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Review

Metastasis and the Microbiome: The Impact of Bacteria in Disseminated Colorectal Cancer

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Abstract

Metastasis remains a leading cause of mortality for patients with solid tumors. An expanding body of literature suggests interplay between the host, gut, and tumoral microbiomes may play a role in cancer initiation and distant dissemination. These associations have been particularly well-studied in colorectal cancer, where gut dysbiosis and an endotoxin-induced inflammatory milieu foster premalignant polyp formation, setting the stage for carcinogenesis. Subsequent violation of the gut vascular barrier enables dissemination of bacterial agents to sites such as the liver, where they contribute to establishment of pre-metastatic niches, which promote tumor cell extravasation and metastatic outgrowth. Intriguingly, breakdown of this vascular barrier has been shown to be aided by the presence of tumoral bacteria. The presence of similar species, including *Fusobacterium nucleatum* and *Escherichia Coli*, in both primary and metastatic colorectal tumors, supports this hypothesis and their presence is associated with chemotherapy resistance and an overall poor prognosis. Specific gut microbial populations are also associated with differential response to immunotherapy, which has a growing role in microsatellite unstable colorectal cancers. Recent work suggests that modulation of gut microbiome using dietary modification, targeted antibiotics, or fecal microbiota transplantation may improve response to immunotherapy and oncologic outcomes. Elucidation of the precise mechanistic links between the microbiome and cancer dissemination will open the doors to additional therapeutic possibilities.

Keywords: colorectal cancer; microbiome; metastasis

1. Introduction

Approximately 90% of cancer-related mortality can be attributed to metastatic disease [1,2]. The metastatic cascade is a complex, multifaceted process that involves interplay between the primary tumor, tumor microenvironment, host immune system, and target organ. The microbiome, comprised of the collective bacteria, viruses, and fungi in a person's body, plays an underappreciated role in this process. The average human hosts over 38 trillion bacteria and up to ten times as many viral particles, far exceeding the number of human cells [3–5]. With this colossal biomass, it is no surprise that microbial agents have been implicated in up to 20% of malignancies [6]. Advances in next generation sequencing technology and data processing algorithms have enabled an increasingly thorough characterization of the cancer-associated microbiome. Given the extensive study of the impact of the gut and tumoral microbiome on carcinogenesis, dissemination, and metastatic outgrowth in colorectal cancer (CRC), this will be our focus of review.

2. The Microbiome and Primary Tumor Initiation

The colon and rectum contain the highest microbial density and diversity of all human organ systems. While the precise components of a "healthy" colorectal microbiome have not been defined, both culture-based methods and 16S rRNA gene sequencing have identified Bacteroides, Proteobacteria, Firmicutes, Verrucomicrobia, and Actinobacteria as the most common phyla in the normal human gastrointestinal tract [7–9]. Importantly, these gut microbial populations are dynamic, with numerous environmental factors shaping the gut microbiome including diet, antibiotic use, exposure to chemicals or toxins, and social interactions [10–14]. Imbalance in gut microbiome composition and function, termed dysbiosis, has been linked to the pathogenesis of multiple diseases, including colorectal cancer [15–17].

The relative overabundance of specific bacterial taxa in a dysbiotic colon have been linked to colorectal tumor initiation (Table 1, Ref. [18–38]). *Escherichia coli* was one of the earliest microorganisms linked to CRC. In 1998, Swidsinski and colleagues [18] using 16S rRNA sequencing in combination with a gentamicin protection assay, identified intracellular *E. coli* in colonic adenomas not present in healthy colorectal epithelium. This finding was replicated in colorectal tissues from patients with Crohn's disease and CRC by Dejea *et al.* [19] using dithiothreitol mucolysis followed by bacterial culture to show *E. coli*'s ability to penetrate the mucus layer and adhere to the underlying colonic epithelium. They also treated tumor cells with gentamycin to deplete the extracellular microbiota and identified intracellular *E. coli* in Crohn's and CRC epithe-

Table 1. Select studies evaluating links between specific bacterial taxa and colorectal cancer.

Study	Subject of study	N (human patients)	Findings
Eschericia coli			
Swidsinski, *et al.* 1998 [18]	Patients	65 healthy controls 29 with adenomas 31 with CRC	Intracellular *E. coli* identified in patients with colonic adenomas and CRC but not healthy controls.
Martin, *et al.* 2004 [33]	Patients, cell lines	14 with Crohn's disease 21 with ulcerative colitis 24 healthy controls 21 with CRC	Mucosa-associated and intramucosal bacteria more commonly cultured from CRC and Crohn's disease, of which *E. coli* accounts for majority of isolates.
Arthur, *et al.* 2012 [20]	Patients, mouse model	21 with CRC 35 with IBD 24 healthy controls	Colonization with pks+*E. coli* promotes CRC in colitis-susceptible mice. Mucosa-associated pks+*E. coli* enriched in patients with CRC and IBD.
Dejea, *et al.* 2018 [19]	Patients, mouse model	25 with FAP 23 healthy controls	*E. coli*-derived colibactin enriched in colonic mucosa of FAP patients. Mice co-colonized with pks+*E. coli* and ETBF exhibit faster tumor growth.
Bacteroides fragilis			
Wu, *et al.* 1998 [22]	Mouse model, cell line	N/A	*B. fragilis* toxin cleaves E-cadherin, producing morphologic changes dependent on target-cell ATP.
Wu, *et al.* 2009 [21]	Mouse model	N/A	ETBF, but not NTBF, triggers colitis and tumor growth in mice through Stat3 activation and mediated by a T_H17 response.
Purcell, *et al.* 2017 [34]	Patients	150 undergoing colonoscopy	ETBF associated with pre-cancerous colonic lesions and more common in descending colon biopsies.
Dejea, *et al.* 2018 [19]	Patients, mouse model	25 with FAP 23 healthy controls	ETBF toxin enriched in colonic mucosa of FAP patients. Mice co-colonized with pks+*E. coli* and ETBF exhibited faster tumor growth.
Chung, *et al.* 2018 [35]	Mouse model, cell line	N/A	IL-17, NF-κB, and Stat3-mediated inflammation triggered by ETBF induces myeloid-cell dependent colon tumorigenesis.
Fusobacterium nucleatum			
Kostic, *et al.* 2013 [24]	Patients, mouse model	20 healthy controls 29 with adenomas 27 with CRC	Fusobacterium spp. are enriched in adenomas and stool from adenoma and CRC patients. *F. nucleatum* increases tumor growth through myeloid cell recruitment and creation of a proinflammatory microenvironment.
Rubinstein, *et al.* 2013 [23]	Patients, mouse model, cell lines	14 healthy controls 16 with adenomas 19 with CRC	The FadA adhesin produced by *F. nucleatum* binds E-cadherin and activates oncogenic signaling via β-catenin. High FadA levels are associated with adenomas and CRC.
Bullman, *et al.* 2017 [32]	Patients, mouse model, cell lines	833 with CRC from multiple cohorts	*F. nucleatum* and associated taxa (*Bacteroides*, *Selenomonas*, and *Prevotella*) are maintained in CRC metastases. Metronidazole reduces *Fusobacterium* load and tumor growth in mice.
Yu, *et al.* 2017 [30]	Patients, mouse model, cell lines	16 with recurrent CRC 15 with non-recurrent CRC	*F. nucleatum* is associated with recurrence post-chemotherapy and promotes chemotherapy resistance through activation of autophagy.
Yang, *et al.* 2017 [36]	Patients, mouse model, cell lines	105 with CRC	*F. nucleatum* increases CRC proliferation and invasion *in vitro*. *F. nucleatum*-induced TLR4 signaling leads to increased miR21. High levels of *F. nucleatum* DNA and miR21 associated with shorter OS in patients.
Serna, *et al.* 2020 [37]	Patients	143 with rectal cancer	*F. nucleatum* persistence after neoadjuvant chemoradiation associated with decreased $CD8^+$ T cell induction and increased risk of recurrence.

Table 1. Continued.

Study	Subject of study	N (human patients)	Findings
Chen, *et al*. 2022 [25]	Patients, mouse model, cell lines	380 with CRC	*F. nucleatum* activates YAP signaling, reducing METTL3 expression and increasing KIF26B expression. High KIF26B expression associated with shorter OS in CRC patients.
Jiang, *et al*. 2023 [31]	Patients, mouse models, cell lines	42 with CRC	*F. nucleatum* metabolite succinic acid reduces $CD8^+$ T cell infiltration and is associated with decreased response to immunotherapy.
Akkermansia muciniphila			
Wang, *et al*. 2020 [28]	Patients, mouse model, cell lines	72 healthy controls 58 with ulcerative colitis 18 with adenomas 22 with CRC	*A. muciniphila* was reduced in IBD patients and mice with colitis or CRC. *A. muciniphila* associated with cytotoxic T lymphocyte activation and decreased tumorigenesis.
Jiang, *et al*. 2023 [29]	Mouse model, cell lines	N/A	Acetyltransferase of *A. muciniphila* promote a cytotoxic T cell response and blunts tumorigenesis in mice.
Peptostreptococus anaerobius			
Nakatsu, *et al*. 2015 [38]	Patients	61 healthy controls 47 with adenomas 52 with CRC	Metagenomic profiling linked multiple taxa, including *Peptostreptococcus* and *Parvimonas*, to CRC development and progression.
Tsoi, *et al*. 2017 [26]	Patients, mouse model, cell lines	49 healthy controls 45 with adenomas 50 with CRC	*P. anaerobius* enriched in CRC patient stool and biopsy specimens. *P. anaerobius* exposure promoted cholesterol biosynthesis and cell proliferation *in vitro* and intestinal dysplasia in mice.
Long, *et al*. 2019 [27]	Mouse model, cell lines	N/A	*P. anaerobius* adheres CRC cell integrins, activating the PI3K-Akt pathway and stimulating proliferation and myeloid cell recruitment.

Abbreviations: PCR, polymerase chain reaction; CRC, colorectal cancer; qPCR, quantitative PCR; pks, polyketide synthase; IBD, inflammatory bowel disease; FISH, fluorescence *in situ* hybridization; ETBF, enterotoxic Bacteroides fragilis; FAP, familial adenomatous polyposis; N/A, not applicable; ATP, adenosine triphosphate; NTBF, non-toxigenic Bacteroides fragilis; TLR4, toll-like receptor 4; miR21, microRNA21; OS, overall survival; MDSC, myeloid-derived suppressor cell; KIF26B, kinesin family member 26B; ICAM1, intercellular adhesion molecule 1.

lium that was not present in healthy control tissue. Moreover, the phenotype of *E. coli* isolated from Crohn's and CRC patients was distinct, exhibiting upregulation of hemagglutinins, corresponding to increased mucosal adherence [19]. *E. coli* strains expressing polyketide synthases (pks), an enzymatic complex that synthesizes the genotoxin Colibactin, have particularly potent carcinogenic potential. Deletion of pks from these strains results in decreased tumor multiplicity and invasion in azoxymethane treated Il-10 deficient mice. Pks+*E. coli* have been found in colorectal tissues from a 40% of IBD patients and 67% of CRC patients [20]. As such, this and continued work studying Pks+*E. coli* have begun to demonstrate the importance of bacterial function as well as taxal associations in carcinogenesis.

Similarly, a subgroup of the ubiquitous *Bacteroides fragilis* species, enterotoxic B. fragilis (ETBF) is also strongly linked to colorectal carcinogenesis. ETBF may present with asymptomatic colonization but can also cause acute diarrheal illness. A study of ETBF in *Apc*-deficient multiple intestinal neoplasia (Min) mice found that ETBF colonization leads to Stat3 activation and a T_H17-driven immune response that precipitates colonic tumor formation. This pro-tumor inflammatory pattern did not occur in mice that were colonized with non-enterotoxigenic *B. fragilis* [21]. ETBF toxin also leads to degradation of E-cadherin, β-catenin nuclear localization, and *c-myc* transcription, increasing the permeability of the gut epithelial barrier, which promotes invasion and metastasis by mechanisms detailed in later sections [22,39,40].

Perhaps more than any other species, *Fusobacterium nucleatum* has been closely associated with CRC at every stage of disease. *F. nucleatum* is an oral commensal bacterium that reaches the colon through the digestive tract and by hematogenous spread [41]. Indeed, a study evaluating paired saliva and CRC samples found identical *F. nucleatum* strains at both sites [42]. In an evaluation of the CRC-associated gut microbiome, Ahn and colleagues found that feces from patients with CRC were enriched in *F. nucleatum* and *Porphyromonas* mRNA when compared to healthy controls [43]. Flanagan and colleagues similarly performed a qPCR-based evaluation of resected colorectal tumors and benign biopsy specimens and found higher levels of *F. nucleatum* in CRC patients. They further demonstrated an inverse correlation between *F. nucleatum* level and overall survival (OS). Importantly, a subgroup analysis of patients with pre-cancerous adenomas demonstrated *F. nucleatum* enrichment in specimens with high-grade dysplasia, suggesting that the carcinogenic impact of *F. nucleatum* occurs early in the adenoma-carcinoma progression [44]. Mima *et al*. [45] confirmed that *Fusobacterium* level was independently associated with shortened OS (Hazard ratio (HR) 1.58 [95% confidence interval (CI) 1.04–2.39]) and additionally noted that *BRAF* mutant tumors were enriched in *F. nucleatum*, linking the bacteria to a more aggressive phenotype.

Multiple mechanisms connecting *F. nucleatum* to CRC initiation have been studied. The *Fusobacterium* virulence factor, FadA, has been shown to act by binding to E-cadherin and activating Wnt/β-catenin signaling, leading to multiple downstream inflammatory and oncogenic processes [23]. The presence of *Fusobacterium* in colorectal tissues is also associated with the selective recruitment of myeloid-derived immune cells, making for an immunosuppressive milieu favoring carcinogenesis [24]. Moreover, Chen and colleagues found that *Fusobacterium* drives CRC progression through downregulation of METTL3/m^6A, a prominent epitranscriptomic axis involved multiple cancers, through activation of the Hippo-YAP signaling pathway [25].

Other genera including *Peptostreptococcus*, *Parvimonas*, *Akkermansia*, and *Desulfovibrio* have been linked to CRC initiation [26–29,46,47]. However, dysbiosis-related CRC is not always driven by a single bacterial taxon. A generalized decrease in microbial diversity has also been linked to CRC in multiple studies. Wong *et al.* [48] found that oral gavage of fecal samples from CRC patients, but not healthy controls, promoted colonic polyp formation in germ-free and conventional mice treated with azoxymethane. The resultant gut microbiome in mice treated with CRC-derived stool was characterized by lower Fisher and Shannon-Weaver alpha diversity [48]. A fecal metagenomic comparison of patients with CRC and healthy controls similarly demonstrated reduced gene richness and alpha diversity in patients with CRC. Interestingly, control-enriched microbial genes occurred at a higher frequency and abundance than CRC-enriched genes, suggesting that CRC carcinogenesis is more commonly driven by an imbalanced gut microbiome rather than a dominant pathobiont [46].

3. The Microbiome and the Metastatic Cascade

The association between the microbiota and CRC extends beyond development of the primary tumor. Sun *et al.* [49] collected fecal samples from 30 patients with early-stage CRC and 30 with metastatic CRC and found consistent, generalized differences in fecal microbiome composition at both the genus and species levels. Bullman and colleagues [32] further demonstrated that *Fusobacterium*, and co-occurring anaerobes, were present in both primary colon tumors and matched liver metastases, suggesting that these agents may co-migrate with tumor cells to the metastatic target organ. The mechanisms by which the microbiome affects metastasis are multifactorial and have only begun to be understood but appear to involve both the tumor and gut microbiome. Current research links the microbiome to almost every stage of the metastatic cascade, including gut barrier penetration, pre-metastatic niche formation, epithelial-mesenchymal transition, intravasation, extravasation, and outgrowth (Fig. 1).

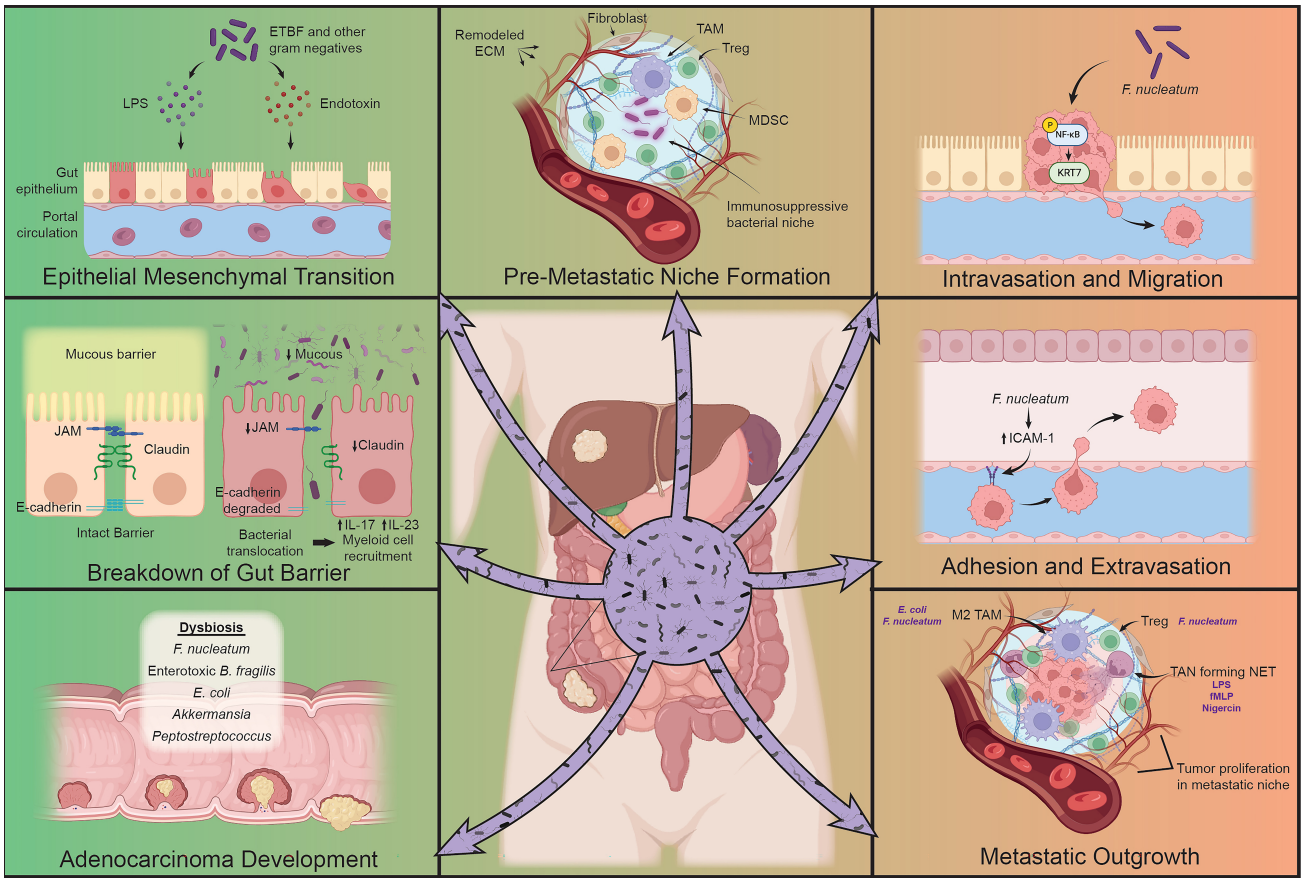

Fig. 1. The microbiome and colorectal cancer metastasis. The gut and tumoral microbiomes modulate colorectal cancer metastasis at multiple levels, including primary tumor development, breakdown of the gut vascular barrier, epithelial mesenchymal transition, pre-metastatic niche formation, intravasation and migration, adhesion and extravasation, and metastatic outgrowth. LPS, lipopolysaccharide; ETBF, enterotoxic bacteroides fragilis; JAM, junctional adhesion molecule; ECM, extracellular matrix; TAM, tumor associated macrophage; MDSC, myeloid derived suppressor cell; NF-κB, nuclear factor kappa B; KRT7, keratin 7; ICAM-1, intracellular adhesion molecule; TAN, tumor associated neutrophil; NET, neutrophil extracellular trap. Figure created using BioRender.com.

3.1 Gut Vascular Barrier Penetration

The gut vascular barrier is a complex system that acts as both a physical and chemical defense, preventing harmful intestinal microbes and antigens from entering the host circulation [50]. Penetration of the gut vascular barrier is a critical step in CRC invasion and metastasis and is mediated, in part, by the gut microbiome. Wu and colleagues [39] found the metalloproteinase enterotoxin of ETBF cleaves the extracellular domain of E-cadherin, a critical zonula adherens protein. This process increases gut barrier permeability and activates the β-catenin oncognic pathway, leading to increased cellular proliferation [39]. Corroborating these findings, Grivennikov *et al.* [51] found that pre-malignant colorectal adenomas are characterized by increased epithelial permeability due to the decreased expression of multiple barrier components, including mucin 2 (Muc2); junctional proteins JAM-A and JAM-B; and claudin. Microbial products are thus able to penetrate defective tumor-associated tight junctions and activate myeloid cells, leading to upregulated IL-23, which enhances tumor growth through IL-17 signaling. Translocation of gut microbes through a leaky gut vascular barrier sets the stage for the hepatic pre-metastatic niche, discussed further below.

3.2 The Hepatic Premetastatic Niche

The liver is the most common site of CRC metastasis, with colorectal liver metastases (CLM) developing in up to 50% of CRC patients at some point during their disease course [52]. Recent evidence suggests that a hepatic pre-metastatic niche (PMN) is formed prior to the development of overt CLM, and implicate the gut and tumoral microbiomes in its formation.

PMN are organ specific microenvironments that are primed for the implantation and outgrowth of disseminated tumor cells prior to their arrival [53]. PMNs are modulated by soluble secreted factors and extracellular vesicles from the primary tumor and are characterized by vascular permeability, extracellular matrix remodeling, angiogene-

sis, and an immunosuppressive microenvironment rich in regulatory T cells (Treg), myeloid derived suppressor cells (MDSCs) and fibroblasts [54].

Recent work by Bertocchi *et al.* [55] elucidated the role of the gut and tumoral microbiomes in hepatic PMN formation. The authors showed that gut vascular barrier impairment triggered by *E. coli* at the primary tumor site, as indicated by plasmalemma vesicle-associated protein-1 (PV-1) expression, correlated with bacterial levels in paired CLM samples. They further demonstrated that targeted antibiotic treatment with neomycin reduced the recruitment of innate immune cells to the liver and abrogated CLM formation [55]. Dysbiosis secondary to a high-fat diet has also been linked to liver metastasis with increased expression of multiple PMN markers, including matrix metallopeptidase 2 (MMP2), matrix metallopeptidase 9 (MMP9), fibronectin, and C-X-C chemokine ligand 12 (CXCL12), in non-tumoral hepatic tissue. This pro-tumoral cytokine signature was abrogated by antibiotic treatment, suggesting that its formation is microbiome-dependent [47]. The role of bacteria in PMN establishment was further supported in a recent study by Galeano Niño *et al*. [56], using spatial transcriptomics to show that tumor-associated microbial communities are not distributed randomly, but comprise organized, immunosuppressive microniches that promote tumor progression.

3.3 EMT and Cancer Cell Migration

Epithelial-mesenchymal transition (EMT) is a highly complex process by which malignant epithelial cells lose their normal polarity and assume a mesenchymal stem cell phenotype, enabling invasion, migration, and metastasis. Studies across multiple tumor types suggest that gram negative bacteria interface with the EMT program to promote cancer dissemination.

Zhao and colleagues found that lipopolysaccharide (LPS) in the gram-negative bacterial cell wall can induce EMT in the liver. Benign biliary epithelial cells cultured in the presence of LPS exhibited upregulation of mesenchymal markers including S100A and α-smooth muscle actin as well altered polarity and mesenchymal morphology in a process dependent on TGF-β1/Smad2/3 signaling [57]. Kim *et al*. [58] confirmed a causative link between LPS and EMT and found that simvastatin treatment could inhibit mesenchymal transformation by reducing toll-like receptor 4 (TLR4) expression and NF-$\kappa\beta$ signaling. In breast cancer cell lines, exposure to ETBF toxin led to β-catenin-dependent upregulation of EMT-related transcription factors, including those of the *TWIST* and *SNAI* family, as well as cancer stem cell markers, including OCT4 and NANOG [59]. In CRC, consensus molecular subtype 4 (CMS4) tumors are characterized by the overexpression of EMT-associated transcription factors including *SLUG*, *TWIST*, and *ZEB1/2*, and are associated with high rates of metastasis and a poor prognosis [60]. However, in a CRC mouse model driven by colonic expression of *Zeb2*, microbiota depletion with antibiotics was able to completely prevent cancer development, further supporting a link between the microbiome and EMT [61].

The potential for CRC cells to migrate and metastasize is also increased in the presence of *F. nucleatum*. Chen *et al.* [62] found that *F. nucleatum* infection leads to upregulation of the non-coding antisense RNA *KRT7-AS* via NF-$\kappa\beta$ signaling. This led to increased KRT7 expression, which was associated with increased transwell migration capacity in a CRC cell line, a higher rate of metastasis *in vivo*, and higher rates of nodal disease in human patients [62].

3.4 Endothelial Adhesion and Extravasation

Fusobacterium nucleatum plays yet another role in CRC progression at the juncture of circulating tumor cell endothelial adhesion and extravasation. Zhang *et al*. [63] serendipitously found that the human CRC cell line HCT116 exhibited markedly increased adherence to vascular endothelium in the presence of *F. nucleatum* as compared to *E. coli* or PBS. *F. nucleatum* infection also enhanced cell migration relative to *E. coli* and *Akkermansia muciniphilia* through the upregulation of ICAM1 via ALPK1-mediated NF-$\kappa\beta$ activation. The authors confirmed the activation of this signaling pathway *in vivo* using murine tail vein injection to model lung metastases and in a human CRC tissue microarray [63].

3.5 Dormancy and Metastatic Outgrowth

The process by which extravasated tumor cells survive and proliferate in the target organ is exceedingly complex and involves interplay between tumor cells, the target organ microenvironment, and the host immune system. Studies in breast cancer have demonstrated that tumor cells may disseminate early in the disease course and remain in a dormant/quiescent state for years before metastatic outgrowth is triggered [64,65]. In fact, stress from surgery to remove the primary tumor may, in some cases, precipitate metastatic outgrowth in patients with no clinical evidence of metastatic disease [66,67].

Cytotoxic necrotizing factor 1 (CNF1), a bacterial toxin produced by *E. coli*, plays a role in the quiescence of disseminated CRC cells. CNF1 blocks cytokinesis, elicits endoreplication and polyploidization, and drives cells into a reversible dormant state [68]. Dormant tumor cells exhibit intrinsic resistance to chemotherapy agents that are conventionally designed to target and eradicate rapidly proliferating tumor cells [69,70].

The multifarious triggers that cause tumor cells to exit dormancy and proliferate have not been fully elucidated, but it is known that a favorable tumor immune microenvironment (TIME) is required [71]. The gut and tumor-associated microbiomes interact with both the innate and adaptive immune systems to produce an inflammatory milieu that favors metastatic outgrowth.

Tumor-associated macrophages (TAMs) and neutrophils (TANs) play important roles in the promotion of metastatic outgrowth. TAMs exist in a state of flux between the M1 and M2 polarization states. M1 macrophages promote cytotoxicity and effectuate a tumor-suppressive microenvironment while M2 macrophages are associated with immunosuppression and the expression of cytokines that promote tumor survival and proliferation [72]. Both *E. coli* and *F. nucleatum* have been shown to shift this balance in favor of the M2 state. Li and colleagues [73] found that *E. coli* gavage stimulated the secretion of cathepsin K from MC38 cells implanted in the cecal mesentery of antibiotic treated mice. Binding of cathepsin K to TLR4 produced M2 polarization of TAMs and was associated with more numerous liver metastases [73]. *F. nucleatum* infection similarly facilitates metastasis through the downregulation of miR-1322, leading to increased CCL20 expression and M2 macrophage differentiation [74].

Activated TANs also support metastatic outgrowth through the expression of multiple cytokines including MMP-9, VEGF, CXCL4, and CCL5, and through the formation of weblike structures known as neutrophil extracellular traps (NETs). Bacteria residing in the metastatic niche activate NETosis through pathogen-associated molecular patterns (PAMPs), including LPS, fMLP, and Nigercin [75–77]. Cleavage of the extracellular matrix protein laminin by NET-associated proteases then activates dormant tumor cells through integrin signaling [75,78]. NETs can further promote metastasis by shielding circulating tumor cells, stimulating angiogenesis, and promoting the formation of tumor thrombi [75].

Finally, tumor-associated microbes also modulate the adaptive immune response to promote metastatic outgrowth. Sakamoto *et al*. [79] found that *F. nucleatum* levels were associated with significantly lower cytotoxic ($CD8^+$) T cell density in a sample of 181 CLM specimens, suggesting that anti-tumor inflammation is blunted in the presence of this bacteria. Oral gavage with *F. nucleatum* also resulted in decreased NK, $CD3^+$, $CD4^+$, and $CD8^+$ T cells, and a significant increase in Tregs in a mouse model of CLM [80].

4. Response to Therapy

Systemic therapy plays an indispensable role in the modern management of metastatic CRC. In addition to mediating therapy resistance through quiescence, the microbiome can have a direct impact on response to both cytotoxic chemotherapy and immunotherapy.

4.1 Response to Cytotoxic Therapy

Systemic chemotherapy is known to alter gut microbial composition, diversity, and function [81]. However, the gut and tumoral microbiomes can also mediate cytotoxic therapy metabolism and response. Multiple chemotherapeutic agents, including cyclophosphamide, oxaliplatin, irinotecan, and 5-FU are known to interact with the intestinal microbiome [82–85]. With regard to CRC, Yu *et al*. [30] found that colon cancer cell lines attained resistance to both 5-FU and oxaliplatin when cocultured with *F. nucleatum*. Chloroquine assays revealed that this process is dependent on autophagy driven by TLR4 and MYD88 signaling [30]. *F. nucleatum*-induced TLR4/NF-$\kappa\beta$ signaling has also been shown to incite 5-FU chemoresistance through upregulation of baculoviral IAP repeat C3 (BIRC3) [86]. Interestingly, a specific gut microbial metabolite, urothilin A, has been associated with increased 5-FU sensitivity and reduced murine xenograft tumor growth in both CRC and pancreatic adenocarcinoma, supporting the importance of a balanced microbiome in cancer patients [87,88].

4.2 Response to Immunotherapy

Immune checkpoint blockade (ICB) has revolutionized the treatment of multiple solid tumors. Several important studies have linked the gut microbiome to ICB response and toxicity in melanoma and non-small cell lung cancer. In these investigations, increased relative abundance of specific bacterial taxa, including *Akkermansia muciniphila*, Bacteroidaceae, Ruminococcaceae, and *Bifidobacterium*, were associated with improved ICB response [89–92].

In CRC, the role of ICB has been largely limited to patients with microsatellite unstable tumors [93]. These cancers, characterized by a high mutational burden and increased expression of immune checkpoints, exhibit increased immunogenicity and response to both CTLA-4 and PD-1 inhibition [94–96]. The impact of the microbiome in this setting and the potential for microbe-based sensitization of microsatellite stable tumors to ICB has not yet been established; however, early preclinical studies provide promising results. Destefano and colleagues found that $BRAF^{V600E}$ mutant ETBF-induced colon tumors were uniquely sensitive to PD-L1 inhibition compared to *BRAF* wild type tumors, suggesting a unique interaction between a known driver mutation, microbiome-mediated carcinogenesis, and ICB response [97]. Jiang *et al*. [31] demonstrated that succinic acid from *F. nucleatum* confers resistance to anti-PD-1 therapy that could be abrogated by fecal microbiota transfer from *F. nucleatum*-low responders or the antibiotic metronidazole.

5. Future Directions

5.1 Unraveling Peritoneal Metastases

Peritoneal metastases (PM) occur in 5 to 10% of CRC patients and are associated with worse clinical outcomes than any other metastatic site [98]. The peritoneal microenvironment and the role of the microbiome in CRC carcinomatosis have not been well studied or defined. In ovarian cancer, another solid tumor with a strong connection to dysbiosis, local gram-negative peritoneal colonies and decreased microbiome diversity have been associated with

peritoneal spread [99,100]. In PM from appendiceal cancer, enteric bacteria, including those of the Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes phyla, have been identified in both PM and associated mucinous ascites, implicating these taxa in the pathobiology of this disease site. Ongoing studies will reveal more about the unique TIME of CRC PM and the potential role of gut and tumoral microbes in its development.

5.2 Potential for Therapeutic Microbiome Modulation

Strong correlations between the gut microbiome and oncologic outcomes have led to the study of microbiome modulation as a therapeutic strategy. In their aforementioned study of paired CRC and CLM, Bullman and colleagues found that treatment with the antibiotic metronidazole led to reduced Fusobacterium levels and decreased tumor cell proliferation [32]. Two forthcoming trials are now evaluating the efficacy of neoadjuvant metronidazole in patients with CRC (NCT04264676, NCT05748145).

Dietary modification also presents a low-risk means to modulate the gut microbiome as well as CRC outcomes. CRC patients who consume a high-fat diet are known to have significantly shorter 5-year disease-free survival [101]. On the other hand, work by Spencer *et al*. [102] showed that a high-fiber diet produced taxonomic and structural changes in the gut microbiome that correlated with anti-PD-1 response in conventionally housed mice and in human patients who reported sufficient dietary fiber intake. Numerous trials are now evaluating the effect of diet-based microbiome modulation on CRC outcomes. At our institution, the Beans to Enrich the Gut Microbiome vs Obesity's Negative Effects (BEGONE) trial is evaluating the longitudinal effect of dietary fiber on the gut microbiome and risk of CRC recurrence.

Fecal microbiota transplant (FMT) is the most direct means of altering the gut microbiome and is now widely accepted as an effective treatment for refractory *Clostridium difficile* colitis [103]. FMT has also been used to successfully treat inflammatory bowel disease and is under investigation in the treatment of other autoimmune disorders [104,105]. In oncology, FMT has been used to overcome melanoma resistance to ICB in multiple preclinical studies and at least two clinical trials [106,107]. Multiple ongoing trials are investigating the role of FMT in modulating toxicity and improving response to ICB in other solid tumors, including CRC.

While still in the preliminary stages of investigation, study of both additive (FMT) and subtractive (antibiotics and novel therapeutics) modulation of the gut and tumoral microbiome in CRC carries enormous clinical potential. As suggested by the preclinical studies covered in this review, these efforts may provide new avenues through which to improve CRC detection and increase the efficacy of existing standard treatments. As our understanding of the microbiome's role in gut vascular permeability and modulation of the host immune response deepens, we anticipate a new class of microbiome-based interventions that may even stymie CRC dissemination and metastatic outgrowth.

Author Contributions

RIA outlined, wrote, and edited the manuscript and created the figure. MGW was invited to contribute this review, outlined the concepts to be covered, wrote and edited the manuscript. Both authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity. Both authors read and approved the final manuscript. Both authors contributed to editorial changes in the manuscript.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

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