

WJG 20th Anniversary Special Issues (5): Colorectal cancer**Inflammation and colorectal cancer, when microbiota-host mutualism breaks**

Marco Candela, Silvia Turrone, Elena Biagi, Franck Carbonero, Simone Rampelli, Carla Fiorentini, Patrizia Brigidi

Marco Candela, Silvia Turrone, Elena Biagi, Simone Rampelli, Patrizia Brigidi, Department of Pharmacy and Biotechnology, University of Bologna, 40126 Bologna, Italy

Franck Carbonero, Department of Food Science, University of Arkansas, Fayetteville, AR 72704, United States

Carla Fiorentini, Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, 00161 Rome, Italy

Author contributions: Candela M conceived and designed the article, and wrote the manuscript; Turrone S, Biagi E, Carbonero F, Fiorentini C reviewed literature, wrote the manuscript, revised and edited the draft; Rampelli S performed bioinformatics analysis; Brigidi P reviewed and edited the final version of the article; all Authors read and approved the final version of the manuscript.**Correspondence to:** Marco Candela, PhD, Department of Pharmacy and Biotechnology, University of Bologna, Via Belmeloro 6, 40126 Bologna, Italy. marco.candela@unibo.it

Telephone: +39-51-2099727 Fax: +39-51-2099734

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Abstract

Structural changes in the gut microbial community have been shown to accompany the progressive development of colorectal cancer. In this review we discuss recent hypotheses on the mechanisms involved in the bacteria-mediated carcinogenesis, as well as the triggering factors favoring the shift of the gut microbiota from a mutualistic to a pro-carcinogenic configuration. The possible role of inflammation, bacterial toxins and toxic microbiota metabolites in colorectal cancer onset is specifically discussed. On the other hand, the strategic role of inflammation as the keystone factor in driving microbiota to become carcinogenic is suggested. As a common outcome of different environmental and endogenous triggers, such as diet, aging, pathogen infection or genetic predisposition, inflammation can

compromise the microbiota-host mutualism, forcing the increase of pathobionts at the expense of health-promoting groups, and allowing the microbiota to acquire an overall pro-inflammatory configuration. Consolidating inflammation in the gut, and favoring the bloom of toxigenic bacterial drivers, these changes in the gut microbial ecosystem have been suggested as pivotal in promoting carcinogenesis. In this context, it will become of primary importance to implement dietary or probiotics-based interventions aimed at preserving the microbiota-host mutualism along aging, counteracting deviations that favor a pro-carcinogenic microbiota asset.

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Key words: Colorectal cancer; Inflammation; Gut microbiome; Co-abundance groups**Core tip:** By performing the co-abundance groups analysis of the publicly available datasets from microbiome surveys in colorectal cancer (CRC) patients, we have been successful in identifying pro-carcinogenic and protective groups of microorganisms, showing the potential to modulate the fate of CRC onset and progression. Possible mechanisms involved in microbiota-dependent carcinogenesis are reviewed, and the central role of inflammation as a trigger forcing the microbiota from a mutualistic configuration to a CRC-promoting asset is discussed. Finally, possible intervention strategies for modulating microbiome in order to preserve its mutualistic configuration along life span are suggested.Candela M, Turrone S, Biagi E, Carbonero F, Rampelli S, Fiorentini C, Brigidi P. Inflammation and colorectal cancer, when microbiota-host mutualism breaks. *World J Gastroenterol* 2014; 20(4): 908-922 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v20/i4/908.htm> DOI: <http://dx.doi.org/10.3748/wjg.v20.i4.908>

HUMAN INTESTINAL MICROBIOTA

Structure of the human intestinal microbiota

Outnumbering human cells 10 to 1, over 100 trillion microbes are hosted in the human body, with the majority of them residing in the gut, in a continuum of dynamic ecological communities, referred to as microbiome^[1]. From 10¹ to 10³ microbes per gram of content in the stomach and duodenum, the human gut microbiota reaches a microbial density of 10⁴ to 10⁷ cells per gram in the jejunum and ileum, culminating with 10¹³-10¹⁴ cells in the colon and feces^[2,3].

Metagenomic surveys of the intestinal microbiota revealed an immense phylogenetic diversity, estimating more than 1000 species-level phylotypes across the human population, with at least 160 prevalent species per individual^[4]. While phylogenetic diversity is high at the species level, most of the endogenous bacteria in healthy adults belong to just two phyla, *Firmicutes* and *Bacteroidetes*, which account for > 90% of the known phylogenetic categories of the human gut. Members of *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, *Verrucomicrobia*, *Spirochaetes* and *Lentisphaerae* are regularly present but scarce (< 1%-15%)^[5-9].

Since the first application of culture-independent methods a large inter-individual variability in microbial compositions was apparent^[4], with twins sharing less than 50% of their species-level microbial taxa^[10]. The multiple genetic and environmental factors that contribute to shape the individuality of the gut microbiota composition are now beginning to be understood, reflecting interpersonal, geographical, lifestyle and temporal differences^[11-13], and not least, perturbations caused by disease. Recent work has established that despite the unique fingerprint of microbial taxa per individual, a core of > 50 taxa can be found in nearly half of the human subjects sampled^[4,8]. It has been suggested that individuals can be categorized into one of three predominant variants or “enterotypes” based on the abundance of dominant genera (*Bacteroides*, *Prevotella* or *Ruminococcus*)^[14], though some researchers are now favoring the concept of a continuum or gradient of species functionality rather than a discontinuous variation with segregated types^[15]. Individuals have also been shown to share a set of microbial genes involved in central metabolic pathways, and deviations from this functional core have been associated with altered physiological states^[16]. However, the subject-specific genetic diversity is remarkable and still remains largely unassigned, with a probably unique metagenomic genotype per individual^[17].

Human gut microbiome and role in host physiology

The collective genome of the human intestinal microbiota-microbiome-contains 3.3 million microbial genes, 150-fold more than the human genome^[4]. Adding this immense gene catalogue to host genetics, intestinal

microorganisms are expected to exert a profound influence on human physiology and metabolism. In fact, gut microbes complement several gaps in our metabolic pathways, *e.g.*, producing essential vitamins and oligo-elements, as well as affording the extraction of energy from otherwise indigestible carbohydrates^[18], playing a major role in host energy balance and nutrition^[19]. This function has probably been the initial evolutionary force toward the microbiota establishment as an animal and human symbiotic partner^[20]. Other recognized functions include the support for colonization resistance against incoming enteropathogens. Mechanism involved in this barrier effect are: competition for food resources^[21], inhibition of pathogen growth by means of acetate production^[22], killing with bacteriocins^[23], and immune response stimulation^[24,25]. The gut microbiota also acts as an integral component of the human immune system, finely calibrating the immunological potential and responses at different host ages^[26,27]. The intimate interplay between gut microbes and the mucosal immune system has indeed proved to be crucial for immune education during our infancy as well as for maintaining a well-balanced immune homeostasis along the adult life^[26,28]. Of note, accumulating data are also supporting the emerging concept of a microbiota-gut-brain axis with a role in the regulation of anxiety, cognition, pain and behavior, and a possible contribution to the pathophysiology of central nervous system disorders^[29-32].

Microbiota dynamics in response to diet-inflammation-age

The intestinal microbiota composition was believed to be stable throughout adulthood until few years ago^[33,34]. More recently, with the bloom of longitudinal studies in humans, the plasticity of this ecosystem has become evident, highlighting that diet, environment, and physiological changes can impact on both composition and functionality of the gut microbiota^[12,27]. Faith *et al*^[35] investigated the normal long-term plasticity of the human gut microbiota in healthy subjects. By applying a low-error amplicon sequencing approach, the Authors demonstrated that 40% of the individual microbiota was variable over the time course of 5 years.

The effect of changes in dietary habits is the plainest manifestation of the ability of the microbiota to adapt its architecture in response to environmental stimuli, with the speed and efficacy required for the maintenance of the nutritional function of the host-microbiota symbiosis. Indeed, short-term dietary responses of the microbiota composition were detected after 24 h and seemed to be driven principally by the type of ingested fermentable carbohydrates^[36,37]. These fluctuations could be considered a necessary feature of an intestinal microbial ecosystem able to rapidly adapt itself to the host requirements, maximizing the efficiency of nutrient extraction and supporting health. Remarkably, the same changes in diet in different persons did not result in the same final microbiota configuration since the diet-related variations did not overcome the inter-individual differences. Conversely,

in the long term, people with similar dietary patterns may end up sharing a similar architecture of the gut microbiota; in fact, it has been shown that the presence or absence of several bacterial taxa can be associated with the intake of different nutrients^[37].

Along with the dietary influence, a certain plasticity of the human intestinal ecosystem is being observed in response to less obvious environmental stressors, such as climate and geography^[38,39], as well as the degree of exposure to environmental bacteria, the latter being of primary importance for the education and the maintenance of the functionality of the immune system from birth to adulthood^[40-42]. Moreover, the consumption of drugs, especially antibiotics but also anti-inflammatory medicines, impacts on the gut microbiota composition^[43-45] and different configurations of the microbiota, in turn, have the ability to promote or reduce the metabolization and effectiveness of drugs^[46]. Along adulthood and later in life, natural physiological changes add themselves to the list of drivers of modification in the microbiota structure, both temporarily (*i.e.*, pregnancy or lactation^[47]) and permanently, as in the aging process.

Aging can impact on the gut microbiota structure directly, by means of age-related physiological processes involving local and systemic inflammation (*i.e.*, immunosenescence and inflamm-aging; see below), and indirectly, causing changes in dietary habits and lifestyle^[48]. Increased threshold for taste and smell, together with chewing problems caused by teeth and muscle loss, can lead to the consumption of a restricted diet, poor in fibers and proteins that are known to strongly impact on microbiota composition^[36,37]. Moreover, poor diet and diminished physical activity contribute to increase the chances of constipation and, consequently, of slower intestinal transit time, which may impact on the composition of the colonic microbiota due to the reduced bacterial excretion^[48]. The age-related increased drug consumption^[49] and the interaction between different medicines can also be listed among the possible factors that rule changes in the gut microbiota. The subject-specific combination of all these impacting environmental variables may be responsible for the inter-individual variability of the gut microbiota composition that is known to increase along with aging^[50,51].

The aged-type gut microbiota is typically characterized by a reduced biodiversity, an increased abundance of opportunistic facultative anaerobes, and a decreased abundance of species with anti-inflammatory properties (*i.e.*, *Faecalibacterium prausnitzii* and other butyrate producers)^[7,44,52-55]. Interestingly, these deviations from the healthy adult-like profile overlap with those known to accompany several disorders characterized by systemic and/or chronic inflammation, such as obesity, metabolic syndrome and inflammatory bowel diseases^[21,56,57]. Indeed, aging itself involves chronic immune and inflammatory unbalances. Elderly are generally affected by a process called “immunosenescence” that causes a decline in immune system functionality, and a chronic inflammatory status (“inflamm-aging”) characterizing the whole organism^[58,59]. At the level of the gut, inflamm-aging could be

responsible for an increased stimulation of the inflammatory response, allowing opportunistic pathogens (pathobionts) to thrive to the detriment of symbionts^[60,61]. The age-related proliferation of opportunistic bacteria could both contribute to and be nurtured by inflamm-aging, in a sort of self-sustaining loop^[55], possibly creating a predisposing environment for diseases the risk of which is known to increase along with age, such as colorectal cancer.

INTESTINAL MICROBIOTA AND COLORECTAL CANCER

Colorectal cancer (CRC) is the fourth most commonly diagnosed cancer in the Western world^[62,63]. With more than one million of new cases and 600000 deaths per year, CRC undoubtedly constitutes a significant burden for public health in Western world.

CRC is the result of a multistep process whose progression is associated with the gradual accumulation of genetic and epigenetic mutations. Sporadic in more than 90% of the cases, CRC develops gradually, proceeding from normal epithelium to adenomatous polyps and invasive carcinoma, defining a process that can be slow, taking more than 10 years depending on the mutation frequency^[64]. Several genetic predispositions which can increase cancer risk have been identified. The principal driver mutations involved in CRC include tumor suppressors adenomatous polyposis coli gene, β -catenin gene, deleted in colorectal cancer gene and *p53*^[64], as well as the oncogenes Kirsten rat sarcoma^[65] and myelocytomatosis oncogene^[66,67]. However, even if within the last years a growing number of acquired genetic mutations have been described in CRC, trigger factors leading to their accumulation remain to be determined.

Environmental factors have been reported as the leading causes involved in CRC onset^[68]. Chronic inflammation and diet have been historically recognized as the prominent CRC drivers^[69,70], however, recently, a new potential factor in CRC is emerging: the human intestinal microbiota^[71-73]. For instance, the relevance of a compromised microbiota-host homeostasis in CRC onset has been highlighted by the recent finding that mice defective in the inflammasome function have an increased risk to develop CRC^[74]. While the involvement of diet and inflammation in CRC has been proved by “traditional” observational and epidemiological studies^[70,75-77], only the recent widespread of next-generation sequencing (NGS)-based approaches for gut microbiota characterization allowed to identify characteristic ecosystem changes associated with CRC. Comparative NGS studies of the gut microbiota structure in stools, luminal samples and swabs from CRC patients and age-matched healthy controls have been carried out^[78-80]. With respect to healthy controls, CRC patients were significantly enriched in fecal *Fusobacterium*, *Enterococcaceae*, *Campylobacter*, *Erysipelotrichaceae*, *Collinsella*, *Peptostreptococcus* and *Anaerotruncus*, and depleted in members of the *Clostridium* cluster IV, such as *Faecalibacterium prausnitzii* (*F. prausnitzii*) and *Rose-*

buria. On the intestinal mucosa, CRC patients showed an increase of *Porphyromonas*, *Fusobacterium*, *Peptostreptococcus* and *Mogibacterium*, whereas *Faecalibacterium*, *Blautia* and *Bifidobacterium* were depleted. This CRC-associated microbiome is enriched in pro-inflammatory opportunistic pathogens, e.g., *Fusobacterium*, *Enterococcaceae* and *Campylobacter*^[81-85], and microorganisms commonly associated with metabolic disorders, such as *Erysipelotrichaceae*^[86,87], while depleted in microbial partners strategic to preserve the intestinal homeostasis^[88], such as well-known butyrate producers (i.e., *F. prausnitzii* and *Roseburia*) and protective bifidobacteria^[22,89]. These NGS data reflect an overall pro-inflammatory configuration for the CRC-associated gut microbial ecosystem, which can concur in compromising the microbiota-host mutualism and, eventually, consolidate the disease state. Very recently, comparative analyses of mucosal microorganisms on cancerous tissue and matched non-cancerous tissue have been carried out, allowing to detect microorganisms specifically enriched on CRC tumor sites^[79,84,85]. Cancerous mucosa showed an overall decrease in bacterial diversity with respect to non-cancerous tissues, and was characterized by a reduction in *Faecalibacterium* and higher abundances of *Fusobacterium*, *Bacilli* and *Phascolarctobacterium*. These pro-inflammatory microorganisms can modulate the tumor microenvironment, affecting the course of CRC progression.

In order to explore dysbiosis of the gut microbiota in CRC at the community level, we sought associations between individual genera. To this aim, we obtained co-abundance groups (CAGs), groups of microorganisms which correlate and cluster together, by a bioinformatics analysis^[90] of the publicly available dataset from Wu *et al.*^[80], a well characterized case-control study of the CRC-associated microbiome. Six CAGs displaying significantly different inter-relationships from each other ($P < 0.001$) have been identified: *Fusobacterium* CAG, *Prevotella* CAG, *Barnesiella* CAG, *Coprobacillus* CAG, *Faecalibacterium* CAG and *Bifidobacterium* CAG. Significant associations between bacterial genera have been calculated and represented in a Wiggum plot (Figure 1). This network analysis allowed us to describe - to our knowledge for the first time - microbial co-abundance networks which include microorganisms previously associated with CRC risk or protection. According to our analysis, the CRC-associated microorganisms *Fusobacterium* and *Erysipelotrichaceae* belong to the same CAG (*Fusobacterium*). Analogously, CRC-associated groups as *Enterobacteriaceae*, *Escherichia*, *Shigella* and *Klebsiella* co-vary within the same cluster (*Prevotella*). On the other hand, a common CAG (*Bifidobacterium*) is shared by non-CRC-associated groups as *Bifidobacterium* and *Lachnospiraceae* (a family member of the *Clostridium* cluster IV). Other health-promoting mutualists belonging to the *Clostridium* cluster IV, such as *Faecalibacterim*, *Blautia*, *Roseburia*, *Dorea* and *Lachnospiraceae*, group together in *Faecalibacterim* CAG. Finally, we identified one CAG (*Barnesiella*) including both pro-carcinogenic microorganisms as *Porphyromonadaceae* and *Eubacterium*, as well as protective members of the *Clostridium* cluster IV (*Ruminococcus*, *Butyrococcus* and *Oscillibacter*). Even if data from this computational

analysis must be taken with caution since based on a limited dataset, we can hypothesize the existence of 3 pro-carcinogenic CAGs (*Fusobacterium* CAG, *Prevotella* CAG and *Coprobacillus* CAG) and 2 CRC protective CAGs (*Bifidobacterium* CAG and *Faecalibacterium* CAG).

Suggesting the involvement of specific microbiota dysbiosis in CRC, NGS-based microbiome studies are imposing a more holistic vision of the interplay between environment and genetics in CRC, where dietary factors and inflammation need to be considered against the background in the microbiota-host interaction process (Figure 2). However, the static nature of these studies did not permit to comprehend whether dysbiosis are a cause or a consequence of the disease onset. Further, these descriptive studies did not provide information on either the mechanisms by which members of the gut microbial ecosystem can influence the CRC, or, more importantly, the triggers that shift the microbiota towards a carcinogenic configuration. With the attempt to deal with these questions, a new approach to study the role of microorganisms in CRC onset is emerging. Pairing NGS-based microbiota surveys and the usage of germ-free (GF), conventionalized and mono associated mice to test mechanistic hypotheses, new insights on the microbial ecology of CRC have been provided^[73].

Bacterial driver-passenger model

Recently, a first dynamic model of the microbial ecology involved in CRC onset and progression has been proposed by Tjalsma *et al.*^[73]: the bacterial driver-passenger model. According to this model, CRC development is initiated by indigenous bacteria with pro-carcinogenic features - defined as bacterial drivers - that drive epithelial DNA damage and contribute to CRC initiation. In a subsequent step, the local microenvironment is altered as a consequence of the ongoing tumorigenesis and bacterial drivers are replaced by bacterial passengers, microorganisms showing a competitive advantage in the tumor microenvironment and being capable of nurturing tumor progression. For instance, nutrients and co-factors specific of the tumor microenvironment - such as the presence of reactive oxygen species - can be selectively utilized by specific bacterial passengers^[91].

Bacterial drivers are defined as intestinal bacteria showing pro-carcinogenic features - either transient or autochthonous microbiota components - that may initiate the process of carcinogenesis. Several candidate bacterial drivers have been identified (Table 1), such as superoxide-producing strains of *Enterococcus faecalis*^[92], genotoxin-producing *Escherichia coli* strains^[93], and toxigenic strains of *Bacteroides fragilis*^[94]. Furthermore, pro-inflammatory members of *Enterobacteriaceae*, such as *Shigella*, *Citrobacter* and *Salmonella* have been associated with early stages of CRC as possible bacterial drivers^[95,96]. Occasionally, bacterial drivers act in concert with helper bacteria (or α -bugs) in carcinogenesis promotion^[97]. Generally belonging to pro-inflammatory *Enterobacteriaceae*, these microorganisms are proposed to crowd out symbiont CRC-protecting anti-inflammatory microbiota components, such as *F.*

Table 1 Microorganisms involved in colorectal cancer

| Microorganism | Role in CRC | Mechanism | Ref. |
|---------------------------|-------------|---|-------|
| <i>E. faecalis</i> | Driver | Production of superoxide | [92] |
| <i>E. coli</i> NC101 | Driver | Genotoxin production (colibactin) | [122] |
| <i>B. fragilis</i> | Driver | Genotoxin production (fragilisin) | [94] |
| <i>Shigella</i> | Driver | Induction of inflammation | [73] |
| <i>Citrobacter</i> | Driver | Induction of inflammation | [73] |
| <i>Salmonella</i> | Driver | Induction of inflammation | [73] |
| <i>Enterobacteriaceae</i> | Helper | Induction of inflammation | [73] |
| <i>Fusobacterium</i> | Passenger | Induction of inflammation | [84] |
| <i>S. gallolyticus</i> | Passenger | Induction of inflammation | [98] |
| <i>C. septicum</i> | Passenger | Induction of inflammation | [99] |
| <i>F. prausnitzii</i> | Protective | Butyrate production; anti-inflammatory properties | [78] |
| <i>Roseburia</i> | Protective | Butyrate production; anti-inflammatory properties | [78] |
| <i>Bifidobacterium</i> | Protective | Protection from pathogens; anti-inflammatory properties | [71] |
| <i>Corynebacteriaceae</i> | Protective | Anti-inflammatory properties | [78] |

Microorganisms involved in colorectal cancer (CRC), their role as driver, passenger or protective bacteria and the mechanisms involved in CRC induction or protection. *E. faecalis*: *Enterococcus faecalis*; *E. coli*: *Escherichia coli*; *B. fragilis*: *Bacteroides fragilis*; *S. gallolyticus*: *Streptococcus gallolyticus*; *C. septicum*: *Clostridium septicum*; *F. prausnitzii*: *Faecalibacterium prausnitzii*.

prausnitzii, *Roseburia* or *Bifidobacterium*, favoring the subsequent tissue colonization by drivers.

Passenger bacteria are always autochthonous members of the gut microbial community. Relatively poor colonizer of a healthy intestinal tract, passengers show a competitive advantage in the tumor microenvironment (Table 1). However, differently from drivers, which are always pro-carcinogenic, passenger bacteria can be of either pro-carcinogenic or protective nature, depending on the microorganism. While in some cases the carcinogenic tissue has been shown to be selectively colonized by opportunistic pathogens, such as *Fusobacterium*^[78,83,84], *Streptococcus gallolyticus*^[98] and *Clostridium septicum*^[99], which can be involved in CRC progression, in other circumstances the tumor sites were enriched in passenger bacteria belonging to well-known mutualistic microbiota components, as *Corynebacteriaceae*, *Roseburia* and *Faecalibacterium*, suggesting a possible protective role for these microorganisms as CRC quencher^[78].

Mechanisms possibly involved in microbial CRC promotion

Gut microorganisms may promote CRC onset and progression by different processes (Table 1)^[71], such as (1) the induction of a chronic inflammatory state; (2) the biosynthesis of genotoxins interfering with the cell cycle regulation or directly damaging DNA; (3) the production of toxic metabolites; and (4) the activation of dietary heterocyclic amines to pro-carcinogenic compounds. Here we will specifically discuss the role of three of these factors - inflammation, genotoxins and toxic metabolites - in CRC onset and progression.

Chronic inflammatory disorders are associated with a higher risk of cancer development^[100]. Inflammation can nurture carcinogenesis by inducing gene mutations, inhibiting apoptosis or stimulating angiogenesis and cell proliferation. By regulating cell survival, inflammation and immunity, nuclear factor (NF)- κ B is at the connection between inflammation and cancer. In particular, experi-

ments carried out in mouse models of colitis-associated cancer have been successful in demonstrating a dual role for NF- κ B in carcinogenesis, which depends on the cell type^[101]. While in enterocytes NF- κ B contributes to tumor initiation by suppressing apoptosis, in myeloid cells it is involved in the promotion of tumor growth by means of the production of inflammatory mediators. Further, it has been recently demonstrated that elevated NF- κ B signaling can activate mutations in the Wnt pathway, leading to the differentiation of epithelial non-stem cells into tumor-initiating cells^[102]. Generally, the activation of NF- κ B results in the expression of inflammatory cytokines [e.g., tumour necrosis factor-alpha, interleukin (IL)-1, IL-6 and IL-8], adhesion molecules, enzymes involved in prostaglandin synthesis, nitric oxide synthase, angiogenic factors and anti-apoptotic genes, providing survival advantages to precancerous or tumor cells in the gut^[75,103]. The activation of NF- κ B as a result of microbial sensing *via* the host Toll-like receptors (TLRs) has been proposed to support intestinal tumor growth under steady-state conditions^[104,105]. Several evidences have been reported in support of the role of the gut microbiota in the inflammation-dependent carcinogenesis in the gut. Crohn's disease and ulcerative colitis are often associated with an increased risk of developing CRC and epidemiological data suggest that duration and severity of chronic colitis represent a significant risk factor for colitis-associated CRC^[106,107]. Furthermore, microbiota unbalances in favor of pro-inflammatory opportunistic pathogens as *Enterobacteriaceae* and *Clostridium difficile* have been indicated to be involved in tumor progression^[108,109] and, in the context of the bacterial driver-passenger model, several bacterial drivers, such as *Shigella*, *Citrobacter*, *Salmonella* and toxigenic *Bacteroides fragilis* (*B. fragilis*), as well as the passengers *Fusobacterium* and *Streptococcus gallolyticus* and *Clostridium septicum*, have been reported to support carcinogenesis by the induction of a pro-inflammatory response^[73]. Strikingly, by inducing azoxymethane (AOM)-colitis in conventional and GF IL-10 knockout (*Il10^{-/-}*) mice, Uronis *et al.*^[110] were

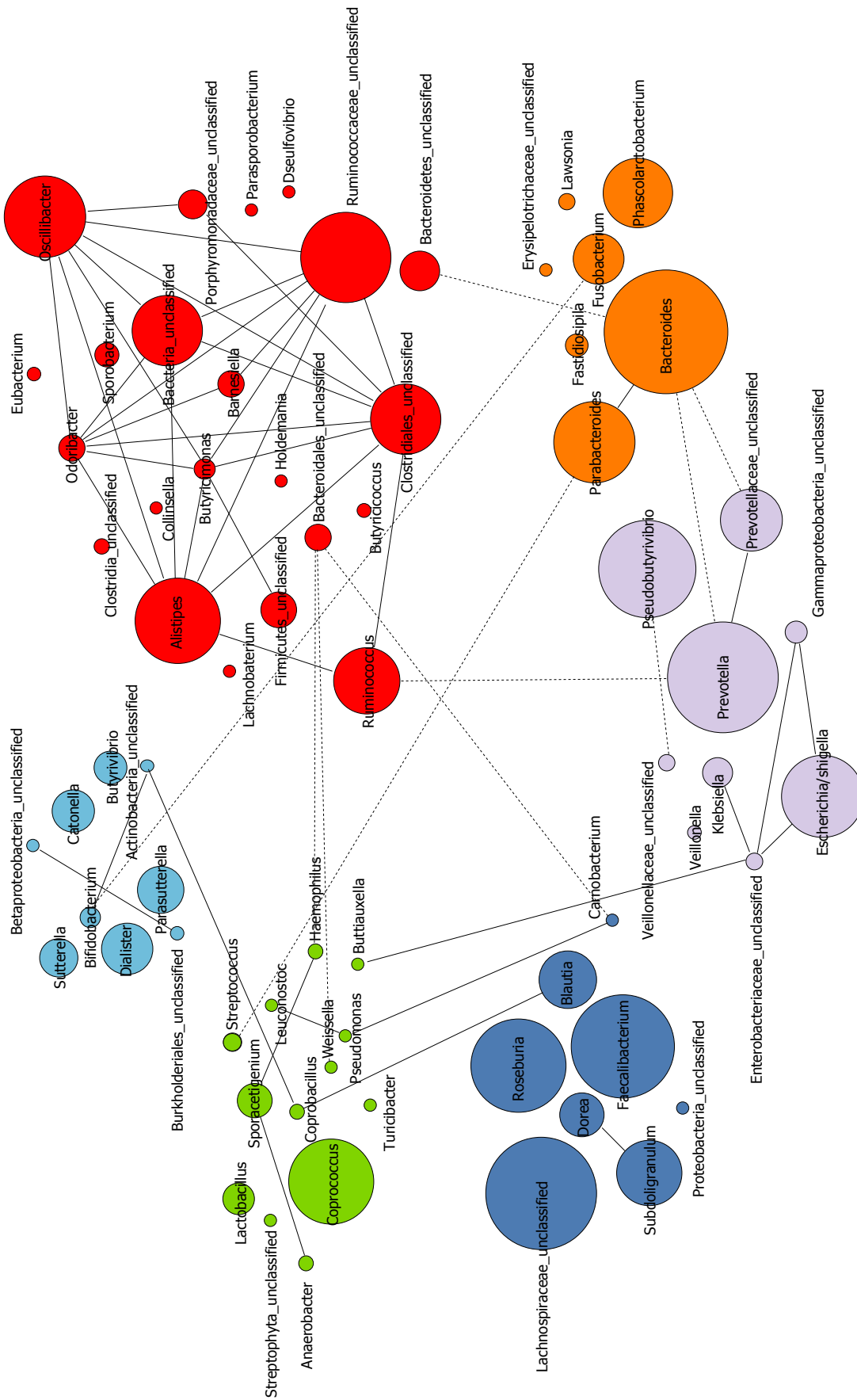


Figure 1 Network plot showing correlation relationships among clusters of bacterial genera for the Wu *et al*⁽⁹⁰⁾ dataset. Each node represents a taxon co-abundance group and each line highlights a significant correlation between two bacterial genera. Circle size is proportional to genus abundance. Solid lines indicate positive correlation, whereas dot lines indicate negative correlation. Thickness of the lines is proportional to correlation strength.

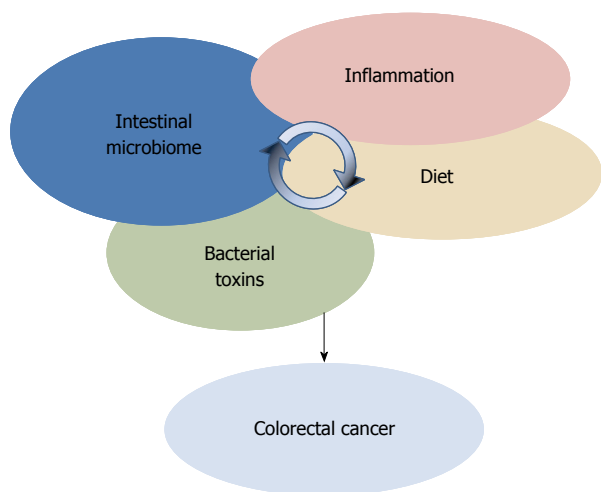


Figure 2 Colorectal cancer arises from the interplay between endogenous and exogenous factors, such as inflammation, diet, intestinal microbiome structure, and transcription and activity of bacterial genotoxins.

successful in demonstrating that microbial sensing *via* TLRs is essential to develop colitis-associated CRC.

Inflammation also represents a molecular link between host immune response, intestinal microbiota and genotoxic events in the inflammation-associated CRC^[111]. Several bacterial taxa that belong to the human gut microbiome in a subset of the healthy population contain toxin-producing strains^[5]. The long-term effects of chronic exposure to low doses of such bacteria as well as the eventual contribution to the carcinogenic process of bacterial toxins remain to be elucidated. Toxins impinge on key eukaryotic processes, such as cellular signaling, and some directly attack the genome^[112] these last by damaging DNA, either directly, by enzymatic attack, or indirectly, by provoking an inflammatory reaction that produces free radicals. Also, they can affect DNA repair mechanisms.

The capacity of the *B. fragilis* toxin (BFT)-producing strains to promote colon tumorigenesis is mediated by the increased expression of STAT3 that leads to the recruitment of the highly pro-inflammatory subset of T helper type 17 lymphocytes, suggesting that the pro-carcinogenic role of BFT is to promote a de-regulated inflammatory response^[113]. BFT is a metalloprotease known to bind to colonic epithelial cells and stimulate cleavage of E-cadherin, thus increasing intestinal barrier permeability and augmenting cell signalling *via* the β -catenin/Wnt pathway, which is constitutively activated in essentially all CRC. As a result, BFT stimulates proliferation and migration of human colon cancer cells *in vitro*^[114]. It is worth noting that the enterotoxigenic form of *B. fragilis* (ETBF) is only present in approximately 10%-20% of the healthy population whereas the fecal carriage of ETBF is increased of about 40% in CRC patients^[94,115].

However, although the *B. fragilis* toxin has been proposed as one of the main CRC driving suspects on the basis of experimental work^[113,116], very recent studies show that the most actively transcribed toxins in tumor

tissue and surrounding mucosa from CRC patients are those derived from *Escherichia coli* (*E. coli*), *Salmonella enterica* and *Shigella flexneri*. This suggests a strong involvement of enterobacterial toxins in tumorigenesis. Also in this context, inflammation has been shown to increase toxigenic *E. coli* strains, promoting their adhesion to the host epithelia^[111]. A number of *E. coli* strains produce a wide array of toxins, some of which are turning out to be potentially harmful in humans, either directly damaging DNA or specifically disrupting cell signaling.

The cytolethal distending toxins (CDTs), which comprise a family of intracellular-acting bacterial protein toxins produced by several gram-negative bacteria, belong to the first group. Their activity upon eukaryotic cells results in several consequences, the most characteristic of which is the induction of G(2)/M cell cycle arrest^[117]. Active CDTs consist of three subunits: CdtA and CdtC, which guide internalization, and CdtB, which enzymatically induces DNA double-strand breaks that recruit and activate the ataxia telangiectasia mutated kinase, thus triggering a DNA damage response (DDR). The DDR provides an efficient barrier to tumorigenesis through induction of cell death or senescence^[118]. Cells exposed to sub-lethal doses of the CDTs from *Helicobacter hepaticus* (*H. hepaticus*) or *Haemophilus ducreyi* exhibit increased frequency of mutations, accumulation of chromosomal aberrations and enhanced anchorage-independent growth^[119]. Furthermore, chronic infection of mouse liver and intestine with CDT-producing *H. hepaticus* or *Campylobacter jejuni*, respectively, is associated with dysplasia^[119], confirming the capacity of CDT-producing bacteria to induce pre-neoplastic lesions *in vivo*. Very recently, Buc *et al.*^[120] demonstrated a high prevalence of genotoxin- and cyclomodulin-producing mucosa-associated *E. coli* strains in CRC patients.

Furthermore, some commensal *E. coli* strains of the phylogenetic group B2 harbour a 54 kb polyketide synthase (pks) pathogenicity island encoding the enzymes required for the synthesis of a putative hybrid peptide-polyketide genotoxin, named colibactin^[121]. Infection of mice with a pks+ *E. coli* strain has been linked to the expression of pks genes required for colibactin production as well as to DNA damage induction^[122]. The capacity of colibactin to promote tumorigenesis *in vivo* has been recently proven in an animal model of colitis-associated CRC. GF IL-10 knockout mice treated with the colon-specific carcinogen AOM and monocolonized with pks+ *E. coli* showed a high incidence of invasive adenocarcinoma if compared to mice infected with an isogenic pks-deficient strain or the control commensal bacterium pks-*E. faecalis*^[93]. The detection of *E. coli* isolates carrying the pks island in 66.7% CRC patients compared to 20% found in non-inflammatory bowel disease/non-CRC controls suggests a concerted action of host inflammation and *E. coli*-derived pks in giving rise to a host microenvironment that promotes DNA damage and tumorigenesis^[93]. These authors also showed that optimal colonization by colibactin-producing *E. coli* strains is established in an

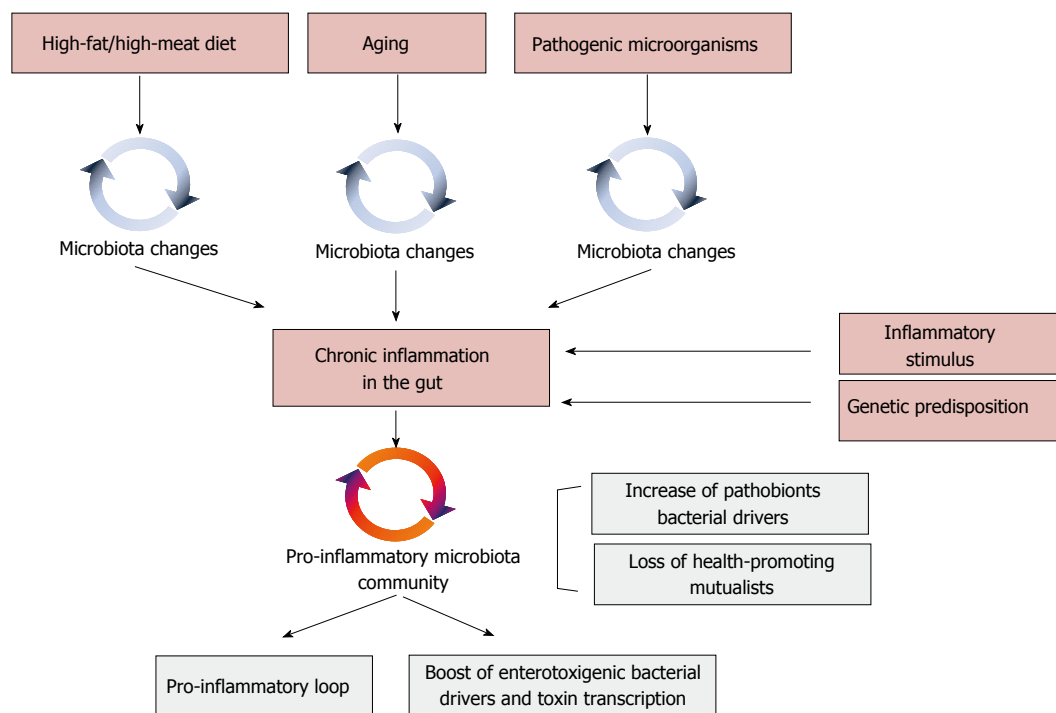


Figure 3 Environmental triggers, such as diet, aging and pathogen infections, can force microbiota changes that, in a genetically susceptible host, can drive to chronic inflammation in the gut. Inflammation shifts the gut microbiota towards a pro-inflammatory configuration, supporting colorectal cancer (CRC) drivers as pathobionts at the expense of health-promoting CRC-protective microbiota components. As a consequence, a pro-inflammatory loop is established in the gut, directly supporting CRC onset and favoring colonization by toxigenic bacterial drivers directly involved in CRC promotion.

already-inflamed gut. In fact, by remodeling the intestinal immune response and shifting the colonic bacterial community to one that further promotes CRC, bacterial drivers permit the colonization of colibactin-producing *E. coli* strains that actively contribute to disease progression.

A second group of toxins includes those disrupting the cell signaling that regulates cell proliferation or induces inflammation. The *E. coli* cytotoxic necrotizing factor 1 (CNF1), which is expressed by many human isolates, activates the Rho GTPases^[123], inducing dysfunctions in already transformed epithelial cells, such as apoptosis counteraction, pro-inflammatory cytokines' release, COX2 expression, NF- κ B activation and boosted cellular motility. Also, CNF1 induces quiescent cells to enter the cell cycle and undergo DNA synthesis^[124], interferes with normal cytokinesis, resulting in the production of multinucleated cells and in the onset of aneuploidy. As cancer may arise when the same regulatory pathways are affected, it is conceivable that CNF1-producing *E. coli* infections can contribute to cancer development^[125]. Our hypothesis is that these bacteria may act as passengers, reinforcing and favoring but not causing the development of colorectal cancer. The pro-inflammatory capacity of CNF1 has recently been confirmed in *Drosophila*, where the toxin could activate one of the key transcription factors of the innate immune response, namely NF- κ B, independently of the triggering of pathogen recognition receptors. Indeed, the CNF1-mediated activation of the Rac2 GTPase triggers protective immunity *via* the innate Rip kinase signaling that functions upstream of NF-

κ B^[126]. Taken altogether, these data support the strategic role of toxigenic *E. coli* strains in CRC onset and progression.

The gut microbiome is a major driver in shaping the gut metabolome^[127]. Among microbial metabolites, several have been identified as potentially important carcinogens or protective. Secondary bile acids in particular have been detected in elevated levels in CRC patient stools and have been shown to have carcinogenic properties *in vitro*^[128]. A long list of other metabolites are suspected at varying degrees to be implicated in CRC development, such as hydrogen sulfide^[129], proteolysis products (ammonia, amines, phenols)^[130], and acetaldehyde^[131]. Butyrate is the most sought-after beneficial metabolite as it is a major energy source for colonocytes and more importantly has an anti-proliferative activity and induces apoptosis of CRC cells *in vitro*^[132].

Triggering factors that force microbiota to become carcinogenic

Besides its role in CRC onset, inflammation surely exerts a central role in triggering the carcinogenic potential of the gut microbial ecosystem (Figure 3)^[93]. Experiments relying on mice defective in components of the immune system successfully demonstrated that chronic inflammation alters the intestinal microbial community composition towards a configuration that predisposes to the disease^[133]. According to Garrett *et al.*^[134], *Tbet*^{-/-}/*Rag2*^{-/-} mice, which are deficient in adaptive and innate immune function, developed a colitis phenotype transmissible

to wild-type mice by the adoptive transfer of their gut microbiota. Analogously, mice lacking the bacterial flagellin receptor TLR5 exhibited a syndrome encompassing insulin resistance, hyperlipidemia, and increased fat deposition associated with microbiota alterations. Strikingly, these metabolic changes were transferable to wild-type mice by acquiring the *Tlr5*^{-/-} gut microbiota^[134]. In this context, Arthur *et al*^[93] specifically demonstrated that intestinal inflammation can boost the cancer-inducing activity of the gut microbiota. According to the Authors, chronic inflammation in *Il10*^{-/-} mice was sufficient to prompt microbiota shifts, supporting the AOM-induced carcinogenesis. Favoring the adhesion of driver bacteria with genotoxic potential to the colonic mucosa - as well as the overall expansion of pro-inflammatory *Enterobacteriaceae* in the gut - inflammation creates the environment that supports a bacteria-mediated carcinogenesis process. In particular, Arthur *et al*^[93] showed that chronic colitis in *Il10*^{-/-} mice was sufficient to favor a dramatic expansion of *E. coli* NC101 on the intestinal mucosa. Harboring a pks pathogenicity island, *E. coli* NC101 codes for the genotoxin colibactin^[121] that allows this microorganism to accelerate progression from dysplasia to invasive carcinoma. Inflammation in the gut is also pivotal to initiate a microbiota-dependent pro-inflammatory loop detrimental for host health^[71]. An aberrant inflammatory response in the gut can shift the balance between protective mutualists and pathobionts in favor of the latter^[135,136]. By inducing a pro-inflammatory loop, these microorganisms can work as bacterial drivers, consolidating the inflammatory state^[28] and resulting in a self-sustained pro-inflammatory response that affects the microbial ecology of the human gut, further compromising the microbiota-host mutualism and supporting CRC.

Abnormal dietary inputs can lead to the expansion of pro-inflammatory microbes in the gut^[137]. For instance, a diet rich in saturated milk fat has been reported to induce the expansion of *Bilophila wadsworthia*, which may favor carcinogenesis in the gut by promoting pro-inflammatory Th1^[138]. Indeed, high-fat diet impacts on gut microbiome have seen increased interest in the recent years as fat has been linked epidemiologically to intestinal inflammation and diseases. While as expected a high-fat diet modifies the microbiome, the fact that different fat compositions induced different changes in animal models calls for a more controlled dietary intervention in humans. For example, observational data suggested that Western diet (protein- and fat-enriched) and African diet (polysaccharide-enriched) drive strikingly different microbiomes, possibly explaining different CRC rates^[13,38,139]. Reciprocal diet exchanges indeed demonstrated that the microbiome and metabolome were rapidly responsive towards respective “beneficial” and “detrimental” states, as well as markers of mucosal proliferation^[13].

The process of human aging has a well-documented impact on the gut microbiota structure^[48,140], raising the question of whether age-related microbiota dysbioses can trigger a microbiota-dependent carcinogenic process in the gut. Showing a pro-inflammatory configura-

tion, the aged-type gut microbial ecosystem can force a microbiota-dependent pro-inflammatory loop in the gut, compromising the microbiota-host mutualism and supporting carcinogenesis. Strengthening this hypothesis, the incidence of CRC has been reported to increase in the elderly; about 50% of the Western population develops colorectal polyps at the age of 70 and 5% of these polyps progress to cancer^[141].

The pervasive role of genotoxins in CRC onset and progression, led researchers to investigate triggering factors that govern toxin biosynthesis and activity. Environmental changes in the gut ecosystem, such as changes in pH, in oxygen availability or the presence of a specific metabolite, have been suggested to have a role in the modulation of toxin transcription. Intriguingly, interspecies quorum sensing resulting from microbe/microbe interaction processes has been suggested to play a role in governing bacterial toxin production in the gut^[72,142]. Even if research in this field is still in its infancy, recent experimental works demonstrated the strategic role of microbe/microbe, microbe/host and microbe/environment interaction processes in regulating bacterial virulence and toxin activity. In a recent experimental research based on GF and conventional mice, Kamada *et al*^[143] demonstrated that changes in dietary substrates can result in a microbiota-dependent regulation of virulence factors. According to the Authors, dietary changes can boost commensals capable to outcompete toxigenic pathogens for food sources, resulting in the down-regulation of virulence genes and eventually pathogen clearance. Further, Marks *et al*^[144] demonstrated that interkingdom signaling as a result of the host response to the influenza A virus infection was sufficient to trigger the expression of *Streptococcus pneumoniae* virulence genes, resulting in the transition from commensalism to pathogenicity. Even if *S. pneumoniae* is a common human nasopharyngeal opportunistic bacterium, these findings allow us to hypothesize the existence of analogous processes in the gut ecosystem, resulting in the activation of a virulence phenotype and toxin transcription of enterotoxigenic CRC drivers.

CONCLUSION

The worldwide diffusion of NGS-based microbiota surveys in CRC patients, alongside the utilization of GF, mono associated and humanized mice, led to an increasing perception of the pivotal role exerted by the gut microbiota in CRC onset and progression. Lights on the microbial ecology of the process have been provided, and possible mechanisms involved suggested. This brought the researchers to focus their attention on triggering factors that turn the intestinal microbiota from a mutualistic configuration to a CRC-promoting asset. Inflammation has undoubtedly a central role in this process, being a common outcome shared by different triggering factors, such as diet, aging, microbe-microbe and microbe-host interactions (Figure 3). In fact, changes in diet, aging, as well as pathobiont-dependent pro-inflammatory dysbiosis of the gut microbiota, can force the gut microbiota to

a pro-inflammatory asset, changing the microecology of the gut ecosystem and activating toxigenic CRC bacterial drivers. In this context, of extraordinary importance will be the development of strategies able to interfere and/or block these triggering factors, preserving the microbiota-host mutualism along the entire life span. Different approaches can be implemented. Since diet represents the pivotal strategy to modulate composition and functionality of the gut microbiota, the most promising approach to preserve microbiota-host mutualisms relies on dietary interventions. For instance, diet can be modulated to boost health-promoting microbiota groups, such as anti-inflammatory members of the *Clostridium* cluster IV or short chain fatty acid producers of the *Clostridium* cluster XIVa. Strengthening this perspective, in a life-long longitudinal study carried out in mice, Zhang *et al.*^[145] demonstrated that different diets modulated differently the microbiome trajectories along with aging. In particular, according to the Authors low-fat diet and caloric restriction increased the relative abundance of phylotypes positively associated with the life span in the middle-life, and, at the same time, lowered the abundance of opportunistic pro-inflammatory pathogens, which could represent CRC bacterial drivers.

A second approach for CRC prevention surely relies on the usage of probiotic bacteria, such as *Bifidobacterium* and *Lactobacillus*. Probiotics have been demonstrated to be effective in reducing CRC risk in humans^[146-149]. Showing immunomodulating properties, antimicrobial activities, as well as the capacity to interfere with toxin synthesis and activity, probiotic bacteria can act simultaneously on different CRC triggering factors. In fact, probiotics have been reported as effective in quenching host inflammatory response^[150], in inhibiting the colonization of known CRC drivers^[22,151] and in inactivating bacterial toxins^[152] or interfering with their production^[153,154].

Even if significant steps forward have been carried out, we are still far from fully appreciating the multifactorial role of the intestinal microbiota in CRC. More longitudinal microbiome surveys need to be carried out, and intestinal polyps as well as adenocarcinoma tissues must be sampled, in order to follow the gut microbiota dynamics over time for the development of colonic neoplasia. Microbiota on tumor sites needs to be compared with off-tumor matched tissues, as a better comparison than mucosal samples from healthy patients. Associations between structure and dynamics of the gut microbiome and the different stages of colonic neoplasia need to be better defined, causality should be further explored, possibly by using GF, mono associated as well as humanized animal models. Finally, meta-analysis integrating epidemiological studies with microbiome datasets will allow us to better define triggering factors that force the microbiota to become carcinogenic, so that hypotheses can be verified in mice where possible intervention strategies can be tested.

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