

## Chapter 2

# Microbes in Colon Cancer and Inflammatory Bowel Disease

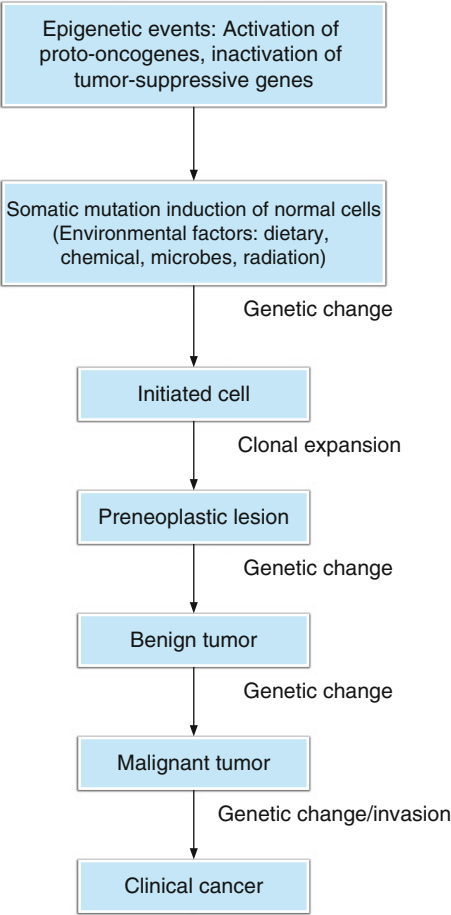
### 2.1 Introduction

Colorectal cancer [CRC] is the third most common tumor and fourth most common cause of cancer death in the world [1]. It is prevalent in many countries but more common in developed nations. In the United States [US] CRC estimated new cases in 2008 were 77,250 in men and 71,560 in women [1]. The higher incidence of CRC in developed countries has been attributed to dietary and lifestyle habits, and the influence of genetic predisposition.

#### 2.1.1 Risk Factors

The vast majority of CRC occur in the population over age 50 and continue to increase with further aging. The highest incidence and mortality in the US has been found in people of African American ethnicity when compared to other ethnic populations [1]. Genetic factors likely play a strong role, as a family history of CRC in a first-degree relative increases the risk of CRC by two- to threefold, and even second-degree relatives increases the risk by 25–50 % over the general population [1]. Genetic and environmental factors are associated with the development of CRC, see Fig. 2.1 [2]. Three major categories of genes have been implicated in the development of CRC, such as K-ras [retrovirus-associated DNA sequence], tumor suppressive genes, and the mismatch repair genes [1]. Mutations or alterations of the tumor suppressive T53 gene are present in up to 75–85 % of CRC [2, 3]. The transition from normal to malignant colonic mucosa involves a multistep cascade of genetic mutations involving the deleted in colorectal cancer [DCC] gene and the T53 suppressor genes, and the K-ras oncogene mutation leading to tumor formation [47–50 %] [1, 2].

**Fig. 2.1** Multistage development of colon cancer



It is generally believed that westernized dietary habits, including increased consumption of red meat and fat with decreased consumption of vegetables and fruits, is largely responsible for the increased rates of CRC in developed nations. There is conflicting data on the protective effect of fruits and vegetables on the development of CRC, while high consumption of these natural products may not directly protect against CRC, low consumption is related to increased risk of CRC [1].

During the progression of normal epithelial cells to CRC there is a succession of clonal expansions and loss of control or inhibition of cell division. Changes in colonic crypt cell proliferation have been shown to proceed and accompany neoplasia. Ingredients in the diet that stimulate cells to divide are vulnerable to effects of carcinogens to promote cancer, and dietary factors that enhance differentiation or apoptosis protect against CRC. The contents of fiber in the human diet consist of

soluble fibers, more readily fermented by colonic bacteria, and insoluble fibers [wheat bran, cellulose, etc.] which remain in the fecal stream as diluents, are believed to be important in the tumorigenesis of CRC. Insoluble fibers create bulk and greater moisture content that decrease transit time in the colon and may have a diluting effect on carcinogens and their constant exposure to epithelial cells [4]. Soluble fibers can be metabolized by the intestinal bacterial flora and these metabolic by-products may influence carcinogenesis. Inflammatory bowel diseases [IBD], such as ulcerative colitis [UC] and Crohn's disease [CD], are well-known risk factors for CRC. The extent and duration of the disease are directly correlated to the risk of CRC. There is greater risk of CRC for pancolitis compared to left-sided colitis [5- to 15-fold increase versus threefold increase], and the risk increases from 2 to 8 % at 10–20 years versus 18 % after 30 years of UC [1]. Obesity has also been shown to be associated with increased risk of CRC and increase in physical activity may be protective [5]. However, the exact mechanisms for carcinogenesis and the association with these conditions are not exactly clear. IBD are putatively linked to CRC through the inflammatory cascade involving proinflammatory cytokines, stimulation of the cyclooxygenase, and prostaglandin pathways. This has led to the potential use of aspirin and other nonsteroidal anti-inflammatory drugs [NSAIDS] as preventative agents for CRC, through their inhibitory effect on the cyclooxygenase pathway [6].

It is unclear at present whether the association of obesity and increased risk of colonic adenomas and CRC can be explained alone on dietary factors, consumption of foods with higher levels of sugar and saturated fats. Visceral adiposity is a stronger risk factor for CRC than increased body mass index alone [7]. There are several elements that are secondarily increased in obesity that have been proposed to participate in the pathogenesis of CRC. Chronic low-grade inflammation with persistent activation of the nuclear transcription factor NK-kB may result in transcription of genes that promote tumorigenesis in visceral adiposity [8]. Hyperinsulinemia, insulin resistance, and insulin growth factor are increased in the metabolic syndrome and obesity, and all of these factors may promote tumorigenesis by increasing colonic cell proliferation and angiogenesis and inhibition of epithelial cell apoptosis, as shown in cell lines and animal models [9].

Lifestyle risk factors for CRC are also associated with obesity and increased risk of cardiovascular disease such as physical inactivity, smoking, and excessive alcohol [9]. Despite the close relationship with physical inactivity and obesity, association remains strong for CRC even after adjustment for age, diet, and obesity [10]. Smoking has been reported to increase the relative risk of CRC in observational studies [10], possibly by inducing genetic alterations in the colonic epithelium [11], but there was insufficient evidence for a causal relationship [9]. Heavy alcohol consumption has been associated with increased relative risk [RR 1.41] of CRC from the pooled analysis of eight cohort studies [12]. This observation may be due to poor nutritious diet in alcoholics, especially to the low intake of folate-containing foods [13].

## 2.2 Microbes and Colorectal Cancer

There has been increasing evidence over the past two decades or more that the intestinal commensal flora is very important in maintaining a regulated immune homeostasis of the gastrointestinal [GI] tract. Moreover, dysregulation of the normal balance or symbiotic relationship of the microbiota may play a role in the pathogenesis of IBD and colon cancer. Preliminary small case series using routine microbiological methods had suggested differences in bacterial composition and metabolites in feces of patients with CRC compared to healthy controls in 1996 [14]. Probably the most convincing evidence to date of a role of microbes in the pathogenesis of bowel cancer is demonstrable in animal models. There are also precedent, as GI cancers such as gastric cancer and mucosa-associated lymphoma of the stomach are strongly associated or established to be secondary to chronic *Helicobacter pylori* infection in at risk individuals.

### 2.2.1 Animal Models of Colorectal Cancer

Genetically modified mice with deletion of genes to regulate inflammation or modify innate immune responses are susceptible to multiple neoplasms of the colon in the presence of normal GI flora, but fail to develop tumors in germ-free animals with the same defects. This was demonstrated in T-cell receptor chain and P53 double-knockout mice, with conventional GI flora colonized mice developing ileocecal adenocarcinoma in 70 % of animals at 4 months of age versus none in germ-free mice [15]. Specific murine enteric pathogens that produce epithelial inflammation have also been shown to promote colonic tumors in mice with mutation in tumor suppressive gene, but a fourfold decrease in similar mice without infection [16]. Others have also shown that the specific murine pathogen *Helicobacter hepaticus* will induce colon cancer in 50–60 % of mice deficient in transforming growth factor-beta [TGF- $\beta$ ] signaling pathway [SMAD-3], but not in those without infection [17]. In a pathogenesis study using the same bacteria to induce inflammation and neoplastic changes, it was found that the regulator T-cells [RTC] require interleukin [IL]-10 to inhibit inflammation and early neoplastic changes [18].

Studies in rats, utilizing chemical carcinogens [1,2-dimethyl-hydrazine] to induce early cellular neoplastic changes with aberrant crypt foci, could be influenced by specific species of intestinal flora, and some bacteria might behave as promoters and others as antipromoters in carcinogenesis [19]. For instance, *Bifidobacterium breve* inoculated orally to gnotobiotic rats had lower rates of aberrant crypt foci than with other bacteria after treatment with a carcinogen. Similarly, in male Sprague–Dawley rats injected with azoxymethane [ADM] carcinogen or saline development of colonic tumors fed different fiber diets had different rates of neoplasm associated with different intestinal bacterial population [20]. Rats receiving ADM consuming high cellulose diet and not developing tumors possessed larger amounts of anaerobes in their feces at 10 months [ $p < 0.05$ ].

Investigators utilizing human-flora rats fed high-risk diet [high in fat, sucrose, low in calcium and fiber] compared to rats on low-risk diet [low in fat, high in starch, calcium, and fiber] found significant changes in gut microflora and associated biomarkers of colon cancer [21]. Rats fed high-risk diet had significant altered cecal bacteria, with 2.5-fold increase in beta-glucuronidase activity, increased cecal ammonia concentration, and enhanced genotoxic risk from 7-hydroxy-imidazole quinolone, 3 putative biomarkers of colon cancer. Review of the pathogenic mechanisms in colon cancer about the same time [1997] concluded that CRC was caused by increased mutagenic actions of free radicals produced during oxidation reaction, and that dietary factors and intestinal bacteria produce endogenous metabolites that contribute to free radicals in the colon [22]. It is believed that polyunsaturated fat can be oxidized in the bowel by bacteria to produce mutagens [lipid hydroperoxides and malondialdehyde], and that fecal bacteria can generate high flux of reactive oxygen species [superoxide radicals] on the surface of the intestinal mucosa, and inflammatory cells in the colon can produce reactive nitrogen species [nitrogen dioxide]. Theoretically diets rich in antioxidants [i.e. vitamin E] can reduce these harmful effects.

Further studies in gnotobiotic mice treated with chemical carcinogens, 1,2-dimethyl-hydrazine [DMH], to induce tumors have examined the effect of specific bacteria [mono-associated GI colonization] compared with conventional mice treated with DMH [23]. The incidence of colonic adenomas between gnotobiotic and conventional mice after treatment with DMH was similar [74 and 69 %], but the tumors were larger in the conventional mice. The incidence of tumors in gnotobiotic mice with single GI colonization of different bacteria was similar for *Mitsoukella multacida*, *Clostridium butyrican*, and *Bifidobacterium longum* [63–68 %] and significantly lower for colonization with *Lactobacillus acidophilus* [30 %]. Clostridia colonization was associated with larger adenomas and significantly higher concentration of fecal bile acids, whereas *L. acidophilus* was associated with significantly lower levels of bile acids [23]. A subsequent study was performed by the same group of investigators to examine the changes in the immunological environment in gnotobiotic mice with single species of bacterial GI colonization without DMH treatment [24]. These findings suggested that activation of T-cells in the liver and granulocytes in the colonic mucosa may be related to the antineoplastic effect of *L. acidophilus* in this model.

Other investigators have reported that *B. longum*, lactic acid producing intestinal bacteria, fed to male F34C-rats as a probiotic compared to control diet in ADM-treated rats reduced tumor burden and exerted strong antitumor activity [25]. The probiotic inhibited ADM-induced cell proliferation, oncogenic activity, and expression of ras-p21 oncoprotein compared to control diet. Subsequent studies on the impact of probiotics on microbial flora, inflammation, and tumor development have been performed on IL-10 knockout C57BL/6 mice [26]. Twenty-one mice were fed *Lactobacillus salivarius* in milk compared to 10 control mice, fed a modified milk for 16 weeks. Two of 10 [20 %] control animals died of fulminant colitis versus none in the probiotic group. Fifty percent [5 of 10] control mice develop colonic

neoplasms versus just less than 10 % [2 of 21] in the treated group. Fecal coliforms and enterococcus species were significantly decreased in the probiotic versus control mice [ $p < 0.05$ ]. Also at sacrifice there was significantly decreased amount of *Clostridium perfringens* [ $p < 0.05$ ] in the bowel of the treated group. Thus, probiotic significantly reduced inflammation and colon cancer in IL-10 deficient mice [26]. Others have also noted recently that *Bifidobacterium lactis* and resistant starch products had combined effect in protection against CRC in rat model induced by AOM [27].

### 2.2.2 Mechanisms of Probiotics and Favorable Commensal Bacteria

The protective effect of favorable commensal bacteria and probiotics on modifying carcinogenesis in the bowel has been examined both in vitro using human intestinal cell lines and animal models. In vitro probiotics can ameliorate expression of Cox-2 and prostaglandin E-2 secretion in intestinal epithelial cells, which are considered important in the inflammatory cascade for tumor development [28]. The combination of probiotic and resistant starch may also facilitate apoptotic deletion of carcinogen damage cells, as demonstrated in the rodent model [29]. Apoptosis provides an innate cellular defense against oncogenesis by removing cells with genetic instability or with DNA mutation or damage from carcinogens in the process of carcinogenesis [30]. The probiotic may act via fermentation of resistant starch to produce butyrate and together exert an immunomodulating effect [31]. More recent investigation in both mice and colonic cancer lines supports the paradigm that bacterial fermentation of dietary fiber in the colon generates short-chain fatty acids which protects against some CRC and IBD [32]. Among the bacterial metabolites butyrate appears to be the most important. GPR109A, a G-protein-couple to receptor for nicotinate but recognizes butyrate at low affinity and function as a tumor suppressor in colon [32]. Others, also using experimental models, have concluded that probiotic and favorable commensal bacteria function as “physiologic cancer surveillance” by preventing proliferation of dysplastic cells by induction of apoptosis [33].

Some probiotics may have different mechanisms of antineoplastic effect, which may be species dependent. Recently, studies utilizing human colon cancer cells and xenograft model [CD-1 nude mice] of human colon cancer determined that *Bacillus polymycticus* [commercially available probiotic bacterium] anticancer effect was mediated by inhibition of proto-oncogene ErbB2 and ErbB3 protein expression [34]. The ErbB receptor family consists of four members including EerB1/epidermal growth factor receptor [EG FR/HER1, ErbB2/HER2/, Neu, ErbB3/HER-3, and ErbB4/H ER-4]. ErbB2 is the most oncogenic member of the family and overexpression is observed in many human cancers, including breast, colon, bladder, and lung cancers [35].

### 2.2.3 Harmful Effects of Some Commensal Bacteria

Some commensal enteric bacteria are cancer promoters as shown in animal models of germ-free and gnotobiotic mice colonized with specific bacteria. Infection-associated inflammation has been well established as risk factors for cancers in various organs, i.e., chronic hepatitis B and C predispose to hepatocellular carcinoma. Genetic modified mice that develop greater burden of CRC with specific murine pathogens is through the induction of inflammation and colitis, e.g., *Helicobacter hepaticus* and *Citrobacter rodentium*. However, the human intestinal commensal *Enterococcus faecalis* can induce colitis and colonic tumors in IL-10-knockout mice in mono-microbe associated model, but other commensal and pathogenic bacteria and yeast failed to produce any intestinal pathology [36]. *E. faecalis* can induce chromosomal changes in colonic epithelial cells that may predispose to neoplastic changes [37]. This commensal can cause DNA damage and instability, potentially transforming events and tumorigenesis analogous to radiation-induced effect [37].

Other human bowel commensal universally present, such as *Bacteroides fragilis*, has been shown to induce colonic tumors in multiple intestinal neoplasia [MIN] mice [38]. However, only enterotoxigenic strains of *B. fragilis* [ETBF] induce robust selective colonic signal transducer and activator of transcription-3 [STAT-3] activation of T-helper-type-17-cell response and can trigger colitis and tumors [38]. It was also found that ETBF tumorigenesis in MIN mice is through the contribution of polyamine catabolism [39]. These animal models have also demonstrated the importance of the natural immune defense mechanisms to counteract the harmful effects of some commensal and pathogenic microbes. Immunocompetent regulatory T-cells are important in preventing pathology and remodeling of intestinal mucosa following tumorigenic microbial insults [40]. The anti-inflammatory cytokine pathway is also important to counteract inflammation-induced tumors, as evident in the IL-10 knockout mice model [18]. The innate immune receptor Nod1 also appears to play protective role in the intestine from inflammation-induced tumorigenesis [41].

In vitro experiments have also demonstrated that some species of commensals including *Lactobacillus*, *Streptococcus*, and *enterococcus* species can generate hydrogen peroxide [ $H_2O_2$ ] [42]. The strong influx of  $H_2O_2$  leads to stimulation of immune cells to produce proinflammatory cytokines which may predispose to IBD, by perpetuating the inflammatory reaction and increasing apoptosis and necrosis [43]. Although apoptosis plays a favorable regulatory role in controlling tumorigenesis, protracted apoptosis in IBD subjects may cause disruption of the epithelial integrity and possibly impair healing of the mucosa that could predispose to neoplasm [43]. Some commensal bacteria may also convert dietary procarcinogens into DNA damaging chemicals, i.e., ethanol and heterocyclic amines, or directly produce carcinogens such as fecapentaenes [43]. Many colonic commensals express alcoholic dehydrogenase [ADH] that can convert sugars to ethanol by the fermentation process. In the presence of excessive alcohol intake even by moderate alcohol consumption, the microbial ADH activity can be reversed and lead to aldehyde production [44]. Aldehyde is a known carcinogen that promotes mutagenesis by

inactivating cellular proteins important in DNA repair. Fecapentaenes are a family of ether-linked polysaturated lipid with potent mutagenic effect [45]. Fecapentaenes are produced by *Bacteroides* species at detectable concentration in the bowel and may produce oxidative damage to DNA by generation of radicals [44].

Endogenous reactive oxygen radicals that damage DNA are considered an important mechanism for somatic mutations that give rise to cancer [46]. The most important reactive oxygen species are superoxide, hydrogen peroxide, hydroxyl radical, and peroxynitrite. Several of these reactive oxygen species can damage DNA, but  $H_2O_2$  is the only one stable enough to diffuse into cells where hydroxyl radicals can be generated [47]. Abundant hydroxyl radicals production occur in normal feces, especially in diet rich in fat and poor in fiber, and likely produced by certain commensal bacteria, i.e., *E. faecalis* [48–51]. Sulfate reducing bacteria are members of the normal colonic flora that use sulfate as an oxidant for the degradation of organic matter and produce hydrogen sulfide [ $H_2S$ ]. Biochemical and functional genomic data suggest that  $H_2S$  may impair the balance between cell differentiation, proliferation, and apoptosis of the intestinal epithelium [52].  $H_2S$  may be tumor promoting as it is directly or indirectly involved in the signaling and upregulation of genes involved in the mitogenic activated protein kinase [MAPK] signaling [52] and the oncogenic activation of *Ras* pathway [*Ras*/Raf/MEK/ERK] [53]. There is also evidence that  $H_2S$  stimulates nitric oxide [NO] production of intestinal epithelial cells [44] that have variable mitogenic and apoptotic elements, and activate several neoplastic-associated genes including vascular endothelial growth factor [VEGF], which plays a role in tumor progression and metastases [52, 54]. Most of the colonic sulfidogenic bacteria are gram-negative bacteria in the delta subdivision of the Proteobacteria phylum, but others such as *Desulfotomaculum* are grouped with gram-positive bacteria of the Clostridium subdivision [44].

## 2.3 Human Studies

There are only a few studies of the potential role of microbes in the tumorigenesis of CRC in humans. In a relatively small study of randomly selected subjects, ages 50–65 years, three groups were identified: African Americans [ $n=17$ ], native Africans [ $n=18$ ], and Caucasian Americans [ $n=17$ ] [55]. Comparisons were made for diet, hydrogen and methane breath responses to oral lactulose, culture of fecal samples for 7- $\alpha$  hydroxylating bacteria and *Lactobacillus plantarium*, and mucosal biopsies of colon to measure cell proliferating rates. The aim of the study was to identify factors that predispose African Americans to greater risk of CRC [60 per 100,000] versus native Africans [less than 1 per 100,000]. Similar to Caucasian Americans, the African Americans [AA] consume more meat protein, total fat and saturated fat, cholesterol, vitamins A and C than native Africans [NA],  $p<0.05$ –0.01 [55]. However, fiber intake was about the same, but hydrogen breath test was higher and methane breath test lower in AA compared to NA. Fecal colony counts of 7- $\alpha$  hydroxylating bacteria were higher and *L. plantarium* lower in AA versus NA.



The colony crypts cell proliferation rates [reflecting propensity for neoplastic changes] were dramatically increased in AA versus NA,  $p < 0.001$ . The conclusion of the investigators was that higher animal products in diet increase potentially toxic hydrogen and by bile salt reducing bacteria that predisposed to CRC [55]. The limitations of this study included small sample size, lack of molecular microbiology to assess differences in ratios or quantization of various phyla of the bowel microbiome, and lack of chemical analysis for putative carcinogens associated with differences in the intestinal microbiota profile.

In a previous study of only 13 male patients with recurrent neoplasia of the colon following surgery compared to 14 healthy males of similar age,  $H_2S$  concentrations were significantly higher secondary to sulfate reducing bacteria [56]. However, the design of the study cannot exclude an effect of alteration in bowel microbiota from previous surgery or inflammation. A recent study examined the concentration of mucosal bacteria in 51 patients, by analysis of colonic biopsies taken from adenoma polyps and normal mucosa [57]. There was a 20-fold reduction of mucosa-adherent bacteria from polyps [associated with overproduction of antibacterial molecules  $\alpha$ -defensins] compared to normal mucosa. The authors postulated that microflora dysbiosis at the mucosal surface in colonic adenomas may be a potential factor for dysplastic cell proliferation [57]. The cause-and-effect relationship still remains unknown and larger prospective studies are needed. Conversely, a previous clinical study had reported increased mucosal adherence and invasion by *Escherichia coli* in patients with colon cancer [ $n=21$ ] and in Crohn's disease [ $n=14$ ], but not in ulcerative colitis [ $n=21$ ] and controls [ $n=24$ ] [58]. Thus, the data on adherent bacteria in CRC is conflicting.

Two recent studies have reported on the association of *Fusobacterium* species in human CRC, using quantitative PCR and 16S r DNA sequence analysis [59, 60]. In one study from Boston the composition of the microbiota, using whole genome sequences, was determined from nine specimen pairs of tissues from CRC and normal mucosa [59]. *Fusobacterium* sequences were significantly enriched in the cancer metagenomes, ranging over 20 % of total bacterial sequences. Further examination of a larger cohort of 95 pairs of specimens of colon cancer and normal colonic DNA, *Fusobacterium* species were enriched in cancer DNA and tissue, while *Bacteroides* and *Firmicutes* phyla were depleted in tissues [59]. In another study reported at the same time from British Columbia, an overabundance of *Fusobacterium nucleatum* sequences in colon tumors was found compared to matched normal adjacent control mucosa in 99 subjects [60]. A *Fusobacterium* isolate was also cultured from a tumor specimen and abundant *Fusobacterium* sequences in tumor were positively associated with lymph node metastases.

These two exciting studies strongly link a single species of bacteria, *Fusobacterium*, with CRC. However, this microbial association does not prove cause and effect and could represent an innocent bystander effect. The fact that *Fusobacterium* species is part of the normal oral flora and not an abundant constituent of the colonic microbiota [61] favors a pathobiologic role in the development of CRC. A previous report of r RNA sequence in a small number of colorectal tumors and control samples [6 pairs] had found an increased trend of coribacteria in tumors and suggestion of high amounts of *Fusobacterium* [62].

## 2.4 Summary of Colorectal Microbial Pathogenesis

Although there is increasing evidence that the bowel microbiota plays a role in the pathogenesis of CRC, this is likely a complex interaction with genetic predisposition, dietary factors, and alteration of the delicate balance between favorable and unfavorable commensal flora. The protective effect of favorable commensals and the mechanisms of cancer promoting flora are summarized in Tables 2.1 and 2.2. Recent evidence demonstrating the association of *Fusobacterium* with colonic tumors in humans is intriguing but requires further confirmatory studies. At present the animal studies are more convincing of the role of the bowel microbiome in carcinogenesis of CRC than the human data.

Future research is needed to explore the role of microbes in the pathobiology of CRC in both animal models and humans. In the field of animal experimentation rodent models with humanized bowel flora and genetic alterations, utilizing common mutations and polymorphisms associated with human CRC are best used. Other animal models such as primates and pygmy pigs would help strengthen causality role. Animals with humanized bowel flora could be colonized with high concentrations of *Fusobacterium nucleatum* compared to other species in the presence of a high- and

**Table 2.1** Cancer-promoting commensals and mechanisms

Microbe	Mechanism	Reference
<i>E. faecalis</i>	DNA damage and instability, H <sub>2</sub> O <sub>2</sub> , superoxide radicals	[36, 37, 51]
<i>B. fragilis</i> [ETBF]	Inflammation, polyamine catabolism → fecapentaenes tend to mutagenesis	[38, 39, 44]
Lactobacillus, Streptococcus and Enterococcus spp.	H <sub>2</sub> O <sub>2</sub> generation, inflammation, and disruption of epithelium	[42, 43]
Proteobacterium phylum [sulfide reducing bacteria]	H <sub>2</sub> S upregulation of oncogenes	[52]
Undefined flora	Conversion of procarcinogens to carcinogens by aldehyde and amines	[44]
<i>Fusobacterium</i> spp.	Inflammatory cytokines pathway	[61, 62]

**Table 2.2** Anti-cancer commensals and mechanisms

Microbe	Mechanisms	Reference
Bifidobacterium spp.	? Metabolize carcinogens	[19, 20]
<i>B. longum</i>	Decrease cell proliferation	[25]
<i>L. acidophilus</i>	? Alter bile acid metabolism activation of T-cells, granulocytes	[23, 24]
<i>L. salivarius</i>	Reduce inflammatory pathway	[26]
Probiotics	Reduce COX2, PGE-2 expression and inflammation, enhance apoptosis; increase butyrate and immunomodulation, physiologic “cancer surveillance” Inhibition of protooncogene	[28, 29, 31–34]

low-risk diet, as well as the effect of selective probiotics on the development of CRC. Larger prospective studies of the colonic microbiome are needed in high- and low-risk human subjects over several years to map the genome sequence of feces, normal mucosa, polyps, and cancerous tumors.

Preventative large multicenter, randomized trials of hundreds or thousands of high-risk subjects, such as African Americans over 50 years of age and overweight with the first colonic polyp, could be implemented to compare a daily mixture of suitable probiotics versus placebo [e.g., yogurt without probiotics] given for 5–10 years and monitored for recurrence of adenomas or CRC as end point. A less rigorous but acceptable study could randomize patients to daily yogurt with probiotics versus standard diet with no yogurt. These trials although expensive and time–resource consuming should be worthwhile, as CRC is a major disease of the Western world, which is likely to increase in incidence worldwide with the increase in the aging and overweight/obese populations characteristic of most developed countries in recent years. Moreover, as developing nations such as China and India become more affluent societies it is likely that the rates of colorectal cancer in these nations will also increase from adopting westernized customs and diet.

## 2.5 Microbes in Inflammatory Bowel Diseases

### 2.5.1 *Background*

Crohn's disease [CD] and ulcerative colitis [UC] are the two major forms of idiopathic IBD, characterized by chronic or relapsing immune activation and inflammation of the intestines. Although these conditions are not common diseases they are not extremely rare in westernized countries. The incidence of IBD varies with the geographic regions and similar to CRC may be considered a disease of civilization, with the highest incidence in Europe and North America. In North America the incidence rate ranges from 2.2 to 14.6 cases per 100,000 person-years for UC and from 3.1 to 14.6 cases per 100,000 years for CD [63]. IBD is rare in developing countries or regions in tropical and subtropical climate except for Caucasians in Israel, Australia, and South Africa. This may reflect genetic predisposition inherited from their ancestors, arising mainly from Europe. There is evidence, however, of increasing incidence of IBD, especially UC in Japan, South Korea, Singapore, Latin America, Hong Kong, and Northern India, areas of the world previously with low incidence [63]. It is unclear at present whether or not the increasing incidence of IBD in these regions reflects environmental changes or dietary influence with affluence, or genetic mixing of the populations, or theoretically changes in bowel microbiota.

Ethnicity is an important influence on the occurrence of IBD, greater in Jewish populations [two- to fourfold increase] in Europe, North America, and South Africa, with decreasing prevalence in the non-Jewish white, African Americans, Hispanic, and Asian populations [63].

### 2.5.2 Pathobiology of IBD

There is general consensus that IBD occurs in genetically predisposed individuals, with increased risk in family members up to 14- to 15-fold greater in first-degree relatives than the general population [64]. However, in most patients there is an absence of family history and IBD is a familial disease in only 5–10 % of patients [63]. IBD is associated with certain genetic syndromes [i.e. Turner's syndrome] as well as inherited immune deficiency disorders, including Wiskott–Aldrich syndrome, chronic granulomatous disease, hypogammaglobulinemia, selective IgA deficiency, and immune dysregulation [63]. This alone would suggest that the immune response to microbes could be important in the pathogenesis of IBD, besides just genetic predisposition. Recent studies have found association of CD with genetic variants of the nucleotide-binding oligomerization domain-2 [NOD-2] gene, also known as CARD 15 [caspase-recruitment domain 15], of which the gene product [a cytosolic protein] functions as an intracellular sensor for bacteria [64]. It has been estimated that up to 20–30 % of patients with CD may carry abnormal NOD-2/CARD 15-gene. The NOD-2/CARD 15 protein is expressed in monocytes and enterocytes within intestinal crypts and produces an endogenous antimicrobial peptide [defensins] by binding to bacterial peptidoglycan of gram-positive and gram-negative bacteria [64]. The NOD-2/CARD 15 gene mutations have not been associated with UC. However, IBD is a polygenic disorder with multiple clinical subgroups, and with about 100 disease-associated loci on many different chromosomes, of which about one third are shared between UC and CD [63]. An important aspect of the genetic factors that predispose to IBD is their association with innate immunity and autophagy, utilizing immune cells to respond to bacteria, mycobacteria, and viruses [e.g., NOD-2, ATG 16L1, IRGM, JAK-2, STAT-3]; other genes regulate the inflammatory response associated with the regulation of adaptive immunity [i.e., IL-23R, IL-12B, IL-10, PT PN2], through cytokines, leukocyte recruitment, and inflammatory mediator production [63]. Regulatory T cells [RTC] are important in maintaining homeostasis in response to food and microbial antigens. Targeted deletion of certain genes expressed by RTC, which results in colitis with conventional gut flora, includes those that encode IL-10, IL-2, IL-10 R I, TGF beta, TGF BR II, and Fox p3 [64].

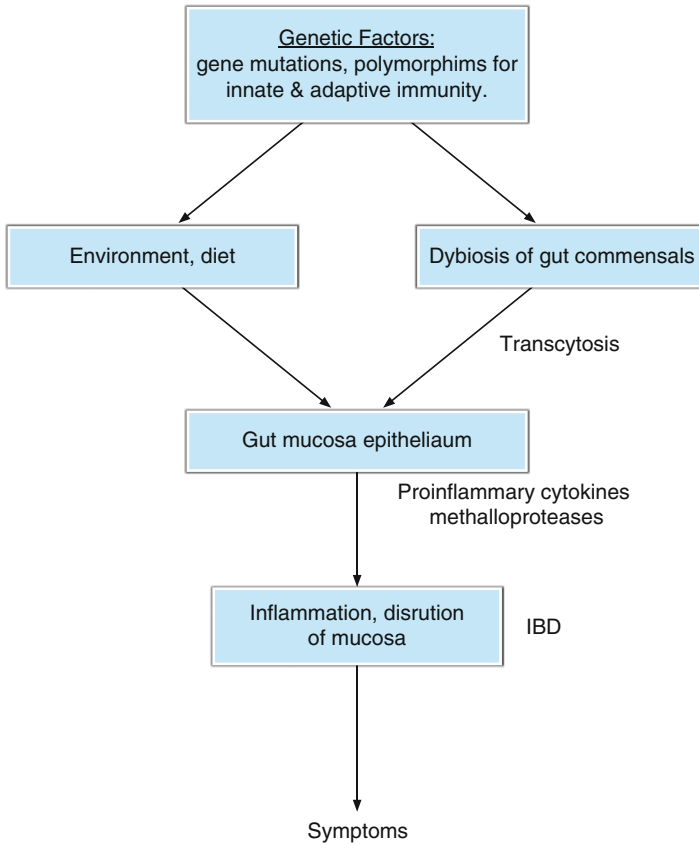
It is generally accepted at present that the pathogenesis of IBD is a result of continuous antigenic stimulation by commensal bowel flora that lead to chronic inflammation of the mucosa of the intestines in genetically susceptible individuals [65]. However, although several infectious agents have been implicated in the pathogenesis of IBD, no specific agents have been identified or established to be the cause of CD or UC. The pathogenesis of IBD is characterized by the introduction, maintenance, and intermittent flares of proinflammatory cytokine responses followed by inflammatory changes in the bowel leading to symptoms. The nature of these responses elucidated in the past two decades has been recently reviewed [66]. The initial cytokine responses are controlled by the T-cell differentiating patterns of the disease. In CD the major cytokines are derived from T-helper cell [Th]-1 and

Th-17 CD4 positive T-cell differentiation and generation of interferon gamma [IFN- $\gamma$ ] and interleukin [IL]-17/IL-22 cytokines. In contrast, in UC there is mainly Th-2 cell differentiation with heightened expression of natural killer cells and generation of IL-13 and possible IL-5 [66]. Secondary inflammatory mediators are stimulated by disease-specific cytokines to produce tumor necrosis factor-alpha [TNF- $\alpha$ ], IL-1B, IL-6, and tumor necrosis factor-like ligand [TL1A].

There is increasing evidence of the interaction of three fundamental cell biological pathways involved in the pathogenesis of IBD [67]. These include: (1) autophagy, as revealed by the identification of ATG 16LI and IRGM as major genetic risk factors for CD; (2) intracellular bacterial sensing, demonstrated by the importance of NOD-2 in autophagy induction by entry of bacteria intracellularly; and (3) unfolded protein response initiated by endoplasmic reticulum stress, due to accumulation of misfolded proteins [closely linked to autophagy and innate immunity] [67]. These three pathways are increasingly recognized as being important in the pathogenesis of IBD and provide a link between genetic and environmental influence with altered epithelial cell function as the initiating critical event. Autophagy is a process of segregation of the cell's cytoplasmic material within a membrane and its digestion after fusion of the segregated vacuole with a lysosome. Currently, there is cumulative evidence that the intestinal microbes influence host immune development, immune responses, and susceptibility to a variety of diseases, including IBD, CRC, IBS, diabetes mellitus, and obesity [68]. Although the intestinal immunity defends against invading pathogens it maintains a state of immune tolerance [symbiosis] with resident commensal microbiota. Perturbation of the balance is associated with intestinal inflammation as shown by animal models of IBD. A role of microbes in the pathogenesis of IBD has been demonstrated by the strong association between IBD and genes that regulate microbial recognition and innate immune pathology, i.e., NOD-2 gene, genes that control autophagy [ATG 16LI, IRGM], and genes for IL-23-Th 17 pathway that regulate gut immune homeostasis [68]. Intestinal epithelial cell barrier functions are critical for host-microbiota mutualism, and genes regulating mucous secretion [Muc1, Muc2], Paneth cells producing antimicrobial peptides or affect epithelial proliferation and integrity can predispose to IBD [69].

### ***2.5.3 Microbes and Inflammatory Bowel Diseases***

The current paradigm in the pathogenesis of IBD involves disturbances in the balance or homeostasis of host genetic factors, barrier function of the gut epithelium, innate and adaptive immunity, and changes in the composition [qualitative and quantitative] of the gut microbiota [Fig. 2.2]. Although dysbiosis of the commensal bacteria, with selected microorganisms have been observed in studies of both CD and UC, it is still unclear whether these perturbations are cause and effect or secondary to changes in the bowel mucosa [70]. The cumulative data will be reviewed in this section.



**Fig. 2.2** Paradigm of the pathobiology of IBD

*Mycobacterium avium* subspecies paratuberculosis [MAP] causes a disease in cattle [Johne’s disease] that resembles to a great degree the features of CD. It has been postulated for several decades that MAP could be the cause of CD. However, the data is inconsistent and variable and currently MAP is not considered as a strong candidate for the etiology of CD, recently reviewed by Over et al. [71]. MAP infections have been reported in a wide variety of wild and domesticated animals [ruminants and nonruminants], and it can survive in the environment. In a previous systematic review and meta-analysis of the association of CD and MAP, 28 case–control studies were analyzed and although the pooled odds ratio [OR] from studies using PCR in tissue samples showed a strong association with CD compared to healthy controls, similar findings were found in UC [72]. However, in a more recent study of the fecal detection of MAP using IS900 DNA sequences, the results were similar in CD, UC, and healthy subjects, 68 %, 65 %, and 48 %, respectively [73]. The precise mechanisms by which normal commensal bacteria, even in the presence of altered equilibrium, could cause IBD in genetically susceptible subjects still

remain unclear. One of the promising postulates with some supportive evidence involves early state of antigenic overload of the lamina propria due to unregulated transcytosis [translocation] of bacteria across the epithelium, producing innate immune response and chronic inflammation [74]. It has been proposed that dendritic cell function underlies dysregulated T-cell responses in CD, which may be altered by the composition of the intestinal microbiota. In a recent study of 28 CD patients and 10 controls, rectal tissue samples were obtained and analyzed for dendritic cell response and intestinal microbiota composition [75]. IL-6 production by intestinal dendritic cells increased in CD and correlated with the disease activity and composition of the commensal microbiota. Thus, the authors concluded that bacterially driven IL-6 production by dendritic cells may overcome regulatory activity, resulting in uncontrolled inflammation and tissue damage [75].

Some studies have implicated colonization of pathogenic adherent invasive *E. coli* [AIEC], which adhere and invade intestinal epithelial cells [IEC] and survive in macrophages, in the pathogenesis of CD [76–79]. Decreased levels of protective proteases [meprin] that counteract bacterial colonization have been found in the ileum of CD patients and may contribute to increased colonization by AIEC [80]. However, the most promising data on this topic has been from one center and is not very robust. Darfeuille-Michaud et al. [76] analyzed ileum mucosal biopsies from 63 CD patients and 16 healthy controls, as well as colonic specimens from 27 CD and 8 UC patients and 102 normal controls for AIEC. Specimens from ileum were positive for AIEC in 21.7 % of CD lesions and 6.2 % of controls, but in 36.4 % of new lesions [ $p=0.034$ ] and 22.2 % of healthy mucosa of CD patients. In colonic specimens AIEC were found in 3.7 % of CD and 0 % of UC patients and 1.9 % of healthy controls. If AIEC were an etiologic agent of CD one should expect to detect the organism in a higher proportion of patients, both from lesions of the ileum and colon. However, the ability of AIEC to induce granulomas in vitro [78], and the association of a similar *E. coli* with canine granulomatous colitis, a rare form of specific inflammatory bowel disease of young Boxer dogs, are intriguing and does suggest that AIEC may play a pathogenic role in a subgroup of CD patients. AIEC express an adherence factor that aids in the binding to M cells overlying Peyer's patches and subsequent entry into lymphoid tissue, but it is unclear whether this is causal or secondary to underlying immune deficiencies in CD patients [81]. In an ex vivo study of the mucosa of the terminal ileum and colon of pediatric CD patients compared to the mucosa of healthy controls, there was inappropriate and aberrant response to commensal nonpathogenic bacteria. i.e. *Bacteroides* species [82]. There is a large diversity of commensal bacteria in the gut and it may be that an overabundance of certain genus or species, predominantly with proteolytic activity, participates in the pathogenesis of IBD by disturbing the mucosal integrity and homeostasis [83].

It has been postulated that a novel or unique strain of *Helicobacter* bacteria may play a role in UC and investigation in this area is continuing. In a recent study archived and prospectively collected, colonic samples were analyzed by molecular methods [FISH analysis and PCR] for *Helicobacter* species from UC patients and healthy controls [84]. *Helicobacter* genus was significantly higher in UC versus controls [32 of 77 versus 11 of 59,  $p=0.004$ ]. *H. pylori* which has been

well established to cause peptic ulcer and gastric cancer is found in over 90 % of these ulcers, whereas Enterohepatic *Helicobacter* species are detected in less than 50 % of UC patients. *Helicobacter* species, however, has been recognized as an important cause of colitis in rodents and primates; some of these bacteria [e.g., *H. hepaticus*, *H. trigonum*, and *H. bilis*] are routinely used in immunocompromised rodent models of IBD [85]. *H. cinaedi* and *H. fennelliae* have been associated with proctitis in homosexual males, but none of the *Helicobacter* species have been cultured from UC lesions and are not strong candidates for causing UC [85].

In the past few years *Campylobacter concisus* has been implicated in the pathogenesis of CD [86]. The organism has been isolated from children with newly recognized CD and can invade intestinal epithelial cells and disrupt barrier function [87, 88]. Although the detection of *C. concisus* is higher in children with CD [34 of 54, 65 %] versus in healthy controls 11 of 33, 33 % [89], and the bacteria can cause weight loss in immunocompetent BALB/CA mice, it did not produce inflammation of the gut [90]. Thus, the data on *C. concisus* as possible causal agent in the pathogenesis of CD is weak.

### 2.5.4 Dysbiosis of Intestinal Microbiota in IBD

The leading hypothesis of microbial pathobiology in IBD is related to dysbiosis of the intestinal microbiota, which leads to ineffective control of commensal bacterial invasion, as a result of impaired antibacterial response, resulting in chronic inflammation. For instance, the impairment of clearing intracellular bacteria by the cellular process of autophagy appears to be the main defect in CD [91]. There is cumulative data to support the concepts that defects in the innate immunity are responsible for the robust proinflammatory response to commensals that resulted in IBD [92]. Recent studies have also confirmed that disease phenotype [CD or UC] and genotype [NOD-2 and ATG16LI risk alleles] are associated with compositional changes of the intestinal microbiota [shift in the relative frequencies of *Fecalibacterium* and *Escherichia* taxa [93]. There is increasing evidence that the normal commensal bacteria protect the intestinal epithelium from toxic injury and provide an anti-inflammatory effect [94]. However, disturbances in the immune system or epithelial homeostasis can affect the delicate balance between the gut microbiota and epithelium, which can lead to inflammation. Under these situations the commensal flora appears to enact as a substitute foreign pathogen, which the host response is unable to eradicate and thus results in lifelong inflammation [94]. The immune activation leads to increased concentrations of cytokines, lipid mediators of inflammation, and free radicals with influx of inflammatory cells, the upregulation of matrix metalloproteases from fibroblasts, producing degradation of matrix and eventual ulcerations [95]. Hence, in IBD the homeostatic mechanism that results in coexistence of the host and commensal flora is disrupted, generally by mutations of genes that control innate and adaptive immunity and epithelial barrier function [96].



Much of the evidence that commensal bacteria can induce gut inflammation is derived from animal models. In many of these models the absence of commensal flora in germ-free conditions resulted in absence of disease or decreased risk even with genetic predisposition [94]. There are over 30 models of IBD in rodents which can be divided into four major groups: (1) colitis that developed spontaneously, (2) chemically induced colitis, (3) animals with defect in epithelial barrier function, and (4) colitis that develops in genetically engineered mice with defects in the immune system or regulating cell function [97]. These studies, however, do not show any specific pattern of the bowel flora component that is important in IBD. Although the propensity of the normal gut flora to induce inflammation is not the same for all species, this can vary with the defect or the model used. For instance, in the HLA-B27 transgenic rats *Bacteroides vulgatus* induces colitis while *E. coli* does not [98], but in the IL-10 deficient mice commensal *E. coli* induces disease but *B. vulgatus* does not [97]. Whether or not this could also apply to humans with IBD and different genetic predisposition or defects is unknown.

Dysbiosis [disturbance of the symbiotic or mutual benefit between microbiota and host] toward selected microorganisms and decreased complexity by the gut flora has been observed in both CD and UC, but it is still unclear whether the dysbiosis causes IBD or is the result of the epithelial pathology [99]. A full understanding of the composition of the gut microbiota, complexity of the diversity, and appreciation of dysbiosis in IBD have only been appreciated in the past several years with the development of culture-independent molecular techniques. Previous studies on mucosal microbiota of patients with IBD and controls using standard culture methods failed to detect differences in composition of mucosal flora but reported marked changes in the concentration of bacteria between normal mucosa [which is almost sterile] and those with IBD [100]. Ileocollectomy induced significant increase in bacterial counts and variety [assessed by standard culture methods] in the neoterminal ileum in CD patients and controls, with greater numbers of *E. coli* and enterococci in CD, but higher quantities of bifidobacteria and ruminococci in controls [101]. Early recurrence of disease was associated with high counts of *E. coli* and *Bacteroides* with frequent isolation of *Fusobacterium*.

Recent studies using molecular methods to analyze the intestinal microbiota [mucosa or feces] have in general shown compositional differences between healthy controls and patients with IBD. Table 2.3 summarizes the results of 26 studies performed in the past decade [102–127]. These studies included a total of 462 patients with CD, 308 with UC, and at least 450 healthy controls. The sample size per group varied from 6 to 63 subjects. Three studies [107, 116, 117] did not include healthy controls but were comparing mucosa-associated microbiota from different sites [ileum, colon, and rectum] and from abnormal or ulcerated areas to normal or noninflamed mucosa. A few studies [103, 118, 125] examined the microbiota during active disease and remission, 1 during remission only [119], but the majority of studies did not specify the patients' status and were presumed to be during the active stages of disease. Eight of the studies analyzed fecal samples only, and one study collected samples over a year in UC patients in remission and

**Table 2.3** Gut microbiota [by molecular methods] in IBD

Ref./year	CD	UC	Controls	Findings	Method/sample
1. Kleesen [102], 2002	12	12	14	↑ Bacteroides in mucosa of CD [25–55 %], UC [83.3 %] vs controls [0 %]	FISH/ileum colon mucosa
2. Seksik [103], 2003	17 [active and inactive]	–	16	↑ Enterobacteriaceae in CD vs. controls	Dot blot hybr feces
3. Ott [104], 2004	26	31	46	↓ Diversity of microflora in CD by 50 % & UC by 30 % due to loss of anaerobes, $p<0.0001$	RT-PCR, SSCP colon mucosa
4. Mylonaki [105], 2005	33	6	14	↓ Bifidobacteria, ↑ <i>E. coli</i> and Clostridia in IBD	FISH/rectal mucosa
5. Lepage [106], 2005	20	11	4	MAM differ from feces but stable from ileum to rectum	TTGE-16S r DNA/feces ileum-rectal mucosa
6. Seksik [107], 2005	15 [75 specimens]	–	–	MAM similar between ulcerated and nonulcerated mucosa, but biodiversity greater in ulcers	TTGE-16S r RNA/ileum-rectal mucosa
7. Manichanh [108], 2006	6	–	6	↓ Complexity of Firmicutes phylum in CD	Genomic DNA/feces
8. Conte [109], 2006	12 [children]	7	7	↑ Aerobes in IBD decreased <i>B. vulgatus</i> in UC and CD	PCR, culture ileum-rectal mucosa
9. Martinez-Medina [110], 2006	19	–	15	↑ Clostridia, Ruminococcus and <i>E. coli</i> in CD	PCR-DGGE/ileo-colonic mucosa
10. Sokol [111], 2006	14	16	13	↓ Fecal bacteria IBD, ↓ <i>C. coccooides</i> in UC, ↓ <i>C. lepticum</i> in CD [ $p<0.001$ ]	FISH-flow cytometry/feces.
11. Kotlowski [112], 2007	13	19	15	↑ Enterobacteriaceae B2+D phyla in IBD	RISA, DNA sequence, colon mucosa
12. Baumgart [113], 2007	12	–	7	↑ <i>E. coli</i> , ↓ Clostridiales in CD	16S rDNA libraries/ileo-colonic mucosa
13. Andoh [114], 2007	–	44	46	Diversity of microbiota vary between UC and controls, ↑ Fusobacterium in active UC	T-RFLP/feces
14. Frank [115], 2007	63	63	63	Depletion of Firmicutes and bacteroidetes in IBD	PCR-broad range/ileo-colonic mucosa

15. Vasquez [116], 2007	15				Microflora similar for inflamed and noninflamed sites	FISH, TTGE/normal and affected ileal mucosa
16. Sokol [117], 2007	–	10			Dominant MAM similar for injured and healthy mucosa	TTGE/colo-rectal mucosa
17. Ott [118], 2008	–	13 [remission and relapse]	5		↓ MAM, temporal instability, ↓ bacterial richness with relapse	16S r RNA PCR/colonic mucosa
18. Martinez PCR/[119], 2008, 1 year	–	16 [remission]	8		Low diversity and temporal instability in UC	16S r RNA feces over
19. Sokol [120], 2008	26	–	21		↓ Firmicutes, <i>F. prausnitzii</i> in relapse in CD	FISH/before and 6 months after surgery
20. Nishikawa [121], 2009	–	9	11		↓ Diversity in active UC vs. controls and inactive UC	T-RFLP/colonic mucosa
21. Willing [122], 2010	29	16	35		↓ Faecalbacterium and roseburia in ileal CD, and Incr. Enterobacteriaceae/Ruminococcus	Pyrosequencing/feces and mucosa
22. Mondot [123], 2011	16	–	16		↑ <i>E. coli</i> , <i>E. faecium</i> and Proteobacteria in CD dysbiosis in CD	RT-PCR, DNA extracted/feces
23. Walker [124], 2011	6	6	5		↓ Firmicutes, diversity in IBD, ↑ Bacteroides [UC, CD] ↑ Enterobacteriaceae in CD	High throughput PCR/paired mucosal specimens [ <i>n</i> = 29]
24. Joossens [125], 2011	68	–	55		↓ <i>F. prausnitzii</i> , Bifidobacteria, Clostridia cluster and ↑ ruminococcus in CD	DGGE finger printing/feces
25. Andoh [126], 2011	31	31	30		↓ Clostridia in active UC and all CD; ↑ bacteroides in CD	PCR, T-RFLP, feces
26. Lepage [127], 2011	–	8+ disc. twins	17 twins + 10 unrelated		↓ Diversity in UC, unusual aerobic bacteria, lower protective bacteria	16S r DNA, microarray analysis/colon mucosa

CD Crohn's disease, DGGE denaturing graded gel electrophoresis, Disc discordant, FISH fluorescent in situ hybridization, Hybr hybridization, MAM mucosa-associated microbiota, PCR polymerase chain reaction, RISA ribosomal intergenic spacer analysis, RT real time, T-RFLP terminal restriction length polymorphism, TTGE temporal temperature gradient gel electrophoresis, UC ulcerative colitis

control subjects [119]; 16 studies process mucosal samples only, 1 at the time of an ileum resection and 6 months after surgery [120], and two studies analyzed both feces and mucosal samples [106, 122].

The molecular methods in these studies varied widely and included quantitative real-time PCR [RT-PCR], terminal restriction fragment length polymorphism [T-RFLP], temporal temperature-gradient gel electrophoresis [TTGE], quantitative fluorescent in situ hybridization [FISH], PCR-denaturing gradient gel electrophoresis [PCR-DGGE], ribosomal intergenic spacer analysis [RISA], 16S r DNA-single-strand confirmation polymorphism [SSCP], and 454 pyrotag-sequencing, or combination of two or more methods. Despite the differences in methodologies between studies, which make comparison of findings difficult to interpret, there has been a remarkable similarity in the overall trends. It does appear that mucosa-associated microbiota differ from feces but remained stable from ileum to rectum in the same individuals [106]. Thus, it is important to analyze data of fecal microbiota separately from studies assessing mucosa-associated microbiota [MAM].

Of the 15 studies primarily analyzing MAM 3 did not include samples from healthy controls, but compared ulcerated or inflamed mucosa to nonulcerated healthy mucosa in the same individuals [107, 116, 117]. In two of these studies assessing patients with CD, the MAM were similar between ulcerated and nonulcerated or noninflamed mucosa, which was also similar to the findings in patients with UC [117]. Nearly all the studies comparing MAM of intestinal mucosa of IBD patients compared to controls found significant differences but of variable pattern. Two groups reported increased Bacteroidetes in the mucosal biopsies of both CD and UC patients [greater in the latter group] compared to controls, 18 subjects per group [102, 124]. However, in the largest single study depletion of Bacteroidetes and Firmicutes were found in both CD and UC patients' intestinal mucosa compared to controls, 63 subjects per group [115]. Six studies [105, 109, 110, 112, 113, 124] reported significant increase in *E. coli* or Enterobacteriaceae in the ileal or colonic mucosa of CD patients [ $n=104$ ], and less consistently with UC patients [ $n=38$ ] compared to controls [ $n=63$ ]. Decreased diversity of the microflora of the colonic mucosa has also been reported in CD [by 50 %] and UC [by 30 %] subjects primarily due to loss of the anaerobes [104], and decreased bacterial richness has been associated with relapse in UC [118]; in a discordant twin study UC was associated with decreased biodiversity, different gene expression, unusual aerobic bacteria, and lower protective bacteria [127]. The microbiota of the ileal mucosa before surgical resection and 6 months later demonstrated decreased Firmicutes and *Fecalbacterium prausnitzii* with relapse in a study of 26 CD patients [120]. Moreover, in vitro *F. prausnitzii* demonstrated anti-inflammatory properties and may be protective against active disease.

In fecal samples of 68 CD patients compared to 55 controls there was also evidence of decreased *F. prausnitzii*, bifidobacteria, clostridia, and increased Ruminococcus [125]. In comparison with healthy relatives [ $n=85$ ], CD patients' feces had relatively different microbiota composition [125]. Others have also reported decreased *Fecalbacterium* and clostridia species in the feces of both CD and UC patients compared to controls [111, 122]. Decreased biodiversity of the fecal flora has also been reported in UC subjects with high concentration of *Fusobacterium* in

active disease [114], and temporal instability has been noted on analysis of feces over a year [119]. In a more recent study utilizing standard culture methods *Fusobacterium nucleotum* from colonic mucosa has been found to correlate with IBD status from a study of 110 subjects, 22 with IBD. Furthermore, strains of *F. nucleotum* from IBD patients were more invasive than strains from healthy mucosa assessed by Caco-2 cell invasion assay [128].

### 2.5.5 Probiotics in IBD

The value of probiotics in human IBD has recently been reviewed in 2011 by Meijer and Dieleman [129]. Only randomized, controlled studies reported in English were analyzed, including patients with postoperative pouchitis, and included trials before 2007 through to December 2010. Although the trials since 2007 compared to before this period were more robust, there have been several methodological limitations identified. This included small sample sizes of the cohorts, with only 6 of 22 [27.2 %] trials had a sample size of greater than 100 patients [130–135] and only one study had more than 200 subjects [130]. Other limitations noted by the reviewers were the study of a wide range of probiotic strains or combinations, variation in dose and treatment duration, and inconsistent use of conventional adjuvant medicines or comparators [129]. None of the five small controlled studies in CD showed significant clinical benefit of the probiotics [lactobacillus species used in four studies], but a small study with *Saccharomyces boulardii* in CD patients [15 per group] showed improved intestinal permeability and maintenance of remission [131].

The trials in UC on induction or maintenance of remission with probiotics compared to standard therapy have produced mixed results [129]. The single largest study [ $n=327$ ], however, found that *E. coli* Nissle 1917 was as effective as mesalazine at maintaining remission for 1 year [130]. Two small studies from the same group of investigators reported that VSL#3 [a commercial mixture containing several *Bifidobacterium* species, lactobacillus species, and *Streptococcus salivarius* subsp. thermophilus] increases the duration of remission by 9–12 months in patients with pouchitis [132, 133]. A larger study of 117 patients with pouchitis treated for 3 years also found that *Lactobacillus rhamnosus* GG increased the duration of remission [134]. Two relatively large studies in UC patients reported less effective induction or maintenance of remission with VSL#3, but assessment was only for 8–12 weeks [135, 136].

Since the review of probiotics in IBD in 2011 [129], the preliminary results of a trial in UC with a promising symbiotic [combination of a probiotic and prebiotic], consisting of *Bifidobacterium* and galacto-oligosaccharide, have been reported in a study from Japan [137]. Forty-one patients with mild-to-moderately active UC were randomized to receive the symbiotic versus placebo three times a day for 1 year. There were clinical and colonoscopic indices improvement on the symbiotic, as well as decrease in the amount of myeloperoxidase from colonic lavage, which was used as a surrogate marker of intestinal inflammation. Another recent placebo-controlled

trial of a prebiotic alone [fructo-oligosaccharide] for 4 weeks in CD patients showed no clinical benefit, and there was no change in the fecal flora such as *Bifidobacterium* or *F. prausnitzii* [138].

### 2.5.6 Conclusion and Future Directions

The cumulative bulk of evidence over the past decade more strongly support a role of the intestinal microbiota in the pathogenesis of IBD, together with the presence of immune disturbances from genetic predisposition or spontaneous mutations in the genes controlling innate and adaptive immunity. It is unlikely that a single microorganism will be found responsible for the etiology of CD or UC, and more likely that there is a shift in the balance between harmful microbes and protective ones. Although at present, it cannot be definitely stated that dysbiosis of the intestinal flora is the cause of IBD rather than the result, the evidence is more in support of a causative role. The studies to date, however, do not clearly distinguish between the types of IBD based on the bowel microbiota, and predisposition to CD or UC is more likely related to the specific genetic and immunologic disturbances.

Future studies on the pathobiology of IBD should continue to adopt the latest specific and sensitive molecular techniques, but also require larger samples of patients and controls, with analysis of bowel mucosa and fecal specimens multiple times over many months to years. Much larger multicenter, randomized, controlled trials are needed to assess the utility of probiotics for longer duration of time, i.e., 2–4 years. These trials should employ a standardized mixture of probiotics and dosages compared to a standard regimen of comparators. The clinical and colonoscopic indices end points should be well established and accepted by the gastroenterology community. The combination mixture of probiotics should be chosen based on promising preliminary results of clinical trials [i.e., *Bifidobacterium* symbiotic combination], in vitro biologic studies [such as with *F. prausnitzii*], and animal studies. For instance, in a recent study in mice of *Bifidobacterium bifidum* S 17 was shown to partially protect animals from Th1-driven inflammation [significant reduction of histological score and levels of proinflammatory cytokines] in a chemically induced model of colitis [139].

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