 was shown to be directly affected, thus leading to the hypothesis by Goldman *et al* (1997), that increased pulmonary infections in CF might be also due in part to the inactivation of antimicrobial peptides. A primary defect in β-defensin production or secretion was not found when comparing bronchoalveolarlavage fluid from CF patients and healthy subjects, supporting the view that not the quantity but the quality i.e. functionality of hBD-2 was affected (Singh 1998). Despite this preliminary evidence, a role for defensins in CF pathogenesis remains speculative.

Psoriasis and atopic dermatitis

The skin is frequently exposed to bacterial attacks. However, patients with psoriasis (a non-infectious inflammatory skin-disease) rarely suffer from skin infections. Harder *et al* (1997a) hypothesized that lesional psoriatic skin might contain antimicrobial peptides (AMP) which served as a "chemical shield" that resists infections. After purification of AMPs from psoriatic-scale extracts they discovered the presence of hBD-2, HNP 1-3, lysozyme and other antimicrobial peptides (Harder 2005). Liu *et al* (1998) found increased amounts of hBD-2 mRNA in inflamed skin epidermal cells whereas keratinocytes from healthy skin areas did not express hBD-2. In contrast to psoriasis, patients with atopic dermatitis had lower amounts of the AMPs LL-37 and hBD-2, and these patients were frequently subject to skin infection by microorganisms such as *S. aureus*. This strain was able to colonize and infect skin from patients with atopic dermatitis but not patients with psoriasis or normal skin that had become inflamed from some other cause, probably due to the reduced production of AMPs in atopic disease (Ong 2002).

Gastrointestinal diseases

Increasing evidence has arisen in recent years that a dysregulated defensin expression correlates with diseases of the gut. Elson (1998) hypothesized, especially for Crohn´s disease, a dysregulated immune response of the mucosal immune system to commensal enteric bacterial antigens. Human α-defensins in intestinal epithelium are exclusively expressed by Paneth cells in the crypts of the small intestine (Fig. 5I) (Jones 1992; Ouellette 1997), but can be aberrantly produced in metaplastic Paneth cells in the colon (Fig. 5E) or stomach

(Fig. 5H) during inflammatory bowel diseases (Cunliffe 2001; Wehkamp 2002b; Fahlgren 2003).

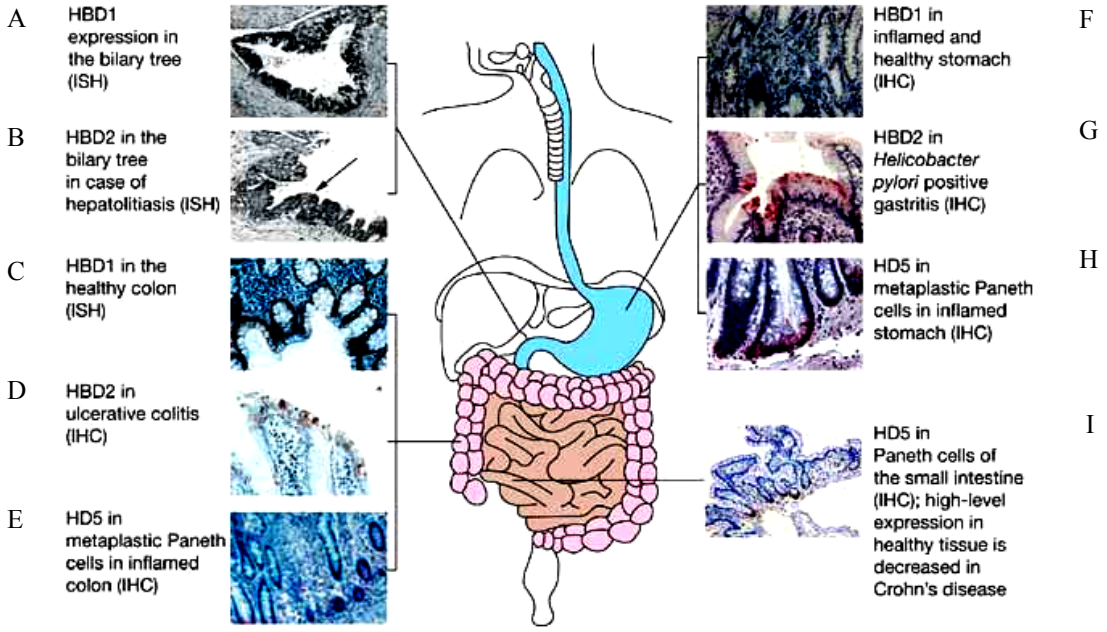


Figure 5: Expression of defensin peptide and mRNA at various sites of the GIT (Source: Wehkamp 2005) IHC, immunohistochemistry; ISH, in situ hybridization.

HBD-1 is constitutively expressed in almost all tissues in the gastrointestinal tract (Fig. 5A biliary tree, 6C colon, 5F stomach). In contrast, hBD-2 expression is induced by inflammation such as ulcerative colitis (Fig. 5D) or *H. pylori* induced gastritis (Fig. 5G) (O´Neil 2000; Wehkamp 2003a). Furthermore, Wehkamp *et al* (2003b) demonstrated an impaired hBD-2 and hBD-3 induction in Crohn´s disease in opposite to ulcerative colitis

Consequently, it has been hypothesized that a defect in the antimicrobial defence system of defensins might lie at the root of a persisting but slow bacterial invasion triggering the inflammatory process (Fellermann 2003).

1.5 Signalling pathways involved in the innate immune response

HBD-2 can be induced by two different activation pathways: Firstly, endogenous stimuli like proinflammatory cytokines (IL-1β, TNF-α, IL-22) which are mostly produced by macrophages and monocytes of the host. Secondly, exogenous stimuli such as *E. coli* bacteria where specific prokaryote motifs called PAMPs (pathogenic associated molecular

patterns) are recognized by cellular receptors. In the second case the so-called Toll-like receptors (TLRs), named for their homology to the drosophila toll protein, form the best-characterized family of mammalian PAMP receptors (Takeda 2003). They recognize a wide array of conserved microbial products, such as lipopolysaccharide (LPS), lipoprotein, peptidoglycan, unmethylated CpG motifs occurring in microbial DNA or bacterial flagellin (Takeda 2003; Akira 2001; Athman 2004) (Fig. 6).

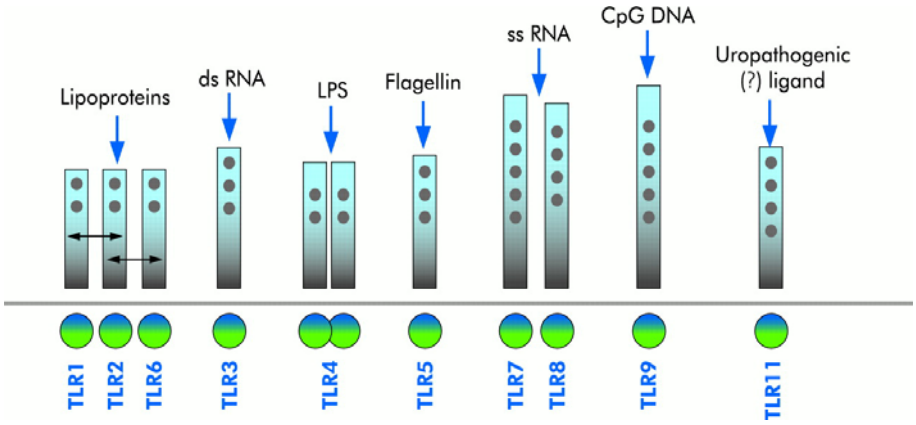


Figure 6: TLR ligand diversity. TLR1 to TLR11 have been identified and characterized to date. TLR1, 2, 4 and 6 have lipid ligands, TLR3, 7, 8 and 9 have nucleic acid ligands and TLR5 has a protein ligand. TLR11 recognizes uropathogenic bacteria as its ligand. The ligand for TLR10 is unknown. (Source: Cario 2005)

These prokaryote motifs are shared by both pathogenic and commensal bacteria, giving rise to the affirmation that either type of bacteria might trigger innate immune responses in IECs.

Certain TLR´s need adaptor proteins for proper signal transduction as in the case of TLR4 which forms a complex with MD-2, that is necessary for surface expression and LPS-regulated activation of TLR4 (Yang 2000). RP105 acts also as an LPS sensor in B cells and requires MD-2-related protein MD-1 for its surface expression (Ogata 2000; Miura 1998).

In addition, some TLR´s (e.g., TLR1, TLR6) act as coreceptors with other TLR´s (e.g., TLR2) (Ozinsky 2000). TLR´s have an intracellular domain that is homologous to the IL-1 receptor and known as TIR (Toll/IL-1R homology). Its activation results in the recruitment of the adaptor protein MyD88, activation of the IRAK family, TRAF6 and of TAK-1, which subsequently activates NF-κB through IκB kinases and is involved in the activation

of the AP-1 transcription family members Jun and Fos, by way of diverse MAP kinases (Aderem 2000).

The exact mechanisms by which inflammatory mediators upregulate hBD-2 have been intensively investigated. Cytokines and bacterial factors seem to induce hBD-2 expression by influencing the activities of protein kinase C (PKC), mitogen-activated protein kinases (MAPKs) as well as transcriptional factors e.g. the nuclear factor-κB (NF-κB) (Table 4).

Table 4: Transcriptional regulation of hBD-2 gene expression and involved MAPkinases

Cell type	Involved …	Not involved	Stimuli	Reference
Madin–Darby bovine kidney epithelial cells	NF-κB		Isoleucine	Fehlbaum, 2000
MKN45 epithelial tissue cells (stomach mucosal tumor cells)	NF-κB (p65/p65)	p50/p50 p65/p50	H. pylori	Wada, 2001
Gingival epithelial cells	p38, JNK	NF-κB	Fusobacterium nucleatum	Krisanaprakornkit, 2002
Human middle ear epithelial cells	ERK1/2 MAPK		IL-1α	Moon, 2002
Airway epithelial cells (LC2/ad)	NF-κB, AP-1, intracellular Ca^{2+}		LPS	Tomita, 2002
mononuclear phago-cytes (RAW264.7)	NF-κB	STAT, NF-IL-6, AP-1	LPS	Tsutsumii-Ishii, 2002
Corneal epithelial cells	NF-κB, JNK, p38	ERK1/2	IL-1β	McDermott, 2003
Alveolar epithelial adenocarcinoma cells	PKC, p38, JNK, PI3K, NF-κB	ERK1/2	IL-1β	Jang, 2004
Human airway epithelial cells	NF-κB	JAK	IL-17	Kao, 2004
Colonic epithelial cells (Caco-2)	NF-κB, AP-1, JNK	ERK1/2, p38	*E. coli* Nissle	Wehkamp, 2004

NF-κB is ubiquitously expressed and governs the expression of multiple genes encoding cytokines, chemokines, growth factors or some acute phase proteins. Several agents such as cytokines, oxidant free radicals and bacterial or viral products activate NF-κB. Inappropriate activation has been linked to inflammatory events associated with autoimmune arthritis, asthma and AIDS. In contrast, complete and persistent inhibition of NF-κB is associated with apoptosis, inappropriate immune cell development and delayed cell growth (Chen 1999).

In resting cells, NF-κB resides in the cytoplasm in an inactive form bound to an inhibitory protein known as IκB (Fig. 7). Upon cellular activation by extracellular stimuli, IκB is phosphorylated which induces the polyubiquitination of IκB and further degradation by the 26S-proteasome, thereby activating NF-κB, which subsequently translocates into the nucleus. NF-κB initiates early-response gene transcription by binding to decameric κB motifs, found in the promoter or enhancer regions of specific genes (Chen 1999).

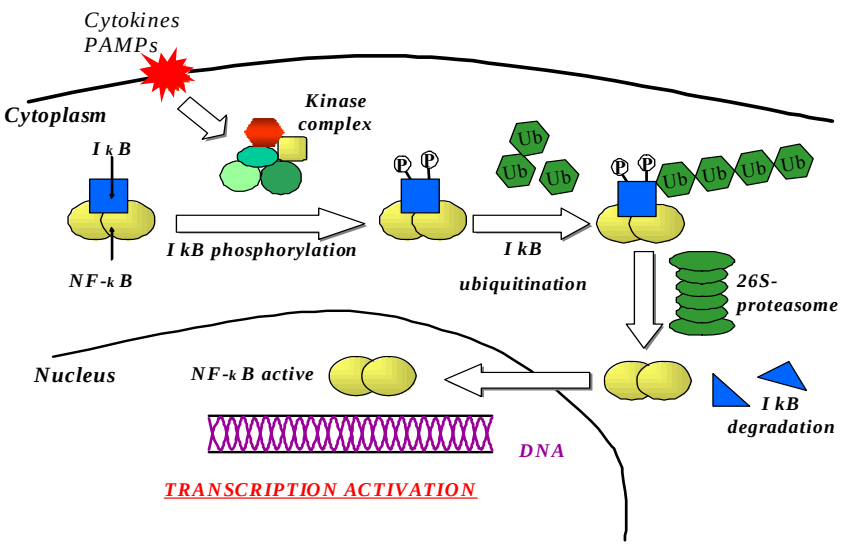


Figure 7: NF-κB activation (adapted from Figure 1, Makarov *et al.*, 2000)

NF-κB is regarded as a genetic switch, which controls early-response gene expression; the synergistic interaction of NF-κB with other transcription factors such as STAT, AP-1, or NF-IL-6 might also be required to achieve a purposeful induction of a particular gene. Although NF-κB binding sites have been identified in the promoter regions of many genes (including defensins) not all of them are upregulated by NF-κB in a given cell type under every stimulatory condition.

We have recently demonstrated that hBD-2 expression stimulated by the probiotic strain *E. coli* Nissle requires NF-κB and AP-1 but one cannot eliminate the possibility that further transcription factors are of relevance, too (Wehkamp 2004).

1.6 The *E. coli* strain Nissle and its characteristics

In 1916, Alfred Nissle attempted to implant non-lactic acid bacteria such as *Escherichia coli* for "causal fighting against pathological intestinal flora" (Nissle 1916). The administration of the probiotic *E. coli* Nissle has been reported since as an advantageous alternative treatment for remission maintenance in ulcerative colitis as this strain colonizes well (48 weeks after withdrawal bacterial DNA was still detectable in faecal samples) and is not killed by simultaneous mesalamine administration (Nguyen-Xuan 2006). Further, recent results state a clearly reduced duration of unspecific prolonged diarrhoea in infants and toddlers by administration of this strain with a treatment efficacy of higher statistical significance than any of the preceding studies with lactobacilli (Isolauri 1991, Guandalini 2000, Szajewska 2005). Despite the successful therapeutic applications of *E. coli* Nissle, only limited information is available about the beneficial traits contributing to the strains' probiotic character.

The absence of defined virulence traits (i.e. alpha-hemolysin, P-, M- and S-fimbrial adhesins), its non-invasiveness, the lack of invasion-associated type III protein secretion system and the missing toxin production (enterotoxins, cytotoxins, shiga-like toxins) contribute to *E. coli* Nissle´s safe character. Further, this bacterium exhibits many known fitness factors (Fig. 8) which promote its competitiveness and effective colonization of the host (Lodinová-Zádníková 1997, Nguyen-Xuan 2006). It secretes the microcins M and H47 (Patzer 2003), peptide antibiotics, that are insensitive to proteases and active against gram-negative bacteria. *E. coli* Nissle attaches to the gastrointestinal epithelium by means of specific fimbriae (type I, F1C, curli) which bind to glycoprotein on the epithelial cell surface. *E. coli* Nissle is able to produce a bacterial biofilm on the tissue surface by use of its cellulose synthesis genes, which form parts of the extracellular matrix. The biofilm might have influence on the signal transduction of pathogens by masking cellular receptors.

In addition to its main function in providing bacterial motility, the *E. coli* Nissle flagellum plays a role for adhesion and biofilm formation on the mucosa. About 50 genes are required for flagellar synthesis and function. The flagellar apparatus consists of several distinct proteins: a system of rings embedded in the cell envelope (the basal body), a hook-

like structure near the cell surface, and the flagellar filament. The basal body and hook anchor the whip-like filament to the cell surface.

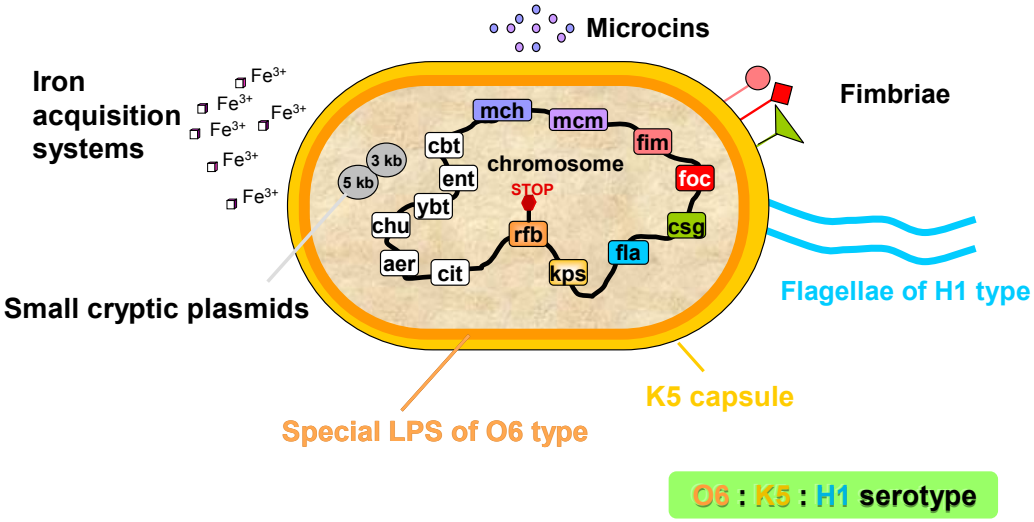


Figure 8: Characteristics of *E. coli* Nissle (fitness factors) Source: Ardeypharm

The availability of Fe (II) ions, an essential factor for bacterial growth, is limited in the mammalian host; therefore, the six iron-uptake systems (e.g., yersiniabactin, enterobactin or aerobactin) of *E. coli* Nissle contribute to its competitiveness amongst other bacteria in the GIT (Grozdanov 2004). *E. coli* Nissle has two specific small "cryptic" plasmids. These plasmids do not carry any genes other than those necessary for their own maintenance and transmission whereas plasmids generally carry genes, which increase the "fitness" of bacteria such as antibiotic resistance genes and support the survival of the bacterium (selection advantage). *E. coli* Nissle is serum sensitive in contrast to probiotic lactobacilli.

The strain exhibits an unusual lipopolysaccharide structure due to a mutation in the *wzy* gene which encodes the O6 antigen polymerase. The absence of this polymerase leads to a semirough phenotype (O6-sidechain contains only one repeating unit), which is responsible for its serum sensitivity (Grozdanov 2002) and might explain its apathogenic and immunostimulatory characteristics.

3 OBJECTIVES

The aim of the present study was, to investigate the mechanism that probiotic bacteria use to stimulate the chemical defence system of the human intestine. Furthermore, the missing link between the recognition of the bacterial ligand by its specific cell receptor and the further signalling pathway leading to the final defensin expression was a main focus of our investigations. Since *E. coli* Nissle (Mutaflor®) is a well-characterized probiotic agent in medicine, we were further interested to identify the hBD-2 inducing bacterial component of this strain.

Consequently, the goal of the current work was to investigate the following:

- Find a suitable *in vitro* model to study the defensin expression induced by probiotics
- Investigate the expression and transcriptional regulation of the human β-defensin 2 gene activated by different probiotic strains
- Elucidate the signalling pathways by which probiotics induce hBD-2 gene expression in Caco-2 cells
- Identify and isolate the unknown hBD-2 inducing factor of the probiotic strain *E. coli* Nissle

4 MATERIAL AND METHODS

4.1 Material

4.1.1 Chemicals and reagents

Agarose	Roth (Karlsruhe)
AG126	Calbiochem (Darmstadt)
Ampicillin	Sigma (Deisenhofen)
Bromphenolblue	Merck (Darmstadt)
Chloroform	Roth (Karlsruhe)
Complete, Mini, EDTA-free protease inhibitor cocktail tablets	Roche (Mannheim)
Coomassie brilliant blue R-250	Biorad (München)
DEPC	Roth (Karlsruhe)
DMSO	Sigma (Deisenhofen)
EDTA	Roth (Karlsruhe)
Ethidiumbromid	Sigma (Deisenhofen)
Ethylenediamintetraaceticacid	Sigma (Deisenhofen)
Fetal calf serum	Biochrom AG (Berlin)
Gentamicin-sulfate	Gibco (Karlsruhe)
Guanidinhydrochloride	Roth (Karlsruhe)
Interleukin-1ß	Sigma (Deisenhofen)
Isopropanol	Roth (Karlsruhe)
LPS of E. coli, Serotype O111:B4	Sigma (Deisenhofen)
Macroprep® CM Support	Biorad (München)
$(NH_4)_2SO_4$	Sigma (Deisenhofen)
SB203580	Tocris (Ellisville, MO, USA)
Skim milk powder	Fluka (Buchs, CH)
SP600125	Tocris (Ellisville, MO, USA)
SuperSignal® West Dura Extended Duration Substrate	Pierce (Rockford, IL, USA)
Tris-Base	Roth (Karlsruhe)
Triton-X 100	Sigma (Deisenhofen)
TRIzol Reagent	Invitrogen (Groningen, NL)
Tween-20	Merck (Darmstadt)
U-73122	Calbiochem (Darmstadt)
U-73343	Calbiochem (Darmstadt)
X-Gal	Roth (Karlsruhe)
Xylenecyanol	Merck (Darmstadt)

All non-listed chemicals were obtained from Sigma, Roth and Merck. Deionised water was used to prepare buffers and solutions.

4.1.2 General material

Product	Manufacturer
Combitips	Eppendorf (Hamburg)
Cryotube vials	Nunc (Roskilde, Denmark)
Dialysis tubing Servapor®	Serva (Heidelberg)
Gel blotting paper	Schleicher & Schuell (Dassel)
Luminometer tubes	Sarstedt (Nümbrecht)
Microcuvettes	Eppendorf (Hamburg)
Multiwell plates	Becton Dickinson (Heidelberg)
Nalgene® Cryo 1 °C Freezing Container	Nalge Nunc (Rochester, NY, USA)
Neubauer Counting cell chamber	Roth (Karlsruhe)
Nitrocellulosemembrane Protran BA 85	Schleicher & Schuell (Keene, USA)
NuPAGE® Bis-Tris SDS-PAGE	Invitrogen (Carlsbad, CA, USA)
Tissue culture flasks 75 cm²	Greiner (Frickenhausen)

4.1.3 General technical tools

Product	Manufacturer
Bio Photometer	Eppendorf (Hamburg)
Blotting chamber Trans-Blot® SD Semi-Dry Transfer Cell	Biorad (München)
Chemoluminescence camera CCD LAS-1000	Fuji (Tokyo ,Japan)
Electrophoresis chamber B 1A	Peqlab (Erlangen)
Geldrying apparatus Model 583	Biorad (München)
Heating block DB-2A	Techne (Duxford, UK)
Incubator	Heraeus (Stuttgart)
Luminometer Autolumat Plus	Berthold (Bad Wildbad)
Nitrogen tank Union carbide	Messer (Griesheim)
Optima™ MAX-E Ultra Centrifuge	Beckmann Instruments (Fullerton, USA)
Power supply Power Pac®	Biorad (München)
Precision balance AC 100	Mettler-Waagen GmbH (Gießen)
Thermomixer	Eppendorf (Hamburg)
Sterile working bench	Gelaire Flow Laboratories
Thermocycler MJ Research PTC-225	Biozym (Oldenburg)

UV-Emissiontable and camera for gel documentation	Cybertech (Berlin)
Victor^{TM} 1420 multilabel counter (photometer for 96 well plates)	Wallac (Turku, Finland)
Vortexer M2 Minishaker	IKA (Wilmington, NC, USA)
Waterbath HAAKE	Thermo Haake (Karlsruhe)
XCell SureLockTM Mini-Cell	Invitrogen (Groningen, NL)

All other non-listed material was standard laboratory equipment.

4.1.4 Standards

Product	Manufacturer
100 bp DNA-Ladder	Gibco (Karlsruhe)
1 kb DNA-Ladder	Gibco (Karlsruhe)
DNA Molecular Weight Marker VI	Roche (Mannheim)
DNA Molecular Weight Marker VIII	Roche (Mannheim)
Multi Mark Multi-Coloured Standard (Protein)	Invitrogen (Groningen, NL)
Sea Blue® Plus 2 Pre-Stained Standard (Protein)	Invitrogen (Groningen, NL)

4.1.5 Protein, enzymes and peptides

Product	Manufacturer
AMV Reverse Transcriptase	Promega (Mannheim)
BSA	Sigma (Deisenhofen)
HBD-2 peptide	Peptide Institute (Osaka, Japan)
MEM Non essential amino acids	Gibco (Karlsruhe)
Proteinase K	Qiagen (Hilden)
Restrictionenzymes Hind III, Sph I, Xho I, Xba I	New England BioLabs (Beverly, MA, USA)
Trypsin-EDTA	Gibco (Karlsruhe)

4.1.6 Antibodies

Primary and secondary antibodies

Antibody	Type	Source	Dilution
Anti H1 *E. coli*	Polyclonal rabbit	Statens Serum Institute, Copenhagen, Denmark	1:100
Anti H7 *E. coli*	polyclonal rabbit	T.K. Korhonen (General Microbiology, Faculty of Biosciences, FIN-00014 University of Helsinki, Finland)	1:5000
HRP-conjugated IgG	Anti-rabbit	Calbiochem	1:5000
HRP-conjugated IgG	goat anti-rabbit	Dianova, Hamburg	1:5000

4.1.7 Buffers and solutions for SDS-PAGE and Western blot analysis

Product	Ingredients		Manufacturer
Loading buffer	4 x NuPage LDS Sample Buffer		Invitrogen
Running buffer	NuPAGE® MES SDS Running Buffer		Invitrogen
Fixing solution:	Methanol	50 % (v/v)	
	Acetic acid	10 % (v/v)	
	H_2O	40 % (v/v)	
Coomassie blue staining solution:	50 % Methanol	50 % (v/v)	
	Coomassie brilliant blue R-250	0.05 % (v/v)	
	Acetic acid	10 % (v/v)	
	H_2O	40 % (v/v)	
Transferbuffer			Invitrogen
10 x TBS (Washbuffer)	Tris-Base	0.25 M	
	NaCl	1.4 M	
	KCl	27 mM	
	pH 7.4		
TBST:	10 x TBS	10 % (v/v)	
	Tween-20	0.1 % (v/v)	
Blocking buffer	TBST		
	Skim Milk powder	5 % (w/v)	

4.1.8 Reagents and solutions for isolation and analysis of RNA

Name	Ingredients	Concentration
10 x Formaldehyde-buffer	MOPS	200 mM
	Sodiumacetate	50 mM
	EDTA	10 mM
	pH 7.2 adjusted with acetic acid	
6 x RNA-Loadingbuffer	Bromphenolblue	0.25 % (w/v)
	Xylenecyanol	0.25 % (w/v)
	Saccharose	40 % (w/v)
	DEPC- H_2O	49.5 % (v/v)
50 x TAE-Buffer	Tris	2 M
	Eisessig	5.1 %
	EDTA	50 mM
Ethidiumbromidestock	Ethidiumbromide	0.5 µg/µl
	RNAse-free water	

4.1.9 Plasmids and oligonucleotides

Plasmids for transfection experiments or used as PCR standard are listed in Table 5.

Table 5: Plasmids

Plasmids	Origin
pNF-κB-luc	Clontech BD Biosciences (San Diego, USA)
pAP-1-luc	Clontech BD Biosciences (San Diego, USA)
pGL3-Basic Vector:	Dr. Jürgen Harder, Clinic of Dermatology, Kiel
hBD-2-luc	
hBD-2 NF-κB-mut1+2-luc	
hBD-2 NF-κB-mut1+2+3-luc	
hBD-2 AP-1-mut-luc	
hBD-2 AP-1+NF-κB-mut-luc	
phRG-TK (Renilla)	Promega (Madison, USA)

Oligonucleotides (Table 6) were ordered from MWG Biotech (Ebersberg) in HPSF-purified form, solubilized with the respective amount of nuclease-free water and stored at -20 °C. For the PCR the oligo´s were adjusted to a concentration of 0.5 µM and stored short-term between succeeding PCR runs at 4-8 °C.

Table 6: Oligonucleotides used in the present study

Name	mM $MgCl_2$	bp product	Sequence (5´→ 3´)	
hBD-2	3	172	for	ATCAGCCATGAGGGTCTTGT
			rev	GAGACCACAGGTGCCAATTT
GAPDH	3	360	for	CCAGCCGAGCCACATCGCTC
			rev	ATGAGCCCCAGCCTTCTCCAT
TLR-1	3	105	for	CAGTGTCTGGTACACTCATGGT
			rev	TTTCAAAAACCGTGTCTGTTAAGAGA
TLR-2	4.5	67	for	GGCCAGCAAATTACCTGTGTG
			rev	AGGCGGACATCCTGAACCT
TLR-3	6	82	for	CCTGGTTTGTTAATTGGATTAACGA
			rev	TGAGGTGGAGTGTTGCAAAGG
TLR-4	3	135	for	TGCTTCTTGCTGGCTGCATA
			rev	ATACGTCGGTCGTTCTTCGT
TLR-5	3	77	for	TGCCTTGAAGCCTTCAGTTATG
			rev	CCAACCACCACCATGATGAG
TLR-6	5.5	88	for	GAAGAAGAACAACCCTTTAGGATAGC
			rev	AGGCAAACAAAATGGAAGCTT
TLR-7	5.5	72	for	TTACCTGGATGGAAACCAGCTA
			rev	TCAAGGCTGAGAAGCTGTAAGCTA
TLR-9	3	151	for	GGACCTCTGGTACTGCTTCCA
			rev	AAGCTCGTTGTACACCCAGTCT
TLR-10	2	102	for	CTCCCTGGATGCAGTCATTT
			rev	AACTTCCTGGCAGCTCTGAA
MD-1	2	101	for	TAGCTCTCATGTCTCAAGGCTCAT
			rev	ATCTGCTCTCCTTTCCTTCTTCC
MD-2	2	99	for	ATTCCAAGGAGAGATTTAAAGCAATT
			rev	CAGATCCTCGGCAAATAACTTCTT

Name	mM $MgCl_2$	bp product	Sequence (5´→ 3´)	
MyD88	4	100	for	TGAGGGAGCCTAACCATGTC
			rev	TTGGTCCTTTCCAGAGTTTG
RP105	4	142	for	CTGGACTGCACTTGCTCGAATAT
			rev	CACAGGAAAGCTTTGACATCAGATAG
CD14	2	90	for	CGCTCCGAGATGCATGTG
			rev	TTGGCTGGCAGTCCTTTAGG

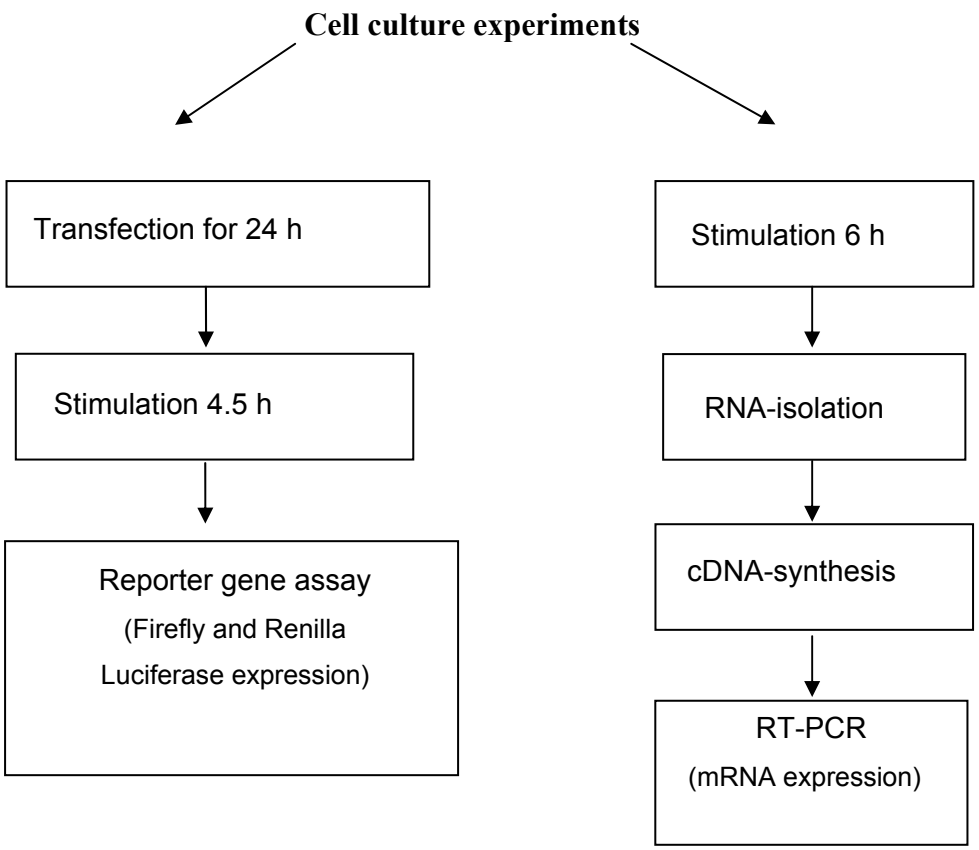
Table 7: PCR-programs for the different PCR targets

Oligonucleotides	Annealing/Melting temperature
hBD-2	62 °C/95 °C
GAPDH	66° - 60 °C/95 °C
TLR-1, -2, -3, -5, -6, -7 MyD88, RP105, CD14, MD1, MD2	68° - 60 °C/96 °C
TLR-4, -9 and -10	68° - 64 °C/95 °C

4.1.10 Commercial Kits

Product	Manufacturer
Dual Luciferase® Reporter Assay System	Promega (Madison, WI, USA)
Human IL-8 ELISA Kit II	BD Biosciences (San Diego, USA)
LightCycler-FastStart DNA Master SYBR Green I mix	Roche Diagnostics GmbH (Mannheim)
MTT Cell Proliferation Assay	ATCC (Manassas, VA, USA)
Quantum Prep™ Plasmid Midiprep Kit	Bio-Rad (Hercules, CA, USA)
QIAprep Spin Miniprep Kit	Qiagen (Hilden)
Reverse Transcription System	Promega (Madison, WI, USA)
VenorGeM® mycoplasma detection kit	Minerva Biolabs GmbH

4.2 Methods



4.2.1 Cell cultivation

Cellline	No.	Medium	Source
Caco-2	ACC 169	DMEM	DSMZ
T84	ATCC CCL-248	Ham's F12/DMEM	ATCC
SW480	ACC 313	RPMI 1640	Dr. Oelschlaeger, Würzburg
SW620	ATCC CCL-227	DMEM	ATCC

For studying the expression of the antimicrobial peptide hBD-2, we used four different human colonic adenocarcinoma cell lines. It is known that Caco-2 cells express the antimicrobial peptide α_1-antitrypsin (Perlmutter 1989; Molmenti 1993). This cell line has also been a useful tool to study functions of mature enterocytes. SW620 cells display high metastatic potential compared to SW480 cells with low metastatic potential.

4.2.1.1 Cell culture media

Cell line	Media
Caco-2	DMEM 25 mM HEPES
SW620	+ 10 % fetal calf serum (FCS)
	+ 50 µg /ml gentamicin
	+ 1 % non essential amino acids
T84	DMEM/Ham´s F12 1:1
	+ 15 mM HEPES
	+ 14 mM $NaHCO_3$
	+ 5 % fetal calf serum
	+ 100 U/ml penicillin
	+ 100 µg/ml streptomycin
SW480	RPMI
	+ 10 % fetal calf serum
	+ 2 mM glutamine
	+ 14 mM $NaHCO_3$
	+ 100 U/ml penicillin
	+ 100 µg/ml streptomycin

Cultivation was carried out in a humidified atmosphere at 37 °C and 5% CO_2/95 % O_2. Cells were subcultured weekly and split with a density of 1:10 or 1:20 according to need. Cells were used between passages 5 and 35. To detect any contamination with mycoplasma, which could be disguised by the use of antibiotics we used the VenorGeM® mycoplasma detection kit. This test is based on a fast and very sensitive detection of mycoplasma-DNA in biological samples via PCR. Cells were prepared for this test by cultivation for 2 weeks without any antibiotics and further prepared as mentioned in the supplier's protocol.

4.2.1.2 Treatment of cells with stimulants

For stimulation experiments, cells were seeded at a density of 2.8 x 10^5 cells/4 cm² in 12-well culture plates (Becton Dickinson GmbH). Cells grown to ~70 % confluence on culture wells were incubated with serum- and antibiotic-free medium for at least 12 h to eliminate serum-induced IL-8 release or hBD-2 expression and prevent any influence of antibiotics on the immune response. Cells were stimulated at 37 °C for the specified times.

4.2.1.3 Cell differentiation

We investigated in Caco-2 cells whether the expression of hBD-2 is influenced by the degree of differentiation. This cell line, differentiating spontaneously in culture (Rousset 1985) was grown for 1, 2, 3, 6, 9 or 12 days to achieve different stages of differentiation (Table 8) before stimulation with 5 ng/ml IL-1β for 6h after an overnight incubation in FCS-/AB-free medium. The culture medium was changed every 2 days during the growth phase. The expression of hBD-2 and GAPDH was determined via RT-PCR.

Table 8: Differentiation grade (Source: Bernet-Camard 1996)

Days of growth	Characteristics
Up to 5 days (2-3 days in our experiment)	Confluence, undifferentiated state
Up to 7 days (4 days ")	Postconfluence, differentiation starts
Up to 10 days (8 days ")	Differentiation develops
Up to 15 days (12 days ")	Differentiation complete

4.2.2 Preparation of human PBMC´s

Since leukocytes contain a number of Toll-like receptors they served as a positive control in comparison to the TLR expression in the epithelial cell lines. PBMC´s were isolated from whole blood by Ficoll-density-gradient-centrifugation. After centrifugation at 400 x g for 25 min (without brake) the interphase was carefully collected and diluted 1:1 with PBS. Cells were centrifuged for 5 min at 400 x g and 4 °C. Supernatant was removed, PBMC-pellets were dissolved in PBS with protease inhibitor cocktail (Complete Mini, EDTA-free) and stored at -20 °C. RNA was isolated from the cells, reverse transcribed into cDNA and the expression of TLR´s and adaptor molecules determined.

4.2.3 TLR spectrum

Caco-2, T84, SW480 and SW620 cells were cultured until confluency and RNA was isolated. For the detection of the different TLR´s and adaptor proteins PCR programs (Table 7) were established. $MgCl_2$ concentration was tested from 2, 3, 4 to 5 mM for each primer pair (Table 6) and annealing temperatures adjusted to measure several TLR´s in one run. We further determined, whether TLR expression could be induced by stimulation of Caco-2 cells with IL-1β (5ng/ml) for 6 h. The PCR data was evaluated qualitatively by ethidium

bromide staining of the PCR products run on a 2 % gel for 1 h. Further, the melting curves were analysed to test for specificity of the oligonucleotides.

4.2.4 Bacteria cultivation

Bacteria strains used in the present study are shown in Table 9. For cultivation 10 ml of sterilized Trypticase soy broth (TSB) (Table 10) was inoculated with 1 pearl of bacteria from the frozen stock and grown overnight with shaking at 200 rpm at 37 °C. To obtain bacteria in a linear growth phase, 100 µl of the bacterial suspension was added to 10 ml of fresh TSB medium and grown with constant shaking for 5 h. Preliminary studies by Wehkamp *et al* (2004) showed that living bacteria induced hBD-2. However, using viable bacteria would have the disadvantage of overgrowth in the cell culture wells; hence, we decided to heat-kill the bacteria to maintain stable culture conditions and bacterial concentrations. Heat-inactivation was carried out in a water bath at 65 °C for 1h. Bacterial culture supernatants were collected by centrifugation at 4000 × g for 10 min. The bacteria pellet was washed with PBS, and after another centrifugation step adjusted to 3×10^8 cells/ml. Since this cell number corresponds to an optical density (OD) of 0.3, we controlled the concentration of bacteria by absorption measurements with a spectrophotometer at 595 nm. The supernatant was diluted by the same dilution factor as the bacteria in FCS- and antibiotic-free cell culture medium. The bacteria pellet and supernatant were stored at -20 °C until use.

Lactobacilli and *Pediococcus* are facultative anaerobes (or facultative aerobes) defined as organisms that can switch between aerobic and anaerobic types of metabolism. Under anaerobic conditions, they grow by fermentation or anaerobic respiration, but in the presence of O_2 they switch to aerobic respiration. *Lactobacilli, Pediococcus* and *Leuconostoc* were cultivated according to De Man, Rogosa and Sharpe in MRS Broth (Table 10) for 18-24 h in a CO_2 enriched atmosphere in an anaerobic jar. The anaerobic environment was generated by placing an AnaeroGenTM sachet (OXOID, Basingstoke, Hampshire, England) in the jar, which resulted in a rapid absorption of oxygen and simultaneous generation of CO_2. In the stationary phase, 300-500 µl of bacteria were transferred into fresh media and grown for another 5-7 h depending on the individual growth rate. The bacteria were heat-inactivated, washed and adjusted to the same cell concentration as mentioned above. Since

the light absorption varies with the geometry of the dispersing particle size of the microorganisms, different OD values are obtained between cocci and rods. Therefore, we had to calculate which OD refers to the bacterial cell number of 3 x 10^8/ml for each lactobacilli strain. Bacteria were diluted and 10 µl were added onto a Fuchs-Rosenthal counting chamber (depth: 0.2 mm; size: 0.0625 mm^2). The obtained cell number was multiplied with the factors 64 and 312.5.

Table 9: Bacteria strains. EcNΔ strains are *E. coli* Nissle strains with deletions of the traits listed below.

Strain	Serotype[c]	Characteristics/Isolatetype	Source
E. coli Nissle 1917 (DSM 6601)	O6:K5:H1	Apathogen, pharmaceutical strain	ACS
EcNΔ*HPI*		Yersiniabactin-gene cluster	1
EcNΔ*fim*		type 1 pili (=fimbriae)	
EcNΔ*foc*		F1C pili (=fimbriae)	
EcNΔ*fim*Δ*foc*		type 1 pili + F1C pili	
EcNΔ*csgBA*		curli (= fimbriae)	
EcNΔ*bcs*		cellulose-synthesis-gene cluster	
EcNΔ*mcmDAB*		microcin	
EcNΔ*K5*		capsule 5–gene cluster	
EcNΔ*c*		plasmid	
EcNΔ*fliA*		sigma factor of flagella genes	
EcNΔ*fliC*		flagellin filament protein	
EcNΔ*flgE*		hook	
EcNΔ*fliCpDB2*		complementant of fliC	
EcNΔ*flgEpDB3*		complementant of flgE	
E. coli JM109 ATCC 53323	Orough:H48	Control strain	
CFT073Δ*hly*–CFT073 WT	O6:K2:H1	Uropathogenic *E. coli* with deleted haemolysin gene	1
E. coli PZ720	O83:K24:H31	Probiotic strain	ACS
E. coli PZ830	O4:H-	Fecal isolate	ACS*
E. coli ATCC 25922		Clinical isolate, Reference strain	KaE
E. coli K12 (DSM498)		Reference strain	DSMZ
E. coli K12 (DH5α)		Reference strain	DSMZ
Salmonella enterica serovar Enteritidis		Intestinal isolate	2
One Shot® Top 10F´ *E. coli*		Competent cells for transformation	Invitrogen

Strain	Strain designation	Isolatetype	Source
L. acidophilus	PZ 1030 (DSM 20079)	Reference strain	ACS (DSMZ)
L. acidophilus	PZ 1041	Paidoflor®	ACS
L. acidophilus	PZ 1129	Industrial probiotic strain	ACS
L. acidophilus	PZ 1130 (JCM 1132)	Reference strain	ACS (GR)
L. acidophilus	PZ 1138	Industrial probiotic strain	
L. gasseri	PZ1144 (DSM 20243)	Reference strain	ACS (DSMZ)
L. gasseri	PZ 1160	Intestinal isolate	ACS
L. fermentum	PZ 1162	Intestinal isolate	ACS
L. plantarum (2592)	LMG P-20606	Growing rye	24a, BCCM
Leuconostoc mesenteroides (77:1)	LMG P-20607	Growing rye	24a, BCCM
Pediococcus pentosaceus (16:1)	LMG P-20608	Intestinal isolate	24a, BCCM
L. paracasei subsp. paracasei (F19)	LMG P-17806	Growing rye	24b, BCCM
VSL#3		Industrial probiotic mixture	3

ACS, Ardeypharm Collection of Strains, Herdecke *, strain obtained from A.M. Snelling and P. Hawkey, Department of Microbiology, University of Leeds, United Kingdom

GR, strain collection of G. Reuter (strain deposited by Mitusoka at the Japanese Collection of Microorganisms)

BCCM, Belgian Coordinated Collection of Microorganisms

DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig

KaE, Collection of strains, Klinik am Eichert, Göppingen

1 Collection of strains University of Würzburg

2 Patient isolate from the Robert Bosch Hospital, Stuttgart

3 sigma-tau Arzneimittel GmbH, Düsseldorf

[c] Serotyping of strains was performed by S. Aleksic, Institute of Hygiene, Hamburg. H-, no H antigen (cells nonmotile).

For testing dose dependency, bacteria were used at concentrations between 10^6 and 10^9 bacteria/ml. Bacteria supernatant was used undiluted or was adjusted with DMEM (--+) to the following dilutions 1:2, 1:4, 1:10, 1:100 and 1:1000.

Table 10: Bacteria culture media and solutions

Bacteria	Media	Source
TOP 10F´ competent *E. coli*	Luria Bertani (LB)	Sigma
TOP 10F´ competent *E. coli*	SOC medium	Invitrogen
TOP 10F´ competent *E. coli*	X-Gal:	Roth
	40 mg/ml in dimethylformamide	
Lactobacilli (anaerobic growth)	MRS Broth:	Fluka (Buchs, CH)
	51 g MRS/L distilled water	
	0.1 % Tween 80	Fluka
E. coli, Salmonella	TSB: pH 7.3	
	1.7 % Peptone from casein	Roth
	0.3 % Peptone from soy	Roth
	0.25 % Glucose	
	0.5 % NaCl	
	0.25 % Dipotassiumhydrogen-phosphate	

4.2.5 Molecularbiological methods

4.2.5.1 RNA isolation and cDNA synthesis

At the end of the stimulation experiments, cells were washed twice with phosphate-buffered saline (PBS), lysed with 500 µl TRIzol reagent/4 cm² well surface for 5 minutes, transferred to a tube and frozen at -80 °C. RNA was isolated according to the supplier's protocol. The RNA pellet was dissolved according to its size in 15-25 µl DEPC-H_2O. RNA quality and quantity were determined by gel electrophoresis and photometry. Subsequently 1 µg of total RNA was reverse transcribed into cDNA with oligo (dT) primers and 15 U/µg AMV Reverse Transcriptase according to the manufacturer's protocol (Promega).

4.2.5.2 Real-time RT-PCR

Real-time reverse transcription PCR (RT-PCR) analyses were performed in a fluorescence temperature cycler (LightCycler; Roche) according to the manufacturer's instructions. This

technique continuously monitors the cycle-by-cycle accumulation of the fluorescently labelled PCR product. As a template, cDNA corresponding to 10 ng of RNA in a 10 µl reaction mixture containing the specific concentration of $MgCl_2$, 0.5 µM of each primer and 1 × LightCycler-FastStart DNA Master SYBR Green I mix was used.

Samples were loaded into capillary tubes and placed in the fluorescence thermocycler (LightCycler). Initial denaturation at 95 °C for 10 min was followed by 45 cycles, each cycle consisting of 95 °C for 15 s, the primer-specific annealing temperature (Table 7) for 5 s and 72 °C for 15 sec. At the end of each run melting curve profiles were obtained by cooling the sample to 65 °C for 15 s and then heating slowly at 0.20 °C/s up to 95 °C with continuous measurement of fluorescence to confirm amplification of specific transcripts. Cycle-to-cycle fluorescence emission readings were monitored and analyzed using LightCycler software (Roche Diagnostics GmbH).

The specificity of the amplification products was verified by subjecting the amplification products to electrophoresis at 100 V for 1 h on a 2 % agarose gel containing ethidium bromide. All quantifications were normalized to the housekeeping gene GAPDH. Relative expression is given as a ratio between target gene expression and GAPDH gene expression.

4.2.5.3 Calculation of the mRNA copy number

For the quantitative evaluation of the hBD-2 gene expression by Real Time PCR the calibration with DNA-solutions of known copy number was necessary. Therefore, the concentration of isolated plasmid-DNA (QIAprep Spin Miniprep Kit) was measured at 260 nm in the photometer and adjusted to 1 ng/µl and the copy number in the plasmid-solution was calculated using the following formula:

$$\text{Plasmid copy number (copies/ml)} = \frac{A \times 6.023 \times 10^{23}}{(B + C) \times D \times 2}$$

A, plasmid concentration calculated by 260 nm absorbance (g/ml); B, basepairs of plasmid vector; C, basepairs of insert; D, average molecular weight of desoxy-nucleosidemonophosphates (NMP´s): 330 g/mol

Molecular weight of the vector:

Basepairs (bp) of vector pCR-Blunt II-TOPO:	3519
bp of insert hBD-2:	172
bp of vector with insert:	3691
bp of double-strand vector + insert:	7382
Molecular weight of double strand vector + insert:	330 g/mol x 7382
	= 2436060 g/mol

Amount of substance [mol] of the stock solution of hBD-2:

Spectrophotometric measurement of hBD-2 DNA-concentration:	1 ng/ml
1 mol double strand vector + insert:	2436060 g
1 g double strand vector + insert:	4.104989×10^{-7} mol
1 ng double strand vector + insert:	4.104989×10^{-16} mol

Conversion of the amount of substance to the amount of particles [= copy number]:

1 Mol = 6.023×10^{23} particles [Avogadro constant]

4.104989×10^{-16} mol = 2.472435×10^{8} copies/ng

The stock solution of the clone hBD-2 contains 1 ng/µl DNA, which matches ~ 2.47×10^{8} copies/µl. For the creation of standard curves, dilution series were made starting from 1 ng plasmid DNA (10^{1} to10^{7} copies). In each run, two samples of the standard curve were included as a control. To avoid variations in DNA-concentrations by repeated freezing and thawing of the standard samples, 5-10 µl aliquots for all dilutions were prepared, thawed once and then stored at 4 °C.

4.2.5.4 Chemical transformation and isolation of plasmid-DNA

For the transformation of competent *E. coli* with plasmids for transfection or the utilization as standards for the PCR, 1 µl plasmid was added to 1 vial of competent *E. coli* Top 10 F´ cells and incubated for 15-30 min on ice. Then cells were heat-shocked for exactly 30 sec in a water bath at 42 °C and immediately placed on ice for 2 minutes. 250 µl of SOC medium was added and tubes shaken for 1 h at 37 °C. 25 µl of the transformation reaction was plated on LB agar plates containing 50 µg/ml ampicillin and 40 µg/ml X-Gal in di-

methylformamide. After incubation overnight at 37 °C, recombinants were identified by blue/white colour screening. White colonies were picked and grown for about 14 h in 40 ml (for transfection) or 5 ml (for PCR) LB medium (containing ampicillin). Plasmid DNA was purified according to the manufacturer's protocol (Quantum Prep™ Plasmid Midiprep (transfection) or QIAprep Spin Miniprep Kit (PCR)) and eluted in Tris-Cl or DNAse free water. DNA concentration was determined photometrically at a wavelength of 260 nm (extinction maximum of nucleic acids). Contamination was identified by a low ratio of the 260 nm/280 nm value; pure samples should have a ratio between 1.8 and 2.0.

4.2.5.5 Restriction enzyme analysis and agarose gel electrophoresis

0.5 µg of plasmid-DNA was digested by restriction enzymes as described by the manufacturer (New England BioLabs). For hBD-2 pGL3 Hind III + Xho I was used, for NF-κB Hind III + Xba I and for AP-1 Hind III + Sph I. After at least 2 h of incubation at 37 °C the digested DNA was analyzed in 0.8 % agarose gels prepared in 1 x TAE buffer and containing 5 % ethidium bromide. Prior to electrophoresis, DNA was mixed with six fold concentrated loading buffer. Electrophoresis was performed in 1 x TAE buffer at a constant voltage of 90 V for 1.5 h. The DNA in the agarose gels was visualized using ultraviolet light (302 nm) and photographed subsequently with a videodocumentation system (Cybertech, Berlin).

4.2.5.6 Luciferase reporter gene assay

To assess hBD-2 promoter activity, Caco-2 cells were seeded into 12-well culture plates (2.8×10^5 cells/ml/well) and transfected upon 70 % confluency. The luciferase reporter constructs for hBD-2-2338-luc, NF-κB-mut1+2-luc, NF-κB-mut1+2+3-luc, AP-1-mut-luc and AP-1+NF-κB-mut-luc were kindly provided by Dr. Jürgen Harder (University of Kiel) and were constructed as described by Wehkamp *et al* (2004). Cells were transfected with 0.5 µg hBD-2 reporter plasmid and 0.05 µg of an internal control *Renilla* luciferase expression plasmid (phRG-TK) by using 1 µl of FuGENE 6 transfection reagent (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. 24 h after transfection, cells were treated for 4.5 h with various stimuli.

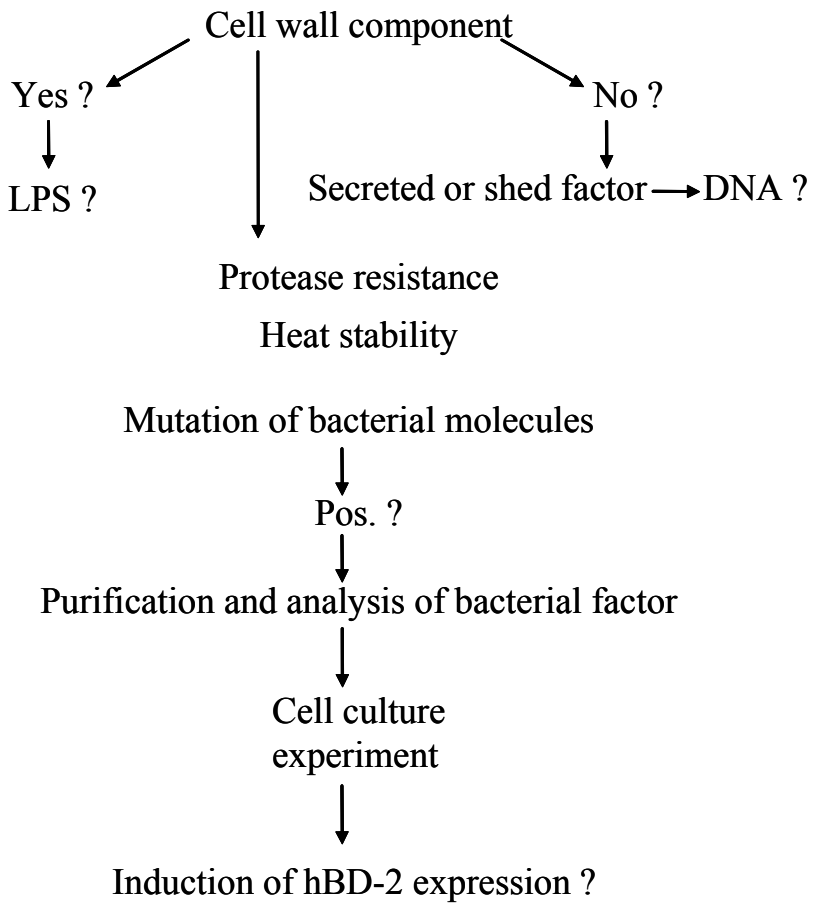
For signalling pathway studies mitogen-activated protein (MAP) kinase inhibitors AG126, SB203580 and SP600125 were used and resuspended in dimethyl sulfoxide (DMSO).

Cells were pre-treated with the specific ERK ½, p38 and JNK inhibitors 1 h prior to stimulation. At the end of the experiment, cells were washed with PBS and 250 µl of passive lysis buffer (Promega) was dispensed into each well. The culture plate was placed on a rocking platform for 15 min and the lysate transferred to a tube for the storage at -80 °C. Before the measurement of luciferase activities, the lysate samples were cleared by centrifugation for 1 min at 10 000 rpm in a refrigerated microcentrifuge. Firefly luciferase activity from the hBD-2-pGL3, NF-κB or AP-1 reporter vector and *Renilla* luciferase activity were analyzed as described for the Dual-Luciferase® Reporter Assay System Kit using a luminometer. Promoter activity was determined as a ratio between Firefly and *Renilla* luciferase activities. Means were normalized to basal unstimulated luminescence of controls, set at 1. In MAPkinase studies or bacteria mutant studies the bacterial supernatant was set at 100 and the inhibited or mutant stimuli were adjusted to this value in %.

4.2.5.7 Cell viability test

To confirm that pharmacological inhibitors were not toxic for Caco-2 cells, cell viability was assessed using the MTT Cell Proliferation Assay, which measures the mitochondrial function. Briefly, metabolically active cells reduce the yellow tetrazolium MTT to the purple formazan product by dehydrogenase enzymes. Caco-2 cells were seeded into a 96 well plate with a density of 0.35×10^5 cells/well. 24 h later cells were incubated in the presence of MAP kinase inhibitors, DMSO or anti-inflammatory drugs for 6-12 h. Then media was removed and DMEM (--+) was added to the cells. After 24 h of incubation 10 µl of MTT (10 mg/ml) was added to each well for a 2 h period. After the formation of formazan crystals 90 µl lysisbuffer/well was added and cells were lysed overnight at room temperature under soft shaking packed into aluminium foil to protect them from light. The absorbance was measured at 550 nm using the photometer Victor. It has been shown previously that viable cell numbers correlate with optical density, as determined by the MTT assay (Twentyman 1987).

4.2.6 Methods to determine the hBD-2 inducing factor from *E. coli* Nissle



4.2.6.1 LPS from *E. coli* Nissle

LPS from *E. coli* serotype O111:B4 (Sigma-Aldrich) and highly purified LPS isolated from *E. coli* Nissle kindly provided by Dr. Zähringer (Research Center Borstel) were diluted in FCS-/ and AB-free DMEM and adjusted to different concentrations (0.1, 0.5, 1 and 5 µg/ml) and Caco-2 cells were incubated for 6 h with these endotoxins.

4.2.6.2 DNA isolation from bacteria

For isolation of bacterial DNA, Protocol D on p. 53 of the QIAamp® DNA Mini kit manual (Qiagen) and continued as described for the DNA isolation from tissue, was used. To increase the DNA concentration in the obtained DNA yield, the DNA was precipitated with ethanol, frozen at -80 °C, centrifuged and the pellet dissolved in Tris-Cl buffer. Synthetic oligodeoxynucleotides (ODN) with immunostimulatory CpG-motifs were commercially synthesized by MWG Biotech (Ebersberg) in a phosphothioate protected form. The phos-

phothioate modification replaces 1 of the 2 nonbridging oxygens of the internucleotide phosphate with sulphur, thus preventing nuclease activity and extending greatly the half-life (Crooke 1995). The sequence of ODN-2006 GC (negative control) differs from ODN-2006 (positive control) by the methylated cytosines at positions 2, 5, 13 and 21 (see below). With hBD-2 transfected Caco-2 cells were stimulated with *E. coli* Nissle DNA, ODN-2006 and ODN-2006 GC and hBD-2 promoter activation was monitored after 4.5 h.

Name	Sequence (5´→ 3´)
ODN-2006	5´-TCGTCGTTTTGTCGTTTTGTCGTT-3´
ODN-2006 GC	5´-T**G**CT**G**CTTTTGT**G**CTTTTGT**G**CTT-3´

Reference: Bauer 2001

4.2.6.3 Heat stability test

To characterise further the hBD-2 inducing factor, *E. coli* Nissle supernatant was heated for 20 min at 100 °C. TSB was treated accordingly as a negative control. Non-boiled, boiled bacteria supernatant and TSB were added to Caco-2 cells for 6 h to observe whether the heat treatment had any deteriorating effect on hBD-2 inducing capacity.

4.2.6.4 Protein digestion in bacterial culture supernatant

Bacterial supernatant diluted to the appropriate concentration in DMEM was subjected to 10 µl proteinase K (>600 mAU/ml)/100 µl supernatant or an aliquot of TSB/DMEM as a control for effects of proteinase K for 1 h at 56 °C. To terminate further proteolysis the suspension was heated for 20 min at 100 °C. Protein content was determined in treated and non-treated bacterial supernatant, which was then placed onto Caco-2 cells for 6 h.

4.2.6.5 Construction of bacteria mutants

The deletion of specific bacterial genes from *E. coli* Nissle was carried out by Artur Altenhoefer (University of Würzburg). The *isogenic* mutant strains Δ*HPI*, Δ*fim*, Δ*foc*, Δ*fim*Δ*foc*, Δ*csgBA*, Δ*bcs*, Δ*mcmDAB*, Δ*K5*, Δ*c*, Δ*fliA*, Δ*fliC* and Δ*flgE* (see Table 8) were constructed according to a method previously described (Datsenko 2000). The single gene clusters were replaced by a *cat* antibiotic resistance cassette, which was part of a PCR product,

generated with plasmid pKD3 as the template and the respective primer pair; here illustrated for the flagellin mutants:
p1 (5´-AAGATAGCGGCTTAATGGCCGTGTGTAGGCTGGAGCTGCTT-3‘) and p2 (5´-CGTTCATCCCTGGGGGCTATCCCATATGAATATCCTCCTTAGTTCCTA-3‘) for Δ*fliA*, p3 (5´-GGCAATTTGGCGTTGCCGTCAGGTGTAGGCTGGAGCTGCTT-3‘) and p4 (5´-ACGGCGATTGAGCCGACGGGTGCATATGAATATCCTCCTTAGTTCCTA-3‘) for Δ*fliC* or p5 (5´-ACGCTGGATCTCGGCACTTACGGTGTAGGCTGGAGCTGCTT-3‘) and p6 (5´-TGTTGATTCAGCGTCTGGCTGGCATATGAATATCCTCCTTAGTTCCTA-3‘) for Δ*flgE*.

The construction of the microcin-negative (Δ*mcmDAB*) and the fim- and foc-negative mutants was described by Altenhoefer *et al* (2004). EcNΔ*c* (Nissle 1917 cured of both plasmids) was generated according to the German Patent Nr. 103 28 669. From the resulting chloramphenicol resistant *E. coli* Nissle 1917 derivate the *cat* cassette was not deleted after transformation for better selection of the derivates.

The flagellin mutant *E. coli* Nissle 1917 Δ*fliC* and Δ*flgE* strains were complemented with a PCR-product of the lacking gene cloned into the pGEM®-T Easy Vector (Promega, Madison, USA), corresponding complementants were labelled Δ*fliC*pDB2 and Δ*flg*EpDB3. The primers used for PCR corresponded to those for the deletion method in the underlined sequences. All mutant and complemented strains were tested for their ability to induce hBD-2 expression either regarding promoter activation or mRNA expression as described above.

Swarm Plate Assay

To evaluate the effect of the deleted flagellin genes on bacterial motility, swarm plate assays were performed by Artur Altenhoefer (University of Würzburg). Swarm agar (0.3 %), containing 10 g NaCl, 5 g yeast extract, 10 g peptone and 3 g agar, was used to evaluate the motility of *E. coli* Nissle wild type, Δ*fliC*, Δ*flgE*, Δ*fliA* and complemented Δ*fliC*pDB2 and Δ*flgE*pDB3 strains. Small wells were punched out in semisolid LB agar plates, loaded with 5 µl of each strain and incubated for 10 h at 37 °C.

Western blot of whole bacteria

Heat killed bacterial samples from overnight cultures of *E. coli* Nissle wild type, Δ*fliC*, Δ*flgE*, Δ*fliA* and complemented Δ*fliC*pDB2 and Δ*flgE*pDB3 were used to analyse flagellin expression by the Western blot technique, which was kindly performed by Artur Altenhöfer (University of Würzburg). Briefly, the resultant sample was separated in a Bio-Rad Modular Mini Electrophoresis System (Hercules, CA, USA) on a 12.5% polyacrylamide gel and then transferred to a 0.2 µm nitrocellulose membrane (Bio-Rad, München). After blocking of unspecific binding sites the blot was incubated overnight at 4 °C with an anti-H7 *E. coli* rabbit polyclonal antibody diluted 1:5000 in blocking buffer under gentle agitation. Following several washing steps with TBS, the blot was incubated for 1h with horseradish peroxidase (HRP)-conjugated anti-rabbit Ig (Calbiochem) diluted 1:5000 in blocking buffer. HRP was revealed with ECL light detection reagents (Amersham Biosciences, Buckinghamshire, UK) and exposed to film for 60 s (Kodak, Rochester, N.Y., USA).

4.2.6.6 Purification of flagellin

Flagellin from diverse bacterial strains were isolated as previously described (Ogushi 2001). Briefly, 1 liter of TSB was inoculated with 10 ml of the bacteria and cultured for 16 h at 37 °C, pelleted by centrifugation (4400 x g, 30 min, 4 °C) and resuspended in 20 ml of PBS. The suspension was adjusted to pH 2 with 1 M HCl and maintained at that pH with constant stirring at room temperature for 30 min. After centrifugation (100 000 x g, 4 °C, 1 h) the pH of the supernatant, containing soluble monomeric flagellin, was adjusted to 7.2 with 1 M NaOH. Solid $(NH_4)_2SO_4$ was added slowly with constant stirring to obtain 65 % saturation. After an overnight incubation at 4 °C, the precipitate was pelleted by centrifugation (15000 x g, 4 °C, 15 min). The precipitate was dissolved in 1-3 ml of distilled water and transferred to a dialyse tubing (MWCO 12.000 – 14.000, pore diameter ~ 25 Å) for concentration and desalting of the protein solution.

For dialysis, the tubing was placed in a vessel with 1-3 liter of distilled water. Under constant stirring, the solution was dialysed for 16 h at 4 °C with three water changes. To remove heat labile proteins the dialysate was heated at 65 °C for 15 min, placed on ice and centrifuged (100 000 x g, 4 °C, 1 h). 0.7 M solid $(NH_4)_2SO_4$ was added to the supernatant, which contained depolymerised flagellin. After incubation overnight at 4 °C, polymerized flagellin was collected by centrifugation (100 000 x g, 4 °C, 1h) and dissolved in PBS.

The concentration of the isolated protein was determined using the Bradford assay (Biorad Laboratories, Hercules, CA, USA) with bovine serum albumine as a standard. The molecular size of the flagellin was identified by mass spectrometry performed by Dr. Harder (University of Kiel). The samples were diluted 1:10 in 50 % acetonitrile containing 0.02 % formic acid and analyzed by electrospray mass spectrometry (ESI-MS) in the positive ionization mode with a quadrupol-orthogonal-accelerating-time-of-flight-mass spectrometer (QTOF-II-hybrid-mass-spectrometer (Micromass, Manchester, U.K.)). The spectra were evaluated using the program MassLynx 3.5. (Micromass).

SDS-PAGE of isolated flagellin

To verify the purity of the flagellin, 4x NuPage LDS Sample buffer was added to the isolated protein and heated for 10 min at 70 °C. Prestained marker and samples were pipetted into the wells of a 4-12 % NuPAGE® Bis-Tris gel and run for 1½ h at 100 Volt. After electrophoresis, the separated fractions were treated with fixing solution for 30 min, stained for protein with Coomassie brilliant blue, washed with 10 % acetic acid and scanned.

Western blot of flagellin isolates

For further immunologic characterization the protein gel was transferred to a 0.45 µm nitrocellulose membrane in a blotting chamber and the protein blotted for 40 min at 1 mA/cm² gel size on to a 0.45 µm nitrocellulose membrane (Schleicher & Schuell, Keene, USA) (Towbin 1979). To block free binding sites, membranes were incubated with 5 % skimmed milk powder in TBST for 1h. *E. coli* H1-antiserum (Statens Serum Institute, Copenhagen, Denmark) which served as primary antibody was diluted in TBST and added to the membrane. Following 1.5 h of incubation, strips were washed five times for 5 min each and incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany), diluted 1:5000 in TBST. After five washes with TBST, the membrane was incubated for 5 min in SuperSignal® West Dura Extended Duration Substrate (Pierce, Rockford, IL, USA). Developing bands were detected with a chemoluminescence camera CCD LAS-1000 (Fuji) and analysis was performed with the software AIDA 2.1 (Raytest, Straubenhardt, Germany).

4.2.7 IL-8 ELISA

At the end of the cell culture experiments supernatants were collected and stored at -80 °C. IL-8 secretion in response to IL-1ß and *E. coli* Nissle supernatant was measured in cell culture supernatants from Caco-2, T84, SW480 and SW620 cells by enzyme-linked immunosorbant assay (OptEIA Human IL-8 ELISA Kit II, BD Biosciences, San Diego, CA) according to the supplier's protocol. Supernatants were diluted 1:10 or 1:50 for determination of IL-8.

4.2.8 HBD-2 purification from cell culture supernatant for hBD-2 ELISA

To determine the secretion of hBD-2 peptide by Caco-2 cells upon stimulation with probiotic bacteria, cell culture supernatants were collected at the end of the experiment, 1% BSA was added as a carrier and samples were stored at -20 °C. Cationic proteins were extracted overnight at 4 °C under constant gentle agitation with a weak cation exchange matrix (Macroprep® CM Support beads), added at a 1:40 ratio of matrix to cell culture supernatant including protease inhibitor cocktail (PIC) (PMSF 10 µM, Pepstatin 10 µg/ml, Leupeptin 10 µg/ml).

Subsequently the beads were washed with ammonium acetate for 10 min, the supernatant discarded and the absorbed cations were eluted twice with 5 % acetic acid (Porter 1998). The eluate was collected, lyophilised under vacuum and resuspended in 0.01 % acetic acid (+PIC). The presence of hBD-2 peptide in the solution was finally determined by ELISA by Dr. Jürgen Harder as described by Wehkamp *et al* (2004).

4.3 Statistical evaluation

The statistical evaluation was performed with Microsoft Excel and the programs SigmaPlot 8.0 or GraphPadInstat (Version 3.1, GraphPad Software, San Diego, CA, USA). To test for normality the Kolmogorov-Smirnov test was used. The data was considered as normally distributed when the P value was > 0.1 (10%-niveau) according to Dallal and Wilkinson's approximation to Lilliefor´s method (1986). When data failed to follow a Gaussian distribution, nonparametric ANOVA was performed using Kruskal-Wallis or Mann-Whitney Test. Means were regarded as significantly different if the null hypothesis was rejected with a probability of error of 5 % ($p < 0.05$).

5 RESULTS

5.1 Expression studies in colonic epithelial cells

5.1.1 Differential human beta defensin-2 and IL-8 expression

In order to identify the most suitable model for studying the hBD-2 inducing effect of *E. coli* Nissle 1917, various intestinal epithelial cell lines (Caco-2, T84, SW480 and SW620 cells) were stimulated with IL-1β and *E. coli* Nissle 1917 culture supernatant (Table 11). The positive control IL-1β induced a strong increase of hBD-2 mRNA expression in Caco-2 cells. This increase was ~10 fold less pronounced in T84 cells. SW480 and SW620 cells lacked to express hBD-2 after incubation with IL-1β.

Table 11: Differences of hBD-2 and IL-8 expression in intestinal epithelial cells. Relative HBD-2 mRNA expression in IECs after 6 h stimulation with IL-1β or *E. coli* Nissle supernatant (sn) was measured by RT-PCR. The data represent the means ± the SD of three independent experiments performed in duplicate.

Cell line	Treatment	hBD-2 mRNA expression	IL-8 protein expression
Caco-2	Unst.	0	39.5 ± 6.35
	IL-1ß	584.53 ± 200.86	5510.18 ± 3730.27
	EcN sn	52.06 ± 15.06	2852.38 ± 1145.3
T84	Unst.	0	295.1 ± 34.08
	IL-1ß	48.38 ± 32.32	336 ± 334.92
	EcN sn	28.63 ± 38.22	981.68 ± 852.66
SW480	Unst.	0	10.65 ± 2.28
	IL-1ß	0	32.5 ± 2.12
	EcN sn	21.01 ± 8.4	6379.2 ± 76.65
SW620	Unst.	0	122.5 ± 14.85
	IL-1ß	0	141 ± 18.38
	EcN sn	13.84 ± 12.04	4441.86 ± 873.23

A similar expression pattern was found after incubation with *E. coli* Nissle supernatant. The highest amount of hBD-2 mRNA was expressed by Caco-2 cells, half as much by T84, followed by SW480 cells. SW620 cells expressed only a small amount of hBD-2 after treatment with the bacterial supernatant (sn). Bacterial invasion or proinflammatory cytokines induce in colonic epithelial cells the secretion of the cytokine IL-8 (Eckmann 1993a).

As hBD-2 expression was determined to be NF-κB-dependent (Wehkamp 2004; Tsutsumi-Ishii 2002) the transcriptional regulation of IL-8 and hBD-2 gene expression might act in concert. We therefore determined the expression of the pro-inflammatory cytokine IL-8 to demonstrate that differences in hBD-2 expression between Caco-2, T84, SW480 and SW620 cells are not caused by an experimental artefact. Both stimulants, IL-1β and *E. coli* Nissle supernatant, induced the secretion of IL-8 by Caco-2 cells to a high extent as shown in Table 11. In SW480 and SW620 cells, only the bacterial supernatant evoked a strong expression of IL-8, which was absent following stimulation with IL-1β. In T84 cells, EcN supernatant induced IL-8 only weakly and IL-1β lacked any inducing effect.

5.1.2 Toll-like receptors

Toll-like receptors recognise microbial products during infection and initiate signalling pathways that culminate in the increased expression of immune and inflammatory genes. To identify potential receptors involved in *E. coli* Nissle signalling, we used real-time PCR to examine the qualitative expression of mRNAs encoding all known TLR´s and several adaptor proteins (e.g., CD14, MD-1, MD-2, MyD88, RP105) involved in TLR functions.

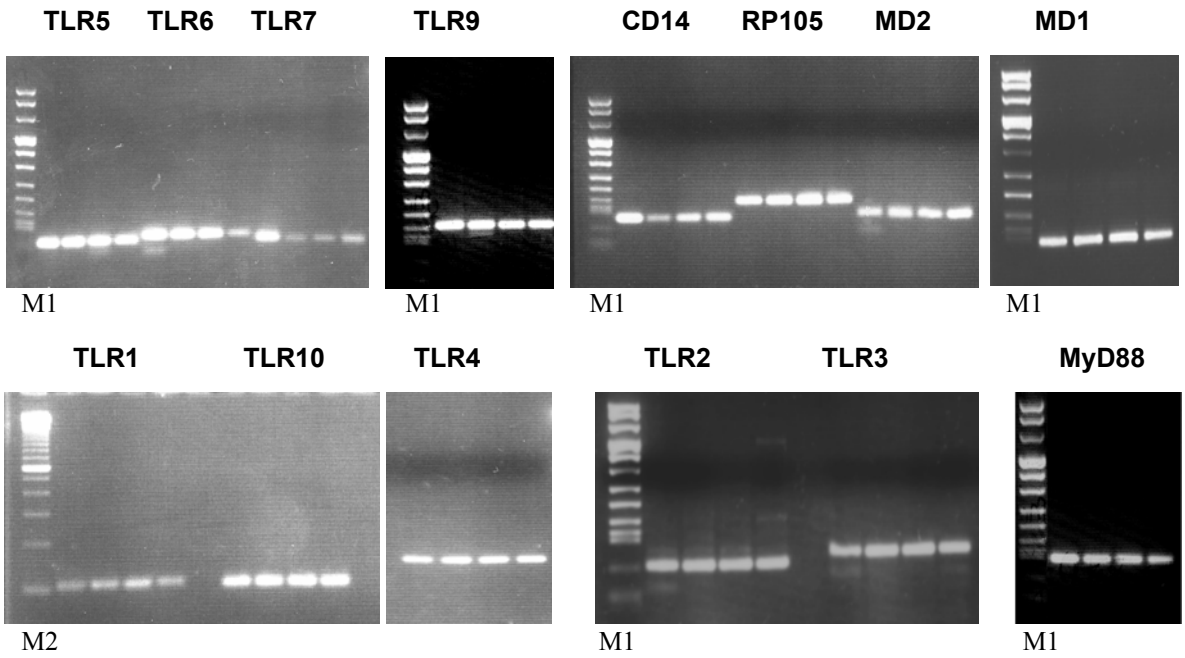


Figure 9: TLR expression pattern in Caco-2 (1st band), T84 (2nd band), SW480 (3rd band) and SW620 (4th band) cells. TLR expression was assessed by real-time PCR. M1 = Mol. Weight Marker VIII (Roche), M2 = 100 bp DNA Ladder (Gibco)

The four cell lines expressed all TLR´s including their adaptor proteins (chaperones) (Fig. 9). Peripheral blood mononuclear cells (PBMC) were used as a positive control. We investigated, whether TLR expression in Caco-2 cells was influenced by stimulation with IL-1β (Fig. 10). All TLR´s were expressed with similar strength whether unstimulated or stimulated, suggesting a constitutive mRNA expression. Only MD-1 and -2 displayed an increase after IL-1β treatment.

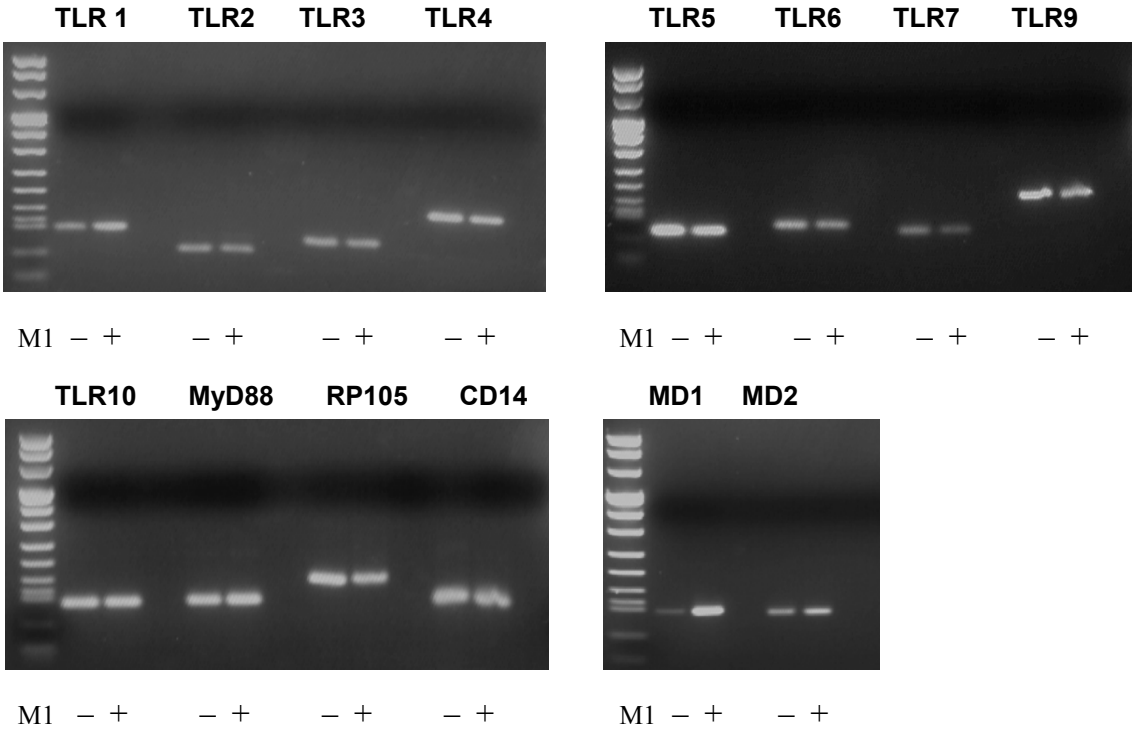


Figure 10: TLR expression basal and IL-1β stimulated in Caco-2 cells. Caco-2 cells were stimulated with IL-1β (5ng/ml) for 6 h. Stimulated and nonstimulated treatment is indicated with (+) or (-) respectively. M1 = Mol. Weight Marker VIII (Roche)

5.2 Time- and dose-dependent hBD-2 mRNA expression

To optimize experimental conditions Caco-2 cells were stimulated with increased concentrations or for different times with IL-1β bacteria pellet or supernatant to establish a dose-response or time-dependent effect on hBD-2 expression.

5.2.1 Induction by IL-1β

According to previous studies, Interleukin-1 can serve as a positive control for the induction of hBD-2 (O´Neil 1999). Caco-2 cells were stimulated with 5 ng/ml IL-1β for 3 h, 6 h, 12 h and 24 h and hBD-2 expression was measured by real-time PCR. hBD-2 mRNA ex-

pression induced by IL-1β followed a time-dependent pattern which increased from 3 h (195.7 ± 49.2) to 6 h (273.6 ± 92.9) of incubation, peaked at 12 h (420.8 ± 32.9) and decreased after 24 h (245.4 ± 111.3). IL-8 expression was determined from the cell culture supernatant and was determined to accumulate from 3 h to 12 h with no further increase after 24 h of stimulation with 5 ng/ml IL-1β (IL-8 secretion (pg/ml) after incubation for 3 h: 5320.9; 6 h: 8708.4; 12 h: 11397.3; 24 h: 12295.2).

For dose-dependence experiments, Caco-2 cells were stimulated for 6 h with increasing concentrations of IL-1β. The induction of hBD-2 mRNA expression by IL-1β was dose-dependent as seen in Table 12 and was maximal when 5 ng/ml IL-1β were applied with a decrease at the highest IL-1β concentration used.

Table 12: Dose-dependent hBD-2 expression in Caco-2 cells by IL-1β stimulation. After 6 h of stimulation with ascending concentrations of IL-1β cells were lysed with TRIzol, RNA was isolated and reverse-transcribed and hBD-2 expression determined via RT-PCR. The data represent the means ± the SD of two independent experiments.

IL-1β concentration (ng/ml)	hBD-2 copies/10 ng mRNA
0	1.01 ± 1.44
0.1	9.8 ± 2.08
1	227.2 ± 76.68
5	325.32 ± 3.6
10	242.66 ± 21.12

5.2.2 Expression of hBD-2 by Caco-2 cells as a function of cell differentiation

We determined that stimulation of Caco-2 cells with IL-1β resulted in a diminished hBD-2 expression when the treatment followed more than 2 days after seeding (Fig. 11). At that time, cells had reached full confluency. After 3 days of growth until the 12th day, IL-1β induced hBD-2 expression remained stable with 400 copies/10 ng mRNA. There was a 4 fold higher increase of the hBD-2 copy number when cells had grown to ~ 70 % confluency at the time of stimulation. According to these results, our experiments were carried out in the subconfluent state when cells had grown for < 2 days.

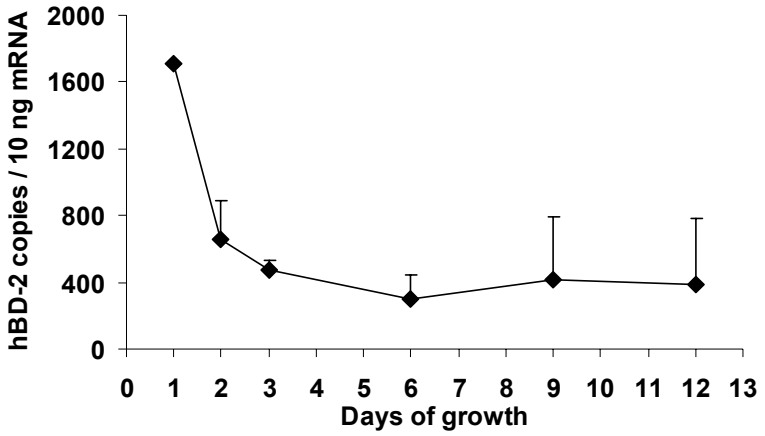


Figure 11: HBD-2 expression with time of cultivation. Cells were cultured for 1, 2, 3, 6, 9 and 12 days before stimulation with 5 ng/ml IL-1ß for 6 h. The data represent the means of two independent experiments ± the SD.

5.2.3 HBD-2 induction by different probiotic strains

Previous data have suggested that probiotic bacteria enhance the barrier function of the gut. We investigated, whether they could exert this barrier stabilizing effect also by the induction of hBD-2. For all tested probiotic strains, the hBD-2 induction followed a time-dependent pattern. The maximum of hBD-2 expression for all strains was determined at 6 h, decreased remarkably at 12 h and reached basal levels after 24 h of incubation (Fig. 12).

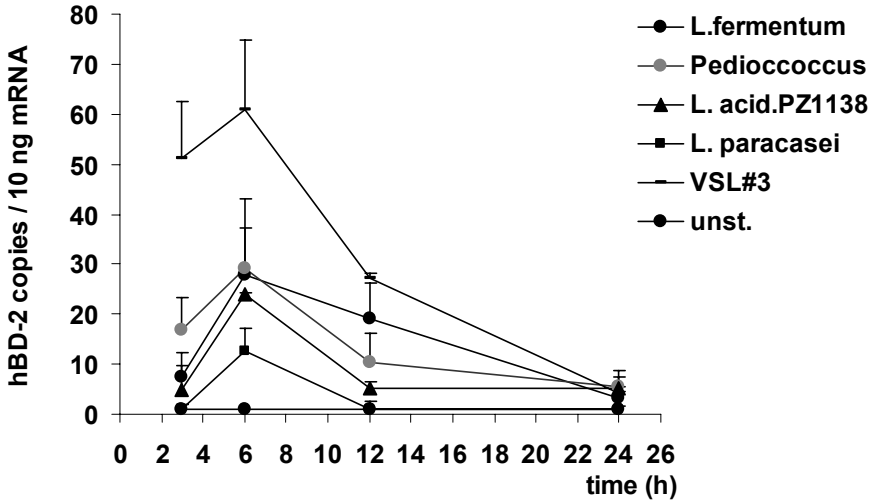


Figure 12: Time-course of lactobacilli induced hBD-2 expression. Caco-2 cells were treated with six different bacterial strains for 3, 6, 12 and 24 h. The data are means ± the SD of at least three independent experiments.

Thus, we defined 6 h of incubation as the optimal time for further experiments regarding hBD-2 mRNA expression.

The highest induction of hBD-2 was elicited by the probiotic cocktail VSL#3, followed with half as much activation by *L. fermentum* and *Pediococcus pentosaceus*. *L. acidophilus* and *L. paracasei* had only weak capacities to induce hBD-2.

In a dose-dependence experiment, we tested the optimal dose at which the probiotic strains induced the highest amount of hBD-2 (Fig. 13).

At a concentration of 1 x 10^6 bacteria cells/ml no hBD-2 induction was detectable. Except for VSL#3, no other bacteria had an inducing effect at the concentration of 1 x 10^7 cells/ml. *Pediococcus*, *L. fermentum* and VSL#3 induced clearly hBD-2 mRNA expression at a concentration of 1 x 10^8 cells/ml, which was further enhanced at the highest bacteria cell number used. *L. acidophilus* induced only with 1 x 10^9 cells/ml hBD-2. *L. paracasei* had only weak capacity to influence hBD-2 expression in Caco-2 cells.

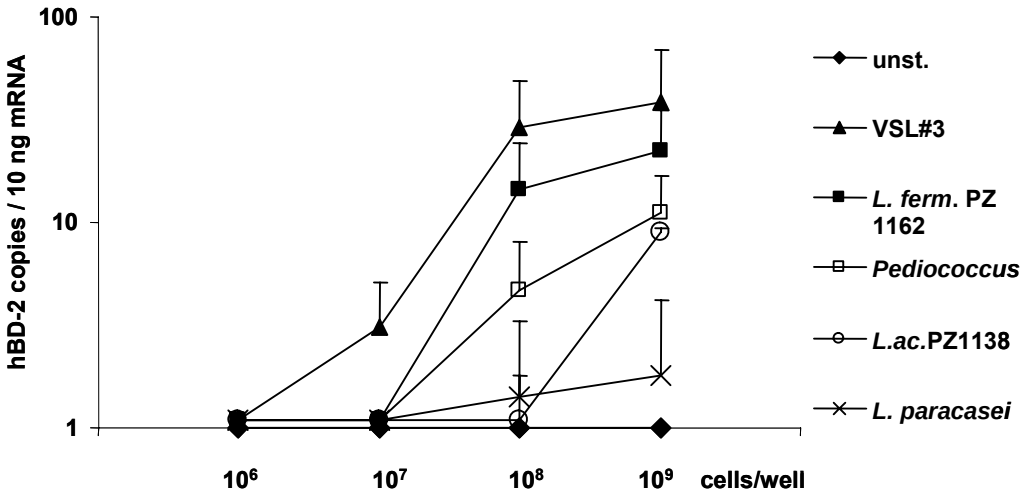


Figure 13: Dose-dependent stimulation of hBD-2 expression in Caco-2 cells by lactobacilli. Cells were stimulated for 6 h with 10^6, 10^7, 10^8 and 10^9 bacteria cells/ml and hBD-2 expression was determined by RT-PCR. The data represent the means ± the SD of three independent experiments.

We tested nine different probiotic strains to clarify whether the induction of hBD-2 is a general feature of probiotics (Fig. 14). Amongst these strains, four were mixed to a cocktail for the investigation of any cumulative effects. *Lactobacillus fermentum* and *Pediococcus pentosaceus* stimulated the expression of hBD-2 similarly strong as *E. coli* Nissle.

The incubation of Caco-2 cells with *L. plantarum*, *L. paracasei* and *L. acidophilus* PZ 1129 did also activate hBD-2 expression but much less pronounced than with *L. fermentum* or *Pediococcus*. There was no difference between *E. coli* K12, *L .gasseri*, *L. acidophilus* PZ 1030 and *Leuconostoc,* which induced only very low levels of hBD-2. The cocktail of the four strains shown in Fig. 14 induced distinctly hBD-2 but this effect seemed not to be cumulative compared to the different strains added individually.

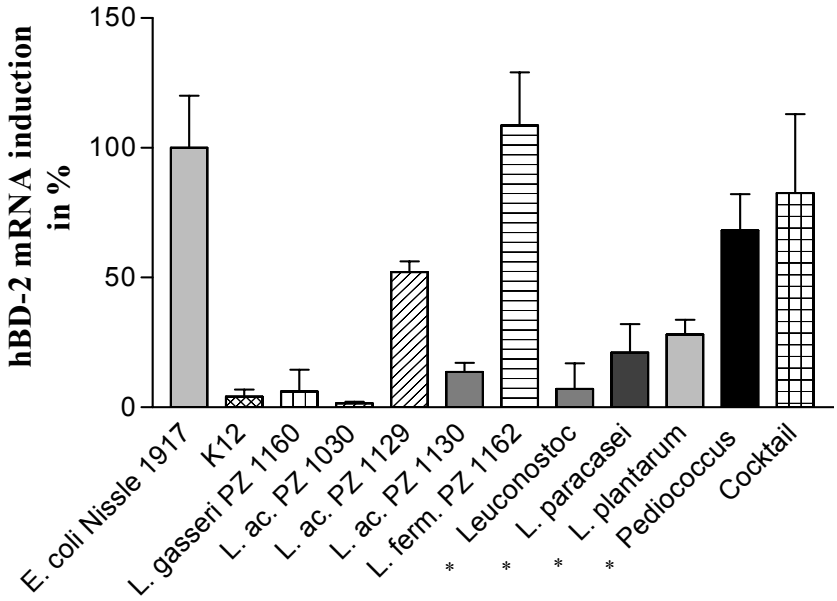


Figure 14: HBD-2 gene expression after stimulation with various lactobacilli. Caco-2 cells were incubated for 6 h with equal amounts of bacteria. Data are set in relation to *E. coli* Nissle (set as 100 %). *bacteria which are part of the cocktail. L. ac., *L. acidophilus*; L. ferm., *L. fermentum*

These data indicate that other probiotic strains besides *E. coli* Nissle increase significantly hBD-2 gene expression but with limitation to a few strains.

Additionally, the probiotic-stimulated secretion of the functionally relevant hBD-2 peptide could be detected in the cell culture supernatant (Fig. 15).

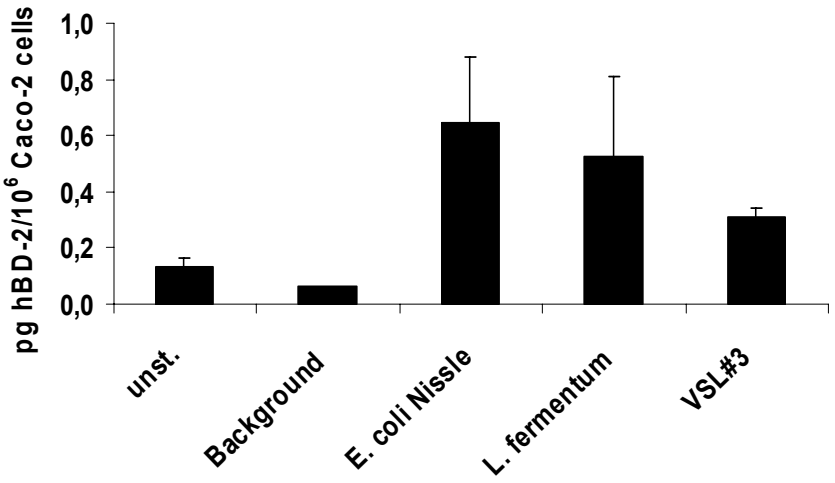


Figure 15: Detection of hBD-2 peptide in culture supernatants from Caco-2 cells stimulated with probiotics. Caco-2 cells were incubated for 24 h with equal amounts of bacteria. Secreted hBD-2 peptide was concentrated and detected by ELISA.

5.2.4 Induction of hBD-2 by culture supernatant of *E. coli* Nissle

Caco-2 cells were stimulated for 1.5, 3, 6, 12 and 24 h with *E. coli* Nissle supernatant (diluted 1:2). Relative HBD-2 expression increased from 1.5 h (6.3 ± 0.1) to 6 h (8.02 ± 0.96) remained stable at 12 h (8.04 ± 2.79) and decreased remarkably after 24 h (0.36 ± 0.16) of incubation.

IL-8 secreted into the cell culture supernatant was determined via ELISA. IL-8 protein (pg/ml) was expressed in a time-dependent manner, increasing highly from 1.5 h (109 ± 9.94) to 3 h (1487 ± 125.39) and further accumulating after 6 h (2583.1 ± 1294.94) until 12 h (3083.1 ± 255.58) of incubation reaching a plateau at 24 h (3224.7 ± 63.9). HBD-2 expression by *E. coli* Nissle supernatant was dose-dependent (Fig. 16).

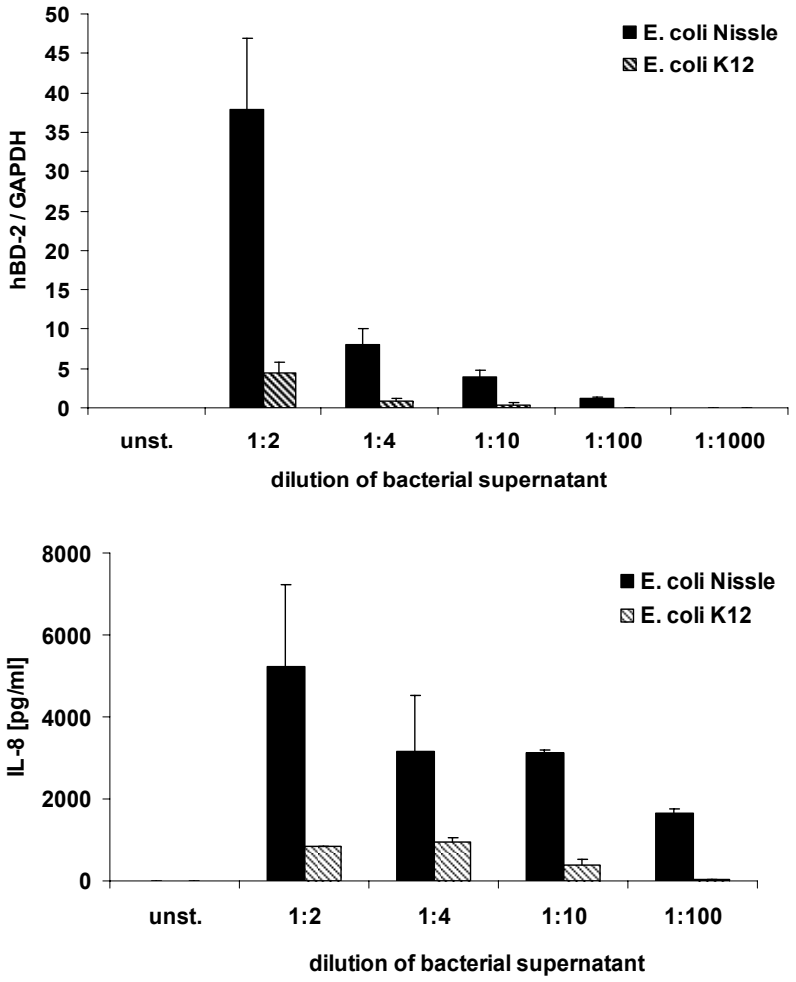


Figure 16: Dose-dependency of bacteria supernatant mediated hBD-2 and IL-8 expression. Caco-2 cells were stimulated for 6 h with EcN and *E. coli* K12 supernatant diluted in AB-/FCS-free DMEM. RNA was isolated and hBD-2 analyzed via RT-PCR. IL-8 secreted into the cell culture supernatant was determined via ELISA.

The highest concentration of the supernatant (dilution 1:2) induced 4.7 fold as much hBD-2 as the dilution 1:4. *E. coli* K12 was 8.5 fold weaker in inducing hBD-2 than *E. coli* Nissle. Since the smallest dilution of supernatant (1:2) might induce cell stress during the incubation time caused by a lack of nutrients, we decided to continue our experiments with supernatant diluted 1:4.

In accordance with our observations of hBD-2 induction, IL-8 was expressed by Caco-2 cells in a dose-dependent manner after *E. coli* Nissle supernatant treatment and decreasing gradually with reduced concentrations of the bacterial supernatant (Fig. 16).

5.2.4.1 HBD-2 induction by bacterial supernatant is stronger than by the pellet

From previous studies we know, that bacterial cell wall components of *E. coli* Nissle are recognized by epithelial cells and induce hBD-2 (Wehkamp 2004). However, it remained unknown, whether a secreted or shed factor present in the culture supernatant of this bacterium, could also activate the expression of the human beta-defensin 2.

In reporter gene studies, we compared the hBD-2 inducing activity of bacterial cells (= crude pellet) and the corresponding bacterial culture supernatant (Fig. 17). Bacteria supernatants exposed a markedly stronger hBD-2 promoter activating capacity than bacterial pellets of the strains tested.

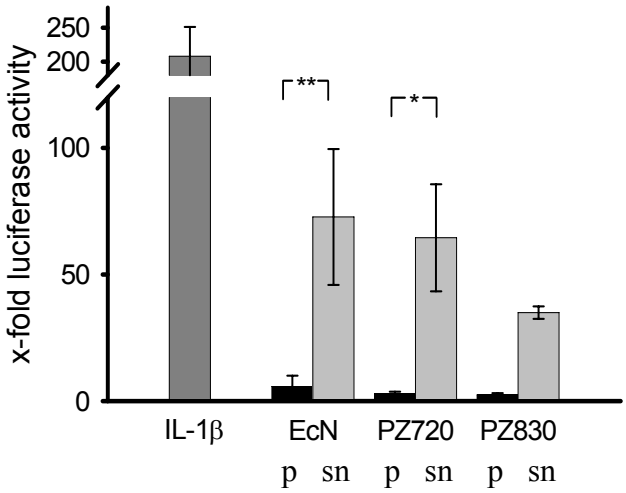


Figure 17: Effect of crude bacterial pellet versus supernatant on hBD-2 reporter gene activity. Transfection of Caco-2 cells with an hBD-2 promoter construct (2338 bp) for 24 h was followed by incubation for 4.5 h with 5 ng of IL-1β or bacterial preparations/ml. The probiotic fecal *E. coli* isolates Nissle (EcN), PZ720, and *E. coli* PZ830 were adjusted to a density of 3×10^8 bacteria/ml and tested either as crude bacterial pellets (p) or corresponding supernatants (sn) in the same amount of volume. hBD-2 promoter activation was determined as a ratio between firefly and *Renilla* luciferase activities. The data represent the median with minimum and maximum values of three to five experiments performed in triplicate. The median was normalized to the basal luminescence of unstimulated controls, set at 1. IL-1β served as positive stimulatory control (left column). A statistical comparison between the crude pellet and the supernatant was performed by using the Mann-Whitney test. * ($p < 0.05$), ** ($p < 0.01$).

We also compared the hBD-2 inducing effect of pellets and supernatants of the strains *L. fermentum*, *Pediococcus* and *L. acidophilus* PZ 1129. However, these strains did not exert relevant differences in hBD-2 expression after stimulation with either pellet or supernatant

(2.2 fold (*L. fermentum*) or 1.3 fold (*L. acidophilus*) higher induction than with the pellet) except for *Pediococcus* (sn: 7 fold more than the pellet).
These results suggest an immunostimulatory role for a secreted or shed factor from some bacterial strains but not for probiotics in general.

5.3 Transcriptional regulation and signalling pathways of hBD-2 gene expression induced by probiotics

5.3.1 Role of the transcription factors NF-κB and AP-1

NF-κB and AP-1 play both a role for *E. coli* Nissle mediated hBD-2 gene transcription (Wehkamp 2004). We investigated whether the unknown factor in the bacterial supernatant induces hBD-2 transcription in the same way as the pellet.

First, we investigated whether a NF-κB reporter gene, which is an artificial construct containing several tandemly repeated NF-κB binding sites would be activated by bacterial stimulation after transfection into Caco-2 cells. Incubation with *E. coli* Nissle supernatant enhanced luciferase activity 5.1 fold compared to unstimulated control, with the pellet 1.4 fold (n=3). The difference between bacterial pellet and supernatant stimulated promoter activation was statistically significant ($p < 0.05$). A similar activation profile was found for *E. coli* PZ720 (pellet: 1.2 fold, supernatant: 4.3 fold).
In contrast, an AP-1 reporter gene was not activated by any of the stimulants indicating that activation of the transcription factor AP-1 alone might not be sufficient for luciferase expression induced by the bacteria.

The importance of NF-κB and AP-1 binding sites in the promoter region of hBD-2 was analyzed by transfecting Caco-2 cells with three mutant hBD-2 promoter constructs encompassing the 5´-untranslated region upstream of the start of gene transcription (2338 bp). Binding sites for the two 5´-upstream NF-κB sites (pos. –205 to –186 and –596 to –572) and AP-1 (pos. –127 to –121) were mutated separately or together (Fig. 18). Maximal activity was observed with the complete promoter encompassing all known NF-κB and AP-1 sites (Fig. 19). Sequential loss of transcription factor binding sites within the hBD-2 promoter resulted in a stepwise decrease in activation upon IL-1β, as well as pellet (EcN) and supernatant (EcNsn) treatment.

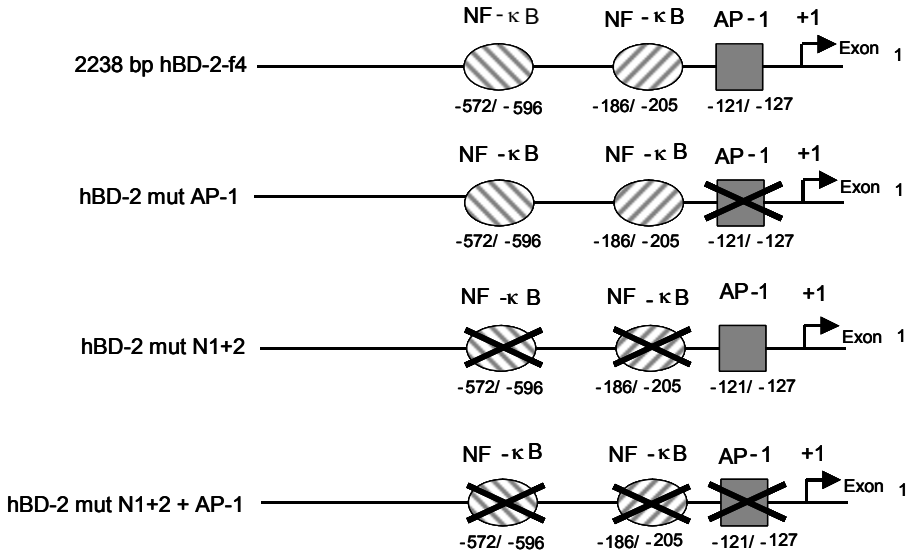


Figure 18: hBD-2 promoter constructs used to investigate the role of NF-κB and AP-1 binding sites. (Source: Wehkamp 2004) Symbols represent positions of putative transcription factor binding sites: open ovals: NF-κB sites (-186/-205; -572/-596); grey boxes: AP-1 site (-121/-127)

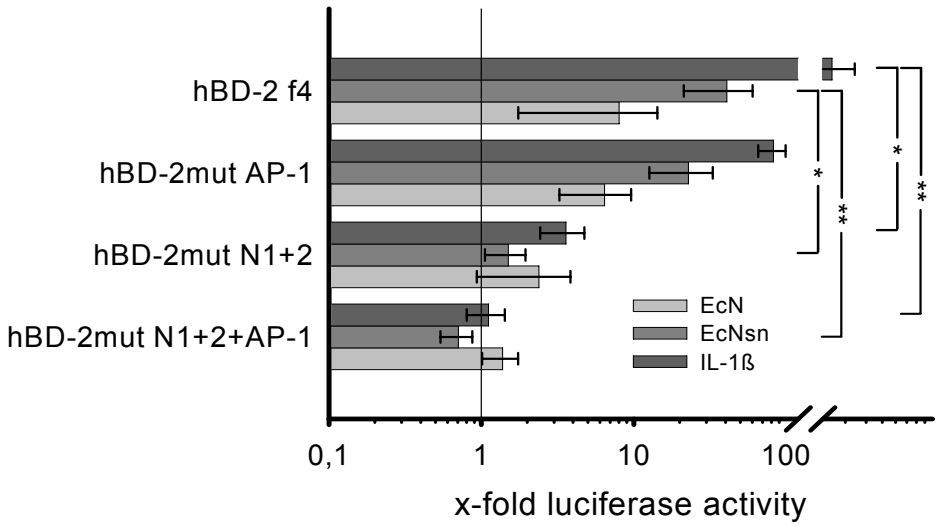


Figure 19: Contribution of NF-κB and AP-1 binding sites to *E. coli* Nissle mediated hBD-2 promoter activation. Various mutant promoter constructs were tested for their ability to respond to IL-1β, as well as *E. coli* Nissle culture supernatant (EcNsn) or crude bacterial pellet (EcN), in luciferase reporter gene assays as outlined in Fig. 17. NF-κB (positions –205 to –186 and –596 to –572) and AP-1 (positions –127 to –121) were mutated separately (hBD-2mut N1+2 or hBD-2mut AP-1) or in combination (hBD-2mut N1+2+AP-1). The data represent the median with minimum and maximum values of duplicate samples of three to four experiments. Statistical evaluation was performed by using Kruskal-Wallis analysis with post hoc Dunn´s test. * ($p < 0.05$), ** ($p < 0.01$).

More precisely, mutation of the two NF-κB binding sites reduced promoter activation by *E. coli* Nissle pellet from 7 fold to 1.8 fold, by the supernatant from 41.3 fold to 1.5 fold. In addition, the IL-1β mediated activation was almost completely blocked (from 255.6 to 3.6). In summary, the deletion of the –205 to –186 and –596 to –572 sites resulted in the complete loss of stimuli responsiveness. Further, we determined whether a third NF-κB binding site (-2187) on the promoter is also relevant for transcriptional regulation. Deletion of the third and most distant NF-κB binding site resulted in no further loss of the basal transcription activity. Thus, it does not appear to be functionally relevant for hBD-2 transcription. Mutation of the AP-1 site alone decreased hBD-2 promoter activation by the supernatant by 30 %. This suggests a limited role for AP-1, as it cannot induce promoter activation without activated NF-κB binding sites in its neighbourhood. After the mutation of AP-1 and the two NF-κB sites, promoter activation by any of the stimulants was completely abolished. Although NF-κB alone contributed greatly to the hBD-2 promoter activation, hBD-2 gene expression seemed to depend on both transcription factors.
Whether the transcription factors NF-κB and AP-1 are also both essential for the expression of the human β-defensin-2 induced by other probiotics was further clarified (Fig. 20).

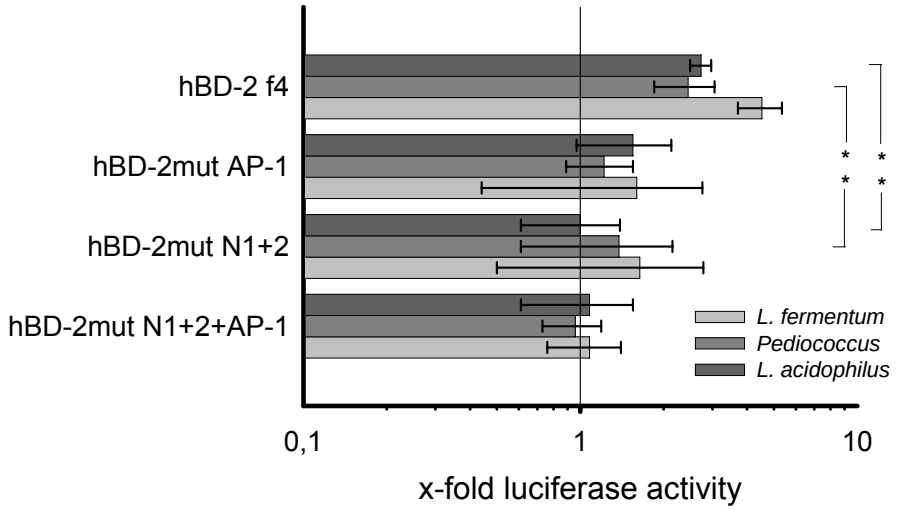
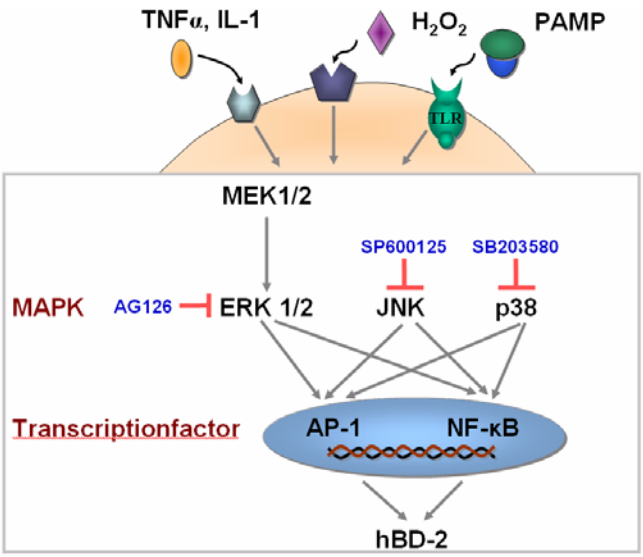


Figure 20: Relevance of the NF-κB and AP-1 binding sites for hBD-2 promoter activation by the strains L. fermentum, Pediococcus and L.acidophilus PZ 1129. Transfected cells were stimulated for 4.5 h with IL-1β or 3 x 10^8 cells/ml and Firefly and Renilla expression was determined. Data represent the median with minimum and maximum values of duplicate samples of three to four experiments. Statistical evaluation was performed by Kruskal-Wallis analysis with posthoc Dunn´s test, ** ($p < 0.01$).

First, we investigated the effect of the deletion of the two NF-κB binding sequences closest to the transcription start. Activation of the promoter by *L. fermentum* was reduced by 64 %, by *Pediococcus* 44 % and by *L. acidophilus* PZ 1129 it was completely abolished. The deletion of the binding site for the transcription factor AP-1 diminished the promoter activation by *L. fermentum*, *Pediococcus* and *L. acidophilus* by 65 %, 50 % or 43 % respectively.

This suggests that AP-1 might be more important for hBD-2 transcription initiated by *L. fermentum* and *L. acidophilus* and *Pediococcus* than by *E. coli* Nissle. Mutation of all transcriptionfactor binding sites completely abolished any promoter activation.

5.3.2 Role of MAP kinases in probiotic cell signalling



Streptococci, *Staphylococci* and *S. enteritidis* have been reported to increase hBD-2 production by epithelial cells by activating one or more MAP kinase signalling pathways (Chung 2004; Ogushi 2004). To determine if those pathways are activated by the hBD-2 inducing factor in *E. coli* Nissle supernatant, we inhibited three main MAP kinase pathways with specific inhibitors: p38 mitogen activated kinase (p38 MAPK), c-Jun amino-terminal kinases (JNK kinase), and the extracellular signal-regulated kinases (ERK½). Blocking of ERK½ by AG126 (AG) and JNK by SP600125 (SP) resulted in almost complete abrogation of *E. coli* Nissle supernatant- mediated hBD-2 promoter activation. Inhibition of p38 MAP kinase by SB203580 (SB) had the slightest effect (50 % (at 10 µM SB) or 75 % (at 50 µM SB) reduction of promoter activation respectively) (Fig. 21). For all experiments, IL-1β stimulation was used as positive control for hBD-2 promoter activation.

The results indicate an important role for ERK½ and JNK and a limited role for p38 in the signalling pathway of the bacterial supernatant of *E. coli* Nissle.

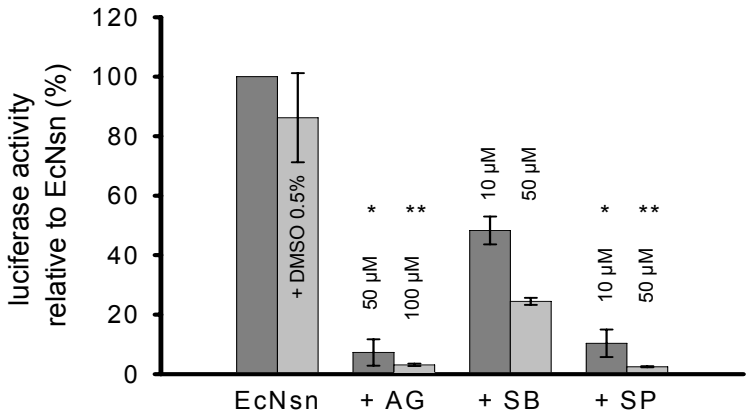


Figure 21: Effects of MAP kinase inhibition on *E. coli* Nissle supernatant mediated hBD-2 promoter activity. The hBD-2 promoter construct was transfected into Caco-2 cells. Specific MAP kinase inhibitors (diluted in DMSO) at the indicated concentrations were added 1 h prior to 4.5 h stimulation with bacterial supernatant, or the corresponding supernatant including 0.5 % DMSO. The data are expressed as a percentage of the control response (EcNsn with DMSO 0.5 %) and represent the median with minimum and maximum values of duplicate determinations of three experiments. Each inhibitor was compared to the DMSO control by Kruskal-Wallis analysis and post hoc testing. * ($p < 0.05$), ** ($p < 0.01$).

Focussing on hBD-2 promoter activation induced by NF-κB binding other MAP kinases might be involved than by AP-1.

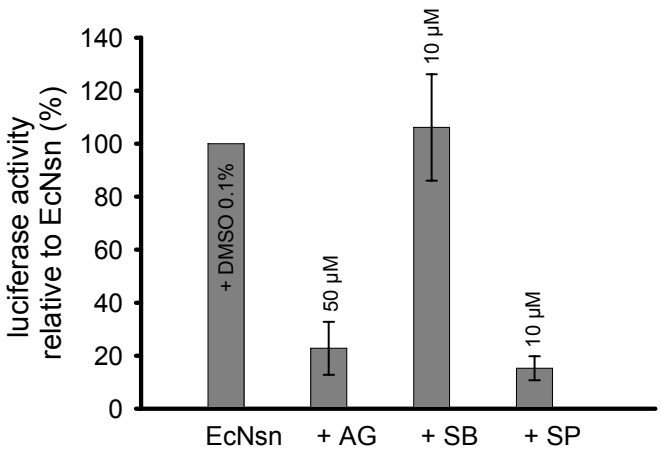


Figure 22: EcNsn induced MAPkinase signalling after deletion of the AP-1 binding site. Caco-2 cells were transfected with an hBD-2mut AP-1 plasmid and pretreated for 1h with the specific MAP kinase inhibitors before stimulation with EcNsn. The data represent the median with minimum and maximum values of duplicate determinations of three experiments.

After deletion of the AP-1 binding site, treatment with the ERK½ inhibitor AG and the JNK inhibitor SP, but not with the p38 inhibitor SB resulted in a distinct decrease of luciferase activity (Fig. 22).

MAP kinase involvement in the signal transduction of two other probiotic strains was also determined (Fig. 23). Inhibition of ERK ½ reduced hBD-2 promoter activation by *L. fermentum* and *Pediococcus* by 55 %. Inhibition of p38 MAP kinase resulted in 20 % or 25 % decreased promoter activation through *L. fermentum* or *Pediococcus* respectively. Normal luciferase activation by *Pediococcus* was inhibited by 2/3 when incubated with the JNK inhibitor. Thus, JNK certainly plays a role for the signalling pathway of *L. fermentum* and *Pediococcus*, ERK½ is also included but p38 seems to be less important.

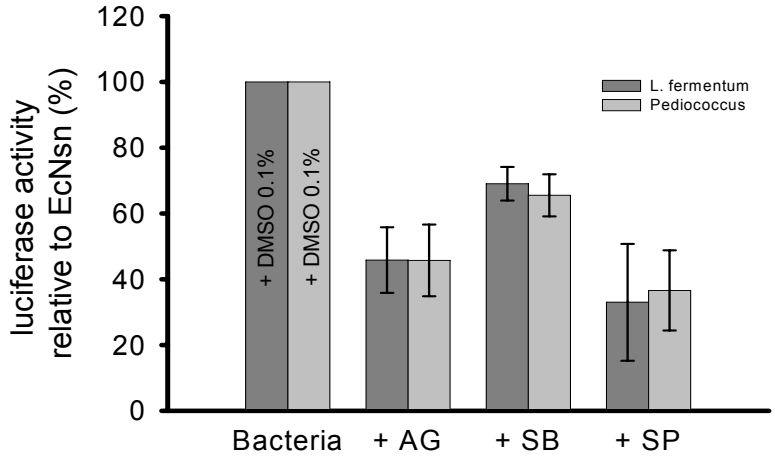


Figure 23: Signaltransduction of *L. fermentum* and *Pediococcus* mediated hBD-2 promoter activation. Caco-2 cells were transfected with a wildtype hBD-2 (-2338) promoter construct and stimulated with L. fermentum (dark grey) or Pediococcus (light grey) for 4.5 h after pretreatment with the MAP kinase inhibitors. The data represent the median with minimum and maximum values of duplicate determinations of three experiments.

To ensure, that the applied inhibitor concentrations dissolved in DMSO had no cytotoxic or growth inhibiting effect we performed the MTT cytotoxicity assay as described in Methods. The concentrations of the MAPkinase inhibitors AG126, SB203580 and SP600125 used for the cell culture experiments caused no significant changes of cell viability. This is indicated by the equivalent mitochondrial activities between untreated cells (DMEM) and inhibitor-treated or DMSO treated cells (Fig. 24).

The MTT cytotoxicity assay revealed that the decrease of hBD-2 inducibility was not due to cytotoxic side effects of the inhibitors as seen with Triton-X.

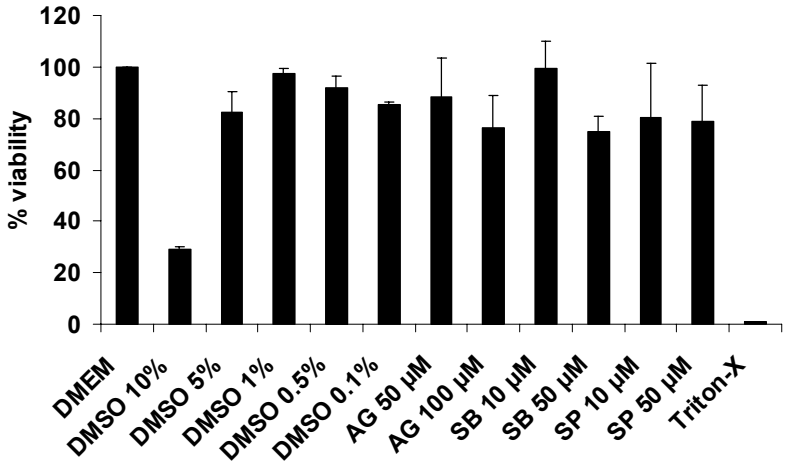


Figure 24: Effects of MAPkinase inhibitors and DMSO on cytotoxicity. Caco-2 cells were treated after incubation with the reagents, with MTT as described in Methods. Results are shown as a percentage of viable cells compared to untreated cells (DMEM). Data are mean ± SD of two experiments performed in duplicate.

5.4 Determination of the hBD-2 inducing factor of *E. coli* Nissle

Secondly, the present study placed emphasis on the determination of the hBD-2 inducing factor of the bacterial strain *E. coli* Nissle. We focused our search on the elucidation of the role of certain cell wall components (e.g., lipopolysaccharide, adhesins, K5-capsule) as well as secreted (e.g., microcins) or shed (e.g., flagella) factors (see Fig. 8) which might be responsible for the stimulatory effect regarding hBD-2.

LPS responsiveness

The following experiment was initiated to define whether the specific *E. coli* Nissle LPS plays a role in the hBD-2 induction by the *E. coli* Nissle strain. We compared LPS responsiveness among four human intestinal epithelial cell lines (Caco-2, T84, SW480 and SW620) which we demonstrated as expressing all TLR´s and adaptor molecules relevant for LPS signalling. We cultivated cells with 0.1, 0.5, 1 and 5 µg/ml LPS (*E. coli* serotype O111:B4) or *E. coli* Nissle LPS respectively. The expression of IL-8 was measured in supernatants of cells stimulated with 1 µg/ml LPS or EcN-LPS.

In all cell lines, neither LPS concentration had any influence on hBD-2 expression (data shown only for 1µg/ml LPS in Table 11). Two cell lines released smaller (Caco-2 and SW480 cells) amounts of IL-8 and two cell lines higher (T84 and SW620 cells) amounts of IL-8, as determined by ELISA (Table 13). In Caco-2 cells, IL-8 generation was slightly upregulated after stimulation with EcN-LPS and even more so with LPS. In contrast, with T84 cells neither of the two LPS types could induce IL-8 protein expression. Surprisingly, SW480 cells responded to LPS stimuli with an 8-fold IL-8 increase of the unstimulated value and even 16 fold by *E. coli* Nissle LPS.

Table 13: LPS responsiveness of IEC´s. The four cell lines were stimulated with 1 µg/ml LPS and relative hBD-2 mRNA expression was determined via RT-PCR. IL-8 (ng/ml) secreted into the cell culture supernatant was measured by ELISA. Values represent means ± SD of three (for hBD-2) and of two experiments (for IL-8).

Cell line	Treatment	hBD-2 mRNA expression	IL-8 protein expression
Caco-2	Unst.	0	16.7 ± 7.3
	EcN-LPS	1 ± 0.1	36.6 ± 0.9
	LPS	1.4 ± 0.5	53.6 ± 0.9
T84	Unst.	0	198.5 ± 147.4
	EcN-LPS	0	105.9 ± 26
	LPS	0	243.6 ± 0.4
SW480	Unst.	0	10.7 ± 14.6
	EcN-LPS	0	166 ± 77.6
	LPS	0.5 +/- 0.8	81.9 ± 19.1
SW620	Unst.	0	122 ± 14.6
	EcN-LPS	0	345.8 ± 65.7
	LPS	0	151.5 ± 8.6

SW620 cells showed only after stimulation with *E. coli* Nissle LPS an almost 3-fold upregulation of IL-8, which was missing with the standard LPS tested.

In summary, LPS is not responsible for inducing hBD-2 in intestinal epithelial cells, but shows a diverse pattern of responsiveness regarding IL-8 expression in the epithelial cells.

Bacterial DNA

Since Caco-2 cells constitutively express TLR-9 mRNA they might have the ability to respond to the respective ligand of CpG-rich bacterial DNA. To explore the potential effect of bacterial DNA on hBD-2 promoter activation, Caco-2 cells were treated for 4.5 h with 25 µg/ml DNA isolated from *E. coli* Nissle and two different artificial immunostimulatory oligodeoxynucleotides (ISS-ODN) as positive and negative control (see Methods). Neither bacterial DNA nor ISS-ODN had any effect on hBD-2 promoter activation.

Stability of hBD-2 inducing factor activity in *E. coli* Nissle supernatant

To assess whether the hBD-2 inducing factor exhibits heat-sensitivity, the bacterial supernatant was heated for 20 min at 100 °C. Compared to the stimulation by supernatant from heat-killed bacteria (set at 100 %) hBD-2 promoter activity was not reduced remarkably after the boiling treatment (91.1 ± 18.2 %).

Next, we investigated whether the hBD-2 activating molecule is a protein by treating the *E. coli* Nissle supernatant with proteinase K. HBD-2 promoter activity was reduced from 100 % (untreated *E. coli* Nissle supernatant) to 28.4 ± 3.4 % when the bacterial supernatant was pretreated with proteinase K. TSB media treated with proteinase K, boiled for 20 min and added to the cells for 4.5 h had no negative effect on cell viability, showing that the proteinase was really inactivated after the heat treatment. These data indicate that the hBD-2 inducing factor in the supernatant is a heatstable and proteinase-sensitive molecule.

HBD-2 promoter activation by mutant bacteria

To clarify which factor in the bacteria supernatant is responsible for hBD-2 induction, Caco-2 cells were stimulated with either wild-type EcN or isogenic mutants with deletions of known fitness factors (e.g., adhesins, microcin, iron siderophores or flagellin). Only the supernatant of the flagellin mutant (EcNsn Δ*fliC*), which lacks the production of the flagellin filament protein, showed a decrease in activation capacity. The other mutants displayed no change compared to wild-type *E. coli* Nissle (EcNsn) (Fig. 25a).

To focus further on the role of flagellin in *E. coli* Nissle signalling two other flagellin mutants (Δ*flgE* and Δ*fliA*) were constructed. Stimulation of Caco-2 cells with supernatants of the flagellin mutants' Δ*flgE* (hook mutant i.e. "anchor" protein of the flagellum) as well as Δ*fliA* (sigma factor) resulted in a similar decrease in hBD-2 promoter activation (wild-type

EcNsn: 15.5 ± 5.2 fold, Δ*flgE*: 2.7 ± 0.7 fold, Δ*fliA*: 2.1 ± 1.0 fold). Consistent with this, the flagellin mutants of *E. coli* Nissle completely failed to stimulate hBD-2 mRNA expression (Fig. 25b).

In order to verify that this remarkable loss of hBD-2 induction by the flagellin mutants was specifically attributable to the lack of flagellin, deleted genes were complemented in trans by transforming the respective mutant with a recombinant plasmid harboring the corresponding gene (= Δ*fliC*pDB2 and Δ*flgE*pDB3).

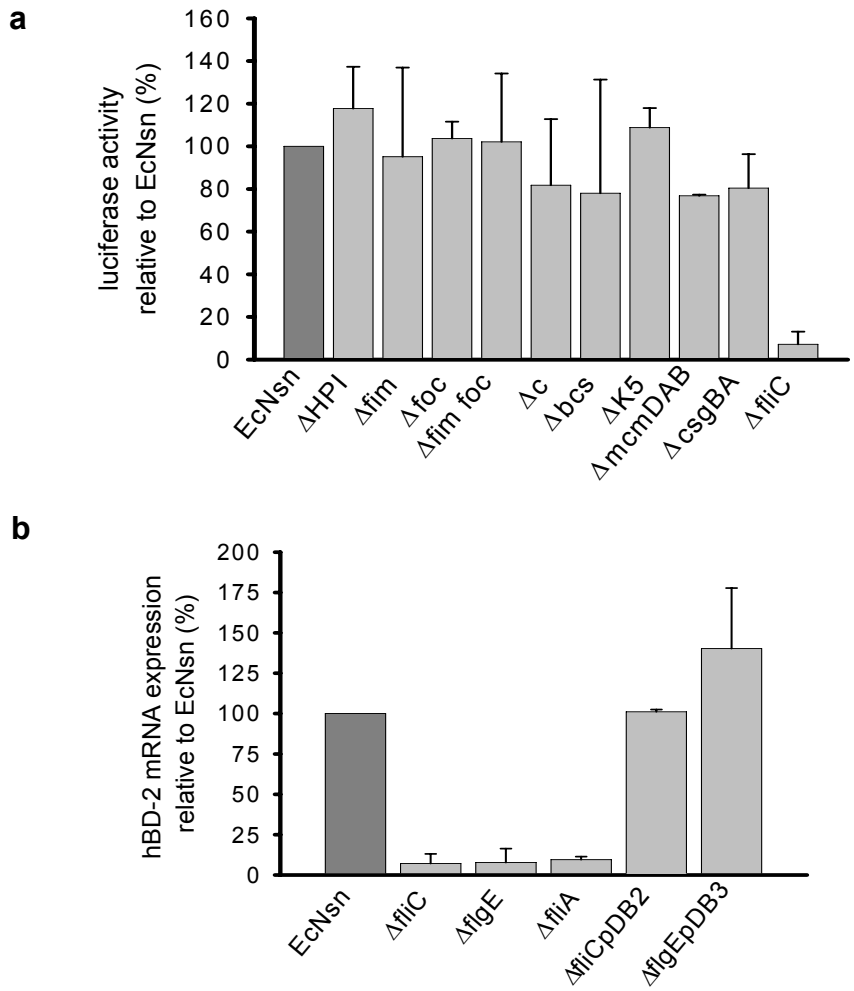
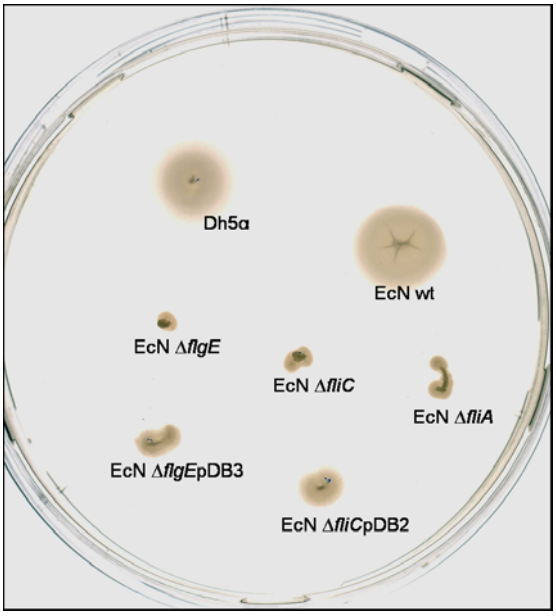


Figure 25: Effect of *E. coli* Nissle fitness factor mutants on hBD-2 expression. a) Transfected Caco-2 cells were incubated for 4.5 h with supernatants from various *E. coli* Nissle mutant strains. hBD-2 inducing activity was determined by the luciferase reporter gene assay. The data are means ± SD of three independent experiments assayed in duplicate. b) Induction of hBD-2 mRNA expression by flagellin-deficient (Δ*fliA*, Δ*fliC* and Δ*flgE)* and complemented (Δ*fliC*pDB2 and Δ*flgE*pDB3) *E. coli* Nissle. Gene expression was analyzed by real-time PCR. The data represent means ± SD of four experiments in duplicate.

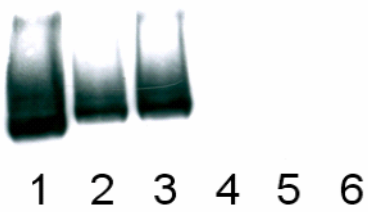
After complementation, the *fliC* and the *flgE* mutants regained their capacity to induce hBD-2 mRNA. Several attempts were made to clone the Δ*fliA* gene without success. Therefore, we continued our experiments only with the Δ*fliC* and Δ*flgE* complementants.

Protein and functionality

As a further proof for the loss of flagellin synthesis, LB swarmagar plates were inoculated with the different strains (Fig. 26a). Visually determined swarming was normal in case of DH5α and wild-type *E. coli* Nissle 1917 (EcN) strains. All flagellin mutants (Δ*flgE*, Δ*fliC* and Δ*fliA*) showed an abolished swarming. The complemented mutants displayed an intermediate swarming (Δ*fliC*pDB2 and Δ*flgE*pDB3).



a



b

Figure 26: **Analysis of flagellin-mutant and complemented *E. coli* Nissle strains. a)** Characterization of motility by swarming in semisolid agar. Cultures of the wild-type *E. coli* Nissle and the flagellar mutants and their complements were plated into wells punched into 0.3 % LB agar plates and incubated for 10 h at 37 °C. Lack of motility is indicated by growth restricted to the spot of inoculation, whereas motility is indicated by growth throughout a larger circle. **b)** Flagellin expression assessed by Western blot analysis. Heat-killed overnight bacterial cultures were subjected to SDS-PAGE electrophoresis, and flagellin expression was analyzed with H7 flagellin antiserum. *Lane 1*, EcN (wild type); *lane 2*, EcNΔ*flgE*pDB3 (complemented Δ*flgE* mutant); *lane 3*, EcNΔ*fliC*pDB2 (complemented Δ*fliC* mutant); *lane 4*, EcNΔ*flgE*; *lane 5*, EcNΔ*fliC*; *lane 6*, EcNΔ*fliA*.

This shows that the complementation with the flagellin or the hook gene respectively was successful regarding the regain of the hBD-2 inducing capacity but failed to accomplish the full motility function of the flagella. The lack of flagellin synthesis after the mutation was also confirmed by Western blot (Fig. 26b).

Flagellin mutants (Δ*flgE*, Δ*fliC* and Δ*fliA*) lacked the bands specific to flagellin whereas complemented mutants (Δ*fliC*pDB2 and Δ*flgE*pDB3) displayed immunoreactivity.

5.5 Identification and isolation of the bacterial hBD-2 inducing factor

Flagellin might act in concert with other molecules, which may be also essential for proper hBD-2 induction. Thus, we had to investigate whether isolated flagellin is capable of sufficiently inducing hBD-2, when the flagellin is seperated from other particles of the bacteria with stimulatory potential. The purity of the isolated *E. coli* Nissle flagellin was assessed by SDS-PAGE and Coomassie staining (Fig. 27). A clear band between 49 and 62 kDa was detectable after staining. After $(NH_4)_2SO_4$ precipitation, dialysis and heat treatment a clear band presumably containing the flagellin was also seen.

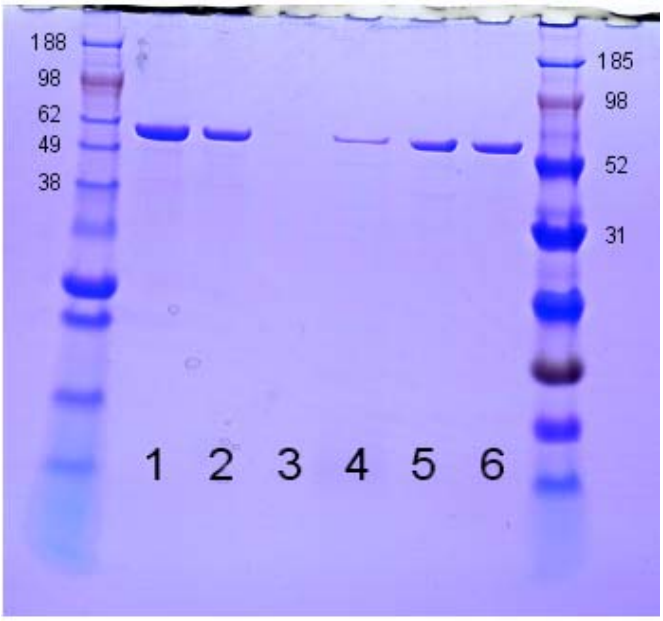


Figure 27: **Analysis of isolated *E. coli* Nissle flagellin by Coomassie blue staining.** SDS-PAGE analysis of the purified flagellin preparation by Coomassie brilliant blue. The relevant positions of the molecular mass standard (SeeBlue Plus 2, Invitrogen) are marked on the left or right (Multi Mark, Invitrogen). *Lane 1 + 2*, final step: flagellin isolate (1 and 0.5 µg/µl); *lane 3*, PBS (Blank); *lane 4*, final step: flagellin residue in supernatant; *lane 5+6*, pellet and turbid supernatant after heating to 65 °C and ultracentrifugation (100 000 x g)

Lane 4 showing the protein found in the supernatant from the last isolation step indicates that some flagellin residue was still present and not all of this protein was precipitated by the lower $(NH_4)_2SO_4$ concentration.

Analysis of the bacteria isolate by mass spectrometry was kindly performed by Dr. Jürgen Harder (University of Kiel). The detected molecular mass of the flagellin confirmed the expected flagellin size of 60.81 kDa (Fig. 28).

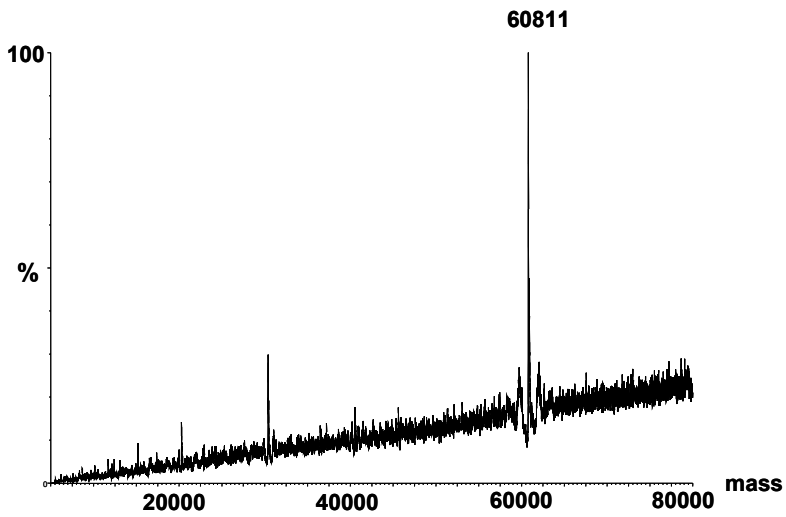


Figure 28: Mass spectrometric analysis of isolated *E. coli* Nissle flagellin. The sample was diluted 1:10 in 50 % acetonitrile containing 0.02 % formic acid and analyzed by electrospray mass spectrometry.

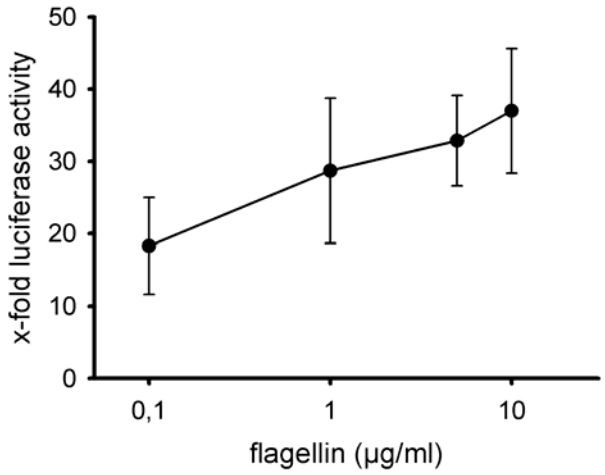


Figure 29: Dose response of hBD-2 promoter activity by E. coli Nissle flagellin stimulation. Caco-2 cells were stimulated for 4.5 h with 0, 0.1, 1, 5 or 10 µg/ml of isolated flagellin. The hBD-2 inducing activity was determined by using a luciferase reporter gene assay with basal activity of the promoter set at 1. The data are means ± the SD of three independent experiments in duplicate.

As shown in Fig. 29, the isolated *E. coli* Nissle flagellin induced a dose-dependent increase of hBD-2 promoter activation.

Moreover, the preincubation of *E. coli* Nissle culture supernatant with H1 flagellin antiserum inhibited hBD-2 mRNA expression by almost 70 % (Fig. 30). Consistent with this observation, the hBD-2 induction by isolated H1 flagellin was inhibited by 50 % in the presence of the corresponding antiserum. The antibody alone had no effect on hBD-2 mRNA expression.

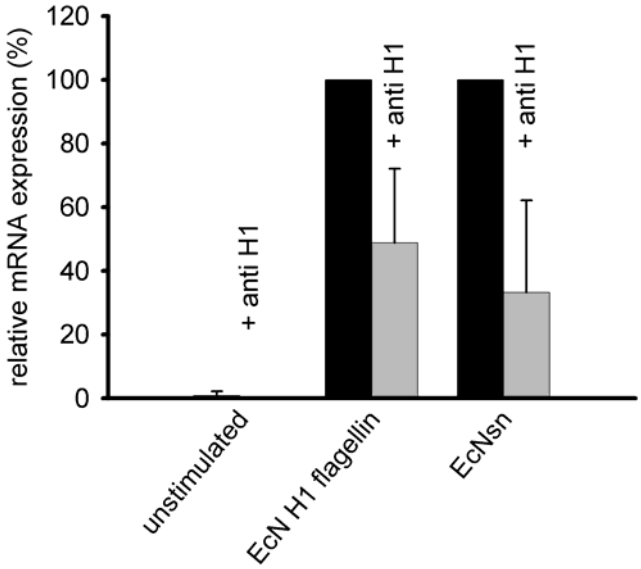


Figure 30: **Effect of anti-H1 flagellin antiserum on activity of flagellin and *E. coli* Nissle supernatant.** Isolated flagellin (0.1µg/ml) or *E. coli* Nissle supernatant (grey columns) were mixed with H1-flagellin antiserum (1:100) in FCS-antibiotic-free DMEM and added to Caco-2 cells. The untreated flagellin and supernatant were used as a reference (black columns). After 6 h of exposure, RNA was isolated followed by determination of hBD-2 mRNA expression by real-time PCR. The data are means ± the SD of three experiments in duplicate.

Isolation of different flagellins

Since flagellins from different *Salmonella* species have been shown to induce hBD-2 expression in epithelial cells (Ogushi 2001), we isolated flagellin from *Salmonella enterica* serovar Enteritidis, as well as from the apathogenic *E. coli* strain ATCC 25922 and the *E. coli* strain JM109 as controls.

The genome structure of *E. coli* Nissle was shown to resemble closely that of the uropathogenic *E. coli* CFT073Δ*hly* strain (see Appendix).

Hence, we compared the hBD-2 inducing capacity of the serotype-identical CFT073Δ*hly* with that of the *E. coli* Nissle flagellin. Only flagellin from *S. enteritidis* induced hBD-2 mRNA significantly (Fig. 31a + b) in contrast to the flagellin of the apathogenic *E. coli* strain ATCC 25922 (Fig. 31b) that lacked an induction.

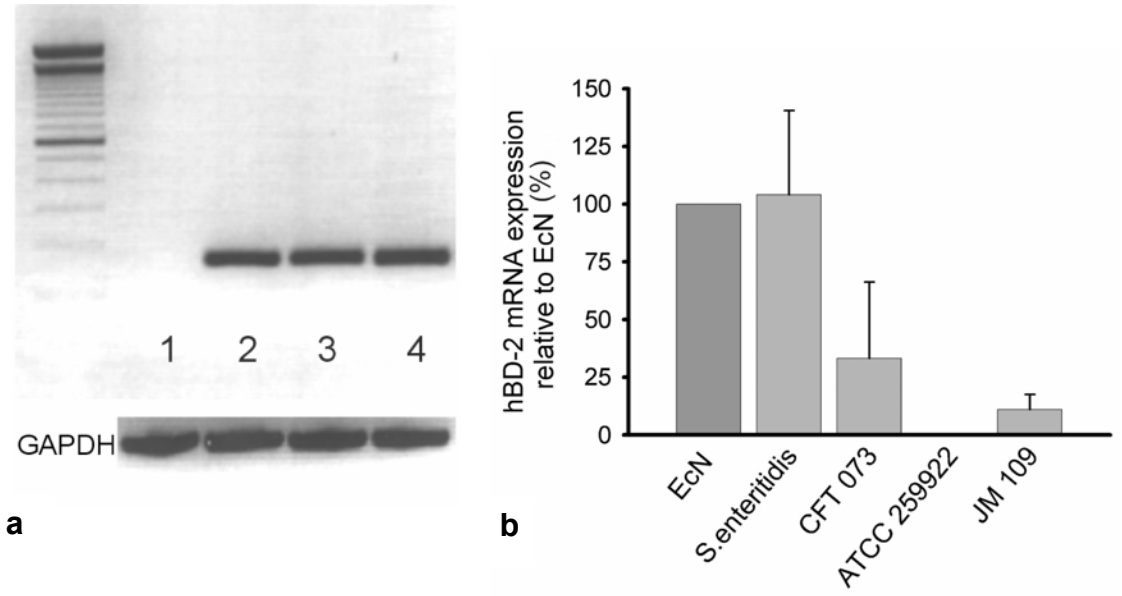


Figure 31: **Flagellin isolates induce hBD-2 mRNA expression. a)** Qualitative hBD-2 mRNA expression induced by *E. coli* Nissle flagellin. The results of agarose gel electrophoresis (2 %) of RT-PCR products from Caco-2 cells incubated for 6 h with medium (*lane 1*), *E. coli* Nissle supernatant (*lane 2*), *E. coli* Nissle flagellin (*lane 3*) or *S. enteritidis* flagellin (*lane 4*) are shown. hBD-2 fragments are given in the upper panel, GAPDH controls of each sample are shown in the bottom panel (molecular mass marker, 100 bp). The product sizes were as follows: hBD-2, 172 bp; and GAPDH, 360 bp. **b)** Quantitative hBD-2 mRNA expression induced by flagellins from different bacterial strains. Caco-2 cells were stimulated for 6 h with flagellin isolates from *E. coli* Nissle, *Salmonella enteritidis*, CFT073Δ*hly*, *E. coli* ATCC 25922 and *E. coli* JM109. hBD-2 mRNA expression was assayed by RT-PCR. The data are means ± the SD of three experiments in duplicate.

The *E. coli* JM109 flagellin of the serotype H48 hardly induced hBD-2, whereas CFT073Δ*hly* flagellin was able to induce hBD-2 but not as strongly as the flagellin of *E. coli* Nissle (Fig. 31b).

The isolated flagellins were also subjected to Western blot analysis with an anti-H1 antiserum. Since serologically distinct flagella possess a highly conserved primary sequence of flagellin monomers within the exposed central region, all flagellins could be detected by Western blotting despite their different flagellin serotypes (Fig. 32). Interestingly, the CFT073Δ*hly* flagellin of the uropathogenic *E. coli* appeared to exhibit a smaller molecular size than the EcN flagellin despite having the same flagellin serotype.

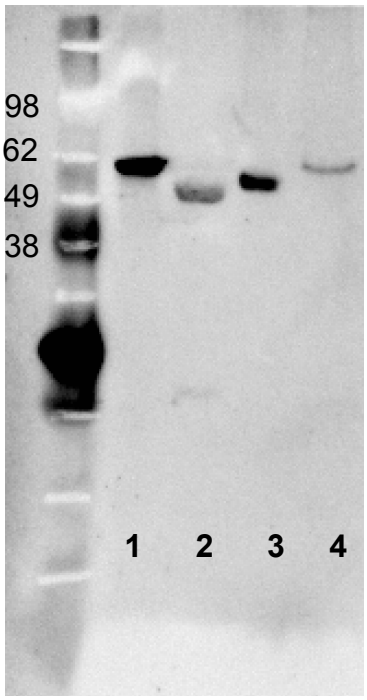


Figure 32: Western blot of flagellins from different bacterial strains. Flagellins from *E. coli* Nissle *(lane 1), Salmonella enteritidis (lane 2),* CFT073Δ*hly (lane 3),* and *E. coli* ATCC 25922 *(lane 4)* were separated by electrophoresis on SDS-PAGE gels and incubated with H1 flagellin antiserum overnight.

6 DISCUSSION

The evidence of a major role of the luminal flora in the development of IBD together with the effective use of antibiotics in the treatment of CD patients gave rise to alternative strategies to manipulate the intestinal flora. The administration of probiotics appeared to be a promising approach as it is principally free of systemic side effects. The question arose concerning how probiotics exert their numerous beneficial effects. These can hardly be explained by a unifying hypothesis that is based on a single quality or mechanism and remains valid for all microorganisms exerting one or the other effect mentioned before. The objective of the present study was to gain more insight into the influence of probiotics on the epithelial barrier function with respect to the induction of the antimicrobial peptide hBD-2. We therefore investigated the ability of probiotics to induce this defensin and examined essential signalling pathways and the transcriptional regulation. Our aim was further, to clarify which bacterial factor of the probiotic strain *E. coli* Nissle was responsible for inducing hBD-2 gene expression in intestinal epithelial cells.

6.1 Different cell lines as a model to investigate hBD-2 expression

To investigate whether different intestinal epithelial cell lines similarly express hBD-2 upon stimulation with *E. coli* Nissle or IL-1β we used four well-characterized human colonic cancer cell lines i.e. Caco-2, T84, SW480 and SW620. We chose to use cultured epithelial cell lines instead of freshly isolated enterocytes for several reasons. First, in contrast to harvested enterocytes, cultured epithelial cells are not contaminated by the presence of other cell populations, intestinal bacteria or endotoxins that could complicate data interpretation. Second, the viability of harvested enterocytes *in vitro* would have been limited and the harvesting process might have caused cell injury.

The hBD-2 inducing effect varied amongst the different intestinal cell lines tested. The highest expression pattern was measured in Caco-2 cells in the case of both stimulants. IL-8, which is induced upon contact with gram-negative bacteria, served as a control response gene and was also differentially strong secreted. This confirms earlier results from Schuerer-Maly *et al* (1994) who found a variable IL-8 response depending on the cell line tested with a missing IL-8 release from T84 cells upon stimulation with IL-1β. Treatment with *E. coli* Nissle supernatant had little effect on IL-8 secretion by T84 cells in contrast to

Caco-2, SW480 and SW620 cells, which released IL-8 significantly. This diversity may be related to differences in the recognition of specific hBD-2 but not IL-8 inducing ligands and variable signalling events upstream of NF-κB.

Taken together these results suggest Caco-2 cells as a model best suiting further investigations on hBD-2 expression induced by bacteria. However, the expression of antimicrobial peptides may not necessarily reflect the expression in normal cells of the human intestine. Successful culture of small intestinal mucosa specimens obtained from patients with IBD has been previously reported by Reimund *et al* (1996). Thus, we also tried to culture biopsies freshly obtained from either control or UC patients to imitate more closely the *in vivo* state. Previous *in vitro* studies of the function and metabolism of intestinal mucosa have been hampered by the rapid epithelial destruction which occurs after excision of intestinal mucosal samples and detachment from the basal membrane. Likewise, our model which was intended to be closer to the *in vivo* state than our experiments with cancer cell lines failed to demonstrate an hBD-2 inducing effect by *E. coli* Nissle or IL-1β treatment, probably also due to necrosis.
Considering the malignant characteristics of Caco-2, T84, SW480 and SW620 cells, it is tempting to speculate that the expression of antimicrobial peptides could be also due to abnormal Paneth cell-like functions expressed by these cells in culture.

6.2 TLR expression

The Toll-like receptor expression pattern in different intestinal cell lines was investigated to gain insight for possibly important bacterial components playing a role in hBD-2 induction. Since all cell lines expressed the investigated range of TLR´s and the associated adaptor molecules, we could not exclude any of the known pathogen-associated molecular pattern (PAMPs) as irrelevant for *E. coli* Nissle signalling. We did not compare the quantitative expression of TLR´s in the four cell lines, since our main interest was the identification of the bacterial inducers or ligands according to the “all-or-nothing” principle. The varying expression of IL-8 and hBD-2 in the different cell lines is obviously not related to a cell type specific TLR expression pattern since the receptors were constitutively expressed in each of the tested cell lines.

6.3 Time- and dose-dependent hBD-2 expression in Caco-2 cells

Several studies have demonstrated that IL-1β is a potent inducer of hBD-2 expression (Harder 2000; McDermott 2003). Furthermore, the up-regulation of hBD-2 expression is detected in surrounding areas of inflammation, but not adjacent non-inflamed regions (Liu 1998, Mathews 1999). Our data identify the proinflammatory cytokine IL-1β as a major agonist for hBD-2 induction in Caco-2 cells, which was found to up-regulate hBD-2 mRNA in a time- and dose-dependent manner.

Interestingly, hBD-2 expression in Caco-2 cells depended on the degree of cell differentiation or rather growth duration before stimulation. Since treatment of confluent cells with IL-1β resulted in a diminished hBD-2 expression we used subconfluent cells for our experiments. Eckmann *et al* (1993b) explains that more differentiated cells are hyporesponsive, which reflects the physiological situation. Controversial to our results Hase *et al* (2002) reported a shared coordination of the antimicrobial protein cathelicidin LL-37/hCAP18 gene expression in colon epithelium with augmented cell differentiation. Ca^{2+} induced differentiation of keratinocytes was also shown to increase hBD-2 expression in response to IL-1α stimulation (Liu 2002). These results suggest that the varying influence of differentiation on gene expression should also be taken into account when investigating hBD-2 inducibility in different cell models.

Based on our previous studies with *E. coli* Nissle, we further investigated whether other probiotic strains might also have the capacity to stimulate hBD-2 expression. A more or less distinct time- and dose-dependent hBD-2 induction by the diverse probiotic strains tested was determined; some strains also lacked any hBD-2 inducing effect. These results suggest that different strains of probiotic bacteria may exert different effects based on specific capabilities and enzymatic activities, even within one species. *L. plantarum* 299v for instance induced mucin gene expression (Mack 1999) thereby inhibiting adherence of enteropathogenic and enterohemorrhagic *E. coli* strains to epithelial cells. Furthermore, this strain also reduced elevated intestinal permeability due to *E. coli* exposure in rats (Fak 2006). However, in our experiments *L. plantarum* exerted only restricted hBD-2 transcription activation.

The probiotic VSL#3, a preparation consisting of 8 different bacterial species, seems to be effective in the treatment of pouchitis, which might be caused at least in part by the induction of the antimicrobial defence system of the gut which we determined. This hypothesis is confirmed by recent unpublished investigations of Otte *et al* (2005) who found that VSL#3, *L. acidophilus* and *E. coli* Nissle significantly induced the expression of diverse antimicrobial peptides (AMPs) such as hBD-2, -3 and -4, LL-37 and RNAse7. Another proof for a beneficial effect of VSL#3 via an induction of AMPs is given by Wang *et al* (2003) who specifically investigated the VSL#3 strain *Bifidobacterium longum*, which stimulated significantly hBD-2 gene expression in cultured human intestinal epithelial cells.

6.4 Bacterial supernatant is more potent than bacterial pellet to induce hBD-2

We further investigated whether Caco-2 cells express hBD-2 in the presence of soluble factors from the bacterial culture supernatant or predominantly in direct contact with the heat-killed bacteria. Indeed, the supernatant of bacteria expressed relevant hBD-2 inducing capacity with a maximal induction between 6 h and 12 h that was greatly attenuated after 24 h of incubation. This was a delayed response compared to the kinetic of hBD-2 expression mediated by the bacterial pellet. Moreilhon *et al* (2005) analyzed the influence of bacterial supernatant on epithelial cells using a pangenomic microarray. He determined also a diminished transcriptional response of genes implicated in inflammation and apoptosis to *Staphylococcus aureus* supernatant when stimulation exceeded 9 h up to 24 h.

Interestingly, when comparing the extent of hBD-2 induction by bacterial pellet or supernatant, a strikingly higher activation by soluble or shed products in the supernatant was determined by reporter gene assay, which was further confirmed on the mRNA level. However, this difference was not a general characteristic as two out of three supernatants from other probiotics, induced similarly strong hBD-2 than the corresponding pellet. The expression of chemokines e.g., CCL20, interleukins (IL-1, IL-6 and IL-8) and activation of NF-κB pathways was also shown to be upregulated in epithelial airway cells when treated with bacterial supernatant of *S. aureus*, but was only marginally altered after direct bacterial contact (Moreilhon 2005).

Several studies have further demonstrated that *E. coli* Nissle, VSL#3 or *L. acidophilus* LA1 respectively, were able to inhibit adhesion to and invasion of human intestinal epithelial cells by enteroinvasive pathogens via a secreted component independent from any direct physical contact (Bernet 1994; Madsen 2001; Boudeau 2003; Altenhoefer 2004). This effect might be due to non-specific steric hindrance of the apical enterocytic receptors for pathogens (e.g. by biofilm formation) and/or additional secretion of hBD-2 which further kills pathogens. Our data strongly suggest that a soluble factor or factors secreted by *E. coli* Nissle might be the predominant means by which hBD-2 transcription is regulated. Still we cannot exclude that bacterial lysis caused by the heat-killing procedure might result in a release of cytosolic proteins into the medium.

Focussing on the heat-treatment of our bacteria, the definition that probiotics have to be viable microorganisms to exert their beneficial effects might need to be revised, since bacteria, which did not survive, still exerted immunostimulatory effects. Dead bacteria could have an even stronger effect than living microorganisms. This assumption could be supported considering the capacity of dead lactobacilli to compensate lactase insufficiency in lactose maldigestion (de Vrese 2001) since dead bacteria in the small bowel are ingested and release β-galactosidase into the upper intestine. In addition, bacteria killed by irradiation, leaving the cell wall intact and therefore enabling protection during gastric transit, could also exert positive immunostimulatory effects (Rachmilewitz 2004).

6.5 Transcriptional regulation of hBD-2 expression by probiotics

NF-κB and AP-1 both participate in the regulation of a variety of genes including those encoding proinflammatory cytokines that are involved in the first line of defense to a variety of insults. Consequently, we concentrated our investigations on these two transcription factors although the hBD-2 gene is known to also include consensus sequences for the transcription factors STAT and NF-IL-6 (Tsutsumi-Ishii 2002).

The transcription factors NF-κB and AP-1 appeared to be both necessary for full hBD-2 promoter activation by the bacterial supernatant of *E. coli* Nissle as well as by *L. fermentum*, *Pediococcus pentosaceus* and *L. acipdophilus* similar to our previous findings with *E. coli* Nissle pellet (Wehkamp 2004). Tomita (2002) has also suggested that NF-κB and AP-

1 as well as intracellular calcium are essential for LPS-mediated hBD-2 expression in a human airway cell line (LC-2/ad). Diamond *et al* (2000) have demonstrated that transcription of the bovine β-defensin TAP gene is cooperatively regulated by NF-κB and NF-IL-6 in response to LPS. Since the promoter activation by *E. coli* Nissle supernatant depended in the present study also partly on AP-1, synergistic effects between NF-κB and AP-1 seem to be also quite likely. We suggest that AP-1 is not able to elicit hBD-2 gene activation without the contribution of NF-κB.

Many genes are regulated through synergistic effects of these two transcription factors, which were first described by Stein *et al* (1993), still we did not further investigate whether NF-κB and AP-1 interact directly or exert an additive effect on the promoter. In contrast to our findings, Tsutsumi-Ishii *et al* (2003) determined that AP-1 was not crucial to the LPS-mediated transcriptional regulation of the hBD-2 gene in mononuclear phagocytes since they determined no significant differences in promoter activity after deletion of the AP-1 binding site. They demonstrated further that the two tandemly repeated binding sites for NF-κB (at -577 and -188) were essential for the LPS responsiveness of the hBD-2 gene. Adjacent binding sites for NF-IL-6 within the hBD-2 promoter did not contribute to the LPS-induced hBD-2 transcription. In particular, the NF-κB binding site closest to the transcription start was shown to have the highest impact on hBD-2 promoter activation by *E. coli* Nissle or *H. pylori* respectively (Wehkamp 2004, Wada 2002).
We emphasize that despite the clear role of NF-κB and AP-1 in hBD-2 induction mediated by our tested probiotics a certain impact of other transcription factors cannot be excluded. In addition, posttranslational modifications might still interfere with the functionally relevant expression of hBD-2 protein.

The prominent role of NF-κB for the hBD-2 induction by diverse stimuli is listed in the introduction part (page 19). Because IECs are surrounded by commensal bacteria and are the first cells affected by pathogens, the effect of bacteria on NF-κB activation in IECs has been intensively examined. Savkovic *et al* (1997) reported that T84 cells infected with enteropathogenic *Escherichia coli* secrete IL-8 through an NF-κB-dependent mechanism. However, IL-8 gene expression was not induced in IECs infected with non-pathogenic *E. coli*, suggesting that IECs discriminate between non-pathogenic and pathogenic bacteria

with respect to NF-κB activation (Savkovic 1997). Hobbie *et al* (1997) have demonstrated that *Salmonella* infection of IECs triggers IκBα degradation and IL-8 release.
A number of invasive bacteria induce NF-κB dependent chemokines in a wide variety of IEC lines (Kagnoff 1997). The requirement of bacterial invasion is controversial, since non-invasive bacteria can also trigger NF-κB activation (Eaves-Pyles 1999) as we have also demonstrated in the present study.

Probiotic bacterial strains, relevant for industrial purposes, have been frequently presented as inhibitors of proinflammatory responses with a concomitant absence of NF-κB activation. For example, the probiotic strain *L. casei* was suggested to reduce inflammation by enhancing the activation of PPARγ, which exerts an anti-inflammatory role by the inhibition of the NF-κB pathway. Hence, *L. casei* also diminished LPS-induced expression of COX-2, IL-8 and TLR-4 (Eun 2006). In contrast to the widely claimed attenuation of inflammatory responses by probiotics on one hand and the strong proinflammatory effect of pathogens, *E. coli* Nissle might induce a proinflammatory response just above the "threshold level" preventing an inflammatory outcome, but inducing a higher alertness of the intestinal epithelial cells host defense system.

6.6 Signalling pathways involved in hBD-2 expression mediated by probiotics

In an attempt to identify some key elements that constitute the link between stimulation with probiotics and the expression of hBD-2, we evaluated whether three major subgroups of MAP kinases, ERK½, p38 and JNK were activated by these probiotics. The ERK signalling module was the first MAP kinase cascade to be characterised. Although, its primary role has long been thought to be restricted to cell growth and proliferation, it has become clear that several inflammatory processes involve ERK½ activation. ERK-1 deficient mice, for instance, expressed defective thymocyte maturation and reduced expression of α and β chains of the T cell receptor (Pages 1999). This indicates that ERK activation is crucial for T cell activation and appears to be mediated by the AP-1 family of transcription factors. The JNK signalling pathway is activated by cell stress and is involved in the regulation of cell proliferation and apoptosis. It appears in parallel with ERK½ to be essential for AP-1 activation due to stress and exposure to various cytokines. JNK has been impli-

cated in a large variety of pathological conditions, including cancer, stroke and inflammatory disorders.

Murine p38 MAP kinase was first identified in 1994 as a kinase activated in response to bacterial lipopolysaccharide (LPS) (Han 1994). Extracellular stimuli of p38 include a variety of cytokines (IL-1, IL-2, IL-18, TGF-β, TNF-α etc.) and a number of pathogens that activate p38 through the different Toll receptors. These involve LPS, peptidoglycan and enterotoxin from *Staphylococcus ssp.* as well as growth factors like GM-CSF (granulocyte macrophage-colony stimulating factor). The downstream targets of p38 are either other kinases or transcription factors such as ATF-2, which can form heterodimers with Jun transcription factors (both belong to the subfamily of AP-1). The MAP kinase p38 has also been associated with activation of NF-κB, as a p38 inhibitor (SB203580) attenuated NF-κB dependent transcription (Bergmann 1998).

Previous experiments regarding signalling events upstream of NF-κB mediated by whole *E. coli* Nissle bacteria (Wehkamp2004) revealed the explicit involvement of the c-Jun N-terminal kinase (JNK), whereas in the present study the supernatant of *E. coli* Nissle transmitted its signal also via ERK½ and to a smaller extent via p38. *L. fermentum* and *Pediococcus pentosaceus* induced hBD-2 promoter activation also via the three MAP kinases although the involvement of ERK½ and JNK seemed to be less pronounced than after *E. coli* Nissle supernatant stimulation. Downstream, ERK½ may be involved in the regulation of NF-κB through MEK, which has been shown to activate both IKK-α and IKK-β (Nakano 1998). Moon *et al* (2002) described also a role for ERK½ in IL-1α mediated hBD-2 induction in human middle ear epithelial cells.

Using an hBD-2 promoter construct with a mutated AP-1 binding site, which creates an almost exclusive activation via NF-κB, revealed that p38 might be of no importance for the NF-κB mediated activation of hBD-2 expression. The moderate inhibitory influence of SB203580 on p38-mediated hBD-2 activation suggests that p38 might play a more decisive role for AP-1 activation leading to hBD-2 transcription.

Here we demonstrate that the stimulation of the MAP kinase pathways by probiotics is directly responsible for the probiotic-induced activation of the AP-1 and NF-κB transcription factors and subsequent synthesis of hBD-2.

Is it possible that both transcription factors are activated by the same MAP kinases?

The answer is to be found in the upper signalling part, where the pathway diverges at the level of MEK kinases or MAP kinase kinases (MEKK´s 1-3), which further activate by phosphorylation:

a) MKK´s leading to the activation of p38 and JNK and additional members of the AP-1 family or

b) I-κBα kinases which regulate transient NF-κB activation (Hobbie 1997).

Thus, common pathways between NF-κB and AP-1 might exist, although the exact links of mutual activation via MAP kinases remain elusive. A probable synergy between NF-κB p65 and Jun/Fos of the AP-1 family might have important implications for both immune and inflammatory responses, since both transcription factors are responsible for immediate early gene expression.

Our results indicate that distinct probiotics share with several bacterial pathogens including *H. pylori,* enteropathogenic *(EPEC)* and enterohaemorrhagic *E. coli* (*EHEC)* the ability to activate MAP kinase signalling pathways and epithelial proinflammatory responses such as IL-8, but in a noninvasive manner. However, considering the great variety of species and strain characteristics, it becomes clear that a proven probiotic effect on one strain or species cannot be transferred to another strain or species.

6.7 Analysis of the hBD-2 inducing factor in the *E. coli* Nissle supernatant

6.7.1 LPS

The major molecular determinant of gram-negative bacteria responsible for NF-κB activation is the glycolipid lipopolysaccharide (LPS) which is found in high concentrations in the colon. This bacterial product signals through the IκB/NF-κB system by binding to its cell surface toll-like receptor (TLR) 4 and co-receptor MD-2 which then utilizes downstream components of the IL-1 signalling cascade (Zhang 2001; Faure 2000). Haller and colleagues (2002) have demonstrated that, following recognition of LPS by TLR4, the non-pathogenic commensal enteric bacteria *Bacteroides vulgatus* activates NF-κB nuclear translocation. This is followed by an induced proinflammatory gene expression in IEC

lines as well as in primary enterocytes. Since *E. coli* Nissle exhibits a special kind of LPS of the semi-rough type, it might mediate its beneficial effect of hBD-2 induction by this structure.

Although LPS has been reported to stimulate hBD-2 expression in gingival tissue and epidermal keratinocytes (Mathews 1999; Harder 1997a) other studies demonstrated that LPS did not trigger hBD-2 gene transcription in colonic epithelial cell lines (O´Neil 1999, Fahlgren 2003). Accordingly, we failed to detect hBD-2 after exposure of Caco-2 cells to either a control LPS (*E. coli* O111:B4) or *E. coli* Nissle LPS. The lack of response was not due to the absence of LPS-binding protein in our FCS-free experimental conditions, since addition of serum did also not induce hBD-2 expression. Furthermore, the absence of expression of the LPS receptor CD14 in the investigated cell lines cannot account for the missing hBD-2 induction. Though we confirmed only CD14 mRNA expression and not the functionally relevant CD14 protein expression a lack of the latter seems to be unlikely.

The failure of LPS to induce hBD-2 expression in Caco-2 cells may reflect an adaptation of the intestinal epithelial cells to the high levels of bacterial LPS that are normally present in the human colon compared to the lower levels in oral mucosa.
How is it then possible that LPS can exert the hBD-2 inducing effect in other tissues?
In vivo LPS penetrating deep into the skin might be detected by macrophages. These could serve as intermediators by the release of cytokines, which stimulate the hBD-2 expression (Liu 2002). When determining the IL-8 response on LPS stimuli we detected no increase of IL-8 release from T84 cells. Caco-2 cells secreted a 2-3 fold IL-8 release upon LPS stimuli compared to unstimulated cells similarly to SW620 cells. Schuerer-Maly *et al* (1994) also determined a missing IL-8 response of T84 cells upon LPS stimulation. In contrast to our results, Caco-2 cells were completely unresponsive to LPS regarding IL-8 secretion. SW620 cells but on the other hand were reported to be highly LPS responsive, which we found to be only weak. However, in our study, the IL-8 responsiveness to LPS was strong in SW480 cells.
Suzuki *et al* (2003) explains the differences between the cell responses by a classification of commonly studied intestinal epithelial cell lines into three different types of LPS responsiveness, TLR4 expression and the effect of priming with interferon-γ (IFN-γ).

Accordingly, Caco-2 cells belong to the phenotype, which is characterized by hyporesponsiveness to LPS, and reduced TLR4 protein expression, which is not influenced by pretreatment with IFN-γ. SW480 cells in contrast, exhibit a highly LPS-responsive phenotype and a clear TLR4 protein expression on the surface even in unprimed condition, whereas the rather LPS-hyporesponsive HT-29 cells, respond to LPS by IFN-γ induction via an increase of LPS uptake, MD-2 mRNA and intracellular TLR4 protein expression.
Furthermore, the degree of differentiation might also account for differences in LPS responsiveness, since PMA-treated leukemic THP-1 cell lines differentiate to a monocyte-like phenotype, which leads to an upregulation of TLR´s and an increased sensitivity to LPS (Zarember 2002).

6.7.2 Immunostimulatory bacterial DNA

We determined a constitutive expression of TLR-9 in Caco-2 cells which might thus have the ability to respond to CpG-rich bacterial DNA. Similar to the Toll-like receptor ligand LPS, immunostimulatory DNA sequences (ISS-DNA) exert a broad range of activities on the mammalian innate immune system. Numerous studies have shown that bacterial DNA elicits a NF-κB response, perpetuates inflammation and downregulates the antibacterial defence effectors HBD-1 and LL-37 (Obermeier 2005, Islam 2001). Akhtar *et al* (2003) provide evidence that exposure of HT-29 cells to *E. coli* DNA resulted in an exclusive AP-1 mediated induction of IL-8 mRNA and protein without the involvement of NF-κB. Several studies reported a diminished pathogen-induced proinflammatory response following the immunostimulatory effects exerted by probiotic DNA (Jijon 2004, Ewaschuk 2006). VSL#3 DNA exhibited for instance inhibitory effects on IL-8 secretion, p38 and NF-κB activation in HT-29 cells and reduced mucosal TNFα and IFN-γ secretion from IL-10-deficient mice after the oral intake of DNA (Jijon 2004). Furthermore, VSL#3 DNA protected mice from experimentally induced colitis when given intragastrically or subcutaneously (Rachmilewitz 2004). The administration of DNA from *Escherichia coli* also ameliorated colitis.

For our experiments we used phosphothioate ODNs as positive controls, which are nuclease resistant and can be as much as 200-fold more potent than natural phosphodiesters ODNs (Krieg 1995). Genomic DNA isolated from *E. coli* Nissle had no influence on hBD-

2 promoter activation and also the synthetic oligonucleotide analogs of immunostimulatory DNA sequences lacked to induce hBD-2 in Caco-2 cells. As a proof for intact immunostimulatory DNA, we could have monitored its effect on IL-8 production, which was reported to be increased in human embryonic kidney cells upon stimulation with CpG DNA (Bauer 2001). However, despite a potentially positive IL-8 response by Caco-2 cells to the bacterial DNA stimuli one cannot generally infer an association between an IL-8 induction and an hBD-2 induction.

Another reason for the missing stimulation of hBD-2 expression could be that signalling of CpG DNA might occur in intracellular compartments, since immobilized CpG DNA has been reported to be inactive (Krieg 1995). Further, stimulatory activity of CpG DNA was enhanced by lipofection (Gursel 2001); therefore, it might be possible, that our DNA could not initiate any immune reaction due to a defective internalization, which occurs normally via a clathrin-dependent endocytic pathway. TLR9 is located in the endoplasmatic reticulum (ER) of resting cells, but it translocates to a CpG DNA-containing lysosomal compartment for ligand binding and signal transduction (Latz 2004). If the proper distribution of TLR9 between the ER and the lysosomal compartment is not ensured, the binding of the CpG motif by TLR9 might be decreased and the successive signal transduction fails to be initiated. Nevertheless, according to Latz *et al* (2004) a small portion of TLR9 becomes surface accessible after exposure to CpG DNA, suggesting that a certain, albeit small DNA-related response could be transmitted by TLR9.

6.7.3 Heat and proteinase treatment

In our next attempt to identify the characteristics of our hBD-2 inducing component, we either boiled the supernatant or treated it with proteinase K. The heat treatment caused no significant change in hBD-2 induction capacity proposing a heat-stable factor to be responsible. This factor was revealed to consist mainly of protein, since protein digestion resulted in a strong down regulation of the normal hBD-2 response by Caco-2 cells.

6.7.4 Mutant *E. coli* Nissle strains

To characterize the responsible factor for hBD-2 induction further, we tried to determine known genes that code for surface expressed or secreted proteins. Since the whole genome

of *E. coli* Nissle has been partially sequenced (Grozdanov 2004; Sun 2005) some differences between this strain and other non-pathogenic or pathogenic bacteria contributing to its successful competition and lack of virulence traits are known. The genes for known fitness factors, distinct from other strains, were therefore deleted to evaluate their relevance for hBD-2 expression. Flagellin, the flagella filament structural protein, triggers native immune responses. Flagellins from *Salmonella typhimurium* (Eaves-Pyles 2001; Hayashi 2001) enteroaggregative *E. coli* (Steiner 2000) as well as *P. aeruginosa* (DiMango 1995) have been shown to stimulate epithelial cells to express interleukin-8 (IL-8), nitric oxide (Eaves-Pyles 2001), and human beta-defensin 2 (Ogushi 2001). Further, the secretion of flagellin into bacterial culture supernatants has been examined. Hence, we might expect flagellin from *E. coli* Nissle to be present in the supernatant, possibly playing a role as hBD-2 inducer.

It has been also reported, that gram-negative *H. pylori* were able to activate NF-κB in epithelial cells by their type IV pilus secretion system (Naumann 2000). *E. coli* Nissle exhibits two different type II secretion apparatus (Sun 2005); however this kind of secretion system functions only in live bacteria, still the supernatant of the heat inactivated bacteria might contain immunostimulatory pili or adhesins. Further, a recent study has demonstrated that *Pseudomonas aeruginosa* IV pilus was necessary to activate host genes in infected epithelial cells (Ichikawa 2000).

Interestingly, among the constructed deletion mutants only flagellin-negative mutants turned out to have a diminished capacity for hBD-2 promoter activation. Complementation of the Δ*fliC* and Δ*flgE* mutation in the mutant strains by addition of the cloned *fliC* and *flgE* genes restored the ability to normal hBD-2 induction. This strengthens the proof that this bacterial component alone already exhibits a strong defensin-stimulating trait. Similar results were obtained by Reed *et al* (2002) who demonstrated that a Δ*fliE*-deficient *S. typhimurium* mutant failed to secrete flagellin and lacked any surface assembly of flagellae. Complementation of the mutant strain also recovered the wild-type phenotype and elicited a proinflammatory response in host cells. Since *fliE* has been shown to interact with the flagellar hook protein *flgB*, a component of the basal body, it is thought to be required for the export of flagellar hook components (Minamino 1999, Minamino 2000). In T84 cells,

Δ*fliC* mutants of enteropathogenic *E. coli* induced significantly smaller amounts of IL-8 than the wild-type strains. Complementation restored the IL-8 inducing capacity of EPEC flagellin (Zhou 2003).

Despite the convincing results described above we cannot exclude that flagellin acts in concert with other molecules, which may also be essential for proper hBD-2 induction. Thus, we had to prove next, whether isolated flagellin, seperated from any potentially, synergistically acting molecules of the bacteria, would be capable to induce hBD-2 efficiently. Otte *et al* (2005) have reported that in comparison to other TLR ligands such as peptidoglycan, lipotaichonic acid or LPS only flagellin exposed the capacity to induce significantly the expression of the antimicrobial peptides hBD-2, LL-37 and RNase7. Isolation of the *E. coli* Nissle flagellin by previously reported methods revealed a unique protein of 60.81 kD as determined by mass spectrometry which was stainable by anti-H1 flagellin antiserum. The isolated protein was able to induce hBD-2 in a dose-dependent fashion further substantiating that flagellin alone is a sufficient stimulant. As reference served *Salmonella* flagellin which was also detectable with the anti H1-flagellin antiserum. However, the missing negative pathogenicity of *E. coli* Nissle, favours positive effects by this strain rather than by *Salmonella.*

Despite some diversity in glycosilation degree and serotype of flagellins isolated from different bacteria, all proteins were detectable by H1 antiserum in the Western blot. This serological cross-reaction might be explained by the remarkable conservation of the sequences that mediate filament assembly, which indicates that all bacterial flagellins are likely to be packed into filaments in a comparable way (Beatson 2006). The band size of the uropathogenic *E. coli* CFT073Δ*hly* flagellin in the Western blot differed compared to the band of *E. coli* Nissle flagellin, despite their flagellin serotype identity, possibly due to post-translational modifications i.e. glycosilation. Whether surface differences are the cause for the determined reduced hBD-2 inducing activity of CFT073Δ*hly* H1-flagellin compared to that of *E. coli* Nissle has to be further investigated.

Addition of anti-H1 to Caco-2 cells was able to suppress hBD-2 induction by isolated flagellin up to 50 %. Since we had used a polyclonal H1-flagellin antibody instead of a mono-

clonal antibody, the concentration of specific anti-H1 antibodies was too low to bind all flagellin. Consequently, the abrogation of flagellin induced hBD-2 induction was incomplete. We conclude that flagellin is the responsible factor of *E. coli* Nissle mediated hBD-2 induction in Caco-2 cells.

The role of flagellin in the development of IBD might also be an important subject for further investigation in this context. Previous studies suggest that bacterial flagellin is a dominant antigen in some patients with CD and mutations of innate receptors increase susceptibility to IBD (Maeda 2005, Lodes 2004). An aberrant innate response to unknown bacterial antigen has been proposed as a contributing factor in IBD. Since human colonic intestinal epithelial cells are hyporesponsive to LPS, bacterial flagellin might be an important inducer of proinflammatory response in IEC´s in the colon. In a study from Subramanian *et al* (2006) mucosal *E. coli* isolates from CD, UC and Colon cancer patients were all capable of inducing IL-8 release in colon epithelial cells, not only by the whole organism but also by the bacteria-free supernatant. Protease and leukocyte elastase pretreatment abolished the IL-8 response, which suggested a role for *E. coli* flagellin in mediating IL-8 secretion. Interestingly, ultracentrifugation of the supernatant from *E. coli* showed that 99 ± 0.5 % of the IL-8 stimulating activity sedimented with the outer membrane vesicle fraction, which contained flagella. Pretreatment with an anti-flagellin antibody blocked IL-8 secretion by 50 ± 19 % compared to untreated supernatant. Centrifugal membrane filtration of the supernatant revealed that 70 ± 30 % of the IL-8 release was in response to components > 30 kDa. Flagellin might hence induce the onset of inflammation in IBD.

The role of flagellin (*fliC*) in stimulating the proinflammatory response through TLR5 receptors has been reported (Gewirtz 2001, Hayashi 2001). The signal of *E. coli* Nissle flagellin might also be transmitted by TLR5, which is expressed in Caco-2 cells, but other pathways may also be relevant. A recent investigation has determined that NOD2, the intracellular receptor for muramyldipeptide, a fragment of bacterial peptidoglycan, is involved in the induction of hBD-2 (Voss 2006). Further, flagellin from enteroinvasive *E. coli* strains activated p38, ERK and JNK MAP kinase phosphorylation and induced IL-8 and GM-CSF release of IEC´s through NOD1 signalling (Miyamoto 2006). Since NOD1

has been implicated in NF-κB-mediated hBD-2 expression (Boughan 2006) *E. coli* Nissle flagellin might also induce hBD-2 via NOD1.
Furthermore, Ren *et al* (2006) suggests that in macrophages which did not express TLR5, the murine Naip5 (neuronal apoptosis inhibitory protein 5) (Wright 2003) might be responsible for flagellin recognition. In addition, Ipaf, the closest homologue of Naip, has been proposed as an intracellular microbial sensor involved in *Salmonella* initiated caspase-1 pathway activation (Mariathasan 2004). Recently, a direct association between cytosolic flagellin and Ipaf mediated caspase-1 cleavage, without any contribution of TLR5 was reported in macrophages (Miao 2006; Franchi 2006). Whether Ipaf might also be involved in flagellin signal transduction in intestinal epithelial cells is questionable. However, the scope of the present study was the identification of the bacterial factor rather than the elucidation of recognition mechanism by intestinal epithelial cells.

If flagellin is the main stimulatory component of *E. coli* Nissle the question arises why the hBD-2 induction by the bacterial pellet was so much lower than by the supernatant, as the flagella is normally integrated in the bacterial membrane. It is well established that flagellated *Salmonella* serovar *typhimurium*, grown *in vitro*, sheds a remarkable proportion of its flagellar subunits into the culture supernatant, of which *fliC* seems to be the most abundant protein under this circumstances (Komoriya 1999). Whether *E. coli* also sheds high concentrations of flagella during cultivation into the culture liquid is not known, but the heat treatment of our bacteria could provoke the release of the flagella into the supernatant, explaining its higher hBD-2 inducing potential.

The main obstacle to engage the epithelium is the distribution of TLR´s on the cell surface. Several lines of evidence indicate exclusive basolateral recognition of flagella by TLR5 (Gewirtz 2001; Gewirtz 2003) in the normal state thereby eliminating the chance of non-pathogenic flagellates to interact with intestinal epithelial cells. This may change in circumstance of inflammation when the epithelium is already breached and the tight junctions are leaky. Pathogenic strains such as *Salmonella* with a type III secretion mechanism obviously displace flagellin directly into the cell and have the potential to invade the epithelial lining thereby gaining contact to basolaterally expressed TLR5. Once the TLR5 recognition has taken place, the proinflammatory epithelial response is about to start with the sub-

sequent engagement of immune cells. However, flagellin synthesized or secreted by many nonpathogenic bacteria does not incude a proinflammatory response. Interestingly, *E. coli* Nissle as an apathogenic strain induces a proinflammatory response despite its lack of ability to translocate its flagellin across the epithelial membrane to a basolaterally expressed TLR5. Since we used undifferentiated cells for our flagellin experiments, the ligand might still be able to bind to TLR5 despite its exclusive basolateral expression, making a translocation unnecessary for signalling. Most ligands induce TLR expression in polarized cells on the basolateral side whereas unpolarized cells show a different pattern. Raz (2006) demonstrated that the activation of apical and basolateral TLR9 induced intracellular tolerance in IEC´s via distinct mechanisms. He determined that apical TLR9 triggering leads to a lack of IκBα degradation, an inhibition of TLR9 re-stimulation and the subsequent IL-8 production by the basolateral TLR9. Lack of apical TLR9, by a loss of polarization, facilitates NF-κB activation and provokes colitis. Hence, apical TLR9 activation in polarized cells sustains a peaceful coexistence with commensals. In contrast, another study demonstrated an almost 3-fold greater IL-8 release after apical compared to basolateral stimulation of cells with enterohaemorrhagic *E. coli* (Berin 2002). Nevertheless, signalling differences might similarly occur when TLR5 is activated basolaterally or apically by flagellin, therefore resulting in protective or inflammatory response. An increased mucosal permeability during inflammatory bowel disease, could also give commensal bacteria access to basolateral TLR5.

6.8 Outlook

With our results of *E. coli* Nissle flagellin, we provide the first evidence that flagellin from an apathogenic, probiotic bacterial strain specifically stimulates the expression of an antimicrobial peptide by epithelial intestinal cells. Since this strain, interestingly, inherits considerable genomic identity with the uropathogenic *E. coli* strain CFT073Δ*hly* it might mimic on the one hand its proinflammatory effects by NF-κB and IL-8 induction but on the other hand, it fails to cause any real inflammation in the patient. It remains to be shown, whether isolated *E. coli* Nissle flagellin exerts its defensin-stimulating effect also *in vivo* and might ameliorate human inflammatory bowel disease. In general, flagellin induces inflammatory processes *in vivo* e.g. *Salmonella* flagellin injected into mice elicited at higher concentrations a shock-like state associated with hypotension, vascular dysfunction

and mortality (Eaves-Pyles 2001). A recent report in DSS colitis has demonstrated an aggravation by flagellin isolated from a non-pathogenic *E. coli* strain (Rhee 2005). Although this might implicate a major drawback for the use of probiotics in inflammatory bowel disease, *E. coli* Nissle has proven its efficacy in remission maintenance not in active disease.

However, the administration of isolated *E. coli* Nissle flagellin might exert an attenuated immune response compared to the complete viable strain, since living bacteria exhibit still many unknown effects e.g. the occupation of ligand binding sites of TLR´s to prevent the translocation of pathogens, which cannot be executed by an isolated flagellum. Hence, an aflagellate *E. coli* Nissle strain carries still the potential to exert a range of other probiotic effects.

Focareta *et al* (2006) presented another application field of probiotics for the treatment and prevention of cholera, in form of a molecular mimicry of the ganglioside GM_1 receptor for cholera toxin (Ctx) expressed on the surface of a non-pathogenic *E. coli* strain, which was able to bind and neutralize more than 99 % of Ctx. In an *in vivo* experiment, the recombinant probiotic strain protected infant mice from challenge with virulent *Vibrio cholerae* even when the probiotic was given 4 h post-challenge.

E. coli Nissle flagellin mimicking pathogenic flagellin and occupying TLR5 might have similar effects in the prevention of pathogenic flagellin induced inflammation. Similarly, the construction of *E. coli* Nissle strains that express normal gut epithelial cell receptors for virulence factors might be an alternative means of preventing enteric infectious diseases in addition to other defined probiotic effects. Recombinant *E. coli* Nissle might be able to interfere with the interactions between for instance bacterial toxins (or adhesins) and host cell oligosaccharide receptors thereby interrupting a crucial step in pathogenesis. An advantage of a receptor mimic probiotic treatment would be its neutralization of virulence factors, without inhibiting the growth of the bacteria. Since resistance commonly occurs in response to a reduction in bacterial viability, the acquisition of resistance by pathogens to a probiotic receptor mimic is anticipated to be unlikely (Kuehn 2006).

Nevertheless, the hazard of an induction of autoreactive antibodies directed against these receptors remains. Furthermore, most pathogenic strains exhibit many different virulence factors, which can impossibly be neutralized by recombinant bacteria carrying only some of the recognizing receptors.

Finally, the administration of probiotics to patients susceptible to infections might be useful for prevention, since non-pathogenic strains often exhibit the metabolic capacity to occupy the same host niche as pathogenic strains. Thus, they could co-exist in the intestinal tract for a sufficient length of time to be therapeutic, particularly if they outnumber the pathogen. The necessity of a preventive administration of probiotics might be further supported by a study carried out in the IL-10 knockout mice model of IBD, where a reduced expression of the Paneth cell defensins cryptidin 1, 4 and 5 was determinable before any histological changes of colonic inflammation appeared (Inaba 2006).

Thus, an induction of antimicrobial peptide expression by probiotics might be a new therapeutic strategy to be applied in the remission state of diseases, to strengthen the defense mechanisms of the intestinal tract and extend the relapse-free time. For the acute state of inflammatory diseases, standard treatment should still be relied on, rather than switching too early to a probiotic treatment.

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8 PUBLICATIONS

Original work:

Schlee M, Wehkamp J, Altenhöfer A, Ölschläger TA, Stange EF, Fellermann K. Induction of human β-defensin 2 by the probiotic Escherichia coli Nissle 1917 is mediated through flagellin. ***Infect Immun* 2007*;* published in May in volume 75 (5)**

Wehkamp J, Harder J, Wehkamp K, Wehkamp-von Meissner B, Schlee M, Enders C, Sonnenborn U, Nuding S, Bengmark S, Fellermann K, Schröder JM, Stange EF. NF-κB- and AP-1-mediated induction of human beta Defensin-2 in intestinal epithelial cells by *Escherichia coli* Nissle 1917: a novel effect of a probiotic bacterium. ***Infect Immun* 2004; 72 (10): 5750-5758**

Wehkamp J, Harder J, Weichenthal M, Schwab M, Schäffeler E, Schlee M, Herrlinger KR, Stallmach A, Noack F, Fritz P, Schröder JM, Bevins CL, Fellermann K, Stange EF. NOD2 (CARD15) mutations in Crohn's disease are associated with diminished mucosal alpha-defensin expression. ***Gut* 2004; 53 (11):1658-64.**

Oral presentations:

Schlee M, Wehkamp J, Harder J, Wehkamp K, Schröder JM, Stange EF, Fellermann K. Das Probiotikum *E. coli* Nissle 1917 induziert humanes beta Defensin-2 in Abhängigkeit von NF-κB in intestinalen Epithelzellen. **DGVS-Tagung, Nürnberg (09/2003) *Z Gastroenterol* 2003; 41:73**

Schlee M, Wehkamp J, Harder J, Wehkamp K, Schröder JM, Stange EF, Fellermann K. The probiotic strain *E. coli* Nissle 1917 induces Human beta defensin-2 by a NF-κB dependent mechanism in Caco-2 cells. **WCPGHAN, Paris (07/2004) *J Pediatr Gastroenterol Nutr*, Vol. 39, Suppl. 1, June 2004**

Poster presentations:

Schlee M, Wehkamp J, Harder J, Wehkamp K, Schröder JM, Stange EF, Fellermann K. The probiotic strain *E. coli* Nissle 1917 induces Human beta defensin-2 by a NF-κB dependent mechanism in Caco-2 cells. **AGA, New Orleans, Louisiana (05/2004)**

Schlee M, Wehkamp J, Harder J, Nuding S, Fellermann K, Stange EF. Probiotics exert immunomodulatory effects by induction of Human beta defensin-2 in Caco-2 cells. **Gordon-Conference (Antimicrobial Peptides), Ventura, CA (03/2005)**

Schlee M, Nuding S, Altenhoefer A, Oelschlaeger TA, Wehkamp J, Stange EF, Fellermann K. *Salmonella enteritidis* and *E. coli* Nissle flagellin induce human ß-defensin-2 in Caco-2 cells. **Inflammatory Bowel Disease-Symposium, Stuttgart (11/2005)**

Schlee M, Wehkamp J, Altenhoefer A, Oelschlaeger TA, Stange EF, Fellermann K. Flagellin is the essential factor of *E. coli* Nissle mediated human ß-defensin-2 expression in CaCo-2 cells. **AGA, Los Angeles, CA (05/2006)**

Schlee M, Wehkamp J, Altenhoefer A, Oelschlaeger TA, Stange EF, Fellermann K. Flagellin ist der essentielle Faktor der *E. coli* Nissle vermittelten Expression von humanem beta-Defensin-2. **DGVS-Tagung, Hannover (09/2006) *Z Gastroenterol* 2006;**

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9 ACKNOWLEDGEMENTS

I thank Prof. Bode for introducing me to the research group of Prof. Stange. Although I worked externally, she included me always as fully belonging to her group and followed my progress with great interest. I thank her for the steady mental support and the helpful comments regarding my work.

I sincerely thank Prof. Stange, my thesis „father"; his enthusiasm, engagement and patience have been outstanding. I am grateful for his continuous support of the process of my studies, keeping the overview when things tended to turn too much into detail.

I wish to express my sincere gratitude to my supervisor Klaus Fellermann whom I confronted with my daily lab questions. I thank him for numerous hours of supervision, correction and helpful comments regarding the interpretation and publication of results. He supported the quality of this work by his sharp-wittedness and intellectual contribution.

Jan Wehkamp infected me with his fascination for defensin research. I acknowledge his support to present early my first results and profited from his important contacts to other research groups.

Sabine Nuding has contributed well with her broad knowledge of microbiology, which she shared with me. I appreciated our times of conversation with either serious but also humorous content, which refreshed my strained lab-brain.

I acknowledge highly the cooperation with Jürgen Harder and Kai Wehkamp for providing the hBD-2 luciferase reporter plasmids, executing hBD-2 ELISA measurements and MS spectrometric analysis. I was grateful for the excellent possibility of training during 4 weeks in their lab and the helpful and interesting discussions since.

Without the cooperation with Tobias Ölschläger and Artur Altenhöfer, who constructed the flagellin mutants and proved the lack of flagellin expression by Western blot analysis and swarm agar assay, we might still search for the needle in the haystack of *E. coli* Nissle mediated defensin induction.

Kathleen was a wonderful colleague and comrade who introduced me to the LightCycler world and many other methods with patience, humour and exactness.

Tabea, the cell-culture expert, gave me many hints to improve my cells well being.

I want to thank very much Silke, Dagmar and Petra and many others who shared with me their knowledge regarding Western blot, cell culture, and other lab questions.

A warm thank to my office and lab mates Alexander, Simone, Olga, Irmgard, Yoshio and especially Guoxing who were wonderful colleagues to me.

My PhD mate Monica Istrate shared with me late hours in the lab as well as nice barbecue evenings (outside the lab!) and discussed important subjects of life next to the lab world.

Bernd Borstel was an irreplaceable, patient help for all tricky computer problems, always quick without mourning at hand. I owe him good clues resulting in nicely formatted presentations and posters and enjoyed interesting discussions with him about different aspects of life.

Thank´s be to all my faithful friends who saw very little of me in the past years and tolerated my excuses of lack of time especially Ulrike, Herbert, Evelyn, Sabine, Tanja and many others.

The virus-free computer of Karl Möndel, which he borrowed me during my time of writing at home, was a very kind gesture, which I appreciated highly.

Spontaneously, my English friend Rob Tranter offered his help in correcting the style of my writing, which he did with enormous speed.

I thank my parents who prayed for me during my thesis and especially during times of exhaustion.

Importantly, I thank Alexander for his endurance and patience to support me during the thesis with all it’s up and down phases. He believed always in me and helped me to refresh my strength during hours spend in the beauty of the mountains, lakes, vineyards and fields.

I thank God who carried and comforted me in times of storms and deserts and rejoiced with me at flourishing times of productiveness.

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11 APPENDIX

Comparison of amino acid sequences of a H1 flagellin with *E. coli* K12, CFT073 flagellins

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                                                   fliC

                                                                                                                Section 1
                    (1) 1         10        20        30        40        53
fliC E.coli H1      (1) MAQVINTNSLSLITQNNINKNQSALSSSIERLSSGLRINSAKDDAAGQAIANR
fliC CFT073         (1) MAQVINTNSLSLITQNNINKNQSALSSSIERLSSGLRINSAKDDAAGQAIANR
fliC E.coli K12     (1) MAQVINTNSLSLITQNNINKNQSALSSSIERLSSGLRINSAKDDAAGQAIANR
Consensus           (1) MAQVINTNSLSLITQNNINKNQSALSSSIERLSSGLRINSAKDDAAGQAIANR
                                                                                                                Section 2
                   (54) 54      60        70        80        90       106
fliC E.coli H1     (54) FTSNIKGLTQAARNANDGISVAQTTEGALSEINNNLQRIRELTVQASTGTNSD
fliC CFT073        (54) FTSNIKGLTQAARNANDGISVAQTTEGALSEINNNLQRIRELTVQASTGTNSD
fliC E.coli K12    (54) FTSNIKGLTQAARNANDGISVAQTTEGALSEINNNLQRVRELTVQATTGTNSE
Consensus          (54) FTSNIKGLTQAARNANDGISVAQTTEGALSEINNNLQRIRELTVQASTGTNSD
                                                                                                                Section 3
                  (107) 107       120       130       140       159
fliC E.coli H1    (107) SDLDSIQDEIKSRLDEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGQTITID
fliC CFT073       (107) SDLDSIQDEIKSRLDEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGQTITID
fliC E.coli K12   (107) SDLSSIQDEIKSRLDEIDRVSGQTQFNGVNVLAKNGSMKIQVGANDNQTITID
Consensus         (107) SDLDSIQDEIKSRLDEIDRVSGQTQFNGVNVLAKDGSMKIQVGANDGQTITID
                                                                                                                Section 4
                  (160) 160       170       180       190       200       212
fliC E.coli H1    (160) LKKIDSDTLGLNGFNVNGSGTIANKAATISDLTAAKMDAATNTITTTNNALTA
fliC CFT073       (160) LKKIDSDTLGLNGFNVNGSGTIANKAATISDLTAAKMDAATNTITTTNNALTA
fliC E.coli K12   (160) LKQIDAKTLGLDGFSVKN-----NDTVTTSAPVTAFGATTTNNIKLTGITLST
Consensus         (160) LKKIDSDTLGLNGFNVNGSGTIANKAATISDLTAAKMDAATNTITTTNNALTA
                                                                                                                Section 5
                  (213) 213     220       230       240       250       265
fliC E.coli H1    (213) SKALDQLKDGDTVTIKADAAQTATVYTYNASAGNFSFSNVSNNTSAKAGDVAA
fliC CFT073       (213) SKALDQLKDGDTVTIKADAAQTATVYTYNASAGNFSFSNVSNNTSAKAGDVAA
fliC E.coli K12   (208) EAATDTG-G--TN-----PASIEGVYTDNGNDYYAKITGGDNDGKYYAVTVAN
Consensus         (213) SKALDQLKDGDTVTIKADAAQTATVYTYNASAGNFSFSNVSNNTSAKAGDVAA
                                                                                                                Section 6
                  (266) 266         280       290       300       318
fliC E.coli H1    (266) SLLPPAGQTASGVYKAASGEVNFDVDANGKITIGGQEAYLTSDGNLTTNDAGG
fliC CFT073       (266) SLLPPAGQTASGVYKAASGEVNFDVDANGKITIGGQEAYLTSDGNLTTNDAGG
fliC E.coli K12   (253) ------------------------------------------DGTVTMATGAT
Consensus         (266) SLLPPAGQTASGVYKAASGEVNFDVDANGKITIGGQEAYLTSDGNLTTNDAGG
                                                                                                                Section 7
                  (319) 319       330       340       350       360       371
fliC E.coli H1    (319) ATAATLDGLFKKAGDGQSIGFNKTASVTMGGTTYNFKTGADAGAATANAGVSF
fliC CFT073       (319) ATAATLDGLFKKAGDGQSIGFNKTASVTMGGTTYNFKTGADAGAATANAGVSF
fliC E.coli K12   (264) ANATVTD----------AN-TTKATTITSGGTPVQIDN--TAGSATANLGAVS
Consensus         (319) ATAATLDGLFKKAGDGQSIGFNKTASVTMGGTTYNFKTGADAGAATANAGVSF
                                                                                                                Section 8
                  (372) 372     380       390       400       410       424
fliC E.coli H1    (372) TDTASKETVLNKVATAKQGTAVAANGDTSATITYKSGVQTYQAVFAAGDGTAS
fliC CFT073       (372) TDTASKETVLNKVATAKQGTAVAANGDTSATITYKSGVQTYQAVFAAGDGTAS
fliC E.coli K12   (304) ---------LVKLQDS-------KGNDTDTYALKDTNGNLYAADVNETTGAVS
Consensus         (372) TDTASKETVLNKVATAKQGTAVAANGDTSATITYKSGVQTYQAVFAAGDGTAS
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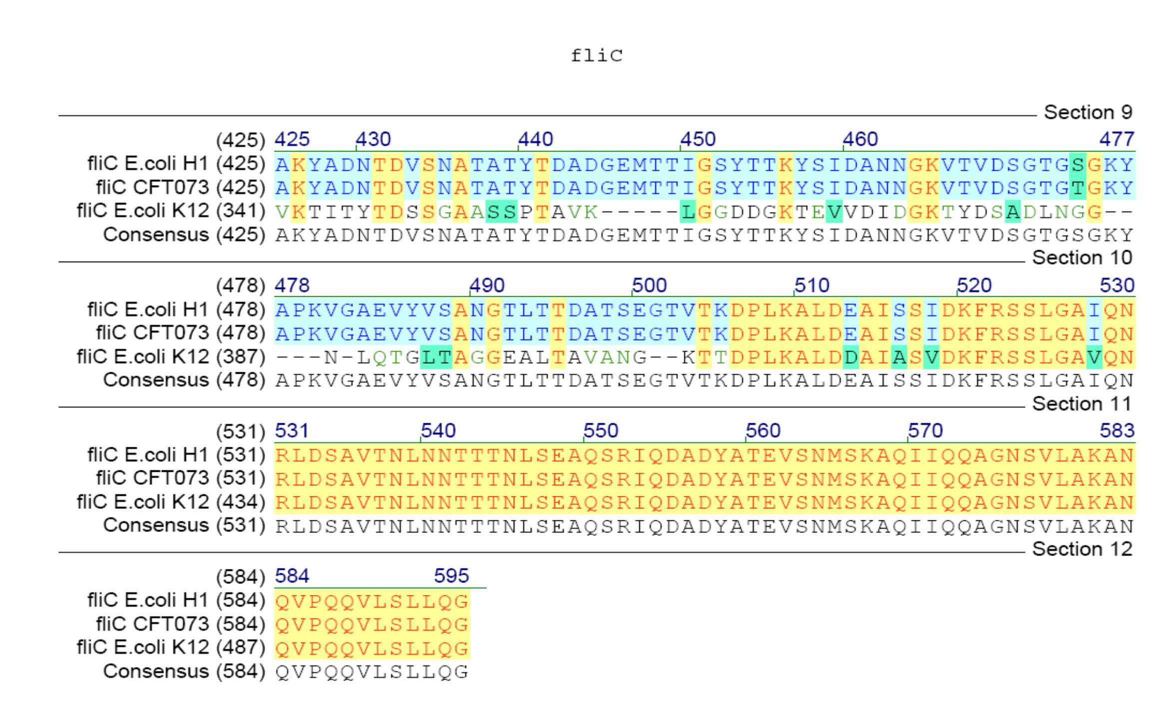

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                                     fliC

                                                                        Section 9
          (425) 425    430             440             450             460              477
fliC E.coli H1 (425) AKYADNTDVSNATATYTDADGEMTTIGSYTTKYSIDANNGKVTVDSGTGSGKY
fliC CFT073 (425) AKYADNTDVSNATATYTDADGEMTTIGSYTTKYSIDANNGKVTVDSGTGTGKY
fliC E.coli K12 (341) VKTITYTDSSGAASSPTAVK-----LGGDDGKTEVVDIDGKTYDSADLNGG--
   Consensus (425) AKYADNTDVSNATATYTDADGEMTTIGSYTTKYSIDANNGKVTVDSGTGSGKY
                                                                        Section 10
          (478) 478         490             500             510             520           530
fliC E.coli H1 (478) APKVGAEVYVSANGTLTTDATSEGTVTKDPLKALDEAISSIDKFRSSLGAIQN
fliC CFT073 (478) APKVGAEVYVSANGTLTTDATSEGTVTKDPLKALDEAISSIDKFRSSLGAIQN
fliC E.coli K12 (387) ---N-LQTGLTAGGEALTAVANG--KTTDPLKALDDAIASVDKFRSSLGAVQN
   Consensus (478) APKVGAEVYVSANGTLTTDATSEGTVTKDPLKALDEAISSIDKFRSSLGAIQN
                                                                        Section 11
          (531) 531          540             550             560             570           583
fliC E.coli H1 (531) RLDSAVTNLNNTTTNLSEAQSRIQDADYATEVSNMSKAQIIQQAGNSVLAKAN
fliC CFT073 (531) RLDSAVTNLNNTTTNLSEAQSRIQDADYATEVSNMSKAQIIQQAGNSVLAKAN
fliC E.coli K12 (434) RLDSAVTNLNNTTTNLSEAQSRIQDADYATEVSNMSKAQIIQQAGNSVLAKAN
   Consensus (531) RLDSAVTNLNNTTTNLSEAQSRIQDADYATEVSNMSKAQIIQQAGNSVLAKAN
                                                                        Section 12
          (584) 584        595
fliC E.coli H1 (584) QVPQQVLSLLQG
fliC CFT073 (584) QVPQQVLSLLQG
fliC E.coli K12 (487) QVPQQVLSLLQG
   Consensus (584) QVPQQVLSLLQG
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Declaration/Selbständigkeitserklärung

I hereby certify that this thesis is entirely my own work. All material and references, which were required for this work, are indicated.

Hiermit bestätige ich, dass ich die vorliegende Doktorarbeit selbständig verfasst und nur die angegebenen Quellen und Hilfsmittel verwendet habe.

Stuttgart, den 20. November 2006

(Miriam Schlee)