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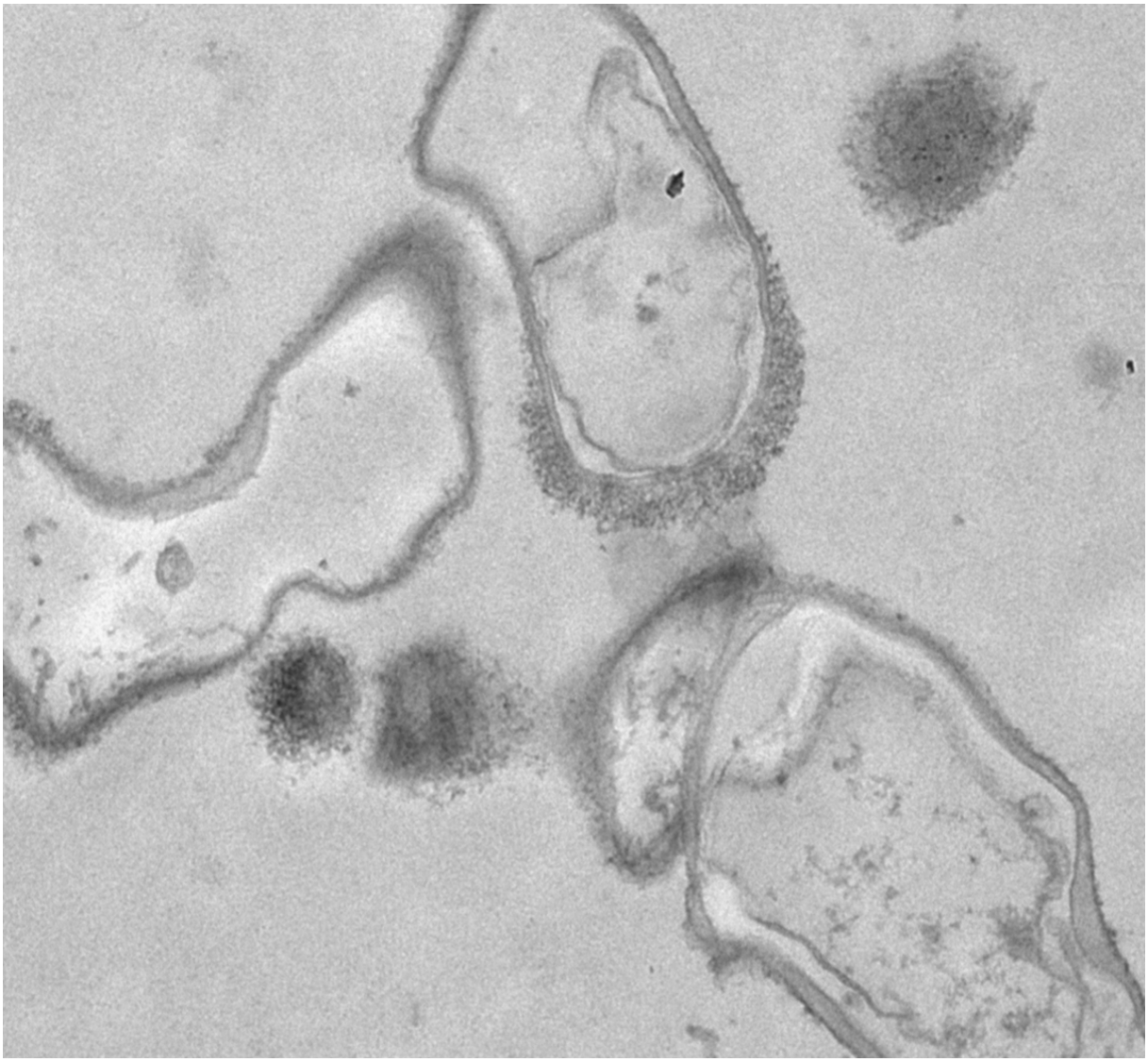
THE RELATIONSHIP BETWEEN HUMAN BETA-DEFENSINS AND ANAEROBIC COMMENSAL GUT MICROBIOTA



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The Relationship between Human Beta-defensins and anaerobic commensal Gut Microbiota

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I Summary

Humans are permanently confronted with microorganisms, including bacteria, fungi and viruses. They do not only colonize outer surfaces but also grow in large numbers in the human intestine. In the gut, the majority of microbes are commensal bacteria, which have beneficial effects on the host. They assist in degrading complex carbohydrates, provide vitamins and modulate the intestinal immune system. As important components of the innate immune system, endogenous antimicrobial peptides (AMPs) defend against bacteria, fungi and some viruses. They are small, cationic peptides produced by multicellular organisms, including vertebrates, invertebrates and plants. One of the major classes of AMPs are the defensins, which are characterized by a conserved array of disulphide bridges. Depending on their disulphide-connectivity, they are sub-grouped into α- and β-defensins.

Probiotics, which are live nonpathogenic microorganisms that confer health benefits on the host when administered in adequate amounts, have been found to stimulate β-defensin production in different cell culture experiments. They exhibit a beneficial effect after application in different diseases, but the molecular basis of their *in vivo* effect was unclear. In this work it is described that the probiotic *E. coli* Nissle stimulates the secretion of human β-defensin 2 (hBD-2) into the feces of healthy volunteers. The probiotics used are effectively killed by hBD-2 and other antimicrobial peptides, explaining the requirement for the continuos application of probiotics.

Since most commercial probiotic mixtures include *Bifidobacteria* and *Lactobacilli,* which are also normally part of the commensal intestinal flora, we investigated the effect of the constitutively produced human β-defensin 1 (hBD-1) on these and other microorganisms. Because we wanted to reproduce the natural conditions in the gut as close as possible, we used a reducing environment like that present in the gut due to extensive bacterial metabolism. It is described in this work that only the reduced hBD-1 peptide but not the oxidized form is antimicrobially active against commensal *Bifidobacteria* and *Lactobacilli* and the opportunistic pathogenic fungus *Candida albicans*. Besides the surrounding medium, reduction of hBD-1 can be performed enzymatically in the presence of oxygen by the constitutively expressed oxido-reductase thioredoxin. Consequently, we found redox-modulation as a novel mechanism of regulating antimicrobial host defense.

In summary, human β-defensins and anaerobic commensal gut bacteria are in a well-balanced and tightly regulated relationship in which neither site favors too much closeness.

II Zusammenfassung

Der menschliche Körper ist permanent Mikroorganismen wie Bakterien, Pilzen und Viren ausgesetzt. Diese besiedeln nicht nur äußere Oberflächen, sondern sind auch in großen Mengen im menschlichen Darm zu finden. Dort besteht die Mehrzahl der Mikroorganismen aus kommensalen Bakterien, welche positive Effekte für den Wirt besitzen. Sie unterstützen den Abbau komplexer Kohlenhydrate, synthetisieren Vitamine und beeinflussen das intestinale Immunsystem. Ein wichtiger Bestandteil des angeborenen Immunsystems sind antimikrobielle Peptide (AMPs), welche Bakterien, Pilze und einige Viren eliminieren. AMPs sind kleine, kationische Peptide, welche von Wirbeltieren, wirbellosen Lebewesen sowie Pflanzen produziert werden. Eine der Hauptklassen der AMPs sind die Defensine, welche durch eine besondere Anordnung von Disulphidbrücken charakterisiert sind und abhängig davon in α- und β-Defensine eingeteilt werden.
Probiotika sind lebende, nicht-pathogene Mikroorganismen, die einen positiven Effekt auf die Gesundheit des Wirts besitzen, wenn sie in entsprechenden Mengen verabreicht werden. In Zellkultur-Experimenten wurde gezeigt, dass Probiotika die Produktion von β-Defensinen stimulieren. Auch wurden Probiotika erfolgreich in einigen Krankheiten eingesetzt, die genaue Wirkung *in vivo* war bislang allerdings nicht geklärt.

In dieser Arbeit wird beschrieben, dass die Verabreichung von Probiotika an gesunde Probanden eine Sekretion des humanen β-Defensins 2 (hBD-2) in den Stuhl bewirkt. Die verabreichten Probiotika sind nicht resistent gegen hBD-2 und andere AMPs, was erklären könnte, warum Probiotika kontinuierlich eingenommen werden müssen.
Da die meisten kommerziellen Probiotika Bifidobakterien und Laktobazillen, welche auch Teil der kommensalen Flora des Darms sind, enthalten, interessierte uns die Wirkung des konstitutiv produzierten humanen β-Defensins 1 (hBD-1) auf diese und andere Mikroorganismen. Um den Bedingungen im Darm möglichst zu entsprechen, führten wir die Versuche unter reduzierenden Bedingungen durch, wie sie im Darm durch mikrobiellen Stoffwechsel herrschen. In dieser Arbeit wird beschrieben, dass nur das reduzierte, aber nicht das oxidierte hBD-1 gegen Bifidobakterien und Laktobazillen sowie gegen den opportunistisch pathogenen Pilz *Candida albicans* wirkt. Neben dem reduzierenden Milieu kann auch die Oxidoreduktase Thioredoxin die Reduktion von hBD-1 katalysieren. Somit konnten wir die Redox-Modulation als einen neuen Mechanismus der antimikrobiellen Abwehr gegen Bakterien und Pilze identifizieren.

Zusammenfassend lässt sich feststellen, dass humane β-Defensine und anaerobe Darmbakterien eine gut ausbalancierte und eng regulierte Beziehung pflegen, in welcher beide Seiten keine zu große Nähe bevorzugen.

III Introduction

1 Man and Microbes

Humans are permanently confronted with bacteria. In the body, estimations assume a total number of up to 100 trillion bacterial cells, which is tenfold the number of human cells [Ley et al., 2006]. While only minor amounts of microbiota can be found at external body sites as the skin, major numbers reside in the human intestine. With up to 10^{12} cells per gram of luminal content the colon is one of the most densely populated microbial ecosystems [Garrett et al., 2010]. In contrast, the bacterial load in the small intestine is only about $10^{3\text{-}9}$ bacteria per gram of content [Hao and Lee, 2004].
Microbial colonization starts immediately after birth by maternally acquired bacteria and is continued by exposure to environmental microbes taken up during nutrition. Although the specific composition differs between individuals, generally *Firmicutes spp.* and *Bacteroidetes spp.* form the two major phylotypes, constituting over 90% of the known intestinal population [Ley et al., 2008, Qin et al., 2010].

For a long time it has been a challenging task to characterize bacterial communities in the human gut since most species are not easily cultivable. Only recently novel techniques allowed a more detailed categorization of the complex ecosystem. Culture-independent 16S ribosomal RNA (rRNA) gene based methods generated initial impressions of the complexity of the microbial composition. Today, such analyses can be performed by metagenomic sequencing, allowing to handle large data sets [Riesenfeld et al., 2004,von Mering et al., 2007]. With these methods it became clear that the gut microbial gene set is about 150 times larger than the human set and that more than 99% are of bacterial origin, representing more than 1,000 different species [Qin et al., 2010]. Nevertheless, representatives from the domains Archaea, Eukarya and their viruses are also present.

During evolution, an effective symbiosis has evolved between the human host and his microbiota. While the intestinal bacteria benefit from nutritional supply and comfortable accommodation, the host has some advantages from this relationship, too. Microorganisms are important contributors to the catabolization of glycans and synthesis of vitamins [Moran et al., 2008, Gill et al., 2006]. Furthermore, the presence of intestinal bacteria influences metabolic processes of the host, promoting nutrient uptake and digestion [Bäckhed et al., 2004,Samuel et al., 2008]. Because of these beneficial effects and since intestinal microorganisms expand the genetic repertoire of the host, a human body can be regarded as a superorganism [Gill et al., 2006, Cerf-Bensussan and Gaboriau-Routhiau, 2010].

2 Immunity in the Gut

Despite all the beneficial effects mentioned before, the tight relationship between man and microbes has its risks. The enormous bacterial load in the gut permanently challenges the intestinal immune system: invasion of microbiota into tissue has to be prevented and pathogenic microorganisms need to be eliminated, but at the same time an overreaction of the immune system should be avoided [Hooper and Macpherson, 2010]. Therefore, immunity in the gut is a tightly regulated system tolerating the presence of one group of microorganisms but at the same time fighting the presence of others.

The intestine is a primary immune organ composed of different specialized cell types. The adaptive immune system is thereby referred to as gut-associated lymphoid tissue (GALT). Peyer's patches, which are groups of organized lymph nodules located in the mucosal lamina propria, function as immune surveillance by sampling antigens from the intestinal lumen [MacDonald, 2003, Round and Mazmanian, 2009]. The process is executed by specialized epithelial microfold cells (M cells) which deliver antigens to dendritic cells (DCs) of the subepithelial parts of Peyer‘s patches. Activated DCs then migrate to mesenteric lymph nodes where production of immunoglobulin A (IgA) from naïve B cells is induced [Macpherson and Uhr, 2004]. Secreted IgA finally accumulates in the mucus and prevents adhesion of intestinal bacteria to the epithelial surface [Fagarasan and Honjo, 2003]. In addition, activated DCs trigger the maturation of pathogen specific T-cells, leading to a clearance of invading microorganisms [Autenrieth and Schmidt, 2000].

Since the successful initiation of an adaptive immune response lasts up to several days, an effective innate immune response is required for an immediate defense. Within the lamina propria, invading bacteria that have passed the epithelial barrier can be detected and eliminated by circulating macrophages by use of phagocytosis, antimicrobial effector molecules as well as reactive oxygen species [Kelsall, 2008]. In contrast to macrophages residing in other tissues, intestinal macrophages have adopted to the constant stimulation by bacteria and their products. Thus, to prevent an overreaction of the immune system, they do not release large amounts of pro-inflammatory cytokines leading to recruitment of neutrophils and T-cells [Smythies et al., 2005].

As it has already become clear, an important role in barrier defense is played by the intestinal epithelium. This single-cell layer forms a tightly sealed physical barrier preventing mechanically the invasion by microorganisms. A crucial function here is held by the tight junctions, which link adjacent epithelial cells to form an impermeable network [Staehelin et al., 1969]. Moreover, the intestinal epithelium is covered by a thick layer of mucus, produced by Goblet cells located in the crypts and villi. The mucins, sticky

glycopeptides comprising the mucus, can interact with microbial surfaces to trap bacteria and accordingly impede access to the epithelial surface [Wadolkowski et al., 1988, Gusils et al., 2004]. Indeed, it was shown by mouse experiments that mucus consists of two layers: a sterile, densely packed inner layer covering the epithelial cells and an expanded outer layer which is colonized by bacteria [Johansson et al., 2008].
Besides being a physical and mechanical barrier the intestinal epithelium also holds chemical defense mechanisms. The most effective cell type to mention here is the Paneth cell, located at the bottom of small intestinal crypts of Lieberkühn. This specialized epithelial cell is an effective producer of antimicrobial peptides and enzymes [Jones and Bevins, 1992, Ouellette, 1999, Porter et al., 2002] which will be described later in detail.

The first step in defending epithelia against microorganisms is their recognition. This is accomplished by epithelial cells through a diverse set of pattern recognition receptors (PRR), which are able to sense microbe-associated molecular patterns (MAMPs). These have been historically referred to as pathogen-associated molecular patterns (PAMPs), although they are also present on non-pathogenic microbes. P/MAMPs like lipopolysaccharide (LPS), lipoprotein, peptidoglycan (PGN), lipoteichoic acid, flagellin and CpG-containing, unmethylated DNA, are exclusively produced by prokaryotes and allow the distinction between infectious nonself from noninfectious self [Janeway, 1992]. The best-known PRRs are comprised by the group of Toll-like receptors (TLRs), which are expressed by myeloid and lymphoid cell lineages, epithelial and endothelial cells [Aderem and Ulevitch, 2000]. Up to now, 11 human TLR encoding genes have been discovered where TLR-1 to -9 each seems to bind a specific microbial pattern [Medzhitov et al., 1997]. Toll-like receptors can be classified into two groups according to their subcellular localization. TLR-1, -2, -4, -5 and -6 are found at the plasma membrane while TLR-3, -7, -8 and -9 are restricted to intracellular compartments like endosomes [Pasare and Medzhitov, 2004]. After recognizing bacterial structures TLRs trigger intracellular signaling cascades, leading to the activation of pro-inflammatory pathways [Schwandner et al., 1999, Bauer et al., 2001].
A different group of PRRs is the family of nucleotide-binding oligomerization domain (NOD) proteins. These receptors are expressed intracellularly and detect PGN. While NOD1 recognizes muramyl tripeptides derived from Gram-negative bacteria, NOD2 recognizes muramyl dipeptide characteristic for both Gram-positive and -negative bacteria [Girardin et al., 2003a, Girardin et al., 2003b, Chamaillard et al., 2003]. Recognition of bacterial products by these receptors will trigger a cascade leading to an activation of the innate and adaptive immune system [Kobayashi et al., 2005].

The question arises why stimulation of PRRs by pathogens leads to an effective immune response whereas MAMPs of nonpathogenic bacteria do not necessarily cause a similar activation. A final answer has yet to be found, but several hypotheses exist. Since most of the anaerobic bacteria of the normal flora are not well characterized, it may be that they do express fewer MAMPs than their pathogenic counterparts or that their MAMPs are less biologically potent. Second, intestinal epithelial cells might have reduced expression of some PRRs leading to a non-responsiveness against permanent stimulation by the normal flora. And third, commensal bacteria are possibly not in direct contact with the intestinal epithelium due to the defense mechanisms mentioned before [Didierlaurent et al., 2002].

3 Antimicrobial Peptides in the Intestine: Defensins

Upon recognition of bacterial products via their receptors, Paneth cells and other epithelia secrete antimicrobial peptides into the intestinal lumen. Antimicrobial peptides are endogenous, naturally produced peptide antibiotics which are able to kill a wide range of microorganisms including bacteria, fungi and viruses [Zasloff, 2002, Boman, 2003]. They are produced by plants, vertebrates, invertebrates and possibly by all multicellular organisms, demonstrating their important role in innate immune function [Martin et al., 1995, Bevins et al., 1999]. Most antimicrobial host peptides have a cationic charge (cationic antimicrobial peptides, CAMPs) to effectively bind microbial surface structures [Weidenmaier et al., 2003]. They include bactericidal proteins as defensins, cathelicidins, cryptdin-related sequence peptides, some chemokines, bactericidal/permeability-increasing protein (BPI) and antimicrobial enzymes as lysozyme and group IIA phospholipase A2 (PLA2) [Müller et al., 2005]. An overview of intestinal antimicrobial peptides is given in table 1.
Although the exact mode of action of AMPs is not clear yet, it is obvious that they target essential components required for bacterial structure or metabolism. Consequently, microbial resistance against CAMPs is found only very rarely. Nevertheless, by modification of their surface structure or secretion of AMP-specific proteases, some bacteria manage to cope with permanent exposure to host AMPs [Peschel, 2002, Peschel and Sahl, 2006].

Humans produce a wide spectrum of antimicrobial peptides with the defensins forming one of the major groups [Wehkamp et al., 2005a, Harder et al., 2007]. Defensins are characterized by their small size of 28-47 amino acids and a molecular weight of 3-5 kDa and a cationic charge [Ganz, 2003]. Most of them are sub-grouped into α- and β-defensins, depending on the pattern of their three characteristic intramolecular disulphide bridges. Both groups share a similar β-sheet-like three-dimensional structure, are encoded by a

gene cluster on chromosome 8p23 and are produced as inactive pre-pro-peptides [Skalicky et al., 1994, Diamond et al., 1996, Harder et al., 1997, Liu et al., 1997].

Intestinal Antimicrobial Peptides and Polypeptides			
AMP	Producing cell	Molecular weight [kDa]	Charge
α-Defensins			
Human Defensin 5 (HD-5)	Paneth cell	3.3	+3
Human Defensin 6 (HD-6)	Paneth cell	3.3	+2
β-Defensins			
Human Beta-Defensin 1 (hBD-1)	intestinal epithelia	3.9	+4
Human Beta-Defensin 2 (hBD-2)	intestinal epithelia	4.3	+6
Human Beta-Defensin 3 (hBD-3)	intestinal epithelia	5.1	+11
Lysozyme	Paneth cell	14.7	+8
Cathelicidin			
LL-37	intestinal epithelia	19.3	+8
Bactericidal/ permeability-increasing protein (BPI)	intestinal epithelia	50.7	+14
Group IIA phospholipase A2 (PLA2)	Paneth cell	13.9	+15

Table 1: Selection of intestinal antimicrobial peptides and polypeptides. Molecular weight and charge values were obtained from NCBI database (www.ncbi.nlm.nih.gov/protein) and ExPASy Proteomics Server (www.expasy.ch/tools/protparam).

Today, six α-defensins have been described: Human Neutrophil Peptides 1-4 (HNP1-4) and Human α-Defensins 5 and 6 (HD-5 and -6) [Ganz et al., 1985, Bevins, 2006]. Whereas HNPs are mainly produced by circulating neutrophils, HD-5 and HD-6 are expressed constitutively and exclusively by small intestinal Paneth cells. The ratio of HD-5 to HD-6 is about 3:1 while the expression level of HD-5 outnumbers those of lysozyme and PLA2 by a factor of up to 100 [Bevins, 2004, Wehkamp et al., 2006]. HD-5 is sto-

red as an inactive precursor in cytoplasmatic granules of Paneth cells and is activated by cleavage through the protease trypsin. Upon bacterial stimulation HD-5 is released into the intestinal crypts where it displays potent antimicrobial activity [Ghosh et al., 2002, Ayabe et al., 2000, Ericksen et al., 2005]. Further evidence for the relevance of this human α-defensin was obtained from mouse studies in which transgenic mice expressing human HD-5 were protected against enteric salmonellosis [Salzman et al., 2003]. In contrast, the role of HD-6 in innate immunity is not clear yet but an antiviral function is discussed [Klotman and Chang, 2006].

β-Defensins resemble α-defensins in size and structure but differ in the pattern of their characteristic disulphide-bridges. While 28 β-defensin genes have been identified so far and eight of them seem to be expressed [Schutte et al., 2002], major research focus remained restricted to hBD-1, hBD-2 and hBD-3.

HBD-1 was originally isolated from human blood filtrate as a cationic peptide containing 36 amino acids and three disulphide bridges [Bensch et al., 1995]. In subsequent studies different N-terminal truncated variants of hBD-1 were isolated from urine, ranging from 36 to 47 amino acid length [Valore et al., 1998]. All these peptides showed antimicrobial activity against laboratory strains of *Escherichia coli* (*E. coli*) in micromolar concentrations. Low pH did not alter antimicrobial activity of hBD-1 while high salt concentration inhibited the microbicidal effect. Besides expression by the kidney, hBD-1 mRNA expression was detected in the salivary glands, trachea, prostate, small intestine, colon, skin, respiratory tract, urogenital tract and oral cavity [Zhao et al., 1996, Zucht et al., 1998, Singh et al., 1998, Krisanaprakornkit et al., 1998, O'Neil et al., 1999, Harder et al., 2004]. Since hBD-1 is produced constitutively by these epithelial cells and mucosal surfaces, it seems plausible that it is essential for effective protection of these sites against microbial colonization. Functional studies found only minor antibiotic activity of hBD-1 in comparison with other defensins [Bensch et al., 1995, Tollin et al., 2003, Nuding et al., 2009], so it has been a controversy why an organism produces high amounts of an ineffective defense molecule.

While hBD-1 is produced constitutively, hBD-2, the second human β-defensin discovered, was identified as an inducible antimicrobial peptide from human skin [Harder et al., 1997]. The peptide consists of 43 amino acid residues and has the classical disulphide-bond motif. Its expression can be induced by pro-inflammatory cytokines, bacterial products and interleukins and is mediated by the transcription factors nuclear factor kappa-B (NF-κ-B) and activator protein 1 (AP-1), which can bind to the promotor region of hBD-2 [Wehkamp et al., 2004, Harder et al., 2007]. Broad-spectrum bactericidal activity of hBD-2 was

found against *E. coli, Pseudomonas aeruginosa, Staphylococcus epidermidis* and *Candida albicans* while the effect against *Staphylococcus aureus* was only bacteriostatic [Liu et al., 2002]. Similar to hBD-1, antibiotic killing activity of hBD-2 is abolished in the presence of high salt concentration.
Human β-defensin 3 (hBD-3) was isolated as a 45 amino acid-residue peptide from lesional psoriatic skin in a functional screen for antimicrobial peptides [Harder et al., 2001]. In addition, mRNA expression of hBD-3 was found in human skin, gingival keratinocytes, tonsils, esophagus, trachea, placenta, heart, skeletal muscle and fetal thymus and can be induced by bacterial products and cytokines [Harder et al., 2001, García et al., 2001]. In comparison with other defensins, hBD-3 seems to be the most potent, having a spectrum that includes several pathogenic and nonpathogenic bacteria, fungi and viruses [Hoover et al., 2003, Midorikawa et al., 2003, Nuding et al., 2009].

Since defensin activity requires a sufficient peptide concentration, a local enrichment at the intestinal surface would be favourable. As mentioned before, gut epithelia are covered by a layer of mucus. Besides only trapping bacteria, the sticky mucins have been shown to bind secreted antimicrobial peptides, including defensins, in mouse experiments [Meyer-Hoffert et al., 2008]. Probably, this interaction is caused by opposite electric charges of mucins and defensins, leading to an effective border control of mucosal surfaces.

4 Probiotics and Intestinal Immunity

With respect to host defense against intestinal microorganisms it has to be emphasized that not all of them are necessarily "bad". As stated earlier, several bacteria live as commensals in the intestine and have beneficial effects on the host. This has been exploited therapeutically by the application of probiotics. Probiotics are defined as live nonpathogenic microorganisms that confer health benefits on the host when administered in adequate amounts [Havenaar and Huis in't Veld, 1992, FAO/WHO, 2001]. They are mostly selected from *Lactobacillus* and *Bifidobacterium* genera but also some *E. coli* and *Saccharomyces* species are found [Saavedra, 2001, Marco et al., 2006]. Remarkably, the characteristic of having a probiotic effect is not defined by the species but by the strain level, making it difficult to relate it to specific molecular factors.
Most probiotics used today have been originally isolated from the human commensal flora. In 1885 Theodor Escherich isolated *Bacterium coli commune* from human colon and in 1890 Albert Döderlein characterized *Lactobacilli* from the vaginal flora. In 1919 Sigurd Orla-Jensen identified *L. bifidus* from the intestine of an infant, thereby completing the

base for todays probiotic *E. coli, Lactobacillus* and *Bifidobacterium* species.

Probiotics are used as dietary supplements or pharmaceutical products to treat or prevent gastro-intestinal disorders and have been successfully applied in gastro-intestinal infections, inflammatory bowel disease (IBD), allergic disease and acute diarrhea [Marteau et al., 2001, Borchers et al., 2009]. The mode of action of probiotics is diverse and only partly understood yet. Very likely, one mechanism includes the displacement of pathogenic microbiota by colonizing ecological niches and competing for nutrients [Lebeer et al., 2008]. This strategy might be effective for treatment and prevention of disorders caused by pathogens as acute and traveller's diarrhea. Furthermore, probiotics might affect the host adaptive immune system by modulating DC and T-cell responses through the induction of anti-inflammatory cytokines like IL-10 and transforming growth factor beta (TGF-β). This is important for preventing allergic diseases, excessive inflammation and autoimmune diseases [Smits et al., 2005].
A third mechanism of probiotic action involves the modulation of intestinal epithelial barrier function. *Lactobacilli* were found to stimulate mucus secretion *in vitro* after adherence to intestinal epithelial cells while *Lactobacillus rhamnosus* GG protected the intestinal epithelial tight-junctions from hydrogen peroxide-induced injury [Mack et al., 2003, Seth et al., 2008]. Moreover, probiotics are able to modulate innate immune function: *E. coli* Nissle 1917 (Mutaflor), different *Lactobacilli* as well as the commercial probiotic VSL#3, containing four different *Lactobacillus*, three *Bifidobacterium* and one *Streptococcus* species, induced expression of hBD-2 in Caco-2 cells. This effect was confirmed on protein level and found to be dependent on pro-inflammatory pathways. In contrast, 40 clinical *E. coli* isolates had no such effect, highlighting its specificity [Wehkamp et al., 2004, Schlee et al., 2008]. Consecutive studies on the underlying mechanism found that in the case of *E. coli* Nissle 1917 defensin induction was mediated by a specific flagellin. This was clearly demonstrated as isolated flagellin from *E. coli* Nissle 1917 was a potent inducer of hBD-2 [Schlee et al., 2007].

A drawback of all these studies is the use of cell culture-models while the molecular mechanism of the *in vivo* effect of probiotics remained unclear. Thus we planed to investigate how the administration of a viable *E. coli* probiotic mixture (Symbioflor 2) affected intestinal hBD-2 secretion into the feces of healthy humans [Möndel et al., 2009](Manuscript 1). Moreover, we investigated the effect of different antimicrobial peptides on probiotic *E. coli* strains to evaluate if an integration into the human normal flora is likely and if they can resist antimicrobial action.

5 Commensal Bacteria, Oxygen and Beta-Defensin 1

When dealing with intestinal microbes including commensals/probiotics and pathogens, the conditions in their natural environment have to be taken into account. Concerning the small intestine, oxygen concentration is very low and only about 22% of airborne oxygen content at the luminal surface. In the lumen only little information about oxygen content is available but it has been found that due to microbial metabolism the redox potential is only -150 mV. This issue is even more pronounced in the anaerobic colonic lumen with a redox potential between -200 mV and -300 mV [Wilson, 2005]. Consequently, to obtain a better picture of intestinal defense mechanism, these facts have to be considered.

Antimicrobial peptides have been mostly studied by testing „classical" bacteria tolerating the presence of oxygen, including *E. coli, S. aureus* and *P. aeruginosa*. However, in the human intestine anaerobic bacteria outnumber aerobes by a factor of 1000:1 to 10000:1 [Nagy, 2010]. Thus, when characterizing antimicrobial peptides of the intestine, (facultative) anaerobic bacteria including *Bifidobacterium, Lactobacillus, Bacteroides* and other genera, should be included in those studies.

Only recently intestinal microbes have been analyzed comprehensively, although antimicrobial testing was performed in the presence of oxygen [Nuding et al., 2009]. Thus we aimed to study antimicrobial activity of defensins against anaerobic bacteria of the human normal flora under reducing conditions as present in the intestine [Schroeder et al., 2011] (Manuscript 2). Therefore, we modified a classical antimicrobial test in a way that testing of anaerobic bacteria is possible (detailed protocol: Manuscript 3). Since we were primarily interested in the defense under non-inflammatory conditions, we focused on hBD-1, which is constitutively produced by intestinal epithelial cells and thus present regardless of inflammation or infection.

IV Probiotic *E. coli* treatment mediates antimicrobial human β-defensin synthesis and fecal excretion in humans. *Mucosal Immunology* 2009, 2(2):166-72.

1 Aims and hypothesis

Probiotics are live non-pathogenic microorganisms that confer health benefits on the host, when administered in adequate amounts. They are either used as dietary supplements or as pharmaceutical products. It was shown that some probiotic bacteria induce human β-defensin 2 (hBD-2) in cell culture experiments *in vitro*. Our aim was to test whether administration of a viable, probiotic *E. coli* culture (Symbioflor 2) stimulates secretion of hBD-2 into the feces of healthy individuals *in vivo*. Furthermore we wanted to investigate if probiotics are resistant to human antimicrobial peptides.

2 Methods

Symbioflor 2, which contains three genotypes of one strain *E. coli,* was administered to 23 healthy volunteers for three weeks while 5 individuals received a placebo. HBD-2 peptide concentration was determined in fecal samples before probiotic treatment, after 3 weeks, and for some individuals after 12 weeks (9 weeks after treatment) with ELISA. All three genotypes were analyzed on their induction of hBD-2 mRNA in cell culture experiments. Antimicrobial activity of hBD-2, -3 and lysozyme was tested against different probiotic *E. coli* strains including the three Symbioflor 2 genotypes.

3 Summary of findings

Administration of Symbioflor 2 caused a 3.7-fold increase of hBD-2 concentration in human feces after three weeks of treatment. The concentration was still elevated 9 weeks after end of treatment. Cell culture experiments revealed that only one Symbioflor 2 *E. coli* genotype out of three was able to induce hBD-2 expression while the other two had no effect. To study functional relevance it was tested if the three *E. coli* genotypes and the probiotic *E. coli* Nissle1917 resisted incubation with the antimicrobial peptides hBD-2, hBD-3 and lysozyme. All three peptides efficiently killed the probiotics, regardless if they induced hBD-2 expression or not. Concerning hBD-2, this might explain why probiotics have to be administered continuously: Probiotic bacteria induce production of this human beta-defensin, which is able to eliminate the bacterium.

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ARTICLES

Probiotic *E. coli* treatment mediates antimicrobial human β-defensin synthesis and fecal excretion in humans

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Inducible epithelial β-defensins (hBD) play an important role in intestinal barrier function. *In vitro* studies showed that clinically effective probiotics induce antimicrobial hBD-2. Here, we aimed to assess the *in vivo* effect in healthy volunteers and also addressed how defensins affect probiotic survival. Symbioflor 2® containing one strain of several viable genotypes of *E. coli* was administered to 23 healthy individuals. After 3 weeks, fecal hBD2-peptide was increased in 78% (mean 3.7 fold; p<0.0001). Interestingly, the fecal hBD-2 peptide was still elevated 9 weeks after treatment (p=0.008). *In vitro* studies revealed that this effect was mediated by only one out of three tested *E. coli* genotypes and comparable to probiotic *E. coli* Nissle 1917 (10-15 fold). Functional assays showed that all tested bacteria were similarly killed by defensins allowing to speculate about a suicidal character of this effect. Defensin induction seems to be a common and important mechanism of probiotic treatment.

INTRODUCTION

Probiotics are commonly defined as live non-pathogenic microorganisms that confer health benefits on the host, when administered in adequate amounts (1). General formulated expectations include that they should resist gastric, bile, and pancreatic secretions, attach to epithelial cells and colonize the human intestine. Probiotic microorganisms which are currently in use include most commonly lactic acid bacteria of the genera *Lactobacillus* and *Bifidobacterium* but also *E. coli* or yeasts, such as the *Saccharomyces boulardii* strain (2). Probiotics are either used as dietary supplements or as pharmaceutical products for therapeutic purposes.

Most recent clinical studies substantiate reliable evidence for the therapeutic efficacy of some, yet not all, probiotic organisms. The probiotics *Bifidobacterium bifidum*, *Streptococcus thermophilus* or *E. coli* Nissle 1917 (Mutaflor®) administered to toddlers highly significantly reduced the incidence of acute diarrhoea and rotavirus shedding (3,4). In adults, the efficacy of probiotics was demonstrated by shorter durations of traveller's diarrhea and other self-limited gastrointestinal infections (5). In recent years several studies substantiate that the intestinal flora plays a key role in the development of inflammatory bowel disease (IBD) (6). This chronic inflammation of the intestine is often grouped into two major entities, Crohn's disease (CD) and ulcerative colitis (UC), based on clinical features and histopathology. While inflammation in UC is typically restricted to the colon, that of CD occurs at many sites, most commonly in the small intestine and in the colon. In addition to genetic factors in IBD, numerous studies have implicated a key role of the intestinal microbiota in the pathogenesis of this disease (7,8). Especially for ulcerative colitis patients, probiotics offer a safe alternative to current therapy. Three independent studies have found an equal efficacy on the remission maintenance of ulcerative colitis after administration of the probiotic *E. coli* Nissle 1917 compared to the standard treatment with 5-aminosalicylate (9-11). The advantage of probiotic treatment was a prolongation of remission without any adverse effects in contrast to conventional treatment. The probiotic mixture VSL#3, composed of 8 different

bacterial species, also diminished symptoms in patients suffering from inflammatory bowel diseases (12).

We have recently hypothesized that the beneficial effect of *E. coli* Nissle 1917 and other probiotic bacteria in the intestinal tract might be at least partly due to the induction of protective host antimicrobials. An up-regulation of a diminished antimicrobial defense as seen in Crohn´s disease (13) could be one important mechanism by which some probiotics exert their beneficial effect in IBD patients and thus inhibit the invasion of the mucosal surface by commensals and other microorganisms. *In vitro* experiments showed that incubation of intestinal epithelial cells with these bacteria provoked a strong induction of inducible human β-defensin-2 (hBD-2) expression (14).

Defensins are components of the armory of endogenous antimicrobials, which are part of the innate immune system (15). They are small, cationic (positively charged) peptides of which most are classified into α- or β-defensins. The human β-defensins provide a first line of defence against potentially pathogenic microbes at the body's mucosal frontiers as they are produced by epithelial cells of the lung, skin, and intestinal tract (16). Defensins, produced by the mucosal epithelium, have a broad antibiotic spectrum against Gram-negative (*E. coli*, *Salmonella*) and -positive (*Staphylococcus aureus*) bacteria as well as fungi (*Candida albicans*), and viruses (HIV).

Despite the known induction of antimicrobial peptides in cell culture, nothing is known about the *in vivo* influence and especially if the real treatment under normal doses is sufficient to induce antimicrobial peptide secretion in the intestinal lumen. Thus, we investigated whether the administration of a known probiotic *E. coli* viable bacterial culture (Symbioflor 2®) might impact on the hBD-2 peptide secretion in the feces. In order to exclude other possible influences caused by disease, we used healthy individuals. In a second step, after we observed *in vivo* secretion upon treatment with Symbioflor, we tested which viable *E. coli* genotype induced defensin expression in cell culture. Since it is known that different clinically used probiotic *E. coli* strains do not survive in the normal flora and have to be administered daily, we also tested if these bacteria are resistant to antimicrobial defensin killing.

RESULTS

Symbioflor 2® induces hBD-2 fecal peptide secretion in man

The administration of Symbioflor 2® was well tolerated by all study participants and no adverse events were observed. Five individuals with placebo treatment did not show any significant changes in hBD-2 peptide levels (Fig. 1a). Taken together, the overall increase of hBD-2 protein fecal level after 3 weeks of probiotic treatment was 3.7 fold as compared to before treatment ($p<0.0001$) (Fig. 1b). To assess whether the probiotic uptake results in increased hBD-2 secretion, we also determined the percentage of study participants who expressed a higher hBD-2 secretion into the feces after the intake of Symbioflor 2®. After 3 weeks, 78% showed an increase of more than 20% and 74% more than 30% of hBD-2 secretion as compared to before treatment. At week 12, nine weeks after the end of the probiotic administration, the hBD-2 fecal levels were determined in a smaller subset and compared with the baseline values of the same individuals (Fig. 1c). Even though the levels were lower than at week 3, fecal hBD-2 protein was still significantly higher than at the beginning of the study ($p=0.008$) (Fig. 1c).

hBD-2 induction by probiotics in colonic epithelial cells

To confirm the *in vivo* studies and to identify the inducing bacterium, we performed cell culture experiments. We tested the hBD-2 stimulating effect of different probiotics including different genotypes of the Symbioflor *E. coli* DSM 17252 (G1, G2 and G3) as well as a positive (*E. coli* Nissle 1917) and negative control (*E. coli* K12). In previous studies we have shown that different probiotic strains including *E. coli* Nissle 1917, *L. fermentum*, *P. pentosaceus* and *L. acidophilus* PZ 1129 consistently induce hBD-2 mRNA and protein secretion *in vitro* (14,19).

In contrast, more then 35 *E. coli* clinical isolates as well as *E. coli* K12 showed no effect (14). Herein, only one of the three Symbioflor® 2 genotype strains (G2) showed a strong induction of hBD-2 in Caco-2 cells after 6 hours of incubation which was comparable to *E. coli* Nissle 1917 (Fig. 2a). When we tested the hBD-2 induction of G2 at different time points, we observed the highest value after 12 hours of incubation (Fig. 2b). However, the induction of hBD-2 reached a significant extent at all tested time points demonstrating the consistency of the induction capacity. The housekeeping gene expression (GAPDH) remained stable at all time points. The induction of hBD-2 was also shown to be dose-dependent with a maximum at an optical density of 0.3 (Fig. 2c). In contrast, the other *E. coli* G1 and G3 did not induce hBD-2 in Caco-2 cells at any time (Fig. 2b) or any tested bacterial concentration (data not shown).

Probiotic *E. coli* bacteria strains are not resistant to antimicrobial peptides

Next it was studied whether the same strains are sensitive to antimicrobial killing by natural defensins. In plate diffusion assays, hBD-2 showed a strong potency to kill *E. coli* bacteria which is consistent with previous reports. Interestingly there were no differences between any of the tested strains. The two probiotic *E. coli* bacteria which strongly induced hBD-2 (*E. coli* Nissle, DSM 17252 G2) were killed in an almost equal extent as compared to *E. coli* K12 as well as Symbioflor 2® bacterial genotypes with negative defensin induction (DSM 17252: G1 and G3) (Fig. 3a). We also tested if the same bacteria were different towards resistance to other antimicrobial peptides. Similar to hBD-2 killing, all tested bacteria were potently eliminated by hBD-3 with no differences between any of the strains (Fig. 3b). In addition, lysozyme, which is another known antimicrobial host molecule, equally showed antibiotic activity with no difference between probiotic and other tested *E. coli* bacteria (Fig. 3c).

DISCUSSION

Herein we show for the first time that oral administration of regular doses of a probiotic *E. coli* preparation (Symbioflor 2®) induces mucosal human β-defensin peptide secretion into the feces of healthy individuals. Consistent with these *in vivo* studies, we identified one *E. coli* strain which is part of the commercial mixture as a stimulatory agent *in vitro*. Since the tested probiotic

bacteria are not resistant to antimicrobial host factors such as hBD-2, these functional studies presented here allow to speculate about a possible suicidal character at the place of defensin secretion.

It was recently demonstrated that *E. coli* Nissle 1917, as well as *L. acidophilus*, *Lactobacillus fermentum* and *Pediococcus pentosaceus* as well as VSL#3 and other probiotic strains highly specifically activate colonic cells to synthesize defensins *in vitro* (14,19). In contrast 40 other clinical *E. coli* isolates lacked this effect (14). This specific effect is consistent with the observation that inducible β-defensins are normally - despite the presence of numerous *E. coli* species in the gut microbiota - absent in the healthy intestine. However, the major limitation of all these studies is the exclusive focus on *in vitro* experiments as performed in intestinal (tumor) epithelial cells.

Unfortunately there are many examples of experimental effects which can not be confirmed *in vivo*. For this reason we studied if a regular dose of a commercial probiotic preparation (Symbioflor 2®) can induce active antimicrobial peptide secretion in healthy individuals and how this *in vivo* effect translates back to experiments using cell culture. Interestingly, hBD-2 was still increased even 9 weeks after the administration of Symbioflor 2® was stopped, suggesting additional stimulatory mechanisms which cannot be clearly explained by the presented data. Compared to the controls *E. coli* Nissle 1917 and PZ720, only one of the Symbioflor 2® genotypes had a similarly strong capacity to induce hBD-2 in cell culture. This induction of hBD-2 was further enhanced by an increased contact time of the bacteria with the colonic epithelial cells which differed from the induction pattern of *E. coli* Nissle 1917 (14). Based on recent observations with *E. coli* Nissle 1917 we also expected an increase of the effect by increasing the bacterial concentration. However, all doses except the smallest reached the same level in inducing hBD-2 expression with no dose dependence.

Together these time and dose data suggest that the probiotic stimulatory strength is similar to other probiotic strains (e. g. Nissle) but the mechanisms of induction may be different. In case of *E. coli* Nissle 1917 the hBD-2 induction mechanism is mediated by a specific flagellin (20). In these studies genetically manipulated *E. coli* Nissle 1917 deficient in flagellin protein failed to induce defensin expression. On the other hand, isolated flagellin from *E. coli* Nissle 1917 strain was effective in contrast to similar doses of flagellin isolated by another apathogenic *E. coli* strain. Thus it is likely that the flagellin structure of the *E. coli* strain Nissle 1917 exhibits some important modifications compared to other *E. coli* strains which also express flagella but lack the ability of hBD-2 induction. Interestingly, the *E. coli* Symbioflor 2® strains including G2 do not express Flagella protein (20) despite similar hBD-2 induction. Similarly other defensin stimulating probiotic strains like the bacterial mix VSL#3 or other *Lactobacilli* also lack flagella. Thus, even if the result of hBD-2 induction can be found by all tested probiotic strains, the relevant factors and mechanisms mediating this immune stimulatory effect appear to differ from strain to strain. These factors which are likely to be different between different *E. coli*, *Lactobacilli* and other probiotic species still remain to be investigated.

Another important question concerns the biological relevant concentrations of hBD-2. In this study, the hBD-2 protein concentration was measured in overall stool samples. Of note, the main biological locus of action of antimicrobial defensins is probably the mucus which is attached to the mucosal layer (21). The defensin concentration as measured here likely reflects the luminal rather then the mucus concentration. Thus, the amounts in the mucus would be much higher since the obtained values of hBD-2 are a dilution of the original amount as secreted by mucosal epithelial cells.

To study the possible functional consequence of defensin induction, we tested if Symbioflor 2® bacterial cultures are able to resist antimicrobial killing. We found that there were no differences between the genotypes and probiotic and normal *E. coli* were similarly killed by hBD-2 *in vitro*. The same observation was found for other antimicrobial peptides such as hBD-3 and lysozyme. Thus, even though the probiotic strains are very specific in provoking an antimicrobial host response, they do not seem to be provided with protective factors to resist an antimicrobial attack by the host. Possibly, this could at least partly explain why both, *E. coli* Nissle as well as Symbioflor 2® have to be continuously administered. On the other hand, it is quite possible that *E. coli* strains that do not induce hBD-2 also will not stably colonize the gut. Thus, there may or may not be a cause and effect with hBD-2 induction.

Another functional question is the effect of probiotic hBD-2 induction, especially that of G2 on the luminal intestinal microbial flora. Unfortunately there are no direct data available. Our own recent studies have shown that very small changes in antimicrobial defensins (Human defensin 5) significantly alter the composition of the downstream luminal microbiota (22). In order to mechanistically address the question we used a HD-5 transgenic mouse model in which we compared heterozygotic (+/-) with homozygotic (+/+) mice (2 fold difference in small intestinal HD5 expression)(22). It would be attractive to create a human beta defensin 2 transgenic mouse model and also analyze the composition of microbiota in these mice.

In addition future studies should systematically analyze the composition of the microbiota after treatment with G2 and the other probiotic components in rodent models as well as in humans. The elucidation of probiotic actions is still on an initial stage, despite increasing numbers of studies confirming their beneficial effects on animals and humans. More insights into the probiotic-induced immune regulation are relevant to fortify the application of this promising treatment strategy.

Taken together, this study suggests that the stimulation of protective host factors such as defensins is an important and broad mechanism of probiotic action *in vivo* even though the precise mechanisms seem to vary between individual probiotic strains.

METHODS

Human study and sample collection

31 healthy individuals who gave informed consent were included in the study. Of those, five individuals were treated with placebo and 26 individuals received Symbioflor 2® (containing viable commensal *E. coli* bacteria). From the initial verum group of 26, three individuals were excluded from the study, one because

of irregular drug intake and two because of study discontinuation due to compliance. Thus, the final verum group contained 23 individuals for a total of 21 days of therapy. Symbioflor 2® was administered in standard dosage starting with 2 times 5 drops daily and a daily increase by 2 times 1 drop until 15 drops daily were reached. 14 drops are one ml containing 1.5 to 4.5 x 10^7 viable bacterial counts. The total duration of treatment was 21 days. Stool samples were taken at baseline and after 3 weeks (stop of treatment). From some subjects a third sample was collected after 12 weeks (9 weeks after stop of treatment). Due to lack of compliance, the latter sample was not obtained in all cases (n = 10 out of the total of 23). The study was approved by the local ethical committee and all volunteers gave written informed consent.

hBD-2 protein concentration (hBD-2 ELISA)
The protein assessment of hBD-2 secretion was performed as described (17). After collection, stool samples were frozen at -20 Degrees Celsius. Before analysis 100 mg of stool were prepared using the commercially available stool preparation kit (Roche Diagnostics, Mannheim, Germany; cat # 745804). HBD-2 protein concentration was assessed in stool samples at different time points in all participants of the study. The protocol was used according to the supplier's recommendation (ALPCO, Windham, Germany). In brief, the β-defensin-2 in standard and samples is bound to an available excess of polyclonal antibodies against β-defensin-2, which are immobilized on the surface of the microtitre plate. After a washing step, to remove all interfering substances, the quantification of bound β-defensin 2 is carried out by adding a biotinylated polyclonal anti-β-defensin-2 antibody. This antibody is detected with horseraddish peroxidase labeled streptavidin. The amount of converted substrate by peroxidase is directly proportional to the amount of bound β-defensin-2 and can be determined photometrically at 450 nm.

Bacterial strains and growth conditions
Bacterial strains used in this study are shown in Tab. 1. The probiotic strains were grown overnight at 37 °C under gentle agitation at 200 rpm in trypticase soy broth (TSB). To obtain bacteria in a linear growth phase, 100 µl of the bacterial suspension were added to 10 ml fresh TSB medium and grown under permanent shaking for 5 h. Heat inactivation of the bacteria was carried out in a water bath at 65 °C for 1 h. Bacteria were concentrated by centrifugation, washed with phosphate buffered saline (PBS) and adjusted to a density of 3×10^8 cells/ml with FCS- and antibiotic-free cell culture medium. For testing of dose-dependency, bacteria were adjusted to the optical densities 0.1, 0.3, 0.6, 0.9, and 1.2.

Cell culture
Caco-2 cells (German Collection of Microorganisms and Cell cultures (DSMZ) ACC 169) were cultivated in DMEM containing 25 mM HEPES, 2 mM glutamine, 10% FCS, 50 µg/ml gentamicin and 1% nonessential amino acids as described (14). Cells were grown as monolayers in 75-cm² flasks (Greiner, Frickenhausen, Germany) at 37 °C in a 5% CO_2 - 95% air atmosphere with 90% humidity. For stimulation experiments, cells were seeded at a density of 3.2×10^5 cells/well in 12-well culture plates (Becton Dickinson GmbH, Heidelberg, Germany). Cells grown to ~70% confluence in culture wells were incubated overnight with serum- and antibiotic-free medium to eliminate serum-induced hBD-2 expression and prevent any influence of antibiotics on the immune response. To determine hBD-2 mRNA expression, Caco-2 cells were incubated with the bacteria for different time points including 3, 6 and 12 hours.

RNA isolation and cDNA synthesis
After stimulation cells were washed with PBS and harvested with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the supplier´s protocol (14). Subsequently 1 µg of total RNA was reverse transcribed into cDNA with oligo (dT) primers and 15 U/µg AMV Reverse Transcriptase (Promega, Madison, USA) according to standard procedures.

Real-time RT-PCR
Real-time reverse transcription polymerase chain reaction (RT-PCR) analyses were performed in a fluorescence temperature cycler (LightCycler; Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer's instructions (14). As a template served cDNA corresponding to 10 ng of RNA in a 10 µl reaction mixture containing 3 mM $MgCl_2$, 0.5 µM of each primer and 1 × LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics GmbH). 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 for 5 s and elongation at 72 °C for 15 s. For hBD-2 (sense 5`-ATCAGCCATGAGGGTCTTGT-3`; antisense 5`-GAGACCACAGGTGCCAATTT-3`) the annealing temperature was set to 60 °C. Amplification using these primers resulted in a 172-base pair fragment. As an internal control gene we used the common housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase). For GAPDH (sense 5´-CCAGCCGAGCCACATCGCTC-3´; antisense 5`-ATGAGCCCCAGCCTTCTCCAT-3`) we used a touchdown protocol with a primary temperature of 66 °C and a target temperature of 60 °C. At the end of each run melting curve profiles were achieved 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). Melting curves were generated after each run to confirm amplification of specific transcripts. The specificity of the amplification products was verified by subjecting the amplification products to electrophoresis on a 2% agarose gel and visualization by ethidium bromide staining. Relative mRNA expression is given as a ratio between target gene and GAPDH gene expression.

Antimicrobial Assay
To test if different *E. coli* strains including probiotic bacteria are susceptible to antibacterial host peptides, we used human β-defensin-2 and -3 as well as lysozyme. Antimicrobial activity was determined by using a radial diffusion assay as described by Lehrer *et al.* (18) with minor modifications. Briefly, 1 x 10^7 bacterial CFU were

inoculated into 10 ml of warm sterile underlay agar (0.03% w/v trypticase soy broth (TSB, Becton Dickinson GmbH), 10 mM sodium phosphate, pH 7.4, 1% low-electroendosmosis-type agarose (Sigma-Aldrich, Steinheim, Germany) and 0.02% v/v Tween 20 (Merck, Darmstadt, Germany)). After strong vortexing the liquid agar was poured into square Petri dishes (Becton Dickinson GmbH) and small wells were punched with a biopsy-puncher. Test substances lysozyme (1µg), hBD-2 (1µg) and hBD-3 (1µg) were diluted in 5 µl of 0.01% acetic acid, which also served as vehicle control, and added into the wells. After incubation for 3 hours at 37°C the gel was overlayed with 10 ml of full-nutrition overlay agar (6% w/v TSB, 1% low-EEO agarose) and incubated for 16 hours at 37°C. The gel was stained as described by Lehrer *et al*. and diameter of inhibition zone was measured; indicated values were obtained from triplicates.

Statistics

Data were analyzed using GraphPad Prism (Version 3.1 for Windows, GraphPad Software, San Diego, CA, USA). For the description of random samples, the mean ± standard error of the mean (s.e.m.) was used. Paired t-tests were used to evaluate differences in hBD-2 levels before and after treatment in stool samples (Figure1). Data from hBD-2 induction experiments in colonic epithelial cells were analyzed using unpaired t-tests (Figure 2). For statistical analysis of the antimicrobial plate assays the one sample t-test was used. $P<0.05$ was considered as statistically significant.

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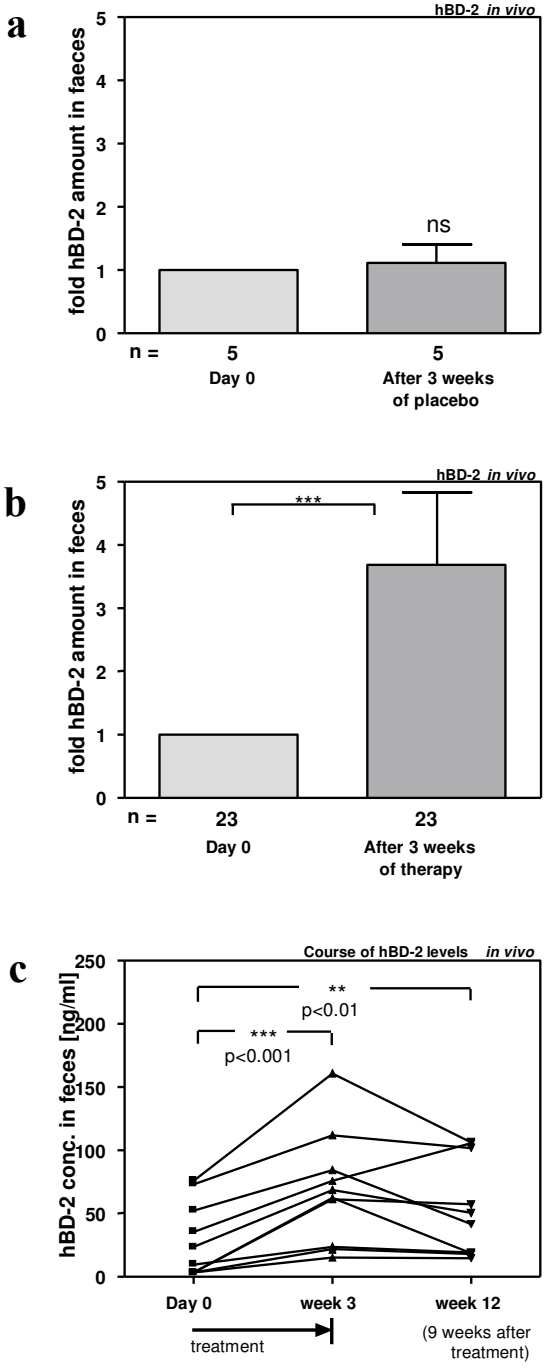

Figure 1: Probiotics stimulate hBD-2 protein secretion *in vivo*. (a) hBD-2 peptide secretion after 3 weeks of placebo treatment in 5 individuals. (b) In total, 23 individuals received preparations of Symbioflor 2® for 3 weeks. HBD-2 peptide was determined by ELISA in stool samples. Data were normalized to hBD-2 levels before treatment which were set as 1 and represent the mean ± s.e.m. (c) Probiotics exert a post-treatment effect on hBD-2 secretion. HBD-2 peptide levels are shown in a subset at day 0, after 3 weeks of probiotic treatment and 9 weeks after stop of treatment. Paired t-tests were used for the comparison of values before and after treatment. The data represent the means ± s.e.m. $^{**}P<0.01$; $^{***}P<0.001$;

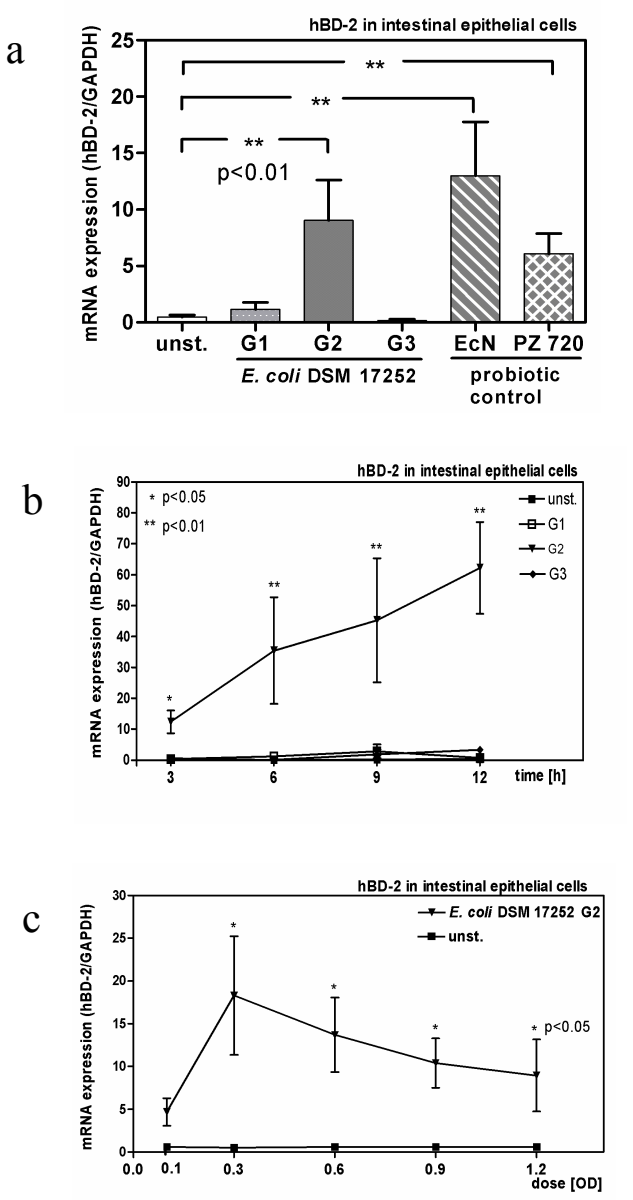

Figure 2: Symbioflor 2® induces hBD-2 in intestinal epithelial cells *in vitro.* (a) Incubation of Caco-2 cells with three genotypes of Symbioflor 2® *E. coli* strain (G1, G2, G3). Caco-2 cells were incubated for 6 hours with the heat-killed test bacteria and two probiotic strains as controls (optical density of 0.3). RNA was isolated, reverse-transcribed into cDNA and the amount of hBD-2 copies was determined by real-time PCR. (b) Time course of hBD-2 induction by Symbioflor 2®. Caco-2 cells were treated with the three genotypes (G1, G2, G3) of Symbioflor 2® *E. coli* strain for 3, 6, 9 and 12 hours. (c) Dose dependence, Caco-2 cells were treated for 6 hours with elevating doses of the genotype G2 of the Symbioflor 2® *E. coli* strain. The data represent the mean ± s.e.m. of three independent experiments performed in duplicates. Unpaired t-tests were used to evaluate the statistical significance for comparisons between the groups. The data represent the means ± s.e.m. $^{*}P<0.05$; $^{**}P<0.01$;

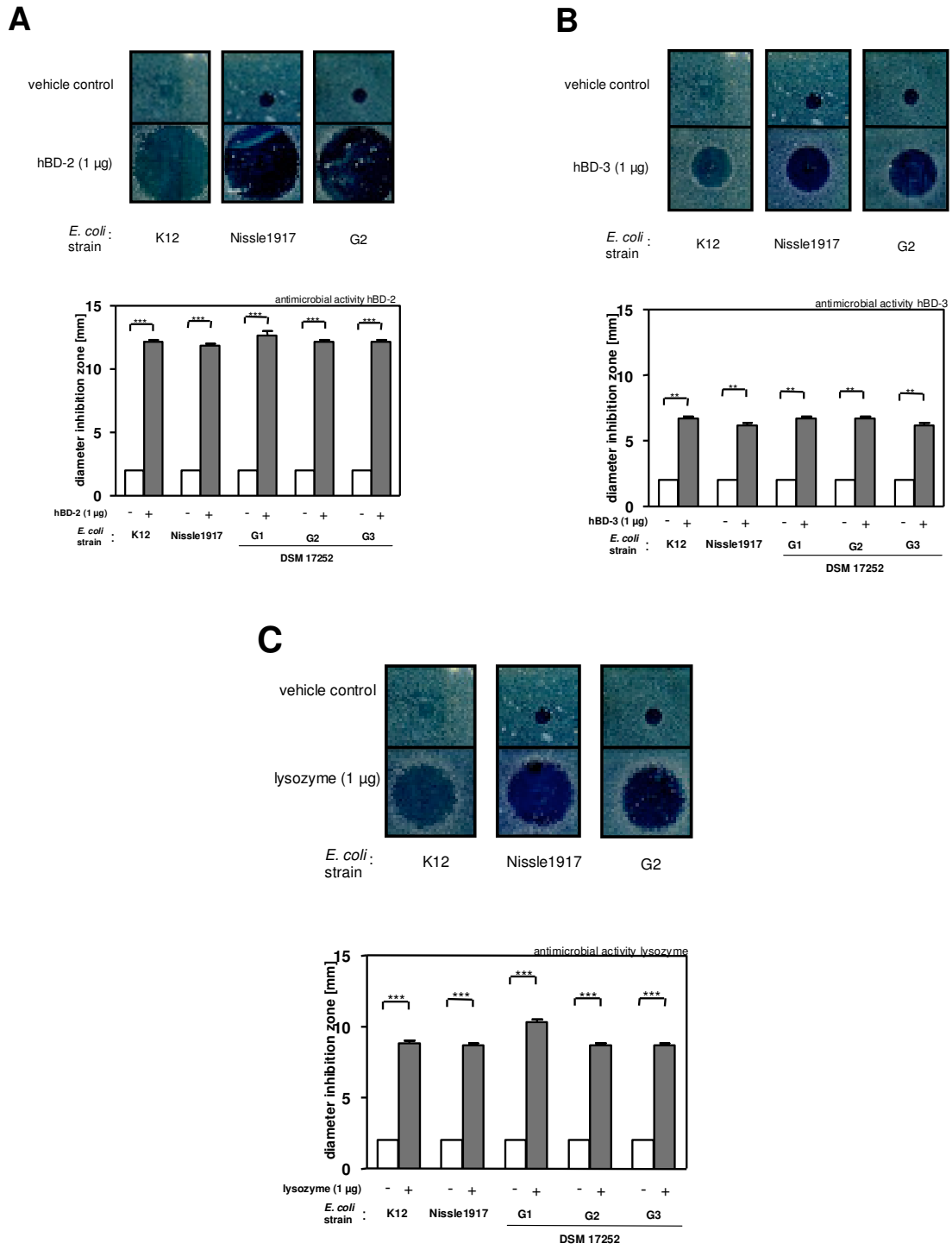

Figure 3: Antimicrobial resistance of probiotic and control *E. coli* toward antimicrobial peptides. (a) hBD-2, (b) hBD-3, (c) lysozyme. Representative antimicrobial plate assays are shown for each antimicrobial (upper part). The data represent the mean ± s.e.m. and were analyzed for statistical significance using one sample t-test. **P<0.01; ***P<0.001.

TABLE 1: Bacterial strains used in this study

Strain	Strain designation /Serotype	Type of isolate	Source
E. coli Nissle 1917 (EcN)	O6:K5:H1	Pharmaceutical	1
E. coli PZ 720	O83:K24:H31	Probiotic strain	1
E. coli DSM 17252			2
S2 G1: *E.coli* Genotype 1/2	Osp.:H-		2
S2 G2: *E.coli* Genotype 3/10	O13.:H-		2
S2 G3: *E.coli* Genotype 4/9	Osp.:H-		2
Enterococcus fecalis			
Klebsiella spec.			

Osp = spontaneously agglutinating i.e. the classical rough *E. coli* type
1 Ardeypharm Collection of Strains, Pharma-Zentrale GmbH, Herdecke, Germany
2 SymbioPharm GmbH, Germany

V Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1. *Nature* 2011, 469, 419-23.

1 Aims and hypothesis

In the gut the number of microbes is about tenfold higher than the total number of human cells. Due to the limited presence of oxygen the major fraction of gut microbes are facultative or strict anaerobes. To prevent infection by these microbes the host holds several defense mechanisms, including epithelial production of antimicrobial peptides. Antimicrobial peptides have been mostly studied under standard air oxygen conditions which do not necessarily reflect the conditions in nature. Therefore the aim was to analyze antimicrobial activity of the constitutively produced human β-defensin 1 (hBD-1) against anaerobic bacteria of the human normal flora under reducing conditions as present in parts of the intestine.

2 Methods

To analyze antimicrobial activity of hBD-1 against anaerobic bacteria under reducing conditions we modified the classical radial-diffusion assay developed by Lehrer *et al.* [Lehrer et al., 1991]. Most importantly, minor amounts of the reducing agent DTT were added to assay medium. Further biochemical methods, including RP-HPLC analysis, MALDI-TOF-MS, CD spectroscopy and NMR spectroscopy were performed to investigate molecular properties of reduced hBD-1. Enzymatic reduction-assays, electron microscopy, immunohistochemistry and real-time PCR were used to study biological and functional aspects of reduced hBD-1.

3 Summary of findings

Human β-defensin 1 showed antimicrobial activity against several *Bifidobacteria* and *Lactobacilli* only under reducing conditions. The effect was attributable to the reduced, linear peptide which differed structurally from the oxidized form. Besides the commensal, Gram-positive anaerobic bacteria, reduced hBD-1 was antimicrobially active against clinical isolates of the opportunistic pathogen *Candida albicans* while the oxidized peptide was not.

The constitutively produced oxido-reductase thioredoxin was able to catalyze reduction of hBD-1 and co-localizes with reduced hBD-1 in human epithelia of skin and intestine. Incubation of *Bifidobacteria* with reduced hBD-1 lead to bacterial membrane

depolarization and the loss of stainable cytoplasmatic content, as observed in transmission electron microscopy.

Taken together, these findings raise novel aspects on the biological relevance of hBD-1, which was thought to be only of minor importance. Furthermore, by redox-modulation a human antimicrobial peptide can be activated, giving new insights into the molecular regulation of innate immunity.

LETTER

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Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1

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Human epithelia are permanently challenged by bacteria and fungi, including commensal and pathogenic microbiota[1,2]. In the gut, the fraction of strict anaerobes increases from proximal to distal, reaching 99% of bacterial species in the colon[3]. At colonic mucosa, oxygen partial pressure is below 25% of airborne oxygen content, moreover microbial metabolism causes reduction to a low redox potential of −200 mV to –300 mV in the colon[4]. Defensins, characterized by three intramolecular disulphide-bridges, are key effector molecules of innate immunity that protect the host from infectious microbes and shape the composition of microbiota at mucosal surfaces[5–8]. Human β-defensin 1 (hBD-1) is one of the most prominent peptides of its class but despite ubiquitous expression by all human epithelia, comparison with other defensins suggested only minor antibiotic killing activity[9,10].

Whereas much is known about the activity of antimicrobial peptides in aerobic environments, data about reducing environments are limited. Herein we show that after reduction of disulphide bridges hBD-1 becomes a potent antimicrobial peptide against the opportunistic pathogenic fungus *Candida albicans* and against anaerobic, Gram-positive commensals of *Bifidobacterium* and *Lactobacillus* species. Reduced hBD-1 differs structurally from oxidized hBD-1 and free cysteines in the carboxy terminus seem important for the bactericidal effect. *In vitro*, the thioredoxin (TRX) system[11] is able to reduce hBD-1 and TRX co-localizes with reduced hBD-1 in human epithelia. Hence our study indicates that reduced hBD-1 shields the healthy epithelium against colonisation by commensal bacteria and opportunistic fungi. Accordingly, an intimate interplay between redox-regulation and innate immune defence seems crucial for an effective barrier protecting human epithelia.

We modified the radial diffusion assay[12] to analyse antimicrobial activity of synthetic hBD-1 against anaerobic bacteria of the normal flora under anaerobic conditions. Increasing concentrations of the reducing agent dithiothreitol (DTT) were added to assay medium. In medium without DTT hBD-1 did not affect growth of Gram-positive *Bifidobacterium adolescentis* (Fig. 1a). Surprisingly, addition of increasing amounts of DTT led to an increase of inhibition zones in size and sharpness. In contrast, human β-defensin 3 (hBD-3) and lysozyme showed less antimicrobial activity upon addition of DTT (Fig. 1b and Supplementary Fig. 1). Thus, increased antimicrobial activity of hBD-1 in reducing environment was specific for hBD-1 and not caused by impaired bacterial fitness. Remarkably, at concentrations of 2 mM DTT hBD-1 became as effective as hBD-3 against *B. adolescentis* (Fig. 1c). This finding is crucial as hBD-3 is one of the most powerful antimicrobial peptides in oxygen-rich environment, whereas hBD-1 was thought to be one of the weakest.

For the Gram-negative anaerobe *Bacteroides vulgatus* we found no antimicrobial effect of hBD-1 and lysozyme under any conditions, whereas hBD-3 showed concentration-dependent inhibition zones (Supplementary Fig. 2).

Because β-defensins contain three intramolecular disulphide-bridges[13] we investigated the involvement of cystines for the observed antimicrobial effect. Therefore, we incubated hBD-1 with increasing concentrations of DTT and analysed samples using matrix-assisted laser desorption/ionisation (MALDI)-mass spectroscopy (MS). A single signal could be detected for oxidized hBD-1 (oxhBD-1) at *m*/*z* 3,926.8 (Fig. 2a). DTT-treatment and carboxamidomethylation resulted in a second signal, corresponding to the completely reduced form (redhBD-1) with all six cysteine residues alkylated. These results suggest that all three cystines are either present or absent, without the existence of intermediate states containing one or two disulphide-bridges.

Reversed-phase high performance liquid chromatography (RP-HPLC) showed that oxhBD-1 eluted after 30 min (Fig. 2b). Incubation with increasing concentrations of DTT resulted in a shift towards a peak at 33.5 min, representing the completely reduced hBD-1 (confirmed by MALDI-MS, data not shown) and indicating an increase of hydrophobicity of redhBD-1 in solution.

To elucidate structural changes further, nuclear magnetic resonance (NMR) experiments on recombinant, uniformly ^{15}N-labelled reduced and oxidized hBD-1 were performed. oxhBD-1 showed a well-dispersed ^{15}N-heteronuclear single quantum coherence (HSQC) spectrum as expected for a folded protein (Fig. 2c). In addition, the detected cross peaks in the HNH- and NNH-nuclear Overhauser enhancement spectroscopy (NOESY) spectra as well as the restricted dynamics observed in the ^{1}H-^{15}N-heteronuclear NOE (hetNOE) experiment indicated a highly structured peptide (data not shown). In contrast, the dispersion of the HSQC spectrum of redhBD-1 indicated a lack of hydrogen bonds for most parts of the protein (Fig. 2c). Accordingly, the HNH- and NNH-NOESY spectra showed significantly less cross peaks and the dynamics of the hetNOE experiment clearly support an unstructured, highly flexible polypeptide chain. Similar findings were obtained by circular dichroism (CD) spectroscopy (Fig. 2d). oxhBD-1 displayed a characteristic minimum at 209 nm for alpha-helices and a significant signal at 218 nm, corresponding to the beta-sheet content, indicating a well-folded peptide. In contrast, redhBD-1 did not show these minima but displayed a minimum around 195 nm, indicating a random coil-like structure, being consistent with previous data[14]. Indeed, calculation of secondary structure elements proposed an increase in the random coil fraction for the reduced peptide (Supplementary Table 1). From these structural observations we propose that antimicrobial activity of hBD-1 in a reducing environment is attributable to its reduced, unstructured form.

To exclude artificial effects of the reducing agent DTT we compared the antimicrobial activity of oxhBD-1 and redhBD-1 against different bifidobacteria under anaerobic conditions in medium lacking DTT. Synthetic, oxidized hBD-1 did not affect growth of bifidobacteria whereas linear hBD-1 showed antimicrobial activity against all examined strains (Fig. 3a and Supplementary Fig. 3). Similar results were obtained for Gram-positive lactobacilli, whereas we detected no effect of any hBD-1 against Gram-negative *Bacteroides vulgatus* (Fig. 3b). For the facultative anaerobe *Escherichia coli* K12 we found antimicrobial activity of both oxhBD-1 and redhBD-1, which is in accordance with literature[14–16]. When testing the commensal, facultative pathogenic fungus *Candida albicans* we found potent antifungal activity of redhBD-1 but not oxhBD-1 against four out of five strains (Fig. 3c) when using a flow cytometric antimicrobial killing assay[17]. Consequently, reduced hBD-1 is not only antimicrobial against Gram-positive anaerobes of the human normal flora but also against a facultative pathogenic fungus of clinical importance.

Subsequently, we generated recombinant Ala/Ser-variants of hBD-1 in which all cysteine residues were substituted by alanine or serine to investigate if a linear structure itself accounts for the augmented activity (Fig. 3d). All variants were either inactive or only weakly active against bifidobacteria and lactobacilli (Supplementary Fig. 4). The only bacterium being sensitive was *E. coli* K12, which was also sensitive to oxhBD-1 (Fig. 3b). Since redhBD-1 showed increased hydrophobicity, we performed RP-HPLC analyses of the Ala/Ser-variants (Fig. 3d), revealing that these eluted earlier, indicating a lower surface hydrophobicity compared to redhBD-1.

A truncated variant of hBD-1 (hBD-1$_{\Delta 30–36}$) lacking the seven C-terminal amino acids *GKAKCCK* did not inhibit growth of *B. adolescentis* under normal nor reducing conditions whereas the C-terminal seven amino acid peptide itself was antimicrobially active (data not shown). Comparable activity was observed when reversing the amino acid sequence of the C-terminal peptide while substituting cysteines by alanines or serines abolished antimicrobial activity completely. Accordingly, unfolding itself is not sufficient for antimicrobial activity of redhBD-1; rather hydrophobicity and free cysteine residues located in the C-terminus are important features (Supplementary Fig. 5).

By using a flow cytometric antimicrobial killing assay[17] we found that redhBD-1 had a rapid bactericidal effect*:* redhBD-1, but not oxhBD-1, caused bacterial membrane depolarization of *B. adolescentis* after incubation for only 30 min (Fig. 3e). Correspondingly, when performing transmission electron microscopy with *B. adolescentis* (Fig. 3f), oxhBD-1 did not cause any morphological damage of bacterial cells. In contrast, incubation with redhBD-1 caused loss of stainable cytoplasmic content through a process which seemed to be initiated intracellularly. Hence, we assume that linear hBD-1 kills bacteria by using a mechanism being different from that of hBD-3, which primarily attacks the bacterial membrane (Fig. 3f).

Physiologically, enzymatic redox-regulation is mainly controlled by thioredoxin (TRX), a multifunctional and ubiquitously expressed oxidoreductase[11,18]. Because TRX is expressed by mucosal surfaces and shows extracellular functions[19], we tested if hBD-1 is a natural substrate for thioredoxin. Therefore we incubated oxhBD-1 with different concentrations of TRX in the presence of nicotinamide adenine dinucleotide phosphate (NADPH) and thioredoxin reductase (TrxR), composing the natural thioredoxin system[11], and analysed samples by RP-HPLC to evaluate oxidized and reduced hBD-1 fractions. hBD-1 was reduced by thioredoxin in a concentration-dependent fashion (Fig. 4a). Likewise, increasing TrxR concentration increased the amount of redhBD-1 while omitting TrxR prevented reduction (data not shown). These findings were confirmed by a sensitive spectrophotometric protein disulphide reduction assay[20] measuring the consumption of NADPH (Supplementary Fig. 6). Although minor amounts of NADPH were consumed when using protein disulphide isomerase (PDI) instead of TRX or by using TrxR without TRX (data not shown), we propose that the complete thioredoxin system might be a physiological mediator catalysing effective reduction of oxhBD-1.

To document the presence of reduced hBD-1 *in vivo*, we generated a redhBD-1-specific antibody. This antibody recognized reduced hBD-1 and alkylated hBD-1, but not correctly folded synthetic or recombinant hBD-1 in an immunodot assay as well as during western blot analyses (Supplementary Fig. 7). redhBD-1 was observed in human colonic mucosa and at the bottom of small intestinal crypts by immunohistochemistry staining (Fig. 4b). Additionally, redhBD-1 staining was observed in human skin epidermis, whereas the protein

G-purified pre-immune IgG fraction did not cause any staining. Analyses with a TRX-specific antibody revealed a similar staining pattern, indicating that redhBD-1 and TRX co-localize and thereby strengthened the hypothesis that TRX is a physiological mediator catalysing reduction of hBD-1 in human epithelia.

In inflamed tissue from rodent models, application of recombinant thioredoxin has a beneficial effect and ameliorates colitis[21]. We therefore studied TRX mRNA in human inflammatory bowel disease and found it to be decreased in inflamed colonic tissue (Supplementary Fig. 8). It remains unclear whether this relative lack of thioredoxin could perpetuate inflammation via a reduction of intestinal antibiotic barrier function. Unfortunately, due to differences in the antimicrobial peptide repertoire in rodents compared to humans definite proof of such a concept will be difficult to obtain.

Anaerobic niches can be found in cutaneous sweat- and sebaceous glands, wounds, infectious sites as well as mucosal membranes of intestine, vagina, oral cavities and others[22,23]. It seems plausible that hBD-1 and thioredoxin are constitutively produced by these epithelia to provide a hostile environment for (facultative) anaerobic pathogens or commensals. A disease relevance of these mechanisms appears likely, since single nucleotide polymorphisms in the hBD-1-encoding *DEFB1* promoter region have been associated with increased risk of caries, periodontitis, *Candida* infection and Crohn's Disease[24–28].

Because antimicrobial activity of hBD-1 was found to be comparably low[16,29] it was paradoxical why an organism produces high amounts of an ineffective defence molecule. Here we show that reducing disulphide bonds unmasks potent antimicrobial activity of hBD-1. Although its definite *in vivo* relevance remains to be demonstrated, these findings provide a new mechanism: redox modulation depending on enzymatic and environmental factors strongly augments antibiotic killing, a finding which opens new avenues in understanding the complex process of mucosal as well as skin host protection against commensals and pathogens.

METHODS SUMMARY

Antimicrobial assay
Antimicrobial assay was modified from ref. 12. Bacteria (except *E. coli* K12) were grown anaerobically and the assay was performed under anaerobic conditions with antimicrobial peptides or its variants/fragments and 0, 1 and 2 mM DTT.

Generation of recombinant hBD1 and its variants
Generation of hBD-1 and its variants is described in the Supplementary Methods and Supplementary Table 2.

Nuclear magnetic resonance (NMR) spectroscopy
Uniformly ^{15}N-labelled reduced and oxidized hBD-1 was solubilised in H_2O containing 10% D_2O to a final concentration of 0.5–0.7 mM. All spectra were recorded at 298 K.

Circular dichroism (CD) spectroscopy
CD spectroscopy was carried out at 25 °C in 10 mM sodium phosphate, pH 7.4. Spectra were recorded at 0.1 mg ml^{-1} in the far-ultraviolet range.

Transmission electron microscopy of bacteria
Bifidobacterium adolescentis was incubated with 200 μg ml^{-1} synthetic defensin. Bacteria were fixed in Karnovsky's fixative, embedded in agarose, coagulated, cut in small blocks and fixed again in Karnovsky's solution. After post-fixation and embedding in glycid ether blocks were cut using an ultra microtome. Sections (30 nm) were mounted on copper grids and analysed using a Zeiss LIBRA 120 transmission electron microscope.

Generation of anti-reduced-hBD-1-specific antibodies
Generation of anti-reduced-hBD-1-specific antibodies and its use for immunohistochemistry and dot blot is described in Methods and Supplementary Fig. 7.

Western blot analysis
Western blot analysis of reduced/alkylated hBD-1 is described in ref. 30 and Methods.

Thioredoxin reduction assay
Thioredoxin reduction assays were performed analogous to ref. 20. Briefly, oxhBD-1 was incubated with 0.5–2.0 μM thioredoxin or protein disulphide isomerase in the presence of 100 nM thioredoxin reductase and 0.8 mM NADPH in 0.1 M potassium phosphate buffer-2 mM EDTA. Incubation mixtures were analysed with RP-HPLC. For spectrophotometric disulphide reduction assay similar conditions were used in a volume of 100 μl, absorbance decrease was monitored at 340 nm.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions B.O.S. performed antimicrobial activity assays, HPLC analyses, MALDI-MS and TRX assays, designed and evaluated experiments, generated figures and wrote the manuscript. Z.W. generated and purified recombinant hBD-1, its ^{15}N-labelled forms and hBD-1-variants, generated alkhBD-1-affinity columns and affinity-purified the red/alkhBD-1-antibody. S.N. performed flow cytometric analyses, S.G. performed NMR spectroscopy and analysed data, M.M. performed CD spectroscopy and analysed data together with J.Bu., J.Be. performed RT-PCR and M.S. was in charge of electron microscopy. E.F.S. and J.W. designed and evaluated experiments and wrote the manuscript. All authors were involved in data discussions and the final version of the manuscript.

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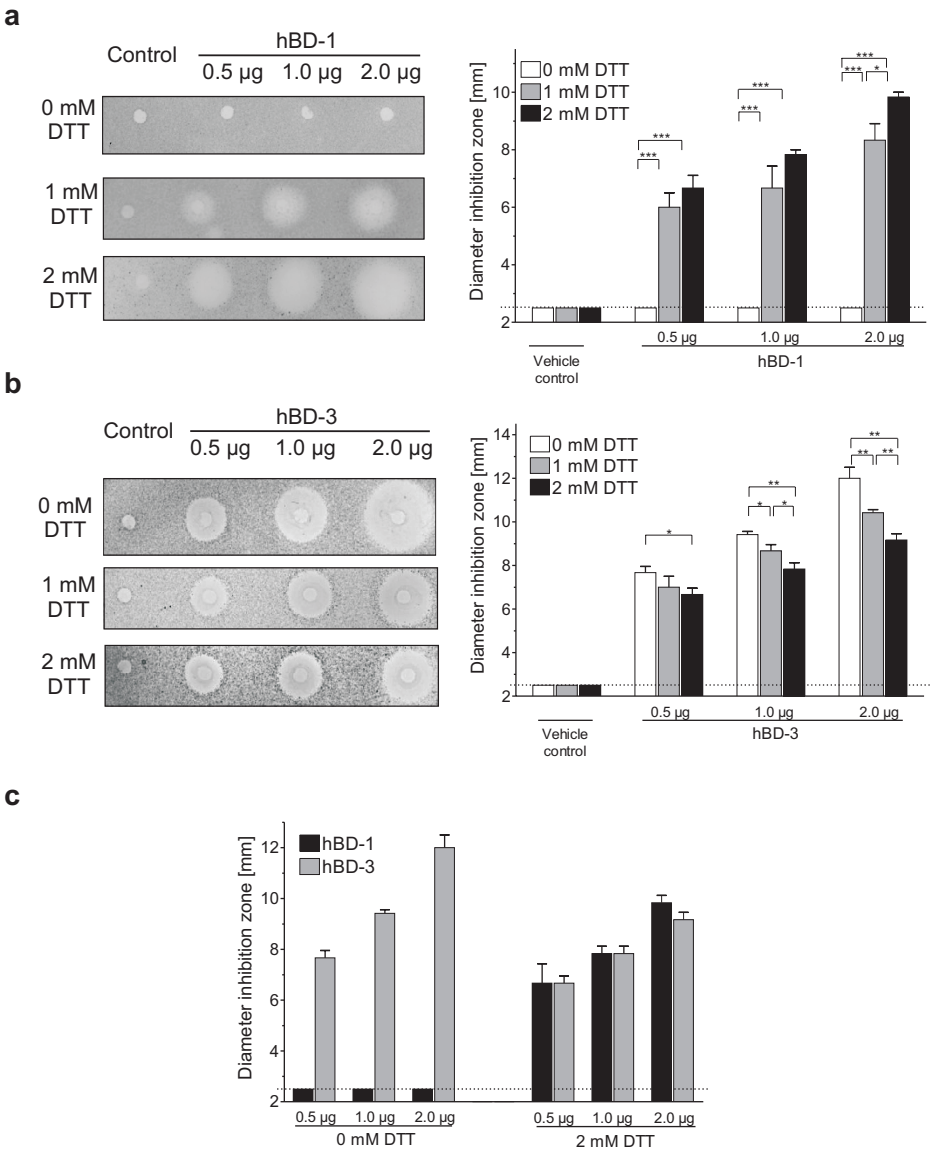


Figure 1: hBD-1 shows antimicrobial activity under reducing conditions. a, **b**, *Bifidobacterium adolescentis* was incubated with up to 2 mM DTT under anaerobic conditions with hBD-1 (**a**) or hBD-3 (**b**). Inhibition zones were measured and statistically evaluated using student's *t*-test with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Representative radial diffusion assays of three experiments are shown. Data are presented as means, error bars indicate standard deviation. **c**, Comparison of inhibition zones between hBD-1 and hBD-3 in growth medium without or with 2 mM DTT. Dotted lines at 2.5 mm represent base line diameter of punched wells.

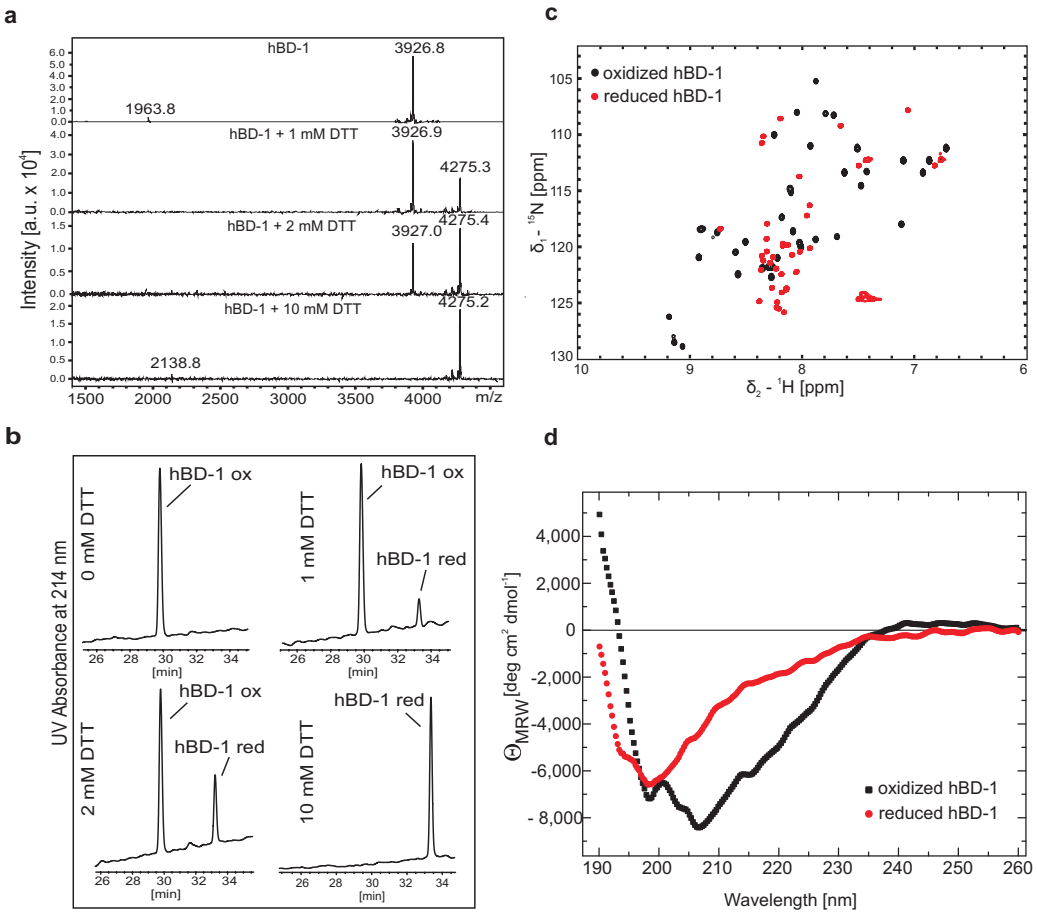


Figure 2: Reduced hBD-1 differs structurally from oxidized hBD-1. a, hBD-1 (1 µg) was incubated with different concentrations of DTT, alkylated and analysed by MALDI-MS. **b**, hBD-1 (1 µg) was incubated with different concentrations of DTT and analysed by RP-HPLC. oxhBD-1, oxidized hBD-1; redhBD-1, reduced hBD-1. **c**, Nuclear magnetic resonance (NMR) analysis of hBD-1. Superimposed ^{1}H-^{15}N-heteronuclear single quantum coherence (HSQC) spectra of oxidized (black) and reduced (red) hBD-1. δ_1-^{15}N, chemical shifts of ^{15}N nuclei; δ_2-^{1}H, chemical shifts of protons. **d**, Circular dichroism spectroscopy of oxidized (black) and reduced (red) hBD-1. Θ_{MRW}, molar ellipticity normalized to mean amino acid residue weight.

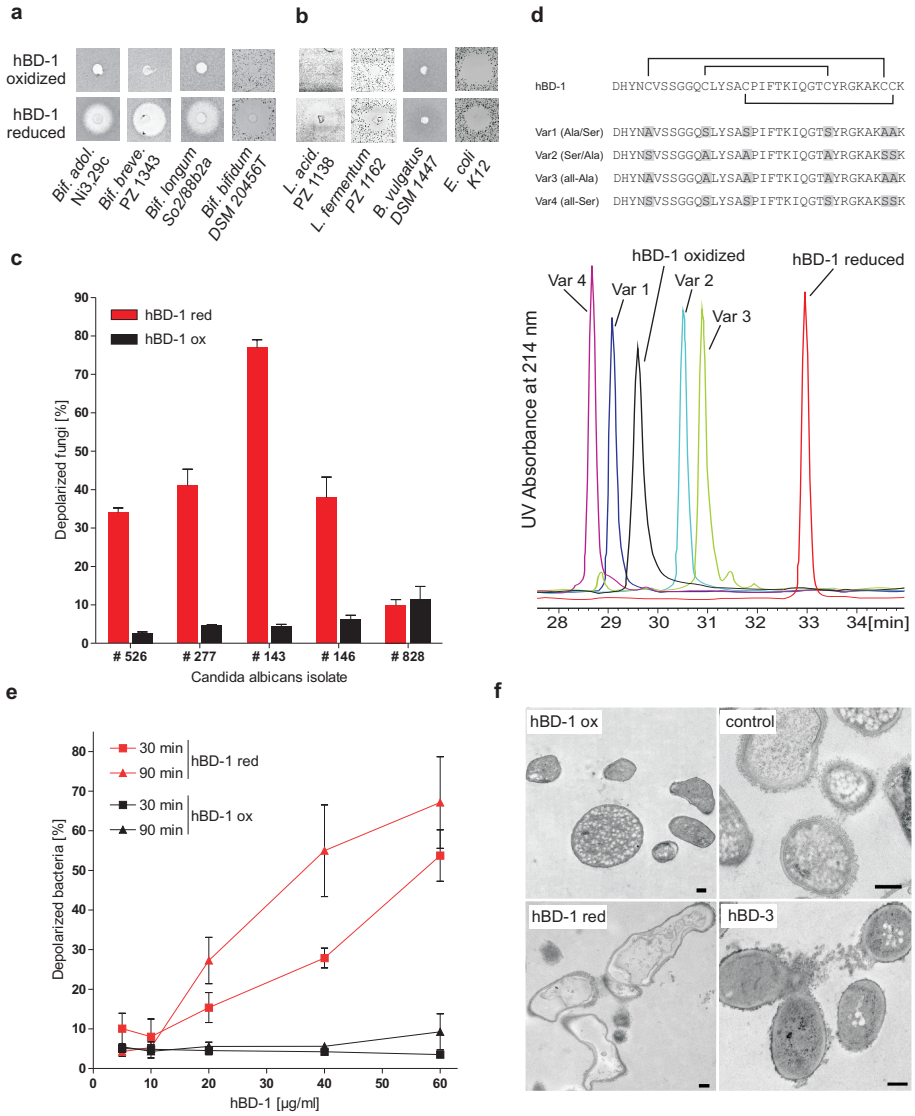


Figure 3: Reduced but not oxidized hBD-1 has a microbicidal effect. a, **b**, oxhBD-1 and redhBD-1 (1.5 µg) were incubated with bifidobacteria (**a**) and lactobacilli (3 µg defensin), *Bacteroides vulgatus* and *Escherichia coli* (**b**). *Bif. adol.*, *Bif. adolescentis*; *L. acid.*, *Lactobacillus acidophilus*. **c**, Flow cytometric antimicrobial killing assay of *C. albicans* incubated with reduced (red) or oxidized (black) hBD-1. Data are presented as mean + s.e.m. of two independent experiments each done in duplicates. **d**, RP-HPLC of oxidized and reduced hBD-1 and alanine/ serine variants. **e**, Flow cytometric antimicrobial killing assay of *Bif. adolescentis* incubated with reduced (red) or oxidized (black) hBD-1 for 30 (squares) or 90 (triangles) minutes. Results are presented as mean ± s.e.m. of two independent experiments each done in duplicates. **f**, Transmission electron microscopy of *Bif. adolescentis* incubated with oxidized hBD-1, without defensin (control), reduced hBD-1 or with hBD-3. Scale bars, 200 nm.

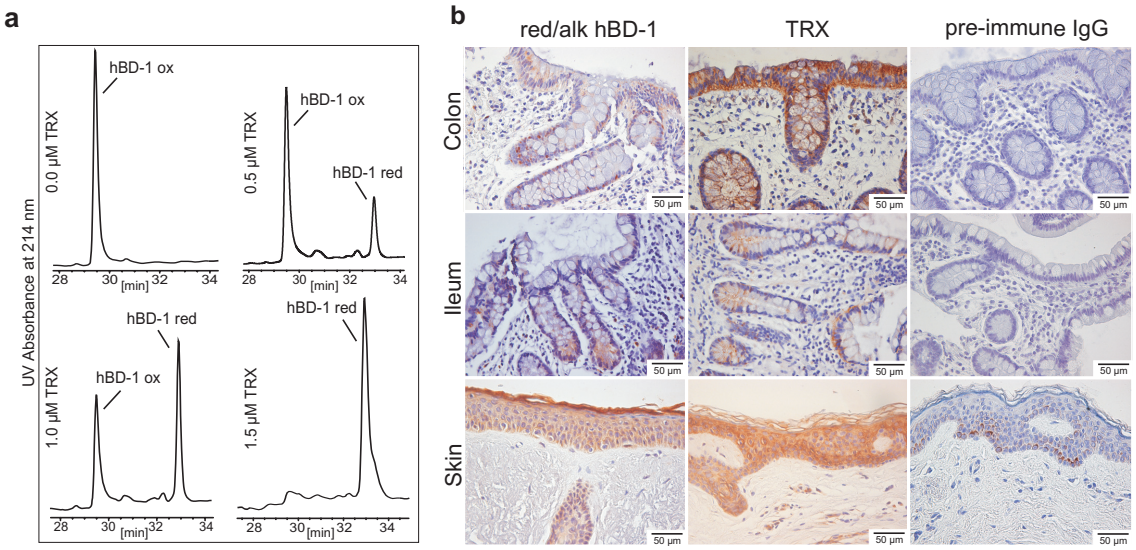


Figure 4: Thioredoxin (TRX) catalyses reduction of oxidized hBD-1 and co-localizes with redhBD-1 *in vivo*. **a**, Oxidized hBD-1 was incubated with human thioredoxin, rat thioredoxin reductase and NADPH. Incubation mixtures were analysed by RP-HPLC. **b**, Immunohistochemical analysis of reduced/alkylated hBD-1 (red/alkhBD-1; left), thioredoxin (TRX, centre) and pre-immune IgG fraction (right). Staining of human colon (upper row), ileum (middle row) and skin sections (bottom row) are shown. In the pre-immune IgG-treated skin (bottom row, right), brownish cells in the basal epidermal layer are melanocytes. Scale bars, 50 µm.

METHODS

Bacterial and fungal strains

Bacterial strains *B. adolescentis* Ni3,29c (clinical isolate), *B. adolescentis* DSM20038T (reference strain from German Collection of Microorganisms and Cell Cultures (DSM, Germany), *B. adolescentis* PZ 4009 (clinical isolate), *Bifidobacterium breve* DSM20213T (DSM reference strain), *B. breve* PZ 1343 (from probiotic VSL#3), *B. breve* Ha6/14c (clinical isolate), *Bifidobacterium longum* DSM 20219T (clinical isolate), *B. longum* So2/88b2a (clinical isolate), *Lactobacillus acidophilus* PZ 1138 (clinical isolate), and *Lactobacillus fermentum* PZ 1162 (clinical isolate) were obtained from Ardeypharm (Germany) and *Bacteroides vulgatus* DSM1447 was obtained from the German Collection of Microorganisms and Cell Cultures (DSM). *Candida albicans* strains 143, 146 and 526 were isolated from faeces, strains 277 and 828 from tracheal secretions and provided by the Institute of Laboratory Medicine, Klinik am Eichert (Göppingen, Germany).

Antimicrobial assay

Antimicrobial radial diffusion assay for anaerobic bacteria was modified from ref. 12 (see ref X1). Bacteria were grown anaerobically (AnaeroGen, Oxoid) for 24 h at 37 °C on Columbia Blood agar plates, inoculated into liquid trypticase soy broth (TSB) medium and cultivated for another 24 h. Bacterial cultures were washed and diluted to optical attenuance (D_{620nm}) = 0.1, 150 µl were used for killing assay. Incubation was carried out in 10 ml of 10 mM sodium phosphate containing 0.3 mg ml^{-1} of TSB powder and 1% (w/v) low EEO-agarose (Sigma-Aldrich) with 0–2 mM DTT (Sigma-Aldrich) under anaerobic conditions with synthetic hBD-1, hBD-3 (both Peptide Institute), lysozyme (Sigma-Aldrich), hBD-1 variants (recombinantly expressed as described) or synthetic C-terminal heptapeptides (emc microcollections GmbH). Reducing milieu was monitored by addition of redox indicator resazurin (1 mg l^{-1}, Sigma-Aldrich). Reduced hBD-1 was obtained by reduction with 20 mM DTT, purification with RP-HPLC as described, drying with vacuum-centrifuge and storage under argon gas at −20 °C. An overlay-gel containing 6% (w/v) TSB powder, 1% agarose and 10 mM sodium phosphate buffer (pH 7.4) with or without DTT was poured onto the plates after 3 h and after incubation for 48 h at 37 °C (*Bifidobacterium bifidum* was incubated for up to 4 days) the diameter of inhibition zones was measured and in part statistically evaluated using GraphPad Prism 4.03 (Graphpad Software) and student's *t*-test with $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Matrix-assisted laser desorption/ionization mass spectroscopy (MALDI-MS)

hBD-1 (1 µg) was incubated with different concentrations of DTT in 10 mM sodium phosphate buffer, pH 7.4, for 30 min at 37 °C, followed by alkylation with 20 mM iodoacetamide for 30 min at 25 °C and co-crystallized with α-cyano-4-hydroxy cinnamic acid. MALDI-MS was carried out at an ultraflex TOF/TOF machine (Bruker).

High performance liquid chromatography (HPLC) analysis

hBD-1 (1 µg) was incubated with different concentrations of DTT in 10 mM sodium phosphate buffer, pH 7.4, for 30 min at 37 °C. HPLC analysis was carried out with an Agilent 1200 series system (Agilent) and a Vydac 218TP-C18 Column (250 × 4.6 mm, 5 µm, Grace). Gradient increased from 2% B to 35% B in 33 min (solvent A, water + 0.18% (v/v) trifluoroacetic acid (TFA); solvent B, acetonitrile + 0.15% (v/v) TFA) at 25 °C and 0.8 ml min^{-1}.

Generation of recombinant hBD1 and its variants

Generation of recombinant hBD-1 and its variants is described in the Supplementary Methods part and Supplementary Table 2 (see ref X2).

Nuclear magnetic resonance (NMR) spectroscopy

Lyophilized uniformly ^{15}N-labelled reduced and oxidized hBD-1 was solubilised in H_2O containing 10% D_2O to a final concentration of 0.5–0.7 mM. The reduced peptide was kept under nitrogen atmosphere during measurement. All spectra, the ^{1}H-^{15}N-heteronuclear single quantum coherence (HSQC), the heteronuclear edited NNH-nuclear Overhauser enhancement spectroscopy (NOESY), the conventional ^{15}N-HSQC-NOESY and the ^{1}H-^{15}N-heteronuclear NOE spectrum, were recorded at 298 K on a Bruker AVIII-600 spectrometer (Bruker).

Circular dichroism (CD) spectroscopy

CD spectroscopy was carried out on a Jasco J-715 spectropolarimeter (Jasco Corporation, Japan). Reduced hBD-1 peptide was obtained by reduction with 20 mM DTT and purification with HPLC analysis as described. Reduced peptide was kept under argon atmosphere until CD measurement and spectra were recorded in 10 mM sodium phosphate buffer, pH 7.4 at a concentration of 0.1 mg ml^{-1} in the far-ultraviolet range at 25 °C. Spectra were deconvoluted with CDSSTR and Selcon3 algorithms.

Flow cytometric antimicrobial assay

Flow cytometric antimicrobial assay measuring membrane depolarization of bacteria and fungi was carried out as described elsewhere[17]. Briefly, 1.5 × 10^6 cells per ml were incubated in 1:6 diluted Schaedler broth at 37 °C with hBD-1 peptide in a final volume of 50 µl. hBD-3 served as positive control. Defensins were dissolved in 0.01% acetic acid and added to bacterial/fungal suspensions at indicated final concentrations (40 µg ml^{-1} for *C. albicans*). Bacterial or fungal suspensions incubated with solvent (0.01% acetic acid) served as controls for viability. After 30 and 90 min the suspensions were incubated for 10 min with 1 mg ml^{-1} of the membrane potential sensitive dye [bis-(1,3-dibutylbarbituric acid) trimethine oxonol] (DiBAC4(3)) (Invitrogen, USA). Suspensions were centrifuged and the sediments were resuspended in 300 µl phosphate-buffered saline. The percentage of depolarized fluorescent bacteria or fungi in suspension was determined by a FACSCalibur flow cytometer (BD, USA) using Cell Quest software (BD) for 30 000 events per sample.

Transmission electron microscopy of bacteria
Approximately 2 × 10^8 CFU of *Bifidobacterium adolescentis* Ni3,29c were incubated for two hours at 37 °C in an anaerobic jar in 10 mM sodium phosphate buffer (pH 7.4) containing 0.3 mg ml^{-1} of TSB-powder in the absence or presence of 200 µg ml^{-1} synthetic oxidized hBD-3, oxidized hBD-1 (Peptide Institute Inc.) and reduced hBD-1. For transmission electron microscopy, bacteria were centrifuged, fixed in pre-warmed Karnovsky's fixative for 1h at room temperature and stored at 4 °C for 24h. After centrifugation, the sediment was embedded in 3.5% agarose at 37 °C, coagulated at room temperature, cut in small blocks and fixed again in Karnovsky's solution for at least 1h. Post-fixation was based on 1.0% osmium tetroxide containing 1.5% K-ferrocyanide in 0.,1 M cacodylate buffer for 2 h. After embedding in glycide ether the blocks were cut using an Ultracut microtome (Reichert, Austria). Ultra-thin sections (30 nm) were mounted on copper grids and analysed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss, Germany) operating at 120 kV.

Generation of anti-reduced-hBD-1-specific antibodies
For a detailed protocol see ref. X2. Briefly, polyclonal anti-red-hBD-1 antibodies were generated in rabbits against a mixture of reduced and alkylated recombinant full-length hBD-1 as antigens. For each rabbit, a total of 0.9 mg of a protein mixture including 600 µg of HPLC-purified carboxamidomethylated hBD-1 and 300 µg of HPLC-purified reduced hBD-1 was conjugated to glutaraldehyde-treated maleimide-activated keyhole limpet hemocyanin (KLH) (protein-KLH 1:1, w/w) in phosphate buffered saline (PBS, pH 7.2) and used as immunogens. Immunization was carried out four times on days 0, 14, 28 and 35. Rabbits were bled 2 weeks after the last booster. Antisera were first applied to a HiTrap Protein G HP column (GE Healthcare, Germany) to separate IgG fraction. Next, antibodies were further purified by affinity chromatography using an alkylated hBD-1-column which was prepared from 1 mg highly HPLC-purified alkylated hBD-1-pet32-fusion protein that was covalently bound to a HiTrap N-hydroxysuccinimide (NHS)-activated HP 1 ml column (GE Healthcare). To deplete any cross-reacting antibodies recognizing the correctly folded oxhBD-1, the purified antibodies were further applied to an ox-hBD-1-column, which was generated from a HiTrap NHS-activated HP 1 ml column, where correctly folded oxhBD-1 (prepared as described) was covalently bound. Specificity was tested by immunodot and western blot analyses (Supplementary Fig. 7). As antigens alkylated hBD-1, synthetic, correctly folded oxhBD-1, purified recombinant oxhBD-1 and freshly prepared redhBD-1 were used. Upon immunodot and western blot-analyses purified antibody preparations recognized specifically redhBD-1 and alkylated (alk)hBD-1, but neither synthetic, correctly folded nor purified, recombinant oxhBD-1. In addition, pre-incubation of the antibody with alkylated hBD-1 prevented immunohistochemical staining (Supplementary Fig. 7).

Thioredoxin reduction assay
Thioredoxin reduction assays were performed analogous to A. Holmgren[20]. For RP-HPLC analysis 10 µM synthetic, oxhBD-1 was incubated with 0.8 mM NADPH (biomol, Germany), 100 nM rat thioredoxin reductase (IMCO, Sweden), 0.0 – 1.5 µM human thioredoxin (Sigma Aldrich) or up to 2.0 µM bovine protein disulphide isomerase (PDI, Sigma-Aldrich) for 30 min at 37 °C in 0.1 M potassium phosphate-2 mM EDTA (EDTA), pH 7.0 buffer. Incubation mixtures were acidified with TFA, mixed with HPLC solvent and analysed with HPLC as described and conversion from oxidized into reduced hBD-1 peptide was followed by retention time. For spectrophotometric disulphide reduction assay similar conditions were used with the following exceptions: oxhBD-1 concentration was either 25 µM while TRX concentration was 0.5 – 2.0 µM or hBD-1 concentration ranged from 12.5 to 100 µM while TRX concentration was constant at 2.0 µM. Absorbance decrease was monitored at 340 nm for up to 60 min at 37 °C in a 96-well plate reader and a final volume of 100 µl. All values were corrected against a control which contained buffer instead of hBD-1; experiments were repeated at least twice.

Immunohistochemistry
Fixation of tissue samples and biopsies was performed in 4% formalin containing 20 mM iodoacetamide to conserve open disulphide bonds and a protease-inhibitor cocktail (Complete, Roche, Germany). Paraffin sections (5 µm) of tissue samples were deparaffinised and rehydrated before heat-induced antigen retrieval was performed in 0.01 M citrate buffer (pH 6.0). Slides were blocked with 12% bovine serum albumin in Tris-buffered saline, pH 7.4, before staining. Immunohistochemical staining was performed at room temperature for one hour using either affinity-purified polyclonal rabbit reduced/alkylated-hBD-1-antibody (5 - 20 µg ml^{-1}) or rabbit thioredoxin antibody (intestinal sections, Dako, 1:400) or goat thioredoxin antibody (skin sections, R&D Systems, 1:500). A biotinylated secondary pig anti-rabbit IgG antibody (1:300, Dako Cytomation) or rabbit anti-goat IgG antibody (1:500, Jackson Immuno Research Lab) was used, followed by incubation with Vectastain ABC Kit Elite Pk-6100 (Vector, USA) developed with Vector NovaRED substrate kit for peroxidase Sk-4800 (Vector) and counterstained with hematoxylin. Specificity test of the reduced/alkylated hBD-1-antibody was performed by using protein G-purified pre-immune IgG (Fig. 4b) and blocking with alkylated hBD-1 (Supplementary Fig. 7).

Immunodot blot analysis
Proteins (synthetic, correctly folded oxidized hBD-1 in the absence or presence of 2 mM (tris(2-carboxyethyl) phosphine (TCEP); recombinantly expressed, refolded and HPLC-purified oxidized hBD-1 in the absence or presence of TCEP as well as HPLC-purified alkylated hBD-1) were dissolved in 0.1% (v/v) formic acid and were dotted to a Protran-nitrocellulose membrane (Schleicher & Schuell BioScience, Germany), blocked for 1 h in 5% (w/v) nonfat powdered milk in PBS + 0.05% Tween (PBST) and incubated for 18 h at 4 °C in 3% (w/v) nonfat powdered milk in PBST containing 8.8 µg ml^{-1} affinity-purified polyclonal reduced/alkylated rabbit hBD-1-antibody. The membrane was washed with PBST six times for 5 min each, then incubated for 1 h in 3% (w/v) nonfat powdered milk in PBST containing 1:20.000 dilution of goat anti-rabbit IgG HRP conjugate (Dianova, Germany). After six washing steps the membrane was

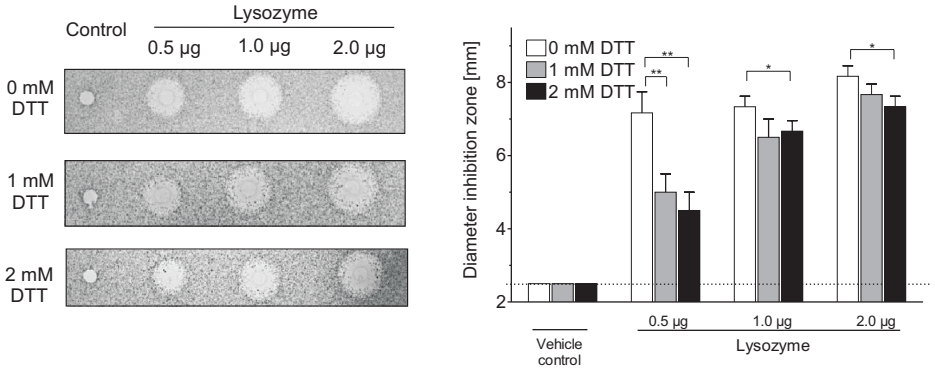
incubated for 5 min with chemiluminescent peroxidase substrate (Sigma) and visualized using a Diana III cooled CCD-camera imaging system (Raytest, Germany). Densitometric quantifications were performed using AIDA evaluation software (Raytest). Quality control experiments for the reduced/alkylated-hBD-1-antibody in the immunodot system (performed by pretreatment of the antibody with 5 µg/ml alkylated hBD-1) revealed no staining with oxidized, reduced as well as alkylated hBD-1 (not shown).

Western blot analysis
Samples were boiled in sampling buffer in the absence or presence of 2 mM DTT as indicated and electrophoresed in a system optimized for the analysis of 1-10 kDa-peptides as previously described for AMPs[30]. Briefly, samples were electrophoresed in a 16.5% SDS-tricine polyacrylamide gel containing 8 M urea but no reducing agent. Proteins were transferred to a Protran-nitrocellulose membrane (Schleicher & Schuell BioScience, Germany) and then treated analogue to immunodot analysis.

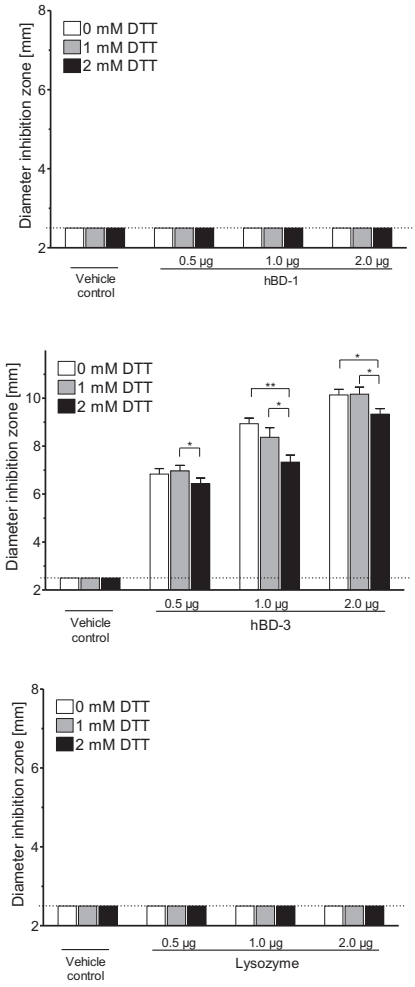
Isolation of total RNA and real-time PCR
Frozen biopsies were disrupted mechanically and total RNA was isolated using TRIzol reagent according to the supplier's protocol. RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) into cDNA, according to the standard protocol of the manufacturer. cDNA derived from 10 ng of total RNA served as a template for real-time PCR reaction. The expression levels of thioredoxin mRNA were quantified by real-time PCR using a fluorescence detection monitor (Light-Cycler; Roche Diagnostics) using oligonucleotides as shown in Supplementary Table 2. All expression levels were normalized with respect to the expression of beta-actin. To calculate statistically significant differences between groups, samples were analysed by Mann – Whitney test. Gaussian distribution was determined using the Kolmogorov – Smirnov test. Results are presented as mean ± s.e. Values of $P < 0.05$ were considered to be statistically significant. Data were analysed using Graphpad Prism version 4.

X1. Schroeder, B.O. & Wehkamp, J. Measurement of antimicrobial activity under reducing conditions in a modified radial diffusion assay. *Protocol Exchange* doi:10.1038/protex.2010.204 (2011)

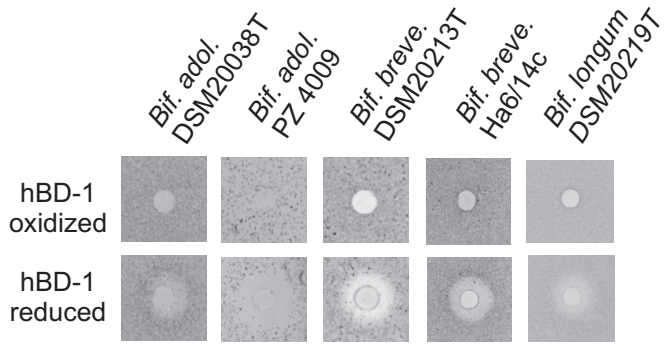
X2. Wu, Z., Schroeder, B.O., Schroeder, J.-M. & Wehkamp, J. Production of recombinant hBD-1 in *Escherichia coli* and its specific polyclonal antibody in rabbits. *Protocol Exchange* doi:10.1038/protex.2010.205 (2011)



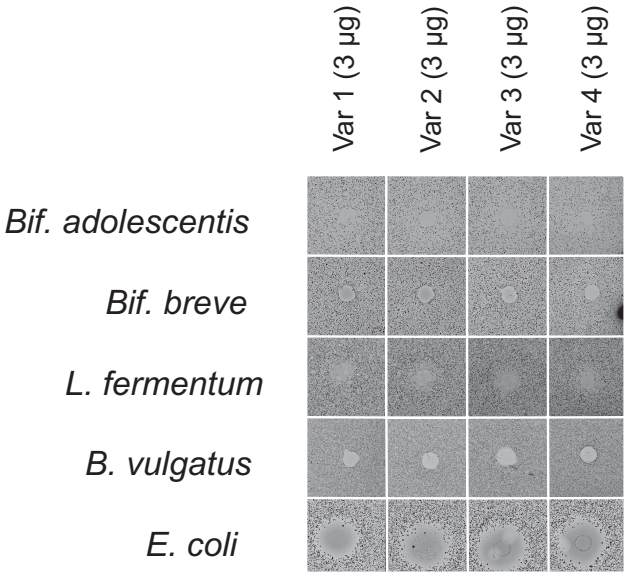
Supplementary Figure 1. Lysozyme shows slightly diminished antimicrobial activity against *Bif. adolescentis* under reducing conditions. Colonies within the inhibition zone might be caused by selective impairment of enzymatic but not non-enzymatic lysozyme function through DTT as shown for *Streptococcus faecalis*[31]. *Bif. adolescentis* Ni3,29c was incubated without DTT or with 1 mM or 2 mM DTT under anaerobic conditions with 0.5 µg, 1.0 µg or 2.0 µg of lysozyme in a modified radial diffusion assay. After incubation for 48 hours at 37°C the diameter of inhibition zone was measured and statistically evaluated using GraphPad Prism 4.03 and student's t-test with (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$. Data are presented as means, error bars indicate standard deviation. Representative radial diffusion assay pictures are shown while experiments were carried out three times. Dotted line at 2.5 mm in the right panel represents base line diameter of punched wells.



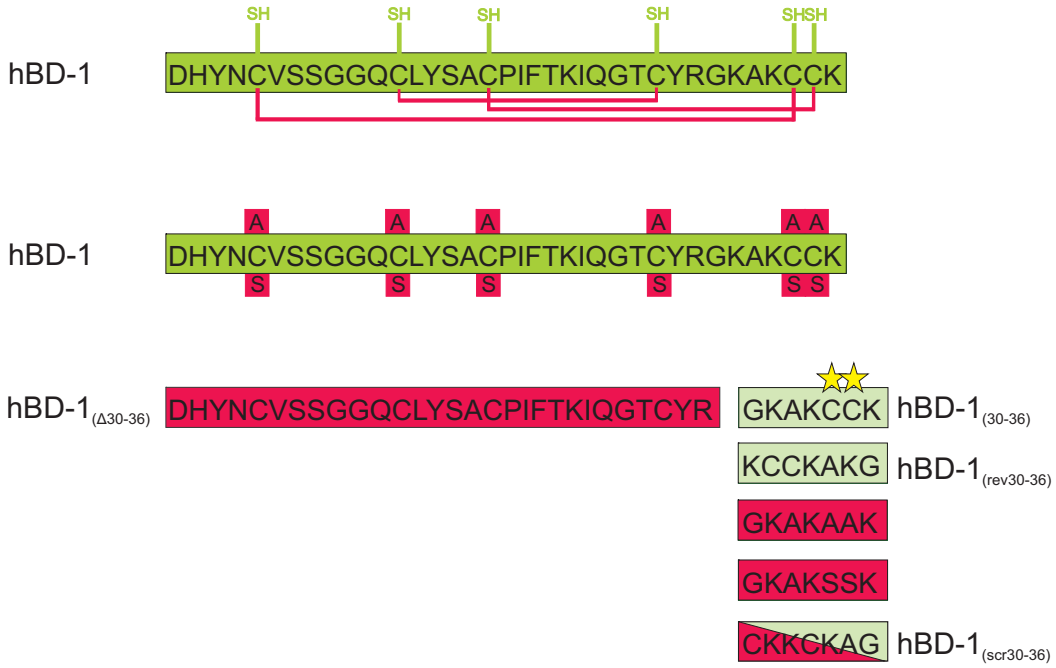
Supplementary Figure 2. hBD-1 does not show antimicrobial activity against *Bacteroides vulgatus* under reducing conditions. *B. vulgatus* was incubated without DTT or with 1 mM or 2 mM DTT under anaerobic conditions with 0.5 µg, 1.0 µg or 2.0 µg of hBD-1 (upper panel), hBD-3 (center) or lysozyme (bottom) in a modified radial diffusion assay. After incubation for 48 hours at 37°C the diameter of inhibition zone was measured and statistically evaluated using GraphPad Prism 4.03 and student's t-test with (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$. Data are presented as means, error bars indicate standard deviation. Experiments were carried out three times. Dotted lines at 2.5 mm represent base line diameter of punched wells.



Supplementary Figure 3. Reduced hBD-1 is active against different *Bifidobacteria* while oxidized peptide is not. Modified radial diffusion assay was carried out as described. hBD-1 peptide was reduced in 20 mM DTT, purified by HPLC, dried under vacuum and stored under argon-atmosphere. 1.5 µg of oxidized (upper panel) and reduced hBD-1 (bottom) were incubated with *Bifidobacterium adolescentis* DSM20038T, *Bif. adol*. PZ 4009, *Bif. breve* DSM20213T, *Bif. breve* Ha6/14c as well as *Bif. longum* DSM20219T in assay-medium without DTT and generation of inhibition zones was evaluated after 48 hours. Representative radial diffusion assay pictures are shown while experiments were carried out three times.

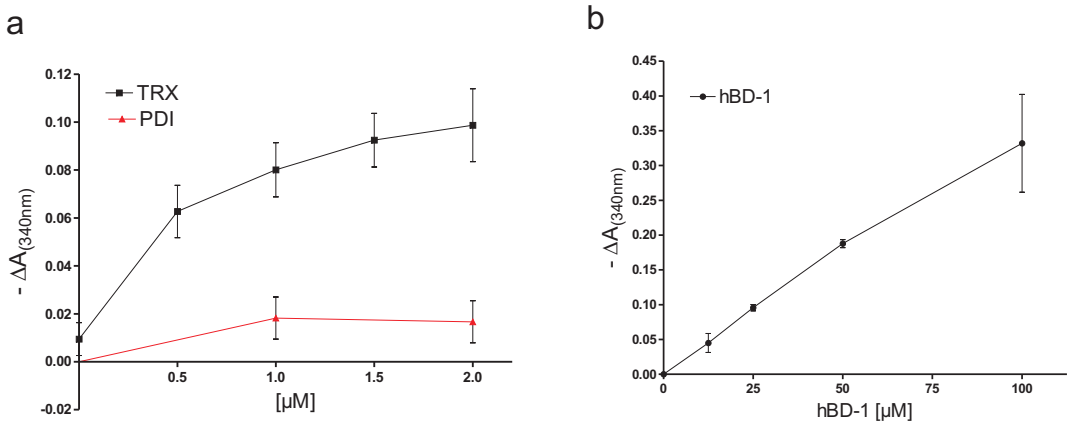


Supplementary Figure 4. Antimicrobial activity of Ala/Ser variants of hBD-1 (3 µg) against *Bifidobacterium adolescentis* Ni3,29c, *Bif. breve* PZ 1343, *Lactobacillus fermentum* PZ 1162, *Bacteroides vulgatus* DSM1447 and *Escherichia coli* K12 measured by modified radial diffusion assay without DTT as described in the methods part. Generation of inhibition zones was evaluated after 48 hours. Representative radial diffusion assays are shown.

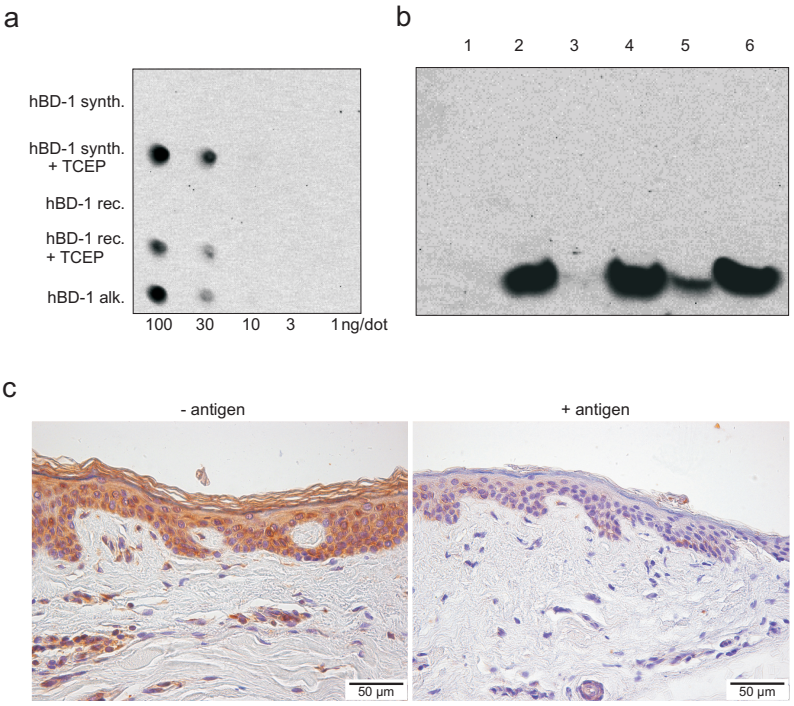


Supplementary Figure 5. Schematic model for the antimicrobial activity of reduced hBD-1 against *Bifidobacterium adolescentis* using the amino acid one-letter code. Full-length, reduced hBD-1 is active (depicted in green) while disulfide-bridged, oxidized hBD-1 (indicated by red lines between cysteines) is not. Changing cysteine residues to alanine or serine abolishes activity. The N-terminal 29 amino acid-peptide of hBD-1 does not show antimicrobial activity while the C-terminal seven amino acids *GKAKCCK* do. The same is true when reversing the amino acid order. Changing cysteine residues in the C-terminus to alanine or serine abolishes activity, indicating a crucial role for these two cysteines, depicted by yellow stars. A peptide with scrambled amino acid sequence of the C-terminus shows only weak antimicrobial activity.

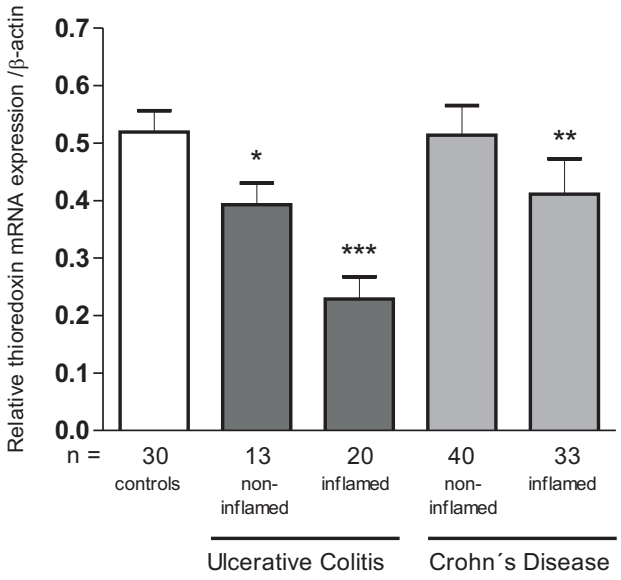
Notably, isolated C-terminal peptides do not show full activity of comparable amounts of the full-length-peptide (depicted by light green colour), indicating that either antimicrobial activity located in the C-terminus of hBD-1 is positively modulated by the N-terminal fragment hBD-$1_{(\Delta 30\text{-}36)}$ or that the small peptides behave different in our assays.



Supplementary Figure 6. The thioredoxin-system is able to reduce oxidized hBD-1 in a dose-dependent manner. **a,** 25 µM hBD-1 was incubated with different concentrations of thioredoxin (TRX, black squares) or protein-disulfid-isomerase (PDI, red triangles) for 30 minutes in the presence of 0.8 mM NADPH in 0.1 M potassium phosphate buffer -2 mM EDTA in a final volume of 100 µl. After addition of 0.1 µM thioredoxin reductase decrease of absorbance at 340 nm was monitored against a buffer control without hBD-1. Data are represented as mean± s.d. from three independent experiments. **b,** The thioredoxin reduction assay was performed as described above while keeping thioredoxin concentration constant at 2.0 µM and varying hBD-1 concentration (black circles). Decrease of absorbance at 340 nm was monitored after 60 minutes; data are presented as mean ± s.d. from two independent experiments.



Supplementary Figure 7. Specificity of polyclonal affinity-purified rabbit anti-reduced/alkylated hBD-1 antibody. **a**, Immuno dot blot analysis of oxidized hBD-1 (synthetic (synth.) or recombinant (rec.)), reduced hBD-1 and alkylated hBD-1 incubated with a 8.8 µg/ml of anti-redhBD-1 antibody. hBD-1 was reduced by incubation with 2 mM reducing agent tris(2-carboxyethyl) phosphine (TCEP) for 15 minutes in 0.1% formic acid. **b**, Western blot analysis of oxidized, reduced and alkylated hBD-1 incubated with 8.8 µg/ml anti-redhBD-1 antibody. Lane 1: 100 ng synthetic oxidized hBD-1, lane 2: 100 ng synthetic oxidized hBD-1 boiled with 2 mM DTT, lane 3: 100 ng recombinant oxidized hBD-1, lane 4: 100 ng recombinant oxidized hBD-1 boiled with 2 mM DTT, lane 5: 30 ng recombinant oxidized hBD-1 boiled in 2 mM DTT, lane 6: 100 ng alkylated hBD-1. **c,** 10 µg/ml of antibody was either used directly for immunohistochemical analysis (left) or pre-incubated with 1.25 µg/ml antigen (alkylated hBD-1, right) to block specific binding sites. Representative staining of human skin sections are shown, pre-incubation with antigen strongly reduces staining intensity.



Supplementary Figure 8. Expression of thioredoxin mRNA in human colonic biopsies from healthy controls and patients with inflammatory bowel disease (non-inflamed and inflamed). Thioredoxin mRNA expression was analysed using real-time PCR, compared with healthy controls and normalized to beta-actin. Data were statistically evaluated using GraphPad Prism 4.03 and Mann-Whitney test with (*) $p < 0.05$, (**) $p < 0.01$ and (***) $p < 0.001$. Data are presented as mean ± s.e.

Representative secondary structure fraction calculation for oxidized hBD-1						
Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
0.31	0.11	0.13	0.06	0.13	0.26	1
Representative secondary structure fraction calculation for reduced hBD-1						
Helix1	Helix2	Strand1	Strand2	Turns	Unordered	Total
0.02	0.05	0.18	0.11	0.15	0.49	1

Supplementary Table 1. Representative secondary structure fraction calculation from Circular Dichroism (CD) spectra of oxidized and reduced hBD-1 using CDSSTR algorithm[33] by DichroWeb (http://dichroweb.cryst.bbk.ac.uk). Use of different algorithms (i. e. Selcon3[34]) and different reference protein sets lead to slightly different results (data not shown). Calculation takes account of the sum of the spectral contributions from all secondary structural elements of the peptide. Off note, alpha-helical signals in two-dimensional CD-spectroscopy are 3 to 4 fold more intense than beta-strands, resulting in the observed high alpha-helical content and explaining the differences to published three-dimensional structure analyses of oxidized hBD-1 (PDB-ID: *1KJ5*[35] and *1IJU*[36]).

	template amplification	insert amplification
hBD-1 wt	hBD1-f 5'-ccagtcgccatgagaacttcctacc-3' hBD1-r 5'-ctgcgtcatttcttctggtcactcc-3'	pet-hBD1-f 5'-ACTGAGATCTGGGTACCGACGACGACGACAAGgatc-attacaattgcgtcagcagtggt-3' pet-hBD1-r 5'-ATTTGCGGCCGCTCActtgcagcacttggccttccctcg-3'
Var 1 (Ala/Ser)	hBD-1S-f 5'-gatcattacaat**tct**gtcagcagtggtgggcaa**tct**ctctattctgcc**tct**ccg-3' hBD-1S-r 5'-tcactt**agaaga**cttggccttccctcggta**aga**ggt-3'	Su3-hBD1A-f 5'-AAGGTCTCAAGGTgatcat**tac**aatgccgtcagc-3' Su3-hBD1A-r 5'-AAGGATCCTCActt**ggcggc**cttggccttccctcg-3'
Var 2 (Ser/Ala)	hBD-1A-f 5'-gatcattacaat**gcc**gtcagcagtggtgggcaa**gcc**ctctattctgcc**gcc**ccg-3' hBD-1A-r 5'-tcactt**ggcggc**cttggccttccctcggta**ggc**ggt-3'	Su3-hBD-1S-f 5'-AAGGTCTCAAGGTgatcattacaat**tct**gtcagc-3' Su3-hBD-1S-r 5'-AAGGATCCTCActt**agaaga**cttggccttccctcg-3'
Var 3 (all-Ala)	hBD-1A-f gatcattacaat**gcc**gtcagcagtggtgggcaa**gcc**ctctattctgcc**gcc**ccg hBD-1A-r tcactt**ggcggc**cttggccttccctcggta**ggc**ggt	Su3-hBD-1A-f AAGGTCTCAAGGTgatcattacaat**gcc**gtcagc Su3-hBD-1A-r AAGGATCCTCActt**ggcggc**cttggccttccctcg
Var 4 (all-Ser)	hBD-1S-f gatcattacaat**tct**gtcagcagtggtgggcaa**tct**ctctattctgcc**tct**ccg hBD-1S-r tcactt**agaaga**cttggccttccctcggta**aga**ggt	Su3-hBD-1S-f AAGGTCTCAAGGTgatcattacaat**tct**gtcagc Su3-hBD-1S-r AAGGATCCTCActt**agaaga**cttggccttccctcg
TRX (rt-PCR)	TXN-f 5'-tggtgtgggccttgcaaaatga-3' TXN-r 5'-ttcacccacctttgtcccttc-3'	
β-actin (rt-PCR)	bAct-f 5'-gccaaccgcgagaagatga-3' bAct-r 5'-catcacgatgccagtggta-3'	

Supplementary Table 2. Oligonucleotides used for generation of wild-type and alanine/ serine variants of hBD-1 and real-time PCR analysis of human colonic samples. For recombinant expression in *E. coli* the codon gga for glycine was changed to ggt; aga for arginine was changed to cga. Letters in bold highlight modified codons.

SUPPLEMENTARY METHODS

Construction of expression vectors. Five expression vectors were constructed to generate recombinant hBD-1 and its four different variants (Figure 3d).

Construction of pET32a-hBD1: Total RNA was isolated from cultured foreskin-derived keratinocytes using the RNeasy Mini kit (Qiagen, Germany). 2 μg of total RNA was reverse transcribed with an oligo$(dT)_{18}$ primer and Superscript II RNaseH$^-$ RT (Invitrogen, Germany). To retrieve a general hBD-1 cDNA template, PCR was performed with one pair of primers, hBD1-f and hBD1-r (Supplementary Table 2), and Taq DNA polymerase (Invitrogen) under the following conditions: 95°C for 2 min; 32 cycles at 95°C for 20 s, 64°C for 30 s, 72°C for 30 s; and finally 72°C for 10 min. The PCR product was cloned and confirmed by sequencing before its plasmid DNA was used as the template for the following PCRs. To generate hBD-1 insert, PCR was performed with *Pfu* Hotstart Turbo DNA Polymerase (Stratagene, USA) and the primer pair, pet-hBD1-f and pet-hBD1-r, containing appropriate *Bgl*II or *Not*I digestion site (Supplementary Table 2), under the following conditions: 95°C for 2 min; 5 cycles (95°C for 20 s, 56°C for 30 s, 72°C for 30 s); 25 cycles (95°C for 20 s, 72°C for 45 s); and finally 72°C for 10 min. The PCR product was double digested with *Bgl*II and *Not*I prior to ligation into the pET32a vector (Novagen, Australia; double digested with *Bgl*II and *Not*I) with T_4 DNA ligase (Invitrogen). The expression construct was amplified in *E. coli* Top10 (Invitrogen) and confirmed by sequencing.

Construction of pSumo3-hBD-1-variants: To generate templates for hBD-1 variants, PCR was performed with hBD-1 cDNA, one pair of specific primers (Supplementary Table 2), and Taq DNA polymerase (Invitrogen) under the following conditions: 95°C for 2 min; 5 cycles at 95°C for 20 s, 56°C for 30 s, 72°C for 30 s;25 cycles at 95°C for 20 s, 72°C for 45 s; and finally 72°C for 10 min. To generate inserts for subcloning into pSumo3 vector (Invitrogen), PCR was performed with *Pfu* Hotstart Turbo DNA Polymerase (Stratagene) and one pair of primers containing appropriate *Bsa* I or *BamH* I digestion site (Supplementary Table 2) under the following conditions: 95°C for 2 min; 5 cycles (95°C for 20 s, 58°C for 30 s, 72°C for 30 s); 25 cycles (95°C for 20 s, 72°C for 45 s); and finally 72°C for 10 min. The PCR product was double digested with *Bsa* I and *BamH* I prior to ligation into the pSUMO3 vector (Invitrogen). The expression construct was amplified in *E. coli* Top10 (Invitrogen) and confirmed by sequencing.

Protein expression. The constructed plasmid pET32a-hBD-1 was transformed into *E.coli* BL21(DE3)pLysS competent cells (Novagen) and plated on LB plates with corresponding antibiotics. Transformants were grown at 37°C and 250 rpm in LB medium or M9 medium containing appropriate antibiotics to an OD_{600} of 0.6 to 0.7. To generate uniformly ^{15}N-labeled hBD-1, bacteria were grown in M9 minimal medium using $^{15}NH_4Cl$ (Cambridge Isotope Laboratories, USA) as the sole nitrogen source. Protein expression was induced with 1 mM IPTG (isopropyl-beta-D-thio-galactopyranoside) for 4 h. After incubation, cells were harvested by centrifugation and resuspended in 1×LEW buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 5 mM imidazole). Resuspended cells were subjected to one cycle of freeze-thawing and sonicated on ice. After centrifugation at 13,500×g for 45 min, the clarified supernatant was applied to a HisTrap^{TM} HP prepared column (GE Healthcare, UK) and the polyhistidine-tagged protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The fusion protein was further purified by RPHPLC using a preparative SP250/10 Nucleosil 300-7 C8 column (Macherey-Nagel) that was previously equilibrated with 0.05% (v/v) TFA in HPLC-grade water containing 10% isopropanol. Proteins were eluted with a gradient of increasing concentrations of isopropanol containing 0.05% (v/v) TFA (flow rate, 3 ml/min). Fractions containing UV_{215nm}-absorbing material were collected, analyzed by ESI-QTOF-mass spectrometry (Micromass, UK) and lyophilized.

The pSUMO3-derived expression constructs were transformed into *E. coli* BL21(DE3)*pLys*S cells (Novagen) and selected on LB agar plates containing kanamycin (50 μg/ml) and chloramphenicol (34 μg/ml). Transformants were grown at 37°C and 200 rpm in LB medium containing appropriate antibiotics to an OD_{600} of 0.6. Protein expression was induced with 1 mM IPTG for 3 h. After incubation, cells were harvested by centrifugation and resuspended in 1xLEW buffer. Resuspended cells were subjected to three cycles of freeze-thawing and sonicated on ice. After centrifugation at 13,500×g for 45 min, the clarified supernatant was applied to Protino-Ni-prepared columns (MachereyNagel, Dueren, Germany) and the polyhistidine-tagged protein was eluted with elution buffer. The fusion protein was further purified by HPLC using preparative wide-pore RP-C8 HPLC with a column (SP250/10 Nucleosil 300-7 C8; Macherey-Nagel) that was previously equilibrated with 0.1% (v/v) TFA in HPLC-grade water containing 10% acetonitrile. Proteins were eluted with a gradient of increasing concentrations of acetonitrile containing 0.1% (v/v) TFA (flow rate, 3 ml/min). Fractions containing $UV_{215\ nm}$-absorbing material were collected, analyzed by ESI-QTOF-mass spectrometry (Micromass, Manchester, U.K.) and lyophilized.

Refolding of the pET32a-hBD-1 fusion protein. We had observed that enterokinase-treatment of the pET32a-hBD-1 protein led to a truncated hBD-1 form, missing seven C-terminal amino acids (hBD-$1_{\Delta 30\text{-}36}$) and nearly no full-length form. With the finding that the correctly folded, compact form of hBD-1 is protease-resistant we aimed to refold the pET32a-hBD-1 protein prior to enterokinase digestion. This was performed with the two-step quick dilution method adapted from Lu *et al*[32]. Recombinant fusion protein was adjusted to 7.5 mg/ml with PBS and quick diluted into 37.5-fold refolding buffer containing 100 mM Tris-HCl, pH 8.5, 1 mM EDTA, 1.5 M urea, 0.3 mM oxidized glutathione (GSSG) and 3.0 mM glutathione (GSH). After incubation for 30 min at room temperature, the protein solution was further diluted by 37.5-fold refolding buffer containing 100 mM Tris/HCl, pH 8.5, 1 mM EDTA, 1.5 M urea, 5.7 mM GSSG and 3.0 mM GSH and then incubated under shaking at room temperature. After 16-h incubation, protein was concentrated with an Amicon 3-

kD membrane and dialyzed against enterokinase buffer (50 mM Tris/HCl, pH 8.0, 1 mM $CaCl_2$).

Purification of recombinant proteins. For purification of recombinant wild-type hBD-1, the dialyzed pET32a-hBD-1 protein solution was adjusted to 0.5 mg/ml and incubated for 12 h at 37 °C with enterokinase (EKMax™, Invitrogen) after addition of 0.1% (v/v) Tween-20 according to the manufacturer's instructions. The sample was loaded onto a MonoS FPLC HR5/5 column (Pharmacia, Sweden) which was washed with starting buffer containing 20 mM Tris/HCl (pH 8.2) and eluted with a gradient of buffer containing 20 mM Tris/HCl (pH 8.2) and 1.0 M NaCl. Fractions containing hBD-1 (as determined by ESI-MS) were pooled and either reduced (to obtain linearized, reduced hBD-1) or further purified by high resolution RP-HPLC (Jupiter, Phenomenex C18, 30 nm, 5 µm, 150×2.0 mm; eluent: acetonitrile/TFA (solvent A 0.1% TFA; solvent B 80% CH_3CN, 0.1% TFA; gradient 25–70% solvent B in 30 min)) to obtain correctly folded oxidized hBD-1 and separate alternatively folded ox-hBD-1 variants. Fractions containing the desired peptide, as verified by SDS-PAGE and ESI-MS, were combined and lyophilized.
For purification of recombinant hBD1 variants, the SUMO-His-tagged fusion proteins purified from RP-C8 HPLC were dissolved at 0.2 mg/ml of 1×PBS buffer containing 2 mM DTT. The digestion was performed for 4 h at 30 °C with SUMO protease 2 according to the manufacturer's protocol (Lifesensors Inc., USA). The sample was loaded onto a Jupiter-5µ-C18-300A (150×2.0mm) HPLC column (Phenomenex, Germany) equilibrated with 0.05% (v/v) TFA and 10% acetonitrile in water. Peptides were eluted with a gradient of increasing concentrations of acetonitrile containing 0.05% TFA (flow rate, 0.5 ml/min). Fractions of each peak were collected, analyzed by ESIQTOF-mass spectrometry and lyophilized.

Reduction and alkylation of recombinant hBD-1. The lyophilized mixture of recombinant oxhBD-1-variants was dissolved in 50 mM NH_4HCO_3 buffer (pH 8.0), reduced with 20 mM DTT (2h, 37 °C) and then alkylated with 50 mM iodoacetamide (IAA), dissolved in 50 mM NH_4HCO_3 for 1 h at 37 °C in the dark. Purification of the alkylated sample was performed by RP-HPLC as described above at a flow rate of 150 µl/min. Fractions containing the desired peptide, as verified by SDS-PAGE and mass spectrometry, were combined and lyophilized. ^{15}N-labeled redhBD-1 was generated from correctly folded ^{15}N-oxhBD-1, which has been obtained from refolded ^{15}N-pET32a-hBD-1 fusion protein. This cumbersome procedure was necessary because only digestion of the refolded pET32a-hBD-1 fusion protein led to the full-length hBD-1. Un-refolded fusion protein leads to the truncated hBD-$1_{\Delta 30\text{-}36}$ peptide. Lyophilized ^{15}N-oxhBD-1 was dissolved in 0.01 % acetic acid and incubated with 10 mM Tris-carboxyethyl-phosphin (TCEP) for 30 min. After adding 50 mM NH_4HCO_3 buffer (pH 8.0) 20 mM DTT was added, the mixture further incubated (2h, 37 °C) and then separated by RP-HPLC (Jupiter, Phenomenex C18) as described. Fractions containing the labelled redhBD-1 (as assessed by ESI-MS) were lyophilized and stored at -20°C until further use.

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VI Measurement of antimicrobial activity under reducing conditions in a modified radial diffusion assay. ***Protocol Exchange*** **2011, DOI: 10.1038/protex2010.204.**

1 Aims

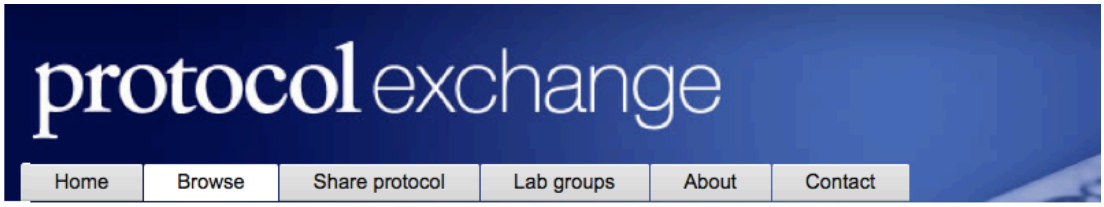
The testing of antimicrobial peptides, including defensins, has been established for bacteria like *E. coli, S. aureus, P. aeruginosa* and others, which mainly live in the presence of oxygen. Similarly, it was initially thought that disulphide bridges, a characteristic feature of defensins, are essential for their antimicrobial activity. However, this conclusion has been drawn from the testing of only few antimicrobial peptides and then generalized.
Our aim was therefore to test antimicrobial peptides under reducing conditions as they occur in the intestine. This would also allow to analyze anaerobic gut bacteria, which do not tolerate high concentrations of oxygen. Moreover, these conditions resemble better the natural environment in the gut.

2 Method

A well established method for testing antimicrobial activity of proteins and peptides is the radial diffusion assay developed by Robert Lehrer *et al.* [Lehrer et al., 1991]. We therefore modified the assay by the addition of low amounts of the reducing agent dithiothreitol (DTT) to the assay medium and incubated the bacteria under anaerobic conditions in an anaerobic jar. We focused in our testings on commensal gut bacteria including *Bifidobacteria, Lactobacilli, Bacteroidetes* and *Escherichia coli.* Due to the prolonged growth phases of most of these bacteria, the incubation times during the assay had to be modified as well.

3 Summary of findings

The modified antimicrobial radial diffusion assay worked well to allow growth of *Bifidobacteria, Lactobacilli, Bacteroidetes* and *E. coli.* Furthermore, we found that some antimicrobial peptides indeed lose their potency to kill bacteria under reducing conditions, on the other hand for one antimicrobial peptide, hBD-1, we found strongly enhanced antimicrobial activity under these conditions. By variation of the DTT concentration we could optimize growth of bacteria and modulate antimicrobial activity of some defensins.



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Measurement of antimicrobial activity under reducing conditions in a modified radial diffusion assay

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This protocol describes the measurement of antimicrobial activity under reducing conditions against anaerobic bacteria of the human normal flora. The protocol is modified from the original description by Robert Lehrer *et al.* (1), which is well established for the analysis of antimicrobial peptides with aerobic bacteria. To perform analyses for facultative and strict anaerobes we modified the assay by the addition of the reducing agent DTT to assay medium and performed incubation steps in an anaerobic jar. It was successfully used with analysis of *Bifidobacteria, Lactobacilli, Bacteroides* and *Escherichia coli* (2) and might be adapted to other anaerobic bacteria. Several antimicrobial peptides have been tested with that system: while most of them showed less antimicrobial activity under reducing conditions, human beta-defensin-1 was only active under these conditions against some of the mentioned strains.

Subject terms: Microbiology, Immunological techniques

Keywords: radial diffusion assay, antimicrobial activity, reducing conditions, anaerobic bacteria

Reagents

- Type I low EEO-agarose (Sigma-Aldrich, #A6013)
- Tryptic Soy Broth (TSB) powder (Difco, #211825)
- monobasic sodium phosphate buffer $NaH_2PO_4 \cdot H_2O$ (MW = 137.99)
- dibasic sodium phosphate buffer $Na_2HPO_4 \cdot 2 H_2O$ (MW = 177.99)
- Dithiothreitol (DTT) 1 M stock solution in H_2O from powder (Carl Roth, #6908.1)
- HCl (1:10 and 1:100 dilutions in H_2O)
- Resazurine (Sigma-Aldrich, #R7017) stock solution 1 mg/ ml in H_2O
- Bacteria to analyze
- Peptides to analyze (dissolved in H_2O or 0.01% acetic acid)
- Columbia Blood Agar plates (or similar growth medium)

Equipment

- three 100 ml bottles
- five 500 ml bottles
- 50 ml Falcon tubes
- square-dish 100×100 x 15 mm (BD Falcon, #351112)
- sterile glass pipettes 1 ml, 5 ml, 10 ml
- gel puncher (for instance biopsy punch (Stiefel))
- 37°C anaerobic incubator or system (Oxoid #AG0025, #AN0025, #BR0055)
- centrifuge for 50 ml Falcon tubes
- autoclave
- microwave
- water-bath maintained at 46°C
- spectral-photometer at 620 nm

Procedure

A. Prepare media and reagents

1. *1x Tryptic Soy Broth (TSB):* Dissolve 15 g of TSB powder in 500 ml ddH_2O, autoclave

2. Phosphate buffer 100 mM, pH 7.4

– Prepare 200 mM monobasic phosphate buffer ($NaH_2PO_4 \cdot H_2O$) (MW = 137.99): dissolve 13.8 g of NaH_2PO_4 in 500 ml ddH_2O
– Prepare 200 mM dibasic phosphate buffer ($Na_2HPO_4 \cdot 2\ H_2O$) (MW = 177.99): dissolve 17.8 g of Na_2HPO_4 in 500 ml ddH_2O
– Mix 47,5 ml of monobasic NaP with 202,5 ml dibasic NaP, add 250 ml H_2O, autoclave

3. Phosphate buffer 10 mM, pH 7.4

Dilute 100 mM Phosphatpuffers 1:10 with H_2O, final volume of 100 ml, autoclave

4. Underlay "low nutrition" gel (5 gels)

– add 0.5 g EEO-agarose into 100 ml bottle
– add 0.5 ml 1x TSB
– add 5 ml of Phosphate buffer 100 mM, pH 7.4
– add ddH_2O to a final volume of 50 ml (Note: If melting of the gel in the microwave causes significant loss of liquid it is possible to add more ddH_2O in order to keep a remainder of 50 ml after microwaving.)
– adjust pH to 7.4 with diluted HCl (1:100 or 1:10) (Note: depending on the number of gels you prepare only few drops might be required.)
– autoclave in 100 ml bottle for 20 min at 121°C, store for short term at room temperature

5. Overlay „high nutrition" gel (5 gels)

– add 0.5 g EEO-agarose into 100 ml bottle
– add 3 g TSB powder
– add 5 ml of Phosphate buffer 100 mM, pH 7.4
– add ddH_2O to a final volume of 50 ml (Note: If melting of the gel in the microwave causes significant loss of liquid it is possible to add more ddH_2O in order to keep a remainder of 50 ml after microwaving.)
– autoclave in 100 ml bottle for 20 min at 121°C, store for short term at room temperature

B. Bacterial Preparation

1. Prepare anaerobic bacteria from kryo-culture
2. Incubate on Columbia Blood-Agar plates in an anaerobic jar for about 48 hours
3. Transfer bacteria into 50 ml falcon containing 10 ml 1x TSB
4. Place Falcon without lid into anaerobic jar, incubate for about 16 hours at 37°C without shaking. (Note: Since different bacterial strains behave differently optimal culture conditions should be optimized. Some bacteria might require shorter incubation times while others might require more time to produce optimal numbers of cells required for the assay.)

C. Modified Radial Diffusion Assay

1. Pre-chill centrifuge at 4°C
2. Place sterile phosphate buffer 10 mM, pH 7.4 on ice
3. Melt Underlay gel in microwave (Note: Mix gently to produce homogenous preparation.)
4. Transfer to 50 ml Falcons with 10-ml aliquots each; maintain gel liquid by placing in 46°C water bath
5. 5 – 20 µl DTT can now be added into liquid gel from the 1 M stock solution. (Note: It is important to use freshly prepared DTT since repeated freezing and thawing impairs its function. Furthermore, some bacterial strains tolerate higher amounts of DTT while others do not. We obtained best results between concentrations of 0.5 and 2 mM (final) whereas 10 mM were mostly lethal. Depending on bacteria used different amounts of DTT might lead to best results.
6. 10 – 20 µl resazurine can be added into liquid gel (final concentration 1 – 2 µg/ ml). (Note: The addition of this redox-indicator is a good opportunity to control reducing conditions in the underlay-gel. A "classical gel" should stain light blue while a reduced gel should look pink.)
7. Centrifuge bacterial overnight-culture in Falcons for 10 minutes at 4°C and 900 x g
8. Wash bacterial sediment in 10 ml ice-cold sterile phosphate buffer 10 mM, pH 7.4
9. Centrifuge bacterial culture for 10 minutes at 4°C and 900 x g
10. Resuspend sediment in 5 ml ice-cold sterile phosphate buffer 10 mM, pH 7.4
11. Determine Optical Density at 620 nm against a buffer control (10 mM phosphate buffer)
12. Adjust to OD_{620nm} = 0.1 and use 150 – 300 µl for assay (Note: The optimal volume of bacterial culture should be determined after the first experiments depending on the bacterial lawn obtained after the over-night incubation of the assay plates. In our hands it was optimal to use 150 µl for *Bifidobacteria* and 300 µl for *Lactobacilli*. For fast-growing bacteria like *E. coli* the user should refer to the classical protocol of Lehrer *et al.* (1))
13. Add bacterial suspension into 10 ml of warm, prepared underlay gel, mix gently. (Note: It is important that the gel is not too hot, which would have an effect on or kill bacterial cells, and not too cold since the gel would solidify. A temperature range between 42°C and 46°C should be fine for mixing with bacterial cells.)
14. Quickly pour the gel containing bacteria into square-petri-dishes, placed on an even surface, and let it solidify for about 30 minutes at room temperature. (Note: By letting a small opening between plate and its lid moisture level may be reduced, which improves punching of the wells later on. For strict anaerobic bacteria solidification time should be minimized).
15. Punch 6×6 wells into the gel and discard gel pieces with a sterile pipette tip or Pasteur-pipette connected to a vacuum pump
16. Fill up to 5 µl of proteins, dissolved in H_2O or 0.01% acetic acid, into the wells. Include

negative control (H_2O or 0.01% acetic acid) and positive control (for example lysozyme, depending on bacteria tested)

17. Let protein solutions diffuse into the gel (short storage at 37°C or room temperature) and place plates into anaerobic jar for 3 hours.
18. Melt overlay gel in microwave, transfer to 50 ml falcons, 10 ml each, maintain at 46°C
19. Add DTT and resazurine, according to corresponding underlay gel
20. Pour 10 ml of nutrient-rich overlay gel onto each underlay gel and let solidify
21. Place plate upside-down in anaerobic incubator until bacterial lawn is clearly visible (16 to 48 hours)

Troubleshooting

1) No bacterial lawn after incubation.
   – Bacteria added to liquid gel might not have been viable. Plate some remaining microliters of the over-night culture on Columbia Blood Agar plates and incubate anaerobically as a control
   – Liquid gel might have been too hot. Reduce maintaining temperature or wait a little bit longer before mixing with bacteria
   – For strict anaerobic bacteria oxygen exposure might have been to long
   – DTT concentration might have been too high

2) Bacterial lawn is too thick, no inhibition zones are visible
   – Use less volume of bacterial culture for underlay gel
   – Reduce incubation time for the primary overnight culture

3) No inhibition zones are visible
   – Inappropriate positive control (for example lysozyme does not work for *Bacteroides vulgatus*)
   – Proteins/ Peptides are not functional under reducing conditions
   – Too much moisture in the underlay gel has pressed protein solution out of the wells

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Associated Publications

This protocol is related to the following articles:
Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1

Author information

Competing financial interests:
The authors filed a patent application on the therapeutic use of reduced human beta-defensin 1.

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VII Discussion

1 Application of Probiotics in Inflammatory Bowel Disease

During the last two decades it has become clear that human antimicrobial peptides are important contributors to innate immune defense against microorganisms. They are produced by nearly all cells that face contact with elements coming from outside the body. On the other hand, microorganisms are no longer considered as generally bad by the public, allowing their application in „functional food“ or as therapeutics.
The concept of probiotics combines these ideas to utilize microorganisms as stimulators of the host immune defense system. While previous studies focused on *in vitro* experiments using cell culture models, we could show for the first time that administration of viable *E. coli* cultures to healthy volunteers induced secretion of hBD-2 into the feces [Möndel et al., 2009]. Thus, defensin induction is one important mechanism of probiotic action *in vivo,* suggesting therapeutic use for patients suffering from a disturbed intestinal barrier function.
During the last years it has become increasingly clear that inflammatory bowel disease (IBD) is a defect of the intestinal barrier function. The two major forms of IBD are Crohn's disease (CD), a chronic disease of the intestine, characterized by transmural inflammation of the gut, and ulcerative colitis (UC), a chronic disease with inflammation limited to the mucosa. While for UC patients inflammation is restricted to the colon, in CD the disease can occur anywhere in the gastro-intestinal tract [Stange et al., 2001].

Analyses of the microbial flora of CD and UC patients revealed that there was a difference in composition, but not in the total number of bacteria in both patients groups in comparison with healthy controls [Sartor, 2008]. Furthermore, in a functional assay Nuding *et al.* could show that antimicrobial activity of biopsy extracts from CD patients against several bacteria was reduced in comparison with healthy controls while extracts from UC patients had an even higher killing potency [Nuding et al., 2007]. From these findings, it is likely that a disregulated mucosal barrier protection mediated by antimicrobial peptides plays a crucial role in these diseases [Wehkamp et al., 2007a]. Indeed, application of the probiotic *E. coli* Nissle 1917 was effective in maintaining remission status in UC patients [Kruis et al., 2004], indicating a beneficial role of these microorganisms to modulate mucosal barrier function. In contrast, application of probiotics to CD patients did only have a limited effect, probably due to a genetic defect in induction of defensin synthesis in CD patients [Wehkamp et al., 2005b, Wehkamp et al., 2007b].

2 Defensins in the Intestine

As probiotic bacteria can modulate defensin production, the same is true *vice versa*. Transgenic mice expressing the enteric human α-defensin HD-5 were found to have changes in their microbial composition, but not in the total number of bacteria [Salzman et al., 2010]. This experiment clearly shows that the intestinal microbial composition is influenced by defensins *in vivo*.
While the functional importance of the constitutively produced Paneth cell defensin HD-5 was clear, the role of hBD-1 has been controversial since it is produced constitutively by intestinal and other epithelia, but its antimicrobial activity was comparably low [Bensch et al., 1995, Tollin et al., 2003, Nuding et al., 2009]. Most strikingly, there was no antibiotic activity observed against Gram-positive anaerobes like *Bifodobacteria* and *Lactobacilli* which make up an important contribution to the intestinal flora. On the contrary, hBD-3, which is induced during inflammation by cytokines and bacteria, shows strong activity against these commensal bacteria [Nuding et al., 2009]. So in the scientific community there has always been the question what hBD-1 really does in the intestine.

We found that by reduction of the three disulphide bridges hBD-1 becomes much more active against *Bifidobacteria, Lactobacilli* and the fungus *C.albicans*. The reduction can be performed either by the surrounding environment or by the constitutively expressed oxido-reductase thioredoxin [Schroeder et al., 2011]. Additional *in vitro* analyses have been performed with oxidized and reduced hBD-1 against *E. coli, P. aeruginosa, Enterococcus fecalis* and herpes simplex virus type I [Scudiero et al., 2010]. For the virus and all bacteria tested, there was no difference between the oxidized and the reduced peptide in their antibacterial or antiviral activity. So the effect of increased antimicrobial activity of linear hBD-1 seems to be restricted to a specific subset of microorganisms. Remarkably, neither we nor others found microorganisms which were killed only by the oxidized peptide but not by the reduced form. Consequently, the reduced peptide is a more potent antimicrobial agent than the oxidized one.

Very recently, an analogue finding has been observed in a mouse model. Mouse Paneth cell α-defensins are termed cryptdins and resemble their human orthologs in functional aspects. Among the cryptdins, Crp4 was found to be the most potent one, indicating an important role in mouse intestinal mucosal defense [Ouellette et al., 1994]. Similar to our approach, oxidized and reduced Crp4 were analyzed on their antimicrobial activity against different commensal and noncommensal bacteria. Both forms were comparably active against noncommensal bacteria including *Salmonella enterica* serovar Typhimurium, *S. aureus, Yersinia enterocolitica* and *Listeria monocytogenes* [Masuda

et al., 2010]. In contrast, when testing commensal bacteria, reduced Crp4 showed increased potency to kill different *Bifidobacteria* and *Lactobacilli, Bacteroides fragilis* and *B. ovatus* as compared to the oxidized peptide. In agreement with experiments performed by Scudiero et al. [Scudiero et al., 2010], both peptides did not differ regarding their antibiotic activity against *Enterococcus faecalis* and *E. faecium.*

Taken together, these experiments show that redox status of defensins is an important regulatory feature of their activity (Figure 1). Both α- and β-defensins can be activated against a subset of commensal bacteria and fungi by opening their disulphide bridges. As this observation was made in men and mice, it seems to be a general mechanism modulating innate mucosal barrier function.

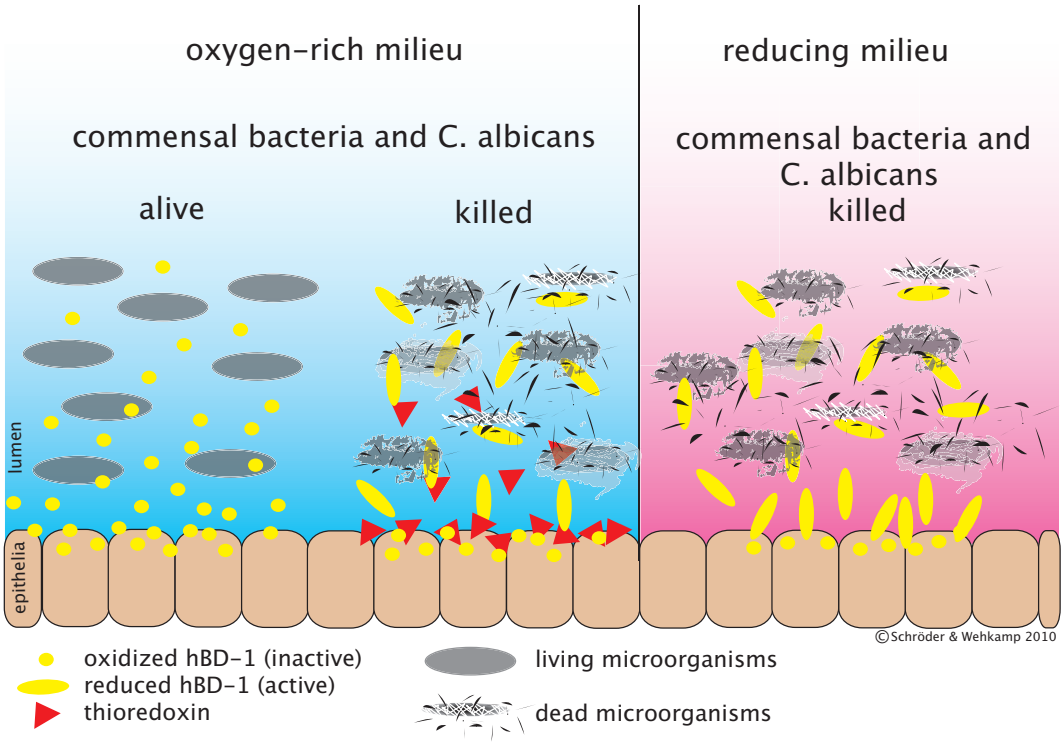

Figure 1: Schematic model of redox-modulation of hBD-1 against *Bifidobacteria, Lactobacilli* and *Candida.* In oxygen-rich milieu epithelial hBD-1 itself is not active against these microorganisms. In the presence of oxygen, the constitutively produced oxido-reductase thioredoxin, which is also expressed by epithelia, is able to reduce hBD-1 and activate it against commensal *Bifidobacteria, Lactobacilli* and *Candida albicans.*
In reducing environment the mileu itself is sufficient to activate hBD-1; reduction of disulphides by thioredoxin is not neccessary.

3 Anaerobic Locations in Health and Disease

From the mentioned results it is evident that a broad spectrum of bacteria is susceptible only to the reduced form of either hBD-1 or Crp4. They include Gram-negative (minority) as well as Gram-positive (majority) bacteria, different genera and species as well as strict and facultative anaerobes. Furthermore, we identified four out of five clinical isolates of the opportunistic pathogenic fungus *C. albicans* as being susceptible to reduced, but not oxidized hBD-1. An obvious similarity of all these microorganisms is that they prefer, or at least tolerate, anaerobic conditions and occur as commensals in the human intestine. Thus it makes perfect sense that the constitutively produced hBD-1 protects the intestinal epithelium against these commensals preferably under reducing conditions.

Besides the intestine, the oral cavity is another warm, moist and partly anaerobic niche providing excellent growth conditions for bacteria and fungi. Oral microbes include different *Streptococci, Lactobacilli, Candida spp.* and others [Dale and Fredericks, 2005]. Accordingly the oral epithelium expresses an arsenal of antimicrobial peptides including α- and β-defensins, the cathelicidin LL-37, histatin, calprotectin and adrenomedullin. When comparing the activity of β-defensins against oral microbes, hBD-3 was the most potent one, followed by hBD-2 and finally hBD-1. The activity of hBD-2 and -3 was stronger against aerobes than against anaerobes [Joly et al., 2004, Dale and Fredericks, 2005]. It has to be emphasized here that all these experiments have been performed under „classical“ conditions in the presence of oxygen. So we hypothesize that hBD-1 would play a much more important role when testing under reducing conditions. Nevertheless, experimental data proving this idea have not been obtained yet. While the ratio of aerobic to anaerobic bacteria in the intestine is between 1:1000 - 1:10000 and in the oral cavity 1:10, the vagina is another body site where anaerobic bacteria dominate over aerobes by a factor of 10 to 100 [Nagy, 2010]. The main constituents here are the Gram-negative *Prevotella* and *Porphyromonas spp.* as well as Gram-positive *Lactobacilli* and *Peptostreptococcus spp.* As hBD-1 has been isolated from vaginal mucosal secretions [Valore et al., 1998] a functional role of this defensin under reducing conditions is plausible here.

Although high numbers of anaerobic bacteria colonize the human body, infections by these microorganisms are rather the exception than the rule. As opportunistic pathogens they do not normally cause infections but are able to invade sterile body sites in immunocompromised hosts or in wounds. In the human body, most anaerobic infections occur in the bloodstream, head and neck, thorax, the intra-abdominal region, the intestine, the female reproductive tract and in skin and soft-tissue. The outcome of these infections can be a mild infection but also severe to life-threatening [Nagy, 2010].

4 hBD-1 and Disease

As stated above, immunocompromised hosts have a higher risk of bacterial and fungal infection. While a weakened immune status can be caused by acute events, also a permanent impairment of the mucosal barrier by genetic factors is possible. For determination of individual susceptibility, analyses of single nucleotide polymorphisms (SNPs) have been established as a powerful technique. Indeed, as part of the constitutive innate immune defense, several SNPs in the hBD-1 encoding *DEFB1* promotor have been associated with increased risk of diseases.

In the oral cavity candidiasis is the most common fungal infection in humans [Samaranayake, 1990]. By investigating diabetic and nondiabetic subjects, a SNP at position -44 (rs1800972, C-44G) was associated with lower *Candida* carriage levels and had thus a positive effect in both study groups [Jurevic et al., 2003]. The SNP is located in the 5'-untranslated region of *DEFB1* and does consequently not cause an amino acid exchange. Moreover, the reason for the advantageous effect was the formation of a putative binding site for the transcription factor NF-κB, resulting in enhanced expression of *DEFB1* [Prado-Montes de Oca et al., 2007]. Nevertheless, since hBD-1 was reported to have only minor anti-fungal activity against different *Candida* strains or to be effective only in unphysiological high concentrations [Feng et al., 2005, Dale and Fredericks, 2005], the functional consequence of this SNP regarding *Candida* infection was unclear. These findings can now be regarded from a different perspective as we now found that reduced hBD-1 is much more active against *C.albicans* strains than the oxidized peptide [Schroeder et al., 2011]. So providing the existence of reduced hBD-1 in the oral cavity, which is speculative but likely, the identified SNP might cause an increased production of hBD-1, leading to more reduced hBD-1, which is able to restrict the growth of *Candida spp.* in the oral cavity.

Two additional infectious diseases in the oral cavity are caries and periodontitis. The underlying mechanisms of both diseases are not fully understood yet, but for both a genetic influence of about 50% is discussed [Michalowicz et al., 2000, Bretz et al., 2005]. As in both cases microbial infections are the causative factors, genetic analyses of innate defense molecules are reasonable. While genetic associations of *DEFB1* and periodontitis are contradictory [Wohlfahrt et al., 2006, Ozturk et al., 2010, Schaefer et al., 2010], an association between *DEFB1* and caries seems to exist [Ozturk et al., 2010]. The SNP rs11362 (G-20A) in the promotor region correlated with higher susceptibility to caries whereas rs179946 (G-52A) was associated with lower risk.

Correspondingly to *Candida* infections, the functional consequence of the *DEFB1*-SNP

in relation with caries was unclear since hBD-1 was found to have the least antimicrobial activity against cariogenic bacteria in comparison with other antimicrobial peptides [Ouhara et al., 2005]. Although the antibiotic activity against *Streptococci* and other oral bacteria remains to be elucidated, we found that only reduced, but not oxidized hBD-1 kills *Lactobacilli,* which are also part of the oral flora [Schroeder et al., 2011]. So again, the functional consequence of this caries-associated SNP might be mediated by reduced hBD-1 and not the oxidized peptide, which has been studied in previous experiments.

Decreased α-defensin levels have already been related with the pathophysiology of ileal Crohn's disease (CD) [Wehkamp et al., 2005b]. Furthermore, it was suggested that different regional localizations in the disease might be characterized by different defensin expression profiles. Accordingly, the *DEFB1* gene was analyzed for SNPs associated with CD. As already reported for caries, rs11362 (G-20A) in the promotor region of *DEFB1* was associated with a higher risk of developing colonic, but not ileocolonic or ileal CD. A second SNP, rs1800972 (C-44G), which has been found protective for *Candida* infection (see above), seemed also beneficial in CD since a higher frequency was observed in the control group in comparison with CD patients [Kocsis et al., 2008, Peyrin-Biroulet et al., 2010]. Taken together, α- and β-defensins seem to play an important role in ileal as well as colonic Crohn‘s disease and highlight the relationship between intestinal microbes and mucosal antibacterial defense.

5 Structural and Functional Aspects of Human Beta-Defensins

Beta-defensins share a common motif of three disulphide-bridges, formed by Cys1-Cys5, Cys2-Cys4 and Cys3-Cys6 [Tang and Selsted, 1993]. The carboxy-terminal part forms three β-strands whereas the amino-terminal region is characterized by a short α-helical domain [Hoover et al., 2001]. Human β-defensins show a cationic charge, differing between +4 for hBD-1, +6 for hBD-2 and +11 for hBD-3. Furthermore, antimicrobial activity of hBD-1 and -2 is inhibited by high concentrations of NaCl while hBD-3 remains active [Goldman et al., 1997, Tomita et al., 2000, Harder et al., 2001]. So regarding these characteristics, several studies have been performed to dissect those features being essential for the antimicrobial action.

Due to the high conservation of disulphide bonds they have long been thought to be essential for antimicrobial activity. However, it was demonstrated that a variant of hBD-3, in which all cysteine residues have been replaced by α-aminobutyric acid, retains its antimicrobial activity, but loses only its chemotactic activity [Wu et al., 2003]. Furthermore, the group found that hBD-3 did not preferentially fold into the „native“

disulphide pairing under different oxidative conditions *in vitro*. Moreover, Taylor *et al.* found that hBD-3 with all cysteine residues blocked with iodoacetamide was similarly active as the disulphide-bridged variant [Taylor et al., 2008]. Consequently, at least for hBD-3, cysteine bridges seem not essential for antimicrobial activity, rather might they have a protective effect against proteolytic digestion.

These findings are in line with our results for hBD-1 as we found even increased activity of the linear peptide without any disulphide bridges. In our experiments replacing cysteine residues by alanine or serine abolished antimicrobial activity against *Bifidobacteria* and *Lactobacilli* but not against *E. coli*. This clearly suggests that there is not one single characteristic but that one molecule can hold different properties required for antibiotic activity against different bacteria.

The hypothesis was further supported as we found that an isolated carboxy-terminal heptapeptide of hBD-1 showed antimicrobial activity against *Bifidobacteria* but not *E. coli* and that this effect was mediated by free cysteines. In contrast, antimicrobial activity against *E. coli* was abolished by amino-terminal truncation since a 33-amino acid carboxy-terminal peptide of hBD-1 did not show any activity [Valore et al., 1998]. Also, by generating single-site variants of hBD-1, Pazgier *et al.* found that when mutating specific amino acids in the amino-terminus or cationic amino acids in the carboxy-terminus, anti-*E. coli*-activity was either abolished or strongly reduced [Pazgier et al., 2007]. Accordingly, both terminal domains of hBD-1 seem to carry elements exhibiting antimicrobial function against *E. coli,* indicating important functions of the α-helical domain as well as the positive charge (Figure 2).

It has often been observed that high concentrations of NaCl inhibit function of antimicrobial peptides. An explanation was that electrostatic interactions are required for the initial contact between the peptide and the bacterial membrane. So it was assumed that most antimicrobial peptides are only functional in low-salt environment. This general hypothesis was challenged by Dorschner *et al.*, who could show that medium containing bicarbonate could compensate the inhibitory effect of NaCl on the cathelicidin LL-37 [Dorschner et al., 2006]. Also for the β-defensin hBD-2 and the mouse α-defensin Cryptdin-4 it was shown that antimicrobial activity was highly increased in medium containing bicarbonate in comparison with medium lacking this component. Hence, the surrounding environment is able to directly influence the antimicrobial capacity of several antimicrobial peptides and has to be considered when performing antimicrobial assays.

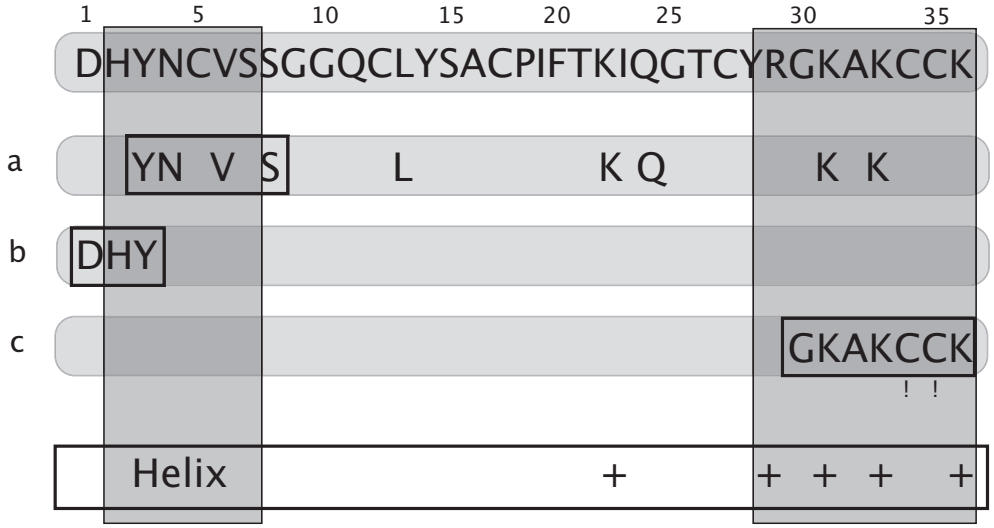

Figure 2: Schematic model of hBD-1 with regions being important for its antimicrobial activity. Letters indicate one-letter amino acid code of wild-type hBD-1 (upper row) and amino acid residues crucial for antimicrobial activity of hBD-1 (rows a-c). a, Results from Pazgier *et al.* [Pazgier et al., 2007] testing antimicrobial activity of hBD-1 single-site mutants against *E. coli*. b, Results from Valore *et al.* [Valore et al., 1998] testing an amino-terminal truncated variant of wild-type hBD-1 against *E. coli*. c, Our own results [Schroeder et al., 2011] testing the isolated carboxy-terminus of hBD-1 and cysteine-mutated variants against *Bifidobacterium adolescentis*. Exclamation mark indicates important functional role of cysteines within the carboxy-terminal fragment. Bottom row summarizes findings, highlighting importance of amino-terminal helix and positive charge of the carboxy-terminus of hBD-1.

6 Conclusion

Antimicrobial peptides are important contributors to innate host defense against microbes. They do not only kill invading microbes but can also modulate the composition of the intestinal flora. On the other hand, commensal and probiotic bacteria are able to modulate the host defense, including the production of antimicrobial peptides. Thus, a well-balanced relationship between gut bacteria and host defense mechanisms is crucial to maintain a healthy status of the intestine. The use of probiotics to modulate innate immune functions is a consequent therapeutic concept exploiting this idea and providing an alternative therapy for the treatment of patients.

Redox-modulation of innate immune functions has not been investigated in detail yet. Although it has been shown that the mammalian enzyme thioredoxin-reductase

was able to alter cytolytic activity of granulysin, an antibacterial protein of cytolytic cells [Björkhem-Bergman et al., 2004], the link to antibacterial function has not been drawn. Since bacterial metabolism in the intestine produces a strongly reducing environment, it was possible that this condition influences bacterial defense. We found that not only reducing environment but also the oxido-reductase thioredoxin is able to reduce hBD-1. Thus, besides passive reduction by the intestinal environment, active enzymatic reduction in oxygen-rich milieu can activate this human defensin. These findings will contribute novel aspects to the understanding of the complex regulation of innate immunity and will hopefully help to adequately treat patients suffering from disturbances in innate antibacterial functions in the future.

VIII References

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IX Acknowledgement

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Also I want to thank him for the great discussions we had in company with tasty wines. They were the origin of several good ideas, not only for the lab.

Furthermore, I want to thank Michelle Katajew for her excellent technical assistance. Her humor and constant happiness were additional motivation when experiments did not work as they were supposed to.
Of course, I also want to mention all the others members of the Wehkamp-Group and Stange-Group for their continuous support and helpful discussions as well as enjoyable coffee breaks during the afternoon.

My sincere thanks is given to Eduard Stange for his helpful discussions and good advices on my project, Andreas Peschel for interesting discussions and supervising my thesis, and Matthias Schwab for giving me the opportunity to complete my PhD-thesis in his institute.

I also want to thank all our partners and friends for successful collaborations and the Robert-Bosch-Foundation for financial support.

My special thanks goes to my family and especially my parents. With their discussions and continuous support in any situation during my thesis they certainly contributed to its successful completion.

X Declarations

1 Declaration of individual contributions to the included manuscripts

„Probiotic *E. coli* treatment mediates antimicrobial human beta-defensin synthesis and fecal excretion in humans", Möndel M, Schroeder BO, Zimmermann K, Huber H, Nuding S, Beisner J, Fellermann K, Stange EF, Wehkamp J. *Mucosal Immunol.* 2009 Mar;2(2):166-72

- Miriam Möndel
  - generation of data leading to figures 1 a,b,c and 2 a,b,c
  - preparation of figures 1 and 2
  - preparation of the manuscript
- Björn Ole Schröder
  - establishing antimicrobial activity measurement with the radial diffusion assay in the working group
  - generation of data leading to figure 3 a,b,c, and preparation of the figures
  - involved in data discussions and preparation of the manuscript
- Klaus Zimmermann
  - involved in the design of the study and provided samples
- Hans Huber
  - involved in the design of the study and provided samples
- Sabine Nuding
  - involved in cultivation of bacterial strains
- Julia Beisner
  - involved in data discussions and preparation of the manuscript
- Klaus Fellermann
  - involved in data discussions
- Eduard F. Stange

  - involved in design of the study and data discussion

- Jan Wehkamp
  - involved in design of the study
  - involved in data analysis and discussion
  - preparation of the manuscript

My personal part for this manuscript involved a functional assay, which I established in the laboratory of PD Dr. Jan Wehkamp, to test the effect of antimicrobial peptides against different probiotic *E.coli* strains. I found that probiotic bacteria, which are able to induce human β-defensin-2 *in vitro* or *in vivo*, are not resistant to this antimicrobial peptide. Consequently, these probiotics are not able to colonize the human intestine, thereby maybe explaining why probiotics need to be administered continuously to develop their beneficial effect.

For the manuscript I prepared the figure generated from my data, wrote the methods-part of this experiment and was involved in data discussions of the whole manuscript.

„Reduction of disulphide bonds unmasks potent antimicrobial activity of human β-defensin 1", Schroeder BO, Wu Z, Nuding S, Groscurth S, Marcinowski M, Beisner J, Buchner J, Schaller M, Stange EF, Wehkamp J. *Nature*, 469(7330):419-423

- Björn Ole Schröder
  - decisively involved in initial hypothesis generation
  - decisively involved in planning, establishing and accomplishment of experimental setup
  - generation of data (antimicrobial activity assays, HPLC analyses, MALDI-MS and TRX assays), leading to figures 1 a,b,c; 2 a,b; 3 a,b,d, 4 a, S1, S2, S3, S4, S5, S6
  - performing bacterial preparations and initial fixation for transmission electron microscopy (figure 3f)
  - preparing material for generation of data for CD-spectroscopy (figure 2d) and flow cytometric antimicrobial measurements (figures 3c and e)
  - decisively involved in all data discussions
  - coordination of cooperation partners
  - preparation of all figures
  - writing of the manuscript

- Zhihong Wu
  - generated and purified recombinant hBD-1, its ^{15}N-labeled forms and recombinant alanine/ serine-hBD-1-variants
  - generated alkylated-hBD-1-affinity columns
  - affinity-purified the red./alk.hBD-1-antibody

- Sabine Nuding
  - performed flow cytometric analyses (figures 3c and 3e)

- Sandra Groscurth
  - performed NMR spectroscopy and analysed data (figure 2c)

- Moritz Marcinowski
  - performed CD spectroscopy and analyzed data (figure 2d)

- Johannes Buchner
  - analysed data from CD spectroscopy

- Julia Beisner
  - performed RT-PCR and analyzed data (figure S8)
- Martin Schaller
  - responsibility for transmission electron microscopy (figure 3f)
- Eduard F. Stange
  - involved in critical data discussions and the final version of the manuscript
- Jan Wehkamp
  - supervised the project
  - decisively involved in initial hypothesis generation
  - decisively involved in all data discussions and analyses
  - manuscript preparation and its final version

My personal part for this manuscript involved performance of all initial experiments leading to the conclusion that human β-defensin 1 (hBD-1) becomes antimicrobially active against anaerobic, Gram-positive bacteria of the human normal flora only under reducing conditions, that thioredoxin is able to catalyse reduction of hBD-1 and that biochemical parameter differ between oxidized and reduced hBD-1. From these results me and PD Dr. Jan Wehkamp designed further experiments strengthening our generated hypothesis. Both of us were involved in recruiting co-operation partners and planning of experiments.

The results reported in this manuscript contribute novel insights into the field of innate immunity. They describe for the first time that redox-regulation can activate an antimicrobial peptide. This modulation can be accomplished either by chemical means through the environment or enzymatially by an oxido-reductase.

For this manuscript I wrote major text parts, generated figures and was in charge of the final version.

„Measurement of antimicrobial activity under reducing conditions in a modified radial diffusion assay", Schroeder BO and Wehkamp J. *Protocol Exchange* 2011, DOI: 10.1038/protex2010.204

- Björn Ole Schröder
  - decisively involved in planning, establishing and accomplishment of experimental setup
  - testing the modified assay under different conditions and with different bacteria
  - writing the protocol

- Jan Wehkamp
  - supervised the project
  - decisively involved in initial hypothesis generation

My personal part for this protocol was the initial focus on the problem that intestinal microbes and defensins have not been analysed under reducing conditions as they occur in the intestine. The classical assays focused on measurements performed under standard conditions, requiring a modification of established protocols to use them for anaerobic bacteria. Therefore I developed a modified radial diffusion assay by the addition of a chemical reducing agent and bacterial incubation under anaerobic atmosphere and provided a detailed protocol.

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TÜBINGEN

Mathematisch-Naturwissenschaftliche Fakultät

Erklärungen gem. §§ 5 Abs. 2 und 6 Abs. 2 der Promotionsordnung der Mathematisch-Naturwissenschaftlichen Fakultät zum Antrag auf Zulassung zum Promotionsverfahren

Bitte nur die zutreffenden Erklärungen ankreuzen, Nichtzutreffendes streichen

☐ Ich erkläre hiermit, dass ich die zur Promotion eingereichte Arbeit selbständig verfasst, nur die angegebenen Quellen und Hilfsmittel benutzt und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe.

☐ Es hat noch kein Promotionsverfahren oder entsprechendes Prüfungsverfahren stattgefunden (bisherige, abgebrochene oder abgeschlossene Verfahren).
(Gegebenenfalls eine Erklärung beifügen, wann und wo, in welchem Fach und mit welchem Ergebnis.)

☐ Die vorgelegte Dissertation ist noch nicht ganz oder teilweise veröffentlicht worden *(gemeint ist: als Dissertation)* und sie ist noch nicht ganz oder teilweise als Dissertation oder sonstige Prüfungsarbeit eingereicht worden.
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(Gegebenenfalls eine Erklärung über strafrechtliche Verurteilungen, Disziplinar-maßnahmen und anhängige Straf- und Disziplinarverfahren, soweit die Auskunftspflicht nicht durch § 51 des Bundeszentralregistergesetzes ausgeschlossen ist, beifügen.)

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