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Microbiome in Colorectal Cancer: How to Get from Meta-omics to Mechanism?

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Mounting evidence from metagenomic analyses suggests that a state of pathological microbial imbalance or dysbiosis is prevalent in the gut of patients with colorectal cancer. Several bacterial taxa have been identified of which representative isolate cultures interact with human cancer cells *in vitro* and trigger disease pathways in animal models. However, how the complex interrelationships in dysbiotic communities may be involved in cancer pathogenesis remains a crucial question. Here, we provide a survey of current knowledge of the gut microbiome in colorectal cancer. Moving beyond observational studies, we outline new experimental approaches for gaining ecosystem-level mechanistic understanding of the gut microbiome's role in cancer pathogenesis.

Linking the Gut Microbiome to Colorectal Cancer

The complex interplay between colorectal cancer (CRC) and the human gastrointestinal tract (GIT) **microbiome** (see Glossary) has become a highly topical area in current CRC research. The microbiome represents the interface and translating agent of environmental factors for the human body. Its genomic complement outnumbers unique human genes by at least 150-fold [1]. Primarily, this vast genetic repertoire delivers molecules, which support host homeostasis and health. It aids digestion and educates the host's immune system. Furthermore, a healthy microbiome inhibits the proliferation of pathogenic bacteria and their colonization of the gut by occupying intestinal niches and competing for nutrients. In CRC patients, however, an imbalance of the gut microbiome (dysbiosis) is present. It has therefore been suggested that bacteria interfere with the molecular mechanisms underlying CRC.

Many effects of CRC-associated bacteria have been described (Box 1); however, the underlying pro-oncogenic mechanisms remain elusive. Therefore, further efforts are required to resolve the mechanistic roles of CRC-associated bacteria in tumor initiation and progression. In essence, bacteria may affect CRC development by directly or indirectly affecting host cells or their associated microenvironment by different means: (i) Bacterial metabolism and its secreted molecular complements [e.g., extracellular superoxide, **genotoxins**, or **short-chain fatty acids (SCFAs)**]; (ii) attachment, invasion, and translocation; (iii) host defense modulation (e.g., bacteria–immune cell interactions; Figure 1).

In this article, an overview of the aforementioned purviews is provided by covering the three main areas of (i) identification of CRC-associated bacteria, (ii) host–microbe interactions in disease progression and treatment, and (iii) host–microbe interaction study models.

CRC-Associated Bacteria

Over the past few years, culture-based methods and quantitative real-time polymerase chain reaction (qRT-PCR) on DNA extracted from colorectal tissue biopsies, as well as patients' stool, have allowed us to identify enrichments in particular bacterial species. In addition, present-day next-generation sequencing techniques enabling 16S rRNA gene and **metagenomic** profiling

Highlights

A pathological imbalance of the gut microbiome (dysbiosis) is present in colorectal cancer (CRC) patients.

Bacteria may affect CRC directly or indirectly, by secreting metabolites, by invading tissues, and by modulating the host immune response.

The underlying pro-oncogenic mechanisms of many CRC-associated bacteria remain undescribed.

Fusobacterium, Peptostreptococcus, Porphyromonas, Prevotella, Parvimonas, Bacteroides, and *Gemella* are among the most prominent CRC-associated bacteria.

Many *in vitro* and *in vivo* models are currently used for studying the microbiome in CRC, such as gut-on-chip models, 3D intestinal organoids, and gnotobiotic mouse models, each offering its own advantage.

The microbial metabolism plays an important role in CRC. Computational modeling is a promising approach for studying genotype-to-metabolic-phenotype relations or microbe–microbe and host–microbe metabolic interactions.

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Box 1. Theories on Bacterial Involvement in CRC Tumorigenesis

Theories behind bacteria-driven tumorigenesis in CRC have been put forth since the middle of the 20th century, when McCoy and Mason first suggested a link between *Enterococcus* and carcinoma of the sigmoid [157]. In 2011, Sears and Pardoll formulated the 'alpha-bug' hypothesis, in which species such as *Bacteroides fragilis* exert a central pro-oncogenic, enterotoxigenic role, thereby contributing to the onset of CRC [158]. Subsequently, Tjalsma *et al.* proposed the driver–passenger model in 2012, in which driver bacteria (e.g., *B. fragilis*) lead to a multistep development of colorectal tumorigenesis including inflammation, increased cellular proliferation, and/or the production of genotoxins [159]. An extension of this is the 'Keystone hypothesis' by Hajishengallis *et al.*, in which key pathogens, even at low abundance, facilitate colonization by accessory pathogens [160]. This is followed by the subversion of host responses, resulting in a dysbiotic microbiota in which further pathobionts overstimulate the inflammatory response [160]. Another theory, which has been applied to *Helicobacter pylori* infections in gastric cancers, suggests a hit-and-run action of tumor-initiating bacteria, whereby the pro-oncogenic action of the bacterial toxin CagA leads to genetic and epigenetic alterations [161]. This suggests that potentially transient pathogenic bacteria are required for the initiation, but not for the maintenance, of a neoplastic phenotype in cancer cells.

of the CRC-associated microbiome provide fundamental data for follow-up mechanistic studies. Here, we provide a comprehensive list of the sequencing-based identification of CRC-associated bacteria (Table 1, Key Table, and Supplemental Table S1 online). Although 16S rRNA gene

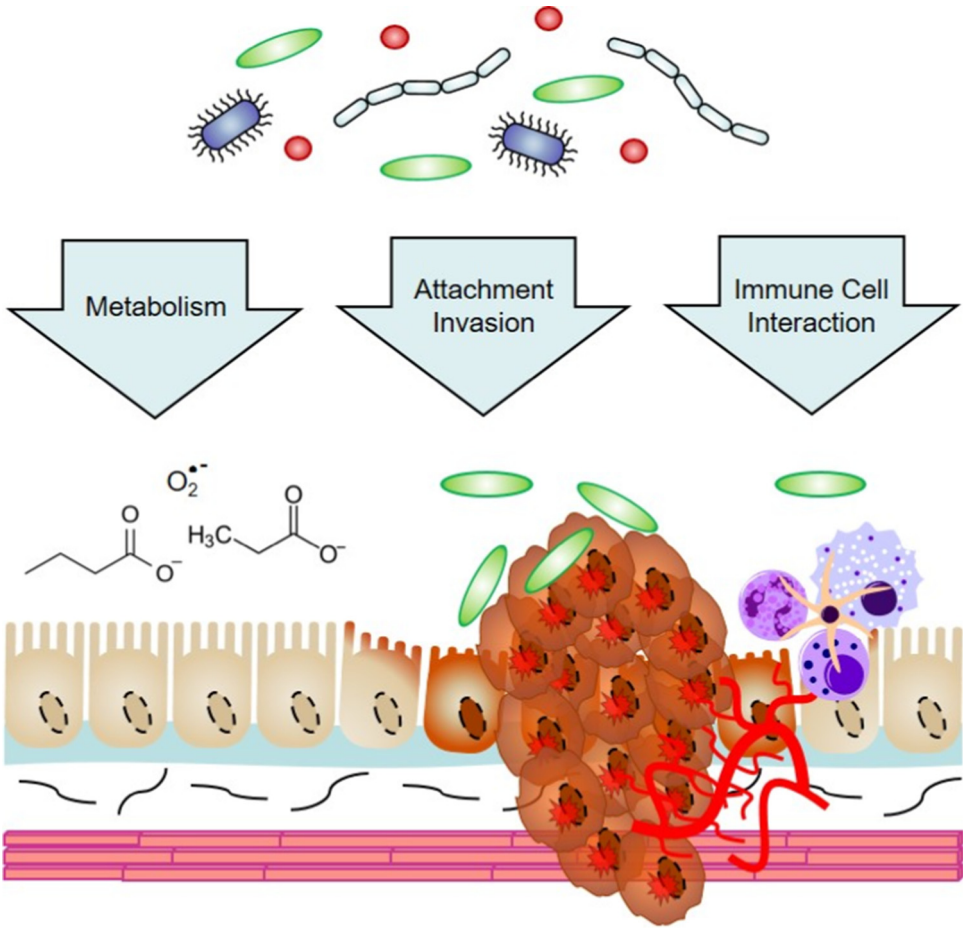

Figure 1. Potential Bacteria–Host Interactions in Colorectal Cancer (CRC). Bacteria may affect CRC development by exerting a direct or an indirect impact on the host cells or their associated microenvironment: (i) Bacterial metabolism and its secreted molecular complements (e.g., extracellular superoxide, genotoxins, or short-chain fatty acids); (ii) attachment, invasion, and translocation; (iii) host defense modulation (e.g., bacteria–immune cell interactions).

Glossary

CIMP: The CpG island methylator phenotype is characterized by a switch-off of tumor suppressor genes due to global genome hypermethylation.
CMS: The Consensus Molecular Subtype classification system was established to resolve inconsistencies between six independent classification systems of colorectal cancer subtypes. Four groups were established: CMS1 (hypermutated, unstable microsatellite sequences, and high immune activation), CMS2 (canonical, WNT, and MYC signaling activation), CMS3 (metabolic dysregulation), and CMS4 (TGF-β activation, stromal invasion, angiogenesis).
Environmental filtering: conceptual understanding of the relationship between an organism and the environment, which provides a selective force that eliminates species unable to tolerate conditions at a particular location.
FBA: flux balance analysis is a mathematical method for simulating the flow of metabolites through a genome-scale reconstruction of a metabolic network. It can calculate metabolic fluxes in a steady state, allowing to predict the growth rate of an organism or the production rate of a metabolite.
Genotoxin: chemical agent that can damage DNA, causing mutations and possibly cancer.
Gnotobiotic model: all microorganisms present in and on the animal are known and can be accounted for. The most common types of gnotobiotic models are germ-free mice, in which no microorganisms are present, and gnotophoric mice, in which a single known contaminant is present.
Metabolome: the complete set of metabolites (small molecules – amino acids, fatty acids, vitamins, etc.) found in a biological sample (cell, tissue, organ, organism).
Metagenome: the collective genome (total of genetic material present in a cell or organism) of all microorganisms found in a given environment – for example, the gut metagenome comprises all of the genomes of the microorganisms present in the gut, excluding the host genome.
Metatranscriptome: the collective transcriptome (total of all messenger RNAs present in a cell or organism) of all microorganisms found in a given environment.
Microbiome: the ensemble of all microorganisms (microbiota) and their

Key Table

Table 1. Top 20 Enriched Bacterial Genera and Species in Colorectal Adenoma and CRC Patients[a]

Genus	Species	Refs	Number of hits
Fusobacterium		[5–31]	31
	nucleatum	[5,7,13,16,21,27,32–35]	
	gonidiaformans	[16]	
	mortiferum	[34]	
	necrophorum	[34]	
	peridonticum	[16]	
Peptostreptococcus		[6,8,10,11,19,20,22,24–26,28–30,36]	18
	stomatis	[16,32,34,35]	
	anaerobius	[34,35]	
	endodontalis	[22]	
Porphyromonas		[8,10,11,15,25–31,37]	16
	asaccharolytica	[16,33,34]	
	uenonis	[34,35]	
	somerae	[34]	
Bacteroides		[8,19–21,31,38,39]	14
	fragilis	[10,16,22,32–34]	
	ovatus	[19,22]	
	caccae	[19]	
	dorei	[19]	
	eggerthii	[19]	
	massiliensis	[19]	
	salyersiae	[34]	
	splanchnicus	[19]	
	vulgatus	[19]	
	xylanisolvens	[19]	
Parvimonas		[17,19,20,22,25,26,28]	13
	micra	[16,24,32–35]	
Prevotella		[8,9,20–22,25,26,28,38,40]	13
	intermedia	[22,33,34]	
	nigrescens	[16]	
Gemella		[8,9,17,20,22,25,30]	12
	morbillorum	[10,19,32,34,35]	
Streptococcus		[8,10,17,23,26,28,37]	11
	anginosus	[16,35]	
	dysgalactiae	[34]	
	constellatus	[34]	
	gallolyticus	[34]	
	thermophilus	[19]	
	tigurinus	[34]	

(continued on next page)

genes (genomes) found in a particular environment; for example, the gut microbiome comprises all microorganisms (bacteria, virus, fungi, etc.) found in the large intestine.
Microenvironment: the immediate small-scale environment in close contact with a given entity (cell, tissue, organism) – for example, a tumor microenvironment is the close environment surrounding a tumor, which is different from the microenvironment in the surrounding healthy tissue.
MSI: The microsatellite instability is a hypermutable phenotype, characterized by a high number of mutation in microsatellite regions, caused by an impaired DNA mismatch system.
OTU: When performing 16S sequencing, an operational taxonomic unit is an operational definition assigned to a cluster of similar genetic sequences, which allows for the identification and classification of bacteria into species, genera, or phyla.
Proteome: the complete set of proteins expressed by a cell or an organism.
SCFA: short-chain fatty acids are fermentation products of gut microbes. They are nutrients for colonocytes and can affect host cellular metabolism
Spatial resolution: the term refers to the precision of bacterial detection with respect to location/space (proximal–distal, tissue–mucosa–lumen). In other words, it describes the possibility to trace back and localize bacteria in their gut niche.
Stoichiometric matrix: matrix containing binary information about all metabolic transformations in a metabolic reconstruction.

Table 1. (continued)

Genus	Species	Refs	Number of hits
Clostridium		[15,26,28,29,31,39,41]	9
	symbiosum	[16,19,34]	
	hylemonae	[16]	
Escherichia		[10,20,21,23,26,28,36]	9
	coli	[19,34]	
Bilophila		[9,29,39,42]	8
	wadsworthia	[16,19,34,35]	
Campylobacter		[17,22,26,28]	8
	gracilis	[34]	
	rectus	[16]	
	showae	[13,34]	
	ureolyticus	[34]	
Phascolarctobacterium		[15,26,31,39–41,43]	8
	succinatutens	[35]	
Selenomonas		[8,11,29,30]	8
	sputigena	[13,16,34,35]	
Ruminococcus		[16,25,26,29,34,39,41]	7
	torques		
Shigella		[10,20,21,23,26,36,41]	7
Akkermansia		[9,29,31,41]	6
	muciniphila	[21,43]	
Desulfovibrio		[8,16,28,39]	6
	desulfuricans	[34]	
	longreachensis	[35]	
	vietnamensis	[35]	
Eubacterium		[6,8,26,29,41]	6
	infirmum	[34]	
	limosum	[34]	
Leptotrichia		[22,28,34]	6
	hofstadii	[13,16]	
	buccalis	[16]	

[a]The ranking was based on the number of studies (referred to as 'hits'), reporting elevated abundance per bacterium in CRC. Table continued in Supplemental Table S1 online.

sequencing is helpful for the identification of bacterial genera, it lacks resolution at the strain level and fails to resolve intraspecies diversity, in contrast to metagenomic and **metatranscriptomic** approaches. Nevertheless, it provides information on the most prevalent genera, providing a starting point for other highly sensitive approaches, such as qRT-PCR-based and RNA/whole-genome-sequencing-based studies.

When comparing different studies (Supplemental Table S2 online), often, control patient groups differ from CRC patient groups in terms of basic parameters, such as male-to-female ratios or age [2]. In addition, results can differ between ethnic groups. Regarding the analysis, the choice

of the hypervariable region for the 16S rRNA gene can affect the analysis depth and generate bias in microbial diversity. Furthermore, the use of different databases or computational pipelines for the analysis and annotation of **OTUs** can give rise to different annotations. Here, improvements can be achieved by the use of a reproducible and modular short-read library 16S rRNA gene sequencing pipeline 'sl1p' [3].

Finally, the analysis of microbial communities in fecal samples may reflect a disease state, without fully reflecting the tumor microenvironment, the tissue-adhering bacteria, and the topology of the GIT. One must also focus on the cancer proximate/adherent microbiome, its potential source such as the oral cavity, and the microenvironment itself, along with all its players (e.g., immune cells, fibroblasts, telocytes). The **spatial resolution** of certain CRC-associated bacteria can provide hints, as to where exactly particular bacteria occur (lumen/stool, mucus, or tissue) [4].

Studies have also shown that microbiome composition can influence chemotherapy efficacy [44]. In this context, a recent paper by Tanoue and colleagues identified a consortium of 11 bacterial strains, which enhanced resistance against pathogenic infections and improved the therapeutic efficacy of immune checkpoint inhibitors in mice [45].

Despite the many roles which the gut microbiome may play in CRC initiation, progression, diagnosis, and treatment, here we primarily review the most recent evidence implicating the gut microbiome in CRC pathogenesis. In addition, we explore their roles in other cancers and diseases, as well as their roles in CRC detection (Box 2) and therapy (Table 2).

In the following section, we explore the empirical and potential roles of the most relevant, but also some previously unresearched CRC-associated bacteria, which were identified as higher ranked in Table 1.

Box 2. The Microbiome as a Biomarker – Clinical Relevance

Given the link between microbial dysbiosis and CRC, the gut microbiome is currently being explored as a source of diagnostic and prognostic markers [15,16]. State-of-the-art CRC detection primarily relies on guaiac fecal occult blood tests (gFOBTs), fecal immuno-chemical tests (FITs), multitarget stool DNA (sDNA) testing, and sigmoidoscopy or colonoscopy [162,163]. It is worth mentioning that nine of 10 people can be cured of CRC, if diagnosed in time [163]. The 5-year survival rate is > 85% but this rapidly decreases to <10% for late diagnosis of metastasized cancer [164]. Consequently, patient prognosis and treatment options highly depend on the stage of the disease.

gFOBT is based on blood detection in feces and despite its high specificity (87%–98%), the low sensitivity of 9%–12% leads to a high number of false negatives [165]. FIT, also a stool-based screening test, provides a more sensitive, quantitative measure of hemoglobin concentrations and is on the verge of largely replacing gFOBT clinically. However, it still shows sensitivity drawbacks for early and advanced neoplasia (7.6% [162] and 38% [166]). sDNA, a molecular assay for detecting *KRAS* mutations, aberrant methylation of *NDRG4*, and *BMP3* methylation, shows the highest sensitivity for detecting advanced precancerous tissue (42.4%), but has a higher false-positive rate compared with gFOBT and FIT [162].

Given the need for improvement of noninvasive screening methods for early stage detection of CRC and the potential to harness polymicrobial signatures associated with CRC, recently developed metagenomics-based classification models are combined with the standard gFOBT or FIT. This approach led to marked improvements in CRC-detection efficiency by gFOBT (a relative improvement in sensitivity of >45% over the FOBT alone at identical specificity [16]). This classification model largely depends on abundance changes of four species to accurately discriminate between CRC cases and controls: *Fusobacterium nucleatum* subsp. *vincentii*, *F. nucleatum* subsp. *animalis*, *Porphyromonas asaccharolytica*, and *Peptostreptococcus stomatis*, all of which were found to be enriched in tumor and stool samples from CRC patients [16] (Table 1).

The prognostic value of bacterial biomarkers has recently been highlighted by Mima *et al.*, who found *F. nucleatum* signatures in colorectal cancer tissue of US patients correlating with proximal tumor location, shorter patient survival, and molecular alterations such as MSI-high, CIMP-high, LINE-1 hypomethylation, and BRAF mutations [116]. These alterations usually influence the clinical outcome for patients and therefore serve as predictive and prognostic markers [167].

Table 2. Roles of CRC-Associated Bacteria in Other Cancer and Other Diseases; Roles in CRC Detection and Therapy

	Fusobacterium nucleatum	*Peptostreptococcus* ssp.	*Porphyromonas* ssp.	*Prevotella intermedia*	*Parvimonas micra*	*Bacteroides fragilis*	*Gemella morbillorum*
Characteristics	• Gram negative • Rod or spindle shaped	• Gram positive	• Gram negative • Nonmotile • Nonspore forming	• Gram negative • Rod shaped	• Gram positive	• Gram negative • Nonspore forming • Rod shaped	• Gram positive • Nonmotile • Nonspore forming • Single coccus, pairs, or small aggregates
Nutrition	• Obligate anaerobe	• Anaerobe	• Obligate anaerobe	• Anaerobe	• Anaerobe	• Anaerobe	• Facultative anaerobe
Localization in healthy state	• Oral and intestinal regions; • Weak gut colonizer	• Oral cavity • Commensal	• Oral cavity	• Oral cavity	• Oral cavity and GIT	• GIT • Commensal	• Oropharynx, GIT and female genital tract
Involvement in cancers other than colorectal cancer		• Oral squamous cell carcinoma (OSCC) - *P. stomatis* highly localized at the tumor site [102]	• OSCC - Increased invasiveness [104,105] - Increased metastatic potential and chemoresistance [106] - *P. gingivalis* disrupts immune surveillance by supporting myeloid-derived dendritic suppressor cell generation [107]. • Head and neck squamous cell carcinoma - *P. gingivalis* triggers the noncanonical activation of β-catenin in epithelial keratinocytes and promotes the disassociation of the β-catenin destruction complex using its proteolytic enzymes gingipains RgpA/B [108]. - *P. gingivalis* LPS-induced macrophages demonstrated increased nitric oxide production and their conditioned media fueled proliferation and invasion of tumor cells [61,109].	• Pancreatic cancer *Effect on cancer cells* - *P. intermedia* possesses peptidyl arginine deaminases, which may play a role in causing p53 mutations [61].			• OSCC *Effect on cancer cells* - Enrichment of *Gemella* at tumor sites and in cervical lymph nodes [103].
Involvement in other diseases	• Periodontal disease; pregnancy complications	• Oral infections; female genital tract infections	• Chronic periodontitis	• Persistent endodontic infections - *P. intermedia* disables and kills	• Sporadic infections of the oral cavity, knee, joints, heart, lung, and brain;		• Opportunistic pathogen • Often incorrectly identified • Pericarditis; arthritis;

Table 2. (continued)

	Fusobacterium nucleatum	*Peptostreptococcus* ssp.	*Porphyromonas* ssp.	*Prevotella intermedia*	*Parvimonas micra*	*Bacteroides fragilis*	*Gemella morbillorum*
				tissue-infiltrating neutrophils [60]. - *P. intermedia* suppresses neutrophil phagocytosis [110,111].	• Periodontitis - *In vitro*, *P. micra* promotes innate immune responses [63,112].		pneumonia; infections of the central nervous system; postoperative wound infections; liver abscesses in Crohn's disease • Oral infections - *Gemella* has been shown to reduce IL-12 levels in oral infections in mice [113].
Involvement in therapy	• *F. nucleatum* promotes (chemo) resistance of CRC cells by triggering autophagy activation [52]. • *F. nucleatum* reduces chemosensitivity in CRC cells through NF-κB, followed by apoptosis inhibition [53].	Not addressed yet	Not addressed yet	Not addressed yet	Not addressed yet	• *B. fragilis* enhances the efficacy of anti-CTLA-4 therapy by triggering a Th1 response and promoting dendritic cell maturation [114].	Not addressed yet
Involvement in CRC detection (Biomarker, Box 3)	• A metagenomics-based classification model, using abundance changes of *F. nucleatum* ssp. *vincentii* and *animalis*, *P. stomatis*, and *P. asaccharolytica* in CRC patients versus healthy controls combined with standard CRC diagnostics improved CRC-detection efficiency for gFOBT (> 45%) [16]. • A microbiota-based random forest model using abundance changes of *Fusobacterium, Peptostreptococcus, Porphyromonas, Prevotella, Parvimonas, Bacteroides,* and *Gemella* complemented FIT, thereby improved FIT sensitivity for adenoma detection to 45.5% versus 15.7% for FIT alone [25]. • A random forest-based model using abundance changes of *F. nucleatum, P. stomatis, P. asaccharolytica, Prevotella* ssp., *Parvimonas* ssp., *G. morbillorum*, and other bacteria, combined with FOBT, improved the sensitivity/specificity of CRC detection [34].						
	• The combination of *F. nucleatum* as a microbial marker with the FIT-based CRC detection improved detection sensitivity for advanced adenoma by >23% over the FIT alone [115]. • *F. nucleatum* signatures in colorectal cancer tissue correlate with proximal tumor location, shorter patient survival, and molecular alterations [116]. • *Fusobacterium* abundance changes improved diagnostic performances of FIT-based CRC detection [117].					• *Bacteroides* abundance changes improved diagnostic performances of FIT-based CRC detection (sensitivity 92.8%, specificity 81.5%) [117].	

Fusobacterium nucleatum in CRC

Today, overabundance of *Fusobacterium* in the gut is considered a potential CRC biomarker [46]. Enrichment in *Fusobacterium* is observed in CRC stool and tissue. Moreover, the fusobacterial virulence factor FadA is known to bind to E-cadherin's extracellular domain, promoting the proliferation of cancer cells via Wnt signaling [47]. Furthermore, higher FadA expression levels are found in patients with adenomas and adenocarcinomas than in healthy adjacent tissues [47].

Fusobacterium has been shown to expand myeloid-derived immune cells, while inhibiting T-cell responses [48]. A link between *Fusobacterium* and oncogenic and inflammatory responses was also observed in another study, in which *Fusobacterium* enabled the expansion of $FOXP3^{lo}$ non-T_{reg} cells and suppressed antitumor immune responses in CRC [49]. *Fusobacterium* was also associated with macrophage activation following an upregulation of certain microRNAs, in particular miR-21. miRNA-21 upregulates interleukin-10 (IL-10) and prostaglandin E_2, and causes a decrease in T-cell antitumor suppressor functions [48]. A similar immunosuppressive mechanism, involving the direct binding of fusobacterial Fap2 protein with the TIGIT inhibitory receptor on natural killer cells, drives immune cell evasion [50]. Fap2 was also found to target the host epithelial Gal-GalNAc, which mediates *Fusobacterium nucleatum* localization and enrichment in CRC [51].

A recent study found that *Fusobacterium* promotes chemoresistance in CRC by targeting the innate immune receptors TLR4 and MYD88, as well as specific miRNAs (miR18a and miR-4082) responsible for autophagy activation [52]. Thus, patients presenting high levels of *Fusobacterium* are more susceptible to chemotherapy failure and disease relapse. These results are in line with another study showing that upon *Fusobacterium* infection, chemosensitivity of CRC cells was reduced due to a TLR4/NF-κB-mediated upregulation of BIRC3. Subsequent inhibition of apoptosis led to drug resistance [53].

Although *F. nucleatum* is one of the best studied bacteria in CRC, its mechanistic role in driving CRC needs to be further investigated. It remains unclear whether the bacterium has a causative role, since, depending on the strain, it does not always induce cancer formation *in vivo* [54], nor cause hyperproliferation in all cancer cell lines *in vitro* [47]. It seems to require 'two hits': first a somatic mutation and then *F. nucleatum* exposure [55]. Here, a more holistic approach, including *Fusobacterium*'s vast **metabolic** potential, as well as its effect on epigenetic modifications, may provide further insights.

Peptostreptococcus ssp. in CRC

A recent study showed that patients with *Peptostreptococcus* bacteremia have an increased risk of developing CRC [56], in particular *Peptostreptococcus stomatis* and *Peptostreptococcus anaerobius* [57,58].

P. stomatis is a mild saccharolytic and fermented product producer, including acetic, isobutyric, isovaleric, and isocaproic acids. *P. stomatis* enrichment in CMS1 tumor tissues has been validated by targeted qRT-PCR [57]. Studies have suggested that *P. stomatis* might contribute to the acidic and hypoxic tumor **microenvironment**, which promotes bacterial colonization; however, no functional study on *P. stomatis* in colorectal tumor development exists to date.

P. anaerobius has been found to be highly enriched in CRC patient stool and tissue. In an *in vitro* and *in vivo* study by Tsoi *et al.*, a protumorigenic effect of *P. anaerobius* via TLR2 and TLR4 led to reactive oxygen species accumulation, which supported cholesterol synthesis and cellular proliferation [58].

Porphyromonas ssp. in CRC

Porphyromonas gingivalis has been found to be involved in several different types of cancer. Future mechanistic studies may relate *P. gingivalis* function to CRC, as a significant overabundance of *P. gingivalis* was found in fecal samples from CRC patients [29].

Prevotella intermedia in CRC

A study with African-American cohorts showed that *Prevotella intermedia* was associated with a higher risk of developing CRC [59]. In addition, *P. intermedia* was identified in a multinational multicohort study of 526 metagenomic CRC fecal samples [33].

Although it is not yet clear how *P. intermedia* correlates with CRC, one may speculate its role in CRC progression, either by modulation of the innate immune response via neutrophil suppression, as has been observed in endodontic infections [60], or by causing p53 mutations, as has been shown in pancreatic cancer [61].

Parvimonas micra in CRC

Overabundance of *Parvimonas micra* has been reported in CRC patient stool [62]. *P. micra* has been shown to disrupt the normal functioning of the NOD2 signaling pathway in periodontitis [63]. This could potentially give rise to a protumorigenic and inflammatory environment. Thus, a mechanistic study on *P. micra*'s involvement in CRC may be particularly interesting.

Bacteroides fragilis in CRC

Enterotoxigenic *Bacteroides fragilis* strains (ETBF) have been associated with CRC [64]. Dejea *et al*. showed that *B. fragilis* is associated with sporadic CRC, and tissues of patients with familial adenomatous polyposis (FAP) carry *B. fragilis* and *Escherichia coli* biofilms [65]. Patients with a medical history of *B. fragilis* bacteremia were also shown to be at a higher risk of developing CRC [56].

In mouse models of CRC, a synergistic oncogenic potential between ETBF and pks+ *E. coli* has been demonstrated. Co-colonization by ETBF and pks+ *E. coli* results in increased tumor burden compared with monocolonization, as ETBF promotes mucin degradation, favoring colonization by pks+ *E. coli* that leads to genotoxicity [65]. Furthermore, ETBF mediates carcinogenesis through IL-17-dependent STAT3 and NF-κB activation, supporting the differentiation of myeloid-derived suppressor cells and tumor-associated macrophages [66,67]. Finally, the metalloprotease and enterotoxin *B. fragilis* toxin (BFT) is able to cleave E-cadherin, leading to morphologic changes in epithelial cells [68]. It damages the colonic mucosa [69] and induces the expression of proinflammatory chemokines in colonic epithelial cells [67]. By contrast, nontoxigenic *B. fragilis* has a protective role against colitis-associated CRC, which is mediated by TLR2 signaling in mice [70]. Parallel to this, Ahmadi Badi *et al.* have observed an immunoprotective role of the outer membrane vesicles of *B. fragilis*, which suppress TLR2 and IFN-γ, while increasing IL-4 and IL-10 expression in Caco-2 treated cells [71].

In summary, the pathogenicity of *B. fragilis* highly depends on the expression of certain virulence factors or pathogenicity islands. A recent study demonstrated that the BTF-containing pathogenicity island of *B. fragilis* regulates bacterial competition and shapes the niche for pathogenic and commensal *B. fragilis* strains [72]. Further studies are needed to reveal the interactions taking place between *B. fragilis* strains and other bacterial strains.

Gemella morbillorum in CRC

Although there are no studies directly linking *Gemella* to cancer progression, it might be interesting to investigate how *Gemella* influences IL-12 production and subsequent IL-12-dependent

immunoregulation in a CRC setup, as it has been shown that *Gemella* reduces IL-12 levels in oral infections in mice [73]. Furthermore, several *Gemella* species have the ability to cleave IgA1, allowing bacteria to evade the protective functions of adaptive immune responses at mucosal surfaces [74]. Since *Gemella* mainly infects immunodeficient patients and localizes around the tumor, its potential interference with the host's immune system makes it an interesting target for CRC–microbiome studies.

Streptococcus gallolyticus in CRC

In vitro co-cultures of CRC cell lines (HCT116, HT29, or LoVo) with *S. gallolyticus* as well as bacteria-gavaged mouse models demonstrated elevation of β-catenin-dependent tumor cell proliferation [75]. Similar to *F. nucleatum*, *Streptococcus gallolyticus* was recently shown to influence the tumor microenvironment. Zhang and colleagues observed recruitment of tumor-infiltrating immune cells after *S. gallolyticus* exposure, generating an immune-suppressive microenvironment driving neoplasia [76]. However, proinflammatory states, marked by high NF-κB and IL-8 messenger RNA tissue expression, are also associated with *S. gallolyticus*-positive patients [77]. While *S. gallolyticus* draws increasing attention in the field of microbiome–CRC association, there is a need for subspecies-level resolution when studying host–microbe interaction. In this regard, Kumar and colleagues showed that only certain subspecies of *S. gallolyticus* were able to stimulate host cell proliferation [78].

E. coli^{pks+} in CRC

E. coli strains implicated in CRC are often characterized by their expression of genotoxins, such as the cyclomodulins CIF (cycle inhibiting factor), cytolethal distending toxin, cytotoxic necrotizing factor (CNF-1), or colibactin. CIFs are capable of blocking mitosis independently of DNA damage [79], and inducing apoptosis in epithelial cell lines [80]. In colonocytes, CNF-1 affects the actin cytoskeleton, causing reversible cellular senescence, which is potentially linked with chromosomal aberrances and genomic instability [81]. The genotoxin colibactin is produced by polyketide synthase-positive *E. coli* and induces DNA double-strand breaks *in vitro* and *in vivo* [82]. It increases tumor formation *in vitro* [54] alone, or in co-colonization with ETBF in FAP patients [65].

CRC-Associated Bacteria and (Onco-) Metabolites

While some bacteria, such as *F. nucleatum*, *E. coli*$^{pks+}$, or *B. fragilis* directly interact with host receptors on tumor or immune cells, many bacteria-driven effects might be due to secreted metabolites. The gut microbiome is a vast source of secretory proteins (secretome) and metabolites (metabolome) [83], feeding into a common metabolite reservoir of the tumor microenvironment. This metabolic pool includes growth factors, cytokines, proteases [84], but also oncometabolites, which are implicated in cancer progression. Oncometabolites are metabolic intermediates that accumulate in cancer, either upstream (e.g., L-2-hydroxyglutarate, succinate, fumarate) or downstream (e.g., D-2-hydroxyglutarate, lactate) of metabolic defects [85]. Along this line, the CRC-associated microbiome can be a source for such metabolites. For example, *B. fragilis* and some *Prevotellaceae*, but also *F. nucleatum*, have been shown to produce succinate [86,87], an inducer of proinflammatory pathways via succinate receptor 1 on immune cells [88]. *E. coli* catabolism of lysine to succinate involves the intermediate L-2-hydroxyglutarate, an oncometabolite that is involved in epigenetic deregulation in certain cancers [89]. Although it is a major product of beneficial probiotics [90], lactic acid can also fuel cancer cells [91]. Increased levels of SCFA, namely, acetate, butyrate, and propionate in CRC are usually linked with lower risk and improved prevention or therapy [92], so microbial-derived butyrate rather counteracts tumor development [93]. Butyrate suppresses proinflammatory genes and tumor growth, the latter via histone deacetylase inhibition, which downregulates oncogenic signaling pathways [94]. However,

depending on local concentration, SCFAs can also play a dual role in cancer. Butyrate, for instance, also inhibits proliferation of healthy intestinal progenitor cells [95]. Exogenous formate might even fuel cancer invasion [96]. In conclusion, understanding the metabolite-driven crosstalk between host and bacteria, metabolite sources and sinks, might therefore help in identifying new therapeutic strategies, for instance, by shifting the metabolic state of the tumor microenvironment into a lesser oncometabolite-containing state.

Tumoral versus Bacterial Spatialization in CRC

The large intestine possesses a great microbial abundance that increases along the GIT (10^3–10^4 bacteria/mL in the small intestine to 10^{11} bacteria/mL in the colon [97]) and mirrors the distribution of cancer risk [54]. There, **environmental filtering** and competitive exclusion between microbes are the factors responsible for shaping microbial diversity. Consequently, different microecologies are established [98], in which microbes might drive tumor progression in different ways. This aspect gains importance when we consider the difference in biology, pathology, and epidemiology of right- versus left-sided CRCs (RCC vs LCC). There are controversial findings regarding CRC patient survival rates when it comes to tumor location. Several studies have shown a worse prognosis of tumors located on the right side (caecum to ascending and transverse colon), while others a worse prognosis of tumors located on the left side (splenic flexure and descending colon to rectosigmoid junction; Box 3). The differing results of the studies might be explained by the use of varying statistical tools and different cohort properties (cohort size variation and age differences).

In CRC, hypermutable **microsatellite instability (MSI)**-high and **CpG island methylator phenotype (CIMP)**-high phenotypes, as well as *BRAF* mutation rates, are known to decrease from the ascending colon to rectum [99]. This may alter the potential pathogenic influence of intestinal microbiota along the proximal–distal axis. Along this line, Dejea *et al*. showed that bacterial organization into biofilm structures in the mucus layer of the gut is a consistent feature of RCC, but not LCC [100]. Interestingly, RCC biofilms invade the colonic crypts [101] and mainly consist of known CRC-associated bacteria [32]. Finally, Purcell *et al.* showed that these bacteria are associated with the consensus molecular subtype (CMS) 1, which is called MSI immune subtype, marked by MSI, CIMP-high, *BRAF* mutations, and immune cell infiltration [57]. Consequently, it is important to consider RCC and LCC microbiomes separately in order to evaluate their distinct features.

Nevertheless, bacterial spatial distributions do not always show direct interactions with the tumor. For example, *B. fragilis* is abundant in the proximal colon, however, its IL-17-dependent NF-κB activation induces a proximal-to-distal mucosal gradient of chemokines, thus mediating immune cell infiltration along with distal colon tumorigenesis [67].

Taken together, tumor localization goes hand in hand with spatial resolution of bacteria and gains importance when it comes to patient prognosis and treatment. Consequently, to better understand the spatial resolution of bacteria, multiple questions need to be addressed in the future: Which ecological abiotic niche factors (physical and chemical factors) support bacteria–tumor co-localization? To what extent does host–microbe mutualism (bilateral cross-feeding) take place? Furthermore, what is the effect of presampling bowel preparation procedures on the microbial composition on a (sub-) species level? Finally, can CRC-associated microbial communities be linked with different tumor microenvironmental metabolic states (following up on the work from Yachida *et al*. [35])? Here, the identification of cancer-specific metabolic niches in the gut (e.g., gradients of oncometabolites lactate or 2-hydroxyglutarate) can further be a point of interest.

Box 3. Right- versus Left-Sided Colorectal Cancers

There is evidence of differences in CRC patient survival rates when tumors are located on the right side (RCC, caecum to ascending and transverse colon) in comparison with the left side (LCC, splenic flexure, and descending colon to the recto-sigmoid junction) (Figure I). A meta-analysis of 66 studies, including over 1.4 million patients in total, showed that RCCs were associated with a significantly higher risk of death, independent of the study year, the number of participants or their race, tumor stage, or adjuvant chemotherapy [168]. According to a population-based study including 53 801 individuals, medium average survival was also significantly longer for patients with Stage III LCC tumors, than for patients with RCC tumors (60 vs. 46 months) [169]. Other studies have shown similar results [170,171], potentially in line with the fact that LCC CRC patients show favorable treatment outcomes [172–174].

Controversially, in agreement with a study by Weiss *et al.* [175] on Stage I CRCs, Moritani *et al.* showed a higher 5-year-survival rate in RCC patients over LCC patients, although for Stages II, III, and for the overall population, no differences were observable [176]. In the Weiss *et al.* study, a lower mortality rate was observed in Stage II RCCs than in LCCs; however, in Stage III RCCs, it was higher [175]. Especially in younger patients (20–39 years old) Stage II RCC seems to have lower mortality [177]. Finally, a more recent study by Warschkow *et al.* showed that the prognosis of RCC was better regarding overall survival. In Stages I and II, the RCC prognoses were better and in Stage III, similar prognoses were achieved [178]. Of note, the differing results of the studies might be explained by the use of varying statistical tools and different cohort properties (cohort size variation and age differences) [176].

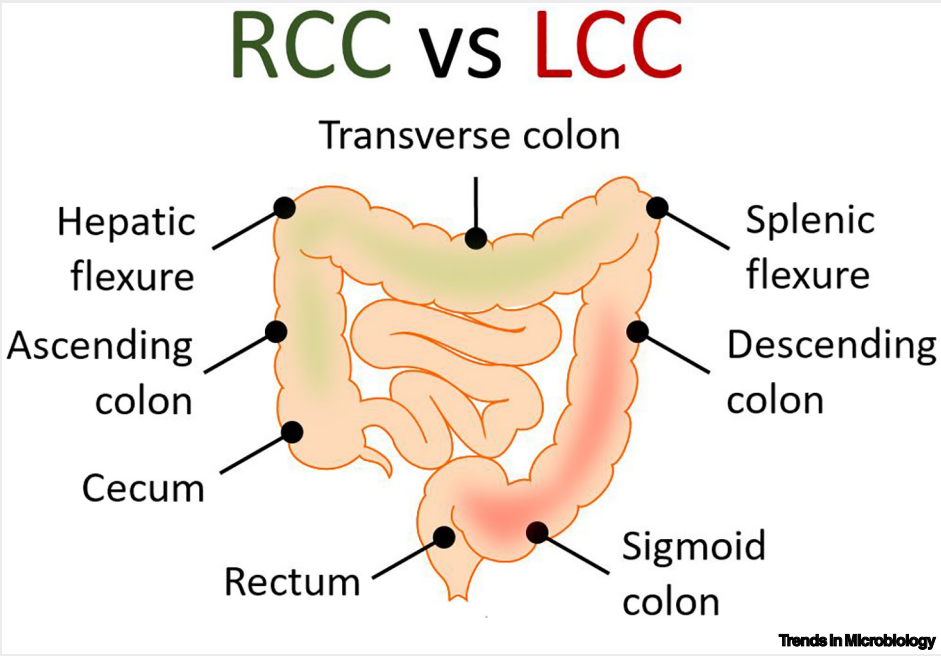

Figure I. Colon Anatomy.

In summary, the distinct spatial resolution of bacterial species can serve not only as a prognostic marker, but can also be targeted for better treatment outcomes. Finally, one must consider bacterial community structures and understand how they modulate their niches, in order to learn how to modulate them into communities, which may ultimately improve patient outcome.

Models for Investigating Host–Microbe Interactions

Recapitulating complex native physiological conditions is a key feature for a successful research model. 2D cell monolayers are still the most commonly used research model, although recent studies have furthered *in vitro* models to better reflect a 3D *in vivo* state. Nevertheless,

mouse models remain the golden standard, as they allow for the study of tumors and their microenvironment.

In Vitro Models

In vitro models provide a cheap, fast, and reliable method for testing direct or indirect links between microbes, their secreted molecules and cancer initiation or progression. Compartmentalized short-term direct co-culture systems (transwell system) are widely used, as well as experiments exposing human cells to bacteria-free supernatants, bacterial lysates, or live bacteria. However, among other 2D or 3D models, these *in vitro* methods have limitations, as they are typically constrained in maintenance of required anaerobic conditions, a physiological pH, or constant nutrient supply. In this respect, *in vitro* gut-on-chip models [118], which simulate human gut physiology are very promising, as they allow the probing of molecular exchanges between microbial and human cells and their repercussions in a representative manner [119]. Gut-on-chip models such as the 'Gut Chip' model from Kim and colleagues [120] was recently used for disease modeling in order to analyze the contribution of the microbiome to the intestinal pathophysiology of ileus and inflammatory bowel disease [121]. The HuMiX device (Human microbial crosstalk), a perfusion bioreactor system, provides a promising tool for integrated analysis of the effects of microbiome interactions on human colorectal adenocarcinoma enterocytes [122]. HuMiX mimics the human gut and has the advantage of allowing molecular crosstalks between patient-derived enterocytes and microbes, while providing a physical barrier similar to the mucosa. The HuMiX setup can be further enhanced by attaching additional compartments for immune, neuronal, or other cells. Recently, HuMiX was able to recapitulate transcriptional, metabolic, and immune responses in Caco-2 cells after co-culturing with probiotic *Lactobacillus rhamnosus* GG [122] and demonstrated potential of modeling synbiotic-based treatments [123]. Further developments of gut-on-chip models include microphysiological systems for pharmacology studies, such as the '10-MPS' system or the 'OrganoPlate' platform [124,125]. Finally, with the 'SynVivo' (SynTumor) model, as well as the 'Quasi-Vivo' (Kirkstall) model, more and more microfluidics-based chip platforms are emerging, as reviewed by Marchesi *et al*. [126].

3D Intestinal Organoids

The 3D organoid model system has gained ongoing attention in the fields of developmental, regenerative, and disease biology [127], as it provides a middle ground between 2D cell cultures and *in vivo* models. Organoids are generated from multiple sources such as adult and fetal tissues, embryonic stem cells, or induced pluripotent stem cells. They consist of multiple organ-specific cell types embedded in extracellular matrices, which facilitate self-organization through cell sorting and spatially restricted lineage commitment [128]. Organoids also bring advancements for studying host–pathogen interactions. For example, they have been used as an infectious model by microinjection of pathogenic bacteria (*Helicobacter pylori*) into the organoid's lumen [129].

Patient-derived organoids (PDOs) appear to be an encouraging CRC disease model. PDOs bring us closer to clinically relevant models and to personalized medicine, as gene-editing tools are easily applicable [130]. Despite the promising features of organoids, they also have limitations. Matrigel is the main organoid matrix used, but its heterogeneity and ill-defined components can pose a problem, since tissues require different matrix cues [131]. Regardless, intestinal organoids are a powerful and developing experimental model for understanding CRC development and host–microbe interactions. For example, recent developments in the organoid field now allow for the expansion of the immune cell compartment within organoids.

This paves the way for future studies on the interaction between the microbiome and the host immune system [132].

CRC Mouse Models

Mouse models have been widely used to study the microbiome in CRC and are able to provide valuable insights into CRC research, from tumor initiation and progression to metastatic processes. Mouse models offer a physiologically relevant setting, allowing for experimental repetition with reproducible results. They are indispensable tools for the discovery and validation of novel therapeutic targets. Both chemically induced and genetically engineered mouse models have their advantages and limitations. Among the chemically induced models, there is the inflammation-associated CRC model [azoxymethane (AOM)/dextran sodium sulfate (DSS)]. Here, inflammation is the main driver of cancer incidence. Alternatively, AOM can be used alone, which induces sporadic mutations over time. However, mutations in the latter model do not always recapitulate the mutational sequence observed in human CRC [133], although, for instance, early mutations in APC or β-catenin signaling are observed [134]. In CRC microbiome studies, both models are used. Nevertheless, in the inflammation-associated model, the bacterial effects might be overwhelmed by the robust chemically induced inflammation [135]. This is why microbiome studies often use the AOM model, in which the bacterial effect on CRC incidence can be explored in the absence of DSS-induced inflammation. Several studies have shown that the administration of certain CRC-associated bacteria in AOM models promotes colonic tumor formation (Table 3). Besides the chemically induced models, the genetic models, among which the widely used is the $APC^{min/+}$ mouse model, are often used to address the effect of specific bacteria or a specific microbiome in CRC (Table 3). This model also has its own disadvantages, as mice adenomas are mostly formed in the small intestine, whereas in humans tumors are found in the large intestine [136].

The aforementioned models can either be used in specific (and opportunistic) pathogen-free (SOPF/SPF) conditions or in germ-free (GF) conditions. Mice housed in GF conditions are a powerful model, which allow us to study the effects of a single microorganism or a predefined microbial community [137], without competition from the host microbiome. Nevertheless, high technical costs and translatability into the clinic are two major concerns. Indeed, it has long been reported that GF mice display changes in immune responses compared with their non-GF counterparts [138]. As the microbiome changes over time, one should carefully consider the study target (inflammation or genetic mutation) and the stage of CRC one wishes to study [139]. In addition, successful bacterial colonialization needs to be confirmed after bacterial gavage. Colonization depends on several factors such as viability in the upper digestive tract, host immune reactions, or specific bacterial factors.

The genetic models $IL\text{-}10^{-/-}$ and $RAG^{-/-}$ are commonly used to study immune-related mechanisms [140]. Yet, under GF conditions, studies have shown that several immunodeficient mouse strains ($IL\text{-}2^{-/-}$, $IL\text{-}10^{-/-}$, T-cell receptorβ/p53, or TGFβ1/Rag-2 mice) do not optimally develop chronic inflammation and tumors [140], suggesting the essential role of intestinal microflora. In CRC, a recent study showed that GF $APC^{min/+}$/$IL\text{-}10^{-/-}$ mice, when conventionalized with an SPF microbiome, had an increased colonic tumor incidence when compared with their GF counterparts or SPF-conventionalized GF $APC^{min/+}$ [54]. Along the same line, Apc^{468}/$IL\text{-}10^{-/-}$ mice under SPF conditions showed elevated levels of *Bacteroides* and *Porphyromonas* genera, correlating with increased inflammation and colon polyposis compared with Apc^{468} mice [141], thus demonstrating that colon cancer is driven by inflammation and the microbiome in addition to genetic factors.

In summary, using genetic and inflammation-associated CRC mouse models housed in both SPF/SOPF and GF conditions might be ideal, as it allows to observe subtle changes induced by bacteria, without missing their relevance in the context of the study target.

Table 3. Studies of CRC-Associated Bacteria in Various Mouse Models

Bacterial strain	Mouse model	Housing and pre-treatment	Dose and time points of bacterial infection	Proposed mechanisms		Refs
				Direct	Indirect	
Fusobacterium nucleatum						
EAVG_002 *F. nucleatum*	$Apc^{min/+}$	SPF	Daily gavage of 10^8 CFU/mouse for 8 weeks		Tumorigenesis is promoted by infiltration of $CD11^+$ myeloid-derived immune cells	[142]
F. nucleatum 12230 + $fadA^-$ mutant US1	Nude	SPF	Intratumoral bacteria injection into subcutaneous (SC) tumor	Binding of FadA into E-cadherin of the host modulates Wnt signaling		[47]
Clinically isolated *F. nucleatum*	$Apc^{min/+}$, $Apc^{min/+}$; IL-10 AOM/$IL\text{-}10^{-/-}$	GF bred, then transferred to SPF	One-time gavage of 10^8 CFU/mouse		Tumorigenesis is inflammation driven, with colibactin-producing *Escherichia coli* but not with *F. nucleatum* ($FadA^+$ or $Fap2^{+/-}$).	[54]
ATCC 25586	$APC^{min/+}$, AOM/DSS	SPF; pretreated with streptomycin (2 mg/mL) (drinking water) for 3 days	Daily gavage of 10^9 CFU/mouse for 5–6 weeks	Tumorigenesis by regulation of miR-21 through TLR4/MYD88/NF-κB		[143]
ATCC 25586	BALB/c nude	SPF	Subcutaneous injection of *Fusobacterium*-infected colorectal cancer cells (1×10^7 SW480 cells or 5×10^6 HCT116 cells)	Tumorigenesis by autophagy-induced chemoresistance through miR-4802 and miR-18a via TLR4/MYD88		[52]
Fn(F01) clinical isolated strain	C57BL/6, $Apc^{min/+}$	SPF; treated with ampicillin (1 g/L), metronidazole (1 g/L), vancomycin (0.5 g/L), and neomycin sulfate (1 g/L) (drinking water)	Daily gavage of 10^9 CFU/mouse for 8 weeks	TLR4/p-PAK1/p-β-catenin S675 cascade drives tumorigenesis		[144]
Fn(F01) clinically isolated strain, Fn (ATCC 10953)	$C57BL/6^-$ $Apc^{min/+}$	SPF	Daily gavage of 10^9 CFU/mouse for 8 weeks		Bacteria induces M2 macrophage polarization via TLR4, promoting tumorigenesis	[145]
ATCC 25586	BALB/c nude mice	SPF	Multipoint intratumoral bacteria injection into SC injection. Total of five injections every 3 days	Fuso-driven chemoresistance of 5-fluorouracil in CRC is regulated by BIRC3 genes via TLR4/NF-κB pathway		[53]
F. nucleatum 12230 + $fadA^-$ mutant US1	$Apc^{min/+}$	SPF	2×10^{10} CFU/mice was gavaged three times a week for 8 weeks.	Upregulation of ANXA1 gene via Fad-A and E-cadherin promotes tumorigenesis		[55]
Bacteroides fragilis						
NCTC9343 or PSA	DSS and AOM/DSS	SPF	Three times a week of 5×10^9 to 7×10^9 CFU prior to DSS or AOM administration until end point	Antitumorigenic properties in colitis-associated CRC are mediated through TLR2/CCR5 and production of polysaccharide A		[70]
ETBF 086-5443-2-2	AOM and $Apc^{Min\Delta 716/+}$	SPF	Mono- or co-colonization of ETBF and pks+ *E. coli*		Tumorigenesis is mediated through upregulation of IL-17 and DNA damage	[65]
Nontoxigenic *B. fragilis* (NTBF) TM4000, ATCC 43858/43859 (ETBF)	C57BL/6	SPF; pretreated with clindamycin (100 mg/L) (drinking water) for 1 day prior to and	First gavage with 10^8 CFU/mouse (TM4000). Seven days postcolonization, second	Lack of type IV secretion system and *B. fragilis* pathogenicity island promotes ETBF colonization		[72]

(continued on next page)

Table 3. (continued)

Bacterial strain	Mouse model	Housing and pre-treatment	Dose and time points of bacterial infection	Proposed mechanisms		Refs
				Direct	Indirect	
		throughout the course of infection	gavage with either ETBF (ATCC 43859) or an isogenic mutant (bfpai)			
ETBF 86-5443-2-2 and ETBF (bft2)	C57BL/6; $Apc^{Min\Delta716/+}$	SPF; pretreated for 5 days with clindamycin and streptomycin (drinking water) prior to gavage	One-time gavage of 10^8 CFU/mouse		ETBF-mediated tumorigenesis via IL-17-dependent NF-κB and STAT3 activation, and CXCL1, -2, and -5	[67]
ETBF 86-5443-2-2 NTBF 9343 (pFD340)	C57BL/6; $Apc^{Min\Delta716/+}$	SPF; pretreated for 5 days with streptomycin (5 g/L) and clindamycin (100 mg/L) (drinking water) prior to and following bacterial gavage	One-time gavage of 10^8 CFU/mouse for period of 6 weeks	–	Production of spermine oxidase, promoting inflammation-induced tumorigenesis through reactive oxygen species and DNA damage	[146]
Streptococcus gallolyticus subsp. *gallolyticus* (SGG)						
SGG TX20005	A/J mice AOM	SPF; two doses (longer procedure, three times/week for 24 weeks) and four doses (shorter procedure, one time/week for 12 weeks) of AOM, followed by 1-week antibiotic treatment.	10^8 CFU/mouse TX20005, *Lactococcus lactis*, or saline at different frequencies; shorter procedure: once a week for 12 weeks	Wnt/B-catenin, c-Myc, and PCNA activation		[75]
SGG UCN34Δblp mutant, SGG UCN34	Notch and Notch/APC; C57BL/6J RJ	SPF/GF; SPF (short term); Notch and Notch/APC mice were given daily intraperitoneal injection of body weight Tamoxifen (50 μg/g) for 5 days; SPF (long term); Notch and Notch/APC mice were given vancomycin (50 μg/g), neomycin (100 μg/g), metronidazole (100 μg/g), amphotericin B (1 μg/g), ampicillin (1 g/L) for 8 days	SPF; single gavage (short term) or three times consecutive gavage (long term) of 10^{10} bacteria/mouse. GF; single gavage of 10^{10} bacteria/mouse after receiving microbiota transfer (stool) from Notch and Notch/APC mice		Elevated gallocin, class II SGG-specific bacteriocin, leads to the enrichment of intestinal bile-acid favoring CRC development	[147]
ATCC BAA-2069	C57BL/6 AOM/DSS	SPF; AOM injection followed by three cycles of 2.5% DSS (drinking water) for 5 days	Daily gavage of 1.2×10^7 CFU/mouse/day for 2 weeks prior to the experiment		Tumorigenesis via immune suppression, recruitment of tumor-infiltrating immune cells, and upregulation of myeloid-cell-derived proinflammatory cytokines (IL-6, IL-1B, IL-8, CCL2, COX2, TNF-α)	[76]
Other bacteria						
Peptostreptococcus anaerobius (ATCC 27337)	C57BL/6 AOM	SPF; pretreated with ampicillin (0.2 g/L), vancomycin (0.1 g/L), neomycin (0.2 g/L), and metronidazole (0.2 g/L) (drinking water) for 2 weeks before AOM injection	10^8 CFU/mouse of *P. anaerobius* or *E. coli* MG1655 (as control) every 2 days for 6 weeks, starting 3 days after AOM injection	Activation of TLR2 and TLR4	Production of reactive oxygen species and cholesterol biosynthesis	[58]

Table 3. (continued)

Bacterial strain	Mouse model	Housing and pre-treatment	Dose and time points of bacterial infection	Proposed mechanisms		Refs
				Direct	Indirect	
Campylobacter jejuni	$Apc^{Min/+}$ 1% DSS	GF	10^5 CFU/mouse one-time gavage		Genotoxin (cytolethal distending toxin)-mediated DNA damage and tumorigenesis	[148]
Bacteria derived from patient stool						
Mixed strains of bacteria	C57BL/6 and AOM	SPF/GF; pretreated daily for 2 weeks with ampicillin (0.2 g/L), neomycin (0.2 g/L), metronidazole (0.2 g/L), and vancomycin (0.1 g/L) (drinking water)	SPF; 1 week after AOM injection, two times/week gavage for 5 weeks of mixed stool from five CRC patients, five healthy controls, or PBS[a]. GF; one-time gavage of CRC patient stool or healthy controls at week 0.		CRC development is mediated through CXCR1, CXCR2, IL-17A, IL-22, IL-23A, and Th1 and Th17 infiltration	[137]
Mixed strains of bacteria from homogenized pieces of human intestine carrying biofilms or not	C57BL/6 GF 129/SvEv $Apc^{Min\Delta 850/+}$ GF 129/SvEv $Apc^{Min\Delta 850/+}$ $IL\text{-}10^{-/-}$ SPF C57BL/6 $Apc^{Min\Delta 716/+}$	SPF/GF; treated with cefoxitin (500 mg/L) for 48 h (drinking water)	SPF: After removal of antibiotic water for 24 h, mice were gavaged with human colon mucosal homogenates [anaerobically prepared by mincing/homogenizing tissue in PBS in an anaerobic hood to a final dilution of 1:20 (weight/volume)], GF: gavage with human colon mucosal homogenates as described above.		Biofilm-positive human mucosal tissues induce early IL-17 and myeloid cell infiltration	[149]
Mixed strains of bacteria (1 g mixed feces from 10 healthy or CRC patients in 5 mL PBS)	C57BL/6J, $Apc^{Min/+}$	SPF; pretreated for 3 days with antibiotics mixed of ampicillin (200 mg/L), metronidazole (200 mg/L), neomycin (200 mg/L), and vancomycin (100 mg/L) (drinking water)	16 times/8 weeks after antibiotics treatment: one time/week 1, three times/week 2, one time/weeks 3–6; C57BL/6: one group gavaged with mixed fecal samples from healthy controls, the other group gavaged with mixed fecal samples of CRC patients. $Apc^{Min/+}$ (three study groups): group 1 treated with PBS, group 2 gavaged with mixed healthy fecal samples, and group 3 gavaged with mixed CRC fecal samples.	Modulation of Wnt/β-catenin and cyclin D1 cascade		[150]

[a]Abbreviation: PBS, phosphate-buffered saline.

In Silico Models

Computational modeling, particularly constraint-based modeling of bacterial metabolism from a genome-scale network exhibits promising attributes when looking at genotype-to-metabolic-phenotype relations or microbe–microbe and host–microbe metabolic interactions. This approach requires the conversion of a human or microbial genome sequence into a metabolic

reconstruction and further, the transformation into a condition-specific model. Thereby, a biochemical reaction list is converted into a computable, **stoichiometric matrix** format on the basis of existing experimental data [151]. Next, as all cellular networks operate within boundaries of physical and chemical constraints, mathematical constraints are also applied to the model. Finally, via **flux balance analysis**, a computed steady-state flux space contains all feasible steady-state flux distributions for the biochemical network and thereby predicts the metabolic phenotype [151].

In the past, constraint-based modeling has successfully predicted optimal growth rates for various bacteria and the results were further confirmed experimentally [86]. Today, genome-scale metabolic models based on human GIT metagenomics data are used for testing hypotheses of how microbial communities modulate human metabolism and health, potentially leading to predicting dietary-based treatments [152]. In these cases, the publicly available AGORA reconstructions provide a valuable resource [153]. Integration of context-specific host cell models [154] might then help to address the question of how bacterial metabolism affects GIT cell metabolism and vice versa. Ultimately, these models can suggest FDA-approved drugs [155] for treatment of and interference with (microbiome-) cancer-specific pathometabolism [123]. An example study for using *in silico* metabolic models is shown in Figure 2. Despite its advantages, a major constraint of the approach itself is that it strongly depends on experimental data and high-quality metabolic networks requiring extensive computational and manual analysis, which needs experimental validations.

Concluding Remarks and Future Perspectives

Whereas most studies focus on the presence of different bacterial species in CRC, we would like to draw attention to the potential importance of the products they secrete as a target for novel treatments. This should include stepping back from the drastic killing strategy of antibiotic therapies and addressing the more modern approach of disarming and/or replacement of these pathogens. It is particularly important to identify the mechanistic pathways by which bacteria influence CRC in order to then metabolically (interfering with the metabolic state with drugs or diets) or immunologically (e.g., immunogenic/inhibitory peptides against Fap2, FadA, or BFT) target these pathways. Interfering with the microbial metabolome by administering selective drugs or food additives may influence disease outcome and therapy efficiency. The role of community-driven pathogenicity must also be addressed in order to redirect efforts toward the establishment of targeted treatments. Computational modeling is a promising approach for studying genotype-to-metabolic-phenotype relations or microbe–microbe and host–microbe metabolic interactions. Meta-omic approaches combining functional collective approaches such as meta-transcriptomics, meta-**proteomics**, and metabolomics have massively gained pace throughout recent years. Through integration, we have qualitatively and quantitatively advanced our knowledge on the genetic, transcriptional, proteomic, and metabolic potential of microbial communities at given stages of a disease. Nevertheless, we still lack information on individual mechanistic insights and experimental validation of microbes in CRC [156] (see Outstanding Questions).

To reach this goal, we propose an intertwining triphasic experimental approach (Figure 3):

- Parallelized experiments using different *in vitro* assays for hypothesis testing with multiplexed read-outs, followed by *in vivo* experimental validations with appropriate mouse models
- Generation and analysis of multi-meta-omics data
- Integration of meta-omics data into *in silico* models

Starting with the selection of clinically relevant model organisms on the basis of patient samples that are better resolved in time (disease progression) and space (localization and microenvironment

Outstanding Questions

Which microbiome-derived molecular factors are involved in disease initiation and progression?

What microbe–microbe interactions can affect CRC development?

What is the importance of community-driven changes as opposed to single bacteria?

Is there a predominant effect of the tissue-proximal microbiome on CRC and should studies focus more on the tissue-adherent microbiome?

How do fluctuations in the gut microbiome affect cancer treatment? And vice versa, how does cancer treatment affect the microbiome and the further path to recovery?

How can the microbiome as a biomarker be integrated into everyday diagnostics in order to better the current tools?

How can personalized dietary guidelines for recovering CRC patients become a reality?

What is the role of viruses and fungi in CRC progression?

What is the role of the oral microbiome in colorectal cancer?

How can meta-omics data be integrated into microbiome–CRC studies?

How can we gear *in silico* models toward predicting personalized treatment strategies that include prebiotics and probiotics, chemotherapy, and fecal transplantation?

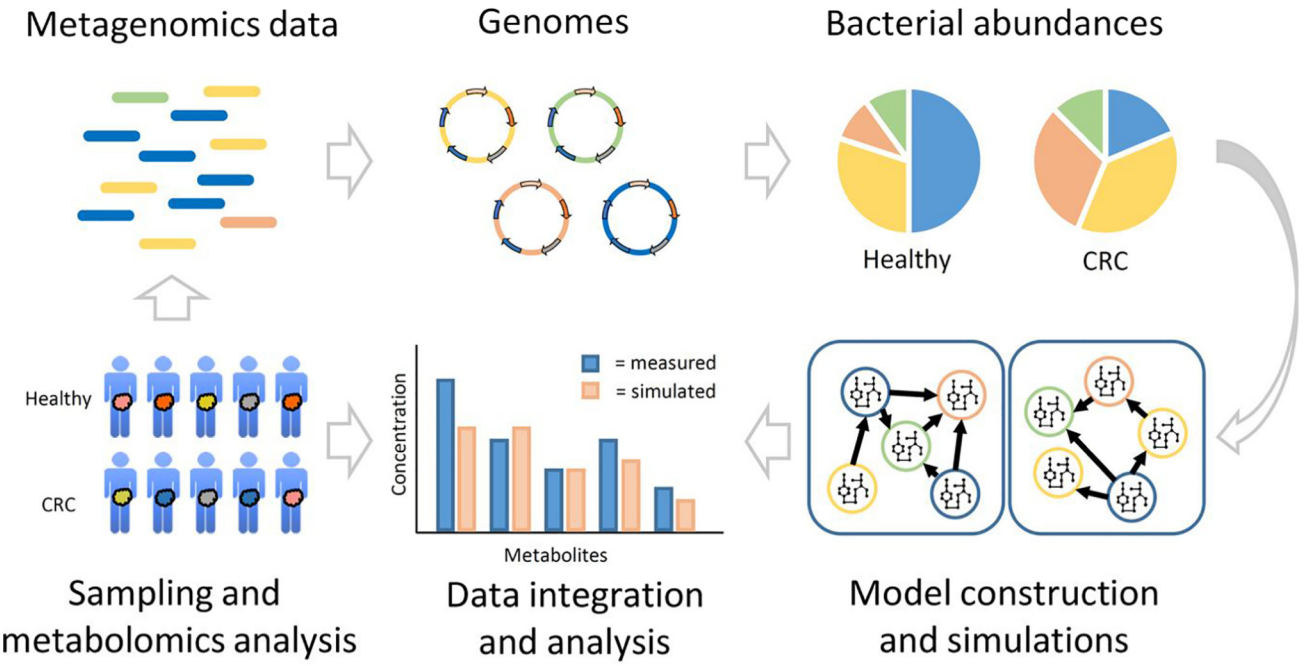

Figure 2. Modeling of the Colorectal Cancer (CRC)-Associated Microbiome with Cell-Type-Specific CRC Model Using Constraint-Based Reconstruction and Analysis. The computational framework uses metagenomic data to construct genomes, which give information about the diverse microbial content of a sample. After transfering the individual genome-scale reconstructions (models) into an *in silico* microbiota for each patient and heathly control, metabolite concentrations can be simulated and compared with metabolomics data. Based on these comparisons, new hypotheses about metabolic states of bacterial communities could manifest holisic perspectives on the role of CRC-associated bacteria and their crosstalk with cancer.

within the GIT), one could use personalized *in vitro* models for rapid detection of phenotype–genotype relations. This process can already be supported by *in silico* hypothesis testing using publicly available data. Next, the use of **gnotobiotic** mouse models would validate and further test the ro-

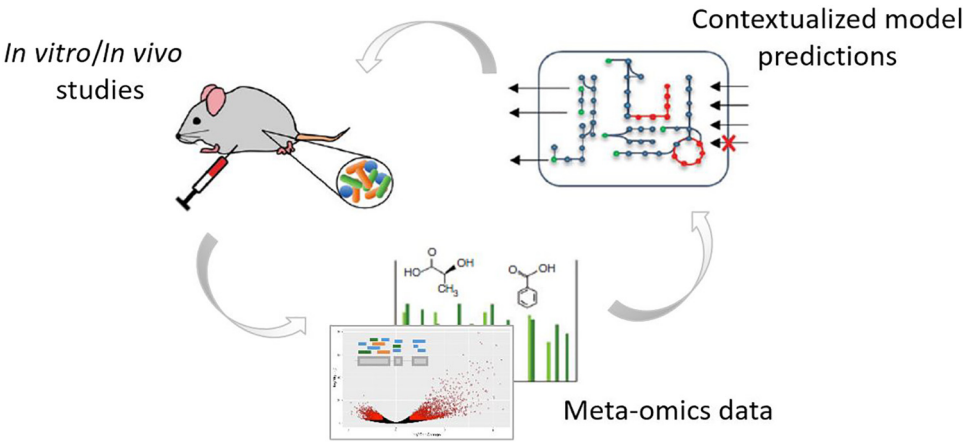

Figure 3. Intertwined Triphasic Experimental Approach. To shed light on individual bacteria-driven mechanisms in colorectal cancer and their experimental validation, we propose an intertwining triphasic experimental approach consisting of parallelized experiments using different *in vitro* assays for hypothesis testing with multiplexed read-outs, followed by *in vivo* experimental validations with appropriate mouse models; generation of multi-meta-omics data and integration of meta-omics data into *in silico* models.

bustness of a phenotype. Resulting omics data would improve *in silico* models, which would then be further used to test hypotheses. This approach can provide insights into how cancer-enriched microbial taxa may be involved in triggering disease-relevant pathways, which in turn will allow for follow-up mechanistically oriented studies.

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