Chapter

Small Animals Gut Microbiome and Its Relationship with Cancer

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Abstract

This chapter aims to discuss recent developments in understanding the small animal gut microbiome's relationship with cancer, focusing on animals as well as a model for studying humans. Based on multidirectional interactions between the microbiome, the environment and the epigenetically/genetically vulnerable host, it intends to address the mechanisms by which microorganisms can contribute to carcinogenesis describing the roles of the microbiome directly in the pathogenesis of the disease through complex interactions between the microbiome and the host's metabolic and immune systems. The feasibility for developing new cancer diagnostic and prognostic methodologies plus treatments based on small animals' microbiome profiles are reviewed.

Keywords: gut microbiome, carcinogenesis, therapy, dog, cancer

1. Introduction

Much recent medical research focuses on understanding the influences of the microbiome on host health and disease progression such as in inflammatory, metabolic, autoimmune and oncologic diseases [1].

In order to introduce the reader to this chapter, it is essential to clarify some common terms such as microbiota, metataxonomics, microbiome and metagenome. The microbiota is defined as the assemblage of living microorganisms present in a certain environment and is composed by bacteria, archaea, fungi, algae and small protists [2, 3]. Metataxonomics defines the high-throughput process used to taxonomically identify microorganisms in the environment and characterize the entire microbiota, creating a metataxonomic tree [2]. The definition of microbiome includes not only the microorganisms community, but also their "theatre of activity" that involves the whole spectrum of molecules produced by them, including their structural elements (nucleic acids, proteins, lipids, polysaccharides), metabolites (signaling molecules, toxins, organic, and inorganic molecules), and molecules produced by coexisting hosts and structured by the environmental conditions [3]. It stands out that all mobile genetic elements, such as phages, viruses, and extracellular DNA should be included in the term microbiome but are not a part of microbiota [3]. Lastly, the term metagenome refers only to the collection of genes and genomes of members of a microbiota [2].

In humans, as well as in small animals, these complex communities of microbes inhabit predominantly the gastrointestinal tract and oral cavity, but other exposed tissues, such as skin, breast, respiratory and urinary tract, can also harbor unique

bacterial communities [4–9]. The host microbiota and immune system must communicate to maintain a balance between tolerance and activation, otherwise a dysbiotic state can be established and may incite or sustain diseases, such as cancer [10]. Epidemiological associations of abnormal microbiome with gastric, esophageal, hepatobiliary, pancreatic, lung, colorectal, lymphoma and other human and canine cancers have been previously established [11–13].

Neoplastic processes are the leading cause of death in adult dogs [14]. The annual cancer incidence rate is 381 per 100,000 dogs with 4 million new cancer cases per year, similar to the reported rate in humans (454 per 100.000) with 18 million new cancer cases annually [15–17]. In these species, naturally occurring cancers share many features, including clinical presentation, biological behavior, histological features, tumor genetics, and treatment response [18, 19]. Coelho and colleagues showed that the dog gut microbiome has a higher taxonomic and functional overlap with the human gut microbiome than pigs or mice and concluded that findings in dogs may be predictive of human microbiome results [20]. In addition, companion animals represent a special human experimental model in microbiomic investigations due to the exchange of microbes between humans and their pets [21].

In humans, there are some reviews involving microbiome and cancer, but they are scarce in veterinary medicine, with the most reviews covering the microbiome gastrointestinal tract and other diseases [22, 23]. The present article reviews the current status of comparative oncology approaches in human and small animals in the field of microbiome with special focus on carcinogenesis, relationship between specific microbiomes as well as the feasibility of new cancer diagnostic tools and therapies based on microbiome profiles.

2. Human and small animal microbiomes

The host's first major exposure to a complex microbiota occurs during birth through contact with the maternal microbiome, which represent a primary mechanism for the intergenerational microbiota transfer in mammals and, afterwards, bacterial colonization progresses from childhood to adulthood [24]. The microbiota development is limited to its niches by the host's immune system, along with the host's chronological development, providing early modulation of the host's physiological development and functions of nutrition, immunity and resistance to pathogens at all ages [24].

The most important group of organisms in microbiome studies is called the dynamic symbionts, whose symbiotic nature may vary along a spectrum from mutualism and commensalism to parasitism and amensalism [25]. Usually, microbes perform synthetic or catabolic metabolic activity through direct microbe-host interactions. Catabolism and bioconversion of compounds from the diet make nutrients more available to the host through the processes of fermentation, hydrolysis, metabolism of drugs and toxins, among others. Some microbiota members can synthesize important cofactors or bioactive signaling molecules such as vitamins and active amines. In addition, this can trigger changes in the host's gastrointestinal epithelial and immune responses [26].

The combination of factors such as age, genetics, physiological status (including innate and adaptive immune system), lifestyle, diet, host environment and disease status can result in variation in microbiomes between hosts [27]. Human gut microbiota is extremely diverse, with an estimated 1,000 bacterial species in the gut with 2,000 genes per species yields an estimate of 2,000,000 genes, which is 100 times the commonly estimated 20,000 human genes [27]. In dogs, gut

microbiome contains around 1,200,000 genes [20] and recent studies suggest that canine and feline gut fecal microbial phylogeny (e.g. predominance of Firmicutes, Bacteroidetes, Proteobacteria and Actinobacteria) and functional capacity (e.*G. major* functional groups related to carbohydrate, protein, DNA and vitamin metabolism, virulence factors and cell wall and capsule) are similar to those of the human gut [28].

3. Microbiome and carcinogenesis

Cancer is a complex disease, in which cumulative genetic, epigenetic physiological, immunological and biochemical changes occur incessantly in the tumor tissue, contributing to the complexity of the understanding, treatment and management of the disease. It is estimated that microorganisms could be associated with 15–20% of cancers [29].

As mentioned, the microbiota has an essential role in host health, in which a beneficial relationship is established, however, dysbiotic states can trigger several diseases, including cancer. Scott and colleagues proposed that in the etiopathogenesis of cancer, dysbiosis should be considered a persistent exit of the host microbiome from the health-associated homeostatic state (consisting of mutualists and commensals), towards a cancer promoting and/or sustaining phenotype (parasitism or amensalism) [25]. Currently, metataxonomic and metagenomics studies have documented and compared the diversity and abundance of microbes in different parts of the body between healthy and diseased patients. In veterinary medicine, it has been demonstrated a significant difference in the microbial communities in dogs with intestinal and multicentric lymphoma and with colorectal tumors comparing to healthy dogs [12, 13, 30]. However, these studies cannot distinguish whether some alterations in microbiota are causes or effects of cancer, describing only the different microbial communities found among the study groups.

The microbiome causative role has been demonstrated by controlled pre-clinical studies utilizing germfree (i.e., devoid of any microbiota) mouse models colonized with selected bacteria. For example, several family members of Enterobacteriaceae, including *Escherichia coli*, harbor an island of polyketide synthase (pks) pathogenicity that synthesizes a genotoxin called colibactin [31]. In an experimental study, knockout mice for IL-10 were mono-associated with two strains of *E. coli* that were pks + or Δpks (with and without pks, respectively) and treated with pro-carcinogenic azoxymethane to induce colorectal tumors to demonstrate that pks play a causal role in tumorigenesis [31]. All mono-associated pks + mice developed invasive carcinoma, in contrast, none of the Δpks mono-associated mice exhibited full invasion [31]. This result suggests that the presence of *E. coli* pks accelerates the progression from dysplasia to invasive carcinoma through the genotoxicity of colibactin, an example of pathway of the microbiota-associated carcinogenesis process.

In a recent consensus on the human microbiome role in carcinogenesis, expert opinion was that the microbiome is one apex of a tripartite, multidirectional interactome alongside environmental factors (such as diet, obesity) and an epigenetically/genetically vulnerable host that combine to cause cancer [25]. Gastrointestinal microbiome, which comprises 99% of the microbial mass, not only has the greatest both local and long-distance effects on overall health and metabolic status, but it is also the best investigated microbiome and serves as a model for understanding host–microbiota interactions and disease [32]. Due to its location, gut microbiome has been well studied as a contributor to colorectal carcinogenesis [33]. Other organs with a well-characterized microbiome include the skin and the vagina [34, 35]. The microbiome of each organ is distinct suggesting that effects

on inflammation and carcinogenesis are likely to be organ specific. Although many organs (e.g. liver and brain), does not have a known microbiome, they may be exposed to pathogen-associated molecular patterns (PAMPs) and bacterial metabolites through anatomical links with the gut [32, 36].

For a better understanding of the microbiome role in carcinogenesis it is important to recognize that bacteria can be found in the tumor tissue itself, in normal adjacent tissue and in tumor sites, such as intestine and genitourinary tract, with overlap between these sites (**Figure 1**). According to Picardo, the microorganisms inside, adjacent and distant from the tumor can play a role in cancer development and progression and interactions between these microbial populations together with the indirect gut microbiome effects have the potential to influence the disease development [37].

At the molecular level, the mechanisms by which microorganisms can contribute to carcinogenesis are multiple and varied, which may broadly be categorized into genomic integration and genotoxicity (by a direct oncogenic effect of microorganisms and their products); promotion of immunological modifications (which disrupts host cancer immunosurveillance through the induction of pro-inflammatory and immunosuppressive pathways); and metabolic reprogramming (by altering circulating metabolites which become pro-carcinogenic and by stimulating the synthesis of trophic factors for cancer cells by the host). Many of these actions can harm the host indirectly, as microbes optimizes conditions for their survival may result in a final common pathway of prolonged host cell survival, enhanced replicative capacity and dedifferentiation [25, 33]. These mechanisms converge to hallmarks of cancer [38] and will be described in more detail below.

3.1 Genomic integration

Although the microbiome viral communities have not been studied as much as the bacterial community, the virus's ability to integrate into the host genome is a causal mechanism of cancer both in dogs and humans.

A remarkable example is the human papilloma virus (high-risk HPV 16 and 18) and its association with human cervix cancer. The key event of HPV-induced carcinogenesis is the integration of two HPV genes (E6 e E7) into the host genomic DNA [39]. In proliferating cells of the basal layer of the uterine cervix, the viral

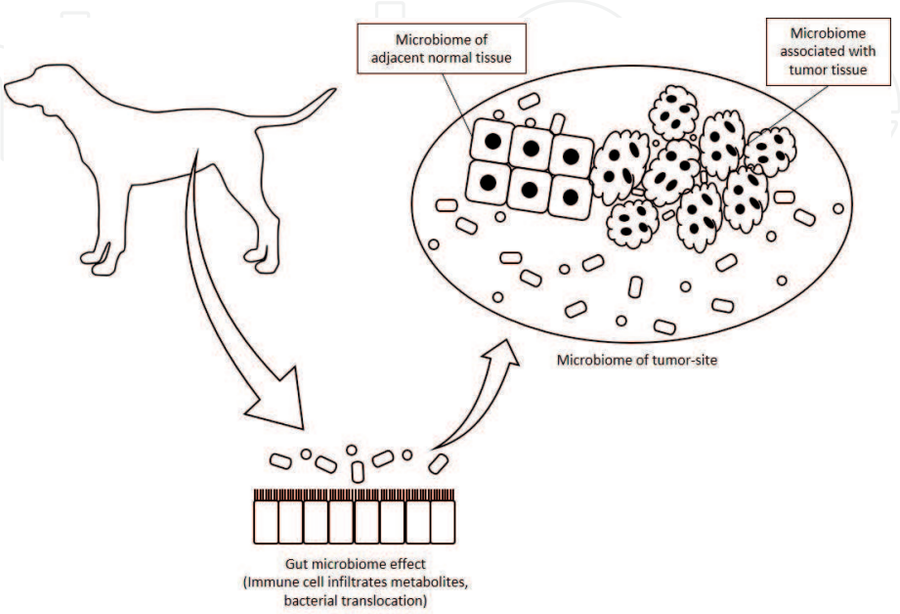

Figure 1.
The relationship between tumor and microbiomes (adapted from Picardo et al., 2019) [37].

genome persists as episomes, replicates in the suprabasal cells and can infiltrate deeper layers [40]. The HPV E6 and E7 genes are regularly present and expressed in the tumor tissue [40]. Their expression and the loss of expression of the E2 region (which negatively regulates E6 and E7) in the integrated HPV genomes cause the disruption of tumor suppressor genes that result in dysregulation of cell growth and inhibition of apoptosis [41]. Therefore, the overexpression of these viral genes synergistically acts to immortalize host cells, a cancer hallmark.

In dogs, investigations with canine papillomavirus (CPVs) have been limited to the association of different CPV genotypes with neoplastic lesions. Up to now, 20 CPVs types have been reported [42]. In skin, most genotypes of CPVs cause benign lesions, such as warts and pigmented/viral plaques or papillomas, which are self-limiting lesions such as those of oral papillomatosis [43].

Dogs that develop extensive papillomatosis may also be predisposed to oral squamous cell carcinoma (SCC) [44]. The detection of CPVs in malignant epithelial lesions is increasing in recent years [42, 45, 46]. CPV 1, 2, 3, 7, 12, 16, and 17 have been reported to cause epithelium neoplastic transformation. In a retrospective study, Thaiwong and colleagues (2018) described 7 dogs bearing benign papillomas associated with CPV1 and also the histological evidence of CPV1 causing malignant transformation of carcinoma *in situ* (ISC) and SCC. Later, the same group showed the expression of p53 and p16 proteins in cells infected with CPV1 in benign papillomas and lesions that progressed to SCC [42].

In a recent retrospective study, CPVs were successfully detected in 11 skin tissue samples and 4 oral tissues obtained from a cohort of canine papillomas and SCCs by PCR and through the detection of intralesional viral antigens using immunohistochemistry [46]. After sequencing, CPV 1, 2 and 6 were detected in the benign lesions, while CPV 9, 15 and 16 were detected in the SCCs, highlighting the risk of these genotypes in the induction of epithelial carcinogenesis [46].

The first report of chromosomal integration of CPV 16 into the host genome was detected in a sample of squamous cell carcinoma, raising the possibility that CPV 16 may be a potential type of high-risk canine papillomavirus [47]. However, the CPVs oncopathogenesis should be further investigated.

3.2 Genotoxicity

The gut microbiota is mainly composed of bacteria, many of which contain toxin-producing strains that can have carcinogenic effects through interfering with the cell cycle regulation, cell growth or directly damaging the host's DNA [48]. Pathogenic bacteria strains produce protein toxins to meet their survival needs, but these bacterial defense factors perturb the host equilibrium and affect tumor suppressor genes or oncogenes and promote host genome instability [49].

Among the large number of bacterial protein toxins, two genotoxins are well known for directly affecting the host's DNA integrity in the host organism target cells: cytolethal distending toxin (CDT), which is produced by several gram-negative pathogenic bacteria (e.g. *E. coli, Shigella dysenteriae, Campylobacter jejuni, Helicobacter sp*.) [50], and colibactin toxin (produced by *E. coli* strains); both trigger double-strand DNA breaks in host cells contributing to carcinogenesis [50, 51]. CDT exerts a pro-carcinogenic effect mainly because it presents a DNase activity. After binding to the host cell membrane, CDT suffers receptor-mediated endocytosis, proceeds to the endoplasmic reticulum and is translocated to the nucleus, where promotes cytotoxicity [50]. Cell CDT intoxication induces DNA damage, which results in the stopping of target cells in the G1 and/or G2 phases of the cycle and activation of DNA repair mechanisms [50]. Subsequently, normal cells that fail to repair the damage and survive the acute phase of CDT intoxication acquire the

cancer hallmark of cellular senescence or undergo apoptosis via the DNA host damage checkpoint pathways [52]. This chromosomal instability supports the notion that CDT might promote tumor initiation and progression [53].

Colibactin-producing *E. coli* colonize frequently the colon mucosa of patients with human colorectal cancer (CRC) being implicated in carcinogenesis and tumor progression [54, 55]. This genotoxin is found in 55–67% of human colorectal cancer compared to less than 20% of controls [31, 54]. Understanding of colibactin's chemical structure and biological activity is limited, but recent studies have shown that these toxins are powerful DNA-damaging agents acting via alkylation and DNA cross-linking, whose lesions activate the DNA damage checkpoint pathway and cells present signs of incomplete DNA repair, G2/M cell cycle arrest and chromosomal instability [51, 56]. In addition, colibactin also supports tumor growth by inducing a secretory phenotype associated with senescence through growth factors secretion [57].

In veterinary medicine, Feng and colleagues identified *E. coli* strains encoding colibactin cytotoxic necrotizing factor (CNF) in the rectal swabs and extra-intestinal samples of macaques, whose can cause clinical and subclinical diseases [58]. Genotoxins in companion animals have not been identified so far, but the fecal microbiota composition in dogs with colorectal epithelial tumors was different from that of control dogs, where Enterobacteriaceae, Bacteroides, Helicobacter, Porphyromonas, Streptococcus and Fusobacteriaceae were overrepresented in those with tumors [13]. Thus, studies are still needed to identify genotoxins produced by bacteria to help understand the carcinogenesis of canine colorectal epithelial tumors.

3.3 Immunological modifications

There is a well-defined bidirectional interaction between the immune system and gut microbiome, playing a role in the entire organism physiology [59]. The gut microbiota is essential for normal development of innate and adaptive immunity at several levels (demonstrated by studies using germ-free mice) and the immune system regulates colonization and abundance of microbiome species, as well as the response to commensal bacteria [60–63].

The host microbiota and the immune system must communicate to maintain a balance between the inflammatory response activation and the immune tolerance preservation [64]. For this, the gut bacterial population presents both a protective and harmful interface. Unlike opportunistic bacteria, other commensals, such as *Bifidobacterium infantiles* and *Faecalibacterium prausnitzii*, induce the development of regulatory T cells that prevent an inadequate immune response and protect the host against intestinal pathogens [65]. A lack of control in pro-inflammatory and anti-inflammatory bacteria (causing an imbalance between Th17 and T-regulatory cells) establishes a dysbiotic state [65, 66]. Immunological intolerance results in a loss of homeostasis that can promote a pro-neoplastic inflammatory environment through chronic inflammation, immune evasion and immune suppression [32, 67].

The gut mucosa consists of a single epithelial cell layer with intraepithelial lymphocytes that facilitates the interaction of bacterial with immune system. The epithelial line contains Paneth cells that secrete anti-microbial molecules and goblet cells that secrete mucus to lubricate the intestinal contents and protect the epithelium, while on the skin, keratinocytes regulate the microbes by secreting antibacterial peptides [68]. The lamina propria is below the mucous layer, which contains a series of other immune cells (including antigen presenting cells and CD4 + and CD8 + T and B cells). This lymphoid tissue is the most important component of body's immune system, capable of influencing immune responses both locally and systematically [1]. Microbe is detected using pattern recognition receptors (PRRs) represented by Toll-like receptors (TLRs) and NOD-like receptors (NLR) [69].

These are widely expressed in intestinal epithelial cells, as well as in intestinal macrophages and dendritic cells. PRRs can either control the microbiota through antibacterial mediators and thus suppress cancer, or they can promote resistance to cell death - a hallmark of cancer [38].

The systemic immune system is prepared (at the epigenetic or transcriptional level) to enact a robust response in the presence of pathogenic bacteria leading to proinflammatory immune responses or to maintain a non-inflammatory state in the absence of threat [70]. A state of disruption of the delicate balance of commensal bacteria (dysbiosis), which is characterized by a less stable microbiota, increases the potential of opportunistic pathogenic bacteria growth [71]. As seen, dysbiosis can promote impaired local, loco-regional and systemic immune responses, being able to generate a profound inflammatory state, both locally and systemically. This process is outlined in **Figure 2**.

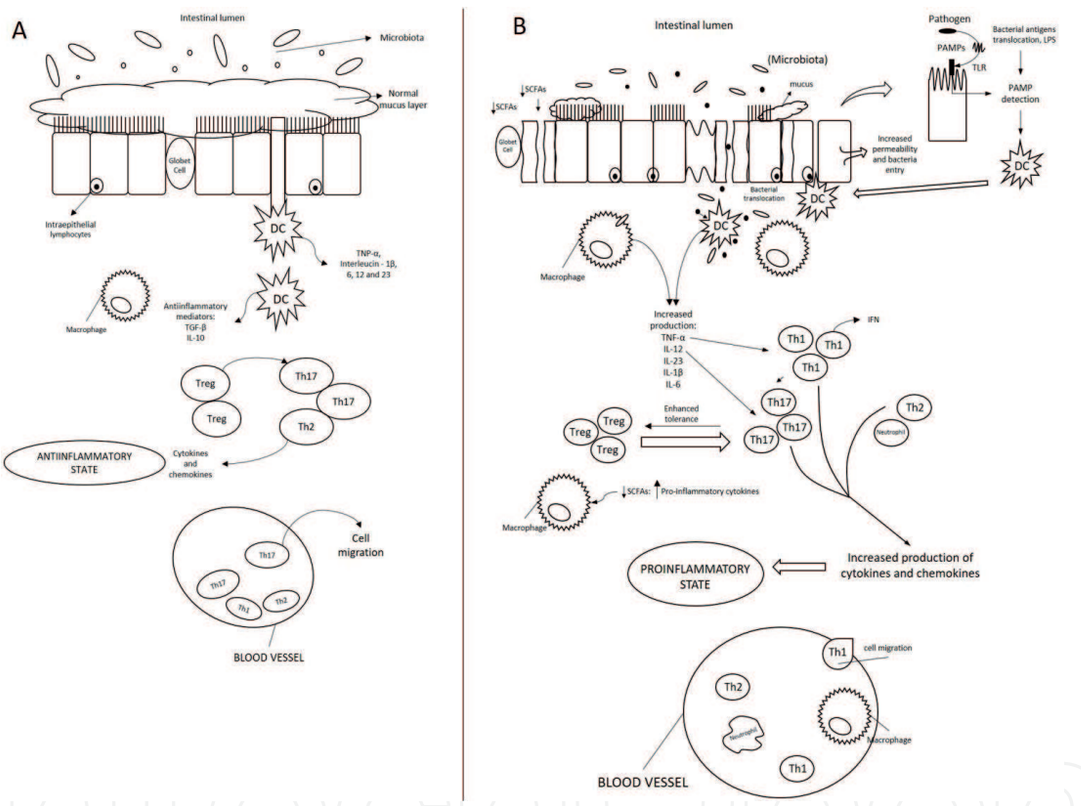

Figure 2.
The gut immune system in healthy and dysbiotic microbiome. (A): In healthy dogs, the lamina propria normally contains immune cells and secreted cytokines. These include anti-inflammatory mediators (transforming growth factor β [TGF-β] and interleukin (IL) -10) that down-regulate immune responses, limit excessive entry of intestinal microbiota and defend against pathogens; and noninflammatory defenses such as phagocytosis by macrophages, that assist in defending against bacteria entering the lamina propria. A homeostatic balance is maintained between regulatory T cells (Treg) and effector T helper cells (Th1, Th2, and Th17). (B): In dogs with gut dysbiosis and secondary gut inflammation, several events contribute to increased bacterial exposure, including mucus layer disruption, dysregulation of epithelial tight junctions, increased intestinal permeability, and increased bacterial adherence to epithelial cells. TLRs initiate the pro-inflammatory stimuli promoting innate local immunity through the recognition of pathogen-associated molecular patterns (PAMPs), present in bacterial antigens, such as lipopolysaccharides (LPS), peptidoglycans, flagella or unmethylated bacterial DNA CpG motifs [72]. The contact of TLRs with PAMPs initiate the innate immune response leading to secretion of cytokines and chemokines and increased expression of adhesion molecules that stimulate and facilitate specialized cells migration responsible for triggering the innate and, subsequently, the adaptive immune response (tumor necrosis factor α (TNF-α), IL-1β, IL-6, IL-12, IL-23, and chemokines) [73]. PAMPs also induce dendritic cells (DCs) maturation that travel to mesenteric lymph nodes and present antigen to naive T cells, which differentiate into Treg and Th17 cells [68]. Tregs also contribute to intestinal homeostasis through the production of immunosuppressive cytokines, such as IL-10. Th17 cells are critical in protecting against bacterial infections because stimulates epithelial cells to secrete anti-microbial proteins and recruit neutrophils from the circulation to the gut microenvironment, resulting in a cycle of inflammation (adapted from Abraham & Cho, 2009 [68]).

In many cases, cancer development is correlated with an inflammatory host response directly to the pathogen (e.g., *Helicobacter pylori* and gastric adenocarcinoma) [74]. But in some cases, cancer progression may be linked to 'sterile' inflammatory causes that are not directly associated with infectious agents, but arising from a response to chronic uncontrolled inflammatory irritation and tissue damage, which adds to the malignant transformation [75]. In these cases, the modulatory roles in cancer development and progression are attributed to commensal or pathogenic agents. It has recently become apparent that commensal community members of microorganisms are crucially involved in tumor-promoting inflammation, which a dysbiotic state stimulates pro-inflammatory properties in the intestinal mucosa [75]. One example is intestinal lymphomagenesis associated to gut microorganism changes in host immune and inflammatory responses affecting lymphocytes [62, 66, 76–78].

The first study for microbiota-induced inflammatory tumorigenesis demonstrated that MyD88-dependent signaling controls the expression of several key modifier genes of intestinal tumorigenesis and has a critical role in cancer progression in mouse model of spontaneous intestinal tumorigenesis and in mice treated with multiple injections of azoxymethane [79]. This revealed that innate immune signaling pathway to intestinal microorganisms is an important factor in intestinal tumorigenesis [79].

It was revealed that mucosal associated invariant T (MAIT) cells from human breast ducts mediate a selective T-helper 17 cell response to human breast carcinoma cells exposed to microbial compounds [80]. This result shows that the presence of bacteria in neoplastic epithelial cells can shape the MAIT cells responses by inflammatory mediators during breast carcinogenesis [80]. Using a mouse model of cutaneous T cell lymphoma (CTCL), it was demonstrated that T cell receptor engagement is critical for the T lymphocytes malignant transformation and that disease progression is also dependent on microbiota [81].

Studies emphasize that inflammatory response to microbial commensal does not occur only in sites of direct contact between the tumor and the microbiota. It was demonstrated an increase of intestinal bacteria translocation associated to inflammation and fibrosis in human chronic liver diseases and also TLR4 activation in non hematopoietic cells in liver carcinogenesis [82]. Thus, there is evidence that intestinal microbiota can affect not only local immunity, but also systemic immune responses.

In veterinary medicine, fecal microbial communities analysis revealed significant lower bacterial diversity and distinct microbial communities in dogs with idiopathic inflammatory bowel disease (IBD) compared to healthy control dogs [30, 83]. This intestinal dysbiosis was correlated with an increase in *E. coli*, a group of particular interest due to its ability to stimulate inflammatory cytokines in human and canine patients with IBD [84–86]. In dogs, the fecal microbiota of patients with intestinal lymphoma, multicentric lymphoma and colorectal tumors showed a significant difference in its composition when compared to clinically healthy dogs microbiota [12, 13, 30]. However, whether these described dysbiotic states play a role in carcinogenesis remains to be determined in dogs.

3.4 Metabolic reprogramming

The metabolome is considered the link between genotypes and phenotypes [87]. It constitutes a set of metabolites synthesized by a biological system, which can be identified by recent "omics" technology called metabolomics, that allows the detection, identification and quantification of intermediate metabolism and, therefore, it can better reflect biological changes in tumorigenesis [88].

Oscillations in microbiota composition induce metabolic changes that can result in host phenotype modifications [89]. Bacterial metabolites production is one of the main signaling pathways between host and its microbiome, and metabolic reprogramming is a central feature of cancer, enabling cells to generate more energy and macromolecules for cancer cell growth, proliferation and division [90].

Microbiome-to-host crosstalk occurs by secreting bacterial metabolites and, after absorption, they enter the circulation and reach the target cells, where they exert their biological effects [91]. Microbial metabolites are detected in peripheral blood (blood metabolome) and feces (fecal metabolome) and have been identified as biomarkers of several diseases, including cancer [92, 93]. The interaction between gut microbiome and fecal and blood metabolome include several mechanisms: a) microbiome can affect gut barrier integrity and alter metabolites absorption (in this case, the same metabolite is associated with a species/pathway in the blood and feces, but the effects directions are opposite); b) direct microbiome-host cell interaction results in host systemic modulation (in this case, the species are associated with blood metabolites, but not fecal metabolites) [94]. In a metagenomic and metabolomic study of 1,004 twins, metabolic pathways were associated with 34% of blood and 95% of fecal metabolites and it was estimated that microbiome was involved in a dialog between 71% of feces and 15% of blood metabolites, highlighting the interaction importance between microbiome and systemic and fecal metabolic environments to identify therapeutic and diagnostic targets [94].

Microbiomes of healthy subjects may share similarities in their metabolic pathways and the fecal metabolome provides a functional readout of microbial activity and can be used as an intermediate phenotype mediating host-microbiome interaction [29, 30, 95]. Zierer and associates (2018) showed that fecal metabolome largely reflects gut microbial composition and fecal metabolic profiling thus is a novel tool to explore links among microbiome composition, host phenotypes, and heritable complex traits [96].

To facilitate understanding, the most investigated bacterial metabolites and enzyme activities can be divided according to the expected effects into more protective or harmful to gut health and carcinogenesis [97].

3.4.1 Protective metabolites (tumor-suppressive metabolites)

3.4.1.1 Short chain fatty acids (SCFAs)

Fermentation of non-digestible carbohydrates from dietary fiber generates SCFAs, such as acetate, butyrate, formate, lactate and propionate [98]. The SCFAs have a key role in gut homeostasis maintenance and epithelial integrity including anti-inflammatory and antiproliferative tumor suppressive effects [98]. Butyrate has been correlated with defense against colon and liver cancer, through its well-known role in regulating inflammation and autophagy [99]. Butyrate production is associated to some Firmicutes, *Eubacterium rectale*, *Roseburia spp.*, *Eubacterium hallii*, *Coprococcus catus, Faecalibacterium prausnitzii* [100]. It is rapidly adsorbed from gut lumen and is preferentially used as an energy source by gut epithelial cells, then its concentration in the systemic circulation is low. Butyrate is fundamental in epigenetic control; once located inside the cell, inhibits activity of histone deacetylases (HDACs) in colonocytes and immune cells, which promotes the hyperacetylation of histones, allowing transcription factors to bind to DNA and genes to be expressed [99]. This has multiple consequences for gene expression and cellular differentiation including: downregulation of pro-inflammatory cytokines (IL-6 and IL-12) in colonic macrophages; induction of differentiation of Treg cells that express transcription factor FOXP3 (crucial role in controlling intestinal inflammation);

and increased acetylation results in higher expression of FOXP3 [99, 101]. As a consequence of HDAC inhibition, butyrate triggers the factor activator protein 1 (AP-1) signaling pathway in the epithelial cell lines that controls cell proliferation and apoptosis [102] (**Figure 3**).

SCFAs modulate several cancer hallmarks, such as cell proliferation, apoptosis and level of expression of certain genes (via inhibition of HDACs), mechanisms that lead to high anticancer activity (**Figure 3**). This protection can affect both stroma and cancer cells, since they have free fatty acid receptors. It was demonstrated that microbial fermentation of high-fiber diet increased concentrations of butyrate in blood and tumor and significantly decreased tumor growth in mouse with lymphoma, suggesting that dietary fiber protects against human lymphoma cancer [104]. A metabolomics-proteomics approach in colorectal cancer provided a mechanistic link between the M2 isoform of a pyruvate kinase (a direct binding target of butyrate) and metabolic remodeling and the antitumorigenic function of butyrate, highlighting an applicable approach to uncovering protein targets for small molecules with biological functions [105].

Studies in veterinary medicine are very scarce. There is one comparative study reporting higher concentrations of β-hydroxybutyrate in blood from dogs with lymphoma than in healthy dogs, but further investigations are essential to understand the significance of this increase [106]. Another research demonstrated that fecal dysbiosis in dogs with acute diarrhea was associated with altered systemic metabolic states, in which concentrations of fecal propionic acid were significantly decreased compared to healthy dogs [107]. In addition, dogs with inflammatory colorectal polyps (ICRP) showed lower amounts of propionic acid and lower proportions of Bifidobacterium compared to feces of control dogs suggesting that the association

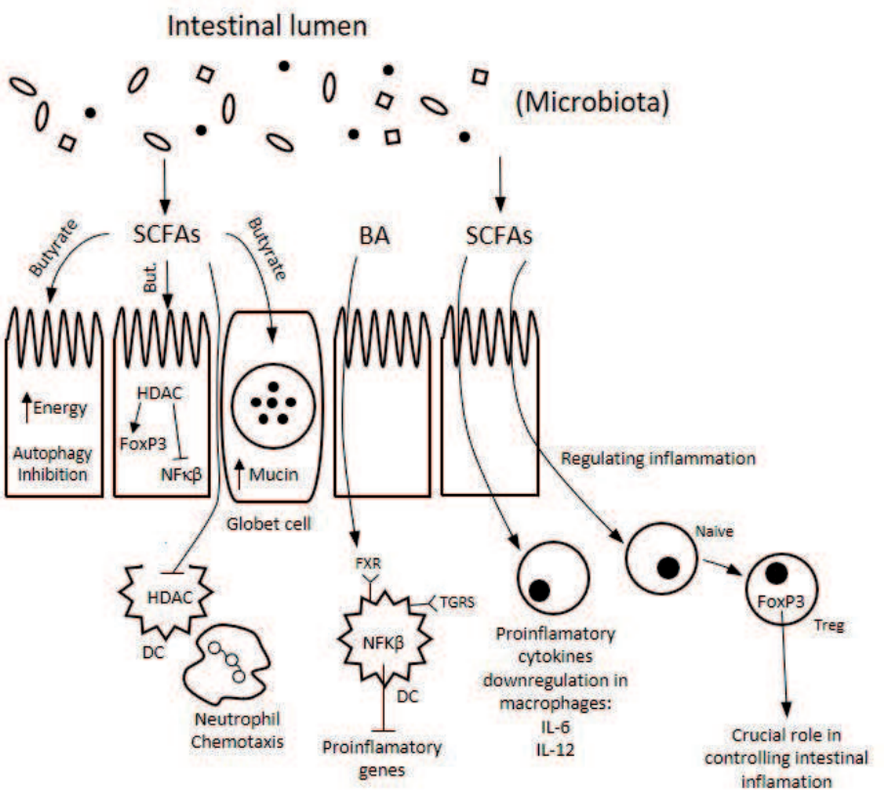

Figure 3.
Modulation of immune signaling through microbial metabolites SCFAs and BA. The metabolic effects directly stimulate the cells of the immune system or are relayed by the intestinal epithelium (adapted from Levy et al., 2019 [103].

between fecal dysbiosis and fecal SCFA concentrations may contribute to ICRP pathogenesis and therapy [108].

3.4.1.2 Phytochemicals

Phytochemicals are bioactive non-nutrient chemical compounds found in fruits, vegetables, grains, and other plant foods, which have biological effects associated with reduced risk of diseases, including cancer [109]. They can be categorized into polyphenols, organosulfur compounds, carotenoids, alkaloids, and nitrogen compounds, but the polyphenols are the most studied ones [109].

Their anti-cancer role includes antioxidant effects, modulation of xenobiotic detoxification pathways and cell proliferation, apoptosis and inflammation [110]. They neutralize reactive oxygen species (ROS) that can damage DNA and predispose to carcinogenesis [97]. A study in human breast cancer cell lines, showed that aqueous extract of the *Pouteria sapota* leaf is rich in phytochemicals with antioxidant properties and significant anti-cancer effects [111]. There is still need for more research and clinical trials in humans and dogs that identify and illustrate the action of phytochemicals.

3.4.2 Harmful metabolite (oncometabolite)

3.4.2.1 Bile acids (BAs)

"Primary" bile acids are synthesized from cholesterol in the liver as cholic acid (CA) and chenodeoxycholic acid (CDCA). When the gallbladder is stimulated after a meal, BA flows into the duodenum and proceeds to the ileum to be actively reabsorbed, returning back to the liver through the portal bloodstream [112]. About 15% of BAs will escape ileum absorption and enter the colon, where the resident microbiota will transform them into secondary BAs (deoxycholic acid, DCA and lithocholic acid, LCA) that have pro and anticancer activity [112]. The enzyme responsible for this conversion is 7α/β hydroxysteroid dehydrogenase (HSDH), and it is produced specially by gram-positive Clostridium species such as *Clostridium scindens* [113].

Quantitative or qualitative BA pool perturbations may greatly affect several BA physiological body functions [113]. The consumption of a high-fat diet changes the gut microbiome and increases the level of DCA, that can promote carcinogenesis in colorectal and liver cancer [114, 115]. Pathways linking BAs to carcinogenesis involve the generation of ROS and reactive nitrogen species (RNS), which cause DNA damage, apoptose and epigenetic changes [112]. Moreover, BAs also exert strong antimicrobial activities, as they damage bacterial cell membranes, contributing to changes in gut microbiota (**Figure 3**). These mechanisms can also be secondary to environmental stimuli (particularly in the context of obesity) and their relationships with human cancer have been recognized as critical in gastrointestinal tract, prostate and breast tissues [116–118].

There are some publications covering changes in the fecal BA profile in canine chronic inflammatory enteropathy and extrahepatic congenital portosystemic shunts, but not in carcinogenesis [119–121].

4. Gut microbiome and therapeutic application

The growing understanding of the microbiome's role in carcinogenesis has allowed the microbiome influence to be linked to the effectiveness of cancer therapies. Microbiome modulation strategies can affect cancer treatment through

inactivation or activation of chemotherapeutic agents, modification of immune responses and interference with side effects [72]. This relationship is bilateral, in which the systemic cancer therapy influences gut microbiota, and gut microbiota influences cancer treatment [122]. Recent publications indicate the gut microbiome manipulation as a new treatment tool or to improve the response to cancer therapy. Some of the proposed mechanisms will be discussed below.

4.1 Roles of microbiome in cancer therapy

4.1.1 Chemotherapy

Iida et al. (2013) demonstrated that microbiota impairs disruption response of subcutaneous tumors to platinum derived chemotherapeutic agents. Tumor-bearing mice that lacked microbiota showed therapy efficacy reduction, given that microbiota was important for activating the innate immune response [123]. In another study, administration of *Ruminococcus gnavus* (bacterial strain depleted by treatment with cisplatin) was able to partially restore intestinal mucosa integrity and reduce systemic inflammation in mice treated with cisplatin [124]. Results indicate that reconstitution of gut microbiome can help healing intestinal epithelium in patients treated with chemotherapy.

On the other hand, Viaud et al. (2013) demonstrated that gut microbiota helps shape anti-cancer immune response of cyclophosphamide (CTX). Using mouse models, it was demonstrated that cyclophosphamide alters intestine microbiota composition and induces translocation of selected species into secondary lymphoid organs, resulting in Th17 cells maturation promoting an adaptive immune response against tumors [125]. Daillere et al. (2016) identified *Enterococcus hirae* and *Barnesiella intestinihominis* species involved in tumor immunosurveillance during cyclophosphamide therapy; *E. hirae* translocates from gut to lymph nodes inducing Th1 and Th17 responses mandatory for anti-tumor activity of CTX, while *B. intestinihominis* increases systemic Th1 and CD8 + cytotoxic T cells, which were associated with an increase of IFN-y-producing γ δ tumor infiltrating-lymphocytes (TILs) contributing also for anti-tumor CTX effect [126]. Therefore, cyclophosphamide immunomodulatory effects require a functional microbiome.

Chemotherapy efficacy can also be impacted by intratumoral bacteria. Geller et al. (2017) showed, in a colon cancer mouse model, that Gammaproteobacteria can metabolize chemotherapeutic gemcitabine into an inactive form inducing chemotherapy resistance and that this effect was reversed by antibiotic ciprofloxacin. Interestingly, about 76% of human pancreatic ductal adenocarcinomas were positive for bacteria, mainly Gammaproteobacteria [127]. Perhaps the treatment for this tumor type may be improved by adding antibiotics to the chemotherapy.

Chemotherapy-induced diarrhea (CD) is a frequent adverse event in dogs, in which changes in gut microbiota appear to play a key role. A recent study of 60 dogs undergoing chemotherapy supported the administration of smectite, a natural medical clay, widely used in acute diarrhea treatment in humans, as a first-line treatment of CD in dogs. Interestingly, smectite has anti-inflammatory properties to decrease intestinal bacterial translocation and stabilize intestinal microbiome [128]. However, studies associating microbiome and effectiveness of chemotherapy are still scarce in veterinary medicine.

4.1.2 Immunotherapy

Several studies have shown a complex crosstalk between bacteria and immune host response in the anti-tumor battle. For example, Paulos et al. (2007) reported

that total body irradiated mice showed a more efficient anti-tumor response to adoptively transferred tumor-specific CD8+ T cells against melanoma after gut microbial translocation to mesenteric lymph nodes. They observed that the radiation induced the release of microbial LPS and activated innate immune response by TLR4 stimulation and then increased anti-tumor CD8+ T cells, while reduction of host microflora using antibiotics, neutralization of serum LPS using polymyxin B, or removal of LPS signaling were associated with a decrease of anti-tumor response [129].

There is also evidence that gut microbiome modulates efficacy of immune checkpoint inhibitors (CIs), that are monoclonal antibodies with inhibitory effect to specific receptors on T cells and tumor cells, blocking signaling pathways that negatively modulate immune system, allowing specific T cells to promote destruction of cancer cells [130]. Those receptors include cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), programmed death 1 protein (PD-1), and programmed death-ligand 1 (PD-L1) [131].

It was demonstrated that oral administration of Bifidobacterium in mice with melanoma was associated with the same degree of antitumor effects as in those mice that received therapy with PD-L1 antibodies, and the combination of both treatments almost abolished tumor growth [132]. In addition, a report of human gut microbiome metagenomic profiling in 39 metastatic melanoma patients treated with anti-PD1 and/or anti-CTLA-4 immunotherapy identified that those who respond to all types of CIs were enriched for *Bacteroides caccae*, enhancing that microbiota may modulate cancer immunotherapy [133]. These correlations have not yet been demonstrated in dogs.

4.2 Therapeutic manipulation of microbiome and its relevance to cancer therapies response

Given the increasing evidence on the significant role that microbiome can play in cancer, microbiota modulation represents a new therapeutic potential capable of altering disease development. These therapies aim to change the microbial community associated with dysbiosis for those associated with health. In small animals, microbiome manipulations are often described as part of gastrointestinal diseases treatment [134]. Mainly as an adjuvant treatment for cancer, these interventions and their effectiveness are not well established and have only recently been described in literature. The following will discuss some ways in which microbiome can be modified:

4.2.1 Prebiotics and symbiotics

Prebiotics are specific chemicals, capable of promoting growth of a selective group of bacteria and their specific metabolites and thus modulating microbiota in a beneficial way, which may help on anti-tumor treatment [135]. These are non-digestible or absorbable dietary fibers and include fructans (oligofructose and inulin), nonstarch polysaccharides found in some cereal grains, algae, disaccharides (lactulose), and polysaccharides including fructooligosaccharides (FOS) [22].

According to Villegér and colleagues, the effect of prebiotics depends on the presence of beneficial bacteria in the host's intestines [33]. Thus, the combination of probiotics and prebiotics, known as symbiotic, looks promising. Dietary treatment with inulin or oligofructose has been demonstrated to selectively stimulate growth of specific bacterial taxa and alter SCFA levels within the gut [136]. Moreover, these prebiotics reduced the incidence of mammary tumors in rats, significantly potentiated chemotherapy effects as well as RT [136]. The perioperative administration of symbiotics, probiotics (strains Lactobacillus and Bifidobacterium) and prebiotic

(fructooligosaccharides), reduced postoperative mortality and complication rates in cancer patients undergoing surgery [137].

The effects of prebiotics were evaluated in dogs, but without focusing on the benefits of cancer treatment. A recent study evaluated the effects of prebiotics in different concentrations in healthy adult dogs and concluded that the galactooligosaccharide prebiotic at 1.0% improved the immunity of healthy dogs [138]. Inulin intervention resulted in a modulation of intestinal bacteria, increase of fecal SCFA and BA in dogs. Given that some studies showed similar dysbiotic states between dogs and humans with cancer [12, 13, 37], it seems relevant that the new approaches to increase anticancer therapy efficiency should include the potential benefits of prebiotic supplementation for both dogs and humans.

4.2.2 Probiotics

Probiotics refers to live bacteria that can be orally administered and confer health beneficial when delivered in adequate amounts [139]. Probiotics colonize the gut temporarily and act modifying colonic environment. Different mechanisms are involved in probiotics protective role: increase in barrier function, epithelial tight junctions integrity, immune response modulation, anti-inflammatory cytokines production, pathogenic bacteria growth inhibition by antimicrobial and antitoxin compound production (i.e. SCFA), and production of enzymatic activities and/or beneficial metabolites to the host [140]. A recent systematic review and meta-analysis investigated probiotics efficacy and safety in patients diagnosed with cancer and concluded that probiotics may be beneficial but further studies are still required [141].

The strains of Lactobacillus and Bifidobacterium are most frequently reported in studies with probiotics. The "protective" effect against colorectal cancer was demonstrated after oral supplement containing *Lactobacillus helveticus* in mice with colonic cancer, in which tumor growth rate and degree of hyperplasia were reduced [142]. These effects were secondary to suppression of NF-κB, increased of anti-inflammatory IL-10 and decreased IL-17-producing T cells [142]. In addition, administration of *L. acidophilus* in mice with breast tumors reduced tumor growth due to altered cytokine production and, in a murine melanoma model, the therapy with aerosolized *L. rhamnosus* promoted immunity against lung metastases, identifying a role for a probiotic cancer "preventing" [143, 144].

Probiotics can also affect patient "outcomes". In a prospective randomized study, after transurethral resection of bladder cancer, the group of patients who received oral supplementation with *L. casei* associated with intravesical epirubicin application had a 3-year recurrence-free survival rate significantly higher than in the isolated chemotherapy group [145]. In addition, some studies demonstrate the action of probiotics on treatment-related toxicity. *L. rhamnosus* decreased diarrhea and abdominal discomfort in patients with colon cancer treated with 5-fluorouracil chemotherapy [146]. Symbiotics (a combination of *Bifidobacterium breve* and *L. casei*) during neoadjuvant chemotherapy in esophageal cancer patients reduced the occurrence of adverse events (diarrhea, neutropenia and lymphopenia) [147].

However, caution should be exercised in their use, since the composition of commercially available probiotics have been inadequately studied, as well as their long-term impact on intestinal microbiota and general health [135]. When investigating the use of pre- and probiotics in dogs, scientific evidence of their benefit is scarce, especially in cancer. Furthermore, the knowledge about appropriate doses and compositions is small in companion animals [148]. Studies suggest that they may have beneficial effects on canine IBD. In a prospective randomized study, 34 IBD dogs received prednisone with or without multi-strain probiotic. Both treatments increased the numbers of total bacteria and were associated with rapid

clinical remission but not improvement in histopathologic inflammation [149]. A protective effect of multi-strain probiotic (strains of Lactobacillus, Bifidobacterium and Streptococcus) was also observed in dogs with IBD compared with a control group (treated with metronidazole and prednisolone), with a significant decrease in clinical and histological scores [150].

A recent study showed that probiotics consumption (*L. casei*, *L. plantarum* and Bifidobacterium) in healthy dogs of different age groups, significantly increased beneficial intestinal bacteria (Lactobacillus and *Faecalibacterium prausnitzii*) and decreased potentially harmful bacteria (*E. coli* and *Sutterella stercoricanisin*) mostly in elderly dogs, suggesting that probiotic treatment improves host health and immunity [151].

4.2.3 Fecal microbiome transplantation (FMT)

In FMT, feces are transferred from a healthy donor to the intestinal tract of a diseased recipient [30]. FMT may be delivered via colonoscopy, enema or oral administration, with equal clinical efficacy [152]. The beneficial mechanisms of FMT are still unknown. Nowadays, FMT has been used in resistant *Clostridium difficile* treatment with high response rates [153]. Contrary to gastrointestinal (GI) diseases, application of FMT in cancer is still limited and data was obtained mainly in animal models. The reconstitution of germ-free mice with fecal material from patients with melanoma responsive to anti-PD-L1 and to anti-PD-1 therapies led to better tumor control in contrast to those that received faces from unresponsive patients [154, 155].

The use of FMT in veterinary medicine was studied mostly in dogs with GI diseases, such as in parvovirus-infected puppies and patients with diarrhea due to IBD and *C. perfringens*, and it was associated with faster resolution of clinical signs [156]. For a deep learning regarding the FMT effects in veterinary non-oncological diseases and the potential applications of FMT in animals, including therapeutic, prophylactic and immunogenic uses, the reader may consult Niederwerder (2018) publication [157].

5. Conclusions

Host-microbiota interactions are crucial in human and animal health and disease development, yet microbiota function and dynamics during disease states are only partially understood. There is growing evidence supporting that immunoregulatory and anti-inflammatory effects of gut and tumor microbiota are essential in the battle of cancer. However, most studies were performed in preclinical models, which have many pitfalls in regard of spontaneous cancer research urging the need for clinical studies benefiting both species.

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Conflict of interest

The authors declare no conflict of interest.

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