

## **Role Of Gut Flora In Inflammatory Bowel Disease- A State Of Art**

**Jaishree Paul\***, Anil Kumar Verma and Ravi Verma

School of Life Sciences, Jawaharlal Nehru University, New Delhi 110067, India

This review helps us to dissect out the underlying mechanism that leads to inflammatory bowel diseases (IBDs, which include ulcerative colitis and Crohn's disease). Two broad line of thoughts have emerged from the study. One body of data suggests that IBD is associated with the genetic make-up of the individual that contributes to evoke the inflammatory response that characterizes the disease. Another body of data suggests that IBD patients have a defective epithelial barrier that enables the proliferation of non pathologic organisms in close juxtaposition to elements of the mucosal immune system, again evoking the inflammatory response that characterizes the disease.

**Keywords:** Gut microbiota; Ulcerative colitis; Crohn's disease; Sulphate reducing bacteria; leaky gut; probiotics; molecular techniques

### **1. Introduction**

There has been a marked resurgence of interest in the gastrointestinal commensal flora and host microbe interactions. This has been generated due to the recognition of the role of intestinal bacteria in the pathogenesis of several intestinal disorders, including Crohn's disease, ulcerative colitis and colorectal cancer. This renewed interest in the flora has also been prompted by increasing evidences of the potential health benefit of therapeutic manipulation of the microenvironment with probiotics during disease condition. We also have started understanding the role of the gut flora in intestinal development, mucosal defence and the relevance of commensal flora on the emerging global problem of antibiotic resistance. Co-evolution of the host and gut flora implies mutually beneficial interactions. Survival of the host requires, however, that the bacterial residents within the human to be contained, without excessive immune reactivity, while retaining the capacity for an effective immune response to challenges posed by pathogens. This is maintained by a precise regulation of the microenvironment [1]. Any disruption of these processes may lead to inappropriate response and chronic inflammatory disease. Many clinical and experimental intestinal inflammatory reactions have been attributed to immune recognition of the intestinal microflora. We intend here to present an overview on the state of the gut flora during IBD, its probable role in modulating immune response during disease and therapeutic strategies tried to manipulate the flora.

### **2. Landmarks achieved so far in IBD research**

Regarding the pathogenesis of IBD, it is recognized that immune tolerance is the normal state of the intestinal immune system. Secondly, it is also understood that a wide variety of cell types are orchestrated in a tightly regulated fashion to maintain immunologic tolerance. Thirdly, the intestinal flora is a key ingredient in the abnormal immune response of IBD and the genetic factors predispose individuals to an abnormal immune response to the flora. Finally, it is recognized that both innate and adaptive immune responses play integrated roles in the homeostasis of the intestinal mucosal response [2]. The normal individuals has the ability to control gut inflammation whereas, individuals susceptible to IBD tend to enter a state of uncontrolled and chronic inflammation with failure to down regulate the inflammation caused by the insult.

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\* Corresponding author: email: [jaishree@mail.jnu.ac.in](mailto:jaishree@mail.jnu.ac.in); Phone: 91-11-26704156

### 3. Role of bacteria in Pathogenesis of IBD

The pathogenesis of inflammatory bowel disease involves interactions between the host susceptibility, mucosal immunity and intestinal microflora. There is therefore great interest in the changes in the endogenous flora in inflammatory bowel disease patients and in the establishment of potential genetic variations in host responses to endogenous bacteria. In this review, we summarize the modifications in the various regional ecosystems in the gastrointestinal tract during inflammatory bowel disease (luminal bacteria in faeces or inside the gastrointestinal tract, bacteria in mucus and bacteria directly attached to the mucosa). Earlier results were obtained following a 'candidate microorganism strategy' and, as is occurring increasingly frequently, following a 'full description strategy', which has progressed largely due to the development of culture-independent techniques.

#### 3.1 Faecal microbiota

In humans, the composition of the flora in an individual is stable, but differs between the stomach and upper bowel, lower small bowel, right colon and rectum. Moreover, the flora recovered from faeces is also different from mucosa-associated or intraepithelial flora.[3] The resident microbiota has a critical role in modulating the immune response of the gut as well as in the initiation and perpetuation of IBD. In the normal host, the protective cell-mediated and humoral immune responses to enteropathogenic microorganisms are allowed to proceed, whereas responses to microorganisms of the indigenous flora are prevented. Differently, under conditions of chronic intestinal inflammation, this homeostasis seems to be disrupted, and the commensal flora seem to act as a surrogate bacterial pathogen: the lifelong inflammation in chronic IBD occurs because the host response is unable to eliminate the flora[4]. Several lines of evidence in adults and in various animal models emphasise the role of the endogenous normal intestinal microflora in the aetiology of IBD.

A study by Rath et al, (2001) convincingly reveals an important but complex effect of luminal bacteria in the acute phase as well as the chronic phase of experimental colitis. The present results in conjunction with their previous observations strongly support the following hypotheses (i) Normal luminal bacteria are required for development of chronic immune-mediated intestinal inflammation. (ii) Commensal enteric bacterial species have unequal proinflammatory capabilities, with some being more aggressive than others. (iii) Various endogenous bacteria have different roles in the inflammatory process. Some, including *Bacteroides* spp. and other, yet-to-be identified species preferentially initiate inflammation, while another, perhaps larger spectrum of intestinal bacteria perpetuate disease. (iv) An initial reduction of the total bacterial load with a broad-spectrum antibiotic combination alters the bacterial composition with a lasting effect on intestinal inflammation, although the total luminal concentration recovers rapidly [5].

The biodiversity of the faecal microflora remains high in patients with CD. Enterobacteria were observed significantly more frequently in CD than in health, and more than 30% of the dominant flora belonged to yet undefined phylogenetic groups [6]. 6 group-specific probes targeting 16S rRNA and spanning the main phylogenetic groups of the fecal microbiota when employed to study IBD, Infectious colitis (IC) vs. Healthy subjects (HS), it was concluded that CD and UC fecal microbiota harbor specific discrepancies and differ from that of IC and healthy subjects [7]. Bibiloni et al, 2006, compared the bacterial community from UC and CD biopsy samples and observed prevalence of unclassified members of the phylum Bacteroidetes in CD than in UC patients by DGGE profiles [8]. Statistical analysis of the composition of 16S rRNA gene libraries showed that the bacterial collections in UC and CD patients differed ( $P < 0.05$ ). When fecal samples of active ulcerative colitis patients were analysed for populations of lactobacilli, bifidobacteria, clostridia, bacteroides, sulphate-reducing bacteria (SRB) and total bacteria using culture independent fluorescence in situ hybridisation (FISH), numbers of lactobacilli were significantly lower ( $p < 0.05$ ) during the active phase of the disease but the other populations tested did not differ [9].

### 3.2 Mucosa associated microflora:

The microbiota close to mucosa which differs from luminal microbiota, has so far received less attention, yet it is very close to inflammatory process. The composition of the mucosal and mid stream/faecal microflora has been shown to be significantly different [10]. How some bacteria may exert inflammatory effect and others a protective role in IBD is yet uncertain. Because IBD is a disorder of mucosal inflammation, the mucosa associated microflora seems of peculiar relevance to the disease process [11]. Differences were observed between the dominant fecal microbiota and the mucosa-associated microbiota of different sites of colon and rectum in IBD vs. healthy subjects, with similarity percentages less than 92% thus confirm that the dominant species differ between the mucosa-associated and fecal microbiota [12]. Colonic biopsies from CD-afflicted patients compared with biopsies from normal control subjects had an increase in facultative bacteria; in small bowel, CD patients had an increase in the *Ruminococcus gnavus* subgroup with a decrease in the *Clostridium leptum* and *Prevotella nigrescens* subgroups [13]. No rDNA sequence, phylogenetic group, or subgroup was consistently associated with CD lesions compared with normal tissues from the same patients. These findings suggested that CD is not caused by invasive pathogens associated specifically with the sites of lesions but that dysbiosis exists in this condition.

A higher number of mucosa-associated aerobic and facultative-anaerobic bacteria were found in biopsy specimens of children with IBD than in controls. An overall decrease in some bacterial species or groups belonging to the normal anaerobic intestinal flora was suggested by molecular approaches; in particular, occurrence of *Bacteroides vulgatus* was low in Crohn's disease, ulcerative colitis and indeterminate colitis specimens [14]. These data underline the central role of mucosa-adherent bacteria in IBD. Ott et al (2004) [15] demonstrated that mucosal inflammation in IBD was associated with loss of normal anaerobic bacteria and identified a number of specific taxa. The reduction in diversity in IBD was due to significant loss of *Bacteroides* species, *Eubacterium* species and *Lactobacillus* species. The reduction in mucosa-associated bifidobacteria and increase in *E. coli* and clostridia in patients with IBD supports the hypothesis that an imbalance between potentially beneficial and pathogenic bacteria may contribute to its pathogenesis [16].

Statistical analyses using incidence-based species richness and diversity as well as the similarity measures on biopsy samples of IBD revealed that the species richness increased from control to noninflamed tissue, and then declined in fully inflamed tissue [17]. Therefore, it was hypothesized that there is a recruitment phase in which potentially pathogenic bacteria colonize tissue, and once the inflammation sets in, a decline in diversity occurs that may be a byproduct of the inflammatory process. It was thus suspected that a better knowledge of the microbial species in the noninflamed tissue, before inflammation sets in, holds the clues to the microbial pathogenesis of IBD. It could therefore be hypothesized that alteration of the bacterial microflora in mucosal inflammation reflects a metabolic imbalance of the complex microbial ecosystem with severe consequences for the mucosal barrier rather than disrupted defense to single microorganism.

Rakoof-Nahonm S., et al, (2004) [18] reported that commensal bacteria were recognised by Toll-like receptors (TLRs) under normal conditions. Interactions of commensal bacteria/bacterial products with those microbial pattern recognition receptors played a critical role in resistance to TLRs under normal conditions. Thus a dysregulated interaction between bacteria and TLR may promote chronic inflammation. Our earlier studies have shown that there is a significant change in the *Lactobacillus* population in a state of amaebiasis when compared with healthy individuals [19]. It has been shown that the biodiversity of this ecological niche remained high during the IBD [6]. *Bifidobacteria* and *Peptostreptococci* have also been implicated in ulcerative colitis [20]. Analysis of the luminal enteric flora, however, has revealed differences in the composition compared to healthy controls. In Crohn's disease, concentrations of *Bacteroides*, *Eubacteria* and *Peptostreptococcus* are increased, whereas *Bifidobacteria* numbers are significantly reduced. Furthermore, in ulcerative colitis, concentrations of facultative anaerobic bacteria increased considerably [21].

### 3.3 Sulphate reducing bacteria

Up to 95 percent of patients with active colitis may harbor SRB [22, 23]. By contrast, up to 50 percent of patients in remission will yield SRB during UC [24]. In addition, the feces of patients with UC have been

shown to have greater than normal levels of SRB and increased levels of sulfate-reducing activity. These observations, coupled with reports of increased fecal hydrogen sulfide (a byproduct of SRB metabolism) in active UC, provide evidence that SRB may play an active role in the pathogenesis of UC. Hydrogen sulfide inhibits the oxidation of butyrate and hence its utilization [25]. Theoretically, the impairment of butyrate metabolism within colonocytes may lead to increased villous atrophy. Increased villous atrophy and crypt cell hyperplasia are characteristic features of both active colitis and pouchitis. The toxic, bacterial metabolite sulfide is implicated in ulcerative colitis. Ulcerative colitis patients taking 5-aminosalicylic acid-containing drugs have lower fecal sulfide levels than those not taking these drugs [26]. Sulfate-reducing bacteria (SRB) belonging to the genus *Desulfovibrio* have been studied extensively in relation to their involvement in the initiation and/or maintenance of UC [23,27] principally through their production of sulphide, which is highly toxic to colonic epithelial cells. However, recent molecular studies have shown that there is little difference in mucosal SRB carriage rates in healthy people and UC patients [28], suggesting that if sulfide is involved in UC, host defects in its detoxication pathways are probably responsible.

### 3.4 Leaky gut

The most important function of the intestinal mucosa is to form a barrier between luminal contents and the interstitium. This intestinal barrier is compromised in a number of intestinal diseases, most notably in IBD [29]. Increased epithelial permeability is not only caused by exogenous factors such as infection; a growing body of evidence suggests that the immune system plays an important role in modulating intestinal permeability. Two cytokines, interferon- $\gamma$  (IFN- $\gamma$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), are found in high levels in intestinal mucosa involved in inflammatory bowel disease [30, 31]. These cytokines have also been found to decrease barrier function of cultured intestinal epithelial monolayers [32-35]. Incubation of intestinal epithelial cell monolayers with both IFN- $\gamma$  and TNF- $\alpha$  leads to reorganization of many tight junction proteins, including ZO-1, junctional adhesion molecule 1, occludin, claudin-1, and claudin-4 [36]. The changes in Paracellular permeability caused by IFN- $\gamma$  and TNF- $\alpha$  are associated with marked increases in myosin light chain phosphorylation and can be reversed using a specific membrane permeant inhibitor of myosin light chain kinase. Hence, a key step in the pathogenesis of inflammatory bowel disease may be myosin light chain kinase activation by IFN- $\gamma$  and TNF- $\alpha$ , leading to intestinal barrier dysfunction [29].

## 4. Abnormal immune responses

Alteration of mucosal and systemic immune responses may play an important role in the pathogenesis of inflammatory bowel disease (IBD). A study carried out by Caradonna et al, (2000) [37] revealed that an impairment of natural immunity exerted by peripheral blood phagocytes and lymphocytes in patients with UC and CD. Colonization of the gut by commensals has a positive effect on the mammalian host [38]. The commensals fight for nutrient and site for attachment on surface of intestinal epithelial cells (IEC) and competitively exclude pathogenic bacteria. Antibacterial products from commensals eg. colicins, also jeopardize the life of pathogens. Gut flora strengthen the monolayer of epithelial cells exposed to various injuries, and provide vitamins and short-chain fatty acids as carbon source for colonic epithelial Cells. These bacteria by metabolizing various food allergen and carcinogen protect IECs. The gut flora also influences gut development and maturation of the mucosal and systemic immune systems [39, 40]. Germ-free mice show reduced expression of enterocytic digestive enzymes, atrophic intestinal vasculature, and enteric nervous system, they also show reduced steady-state activity of the mucosal immune system, with smaller Peyer's patches and lower numbers of intraepithelial lymphocytes [41] and of the systemic immune system, with lower amounts of cytokines and serum antibodies, particularly IgA. Reconstitution of the intestinal flora restores normal immune activation [42, 43]. Patients with Crohn's disease or ulcerative colitis have increased intestinal mucosal secretion of IgG type antibodies against a broad spectrum of commensal bacteria [44].

Immunoinflammatory responses mediated by IgG can damage the intestinal mucosa since, unlike normal IgA responses, they activate the complement and the cascade of inflammatory mediators [45]. Thus,

unrestrained activation of the intestinal immune system by elements of the flora could be a key event in the pathophysiology of inflammatory bowel disease. Matsumoto (2004) demonstrated the specific interaction between GALT(Gut associated lymphoid tissue)-derived CD4<sup>+</sup> αβ T cells and enteric bacteria in the immunopathogenesis of the Crohn's disease-like inflammatory bowel disease (IBD) in SAMP1/Yit mice[46]. The intestinal microflora is a positive health asset that crucially affects development of the normal mucosal immune system. Mucosal immune responses to resident intestinal microflora require precise control and an immuno-sensory capacity for distinguishing commensal bacteria from the pathogenic ones. Increasing evidence indicates that commensals actively dampen “physiological” inflammation normally present in the gut by Inhibition of IκB degradation, manipulation of protein ubiquitination , nuclear export of the NF-κB subunit RelA and transcriptional downregulation of Roc-1 (i.e., subunit of E3-SCF) and of several components of the proteasome, thereby protecting IκBα from degradation [47-49].

Nod1 appears to function in host signaling pathways activated by gram-negative bacterial lipopolysaccharide [50]. This finding strongly suggests that interplay between the gram-negative bacterial flora and intestinal innate immune response is a critical element in the pathogenesis of CD. IBD apparently reflects break of tolerance against the intestinal microbiota. No differences have been identified in immunopathology of the two disease entities except for a more intensive Th1 response in CD and autoimmunity in UC. Various IBD models developed in knockout mice clearly show that the major trigger of local immunological effector mechanisms is the indigenous microbial gut flora [51,52].

In IBD, pro-inflammatory cytokines and chemokines have been detected in elevated amounts in mucosal tissue and/or in peripheral blood, thus suggesting a monocyte /macrophage stimulation by enteric bacteria and/or their constituents (*e.g.* LPS) [53]. From the bulk of data presented, it seems that enteric antigens are able to trigger an exaggerated mucosal immune response in IBD, which may account for the intestinal damage. This hypothesis is also supported by the finding that LPS interacts with TLRs on IECs. In turn, IECs are endowed with antigen processing capacity and interact with T-cells via HLA class I and class II molecules.

#### 4.1 Role of Tumour Necrosis Factor

Tumour necrosis factor (TNF) alpha and TNF-beta are soluble ligands binding to TNF receptors with similar activities; soluble TNF receptors neutralise TNF activity by acting as inhibitors. Experiments carried out by Noguchi et al, 1998 [54] revealed enhanced secretion of TNF-alpha but failure to release enhanced amounts of soluble TNF receptor in lamina propria mononuclear cells of patients with IBD. An imbalance in secretion between TNF and TNF inhibitor may be implicated in the pathogenesis of IBD. Evidence now implicates TNFα in global impairment of intestinal barrier function and may be the link between leaky gut and Chron's disease [55]. This leads to increased uptake of proteins from the lumen and less efficient efflux of foreign substances from the cells, all favouring increased permeation of luminal macromolecules to the lamina propria.

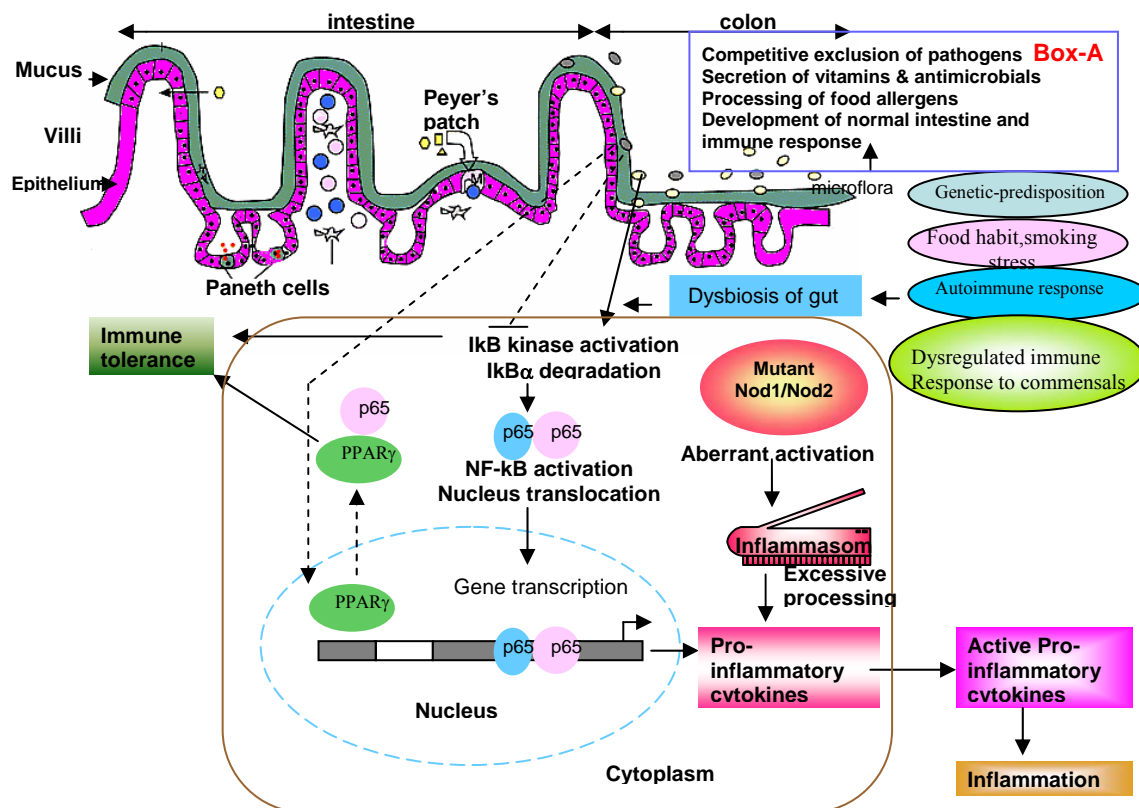
### 5. Genetic make up

Although the causes of inflammatory bowel disease are not yet known, genetic factors certainly play some role. Between 10 - 20% of people with ulcerative colitis have family members with the disease. Several candidate genes and chromosome locations have been identified that might prove to play a role in the development of ulcerative colitis, Crohn's disease, or both. Epidemiologic studies have identified a significant genetic contribution to the etiology of IBD. It is important that even though there are distinct phenotypic differences between CD and UC, studies show that relatives of persons with either CD or UC are at increased risk for developing either form of IBD[56]. This suggests that, although there are phenotype-specific susceptibility loci, some genes will be shared both by patients with CD as well as with UC. One of the most important genetic discoveries to date was the identification of a genetic variant called NOD2, which appears to alter the immune system so that it launches an over-reaction in response to bacteria, causing inflammation. This genetic factor might be involved in 15% of Crohn's disease cases. Those with

one copy of the mutated gene have twice the average risk of developing Crohn's, and those with two defective genes face 20 - 40 times the risk. Several studies have shown that mutations in the LRR region of NOD2 are associated with susceptibility to Crohn's disease [57]. The putative intracellular peptidoglycan receptor NOD2 (CARD15) is a member of the Apaf-1/CARD superfamily and is composed of an N-terminal caspase recruitment domain (CARD), a centrally located nucleotide-binding oligomerization domain (NOD) and 10C-terminal-located leucine-rich repeats (LRRs)[58,59]. NOD2 was found to be expressed in antigen-presenting cells such as monocytes/macrophages, but more recent studies revealed abundant presence of NOD2 in epithelial paneth cells of the small intestine as well as in other epithelial cells [60]. NOD2 has been shown to recognize intracellular peptidoglycan fragments (e.g. muramyl dipeptide, MDP) through its LRR region leading to pro-inflammatory responses through activation of NF- $\kappa$ B. NOD2 serves as an intracellular pattern recognition receptor to enhance host defense by inducing the production of antimicrobial peptides such as human beta-defensin-2. The molecular mechanisms by which mutations in the NOD2 gene cause Crohn's disease are still emerging. However, it is supposed that decreased production of antimicrobial peptides, such as defensins, may promote bacterial-mediated inflammation in Crohn's disease. Recent study demonstrated that NOD2 mutation in CD aggravates NF- $\kappa$ B activity and IL-1 $\beta$  processing, suggesting initiation and/or promotion of mucosal inflammation [61].

It has been shown that genetic variation within a 250-kb haplotype (IBD5) in the 5q31 cytokine gene cluster confers susceptibility to CD and later it was shown that IBD5 may also act as a susceptibility locus for UC. When locus-locus interactions were examined between IBD5 and CARD15 (NOD2), a locus reported to confer risk exclusively to CD, it indicated that the two loci act independently to confer risk to CD but that these two loci may behave in an epistatic fashion to promote the development of UC [62]. Therefore, it was suggested that that IBD5 may act as a general risk factor for IBD, with loci such as CARD15 modifying the clinical characteristics of disease. When CARD15 sequence analysis in a large single-center IBD cohort was carried out in IBD samples [63]. To investigate the impact of different genotypes on disease phenotypes, it was observed that patients homozygous for the CARD15 1007fs (frame shift) mutation had an early disease onset with long-segment ileal stenoses and entero-enteral fistulas. They frequently needed surgical intervention and had a high risk of re-stenosis. Genotyping therefore appears to be an important diagnostic tool in identifying severely affected patients requiring individualized treatment strategies at an early stage of the disease. A highly significant association was observed between Crohn's disease and the IL23R gene on chromosome 1p31, which encodes a subunit of the receptor for the pro-inflammatory cytokine interleukin-23 where an uncommon coding variant (rs11209026, c.1142G>A, p.Arg381Gln) confers strong protection against Crohn's disease, and additional non-coding IL23R variants are found to be independently associated (64)

After evaluating the above issues we propose the following model that will explain the role of various factors involved in modulating the immune responses during IBD state (Figure 1)



**Figure 1** Illustration of the mechanisms by which commensal bacteria limit NF-κB signaling and how environmental factors induce dysbiosis and aberrant activation of inflammatory cytokine genes. Resident microflora prevent the colonisation of intestine by pathogens by the factors described in Box A. In healthy state commensals dampen physiological inflammation mainly by inhibition of IκB degradation, manipulation of protein ubiquitination and nuclear export of NF-κB subunit p65 by PPAR $\gamma$ . But mutation in NLRs (especially in NOD1/NOD2) and some other factors responsible for dysbiosis in gut through activation of NF-κB lead to aberrant immune response & hence inflammation. Dotted lines show the immune-response during healthy state and the solid lines represent the immune-response during dysbiosis. NF-κB, nuclear factor kappa B; NLRs, nod like receptors; PPAR $\gamma$ , peroxisome proliferator-activated receptor; NOD, nucleotide oligomerisation domain.

## 6. Therapeutic interventions

Metronidazole and ciprofloxacin selectively treat colonic Crohn disease, but not ulcerative colitis or ileal Crohn disease, and may prevent recurrence of postoperative Crohn disease. Certain probiotic species decrease relapse of ulcerative colitis and chronic pouchitis and delay onset of pouchitis [65]. A recent review suggests that, both antibiotics and probiotics appear to play a beneficial role in the treatment and prevention of pouchitis and further trials are warranted to fully quantify their clinical efficacy [66].

### 6.1 Probiotics in IBD treatment

An increasing number of novel and alternative therapeutic approaches are in progress [67]. New biologic therapies include the targeting of pro-inflammatory cytokines, enhancement or infusion of anti-inflammatory cytokines, blocking intravascular adhesion molecules, and modifying T-cell functions. Recently, therapeutic approaches to modifying intestinal microflora have been attempted by using prebiotics and probiotics. In addition, antibiotic therapies continue to be used [68,69]. The normal intestinal flora and

the mucosal immune system exist in close spatial proximity. An abnormal host response to the normal intestinal flora leads to chronic intestinal inflammation. Probiotic bacteria may modulate the intestinal flora and the mucosal immune response and are an effective therapy for remission maintenance of ulcerative colitis and pouchitis. Preliminary studies suggest that administration of probiotics may be benefit for experimental colitis and clinical trials for IBD. Introduction of probiotics can balance the aberrant enteric microflora in IBD patients, and reinforce the various lines of intestinal defense by inhibiting microbial pathogens growth, increasing intestinal epithelial tight junction and permeability, modulating immune response of intestinal epithelia and mucosal immune cells, secreting

**Table 1:** Summary of results showing beneficial effect of Probiotic in IBD

Probiotic	No. of Patient	Type of disease	Final effect	Reference
<i>E.coli</i> Nissle 1917	327	UC	Induction of remission	Kruis 2001[75]
	24	CD	Maintaining the remission	Malchow 1997[76]
VSL#3	32	UC	Induction of remission	Bibiloni,R.2005[77]
	20	CD	Very similar to measalamine preventing recurrence	Campieri 2000[78]
	20,20,20	Pouchitis	Remission time increased (p<0.005,0.0001)	Gionchetti 2000[79], Gionchetti 2003[80], Mimura 2004[81],
<i>Lactobacillus</i> GG	65,5	UC	No difference in remission rate combination of L.GG with measlamine gives better results than measlamine alone	Zocco 2006[82], Shultz 2004[83].
	23,39	CD	No difference in remission rates	Prantera 2002[84], Bousvar 2005[85]
	10	Pouchitis	No difference in pouchitis disease activity	Kuisma 2003[86]
<i>Lactobacillus johnsonii</i> LA1	48	CD	No difference in recurrence	Marteau 2006[87]
<i>Bifidobacterium</i> fermented milk	10	UC	Decrease in severity of disease	Kato 2004[88]
<i>Lactobacillus</i> & <i>Bifidobacterium</i> fermented milk	11	UC	Reduction in severity of symptoms	Ishikawa 2003[89]

antimicrobial products, decomposing luminal pathogenic antigens [70]. Probiotic therapies have attempted to modify disease expression by favourably altering bacterial composition, immune status, and inflammation [71].

A synbiotic comprising a probiotic (*Bifidobacterium longum*) isolated from healthy rectal mucosa combined with a prebiotic (oligofructose-enriched inulin - Synergy 1) was developed. Results demonstrated that short-term synbiotic treatment resulted in increased bifidobacterial colonization of the rectal mucosa and induced significant reductions in the expression of molecules that control inflammation in active UC. Steidler et al 2000 observed an inhibition of spontaneous colitis development in IL-10 knockout mice that was mediated by relatively low concentrations of the *Lactococcus*-borne cytokine. These experiments provide the basis for the use of genetically modified organisms designed for delivery of biologically relevant therapeutic molecules [72]. Potential mechanisms of probiotic action include competitive interactions, production of antimicrobial metabolites, influences on the epithelium, and immune modulation [73,74]. However, such changes may be transient, and therefore the implantation of exogenous bacteria has a limited usefulness at present. Restoring the microbial balance using probiotics may be the most physiologic



and non-toxic way to prevent and treat IBD. The possible mechanism of probiotics modulating mucosal immune response in IBD is by down regulating proinflammatory cytokines secretion. This is mediated by 1) Inhibiting NF- $\kappa$ B activation 2) Modulating intestinal apical di-/tripeptide transporter (PepT1) responsible for the uptake of a broad array of small peptides derived from muramyl dipeptide (MDP). 3) Reducing the number of CD4 intraepithelial lymphocytes 4) Regulating anti-inflammatory effect via TLR9 signaling pathway 5) modulating apoptosis and proliferation of immune cell by TLR2 signalling 6) Modulating peroxisome proliferators activated receptor (PPAR) pathway [69].

Though clinical trials so far conducted (table-1) have not demonstrated a robust effect of these agents in IBD. The clearest demonstration of benefit has been with a preparation known as VSL#3 in the treatment and prophylaxis of pouchitis occurring in ileal pouches created as curative therapy for ulcerative colitis. The available studies do not support the use of antibiotics in ulcerative colitis (UC). Antibiotics are effective in treating septic complications of Crohn's disease (CD) but their use as a primary therapy is more controversial, although this approach is frequently and successfully adopted in clinical practice. There is evidence that probiotic therapy may be effective in the prevention and treatment of mild to moderate UC. In contrast, a lack of successful study data at present precludes the widespread use of probiotics in the treatment of CD.

## 7. Applications of molecular techniques in IBD research

Molecular techniques are giving us better insight into the gut microbiota in inflammatory bowel disease that should translate into improved therapies. Today molecular techniques such as PCR amplification, cloning and sequencing of 16S rRNA genes, Denaturing gradient gel electrophoresis(DGGE), Temperature gradient gel electrophoresis (TGGE) analysis, Temporal temperature gradient gel electrophoresis (TTGE), Terminal-restriction fragment length polymorphism (T-RFLP) and fluorescence in situ hybridization (FISH) provide suitable tools for the culture-independent detection and identification of bacteria in complex microbial communities. In a study addressing the composition of the mucosa-associated bacterial flora in colon samples from interleukin-2-deficient mice that developed colitis, investigation was carried out by comparative 16S ribosomal DNA (rDNA) sequence analysis and fluorescence in situ hybridization using rRNA-targeted fluorescent probes to quantify the bacterial populations of the mucosa-associated flora. The investigations revealed distinct differences in the bacterial composition of the mucosa-associated flora between interleukin-2-deficient mice and healthy controls [90].

### 7.1 DGGE and TGGE

In DGGE as well as in TGGE DNA fragments of the same length but with different sequences can be separated. Genomic DNA from biopsy or fecal samples from IBD patients are extracted and subjected to quantitative dot blot hybridisation with six radiolabelled 16S ribosomal ribonucleic acid (rRNA) targeting oligonucleotide probes to measure the proportions of rRNA corresponding to each phylogenetic group [91]. Using colonic biopsies of IBD patients, a loss of bacterial diversity of the mucosal microbiota were observed in a large cohort of patients with IBD applying metagenomic approach where the taxonomical classification of metagenomic fragments is mainly based on 16S rDNA anchor genes [92]. Temporal temperature gradient gel electrophoresis (TTGE) of 16S rDNA was used to evaluate dominant species diversity. Temporal temperature gradient gel electrophoresis (TTGE) of rDNA can be used to evaluate dominant species diversity TTGE profiles subsequently compared using software that measures the degree of similarity [93].

### 7.2 T-RFLP

Terminal restriction fragment length polymorphism (T-RFLP) analysis is a powerful tool to assess the diversity of complexed microbiota. This permits rapid comparison of microbiota from many samples. T-RFLP analysis of the fecal microbiota showed that the diversity of fecal microbiota in patients with UC was different from that in healthy individuals. Unclassified bacteria, as well as known bacteria, can contribute to

alterations in the bacterial diversity of UC patients. The T-RFLP patterns show differences between the active patients and inactive (remission) patients [94]. Automated ribosomal intergenic spacer analysis (ARISA) and terminal restriction fragment length polymorphisms (T-RFLP) were used as molecular tools to investigate the intestinal microbiota from biopsy samples of UC and CD patients where clustering of organisms could be observed based on the inflammation criteria, the majority of biopsies grouped either into inflamed or noninflamed groups [95]. Fluorescently labeled primer for amplification of the 16S rRNA genes are used. This is followed by restriction enzyme digestion; the fluorescent terminal restriction fragments (TRFs) can be visualized and registered by an automated sequencing apparatus. In principle, each TRF corresponds to an individual population in the community, and the peak area corresponds to the abundance of that population. Also, T-RFLP is useful as a quantitative technique to assess changes in microbial communities since the relative abundance of a specific population in a community can be easily compared for different treatments or for different sampling periods [96,97].

### 7.3 FISH

FISH is being increasingly used to study the bacterial composition of the GI tract, and probes have been developed to quantify bacteria belonging to various genera including *Bacteroides*, *Bifidobacterium*, *Streptococcus*, *Lactobacillus*, *Collinsella*, *Eubacterium*, *Fusobacterium*, *Clostridium*, *Veillonella*, *Fibrobacter*, and *Ruminococcus* [98-103]. To facilitate enumeration, FISH has been automated and combined with computerized image analysis. Fluorescent in situ hybridization adapted to flow cytometry was successfully used to analyze the bacterial composition of fecal samples from 13 patients with active CD, 13 patients with active UC, 5 patients with infectious colitis and 13 healthy subjects using 6 group-specific probes targeting 16S rRNA and spanning the main phylogenetic groups of the fecal microbiota [104]. Disadvantages of FISH are that it is dependent on SSU rDNA sequences available in the databases, and that only a few probes can be used per analysis. In addition, FISH is dependent on the permeability of the bacterial cell, the accessibility of the target, and the number of ribosomes per cell.

### 7.4 Real-Time PCR

Real-time PCR with species-specific probes can provide an accurate and sensitive method for quantification of individual species and bacterial populations as well as total bacteria. The use of real-time quantitative PCR (5' nuclease PCR assay) as a tool to study the gastrointestinal microflora that adheres to the colonic mucosa was evaluated [105]. A set of 20 specific molecular probes for detecting the most frequent bacteria of the human gastrointestinal tract and one universal probe detecting the total number of bacteria were designed and optimized [106]. Sawa et Al (2003) [107] analyzed colonic mRNA levels of various cytokines by real-time quantitative polymerase reaction (PCR) to evaluate the comprehensive profile of mucosal cytokines, in order to examine the role of these cytokines in the pathogenesis of IBD. However, concerns are frequently raised, as real-time PCR reproducibility is strongly affected by RNA quality. To obtain a practical approach for clinical usage of real-time PCR in quantifying TNF- $\alpha$  gene expression in inflammatory bowel tissues, calibrators have been constructed by purifying a conventional PCR product that contains the target gene sequence. Calibrators were constructed using dsDNAs that were obtained via the amplification of single-stranded cDNA in conventional PCR. This simple method could be adjusted for clinical use in quantifying cytokines in inflammatory specimens [108].

### 7.5 Microarray

Identification of factors involved in the initiation, amplification, and perpetuation of the chronic immune response and the identification of markers for the characterization of patient subgroups remain critical objectives for ongoing research in inflammatory bowel disease (IBD). Applied to clinical specimens from affected and normal individuals, this methodology has the potential to provide a new level of information about disease pathogenesis not previously possible. Both cDNA and oligonucleotide arrays are interrogated

by hybridization with a fluorescent-labeled cDNA or cRNA representation of the original tissue mRNA. That enabled measurement of the expression levels for thousands of mucosal genes in a single experiment [109]. The expression array data supported a number of widely held concepts about IBD. In particular, the results confirmed increases in a number of mRNA transcripts that previously have been associated with UC. IL-1, IL-1ra, and IL-8 each demonstrated significant overexpression. Distinctions could be drawn between UC and CD along a number of gene expression groupings.

To elucidate the biological dysregulation underlying two forms of inflammatory bowel disease (IBD), ulcerative colitis (UC) and Crohn's disease (CD), global gene expression profiles of inflamed colonic tissue were examined using DNA microarrays [110]. Overall, significant differences in the expression profiles of 170 genes identified UC and CD as distinct molecular entities. The genomic map locations of the dysregulated genes may identify novel candidates for UC and CD genetic susceptibility. The relative expression of 78 cytokines, growth factors, and soluble receptors was determined using proprietary antibody-based protein microarrays amplified by rolling circle amplification in patients with UC and CD during clinical remission [111]. Despite the power of the DNA array method, there is reason for caution. An expansive molecular screen for overexpressed mRNA transcripts frequently may identify secondary changes rather than pathogenic abnormalities. The lack of specificity for the underlying disease may place many overexpressed transcripts into an mRNA equivalent of an acute phase reactant. Yet, combined with knowledge of the molecular pathogenesis gained from a variety of other methods, DNA arrays provide an important means for reexamining previously described disease mechanisms and for looking at new pathogenic pathways.

## 8. Conclusion

Despite inflammatory bowel disease (IBD) is a multi-factorial disease, it is generally assumed that damage of the intestinal barrier results in inappropriate stimulation of the immune system by the endogenous intestinal microflora thus promoting and nourishing the inflammatory process. The knowledge of the distribution of microorganisms isolated from the gut of patients with IBD could be helpful not only to identify antibiotic targets, but also to obtain a therapeutic manipulation of the gut flora by probiotic or prebiotic strategies. Guarner et al, (2006) [112] have discussed how a reduced prevalence of organisms that have been part of human microecology for millennia (including saprophytic mycobacteria, bifidobacteria, lactobacilli, and helminths) and cause little, if any, harm to the host, might explain the increased prevalence of immune-mediated disorders in westernized countries. In summary, despite considerable effort including sensitive PCR-based studies to screen for the presence of microbes, no one particular infectious organism has been definitively associated with IBD. Still, the possibility exists that an as yet unidentified organism that is difficult to detect by current methods is the cause of IBD. Normal, nonpathogenic enteric bacteria induce and perpetuate chronic intestinal inflammation in genetically susceptible hosts with defective immunoregulation, bacterial clearance, or mucosal barrier function. Altering the composition and decreasing mucosal adherence/invasion of commensal bacteria with antibiotics, probiotics, and prebiotics can potentially prevent and treat Crohn's disease, pouchitis, and possibly ulcerative colitis, but optimal treatments have not yet been identified. Identification of multiple susceptibility loci, coupled with an understanding of how these loci interact, may aid in the development of a molecular taxonomy of IBD to improved treatment and outcome predictions in the disease. Perturbation of a tightly controlled cytokine network, with abnormal crosstalk between several mucosal cell types, seems to be an important step in a progressive immunopathological drive of chronic inflammatory mucosal diseases in general. Although no specific bacterium has been singled out as involved in the pathogenesis of IBD, an imbalance between protective and harmful bacteria ("dysbiosis"), has been postulated as a proinflammatory mechanism both in ulcerative colitis and in Crohn's disease. Manipulation of enteric flora by means of either antibacterial agents or probiotics represents a recognized therapeutic measure in ulcerative colitis and Crohn's disease that warrants for more research activities.

## 9. Future prospects

We propose that future research in this area should be directed to resolve the following issues

1. Identification of unique host related events underlying IBD pathogenesis and definition of specific patient sub sets.
2. Identification and manipulation of positive and negative regulators of mucosal responsiveness to prevent or treat IBD.
3. Developments of methods that block proinflammatory and enhance anti-inflammatory cytokines.
4. Acquisition of increased understanding of intestinal microbiota and the host responses they evoke that will help to differentiate the 'protective' and 'pathogenic' bacterial strains for manipulations to prevent and treat IBD.
5. Development of improved methods of gene transfer and delivery of therapeutic macromolecules to mucosal surfaces so that therapies can be targeted to the sites of tissue inflammation and thus minimize the toxicity to non-inflamed tissues.
6. Development of pharmacologic, immunologic and genetic strategies that enhance Intestinal epithelial cell barrier and function and allow its restitution following IBD.

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