The microbiome, its molecular mechanisms and its potential as a therapeutic strategy against colorectal carcinogenesis (Review)

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Abstract. Microbiota is receiving significant attention in the research field, given that it is essential in homeostasis and an indirect modulator of diseases, such as cancer. Humans harbor a multitude of microorganisms that affect both human health and disease. Colorectal cancer is a genetic disease that is composed of distinct subtypes. In all cases, the intestinal microbiota has recently emerged as a crucial factor that promotes tumor growth at all stages through various mechanisms, such as the modulation of inflammation, the stimulation of DNA damage and toxic metabolite synthesis. In this review, we assess the contribution of the gut microbiome to homeostasis, its role as a potentiator or blocker of tumor progression and the underlying molecular mechanisms; we harness human data from both meta-omics analyses and studies using animal models. Furthermore, we evaluate the ways through which microbes can be manipulated for diagnostic and prognostic purposes, and how they respond to chemotherapy or immunotherapy. Mounting evidence suggests that the microbiota may be used for the development of novel therapeutic strategies against colon cancer.

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1. Role of microbiota in homeostasis

The analysis of microbiota composition has become routine in our time, mostly due to the explosion and availability of new technologies [metagenomic sequencing technologies that incorporate next-generation DNA sequencing methods with the computational approach of targeted (16S rRNA hypervariable regions) or whole-genome shotgun sequence reads], that allow for the identification and classification of a great variety of microbial species (1,2). The genome of the microbiota is 100-fold more extensive than the human genome (3). The distribution of microbial cells surpasses the number of all human cells, including somatic and germ cells throughout the human body (4-8). Microbiota is a term that has been established as the sum of bacteria, fungi, parasites and viruses occupying the host (7). The microbiota genome is known as the ‘microbiome’, which acts as 'an indirect genome' with significant functions that are several fold more numerous than those of the human genome (6,7,9,10). The microbiota and host form a complex ‘super-organism’ in which their symbiotic association confers benefits to the host regarding key aspects of life. However, defects in the host regulatory circuits that control bacterial sensing and homeostasis, or alterations in the microbiome, through environmental changes (infection, diet or lifestyle), may disrupt the symbiotic relationship and promote disease. Increasing evidence indicates a key role for bacterial microbiota in carcinogenesis (6,7,9-11). The International Agency for Research on Cancer (IARC) has classified only 10 microorganisms from the total number of $3.7x10^{30}$ microorganisms as pathogenic (12).

The microbiota colonizes any surface in the human body, with significant functional roles in homeostasis. Each surface in different organs (such as the lungs, skin, vagina and oral cavity) is exposed to the external environment and has its distinct microbiome. The highest percentage of microbial mass is located in the gastrointestinal tract (99%), which is the reason why the intestinal microbiota is the most extensively investigated type of microbiota thus far (13,14). In particular, 100 trillion micro-organisms of distinct structures (bacteria,

parasites, fungi and viruses) are colonized in the human intestine, thus termed the intestinal microbiome (15,16). The absolute number of micro-organisms fluctuates between the mouth and rectum (17).

In order to establish the composition of the microbiota, it is only reasonable to consider a human being from the early stages of life, i.e. birth. The microbial composition is acquired during the first three years of childhood; following the initial stages, microbiota development continues with environmental exposure (17-19,29). As a general note, the gut microbiota displays a consistent composition and differs among individuals. During late stages of life, in the elderly, the composition of the microbiota changes again, but retains a stable function (21-24).

With regards to the microbiota of the human gastrointestinal tract, 50 distinct phyla and five hundred bacterial species have been reported as dominant (25). High throughput sequencing techniques have provided deep insight into the composition of the microbiota (26). A high proportion of gut microbiota is divided into three categories: The Firmicutes (30-50%), the Bacteroidetes (20-40%) and Actinobacteria (1-10%) (27). The majority of the microbiota is strictly anaerobic, such as *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Fusobacterium*, *Peptostreptococcus* and *Atopobium*, whereas the minority is facultative anaerobic and comprises Lactobacilli, Enterococci, Streptococci and Enterobacteriaceae (28). The gut microbiota varies in mass and composition along the gut, that is, from the luminal to the mucosal regions (17,25). For example, microbial composition is denser in the large intestine as compared to the small intestine, potentially explaining the higher susceptibility of the large intestine to cancer (30,31). The close association between the gut microbiota and colon cancer was initially revealed in 1975, when researchers observed that the potential of developing colon cancer in a chemically-dependent manner was reduced in germ-free rats (32). Furthermore, the colon is dominated by Bacteroidetes and Actinobacteria (90%), whereas the small intestine is colonized by Bacteroidetes and Actinobacteria (50%). The composition of the microbiota in the small intestine also includes other bacterial phyla, such as Firmicutes species (40%) (33). Despite the fluctuations of microbiota in composition and mass along the gut, the main population of micro-organisms is most certainly composed of bacteria, residing within the gastrointestinal lumen (34), either competing or cooperating with other micro-organisms (35).

The symbiotic association of micro-organisms with the human gut (host) is accomplished after several years of co-evolution and co-adaptation, contributing to a balanced homeostasis in the gut (4,36-38). The microbiota plays a key role in homeostasis, as confirmed by its participation in a wide range of processes, such as wound healing, the maintenance of barrier function, and the modulation of cellular growth and immune system regulation (39). Apart from participating in these processes, the microbiota also expands to the digestion of complex carbohydrates and the establishment of ecological niches that might otherwise be occupied by non-innocuous microorganisms (11). The gut microbiota is characterized by a high self-restitution capacity following perturbation (40).

Other beneficial functions of the microbiota include a significant contribution to the maturation of the immune system and to the protection against infectious agents (41-45). It is important to highlight that the murine gut microbiota displays high homology to the human gut microbiota, paving the way for translational research based on experimental mouse models (46,47). For example, the microbiota has been reported to actively participate in the developmental stages of a healthy immune system, as illustrated by severe immune defects in mice bred under germ-free conditions (18,48). As a general note, the immune system of the gut is responsible for eradicating pathogenic microbes, whereas at the same time it has developed immuno-tolerance mechanisms against classical gut microbes. The mucosal immune system is under the control of the adaptive immune system and it functions in a cell-autonomous manner (39). The disruption of the microbiota has been shown to confer a significant impact on the immune system in many aspects. Importantly, the microbiota has been demonstrated to affect immune structures, in addition to immune cell populations. This was initially illustrated in experimental mice bred under germ-free conditions, which displayed hallmark changes in immunoglobulin A (IgA) secretion and functionality defects in Peyer's patches and draining mesenteric lymph nodes (mLNs) (49,50). Notably, in a previous study, gut-associated lymphoid tissues (GALTs) were efficiently matured with the concomitant activation of T cells and IgA-secreting plasma cells in the presence of the microbiota, which mediated the necessary signals for both epithelial and dendritic cell (DC) activation (51). Furthermore, the gut microbiota appears to be essential for the function of basic immune populations, such as the secretion of interleukin (IL)-22 by type 3 innate lymphoid cells (ILC3 cells). ILC3 cells have been shown to be essential for the growth of T cells in a microbiota status-dependent manner, independently of IL-22, IL-23 or IL-17 synthesis (52). Subsequent scientific evidence has suggested that some bacterial strains are particularly associated with the functions of the immune system. For example, certain microbiota components seem to initiate inflammation and to regulate immune cells within the lamina propria of the intestine. The absence of segmented filamentous bacteria (SFB) causes low enrichments of IgA titers, the reduction of T helper 1 (Th1) and T helper 17 (Th17) cells, and the alleviation of immune responses to classical intestinal pathogens (*Citrobacter rodentium* and *Salmonella* spp.) (48,53,54). Yang *et al* highlighted the significance of SFB in the function of T helper 17 (Th17) cells, through the expression of a T cell receptor (TCR) directed to a specific SFB antigen (55). Despite the beneficial effects mediated by SFB, it was noted that SFB also increases susceptibility to autoimmune disorders (56). Specifically, it has been demonstrated that the induction of SFB to germ-free mice renders them susceptible to the development of collagen-induced arthritis (57). In addition, the expression of the innate-like cytokine IL-17C seems to be under the control of microbiota during intestinal tumorigenesis, as the latter was shown to mediate Toll-like receptor (TLR)/MyD88 signaling, which in turn lead a to IL-17C upregulation during colon cancer progression and ultimately to the uncontrolled proliferation of intestinal epithelial cells (IECs) (58).

On the other hand, the gut microbiota also has the capacity to induce an anti-inflammatory environment, by producing certain metabolic by-products that maintain barrier integrity. For example, the differentiation of IL-10-secreting Tregs has been shown to be absolutely dependent on the signal transduction pathway triggered by *Bacteroides fragilis* (51).

Specifically, polysaccharide A secreted by *Bacteroides fragilis* induces Treg cell expansion via the TLR2 signaling pathway (51). Similarly, it has been reported that *Helicobacter hepaticus* (*Hh*) stimulates T cells to differentiate into T regulatory cells (59). The induction of Tregs was also observed following the incubation of clostridial strains, conferring significant benefits to experimentally-induced colitis (35,60). For example, *Faecalibacterium prausnitzii* is a clostridial organism that has been shown to protect patients from the onset of inflammatory bowel disease (IBD) (61). The gut microbiota therefore appears to be indispensable for the immune system as a whole (62).

Apart from the gut, the skin also harbors a significant number of microbial niches that sustain the recruitment of Th1/Th17 cells and provide protection against pathogens, such as *Leishmania major* (63). Indicatively, the functionality and persistent response of $CD8^+$ T cells has been attributed to the skin microbe, *Staphylococcus epidermidis* (64). The oral cavity also contains microbial communities with key roles in modulating persistent immune responses to various infections (65,66). For example, it has been demonstrated that in the absence of microbiota in the oral cavity, immune cells cannot combat mucosal influenza virus (67). Even though the microbiota exerts its effect on the immune system locally on each surface barrier, the gut microbiome appears to be the most efficient in controlling systemic immune homeostasis (67-69). The insuperable effect of the gut microbiome on the immune system has been attributed to its great variability, the high number of associated micro-organisms and the relatively large surface area that is available to expand on (13). In line with this, it has been demonstrated that the incubation of certain bacterial strains in the skin of germ-free mice does not seem to display systemic effects and to reconstitute Th cell populations in the intestine (63). The significance of the presence of the microbiota in various anatomical sites was established when experimental animal models lacking microbial communities exhibited signs of autoimmune disorders (multiple sclerosis or arthritis) (57,70,71).

However, as described above, the microbiota does not only include populations of bacteria, but also consists of other micro-organisms, such as archaea, fungi, viruses, etc. For example, fungi such as *Candida* appear to be overexpressed in animal models following treatment with antibiotics (72). Nonetheless, the role of microbiota subtypes other than bacteria is still in its infancy and additional studies are required in order to assess their impact on the immune system (72,73).

2. Healthy gut conditions

The protection of the gut against exogenous pathogens is provided by the presence of the epithelial barrier. The integrity of the epithelial barrier is mediated through tight junctions between epithelial cells, the mucous layer, soluble antibacterial factors and distinct cells of innate and adaptive immunity (74-76). Under normal gut conditions, one hundred trillion organisms (particularly microbiota) thrive in the intestine, creating a protected, warm and nutrient-rich microenvironment, which in turn helps the microbiota to be in equilibrium with the host organism (eubiosis). When eubiosis is disrupted, the composition of the microbiota is altered and as a consequence, it is mostly represented by facultative anaerobic bacteria instead of aerobic bacteria (present in healthy conditions) (11). The interaction of the microbiota with epithelial cells is indirect, through the mucous layer, which separates the compartment of commensal bacteria from that of the host (49). If one considers that the gut microbiota is in close proximity to the IECs that line the mucous layer, it is only to be expected that dysbiosis can rupture the mucous layer, thereby leading to inflammatory conditions or even cancer (7,11,62). The mucous layer can be ruptured by microbial translocation, due to specific molecular alterations or abnormal regulatory signals (77).

3. Role of microbiota in colorectal carcinogenesis

One of the hallmarks of cancer is the uncontrolled growth of malignant cells, which ultimately constitutes one of the main causes of mortality. Colorectal cancer is the most predominant type of cancer in the USA and the third most common cause of mortality, therefore presenting a considerable tumor burden (78). Colorectal cancer, as the name implies, is characterized by carcinogenic alterations that occur in the colon and rectal epithelial cells. Different forms of colon cancer arise due to variations in genetic profiles, histological patterns and sensitivity to potential therapeutic drugs (79,80). Indeed, colorectal cancer can be divided into three subtypes, the first of which (35%) is ultimately linked to genetic alterations, the second (65%) is associated with exogenous factors and the third, which accounts for 1% of all colorectal cancer subtypes, is accompanied by chronic IBD (81). Consequently, the majority of patients with colorectal cancer (95%) are not directly genetically vulnerable to cancer, thereby supporting the notion that the gut microbiota is actively implicated in cancer development (82).

The uncontrolled growth of intestinal malignant cells begins with the conversion of normal epithelial cells into hyperplastic cells. In this manner, epithelial cells lose their morphological characteristics and become dysplastic. This is followed by the invasion of hyperproliferative epithelial cells into the gut lumen, where they form adenomas, and the subsequent protrusion of epithelial cells into the gut, which ultimately leads to cancer. From a genetic aspect, IECs are transformed into hyperplastic intestinal cells after the following sequence of genetic events: First the loss of tumor suppressor genes, such as adenomatous polyposis coli (Apc) and subsequently mutations in genes that encode the machinery for DNA repair, such as hMSH2.

Even though significant efforts have been made in order to elucidate the driver mechanisms that cause colorectal carcinogenesis, the landscape remains obscure. The effect of the gut microbiota on carcinogenesis has become a burgeoning issue of research in recent times, considering that the gut microbiota is vital in sustaining the homeostasis and regulation of the immune system. Since colorectal carcinogenesis is a multifactorial cancer type with a genetic basis, it has been proposed that inflammatory processes or perturbations of the intestinal microbiota can lead to the cancer development through genetic alterations. The impact of the gut microbiota appears to be more prominent in colon cancer, independent of the type and cause, where it seems to systemically influence

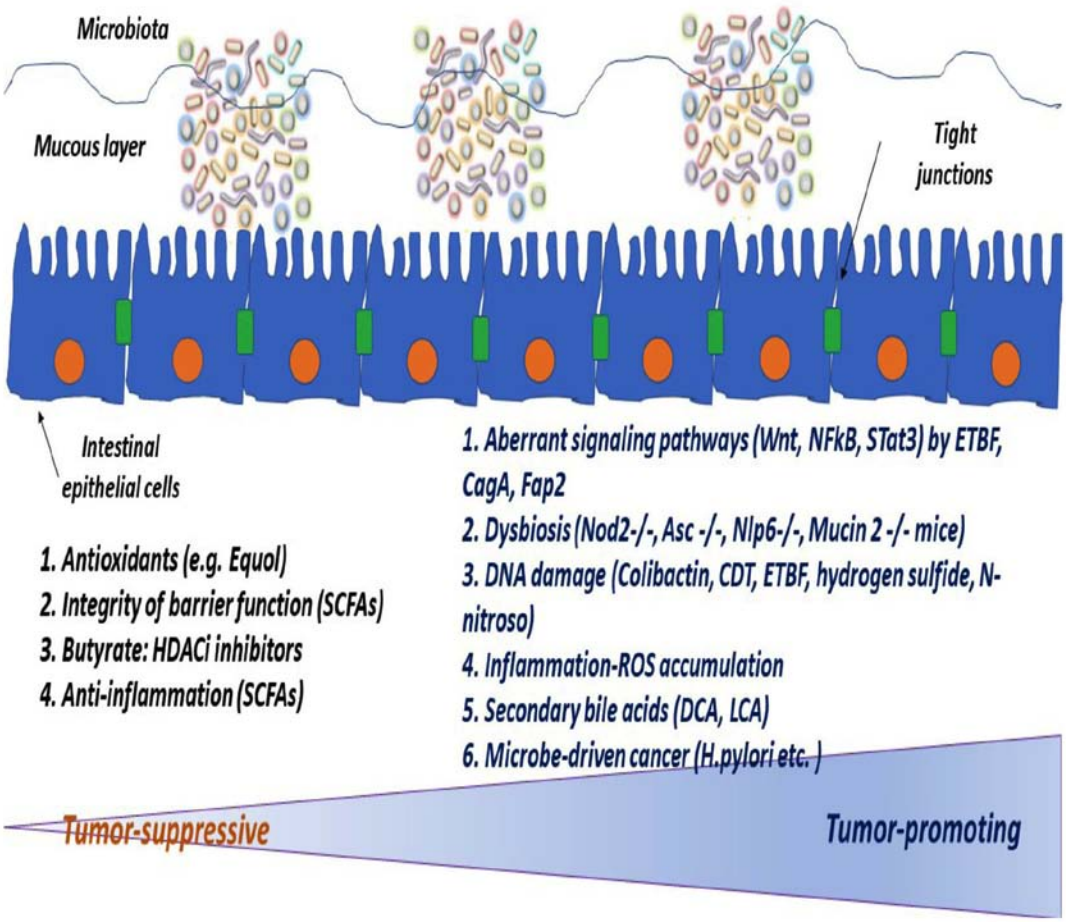

Figure 1. Possible mechanisms through which the gut microbiota induces or blocks colorectal carcinogenesis. The microbiota interacts with the colonic mucosa, creating a pro-inflammatory or anti-inflammatory state with subsequent metabolic profiles, thus leading to cancer progression or remission.

cancer progression either as individual species or as a microbial community (12).

If one considers that microbes create a dynamic symbiotic interplay with the host, it is logical to assume that the gut microbiota can function either as a blocker or as a potentiator of colon cancer (Fig. 1). The first efforts made in the treatment of cancer through the natural effects of the gut microbiota were made in the late nineteenth century, when it was realized that bacterial infections or injections of heat-killed bacteria, such as Coley's toxin prevented the development of sarcoma in patients (83,84). Nowadays, Coley's toxin, which includes a mixture of killed bacteria (e.g., *Streptococcus pyogenes* and *Serratia marcescens*) is regarded a precursor of current immunotherapeutic agents. For example, the injection of *Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) is currently used as the classical therapeutic approach in non-muscle invasive bladder cancer (85). Consistent with the above, experiments have demonstrated that animals bred under germ-free conditions exhibit a stronger likelihood for developing colon cancer than those bred under microbiota-rich environments (86).

Additional *in vivo* experiments have indicated that the microbiota has the capacity to induce cancer in a wider range of organs than previously considered, including the skin, colon, liver, breast and lungs (86-93). In a similar manner, it was shown that the elimination of intestinal microbiota (through antibiotics) ameliorates colorectal cancer or hepatic carcinogenesis (88,94-97).

Consequently, the majority of studies have concentrated on investigating the role of the gut microbiota in colitis-associated colorectal cancers, using either germ-free conditions or antibiotics (90,98). Using genetically engineered experimental models specifically predisposed to cancer, such as TCR-deficient ($TCRb^{-/-}$) and double p53 KO (X $p53^{-/-}$) mice, it was previously demonstrated that the gut microbiota can indeed induce colon cancer. The animals did not develop adenocarcinomas under germ-free conditions (99); however, $TGFb1^{-/-}$ mice gut-colonized with *Helicobacter hepaticus* demonstrated a greater potential for colon cancer development (100). Similarly, the gut colonization of $Rag2^{-/-}$ mice with *Helicobacter hepaticus*, and that of $Tbet^{-/-}$ $Rag2^{-/-}$ mice with microbiota, has been shown to induce colon carcinogenesis (101,102). The resulting phenotype of these experimental animal models ($TGFb1^{-/-}$ and $Tbet^{-/-}$ $Rag2^{-/-}$) under germ-free conditions was very similar to the phenotype following antibiotic treatment (98,103). Additional studies reported that $IL10^{-/-}$ mice developed tumors following treatment with the chemical carcinogen azoxymethane (AOM) and incubation with *Enterococcus faecalis*, as compared to mice bred under germ-free conditions, which remained healthy (90,104).

However, since AOM, in combination with dextran sulfate sodium (DSS), has been shown to induce colon cancer

Table I. Microbes modulate immune responses in murine models of colon cancer.

Bacterial strains	Secreted factor	Molecular mechanism	Murine experimental model predisposed to colon cancer	Refs.
Bacteroides fragilis (*B. fragilis*)	Enterotoxin (ETBF)	Changing host immune system	$Apc^{min/+}$	(116,117,132)
Escherichia coli (*E. coli*)	Unknown	Immune stimulation	$IL10^{-/-}$	(118)
Enterococcus faecalis (*E. faecalis*)	Superoxide	Polarization to M1 macrophages	$IL10^{-/-}$	(118,119)
Fusobacterium nucleatum (*F. nucleatum*)	Unknown	MDSCs, TAMs, TANs, $CD103^{+}$ DC infiltration	$Apc^{Min/+}$	(120)
Fusobacterium nucleatum (*F. nucleatum*)	Fap2	Suppression of immune responses	Allograft of wild-type mice with CT26 cancer cells	(121)
Fusobacterium nucleatum (*F. nucleatum*)	Fap2	Fap2 engagement to TIGIT, avoiding NK cell toxicity	$Apc^{Min/+}$	(127)

MDSCs, myeloid-derived suppressor cells; TAMs, tumor-associated macrophages; TANs, tumor-associated neutrophils; DC, dendritic cells; NK, natural killer.

formation, the role of the gut microbiota in colorectal carcinogenesis in experimental mouse models may be obscured by the effects of chemically induced carcinogenesis (92,105-110). In other words, it is difficult to assess which animal models of colorectal cancer are the most suitable for elucidating the role of the microbiome in cancer development. Despite the seemingly unsurpassed selective pressures displayed by malignant cells, the concept of microbe-driven cancer formation does not cease to exist. A significant number of research studies have explored whether micro-organisms are directly involved in tumor progression or whether they participate indirectly through their metabolites. Recent metagenomic analyses have revealed essential differences between healthy and cancer states and assessed the tumor-promoting effects of microbiota in certain types of cancer. The mechanisms through which specific microbes target certain cancers, contributing to the acceleration of tumor progression remain elusive. For example, human papillomavirus (HPV), hepatitis B virus, hepatitis C virus, human herpesvirus 8 and human T-lymphotropic virus 1 have been shown to trigger carcinogenesis via well-defined processes (5,11). Specifically, HPV can cause anogenital or oropharyngeal carcinomas, hepatic B or C virus can induce hepatocellular carcinoma and human immunodeficiency virus (HIV) or Epstein-Barr virus (EBV) or human T-cell lymphotropic virus type 1 (HTLV-1) can lead to lymphoma (7). As regards individual bacterial species, it has been revealed that specific bacterial strains drive carcinogenesis (111). As highlighted by epidemiological data, the most highly-associated microbe to cancer development, especially gastric cancer, is *Helicobacter pylori* (*H. pylori*) (112). *Helicobacter pylori* releases toxins (CagA or VacA) that cause cytoskeletal rearrangements which cannot be surpassed by the host repair mechanisms (113,114). Notably, the close association of *Helicobacter pylori* with gastric cancer has been regarded a landmark discovery and was awarded a Nobel Prize (115). Furthermore, *Streptococcus bovis*, *Helicobacter pylori*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Clostridium septicum*, *Fusobacterium* spp. and *Escherichia coli* have also been identified as bacterial species that can lead to the development of intestinal neoplasms. Other bacterial strains, including *Bacteroides fragilis*, *Escherichia coli*, *Enterococcus faecalis* and *Fusobacterium nucleatum*, have been identified in experimental animal models chemically predisposed to colon cancer as microbes with an ability to modulate normal immune responses (Table I) (116-121).

An important breakthrough was achieved in the case of *Fusobacterium nucleatum*. In general, *Fusobacterium nucleatum* is localized in the oral cavity. As a resident member of the oral microbiota, *Fusobacterium nucleatum* has been extensively studied and has been found highly associated with periodontitis and appendicitis (122). *Fusobacterium nucleatum* is an anaerobic Gram-negative rod bacterium and one of the leading micro-organisms in intrauterine infections causing premature death. Compared to the gut microbiota of healthy individuals, Fusobacteria are often found in patients with colon adenocarcinoma and IBD, thus suggesting an association between Fusobacteria and the colon inflammatory environment (120,123-126). In the human body, *Fusobacterium nucleatum* can be introduced through the oral cavity and transferred into the gastrointestinal tract, thereby affecting human colon adenocarcinoma. From an immune point of view, *Fusobacterium nucleatum* triggers inflammatory signaling pathways and functions as a shield to tumor cells against an immune attack (12,127). It has been proposed that certain bacterial strains possess many adhesins, mediating their binding to TLR4 and RIG-I, as well as their direct interaction with natural killer (NK) cells via binding to the NKp46 receptor (128-130). In this context, the Fap2 protein of *Fusobacterium nucleatum* has been shown to bind to the human (not mouse) TIGIT [T cell immunoreceptor with Ig and ITIM (immunoreceptor tyrosine-based inhibitory motif)] receptor present on NK cells and T cells in a hemagglutination-dependent manner (Table I) (127). The hemagglutination potency of Fap2 has been tightly linked to TIGIT suppression.

Table II. Microbes modulate DNA damage responses in murine models of colon cancer.

Bacterial strains	Secreted factor	Molecular mechanism	Murine experimental model predisposed to colon cancer	Refs.
Bacteroides fragilis (*B. fragilis*)	Enterotoxin	DNA damage through spermine oxidase action	Apc^{Min}	(133)
Enterococcus faecalis (*E. faecalis*)		Epithelial DNA damage through ROS induction		(119,137)
Escherichia coli (*E. coli*)	Colibactin	DNA damage and senescence activation	IL-$10^{-/-}$, AOM	(134,135)
Helicobacter hepaticus (*H. hepaticus*)	CDT	DNA damage	$Rag2^{-/-}$	(136)

AOM, azoxymethane; CDT, cytolethal distending toxin.

In this manner, *Fusobacterium nucleatum* manages to abolish NK cell-mediated destruction of human cancer cells. Notably, *Fusobacterium nucleatum* has been shown to bind to many tumor cells through interactions of its Fap2 protein with the Gal-GalNac protein of cancer cells. Consistent with the above, the exposure of experimental $Apc^{Min/+}$ animal models to *Fusobacterium nucleatum* has been shown to cause enriched myeloid cell infiltration (predominantly DCs, macrophages) and the activation of the nuclear factor (NF)-κB signal transduction pathway. In addition, *Fusobacterium nucleatum* has been shown to augment the numbers of two types of myeloid-derived suppressor cells (MDSCs), thus inhibiting activated T cell responses and exacerbating small intestinal adenocarcinoma development (Table I) (120). Substantial experimental data have highlighted that *Fusobacterium nucleatum*-fed mice are enriched for tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), $CD103^{+}$ regulatory DCs, thus exhibiting a promotion of neoplastic progression (Table I) (120).

Fusobacterium nucleatum seems to recapitulate tumor progression through its effects on the tumor microenvironment. For example, *Fusobacterium nucleatum* has been shown to synthesize hydrogen sulfide following red meat consumption, thus promoting DNA damage responses and genomic instability in colon epithelial cells, which can in turn lead to tumor development (131,132). The onset of colorectal carcinoma and the extent of tumor progression appear to be more prominent in individuals with mutations or perturbations in the DNA-damage response (e.g., ATR and ATM). In support of this notion, both *in vitro* and *in vivo* xenograft experiments have highlighted the potential of *Fusobacterium nucleatum* to trigger the Wnt/β-catenin pathway, via FadA binding, which is highly associated with the proliferation of neoplastic cells (126).

Furthermore, it has been illustrated that enterotoxigenic *Bacteroides fragilis* (ETBF) can potentiate tumor formation by activating the signal transducer and activator of transcription 3 (STAT3) signaling transduction pathway and recruiting T helper 17 cells (Th17) in $Apc^{Min/+}$ mice (Table I) (132). The oncogenicity of *Bacteroides fragilis* became evident from its capacity to trigger the Wnt/β-catenin pathway, thus aiding in distant site colonization by tumor cells. Overall, *Bacteroides*, *Escherichia coli* and *Enterococcus faecalis* are the bacterial strains found to be DNA damage inducers in animal models (Table II) (119,133-136). *Enterococcus faecalis* has been shown to stimulate the DNA damage response in epithelial cells by secreting high levels of reactive oxygen species (ROS) (119,137), *Bacteroides fragilis* has been shown to trigger the Wnt signaling pathway and *Escherichia coli* is able to initiate double-stranded DNA breaks, thus resulting in increased genomic instability (133-135). *Escherichia coli*, in particular, has been found to contain polyketide synthase pathogenicity islands (pks), which contain the gene responsible for the toxin (colibactin) that triggers DNA damage response in IECs (Table II) (134,138,139). The significant oncogenicity of *Escherichia coli* was highlighted when $IL10^{-/-}$ mice developed intestinal tumorigenesis following treatment with AOM and *Escherichia coli* strains, without any indication of inflammatory sites (134). Therefore, these microbes (*Enterococcus faecalis*, *Bacteroides fragilis*, *Escherichia coli*) are considered to be key mediators of the DNA damage response and tumor progression, without the need for a pro-inflammatory environment (Table II). Last but not least, other bacterial strains have been shown to use other, multifaceted mechanisms in order to induce carcinogenesis in animal models (Table III) (140-143).

4. Role of dysbiotic microbiota in inflammation and colorectal cancer

Even though recent data have suggested that individual micro-organisms specifically influence the formation of cancer, carcinogenesis may also be the result of altered microbiota composition (dysbiosis) (144). The defects in the symbiotic interplay between the host and intestinal microbiota can result in alterations in the composition of the microbiota or in defects in the regulatory signals that orchestrate the normal association of microbiota with the host. In this manner, the balance in the commensal community changes (termed dysbiosis) and colon cancer can become the following event (6,8). Possible causes of dysbiosis can be either pathogenic microorganisms or environmental cues, such as antibiotics, xenobiotics or

Table III. Microbes use various mechanisms in murine models of colon cancer.

Bacterial strains	Secreted factor	Molecular mechanism	Murine experimental model predisposed to colon cancer	Refs.
Bilophila wadsworthia (*B. wadsworthia*)	Sulfide	Uncontrolled growth due to augmented bile acid production	IL-10$^{-/-}$	(140)
Helicobacter spp.	Unknown	ROS generation	Gpx1$^{-/-}$, Gpx2$^{-/-}$	(142)
Helicobacter spp.	Unknown	ROS generation	Smad3$^{-/-}$	(141)
Streptococcus gallolyticus (*S. gallolyticus*)	CDT	Induction of angiogenesis	AOM	(143)

ROS, reactive oxygen species; AOM, azoxymethane; CDT, cytolethal distending toxin.

obesity (11). Other causes may be genetic defects in epithelial, myeloid, or lymphoid cells of the gut, which can stimulate dysbiosis and consequently lead to inflammatory states, such as Crohn's disease, which may in turn confer some predisposition to carcinogenesis (35).

Dysbiotic bacteria appear to be indispensable to the creation of an inflammatory environment in the gut. Nevertheless, additional genetic changes are required for the initiation of colorectal carcinogenesis (11,145,146).

A significant number of studies have investigated the precise mechanisms through which the microbiota can be implicated in tumor development. In general, experimental mouse models chemically predisposed to colon cancer exhibit a lower incidence of tumor formation when treated with antibiotics or when bred under germ-free conditions, as compared to animals bred under conventional conditions (86,92,93,95-97,147,148). It remains to be clarified whether inflammation precedes or follows dysbiosis, before ultimately leading to cancer. On one hand, it has been suggested that a dysbiotic microbial community can lead to carcinogenesis by inducing chronic inflammation (149). For example, IL-18-, IL-18R- and MyD88-deficient mice are unable to mount adequate immune responses due to intestinal dysbiosis that occurs through the expansion of bacterial phyla Bacteroidetes (Prevotellaceae) and TM7, which ultimately leads to colon cancer (108,150,151). Other immune-deficient mice [*Nod2*$^{-/-}$, *Asc*$^{-/-}$ (also known as *Pycard*$^{-/-}$) and *Nlrp6*$^{-/-}$] also display dysbiotic microbiota and exhibit carcinogenesis (152,153). The functional significance of dysbiotic microbiota has been highlighted by the fact that the transfer of dysbiotic intestinal microbiota in healthy mice renders them susceptible to colon cancer (151). Similarly, genetically-edited mice (Tlr5 or IL1- or Tbx1 or Rag2 immunologically ablated) display prominent signs of colon cancer due to dysbiotic microbiota, as compared to immunologically wild-type mice (111). Last but not least, mice deficient for mucin (total Mucin 2 KO) present a defective intestinal barrier and for this they have been classified as models of intestinal neoplasia (154).

On the other hand, it has been proposed that inflammation causes barrier deterioration (dysbiosis), thus facilitating bacterial translocation, which in turn facilitates the creation of amplification feedback loops between intestinal barrier loss and carcinogenesis (145). For example, if mice display defects in pattern recognition receptors which specifically bind to microbial-associated molecular patterns (MAMPs), then these mice are characterized by bacterial translocation, dysbiosis and ultimately exhibit carcinogenesis (134,152,153).

In addition to the local gut microbiota effect on cancer described above, other changes can cause long-distance effects, determining the outcome of neoplasms other than colorectal cancer (e.g., pancreatic, liver and breast cancer) (88,94,155-157). A characteristic example of cancer caused by the distant effect of dysbiotic microbiota is represented by hepatocellular carcinoma. The long-distance effects to host organs are exerted by dysbiotic intestinal microbiota either via the activation of pro-inflammatory MAMPs or via the secretion of bacterial metabolites. For example, the gut microbiota is capable of stimulating hepatocellular carcinoma following entry into the liver through the portal vein (88,94,158,159). Similarly, it has been demonstrated that antibiotics ameliorate the progression of hepatocellular carcinoma (88,159,160). Notably, dysbiotic microbiota have been shown to influence estrogen metabolism, thereby affecting tumors in distant sites (7). In the lungs, *Candida* overgrowth has been observed following antibiotic-mediated gut dysbiosis, with subsequent increases in plasma prostaglandin E_2 levels and macrophage differentiation towards the M2 lineage (72). These findings are in agreement with the results of epidemiological studies supporting a strong link between dysbiosis and the development of extracolonic neoplasms, including breast carcinoma (161,162). It was concluded that bacteria and their products are systemically distributed throughout the body, compromising the integrity of the intestinal barrier (94).

5. Role of microbiota in genotoxic stress

If one considers that cancer is tightly associated with genetic diseases, it makes sense to assume that the microbiota exerts a tumor-promoting role via genotoxic stress. The gut microbiota is highly implicated in colorectal carcinogenesis through the secretion of toxic metabolites as by-products of fermentation. Toxic metabolites bind to specific surface receptors on intestinal cells, thereby affecting key signaling pathways. As a general note, microbiota-secreted toxins trigger DNA damage response, causing cell cycle arrest, which is often followed by apoptosis (116,134,139,163-167).

A particular toxin, termed CagA, secreted by *Helicobacter pylori*, has been shown to induce both inflammation and cancer (168,169). ETBF, another well-described toxin, is secreted by *Bacteroides fragilis* and is also implicated in colorectal carcinogenesis. Specifically, ETBF binds to the epithelial receptor, stimulating the Wnt and NF-κB signal transduction pathways, thus leading to enhanced cell proliferation and DNA damage response (133,171-172). ETBF stimulates IL-17 synthesis in the $Apc^{Min/+}$ mouse model, thereby predisposing it to intestinal neoplasia (116,172,173). The underlying molecular mechanisms of ETBF have been shown to include the epithelial damage through E-cadherin cleavage, which in turn activates the β-catenin/Wnt pathway and the STAT3 signaling pathway (critical for the growth of malignant cells) (116,174).

Since DNA damage is tightly associated with genomic instability, proteins that are responsible for all the changes caused by double-strand DNA breaks, such as cytolethal distending toxin (CDT) and colibactin, can be regarded as true genotoxins (139,165). Colibactin, however, stands out among other toxins, as it is capable of inducing oxidative burst, in addition to causing genome instability (134,139).

Bacteria-secreted toxins may also have a profound impact on the oxidation status of cancer cells (175). *Enterococcus faecalis* seems to be the main bacterial strain producing reactive oxygen intermediates (superoxide and hydrogen peroxide) and inducing harmful changes in epithelial cells and malignant transformation. Notably, these effects are exacerbated in IL-10-deficient mice, suggesting that the microbiota leads to colorectal carcinogenesis via reactive toxins in an established inflammatory environment (104,176). It has also been indicated that IECs are toxically hampered by sulfate-reducing bacteria through production of hydrogen sulfide (H_2S) (177,178). Finally, it has been argued that bacteria can obtain virulence factors and convert them to pathogens. The capacity of bacteria to bind to IECs seems to be facilitated via the acquisition of virulence factors (11,179-181). For example, FadA has been identified as the virulence factor secreted by *Fusobacterium nucleatum* in order to activate colorectal cancer (126,182). Similarly, the afa and eae adhesins have been identified as the virulence factors released by *Escherichia coli* strains in order to drive intestinal malignant transformation (107,183).

6. Role of gut microbiota in metabolism

Recent experimental data have confirmed that the gut microbiota can synthesize an enormous quantity of metabolic by-products that affect tumor progression either positively or negatively, upon interaction with the host. In general, the microbiota is responsible for metabolizing dietary factors into bioactive food components. Commencal bacteria are known to exert their fermentation capacity in the gut, metabolizing non-digestible carbohydrates such as polysaccharides (e.g., resistant starch, cellulose, hemicellulose, pectins and gums), oligosaccharides, and lignins into short-chain fatty acids (SCFAs). The SCFAs are composed of acetate, propionate and butyrate, which are regarded as tumor suppressors with great anti-inflammatory and chemo-preventive properties (184,185). SCFAs are final fermentation products of dietary fiber in gut bacteria and provide the appropriate energy to sustain the health of gut epithelial cells. The type of diet directly influences bacterial abundance and composition, as indicated by technologies, such as metagenomics (effect of diet on microbiota), metaproteomics (microbial gene expression) and metabolomics (microbial metabolites). On the other hand, the microbiota can exert beneficial effects on the host organism, as it is responsible for vitamin synthesis, such as vitamin K and most B vitamins (186). In addition, carbohydrates, branched chain amino acids, ammonia, amines, phenols, indoles and phenylacetic acid are also generated through the actions of gut microbiota (187,188). Several *Bacteroides* spp. and some Firmicutes have been classified as the bacteria responsible for the synthesis of phenylacetic acid, phenols, indoles and *p*-cresol. These metabolites are known to be quite toxic as they cause the nitrogen alkylation of DNA (189,190). For example, *N*-nitroso compounds (NOCs) are exogenously supplied or endogenously synthesized through the nitrosation of amines by gut microbiota. The abundance of NOCs has been positively linked to an increased incidence of colorectal cancer in European populations (94). Some products, such as ammonium are carcinogenic despite being produced at low concentrations (191).

SCFAs, such as acetate, propionate and butyrate are efficiently absorbed by the gut lumen, despite differences in their distribution and their effects on host cell metabolism. Each SCFA has specific characteristics that distinguish it from the other SFCAs. Despite low concentration levels of butyrate in the systemic circulation, IECs predominantly use butyrate to fuel their energy stores (60-70%). Normal colonocytes exploit butyrate as their primary energy source, as butyrate follows the procedure of mitochondrial β-oxidation every 7 days (192-194). With respect to the distribution of other SCFAs, the liver has the greatest metabolizing capacity of propionate, while most of the peripheral blood is occupied by high concentrations of acetate (0.10-0.15 mM) (195).

SCFAs also exert growth-inhibitory effects against pathogens (196). The anti-inflammatory and anti-carcinogenic effects of butyrate attenuate inflammation in IBDs such as colitis and Crohn's disease in both rodent models and humans. More than five microbiome studies have confirmed that butyrate-producing bacteria are diminished in patients with colon cancer, as compared to healthy individuals (197). Particularly, the anti-inflammatory properties of butyrate and propionate (but not acetate) have emerged through their capacity to reduce the activity of histone deacetylases (HDACs) in colonocytes and immune cells. This results in histone hyperacetylation, the recruitment of the transcriptional machinery to specific genes and the downregulation of IL-6/12 signal transduction pathways (198-200). In this context, butyrate and propionate are regarded as powerful stimuli to the differentiation and function of Tregs (201-203). Furthermore, tumor cells use glycose aerobically through the Warburg effect (204) and the majority of butyrate translocate to the nucleus, where it exerts its action (205,206). Consistent with this, colon tumor-bearing mice colonized with wild-type butyrate-producing bacteria do not show any signs of cancer following a high-fiber diet. By contrast, in the absence of butyrate-producing bacteria, the same mice exhibit obvious tumor signs (205,207). Butyrate seems to exert its effects at

specific genomic regions, such as the Fas and p21 genes, which are actively involved in apoptosis and the inhibition of cell cycle progression, respectively (197,208), thereby reinforcing the hypothesis that butyrate is a well-established HDAC inhibitor (197,208). Another example of the beneficial effects of butyrate was demonstrated in experimental $Apc^{Min/+}$ $MSH2^{-/-}$ mice. Polyp formation is abrogated in $Apc^{Min/+}$ $MSH2^{-/-}$ mice following a low carbohydrate diet due to the production of butyrate (185). Apart from functioning as an HDAC inhibitor, butyrate has been found to mediate its signals through certain G protein coupled receptors (209,210).

Butyrate and niacin also constitute main representative metabolites of microbiota-secreted SCFAs that have been shown to influence the immune system through two opposing mechanisms. From one perspective, microbial metabolites may mediate their action through binding to the GPR109A receptor, thereby triggering IL-18 synthesis in IECs and affecting DCs, macrophages and T cells (211). Form another perspective, gut microbial metabolites may exert anti-inflammatory properties, supporting Treg differentiation and expansion, thus establishing an immunosuppressive micro-environment (201).

Importantly, the gut microbiota is significantly implicated in metabolizing certain food supplements and nutrients. For example, berries and nuts involve ellagic acid, which is converted to urolithins by gut microbiota. Urolithins diminish Cox2 levels, thus exhibiting a certain anti-cancer effect (212,213). Daidzen is another nutrient metabolized by gut sulfate-reducing microbiota into equols (214,215). Epidemiological data from Asian populations have reported an association between high urinary or plasma equol concentrations and a decreased breast and prostate cancer risk (216). Another characteristic example is the elimination by certain gut microbiota (Lactobacilli and Bifidobacteria) of linoleic acid levels, which are regarded very toxic as they convert omega-6 to omega-3 and produce prostaglandins (217). Finally, resveratrol constitutes another example of the metabolizing effect mediated by gut microbiota (218).

In contrast to the above, the gut microbiota may promote carcinogenesis through the synthesis of secondary bile acids. Characteristically, a minor portion of primary bile acids (5%) escapes the classical enterohepatic circulation and reaches the colon. The following procedure deconjugates and transforms primary bile acids into secondary bile acids (such as DCA and LCA) though the action of specific bacteria (219). The presence of mutations that are insensitive to apoptosis enables secondary bile salts to act as promoters of tumorigenesis (220). DCA is such a toxic metabolite, provoking epithelial DNA damage and apoptosis in a p53-independent but PKC-ERK1/2-dependent manner, with direct associations to the formation of colon cancer or hepatocellular carcinoma or esophageal cancer (94,221-225). Recent data illustrate that bacteria in Clostridium cluster IX are responsible for enrichment of DCA levels in obese mice, rendering them highly susceptible to cancer formation (144). Similarly, certain types of microbiota that convert ethanol into acetaldehyde have been regarded as major stimulators of carcinogenesis (191).

Last but not least, the cumulative exposure of humans to xenobiotics or pharmaceuticals has helped in the understanding that gut microbiota may have direct or indirect implications in the breakdown of such substances (226).

7. Association of cancer therapy with microbiota

Significant efforts are being made in order to manipulate gut microbiota for preventive, diagnostic and therapeutic purposes. For the diagnostic purposes, identification of specific bacterial strains can offer enormous benefit in the context of new, reliable, non-invasive biomarkers for cancer. For cancer prevention purposes, *Fusobacterium nucleatum* has been proven to be a valuable prognostic biomarker, if one considers the abundance of *Fusobacterium nucleatum* in patients with high-grade colon cancer and adenomas (227,228). This has been supported by elevated fecal levels of *Fusobacterium nucleatum* in patients with colorectal cancer (120,227,229,230). Notably, recent evidence has suggested that the percentage of *Fusobacterium nucleatum* present in fecal samples is inversely associated with the survival of patients with colon cancer (231).

A subset of microbes have also been shown to reduce chronic inflammation or to mitigate malignant transformation (132,232,233). Below, we discuss some potential avenues through which microbiota can be therapeutically exploited.

First of all, the gut microbiota appears to be essential to the effectiveness of classical chemotherapeutic drugs such as oxiplatin, cisplatin, and cyclophosphamide (CTX). In general, chemotherapeutic compounds elicit toxic effects on tumor cells, including ROS activation by myeloid cells, intrinsic mitochondrial apoptosis, and the stimulation of inflammatory genes (132,234,235). The beneficial contribution of the microbiota to chemotherapy has been determined by the composition of the microbiota on myeloid cells (24,236). Similarly, microbiota located on myeloid cells has been found to exert a positive effect on cancer immunotherapy or total body irradiation (TBI). Therefore, the importance of the gut microbiota in cancer therapy emerges from its interaction with anti-neoplastic agents in a bidirectional manner.

On the one hand, many current anti-cancer therapeutic strategies (chemotherapy and radiation therapy) negatively affect microbial composition, by fostering dysbiosis (24,236). Radiation therapy, allogeneic stem cell transplantation and several chemotherapeutic agents, including irinotecan and 5-fluorouracil, appear to negatively affect the composition of the gut microbiota (237-239). On the other hand, a considerable body of evidence has demonstrated that the gut microbiota is of the utmost importance to the efficacy of therapeutic drugs, by eliminating side-effects and by interfering in a pharmacodynamic or immunological manner (132,234,240,241).

Currently, therapeutic interventions based on the gut microbiota are categorized as follows: i) Antibiotics; ii) probiotics; ii) prebiotics; and iv) postbiotics. Each therapeutic perspective of the gut microbiota is distinct. Antibiotics are usually used for the eradication of specific bacterial strains. Probiotics are living bacteria and prebiotics are non-digestible compounds, both of which provide strong support to the host. Postbiotics are non-viable products of microbiota, recapitulating a wide range of functions in the human body. All of these therapeutic categories have been shown to confer significant benefits to the host.

Probiotics and prebiotics are known for their capacity to sustain a balanced microbial community, obviating pro-inflammatory or signaling pathways that lead to carcinogenesis (5-8,11,242,243). Probiotics are usually administered

as a curative strategy for antibiotic-mediated dysbiosis and side-effects in studies with mice and humans (244). Probiotics are innocuous microbes, critical for homeostasis, preventing entry of pathogens by stimulating AMPS, IgA and contributing to intestinal barrier integrity (243,245,246). A number of studies have proposed probiotics as a preventive intervention for inflammatory bowel disease or ulcerative colitis (247-251).

Notably, the chemo-preventive efficacy of probiotics and prebiotics seems to be higher than that elicited by antibiotics, through alleviation of inflammation. Antibiotics are not only insufficient as chemopreventive agents, but they can also eliminate commensal homeostatic bacteria and make certain bacterial strains resistant (252). However, human microbiome reconstitution following antibiotic treatment is defective due to impairment of commensal microbial community (253-255), and a time-consuming process (256). For this reason, efforts have focused on devising therapeutic strategies which sustain the microbial composition and population, thereby conferring benefit to the host. Based on data derived from 20 studies, 36% of patients with IBD who were transplanted fecal-derived microbiota from healthy donors exhibited an alleviation of symptoms (257). In another case, fecal microbiota transplantation has been shown to alleviate diarrhea symptoms in individuals with severe *Clostridium difficile* infections, following the use of antibiotics (258). The most impressive results were derived from the study by Suez *et al*, who demonstrated that autologous fecal microbiome transplantation was able to reconstitute the microbial community in its initial configuration in both murine and human samples following treatment with antibiotics. The rapid and complete recovery of the microbiome niche in aFMT-samples following the use of antibiotics, as compared to incomplete niche following treatment with probiotics, was compelling (259).

Mounting evidence suggests that the microbiota can be a determinant factor in modulating the host immune response. Several studies have demonstrated the crucial role of the microbiota in the response of distinct cancer types to classical immunotherapy (immune checkpoint inhibitors) (260-264). For example, the gut microbiota can positively influence the effectiveness of recently developed immunotherapeutic molecules [cytotoxic T lymphocyte associated protein 4 (CTLA4) or programmed death protein 1 (PD-1) antibodies]. The effects of germ-free state or the effects of colonization with specific bacterial strains on therapies using immune checkpoint inhibitors have been investigated. *Bacteroides* spp. appears to be necessary in the anti-CTLA treatment against sarcomas (265) and Bifidobacterium seems to be essential in anti-PDL1 therapy against melanoma (266). Furthermore, the microbiota seem to elicit an efficient response to immunotherapy [CpG-oligodeoxynucleotides (ODN) with neutralization antibody against IL-10], as indicated by experiments using mice (132). Similarly, mice grown under conventional conditions have exhibited stronger responses to CpG-ODN than TLR4-deficient mice (132).

Additional research efforts are required in order to elucidate the mechanisms through which the gut microbiota modulates the clinical effectiveness of various drugs, thus facilitating the design of appropriate personalized therapies based on the microbiota profile of an individual patient. The binding of the Fap2 protein of *Fusobacterium nucleatum* to the Ig and ITIM domains (TIGIT) of the human inhibitory receptor that is present on NK cells protects tumors from an immune attack by NK cells (127). Therefore, the presence of *Fusobacterium nucleatum* in patients may be a direct determinant and/or predictor of resistance to immunotherapy, and special considerations will have to be taken into account when designing personalized therapies for this particular patient group.

Certain issues will also have to be addressed before we move forward in the design of more personalized therapies. For example, the gut microbiota can be directed towards a specific immune population so as to serve as a tool for enriching the specific immune population against cancer. A better understanding of the microbiome effect on anti-PD1 therapy, currently applied to various types of cancer, may help to address the question (262-264). Another issue may be whether host T cells can be equipped with a TCR that is specific for a bacterial epitope and thereby to orchestrate an appropriate immune response. The ultimate goal will be to use microbiota or microbiota-derived molecules as novel immunotherapeutic approaches that will spare patients from the side-effects associated with systemic immunotherapies. For example, an organized and enriched $CD8^+$ T cell response already looks promising in effectively enhancing the therapeutic action of immune checkpoint inhibitors in melanoma without adverse effects (262).

8. Conclusion

A considerable body of evidence exists nowadays that supports how essential the microbiota is in deciding the fate of neoplastic formations, their progression and their sensitivity to classical therapeutic drugs. The effect of the microbiota on cancer is usually elicited locally but it can also be developed systemically, through alterations in the whole immunological milieu. The knowledge pertaining to the microbiome expands rapidly, however therapeutic interventions of intestinal carcinogenesis are still limited. Further experiments will be critical in understanding the underlying molecular mechanisms of microbiota, using animal models or epidemiological data derived from clinical trials towards inventing new treatments. Nevertheless, the available methodologies need to incorporate new technologies in order to facilitate the growth of microbes in conditions that are a direct replica to those within the gastrointestinal tract of the human body. The combination of metagenomics (effect of diet on microbiota), metaproteomics (microbial gene expression), and metabolomics (microbial metabolites) seems to play an important role in developing strategies for disease prevention.

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Authors' contributions

All authors (SB, MA, DAS, AMK, IC and VZ) were involved in the design and conception of the study and have revised and approved the final manuscript. SB performed the literature search, has written the manuscript, has critically analyzed the existing knowledge and has designed the picture and the tables. MA contributed to editing the manuscript concerning the role of microbiota in metabolism and role of dysbiotic microbiota in inflammation and colorectal cancer, AMK contributed to editing of manuscript concerning the role of microbiota in genotoxic stress, IC contributed to editing the manuscript concerning the role of microbiota in homeostasis, DA contributed to editing of manuscript concerning association of cancer therapy with microbiota. SB, MA, DAS, AMK, IC and VZ were significantly involved in the drafting of the manuscript. All authors have taken the responsibility for publishing this review paper and all authors have read and approved the final manuscript.

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Not applicable.

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Not applicable.

Competing interests

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