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Abdallah Ladaycia, Brigitta Loretz, Catherine Passirani, Claus-Michael Lehr, Elise Lepeltier. Microbiota and cancer: In vitro and in vivo models to evaluate nanomedicines. *Advanced Drug Delivery Reviews*, 2021, 170, pp.44-70. 10.1016/j.addr.2020.12.015 . hal-03129847

HAL Id: hal-03129847

<https://univ-angers.hal.science/hal-03129847>

Submitted on 15 Apr 2022

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Microbiota and cancer: *in vitro* and *in vivo* models to evaluate nanomedicines

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Abstract

Nanomedicine implication in cancer treatment and diagnosis studies witness huge attention, especially with the promising results obtained in preclinical studies. Despite this, only few nanomedicines succeeded to pass clinical phase. The human microbiota plays obvious roles in cancer development. Nanoparticles have been successfully used to modulate human microbiota and notably tumor associated microbiota. Taking the microbiota involvement under consideration when testing nanomedicines for cancer treatment might be a way to improve the poor translation from preclinical to clinical trials. Co-culture models of bacteria and cancer cells, as well as animal cancer-microbiota models offer a better representation for the tumor microenvironment and so potentially better platforms to test nanomedicine efficacy in cancer treatment. These models would allow closer representation of human cancer and might smoothen the passage from preclinical to clinical cancer studies for nanomedicine efficacy.

Published in *Advanced Drug Delivery Reviews* 170 (2021) 44-70

<https://doi.org/10.1016/j.addr.2020.12.015>

1. Introduction

Cancer is a major public health problem. It is the second most frequent cause of death worldwide killing more than 8 million people every year [1]. In 2019, 1,762,450 new cancer cases and 606,880 cancer deaths were registered in the United States [2]. Being one of the biggest priorities in the medical and scientific research fields, studies on oncology made huge progressions in understanding different cancer mechanisms and in developing cancer treatments. For instance, the overall cancer death rate decreased continuously in the last 15 years by 27 %, where it declined annually by 1.4 % among women and 1.8 % among men [2]. These encouraging results do not deny the fact that a lot of work is still to be done in this field. Resistance to chemotherapy, lack of understanding of the full physiopathology of the disease, inefficacy of the treatments and the heavy side effects of the therapies on patients are all current problems that face scientists and doctors in the oncology field. Recent studies have shown the involvement of the human healthy flora, known as microbiota, in the cancer development and the body response to cancer chemo- and immunotherapy [3]. Considering the human microbiota as a potential factor in the cancer development and the therapy efficacy might help moving forward in the process of improving the cancer treatment.

In this review, the human microbiota and its role are explained and its influence on the cancer physiopathology and therapy is detailed. The implication of nanoparticles in the treatment of cancer and infectious diseases is then described. Innovative approaches to use nanoparticles for improving cancer treatment by modulating the microbiota effect are then brought into light. Finally, current *in vitro*, *ex vivo* and *in vivo* models are then reviewed and discussed for their potential as models for testing the anticancer effect of nanoparticles

related to the microbiota. Research in experimental model development in this rather young research field can help to overcome this poor transition from preclinical to clinical studies.

2. Human microbiota

It is nowadays a common knowledge that the healthy human body harbors myriads of microorganisms living in symbiosis with the human cells from various organs. This community of microorganisms, known as microbiota, is not exclusively formed of bacteria but also includes fungus and viruses [4]. The appearance and development of the genetic material sequencing methods allowed better identification and understanding of the different organ microbiota profiles [5]. These techniques showed a different distribution in number and species of the bacteria forming each area microbiota of the human body: a difference that might be attributed to the unique physiological environment that each organ provides and its contact with the external world, leading to a specific shaping of each organ microbiota.

The human microbiota might be classified into four different communities according to its localization: gut, skin, vaginal and lung microbiota (**Table 1**).

2.1. Gut microbiota

Many studies investigate the gut microbiota, offering an important amount of information about its composition and bringing to light its importance. The lion's share of the microbiota populating the GI tract is kept under surveillance by the lion's share of human immune cells also located at the intestinal epithelium. The maintenance of proper cohabitation here is of crucial importance for the overall health and immune condition of humans. More than 500 different bacterial species form the gut microbiota and represent 10 times more than the number of eukaryote

cells in the human body [6]. The deoxyribonucleic acid (DNA) sequencing methods showed that the gut microbiota is colonized by different bacteria species that belong to four essential phyla: *firmicutes*, *Bacteroidetes*, *Proteobacteria* and *Actinobacteria* [7]. Representing almost 50 % of the bacterial phyla forming the gut microbiota, *firmicutes* is the most dominant phyla in the gut microbiota. It is represented by many bacterial families, essentially *Lachnospiraceae*, *Streptococcaceae* and *Lactobacillaceae* [7]. It is important to mention that the gut microbiota might be influenced by different factors causing a changing in its bacterial profile. For instance, the different phyla are not evenly distributed throughout the digestive tube, and different sample collections from different gastrointestinal tract areas might lead to different bacterial profile of the gut microbiota [7,19]. Other factors like age, diet, obesity and genetics have been reported to influence the gut microbiota composition [20–22].

2.2. Skin microbiota

The skin is the largest organ in contact with the outside world, making it as an important interaction zone and a habitat for different microorganisms. Four main phyla represent the bacterial community of the skin microbiota: *Proteobacteria*, *Actinobacteria*, *Firmicutes* and *Bacteroidetes* [11]. *Proctiobacteria* is the major phyla of the skin microbiota and is mainly represented by the bacterial genus: *Pseudomonas* and *Janthinobacterium*. Other genus forming the skin microbiota were identified such as *Corynebacterium*, *Propionibacterium* or *Staphylococcus* [11]. As for the gut microbiota, some factors might impact the skin microbiota, causing a slight or a major changing in the bacterial flora composition. For instance, studies have reported that individuals from different age presented different skin microbiota composition [23,24]. Environmental factors like humidity and temperature also influence

the configuration of the skin microbiota [25]. Finally, some habits like hand washing lead also to a potential change in the skin microbiota [24]. These different factors might explain the potential differences from different studies reporting the composition of the skin microbiota.

2.3. Vaginal microbiota

The healthy flora of the vaginal microbiota is mainly formed of bacterial species that belong to the genus *Lactobacillus* [15]. Other bacterial genus may be found in the healthy female reproductive system. 16S ribosomal ribonucleic acid (rRNA) analysis identified the presence of different genus of strictly anaerobic bacteria in the vaginal microbiota like *Prevotella* or other genus like *Streptococcus* and *Cryptobacterium* or other potential urogenital pathogens [15,26]. Factors like pregnancy, time in menstrual cycle, community class, sexual activity, menopause and hormone therapy, may change the vaginal microbiota or even cause its switching [27–29]. The crucial role of the vaginal microbiota is to protect from urogenital infections [29].

2.4. Lung microbiota

Lungs have been considered as sterile for a long time, and the idea of the healthy lungs being colonized by bacterial flora was far from being taken under consideration for hundreds of years [30]. The sequencing methods of bacteria DNA and RNA proved the presence of bacteria even in the healthy lungs [31]. Originally generated from the higher airway and oral cavity, the lung microbiota is essentially formed of the phyla *Firmicutes* represented by the genus *Veilonella* and *Streptococcus* and the phyla *Bacteroides* represented by the genus *Prevotella* [17]. The idea of healthy deep lungs being colonized by commensal microbiota is still quite new, explaining the lack of studies on the subject.

Table 1: composition and characteristics of the human microbiota.

<i>Microbiota</i>	<i>Composition (phyla/genus)</i>	<i>Properties</i>	<i>Density</i>	<i>Examples of drug delivery strategies for local cancer and effects on microbiota</i>	<i>Ref</i>
Gut	<i>Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria.</i>	- Expression of carbohydrate-active enzymes de novo synthesis of vitamins	From 10 ³ of bacteria per gram of stomach tissue to 10 ¹¹ of bacteria per gram of colon.	Clodronate liposomes for the treatment of colorectal cancer in mice: decrease the abundance of the <i>Firmicutes</i> .	7–10]
Skin	<i>Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes</i>	- Majorly lipophilic (colonizing the sebaceous areas) Production of slime	From 10 ² per cm ² (fingertips, back) to 10 ⁶ per cm ² (forehead, axilla)	Topically applied polyapigenin loaded (lactic-co-glycolide) nanoparticles for the treatment of induced skin cancer in mice: the effect on skin microbiota was not studied	11–14]
Vagina	<i>Lactobacillus, Prevotella, Streptococcus and Cryptobacterium</i>	Lactic bacteria: fermentation of sugar to lactic acid	10 ⁷ to 10 ⁸ of <i>Lactobacillus</i> bacteria in vaginal fluid in healthy premenopausal women	Vaginal administration of paclitaxel loaded poly(lactic-co-glycolic acid) nanoparticles for the treatment of cervical tumors in mice: the effect on skin microbiota was not studied	15,16]
Lung	<i>Veilonella, Streptococcus and Prevotella</i>	-	10 ² of bacteria per mL of bronchoalveolar lavage.	Intratracheal administration of doxorubicin liposomes for the treatment of lung cancer: the effect on lung microbiota was not studied	17,18]

2.5. Role of human microbiota

The bacterial flora forming the human healthy microbiota lives in symbiosis with the human body and so in return of using the body nutrients to survive, it has been shown that the human microbiota plays different local and systemic physiologic and pathologic roles.

Since gut microbiota is the most studied among other organ microbiota, more information is provided about its different roles in the human body. *In situ*, the gut microbiota does not only use nutrients to survive but also helps in the process of nutrition of the human body. More specifically, gut flora produces essential nutrients like vitamin B12, metabolizes complex compounds into molecules ready to be used by the organism and ferment non-digestible nutrients [32–35]. In addition to its nutritional role, the gut microbiota helps to protect the gastrointestinal tract from bacterial infections via different mechanisms: either by competitive exclusion, competition with pathogenic bacteria for nutrients or by synthesizing antibacterial proteins [36–38]. In addition to these local roles, the gut microbiota plays also a systemic immunomodulatory role. It has been proven that the gut microbiota contributes in the maturation of the immune system and in the production of immunoglobulin A and CD4+ T cells [39,40]. Several studies on germ free mice showed that the lack of microbiota led to the appearance of local and systemic anomalies such as a reduced intestinal surface area, a decreased density of villus capillary network, a diminution in the bile acid deconjugation, a reduced cardiac output and an abnormality of the hypothalamic–pituitary–adrenal axis [41–46]. Other than the gut microbiota, other organ microbiota play also crucial roles at different levels. For instance, the skin being the first barrier against the external world, it might be the most exposed organ to pathogenic micro-

organisms. The skin microbiota thus protects against infections by other pathogenic bacteria either by competition for nutrients or by synthesizing antimicrobial compounds that inhibit the pathogenic micro-organisms [47–49]. Vaginal microbiota protects against various urogenital diseases, fungal infections, sexually transmitted diseases and urinary tract infections [50–53].

These different examples show the implication of the microbiota in various physiological and pathological pathways and the key role it plays in the organism equilibrium and health. It is important to specify that the appearance of the Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) sequencing technologies allowed a much better understanding and characterization of the human microbiota and its role in the human health and clarified its implication in human health and disease mechanism. In parallel, cancer is the most known and one of the most lethal diseases of our time. However, with all the difficulties faced, from a poor understanding of its pathogenic mechanisms to the efficacy problems with the anticancer therapy, oncology remains a field where a lot of questions need to be answered in order to improve the anticancer treatments. Recently, it has been suggested that microbiota should be considered in cancer development and cancer therapy efficacy and response.

3. Relationship between microbiota and cancer

Cancer development is a complex mechanism that might be triggered by different factors causing the dysfunction of normal cell life cycle. One of the potential origins of cancer might be secondary to the action of pathogens or commensal bacteria. In fact, infection without proper resolution can cause chronic inflammation and trigger malign cell growth. Autoimmune reactions with chronic inflammation also may weaken the defense system and allow infections to occur. **Figure 1** depicts factors affecting the microbiome

balance and mechanisms contributing to infection-associated pathologies. Such cycles may be difficult to resolve and are known as factors supporting several types of cancer. For example, the growth in a biofilm hinders bacterial clearance by the immune system and antibiotic therapy. A biofilm is a community of bacteria (of one or diverse species) with coordinated behavior, which produces an extracellular polysaccharide matrix for adherence and self-embedding. Biofilms cause

severe infections since the extracellular matrix and the changed metabolic activity render them reduced sensitivity against antibiotics. Biofilm-building bacteria thus may cause chronic infections and contribute in disease pathogenesis. Medical conditions where biofilms are a serious concern are lung infections (CF, COPD), GI diseases (IBD, colorectal cancer) but also chronic wounds and device-associated infections

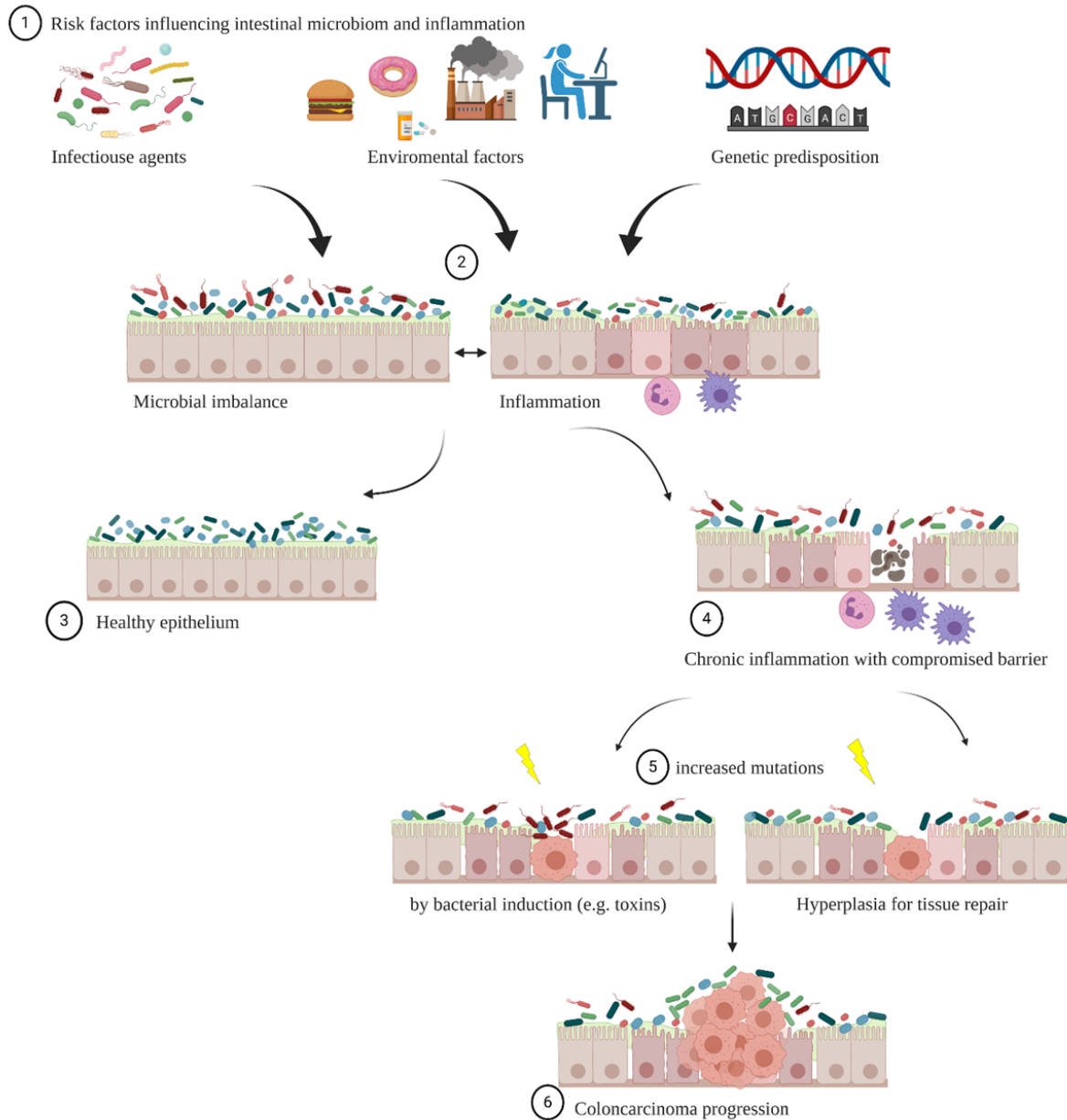


Figure 1: Colorectal cancer development may be influenced by several factors ① as infectious agents, environmental factors (including nutrition, life-style, stress, hazardous substances) and genetic predisposition (like inherited mutations in genes essential in immune response or tumor suppression). ②Microbial imbalance and inflammation happen with some frequency and are usually resolved by our immune response ③ . However, when for severe conditions or combinations of triggers the inflammation/infection cycle cannot be resolved and gets chronic the epithelial barrier integrity gets compromised ④. Chronic inflammation may favor certain pathogenic bacteria capable to escape the immune system e.g. by expression of toxins or virulence factors. Some of these toxins have direct influence of the mutation rate. The continuous tissue repair required to fix the compromised barrier and the inflammation induced level of reactive oxygen species increase the potential of mutagenesis accumulation ⑤. Such hyperplasia can progress into colon cancer ⑥. Certain bacterial species can profit from the tumor microenvironment, accumulate and have influence on the immune response, chemotherapy and tumor growth.

Evidence for the complex interplay between microbiota and tumorigenesis comes from clinical observations and investigational studies of *in vivo* and *in vitro* models [54]. Leystra *et al.* suggested in a review experimental strategies which are meant to “account for the influence of the microbiota on intestinal phenotypes in mouse models of colorectal cancer” [55]. For example, C57Bl/6 APC^{Min/+} mice vs wild type were compared for their colon microbiome by qPCR and 16s rRNA analysis [56]. The mutation of the APC gene was shown to promote the establishment of a dysbiosis.

As the gastrointestinal microbiota is the major constituent of the human microbiome, it was the main subject of the studies describing the interactions between the human microbiota and cancer.

Due to the *in situ* and general role it plays in health or sickness, the implication of the gut microbiota has been studied in both, local and other distal tumors. The microbiota might act as a suppressive or as a promoter on both tumor growth and on cancer therapeutics. These effects might be produced by various different mechanisms that will be described below.

3.1. Bacterial microbiota as driver of cancer

The bacterium *Helicobacter pylori* is a pathogen bacterium of the human stomach. This bacterium was the prime strain reported as associated with the development of human cancer and is even considered by the world health organization (WHO) as a class I human carcinogen bacterium [57,58]. Epidemiological studies have indicated a relationship between *H. pylori* colonization and gastric cancer. Actually, a progressive decline in *H. pylori* acquisition has been reported among people living in developed countries during the last century, a decline reported as associated with decreasing incidence of gastric cancers [59]. Also, different studies have shown that the risk of gastric cancer increased among patients who

tested positive for antibodies to *H. pylori* [60,61]. Furthermore, it has been shown that as the level of antibody to *H. pylori* increased, there was a progressive increase in the risk of gastric carcinoma [62]. The relationship between *H. pylori* and gastric cancer served as an initial link between bacteria and cancer, opening the door to further studies to investigate the implication of other bacteria strains, in the cancer development (**Table 2**).

The first studies showing a relationship between the human microbiota and cancer were based on observations among animals with germ-free status or treated with wide-spectrum antibiotics, and the impact it had on some cancer development.

For instance, many studies showed that germ free models presented less developed tumors compared to control groups with conventional sanitaire status [63–65]. In similar studies, animals that were orally receiving antibiotic treatments, either for infection treatment or using wide spectrum antibiotic cocktail in order to eradicate the healthy microbiota, expressed fewer or reduced colonic, hepatic and gastric tumors compared to non-treated ones [66–74]. In other type of experiments, quantitative polymerase chain reaction (PCR) studies, 16 rDNA sequencing and analysis of *in situ* fluorescence (FISH), detected the presence of elevated levels of *Fusobacterium spp* in colorectal adenomas [75]. These results suggest a pro-oncogenic effect of the healthy microbiota, pushing researchers to understand the assets involved in this relationship and to investigate the mechanisms involved in this effect. One way to explore this was to study the potential effects of the microbiota metabolisms and synthesized molecules. For example, *Bacteroides fragilis* toxin (BFT), secreted by *Bacteroides fragilis*, promotes the cleavage of E-cadherin [76]. E-cadherin is a protein known to be a blood tumor marker: its cleavage leads to a decrease of its blood level, considered as a cancer marker. The oncogenic effect caused by the cleavage of E-cadherin is explained by the

fact that it provokes the nuclear translocation of the β -catenin, subsequent transcription of colonic epithelial hyperplasia [76]. β -catenin is a component of the cell membrane-bound adherens complex, and is involved in the translocation of the protein from the cytoplasm to the nucleus. Many studies suggested its involvement in colorectal cancer. First, a dysregulation of β -catenin protein expression was reported at all stages of adenoma carcinoma sequence [77]. Also, pro-oncogenic factors have been shown to release β -catenin whereas anti-oncogenic factors inhibit its nuclear signaling [77].

In a similar mechanism, *Fusobacterium nucleatum* binds to E-cadherin via the adhesin FadA, a membrane domain, leading to colorectal cancer cell growth, oncogenic and inflammatory responses [78]. In a completely different mechanism, *Escherichia coli* produces colibactin, a substance that damages DNA. Colibactin induces senescence associated secretory phenotypes (SASP), in which senescent cells secrete growth factors that stimulate epithelial proliferation and enhance tumor growth [79]. *Enterococcus faecalis* is a resident bacterium in the oral and gut flora. It has been reported that *E. faecalis* has the capacity to produce reactive oxygen species, inducing DNA breaks and genomic instability. In another mechanism, *E. faecalis* has been linked to macrophage capacity of acting as key effectors in initiation and progression of cancer [89] via potentially linking innate immunity to carcinogenesis[90]. This phenomenon is known as: macrophage-mediated bystander effects. In a study realized by Wang *et al.* [90], it has been shown that *E. faecalis* infected macrophages and caused the production of a macrophage-induced bystander that provoked colorectal cancer development.

Table 2: List of selected pathologic microbiots with at least partly known mechanistic function on tumorigenesis, progression and the malignancies they are associated.

Pathobiont	Malignancy	Mechanism of pathogenicity	Ref
<i>Helicobacter pylori</i>	gastric cancer	Initiation of atrophic gastritis	[62]

In addition to the tumor promoter effect, some studies suggest a tumor-suppressor effect of the human natural flora. In fact, some short chain fatty acids (SCFAs) produced by the gut microbiota have shown anticancer effect. In particular, butyrate and propionate are two SCFAS produced by the gut microbiota via fermentation of dietary non digestible carbohydrates [91]. These two SCFAs have been proved to present an anti-cancerous effect [92,93]. On one hand, butyrate acts as a histone deacetylase inhibitor, favoring apoptosis and so causing an inhibition of the growth and development of lymphoma and carcinoma tumors [92]. On the other hand, propionate causes apoptosis by different cytotoxic phenomena including mitochondrial alterations, caspase activation and nuclear degradation [93]. Another tumor-suppressor pathway of the human microbiota would be its modulator effect of the host immune system against cancer. For instance, gut microbiota has been shown to activate an immune response against cancer cells [94]. Toll-like receptor 4 (TLR 4), expressed in many immune but also tumor cells, has been reported to cause opposite effects on cancer development [95]. While TLR4 activation can promote antitumor immunity, it can also result in an increased tumor growth and immunosuppression [95]. The exact mechanisms causing these contradictory effects are still unclear, but tumor type and bacterial strains involved might be potential factors to condition this effect.

<i>Fusobacterium nucleatum</i>	colorectal cancer	Initiator of polymicrobial biofilms, Fad2 adhesin sustains proliferative signaling	[75,80]
<i>Porphyromonas gingivalis</i>	orodigestive cancers	Induces epithelial-to-mesenchymal transition, activates MMP9 IL-6, IL-8	[81,82]
<i>Enterotoxigenic Bacteroides fragilis</i>	colorectal cancer	Metalloprotease enterotoxin BFT, proliferative signaling, inflammation and mutation	[83,84]
<i>Escherichia coli (group B2)pks⁺</i>	colorectal cancer	Colibactin toxin, sustaining proliferative signaling and causes mutations	[85]
<i>Enterococcus faecalis</i>	colorectal cancer	Extracellular superoxide acts as H ₂ O ₂ oxidative stress to induce DNA damage	[86]
<i>Streptococcus gallolyticus</i>	colorectal -, gallbladder cancer, pancreatic adenocarcinomas, ovarian -, endometrial cancer, nonsolid-organ malignancies	Biofilm formation	[87]
<i>Campylobacter species, in particular C. concisus</i>	esophageal and colonic adenocarcinoma	Biofilm formation, zonula occludens toxin	[88]

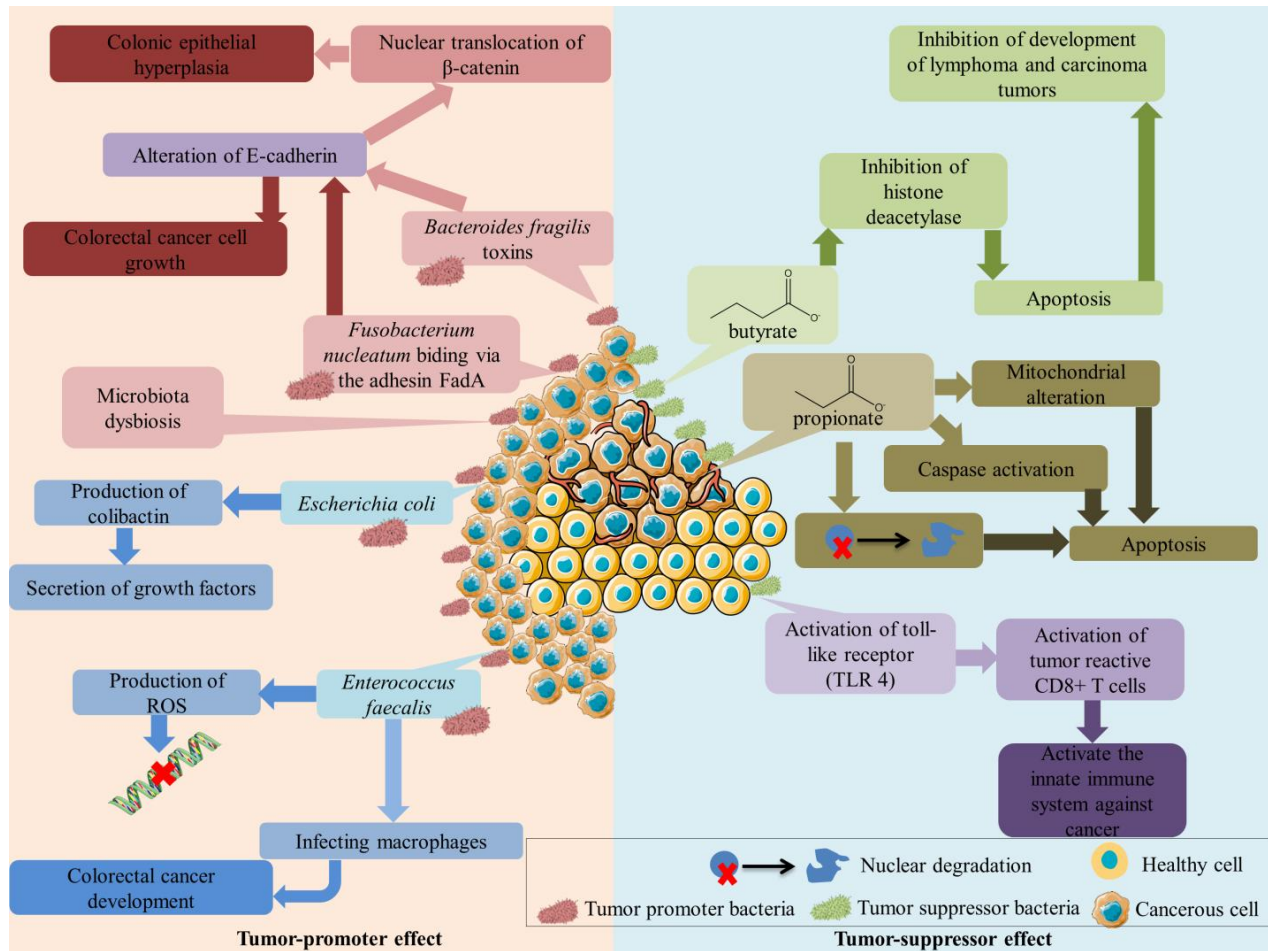


Figure 2: Examples of pro-oncogenic and anti-oncogenic mechanisms of the human microbiota. Bacteria could play a tumor suppressor effect by producing probiotic such butyrate and propionate, provoking cancer cells apoptosis. Another tumor suppressor effect might be the activation of the immune system against cancer via the activation of toll-like receptor. For the tumor suppressor effect, different mechanisms might be adapted: bacteria like *Bacteroides fragilis* and *Fusobacterium nucleatum* cause the alteration of E-cadherin causing colonic and colorectal cancer. Other bacteria like *Escherichia coli* favorite the secretion of tumor growth factors, while *Enterococcus faecalis* produces reactive oxygen species (ROS) leading to DNA damage and infects macrophages leading to colorectal cancer.

Bacterial lipopolysaccharides (LPS) were proven to activate the innate immune system via the engagement TLR 4, thus activating

tumor reactive CD8⁺ T cells [94]. For example, in a study from by Sivan *et al.* [96], commensal *Bifidobacterium*, a resident bacteria of the intestinal flora, enhanced the antitumor immunity in mice with subcutaneous melanoma. More specifically, oral gavage of fecal suspensions enhanced induction and infiltration of tumor specific CD8⁺ T cells, allowing a delay in the tumor growth [96]. 16S ribosomal RNA analysis of the gavaged fecal suspension showed that *Bifidobacterium* was the taxa associated with accumulation of the activated antigen-specific T cells within the tumor microenvironment, responsible for the antitumor effect [96].

In summary, the human microbiota may present a pro-oncogenic or an anti-oncogenic effect, depending on the bacterial strains, the pathways and mechanism involved in this action: **Figure 2**.

3.2. Bacterial microbes as passenger of cancer

Some studies showing the presence of tumor-associated bacteria pushed researchers to investigate if specific strains were associated to specific types of cancer and could explain the relationship between their co-existence. Indeed, recent reports linked the presence of certain bacterial strains to specific types of cancer. Tjalsma *et al.* [97] defined two types of colorectal cancer-associated bacteria: intestinal bacteria with procarcinogenic features that might initiate colorectal cancer development, designed as bacterial drivers, and gut bacteria reported to be present in the gut microbiome of advanced colorectal cancer patients, known as bacterial passengers or cancer passenger bacteria. For example, *Fusobacterium spp* was reported to be the most common passenger bacteria to colonize colorectal cancer tissues [97]. The involvement of this strain in early colorectal cancer is still uncertain, but this finding suggests the existence of a relationship between them. In a study performed by Geng *et al.* [98], 28 location-matched biopsy samples were analyzed by 16S ribosomal RNA pyrosequencing. Samples were a mix of normal, adenoma and tumor biopsies. The obtained results confirmed the aspect of cancer passenger bacteria or driver bacteria. More specifically, members of *Enterobacteriaceae* were defined as potential bacteria drivers and *Streptococcaceae* as potential pro-inflammatory passenger bacteria [98]. These results confirm the ones reported by Tjalsma *et al.* [97] and illustrate the concept of cancer-passenger bacteria.

3.3. Role of microbiota dysbiosis in cancer development

The microbiota dysbiosis has been reported to likely increase the risk of cancer via different mechanisms [99,100]. Dysbiosis designs the disruption of the human microbiota, causing a decrease in its diversity. This phenomenon might be explained by different factors such as

diet, the use of antibiotics, xenobiotics or infectious diseases. Some of these microbiota dysbiosis-causing factors have been linked to cancer. For instance, it has been showed that obesity leads to microbiota dysbiosis by increasing populations of *Firmicutes* and decreasing populations of *Bacteroides* in the gut, observed both among humans and mice [101,102]. It has also been proved that obesity decreases the microbial richness and the associated dysmetabolism in humans [103,104]. A study performed by Yoshimoto *et al.* [66] showed that dietary or genetically obese mice present higher risk of developing hepatocellular carcinoma compared to normal diet fed mice, indicating that obesity promotes hepatocellular carcinoma development. This might be explained by the fact that fat rich diet increases the production of desoxycholic acid, known to favorite the development of hepatocellular carcinoma [66] and to increase the risk of colon cancer [105].

Furthermore, gut microbiota dysbiosis has been reported to promote cancer development *in situ*. For example, in a study performed by Arthur *et al.* [106], it has been shown in mice that microbiota alteration, caused by colon inflammation, can promote tumorigenesis and induce the expansion of microorganisms with genotoxic capabilities. Dysbiosis has been reported not only as a colorectal oncogenic factor, but even as a promoter for other organ cancers. For instance, the hepatic exposure to cancer promoting metabolites has been linked to intestinal dysbiosis. These intestinal bacterial metabolites reach the liver via the portal vein and may promote liver cancer and hepatic fibrosis [67,107]. Li *et al.* [108] used the microbiota from colorectal cancer versus healthy control and transferred it to *Apc^{Min/+}* mice. They found that the dysbiosed microflora promoted cancerogenesis. Such studies provide evidence for a contribution of the microbiota on the cancer progression independent of the priming factors among genetic, environmental or microbial factors. Monitoring or even tuning

of microbiota might be an option for improving cancer therapy. Recent studies also show that a preexisting microbial dysbiosis may lead to inflammation facilitated stronger metastasis of tumors. For example, Rosean *et al.* [109] showed such effect on mouse model of hormone receptor-positive mammary cancer. In this study, first, the mice commensal microbiota was disturbed by gavaging wide spectrum antibiotics. Compared to control groups, the antibiotic gavaged mice showed shifts in commensal bacterial communities at the phylum and genus levels, mimicking a microbiota dysbiosis. In both models, a poorly metastatic syngeneic BRPKp 110 mouse model and a more aggressive and metastatic MMTV-PyMT mouse model, significantly more disseminated cells were detected in the lungs, peripheral blood and tumor-draining axillary lymph nodes of dysbiotic mice [109]. It was shown also that commensal dysbiosis enhanced inflammation both, systemically and within the tissue environment. These results confirm the role the microbiota dysbiosis plays in inflammation and tumor metastasis. Vitiello *et al.* described the challenge of fibrotic, immune-excluded tumor microenvironment for pancreatic cancer, a potential key factor for low therapy successes in pancreatic cancers, failing to be detected in early stages [110]. Peripancreatic inflammation potentially promoted by dysbiosis causes the fibrosis. Evidence for this theory comes from mouse models for pancreatic tumorigenesis (KC mice) where ablation of the microbiome reduced tumor invasiveness and increased therapy efficacy by checkpoint-targeted immunotherapy [111]. The intestinal microbiome was shown as a modulator of pancreatic cancer in studies based on human biopsies and germ-free mice, versus conventional treated mouse model of pancreatic cancer $Kras^{G12D}/PTEN^{lox/+}$ [112].

3.4. Effect of microbiota on cancer therapy

In addition to its direct effects on the cancer development, the microbiota might influence

the chemo- and immunotherapy anticancer drugs. This effect is either directly related to the microbiota ability to metabolize drugs, including anticancer drugs, and so to modulate their efficacy or toxicity [113] or indirectly to its involvement in the immune system response and regulation.

3.4.1 Direct effect on cancer therapy

Human microbiota might influence the chemotherapy efficacy and/or toxicity via different mechanisms: translocation of the commensal or pathogenic bacteria from the gut into the systemic milieu, immunomodulation, metabolism, enzymatic degradation and reduced diversity of the microbiota, caused by these therapeutic agents, which might change its reaction with chemotherapy [114]. These different mechanisms lead to different outcomes: improving the drug efficacy, provoking chemotherapy resistance or changing the drug toxicity. Lehouritis *et al.* [115] tested *in vitro* the effect of two different bacteria, non-pathogenic *E. coli* and *Listeria welshimeri*, on the chemotherapeutic cytotoxicity of 30 anticancer drugs. Drug co-incubation with bacteria showed a decrease of the killing cancer effect of 10 of these drugs, an increase in the efficacy of 6, generally caused by the same bacteria (*Escherichia coli*), and no modification of the anticancer efficacy was observed for 14 drugs [115]. It is important to mention that *E. coli* and *Listeria welshimeri* did not show the same effects on the different drugs. The drugs influenced by bacteria co-incubation were analyzed via high performance liquid chromatography and mass spectrometry. The chromatograms obtained showed the appearance of new pics indicating that all the drugs were biotransformed in the presence of bacteria [115]. Another study from Geller *et al.* [116] showed that bacteria belonging to *Gammaproteobacteria* provoked tumor resistance to an anticancer drug, gemcitabine. Further investigations revealed that this resistance was attributed to the bacterial enzyme cytidine deaminase, that metabolizes

gemcitabine into a deaminated inactive metabolite [116]. These results confirm those obtained by Lehouritis *et al.* [115], where *in vivo* experiments showed reduced gemcitabine antitumor activity in CT26 colon carcinoma tumors containing *Escherichia coli*. On the other hand, other studies showed that microbiota improved the anticancer effect of chemotherapy. For example, Gui *et al.* [117] reported that in a Lewis lung cancer mouse model, a better response to cisplatin-based chemotherapy was observed when it was administered in association with *Lactobacillus* bacteria or when mice had healthy balanced microbiota. The improved response was reflected by a reduced tumor size and an improved survival rate. The opposite was observed when cisplatin was associated with antibiotics to eradicate the host commensal flora.

Beside the impact on the chemotherapy efficacy, microbiota may also influence drug toxicity and side effects. For instance, microbiota was shown to accentuate the toxicity of irinotecan, a chemotherapeutic agent for colon cancer [118]. In fact, irinotecan is metabolized in the liver into the inactive metabolite SN-38G, excreted into the gastrointestinal tract via the biliary ducts. Once in the intestine, the SN-38G is metabolized by β -glucuronidase, an enzyme secreted by the intestinal flora, into SN-38 responsible for a severe intestinal toxicity [118]. Wallace *et al.* [118], demonstrated that the oral administration of a selective inhibitor of this bacterial β -glucuronidase, in association with irinotecan, protected from the intestinal toxicity by preventing either colonic damage or the appearance of diarrhea. Microbiota does not only accentuate the chemotherapy toxicity but was also reported to have a positive effect against it. For instance, a study performed by Frank *et al.* [119] showed that genetic deletion or depletion of the indigenous gut microbiota by antibiotics aggravated chemotherapy, inducing mucositis.

3.4.2 Effect on the immunotherapy

In addition to chemotherapy, microbiota has also an effect on anticancer immunotherapy. Since microbiota has played a key role in systemic immunity, it is likely that immunotherapy response might be influenced by the microbiota.

Immune checkpoint inhibitors are a type of immunotherapy that has proved an efficacy against solid cancer tumors and hematological malignancies. They act by stimulating the T-lymphocyte-mediated immune response against tumor-associated antigens [120]. Programmed death ligand-1 antibody (anti-PD-L1) therapy is one of the immune checkpoint inhibitors. In a study performed by Sivan *et al.* [96], commensal intestinal flora has been shown to improve the anticancer effect of anti-PD-L1 on murine melanoma model, when intravenously administered in association with orally gavaged fecal microbiota. Further investigations, using 16S ribosomal RNA sequencing, identified the taxa *Bifidobacterium* to be responsible for this effect [96]. Once proved on mice, Gopalakrishnan *et al.* [121] tested this effect in human cancer patients. The results obtained confirmed those observed in mice. More specifically, among a group of 112 melanoma patients who received anti-PD-1 immunotherapy, 89 were considered eligible to this study. Two populations were distinguished: responders ($n = 54$) and non-responders ($n = 35$) to the immunotherapy treatment. Buccal and fecal microbiome samples were collected from the patients at the beginning of the treatment and analyzed via 16S ribosomal RNA gene sequencing. The results showed significant difference in alpha diversity of the composition of gut microbiota between the two populations, where responder group showed higher alpha diversity and an enrichment in *Faecalibacterium* genus, while the non-responder group showed a higher abundance of Bacteroidales [121]. These results prove that the difference in the gut microbiota composition could influence

significantly the efficacy of the anti-PD-1 immunotherapy, indicating that patients with a favorable gut microbiota show a better response to anti-PD-1 immunotherapy and suggesting that gut microbiota plays a key role in the effect of anti-PD-1 immunotherapy in human cancer patients. In a similar study, Routy and his colleagues [122] confirmed these results *in vivo* and in a clinical study including 249 patients. First, the use of antibiotics (in order to deplete the gut microbiota) on mice, with established sarcoma and melanoma, or on patients, with advanced non-small cell lung cancer (NSCLC) (n = 140), renal cell carcinoma (n = 67) and urothelial carcinoma (n = 42), reduced the effects of anti-PD-1 and anti-PD-L1 immunotherapy and shorted the survival of both murine and human species [122]. In classifying the patients into responders and non-responders, both groups presented differences in terms of bacterial composition of their commensal flora. The analysis of the patient feces allowed to link the presence of specific bacteria, *Akkermansia muciniphila* and *Alistipes indistinctus*, to a better response to the anti-PD-1 treatment [122]. Finally, in order to confirm the role of the intestinal microbiota in improving the response to anti-PD-1 immunotherapy, antibiotic treated mice were recolonized by fecal microbiota transplantation using patient stools from responder and non-responder groups. Fecal microbiota transplantation from the responder group caused tumor growth delay in mice and up-regulation of PD-L1 splenic T cells when treated with anti-PD-L1 [122]. These studies confirm the role the microbiota plays in improving the response to anti-PD immunotherapy.

Another immune checkpoint inhibitor is the monoclonal antibody against CTLA-4, known as ipilimumab. Vétizou *et al.* [123] showed that, as for the anti-PD-1 immunotherapy, the gut microbiota plays a key role in improving the effect of the anti-CTLA-4 immunotherapy. This theory was first proved in mice. Germ free

mice or mice treated with broad-spectrum antibiotics (in order to deplete their gut microbiota) showed no response to the anti-CTLA-4 immunotherapy, on the other hand, specific pathogen free mice, who presented normal gut microbiota, showed tumor regression when treated with anti-CTLA-4 immunotherapy [123]. These results were verified for the Ret melanoma and MC38 colon cancer models. Further investigations, using 16S ribosomal RNA gene analyses of the feces, linked *Bacteroides* genus and species to the anticancer efficacy of the anti-CTLA-4 immunotherapy [123]. To confirm this result, germ free mice and broad-spectrum antibiotics treated mice were recolonized with *Bacteroides* species in combination with anti-CTLA-4 immunotherapy. These mice recovered the anticancer response to anti-CTLA-4 immunotherapy [123], proving the key role of the intestinal microbiota, and specifically of the genus *Bacteroides*, in improving the response to ipilimumab.

These different studies illustrate the essential role the human microbiota, and more specifically the gut microbiota, plays in modulating and influencing the chemotherapy and immunotherapy, and so taking part in their efficacy, toxicity and the organism response.

3.5. Bacteria as therapeutic concept

Due the proven role they play in cancer development, therapy efficacy and toxicity, bacteria and their derived products could be used to improve anticancer therapeutic outcome.

3.5.1 Direct strategies

Direct approaches consist in the administration of bacteria or its products alone or in association with anticancer treatment to locate and kill cancerous cells. Tumor homing bacteria can be used as delivery systems as described in section 4.5. Probiotics are defined as live microorganisms which, when administrated in adequate amount, confer a health benefit of the host. It has been shown that the co-administration of bacteria with

chemotherapy improved the anticancer effect. For instance, the combination of cisplatin and *Lactobacillus* bacteria in the treatment of lung cancer in mice reduced the tumors volume and improved the survival rate compared to mice treated only with cisplatin [117]. Also, the administration of *Bifidobacterium* in association with anti-PD-L1 immunotherapy nearly abolished melanoma outgrowth in mice [96].

Another way to use microbiota in modulating cancer development might be to benefit from its ability to ferment non-digestible food or else called prebiotics. Some studies investigated the effect of administrating prebiotics on different tumor models. For instance, resistant starch is a non-digestible nutriment that is fermented by the large intestine and produces bioactive metabolites such as SCAFs. Panebianco *et al.* [124] showed that engineered resistant starch diet decreased pancreatic tumor growth in xenograft mouse model [124]. In addition, microbiota prebiotics might influence cancer therapy. Taper *et al.* [125] studied the effect of two different prebiotics, oligofructose and inulin, on six different cytotoxic drugs: 5-fluorouracil, doxorubicin, cyclophosphamide, vincristine sulfate, methotrexate and cytarabine. The study consisted in administrating separately oligofructose and inulin diets to liver cancer model mice groups: each group received different cytotoxic drugs mentioned previously, classically used in human cancer treatment. The results showed that oligofructose and inulin considerably potentiated the therapeutic effects of all six cytotoxic drugs via synergic or additive effect and prolonged the survival compared to the control groups [125]. These non-digestible carbohydrate have been shown to decrease the level of insulin like growth factor and of serum glucose, both important for the tumor growth [125].

A final way to use microbiota to modulate cancer development might be to involve its metabolites, also termed as postbiotics. It has

been reported that postbiotics showed an effect on anticancer therapy. For instance, the supernatant of *Streptococcus thermophilus* has been shown to inhibit 5-fluorouracil-provoked intestinal crypt fission on rats [126]. In a similar study, supernatants from *Escherichia coli* and *Lactobacillus fermentum* partially protected rat intestine from 5-fluorouracil mucositis. As a last example, in a study performed by Swofford *et al.* [127], a protein secreted by *Staphylococcus aureus*, α -hemolysin, has been shown to decrease *in vitro* cell viability by 90 % of MCF-7 mammary carcinoma cells, indicating an anticancer effect of the *Staphylococcus aureus* α -hemolysin.

These different impacts that microbiota has on cancer development and therapy make microbiota essential to be taken under consideration for developing and evaluating innovative anticancer treatments.

3.5.2 Indirect strategies

The indirect approaches can be described as using probiotics and prebiotics to improve the patient health by essentially reducing chemotherapy toxicity and heavy secondary effects. Some studies have shown that the administration of probiotics alleviates the side effects of chemotherapy: it is in correlation with the studies previously described showing the role of microbiota in reducing the toxicity of chemotherapy. In a study performed by Bowen *et al.* [128], a probiotic cocktail (VSL#3) containing four strains of *lactobacilli*, three strains of *bifidobacteria* and one strain of *streptococcus* was administrated in association with irinotecan in rats. Irinotecan is a chemotherapeutic agent that has been shown to provoke chemotherapy induced diarrhea in the chosen model [128]. The results showed that the administration of VSL#3 reduced weight loss, precluded the increase in goblet cell number and prevented moderate and severe diarrhea all induced by irinotecan [128]. In a similar study for patients diagnosed with colorectal cancer, the administration of *Lactobacillus* in association with 5-fluorouracil

chemotherapy, known for causing diarrhea, reduced stage 3 and 4 diarrhea, presented less abdominal discomfort, needed less hospital care and had fewer chemotherapy dose reductions, due to bowel toxicity caused by 5-fluorouracil chemotherapy [129]. Always in the aim of reducing the heavy side effects caused by 5-fluorouracil, the co-administration of *Bifidobacterium bifidum* orally in mice reduced the severity of intestinal mucositis and inflammatory responses and tended to attenuate the clinical symptoms secondary to the treatment by 5-fluorouracil [130]. In a similar study, oral administration in mice of probiotic suspension of *Lactobacillus casei* alone or with *Bifidobacterium bifidum* ameliorated the 5-fluorouracil-induced mucositis in a mouse model [131].

Other studies focused on the effect of prebiotics on chemotherapy toxicity showed that they reduced the toxicity of some anticancer drugs. For example, Funk *et al.* [132] showed that soybean presented a concentration dependent reduction of the methotrexate toxicity symptoms in rats and even alleviated it at high concentrations. In another study, Mao *et al.* [133] showed that pectin supplemented diet on rats significantly decreased methotrexate-induced intestinal injury, body weight loss and improved bowel integrity. Giving the effect observed by administering either probiotics or prebiotics on cancer development, chemo- and immunotherapy, some researchers studied the effect that might be induced by administering combination of pre- and probiotics, or also known as synbiotics. For instance, the administration of *Lactobacilli* with oat fiber in rats reduced the severity of methotrexate-related enterocolitis [134]. The effect observed after the co-administration of *Lactobacilli* and oat fiber was more important than the one observed after the administration of *Lactobacilli* alone, indicating a synergic effect of this association [134].

4. Nanoparticles and microbiota

Nanoparticles are classically defined as materials with a size of 1-100 nm in at least one dimension [135]. A more adapted definition would attribute the term nanoparticle to material up to several hundred nanometers that are developed using top-down or bottom-up formulation strategies. They are characterized by their composition, size, shape, supramolecular structure, surface charge, and formulation process [136,137].

Cancer therapy is one of the fields where nanoparticles have been investigated due to the encouraging results the first studies showed. Since their appearance, nanotechnologies have evolved on many levels and aspects, according to a recent review by Song *et al.* [138] and they can be distinguished into three different generations. The transition from one generation to the next one has been performed by benefiting from the nanoparticle different properties, notably encapsulating and delivering various molecules [139], surface functionalization [140], and engineering and modifying the physical properties. More specifically, first generation nanoparticles were founded on the ability of stealth nanoparticles to passively accumulate in solid tumors, allowing an enhanced permeability and retention effect of the nanoparticles (EPR) [141], a control of the encapsulated drug release [139] and to overcome opsonization via adsorption or grafting of poly(ethylene glycol) (PEG) to the surface of nanoparticles [142]. Second generation nanoparticles, are characterized by their capacity of co-delivering multiple drugs [143], specificity in targeting tissues [144], and stimuli-responsive delivery [145]. Finally the third generation nanoparticles present more pushed characteristics, as promoting immune responses against tumors [146], overcoming biological barriers [147] and self-recognition [148,149].

In addition to their application in cancer therapy, nanoparticles have been used for treatment of infection diseases especially with the appearance of antimicrobial resistance.

First, both inorganic and organic nanoparticles have showed antimicrobial activity against different bacterial species [150–154]. Due to their ability to increase the encapsulated drug intracellular concentration up to 10-fold, the nanocarriers improve the antibacterial activity of the encapsulated antibiotic against intracellular infecting bacteria [155]. Also, nanoparticles protect the encapsulated antibiotics from bacteria resistance phenomena such degradation by β -lactamase bacteria, or thick cell membrane [156,157]. Moreover, different studies have reported that bacterial infections treated with nanoparticle-drug associations showed lower probabilities of inducing resistance [158,159].

Nanoparticles surface charge and the ability to control it depending on the materials and formulation procedure used, find also its utility in adjusting interactions with bacteria. In a study performed by Qiao *et al.* [160], zwitterion silver nanoparticles were formulated by decorating silver nanoparticles with carboxyl betaine groups, leading to pH-responsive transition from a negative to a positive charge. This property made the zwitterion silver nanoparticles compatible with mammalian cells and red blood cells in healthy tissues (pH 7.4) while strongly adherent to negatively charged bacteria at infectious sites (pH 5.5) via electrostatic attraction [160].

Nanoparticle shape might also play a role in their antibacterial effect. In a study performed by Penders *et al.* [161], different shaped gold nanoparticles (nanostars, nanoflowers and nanospheres) were tested for their antibacterial effect against *Staphylococcus aureus*. The results showed a shape-dependent antibacterial effect of the gold nanoparticles towards *Staphylococcus aureus*, where gold nanoflowers showed the most remarkable antibacterial effect among the three different nanoparticles [161].

Another important characteristic of nanoparticles that makes them suitable for microbiome intervention is the ability to

functionalize their surface [162,163]. In fact, recent studies showed that chitosan nanoparticles, known for their antibacterial effect, presented higher efficiency when they are functionally modified with other natural compounds, metallic antibacterial particles or/and antibiotics [164]. This effect was not exclusively reported about chitosan nanoparticles, other studies showed that also silver [165], titanium dioxide [166] and iron oxide nanoparticle functionalization[167] improved their antibacterial effect.

Based on their interactions and effects on different bacterial species, it is obvious to think that administrated nanoparticles could present an effect on human healthy microbiota or tumor associated bacteria. As exposed previously, human microbiota plays crucial role in cancer development and treatment, and so, when using nanoparticles in cancer treatment, these interactions should be considered and studied in order to benefit from the nanoparticle-bacteria interactions to modulate and improve cancer therapy.

Recently, researchers started to become interested in using nanoparticles for modulating microbiota or tumor associated bacteria for improving cancer treatment: however, this application is still at its very beginning. Potential strategies to imply nanotechnology in modulating cancer linked microbiota could be gathered in the following points.

4.1. Modulation of tumor associated bacteria

Targeting bacteria associated tumor is a potential approach to control the microbiota involvement in cancer development and therapy. Two types of tumor associated bacteria can be distinguished: bacteria with pro-oncogenic effect and bacteria with anti-cancer effect.

Due to their antibacterial effect and their use in the infectious disease field for delivering antibiotics, nanoparticles could be used in order to eliminate cancer-causing bacteria. Indeed, due to their targeting properties, nanoparticles have been used to kill specific bacteria strains

[168]. For instance, in a study performed by Angsantikul *et al.* [169], poly(lactic-co-glycolic acid) (PLGA) nanoparticles were coated with plasma membranes of gastric epithelial cells (AGS cells) in order to target *H. pylori*, a bacteria known as a cause for gastric cancer. These nanoparticles were encapsulated with the antibiotic clarithromycin. The results showed that, on a mouse model of *H. pylori* infection, the clarithromycin loaded AGS coated nanoparticles improved *H. pylori* killing in the stomach compared with the free clarithromycin and the non-coated nanoparticles [169]. This type of nanoparticles might be of use to target stomach cancer associated *H. pylori*, without altering the healthy microbiota. A bacteriophage is a virus that specifically infects and kills bacteria. In a study performed by Zheng *et al.* [170], irinotecan-loaded dextran nanoparticles were covalently linked to azide-modified phages, having the ability to inhibit the growth of pro-oncogenic *Fusobacterium nucleatum*. These nanoparticles were orally or intravenously administered in mice with orthotopic colorectal tumors or with spontaneously formed colorectal tumors. The results showed a significant improvement of the anticancer effect of the chemotherapy against colorectal cancer [170]: an improvement that might be explained by the inhibitory effect of the nanoparticle linked phage against the pro-oncogenic *Fusobacterium nucleatum*. This system illustrates the utility of using nanoparticles to target and eliminate cancer causing bacteria and the impact it has on improving cancer chemotherapy.

4.2. Interfering with bacterial metabolites

Considering the various roles that bacterial metabolites play in cancer development, a potential approach might be to use nanoparticles to interact with the bacterial postbiotics. For example, nanoparticles might be used to bind, block or inactivate bacterial secreted products like toxins and carcinogens. In a study performed by Wigginton *et al.* [171],

bare and carbon coated silver nanoparticles were shown to bind to tryptophanase, a protein from *Escherichia coli*, with high affinity and caused the inactivation of the protein. Such effects of the nanoparticles might be used to block bacteria produced toxins and metabolisms with pro-oncogenic effect. In a study by Song *et al.* [172], LPS, one of the most prevalent products in the gut microbiota, have been associated to colorectal cancer development and to resistance to anti-PD-L1 resistance therapy [172]. In order to overcome their effect, Song *et al.* encapsulated the coding sequence of LPS-targeting fusion protein (which blocks specifically the tested LPS) into lipid-protamine DNA (LPD) nanoparticles. In order to formulate these LPD nanoparticles, the coding sequence for the secreted form of LPS trap protein was first cloned. The LPS trap plasmid was then encapsulated into LPD nanoparticles by complexing the plasmid with protamine and cationic liposomes [172]. After administration in orthotopic mice CT26-FL3 model, this system inhibited colorectal cancer liver metastasis [172]. This study represents a concrete example of using nanoparticles to improve cancer treatment by interfering with bacterial products. The promising results obtained encourage exploring this strategy for other bacterial metabolites involved in the tumor evolution.

4.3. Interfering in the crosstalk between bacteria and the immune system

Microbiota has been shown to play a key role in systemic immunity system and more specifically in cancer immune response [94]. Especially with the appearance of third generation nanoparticles and their capacity to promote the immune response, nanotechnology might be used to control or regulate the cancer related immune response or suppress induced by tumor associated bacteria. For example, the previously described study performed by Song *et al.* [172] showed the effectiveness of the nanotrapping LPS system on orthotopic colorectal cancer metastasis model.

Investigations of the mechanism involved showed that these nanoparticles promoted T-cell infiltration into tumor and boosted checkpoint blockade immunotherapy [172].

4.4. Using triggers of tumor-associated bacteria for targeting chemotherapy

An original way to target tumor cells might be to use specific nanoparticles that target tumor associated bacteria. These nanoparticles are said specific because they would be formulated from material allowing a specific liberation of the encapsulated anticancer drug, once in contact with a tumor associated bacteria or its metabolites. Xiong *et al.* [173] illustrated this concept. In a study, a lipase secreting attenuated strain of *Staphylococcus aureus* was used as artificial bacteria associated tumor in H22 tumor-bearing mice model. In parallel, bacteria-sensitive triple-layered nanogel (TLN) was developed. First, it has been confirmed that the TLN was degraded by *Staphylococcus aureus* produced lipase [173]. Second, the intravenous administration of doxorubicin loaded TLN in the H22 tumor-bearing mice model resulted in the specific degradation of TLN in bacteria-accumulated tumor environment triggering specific release of doxorubicin in cancerous cells and so selective cytotoxic effect of the chemotherapy [173]. This approach might be extended by using other bacteria-associated metabolites and products as triggering systems for special targeting of chemotherapy loaded nanocarriers allowing a specific action of the anticancer molecules and so a potential improving of the efficacy and secondary effects.

4.5. Using the affinity of specific bacteria to tumor tissue for targeted delivery

Some bacteria have the ability to accumulate, after its intravenous administration, in hypoxic areas of the tumor and colonize the tumor site as tumor associated bacteria [174]. These bacteria can specifically target tumors and actively penetrate tissues. Due to these properties, it has been already suggested to use bacteria-based systems as drug delivery agents

[175]. Bacteria-nanoparticle association could be potential candidate for such drug delivery systems, especially with the nanoparticle ability of surface functionalization: bacteria could be adsorbed or conjugated to their surface. This property could be used to improve nanoparticle specific targeting for cancer cells. Some bacteria-nanoparticle systems have already been studied for different purposes. In a study done by Akin *et al.* [176], nanoparticle-bacteria complex was developed to deliver DNA-based model drug molecules. To formulate this complex, streptavidin-coated polystyrene nanoparticles were attached to the bacterial outer membrane of *Listeria monocytogenes*. This complex was administrated *in vitro* and *in vivo* into non-phagocytic mammalian cells of human solid organ tumors. In another example [177], biom mineralized gold nanoparticles were used to decorate TNF- α plasmids and the combination was protected with thermally sensitive programmable bacteria. *In vitro* and *in vivo* experiments verified the successful delivery of gold nanoparticles and TNF- α plasmids by the thermally sensitive programmable bacteria [177]. As a last example, Hu *et al.* [178], designed bacteria-nanoparticle delivery model to orally administrate DNA vaccine for cancer immunotherapy. Cationic nanoparticles were formulated by self-assembly of cationic polymers and plasmid DNA. These nanoparticles were used to coat alive attenuated bacteria. Oral delivery of this system had an inhibitory effect against tumor growth [178].

4.6. Improving the efficiency of delivering pro- and prebiotics

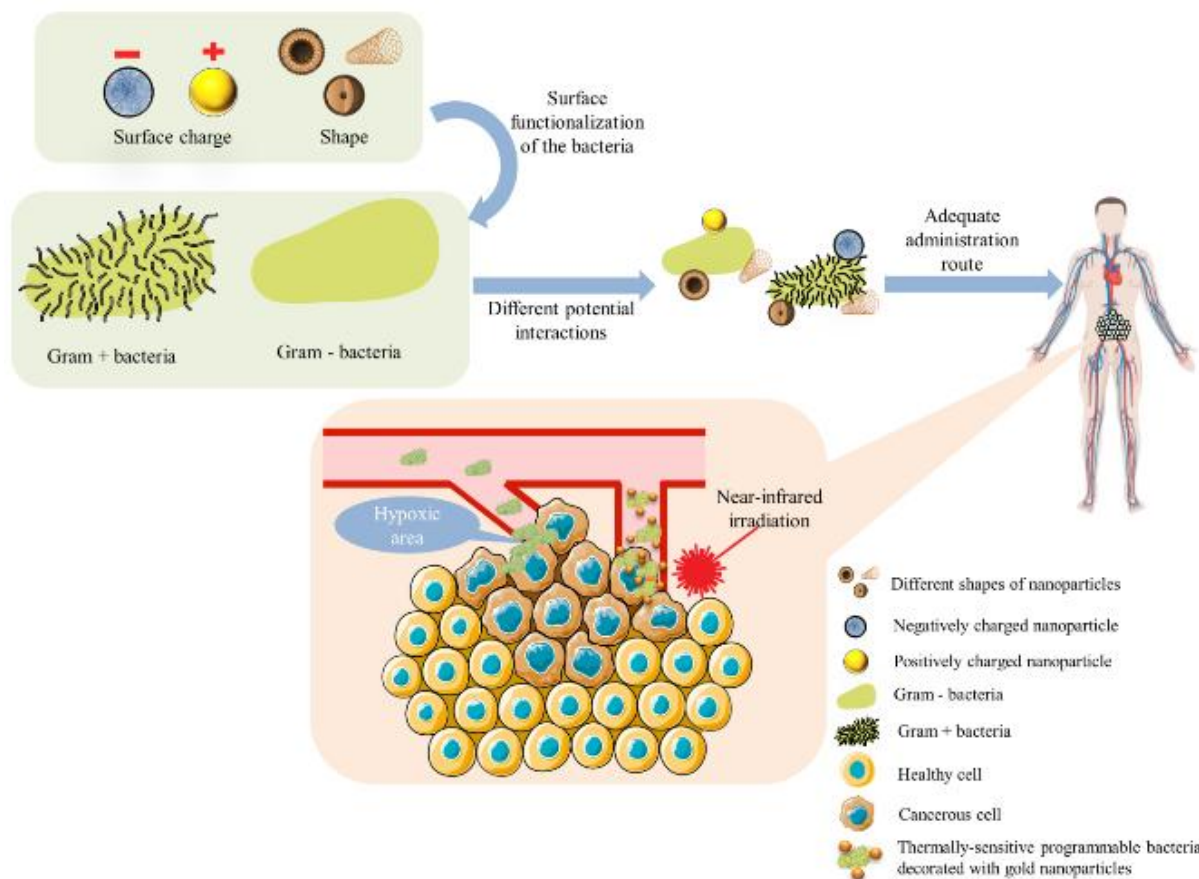
Nanoparticles could be used to promote the growth of tumor suppressor bacteria. One strategy to regulate the growth of tumor suppressor bacteria might be to specifically deliver prebiotics that increase their proliferation. Also, material that might facilitate the growth of tumor suppressor bacteria might be included in the nanoparticle formulation. For example, it has been shown

that dextran-based nanoparticles increased the proliferation of *Clostridium butyricum*, a bacteria known to secrete short-chain fatty acids, allowing to suppress growth of colorectal cancer [138]. This strategy might be applied to promote specifically the growth of bacteria that inhibit the tumor growth without provoking microbiota imbalance.

relevant *in vivo* models will be described and suggested to be used in order to provide potential solutions for improving the transition from preclinical to clinical studies of nanoparticles in cancer therapy.

4.7 Challenges and perspectives

This part of the review described some of the strategies and approaches where drug delivery



One main reason that might explain the poor transition from preclinical to clinical studies of these nanomedicines could be the fact that *in vitro* and *in vivo* models used in these preclinical studies are still far from mimicking the human body's anatomy and physiology. More specifically, despite the demonstrated impact of the human microbiota on cancer development, chemo- and immunotherapy efficacy, this factor has been rarely considered when engineering *in vitro* and *in vivo* models to test the nanoparticle anticancer effect.

In the following parts of this review, examples of bacteria-human cell co-culture models and

systems exploited the microbiota involvement in cancer development in order to improve its treatment. These studies are recent and the concept is in its early beginnings. The first step was to prove that acting on tumor associated bacteria or human microbiota had an effect on cancer development and anticancer therapy, which was shown in many previously, described studies. Moreover, other studies have to be performed, for a better understanding and control of the nanoparticles-microbiota interactions (**Figure 3**).

Figure 3: *Perspectives and challenges facing nanoparticles functionalization of tumor-targeting bacteria. First, depending on the nanoparticle characteristics (e.g shape, surface charge ...) and the bacteria type (gram + or gram -) and shape, the strength and nature of the bounds assuring the bacteria functionalization would differ, opening up numerous possibilities for different decoration procedures. The next challenge would be to choose the adequate administration route for the best bioavailability and efficacy of the nanoparticle-bacteria complex. Finally, the mechanism by which the bacteria targets the tumor environment should be carefully selected, for example, using hypoxic areas-accumulating bacteria [174] or thermally sensitive-bacteria [177].*

First, the toxicity of nanoparticles towards microbiota and tumor associated bacteria should be taken under consideration and studied in parallel with the efficacy in each study. Actually, some studies that investigated the toxicity of nanoparticles on human microbiota almost exclusively talked about inorganic nanoparticles, as they are present in many consumer products [179–181]. Some of these studies showed that inorganic nanoparticles might cause an alteration or an imbalance in the commensal microbiota [181]. Such an effect might be observed with drug delivery systems, especially knowing that some inorganic nanoparticles are used as nanocarriers. In addition, an alteration of the commensal microbiota might lead to its dysbiosis, which has been shown, as described previously in this review, to affect the cancer development and the response to treatment. Finally, such changing in the microbiota might change its interaction with nanoparticles. This confirms the importance of performing toxicology studies while investigating the nanoparticles-microbiota interactions in the treatment of cancer.

Another challenge to overcome is that the commensal microbiota composition may differ due to intra- or inter-individual factors, such as race, age, sex or hygiene. These differences

might result into different interactions and responses toward a same drug delivery system. Giving that such factors are hard to control, the nanomedicines might have different effects and efficacies on different patients. The challenge would be either to ensure the same efficacy of nanoparticles regardless the difference of interactions with commensal microbiota caused by these factors, or to control the different potential nanoparticles-microbiota interactions by taking under consideration the effect of these different factors. This means to adjust the drug delivery systems anticancer therapy depending on the microbiota and its changes due to the factors affecting its composition.

Finally, nanoparticle nature, composition, formulation procedure, size, shape, surface charge, coating, and the bacteria type and shape might control their interactions [182–184]. The effect of each parameter should be studied and taken under consideration for a better understanding and controlling of these interactions and so for a better monitoring of the produced effect.

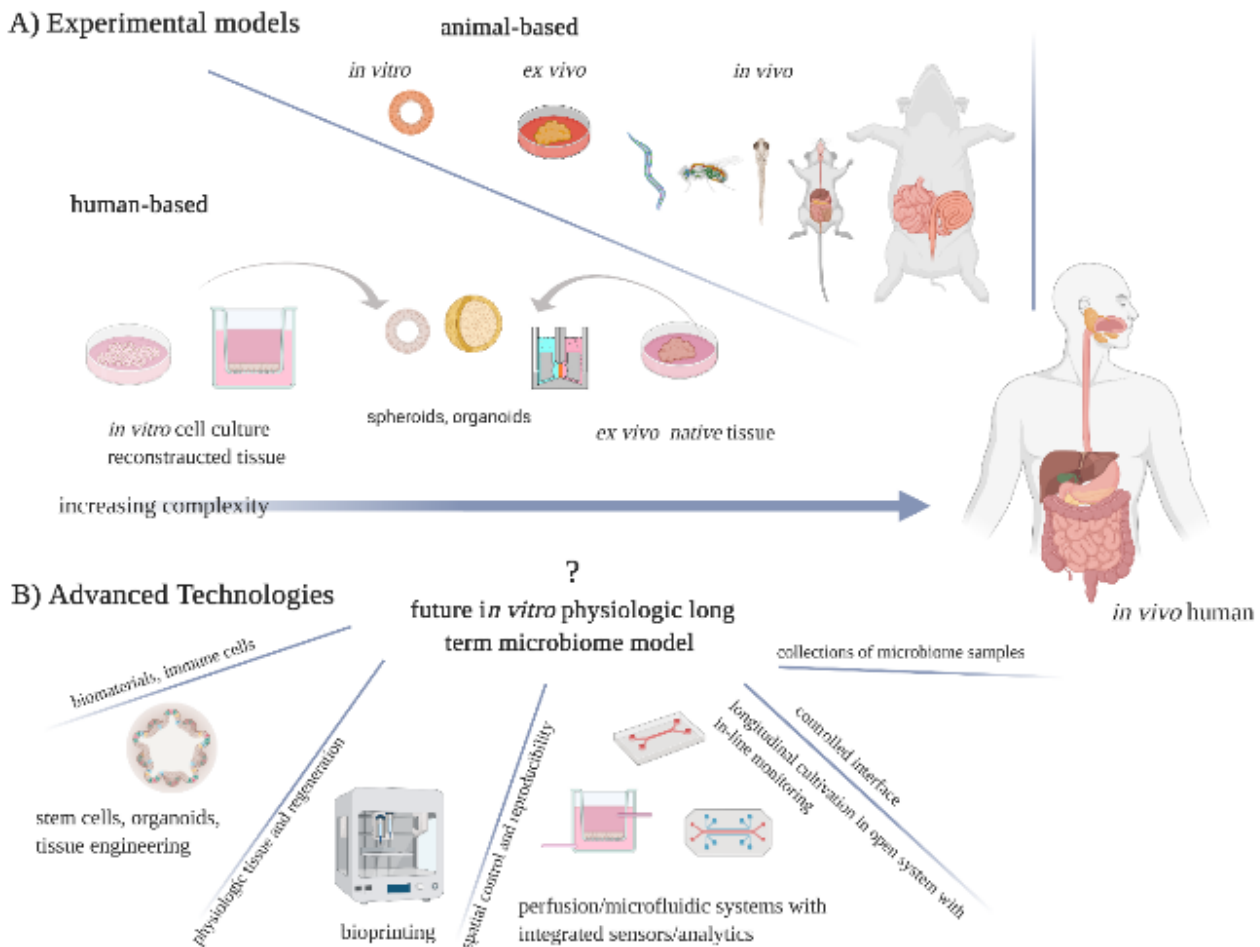
5. Models for investigating the role of microbiota for cancer research and therapy

As said previously, the last decade has seen an enormous rise in the investigation of microbiome effect in human health including cancer. However, investigation of such complex ecosystems is highly challenging. *In vitro* systems are challenged to design tissues mimicking physiology and thus culturability of natural microbioms and their maintenance for sufficient time for investigation. Cancerogenesis is a multiple step progression and needs certain time. Microbial dysbiosis is of most severe health concern when it persists the immune solvation getting chronic. In consequence, models aiming to elucidate such interactions are medium to long-term models. Experimental models are the main source of hypothesis-driven therapy development,

enabling studies for causality in disease initiation, progression and therapy. Clinical observations for microbiome or environmental effects provide candidate factors with increased coincidence. Sequencing approaches (e.g., 16S ribosomal RNA or shotgun diagnosis) determine taxonomic microbial communities' composition and are just descriptive. Experimental models allow a control on the complexity by reducing experimental parameters and thus aim to enable testing hypothesis drawn from clinical observations and statistics as a proof of causality and mechanistically investigations. Accordingly, developed models cover a wide range of complexity to serve various investigatory levels, from screening approaches with maximum reduction in complexity, the testing of isolated, highly specific interactions, *in vitro* models mimicking physiologic condition, to highly complex interaction scenarios of microbiome in animals. *In vitro* models comprise either native tissue, often referred to as *ex vivo*, as well as reconstructed tissue. **Figure 4** provides a schematic summary on

common model systems for humans, enabling interaction studies of increasing complexity. Such models have a tradition in medical testing in tumor therapy development, tumorigenesis studies as well as in studies on microbial pathogen caused diseases and infection models for anti-infective development. Immortalized cell lines are a principal tool for basic studies of molecular investigation, mode of action testing and cytotoxicity studies. They are fast and easy to perform and allow high-throughput screenings, but not representing tissue physiology. More advanced, reconstructed *in vitro* cell and tissue models allow observation of cellular interactions and interactions with the extracellular environment

Figure 4: Biological research models aim to mimic human physiology at sufficient complexity for answering the research question and following set-ups with controllable parameters. **A)** Current experimental models shown by increasing model complexity (from left to right side) for human-based and animal-based models for cancer and



microbiome studies. *In vivo* cancer studies can only be conducted with animal models. Human-based experimental cell and tissue models are an alternative route for investigatory studies and therapy translation in some research fields (*e.g.*, drug absorption, toxicity testing) already. Multiple models independent of their complexity levels contribute to basic and applied research when selected carefully.

B) Technological advances and their anticipated advantage, which provide the tools for current state-of-the-art cancer/microbiome models and further future models of cancer-microbiome interaction. Integration and combination of recent progresses in bioengineering, biotechnology and microbiology into human-based experimental models have the potential to create predictive human models in more controlled systems than animals and thus could progress our understanding of microbiome-tumor-interplay.

over extended time. Native tissue models use tissue biopsies or explant organs kept in experimental set-ups viable. The advantage of physiological tissue is accompanied by the limited cultivation time (hours-days depending on the tissue size and model set-up). *In vivo* animal models range from simple invertebrate to mammalian species and are used in biodistribution, toxicity and functional studies. Comparing simple invertebrate models, lower and higher vertebrate models, the increasing life span and organism complexity allow models of higher relevance from physiological similarity to humans for the price of increased experimental complexity (including effort for required reproducibility allowing statistical power) and costs.

Increasing animal studies, mostly using mouse, on microbiome-cancer dialog were published over the last six years. For the mouse model, the genetic homogeneity of inbred animals is advantageous in repeatability of studies and result interpretation. However, animal models suffer from species differences in physiology and life style/environmental factors and do not represent the human disease faithfully [54,185,186]. Further, the control of all impact

parameters in such animal model is difficult and expensive.

This paper reviews some models already in use cancer-microbiome interaction studies, which are most *in vivo* mouse studies. Concerning *in vitro* models there are models for microbiome studies on the bacterial community only, and some pioneer models for host-microbiome interaction, but not on cancer-microbiome interaction, yet. Future *in vitro* models can, however, profit from technology developed in cancer and infection/microbiome studies. Cancer models are reviewed extensively elsewhere [187–192]. Thus, herein their description is kept very brief. Mainly, they are mentioned in cases with a potential for technology combination/transfer. Some microphysiological models may make use of such technology and are expected to get instrumental in experimental microbiome-cancer interaction studies.

The field of microbiome study and its impact on disease progression is most advanced for the intestinal microbiome, having also the most significant effect on human health and immune reaction. Consequently, most examples presented and discussed are concerning the GI tract microbiome. Still also the epithelial interfaces homing microbiomes, namely skin and airway/lung models, are included.

5.1. *In vitro* models

5.1.1. Native tissue models

Tissue slices and organ culture are used for pharmacodynamics profiling of chemotherapeutics and tumor cell migration studies. Tumorspheroids are derived from cancer tissue either by dissociation into cell suspension or cutting the tissue in pieces allowing to round. The later method allows stromal components (fibroblasts, immune and endothelial cells) longer persistence. Spheroids allow cultivation for extended time (week – month). Spherical tumor models have been

developed for many cancer types, using various cultivation methods as reviewed elsewhere [193–195]. They were used in preclinical tests for chemosensitivity, invasion and tumorigenicity, hypoxia, tumor metabolism analysis, interaction with microorganisms and oncolytic viral therapy.

5.1.2. Infection/microbiome native models

Tissue slices are also used as infection models for viruses, parasites and bacteria. Tissue slides or precision cut tissue slices e.g., for lung tissue used for infection studies of *P. aeruginosa* or *M. tuberculosis*. Skin tissue of pigs or human donors is used for wound infection models. Mucosal tissue in Ussing chambers allows monitoring of short-term effect of infection on permeability. Limitation in such *ex vivo* models is the rather limited time tissue can kept viable and in presence of microbes without outgrowth in absence of clearance (e.g. dynamic flow condition) or functional immune system components. Usual cultivation times for such tissue slices are several days. [187,196] Some microfluidic systems for cultivation of colon explant tissue are also in recent development. [197,198]. Current status is the demonstration of longer cultivation times and reproducible viability of the tissue.

In a recent study, some bacteria-spheroid co-culture were reported. Tumor spheroids derived from HT-29 cells were inoculated with anaerobic bacteria (*F. nucleatum*, *F. prausnitzii* and heat-killed *F. nucleatum* control). It were shown to allow the growth of *F. nucleatum* but not *F. prausnitzii*. [199]. Such co-cultivation could be maintained for up to 48 h and observed biofilm-like structures in microscopical analysis. Such reductionist model allows direct contact without membrane segregation and analysis of gene expression and metabolomics changes. Limitations of such models are i) the non-suitability for cultivation of many GI bacteria and ii) the short co-cultivation time caused by bacterial cytotoxicity on the enterocytes.

5.2 *In vitro* reconstructed tissue models

5.2.1. Tumor models

Tumor spheroids are grown from cancer cell lines cultivated in non-adherent conditions. Rotating or shanking systems or “hanging drop cultivation” prevents surface adherence and promotes cell-cell-adhesion leading for many cancer cell lines to the formation of multicellular aggregates. Stem cell derived organ-specific organoids also contain mesenchyme. They are primary cells without immortalization transformation and self-organize in multicellular structures with cell differentiation. Culture additives can gear differentiation into specific cell types. Stem cells in intestinal crypt biopsies allow long-term culture and certain degree of cell differentiation. Such tissue derived epithelial organoids are called enteroids and do not contain mesenchyme. In general, such enteroids or organoids are spherical with the apical epithelial surface in the inside, which complicates studies mimicking oral route entry. In their study, Langer *et al.* used tumor cell lines (e.g. MCF7) HUVECS, primary mammary or pulmonary fibroblasts as well as primary tumor cells from patients, which were printed in alginate hydrogels on transwell inserts [200]. The alginate hydrogel was cross-linked and the printed structure cultivated for two days before the alginate was removed by enzymatic degradation with alginate lyase. Spheroids remained further in cultivation for 14 days.

Han *et al.* recently published a tumor model where they printed a tissue layer of fibroblasts and enterocytes, which after a few days cultivation served as vasculated tissue layer on which precultivated glioblastoma spheroids were seeded [201]. Within few days of cultivation, they observed faster growth of the tumor spheroids and a beginning infiltration of blood vessels.

5.2.2. Infection/microbiome reconstructed models

For all epithelial surfaces, various infection models exist. In contrast, microbiome models are mostly available for GI-tract, including the oral cavity. First attempts for *in vitro* models in skin microbiome are also made. The lung microbiome, in diversity and number of microbes is much less, did not get comparable investigation and no distinct models for microbiota was suggested. **Table 3** therefore uses the same sequence of GI, skin and lung to provide a summary of *in vitro* models.

Gut fermentation models in perfusion mode are *in vitro* systems with control on pH, temperature, and oxygen. Such systems contains vessels connected in a flow system and can mimic conditions of the diverse gastrointestinal parts. A simple, one-vessel *in vitro* continuous flow system to investigate the anaerobic large intestinal microflora was already used by Rolf Freter [202]

The rising interest in the GI microbiome has led to the development of several GI simulating and multi-stage models. Examples are the multistage fermenter PolyFermS using beads to immobilize and thus stabilize the fecal-origin microbiome [203]; or the model systems developed by TNO called TIM1 (upper GI) and TIM2 (lower GI) [204]. With the InTESTine™ TNO also developed an *ex vivo* system for digestion and absorption studies using fresh porcine GI tissue, in some analogy to the Ussing chamber but for increased throughput in transwell-comparable format [205]. Among the most advanced fermenter systems, the SHIME® (Simulator of the Human Intestinal Microbial Ecosystem) system from the ProDigest company is a validated system for simulation of the full GI-tract (stomach to descending colon). The M-SHIME includes mucin covered agar microcosms for improved persistence of mucin-adhesive GI species such as *Lactobacilli*, reducing the otherwise observed shift in *Bacteroides/Firmicutes* ratios [206].

Fermentation models allow investigation of purely microbial communities over longer time. All host-factors (e.g., enzymes, cells, immune defense, neuroendocrine response) are omitted and such facilitates the analysis of environmental on microbial communities (e.g. nutritional effects; antibiotic therapy, probiotics) or the microbial effect on nutrition, stability of oral drugs.

The cultivation of anaerobic or microaerophilic bacteria requires hypoxia. Continuous low oxygen conditions are created by incubators, anaerobic jars or oxygen absorbers (such as AnaeroPack). Pack-pouch AnaeroPack are also suitable for tissue culture, however fail to create oxygen gradients of GI tissue allowing extended culture of viable mucosa and the microbiome.

The mucus layer is a protective barrier serving to reduce direct contact between the microbial community and the host epithelial cells. However, some bacteria use the mucus layer as habitat and nutrition source. Some commensals having a function in the mucus turnover, but also may assist in certain physiological situations pathogenic bacteria in colonialization, growth and thus dysbiosis establishment. A two-stage continuous culturing system with mucin gels was developed for monitoring bacterial growth from fecal samples in dynamic conditions [207]. Such cultivation may allow investigation of bacteria with special capability to grow on mucosal surfaces.

Pure biofilm models on abiotic surfaces are used to elucidate the special growth mode and antibiotic susceptibility. Flow-based biofilm systems are used to investigate the kinetic of biofilm formation, in particular initial adhesion events, since they are mostly designed to allow CLSM live monitoring. Biofilm models are reviewed elsewhere [208–210]. Biofilm size, structure, species richness of current biofilm models is not representative for biofilms observed in human disease, although even our

knowledge of human observed biofilms is in its infancy. More advanced models use biological substrates (e.g. cells, tissue, or mucus samples for a more realistic mimic of the niche or study polymicrobial biofilms. Some examples of such models are M-SHIME or WinCF (see **Table 3**).

Infection in 2D and 3D cell culture relies mostly on single cell type or co-cultures of cell lines with distinct function e.g. co-culture of epithelial cells with macrophages and dendritic cells. Such models allow the investigation of microbial invasion of selected, single microbes and their survival or molecular toxicity mechanisms. Transwell approaches are frequently used for epithelial cultures. The compartmental set-up enables monitoring of epithelial barrier properties by measurement of the transepithelial electrical resistance. Oral biofilm studies were also conducted in a model called Hanging Basket method, where a pre-grown biofilm is exposed in the basket on top of an oral epithelial monolayer. However, such conventional cell culture does not recapitulate physiological conditions in cell type variance and dynamic condition of nutritional supply and removal of cells and metabolites. Thus, a longer co-cultivation of microbial and host cells is impossible due to the outgrowth of the microbes.

Enteroids (derived from tissue) and organoids (derived from stem cells) of the GI tract contain more of the multiple cell types contributing to the functional architecture of this remarkable barrier tissue. The maintenance of immune homeostasis in presence of the largest part of the human microbiome and nutrition transport, via a selective permeability, is enabled by a number of cell types. These include absorptive enterocytes, Paneth cells for hormone and antimicrobial peptide secretion, mucin-producing goblet cells, microfolded cells and chemosensory Tuft cells as early mediators to sense environment. Organoids contain such epithelial cell types, but lack the supporting cell types of the endothelium, immune cells and

nerve cells. The mucosal associated lymphoid tissue also contains innate (macrophages, dendritic cells) and adaptive immune cells (B and T lymphocytes) for mediating mucosal immunity. The mucosal epithelium, lymphoid structures and the underlying muscle and enteric nervous system functions through a spatial confined design, which is an enormous challenge for *in vitro* models. Thus, organoids represent higher degree of physiologic tissue structure and allow longer cultivation times in devices such as rotating vessels. Bacteria or viruses are applied to enteroids or organoids by microinjection. In some models, organoids are disintegrated from their original structure and grown on transwell filters for easier application and analysis. There are few recent excellent reviews on the use of such 3D *in vitro* models for infection studies [211–213]. Techniques used for the establishment of enteroids and organoids are perfectly suited to grow more physiologic tissue also in microphysiologic devices.

Few most advanced *in vitro* models developed perfusion systems to allow an interaction of tissue mimic of the host (e.g. Caco-2 monolayers or epithelia regrown from human origin spheroid samples) with GI fluids containing bacterial samples. Such attempts were taken from scientists of various fields and therefore, it is not surprising that attention was paid to various aspects. The ProDigest attempt for a host-microbe interaction model starts from the GI fermenter system SHIME. They designed a flow system suitable to test SHIME fluids in an interface with an epithelial cell line layer separated by a microporous membrane and a mucus mimicking mucin gel layer. For studying uptake and transport phenomena, this system called Host-Microbe-Interaction (HMI) module has a basolateral channel allowing to probe permeation [214]. The team of Prof. Wilmes developed a microfluidic system, called HuMiX with oxygen control to create anoxic milieu enabling the growth of the obligate anaerobic GI bacteria at an interface

with enterocyte cell monolayer (Caco-2 or HT-29 cells) [215]. Similar to the HMI module, this system separates the microbial chamber from the enterocyte chamber by a mucin coated porous membrane. In further development, primary T lymphocytes as immune cells were cultivated in the chamber for medium perfusion [216]. The Ingber lab, Wyss Institute at Harvard, are experts in microfluidic. They brought the dynamic of peristaltic in their system by mechanical deformation of the channel. Such mechanical forces were shown to foster the physiologic development into GI tissue including formation of villus structure and mucus secretion [217]. Such system was used to bring GI bacteria into direct contact with the intestinal tissue, without any separating membrane. Pre-cultivation of the enterocytes (for 100 h) for an initial tissue development was followed by a short halt in peristaltic to inoculate LGG bacteria. For one week, a coculture with viable bacteria and viable enterocytes could be maintained [218]. Designed bacterial community of eight members either facultative or obligate anaerobic was tested on the gut-on-a chip set up. The system was used to monitor the tissue damage of enteroinvasive *E. coli*. [219]. Integration of oxygen sensors (e.g. Oxy-4 probe system) in the gut-on-a-chip model achieved a real-time assessment of this important parameter. A transmural hypoxia gradient with luminal O₂ content <0.5 % could be achieved by positioning the chips in anaerobic chamber with humidified atmosphere of 5 % CO₂ in N₂, while the oxygen for the host tissue is provided by the perfusion with oxygenated medium in the lower channel [220]. Such anaerobic intestine-on-a-chip showed an increase in barrier function and the production of a dense mucus layer from the epithelial tissue. It also allowed the cultivation of obligate anaerobe commensal bacteria, human microbiome passaged in gnotobiotic mice and fresh human stool samples at least

over several days with sustained bacterial diversity.

5.3. *Ex vivo* models

5.3.1. Tumor model

Ex vivo models are represented by living functional tissues or organs isolated from an organism and cultivated outside the organism in an artificial environment under highly controlled conditions. The biopsy is then washed with saline or phosphate buffered saline and placed in polarized or non-polarized orientations according to the purpose of the study [261]. They have the advantages of being closer to the organism anatomy than *in vitro* models and at the same time provide more controlled conditions than it is possible *in vivo*. *Ex vivo* models are also relatively cheap, allow real time monitoring, permit to control the experimental conditions and are ethically more accepted by the public than the *in vivo* experiments [262].

The *ex vivo* cancer models permitted a scientific jump in the understanding of tumorigenic growth and resistance. These models being obtained from biopsied organs, they offered the possibility to engineer more complex platform, giving the possibility to include factors as the tissue vascularity and the tumor microenvironment, factors that has been shown to play a key role in the tumor development [263,264]. Despite all these advantages brought by the *ex vivo* models and the successes made in developing the tumor models, the gap between the results obtained in preclinical and clinical studies of the nanomedicines in cancer therapy, might indicate that these models lack other factors that play a key role in the human cancer. As detailed before in this review, the human microbiota is involved in the cancer development at different levels, and to include it in the *ex vivo* models to evaluate nanoparticles anticancer efficacy seems mandatory to reflect closer the human cancer microenvironment.

Some studies evaluated the anticancer effect of nanoparticles on *ex vivo* models. Kutty *et al* [265] developed docetaxel-loaded micelles for the targeting and the treatment of triple negative breast cancer. An *ex vivo model* was prepared by dissection of the tumor tissue from MDA-MB-231/Luc cancer xenograft models. The explant recovered was then treated and prepared for testing the efficacy of the docetaxel-loaded micelles [265]. The results obtained showed that the docetaxel-loaded micelles significantly enhanced the therapeutic efficacy compared to the free docetaxel and to the non-laded non-targeting micellar formulations [265]. These encouraging results should be followed with testing these nanoparticles on an *ex vivo* model of co-culture with bacteria from the human microbiota, to investigate if the human flora plays a role in this type of cancer or modulate this treatment. In another study, Swami *et al* [266] formulated polymeric nanoparticles composed of poly(D,L-lactic-co-glycolic acid) (PLGA), polyethylene glycol (PEG), and a targeting ligand (bisphosphonate). The targeting polymeric nanoparticles were then tested on mice skull bone as an *ex vivo* model. The results obtained confirmed the affinity of the designed targeting polymeric nanoparticles to the bone marrow [266]. These nanoparticles were then used to encapsulate bortezomib, a drug known for its antimyeloma effect but its peripheral neuropathy make it dangerous for direct administration. The encapsulated nanoparticles produced a similar antimyeloma effect with the advantage of the bone-specific drug delivery [266].

5.3.2. Infection/microbiome *ex vivo* models

Ex vivo co-culture models of bacteria and human cells were the subject of some studies that aimed to understand the interactions between the host and its microbiota or to investigate the implication of some bacterial species in infectious diseases (Table 4) but less often in the oncology field. These models may be used as potential *ex vivo* templates for

testing the nanoparticles anticancer effect on more complete models that take into consideration the microbiota involvement and the host response to cancer therapy. For instance, in a study performed by Carol *et al.* [267], non-pathogenic *Escherichia coli* and *Lactobacillus casei* were successfully co-cultured with ileal tissues obtained at surgery from patients with Crohn disease. Such a model could be used to evaluate the effect of nanoparticles on intestinal cancer if the ileal tissue used comes from patients with colon cancer. Moreover, nanoparticles have been reported to present a possible antibacterial effect against *Escherichia coli* and *Lactobacillus casei* causing a possible destabilization of the microbiota [183,268]. Such an effect might influence the therapeutic outcomes of the used nanoparticles in cancer treatment, especially with the essential role the microbiota plays in tumor development and response to therapy. Another example from Keita *et al.* studied *Escherichia coli* co-cultured with ileal biopsies recovered from colonic cancer patients [269]: this model using cancerous tissues, it can be directly used to test the efficacy of nanomedicines on colonic cancer. This model and in addition to the classical *ex vivo* model advantages, would take under consideration the role of the intestinal flora, allowing a better representation of the human tumor microenvironment and so offering a better correlation between preclinical and clinical studies.

Table 3: Selected examples of *in vitro* models of the GI tract, skin and lung as the outer epithelial surfaces (GI, skin, lungs), their advantages/limitations and applications. **Circled numbers relate the description to the schemes in Figure 6.**

<i>Model</i>	<i>Description</i>	<i>Advantages/Limitations</i>	<i>Application</i>	<i>References</i>
<i>Gastrointestinal</i>				
Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) ①	Multi-stage chemostat mimicking the entire GIT fluids in five compartments (stomach, small intestine, ascending, transverse and descending colon)	+ Can use inoculation samples from patients; + Allow monitoring microbiome adaptation over time; + Investigate drug- microbiome interaction or metabolism - Host cells, nerve system etc. are not present	Dysbiosis studies Nutrition, probiotics Drug stability/metabolism	[221,222]
Caco-2 monolayer ②	Colon carcinoma cell line grown on transwell filters to polarized epithelium	+ Most reductionist model for epithelial barrier/transport - Limited to short term infection studies	Transport studies; Effect of toxoids on barrier integrity	[84]
3D coculture with immune cells ②	Cell line model on transwell filter inserts; macrophages and DCs in collagen layer overlaid with Caco-2 epithelial monolayer	+ Reacts on inflammatory triggers - Static system not applicable for long-term infection studies	Transport studies in inflamed conditions	[223,224]
Hanging basked model ③	Coculture of oral bacteria as pre-grown biofilm and oral keratinocyte cells	+ Artificial saliva as medium for multi-species biofilm	Soluble mediators interaction between bacteria and host cells	[225]
Organoids ④	Biopsy-derived spheroids or stem cell derived organoids (with mesenchyme)	+ Long term culture possible + Allows culturing of “non-culturable” organisms (e.g. human viruses)	Infection studies with human viruses and bacteria	[199]
Organoids in 2D	Spheroids or organoids disintegrated and cultured on filter membrane	+ Easier application of pathogens, drugs and analysis	Infection studies Transport studies	[226,227]

Bioprinted GI model	Bilayer of myofibroblasts and human primary epithelial cells printed on transwells	<ul style="list-style-type: none"> + Barrier markers of epithelial cells as mucus production shown; + Study efflux pumps 	Transport studies	[228]
Tissue slice cultivation ⁵	Intestinal tissue slice	<ul style="list-style-type: none"> + Physiological environment maintained (e.g. tumor microenvironment or including enteric nerves); + Patient-derived, human or animal tissue - Limited cultivation time 	<ul style="list-style-type: none"> Incubation in well plates for microscopical observation; Combination with Ussing chambers for sort term transport studies 	[229]
Anerobic villi model	Human stem cell derived enterocytes cultivated for O ₂ gradient creating device	<ul style="list-style-type: none"> + Crypt polarization; + Chemical gradient 	Cocultivation with facultative and obligatory anaerobic gut bacteria	[230,231]
Gut-on-a-Chip ⁶	Microfabricated flow chip with chambers for microbial/lumen compartment, and epithelial compartment	<ul style="list-style-type: none"> + Mimic peristalsis; + Near physiological adherence by ECM coating of cell growth surface; + Seeding with primary or organoid derived cells; + Cocultivation with immune cells or microbes possible - No muscle cells, ENS 	<ul style="list-style-type: none"> Absorption studies Host-microbe interactions Model for drug/therapy development Barrier properties (TEER) 	[232,233]
Tumor-on-a-Chip ⁶	Microfluidic cell culture of normal parenchymal cells and cancer cells	<ul style="list-style-type: none"> + Developed for several organs and cancerogenesis processes; + Possible with cancer cell lines or PDX - Technical complexity, robustness, throughput capacity and long-term use are developable 	<ul style="list-style-type: none"> Modelling processes in cancer cascade (invasion, expansion, angiogenesis, extravasation) Testing delivery strategies 	[234,235]

Anaerobic intestine-on-a chip ^⑦	Gut-on-a-chip model with sensors/control on oxygen	+ Enabling transmucosal hypoxia gradient; + Cultivation of anaerobic GI bacteria	GI microbiome host cell interaction studies	[220,236]
Host-microbe-interaction module (HMI) ^⑧	Device with two-chamber flow system for long term studies of host-microbe interactions	+ Applicable to SHIME system; + Mucin layer applicable - No cells of the immune system integrated; - No peristaltic; - No villi structure	Absorption studied Model for drug/therapy development	[214]
Human-microbial cross talk platform (HuMiX) ^⑨	Module for cultivation of Caco-2 cells as epithelium in aerobic condition and gut bacteria in anaerobic condition in luminal chamber	+ Cultivation of anaerobic GI bacteria; + Integration of immune cells possible - No peristaltic; - No villi structure	Microbiome studies Barrier properties (TEER)	[216]
Perfused intestinal segments with enteric nerve system and muscles ^⑩	Mouse intestinal tissue cannulated and perfused	+ Full physiological tissue - Animal origin	Host-environment interactions Short-term responses	[237,238];
<i>Skin</i>				
Skin mimetic with callus	Agar overlaid with callus	+ Physiological substrate - No living skin cells	Skin microbiome cultivation	[239]
Reconstructed Skin	Differentiated epidermis on membrane cell culture inserts grown from keratinocytes in air interface	+ Physiological epidermis structure; + Commercially available models - Barrier properties weaker than in skin explants	Models in cosmetic and pharmaceutical development (toxicity, penetration) Infection and wound models	[240,241]

- No immune cells

Full-thickness skin models	Differentiated epidermis on fibroblast dermis	+ Commercially available models - Lacking immune cells, adipose tissue, appendages, blood vessels	Skin irritation/corrosion studies, Permeability studies, Bacterial adhesion studies, Thermal wound infection model	[242,243]
Excised skin infection models	Human or porcine origin	+ Structural integrity; + Resident immune cells present - Limited time for viable tissue	Wound infection models Skin permeation study	[244,245]
Printed skin	3D-tissue generated by a bioprinting process of keratinocytes and fibroblasts in ECM matrix	+ Stratification; + Accurate organization, reproducibility - Technical effort and costs; - Lack in reproducibility of skin appendages (hair follicles)	Tissue regeneration, Wound models Models in cosmetic and pharmaceutical development	[246]
Skin-on-a-chip	Microscale tissue, kept in a dynamic perfusion model	+ May use tissue biopsy or reconstructed/engineered skin + Allow studying the migration of immune cells	Percutaneous absorption, toxicity Disease models (cancer, lupus, psoriasis, acne)	[247,248]
<i>Lungs</i>				
WinCF model	Artificial sputum medium in narrow capillary for growing sputum samples of CF patients	+ pH, respiratory activity, biofilm growth and gas production due to microbial activity monitored	Investigation of chemical triggers/gradients	[249,250]

Transwell model lung	Airway or alveolar cells grown at air-interface	<ul style="list-style-type: none"> + Physiological interface; + Mucus or surfactant can be included; + CF patient biopsy originating primary cells; + Air-interface culture - Short term studies only 	<p>Infection model with <i>Pseudomonas aeruginosa</i> /biofilm;</p> <p>Aerosol deposition possible</p>	[251,252]
Lung-on-a-chip	Microfluidic devices for growing lung cells in dynamic flow condition	<ul style="list-style-type: none"> + Mimic breath dynamic; + Air-interface; + Integration of immune cells 	<p>Aerosol medicine testing;</p> <p>Pharmatotoxicology studies;</p> <p>Infection model</p>	[253–255]
Precision cut tissue slices	Agar filled lung tissue for precision cutting	<ul style="list-style-type: none"> + Tissue structure of fragile tissue maintained + Tumor microenviroment maintained 	<p>Pharmacotoxicology;</p> <p>Efficacy tests for chemotherapeutics</p> <p>Infection studies with viruses, <i>P. aeruginosa</i> and <i>M. tuberculosis</i></p>	[256–259]
3D monotypic bacterial coculture	<p>A549 alveolar epithelial cells pregrown on beads in rotating vessels</p> <p>Infection with <i>P. aeruginosa</i></p>	<ul style="list-style-type: none"> - Short term studies only 	<p>Antimicrobial efficacy</p> <p>Biofilm investigation on biotic surface</p>	[260]

Table 4: *Ex vivo* models of human cells-bacteria co-culture.

Source of the biopsies	<i>Ex vivo</i> technique used	Bacterial species	Aim of the study	Ref
duodenal biopsies from ulcer disease patients	Co-culture of the tissue with bacteria in a shaking water bath	Enterotoxigenic <i>Escherichia coli</i> (ETEC)	To study the adhesion of ETEC to host intestinal mucosa causing diarrhea	[270]
Intestines of intestinal bowel disease patients (both inflamed and healthy tissues)	Tissue culture on sterile metal grid on a cylinder support	Strains of <i>Lactobacillus</i> and <i>Salmonella</i>	To test the effects of probiotics on the human health and the role it plays in intestinal bowel disease	[271]
Ileum resection from patients with Crohn disease	Transwell	No pathogenic <i>Escherichia coli</i> and <i>Lactobacillus casei</i>	To investigate the role of bacteria in activated T lymphocytes immune response in the lamina propria of Crohn diseases inflamed mucosa	[267]
duodenal biopsies of patients with intestinal disorders	Modified micro-Snapwell system (pIVOC)	Enteropathogenic <i>Escherichia coli</i> (EPEC)	To study the human intestinal mucosal response to EPEC infection	[272]
Ileum from patients with colonic cancer	Modified Ussing chambers	<i>Escherichia coli</i>	To characterize the normal barrier properties of human follicle-associated epithelium	[269]

5.4. *In vivo* models

5.4.1. Cancer models

In classical cancer research, two large groups of animal experiments are distinguishable (**Figure 5**): i) grafting of tumor cells or tissue either subcutaneous or orthotopic, with the aim for testing chemotherapeutic approaches and drug delivery systems and ii) using various transgenic mouse models carrying oncogene mutations or tumor suppressor gene deletions for tumorigenesis investigations [190,273]. Spontaneous tumor development models in specific organs *e.g.*, by using genetic modified mice, represent tumor heterogeneity in a higher level, are however less frequent used for testing nanomedicines maybe due to the higher effort in showing significance. Recent developments in bioengineering and of genetic tools of higher precision (*e.g.*, genome editing tools like CRISPR Cas) facilitates the establishment of powerful models [190].

A model for genetic induced cancer is the $Apc^{Min/+}$ mouse model, used as representative for human sporadic Familial Adenomatous Polyposis and shows occurrence of intestinal and mammary tumorigenesis [274]. In addition, the DNA mismatch repair Deficiency - $Msh2^{-/-}$ mouse model shows spontaneous cancer development. Chemical induction of inflammation and tumor is done using Azoxymethan (AOM) or Dextrane sulfat sodium (DSS) in mice. Mutagenic agents like AOM or/and DSS are used to mimic environmental factors for non-hereditary tumor development. AOM causes mutations and thus tumor development but with a lack of invasiveness. Co-treatment with DSS increases the inflammation [275].

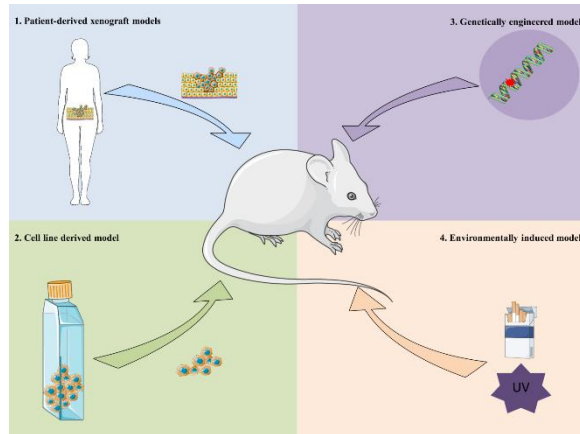


Figure 5: Different available concepts of *in vivo* models for cancer studies. 1. Patient-derived xenograft models: obtained by subcutaneous implantation of fresh, surgically derived human tumor into immunodeficient mice. 2. Cell line derived model: human tumor samples are cultured as cell lines and implanted into mouse models. 3. Genetically engineered model: obtained by alteration of the mice genome, using genetic engineering techniques. 4. Environmentally induced model: using suspected environmental cancer-causing agents to induce cancer in mice.

5.4.2. Infection/microbiome models

Invertebrate models like *Drosophila melanogaster* or *Caenorhabditis elegans* contribute important findings of molecular interactions, allowing also understanding of functional mechanisms (*e.g.* in host-microbe interactions) in higher organisms (Ref). Also, the nematode *C. elegans*, the zebrafish *Danio rerio*, or the wax moth larvae *G. mellonella* are models used in first *in vivo* screens for anti-infective therapy (Ref.). Among the lower vertebrates, the zebrafish is an important investigatory model of increasing usage. Zebrafish larvae allow cost-effective experiments over short timescales and have no ethical issues about animal welfare. Still, the vast majority of animals used in such studies are rodents. In particular inbred mice offer advantages in easy and economic experimentation but as important also control over genetic variation and environmental factor influences. In addition, pigs find some use for their

higher similarity in size, GI-physiology and omnivore nutrition.

Microbial communities are evolutionary conserved with regard to the organismal phylogeny. Thus, the mouse microbiome shares some bacterial commensals on the genus level, but has distinct differences in which bacteria cause disease compared to humans. Infection models thus can decide to use the mouse pathogenic bacteria for disease modelling, investigation of host-pathogen interactions as in particular the immune response. For example, *Citrobacter rodentium* is a murine, mucosal pathogenic bacterium causing colitis. This infection model shows pathology comparable to human pathogens like enterohemorrhagic *E. coli* and Salmonella and thus provides important mechanistic findings on host-pathogen interactions [276,277]

Another approach is to reduce, eliminate or prevent the murine microbiome to allow infection by human pathogens. The nutrient-niche hypothesis by R. Freter suggests a control of bacterial population by the concentration of few limiting substrates (carbon source and micronutrients like Fe) [278]. A balanced, complex bacterial consortium is stable because all niches are occupied and therefore infection by new arriving bacteria is very difficult. Vacant niches, as may occur by microbiome perturbation by antibiotics, facilitate the gaining of an infection or outgrowth of a usually rare species. Antibiotic treatments occasionally in combination with neutropenia are ways to increase the susceptibility for bacteria, which otherwise would not cause infections in mice.

Gnotobiotic mice are grown in germ-free conditions and thus can be used for inoculation with mono-, multi-, design microbiome communities or human sample microbiomes. Gnotobiotic mouse models were shown as suitable tool for investigation of specific defined aspects in

microbiota-host-pathogen interactions. Such relatively well-defined *in vivo* experimental systems is helpful in providing experimental tests for hypothesis origin from clinical observations and bioinformatics/statistical prediction [279–281]. Genetic models such as mice with knockout of important immune mediators are also suitable for infection models. For example, the IL-10 knockout mouse is known to develop colitis. Combinations of gnotobiotic conditions with genetic models can be applied.

The term “humanized” for mouse models is used in two scenarios: humanization is related either to human derived microbes (*e.g.*, stool or biopsy derived microbial communities), or to the immune system. In the later case, mice are made immune-deficient and then engrafted with human peripheral blood mononuclear cells (PBMCs). Such humanization allows studies on the adaptive immune response. However, mechanisms of the initial innate immune response remain murine.

Nagao-Kitamoto *et al.* used an IL-10 ko gnotobiotic mouse to engraft human bacteria with the aim to assess gut dysbiosis in inflammatory bowel disease [282]. Samples of Crohns disease and ulcerative colitis patients were applied. The experimental model showed a decrease in microbial diversity and colitis development.

Another recent study tested a 15-member consortium of human bacteria, which was engrafted in the colon of gnotobiotic mice to test analytical tools enabling a spatial organization of bacteria in such complex environment. Hybridisation technique and spectral imaging analysis provided images to monitor interactions/aggregation tendency of specific bacteria with mucus or food particles [283].

IL-10^{-/-} mice are a model for enterocolitis and show in longer maintenance a strong increase of spontaneous colon cancer development (60 % incidence at six

month) [284]. The model is established in colitis research. The knockout of

used to study effects of selected bacteria at various organs. Intestinal *Enterococcus*

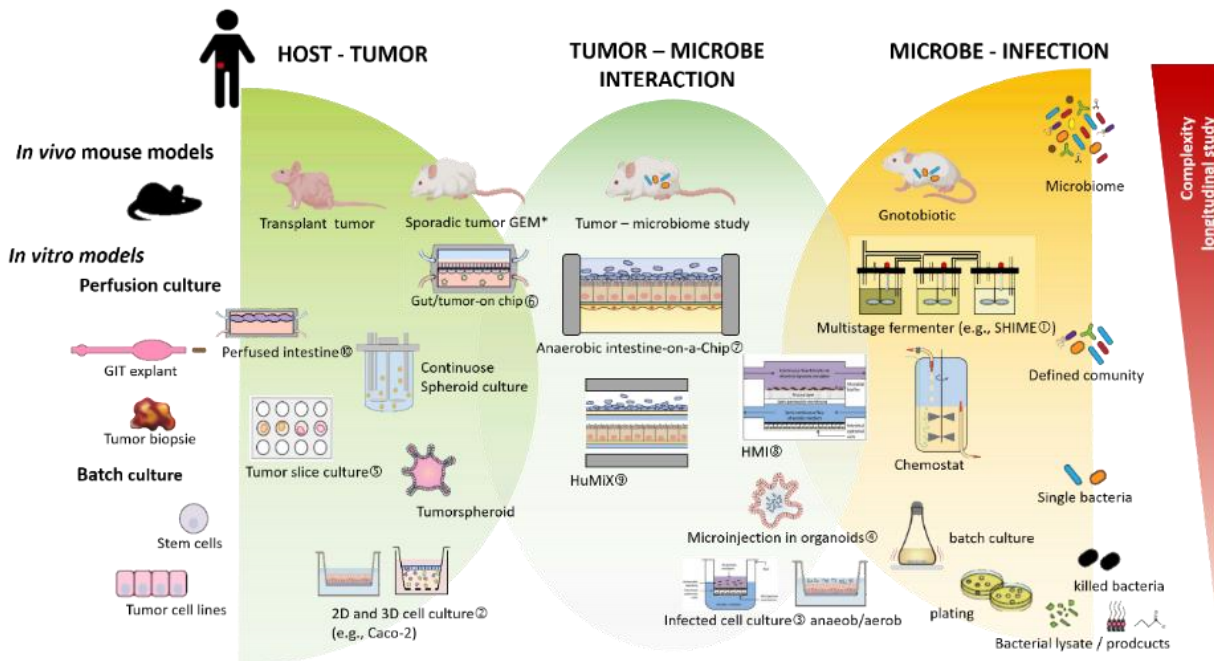


Figure 6: Overview on convergence of tumor models and infection/microbiome models over the full complexity range from mouse in vivo study, in vitro advanced native or reconstructed tissue to maximum-reductionist single cell studies. Left column shows starting material from the host. Right column shows starting bacterial material. Circled numbers relate the schemes of in vitro models to the description in Table 1. *GEM= genetically engineered model e.g. $Kras^{G12D}/PTEN^{lox/+}$; $APC^{Min/+}$ mice. Choice of complexity is done at host cell selection, microbe species or community selection and technical model set-up. Of note, experimental tumor-microbe interaction studies so far are limited to animal studies. The experimental human-based in vitro models (anaerobic-intestine-on-a-Chip, Host-Microbe-Interaction (HMI) module, or human-microbial cross talk platform (HuMiX)) were not used in such scenario yet. However, in particular the anaerobic-Gut-on-Chip model with direct contact between the bioengineered intestinal tissue and the GI bacteria should be suitable.

the anti-inflammatory cytokine IL-10 affects inflammation resolution and thus is a model for inflammation-related disease progression. As such, this model was also

faecalis was shown to promote IBD development and adenocarcinoma [285], while *Lactobacillus salivarius* ssp. *Salivarius* was shown to have some probiotic protective effect and reduced inflammation and colon cancer [286]. The model was also used to study effects of oral *Porphyromonas gingivalis* infection [287] and the effect of bronchus inoculations with *Pseudomonas aeruginosa* [288] on chronic inflammation and disease progression.

Admitted the large progress in model development and the numerous important findings resulting from such in vivo studies, important limitations in mouse models remain, which are simply intrinsic to the different species. There are some distinct differences in the gastrointestinal tract between mouse and man, nutrition and behavior are distinct. These differences, limitations as well as some recommendations for paying attention to in experimental study design were summarized in recent reviews [185,186]. The microbiome is shaped by the environment and thus it is not surprising that also showing quite some homology at the level of bacterial families – there is

Complexity longitudinal study

quite some difference in stains and in abundance. The application route (e.g., oral, intra venous or intraperitoneal) for bacteria as well as for chemotherapy in the selected model is of importance. In such complex experiments, the effect of biological barriers to restrict the bioavailability of chemotherapeutics may complicate the analysis of results.

Cancer and infection/microbiome models can be used as starting set-ups for tumor-microbiome interaction studies. **Figure 6** provides a schematic overview on *in vivo* and *in vitro* models. Complexity selection has to be done at various levels (host cell type(s), extracellular matrix, microbiome/pathogenicity factors, biochemical/biophysical cues) for are indispensable/dispensable parameters with regard to the research goal.

6. Discussion and perspectives

The physiology at outer epithelia is a trilogy between the human tissue (epithelial barrier and associated immune tissue), the microbiome and environmental factors, which constantly influence and shape each other. Our attempts to understand this orchestra is only at the beginning. Simplified models can be instrumental for proving hypothesis and principal findings. Investigations in realistic scenarios would ideally use human cells, since no animal really is able to mimic the diversity of human life-style. Sampling of human microbiota for observation is limited for larger screenings to accessible organs and samples (stool, skin samples). Important organs like the small intestine or the lung are difficult to access and need invasive procedures. We are still in progress to detect the microbiome at some human habitats and finding ways how to culture them for sufficient time span enabling disease progression investigations. Culturomics is an attempt to increase the percentage of culturable organisms and assessing their metabolomics with MALDI based analysis.

Experimental derived stable microbial communities (e.g. by human derived samples going through several passages in gnotobiotic mice with stable diversity) or designed bacterial communities to fulfill the criteria of minimum phylogenetic diversity representing the natural microbiome and community stability may be important tools we need in microbiome research. Models to grow complex microbiome in physiological conditions are needed. Ecological microbiologists started to leave the conventional lab cultivation and proved metabolomics strongly altered as reaction on the environment. *In vitro* models having the physiology of the human epithelial tissues, with human cells and allowing culturing over longer time could boost our understanding of this interaction with the ultimate goal of working with complete isolated microbiota samples for humans (health or suffering from various diseases).

The categorization into commensals, pathogens and symbionts is not straightforward. Most bacteria we are currently referring to as “pathogens” are as well human commensals for a majority of human hosts. Finding a niche for growth in unusual places and manifesting thus a pathological infection, shift in the tissue conditions offering opportunistic pathogen to outcompete the normal commensal flora or to express virulence factors, which are toxic for the host – all this can be events converting a harmless commensal into an opportunistic pathogen. On top of taxonomic analysis of genomics and transcriptomics, deeper studies involving also proteomics and metabolomics are needed to elucidate phenotypes, since often the phenotype (e.g., virulence factor expression, secretion of toxins, specific metabolic trait) is crucial for pathogenicity. Models facilitating continuous sampling and observation (in contrast to endpoint analysis only) will boost our knowledge on interaction and pathology.

Attempts to improve cancer therapy by manipulating the microbiome therefore should be designed with caution. Selective antibiotics are preferable to broad-spectrum. Location matters and in non-life-threatening infections, local therapy could spare the microbiome in other body sides. The microbiome and the human immune response co-evolved and are heavily interconnected. Thus, that antibiotic therapy impacts the immune response and cancer therapies based for example on immune check point inhibitors should not come as a surprise. Selective antibiotic use as adjunctive therapy may help in cancer therapy where bacteria are important cofactors in cancer growth and resistance, but in other cases only be deliberating. Tumor-associated bacteria may also just use the ecological niche (passengers according to the so-called "driver-passenger" theory) with or without affecting tumor progression and (chemo)therapy resistance. In consequence, the pathogen effect is important for drawing conclusions if such organism should be eliminated, can be tolerated or even used as carrier system or therapeutic.

Today, many clinical microbiome observations are from patients already having a disease status. Such studies have the advantage of human disease relevance, but also the limitation of observing the state of already developed pathogenicity; however, being of limited use in the understanding of mechanisms in the disease development and progression. Recent initiatives of following microbiome and health status of cohorts over long time will also assist in establishing links between some specific microorganisms capable to grow at specific physiological niches inducing inflammation, pathophysiologic immune response, tissue damage and disease. Increased understanding of the microbiome role in malignant and chronic diseases has the perspective to use it as biomarker but also

as basis for precision medicine. Pharmacomicrobiomics is now be seen as one cause of variability in response to drugs [289,290]. Beyond chemotherapeutics of established cancers, tumors could be prevented or more efficiently treated by integration of diet, pre and probiotics, adjunctives like antibiotics [291].

In vitro prototype models are available to allow co-culture over hours to weeks, which may be a starting point for development of models allowing longitudinal studies. Caution is needed in the interpretation of all current models since our knowledge in this field is inchoately, which complicates the construction of models and their validation.

Sophisticated combinations of micro-fabricated flow systems and organoid or stem cell based regenerated tissue with physiological mechanical cues, physiological medium conditions (e.g., body fluid mimics), incorporation of immune system components and microbiomes of sufficient complexity may in future allow best mimic of mucosal tissue physiology. Such model may use patient derived sample or be tuned by genetic modification (e.g. CRISPR Cas9 mutation) for inflammation, tumorigenesis or challenged by opportunistic pathogens for infection. Likewise environmental stress factors, colonialization behavior of single pathogens against an established microbiome, or chemotherapeutic regiments could be evaluated. The beauty of such microphysiological approach is the suitability for parallel set-up of disease *vs* health or sample *vs* control, required in the analysis and interpretation of such multi-parameter experiments.

For reaching such model system, however, several challenges need solutions: i) Creating *in vitro* systems with sufficient lifetime (renewing capacity in the model) for studies extending two weeks without

loss in tissue integrity/cell viability. For *in vitro* system, such cultivation times will depend on advanced technology perfusion models. ii) Controlling bacterial growth without artificial separation by membranes to also enable studies of direct interaction. iii) Achieving stability in multi species cultivation. For *in vitro* models, for studying GI microbiome should ideally be able to represent aerobic to anaerobic gradient found in the gut between the epithelial crypt and the gut lumen. Concerning i-iii) the anaerobic-gut-on-a-chip model here seems currently most promising approach to provide solutions. iv) Improvement of material to create systems in more physiologic interface than PDMS chips. Improvement of sensor systems enabling constant monitoring and adjustment of such long-term culture system. v) Adapt systems to needs for drug delivery researchers or physiology researchers to allow application of chemotherapies or environmental stressors. vi) Alliance with bioinformatics researcher to predict/model highly complex interaction networks. Models and their analysis need to be developed, which are at affordable price and manageable from technical skills at the diverse scientific subjects required in complex set-up's to find broader usage.

Bioinformatics and statistical tools are able to generate hypothesis on microbial interaction types (cooperative/synergistic or competitive, antagonistic) from metagenomics data sets. Such tools can help in predicting stable microbiomes, effects of selective microbiome outgrowth, microbiome-targeted approaches. However, simulation derived hypothesis need experimental proof. *In vivo* models in particular mouse, are a gold standard for such experiments. The extraordinary high need for standardization of such experiments, the species difference that cannot be fully eliminated and the ethical issues with animal experiments are constrains for the *in vivo* testing.

Alternative approaches of *in vitro* models have the potential to complement and replace *in vivo* experiments to study host-microbe interactions in tumor or chronic pathology research bypassing difficulties of experimental control and animal welfare issues. Predictive *in vitro* models would be important since they reduce the ethical issues and the translational gap caused by species differences. The development and validation of such models however, is a challenging task, which needs the contributions of diverse experts from engineering, analytics, bioinformatics, microbiology, medicine and pharmacists eventually when they are intended as tools for therapy development.

Development of pathophysiological *in vitro* models aim representing a tissue or organ not only in normal conditions, but also to include disease relevant changes. While it is already quite challenge to build relevant models of either neoplastic OR infectious diseases, modeling the interplay of the two goes even one step further. Such models, however aspire to provide understanding and evidence on a mechanistic basis as source for rational therapeutic intervention. Many questions of drug delivery technology like the interaction of GI microbiome with the nanoparticle could profit from using existing microbiome cultivation models, which is currently still underexplored. Miniaturization, customization and personalization would increase the applicability in the technologist's community. The supreme discipline would be the complex system of host-microbiome-disease model (in a parallel approach with host-microbiome-health control) in a set-up where either the host genomic, the environmental stress factors, the microbiome or pathobiont could be modified in a controlled way – and even allow the treatment and consistent monitoring. Such models do not exist yet, however looking at the progress made in

the last decade does not sound too futuristic anymore.

7. Conclusion

Cancer is the disease of our era. Despite the huge accomplishment scientists made in its understanding and its treatment, this disease still takes lives of hundreds of thousands of people each year. Lately, the human microbiota has been linked to cancer development. The human bacterial flora has been shown to have different effects on the tumor evolution: pro-oncogenic or anti-oncogenic, but also on cancer chemo- and immunotherapy by favoring or blocking their effect. These interactions with the cancer physiopathology and treatments indicate that the microbiota should be considered in every cancer model for a better representation of the cancer microenvironment. More knowledge and consideration of the microenvironment may guide therapy selection, harnessing and tuning interactions in the right direction. Also, with the appearance of the aspect of cancer passenger or driver bacteria, and its potential role in specific cancer initiating, these bacterial species can be identified as cancer risk factors or indicators. This might be a strategy for early cancer diagnostic and prevention.

Nanomedicines proved a remarkable efficacy in preclinical studies but only few of them are approved for human use. The studies show a gap between preclinical and clinical results that might be explained by the fact that preclinical models are always not close enough to represent the human cancer model. This lack of representation might be improved by co-culture models of cancerous cells and bacteria from the human microbiota, in order to take into consideration the attribution of the microbiota in the cancer development and host response to the anticancer treatment. Such models might be a better representation of the human cancer microenvironment, allowing a closer representation of the clinical assays and

more reliable evaluation for the nanoparticle anticancer efficacy.

Acknowledgement

The authors thank the support of the National Association of Research Technology (ANRt) via financing the PhD contract number 2018/1485.

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