JOURNAL OF ANIMAL SCIENCE ADVANCES

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J Anim Sci Adv 2011, 1(1): 1-11

Online version is available on: www.grjournals.com

A Review

Antimicrobial Peptides and its Role in Gastro-Intestinal Diseases: a Review

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Abstract

The extensive use of antibiotics to treat human or animal infections is the cause of an alarming increase in antibiotic resistance among Gram-negative, Gram-positive and fungal pathogens. Therefore, the search continues for new antibiotics that are active in vivo, are fast acting and broad-spectrum, do not induce bacterial resistance and have limited side effects. Synthetic congeners of natural antimicrobial peptides are good candidates. One component of host defence at mucosal surfaces seems to be epithelial derived anti-microbial peptides (AMP). This review summarises the diversity of anti-microbial peptides and their role in gastro-intestinal diseases.

Key words: Anti microbial proteins, defensins, cathelicidins, gastro-intestinal diseases

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Received on: 13 Oct 2011

Revised on: 17 Oct 2011

Accepted on: 20 Oct 2011

Online Published on: 1 Nov 2011

Introduction

The survival of a multicellular organism in a world laden with microorganisms depends on a network of host defense mechanisms, involving several levels of interacting systems. The initial contact of pathogenic microorganisms with the host usually takes place at inner or outer body surfaces (Bals, 2000).

Several possible consequences result from the contact of microorganisms with host tissue:

1. The invading microorganisms are eliminated by innate host defense mechanisms without an inflammatory response or the activation of adaptive immunity. This normally happens in the lower respiratory tract and occurs within a short time.
2. The microbe outgrows the innate host defense. As a consequence, effector mechanisms of the innate immune system are upregulated and have direct antimicrobial activity and mediator function to attract inflammatory cells and cells of the adaptive immune system. These mechanisms finally result in the elimination of the microorganisms. In this scenario, the innate host defense keeps the doubling time of the microorganisms long enough to avoid an overwhelming of the immune system (Figure 1).
3. The microorganism outgrows innate and adaptive immunity. Together with a strong inflammatory response this situation leads to the death of the host.
4. Microorganisms with specific physiological adaptations can colonize the airways for a long time. In this case the activities of the immune system are insufficient to eliminate the invader.

Antimicrobial Peptides

Antimicrobial peptides are evolutionarily ancient weapons. Their widespread distribution throughout the animal and plant kingdoms suggests that antimicrobial peptides have served a fundamental role in the successful evolution of complex multicellular organisms. Despite their ancient lineage, antimicrobial peptides have remained effective defensive weapons, confounding the general belief that bacteria, fungi and viruses can and will develop resistance to any conceivable substance. Antimicrobial peptides target a previously under-appreciated `microbial Achilles heel', a design feature of the microbial cellular membrane that distinguishes broad species of microbes from multicellular plants and animals (Zasloff, 2002).

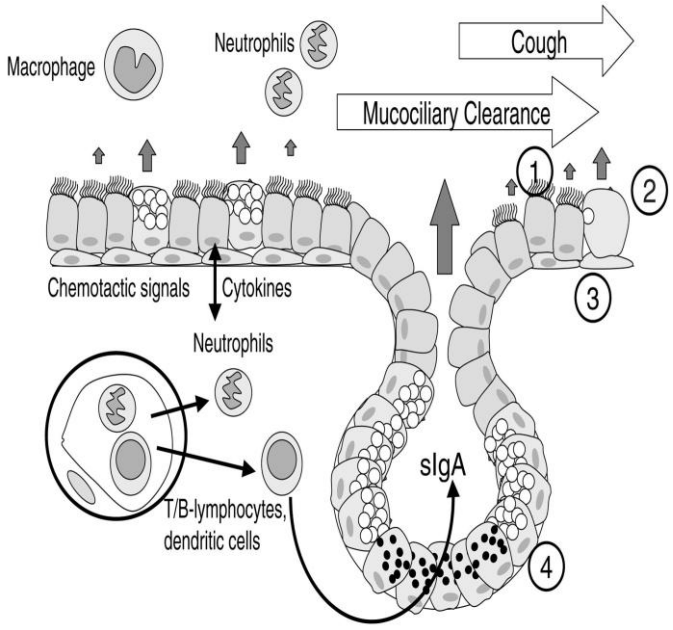

Fig. 1: Host defence mechanisms of the respiratory epithelium. Cells of the respiratory epithelium (1=ciliated cell, 2=goblet cell, 3=basal cell, 4=mucous and serous gland cells) are not passive bystanders of an inflammatory process but secrete effector molecules including antimicrobial peptides. The adaptive immune response with its T cells and B cells (sIgA=secretory immunoglobulin A) is triggered by innate mechanism (Wilmott et al., 1998).

Recent studies have shown that antimicrobial peptides are expressed in wide array human tissues and are involved in the host defence. Antimicrobial peptides are effector molecules of innate immunity with direct antimicrobial and mediator function. They have an important role in all scenarios described above by providing an initial host defense mechanism (Bals, 2000).

Diversity of Anti-Microbial Peptides (AMPs)

A large variety of antimicrobial peptides is found in animals and is expressed in many tissues, polymorphonuclear leukocytes, macrophages and mucosal epithelial cells (Table 1). These peptides are broad-spectrum with potent antimicrobial activity against both human and veterinary pathogens.

Antimicrobial peptides are conserved in their structure, function and mechanisms of action, and thus it is attractive to speculate that synthetic antimicrobial peptides or their congeners might be used to prevent or treat infections (Brogden et al., 2003).

The diversity of antimicrobial peptides discovered so far has been broadly categorized on the basis of their secondary structure. The fundamental structural principle underlying all classes is the ability of the molecule to adopt a shape in which clusters of hydrophobic and cationic amino acids are spatially organized in discrete sectors of the molecule (amphipathic design).

Linear peptides, such as the silk moth's cecropin (Steiner et al., 1981) and the African clawed frog's magainin (Zasloff, 1987), adopt this organization only when they enter a membrane, whereupon they assume an amphipathic α-helical secondary structure (Bechinger et al., 1993). Frog species of the genus Rana modify this design by adding a single loop formed by a disulphide bond at the carboxy end (Simmaco et al., 1998). Peptides such as bactenecin (Romeo et al., 1998) and defensins (Selsted et al., 1985) use a relatively rigid anti-parallel β-sheet constrained by disulphide bonds as the framework, around which segregated patches of cationic and hydrophobic residues are organized.

All antimicrobial peptides are derived from larger precursors, including a signal sequence. Post-translational modifications include proteolytic processing, and in some cases glycosylation (Bulet et al., 1993), carboxy-terminal amidation and amino-acid isomerisation (Simmaco et al., 1998), and halogenations (Shinnar, 1996). A rather complex modification involves the cyclization of two short peptides leading to the fully circular θ-defensin isolated from neutrophils of Rhesus (macaque) monkeys (Tang et al., 1999). Some peptides are derived by proteolysis from larger proteins, such as buforin II from histone 2A (Kim et al., 2000) and lactoferricin from lactoferrin (Ulvatne and Vorland, 2001).

The diversity of sequences is such that the same peptide sequence is rarely recovered from two different species of animal, even those closely related, be they insects, frogs or mammals (exceptions include peptides cleaved from highly conserved proteins, such as buforin II). However, both within the antimicrobial peptides from a single species, and even between certain classes of different peptides from diverse species (Simmaco et al., 1998), significant conservation of amino-acid sequences can be recognized in the preproregion of the precursor molecules; the design suggests that constraints exist on the sequences involved in the translation, secretion or intracellular trafficking of this class of membrane-disruptive peptide. This feature is dramatically illustrated by the cathelicidins (Zanetti et al., 2000).

Role in Gastro-Intestinal Diseases (Figure 2):

Esophagus

Microbial infections of the esophagus represent a rather uncommon event in healthy individuals. Nevertheless, the immunocompromised host quite frequently suffers from infections with *C. albicans*, CMV or HSV, while bacterial infections remain rare. Despite a high expression of numerous antimicrobial peptides, assays with oesophageal tissue showed a weakened potency to kill *C. albicans* (Hosaka et al., 2008), explaining the susceptibility of esophageal tissues to infections with this yeast. Kiehne et al. (2005) observed that Candida colonization induced a high expression of a subset of antimicrobial peptides, especially hBD-2 and hBD-3. The polymorphonuclear leukocytes (PMNs) reinforces the defensin expression in the epithelium. Individuals suffering from neutropenia lack this stimulus for the expression of epithelial antimicrobial peptides and thus, a pathophysiologic explanation for the high incidence of *Candida esophagitis* and *Candida*-related deaths in neutropenic patients can be proposed (Steubesand et al., 2009). Furthermore, even in esophageal reflux disease, an induction of β-defensin expression (hBD-2 and hBD-3) could be found, although to a minor degree (Kiehne et al., 2005).

Table 1: Classes of antimicrobial peptides

Class of peptide	General antimicrobial activity
Anionic peptides	
➢ Rich in aspartic and glutamic acids	Gram-negative, Grampositive bacteria (Brogden et al., 1996, 1997)
Cationic peptides	
➢ Linear and amphipathic helical molecules Seminal plasmin ➢ Myeloid antimicrobial peptides (PMAP, SMAP, BMAP) ➢ Porcine cecropin P1	Broad-spectrum (Sitaram et al., 1997) Broad-spectrum (Travis et al., 2000 Zhang et al., 2000)
➢ eCATH-1, eCATH-2, eCATH-3	Gram-negative, some Gram positive bacteria (Lee et al., 1989, Gazit et al., 1995)
➢ Linear peptides rich in proline Bac5 and Bac7	Broad-spectrum (Scocchi et al., 1999, Skerlavaj et al., 2001)
➢ PR-39	Gram-negative bacteria (Nicolas and Mor, 1995) but can be broad spectrum (Shamova et al., 1999)
➢ Indolicidin (rich in tryptophan)	Gram-negative bacteria (Nicolas and Mor, 1995, Shi et al., 1996)
➢ Prophenin (rich in proline and phenylalanine)	Gram-negative, Gram-positive bacteria (Selsted et al., 1992) Gram-negative bacteria (Harwig et al., 1995)
➢ Cysteine-stabilized sheet molecules α−, β−Defensins	Broad-spectrum (Diamond et al., 1991, Selsted et al., 1993, Lehre et al., 1993, Nicolas and Mor, 1995,)
➢ Rhesus theta defensin-1 (RTD-1) ➢ Protegrins	Broad-spectrum (Tang et al., 1999) Broad-spectrum (Zhang et al., 2000, Kokryakov et al., 1993)

Stomach

The high prevalence and morbidity resulting from colonization by the Gram-negative bacterium *Helicobacter pylori* has emphasised in the role of antimicrobial peptides in the stomach. Though the mucosa exhibits a strong inflammatory response against *H. pylori* bacteria, clearance of the pathogen is unsuccessful in many cases.

Helicobacter infection is known to lead to a significant induction of hBD-2, while the defensin gene expression caused by non-*Helicobacter gastritis* is much less pronounced (Wehkamp et al., 2003b). In a recent study, it was demonstrated that *H. pylori* induces gastric epithelial cells to upregulate the endogenous production of hBD-2 (Grubman et al., 2010), furthermore this is mediated by the cytosolic pattern recognition receptor NOD1 (nucleotidebinding oligomerization domain 1). In the chronic *H. pylori* induced gastritis, intestinal metaplasia (replacement of the normal mucosa by a columnar epithelium with characteristics of intestinal epithelia, e.g., goblet cells, Paneth cells),

is a frequent event. A high HD-5 expression has been observed by Shen et al. (2005), suggesting that in intestinal metaplasia, where α-defensin producing Paneth cells are present, this metaplastic change may strengthen the antibacterial response via production of HD-5. Aside from the defensins, *H. pylori* is reported to induce Cathelicidin LL-37 in gastric epithelial cells (Hase et al., 2003).

Biliary Tree

10% to 20% of adult populations in developed countries suffer from cholelithiasis (gallstones). Though more than 80% of patients remain asymptomatic, infections of the gallbladder or the biliary tree are common diseases, which require antibiotic treatment in many cases. The normal sterility of bile is maintained by the bactericidal effect of bile salts and immunoglobulin A, and a notable expression of hBD-1 and hBD-2 is noted in biliary tract epithelium and in the liver (Harada et al., 2004). Similarly to other anatomic sites, hBD-1 expression is constitutive, while in the large intrahepatic bile ducts, hBD-2 was induced by biliary obstruction or hepatolithiasis, where these peptides contribute to the local antimicrobial defense.

Patients with primary sclerosing cholangitis, especially following endoscopic manipulation, suffer from frequent bouts of infection. The observed lack of induction of hBD-2 and possibly other antibacterial peptides could be implicated in the disease mechanism. D'Aldebert et al. found an intense immunostaining for cathelicidin in human liver biliary epithelium, and showed that bile salts (chenodeoxycholic acid and ursodeoxycholic acid), which also possess intrinsic bactericidal properties, induce cathelicidin expression through different nuclear receptors. It could be concluded that either farnesoid X receptor or vitamin D receptor is involved and upon activation, promote cathelicidin expression in the biliary tract (2000).

Intestine

The microbial colonization of the lumen increases along the intestine, though the number of bacteria is still very low from the duodenum to the proximal ileum. The distal ileum contains up to 10^8 primarily anaerobic bacteria per gram of luminal contents (Berg, 1996), whereas up to 10^{11}–10^{12} bacteria per gram colonize the colon. The bacterial microflora is crucial for the maintenance of human health and the development of the mucosal immune system. Moreover, its contribution to the pathogenesis of the chronic idiopathic inflammatory bowel diseases is widely acknowledged. In these entities, a shift in microbial composition towards less Bacteroidetes and more Firmicutes (Bacilli) has already been observed.

The scarcity of bacteria in the ileum can be attributed to the hostile environment created by acid, bile, and pancreatic secretions as well as to the phasic propulsive motility of this part of the gut (Guarner and Malagelada, 2003). On the other hand, adaptive and innate branches of the immune system contribute as well to maintain a low microbial density. Paneth cells, which are a characteristic epithelial lineage of the small intestine and localize to the bottom of the intestinal crypts, secrete α-defensins in response to bacterial antigens including lipopolysaccharide and muramyl dipeptide (Ayabe et al., 2000). A constitutive expression of exceptionally high levels of α-defensins HD-5 and HD-6 could be demonstrated in human small intestines (Wehkamp et al., 2006). Interestingly, expression of HD-5 exceeds expression levels of other AMPs produced by the Paneth cell (lysozyme and sPLa2) by a factor up to 100 (Wehkamp et al., 2005).

In studies with knockout animals, intestinal extracts from mice deficient for the cryptdin-processing enzyme matrilysin and thus lacking functional mature mouse α-defensins (the mouse homologs to defensins are called cryptdins), show decreased antimicrobial activity (Wilson et al., 1999), and these mice are more susceptible to orally administered bacterial pathogens as well as to DSSinduced colitis. The human α-defensin HD-5 transgenic mice are resistant to infection from orally administered *S. typhimurium* (Bevins et al., 2002). *S. typhimurium* can downregulate HD-5 expression via a type-3 secretion system. In addition, the Paneth cell defensins can shape the composition of microbial species present in the small intestinal lumen, while the total number of bacteria remains unaffected (Wehkamp et al., 2005, Salzman et al., 2010).

In a mouse model with transgenic expression of DEFA5, Salzman et al. demonstrated that the

colonization with segmented filamentous bacteria (termed SFB, from the genus Clostridia) was dramatically decreased when the mice produced the human α-defensins HD-5. Interestingly in this context is the fact that mice colonized with SFB were shown to be more resistant to infection with *Citrobacter rodentium*, a close relative to the well-known *E. coli*. Paneth cells also exert control over intestinal barrier penetration by commensals and pathogenic bacteria (Vaishnava et al., 2008), apparently mediated by TLR (Toll-like receptor) recognition and a subsequent induction of antimicrobial peptides. The signalling was shown to be dependent on the expression of the MyD88 adaptor protein inside the Paneth cell. The release of Paneth cell secretions into the intestinal lumen thus follows stimulation of pattern recognition receptors (PRR, e.g., Toll-like receptors, NOD-like receptors, RIGI- like receptors) with pathogen-associated molecular patterns, termed PAMPs, which are provided by resident and pathogenic bacteria. Corroborating the concept of a host driven composition of the microbial flora, Petnicki-Ocweija et al. showed that in the mouse model, the bactericidal activity of crypt secretions of the terminal ileum was severely compromised by NOD2 deletion, and that NOD2 expression depends on the presence of commensal bacteria (2009).

Other mechanisms leading to diminished Paneth cell α- defensin function in patients with ileal CD are even more complex. The differentiation of crypt stem cells into mature secretory cells is governed by the so-called Wnt pathway. Disruption of this signaling cascade leads to impaired Paneth cell differentiation, an event which manifests as a disordered localization of these cells within the crypts (Pinto and Clevers, 2005). One of the Wnt signaling transcription factors, TCF-4, shows a reduced expression in patients with ileal CD, independent of the extent of inflammation in the biopsies (Wehkamp et al., 2007). As the Paneth cell's foremost activity is the secretion of huge amounts of defensins, an attractive interrelation can be proposed for the decreased defensin functionality in Crohn's disease and the mutation in ATG16L1 (ATG16L1 protein is involved in autophagy, a process which is essentially responsible for the degradation of intracellular structures, but also mediates degradation of phagocytosed or invasive bacteria). Moreover, Cadwell et al. provided evidence that in ATG16L1 knockout mice, granule exocytosis is abnormal (2008, 2009), and recently it has been shown that recruitment of ATG16L1 to the site of bacterial entry in the plasma membrane is dependent on activation of NOD2 by bacteria (Travassos et al., 2010).

Aside from these numerous associations between Paneth cells and defensins in ileal inflammation of CD, the involvement of antimicrobial peptides in active celiac disease, an inflammatory disorder of the small intestine as well, has been investigated. Vordenbaumen et al. assessed a panel of β-Defensins (hBD-1 to 4) and α-defensins (HD5-6) in duodenal biopsies of pediatric celiac disease patients and found a decreased hBD-1 and hBD-4 expression, while the remainder of the antimicrobial peptides did not show differences to healthy controls (Vordenbaumen et al., 2010). The pathophysiologic significance of this expression pattern has yet to be determined.

Colon

The composition of the extensive colonic microflora has been characterized more thoroughly by sequencing of 16S ribosomal DNA of fecal contents. Among the approximately 400 different species harboured by the human colon, two phyla clearly dominate: anaerobic Gram-positive firmicutes (*Clostridium, Bacillus, Lactobacillus*) and anaerobic Gram-negative bacteroidetes (*Bacteroides, Flavobacteria*) (Eckburg et al., 2005).

The first defensin identified in the human large bowel was the β-defensin hBD-1, and in the noninflamed colon, it is the major β-defensin. The peroxisome proliferator-activated receptor (PPAR) gamma is playing a major role in the constitutive expression of hBD- 1 (Peyrin-Biroulet et al., 2010) and reduction of hBD-1 expression in inflamed mucosa of IBD patients was observed (Wehkamp et al., 2003a). Strongly supporting an important role of hBD-1 in colonic IBD, Kocsis et al. have reported a genetic association of hBD- 1 SNPs with colonic Crohn's disease in a Hungarian cohort (Kocsis et al., 2008). These findings challenge the perspective that reduced defensin expression is merely the result

of epithelial loss in inflammatory states (Arijs et al., 2009).

In the healthy colon, hBD-2 and hBD-3 are absent and only induced during inflammation or infection. Stimuli for hBD-2 induction comprise both bacteria and cytokines, like *Campylobacter jejuni* (Zilbauer et al., 2005) or the bacterial component flagellin from the *E. coli* strain Nissle 1917, which is used as probiotic in the maintenance treatment of ulcerative colitis (Kruis et al., 2004). On the cytokine level, the induction is mediated by proinflammatory cytokines such as IL-1β (through NF-κB-dependent and AP-1-dependent pathways) and TNF-α (Schroder and Harder, 1999). Different defensin mRNA expression in the different forms of inflammatory bowel diseases has been noted, as in patients with ulcerative colitis, hBD-2 and hBD-3 are strongly induced in the event of inflammation. In comparison, the induction is attenuated in Crohn's disease (Wehkamp et al., 2002, 2003a, Aldhous et al., 2009) and the colonic mucosa of Crohn's disease patients is compromised in the killing capacity towards different commensal bacteria (Aldhous et al., 2009). The mechanism behind the reduced hBD-2 expression in inflamed colonic Crohn's has not been elucidated up to now.

Voss et al. (2006) demonstrated that the expression of hBD-2 is mediated by NOD2 activation, but a subanalysis stratified for NOD2 mutation status could not identify differences in colonic hBD-2 expression. Further investigations revealed that 1, 25-dihydroxyvitamin D3 and MDP induce expression of hBD-2 and cathelicidin through stimulation of NOD2 expression (Wang et al., 2010). Many lines of evidence thus point to a major role of β-defensins in inflammatory processes of the colon. New data for the α-defensins from a mouse model show that the Paneth cell cryptdins synthesized in the ileum retain their structure and functionality till the colonic lumen (Mastroianni and Ouellette, 2009), suggesting a role for α-defensins in the large bowel as well. Furthermore, an interesting observation by Langhorst et al. (2009) showed a significant elevation of hBD-2 peptide in fecal samples from patients with irritable bowel syndrome, a condition which demonstrates nomacroscopic visible inflammation on colonoscopy.

The antimicrobial peptide elafin shares a similar expression pattern with the inducible β-defensin, LL37 and secreted leukocyte protease inhibitor. Its additional function as an antiprotease balances the proteolytic effects of HNE (human neutrophilic elastase) frompolymorphonuclear cells in healthy tissues. Moreover, it has been found to be reduced in colonic Crohn's disease, which could point to an involvement of protease-antiprotease disbalance explaining in part the penetrating, transmural type of inflammation (Schmid et al., 2007).

Cathelicidin (LL37) shows induction in inflamed tissues of ulcerative colitis, while in active Crohn's disease the induction seems to be attenuated (Schauber et al., 2006). In mutant mice, Cathelicidin restricts colonization with epithelial adherent bacterial pathogens like *Citrobacter rodentium* (Iimura et al., 2005), confirming its vital role in the armamentarium of the innate immune system.

The large intestine harbors a complex ecosystem, where classical immune cells and colonic epithelial cells interact in concert with the dense resident microflora (McCracken and Lorenz, 2001). After recognizing the importance of the microbiota in chronic intestinal inflammation too, the characterization of the enteric luminal flora in inflammatory bowel disease revealed differences in the composition compared to healthy controls. Swidsinski et al. (2002) among others demonstrated that mucosa-associated bacteria are dramatically increased in IBD mucosa. Anaerobic Bacteroides species and aerobic *Enterobacteriaceae* (E. coli) were most prevalent and furthermore early disease recurrence seemed to be accompanied by increased numbers of *E. coli*, *Bacteroides*, and *Fusobacterium*.

Conclusion

Antimicrobial peptides have emerged as effector substances of the innate immune system involving activities not only as endogenous antibiotics but also as mediators of inflammation.

Several important topics will have to be addressed in the future:

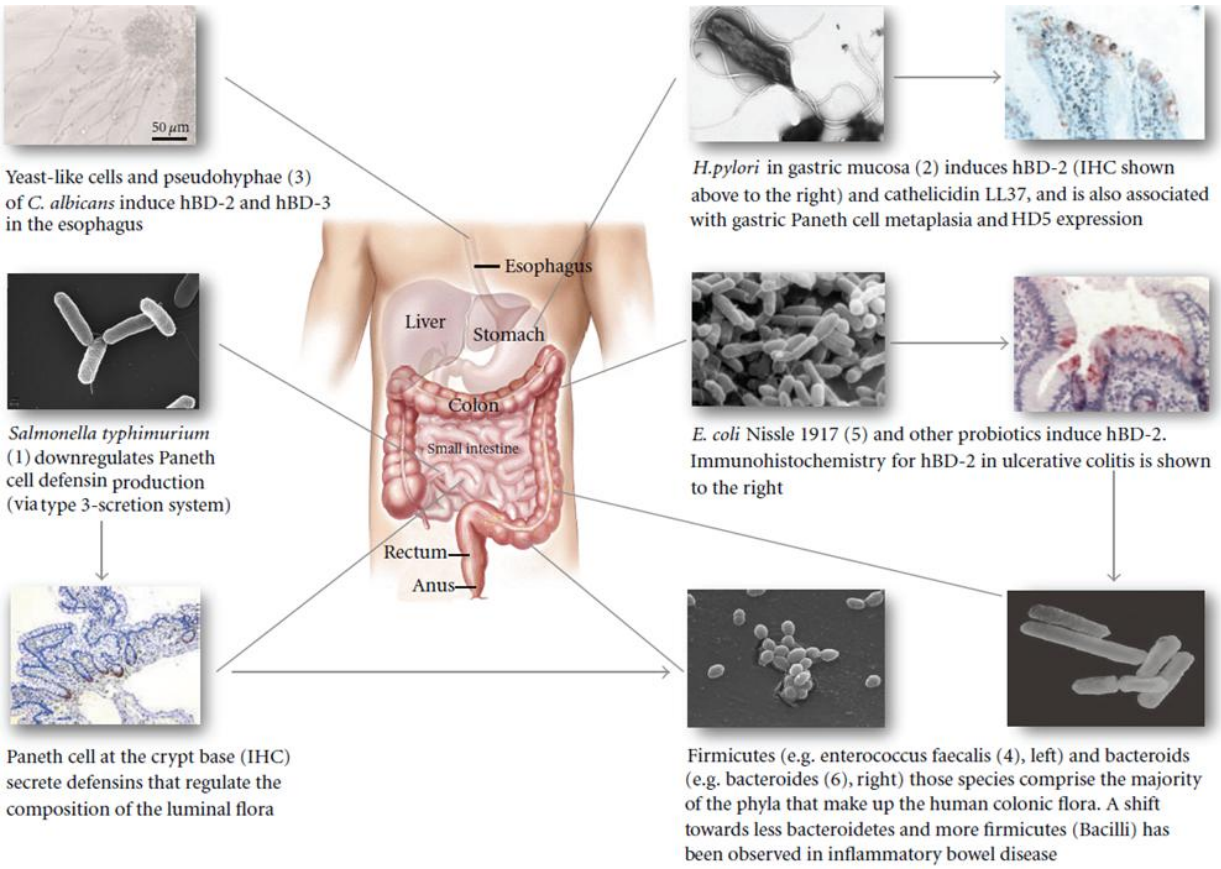

Fig. 2: Interaction of Microbes and Anti Microbial Peptides in the Gastrointestinal Tract

1. The identification of novel antimicrobial peptides. It is likely that human families of antimicrobial peptides consist of multiple molecules. Progress in the Human Genome Project will also reveal ways of shortcutting conventional bioscreening procedures for the identification of host defense substances.
2. Analysis of the biologically relevant functions of antimicrobial peptides. Beside experiments in vitro that give the first molecular insight into the function of peptide antibiotics, a broader approach involving genetic animal models is necessary to interpret results in vitro in the context of a whole organism.
3. Development of antimicrobial peptides as drugs. Studying the biology of antimicrobial peptides might permit the development of novel therapeutics for infectious or inflammatory diseases.

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