Association of the microbiome with colorectal cancer development (Review)

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Abstract. Colorectal cancer (CRC) is the second most common malignancy causing cancer-related mortality globally. It is the third most common type of cancer detected worldwide. The recent concept of the human body supporting a diverse community of microbes has revealed the important role these microbes play synergistically in maintaining normal homeostasis. The balance between the microbiomes and epithelial cells of the human body is essential for normal physiology. Evidence from meta-genome analysis indicates that an imbalance in the microbiome is prominent in the guts of patients with CRC. Several studies have suggested that the gut microbiota can secrete metabolites [short-chain fatty acids (SCFAs), vitamins, polyphenols and polyamines] that modulate the susceptibility of the colon and rectum by altering inflammation and DNA damage. The state of microbiome imbalance (dysbiosis) has been reported in patients with CRC, with an increasing population of 'bad' microbes and a decrease in 'good' microbes. The 'good' microbes, also known as commensal microbes, produce butyrate; however, 'bad' microbes cause a pro-inflammatory state. The complex association between pathological microbial communities leading to cancer progression is not yet fully understood. An altered microbial metabolite profile plays a direct role in CRC metabolism. Furthermore, diet plays an essential role in the risk of gastrointestinal cancer development. High-fiber diets regulate the gut microbiome and reduce the risk of CRC development, and may be fruitful in the better management of therapeutics. In the present review, the current status of the microbiome in CRC development is discussed.

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Contents

1. Introduction

Globally CRC is one of most common malignancies, ranking as the third most frequently diagnosed type of cancer and the second cause of cancer-related mortality worldwide (1). CRC stems from acquiring genetic and epigenetic alterations over the course of several years referred to as the adenoma-carcinoma sequence (2). CRC is a multifaceted and heterogenous disease and its etiology emerges from an interaction between the host and the environment. The role of microbes among environmental factors in carcinogenesis has been recognized. Infectious agents have been estimated to cause >15% of all cancers; for example, *Helicobacter pylori* causes gastric cancer, hepatitis B and C viruses cause hepatocellular cancer and papilloma virus causes cervical cancer. The scientific community has begun to study the role of the host-microbe interaction in cancer progression. Microbiota refers to the diverse community of microorganisms present in a specific environment. This has emerged as an important environmental factor for gastrointestinal cancer and CRC. The gut microbiome consists of a large population of bacteria (>100 billion) interacting with intestinal cells of the host, affecting immunity and the metabolome (3). Microbial composition varies along the gastrointestinal tract; 70% of microbes are located in the colorectum where interaction and crosstalk take place (4).

The large intestine, particularly the colon, is more prone to developing cancer as compared to the small intestine, due to the heavy colonization of the microorganism. Cancer incidence is 12-fold more in the colon as compared to the remainder of the gastrointestinal tract (5).

The microbiota is important for normal physiological functions, such as energy harvest (6) and the immune maturity of the gut (7). Alterations in relative abundance can modulate the balance, leading to pathological conditions, such as obesity, metabolic disorders and autoimmune disease (8-10). Certain microbes and their metabolites may create a microenvironment that is more favorable to cancer growth (11). Emerging evidence suggests that the gut microbiota plays an important role in the initiation and progression of CRC (12). Previous research with germ-free animals have demonstrated a role for the microbiota in a number of models of carcinogenesis (13). The association between the microbiota, inflammation and CRC is well understood; patients with inflammatory bowel disease (IBD) are known to be more susceptible to CRC progression. The composition and diversity of microorganisms varies among different individuals, depending on diet, antibiotic/medicine consumption and chemical exposure (14). Dietary habits and lifestyle are well-established risk factors known to alter the gut microbiota. Alterations in diet have been found to affect the microbiota (15). The gut microbiota releases various metabolites that may have beneficial or damaging effects on the host. The production of metabolites, such as short-chain fatty acids (SCFAs), polyphenols, vitamins and polyamines lead to the pathogenesis of human disease. Recent findings have reported that SCFAs, specifically butyrate, play a critical role in immunomodulatory functions. Alterations in SCFA levels and other amino acid metabolites have been known to play an important role in cancer progression and metastasis (16). The biosynthesis of chemical carcinogens by microorganisms, such as N-nitroso compounds and acetaldehyde are among the potential mechanisms through which the microbiota may play a role in cancer progression. Resarch on the gut microbiota has provided a new direction and hope for the early detection of CRC, specifically during the early stages, increasing the 5-year survival rate, as compared to the late stages (17). The detection of alterations in certain microorganisms has provided a promising strategy for the early diagnosis of CRC (17). Some microbes have been shown to exert a protective effect against CRC by metabolite production, immune tolerance and outcompete with detrimental microbes (18). The better understanding of the microbiota and host interaction would provide novel opportunities for the early detection of CRC and therapeutics targeting the microbiota. The present review focuses on the role of the microbiota associated with CRC development.

2. Bacteria associated with CRC

It is known that an adult human houses approximately 10^{13} bacteria in the colon and other parts of the large intestine, which are chiefly responsible for maintaining gut homeostasis (19). It is the commensal relation with the gut microbiota that inhibits the invasion and colonization of pathogenic bacterial species in the gut and helps boost immunity. The gut microbiota is composed of several hundreds of bacterial species, which are predominantly anaerobic in nature, and include species such as *Lactobacilli,* Enterobacteriaceae, *Streptococci, Bacteroides, Bifidobacterium, Fusobacterium, Enterococci, Peptostreptococcus* and *Atopobium* (20). A few of these bacterial populations have been identified as contributors to CRC and tumorigenesis, exerting a pro-carcinogenic effect, whereas few of these are known to inhibit tumorigenesis in the colon, acting as protective species. Comparative analyses of the metagenomic and metataxonomic profiles of the gut microbiota found in the colon of patients with CRC and healthy individuals revealed that the gut microbiota composition of a patient with CRC differed significantly from that of a healthy individual, where pro-carcinogenic taxa such as *Fusobacterium, Bacteroides, Escherichia* and *Streptococcus* were found in abundance along with a reduction in the population of protective microbes, such as *Bifidobacterium, Lactobacilli* and *Clostridium* (21-24).

A pyrosequencing-based strategy adopted to study microbial dysbiosis in patients with CRC explained that the elevation in the population of *Bacteroides fragilis* (*B. fragilis*) can be linked to the development of cancer in the colon (25). *B. fragilis* is known to be an enterotoxigenic strain producing a bacterial toxin (metalloprotease) known as the *B. fragilis* toxin (BFT) responsible for the virulent properties of the strain (26). In the study by Ahn *et al* the taxonomic analyses of the gut microbiota was carried out by the amplification of 16srRNA sequences obtained from fecal bacterial DNA; it was observed that the increased abundance of the anaerobic Gram-negative genera, *Fusobacterium* and *Porphyromonas*, led to an increased risk of CRC development in the individuals (27). *Fusobacterium nucleatum* (*F. nucleatum*) was found in abundance in the fecal samples of patients with CRC and various clinical and metagenomic studies have highlighted a significant role of *F. nucleatum* in the inflammatory response of IBD and CRC (27,28). Along the similar lines of research i.e., based on the sequencing of 16srRNA, the clinical study performed by Wu *et al* revealed that *Campylobacter* and *Fusobacterium* species were relatively more abundant in CRC samples along with several other families, such as Enterococcaceae, Staphylococcaceae and Eubacteriaceae, exerting pathogenic effects in the colon (28). Another anaerobic commensal bacterium predominant in the gut microbiota is *Escherichia coli* (*E. coli*) and a previous study on mice inoculated with colon cancer associated *E. coli* strains reported an increase in the colonization of tumor-associated and mucosa-associated *E. coli* strains in patients with CRC (29). *E. coli* strains obtained from CRC samples were found to express certain toxin-producing genes, such as colibactin, which confer bacterial cells with properties to induce DNA damage and genomic instability, subsequently causing colorectal carcinogenesis (30). The statistical study by Zhang *et al* also revealed an elevation in the numbers of *Devosia* in the gut microbiota of patients with CRC (31).

Apart from elevated levels of several pathogenic microbes in the mucosal and fecal samples of patients with CRC, Ahn *et al* in their study, also highlighted a decrease in the population of Gram-positive strains of *Clostridia,* specifically *Coprococcus*, increasing the risk of inflammation in the colon followed by tumorigenesis (27). Some butyrate-producing genera, such as *Roseburia, Faecalibacterium* and *Eubacterium* were significantly reduced in the microbiota obtained from CRC samples,

Table I. Bacteria playing a key role in the development of colorectal cancer.

Bacteria	Effector	Mechanisms of action
Escherichia coli	Genotoxins (pks)	DNA damage
Enterotoxigenic *Bacteroides fragilis*	*Bacteroides fragilis* toxin	Inflammation, immune surveillance, EMT
Fusobacterium nucleatum	FadA, Fap2	DNA damage, antitumor immune activity,
Streptococcus gallolyticus	SGG specific bacteriocin	Cell proliferation
Peptostreptococcus anaerobius	Unknown	ROS accumulation, Cholesterol biosynthesis

suggesting that dysbiosis in the fecal microbiota can serve as a marker for CRC detection (28,31). Another notable observation for the dysbiosis of the colon microbiota was made by Marchesi *et al* where upon investigating the tumor tissues of patients with CRC, a significant reduction in the population of *Enterobacteriacea*e was identified, namely in species such as *Citrobacter, Shigella, Serratia, Salmonella* and *Kulyvera* spp. (32). Lactic acid bacteria (LAB), such as *Bifidobacterium* and *Lactobacilli* have also been found to play a negative role in CRC (33). An imbalance in the microbial population can alter the micro-environment of the intestine, which cause an imbalance in crucial intrinsic and extrinsic factors, resulting in the initiation of cancerous growth. A summary of the bacteria playing a role in CRC development is provided in Table I.

3. Bacterial metabolites and CRC development

To date, various metagenomic studies performed on clinical samples obtained from individuals suffering from CRC have pointed towards the abundance of pathogenic bacterial strains of *F. nucleatum*, *Bacteroides fragilis* and *E. coli*. On the contrary, a significant reduction in the population of butyrate-producing strains belonging to the class *Clostridia*, are also considered to be a contributing factor to CRC. Thus, the understanding of the metabolites produced by these species and their mechanisms of action is critical in order to reach to better diagnostic and therapeutic conclusions and to assist in devising novel treatment strategies.

F. nucleatum. Several studies on *F. nucleatum* have suggested the involvement of this bacteria in triggering epithelial-mesenchymal transition (EMT) in colon tissues. *F. nucleatum* is known to adhere and invade epithelial cells by employing several virulence factors, such as *Fusobacterium* adhesin A (Fad A), *Fusobacterium* autotransporter protein 2 and *Fusobacterium* outer membrane protein A responsible for promoting pro-oncogenic signaling and CRC development (34-36). In order to provide further insight into the metabolite alteration leading to EMT induced by these virulence factors in colorectal carcinogenesis, Ma *et al* carried out a detailed analysis on the interactions of these virulence factors with E-cadherin/β-catenin resulting in an altered molecular mechanism upon the infection of epithelial cells with *F. nucleatum* (37). Upon infection, *F. nucleatum* was found to increase the phosphorylation of p65, a crucial component of NF-κB signaling and to enhance the production of interleukin (IL)-6, IL-1β and matrix metalloproteinase (MMP)-13. This activation of NF-κB signaling indicated that *F. nucleatum* induced inflammation in colon tissue, which subsequently led to tumorigenesis (37). From previous studies conducted on cell adhesion, it is a well-known fact that the loss of E-cadherin and β-catenin-mediated cell adhesion is a crucial factor for inflammation and the induction of tumorigenesis (38,39). Surprisingly, *F. nucleatum* did not alter the expression levels of E-cadherin and β-catenin in epithelial cells and was found to interact only with E-cadherin to induce inflammatory response and promote malignant phenotype of CRC (37). Another study by Abed *et al* revealed that Fap2, a Gal-GalNAc binding protein, is expressed on the surface of *F. nucleatum* (36). Fap 2 functions as Gal-GalNAc lectin in CRC cells, where it binds to Gal-GalNAc overexpressed in CRC cells and causes the expansion of CRC by inhibiting antitumor immune activity by targeting T cell immunoglobulin and ITIM domain (TIGIT) (35,36). This indicates that virulence factors, such as FadA along with surface proteins of *F. nucleatum* may be a potential target for the development of an immunotherapy against CRC.

The role of *F. nucleatum* in experimental colitis was recently confirmed and it was found that *F. nucleatum* along with dextran sodium sulfate (DSS) synergistically promoted EMT and the aggressiveness of CRC (40). *F. nucleatum* was found to play a pro-tumorigenic role by activating the EGFR, AKT and ERK signaling pathway in azoxymethane (AOM)/DSS-induced CRC. The *F. nucleatum* cell surface protein, FadA, induces DNA damage by upregulating chk2 in CRC progression. Treatment with *F. nucleatum* resulted in DNA damage in adenomatous polyposis coli (APC) (-/+) mice (41). The number, size and tumor burden were reduced following treatment with $FadA^{-/-}$ *F. nucleatum* as compared to WT, demonstrating a direct role of the cell surface protein of *F. nucleatum* in tumor progression (41). Recently, Okita *et al* described a heavy to moderate load of *F. nucleatum* (Fn) DNA associated with high microsatellite instability (MSI-H) and L/E [L: MSI-L/E: Elevated level of microsatellite alterations at selected tetra-nucleotide repeats (EMAST)] in two CRC cohorts; they further presented evidence that Fn activated factors that promote γ-H2AX, a marker for DNA damage (42). In another study, murine and human enteroid-derived monolayers (EDMs) co-cultured with Fn exhibited a downregulation of a key DNA repair protein, NEIL2 (DNA glycosylase) (43). $NEIL^{-/-}$ EDMs exhibited increased DNA damage and elevated cytokine levels. Furthermore, NEIL2 downregulation is mainly observed in microsatellite stable (MSS) CRC, as compared to MSI CRC, indicating that Fn accumulation induces NEIL2 downregulation, resulting in DNA damage and thus leading to CRC progression. A recent study on

microRNA (miRNA/miR) profiles and proteomic analysis from Fn-infected and non-infected cells found that the generation of miR1246/92b-3p/27a-3p-rich and CXCL16/RhoA/IL-8 exosomes promoted pro-metastasis (44). Bone-marrow derived mesenchymal stem cells (BMSCs) infected with Fn have also exhibited increased tumor growth and an enhanced susceptibility to tumors an APC (-/+) mouse transplantation model. Fn-infected BMSCs also displayed accelerated cancer-initiating potential and invasiveness in a nude murine model by activating Wnt-β-catenin-transforming growth-interacting factor (TGIF) signaling pathways (45). The co-culture of human CRC cell lines (SW480 and HCT116) with Fn was also shown to result in an increased expression of mesenchymal markers, such as Vimentin, Snail and Zeb1. Furthermore, Fn infection increased the capability of intrusion, migration and tumor-sphere formation along with a high expression of CD44, indicating that Fn plays a critical role in EMT and the cancer stem cell phenotype (46). These finding confirmed that the Fn play important role in the progression of CRC.

Bacteroides fragilis. Enterotoxigenic *Bacteroides fragilis* (ETBF) is an enterotoxin producing bacterium found in an abundance in fecal samples obtained from patients with CRC is known to cause virulence with the aid of a metalloprotease holotoxin, BFT, also known as fragylisin (47). BFT is associated with acute diarrhea, IBD and CRC. This BFT binds to the colonic epithelial cell (CEC) surface protein receptor, initiating E-cadherin cleavage (47). E-cadherin in its intracellular domain is bound to β-catenin, which is a very crucial component of the Wnt signaling pathway. The degradation of E-cadherin releases β-catenin and facilitates EMT, and increases cell permeability by decreasing cell adhesion (48). The degradation of E-cadherin also facilitates the production of chemokines, such as IL-6, IL-8 and IL-1β, which activate NF-κB and MAPK signaling in CEC, generating inflammatory response in cells followed by carcinogenesis (49). The binding of BFT to CEC receptor also leads to the activation of key cellular pathways, such as signal transducer and activator of transcription (STAT)3 along with the formation of reactive oxygen species and nitrogen species which are known to cause DNA damage in epithelial cells and this genomic instability caused due to damaged DNA enhances expression of several oncogenes such as c-Myc by multi-folds, leading to the development of adenomas and carcinomas in the colon and rectum (50). Zamani *et al* investigated the mucosal colonization of ETBF to find the potential association of ETBF in benign and malignant lesions. *B. fragilis* was abundantly associated with patient samples as compared to healthy control (51). ETBF was increasingly associated with serrated lesions and with low-grade dysplasia adenoma. Liu *et al* found that ETBF increased the stemness of CRC by upregulating Nanog and Sox2 expression. They found that mechanistically, ETBF significantly elevated JMJD2B by activating TLR4 pathway (52). In a colitis CRC model, with ETBF colonization in wild-type BALB/c mice, the administration of AOM/DSS increased the rapid development of large number of polyps mainly in the colon and rectum (53). Roberti *et al* studied the contribution of gut microbes to the elicitation of the follicular helper T (T_{FH}) response (54). Ileal microbiota (*Bacteroides fragilis* and *Erysipelotrichaceae*) was involved in the protective immune response against colon cancer. The ileal microbiome plays an important role in immune surveillance and in the prognosis of proximal colon cancer and chemotherapy-mediated ileal crypt apoptosis (54). Bacteroides fragilis-associated long non-coding RNA (lncRNA1: BFAL1) mediates ETBF cancer progression in CRC; BFAL1 expression is increased in CRC compared with adjacent normal tissues. ETBF promotes CRC tumor growth through BFAL1 by activating RHEB (Ras homolog)/mTOR signaling. BFAL1 and ETBF are highly expressed in tumor tissues and predict poor outcomes in CRC (55).

E. coli. *E. coli* is a Gram-negative commensal bacterium of the human microbiota and represents the most common cultivable and aero-anaerobic bacteria. A number of studies have demonstrated a clear link with CRC (56-60). According to the acquisition of factors of virulence, there are four *E. coli* phylogenic groups (A, B1, B2 and D). Groups A and B1 are generally not pathogenic; however, groups B2 and D are involved in intestinal and extra-intestinal pathogenesis. Crohn's disease, a chronic IBD known to be a risk factor for CRC, is caused by some strains of phylogroup B2 that are associated with it (60,61). Swidsinski *et al* (58) and Martin *et al* (59) demonstrated that mucosa-associated *E. coli* resided in higher numbers in patients with CRC than in the controls, supporting the central role of these bacteria in CRC development. Pathogenic *E. coli* strains synthesize various virulence factors (62). These factors consist of several toxins known as cyclomodulins, such as cytolethal distending toxins (CDT), cytotoxic necrotizing factor (CNF), cycle inhibiting factor and colibactin. Cyclomodulins are genotoxic and known to alter cell cycle progression, proliferation, cell differentiation and apoptosis (63-68). Cuevas-Ramos *et al* stated that a specific type of bacterial strain belonging to the B2 phylogenetic group possessed a unique gene island termed 'pks', which translates into the genotoxic compound, colibactin, possessing genotoxic properties causing DNA double-strand breaks and chromosomal instability (CSI) in human cells (68). As regards cyclomodulin-producing *E. coli*, a previous study revealed an increased prevalence of cyclomodulin-producing B2 *E. coli* in colon tumor biopsies, suggesting a possible role of such pathogenic *E. coli* in colon carcinogenesis (30).

This genotoxic compound generates double-strand breaks in DNA causing damage to several portions of DNA, rendering it unstable and this instability of the genome paves the way for the upregulation of expression of oncogenes, such as c-Myc, which results in the formation of adenomas and the development of CRC (56,67).

Peptostreptococcus ssp. Recent studies have found that patients with an enrichment of *Peptostreptococcus stomatis* (*P. stomatis*) and *Peptostreptococcus anaerobius* (*P. anaerobius*) have a higher risk of CRC development (69-71). Patient stool and tissue are enriched with *P. anaerobius* in CRC. Tsoi *et al* indicated the pro-tumorigenic role of *P. anaerobius* modulating TLR2 and 4 leading to reactive oxygen species (ROS) accumulation, resulting in cholesterol biosynthesis and proliferation (70). Purcell *et al* found the enrichment of *P. stomatis* in consensus molecular subtype 1 (CMS1) of CRC tumor tissue (71). *P. stomatis* supports bacteria colonization

by promoting acidic and hypoxia around tumor microenvironment (71). Long *et al* found that *P. anaerobius* promoted CRC in APC (-/+) mice and altered tumor immunity by the PCWBR2-integrin α_2/β_1 -PI3K-AKT-NFkB signaling pathways, identifying the PCWBR2-integrin α_2/β_1 axis as a potential therapeutic target in CRC (72).

Streptococcus gallolyticus. *S. gallolyticus* has long been associated with CRC. Kumar *et al* reported significant role of few selected strains of *Streptococcus gallolyticus subsp. gallolyticus* (SGG) in CRC and upon a detailed analysis of its metabolite profile and mechanisms of pathogenesis, *S. gallolyticus* was found to enhance the proliferation of CRC cells by targeting Wnt/β-catenin signaling and upregulating β-catenin in cells, thereby elevating the expression of target oncogenes c-Myc and cyclin-D, resulting in the adenocarcinoma of CRC tissue (73). Kwong *et al* found that CRC diagnosis was associated with *S. gallolyticus* by perturbing barrier function (69). Another study found that tumorigenic conditions promoted the *S. gallolyticus* colonization of gut by activation of SGG-specific bacteriocin and replacing commensal enterococci (74). Some strains of *S. gallolyticus* have been found to induce cell proliferation designated as proliferation-promoting Sg: PP-Sg and others were not found to stimulate cell proliferation and therefore classified as non-proliferation-promoting: NP-Sg. PP-Sg found to higher colonization in mice as compared to NP-Sg, owing to the better interaction of PP-Sg with host epithelial cells. Furthermore, PP-Sg promoted CRC development in an AOM mouse model (73) (Table I).

Apart from these bacterial species mentioned above, there are several other microbes predominantly found in the fecal and mucosal samples of patients with CRC, and play a key role in pathogenesis and carcinogenesis. Microbiota analysis of healthy individuals indicated that several species of LAB namely, *Lactobacillus acidophilus, L. casei*, *L. rhamnosus* exert anti-inflammatory effects in maintaining gut homeostasis (75,76). *L. rahmnosus* was found to reduce the levels of β-catenin and NF-κB p65 proteins, and to induce the expression of tumor suppressor gene p53 and anti-apoptotic factor BAX in colon epithelial cell, thus preventing CRC (76). *Bifidobacterium*, another LAB, exerts a negative effect on CRC proliferation due to its property to reduce β-glucuronidase activity in the gut which enhances the chemotherapeutic efficacy of CPT-11, hence providing a beneficiary role in the treatment of CRC (77). The detailed role of these commensal LABs in the prevention of CRC has yet to be identified and can be explored in the near future in order to gain better insight into potential treatment strategies for CRC. The abundance of *Parvimonas micra* has been found in the stool of patients with CRC. *P. micra* was found to inhibit the NOD2 signaling, giving rise to an inflammatory and pro-tumorigenic microenvironment (78). Xu *et al* found that *P. micra* abundance was elevated in patients with CRC and was low in healthy individuals and patients with colorectal adenoma (79). The overabundance of *Porphyromonas gingivalis* has been found in patients with CRC (80). Yang *et al* reported an association between *Prevotella intermedia* with a higher risk of CRC development (81). Another study identified *P. intermedia* in a multinational cohort of fecal samples of CRC (82). *Gemella morbillorum* has been found to regulate IL-12 production and thereby, immunoregulation in CRC. Other species of *Gemella* regulate the protective function of the adaptive immune response at the mucosal surface by cleaving IgA1 (83,84). Still, however, no direct role of this bacteria has been reported in CRC development. A schematic diagram of the mechanisms of action of major species of bacteria involved in the development of CRC in humans is presented in Fig. 1.

4. Host-microbe interaction

CRC can be caused due to the cumulative effects of several genetic, epigenetic and environmental factors, which modulate microbial composition in the gut, altering the metabolite profile and the immune response of the body accordingly. CRC is not a single-step process, but rather a culmination of several steps which involve a number of changes in the genetic and epigenetic machinery of the host. The development of CRC begins with the transition of a normal epithelium into the hyperproliferative epithelium, which eventually leads to the loss of its structure and function, leading to a condition known as hyperplasia followed by dysplasia, leading to the formation of adenomas. These adenomas are non-malignant in nature and are known as polyps (85). In this section, the host-microbe interaction in the context of epigenomic modifications and immune system alterations is discussed.

Microbiota-genome/epigenome interactions. Allen and Sears highlighted the impact of the dysbiosis of the gut microbiome on genome and epigenomics of the colon epithelial cells which increased cell proliferation and tumor formation, growth and metastasis (86). DNA methylation patterns and histone marks on several promoters and enhancers were found to be dysregulated, leading to the downregulation of tumor suppressor genes (TSGs) and the upregulation of oncogenes. Several of the miRNAs (oncomiRs and anti-oncomiRs) and long non-coding RNAs have also been found to be dysregulated and associated with CRC (86). *B. fragilis* has been found to induce the hypermethylation of several TSGs, such as Hoxa5, Polg, Runx1, Runx3, CD37, Stx11, Tceb2, Lgr6, Cdx1 and Fut4, causing carcinoma development in the colon and rectum (86). Xia *et al* identified *F. nucleatum* and *Hungatella hathewayi* to be highly associated with the upregulation of DNA methyltransferases which cause the hypermethylation of promoter of TSG CDX2 and MLH1 (87). In a previous study, the APC gene and DNA mismatch repair (MMR) system were the prime genetic factors identified, which if altered or silenced, led to the development of CRC (88). In an extensive clinical analysis performed by Gagnière *et al* the MMR pathway was shown to be most affected by entero-pathogenic *E. coli*. They reported that *pks*$^+$ *E. coli* strains downregulated the expression of MLH1 genes and inhibited the formation of MLH1 MMR protein in T-84 cells, which led to MSI and tumorigenesis (89). In addition, methylation in APC and INK4a TSG also promoted tumor development in the case of colitis-associated CRC (90-92). *Streptococcus* species have also been identified to be associated with APC gene hypermethylation (87).

Apart from methylation, dysregulation in the expression of miRNAs serves as a major epigenetic marker responsible for CRC. *F. nucleatum* is often found to increase RASA1 expression by inhibiting miR-21 expression, thus leading to chronic

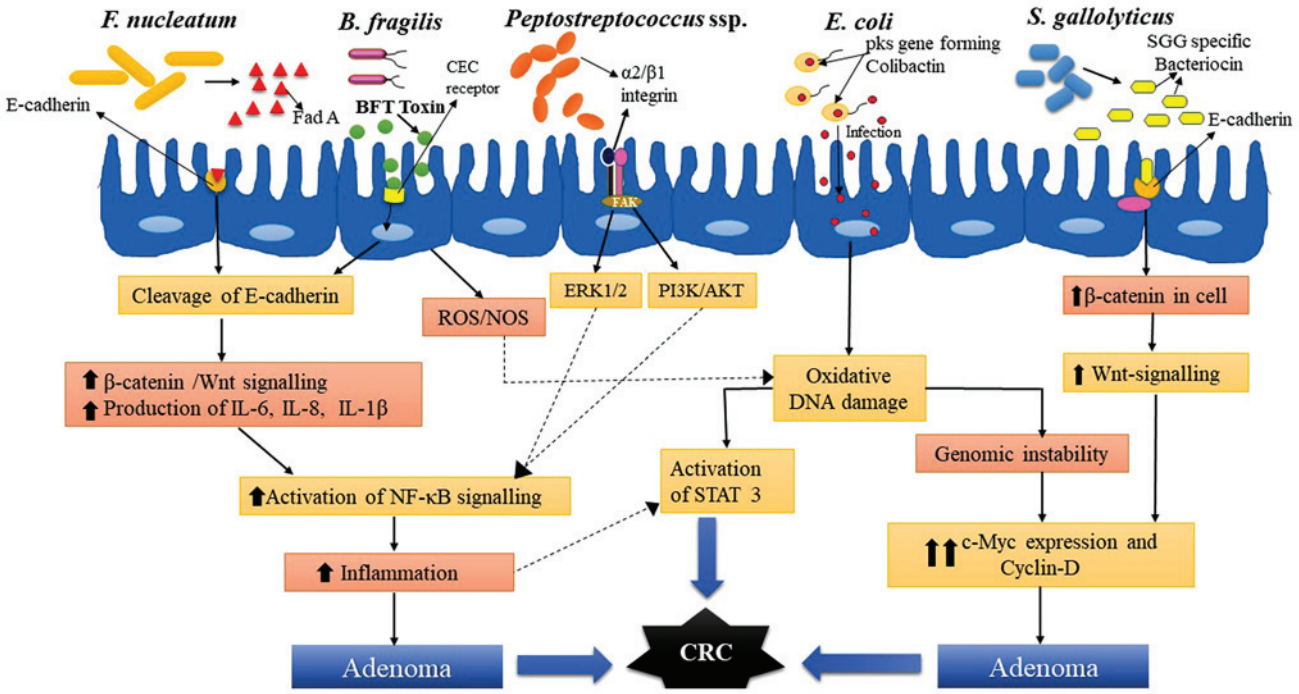

Figure 1. Mechanisms of action followed by major species of bacteria involved in the development of CRC in humans: *F. nucleatum*: Virulence factors, such as Fad A disrupts E-cadherin and promotes the Wnt signaling pathway, resulting in the inflammatory response by colon epithelial cells and adenoma and carcinoma formation. *B. fragilis*: BFT acting as a virulence factor found to be actively associated with cleavage of E-cadherin complex and subsequent activation of NF-κB signaling to generate CRC. Simultaneously, BFT induces oxidative stress, causing DNA damage, and the upregulation of c-Myc oncogene expression to initiate carcinogenesis in the colon. *Peptostreptococcus* spp.: Triggers adenocarcinoma formation by activating α2/β1 integrin, which further activates a cascade involving the ERK1/2 and PIK3/AKT pathways; *E. coli*: Specific *E. coli* strains producing genotoxic compound colibactin bind to DNA and cause damage in DNA sequence, leading to genomic instability and the development of adenocarcinoma in colorectal tissue; *S. gallolyticus*: Actively involved in upregulation of β-catenin and Wnt signaling pathway-overexpressing c-Myc and cyclin D, resulting in CRC. CRC, colorectal cancer; *F. nucleatum*, *Fusobacterium nucleatum*; *B. fragilis, Bacteroides fragilis;* BFT, *Bacteroides fragilis* toxin; *S. gallolyticus, Streptococcus gallolyticus*.

inflammation in the intestine, initiating carcinogenesis (93). *F. nucleatum* has also been found to downregulate the expression of miR-4802 and miR-18a, which lead to resistance against chemotherapeutic drugs administered to patients suffering from CRC (94). Several other oncomiRs and anti-oncomiRs were analyzed from fecal samples and gut mucosa, which revealed differential miRNA profiles in the presence of different gut microbiome, and this variation in miRNA profiles can also serve as a fingerprint for the detection and diagnosis of CRC (95,96).

Microbiota-immune system interaction. The gut microbiota is known to contribute immensely towards the maintenance of the immune system. Fluctuations in the dynamic equilibrium of this microbiota composition leads to defects in the immune system, resulting in inflammation and in tumor initiation. Genotoxins from *B. fragilis* and *pks*$^{+}$ *E. coli* are known to induce inflammation in colon epithelial cells and this inflammatory response by the host induces genetic and epigenetic alterations, which contribute to CRC development (97). Mutations in the tumor suppressor p53 gene are commonly associated with cancer initiation, as well as progression, and they have been found to prolong the effects of NF-κB signaling, which generates the inflammatory response in cells (98). Inflammation triggers oxidative stress, increasing DNA damage and causing mutations in genes, such as APC, directing the Wnt signaling pathway and KRAS, which initiates adenoma formation and is followed by the loss of chromosome 18q and mutations in TP53, resulting in CSI and in the formation of carcinomas (99,100). Inflammation in cells often regulates the production of chemokines and cytokine-driven signaling pathways, such as the NF-κB, PI3K, Akt and ERK pathways. These signaling pathways are responsible for the initiation of tumorigenesis by either upregulating Wnt signaling, which promotes cell proliferation or by inhibiting apoptosis (101). *F. nucleatum* has also been found to enhance pro-inflammatory markers and the infiltration of CD11b^{+} myeloid immune cells and few macrophages, activating Th17 cells and TGFβ signaling, which promotes tumor initiation and angiogenesis (102).

This interaction of *F. nucleatum* with the host immune system suggests its prime role in CRC. *F. nucleatum* induces cancer development by altering the host immune response in the tumor microenvironment. *F. nucleatum* expresses various cell surface proteins (FadA, Fap2 and RadD) which can activate inflammatory factors and favor an environment for tumor growth by recruiting inflammatory cells. *F. nucleatum* can promote the immune suppression of the intestinal mucosa by inhibiting the function of T cells, natural killer (NK) cells and macrophages, resulting in CRC progression (103). Cancer-targeting NK cells are also inhibited by *F. nucleatum*, due to the binding of Fap2 protein on T-cells (35). It is the combination of CSI and MSI mechanisms that govern the development of adenoma-carcinoma in colorectal tissues. CSI is under the influence of immune factors, which is in a synergy with the MSI pathway characterized by changes in the

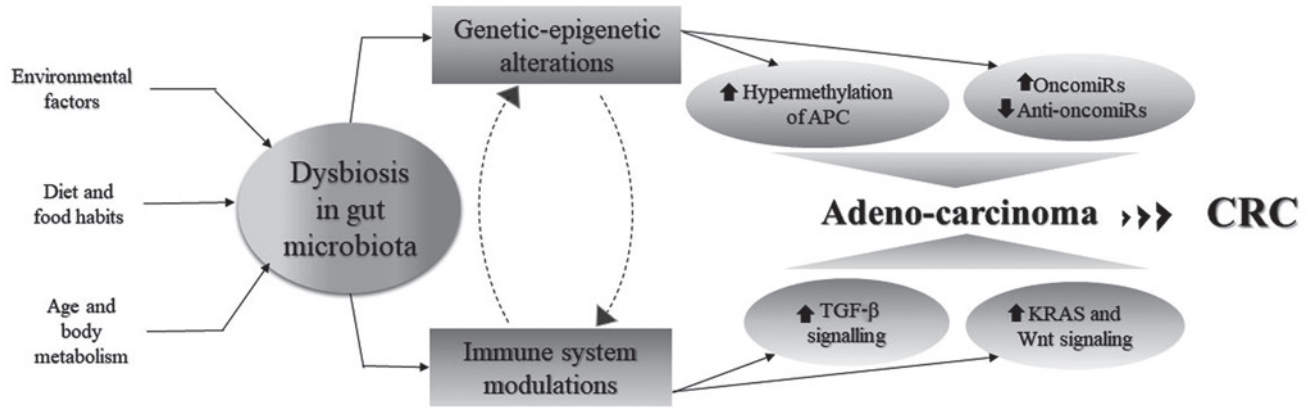

Figure 2. Crosstalk between several factors playing key roles in the development of CRC. Environmental factors, such as smoking/pollution/chemical exposure and food habits (high alcohol intake, high-fat diet, processed foods and red meat consumption), along with metabolic disorders (diabetes and obesity) lead to dysbiosis in the gut microbiota. Dysbiosis becomes the major early event leading to genetic and epigenetic alterations of tumor suppressor genes, proto-oncogenes and DNA repair genes, driving the transformation of the normal colonic epithelium and alteration in immune response leading to CRC development. Hypermethylation and mutations lead to the dysregulation of signaling pathways (TGFβ/KRAS/Wnt) and dysbiosis mediated differential expression of miRNAs, further enhancing the development of CRC and metastasis. CRC, colorectal cancer.

epigenetics of colon epithelial cells (100); the three commonly found pro-carcinogenic microbial strains, *F. nucleatum, B. fragilis* and *E. coli,* have been observed to exert an equal influence on both the pathways driving towards CRC. By contrast, several protective microbial species known to produce butyrate and propionate exert an inhibitory effect on CRC by producing SCFAs and activating cancer-preventing phytochemicals (100). A schematic illustration of the crosstalk between several factors which play a key role in the development of CRC is presented in Fig. 2.

The composition of microbes in the host are a continuous influence on the environment and in order to protect themselves, microbes belonging to specific species tend to form mucosal biofilms. These microbial biofilms exert a protective function for the microbiota from immune factors present in the host (104). The study by Dejea *et al* demonstrated that biofilms were characteristically found in almost all the patients with right-sided CRC (105). The microbial composition of these biofilms was then further studied and it was predominantly found that microbes belonged to *Bacteroides, Fusobacteria, Clostridia, Bifidobacterium* and *E. coli* (104,105). These biofilms in the colon have been found to exert pro-carcinogenic effects by disrupting E-cadherin, and enhancing IL-6, Ki63 and pSTAT3 expression in the colon epithelium (105), more specifically on the right side. The presence of these biofilms often results in a poor prognosis (100). Thus, it can be conveniently concluded that biofilms can serve as a signature biomarker for CRC.

5. Microbiome-associated early diagnosis

The present review thus far discussed several microbial species which act as a driving force behind the occurrence of CRC. This dysbiosis in the microbiota is frequently studied by obtaining fecal samples. The metabolomic study of fecal dysbiosis has pointed towards the utility of microbial dysbiosis as a signature biomarker for the early prognosis and diagnosis of CRC (106). The most pathogenic bacterial strains identified as driving microbes for CRC, *F. nucleatum* and *B. fragilis*, can serve as fecal biomarkers for the early diagnosis of CRC (107,108), since elevated levels of these bacterial species have been found to be associated with an elevation in the levels of major inflammatory mediators (109). *F. nucleatum* has been found to increase the levels of β-catenin and TGF-β, whereas *B. fragilis* upregulates the expression of NF-κB, COX-2 and MMP-9, which can be indicative of early signs of CRC. *Faecalibacterium prausnitzii* (*F. prausnitzii*) was found in low levels in the fecal microbiota of patients with CRC and was responsible for low levels of β-catenin. Hence, the detection of *F. nucleatum, B. fragilis* and *F. prausnitzii* in fecal samples of individuals can help detect signs of CRC at an early stage. In addition, the analysis of these inflammatory mediators along with immunohistochemical markers, such as enhanced KRAS expression and decreased MLH1 expression can serve as effective diagnostic markers for early prognosis of CRC (109). Wu *et al* carried out a 16srRNA based meta-analysis on fecal biomarkers for CRC and adenoma and identified 24 biomarkers sorted into three clusters, out of which first and third cluster were found to have heterogenous population of bacteria and second cluster had relatively homogenous population comprising mainly of members of *Clostridiales* order (110). These clusters were claimed to be distinguishing biomarkers between adenoma and cancer in the colon and rectum (110). Microbes belonging to genera *Porphyromonas, Parvimonas, Hungatella* and *Bacteroides* were CRC-associated biomarkers, whereas *Streptococcus thermophilus TH1435*, *Roseburia intestinalis, Blautia faecis* and *Eubacterium ruminantium* were found to be adenoma-associated biomarkers (110). miRNAs are often found to mediate the crosstalk between microbes and the immune system. Hence, detecting fecal miRNAs and their analysis can prove to be beneficial in the prognosis of CRC and may also provide insight into the early diagnosis, as these fecal miRNAs play a significant role in the fecal dysbiosis of the microbial population (111-113). These fecal miRNAs interact with the microbiota and have been found to help *F. nucleatum* and *E. coli* invade host intestinal cells, disrupting the intestinal homeostasis (114). A preliminary investigation in this context was performed by Li *et al* to find

Table II. Novel therapeutic strategies for the treatment of colorectal cancer and their possible mechanisms of action.

Therapeutic strategy	Mechanisms of action
L. acidophilus and *Bifidobacterium* as Probiotics	Reduces tumor growth by enhancing anti-PD-L1 antibody response.
Probiotics in combination with immunotherapy	Targeting anti-CTLA4 response and activation of dendritic cells
Bioengineering of bacteria as novel therapeutics	Produces shRNA which downregulates Wnt signaling on interaction with β-catenin

a conclusive association between the differential expression of several oncomiRs and anti-oncomiRs, and distinctive microbiome profiles in CRC specimens (115). Fecal miRNAs can be upregulated, as well as downregulated under the influence of bacterial metabolites and virulence factors, exerting a carcinogenic effect (116). Among several dysregulated miRNAs, the ones which are upregulated were miR-17-92 cluster, miR-20a, miR-135, miR-144, miR-221 and miR-92a, and those found to be downregulated were miR-29a, miR-224, miR-143, miR-145 and miR-4478, significantly contributing to CRC and serving as non-invasive fecal biomarkers for the early diagnostics and therapeutic implication in CRC.

6. Exploiting the microbiome for CRC therapeutics

CRC is not a single-step process occurring due to one particular pathway or one individual bacterial strain, but rather an amalgamation of several epigenetic and immunomodulated cascades, which are initiated due to an increased abundance and synergistic effect of characteristic 'Driver and Passenger' bacteria i.e., *F. nucleatum, B. fragilis, E. coli, E. faecalis* and *Streptococcus spp*. Therefore, there are several treatment strategies devised and several of these are on their way from 'bench to bedside' in order to combat CRC. These therapeutic strategies can be broadly classified into chemotherapy and immunotherapy, following two different approaches but reaching to a similar conclusion i.e., a cure for CRC. Chemotherapy involves the administration of potent anticancer drugs (5-fluorouracil, oxaliplatin, irinotecan, etc.) in combination, to eliminate or inhibit tumor cells. Targeted therapeutics have been approved by the FDA for the treatment of CRC, such as cetuximab, a monoclonal antibody against EGFR, bevacizumab, inhibitor of angiogenesis and capecitabine, inhibitor of DNA synthesis (94). The efficacy of these chemotherapies are often found to be affected by gut microbiota-induced toxicity and chemoresistance (117-119); for instance, *F. nucleatum* renders CRC resistant to the chemotherapeutic drugs, oxaliplatin and 5-FU. This chemoresistance by *F. nucleatum* was observed on colorectal cell lines *in vivo* and the induction of autophagy by *F. nucleatum* has been found to be the prime reason behind it (119). A previous study found that *F. nucleatum* was also involved in the risk of recurrence following neoadjuvant chemoradiotherapy in locally advanced rectal cancer (120). Within the tumor microenvironment, the intestinal microbiota was found to regulate the functions of myeloid derived cells, thereby affecting the response to chemotherapy against cancer (85). Since the gut microbiota was often observed to interfere and affect the efficacy of anticancer drugs through its interaction with immune cells (121-123), there is an urgent need to identify alternatives to chemotherapy.

Immunotherapy is a biological method of motivating the immune system to fight by stimulating or suppressing body's own immune cells as per requirement in order to elicit an immune response. Now that microbes have been found to play a key role in development, as well as in the diagnosis of CRC, it was hypothesized by several groups that protective bacterial species if restored/maintained in the intestine, can serve as bio-therapeutic in triggering the host immune response against virulence factors generated by drivers of CRC. Several clinical trials have highlighted the antitumor potential of *L. acidophilus* and *Bifidobacterium*, and suggested their utility as a probiotic in the treatment of CRC, where it can be administered orally to patients. This will help restore the balance of commensal microbial genera in the gut and preventing intestinal toxicity (79). The study by Sivan *et al* detailed the potency of *Bifidobacterium* as a probiotic and its efficacy as a biotherapeutic agent (121). *Bifidobacterium* in the gut, on its oral administration to patients with CRC, reduced tumor growth to an extent similar to that obtained by treatment with anti-PD-L1 therapy, and when the probiotic in combination with therapy was used for treatment, *Bifidobacterium* enhanced the response of anti-PD-L1 antibody therapy and abolished tumor growth (121). Another immune checkpoint therapy involves targeting anti-CTLA4 in the intestine to induce the maturation and activation of dendritic cells and exert antitumor effects; several gut microbiota species are known to have a positive impact on this immunotherapy (122). A positive impact of the gut microbiota in several other immune therapies have been deduced which target induction of $CD8^+$ T-lymphocytes, macrophages and the activation of dendritic cell, and major histocompatibility complex (MHC) driven pathways in order to exert antitumor effects (123). The restoration of the commensal gut microbiota population beneficial for intestinal epithelia, can trigger several immunoregulatory pathways and minimize adverse effects of immunotherapy. Thus, it may prove to be crucial in treatment of CRC and can pave the way for the development of novel therapeutic approaches (22). Another therapeutic approach involves the bioengineering of bacteria to trigger the immune response in the host. Cancer-invading bacterial cells were engineered, such that they produce a short hairpin segment of RNA which can effectively interact with β-catenin to suppress Wnt signaling and inhibit tumor formation in colorectal tissue (124). Thus, the human gut microbiota plays a crucial role in determining the efficacy and toxicity potential of a treatment, and also provides a great future prospect for developing novel biotherapeutics using gut microbiota and its metabolites for the treatment of CRC. A summary of

novel therapeutic strategies for the treatment of CRC and their possible mechanisms of action is presented in Table II.

7. Conclusion

Several studies have revealed that the microbiota composition has been altered in benign lesions and in malignant tumors of the colon and rectum. Moreover, in patients with CRC, a dysbiosis in the gut microbiota has been found as compared to healthy controls. An enrichment in pro-inflammatory microbiota and the depletion in butyrate-producing bacteria has been noted. The dysbiosis of the gut microbiota in CRC results in the impairment of the intestinal epithelial barrier function, the activation of pro-inflammatory responses, genotoxin synthesis and toxic metabolite generation. The gut microbiota should be considered as a prime factor that can contribute to both CRC initiation and development. Dysbiosis can be avoided by the intake of dietary components that can alter the cancer-associated microbiome and suppress intestinal inflammation. This strategy can improve the cancer therapeutic response and prevent the progression of CRC. Furthermore, once the microbiome composition of a given patient is characterized, using a personalized medical approach, a desired bacterial equilibrium could be restored using pre and probiotics and a tailored phage therapeutics. Identifying the mechanistic pathways through which the gut microbiota influences CRC would help in devising more effective strategies for the treatment of CRC. Therefore, targeting metabolome by drugs or diet modulation and developing immunogenic peptides against cell surface proteins would improve the therapeutic efficacy for CRC and overall survival.

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Authors' contributions

DA, MA, RA and SKS conceived the study, and drafted and wrote the manuscript. MKP, JKS and TBT were involved in the critical review of the manuscript. SKS, RA and MKP revised and edited the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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