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Biochim Biophys Acta Rev Cancer. Author manuscript; available in PMC 2022 January 01.

Published in final edited form as:

Biochim Biophys Acta Rev Cancer. 2021 January ; 1875(1): 188490. doi:10.1016/j.bbcan.2020.188490.

Role of Gut Microbiota in Epigenetic Regulation of Colorectal Cancer

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Abstract

Colorectal cancer (CRC) remains one of the most commonly diagnosed cancers and a leading cause of cancer-related deaths worldwide. The stepwise accumulation of epigenetic alterations in the normal colorectal epithelium has been reported to act as a driving force for the initiation and promotion of tumorigenesis in CRC. From a mechanistic standpoint, emerging evidence indicates that within the colonic epithelium, the diverse gut microbiota can interact with host cells to regulate multiple physiological processes. In fact, recent studies have found that the gut microbiota represents a potential cause of carcinogenesis, invasion, and metastasis via DNA methylation, histone modifications, and non-coding RNAs - providing an epigenetic perspective for the connection between the gut microbiota and CRC. Herein, we comprehensively review the recent research that provides a comprehensive yet succinct evidence connecting the gut microbiota to CRC at an epigenetic level, including carcinogenic mechanisms of cancer-related microbiota, and the potential for utilizing the gut microbiota as CRC biomarkers. These scientific findings highlight a promising future for manipulating the gut microbiota to improve clinical outcomes in patients suffering from CRC.

Keywords

Colorectal cancer; Gut microbiota; Diagnostic biomarkers; Epigenetic modifications; non-coding RNAs

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Conflict of Interest: None of the authors has any potential conflicts to disclose.

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1. INTRODUCTION

Colorectal cancer (CRC) remains the third leading cause of cancer-related deaths among men and women in the United States, with an estimated 147,950 new cases and 53,200 deaths projected in 2020 [1]. Even with advances in CRC screenings and therapeutic strategies, CRC still remains one of the most devastating malignancies. Patients with metastatic CRC have a median overall survival of only ~30 months [2]. Comprehensive studies on disease pathogenesis at the molecular level have provided a unique perspective for its detection, surveillance, and therapeutic approaches for improving outcomes and survival in CRC patients [3]. The driving force for CRC tumorigenesis is a combination of multiple factors, with various host and environmental factors involved in tumor formation and growth [4]. To some extent, CRC has been described as a hereditary susceptibility syndrome [5], in which genetic predisposition or familial influences, such as Lynch Syndrome [6], familial adenomatous polyposis [7], and Peutz–Jeghers syndrome [8], influence the overall risk of developing CRC. However, it is estimated that only a minority of CRC cases develop through germline transmission of genetic alterations; while the majority of cases are believed to result from host-environmental interactions [9]. Environmental factors thought to act as either carcinogens or tumor-promoting agents can manifest in the accumulation of epigenetic variations in host cells, among which the gut microbiota as emerged as an important player for its pathogenic role in various cancers, including CRC [10, 11]. Therefore, a better understanding of the epigenetic connection between the gut microbiota and CRC pathogenesis will likely yield novel insights into the impact of environmental exposure in CRC. In this review article, using comprehensive search terms related to the gut microbiota, CRC and epigenetics, we conducted a literature search through the database searches in PubMed, Embase, and Web of Science to search for all relevant articles and abstracts up until Novermber 2020, including both clinical trials and basic research. We highlight the carcinogenic mechanisms of CRC-related microbiota and discuss the potential for utilizing the gut microbiota as biomarkers in this malignancy. Such a knowledge will provide a theoretical basis for the potential use of gut microbiota as biomarkers for cancer screening, diagnosis and risk prediction.

2. THE GUT MICROBIOTA AND CRC

For human beings, from birth, microbiota colonizes the skin, digestive tract, respiratory tract, reproductive tract, and other parts in contact with the external environment, among which the colon and rectum represent ideal habitats for containing the largest number and variety of microbiota [12]. With the most expansive mucosal surface area in contact with the outside environment, the human colorectal epithelium comprises of a considerable gut microbiota ecosystem, comprising of more than 10^{14} microorganisms, that primarily include *Bacteroidetes, Proteobacteria, Actinobacteria, Firmicutes*, and *Fusobacteria* [13]. In this context, the intestinal environment, dietary regulation, and drug metabolism have a rapid and major impact on the communal characteristics of the gut microbiota [14, 15]. The gut microbiota, in turn, plays a fundamental role in overall human health, by assisting in host digestion and absorption, regulating the development and function of the mucosal barrier, promoting the maturation of immune tissues, and affecting tolerance to gastrointestinal antigens [16, 17]. However, at the same time, metabolites of the gut microbiota that have

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toxic effects on the human body, including but not limited to phenol, p-cresol and indole are recognized to be involved in the occurrence and development of CRC [18]. In general, the gut microbiota likely acts as a common denominator during cancer pathogenesis by acting as bridge for various tumor stages, from environmental effects and inflammation of the gastrointestinal mucosa to colorectal mucosal cancerization.

2.1 ADVANCED APPROACHES FOR IDENTIFICATION OF GUT MICROBIOTA

Polymerase chain reaction (PCR), which can identify the presence and abundance of specific bacterial strains among the complex community of microbiota through specific primer design, has been considered to be the most attractive approach for studying the gut microbiota profiles. However, PCR testing is limited to detecting a particular strain using specifically designed primers, and thus cannot fully provide a comprehensive description of the gut microbiome [19]. The emergence of next-generation sequencing (NGS) technologies have enabled large-scale studies of the gut microbiota to identify previously unknown bacterial species and strains, and has revealed high variability in microbiome composition between individuals and between different body sites within the same person [20, 21]. To date, several types of large-scale analyses based on NGS techniques have been used to assess the gut microbiota, including 16S amplicon sequencing, which is based on sequencing hypervariable regions of the 16S ribosomal RNA (rRNA) [22], and shotgun analysis, which is based on direct sequencing of the total DNA (metagenome) and/or total RNA (metatranscriptome) [23]. Such techniques have greatly expanded our knowledge of the diversity and multi-functionality of the gut microbiota (Figure 1).

In this context, 16S amplicon sequencing is a relatively simple, low-cost method to obtain a broad overview of microbiome composition [24], in which specific regions common to all microbiota genomes are first amplified and sequenced. Sequencing reads are clustered into operational taxonomic units (OTUs) based on sequence homology against known 16S rRNA gene databases, which is a commonly used measure of microbial diversity. Population abundance is quantified and the diversity of the microbiome is identified by the resulting OTUs in various specimens [25]. However, data generated by 16S amplicon sequencing is limited in several aspects, due to the lack of information on microbiota function and limited “taxonomic resolution.” For instance, in most cases, microbiota cannot be identified precisely at the species level [26]. To overcome these limitations, metagenomic shotgun sequencing—sequencing all genes presented in the microbiome rather than just a single taxonomic marker gene—can provide information about the abundances of genes at all taxonomic levels by mapping reference genomes/genes. Metagenomic shotgun sequencing can also identify gene content and infer its functional potential of proteins encoding in the microbiome using functional annotated databases [27]. Both 16S amplicon sequencing and metagenomic shotgun sequencing are based on genomic DNA sequences from microbiota samples. RNA-expressing genes express their functional activity better than DNA because many genes are only conditionally expressed. RNA transcripts from the microbiome provide a more comprehensive description of gene expression of the microbiota. Therefore, metatranscriptomic sequencing allows the identification of expressed transcripts, providing different insights from DNA-based microbiome sequencing methods. Transcriptome abundance can also be used to compare gene expression profiles between various

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microbiota. However, it is more expensive to obtain comparable profiles of the microbiota using metagenomic and metatranscriptomic sequencing compared to using 16S amplicon sequencing [25]. In addition, data generated from metagenomic and metatranscriptomic sequencing has been challenging to analyze, owing to its substantial complexity compared to data generated by 16S amplicon sequencing.

2.2 COMPOSITION OF GUT MICROBIOTA IN HEALTHY PEOPLE

The structure and physiological activity of the gut microbiota are closely related to the health of an organism [28]. Gut microbiota are combined in a certain proportion; wherein, each strain is interdependent and competes to maintain a critical ecological balance [29]. In the normal human gastrointestinal tract, the relationship between the host and the gut microbiota is mutually beneficial and symbiotic: the digestive tract provides an environment for the colonization and survival of specific microbiota, and the microbiota in turn plays unique functions in maintaining intestinal health, such as nutrient metabolism and intestinal protection [29]. In addition, the gut microbiota participates in catabolism and synthesis of substances in the intestinal tract and decomposes macromolecular compounds that cannot be otherwise digested by the host into final metabolic products, providing energy for the host as well as nutrition for its own growth and reproduction [30]. For example, gram-positive microbiota, such as *Lactobacillus* spp., *Streptococcus* spp., and *Faecalibacterium* spp. can synthesize B vitamins, vitamin K, niacin, and a variety of amino acids with anti-inflammatory, anti-tumor and anti-bacterial effects [31–33]. Furthermore, epithelial cells, mucosal layers, and intestinal microbiota secretions can form an effective intestinal barrier to prevent the invasion of harmful bacteria and other pathogens [34].

The rapid development of NGS technologies is deepening our understanding of the origin of the gut microbiota and the landscape of its evolution. Due to differences in oxygen content, pH values, antimicrobial peptide levels and intestinal motility at different anatomical sites within the gut, the overall composition of the gut microbiota varies greatly [35, 36]. The gut microbiota colonizes the entire length of the intestine tract, and the load of microbiota generally increases from the duodenum to the distal colon, ranging from 10^3 to 10^4 mL^{-1} content in the stomach, duodenum, and jejunum, to 10^8 mL^{-1} in the ileum, and up to 10^{11} mL^{-1} in the colon [35, 36]. Gut-commensal microbiota can be anatomically defined as: (i) lumen-commensal, (ii) mucus-resident, (iii) epithelium-resident, and (iv) lymphoid tissue-resident populations (summarized in Table 1). The translocation and ecological imbalance of gut commensal microbiota are closely related to carcinogenesis and development of CRC.

2.3 COMPOSITION OF GUT MICROBIOTA IN PATIENTS WITH CRC

The microbial diversity and balance are the key characteristic features of a healthy gut, as a rich gastrointestinal ecosystem can cope with the challenges of various factors that promote disease occurrence [37]. By comparing the gut microbiota of younger individuals (20–39 years old) vs. elderly (>60 years old), the results have revealed that younger individuals tend to gain more microbial taxa, while elderly individuals tend to lose microbiota diversity in a healthy gut[38]. Although CRC incidence and mortality trends have declined overall, these trends in early-onset CRC (EOCRC; in patients <50 years old) are actually on a rise, worldwide[39]. By analyzing healthcare claims data from all geographic areas of the United

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States, it is confirmed that the increased risk of EOCRC is associated with metabolic dysregulation, which is often accompanied by the presence of the gut microbiota dysbiosis [40]. Nowadays, due to various risk factors such as antibiotic use, diet, obesity and stress, the gut microbiota dysbiosis often occurs in younger generations, which may in part explain the increasing risk of EOCRC[41]. Even in the individuals with genetic predisposition to CRC, the gut microbiota still contributes to CRC risk substantially. For example, a significant increase of Bacteroidetes and Proteobacteria as well as a reduction of Firmicutes were observed in Lynch syndrome fecal samples [42]. Leveraging stool meta-transcriptomes, another study showed that the progression toward carcinogenesis of lynch syndrome can be predicted in modest power by gut microbial transcription[43]. Accumulated studies to detect gut microbiota in experimental animal models and in patients have indicated that various stages of CRC disease progression are often associated with significant ecological disorders in CRC tissues and with microorganisms in the adjacent mucosa [44]. [42, 43]Key characteristics of major changes in the gut microbiota associated with CRC are summarized in Table 2. For example, in patients with adenomas, the abundance of *Bilophilia, Desulfovibrio, Corynebacterium* and *Phascolarctobacterium* in the fecal matter was significantly higher, whereas in patients with serrated polyps, *Erysipelotrichia* and *Fusobacteria* were predominantly more, compared with their relatively abundance in healthy people [45]. Both adenomas and serrated polyps may develop into CRC. As adenomas and serrated polyps develop into CRC, toxins produced by pathogenic microbiota present an increasing trend in intestinal mucosal tissues, such as cytotoxic necrosis factor and cycle suppressor produced by *Fusobacterium nucleatum* and *Bacteroidetes fragilis*. In addition, as illustrated in Figure 2, the abundance of some of the invasive microbiota also illustrates a similar upward trend, which includes enteroinvasive *Escherichia coli* (EIEC) [46].

F. nucleatum—*F. nucleatum*, a gram-negative anaerobic bacterium commonly found in the mouth, which was first found to be associated with CRC incidence in 2012 [47, 48]. Fluorescence quantitative PCR (qPCR), fluorescence in situ hybridization [49], and droplet digital PCR [50] were used to demonstrate that *F. nucleatum* was highly enriched in CRC tissues. The qPCR results revealed that the average total abundance of *F. nucleatum* was 415 times higher in 99 CRC tissue specimens compared to the corresponding normal mucosal tissues [49]. Subsequent studies have used metatranscriptomic sequencing and metagenomic analysis to confirm the increased abundance of *F. nucleatum* in CRC tissues vis-à-vis healthy tissues [51–53]. Furthermore, the matched *F. nucleatum* strain was detected in both saliva specimens and CRC tissues in 75% of the CRC-positive patients, suggesting that this bacterium in CRC tissues likely originates in the oral cavity, and digestive tract transmission may be one mechanisms underlying its diffusion [54]. The correlation between *F. nucleatum* and CRC has been widely confirmed; while the potential mechanisms for its mechanistic role in cancer pathogenesis remain unclear but are an area of active investigation.

One mechanism connecting *F. nucleatum* and CRC may be epithelial-to-mesenchymal transition (EMT). Microscopic approaches were used to observe epithelial cells incubated with *F. nucleatum* transdifferentiating into mesenchymal-like cells, showing an enhanced ability to invade [55]. However, western blot analysis of total E-cadherin protein revealed an

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equal expression of this epithelial cell adhesion protein before and after incubation with *F. nucleatum*. Accumulating evidence suggests that the potential toxicity of *F. nucleatum* and its ability to destroy intestinal epithelial cells are primarily due to *F. nucleatum* adhesin A (FadA), a virulence factor that regulates bacterial adhesion and invasion [56, 57]. The FadA gene expression was significantly higher in CRC specimens than in adjacent normal tissues [58]. In another study, *F. nucleatum* emerged as a high-risk factor for CRC metastasis and was found to be bound to E-cadherin-expressing cells via FadA [59]. This is of biological importance, as E-cadherin activates the β-catenin pathway, which promotes CRC cell growth. Recently, *fadA* was shown to up-regulate the expression of the Wnt/β-catenin modulator Annexin A1 through E-cadherin [60]. Dysregulation of the E-cadherin/β-catenin complex leads to CRC cell metastasis. Combined with the regulatory effect of *F. nucleatum* on E-cadherin/β-catenin, this strain may promote occurrence and progression of CRC by reducing E-cadherin-dependent cell–cell adhesion. Additionally, *F. nucleatum* can promote the secretion of the matrix metalloproteinases MMP-9 and MMP-13 by activating the mitogen-activated protein kinase p38, increasing the proliferation and survival of infected epithelial cells [61]. Furthermore, recent studies reported that the microRNA (miRNA) miR-21 plays a key regulatory role in the direct correlation between *F. nucleatum* and proliferation, invasive activity, and xenograft tumor formation in mice [62, 63].

B. fragilis—*B. fragilis*, a common gram-negative obligate anaerobe, is more abundant in fecal samples from patients with CRC compared to controls [64]. Another study used pyrosequencing analysis to confirm that although the absolute abundance of bacteria per gram of feces was similar between patients with CRC and healthy people, *B. fragilis* abundance was significantly higher in patients with CRC than in healthy people [65]. However, it has also been shown that *B. fragilis* is less abundant in CRC tissues than in adjacent non-cancer tissues [66, 67]. These differences may be due to the distinct species of *B. fragilis* existing in the gastrointestinal tracts of patients with CRC. Two main classes of *B. fragilis* that colonize most humans have been described: enterotoxigenic *B. fragilis* (ETBF), which secrete *B. fragilis* toxin (BFT), causing diarrhea, peritonitis, and intra-abdominal abscesses in humans; and non-toxigenic *B. fragilis* (NTBF), which do not secrete BFT [68].

Some investigations have suggested that ETBF may operate as a pathogenic bacteria, triggering an immediate and robust inflammatory response, causing an ecological imbalance in the intestinal microbial community [64, 69]. The key factor for ETBF virulence in CRC is attributed to BFT, a secreted 20-kDa zinc-dependent metalloprotease toxin [70]. Expression of the BFT gene is more common in the intestinal mucosa of patients with CRC compared to healthy individuals, especially in patients with advanced CRC [64]. BFT expression induces cleavage of the extracellular domain of E-cadherin in colonic epithelial cells, leading to increased epithelial cell permeability. E-cadherin stimulates cellular signaling through the β-catenin/Wnt pathway, which is active in certain CRC cases [71]. Another study showed that after BFT treatment of CRC cells, loss of membrane-associated E-cadherin activated the nuclear localization of ß-catenin and induced c-Myc translation, leading to continuous cell proliferation [7]. An additional study revealed that BFT can stimulate the production of spermine oxidase (SPO) in intestinal epithelial cell lines, suggesting that enterotoxin has a

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direct effect on the production of abundant reactive oxygen species (ROS) and thereby causing DNA damage [72].

Escherichia coli—*E. coli*, a member of the Enterobacteriaceae family, is the most common symbiotic, gram-negative anaerobe in the gastrointestinal tract, which causes diverse effects on gut health by different biological components [73]. For example, some strains are known for their probiotic properties, such as the Nissle 1917 strain of *E. coli*, which prevents invasion of human intestinal epithelial cells by various pathogens and has been used as a probiotic to treat gastrointestinal disorders for more than a century [74]. In contrast, some *E. coli* strains show genotoxic activity, which has deleterious effects on host DNA and might ultimately cause colon cancer. Numerous studies have reported elevated colonization levels of colonic mucosa-associated *E. coli* in patients with CRC compared to healthy individuals. For instance, mucosal specimens from patients with CRC contain more than 70% microbiota, generally *E. coli* [75]. One study reported that mucosa-associated or internalized *E. coli* increased significantly in CRC tissues compared to corresponding non-tumor normal tissues [76]. Another study reported that *E. coli* was detected in and accounts for 62% and 77% of patients presenting with adenomas and carcinomas, respectively [77]. In addition, the levels of pathogenic *E. coli* strains that produce the toxin cyclomodulin were more prevalent in stage III and IV CRC tissues than in stage I cancers, indicating that the abundance of pathogenic *E. coli* may be related to CRC stage and prognosis [76].

Various studies have demonstrated a clear link between mucosa-adherent *E. coli* and CRC. Importantly, *E. coli* promotes colon tumorigenesis in CRC mouse models after microbiota transplantation in various CRC mouse models, including $Apc^{Min/+}$ mice [78], azoxymethane (AOM)-treated $IL10^{-/-}$mice [79], AOM/dextran sodium sulfate (DSS)-treated mice [80], and $Apc^{Min/+}$ /$IL10^{-/-}$ mice [81]. The biological roles for *E. coli* in CRC etiology have also been demonstrated. In particular, colibactin, a bacterial toxin synthesized by *E. coli* carrying the polyketide synthase (*pks*) gene, can cause DNA damage and genomic alterations and instability, involved in colorectal carcinogenesis [82]. This conclusion was supported by another study, in which mammalian epithelial cells exposed to *pks*-positive *E. coli* exhibited transient DNA damage, dysfunctional DNA repair, and an increased frequency of gene mutations [83]. Moreover, *pks*-positive *E. coli* induce autophagy and DNA damage repair in intestinal epithelial cells; inhibition of this protective process increases the inflammatory and carcinogenic effects of *E. coli* in susceptible mice [78].

3. EFFECTS OF GUT MICROBIOTA AND THEIR METABOLITES ON EPIGENETIC REGULATION OF CRC

Most CRC cases begin with the growth of polyps in the inner lining of the colon and rectum, which subsequently develop into dysplastic adenomas, and eventually cancer. In addition to multiple genetic mutations, epigenetic modifications also contribute to the pathogenesis of this disease during its initiation and progression, through processes such as tissue invasion and metastasis [84]. Epigenetic modifications broadly refer to phenotypic changes secondary to changes in gene expression that do not involve permanent changes in the DNA sequence. Accumulating data indicate that the gut microbiota can regulate epigenetic modifications in

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the host, thus enabling manipulation of the host's chromatin configuration and functionality. And these modifications can last from one cell division to the next, and hence can be inherited even if the gene sequence is not altered [85]. Therefore, in some sense, epigenetic modifications provide a potentially important interface linking the dynamic interactions between the microbiota and the host genome.

The link between epigenetic modifications and the gut microbiota has been known to be involved in the crosstalk of microbiota-derived metabolites [86]. Microbiota-derived metabolites have received widespread attention for beneficial effects on both cellular energy metabolism and intestinal homeostasis [58]. Short-chain fatty acids (SCFAs) are the most important metabolites of the gut microbiota, as they act as a direct energy source for host cells, stimulate the production of hormones in the body, and play a role in regulating food intake in the brain [87]. Other microbial metabolites, such as bile acids, branched-chain amino acids, indole propionic acid, and endocannabinoids, affect the body's energy expenditure by influencing thermogenesis and adipose tissue browning [88]. In addition to these direct metabolic effects, it is becoming increasingly apparent that microbiota-derived metabolites can be important but indirect regulators of the epigenetic mechanisms. The epigenetic mechanisms that have a role in cancer development include DNA methylation, histone modifications and non-coding RNAs. Subsequently, from these perspectives, we will discuss the impact of the gut microbiota and their metabolites on epigenetic modifications during colorectal carcinogenesis. Studies about gut microbiota involved in CRC epigenomic modifications are summarized in Table 3.

3.1 DNA METHYLATION

DNA methylation is an epigenetic biological process that predominantly occurs through covalent addition of a methyl group (CH_3) to the 5-carbon of a cytosine residue, resulting in 5-methylcytosine (5-mC), which subsequently affects the function and inhibits the transcription of the target gene [89]. Numerous reports have shown that the gut microbiota can modify the methylation pattern of cancer-related genes, thus providing insight into new directions for identifying relationships among the gut microbiota, the epigenetic landscape of host cells, and the occurrence and development of CRC. For example, in a study comparing the methylation profiles of ETBF-induced and spontaneous tumors in the distal colon of *Apc*$^{Min/+}$ mice, greater hypermethylation and reduced hypomethylation of differentially methylated regions were observed in ETBF-induced tumors than in spontaneous tumors [90]. Ecological dysbiosis of the gut microbiota can induce host gene methylation, but to date, studies on specific bacteria regulating CRC methylation and its regulatory mechanisms are very limited. A population-based study reported that a high load of *F. nucleatum* in CRC tissues was associated with high microsatellite instability and a CpG island methylator phenotype [91]. In another study, *F. nucleatum* was correlated with wild-type tumor suppressor *TP53*, methylation of the mismatch repair gene *hMLH1*, genomic hypermutation, and mutation of the chromatin remodelers CHD7/8 [92]. Similarly, comparison of gastric biopsy results between patients infected with *Helicobacter pylori* and healthy individuals revealed that chronic gastritis was associated with hypermethylation of the promoter region of E-cadherin (*CDH1*), the DNA methyltransferase (DNMT) *MGMT*, the Wnt inhibitor *WIF1*, and the *MLH1* gene [93]. Furthermore, fecal bacterial species,

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including several *Parvimonas* species, were recently identified in individuals with a higher cumulative methylation index in the blood [94]. During DNA methylation, S-adenosyl methionine (SAM) acts as a methyl donor to enable DNMTs to form 5-mC [95]. Microbial metabolites such as folate are potentially involved in the synthesis of 5-methyltetrahydrofolate, which is itself a methyl group donor for SAM [96]. Indeed, in human colonic cells, global DNA and p53 region-specific hypomethylation were induced by folate depletion, and the common microbial genera *bifidobacteria* and *lactobacillus* produce folate [97]. Another intriguing study highlighted that volunteers given *bifidobacteria* exhibited high concentrations of folate in the feces [98], which suggests that this probiotic may affect DNA methylation patterns via folate production.

3.2 HISTONE MODIFICATIONS

The acetylation of histone lysine residues is involved in transcription, translation, and DNA repair, and is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs catalyze the transfer of acetyl groups from acetyl coenzyme A (Ac-CoA) to amino-terminal lysine residues on histones, whereas HDACs reverse this process. Consequently, HATs and HDACs collaboratively maintain the balance of histone acetylation *in vivo* to achieve homeostasis [99]. In addition to stabilization of acetylated histones, HDACs can also regulate CRC cell proliferation and apoptosis by modulating the acetylation status of p53 and tubulin protein [100]. In turn, gut microbiota can regulate the activity of HDACs by producing epigenetic metabolites such as SCFAs [86], as illustrated in Figure 3. Many studies surrounding the gut microbiota, CRC, and histone modifications have focused on the roles of SCFAs as HDAC inhibitors. Several different mechanisms by which SCFAs enter colonic intestinal cells have been proposed, including passive diffusion, counter-transport with bicarbonate, transporter monocarboxylic acid transporter 1, and sodium-coupled monocarboxylic acid transporter 1 [101]. The most important SCFA-producing bacteria are *Firmicute, Bacteroidetes, Proteobacteria*, and *Actinobacteria* [102]. How SCFAs regulate tumor formation and histone deacetylation remains unclear and is an active area of investigation. Chromatin maps of colorectal epithelial cells isolated from conventional and germ-free (GF) mice showed a decrease in diacetylated lysine on the histone subunit H3 in GF mice [102]. Supplementation with several SCFAs (acetate, propionate, and butyrate) resulted in a histone profile closer to that of conventional mice, suggesting that these metabolic by-products have the potential to induce histone modifications [103].

3.3 NON-CODING RNAs (NcRNAs)

NcRNAs are important regulators of epigenetic status, and have functional significance in modulating gene expression during CRC tumorigenesis [104]. Whether the gut microbiota modulates ncRNA expression in the host to impact CRC is under investigation. Most studies have used GF and conventional mice to detect differences in long noncoding RNAs (lncRNAs) and miRNA expression in the presence of gut microbiota. Exon microarrays were used to compare lncRNA expression profiles between GF and conventional mice, and distinct changes in lncRNA signatures were observed after GF mice were reconstituted with normal mouse microbiota or with *E. coli* alone [105]. Reduced expression of three miRNAs known to be expressed in intestinal epithelial cells (let-7b, miR-141, and miR-200a) was observed in the feces of GF mice compared to the conventional mice. Following a 6-week

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treatment with antibiotics to deplete the gut microbiota in rats, miRNA expression was also reduced [106]. Other studies have shown that gut microbiota can alter the expression of CRC-related miRNAs; for example, commensal bacteria induce the expression of miR-21–5p in intestinal epithelial cells [107]. To demonstrate the functional impact of microbially mediated miRNA changes on CRC development, global miRNA expression profiling was used to identify several differentially expressed miRNAs between *F. nucleatum*-rich samples from patients with recurrent CRC and low amount of *F. nucleatum* samples from non-recurrent patients. Among the downregulated miRNAs, *F. nucleatum* targeted miR-18a and miR-4802 to alter CRC chemotherapeutic response by activating the autophagy pathway [108].

4. THE VIABILITY OF THE GUT MICROBIOTA AS A BIOMARKER FOR CRC

The progression from normal colonic mucosa to adenoma and eventually to CRC orchestrates in several stages, over at least 10 years, and appropriate measures can be taken to avoid the occurrence of CRC at any stage during this period [109]. Even if cancer is diagnosed, the 5-year survival of patients treated during the early stages of CRC is encouraging, ranging from 72% to 93% [110]. Therefore, early detection of precancerous lesions and CRC is the key to improving the chance of a cure.

Non-invasive screening strategies for early CRC involve either checking the presence of minute quantities of blood in the stool, such as with a fecal immunohistochemistry test (FIT) or a guaiac-based fecal occult blood test, or performing multi-target molecular detection of DNA, RNA, and protein markers of neoplasia in the feces [111, 112]. Single-sample FIT tests are approximately 20%–30% sensitive for advanced neoplasia detection and approximately 80% sensitive for CRC detection [113]. Multi-target molecular detection for high-grade precancerous lesions is approximately 46% sensitive [114]. Therefore, it is necessary to explore novel non-invasive screening methods. Due to differences in the gut microbiota between patients with colon adenoma or CRC and healthy people, characteristic changes in fecal microbiota and metabolites are promising biomarkers for early CRC screening. The possibility of using gut microbiota evaluation as a CRC screening tool via Bayesian statistics was first proposed in 2014 [115]. Targeted detection of CRC-related dysbiosis and microbiota species in fecal samples may represent a promising non-invasive screening method to overcome the limitations and the poor performance of current CRC early diagnosis tools.

Notable advances in the development of non-invasive, early screening methods have been made through the identification of relevant and specific microbial characteristics in patients with CRC. Many studies have assessed the diagnostic accuracy of fecal *F. nucleatum* as a potential non-invasive biomarker for CRC. In a meta-analysis included ten studies comprising 1450 patients with CRC and 1421 healthy individuals, fecal *F. nucleatum* performed well as a diagnostic biomarker for CRC, with 71% sensitivity and 76% specificity [116]. Indeed, in one study of 439 subjects (203 colorectal cancer and 236 healthy subjects), fecal *F. nucleatum* performed well as a diagnostic biomarker, with sensitivity better than FIT for the detection of CRC [117]. By using qPCR to analyze 103 patients with advanced adenoma, 104 patients with CRC, and 102 healthy controls, it is identified that the

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combination of *F. nucleatum* and FIT has a high sensitivity (92.3%) and an area under the curve (AUC) of 0.95 in detecting CRC [118]. The expression levels of the FadA gene is significantly higher in the colon tissue of patients with adenoma and adenocarcinoma than in that of normal individuals; thus, this unique adhesin of *F. nucleatum* may be an ideal diagnostic marker to identify individuals with CRC risk [58]. After metagenomic profiling of fecal microbiota, a non-invasive microbial biomarker panel (*F. nucleatum*, *Peptostreptococcus stomatis*, *Parvimonas micra*, and *Solobacterium moorei*) for CRC was established and validated from ethnically different cohorts in China, Denmark, France, and Austria [119]. Another study also performed fecal metagenomic and metabolomic profiling on fecal samples from a large cohort of 616 participants, providing that the shifts of *F. nucleatum* and its metabolites (branched-chain amino acids, phenylalanine, and bile acids) occurred from the very early stages of CRC development, which is of significance for etiology and diagnosis [120]. Based on an analysis of metagenomic samples from a 526-case CRC cohort, a panel of seven CRC-enriched bacteria (*B. fragilis, F. nucleatum, Porphyromonas asaccharolytica, P. micra, Prevotella intermedia, Alistipes finegoldii*, and *Thermanaerovibrio acidaminovorans*) was identified with an AUC value of 0.80 [121]. Moreover, high abundance of *F. nucleatum* and *B. fragilis* has been identified as independent indicators of poor survival in patients with CRC [122]. Various studies (summarized in Table 4) have used characteristic gut microbiota to construct CRC diagnostic models, which can be divided into the following four categories: (i) individual strains dominated by *F. nucleatum*; (ii) combinations of more than three kinds of microbiota as biomarkers; (iii) inclusion of viruses, fungi, or metabolites as biomarkers; and (iv) prediction models jointly constructed by combination of the above microbiota and FIT or other conventional tests.

In order to translate basic research findings for their clinic utility, clinical trials are currently being performed to determine the translational potential of the gut microbiota as biomarker of CRC. A clinical trial (NCT01778595) was recently conducted to identify a useful diagnostic biomarker Proteobacteria whose median relative abundance was 3-fold higher in colorectal adenoma patients than in healthy individuals. Another trial (NCT02845973) enrolled 1325 participants and identified that one or more gut bacterial species in feces may contribute to early diagnosis of CRC. The results showed that fecal *Clostridium symbiosum*, maybe a promising noninvasive biomarker for early diagnosis of CRC, with a higher diagnostic power than *F. nucleatum*, FIT and CEA. In addition to evaluating the gut microbiota as diagnostic biomarker for CRC, investigators have also studied bacterial diversity for its effectiveness as a prognostic predictor and recurrence monitor for CRC. A clinical trial (NCT04223102) is currently recruiting patients to evaluate the association between the gut microbiota and response to neoadjuvant therapy. Another trial (NCT03385213) is currently underway to discover whether there are differences in the gut microbiota between patients with recurrent CRC and those without. All these data suggest that exploration of the gut microbiota can be used not only for non-invasive and accurate diagnostic approaches for CRC but may also serve as prognostic tools for CRC treatment in the future.

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5. CONCLUSIONS AND PERSPECTIVE

Affected by various factors such as diet, age, and health status, there are natural differences in the composition of the gut microbiota among individuals, but the overall complexity of the human gut microbiota is still relatively conservative. Therefore, it is of universal significance to study the microbiota that play a major role in terms of quantity and function. Specific microbiota (e.g., *B. fragilis, F. nucleatum*, and *E. coli*) have been increasingly found to be carcinogenic in CRC. To conclude, CRC may be the result of enhanced interaction between pathogenic microbiota and a disordered host response at both the genomic and epigenomic level.

Although great progress has been made in investigating the gut microbiota, there are still some unresolved issues. To date, annotations from metagenomic data of microbial taxa mostly reach the level of genus or species, which is not detailed enough to understand which specific strains participate in the pathogenesis of human CRC or the mechanisms by which they do so. Further research identifying microbial strains based on multi-omics and data mining algorithm is urgently needed. In addition, the current state of microbial research on CRC has potential limitations: because most CRC cohorts are cross-sectional, it is difficult to reveal the dynamic balance of gut microbiota and to infer the causal relationship between gut microbiota disorders and CRC. Thus, it is urgent that we integrate multi-omics data across populations for longitudinal microbial profiling to further understand the exact role of the gut microbiota in CRC tumorigenesis.

Along with the development of advanced techniques and the deepening of mechanism research, a more comprehensive and in-depth understanding of the gut microbiota in CRC will undoubtedly contribute to the emergence of novel and precise diagnostic strategies in the foreseeable future. Of note, fecal samples because of their easy availability, repeatable sampling, non-invasive and inexpensive nature, can be used as a suitable substitute to reflect the gut microbiota. However, if contamination occurs or the time interval between sample deposition and collection is too long, the fecal microbiota may become altered[123]. In addition, fecal collection kits, storage conditions, transportation conditions and DNA extraction methods all influence detection results, which makes it difficult to guide clinical diagnosis and treatment decisions [124]. Thus far, it has been challenging to get an accurate result from a somewhat improper specimen. The accuracy of samples has a significant influence on the value of research on the gut microbiota. The current gold standard for fecal sampling is instant freezing of fecal materials at −80°C and without preservatives to preserve microbial integrity[125]. However, in some cases, the ideal conditions for storage of fecal specimens at −80°C are not met, especially for large-scale population studies. Therefore, to ensure the reliability of research, more optimized and precise sampling methods are urgent needed. Overall, it is expected that wider range of standardized specimen collection and preservation, more standardized and rational clinical design, and more powerful database sharing and sequencing analysis techniques will make clinical application of the gut microbiota as a CRC biomarker possible.

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ACKNOWLEDGEMENTS

We would like to thank Dr. Sarah Wilkinson for her important insights, useful suggestions and careful editing of this manuscript.

FUNDING

The present work was supported by the grants CA72851, CA181572, CA184792, CA187956 and CA202797 from the National Institute of Health (NIH) to AG, as well as the China Scholarship Council [grant numbers 201906220156] to YZ.

Abbreviations:

5-mc	5-methylcytosine
Ac-CoA	acetyl coenzyme A
AOM	azoxymethane
AUC	area under the curve
BFT	*B. fragilis* toxin
CRC	colorectal cancer
DNMTs	DNA methyltransferases
DSS	dextran sodium sulfate
EIEC	enteroinvasive *Escherichia coli*
ETBF	enterotoxigenic *B. fragilis*
FadA	*F. nucleatum* adhesin A
FIT	fecal immunohistochemistry test
GF	germ-free
HATs	histone acetyltransferases
HDACs	histone deacetylases
lncRNA	long noncoding RNA
ncRNAs	noncoding RNAs
NGS	next-generation sequencing
NTBF	non-toxigenic *B. fragilis*
OTUs	operational taxonomic units
PCR	polymerase chain reaction
pks	polyketide synthase

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ROS	reactive oxygen species
rRNA	ribosomal RNA
SAM	S-Adenosyl methionine
SCFAs	short-chain fatty acids
SPO	spermine oxidase

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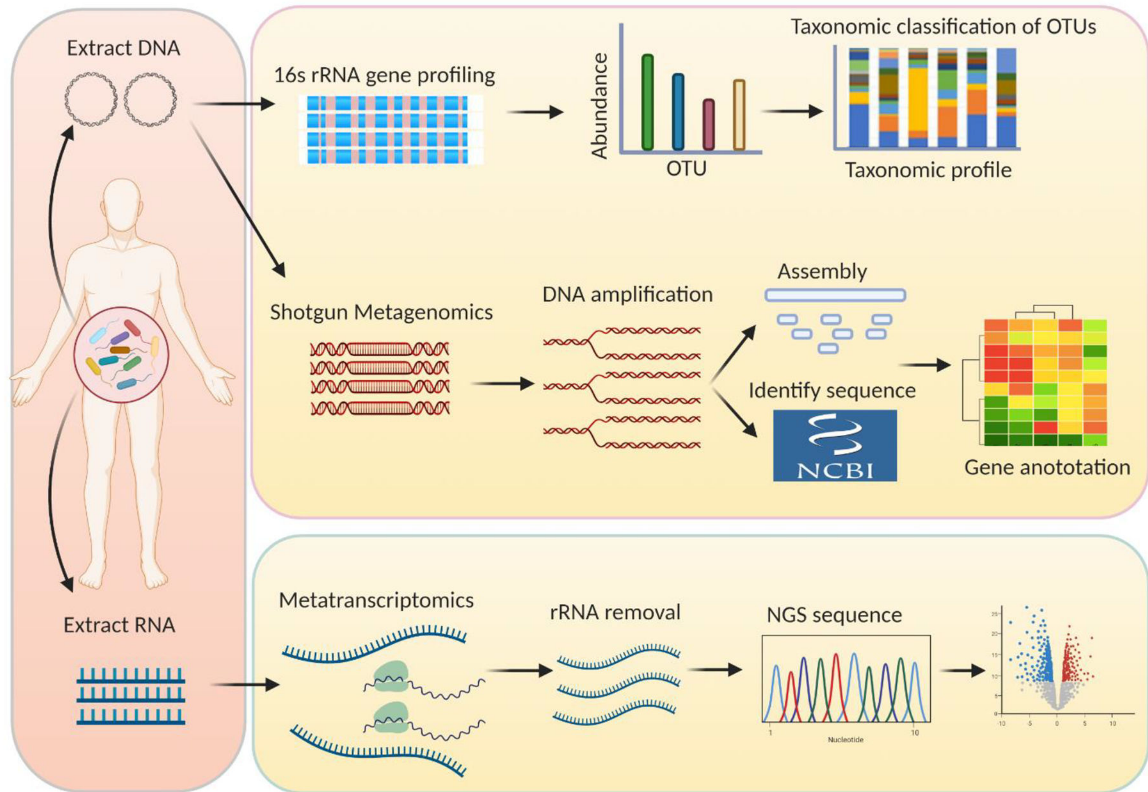

Fig. 1.

General pipeline of different sequencing and bioinformatic strategies for the gut microbiota analysis. Both methods starting from DNA and RNA extraction of the gut microbiota samples. The extracted DNA is either subjected to 16S rRNA gene profiling or sheared into small DNA fragments to perform shotgun metagenomics, and after rRNA removal, the extracted RNA is subjected to metatranscriptomic sequencing. rRNA, ribosomal RNA; OUT, operational taxonomic units; NGS, next-generation sequencing.

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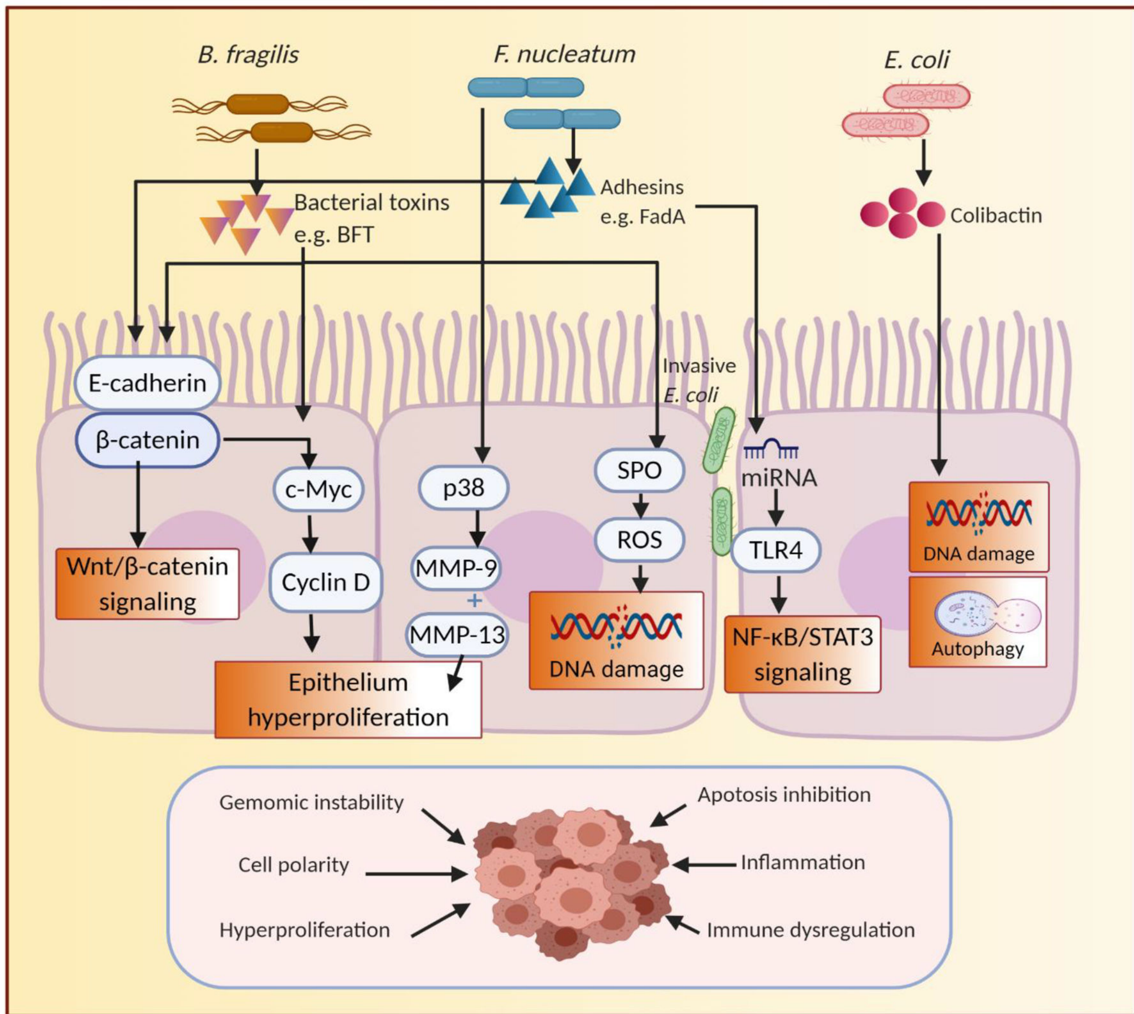

Fig. 2.

Mechanistic links involved in the gut microbiota promoting carcinogenesis of CRC. Interactions between the gut microbiota and host contribute to the alterations at the molecular level, such as the biosynthesis of toxins interfering with the regulation of cell proliferation and apoptosis by Wnt/β-Catenin and NF-κB/STAT3 signaling pathway, or damaging DNA by producing SPO, or regulating autophagy, that ultimately lead to the onset and progression of CRC. BFT, B. fragilis toxin; FadA, F. nucleatum adhesin A; MMP, matrix metalloproteinases; SPO, spermine oxidase; ROS, reactive oxygen species; TLR4, toll-like receptors 4; NF-κB, nuclear factor kappa B; STAT3, signal transducer and activator of transcription 3.

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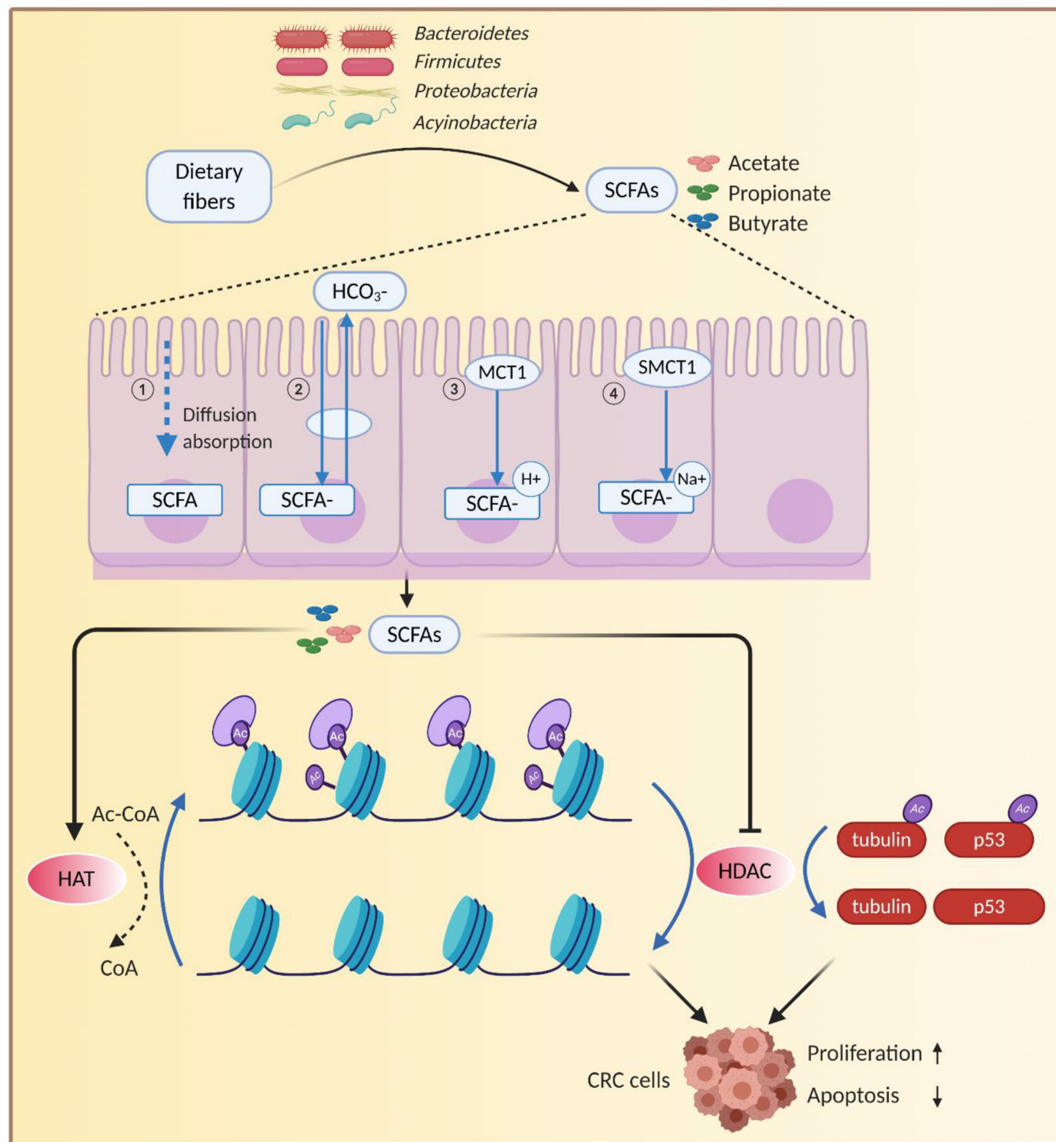

Fig. 3.
Epigenomic interactions between the gut microbiota and CRC via SCFAs. Gut bacteria in the colon or rectum produce a number of low molecular weight SCFAs, which can be absorbed by epithelial cells, and cause epigenetic modifications in DNA methylation and histone acetylation via activation or inhibition of certain enzymes such as DNMTs, HDACs. SCFAs, short-chain fatty acids; MCT1, monocarboxylate transporter 1; SMCT1, sodium-dependent monocarboxylate transporter 1; HATs, histone acetyltransferases; HDACs, histone deacetylases; CoA, coenzyme A; Ac-CoA, acetyl coenzyme A.

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Table 1.

The Composition of Microbiota in Various Anatomical Regions of the Gut

Location	Major microbiota	REFs
Lumen-commensal microbiota	*Bacteroides* spp., *Prevotella* spp., *Mucispirillum* spp., *Lactobacillus* spp., *Ruminococcus* spp., *Oscillospira* spp., *Sutterella*.spp, *Desulfovibrio* spp., *Fusobacterium* spp.	[126]
Mucus-resident microbiota	Streptococcaceae, Actinomycinaeae, Corynebacteriaceae, Mucispirillum spp., Lachnospiraceae, *Lactobacillus* spp., *Veillonella* spp., *Helicobacter* spp.	[127, 128]
Epithelium-resident microbiota	Adherent-invasive *E. coli*, segmented filamentous bacteria, *B. fragilis, Clostridium* spp.	[76, 129]
Lymphoid tissue-resident microbiota	*Achromobacter* spp., *Alcaligenes* spp., *Bordetella* spp. *and Ochrobactrum* spp.	[130]

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Table 2.

Major Gut Microbiota Associated with the Occurrence and Development of CRC

Microbiota	Model	Association with CRC	REFs
F. nucleatum	CRC xenograft mouse model	Promotes metastasis by activating autophagy signaling.	[108]
	$Apc^{Min/+}$ *mice*	Modulates the tumor immune microenvironment via FadA that activates the β-catenin pathway.	[131]
	Human umbilical vein endothelial cells	Secretes FadA that binds endothelial cell surface cadherins.	[132]
Enterococcus Faecalis	$IL10^{-/-}$ mice	Secretes the metalloprotease gelatinase that compromises the epithelial barrier.	[133]
	Rat intestinal colonization model	Produces the reactive oxygen species extracellular superoxide and hydrogen peroxide that cause epithelial cell DNA damage.	[134]
B. fragilis	$Apc^{Min/+}$ mice	Mediates colitis and tumorigenesis.	[135]
	HT29/C1 cells	Secretes the BFT toxin that stimulates E-cadherin cleavage and facilitates CRC metastasis.	[136]
E. coli	$Apc^{Min/+}$ *mice*	Triggers a pro-carcinogenic multi-step inflammatory cascade.	[137]
	Mice intestinal loop model	Induces DNA damage *in vivo* and triggers genomic instability in mammalian epithelial cells.	[138]
	$Apc^{Min/+}$ mice	Secretes colibactin that induces autophagy.	[78]
Streptococcus gallolyticus	AOM CRC model	Increases β-catenin nuclear localization and c-Myc and cyclin D1 expression.	[139]
Escherichia coli NC101	AOM/$IL10^{-/-}$ mice	Produces colibactin that induces CRC.	[140]
Clostridium nexile	Not yet identified	Contributes to the anticancer effect of *pseudomonas aeruginosa.*	[141]
Clostridium septicum	Not yet identified	Produces alpha toxin that binds GPI-anchored cell surface receptors.	[142]
Lactobacillus casei BL23	AOM/DSS CRC model	Downregulates IL-22 that mediates an immunomodulatory effect.	[143]
Faecalibacterium prausnitzii	Colitis mouse model	Induces IL-10 that protects against cancer formation.	[144]
Streptococcus bovis	AOM CRC model	Produces inflammatory cytokines that promotes colon lesions.	[145]
Eubacterium rectale	Colitis-induced CRC mice model	Produces butyrate to induce the anti-inflammatory cytokine IL-10.	[146, 147]

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Table 3.

Gut Microbiota Involved with Epigenetic Alterations in CRC

Epigenetic modification	Microbiota studied	Model	Key findings	REFs
Methylation	Murine gut microbiota	GF mice	The methylation of *WIF1*, *PENK*, and *NPY* were associated with CRC dysbiosis.	[94]
	Murine gut microbiota	*Lgr5-EGFP-CreER* mice	Gene methylation was increased by microbiota transplantation.	[148]
	L. acidophilus, *B. infantis*, and *Klebsiella* species	Human intestinal epithelial cells (IECs)	Microbiota treatment resulted in differential methylation changes in 200 regions of DNA.	[149]
	Murine gut microbiota	GF mice	The number of changes in the methylation status of genes increased with age of GF mice.	[150]
	ETBF	*Min*$^{Apc\ 716+/-}$ mice	ETBF-induced tumors contained methylated tumor suppressor genes.	[151]
Histone modifications	Murine gut microbiota	wild-type mice	Histone marks H3K4me1 and H3K27ac were enriched at poised or active enhancers.	[152]
	Antibiotic-treated murine microbiota	GF mice	De novo generation of oscillating histone marks and rhythmically expressed genes.	[153]
	Murine gut microbiota	wild-type mice	Bacterial presence resulted in numerous changes in histone acetylation in the proximal colon tissue.	[154]
	Murine gut microbiota	GF mice	The location of H3K4 methylation marks was modified when gut microbes colonized.	[155]
	Antibiotic-treated murine microbiota	GF mice	Derived SCFAs promoted H3K18 crotonylation by inhibiting HDACs.	[156]
Non-coding RNAs	*E. coli* strains or fecal-derived murine microbiota	GF mice	Distinct changes in lncRNA signatures occurred after GF mice were reconstituted with normal mouse microbiota or with *E. coli* alone.	[105]
	F. nucleatum	CRC xenograft model	*F. nucleatum* caused resistance to oxaliplatin and 5-FU via downregulation of *miR-4802* and *miR-18a*.	[108]
	Murine gut microbiota	GF mice	A total of 19 miRNAs in IESCs significantly differed in expression depending on microbial status.	[157]
	Murine gut microbiota	GF mice	Microbiota-dependent miR-21–5p expression in IECs regulated intestinal epithelial permeability via ARF4.	[107]
	Antibiotic-treated murine microbiota	GF mice	miRNAs *let-7b*, *miR-141*, and *miR-200a* expression were significantly reduced in GF mice.	[106]
	Murine gut microbiota	IEC-miRNA-deficient mice	The presence of gut microbes was associated with decreased production of miRNAs.	[158]

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Table 4.

Fecal Gut Microbiota as A potential Biomarker for Non-invasive Diagnosis of CRC

Microbiota and Other Tests	Cohort Size		Sample Type	Detection Method	REFs
	Noncancer Controls	CRC			
F. nucleatum	60	188	Fecal samples	16S rRNA gene sequencing and qPCR	[50]
F. nucleatum	370	120	Fecal samples	16S rRNA gene sequencing and qPCR	[159]
Clostridium symbiosum, F. nucleatum, FIT and carcinoembryonic antigen	454	327	Fecal samples	qPCR	[160]
F. nucleatum and FIT	205	104	Fecal samples	Metagenomics and qPCR	[118]
F. nucleatum, clbA-positive bacteria	962	174	Fecal samples	16S rRNA gene sequencing and qPCR	[161]
F. nucleatum, Atopobium parvulum, Actinomyces odontolyticus, Metabolites: branched-chain amino acids, phenylalanine, bile acids	251	365	Fecal sample	Metagenomics and qPCR	[120]
F. nucleatum, Peptostreptococcus stomatis, Parvimonas micra, Solobacterium moorei	187	137	Fecal sample	Metagenomics and qPCR	[119]
B. fragilis, F. nucleatum, Porphyromonas asaccharolytica, Parvimonas micra, Prevotella intermedia, Alistipes finegoldii, Thermanaerovibrio acidaminovorans	271	255	Fecal sample	Metagenomics and qPCR	[121]
F. nucleatum, Enterococcus faecalis, Streptococcus bovis, Enterotoxigenic B. fragilis, Porphyromonas spp	73	20	Fecal samples	16S rRNA gene sequencing and qPCR	[162]
F. nucleatum, Bifidobacterium, Faecalibacterium prausnitzii	367	536	Fecal samples	16S rRNA gene sequencing and qPCR	[163]
F. nucleatum, Bacteroides clarus, Roseburia intestinalis, Clostridium hathewayi	236	203	Fecal samples	16S rRNA gene sequencing and qPCR	[117]