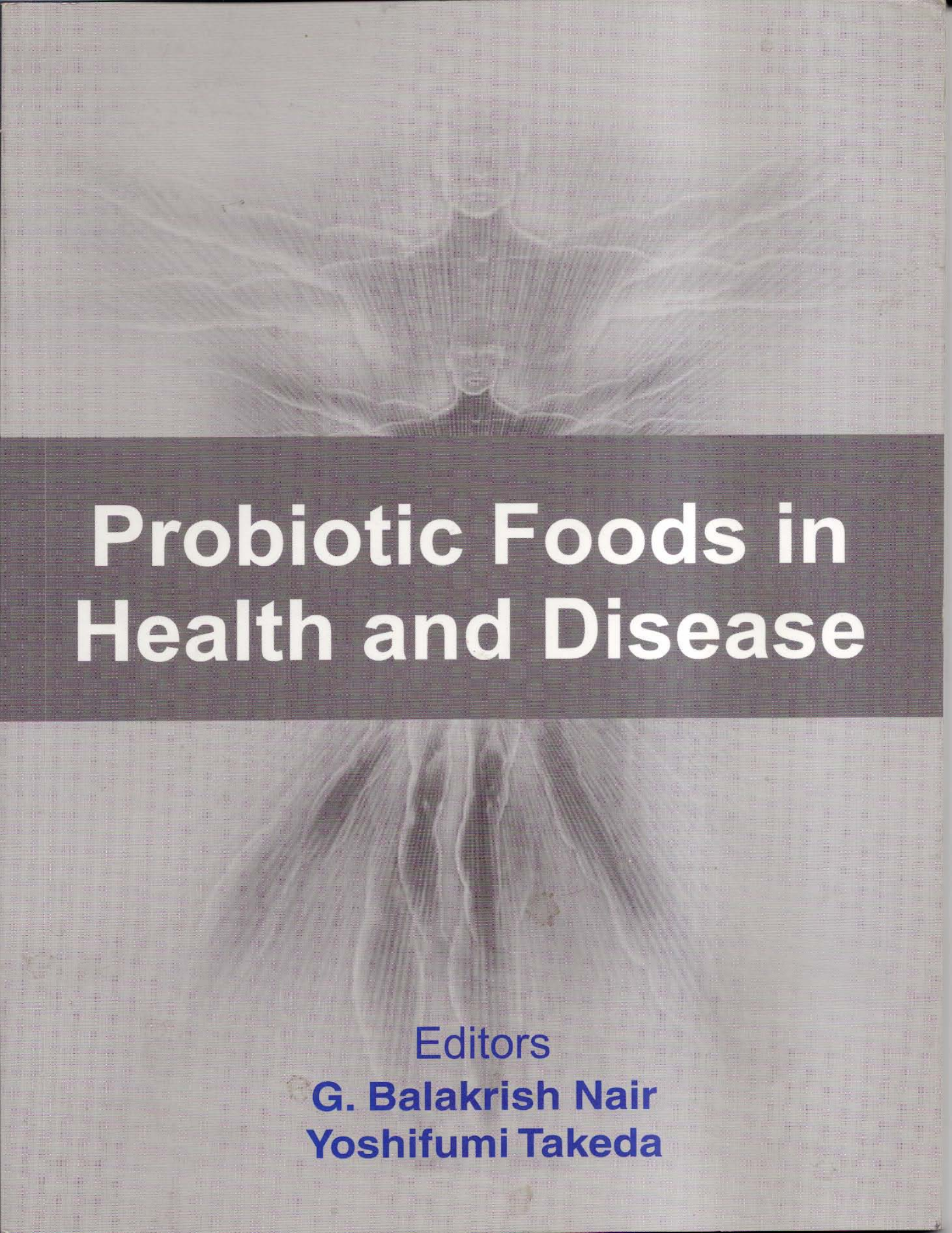

Probiotic Foods in Health and Disease

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PROBIOTIC FOODS IN HEALTH AND DISEASE

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Probiotics and Intestinal Defensins: Augmenting the first line of defense in Gastrointestinal Immunity.

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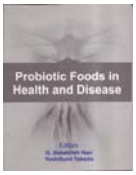
Abstract

Many features of the small intestine which are vital for nutrient absorption, i.e. a thin (single-cell) barrier, very large epithelial surface with numerous villi and crypts, high (membrane) transport rates and nutrient rich milieu ensue inherent vulnerability to bacterial colonization/infections. Host defense of this epithelium is mediated by complex arrays of mucosal innate immune determinants that offer the first line of defense against infectious threat. The intestinal epithelium enjoys diverse innate immune sensors like the 'Toll-like-receptors' (TLRs) and 'Nod Like Receptors (NLRs)'. These receptors recognize 'Pathogen Associated Molecular Patterns' (PAMPs) specific to virulent bacteria. Downstream events include, but are not limited to induction of innate immune effectors, like endogenous antimicrobial peptides and NF-kB mediated inflammatory cascades. Intestinal defensins – two different classes of small (3-4 kDa.), cationic antimicrobial peptides - are the most significant innate immune effectors in the gastrointestinal tract. Paneth cells at the base of the crypts of Lieberkühn store remarkably high levels of the defensins (the Human Defensin 5 and 6 – HD5 and HD6) as proform, along with their processing enzyme – a unique isoform of trypsin (Trypsin 4). Following infectious or cholinergic stimulus, pro-HD5 and trypsin are secreted and activated in the villus crypt. The activated HD5 can independently neutralize severe intestinal infections like Salmonella mediated enteritis and regulate gut flora. Accumulating evidence strongly support that defects in HD5 production and/or Paneth cell innate-immune sensors, directly contribute to Inflammatory Bowel Disorder (IBD) and Small Intestinal Bacterial Overgrowth (SIBO). Therefore, threshold level(s) of intestinal defensins is vital for gut defense and health. Whereas the role of commensal flora in context of intestinal defensins is less understood, emerging reports highlight many interesting relationships. Germ free animals exhibit lower intestinal defensins and enhanced susceptibility to IBD. Further, the commensal flora in IBD and healthy subjects seem genetically different. Interestingly, probiotic bacteria like *Lactobacillus* and *Bifidobacteria* not only exert immunomodulatory effects on cytokine profiles, they also directly interact with innate immune sensors like TLRs and stimulate the defensin-axis in the intestine. Together, this sets an exciting stage for intervention of chronic intestinal diseases like IBD with probiotic technologies that stimulate intestinal defensins and augment host defense.



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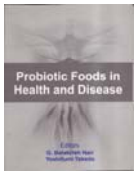
GASTROINTESTINAL INNATE IMMUNITY – THE FIRST LINE OF DEFENSE

From the perspectives of host defense, many features of the gastrointestinal (GI) tract, which are essential for nutrient uptake, concomitantly increase vulnerability to microbes and toxins. For example, to maximize (nutrient) uptake, the GI epithelium presents a very large GI epithelial surface (~300 m^2) packaged into numerous folds, villi and crypts (as in the small intestine)[1]. Together with its nutrient rich contents, this surface provides an ideal niche for microbial colonization. Besides, nutrient uptake mandates a thin, mostly a single-cell (epithelial) barrier with high rates of membrane transport. Not surprisingly, these features are exploited by many pathogens (or their products) which exhibit evolutionary selected mechanisms for the (food-and-water borne) enteric route of systemic infections.

In comparison with the small intestine, the colon presents a far more complex immunological challenge. The epithelial barrier here is closely associated with the largest "non-self" entity in the body: the complex commensal flora. Although non-invasive in nature, these commensals are not completely benign either. Loss of their containment (in the colon) can lead to Small Intestinal Bacterial Overgrowth (SIBO), bacterial translocation and Malabsorption Syndrome (MAS)[2, 3]. Dysregulation of host-commensal balance is also thought to be a major determinant of chronic gastrointestinal diseases like Irritable Bowel Syndrome (IBS) and/or Inflammatory Bowel Disorder (IBD)[4].

Against such complex and significant challenges the gastrointestinal epithelial barrier offers the first line of host defense. The fact that this barrier is rarely compromised in healthy individuals indicates that notwithstanding vulnerabilities, the (gastrointestinal) epithelium is endowed with robust innate immune mechanisms. Over the last two decades, seminal advances in the research on epithelial innate immunity have helped understand some of these processes. At present, it is abundantly clear that epithelial innate immunity is much more than a physical barrier. Many epithelial cells share specific, evolutionarily conserved (innate) immune sensors and effectors, which allow them to (a) identify or discriminate (immune) threat(s) in real time and (b) once stimulated, quickly launch an (innate) immune response to neutralize the threat. These innate immune determinants often lack the specificity and diversity typically exhibited in the mammalian adaptive immune apparatus. Nonetheless, they are extremely effective in countering the vast majority of (immune) challenges to the host. The ensuing concept of the epithelium as a major determinant of mammalian *self vs. non-self* discrimination induced a paradigm shift in immunology [for detailed review refer[5]]. Among many things, this has helped explain the remarkable ability of the gastrointestinal innate immunity to respond to pathogens while practicing immune-tolerance towards the abundant commensal flora. In context of host defense, the fundamental goal of probiotics is to stabilize this immune homeostasis in the gastrointestinal mucosa.



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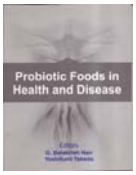
THE COMPONENTS OF GASTROINTESTINAL EPITHELIAL INNATE IMMUNITY

I. The Innate Immune Sensors – *Pattern Recognition Receptors*

Similar to other tissues, the mammalian innate immune sensors in the gut also have a limited repertoire. In order to accommodate this, the system has evolved a simple, yet extremely effective way to discriminate microbial threat. Instead of identifying specific microbial antigens, the innate immune sensors recognize *Pathogen (or Microbe) Associated Molecular Patterns* (PAMPs or MAMPs)[5, 6]. Typically, these include components of the microbial/bacterial cell wall, such as lipopolysaccharide (LPS), peptidoglycan and bacterial DNA. Consequently, these innate immune sensors are designated as *Pattern Recognition Receptors* (PRRs)[5, 7]. At least two major groups of PRRs are known: the 'Toll-like-receptors' (TLRs) and 'Nucleotide-binding domain (Nod) like receptors' (NLRs), which respond to extracellular and intracellular (cytoplasmic) MAMPs respectively[7, 8]. There are 13 different TLRs known in the human genome, each specific for unique class(es) of MAPs from bacteria, fungi and others[8-10]. Structurally, TLRs are transmembrane receptors; they survey the extracellular fluids, including endosomal compartments[8]. In contrast, NLRs are present in the cytosol and respond to intracellular MAMPs. These may be invasive microbial cells or cell products injected through the microbial Type III or Type IV secretion systems; alternately epithelial cell transporters (like the H^+-dependent gastrointestinal peptide transporter that helps muramyl dipeptide entry) can also facilitate this process. The mammalian NLR family is composed of more than 20 members[7, 11, 12]. They exhibit similar LRR (leucine-rich repeat) domains like TLRs that help in MAMP recognition; however NLRs use CARD(s) (caspase activation and recruitment domain) domains for downstream signaling, whereas TLRs use the Toll/interleukin-1 receptor (TIR) domain[7]. Similar to the TLRs, members of the NLR family also exhibit a degree of specificity towards MAMP classes. For example, in the gastrointestinal epithelium the best characterized NLRs are the Nod1 and Nod2; each distinguished by a unique CARD domain[6, 11]. Nod1 (*Card4*) was the first NLR identified as a potent sensor for enteroinvasive *Shigella flexneri*[12]. This was followed by Nod2 (*Card15*)[11]. In terms of specificity towards MAMPs, both Nod1 and Nod2 detect distinct substructures from bacterial peptidoglycan[13]. Nod1 senses peptidoglycan containing *meso*-diaminopimelic acid (*meso*-DAP), which are commonly present in Gram-negative bacteria[14]. In contrast Nod2 detects muramyl dipeptide, the largest molecular motif common to Gram-negative as well as Gram-positive bacteria[11, 13].

<u>Significance of the Gastrointestinal Pattern Recognition Receptors</u>

Downstream events of engagement of either the Nods or TLRs with their respective ligand(s) are complex and exhibit significant redundancy (or cooperation) between these individual receptor pathways (for detailed reviews on the functions and ligands of PRRs please refer[5, 13, 15]). Binding-induced conformational change allows the intracellular



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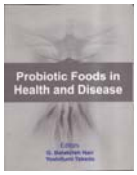
Toll/interleukin receptor (TIR) domain in PRRs to interact with specific adaptor molecules, such as *Myeloid Differentiation Primary Response Protein-88* (Myd88). Subsequent events include activation of major intracellular signaling pathways like the Nuclear Factor – kappa Beta (NF-κB) mediated inflammatory pathways, Mitogen-Activated Protein (MAP) kinases and upregulation and/or secretion of antimicrobial peptides (defensins) and cytokines[1, 11]. TLRs play important roles in (intestinal) epithelial cell proliferation and barrier functions [11, 16]. In the intestine, TLRs are also induce epithelial cells to secrete cholecystokinin, which increases gastrointestinal motility and (gastric) emptying (which help microbial clearance and diffusion of antimicrobial effectors along the intestinal axis) [17]. In a seminal investigation, Weiser and his colleagues recently reported that Nod1 mediates translocation of bacterial peptidoglycan from the gut and activates neutrophils in the bone marrow[14]. This indicates functional roles of intestinal PRRs may well extend beyond early immune responses and modulate systemic immunity. However, in context of bacterial relationships with intestinal PRRs, the feature that has attracted intense interest is ability of the gastrointestinal innate immune apparatus to modulate their responses between immune activation (against pathogens) vs. immune tolerance (against commensals). A large body of evidences support that these two apparently antagonistic processes are vital for immune homeostasis. For example, mice with defects in PPRs, for example TLR5 knockouts (TLR5KO) are actually more susceptible to inflammatory disorders than their normal counterparts [18]. Similarly, blockade of NF-κB activation does not reduce, but exacerbates inflammation in colonic epithelial cells. Significantly, MyD88 knockout mice develop high-titer serum antibodies against gut commensals, which indicate PRR functions are vital to contain exaggerated adaptive immune responses against commensal flora[19]. Therefore, immune homeostasis in the gut involves a degree of innate immune interactions between the flora and the PRRs[20, 21]. Given the extremely complex nature of commensal flora and PRR pathways, the mechanisms and dynamics of these interactions are not completely known. However, a plausible and largely accepted hypothesis for the unresponsiveness of PRRs towards commensal MAMPs is attenuation of PRR activity on the intestinal epithelial surface. Indeed, unstimulated intestinal epithelial cells express significantly lower (compared to other epithelial cells) levels of TLR2, TLR4, TLR11 (mouse) and CD14; all of which are oriented for extracellular recognition of MAMPs[8, 20]. Among others, TLR5, which recognizes bacterial flagellin, is expressed exclusively on the basolateral surfaces; TLR3, TLR7, TLR8 and TLR9 are expressed in endosomes of intestinal epithelial cells[27]. The two intracellular PRRs – Nod1 and Nod2 are also significantly expressed in the intestinal epithelium; the former ubiquitously, whereas the latter (Nod2) only in the Paneth cells of small intestine[13]. Therefore the gastrointestinal innate immune system seem to be adapted to respond to invasive stimuli (pathogens) than extracellular stimuli (offered by



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commensals on the luminal surface), which in part, may explain the attenuated response against the latter[8, 13, 20].

The profound significance of gastrointestinal PRRs in host health and homeostasis is highlighted by series of major breakthroughs that have linked severe gastrointestinal disorders with defects in PRRs and/or PRR- commensal relationships[19, 22, 23]. Genetic defects in several PRRs including TLR4, TLR9, Nod1 and Nod2 have been linked with human Inflammatory Bowel Disorder and Crohn's disease[7, 22, 24, 25].

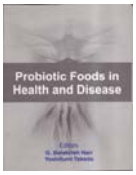
Taken together, these reports indicate that innate immune tolerance is not a passive process but a highly controlled dynamic attenuation of innate immune signals and PRRs present a critical component in functional mutualism between innate and adaptive immune components[19].

II. The Innate Immune Effectors

Arrays of innate immune antimicrobial effectors are synthesized by the gastrointestinal epithelium. Many of these factors are intimately linked with gastrointestinal physiology and exhibit antimicrobial activities as secondary function. A typical example is inorganic acid(s) in the stomach which, other than its digestive role, present a potent antimicrobial milieu against many food and water borne bacteria. Similarly the bile acids, owing to their chaotropic properties, exert potent antimicrobial activities in the proximal intestine [26]. However, there are specialized antimicrobial effectors as well. These are represented by a wide array of cationic antimicrobial peptides, proteins and lectins[6, 26, 27]. The most important of these are lysozyme, cathelicidins (LL-37), secretory phospholipase A2 (sPLA2), (antimicrobial) lectins, and defensins[28]. Each of these are products of distinct genes which may be either constitutive (i.e. alpha defensins or lysozyme) or inducible (i.e. beta defensins) by infectious stimuli.

Defensins – the major effectors of intestinal innate immunity

Defensins – a group of small (~3kDa.) cationic, membrane active, amphipathic molecules are among the most significant mammalian peptide antimicrobial effectors and characterized by a signature six-cysteine motiff [1, 28]. They are generally synthesized as myeloid precursors (~10 kDa.), processed and stored as mature forms in specific tissues and cells. Defensins act by inducing irreversible and lethal damage to target microbial cell membranes. Whereas the mechanism of their action of defensins is not completely known, it appears the antimicrobial activities are critically dependent on charge interactions between defensins and target (microbial) membranes. Both gram negative and gram positive bacteria have highly charged cell walls consisting of peptidoglycan (poly-*N*-acetylglucosamine and *N*-acetylmuramic acid); gram negative bacteria have a further layer of anionic lipopolysaccharides (LPS). Defensins are highly cationic and amphipathic molecules, owing to high levels of arginine and lysine in the primary sequence. This allows electrostatic interactions between defensins and microbial



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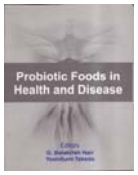
cells under physiologic conditions[29, 30]. Subsequently, the hydrophobic core of defensins induce sequential permeabilization of the outer and inner membrane of the target microbe, leading to irreversible loss of wall integrity and ultimately cell lysis [30, 31]. It is interesting to note that despite their activities against microbial membranes, defensins are largely inactive against eukaryotic cell membranes which are relatively neutral by virtue of their high sterol content, exhibit very little (surface) charge interactions against defensins [29, 32]. Therefore an evolutionarily conserved specificity exists for defensins towards microbial cells, without damaging (eukaryotic) host cells.

Based on their primary sequence: a conserved cysteine motif, pairing between the latter - three different classes of mammalian defensins are known[1, 33, 34]. They are designated as the *alpha, beta* and *theta* defensins. All human defensin genes are located on either chromosome No. 8 or 20. In human, six alpha defensins and eleven beta defensins are expressed. In gastrointestinal epithelium the former (alpha- defensins) are expressed in the small intestine; whereas beta- defensins are predominantly expressed in the large intestine[28, 35].

Four human alpha defensins are produced and stored in neutrophils (often designated as HNP 1- 4; *HNP* being acronym for *Human Neutrophil Peptide*)[1, 34]. In a seminal discovery, Bevins and his group discovered that two members of this family, HD5 and HD6 (*HD* is an acronym for *Human Defensin*) are present in the small intestinal epithelial Paneth cells[36, 37]. The finding indicates interesting parallel evolution of mammalian (innate) immune determinants in professional immune cells and somatic cells. More importantly, this helped extend the concept of epithelial host defense mediated by specific innate immune effectors in the gastrointestinal system[1, 34, 38].

The Alpha Defensins and the Paneth Cell Axis

Paneth cells reside in the base of small intestinal crypts of Lieberku¨hn, often as a cluster of four to six cells[28, 39]. The distinctive feature in these cells is their intracellularly stored azurophilic granules [22, 38, 39]. These granules store abundant antimicrobial effectors like lysozyme, secretory phospholipase A2, antimicrobial lectins and the two alpha defensins HD5 and HD6 [36, 40, 41]. Interestingly, Paneth cell alpha defensins are stored as proform(s) along with unique pattern of trypsin isoforms[42]. In response to cholinergic or innate immune stimuli the Paneth granules are secreted out into the crypt lumen[40, 43, 44]. Both HD5 and HD6 are subsequently processed by Paneth cell trypsin in the crypt lumen[42]. Such post-translational activation of innate immune effectors is not unusual; however, in mammalian defensin families, only the *intestinal* alpha defensins are stored as proforms and activated upon stimulus (HNPs are stored as mature peptides in neutrophils)[42]. Whereas the precise reasons for such elaborate, evolutionary conserved post-translational processing mechanisms are not completely clear; we calculated the secreted HD5 can reach concentrations of 50–250 μg/ml in the intestinal crypt (defensins are



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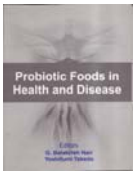
active at 1-10 μg/ml) and 90–450 μg per cm^2 of ileal surface area – ensuing a potent, broad spectrum antimicrobial activity in the small intestine[42]. The significance of this was explicated when we showed that transgenic transfer of HD5 conferred novel resistance against lethal enteric *Salmonella* infection in a murine model[45]. This demonstrated that Paneth cell (alpha) defensins can independently define the outcome of lethal enteric infections[28, 39, 45]. Recently, it was demonstrated that Paneth cell defensins can also modulate the flora of the large intestine[46]. Although we observed HD6 is also subjected to secretion induced processing (Ghosh D. unpublished observations), very little is known about this defensin's activities.

Paneth cell functions are regulated at many levels. The Secretion is controlled cholinergic stimuli that activate G proteins coupled muscarinic receptors[47]. This indicates an evolutionary conserved link between paneth cell secretion/innate immunity and vagal activity, that can ideally be targeted by oral probiotics. However from their position deep inside the base of intestinal crypts, how do Paneth cells sense infectious stimuli? Paneth cells express two major innate immune sensor proteins: MyD88 and Nod2 [48]. Vaishnava et al. reported activation of MyD88 by invasive (translocating) bacteria triggers multiple antimicrobial factors that limit microbial translocation across the epithelial barrier[48]. The fact that Nod2 can respond to muramyl dipeptide, which is conserved in both Gram-positive and Gram-negative bacteria, further expands the sensitivity of Paneth cells. The latter (Nod2) allows the Paneth cell to actively sense and inhibit small intestinal colonization by bacteria[49]. Interestingly, the expression of Nod2 is dependent on the presence of commensal bacteria. Mice re-derived into germ-free conditions expressed significantly less Nod2 in their terminal ilea, and complementation of commensal bacteria into germ-free mice induced Nod2 expression[49]. Therefore, Nod2 and intestinal commensal bacterial flora maintain a balance by regulating each other through a feedback mechanism. Dysfunction of Nod2 results in a break-down of this homeostasis. In part, this is due to the close relationship of Nod2 and Paneth cell defensins. In series of investigations Bevins and his colleagues showed mutations in Nod2 leads to a sharp decrease in Paneth cell defensins and predispose to Crohn's disease (ileal CD)[25]. Together, these powerfully support that Paneth cells are among the most significant effectors of gastrointestinal innate immunity. Not only do these cells possess highly effective innate immune sensors and effectors, the strategic position in the ileum allows them to protect the delicate integrity of the small intestine and control the gut microbiome [46].

The Beta Defensins – effectors of the colon

Despite similarities with alpha defensins, beta defensins exhibit many unique features. Unlike intestinal alpha defensins the beta defensins are predominantly expressed in the colonocytes of the large intestine; few of them are also reported in the upper gastrointestinal tract[35]. All beta defensins are activated at myeloid state and not subject



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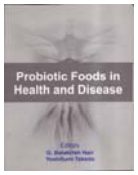
to further post-translational processing[35]. Three beta defensins (called *HBDs*, acronym for *human beta defensin*) are expressed in the intestinal mucosa. In sharp contrast to alpha-defensins, most beta defensins are highly inducible owing to a NF-κB inducible site in the gene[35, 50]. Expression of the enteric beta defensins, HBD-2– 4 are all induced by various inflammatory and bacterial stimuli; although the mechanisms for the induction may be different from one another. The only exception is HBD-1, which is not upregulated by pro-inflammatory stimuli or bacterial infection. In contrast, HBD-2 expression is highly upregulated by MAMPs or inflammatory stimuli in IBD[50, 51]. There is usually little or no expression of HBD-2 in the normal colon, but abundant HBD-2 expression by the epithelium of inflamed colon. Fahlgren et al. investigated HBD-3 and HBD-4 mRNAs in Crohn's and Ulcerative Colitis (UC) patients, using real-time quantitative reverse transcription-polymerase chain reaction (QRT-PCR) and by in situ hybridization[52]. They observed significant upregulation of HBD-3 and HBD-4 in UC patients but none in CD. Interestingly HBD-3 has recently been shown to be the most active defensin against anerobic bacteria in the gut[53]. Nuding et al. have recently demonstrated that major anaerobic gut bacteria *Bacteroides* and *Parabacteroides*, were most effectively controlled by the HBD-3[54].

III. The Innate Immune Ligands – *Microbe Associated Molecular Patterns*

Metagenomic profiling studies using ribosomal DNA (r-DNA) typing have revealed that the human gut flora consists of more than 400 distinct bacterial species adding up to a concentration of $10^{12} – 10^{14}$ bacteria per ml of luminal contents in the adult human large intestine[55]. Based on the innate immune paradigm, this flora would offer a large diversity and concentration of MAMPs to the gastrointestinal PRRs. Remarkably, the innate immune system largely ignores these stimuli. Whereas, some of the host specific mechanism(s) for the immune hypo-responsiveness against commensals are described above, accumulating pool of evidences indicate that the commensal flora may also participate in this process directly. For example, *Bacteroides*, a predominant commensal in the gut engages several mechanisms to modulate innate immune responses. In Gram-negative bacteria, Lipid A, a component of their cell membrane LPS, is a potent MAMP and recognized by TLR4. However, the Lipid A in *Bacteroides* is pentacylated, that largely abrogates its recognition (with TLR4)[56]. *Bacteroides* also inhibit NF-κB mediated inflammatory pathways by increasing the export of the NF-κB subunit RelA from the nucleus and redistributing Peroxisome Proliferator Activated Receptor-gamma (PPAR-gamma)[57].

However not all commensal bacteria offer immunosuppressive MAMPs. *Proteobacteria*, another commensal bacteria in the gut, has a hexacetylated Lipid A that strongly engages TLR4 and induces inflammatory response[58]. Whereas, *Proteobacteria* is vastly outnumbered by *Bacteroides* in healthy individuals, there is significant increase of the former and decrease in the *Bacteroides* population in IBD[59]. In part, this may explain the



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exacerbated intestinal inflammation associated with IBD; an observation that agrees with the current school that at least some subsets of IBD is based on the premise of dysbiosis[60-62]. Paradoxically, absence of MAMPs does not augment of stabilize intestinal homeostasis either. Petnicki-Ocwieja reported, mice introduced into germ-free conditions expressed significantly less Nod2 and exhibited impaired control over small intestinal bacterial colonization[63].

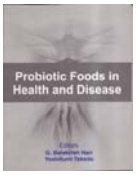
A large body of evidences now indicates that under steady-state conditions the gastrointestinal epithelial PRRs and intestinal commensal bacteria maintain a balance by regulating each other through a feedback mechanism. The gastrointestinal innate immune system recognizes commensal bacteria and elicits signals below an inflammatory threshold that continuously primes the adaptive immune system. Dysfunction of either the epithelial innate immune determinants or, commensal ecosystem results in a break-down of this homeostasis [49]. Therefore the gastrointestinal innate immune system is not based on the principles of exclusion, but inclusion of 'non-self' bacterial (commensal) stimuli that are co-existing participants of (innate) immune adaptation and homeostasis. The latter concept is the fundamental motivation for probiosis, by defined bacterial interventions.

PROBIOTICS AND GASTROINTESTINAL INNATE IMMUNITY

In context of epithelial innate immunity, the inherent hypothesis of probiotics is that bacterial MAMPs would engage the PRRs in the gastrointestinal epithelium and stimulate innate immunity. The caveat to this hypothesis is that the intensity of probiotic immune stimulation probiotics should *not* lead to major inflammatory cascades and tissue damage typically induced by pathogens (PAMPs)[8]. The ideal probiotic model would therefore result in higher antimicrobial potential in the intestinal milieu (owing to upregulation and/or secretion of defensins or antimicrobial probiotic by-products), improved epithelial barrier functions and better infection resistance. Based on the innate immune paradigm, stimulation provided by probiotics would therefore be between the hyporesponsive commensals and proinflammatory stimuli offered by pathogens. The ideal probiotic(s) would also include cytokine profiles that augment immune homeostasis (especially when targeted at inflammation foci like IBD) in the gut. Although there are few systematic studies specifically investigating these areas, many reports validate the basic tenets of the hypothesis.

Direct Antimicrobial Activity of Probiotics

There is evidence that many probiotic strains can directly perform antimicrobial activities, using diverse molecular determinants and mechanisms[64, 65]. These include production of biosurfactants, (lactic) acids, bacteriocins, hydrogen peroxide and other antimicrobial determinants. An example of a specific probiotic antimicrobial - is Reuterin, produced by the vaginal flora *Lactobacillus reuteri.* Reuterin (3-hydroxypropionaldehyde) is a metabolite derived from glycerol and has been extensively



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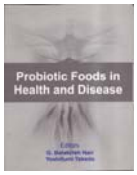
researched for its broad spectrum antimicrobial activities against bacteria, fungi and protozoa. In clinical trials the strain has demonstrated promising results against bacterial vaginosis, however further work is needed to confirm if this was attributed to the activity of Reuterin alone[66]. Interestingly, Reuterin seems to be highly active against enteric bacteria; there are also several reports of its role against enteric pathogens like enterohemorrhagic *E coli*, enterotoxigenic *E. coli*, *Salmonella enterica, Shigella sonnei* and *Vibrio cholera*[65, 67]. This has begun to be exploited by oral probiotic strategies using this bacterium[68]. However, arguably an ever more significant observation is the diversity of antimicrobial factors like Reuterin in different probiotic bacteria, even within the same genus. For example vaginal bacteria like *Lactobacillus rhamnosus* produce Rhamnosin and Lactosin, which (unlike Reuterin) are peptide antibiotics[69]. *In vitro* assays on human organotypic vaginal-ectocervical tissue model (EpiVaginal) showed that Lactosin enjoys excellent activity with minimal side-effects like irritation (usually induced by excess lactic acid) or hemolytic activity[69]. Clearly, significant research is needed to determine the mechanisms of action and roles of probiotic antibiotics, before their commercial applications. However, the diversity of probiotic antibiotics offer interesting potential for their therapeutic use for specific antibiotic production and delivery; indeed several trials in context are underway[70, 71].

Probiotics and Gastrointestinal Innate Immunity: Mechanisms of Homeostasis

Whereas there is not much published reports on the relationships of probiotics with innate immune determinants, a fast growing pool of evidences support that probiotics do work by actively engaging PRRs. For example, it was recently reported that *Lactobacillus plantarum* upregulated HBD-2 RNA and induced secretion in a dose- and time-dependent manner in Caco-2 cells[72]. The HBD-2 RNA was inhibited by anti-TLR-2 neutralizing antibodies, indicating the critical importance of probiotic MAMP engagement with host PRRs for defensin stimulation[72]. Other independent investigations also support that *L. plantarum* and *L. casei* engaged TLR-2, and TLR-4 for their activities[73]. However, one of the most well documented proof-of-principle for probiotics and the innate immune (defensin) axis have come from the studies on *E. coli* Nissle 1917 by Wehkamp and his co-workers[74-77]. The group showed this strain induced HBD-2 whereas, as many as 40 other clinical *E. coli* isolates lacked this capacity[74]. Isolated and purified *E. coli* Nissle 1917 flagellin protein was able to induce hBD-2 in a dose-dependent fashion, whereas flagellin deficient mutants of this bacterium were not able to induce the defensin[75]. Interestingly, LPS (a potent MAMP in other bacteria) of *E. coli* Nissle 1917 did not upregulate hBD-2. Together, this confirmed flagellin is the determining MAMP in this probiotic, that can singularly stimulate HBD-2 production[75]. The flagellin interacted with the functional binding sites for NF-κB and AP-1 in the hBD-2 promoter, which confirmed the genetic basis behind this observation[74].



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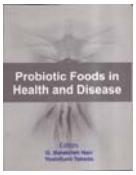
Incidentally, in an independent study Grabig et al. showed *E. coli* Nissle 1917 ameliorates experimental induced colitic inflammation by interacting with TLR2 and TLR4 in murine models [78]. Similar observations on this strain have been reported by others as well, where the outcome of probiotic treatment was often comparable to therapeutic drugs, especially in cases of ulcerative colitis, which share microbial induced inflammatory foci [79]. Indeed, there are substantial evidences supporting that engagement of probiotic MAMPs with intestinal PRRs actually lead to anti-inflammatory outcomes[78, 80]. How do probiotics engage innate immune determinants towards such seemingly contradictory results? At least in part, this is thought to be due to the different cytokine patterns induced by probiotic MAMPs (compared to pathogens) and their target cells. However the details may be more complex and some of these mechanisms are being elucidated. For example, *E. coli* Nissle 1917 was reported to inhibit the expansion of peripheral $CD4^+$ T-cell subsets via TLR2 and limit intestinal inflammation[78]. Similarly, the probiotic yeast *Saccharomyces boulardii* (Biocodex Inc., USA) was shown to downregulate proinflammatory cytokines (such as tumour necrosis factor-alpha and interleukin -6[IL6]) and upregulate anti-inflammatory cytokines (IL-10) in dendritic cells (DC) stimulated with LPS[80].

An interesting prospect emerging from these research and related reports is that probiotic bacteria by virtue of the distinct chemical identities of their MAMPs, as well as their mechanisms of innate immune engagement, may indeed exhibit dramatic redundancy between strains[81]. For example, more than one *E. coli* strain(s) in the commercial preparation *Symbioflor* (Symbiopharm, Germany) does not express flagellar protein. Yet, these strains induce hBD-2 efficiently [75]. This indicates the *Symbioflor* MAMPs are different from *E. coli* Nissle 1917. Similarly, the major MAMP inducing HBD-2 in the probiotic *VSL#3* (Sigma-Tau Pharmaceuticals Inc., USA), was determined as CpG-DNA (deoxycytidylate-phosphate-deoxyguanylate) most strains in this preparation also lack flagella[82]. Yet, VSL#3 also induces hBD-2 via NF-κB and AP-1-dependent pathways similar to *E. coli* Nissle 1917[77]. Significantly, none of these strains carry any infectious risks or severe inflammatory reactions. This indicates innate immune stimulation from an ideal probiotic may truly operate below the threshold level of an active inflammation and thereby affect a protective response. It is interesting to note here many other important probiotic strains, like the widely used probiotic *L. casei (Shirota)* or *Saccharomyces boulardii* is reported to exhibit excellent immunostimulation[73, 83]. However accurate characterization of their MAMPs and/or their mechanisms of defensin (or other innate immune effectors) induction are still awaited.

Innate Immune Stimulation – the diversity of probiotic

Interestingly, the mechanisms of NF-kB or AP-1 for hBD-2 activation seem to vary between probiotic strains. Whereas, in case of *L. acidophilus* hBD-2 promoter activity was stimulated synergistically through NF-kB and the AP-1 binding sites; the latter



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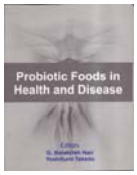
(sites) operated independently in case of *P. pentosaceus* and *L. fermentum*[77]. Differences were reported even within different strains (ATCC27139 and ATCC27139-J1R) of the same *Lactobacillus* species in their ability to induce TLR2, Nod2 and inflammatory cytokines like TNF-alpha, IL-12, IL-18, and IFN-gamma[83]. Together, these indicate different probiotic strains use distinct MAMPs and innate immune stimulation pathways.

A. Stimulation of Defensins and Innate Immune Determinants

Wehkamp et al. was the first to show that the probiotic *E. coli* Nissle 1917, strongly induce the expression of the human beta-defensin-2 (HBD-2) in Caco-2 cells in a dose dependent manner[74]. No induction for HBD-1 or the alpha defensins (HD5 and HD6) was observed. In the L. plantarum model on Caco-2 cells, Paolillo et al. observed selective induction of HBD-2 but not HBD-3[72]. Several other probiotic strains like *L. gasseri, L. acidophilus, L. fermentum, L. plantarum, L. paracasei, Pediococcus pentosaceus* and *Leuconostoc sp.,* also exhibited HBD-2 induction, however at varying and mostly lower than *E. coli* Nissle[74]. Interestingly, the induction was time dependent; hBD-2 levels peaked between 3 and 6 h after exposure, but expression returned to basal values after 12 h[74]. This indicates pharmacokinetics of probiotics may be a significant area of research in future. The probiotic cocktail VSL#3 was shown to induce the secretion of the HBD-2 in Caco-2[77]. VSL#3 is a proprietary probiotic containing lyophilized mixture consisting of eight different Gram-positive organisms (*B. longum*, *B. infantis*, *B. breve*, *L. acidophilus*, *L. casei*, *L. delbrueckii* ssp.*bulgaricus*, *L. plantarum* and *Streptococcus salivarius* ssp. *thermophilus*). Recently, Paolillo et al. independently confirmed *Lactobacillus plantarum* significantly induced HBD-2 secretion in a dose- (16+/-1.4 pg/ml and 31.5+/-2.3 pg/ml at MOI 10 and 50, respectively) and time-dependent manner in Caco-2 cells[72].

The consequences of Innate Immune stimulation and defensin stimulation by probiotic bacteria:

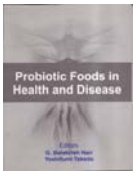
The expected and immediate consequence of defensin stimulation by probiotics is higher antimicrobial potential in the gastrointestinal tract. Consequently improved resistance and or direct remission from (intestinal) infectious diseases is expected from probiotic regimen. Several probiotic Lactobacillus strains like *L. reuteri,* L. johnsonii La1, L. rhamnosus GG, L. casei Shirota YIT9029, L. casei DN-114 001, and L. rhamnosus GR1 showed antimicrobial properties against enteric pathogens *in vitro*[65, 84]. Interestingly this activity was attributable to non-lactic acid molecule(s) present in the Lactobacillus cell-free culture supernatant[84]. Jain et al. recently reported improved resistance to oral Salmonella challenge in mice systematically fed with *L casei* [85]. In a significant study on a defined human microbiota-associated (HMA) mouse model showed oral exposure to probiotic Lactobacilli and Bifidobacteria successfully excluded *Campylobacter jejuni* and reduced the number of intestinal *Salmonella*[86]. Although the effects of human enteric defensin stimulation by probiotics in context of infection resistance has not been studied



in detail; Mondel et al. determined a sustained and significant (78%) upregulation of fecal HBD-2 in healthy human subjects administered with probiotics. It seems rational to speculate that such high levels of intestinal antibiotics would confer improved infection resistance, among other things. The latter (defensin induction) may be one of the reasons why cases of infectious diarrhoea respond to probiotic treatments [87].

The fact that probiotics can directly stimulate intestinal defensins present exciting prospects for extending the current status of their applications in non-infectious gastrointestinal diseases like Inflammatory bowel disease (IBD), a subset of which are linked with dysregulated intestinal flora and/or impaired innate immunity[88]. The associated chronic intestinal inflammation is typically represented by Crohn's disease (CD) and ulcerative colitis (UC)[89]. Commensal induced inflammation has been most frequently associated with UC and many probiotic regimen are reported to ameliorate the disease symptoms[90-93]. Ileal CD, which is attributed to lower defensin levels, maybe another prospective target for probiotic therapy. Another highly relevant scenario for probiotic application is Small Intestinal Bacterial Overgrowth (SIBO), a condition when the bacterial content of the small intestine exceeds >105 cfu/ml[94]. SIBO is frequently associated with malabsorption syndrome (MAS) and/or Irritable Bowel Syndrome (IBS). In any of these cases, enhanced defensins stimulated by probiotics may play a major role in clearing colonizing bacteria in inflamed tissues and thereby reduce disease symptoms.

Whereas augmentation of innate immunity through stimulation of defensins may be a welcome strategy for probiotics, there are many other aspects of upregulating the defensin axis that need careful consideration. Besides antimicrobial activity, HBD-2 can act as a ligand for CCR6, a chemokine receptor for MIP-3 alpha (CCL20). CCR6 is expressed in immature intraepithelial lymphocytes (IELs) and plays vital role in their maturation from the intestinal cryptopatches; CCR6 also recruits dendritic cells and memory T cells in the gut [95, 96]. Thereby beta defensins, particularly HBD-2 can actively modulate adaptive immunity[95, 96]. Introduction/expression of new defensins *in vivo* or upregulation of existing ones is also known to fundamentally alter the host commensal flora [23, 46]. In this background, it is interesting to speculate the effects of high levels (which may reach >300 fold in cell lines and ~78% above normal in human subjects[76]) of probiotic induced defensins (HBD-2) *in vivo*. Even more significantly, in human subjects, the enhanced defensin levels were observed even after 9 weeks following probiotic exposure[76]. Given the multiple roles of HBD-2, such dramatic levels of the same in the gastrointestinal tract may induce profound side-effects. These have not been studied yet and call for detailed research.



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The majority of the tested strains belonging to the dominant anaerobe genera of the gut, Bacteroides and Parabacteroides, were only minimally affected by the constitutively expressed defensins HD5 and HBD-1. The inducible defensin HBD-2 had a limited antibacterial effect, whereas the inducible HBD-3 exhibited potent activity against most strains. The effect of HBD-3 on Bacteroides sp. appeared to be dependent on the presence of oxygen. Bacteroides fragilis strains isolated from blood during bacteremia or from extraintestinal infections were more resistant to HBD-3 than strains from the physiological gut flora. Thus, defensin resistance is not only species- but also strain-specific and may be clinically relevant in the host-bacteria interaction influencing mucosal translocation and systemic infection[54].

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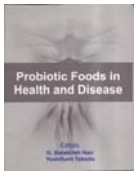

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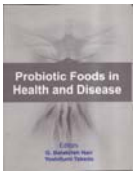

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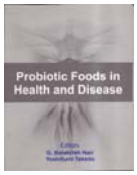

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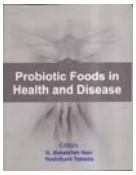

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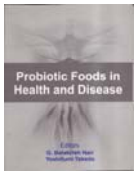

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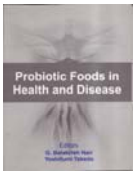

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