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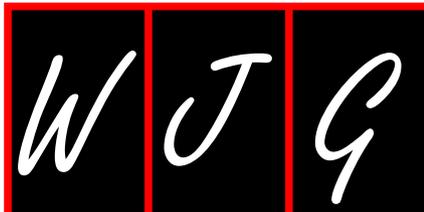
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2016 Colorectal Cancer: Global view

Gut microbiota imbalance and colorectal cancer

Johan Gagnière, Jennifer Raisch, Julie Veziat, Nicolas Barnich, Richard Bonnet, Emmanuel Buc, Marie-Agnès Bringer, Denis Pezet, Mathilde Bonnet

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Abstract

The gut microbiota acts as a real organ. The symbiotic interactions between resident micro-organisms and the digestive tract highly contribute to maintain the gut homeostasis. However, alterations to the microbiome caused by environmental changes (*e.g.*, infection, diet and/or lifestyle) can disturb this symbiotic relationship and promote disease, such as inflammatory bowel diseases and cancer. Colorectal cancer is a complex association of tumoral cells, non-neoplastic cells and a large amount of micro-organisms, and the involvement of the microbiota in colorectal carcinogenesis is becoming increasingly clear. Indeed, many changes in the bacterial composition of the gut microbiota have been reported in colorectal cancer, suggesting a major role of dysbiosis in colorectal carcinogenesis. Some bacterial species have been identified and suspected to play a role in colorectal carcinogenesis, such as *Streptococcus bovis*, *Helicobacter pylori*, *Bacteroides*

fragilis, *Enterococcus faecalis*, *Clostridium septicum*, *Fusobacterium* spp. and *Escherichia coli*. The potential pro-carcinogenic effects of these bacteria are now better understood. In this review, we discuss the possible links between the bacterial microbiota and colorectal carcinogenesis, focusing on dysbiosis and the potential pro-carcinogenic properties of bacteria, such as genotoxicity and other virulence factors, inflammation, host defenses modulation, bacterial-derived metabolism, oxidative stress and anti-oxidative defenses modulation. We lastly describe how bacterial microbiota modifications could represent novel prognosis markers and/or targets for innovative therapeutic strategies.

Key words: Colorectal cancer; Gut microbiota; Dysbiosis; Cyclomodulin; Oxidative stress

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Core tip: The gut microbiota acts as a real organ and many changes in its composition have been reported in colorectal cancer. The pro-carcinogenic properties of bacteria are now better understood. In this review, we discuss possible links between the bacterial microbiota and colorectal carcinogenesis, focusing on the dysbiosis-causing and pro-carcinogenic properties of bacteria, such as genotoxicity, inflammation, and oxidative stress. We lastly detail how microbiota modifications may represent novel prognosis markers and/or targets for innovative therapeutic strategies.

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INTRODUCTION

Colorectal cancer (CRC) is a complex association of tumoral cells, non-neoplastic cells (*i.e.*, stromal cells) and a large number of micro-organisms. The microbiota may be linked to carcinogenesis *via* various mechanisms and could lead to the development of novel prognosis markers and/or targets for innovative therapeutic strategies.

GUT MICROBIOTA IN HEALTH

What is the gut microbiota?

Approximately 100 trillion micro-organisms (including bacteria, viruses and fungi) reside in the adult human gut and constitute the microbiota^[1,2]. The composition of the microbiota is rather stable along the length of the gut, but the absolute number of micro-organisms varies considerably between the mouth and rectum^[3].

The gut microbiota consistently differs among between individuals. It is acquired during the first stages of life *via* the commensal flora from the mother's skin, vagina and feces, and matures primarily during the first two years. Microbiota development is the result of interactions between physiological process in the host and micro-organisms that are introduced from the environment^[4-6]. After the initial stages, the microbiota stabilizes and maintains a consistent composition, despite some fluctuations throughout adulthood in response to environmental, developmental and pathological events^[7,8]. In the elderly, the microbiota composition changes gradually but can maintain similar physiological functions^[9-12]. The early acquisition of a diverse and balanced microbiota is likely critical for the development and maturation of a healthy immune system, as suggested by immune abnormalities in germ-free animals raised in bacteria-free conditions^[4,13]. The colon is colonized by approximately 10³ different microbial species and this colonic microbiota is mostly represented by bacteria^[7,9,14]. Indeed, the colon contains approximately 10¹⁴ bacteria (70% of the host's microorganisms)^[15,16]. This review will therefore focus on the impact of bacteria in CRC.

Most bacteria cannot be cultured, but modern molecular approaches can be used to identify and classify bacteria such as 16S ribosomal RNA (16S rRNA) sequencing from feces or digestive tissues can be used to identify and classify bacteria. The microbiota can be divided according to location in the gut. Specifically, microbes in the lumen are referred to "luminal flora", whereas microbes that penetrate the mucosal layer overlying the intestinal epithelium are referred to "mucosa-associated flora"^[16]. Indeed, thick mucus layers protect enterocytes from excessive exposure to microorganisms and dietary antigens along the length of the intestine, particularly in the colon, thus preventing hypersensitivity responses^[17]. Moreover, the ratio of anaerobes to aerobes is lower at the mucosal surfaces than in the lumen. In addition, the collection of "fecal flora" from the feces is a non-invasive technique that facilitates sampling of colonic microbiota. These bacteria are representative of distal colonic colonization but differ from proximal "associated" flora^[18]. It is essential to note that the composition of murine gut microbiota is quite similar to that in humans, lending translational relevance to mouse experimental models of gastrointestinal disease^[19,20]. Actually, more than 50 different phyla and 500 bacterial species may comprise the human normal commensal gut microbiota. Although the exact number of species and the amount of variability among individuals remain to be characterized^[21,22], these factors are likely highly dependent on lifestyle, diet and host genotype^[23,24]. Some bacterial species are regularly recovered from different individuals, and the human gut microbiota is dominated by 3 primary phyla: *Firmicutes* (30%-50%), *Bacteroidetes* (20%-40%)

and *Actinobacteria* (1%-10%). Strict anaerobes, including *Bacteroides*, *Eubacterium*, *Bifidobacterium*, *Fusobacterium*, *Peptostreptococcus* and *Atopobium*^[25], represent a major portion of the gut microbiota, whereas facultative anaerobes, such as *Lactobacilli*, *Enterococci*, *Streptococci* and *Enterobacteriaceae*, constitute a minority (about 1000-fold lower levels). The fact that composition significantly vary along the gut should also be highlighted, especially given that *Bacteroidetes* and *Actinobacteria* represent more than 90% of bacterial phyla in the colon but only 50% in the small intestine, which contains approximately 40% *Firmicutes* species^[26].

Microbiota and gut homeostasis

The gut microbiota constitutes a natural defensive barrier to infection. Moreover, the microbiota is involved in numerous protective, structural and metabolic roles in the intestinal epithelium and plays a large role in maintaining gut homeostasis. The microbiota is involved in several physiological functions^[27]. The impact of enteric bacteria on intestinal physiology has been studied primarily in germ-free animals raised in bacteria-free conditions. Such animals are more susceptible to infections and have reduced vascularity, digestive enzyme activity, muscle wall thickness, cytokine production and serum immunoglobulin levels, smaller Peyer's patches and fewer intraepithelial lymphocytes^[28]. The reconstitution of a gut microbiota in germ-free mice is sufficient to restore the mucosal immune system^[29] and affects the expression of various host genes that can impact nutrient uptake, metabolism, angiogenesis, mucosal barrier function and development of the intestinal nervous system^[30]. Moreover, commensal bacteria influence the normal development and function of the mucosal immune system^[31,32], such as the humoral components^[33]. These bacteria also modify T-cell repertoires and T-helper-cell cytokine profiles^[34,35]. Such data support a possible influence of gut microbiota composition on individual variations in immunity.

The structural role of gut microbiota on the intestinal epithelium is increasingly evident and has been studied primarily in germ-free mice. These animals present longer intestinal villi, associated with crypt atrophy, slower renewal of epithelial cells and decrease of angiogenesis phenomenon^[3]. Furthermore, it has been reported that mucosa and muscle wall thickness were decreased in these mice^[28] and that microbiota enhanced crypt cell turnover in a CRC-predisposed mouse model^[36].

Furthermore, the gut microbiota is involved in metabolic functions^[27]. For example, the microbiota can participate in (1) anaerobic carbohydrate fermentation through the production of CO₂, H₂, CH₄ and short-chain fatty acids (*e.g.*, butyrate, propionate and acetate); and (2) proteolytic fermentation *via* metabolites such as phenolic compounds, amines,

ammonia, N-nitroso compounds and indoles. These effects can impact on gene expression, intestinal epithelial cell differentiation and proliferation, and can also mediate vitamin synthesis, ion absorption and mucus production^[27,35,37,38]. This complex metabolic activity also increases the yield and storage of energy from dietary sources, regulates fat storage, helps to provide absorbable substrates for both the host and the microbiota, and is involved in bacterial growth and proliferation^[37-40]. Some of produced metabolites, especially during proteolytic fermentation, can be toxic to the host^[27,41,42].

In addition to immune, structural, and metabolic functions, the commensal microbiota inhibits gut colonization of intruding pathogens and ensures "colonization resistance" or "microbial interference"^[43]. The involved mechanisms of these effects remain unclear but likely involve competition with adhesion receptors, stabilization of the gut mucosal barrier, competition for nutrients and the production of anti-microbial substances^[27]. Indeed, alterations in colonization resistance due to, *e.g.*, pathogens or antibiotics treatment, probably increase the risk of gastrointestinal affections.

MICROBIOTA AND COLORECTAL CANCER

Geographic variability of the incidence of CRC highly suggests the involvement of certain environmental risk factors, such as high-fat diets, obesity or living in a Western country^[44,45]. Moreover, Knudson's two-hit hypothesis suggests that host factors play an important role in the predisposition to carcinogenesis. In this scenario, a second environmental hit can lead to uncontrolled cellular proliferation^[46]. Indeed, growing attention has been given to the role of microbial infection in carcinogenesis in recent decades, and microbes are suspected to be involved in approximately 20% of cancers^[47], especially CRC^[48]. Concerning digestive cancers, even if some pathogens, such as *Helicobacter pylori* (*H. pylori*), have been directly and strongly linked to gastric cancer^[49], possible infectious cause in CRC remains controversial. It is becoming increasingly clear, however, that pathogens play a role in colorectal carcinogenesis^[50]. Interestingly, bacteria levels in the colon are one million-fold higher than in the small intestine, and approximately 12-fold more cancers develop in the former than in the latter, suggesting a potential role of gut microbiota in colorectal carcinogenesis^[51]. The first observation linking gut microbiota with CRC was reported in 1975 in germ-free rats that developed less chemically induced colorectal tumor than conventional rats^[52]. These results have been reproduced in CRC-predisposed mice^[36,53]. Contrary to gastric carcinogenesis, which seems to result from a single pathogen, the following differing hypotheses have emerged to explain the

contribution of bacteria to CRC: (1) a dysbiotic microbial community with pro-carcinogenic features are capable of remodeling the microbiome as a whole to drive pro-inflammatory responses and epithelial cell transformation, leading to cancer; and (2) the “driver-passenger” theory, wherein intestinal bacteria, termed “bacteria drivers”, initiate CRC by inducing epithelial DNA damage and tumorigenesis, in turn promoting the proliferation of passenger bacteria that have a growth advantage in the tumoral microenvironment^[50,54]. Studies in mouse models of altered immune and inflammatory responses suggest that dysbiosis could be sufficient to promote cancer^[55,56]. It is thus likely that the immune system is a key factor in the interactions between the gut microbiota and CRC. In addition to the impact of specific pathogens on carcinogenesis, the high redundancy of gut microbiota at a metagenomic level suggests that dysbiosis could exert pro-carcinogenic effects^[57]. These properties could be due to interactions between different emergent bacterial strains activating similar pathways. However, the mechanisms that contribute to dysbiosis and to alterations in microbial richness are not well understood, and it is unknown whether dysbiosis is a cause or a consequence of CRC. Indeed, the CRC microenvironment is characterized by host-derived immune and inflammatory responses that could impact on microbial regulation, alter microbiota composition, and favor the outgrowth of specific bacteria that potentially have carcinogenic effects^[58]. Dysbiosis in CRC could thus result in selection of microbiota composition *via* a tumor-linked microenvironment, with the emergence of “keystone pathogens” that have strong effects on bacterial composition and subsequently amplify dysbiosis^[59].

With the 16S rRNA sequencing of bacteria from the feces or digestive tissues, numerous studies have reported colonic dysbiosis in patients presenting with CRC^[18,60-64]. At present, there is no consensus with respect to the modifications observed in CRC, which are listed in Table 1. However, some bacterial species have been identified and are suspected to play a role in colorectal carcinogenesis^[27,54]. These species primarily include *Streptococcus bovis* (*S. bovis*)^[62,65,66], *H. pylori*^[67-69], *Bacteroides fragilis* (*B. fragilis*)^[61,62,70-72], *Enterococcus faecalis* (*E. faecalis*)^[62,73], *Clostridium septicum* (*C. septicum*)^[74-76], *Fusobacterium* spp.^[77-79] and *Escherichia coli* (*E. coli*)^[80-82].

***S. bovis*/gallolyticus**

S. bovis was the first bacteria indirectly associated with CRC. Indeed, McCoy *et al*^[83] reported the first recognized case of enterococcal endocarditis associated with CRC in 1951. This association was later confirmed by Hoppes *et al*^[84], who reported that approximately two-thirds of *S. bovis* endocarditis cases were associated with gastrointestinal disease. In addition, Klein *et al*^[85] reported a 5-fold increased incidence of CRC in patients presenting such endocarditis.

Interestingly, in this study, most of patients had asymptomatic colorectal tumors that were occasionally benign adenomas, suggesting the involvement of *S. bovis* at an early step of CRC development. However, there are contrasting results in studies of the association between *S. bovis* and CRC; such differences could be due to variations in the collection of feces, sample processing, bacterial culturing or the molecular analysis of fecal samples^[66,86,87].

Studies that confirmed the association between *S. bovis* infection and CRC reported prevalences from 33% to 100% of *S. bovis* with underlying CRC^[88]. It was shown that the mucosal detection of *S. bovis* could be a better tool than fecal level to assess its presence in patients with CRC^[27,89]. More recently, Abdulmir *et al*^[90] used molecular techniques to show increased *S. bovis* in colorectal adenomas and CRC tissues, strengthening the possible involvement of this pathogen in colorectal carcinogenesis. The mechanisms behind this link remain unclear, but these three hypotheses have been reported: (1) *S. bovis* adheres to both normal epithelial and neoplastic cells; (2) this species attains a competitive growth advantage in a tumor microenvironment by foraging on tumor metabolites; and (3) *S. bovis* induces inflammation and/or pro-carcinogenic pathways, leading to tumor progression, especially from pre-malignant tumors^[27]. Thus, the fact that increase in the numbers of *S. bovis* probably occurs at an early step in colorectal carcinogenesis could lead its use for early detection of CRC^[91]. Moreover, these results directly impact clinical practice, leading to recommended colorectal endoscopic exploration in all patients presenting with *S. bovis* infection.

H. pylori

H. pylori has been directly and strongly linked to gastric cancer^[49] and has recently been classified as a carcinogen of the gastrointestinal tract by the International Agency for Research on Cancer. The role of *H. pylori* in colorectal carcinogenesis remains controversial due to differences between studies with respect to the *H. pylori* strains and their specific virulence factors. A meta-analysis of 11 studies conducted between 1991 and 2002 was published by Zumkeller *et al*^[69] and reported a 1.4-fold increased risk of CRC in patients presenting with a *H. pylori* infection. More recently, Guo *et al*^[92] reported a statistical association between *H. pylori* and colorectal adenomas in a meta-analysis of 7679 Asian patients. This result suggested a carcinogenic role of *H. pylori* at an early stage of carcinogenesis. The presence of high levels of *H. pylori* has been reported in CRC tissue using a specific 16S rDNA polymerase chain reaction (PCR) assay and pyrosequencing^[67]. Jones *et al*^[68] analyzed paraffin-embedded tissue blocks of normal colonic mucosa, adenomas and adenocarcinomas from 180 patients. The results indicated a significant

Table 1 Summary of 16S rRNA sequencing and qPCR analyses of colonic microbiota variations in colorectal cancer

Variation in CRC	Phyla	Genus/species	Population	Ref.
Fecal flora				
↑		<i>Enterococcus Faecalis</i>	20 CRC/17 C	[73]
↑	Proteobacteria	<i>Porphyromonas</i> / <i>Escherichia/Shigella</i> <i>Enterococcus</i> / <i>Streptococcus</i> / <i>Peptostreptococcus</i> <i>Bacteroides fragilis</i>	46 CRC/56 C	[62]
↑		<i>Bacteroides/Prevotella</i>	60 CRC/119 C	[61]
↑		<i>Peptostreptococcus/Mogibacterium</i> <i>Anaerococcus/Slakia/Paraprevotella</i> <i>Anaerotruncus/Collinsella/Desulfovibrio</i> <i>Eubacterium/Porphyromonas</i>	21 CRC/22 C	[18]
↑	Bacteroidetes	<i>Atopobium/Porphyromonas</i>	47 CRC/94 C	[63]
	Fusobacteria	<i>Fusobacterium</i>		
↑	Fusobacteria	<i>Fusobacterium/Bacteroides</i>	19 CRC/20 C	[64]
↑		<i>Bacteroides/Fusobacterium</i> <i>Alistipes/Escherichia/Parvoimonas/Bilophila</i> <i>Faecalibacterium prauznitsii</i>	46 CRC/63 C	[214]
↓		<i>Bacteroides vulgatus/Bacteroides uniformis</i>	20 CRC/17 C	[73]
↓	Bacteroidetes	<i>Roseburia/Butyrate-producing bacteria</i> <i>Faecalibacterium prauznitsii/Roseburia</i>	46 CRC/56 C	[62]
↓		<i>Faecalibacterium prauznitsii/Roseburia</i>	19 CRC/20 C	[64]
↓	Firmicutes (clostridia)	<i>Ruminococcus</i>	47 CRC/94 C	[63]
↓		<i>Ruminococcus/Bifidobacterium/Streptococcus</i>	46 CRC/63 C	[214]
Tumor-associated flora				
↑	Bacteroidetes	<i>Coriobacteriae/Roseburia</i> <i>Fusobacterium/Faecalibacterium</i> <i>Butyrate-producing bacteria</i> <i>Bacteroides</i>	6 CRC/6 AM	[14]
↑		<i>Fusobacterium</i>	22 CRC/22 C	[61]
↑		<i>Fusobacterium</i>	95 CRC/95 AM	[106]
↑	Bacteroidetes	<i>Bacteroides/Prevotella/Streptococcus</i>	27 CRC	[18]
	Fusobacteria	<i>Fusobacterium/Peptostreptococcus</i>	27 intestinal lumen	
	Proteobacteria	<i>Morganella/Porphyromonas</i> <i>Fusobacterium</i>	55 CRC/55 AM	[109]
↓	Firmicutes	<i>Shigella/Citrobacter/Serratia/Salmonella</i>	6 CRC/6 AM	[14]
↓	Firmicutes	<i>Lactobacillus/Roseburia/Pseudobutyrvibrio</i>	27 CRC	[18]
			27 intestinal lumen	
↓	Bacteroidetes		95 CRC/95 AM	[106]
	Firmicutes (clostridia)			
Mucosa-associated flora				
↑		<i>Fusobacterium/Porphyromonas</i> <i>Peptostreptococcaceae/Gemella</i> <i>Mogibacterium/Klebsiella</i>	32 CRC 22 C (swab)	[18]
↓		<i>Faecalibacterium/Blautia/Anaerostipes</i> <i>Lachospira/Bifidobacterium</i>	32 CRC 22 C (swab)	[18]

CRC: Colorectal cancer; C: Control patients; AM: Adjacent tumor, normal mucosa of patients with CRC.

increase in colonic mucosa-associated colonization by *H. pylori* in adenomas and adenocarcinomas compared to normal mucosa^[68].

Although the role of *H. pylori* in gastric carcinogenesis has been better studied and described than its involvement in colorectal carcinogenesis, some hypotheses can be extrapolated from the pathophysiology of bacteria-linked gastric cancer. Bacterial cytotoxin-associated gene A (CagA) and vacuolating cytotoxin A (VacA) are encoded by pathogenicity islands in some *H. pylori* strains and may induce the activation of inflammation pathways and cellular proliferation in gastric cancer^[93]. Shmueli *et al.*^[94] reported that patients presenting with seropositivity for CagA-positive *H. pylori* strains had a significantly increased risk of CRC. Another hypothesis is the direct and indirect production of pro-oxidative reactive oxygen

and nitrogen species by some *H. pylori* strains that could participate to colorectal carcinogenesis^[49].

B. fragilis

B. fragilis are common anaerobic bacteria that are detected in up to 80% of children and adults but which represent less than 1% of gut microbiota^[95,96]. There are two molecular subtypes of *B. fragilis*, nontoxigenic or enterotoxigenic, with the latter known to be responsible in diarrheal illnesses in humans, especially children^[97]. Enterotoxigenic *B. fragilis* is easily detected in fecal samples of up to 40% of healthy adults, facilitating studies on the role of these bacteria in gastrointestinal diseases^[98]. Indeed, some studies have reported increased colonic colonization by *B. fragilis* in patients presenting with CRC^[61,62]. For example, Sobhani *et al.*^[61] performed pyrosequencing

on stools from 179 patients with CRC or controls who had a normal colonoscopy. The results indicated more colonization by *B. fragilis* in patients presenting with CRC than in control patients^[61]. Most of enterotoxigenic *B. fragilis* strains detected in mucosal samples from patients with CRC harbored the *bft* gene, which encodes the bacterial toxin *B. fragilis* toxin (BFT). This toxin is involved in the pathogenicity of enterotoxigenic *B. fragilis*^[99,100]. Indeed, the involvement of BFT in colorectal carcinogenesis is becoming increasingly evident as this toxin directly affects pathways that lead to increased cell proliferation, the epithelial release of pro-inflammatory effectors, and DNA damage in *in vitro* and CRC-predisposed mouse models^[70-72].

E. faecalis

E. faecalis is a facultative anaerobic commensal bacterium of the oral cavity and the gastrointestinal tract. Recently, this species has emerged as a human pathogen^[101]. Balamurugan *et al.*^[73] performed 16S rRNA real-time PCR from the feces of patients with CRC and healthy volunteers. The authors reported significantly higher *E. faecalis* fecal populations in patients with CRC compared to healthy controls^[62,73]. These results were confirmed more recently by Wang *et al.*^[62]. The mechanisms linking *E. faecalis* to colorectal carcinogenesis remain unclear, but the production of pro-oxidative reactive oxygen species (ROS) by *E. faecalis* has been described in cellular and animal models^[102,103]. Moreover, *E. faecalis* can trigger colitis, dysplasia and CRC in a susceptible interleukin (IL)-10^{-/-} mouse model^[104].

C. septicum

C. septicum is a rare cause of bacteremia (less than 1%). As for *S. bovis* endocarditis, *C. septicum* infections have been clinically linked to CRC^[76]. Indeed, it has been reported that gastrointestinal disease and/or colorectal malignancies can be found in up to 40% of patients presenting with *C. septicum* infections^[74,75]. Hermsen *et al.*^[75] reported an analysis of 320 cases of *C. septicum* infections, of which more than 40% had a gastrointestinal origin, primarily malignant. The underlying mechanisms of this association remain unknown, but one hypothesis is that the hypoxic and acidic tumor microenvironment favors the germination of *C. septicum* spores *via* ingestion of contaminated food^[27,105]. However, no direct involvement of *C. septicum* in colorectal carcinogenesis has been well defined.

Fusobacterium nucleatum

Fusobacterium nucleatum (*F. nucleatum*) is an anaerobic Gram-negative pathogenic bacterium that recently emerged as a potential candidate for CRC susceptibility. Indeed, recent metagenomic analyses using whole-genome and bacterial 16S rRNA sequencing revealed an enrichment of *F. nucleatum*

in colonic tumor-associated microbiota compared to adjacent normal mucosa in patients with CRC^[106,107]. Moreover, other studies found that luminal *F. nucleatum* colonization was higher in patients presenting with colorectal adenomas compared to healthy patients. An increase of *F. nucleatum* colonization in adenomas compared to adjacent colonic normal mucosa has also been reported^[77,78]. These data strongly suggest that, more than simply being associated with CRC, *F. nucleatum* likely acts at the early steps of colorectal carcinogenesis promotion. This effect may be mediated *via* its FadA adhesion and activates the Wnt/ β -catenin pathway^[79]. This hypothesis has been verified in a CRC-predisposed adenomatous polyposis coli (APC)^{min/+} mouse model. In this animal model, infection with *F. nucleatum* increased tumor multiplicity and selectively recruited tumor-infiltrating myeloid cells, which can promote tumor progression^[77]. Interestingly, *F. nucleatum* is detected in up to 80% of tumor samples. The increased colonic colonization of this species has been correlated with CRC stage, as has been described for other pathogens^[108,109]. Indeed, Viljoen *et al.*^[109] identified significant associations between high-level colonization by *F. nucleatum* and advanced stage III-IV CRC, as well as with microsatellite instability tumor phenotype. These results could lead to further studies that explore (1) the role of *F. nucleatum* in modulating the DNA repair systems involved in colorectal carcinogenesis; and (2) the potential pathogenic interactions between different pathogenic bacterial species.

E. coli

Although *E. coli* is a commensal bacteria of the human microbiota and represents the most common cultivable, Gram-negative, aero-anaerobic bacteria in the gut, various studies have demonstrated a clear link between mucosa-adherent *E. coli* and CRC^[80-82]. *E. coli* belonging to phylogroups B2 and D comprise most of the pathogenic strains that express virulence factors, and some of these species are involved in chronic inflammatory bowel diseases, which are known risk factors for CRC^[110,111].

Some studies have reported higher levels of colonic colonization by mucosa-associated *E. coli* in patients with CRC compared to healthy patients^[80,81]. For example, Swidsinski *et al.*^[81] performed 16S PCR using colonic mucosa tissue samples and detected *E. coli* in only 3% of healthy patient biopsies. However, *E. coli* was detected and predominant in 62% and 77% of patients presenting with adenomas and carcinomas, respectively^[81]. A few years later, Martin *et al.*^[80] reported that more than 70% of mucosa samples from patients with CRC were colonized by bacteria, generally *E. coli*. Increased mucosa- and tumor-associated *E. coli* colonization in CRC patients has been confirmed by several studies, strongly implicating *E. coli* in colorectal carcinogenesis^[82,108,112,113].

CRC samples commonly exhibit colonic mucosal

E. coli that could express genes (*afa*, *lpfA*, *eae* and/or cyclomodulin toxins) that confer characteristics that are relevant to pathogenesis, including M-cell translocation, angiogenesis and genotoxicity^[112,114-117]. Cyclomodulins such as colibactin, are toxins that interfere with the eukaryotic cell cycle and induce DNA damage and genomic instability, which are involved in colorectal carcinogenesis^[118,119]. A pro-carcinogenic effect of B2 phylogroup colibactin-producing *E. coli* has been confirmed in mouse models. Specifically, these bacteria are involved in inflammation pathways and cell proliferation fueling^[82,108,120]. Moreover, possible interactions between *E. coli* and DNA repair system involved in colorectal carcinogenesis, such as the DNA mismatch repair (MMR) system, have been described^[112,121]. Interestingly, these B2 colibactin-producing *E. coli* are preferentially detected in patients with CRC^[82,108,115,120] and were more prevalent in the mucosa of patients presenting with advanced stage III/IV CRC than in those with stage I CRC^[108]. These results suggest that *E. coli* could serve as a prognostic factor in CRC.

SUSPECTED INVOLVED MECHANISMS

As described above, some bacterial species may be involved in colorectal carcinogenesis. Many suspected involved mechanisms, occasionally shared by different species, have been described. These mechanisms include bacterial-derived genotoxins, microbial-derived metabolism, the modulation of host defenses and inflammation pathways, oxidative stress induction, and anti-oxidative defense regulation (Figure 1).

Bacterial-derived genotoxins and other bacterial virulence factors

During their phylogenetic evolution, bacteria progressively acquired virulence factors that conferred pathogenicity. For example, bacteria developed the ability to penetrate the gut mucosal barrier, as well as to adhere to and invade intestinal epithelial cells, specifically through the use of flagellum, pili, and adhesins^[110,122,123]. Most of the disease-promoting and pro-carcinogenic effects of pathogens depend on these virulence factors^[58]. Pathogens can interact with adhesion molecules. For example, *F. nucleatum* uses the FadA virulence factor to adhere to and invade cells^[124], thereby activating β -catenin signaling pathway and promoting CRC^[79]. In the same way, certain CRC-associated *E. coli* strains have acquired virulence factors, such as the *afa* and *eae* adhesins, which confer the ability to adhere to and invade the intestinal epithelium^[112,116].

Toxins may be involved in colorectal carcinogenesis by modulating certain host-derived signaling pathways, resulting in the activation of carcinogenesis-promoting pathways. For example, CagA or VacA are produced by some *H. pylori* strains and increase

inflammation and cancer rates^[125,126]. *B. fragilis* has been associated with CRC^[71,127], and BFT is another well-described example of a bacteria-derived toxin involved in colorectal carcinogenesis. BFT is a 20 kDa zinc-dependent metalloprotease toxin that is grouped into three isotypes, namely, BFT-1, BFT-2 and BFT-3^[100]. These toxins are encoded by a unique *B. fragilis*-specific gene, which was described by Moncrief *et al.*^[128] in 1995. A recent study reported that mucosa-associated BFT-producing *B. fragilis* were more prevalent in late-stage CRC, suggesting possible role of BFT in CRC promotion and progression^[99]. At the molecular level, BFT binds to a specific colonic epithelial receptor, activating the Wnt and nuclear factor-kappa B (NF- κ B) pathways. These effects lead to increased cell proliferation, the epithelial release of pro-inflammatory mediators, and the induction of DNA damage^[97,129-132]. Furthermore, in the CRC-predisposed multiple intestinal neoplasia APC^{min/+} mouse model, BFT promotes IL-17-dependent carcinogenesis^[127].

Other toxins, named cyclomodulins [cytolethal distending toxin (CDT), colibactin, cytotoxic necrotizing factor and cycle inhibiting factor], can induce DNA damage, interfere with the cell cycle and/or apoptosis^[82,118,127,133-136]. Only CDT and colibactin can directly damage DNA and are linked genomic instability; these proteins are therefore considered true genotoxins^[118,133]. Both CDT and colibactin induce double-strand DNA breaks, activate the ataxia telangiectasia mutated (ATM)-checkpoint kinase 2 signaling pathway, and lead to H2AX histone phosphorylation. These effects result in transient G2/M cell cycle arrest and cell swelling.

Most of the Gram-negative bacteria that are involved in CRC produce CDT, which is by far the most well-characterized bacterial-derived genotoxin^[137]. The CdtA and CdtC subunits permit the interaction between pathogens and host cells; subsequently, the cytoplasmic CdtB subunit can translocate to the nucleus, act as a DNase and damage DNA^[133]. CDT also favors persistent gut colonization and induces the production of pro-inflammatory molecules, such as NF- κ B, tumor necrosis factor (TNF)- α , IL-6 and cyclooxygenase (COX) 2. These factors are involved in many carcinogenic processes. CDT has also been reported to induce dysplasia in a *Helicobacter hepaticus*-linked hepatocarcinoma mouse model^[138,139]. When combined, DNA damage, interference with the cell cycle and the modulation of pro-inflammatory pathways can lead to mutations that are involved in genomic instability in CRC.

Colibactin is another bacterial-derived genotoxin that has recently attracted attention. It was first described in 2006 by Nougayrède *et al.*^[119] but has not yet been isolated and purified. Colibactin synthesis and activation requires complex machinery that involves three non-ribosomal peptide megasynthases (NRPS), three polyketide megasynthases (PKS), two NRPS/PKS hybrids and at least eight of nine accessory enzymes encoded in the *pks* island^[119]. The *pks* island is found

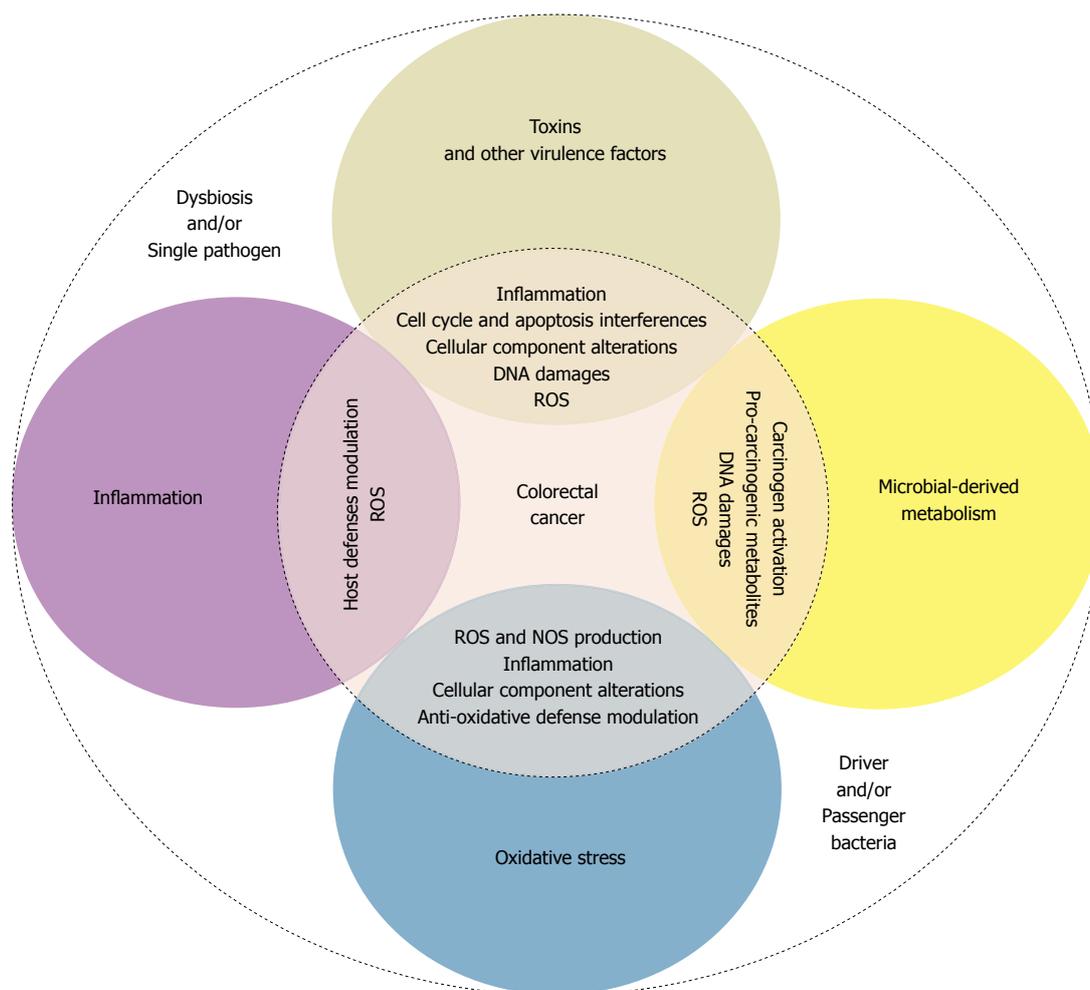


Figure 1 Suspected mechanisms by which the gut bacterial microbiota participates in colorectal carcinogenesis. The bacterial microbiota induces colorectal carcinogenesis through several mechanisms. The primary suspected mechanisms are bacteria-derived genotoxin production, microbial-derived metabolism, the modulation of host defenses and inflammation pathways, oxidative stress induction, and anti-oxidative defense regulation. These mechanisms result in various cellular effects and alterations of host defenses that lead to genomic instability and epithelial cell proliferation, which are involved in colorectal carcinogenesis. ROS: Reactive oxygen species; NOS: Nitrogen species.

primarily in the *Enterobacteriaceae* family, especially in the *E. coli* B2 phylogroup^[115,119]. Interestingly, other micro-organisms, such as *Proteus mirabilis* and *Klebsiella pneumoniae*, which are associated with bacterial-induced colitis and an immunodeficient-CRC-mouse model, also carry the *pks* island^[140-142]. Colibactin induces ROS formation, double-strand DNA breaks, and the associated ATM-mediated DNA damage response, cell cycle arrest and genomic instability^[82,118]. Furthermore, colibactin-producing *E. coli* can enhance tumor growth by inducing the emergence of senescent epithelial cells secreting growth factors and/or by the secretion of pro-tumoral molecules by infiltrating cells^[58,114,120]. Interestingly, even though toxin-producing bacteria constitute a minority of the colonic microbiota, metatranscriptomic analyses performed on human CRC tissues samples reveal a high expression of these toxins in the colon^[143].

Microbial-derived metabolism affecting carcinogenesis

In addition to the involvement of bacterial virulence

factors, such as genotoxin production, it has become increasingly clear that microbial-derived metabolism strongly impacts CRC development^[144]. These metabolic activities may affect colorectal carcinogenesis *via* the following several processes: regulating the generation of CRC-promoting secondary bile acids; the metabolic activation or inactivation of pro-carcinogenic compounds, dietary phytochemicals and xenobiotics; hormone metabolism; and the modification of inflammation pathways^[58].

The interplay between diet, bile acids and gut microbiota is complex. Indeed, high-fat diets are correlated with increased bile secretion and an increased risk of CRC^[145-147]. Primary bile acids excreted into the gut are converted through microbial derived-metabolism, including hydrolase activities, into secondary bile acids. These acids are used by microorganisms as an energy source^[148] but are also known to be involved in many colorectal carcinogenesis-linked process, such as apoptosis, cell proliferation, DNA damage induction and tumor promotion^[27]. Bernstein

et al.^[146] reported a higher incidence of tumors in bile acid-exposed gut in a mouse model. Indeed, bacteria-transformed bile acids can result in DNA damage by the production of pro-oxidative molecules, such as ROS and nitrogen species (NOS)^[149,150]. Therefore, chronic exposure to increased levels of secondary bile acids may favor the induction of DNA damage, leading to genomic instability, which is involved in CRC. Furthermore, strong antimicrobial bile acid activities lead to significant changes in the gut microbiota composition, with a relative increase in some *Gammaproteobacteria* and *Bacteroidetes* species that are associated with CRC^[151].

Some carcinogens are inactivated in the liver by glucuronic acid-mediated conjugation and are excreted *via* bile in the digestive tract. In the gut, and particularly in the colon, this process may be reversed by bacterial β -glucuronidase activity, which can lead to CRC. Indeed, Takada *et al.*^[152] reported a decrease in tumor number with the inhibition of bacterial β -glucuronidase activity in a CRC rat model. Moreover, fecal β -glucuronidase activity is increased in patients with CRC compared to healthy controls^[153]. These results strongly support the involvement of bacterial β -glucuronidase activity in the initiation and progression of CRC *via* the reactivation of toxic components. Moreover, bacterial β -glucuronidase activity plays a major role in the metabolism of xenobiotics and affects the activity and side effects of certain antitumor drugs^[58,154]. For example, irinotecan, a commonly used chemotherapy for CRC that is inactivated in the liver, is locally modified in the gut, generating intermediate molecules without systemic anti-tumor effect and inducing major treatment-limiting side effects (*e.g.*, severe diarrhea). These undesired effects can be prevented with the use of antibiotics or bacterial β -glucuronidase inhibitors^[155].

Contrary to microbial carbohydrate fermentation, which can benefit the host through the generation of short chain fatty acids (*e.g.*, butyrate, acetate, propionate)^[156], microbial protein fermentation generates potentially toxic and pro-carcinogenic metabolites involved in CRC, such as phenols, sulfides, ammonia and nitrosamines^[58]. It has been reported that protein-rich and low-carbohydrate diets can lead to the increased microbial production of toxic metabolites to the detriment of cancer-protective metabolites^[157], increasing the risk for CRC^[158]. However, concentration-dependent effects on CRC have been described for butyrate. At lower concentrations, butyrate appears to stimulate epithelial cell proliferation^[159], while other studies have demonstrated anti-proliferative and anti-cancer properties of this compound^[160]. A subset of bacteria, including *Bacteroidetes* and *Firmicutes* species, produce potentially bioactive substances *via* the degradation of amino acids, especially nitrogenous compounds, in the gut^[161-163]. These compounds can exert carcinogenic effects through DNA alkylation,

leading to mutations that have been reported in Western diet-linked CRC^[161-163]. Moreover, sulfides produced in the gut by the bacterial reduction of dietary sulphate, as well as the metabolism of other compounds^[164], are enterotoxic^[165]. These sulfides have genotoxic effects on human cell lines at physiological concentrations^[166]. These effects occur primarily *via* the induction of ROS formation and DNA damage^[167].

Chronic and/or excessive consumption of alcohol has been found to be an important risk factor for many cancers, including CRC^[168]. Microbial metabolism may contribute to the toxicity of alcohol, especially in the gastrointestinal tract, where aerobic and facultative anaerobic bacteria convert ethanol to acetaldehyde^[169]. Indeed, acetaldehyde is known to be a highly toxic and pro-carcinogenic compound with various negative effects, ranging from DNA damage and impaired DNA excision repair to the degradation of folate. All of these effects have been implicated in colorectal carcinogenesis^[169-171]. The role of microbiota in this process has also been reinforced by Homann *et al.*^[172], who reported that the conversion of ethanol to acetaldehyde was inhibited by the use of antibiotics, such as ciprofloxacin. This drug kills primarily aerobic and facultative anaerobic bacterial populations^[172].

Host defense modulation and inflammation

As previously mentioned, the intestinal mucosa constitute the first line of defense against gut commensal or pathogen bacteria and related microbial molecules. Intestinal epithelial cells need to rapidly detect the presence of pathogens in order to mount a suitable immune response. However, these cells also must maintain a moderate immune response against or tolerance for non-pathogenic bacteria^[173]. The maintenance of gut homeostasis and these interactions between the host and microbiota involve innate immunity receptors, such as Toll Like Receptors (TLRs) and Nod Like Receptors (NLRs), which recognize particular molecular motifs associated with pathogens. The activation of these receptors leads to a cellular response, including the activation of MAPK, NF- κ B or PI3K/AKT signaling pathways^[174]. The activation of these pathways can induce the expression of pro-inflammatory cytokines (*e.g.*, TNF- α , IL-6, IL-8) and/or antimicrobial peptides, all of which are involved in the development of an inflammatory response. Indeed, a decrease in intestinal tumor number has been reported in azoxymethane (AOM)-treated APC^{Min/+} or IL-10^{-/-} mice invalidated for *MyD88* gene, which encodes a TLR signaling adaptor^[175,176]. However, these results remain controversial given that another recent study reported that *MyD88*^{-/-} mice treated with AOM/dextran sulfate sodium (DSS) exhibit increased susceptibility to colorectal tumors. These results suggest that the role of TLR signaling and the host response in CRC differ between colitis-associated and chemically induced CRC^[177]. Moreover, *Nod1*- or *Nod2*-deficient APC^{Min/+}

and AOM/DSS-treated mice exhibit an increase in the number of colorectal tumors compared to control animals, suggesting the involvement of NLRs in colorectal carcinogenesis^[55,178]. These results highlight the major role of the host immune response to gut microbiota in CRC development.

It is now well established that inflammatory bowel disease patients, who are known to have an increased risk for developing CRC, present many changes in their microbiota composition^[179-181]. For these reasons, the involvement of inflammation in the establishment of dysbiosis-related CRC is increasingly evident. Some *in vivo* studies have shown that the gut microbiota composition differs between AOM-treated and untreated *IL-10*^{-/-} mice. Moreover the emergence of dysbiosis after each cycle of DSS treatment in mice treated with AOM has been observed^[82,182]. Taken together, these data constitute strong evidence that inflammation plays an important role in the modulation of the microbiota and dysbiosis emergence during colorectal carcinogenesis.

However, inflammation could also be linked to the host response induced by bacteria during CRC development. It has been previously reported in a CRC-predisposed APC^{Min/+} mouse model that BFT induces a Th17 pro-inflammatory response, which is involved in the development of early-stage tumors^[127]. Furthermore, the contribution of *S. bovis* to colorectal carcinogenesis is associated with the increased expression of pro-inflammatory genes, such as IL-1, IL-8 and COX-2^[90]. Moreover, APC^{Min/+} and *IL-10*^{-/-} mice infected with *F. nucleatum* and *E. faecalis*, respectively, exhibit increased immune cell infiltration in tumors and colonic mucosa and heightened expression of pro-inflammatory cytokines, such as TNF- α , IL-1 β , IL-6 and IL-8^[77,104,183]. *E. coli* is one of the best-characterized bacteria associated with inflammatory bowel disease. An abnormal colonization of the gut mucosa by adherent and invasive *E. coli* in inflammatory bowel disease patients has been reported^[110,111]. Moreover, Raisch *et al.*^[184] showed that CRC-associated *E. coli* can induce the expression of the pro-inflammatory gene COX-2 in macrophages, supporting the bacterial modulation of inflammation in colorectal carcinogenesis.

Oxidative stress and anti-oxidative defenses modulation

ROS induction appears to have a major and central role in microbiota-linked CRC *via* the previously described mechanisms. ROS can be generated by cells during infection and inflammation, as previously discussed, or directly by gut microbiota^[177]. The induction of ROS is known to be a major defense mechanism of infected cells, contributing to the elimination of bacteria^[48,119]. It has been reported in both *in vitro* and *in vivo* studies that some Enterococci species, especially *E. faecalis*, generate hydroxyl radicals^[102,103]. These radicals are powerful mutagens that can cause DNA breaks, point

mutations and protein-DNA crosslinking, all contributing to genomic instability in CRC^[185-187]. Furthermore, this bacterium can induce aneuploidy in colonic epithelial cells, and the use of inhibitors of ROS and NOS can prevent this effect, supporting the role of bacterial-induced oxidative stress in colorectal carcinogenesis^[82]. Moreover, the role of *H. pylori* in gastric carcinogenesis *via* the induction of oxidative stress has been clearly demonstrated^[49], and this species is able to both produce and induce the production of ROS by immune cells. In this manner, *H. pylori* affects many signal transduction pathways in gastric cells and thereby promotes gastric cancer. It could thus extrapolate the fact that existence of colorectal chemical-, bacterial- and/or immune-induced inflammation also induces the recruitment of neutrophils and macrophages, which are major sources of ROS, leading to the genetic and epigenetic alterations involved in CRC^[188-190]. The gut microbiota also promote host-derived production of nitric oxide and its secondary NOS, especially through the activation of macrophages in the inflammation response, which can induce DNA damage. Some bacterial species can directly generate NOS^[191]. Sobko *et al.*^[192,193] reported that *Lactobacilli* and *Bifidobacteria* generate significant levels of NOS in germ-free and monoassociated mice and that a nitrate-enriched diet increased NOS production. However, it is not clear whether in ROS and NOS produced *in vivo* are sufficiently long lived to diffuse from immune cells to the extracellular matrix and subsequently enter the nucleus of epithelial cells to induce damage DNA^[194].

Oxidative stress is defined by an imbalance between the levels of pro-oxidative molecules (*e.g.*, ROS and NOS) and the effectiveness of anti-oxidative defenses^[195]. Oxidative stress results in primarily irreversible direct or organic substrate-mediated cell damage, including DNA breaks and damage, protein aggregation or fragmentation, and cellular membranes dysfunction^[185,195]. The toxic effects of ROS and NOS are thus balanced by various enzymatic and non-enzymatic anti-oxidative defenses, which help to regulate ROS/NOS production and repair mechanisms^[196,197]. DNA repair mechanisms are altered in CRC^[198,199]. Moreover, under specific conditions, the balance between pro- and anti-oxidative compounds is lost, especially in cases of bacterial infection. The bacterial modulation of anti-oxidative defenses, especially DNA repair systems, is becoming increasingly clear. Mangerich *et al.*^[200] reported decreased expression of DNA repair and oxidative response genes in the colons of colitis-induced CRC mice model that were infected with *H. hepaticus*. Moreover, Maddocks *et al.*^[112,121] reported *in vitro* results demonstrating the ability of some enteropathogenic *E. coli* strains to downregulate the DNA MMR system in infected HT-29 intestinal epithelial cells *via* post-transcriptional effect of a secreted effector protein. This downregulation of the MMR system led to the

accumulation of mutations involved in colorectal carcinogenesis^[112,121]. Furthermore, a study on APC^{Min/+} MMR-deficient mice strongly supports a possible role of an interaction between gut microbiota and MMR deficiency in CRC induction^[159]. Indeed, Belcheva *et al.*^[159] reported that (1) altering the microbiota composition reduces tumorigenesis; (2) gut microbes can fuel the hyperproliferation of MMR-altered intestinal epithelial cells; and (3) the MMR pathway has a role in regulating APC/ β -catenin activity and modulating the differentiation of transit-amplifying cells in the colon^[159]. Moreover, Viljoen *et al.*^[109] reported increased colonic colonization by *F. nucleatum* in CRC patients with an MMR deficiency-linked microsatellite instability phenotype. All of these data strengthen the hypothesis of an interaction between the gut microbiota and the DNA repair system in colorectal carcinogenesis.

MICROBIOTA IMBALANCE AND CLINICAL IMPLICATIONS

As previously mentioned, the gut microbiota likely plays a major role in the promotion and progression of CRC *via* several mechanisms, including inflammation, metabolism and genotoxicity. There are therefore many possible ways by which to target the microbiota in terms of CRC prevention strategies. Indeed, the use of probiotics or fecal transplantation protocols could combat CRC-associated dysbiosis and thus restore eubiosis in chronic diseases, helping to reduce microbiota-induced genotoxicity and activation of inflammatory, proliferative and pro-carcinogenic pathways^[58]. However, this microbiota-targeting therapy approach has not been well studied in CRC.

Genotoxins are a target of interest in the context of CRC treatment. For example, supportive evidence has recently been provided that colibactin-producing *E. coli* could be major actors in CRC-related genomic instability^[58,114,115,119,120]. Colibactin synthesis requires the serine enzyme ClbP, which acts as a peptidase to produce colibactin NRPS compounds^[201-203]. On this basis, it has been reported that boronic acid compounds, which are potent inhibitors of active serine enzymes, suppress the genotoxic activity of colibactin-producing *E. coli* *in vitro* and *in vivo*^[204]. In CRC mice model, treatment with such compounds was shown to prevent cell proliferation and genotoxin-induced tumorigenesis compared to water-treated mice^[204].

Changes in gut microbiota composition could also lead to an altered host immune response. On this basis, some authors have studied the impact of the oral administration of probiotics on immunologic signaling. These studies have provided supportive evidence that the gut microbiota plays an essential role in intestinal epigenomic mechanisms of the host^[205,206]. Moreover, it was reported that the deletion of lipoteichoic acid (LTA), a TLR2 ligand, normalizes

innate and adaptive pathogenic immune responses and decreases the number of tumors in a CRC-predisposed murine model. It was also reported that LTA-deficient *Lactobacillus acidophilus* (1) decreased inflammation and protected against CRC^[207]; (2) prevented or induced the regression of established colitis and polyposis^[207,208]; and (3) downregulated downstream signaling^[209], and stimulated tumor suppressor gene expression in CRC cell lines^[210]. Furthermore, some commensal bacteria, such as *Bifidobacterium breve* and *Lactobacillus rhamnosus*, inhibit the production of pro-inflammatory cytokines and decrease host DNA methylation and histone acetylation events that are involved in colorectal carcinogenesis^[211-213]. All of these results underline a feasible microbiota-targeting therapy approach in CRC through the use of probiotics or genetically modified bacteria. Even if fecal transplantation has not been well studied in CRC, future transplantation studies using germ-free CRC animal models likely represent an important next step in this line of inquiry.

To provide adequate treatments as part of modern personalized medicine, accurate prognosis factor have yet to be established. Prognostic factors in CRC primarily depend on morphologic and histologic results. Recently, there has been a significant body of work involving the gut microbiota as a prognosis factor in CRC. Bonnet *et al.*^[108] reported increased colonic colonization by cyclomodulin-producing *E. coli* in advanced stage CRC, that have been ever described by Viljoen *et al.*^[109] for enterotoxigenic *B. fragilis* and *F. nucleatum*. These results support a possible use of microbiota CRC prognosis markers that may be used to improve patient selection for aggressive, suitable treatments.

CONCLUSION

The advent of modern molecular microbiota sequencing techniques has strongly improved the characterization of microbiota variations in CRC. However, a better understanding of the interactions between the host and pathogens in colorectal carcinogenesis requires further microbiota functional studies, especially with respect to metabolomics and RNA sequencing approaches. All of the studies published in this regard have been performed without classifying tumors according to their molecular phenotype. Investigations should also consider the heterogeneity of CRC tumors by studying microbiota imbalances in relation to molecular pathways involved in colorectal carcinogenesis, such as chromosomal and microsatellite instabilities or CpG island methylator phenotypes. In summary, the role of the microbiota in CRC is increasingly evident and perhaps represents a new approach towards the improved therapeutic management of patients with CRC.

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