

REVIEW ARTICLE

Intestinal microbiota and colorectal cancer: a new aspect of research

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Summary

Colon holds a complex microbial community, which is crucial for maintaining homeostasis and regulating metabolic functions, supporting the intestinal barrier and controlling immune responses. Previous studies have supported a link between intestinal microbiota and colorectal cancer (CRC). Based on these findings, the present review analyzed the numerous interactions that occur between microbiota and CRC, starting from the role of intestinal microbiota in colonic homeostasis. Intestinal microbiota is a cause of CRC and involves various mechanisms such as chronic inflammation, the production of genotoxins causing DNA impairment and/or the biosynthesis of toxic compounds. Moreover, basic metabolic factors such as short-chain fatty acids (SCFAs) and bile acids are included in CRC pathogenesis. Different pathogenic pathways have been reported among different CRC regions (proximal or distal). Variations in the microbial populations are reported between the CRC from these colonic sites, possibly reflecting the bacterial dysbiosis and biofilm distribution. Bowel preparation is essential prior to

colonoscopy and surgery; there is, however, minor consensus on the effects of this procedure on intestinal microbiota, notably with regard to the long-term outcomes. With regard to the therapeutic strategy in CRC, the intestinal microbiota is further involved in the modulation of the host response to chemotherapeutic agents (5-fluorouracil and irinotecan) by the interference with drug efficacy and by adverse effects and associated toxicity. In addition, the newly emerged research on CRC immunotherapy reveals an important interplay between intestinal microbiota and the immune system, which includes the possibility of targeting microbiota for the enhancement of anticancer treatment. Additional studies will further clarify the interaction between microbiota and CRC, resulting in the development of alternative therapeutic strategies by manipulating microbiota composition.

Key words: bowel preparation, chemotherapy, colorectal cancer, immunotherapy, intestinal microbiota, pathogenesis

Introduction

The human colon is known for its wide microbiota composition, which consists of many different organisms that interfere with the host, usually in a

symbiotic relation. These microorganisms convert the undigested nutrients that reach the colon as substrates for their metabolism, into some useful

metabolic products, such as vitamins, among other functions including the maintenance of colonic immune system [1].

Nevertheless, this equilibrium is not always preserved and improper interactions, causing immune evasion, as well as chronic inflammation, may induce colorectal carcinogenesis. The presence of microbial dysbiosis in patients with CRC has been demonstrated, although the detailed mechanism remains elusive [2].

CRC is a topic of great concern since it is responsible for numerous deaths related to malignancy globally. The incidence of CRC is increasing mainly in developed countries. Several factors have been proposed as potential risk factors for the development of CRC, such as dietary habits, inflammatory response, DNA alterations, genetic polymorphisms, gene-environment interactions and changes in microbiota composition [3]. Previous studies have suggested that the main pathological mechanisms leading to the CRC refer to aberrant DNA methylation and genomic instability, including chromosomal instability, microsatellite instability and DNA base excision repair defect [4,5]. Furthermore, genome-wide association studies (GWAS) have greatly contributed to the investigation of the genetic basis of CRC and the identification of more than 50 CRC-associated loci [6-8].

The role of microbiota in colonic homeostasis has been thoroughly investigated, in addition to the mechanisms that are responsible for the pathogenesis of CRC. Specific differences in the microbiotic environment have been identified between proximal and distal colon in healthy individuals, as well as in patients with CRC. Based on the recent technical advances towards CRC treatment, the effects of chemotherapy and pre-operative bowel preparation (in view of colonoscopy or surgical treatment) in the subsequent colonic microbiota composition have been studied. Moreover, the novel advances in the field of CRC immunotherapy and their interplay with intestinal microbiota have been investigated.

The aim of the present review was to highlight the various interactions between colonic microbiota and CRC.

The role of intestinal microbiota in colonic homeostasis

Intestinal microbiota provides its host with a physical barrier to incoming pathogens by competitive exclusion. Symbiotic microbes can directly and dynamically interact both with pathogens and immune cells, defining the pathogenesis and outcome of an infection. They can protect the host

through distinct mechanisms including occupation of attachment sites, consumption of nutrient sources, competition for defined metabolites, or by production of antimicrobial molecules and bacteriocins that affect pathogen survival (a process referred to as colonization resistance).

In detail, intestinal microbiota is able to modulate the survival and virulence of potential pathogens either by inducing intestinal epithelial cells to produce and excrete peptides and other molecules with antimicrobial properties or by stimulating enteric dendritic cells and recruiting other innate immune cells both locally and systemically in order to promote anti-pathogen responses of effector T and B cells. However, this immune system stimulation requires specific regulation as it can give rise to inflammatory and autoimmune diseases under certain circumstances of uncontrolled induction [9-14]. Similarly, the capacity of the microbiota for the effective suppression of infection is associated with its unique property in order to promote both innate and adaptive immune responses [15].

The dynamic and complex processes of intestinal microbes include a large number of potential ligands and metabolites in a dynamic balance. There is an increasingly recognized role for members of the intestinal microbiota and their metabolites in shaping immune development and altering immune response [16,17]. A continuous crosstalk between lamina propria, B lymphocytes, intestinal epithelial cells, and the microbiota is of crucial importance for maintaining intestinal homeostasis [18]. The communication between host cells and microbiota and the host-pathogen interactions involves the interplay between various immune cells and receptors, which influence immune homeostasis and inflammation.

Microbiota plays an important role in the induction, education and functioning of the host's immune system and regulates both local and systemic immune responses. The host-microbiota co-evolution has enabled the immune system to preserve the symbiotic relationships of the host with the highly dynamic and complex communities of microbes. If balanced, this immune system-microbial relationship allows both the induction of protective responses to pathogens and concomitantly the tolerance to innocuous antigens via various regulatory pathways. Thus, a state of homeostatic equilibrium is maintained [19]. When homeostasis is well-maintained, both inflammatory and regulatory responses are constantly integrated, the sum of which leads to the establishment of a localized inflammation compatible with tissue immunity [9].

Symbiotic bacteria also play a key role to the maturation of the immune system that in turn

contributes to their restraint. Several studies performed with germ-free (GF) animals, revealed the ability of microbiota to chisel secondary and lymphoid structure development. Interestingly, in addition to its immune-system-regulation ability, members of microbiota can also contribute to the maintenance and restoration of the integrity of the intestinal barrier by numerous mechanisms including the induction of epithelial cell maturation, angiogenesis and reinforcement of tight junctions [20,21].

A main strategy of the host to maintain the homeostatic relationship with the microbiota in the colon is to minimize contact between microorganisms and the epithelial cell surface, thereby limiting tissue inflammation and microbial translocation. This strategy is achieved by the combined action of structural and immunological components such as epithelial cells, mucus layer, IgA, antimicrobial peptides and immune cells (collectively referred to as the “mucosal firewall” [22].

One of the main modes of cross-talking between the host and the microbiota is mediated by the recognition of evolutionary conserved microbial associated molecular patterns (MAMPs) Innate pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs), and NOD-like receptors (NLRs). These receptors recognize damage- and pathogen-associated molecular patterns and activate effector responses that promote both tolerance and activation of immune responses [23-25].

TLRs are expressed on the surface and in the cytosol of phagocytic cells such as dendritic cells (DCs), neutrophils, and macrophages as well as in intestinal epithelial cells [26,27]. Specific microbe-associate molecular patterns (MAMPs) that activate the PRRs include various microbial components such as peptidoglycan, LPS, lipoproteins, flagella, fungal cell wall β -glucans prokaryotic DNA and foreign nucleic acids [28,29]. TLR signaling triggers immune defense mechanisms, which enhance barrier function by strengthening tight junctions and zonula occludens. This ultimately leads to obstruction of paracellular microbial invasion [30]. CpG (cytidine-phosphate-guanosine) motifs of damaged human DNA or apoptotic debris (damage-associated molecular pattern - DAMP) may activate TLR9, an intracellular DNA sensor, and trigger a self-destructive, chronic B-cell immune response. Chronic TLR9 triggering may accelerate growth and dissemination of tumors in the GI tract [31,32].

Signaling through PRRs enables host immune sensing and reactivity toward diverse stimuli. PRRs decode signals from the microbiota and help to shape the homeostatic host-microbiota interface.

The development of deleterious effects to the host occurs when the immune response does not properly control the microbiota alterations of commensal and symbiotic microbial communities [29].

The recognition of various microbial MAMPs by PRRs results in triggering intracellular signaling cascades that in turn leads to activation of the NF- κ B pathway, production of proinflammatory cytokines and chemokines and initiation of the immune response [26,27,33,34]. Specifically, recognition of MAMPs by TLRs with subsequent activation of NF- κ B promotes and enhances tumor cell proliferation and survival [35]. Negative regulation of TLR by IL-1 (Interleukin-1), IRAK-M (receptor-associated kinase-M), TOLLIP (Toll-interacting protein), SIGIRR (single immunoglobulin interleukin-1-receptor-related molecule) prevents an excessive inflammatory response, thus maintaining intestinal homeostasis [36]. All these events can also alter the intestinal microbiota such that it can feed back to TLR signaling augmenting inflammation. Responses to microbial ligands such as LPS, the endotoxin found in the outer membrane of gram negative bacterial walls, condition intestinal epithelial cells to become hypo-responsive to subsequent TLR stimulation.

Thus, the healthy microbiota causes a physiologic low- grade inflammatory response in the host mucosa, controlled by the innate immune system. Alteration of intestinal microbiota may promote inflammation through enhanced MAMP presentation to intestinal immune cells [37-39]. A delicate regulation of PRR signaling in the intestine is crucial in the balance between health and disease [29].

Mechanisms of intestinal microbiota in CRC pathogenesis

The intestinal microbiota interacts with the host and maintains human health via various crucial functions, thus acting like a “metabolic organ” [40]. These metabolic functions allow microbiota to utilize available energy sources such as proteins and carbohydrates. In return, the microbiota synthesizes amino acids, influences the absorption of ions, produces vitamins, is involved in the conversion of dietary polyphenolic compounds, and associates with the biological transformation of bile acids [41-43]. Intestinal microbiota generates energy through fermentation of complex carbohydrates, and as a result forms organic acids, which serve as an important energy supply for colonic epithelium, such as short-chain fatty acids (SCFAs). Some of the most productive microbial species in the colon, regarding SCFA formation, include *Clostridium*

and *Bifidobacterium* which belong to the *Firmicutes* group. Not only do SCFAs serve as an energy source, but they also demonstrate immunoregulation effects, such as suppression of the inflammatory cytokine secretion in mice. Butyrate production inhibits the inflammatory response through suppression of NF- κ B factor [44]. GPR109a is a receptor for this metabolite, coded by the *Niacr1* gene. Its signaling stimulates the anti-inflammatory response, allowing the differentiation of anti-inflammatory cells, namely regulatory T cells and IL-10 producing cells. *Niacr1*^{-/-} mice have demonstrated a higher susceptibility to CRC [45]. Apart from its anti-inflammatory properties, butyrate promotes many of the large intestine functions, such as colon motility, improving visceral blood flow and preventing the overgrowth of pathogens [46-48]. Dietary presence of fiber influences production of SCFAs positively [49]. Consequently, SCFAs significantly increase as a result of a fiber-rich diet, which enhances colonic fermentation, lowers intestinal pH, and deters pathogen colonization [50], thus decreasing CRC risk (Figure 1).

A number of recent studies has indicated that there are multiple bacterial species that contribute to the pathogenesis of CRC. Disruptions in homeostasis inducing sequential phenomena such as inflammation, dysplasia and finally cancer, may be a result of alterations in the allocation, metabolic profile, and composition of the colonic microbiota [51]. A symbiotic relationship between microbiota and the host is the key to maintain the balance of the microbiota in the intestine. Under abnormal conditions, an alteration in that balance may cause adverse consequences to the host resulting in "intestinal dysbiosis". This phenomenon may be associated with the overgrowth of opportunistic pathogens that are normally inhibited by commensal bacteria [52], an observation already been described in inflammatory bowel diseases [53,54], obesity [55,56], colorectal adenomas, and cancer [57-60]. Dysbiosis disrupts homeostasis of the immune system and mucosal barrier. The subsequent inflammatory process results in increased permeability allowing intestinal microbes to drive a continuous state of inflammation. One of the main effects of

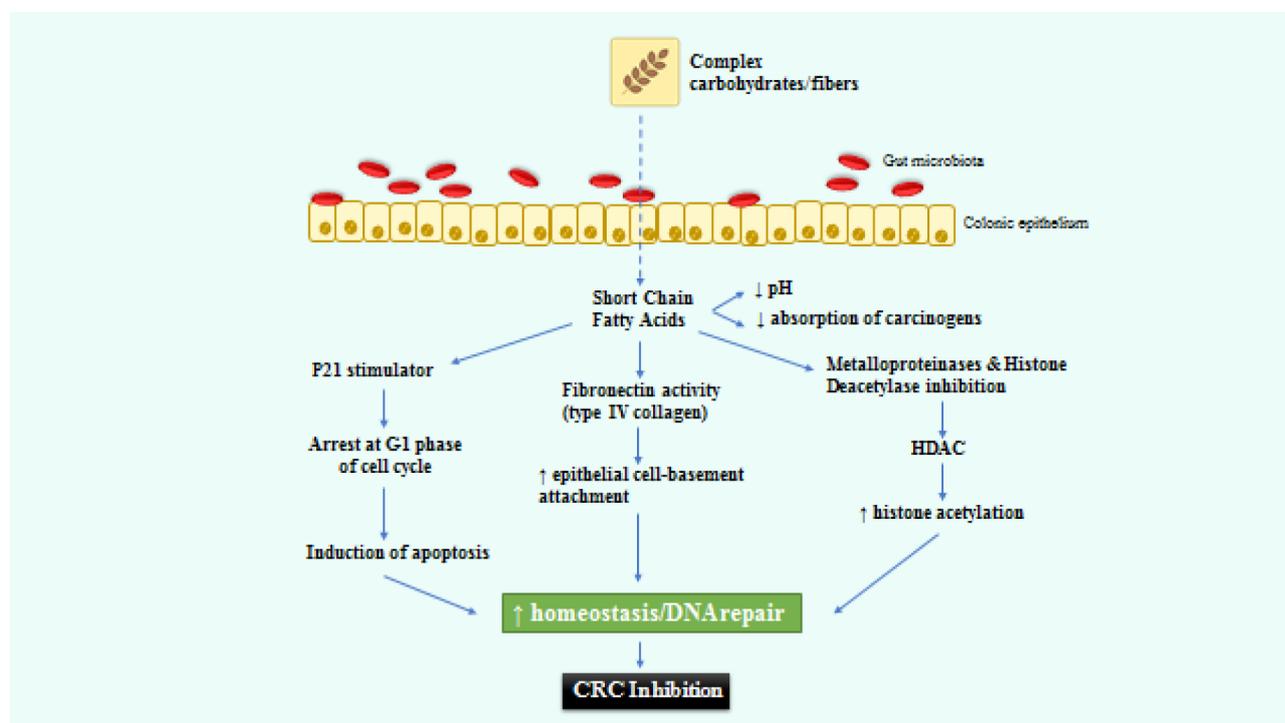


Figure 1. Immunoregulatory effects of SCFAs in CRC inhibition. Fermentation of complex carbohydrates and fibers by intestinal microbiota results in the generation of SCFAs such as butyrate. SCFAs enhance colonic function by promoting visceral blood flow, stimulating intestinal mobility, and preventing the overgrowth of potential pathogens. Butyrate enhances colonic fermentation, reduces the absorption of procarcinogens and intestinal pH and prevents inflammatory response, thus lowering CRC risk. Butyrate may also inhibit HDAC and metalloproteinases, thereby promoting acetylation of histone in the cell. SCFAs also stimulate p21, a key component of cell cycle arrest, inducing apoptosis. SCFAs promote the barrier functions stimulating attachment of epithelial cells to the basement membrane by reducing the activity of type IV collagen. All these mechanisms participate in the maintenance of colonic homeostasis and CRC inhibition.

SCFAs: short-chain fatty acids, CRC: colorectal cancer, HDAC: histone deacetylase.

dysbiosis is chronic unchecked activation of the immune system, thereby creating a proinflammatory milieu, which may favor the development and progression of neoplastic lesions in the intestine [19]. Shifts in the intestinal microbiota composition and density not only affect local immune responses, but also, these changes can also alter immunity and inflammation in organs distal from the intestine [61]. However, the role of intestinal dysbiosis as a cause or a result of the disease is not definite and there is limited information about the various mechanisms which contribute to the progression of CRC. In order to answer this question, the role that microorganisms play at the onset of CRC was investigated. A dynamic “bacterial driver-passenger” model was suggested by Tjalsma et al. [62] in order to clarify the involvement of microbiota in the induction and development of CRC. In accordance with this model, specific bacterial populations (drivers) presenting pro-carcinogenic characteristics are able to advance the progression of the disease through DNA impairment in the intestinal epithelial cells. A change of the intestinal environ-

ment occurs as a response to the tumorigenesis induction, resulting in higher growth rates of opportunistic bacteria (passengers) and a reduction in predominant strains. Passenger bacteria poorly colonize a healthy intestinal tract, are indigenous members of the intestinal bacterial society, and are capable of furthering tumor progression, thus displaying an antagonistic advantage in the tumor microenvironment. For instance, particular bacterial passengers are able to make use of co-factors and substances unique to the microenvironment of the tumor, like reactive oxygen species (ROS). Nevertheless, contrary to drivers, which are mostly pro-carcinogenic, passenger bacteria may serve as protective or pro-carcinogenic, depending on certain species [62].

In recent years, it has been observed that intestinal microorganisms can initiate and further the progress of CRC by different processes (Figure 2). Chronic inflammatory response has found to be associated with a large risk of promoting tumorigenesis by preventing apoptosis, generating mutations or triggering angiogenesis and cell proliferation

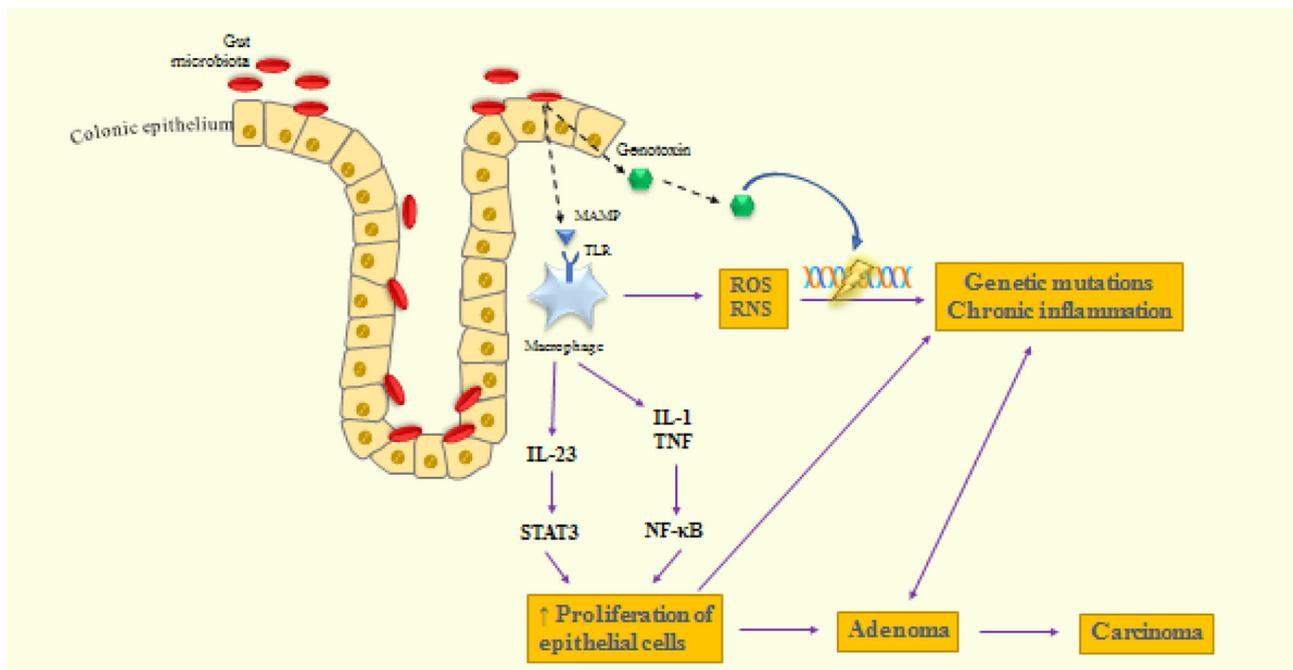


Figure 2. Mechanisms of colonic microbiota in chronic inflammation, adenoma and CRC progression. The colonic microbiota involves various mechanisms to stimulate CRC tumorigenesis. Bacterial dysbiosis weakens epithelial barrier favoring bacterial translocation. Macrophages promote various inflammatory signal cascades, by recognizing MAMPs expressed by commensal bacteria through binding to TLRs. Pro-inflammatory cytokines (IL-1, IL-23, TNF) promote STAT3 and NF-κB pathways leading to stimulation of cell proliferation and suppression of apoptosis, thus progressing chronic inflammation and CRC pathogenesis. Imbalances in ROS/RNS production released from macrophages, as well as genotoxins (colibactin) from bacteria, promote oxidative stress in the colonic epithelium resulting in DNA impairment and inhibition of DNA repair.

CRC: colorectal cancer, MAMPs: microorganism associated molecular patterns, TLRs: toll-like receptors, IL: interleukin, TNF: tumor necrosis factor, STAT3: signal transducer and activator of transcription 3, NF-κB: nuclear factor κB, ROS: reactive oxygen species, RNS: reactive nitrogen species.

[63]. A disturbance of microbiota in favor of opportunistic pathogens contributes to higher mucosa permeability, bacterial translocation, and the stimulation of components involving in adaptive and innate immune systems, resulting in chronic inflammation. The activation of the innate immune system by commensal bacteria results in an increased release of pro-inflammatory cytokines by macrophages, dendritic cells, and “natural killer” cells, such as IL-12, IL-23, TNF- α , and INF γ . This, as a result, stimulates T and B lymphocytes, cells which belong to the adaptive immune system, and a variety of inflammatory mediators [50]. Many studies have mentioned the correlation between CRC development and the up-regulation of IL-17C from CD3 cells, the up-regulation of TLRs (TLR2) and the down-regulation of their inhibitors (TOLL-IP) and the deficiency in TGF- β signaling pathways [45]. The stimulation of transcription factors which involves in particular pivotal signaling cascades in the epithelium, such as STAT3 and NF- κ B [64-66], and the generation of ROS or nitrogen species (RNS), are some of the crucial effects deriving from this inflammatory response. This inevitably results in oxidative stress, DNA damage, abnormal proliferation and, finally, the development of colorectal adenomas and cancer. It has been shown that colonization of germ-free animals with *Enterococcus faecalis* and *Bacteroides vulgatus* leads to the activation of NF- κ B signaling pathways in epithelial cells [67]. Colonic tumorigenesis was greatly enhanced in germ-free mice that colonized with microbiota deriving from tumor-bearing mice, as opposed to mice colonized with normal intestinal microbiota [68]. Therefore, colonization of germ-free mice with human microbiota (CRC patients and healthy individuals) suggests that the primary structure of the microbiome defines susceptibility to tumorigenesis in the colon [69]. Moreover, it has been reported that colonic polyposis is associated with high densities of microorganisms accumulated inside polyps, which elicit the local inflammatory response. The inflammatory responses, as well as microbial density and polyp growth, can be suppressed by IL-10, specifically originated from T and T-reg cells [70].

Another way, through which intestinal microbiota correlates with CRC progression, is by generating toxic or specific genotoxic microbial metabolites. Such metabolites may interact with intracellular signal cascades or result in mutations by binding to particular cell surface receptors [71]. These toxins significantly affect signaling pathways of eukaryotic cells, including other various processes, and occasionally are capable of attempting genomic attacks directly, thus impairing DNA

and initiate an enzymatic or an indirect attack causing an inflammatory reaction, which results in the release of free radicals. Moreover, it has been indicated that they can affect DNA repair mechanisms [72]. Enterotoxigenic strains of *Bacteroides fragilis*, for instance, are able to asymptotically colonize the human mucosa, but they occasionally produce the *B. fragilis* toxin (BFT) that can provoke inflammatory diarrhea. BFT is a zinc-dependent metalloprotease that quickly changes the function and structure of colonic epithelial cells, including the breaking of the E-cadherin, a tumor suppressor protein [73,74]. E-cadherin is a calcium-dependent cell-cell adhesion molecule with crucial roles in tissue formation, epithelial cell behavior and cancer suppression. Loss of this protein increases the permeability of polarized colonic epithelial cells, which constitutes one of primary tumor development steps [75]. BFT also promotes the transcription factor NF- κ B resulting in the generation of cytokines that contribute to mucosal inflammation [76]. However, although BFT is considered one of the main toxins in CRC development, recent studies have suggested that toxins which derive from species such as *Salmonella enterica*, *Shigella flexneri* and *E.coli*, are most vividly expressed in tumorous tissue samples of CRC patients. This indicates the potential involvement of entero-bacterial toxins in tumorigenesis. Certain commensal strains of *E.coli* present the ability to directly produce colibactin toxin in the colon, impairing the DNA of colonic epithelium via double-strand breaks. Cellular repair systems fail to reinstate these DNA lesions. This leads to the accumulation of chromosomal anomalies and, thus, mutations resulting in tumorigenesis [77]. A possible correlated mechanism between *Fusobacterium nucleatum* and CRC is through binding of FadA adhesin to E-cadherin, with subsequent invasion into the epithelial cells and activation of β -catenin pathway, which results in inflammatory response (NF- κ B), release of cytokines (IL-6, IL-8) and expression of oncogenes (Myc, Cyclin D1) [58].

Intestinal microbiota also plays an essential role in the metabolism of bile acids, mainly in the 7 α -hydroxylation process, where the cholic acid is converted into deoxycholic acid, while chenodeoxycholic acid turns into lithocholic acid. This conversion enhances the hydrophilicity of these secondary bile acids [78]. It is shown that deoxycholic acid has a harmful effect in intestinal tract mucosa and contributes to an increase in ROS, thus damaging DNA, stimulating genomic instability, and amplifying tumor development [79]. Strains of *Clostridium IX* convert primary cholic acids into secondary cholic acids, such as deoxycholic acid,

Table 1. Bacteria and related pathogenetic mechanisms linked to colorectal cancer

Microbe	Mechanism
<i>Bacteroides fragilis</i>	BFT toxin
	Activation of STAT3
	Th-17 immune response induction
	Production of IL-1
<i>Bacteroides vulgatus</i>	Activation of β -catenin signaling
	NF- κ B activation
<i>Enterococcus faecalis</i>	ROS production and DNA damage
<i>Escherichia coli</i> (cytotoxic)	Colibactin and DNA damage
<i>Salmonella enterica</i> , <i>Shigella flexneri</i>	Shiga and shiga-like toxins
<i>Fusobacterium nucleatum</i>	FadA binding to E-cadherin
	Activation of β -catenin signaling
	NF- κ B activation
	Production of IL-6, IL-8
<i>Clostridium IX</i>	Myc, Cyclin D1 activation
	Production of DCA
	ROS production and DNA damage

BFT: *Bacteroides fragilis* Toxin, STAT3: signal transducer and activator of transcription 3, Th-17: T helper 17, IL: interleukin, NF- κ B: nuclear factor κ B, ROS: reactive oxygen species, FadA: *Fusobacterium nucleatum* adhesin A, DCA: deoxycholic acid

generating harmful effects through this mechanism [58]. The various pathogenetic mechanisms related to CRC, for each bacterium, are summarized in Table 1.

Microbiota differences between proximal and distal colon in health and CR

Although intestinal microbiota certainly plays an essential role in human homeostasis and disease, this ecosystem has been poorly described and its diversity remains inadequately determined, both on its whole and its possible differences between colonic regions.

As an effort to define the aforementioned factors, Eckburg et al. [80] with the use of comparative analysis of 16S rDNA sequences in colonic mucosal tissue samples from healthy individuals, examined the various differences of the bacterial populations among individuals along with various areas in intestinal mucosa. Phylogenetic analysis showed that most of the inferred organisms were members of the *Firmicutes* (most of the *Firmicutes* sequences were members of *Clostridia* class) and *Bacteroidetes* phyla, yet, relatively few sequences

were associated with the *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* phyla [80]. These findings were coherent with previous studies [81-83]. With the use of diversity coefficients and methods of statistical analysis, it was observed that mucosal samples between individuals (inter-subject) demonstrated a higher diversity profile than those between different large intestine sections in each individual (intra-subject). However, when the ascending colon was excluded from this comparison, the inter-subject and intra-subject diversity profiles were not significantly different [80]. Some studies suggest that diversity indices are significantly higher in the proximal colon than in the distal colon, indicating that more species of intestinal bacteria at higher diversity are detected in proximal colon [84], while others indicate that for 97% of healthy samples, the microbiota of distal and proximal colon was not significantly different in each individual [85].

A recent study reveals that microbiota differences between proximal and distal colon in healthy individuals may be due to the differences in oxygen distribution along colonic mucosa. Proximal colon mainly hosts aerobic bacteria in its mucosa such as *Pseudomonas* and facultative anaerobes like *Enterobacteriaceae* and *Actinomyces* due to its high oxygen concentrations. Adversely, distal colon mostly harbors anaerobic species such as *Finegoldia*, *Anaerococcus*, *Peptoniphilus* and *Porphyromonas* [86].

Understanding microbiota distribution and its differences in healthy individuals is essential for proper investigation of intestinal microbiota in CRC patients. Nowadays, there is growing evidence indicating that various risk factors which are related to CRC development (smoking, IBD, high alcohol consumption, excessive consumption of red meat, diabetes mellitus, obesity, genes) greatly affect the synthesis of intestinal microbiota [87]. Therefore, studying intestinal microbiota distribution will probably clarify the pathogenesis of CRC. For this purpose, Gao et al. [88] performed pyrosequencing-based analysis of 16S rRNA genes in mucosal samples from patients with CRC and healthy controls and also between adjacent neoplastic and non-neoplastic tissues in proximal and distal tumors. The relative abundance of *Fusobacteria* and *Firmicutes* in CRC individuals was reported to be significantly higher than that in the healthy group. Adversely, *Proteobacteria* demonstrated a less abundant profile in cancerous tissues. Moreover, regarding differences within tissue microenvironment, *Lactococcus* and *Fusobacterium* were over-represented in CRC patients, while *Pseudomonas* and *Escherichia-Shigella* were less enriched in adjacent cancerous tissues as opposed to non-cancerous samples [88].

This fact is non-random, as these previously abundant *Pseudomonas* and *Escherichia-Shigella* have shown that they could increase an individual's susceptibility to CRC, with the use of inflammation and toxins, thus resulting in tumorigenesis. Nevertheless, a disruption in the structure of autochthonous microbial communities may occur as a result of the major metabolic and physiological alterations during colonic tumorigenesis. Consequently, some bacterial populations are minimized, whereas other bacteria that are rare colonizers of the colon, such as *Fusobacterium* and *Lactococcus*, show an adaptive behavior to the new environment, thus confirming the hypothesis of a "bacterial driver-passenger" model which plays a key role in CRC pathogenesis [62].

Evaluation of microbial composition in proximal and distal tumors indicates an increasing microbial richness from proximal colon to rectal cancer. Prevalent bacterial genera, such as *Fusobacterium* and *Lactococcus* in CRC individuals, show contiguous abundance between proximal and distal cancers. Moreover, these bacteria share the mutual feature of being both "passenger bacteria". One interpretation of this observation may be that a similar tumor microenvironment develops during colon carcinogenesis in various sections of the colon. In contrast "driver bacteria" *Escherichia-Shigella* which may be involved in CRC pathogenesis, are highly enriched in proximal colon [62,88]. Other bacteria of genus *Bacteroidetes* are highly enriched in distal colon cancer, while bacteria of genus *Prevotella* are highly enriched in proximal colon cancer.

There are also findings from other studies demonstrating the high presence of *Enterococcaceae*, *Erysipelotrichaceae* and *Fusobacteroidaceae* in the distal tumor samples and the high presence of *Enterobacteriaceae*, *Streptococcaceae*, *Verrucomicrobiaceae* and *Pseudomonaceae* in proximal tumor samples in CRC patients, with tumor tissue samples from distal colon exhibiting more microbiota diversity than the tumoral tissue samples from proximal colon [89]. Additionally, another study showed an increased abundance of *Alistipes*, *Akkermansia*, *Halomonas* and *Shewanella* in individuals with rectal and distal cancers, whereas individuals with proximal cancer showed a higher abundance in *Blautia*, *Faecalibacterium* and *Clostridium* [85]. All these findings indicate a considerable difference in intestinal microbiota regarding tumor location in the proximal or distal colon (Table 2).

Recent studies in bacterial biofilms of colonic mucosa give a novel insight into the different pathogenetic mechanisms underlying CRC tumorigenesis between colonic regions. Biofilms consist of densely packed clumps of bacteria enclosed in

the matrix lining the mucosal surface and suggest impairment of the colonic mucous barrier [90,91]. Colonic biofilms may trigger inflammation by promoting a direct interplay between colonocytes and microbiota. Interestingly, recent studies suggest that microbial community structural organization exhibits the potential to contribute to disease progression acting as an early "driver" factor of CRC carcinogenesis, thus inducing malignant alterations in adenomas which further tumorigenesis. Specifically, cancers located in the right colonic regions (cecum, ascending colon and hepatic flexure) generally demonstrate substantial biofilms and present a high frequency of epithelial KRAS and BRAF mutations which are associated with worse prognosis [92-96].

Two recent studies by Dejea et al. and Johnson et al. described the identification of polymicrobial bacterial biofilms and their metabolic contributions toward the colon cancer environment [93,97]. Initially, Dejea et al. demonstrated that biofilms are associated with human colon cancer and linked to cancer location, with virtually all adenomas and cancers located in the right colonic regions being biofilm positive, whereas cancers located in the left colonic regions (distal to hepatic flexure) rarely possess biofilms [93]. According to the authors, invasive complex microbial biofilms interacting with colonocytes are nearly universal on tumors located

Table 2. Bacterial abundance related to CRC site

CRC site	Abundant microbes
Proximal colon	<i>Prevotella</i>
	<i>Enterobacteriaceae</i> (<i>Escherichia</i> , <i>Shigella</i>)
	<i>Streptococcaceae</i> (<i>Lactococcus</i>)
	<i>Verrucomicrobiaceae</i>
	<i>Pseudomonaceae</i>
	<i>Blautia</i>
	<i>Faecalibacterium</i>
	<i>Clostridium</i>
Distal colon	<i>Bacteroidetes</i>
	<i>Enterococcaceae</i>
	<i>Erysipelotrichaceae</i>
	<i>Fusobacteroidaceae</i> (<i>Fusobacterium</i>)
	<i>Alistipes</i>
	<i>Akkermansia</i>
	<i>Halomonas</i>
<i>Shewanella</i>	

CRC: colorectal cancer

in right colonic regions. These bacterial biofilms are related to epithelial changes which characterize progression of tumorigenesis, including diminishment of E-cadherin, increased levels of the angiogenic and pro-inflammatory cytokine, IL-6, as well as activation of the downstream-effector STAT3, and increased epithelial crypt cell proliferation [93].

In a follow-up study, Johnson et al. revealed that bacterial biofilms of colonic mucosa affect the tumor metabolome, to up-regulate cellular proliferation via production of N1, N12-diacetylspermine, possibly interacting with tumorigenesis [97].

Together the findings of the above two studies implicate colonic bacterial biofilms as contributors to a pro-oncogenic state which leads to CRC development [93,94,97].

It should also be mentioned that there is no consensus between studies regarding the accurate definition of “proximal” and “distal” colon. Thus, future studies should apply a common guideline considering this issue, in order for their results to be absolutely comparable.

Effects of pre-op bowel cleanse in post-op composition of intestinal microbiota

Pre-op bowel cleanse is considered as an essential procedure and is performed by almost every surgeon for regular surgical treatment of CRC as well as for successful endoscopic examinations (colonoscopy) [98,99]. Bowel cleanse is associated with reduced incidence of trauma inflammation and anastomotic leak and mostly easier surgical maneuvers during laparoscopic colectomy, as intestine is empty from content and thus maneuvers are more feasible [98]. There are various types of bowel cleanse: a) clear liquid diet for few days pre-op (2-4 days), b) fluid administration through Levine, c) purgative fluid administration (polyethylene glycol/PEG, and sodium phosphate) combined with 2-4L of water per os, d) standard enema at night and early in the morning pre-op and e) intraoperative bowel cleanse [98]. Since PEG administration is considered the gold standard method for bowel cleanse, consequently it was considered appropriate to mostly emphasize how this method affects the colonic microbiota. PEG is a polymeric osmotic agent, which does not undergo metabolization or reabsorption by intestinal microbiota. Due to its ability to accumulate water in the intestinal lumen, it results in diarrhea, especially following high-dose administration. It is used to treat constipation and to cleanse the bowel prior to endoscopy or surgical operation [100]. The administered PEG

solution affects the bacterial ecosystem through depletion of luminal microbiota, diminishment of the nutritional source of the intestinal bacteria and alteration of the typically anaerobic colonic micro-environment by introducing oxygen [99].

There is limited information about alterations in microbiota in patients undergoing bowel preparation, and whether microbiota is restored back to its original composition [101]. Despite the fact that changes in microbiota following bowel cleanse have been observed, a detailed description of the long-term effects has been lacking [99].

The effect of colonoscopy and bowel preparation on the intestinal bacteria was firstly assessed by Mai et al. [101]. These authors investigated alterations in the fecal microbiota in five subjects who underwent screening colonoscopy, with the use of PCR-DGGE and 16S rRNA methods to analyze and contrast a single specimen prior to colonoscopy to two samples collected after colonoscopy 2-4 and 6-8 weeks respectively. The bacterial profiles of fecal samples obtained post-colonoscopy differed from those obtained pre-colonoscopy, as determined by DGGE analysis in 3 of the 5 individuals. Adversely, a lesser degree of diversity was observed between the microbial profiles prior to colonoscopy and after colonoscopy in the two remaining subjects. All three samples obtained from each subject demonstrated a variable relation as observed by 16S rRNA analysis, whereas in many cases DGGE revealed a different relevancy between them. Despite the discordance of results between methods, the authors concluded that the synthesis of the microbiota is altered in individuals subjected to screening colonoscopy. This finding indicates that luminal (fecal) intestinal microbiota is greatly affected by bowel preparations [101].

Harrel et al. also used 16S rRNA methods to investigate short-term effects of a standard bowel preparation (with the use of PEG) on microbiota diversity, richness, and composition of biopsies obtained from 12 healthy individuals [102]. In the first phase of the study, 5 subjects underwent two sigmoidoscopies over two weeks. In the first one, no preparation was preceded, whereas the second sigmoidoscopy was performed following a 24-h liquid diet in combination with a standard bowel preparation with PEG. The results showed divergence, with diversity enhanced in three individuals and reduced in the other two. Regarding the second phase of the study, the initial procedure performed in all subjects without preparation, but concerning the second procedure 3 groups were formed; “control group 1” including 2 subjects with no preceding preparation, “control group 2” including 2 other patients with preceding 24-h liquid diet,

and “experimental group” including the last 3 individuals where PEG preparation in combination with a 24-h liquid diet was preceded. The second procedure was executed for all subjects in less than a week following the first one. The bacterial abundance and diversity of the first biopsy regarding the experimental group were higher compared to the second biopsy, with the two samples demonstrating generic differences in the synthesis of microbiota. No considerable differences, between the two biopsies, were observed concerning these parameters in the control groups. In total, authors reported that the PEG preparation may notably change the intestinal microbiota in a variety of ways in the short-term, instead of liquid diet or the procedure. It has also been mentioned that the use of colonic lavage routinely may considerably change the microbiota related to mucosa of the distal colon, thus recommending that studies of the human intestinal microbiota in the future be conducted on the colon with no preceding preparation where the physiological condition of microbiota associated with both lumen and mucosa is most likely to be retained [102].

In another study Gorkiewicz et al. induced osmotic diarrhea in 4 healthy individuals by administering oral PEG solution in order to estimate how the synthesis of intestinal microbiota is affected by this phenomenon [103]. For this purpose, 16S rDNA analysis was used for the evaluation of the bacterial community in specimens obtained both from mucosa and stools. The results were congruent with previous reports, with *Firmicutes* prevailing in mucosa and *Bacteroidetes* in stools, thus marking the significant differences of bacterial profile between stool and mucosal samples. Moreover, the two most abundant mucosal phyla, matched to *Weissellacibaria* and *Weissella*, which are stable in response to diarrhea demonstrating a potent adhesion capability. In accordance with previous studies, a tendency of higher abundance on the mucosa as opposed to stools was also noted. It was also reported that there is a high degree of personalization in the intestinal microbiota, presenting a great variation in an inter-individual level. This finding was most conspicuous in stools compared to the mucosa, wherein any intra-individual differences were exceeded by inter-individual variations. Decreased phylotype richness was observed in both habitats after PEG administration, which can be subverted by opportunistic pathogens such as *Proteobacteria* that demonstrated important increase on the mucosa, leading to bacterial dysbiosis and chronic inflammation of the colon. Various species of *Firmicutes* and *Bacteroidetes* increased or decreased in stools regarding diarrhea. These

findings demonstrate that PEG treatment, which is utilized prior to endoscopy for bowel preparation, definitely alters the intestinal microbiota [103].

In another study, O’Brien et al. studied the impact of standard bowel preparation on intestinal microbiota [104]. For this purpose, bowel preparation containing a combination of PEG and bisacodyl was administered in 15 out of 20 subjects prior to colonoscopy. Fecal samples were collected in different phases; one month prior to colonoscopy, one week prior to colonoscopy, and one week, one month, and 3 to 6 months post-colonoscopy. The microbiota composition of these samples was assessed using HTS and PCR-DGGE methods. PCR-DGGE comparisons revealed no considerable variations between pre- and final post-colonoscopy. Although alterations in the abundance of main bacterial species belonging to *Firmicutes* and *Bacteroidetes* were indicated at the level of genus via HTS analysis, they did not reach statistical significance compared to those observed with no preparation prior to colonoscopy. The variation observed between samples of individuals who did not undergo colonoscopy was no superior compared to the variation among samples from individuals who were subjected to colonoscopy. In conclusion, the authors reported that the composition of the intestinal microbiota is not permanently affected by bowel preparation [104].

The effect of the two different dosing methods, which are currently utilized in clinical practice, to the intestinal microbiota has not been assessed yet. Recently, Jalanka et al. performed a study in order to shed light on this topic by examining the direct effects of different PEG dosage on the intestinal microbiota and microbial recovery [99]. Twenty three healthy subjects were randomized into two study groups consuming a bowel preparation (Moviprep), either in two separate doses of 1L or as a single 2L dose. Fecal samples were donated by the subjects at the baseline, following bowel preparation and 14 and 28 days post-treatment. Quantitative PCR in addition to phylogenetic microarray analysis were utilized in order to define the synthesis of the intestinal microbiota. Even though intestinal microbiota composition was momentarily affected by the lavage treatment, it recovered from its disturbance rapidly returning to its primary composition. The richness of bacteria belonging to *Clostridium* cluster IV was reduced following bowel cleansing, whereas bacteria belonging to *Proteobacteria* and *Clostridium* cluster XIVa were more abundant. The single dose (2L) of the PEG caused an increased abundance in some microbial species such as *Proteobacteria* and bacteria associated with *Dorea formicigenerans* in the follow-up specimens and less

effective microbial recovery in those subjects than in those who consumed two different doses of 1L. Bowel preparation utilizing two discrete dosages causes few changes to the composition of intestinal microbiota compared to the single dose method, and hence should be the recommended method of bowel cleansing in clinical practice [99].

Effects of CRC chemotherapy in intestinal microbiota

Chemotherapeutic agents, such as 5-fluorouracil, oxaliplatin and irinotecan, are considered to be parts of the standard therapy of CRC. Treatment for advanced stage CRC includes 5-fluorouracil plus leucovorin in combination with irinotecan or oxaliplatin. Consequently, it has been considered appropriate to mostly emphasize on the effects of these chemotherapy agents in colonic microbiota. Various studies have reported how these agents affect microbiota and homeostasis of the colon [105].

Irinotecan, due to the pivotal role of intestinal microbiota in its metabolic path, has been investigated thoroughly compared to other agents [106-108]. Irinotecan (used to treat a variety of solid tumors including CRC) is a topoisomerase-I inhibitor. *In vivo* irinotecan converts to active and toxic metabolite SN-38. SN-38 is further metabolized through glucuronidation to non-toxic metabolite SN-38 glucuronide (SN-38G) [106-108]. This molecule is excreted into the gastrointestinal tract (through bile secretion) where it becomes susceptible to processing by bacterial enzymes. Intestinal microbes produce the enzyme β -glucuronidase, which can cleave the glucuronide molecule from the less toxic metabolite of irinotecan, rendering it re-activated and toxic [106-108]. This phenomenon was observed specifically in the cecum by *E.coli* (a bacterium which produces β -glucuronidase) [106]. Due to this association in the drug's metabolic path and conceivably composite toxicities, the impact of irinotecan on the intestinal microbiota is of great significance. Studies conducted in Dark Agouti rats have shown notable increases in *E.coli*, *Clostridium spp.* and *Staphylococcus spp.* (β -glucuronidase-producing bacteria) following the administration of single-dose irinotecan, by analysis of fecal samples through quantitative 16S rRNA real-time PCR, with significant decreases in *Bifidobacterium spp.* and *Lactobacillus spp.*, which do not produce β -glucuronidase [109,110]. In a more recent study, irinotecan was shown to increase the *Clostridium cluster XI* and *Enterobacteriaceae* groups of bacteria after a dose-intensive therapy, both of which harbor opportunistic pathogens [108].

Antimetabolite 5-fluorouracil, a common therapeutic agent in CRC, has been reported to be related to alterations in the intestinal microbiota. A study reported a variation in the synthesis of the intestinal microbiota from preponderant Gram-positive to ascendant Gram-negative bacteria in Lewis rats after administration of 5-fluorouracil, although no considerable changes were observed in the population of anaerobic bacteria [111]. A later study showed no significant findings, however, peaks were observed following single dose 5-fluorouracil administration in *E. coli*, *Clostridium spp.* and *Staphylococcus spp.* Decreases were observed in *Lactobacillus spp.* and *Enterococcus spp.* following treatment [112]. A combined 5-fluorouracil/irinotecan regimen elevated *Clostridium cluster XI* and *Enterobacteriaceae*, but to a lesser extent than the irinotecan regimen [108].

Mucositis is often presented in CRC patients subjected to chemotherapy as an instant effect of their therapy. This condition is characterized by sore inflammation accompanied by numerous ulcers, affecting the mucosal membranes of the digestive tract. Lately, considerable attention has been paid to the intestinal microbiota in order to reveal the possible pathogenetic mechanisms outlining mucositis. Furthermore, chemotherapy-induced diarrhea (CID) is an ordinary issue, mostly affecting patients with advanced tumors [113]. The aforementioned studies have proposed possible mechanisms and hypotheses about how these mechanisms interfere with these toxic side effects and also the role of microbiota. The current view supports that chemotherapy causes depletion in the mucus layer, usually protecting the epithelial surfaces of the intestine. This allows bacteria to penetrate and activate TLR signaling, which in turn activates NF- κ B signaling. Pro-inflammatory cytokines are activated by NF- κ B and result in the rapid discharge of mucin (a glycoconjugate with a protective role in the intestinal mucosa) stores from goblet cells. Although this rapid discharge is capable of reinstating the mucus layer, it causes exhaustion of the mucin stores in goblet cells. Environmental conditions in the lumen are also altered, affecting the microbiota, reducing the number of commensal bacteria, such as *Lactobacillus spp.*, and allowing other opportunistic species, such as *E.coli*, to proliferate, creating the proper condition for the development of mucositis [114]. Concerning chemotherapy-induced diarrhea, an increase in some bacteria producing β -glucuronidase such as *E.coli*, *Clostridium spp.* and *Staphylococcus spp.* following treatment, may be responsible for its pathophysiology, since these are diarrheagenic species [110].

Intestinal microbiota and immunotherapy in CRC

CRC therapy constitutes a demanding issue. Surgery remains the keystone therapy, with radiotherapy and/or chemotherapy being implemented in cases of invasive tumors, as adjuvant treatment, or metastatic disease as palliative therapy. Lately, major advances have been made in the understanding of the interplay between tumor microenvironment and immune system, since it plays a critical and complex role in tumorigenesis, as well as in the treatment of cancer including CRC [115]. Such advances are promising for patients with inadequate response to chemotherapeutic agents [116]. As a result, various therapeutic approaches have been investigated such as vaccines (autologous, peptide, dendritic cell, viral and bacterial), toll-like receptor (TLR) agonists, cytokine therapy, autologous cell therapy (ACT) and checkpoint inhibition [117]. Immunotherapy has exhibited a clinical advantage in many types of cancer including CRC [118]. Although these approaches have a more tolerable side-effect profile compared to standard chemotherapy, their efficacy is modest [119]. Moreover, the detailed mechanism behind their efficacy is poorly described. It is well known that the long-term outcomes of anticancer agents cannot be considered only autonomous to the targeted cells, but they also include an essential immune component [120,121]. The microbiota seems to be involved here. It is reported that optimal cancer immunotherapy responses rely on an intact commensal microbiota that is able to regulate the tumor microenvironment by modulating myeloid-derived cell mechanisms [122]. Furthermore, the synthesis of microbiota has been reported to influence the response to cancer immunotherapy [122-124].

Major advances in CRC immunotherapy have mainly been focused on the utilization of checkpoint inhibitors, monoclonal Abs targeting co-inhibitory molecules like PD-1/PD-L1 axis and CTLA-4, which suppress the immune system by induction of T-cell apoptosis. PD-1 belongs to a CD28 receptor family, mainly inducing exhaustion in effector T cells, specifically over-expressed in malignancy [125]. PD-L1 is a surface ligand of tumor cells which binds to PD-1 receptors on T cells inducing T-cell apoptosis. CTLA-4 is a surface receptor of CD4+ and CD8+ T cells. It binds to B7 ligands on antigen presenting cells (APCs), thus inhibiting immune stimulation provided by B7 binding to a CD28 receptor on T cells [126]. These pathways are utilized by tumor cells for attaining down-regulation of immune responses [127]. Checkpoint inhibitors have made a

breakthrough in the treatment of specific cancers such as melanoma (blockade of CTLA-4 and/or the interplay between PD-L1 and PD-1) and non-small-cell lung cancer (blockade of PD-L1) [128]. In two recent studies, the efficacy of immunotherapies targeting these pathways was strongly related to particular members of the intestinal microbiota in mouse models [129,130].

Sivan et al. [129] analyzed and compared subcutaneous B16.SIY melanoma growth and response to PD-L1 inhibition, in genetically alike C57BL/6 mice nourished at different facilities; Jackson Laboratory (JAX) and Taconic Farms (TAC). These mice have been reported to demonstrate different intestinal microbiome and immune responses [131]. A major discrepancy in tumor development was observed between mice groups, with a more aggressive tumor growth in TAC mice than in JAX mice. This difference was immune-mediated, as displayed by diminished tumor-specific T cell responses and decreased CD8+ T cell infiltration in the tumors from TAC mice, as opposed to higher immune responses in JAX mice [129]. A hypothesis was made, that the difference in tumor growth could be intervened by commensal microbes. Interestingly, cohousing JAX and TAC mice before melanoma implantation or transferring fecal materials resulted in reverse of the TAC phenotype, ablating the difference in tumor development as well as immune response. This finding suggests that commensal bacteria play a crucial role in facilitating anti-tumor immunity [129]. Administration of PD-L1 blockade demonstrated more favorable outcomes in JAX than TAC mice, as estimated by inhibition of tumor development and anti-tumor CD8+ T cell response. Analyses of microbiota community in fecal samples revealed that anti-tumor T cell response was strongly associated with *Bifidobacterium* species [129]. Interestingly, oral administration of fecal suspension from JAX mice to TAC, containing *Bifidobacterium breve* and *Bifidobacterium longum*, was adequate to delay the tumor development in the latter group compared to untreated mice, and this response was mediated by increased accumulation of antigen-specific CD8+ T cells in the tumor [129]. Notably, the advantage observed in TAC mice after administering the bacterial suspension was equal to the advantage observed by PD-L1 blockade treatment, and combining fecal material transportation with PD-L1 blockade treatment further detained tumor growth to the point of abrogation, similar to JAX mice [129]. Researchers reported that purified dendritic cells from mice treated with *Bifidobacterium* were efficient in presenting a melanoma-derived peptide to T cells, thus enhancing their proliferation and INF- γ secretion. This suggests that

Bifidobacterium improves the antitumor response through a bridging-effect derived from dendritic cells [129].

Vetizou et al. [130] showed that treatment with monoclonal antibody ipilimumab, which inhibits CTLA-4 signaling [132], lost its therapeutic potential in suspending tumor development against MCA205 sarcoma, MC38 colon carcinoma, and Ret melanoma in germ-free mice or in mice being raised in pathogen-free conditions and then treated with various broad-spectrum antibiotics in order to achieve gut sterilization. Specific microbiota composition characterized by distinct bacterial species (*B.fragilis*, *B.thetaiotaomicron*, and *B.cepacia*), which were identified through microbial profiling and functional studies, were found to induce CTLA-4 antitumor immune response and therapeutic efficacy of CTLA-4 blockade, both in mice and in patients [130]. Microbiota composition influences IL-12-dependent Th1 immune responses [133], which facilitate tumor control in mice and patients. Synergizing with TLR2/TLR4 signaling cascades [134,135] they account for the immunomodulatory effects as a result of treatment with CTLA-4 blockade. The lack of treatment effect was explained due to defective antitumor Th1 response. This deficiency was overcome by gavage with *B.fragilis*, by adoptive transfer of *B.fragilis*-specific T cells, or by immunization with polysaccharides deriving from *B.fragilis*. This finding suggests a treatment-related interplay between microbial and tumor antigens identified by the same T cell receptor (TCR) [130]. The intestinal microbiota of patients with metastatic melanoma can be clinically divided into three groups: group A driven by the *Alloprevotella* or *Prevotella*, and groups B and C by distinctive *Bacteroides* spp. They observed that many cluster B patients switch to cluster C following ipilimumab administration. Furthermore, fecal bacterial transplantation from patients to mice favored the increased abundance of *B.fragilis*, which was adversely related to the anticancer potential of CTLA-4 blockade. Thus, CTLA-4 blockade can amend the plethora of immunogenic *Bacteroides* spp. affecting the efficacy of therapy by stimulation of T cell response [130].

Therefore, the basic mechanisms behind the role in the efficacy of checkpoint inhibitors share mutual characteristics but might also differ. Although both studies by Sivan et al. [129] and Vetizou et al. [130] analyzed the intratumoral maturation of dendritic cells depending on intestinal microbiota, the first study (Sivan et al. [129]) proposed a role for cross-reactive T cell epitopes present on microbiota and tumor, the latter (Vetizou et al. [130]) hypothesized an impact on innate immunity prompting

a resetting of antigen-presenting cell capacities relying on intestinal microbiota. These effects absolutely have potential in CRC, where focusing on organisms specifically to the tumor could form immune responses against the tumor and immunotherapy reactions locally. Similarly to the microbial promoters of CRC, such a microbe would postulate an intratumor localize-and-survive mechanism sufficiently enough to influence the immunological environment. Then it could be pharmacologically or genetically neutralized, ideally a protumorigenic bacterium like *F.nucleatum* [136]. This further confuses the line between the positive or negative role of microbiota in the formation of the immune response in the tumor microenvironment.

Three studies also support the concept that the intestinal microbiota synthesis regulates the immune response to immunotherapy with PD-1 or PD-L1 blockade in patients with epithelial tumors [137-139]. Antibiotic-induced dysbiosis in both patients and mice was correlated with the unsuccessful treatment with PD-1 or PD-L1 blockade [137]. Identification of microbial species that positively relate to the clinical response was achieved through fecal microbial analyses in independent patient groups [137-139]. Furthermore, fecal transfer from responder and non-responder patients to mice with adequate immunity, which then were implanted with tumor cells, emulated the responder phenotypes, thus transforming mice into "embodiments" of the patient with respect to the therapeutic efficacy [137-139]. The fecal transfer from responder patients in combination with PD-1, enhanced parameters indicative of a gainful anticancer immune response in these mice [137-139]. Specifically, *Akkermansia muciniphila* was over-observed among responder patients [137,139], and its genus the *Verrucomicrobiaceae* family, is also enhanced among responder patients [138].

Despite the achievement of immunotherapies in treating different tumors, to date, PD-1 blockade monotherapy has had minimal efficacy in CRC therapy [140], mainly in a trial of 41 patients that concentrated on one subset of CRC eminent for a mismatch repair deficit [141]. Ipilimumab was approved by the FDA in 2011 for use in metastatic melanoma when two clinical trials demonstrated that it was related to improved survival [142]. However, these checkpoint inhibitors were unsuccessful in showing similar favorable outcomes in CRC patients [143]. Due to the modest efficacy of such immunotherapies as monotherapy in CRC, recent trials focused on combination therapy. In a recent study, combined nivolumab (PD-1 blockade) and ipilimumab in 53 patients with advanced melanoma induced an objective response in 53% of the

patients achieving 80% or more reduction in tumor growth [144]. Numerous current trials research the use of combination immunotherapy in patients with advanced CRC [117].

Novel targets for treatment in CRC may be revealed through investigation of the relation between dysbiosis and tumorigenesis [145]. For instance, polysaccharide A, produced by commensal microbiota, enhances local Tregs and therefore, IL-10 secretion, an effect induced by TLR2 [146]. Hence, TLR2 might represent a good target for combination immunotherapy. Although most immunotherapeutic strategies in cancer target the adaptive immunity, recent studies have focused on the innate immune system with the use of TLR agonists [147]. TLR-9 has demonstrated a contingent protective role against tumorigenesis in colorectal mucosa [148]. Various sequences of unmethylated deoxycytosine-deoxyguanosine (CpG-ODN) derived from microbial DNA induce TLR9, and clinical trials have shown a non-toxic profile, but with modest efficacy. In many studies, these agents promoted immune responses in cancer patients, but did not induce tumor regression or prolonged survival [149,150]. Iida et al. [122] showed the regulatory interplay between intestinal microbiota and CpG-ODN immunotherapy. Specifically, the researchers found out that, in tumor-bearing mice with EL4 lymphoma, MC38 colon carcinoma and B16 melanoma, treatment with combination immunotherapy consisting of CpG-ODN and anti-IL-10R mediated tumor growth and prolonged mice survival *in vivo*, via induction of TNF-dependent cytotoxic CD8+ T cell response in the tumor microenvironment [122]. However, this effect was deteriorated in mice subjected to administration of broad-spectrum antibiotics or in germ-free mice following diminishment of commensal bacteria. As a result, immunotherapy failed to induce tumor infiltration by TNF- α -producing CD11b+ MHC class II+ myeloid cells in these mice, postulated for potent antitumor response [122]. Identification of specific microbial species, such as *Alistipes shahii* and *Ruminococcus*, was positively related to TNF production from tumor-associated myeloid cells, whereas other species, such as *Lactobacillus fermentum*, diminished this response. These responses were demonstrated by oral administration or gavage of these bacteria in germ-free mice [122].

These results support the idea that intestinal microbiota unequivocally impacts the anticancer efficacy of agents focusing on components of the immune system. Moreover, heterogeneity between patients in the response to anti-tumor immunotherapy is largely related to the intestinal microbiota synthesis, suggesting that manipulation of intestinal microbiota might contribute to the improvement of immunotherapy efficacy. Since these investigations were, for the most part, transacted with xenograft models, the clinical significance of the findings remains to be verified in human clinical trials for future application. However, the intestinal microbiota obviously has a major effect on tumor therapeutics in terms of efficacy-toxicity and this interaction paves the way to a novel field of studies. It is therefore clear that understanding the immune mechanisms of microbial-dependent CRC will open new avenues for cancer prevention and improving cancer therapeutic plans, considering cancer genomics and epigenetics as well as the microbial components that may be forming the tumor microenvironment.

Conclusions

To sum up, colonic microbiota plays a major role in cancer pathogenesis, but also cancer alters colonic microbiota, meaning that there is a bidirectional relation between them. All aforementioned studies indicate various mechanisms through which intestinal microbiota maintains colonic homeostasis and participates in colorectal pathogenesis. They also demonstrate a significant relation between CRC therapy and microbiota alterations, and reveal the double role of microbiota by either enhancing or reducing the efficacy of CRC treatment. However, the available evidence is not sufficient and constantly new hypotheses are generated. Thus, it is necessary to undertake additional studies, in humans and animal models, to decode and understand the mechanisms underlying the interaction between microbiota and CRC, in order to allow us to manipulate microbiota in favor of prevention as well as treatment of CRC.

Conflict of interests

The authors declare no conflict of interests.

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