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# Evidence for Contributions of Gut Microbiota to Colorectal Carcinogenesis

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**Abstract** The contributions of the commensal gut microbiota to the maintenance of human health have long been contemplated. Whereas earlier studies were limited by an inability to analyze microbiota in sufficient depth, recent advances in the application of high throughput sequencing have allowed for an in-depth microbiota analysis in large numbers of individuals. Multiple lines of evidence have been generated that are supportive of an active role of gut microbiota in colorectal carcinogenesis. Although no single microbe has yet been shown to be causally linked to CRC, contributions of the gut microbiota to colorectal carcinogenesis are evident. Further advances in the field, which should include prospective studies in high-risk cohorts, should generate the data needed to start translating findings into microbiota-based screening and prevention regimen that can help to reduce the burden of CRC.

**Keywords** Gut · Flora · Microbiota · Microflora · Commensal · Carcinogenesis · Neoplastic · CRC · Probiotics · Prebiotics · Colon · Cancer · Intestinal

## Introduction

Colorectal cancers (CRCs) remain among the most frequently observed and fatal malignancies worldwide. Genetic predispositions [1] and environmental exposures have been associated with increased CRC risk [2]. Nutrition and lifestyle habits, including exercise, have been suggested to contribute [3]. These factors can affect microbiota activities [4], which might present a mechanism for affecting CRC risk. Chronic inflammation and obesity, which both affect gut microbiota, also have been linked to CRC [5, 6]. A large body of evidence supports the idea that altering gut microbiota can change host physiology [7]. Abnormal immune responses toward commensal microbes are thought to contribute to inflammatory bowel disease (IBD), which is known to increase CRC risk [6]. Changes in host diet, especially the addition of fermentable dietary fiber (DF), can modulate the gut microbiota toward a more protective composition [8, 9].

In recent years, multiple lines of evidence have emerged that are supportive of a role of chronic infections and activities of normal gut microbiota in colorectal carcinogenesis. We initially reviewed aspects of normal colorectal physiology, mucosal immune function, and changes occurring during CRC development that might provide a link to microbiota activities. We then summarized the evidence for associations between CRC and gut microbiota as well as some of the mechanisms that have been investigated.

## Normal Gut Physiology

The large intestine is divided into the cecum, ascending colon, transverse colon, descending colon, and rectum [10]. It is characterized by the presence of several layers, which include, beginning from the most apical layer, the mucosa (which consists of the epithelium and the lamina propria), muscularis mucosae, submucosa, muscularis externa, and serosa. In the healthy gut, commensal microbes are thought to be largely contained to the luminal contents,

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with some penetration into the mucus but little direct contact with the epithelial layer [11].

The large intestine has a large surface area due to crypts and microvilli. Intestinal crypts are composed mainly of absorptive epithelial cells and goblet cells, which secrete mucins. Progenitor cells multiply at the base of crypts and migrate up the crypt-villous axis. After reaching the luminal border, they are sloughed off as exfoliated colonocytes within at most a few days, resulting in a constant cell turnover. Maintenance of structural integrity of the epithelial layer is crucial to avoid leakage of luminal contents. Detailed studies in mice showed that the colonic epithelium provides three distinct layers of physical protection against luminal microbes. A 150-μM viscous mucus coating separated into a diffuse outer layer accessible to bacteria and a dense inner layer resistant to bacterial colonization exists atop a layer of intestinal epithelial cells [12]. Mucus primarily consists of heavily O-glycosylated proteins known as mucins. Mucins form oligomers, which retain water in the glycoprotein matrix [13]. The density of the mucus is thought to affect the ability of bacteria to penetrate [14].

## Normal Commensal Gut Microbiota

The colon harbors up to one hundred trillion bacteria that constitute the commensal human gut microbiota [15]. The human microbiome contains up to 100-fold more genes than the human genome [16]. Microbial communities differ by anatomical site along the colon and their location within the lumen [17, 18]. Whereas the stomach and duodenum harbor roughly $10^1$–$10^3$ organisms per milliliter of luminal contents, the small intestine and large intestine contain an estimated $10^4$–$10^7$ and $10^{11}$–$10^{12}$ organisms per milliliter, respectively [19].

Although most microbes exist in a beneficial balance within the host, opportunistic pathogens also can be present [16]. Whereas microbiota composition differs considerably between individuals, important microbial functions appear more conserved [20••]. Various functions have been attributed to the microbiota, including digestion of complex carbohydrates (such as those in DF), synthesis of vitamins, and modification of primary bile acids [21••]. Microbial products have been detected in the human metabolome, allowing for their analysis in urine or blood samples [22].

Microbiota varies greatly between body sites, and albeit to a lesser extent, within body sites between individuals [20••]. The gut microbiota is dominated by Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria at the phylum level [23••]. Variation increases at lower taxonomic levels, with thousands of operational taxonomic units (OTUs) detected [24]. However, some studies suggest the presence of a microbial core in the majority of individuals [24, 25]. The existence of three distinct enterotypes of microbial colonization pattern has been suggested [26••], but other studies seem to indicate that there is more of a continuum than a clearly distinct pattern.

Dysbiosis of intestinal microbiota has been associated in studies with eczema and IBD [27, 28]. Type 2 diabetes, obesity, and celiac disease also have been linked with aberrant microbiota patterns [29, 30]. Obesity, which is associated with increased CRC risk, has received immense research interest regarding microbiota contributions [25, 31]. The microbiota can offer novel means for modifying energy balance and fat storage. Recent work in prospectively collected samples has linked distortions in normal gut microbiota establishment in preterm infants with necrotizing enterocolitis, a disease characterized by extensive intestinal inflammation [32].

Despite the accumulated wealth of evidence for correlations between various disease states and microbiota composition, the evidence for any causal links is sparse. The large variation in gut microbiota composition between individuals and within individuals over time indicates the requirement for large sample sizes. A nested case/control design, with a prospective collection of a series of microbiota samples from populations at increased risk for the disease of interest, offers the most efficient study design. While animal models have served us well to prove the general principle that microbes can contribute to disease states, the specific findings of microbiota contributions cannot be extrapolated to humans.

## Colorectal Carcinogenesis

Colorectal cancer is thought to initiate in crypt stem cells that form aberrant crypt foci (ACF), regions of cell hyperproliferation at the base of intestinal crypts [33]. In rodent models, ACF, which are thought to be the earliest preneoplastic lesions, correlate with the prevalence of intestinal polyps [34]. Polyps, either adenomatous or hyperplastic, are precancerous legions characterized by hyperproliferation and lack of normal differentiation that vary in their genetic makeup and potential to progress to CRC [35].

The inappropriate activation of the Wnt signaling pathway, important for embryonic development and cell-cycle regulation, is thought to be a frequent initiating event in colorectal carcinogenesis. Various mutations in the APC gene have been established in familial adenomatous polyposis coli (FAP), a CRC syndrome [36]. Many colorectal tumors with intact APC have activating β-catenin mutations, demonstrating the ability of either type of mutation to induce hyper proliferation [37]. Since the initial description of the classic model of colorectal carcinogenesis [38], various specific mutations have been described. Mutations

of *KRAS* and *BRAF* genes, a GTPase and a serine/threonine kinase, respectively, which activate the mitogen-activated protein kinase (MAPK) pathway, are frequently found in colorectal cancers [39, 40]. *PI3KCA* encodes the catalytic subunit of phosphatidylinositol 3-kinase (PIK3), which is important for cell proliferation and survival, and has been found to be mutated in 32 % of colorectal cancers [41].

Mutations in CRC associated genes can affect cell turnover, mucin production, secretion of antimicrobial peptides, etc. Resulting changes in luminal conditions have the potential to affect gut microbiota composition and activities.

### Methylation

Epigenetic modifications have been shown to occur in response to environmental changes and thus also might correlate with activities of the gut microbiota. Loss of function in tumor suppressor genes due to promoter methylation has been observed frequently [42]. Inactivation of *hMLH1* via promoter methylation leads to a condition known as microsatellite instability (MSI) and is associated with sporadic CRC [43]. CpG island methylator phenotype (CIMP) has been associated with *hMLH1* methylation and MSI and is thought to contribute to MSI development.

Studies of potential associations between microbiota composition and methylation status are currently ongoing. Changing microbiota composition might help to reverse methylation patterns.

## Risk Factors for CRC Offer Potential Links with Microbiota Composition

Common factors that influence CRC risk include physical activity, diet, family history, obesity, smoking, and aspirin use [2, 3]. Many risk factors associated with CRC, including African American heritage, can be linked to microbiota composition [9]. Thus, changes in microbiota activities might be a contributing factor to colorectal carcinogenesis.

The case for microbiota contributions is especially strong for DF. By definition, DF reach the proximal largely undigested and form an important substrate for microbial fermentation. DF vary widely in their chemical composition so that different DF enrich for different microbes that are best adapted to utilize the specific DF. They result in different fermentation end products, with effects on the underlying tissue. Individuals with differences in their gut microbiota composition will respond differently to increased intake of the same DF. While some microbial communities generate from DF significant amounts of the beneficial short chain fatty acid butyrate, which helps appropriate differentiation of gut epithelium, other communities produce less beneficial or even detrimental products. Thus, benefits from DF on CRC risk likely depend on the chemical composition of the consumed DF and underlying microbiota and changes in its composition after adaptation to increased DF intake. DF intake has long been suggested to reduce CRC risk and recent reports from large prospective cohort are supportive of this hypothesis [44, 45], although the evidence is not unequivocal. There also is some evidence for inverse associations between fruit, vegetable, and whole grain intake and CRC [46, 47]. Various animal studies have shown inverse associations between DF intake and intestinal carcinogenesis. Experimental evidence from a mouse model suggests that a diet high in DF from fruits and vegetables significantly changed microbiota composition and reduced intestinal carcinogenesis [8]. In that study, unique bacterial signatures were observed in mice with no or few polyps, suggesting that gut environment differed between mice with low and high polyp burden.

Some studies show significant dietary associations with CRC risk only in specific regions, suggesting that diet associated activities of the microbiota, which vary along the gut, might be involved. Location-specific microbiota activities also would indicate a benefit for stratifying studies of associations between CRC and microbiota by location.

It is well established that African-Americans suffer from an increased incidence and mortality of CRC in comparison to Caucasian-Americans. The ethnic differences in CRC risk may be due to observed variations in the diets between groups and resulting differences in microbiota activities [9].

## Microbiota and Mucosal Immunology

CRC is associated with increased colonic inflammation [48], which can be modified by microbiota activities. Through mechanisms of tolerance, the immune system has evolved to maintain a balance that allows for coexistence between microbes and host. The epithelium provides a physical barrier against microbes, whereas the lamina propria contains the immune cells that maintain a tolerogenic response to these microbes. In the lamina propria, microbes that breach the epithelium are recognized and eliminated by the action of various immune cells.

The intestinal lamina propria contains gut-associated lymphoid tissue (GALT), which consists of Peyer's patches in the small intestine, lymphoid aggregates in the appendix and colon, and diffuse immune cells throughout the lamina propria. Dendritic cells (DCs) can project dendrites into the intestinal lumen and present microbial antigens to lymphocytes accompanied by IL-17 secretion to induce antibacterial immunity [49]. B cells secrete immunoglobin A (IgA) into the intestinal lumen via transcytosis [50]. Although no systemic antibody response is induced, bacteria opsonized

with IgA cannot cross the epithelial barrier [51]. However, antibodies against commensal bacteria have been detected in the serum of healthy donors [52].

T-helper ($T_H$) lymphocytes are stimulated by gut microbiota. In mono-associated mice, segmented filamentous bacteria (SFB), or *Candidatus* Savagella, are sufficient to induce signaling of all known T-helper subtypes, similar to responses to conventional microbiota [53]. Several *Clostridia* strains from the clusters IV and XIVa, which contain many important butyrate producers discussed later, induce a $T_{reg}$ phenotype both in the lamina propria and systemically [54]. *Bacteroides fragilis* induces IL-10 production though a polysaccharide A-dependent response that prevents the expansion of $T_H17$ cells [55]. An increase in both $T_{reg}$ numbers and IL-10 expression has been observed in response to PSA from *B. fragilis* in a TLR-2-dependent manner [56]. Although we are still at the beginning of our understanding of the complex interactions between microbiota components and the host immune system, the potential for modifying immune function is already obvious.

## Microbiota, Aberrant Immune Function, and IBD

IBD is a group of inflammatory conditions of the intestine associated with increased risk of developing CRC. Although the etiology is not fully established, an aberrant immune response to gut microbiota in genetically susceptible hosts is thought to be involved [57]. Patients with Crohn's disease exhibit a higher prevalence of adherent-invasive *E. coli,* similar to CRC patients (discussed below) [58]. Firmicutes, especially *Faecalibacterium prausnitzii*, appears underrepresented in IBD patients [59]. Evidence from murine models demonstrates interplay between gut microbiota and IBD in genetically susceptible hosts. For instance, germ-free IL-10 knockout mice did not develop disease; however, specific pathogen-free (SPF)-colonized counterparts rapidly developed IBD [60]. Indirect evidence for a contribution of microbiota can be derived from the detection in IBD cases of mutations in genes important for gut microbiota immune recognition. Mutations in *NOD2*, whose product binds to bacterial lipopolysaccharides and interacts with nuclear transcription factor NF-κβ, are associated with Crohn's disease in humans [61]. Both the addition of potentially beneficial bacteria and the removal of potentially harmful bacteria have been demonstrated to improve disease state [62, 63], indicating a potential for reducing IBD associated CRC.

## Evidence for Correlations of Microbiota with CRC

The discovery that *Helicobacter pylori* is causally associated with non-cardia stomach cancers spurred interest in the potential contribution of this and other infectious agents in intestinal cancers [64]. To date, *H. pylori* has not be linked to colorectal carcinogenesis. Few viral associations have been made with CRC, the associations of polyomaviruses, particularly JC virus, with CRC have been extensively investigated; ongoing research is aimed at utilizing better tools to answer this question in larger population studies [65].

Targeted studies of various bacterial species or groups have yielded both positive associations, most notable with specific *E. coli* types, enterotoxigenic *B. fragilis* (ETBF), and *S. gallolyticus (bovis)*, as well as inverse associations, most notably with Bifidobacteria and butyrate-producing bacteria [66, 67, 68•, 69, 70]. Increased diversity in the *Clostridium coccoides* and *Clostridium leptum* groups has been observed in CRC patients compared with healthy controls [71]. Superoxide-producing *Enterococcus faecalis*, a commensal gut organism, has been shown to induce DNA damage in colon cell lines and rat colonic tissue [72]. Microbial activities that have been associated with CRC risk include those involved in bile acid metabolism and sulfate reduction. Initial 16S rRNA-based microbiota surveys in CRC patients have suggested some dysbiosis, with increased *Coriobacteria* and decreased *Enterobacteria* detected in one study [73•] and differences in *Bacteroidetes/Prevotella* that correlated with increased IL-17 positive cells in another study [74•]. The decreased prevalence of *Enterobacteria* detected in the former study appears inconsistent with other data that show a positive association. However, a hit and run effect, where *Enterobacteria* might have a role in early CRC processes but disappear after the developing lesion changes the gut environment, would explain this observation. In another study that confirmed increased *Coriobacteria* in cancer patients, higher numbers of *Lactobacillales* were detected in cancer tissue [75•], but this could be associated with multiple lifestyle factors, including an increase in the intake of probiotics after diagnosis or due to early CRC symptoms. These observations confirm the need for prospective studies, where microbiota samples are collected from individuals at high risk before the disease develops. Due to differences in study populations, it is to be expected that results of microbiota surveys in CRC patients and controls vary to some extent, but effort should be made to standardize protocols to allow for a comparison between studies and pooling of data where appropriate.

An early emphasis in establishing contributions to CRC has been on proteobacteria, especially γ-proteobacteria, which contain many known enteric pathogens that can cause acute intestinal infections. It is feasible that unresolved acute infections can become chronic, resulting in a proliferative gut milieu that can drive colorectal carcinogenesis. Mucosal adherent proteobacteria have been found to be more abundant in patients with colorectal adenomas compared with normal controls [76]. An increase in intraepithelial *E. coli*

was seen in patients with adenomas compared with healthy controls [70]. Adherent *E. coli* have been shown to decrease expression levels of DNA mismatch repair proteins MLH1 and MSH2 [77]. *E. coli* strains that possess the *pks* pathogenicity island responsible for the production of the bacterial toxin colibactin have been shown to induce DNA damage in mammalian cells lines and lead to chromosomal instability [78]. In an AOM-treated IL-10 knockout mouse model, monocolonization with this strain promoted carcinoma formation [79]. Several pathogenicity islands present in 30-40 % of isolates from CRC patients were previously detected only in uropathogenic *E. coli* strains [80].

*Streptococcus bovis* has been frequently associated with colorectal cancer, but data are conflicting. Fecal carriage of *S. bovis* has been reported to be significantly higher in carcinoma patients compared with healthy controls [81]. However, Potter et al. reported no significant difference between the stool carriage of colorectal cancer patients and healthy controls [82]. This group also reported no significant associations of *S. bovis* with malignant colon tissue using culture techniques, but a study using qPCR detected significantly more *S. bovis* DNA in tumor tissue compared with normal tissue in the same host [83]. An increase in polyamines associated with an increase in ACF was reported after *S. bovis* administration [84]. A recent meta-analysis suggests that a specific biotype, *Streptococcus gallolyticus* (*S. bovis* Biotype 1), is more closely associated with CRC [66]. Although there likely is an association, the evidence for any causal link is weak.

ETBF has been suggested to contribute to intestinal carcinogenesis through the effects of three enterotoxin subtypes on e-cadherin [85, 86]. Nuclear localization of β-catenin has been observed after treating cell lines with *B. fragilis* enterotoxin, subsequently leading to increased expression of *c-myc* and cellular proliferation [87]. Recent data from the same group suggest a $T_H17$-dependent mechanism for carcinogenesis in the APC Min mouse model [88]. Another mechanism by which *B. fragilis* may contribute to CRC development is through the metabolism of heterocyclic amines, which are generated by hot meat practices [89]. *B. fragilis* cultured in meat extract resulted in a twofold increase in mutagenicity [90], suggesting that it may affect genotoxicity of nitrogenous compounds. *B. fragilis* was determined to be enriched in the guts of CRC patients compared with healthy controls [91].

Metabolism of bile acids by the gut microbiota has long been thought to contribute to colorectal carcinogenesis [92]. β-glucuronidase activity, which is involved in bile acid metabolism, has been shown to be increased in CRC patients [93]. Colonic mucosal proliferation has been associated positively with secondary bile acid levels [94]. *In vitro* studies have demonstrated the ability of secondary bile acids to up regulate NF-κβ expression and induce DNA damage through the generation of reactive oxygen species [95]. Bile salt hydrolases (BSHs) are crucial for bile acid modification by the gut microbiota. Bile acids represent signaling molecules that can influence host physiology. BSH activity has been shown to be prevalent in the human gut where it mediates bile tolerance [96]. However, neither detection of fecal bile acids nor bile acid metabolizing bacteria have been convincingly linked to CRC in humans.

Sulfate-reducing bacteria reside in the gut as part of the normal microbiota. Although sulfate-reducing bacteria have not been directly associated with CRC, the production of hydrogen sulfide has been implicated in CRC development. Hydrogen sulfide was determined to be more abundant in subjects who had previously undergone surgery for CRC and developed new neoplasia compared with healthy controls [97]. Hydrogen sulfide also was shown to induce DNA damage mediated by oxidative free radicals. Additionally, COX-2 expression was increased as a result of hydrogen sulfide treatment [98]. Thiosulfate sulfurtransferase (TST), an important enzyme for detoxifying hydrogen sulfide, was found to be decreased in CRC tissue. The abundance of TST was increased in HT-29 cells when cultured with butyrate, suggesting a possible mechanism by which butyrate inhibits CRC development [99].

Butyrate is a preferred fuel for epithelial colonocytes and thought to have a protective effect against CRC. The phylogenetic distribution of butyrate-producing bacteria lies mostly within Clostridium clusters IV and XIVa, also known as the *Clostridium leptum* and *coccoides* groups, respectively [100].

In rodent models, administration of both butyrate-producing bacteria and butyrate metabolic precursors has been shown to increase colonic levels of butyrate and decrease precancerous lesions correlating with increased splenic NK cells and decreased β-glucuronidase activities in fecal cultures, both of which associated with protection against CRC [67, 101]. Butyrate also promotes apoptosis through the Wnt signaling pathway in CRC cell lines [102]. Whereas moderate Wnt levels promote uncontrolled proliferation, high levels appear to induce apoptosis [103]. Butyrate also is a known histone deacetylase inhibitor and has been shown to promote DNA demethylation of pluripotency-associated genes in fibroblasts [104].

Whereas the low pH in the stomach results in low microbiota diversity, the colon harbors some of the most diverse microbial communities. Thus, generating evidence for microbiota associations with CRC requires extensive ecological approaches. Although the evidence of a microbiota/CRC link presented above is as of yet not convincing, there has been progress. There is promise that recent developments in microbiota analysis tools will soon translate into significant progress, especially if the appropriate prospective population studies are performed.

## Means of Influencing Gut Microbiota Composition

A thorough understanding of the contributions of microbiota to CRC can form the foundation for the future development of targeted prevention supplements. Multiple probiotic and prebiotic supplements are already marketed for potential health benefits [105]. Due to the long process of colorectal carcinogenesis, studies in humans often have to be limited to show changes in intermediate CRC markers, such as a decrease in fecal water toxicity with *Bifidobacteria* and lactic acid bacteria supplementation [106]. Most of the experimental evidence of pro- and prebiotic benefits associated with intestinal carcinogenesis is derived from animal models [107]. Nevertheless, there is already a clear indication that supplementation with pro- and prebiotics can modify colonic environment and affect intestinal carcinogenesis. In addition to using naturally derived strains, there also have been efforts to genetically modify lactic acid bacteria for desired characteristics. LAB strains producing human IL-10 and with deletions in the phosphoglycerol transferase gene, responsible for lipoteichoic acid (LTA) biosynthesis, have shown success in IBD and CRC models [108, 109]. Due to the differences in underlying microbiota composition, specific pre- and probiotic supplements are likely to benefit only a proportion of the population. Developing criteria to identify individuals who are most likely to benefit would allow for improved targeting of such interventions.

## Conclusions

Multiple lines of evidence support the notion that gut microbiota can contribute to colorectal carcinogenesis. Diet, particularly DF intake, which has been associated with reduced CRC risk, strongly affects microbiota composition. Thus, changes in microbiota might represent important mechanisms through which diet/DF reduces CRC risk. Various bacteria have been linked with experimental carcinogenesis in animal models or correlated with CRC in human observational studies. Multiple microbiota-based studies suggest differences in mucosa associated and luminal bacteria in subjects with CRC. Various pro- and prebiotic interventions aimed at modifying gut microbiota toward a more beneficial composition have been successful in reducing CRC risk markers, gut inflammation, and preneoplastic lesions. We conclude that although no single, microbial agent has yet been shown to be causally linked to CRC, contributions of the gut microbiota to colorectal carcinogenesis are evident. It can be expected that further advances in the field can soon be translated into the development of microbiota-based CRC screening and prevention regimens. A more detailed understanding of how microbiota can be manipulated also might point us toward novel means to minimize the detrimental effects of surgery, radiation, and chemotherapy treatment on gut health. Due to the established uniqueness of each individual's microbiota, personalized microbiota manipulations, possibly based on the underlying dominant enterotype, will likely offer the best chance for success.

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- •• Of major importance

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