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Corruption of Bacterial-Host Homeostasis as a Potential Risk Factor and Biomarker for Upper Gastrointestinal Carcinogenesis

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Summary

Human resident microbial communities have received increased attention in the context of health and disease because a loss of balance in microbial homeostasis can contribute to disease. While the gut microbiome-host interactions are well studied for their roles in inflammatory bowel disease and colon cancers, little is known about the causative influence of bacteria on upper gastrointestinal tract (UGT) tumorigenesis, including the oral cavity, pharynx, esophagus, and stomach. Risk factors linked to upper gastrointestinal carcinogenesis, such as cigarette smoking, alcohol consumption, poor oral hygiene, and gastroesophageal reflux disease, disrupt the bacterial homeostasis and open a niche for pathogenic bacteria. We present mechanisms including chronic inflammation, disruption of cell signaling, and production of environmental metabolites that help explain how the pathogens Fusobacterium nucleatum, Porphyromonas gingivalis, and Campylobacter concisus, among others, can promote carcinogenesis. We provide examples of bacterial species that could be having a protective role in tumorigenesis, a research area that is less explored. Additionally, we discuss the limitations and challenges during patient sampling and screening, which need to be overcome to help characterize microbiomes associated with UGT cancers. Overall, this review presents an emerging model of synergy and discord of bacteria-host relationships in the UGT.

Introduction

The human microbiome is a diverse network of microorganisms with complex relationships to the human body[1-3]. In this network, the interactions between the host and microorganisms can be categorized into commensalism, mutualism, and parasitism[4]. The commensal population is the principal colonizer and has a protective role against pathogens[3]. Commensals and mutualistic bacteria do not impair the host, and some are beneficial for the host; therefore, they do not activate strong defensive immune reactions. One primary way to attain this balanced state of "immune tolerance" is to maintain an intact epithelial barrier and other components that separate the microbes from the host cells[4]. On the other hand, parasitic or pathogenic bacteria can induce strong inflammatory responses that can harm the host. The loss of bacterial-host homeostasis (dysbiosis) can cause an imbalance, leading to chronic inflammation, which is detrimental to the host[4-6].

Specific bacterial species being enriched or diminished does not prove their direct involvement in cancer development or progression[7]. Nevertheless, microbial changes have been associated with ~25% of cancer cases in developing countries and with ~8% of cancer cases in developed countries[8]. Bacteria and viruses can contribute to tumorigenesis by promoting cancer directly or

indirectly by working synergistically with other risk factors, thereby causing deleterious alterations in physiological host processes[7,8]. Regardless of its role, changes in bacterial abundance have been proposed to be used as a biomarker for early detection of cancer[9].

Tjalsma et al. proposed a driver-passenger bacterial model to explain the possible roles of bacterial strains in the development and progression of cancer[10]. This model proposes that bacterial *drivers* induce DNA damage, contributing to an accumulation of mutations and promoting cancer *initiation*. On the other hand, *passengers* are usually opportunistic pathogenic bacteria with the ability to out-compete the *drivers* and support cancer *progression*[10]. Furthermore, Garrett et al. propose three broad mechanisms through which bacteria could contribute to carcinogenesis. These include the alteration of signaling pathways involved in carcinogenesis, the induction of a chronic inflammatory response, and the microbe metabolization of host- and xenobiotic-factors into oncometabolites (**Figure 1**)[7].

While the classic pathogen theory postulated by Koch proposed that the presence of a specific pathogen can be the cause of a disease[11], a more recent variation of Koch's postulate suggests that entire communities of pathogens or general changes in microbial homeostasis can cause disease[12]. Population-based studies have shown significant differences in the bacterial communities of healthy individuals compared to cancer patients. And while bacterial communities may be the ones driving carcinogenesis, the role of single pathogens needs to be elucidated before broader conclusions can be made.

The upper gastrointestinal tract begins with the oral cavity, anatomically connected to the pharynx (throat) and the esophagus[13,14]. This review will provide examples of bacteria involved in upper gastrointestinal carcinogenesis, focused primarily on the anatomical locations of the oral cavity, the esophagus, and the stomach.

Head and neck cancer locations, epidemiology, and risk factors

Head-and-neck squamous cell carcinoma (HNSCC) comprises 4.9% of cancers[15]. Head and neck locations include the oral cavity, larynx, pharynx, and salivary glands[13]. Cancer in the oral cavity comprises 40% of all HNSCC, including tumors in the lips, tongue, gingiva, cheek

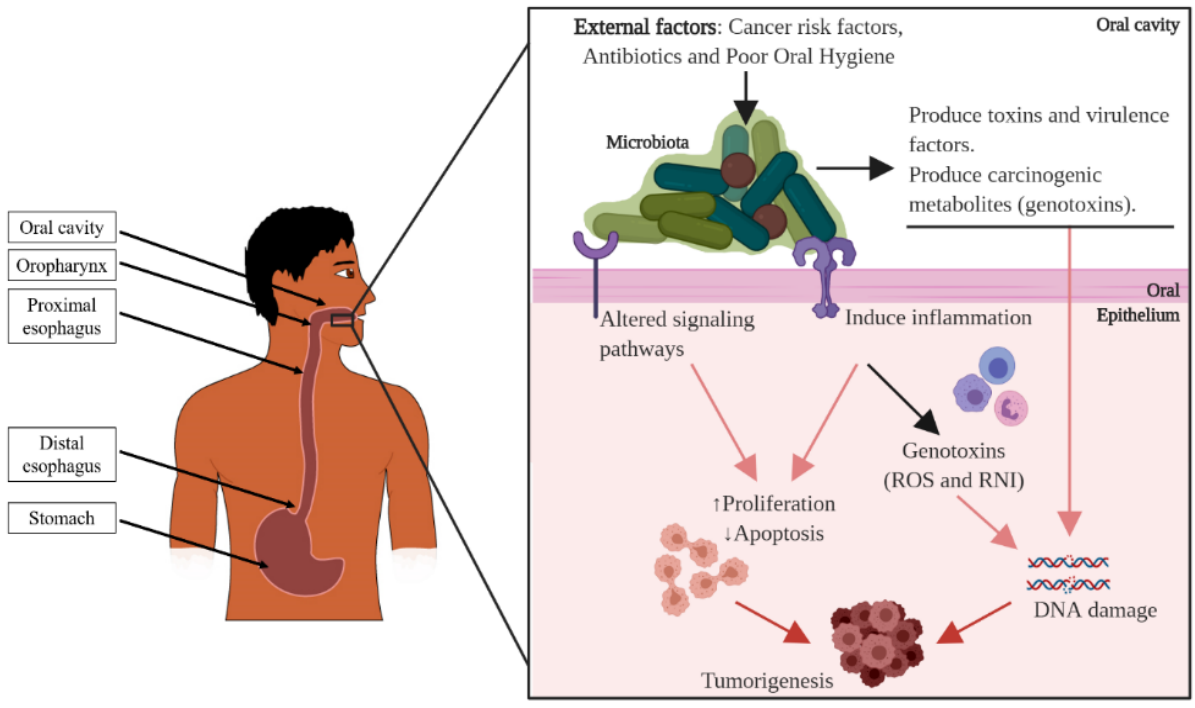

Figure 1. Dysregulated host-microbiome interaction. Changes in the environment, including exposure to risk factors (e.g. tobacco use[24] and alcohol consumption[27]), poor oral hygiene, [18,30] and antibiotic[6,93] use can cause dysbiosis. Dysbiosis is a loss in the host-bacterial homeostasis, which can elicit an inflammatory response and alter signaling pathways in the host. [4–6] The altered signaling pathways, including inflammatory pathways, promote an increase in survival and proliferation, and a decrease in apoptosis. [28] Additionally, inflammatory cells recruited to the area can produce reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) to kill the bacteria. These reactive species are a double-edged sword since they can also induce DNA damage to the host-cells. [6,7,45] Changes in the environment could induce changes in the expression of bacterial toxins and virulence factors; they can also open a niche for specific bacteria that metabolizes host and xenobiotic factors into carcinogenic metabolites. All of these factors have the capacity to induce DNA damage. [7]

mucosa (buccal mucosa), the floor of the mouth, and palate. Cancer in the pharynx comprises 34% of all the HNSCC cases, and this anatomical area includes the nasopharynx (15% of cases), oropharynx (base of the tongue) (10% of cases), and hypopharynx (adjacent to the esophagus) (9% of cases). Finally, laryngeal cancer accounts for 20% of all HNSCC cases[16]. Overall, head and neck cancers account for almost 900,000 cases in the world yearly[16], and an alarming 5-year survival rate that remains around 40%[15]. While their prognoses are similar, every location for HNSCC has different etiologies and major risk factors that need to be considered and explored individually.

Two common and well-established risk factors for all head and neck cancers include tobacco use and alcohol consumption[17]. Human papillomavirus infection (HPV) has been established as a major risk factor for 72% of oropharyngeal cancer cases[18,19], while it is only attributed to 3% of oral cancers[20]. The etiology of many oral squamous cell carcinoma (OSCC) cases remains unknown[18,21,22].

Bacterial Relationship with HNSCC Risk Factors and Bacteria as a Potential Independent Risk Factor

Bacteria can work synergistically with other risk factors and accelerate the tumorigenesis process (**Figure 2**)[23]. Cigarette smoke causes dysbiosis (**Table 2**) by affecting the survival of specific bacteria[24], thereby decreasing the abundance of normal commensals, and consequently leaving an open niche for the growth of pathogens[25]. It also enhances bacterial binding to oral epithelial cell surfaces, accounting for a further increase in pathogenic bacterial colonization[24]. Additionally, it creates a selectively acidic and often anaerobic environment, favoring anaerobic glycolysis over aerobic pathways, which contributes to alterations of the oral microbiome[25,26]. While the bacterial communities of smokers and non-smokers show marked differences, bacterial communities of former smokers and non-smokers are highly similar, suggesting that these bacterial changes are reversible and transient[25]. Nevertheless, the DNA damage caused by the chronic inflammation induced during dysbiosis can still accumulate, promoting tumorigenesis.

Alcohol consumption has been shown to affect the bacterial composition in the oral cavity[27]. A study reported that heavy drinking is associated with an enrichment of *Neisseria, Actinomyces, Leptotrichia*, and *Cardiobacterium*. Also, strains of *Streptococci*, yeast, and communities of

Table 1. Epidemiology of Head and Neck Cancers. Head and neck cancers comprise 4.9% of cases worldwide. Head and Neck Cancers include tumors in the oral cavity, larynx, pharynx (naso-, oro-, and hypo-) and salivary glands. [16]

Types of HNSCC	Incidence of all HNSCC (%)	Mortality (%)
HNSCC general	4.9	51.1
Oral cavity: **lip, tongue, gingiva, check mucosa, floor of mouth and palate**	40	50
Larynx	20	53
Nasopharynx	15	57
Oropharynx	10	55
Hypopharynx	9	43
Salivary glands	6	42

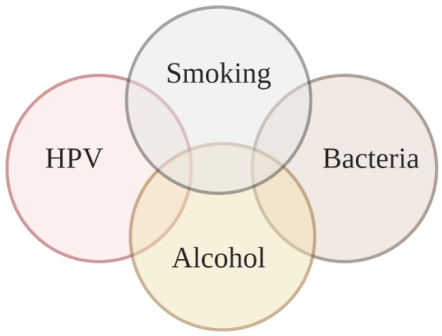

Figure 2. Head and Neck Cancer Risk Factors Overlap. Bacteria can be working synergistically with other risk factors, while it could also be an independent risk factor. [23]

Table 2. Changes in bacterial abundance found in smokers compared to non-smokers.

	General differences in smokers compared to non-smokers	Taxa differences in smokers compared to non-smokers	
Oral cavity (in general)	↑bacteria implicated in periodontitis [98] ↓α-diversity [98]	↑Parvimonas ↑Fusobacterium ↑Bacteroides ↑Prophyromonas ↑*Campylobacter* [24]	
Dental plaque and subgingival plaque	↑anaerobes [25] ↓aerobes [25] ↑bacteria implicated in periodontitis [24]	↑Parvimonas ↑Fusobacterium ↑Bacteroides ↑Campylobacter ↑Dialister ↑Treponema	↓*Veillonella* ↓*Neisseria* ↓*Streptococcus* ↓*Capnocytophaga* [24]
Oropharynx		↑Megasphaera ↑Veilonella	↓Capnocytophaga ↓*Fusobacterium* ↓*Neisseria*[24]

Neisseria contain high levels of alcohol dehydrogenases (ADH)[28], which are secreted by both the human epithelium and oral bacteria[1]. ADH metabolizes ethanol into hydroxyl ethyl radicals and acetaldehyde metabolites that have been shown to promote tumor formation by inducing mutations in the oral epithelial cells[28,29]. Therefore, the role of these bacteria in tumorigenesis needs to be further investigated through *in vitro* and *in vivo* studies.

Poor oral hygiene leads to oral and gum diseases, including periodontitis and gingivitis, which are known to involve bacterial changes that could potentially be an independent risk factor for head and neck cancer[18,30]. While tobacco use and alcohol consumption are known to increase the risk of developing periodontal disease[27,31], poor oral hygiene can be an independent risk factor for gum disease and oral cancer[30,32]. Similarly, good oral hygiene practices, including toothbrushing, visiting the dentist, and no denture use, are associated with a lower risk of head and neck cancer[30].

Periodontitis is an inflammatory gum disease prevalent in ~30% of adults[33], and strongly associated with the enrichment of Gram-negative bacteria[32]. The bacteria associated with periodontal disease are grouped in two complexes. The Red complex, which includes *P. gingivalis, T forsythia*, *and T. denticola*; and the Orange complex, which includes *F. nucleatum, F. periodonticum, Peptostreptococcus micros, Prevotella intermedia,* and *Prevotela nigrescens*[34]. Periodontitis has been identified as a risk factor for HNSCC[32] and for oral leukoplakia, which is a premalignant lesion prevalent in 1.1-3.6% of the population and a precursor for oral cancer[35]. More specifically, leukoplakia has a 1.58-27% probability of transforming into OSCC,[36] which affects 1.96% of the population worldwide[16].

Ganly et al. compared the bacterial communities from healthy individuals, patients with leukoplakia, and OSCC patients[18]. Along with this disease progression, they observed a progressive increase of various periodontal pathogens (*Fusobacterium, Prevotella, Alloprevotella, and Veillonella*) accompanied by a decrease in *Streptococcus* commensals[18]. Upon a quantitative correlation analysis, it was concluded that periodontal pathogens collaborated among themselves, as did non-pathogens, while periodontal pathogens and non-pathogens inhibited each other[18]. Finally, they also observed an increased expression of proinflammatory pathway markers along with disease progression[18]. Since the patients included in this study were non-smokers and were HPV-negative, their findings suggest that poor oral hygiene and the consequential enrichment of periodontal pathogens can be considered independent risk factors for OSCC[18].

Mechanisms Proposed for *Fusobacterium nucleatum* Role in Tumorigenesis

Fusobacterium nucleatum is enriched in OSCC cancer patients compared to healthy individuals, and it is recognized as a driver of oral cancer. A newly-established murine model of periodontitis-associated oral tumorigenesis identified that chronic bacterial infection with the periodontal pathogens *F. nucleatum* and *Porphyromonas gingivalis* promoted oral tumorigenesis[37]. Whether *F. nucleatum* is a commensal bacteria, a pathogen, or an opportunistic pathogen, is still under debate[38], even though this bacteria is one of the most studied in the context of tumorigenesis (**Figure 3**). In colorectal cancer patients, *F. nucleatum* has been associated with tumor stage, poor prognosis, shorter survival, and higher cancer-specific mortality[39].

Known interactions between *F. nucleatum* and host epithelial cells facilitate adhesion, induce inflammation, and promote proliferation, cell growth, invasion, and metastasis[38,40,41]. *F. nucleatum* can cause an inflammatory response and affect host signaling pathways through various mechanisms. The presence of *F. nucleatum* activates the IL6-STAT3 signaling pathway after a direct interaction with oral epithelial cells through Toll-like receptors[37]. The transcription factor, STAT3, enhances cell survival and proliferation, cell migration, and oncogenic transformation of epithelial cells[42]. *F. nucleatum* also induces inflammatory cells in the tumor microenvironment to secrete cytokines, including IL17F, IL22, and CCL20 (MIP3a)[38]. The chemokine CCL20 has been linked to cancer development and progression due to its ability to promote proliferation and migration of cancer cells, and it also induces T_{reg} lymphocyte migration[39].

An imbalance of *F. nucleatum* enrichment in relation to the decreased abundance of *Streptococcus* and other species has been suggested to promote oral cancer early on[43]. A potential mechanism was proposed, as the healthy oral community *Streptococcus, spp* can suppress *F. nucleatum*-induced IL8 and NFkB signaling[43]. This finding highlights the delicate interaction of signaling crosstalk, not just between one bacterial species with the host but also among the different members of the bacterial community. Furthermore, NFkB is a transcription factor that mediates an inflammatory response by promoting the expression of other inflammatory cytokines. Additionally, it can also regulate cell survival, apoptosis, and proliferation. Therefore, its dysregulation plays a crucial role in carcinogenesis[44]. Chronic inflammation can also result in the production of free radicals released as reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), and antimicrobials to combat the infection[6,7,45]. These processes can lead to DNA damage and genome instability in the host cells as an initiating step to tumorigenesis[45].

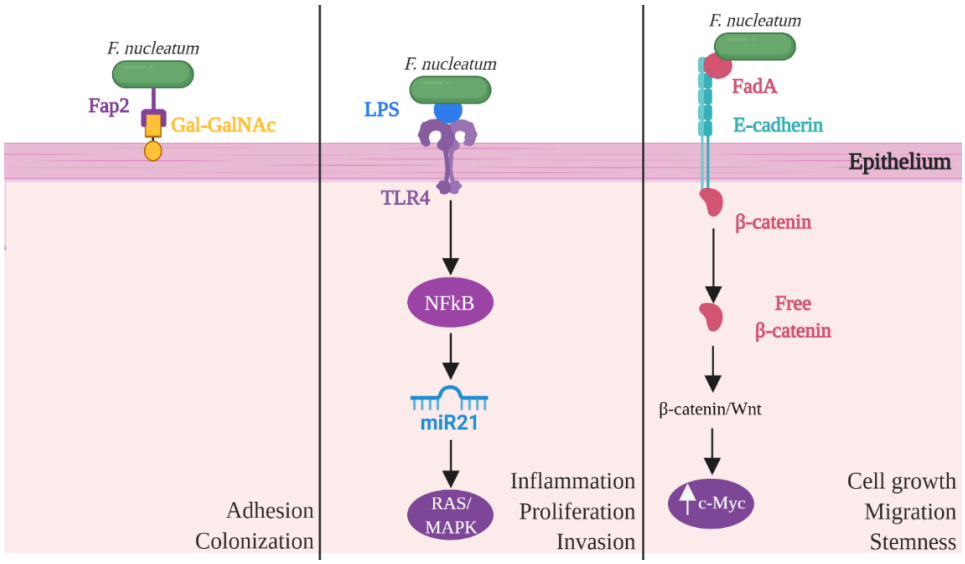

Figure 3. Known interactions of *F. nucleatum* with host epithelial cells. 1) The virulence factor Fap2 (fusobacterium autotransporter protein 2) interacts with Gal-GalNAc, which is overexpressed in colorectal carcinoma cells (CRC). [40] This interaction facilitates the adhesion to the host cell, which could explain why cancer patients have a higher abundance of *F. nucleatum* compared to healthy patients (left panel). 2) Since *F. nucleatum* is a Gram-negative bacterium, it also expresses lipopolysaccharide (LPS) in its outer membrane. LPS interacts with a member of the toll-like receptor family, TLR4, inducing an inflammatory response that promotes the expression of miRNA21 and activates the RAS/MAPK signaling pathway as a result. The RAS/MAPK pathway activation induces host-cell proliferation and invasion (middle panel). [38] 3) Another *F. nucleatum* virulence factor involved in the regulation of cell signaling is the adhesin FadA (adhesin A), which allows the bacteria to bind to E-cadherin. The interaction with E-cadherin frees β-catenin[97] and helps activate the Wnt/β-catenin pathway. [41] Then β-catenin translocates into the nucleus, where it promotes the transcription of the proto-oncogene c-Myc. [41] The Wnt/β-catenin signaling pathway also regulates cell growth, polarity (migration), and stemness. [7] (right panel). 4) More impactful, *F. nucleatum* induces inflammatory cells in the tumor microenvironment to secrete cytokines, including IL17F, IL22, and CCL20 (MIP3a). [38]

Mechanisms Proposed for *Porphyromonas gingivalis* Role in Tumorigenesis

Porphyromonas gingivalis is a periodontal pathogen[34,46]; it is associated with oral cancer [46,47] and has been suggested as an etiological factor for OSCC[48]. To elucidate the role of *P. gingivalis* in the progression from chronic periodontitis to oral cancer, *in vitro* studies provide insight into the mechanisms leading to malignant transformation[49].

P. gingivalis IgG, along with the inflammatory cytokine IL6, has been detected at higher levels in OSCC patients compared to controls[46]. Exposure of oral immortalized epithelial cells and cancer cells to *P. gingivalis* induced an increase of several inflammatory players, including NFkB and TLRs[50], and enhanced the expression of the inflammatory cytokine TNFα[50,51]. Another study using various OSCC cell lines reported a rise in IL8 expression upon 48-hour exposure of *P. gingivalis*[49]. These responses to *P. gingivalis* could lead to the recruitment of inflammatory cells that can contribute to DNA damage[7]. Some cytokines, like TNFα and TGFβ, can also be involved in triggering epithelial-mesenchymal transition (EMT), which is associated with tumor initiation, primary tumor growth, cancer invasion, and metastasis, independently or in synergy with other players[51].

Long-term infection of oral immortalized cells with *P. gingivalis* increased proliferation and augmented S phase (DNA replication phase) length[50]. *P. gingivalis* also produces proteins and lipopolysaccharides that stimulated the proliferation of human fibroblasts *in vitro* by upregulating cyclins and activating cyclin-dependent kinases[1,29]. Furthermore, various studies have shown *P. gingivalis* can induce EMT in oral epithelial cells. EMT is a transformation process that involves changes in cell morphology, increase in self-renewal, motility, migration, and invasion, all contributing to tumorigenesis[52,53]. Exposure of oral primary cells to *P. gingivalis* induced an increase in the expression of Vimentin, a well-known mesenchymal marker, concomitant with a decrease in E-cadherin expression, an epithelial marker[48]. This study also identified an increase in the expression of various EMT transcription factors, including Slug, Snail, and Zeb1[48]. Using human telomerase immortalized gingival keratinocytes, it has been demonstrated that exposure to *P. gingivalis* upregulates the expression of ZEB2, another EMT transcription factor[52]. Additionally, *P. gingivalis* infection has been shown to increase the expression and activation of various metalloproteases that contribute to invasion, including MMP-1[49], MMP-2[48,49], MMP-7[48], and MMP-9[48,50].

Overall, the functional mechanisms that drive *P. gingivalis* to induce certain phenotypes and signaling pathways remain to be elucidated. Nevertheless, these *in vitro* studies are stepping stones towards a better understanding of *P. gingivalis* role in periodontitis and cancer.

Bacteria as a Biomarkers for Earlier Detection of Head and Neck Cancers

Head and neck cancer has a 5-year survival rate of 40% due to its late detection[15]. Specifically, OSCC does not produce any pain during its early stages, leading to late diagnoses that have a 19% 5-year survival rate compared to a 78% 5-year survival rate at early-stage diagnosis. The tumor stage at the time of diagnosis affects the survival rates, prognosis, patient discomfort, therapeutic intervention for treatment, and the recurrence of the disease[9]. There is an urgent need for tools and biomarkers that help achieve an early diagnosis[54].

Biomarkers are indicators of normal and pathogenic processes. These can include proteins, RNA, DNA, lipids, metabolites, antibodies, and microbes[9]. Alterations in the concentration or function of biomarkers can be associated with the development and progression of a disease[55]. The characteristics of ideal biomarkers include non-invasive, efficient, cost-effective, and accurate detection. Current biomarkers require invasive and painful methods, including biopsies and blood draws. On the other hand, salivary-based biomarkers are promising since collecting saliva is a non-invasive, time-saving, and cost-effective process[9]. While microbial biomarkers do not have to be the cause of disease, they have to be associated with it either as a bystander or a result of the disease[9]. A healthy microbial panel should be established to be able to compare it to the disease-associated microbial panel[55]. The eradication of the disease-associated microbial biomarkers should then correlate with an improvement of the patient's health[55].

Many studies have shown differences in bacterial abundance between healthy controls, patients with leukoplakia, and head and neck cancer patients (**Table 3**). Various bacterial marker panels associated with head and neck cancer have been identified and suggested as bacterial biomarkers (**Table 4**)[56–60]. Once these potential biomarkers are validated, clinically evaluated, and approved, they could be used in clinical assays. The use of salivary biomarkers holds promise to positively affect the survival rates for head and neck cancer patients.

Esophageal Cancer Locations, Epidemiology, Risk Factors, and Bacterial Relationship to Main Cancer Risk Factors

Esophageal cancer has an incidence of 3.2% and a mortality of 89% worldwide[16]. The most prevalent type of esophageal cancer worldwide is esophageal squamous cell carcinoma (ESCC), which develops in the proximal esophagus, closer to the oral cavity. Despite multimodal forms of treatment, the prognosis remains poor[61]. Similar to HNSCC, smoking and alcohol consumption are ESCC most important risk factors[62].

Patients with ESCC, or the pre-cancerous lesion esophageal squamous dysplasia (ESD), show alterations in their microbiome compared to controls (**Table 5**). A decrease in microbial complexity and diversity has been associated with the development of ESD[63]. Also, an association between changes in the oral microbiome and an increased risk of ESCC development has been observed[57,64]. The role of the microbiome in ESCC has not been well established yet, nevertheless, these changes suggest an association between the microbiome and cancer progression[63].

The most prevalent type of esophageal cancer in high-income countries is esophageal adenocarcinoma (EAC), which develops in the distal esophagus, closer to the stomach. Similar to the other cancers described here, EAC has a poor prognosis and requiring combined therapies[65–69]. Among the etiological factors proposed for EAC are gastroesophageal reflux disease (GERD), smoking, obesity, and *Helicobacter pylori* infection[65–71]. GERD is a state of chronic inflammation in the gastroesophageal junction, which can lead to Barrett's esophagus (BE), an adaptation of the squamous epithelium to the stomach contents containing bile and acid. BE may become dysplastic and progress to EAC due to constant cytokine release that leads to cell proliferation[65,67,69,70]. Most studies describe smoking and tobacco as an indirect risk factor for EAC since it has

Table 3. Changes in bacterial abundance found in pharynx and larynx cancer patients compared to healthy individuals.

	Differences in bacterial genera compared to healthy individuals	
Oral cavity (in general)	↑*Streptococcus* [96] ↑*Rothia* [96] ↑*Lactobacillus* [96] ↑*Oribacterium* [99] ↑*Actinomyces* [99] ↑*Parvimonas* [99] ↑*Selenomonas* [99] ↑*Prevotella* [99]	↓*Rothia* [99] ↓*Haemophilus* [99] ↓*Corynebacterium* [99] ↓*Paludibacter* [99] ↓*Porphyromonas* [99] ↓*Capnocytophaga* [99]
Oropharynx [24,96,100]	Present in Oral/Oropharyngeal cancer ↑*Rothia* [100] ↑*Haemophilus* [100]	Present in saliva samples from oropharyngeal cancer patients ↑*Actinomyces* [96] ↑*Schwartzia* [96] ↑*Treponema* [96] ↑*Selenomonas* [96] ↓*Prevotella* [96] ↓*Haemophilus* [96] ↓*Neisseria* [96] ↓*Streptococcus* [96] ↓*Veilonella* [96]

Table 4. Suggested bacterial species as potential bacterial biomarkers for OSCC. (↑*enriched,* ↓diminished*,* +present only in tumors*,* -absent in tumors)

Bacterial Marker Species present in OSCC		**References**
↑ *Fusobacterium periodonticum* ↑ *Parvimonas micra* ↑ *Streptococcus constellatus* ↑ *Haemophilus influenza* ↑ *Filifactor alocis*	↓ Streptococcus mitis ↓ Haemophilus parainfluenzae ↓ Porphyromonas pasteri	56
↑ *Fusobacterium nucleatum* ↑ *Pseudomonas aeruginosa* ↑*Campylobacter sp.* oral taxon 44	↓ *Streptococcus mitis* ↓ *Rothia mucilaginosa* ↓ *Haemophilus parainfluenzae*	58
+ *Prevotella melaninogenica* + *Staphylococcus aureus* + *Veillonella parvula* + *Exiguobacterium oxidotolerans*	↓ *Streptococcus mitis* ↓ *Rothia mucilaginosa* ↓ *Veillonella dispar* ↓ *Streptococcus salivarious* ↓ *Actinomyces odontolyticus* ↓ *Propionibacterium acnes* ↓ *Atopobium parvulum* ↓ *Streptococcus parasanguinis* ↓ *Streptococcus oralis* - *Moraxella osloensis* - *Prevotella veroralis*	60
↑ *Streptococcus salivarius* ↑ *Streptococcus sp*. oral taxon 058 ↑ *Streptococcus gordonii* ↑ *Streptococcus parasanguinis* ↑ *Peptostreptococcus stomatis* ↑ *Gemella haemolysans* ↑ *Gemella morbillorum* ↑ *Johnsonella ignava* + *Parvimonas sp.* oral taxon 110 + *Eubacterium [11][G-1] infirmum* + *Eubacterium [X1][G-3] brachy*	↓ *Streptococcus mitis* ↓ *Veillonella dispar* ↓ *Granulicatella adiacens* ↓ *Mogibacterium diversum* ↓ *Parvimonas micra* ↓ *Streptococcus anginosus* ↓ *Streptococcus cristatus* - *Streptococcus sp.* oral taxon 071 - *Selenomonas sputigena*	59
↑ Prevotella melaninogenica ↑ Streptococcus mitis ↑ Capnocytophaga gingivalis		101

Table 5. Changes in bacterial abundance of the oral cavity and the proximal esophagus of ESCC patients compared to healthy individuals.

	Oral cavity	***Proximal esophagus***
Taxa differences in ESD and ESCC patients	↓*Lautropia* [64] ↓*Bulleidia* [64] ↓*Catonella* [64] ↓*Corynebacterium* [64] ↓*Moryella* [64] ↓*Peptococcus* [64] ↓*Cardiobacterium*[64]	↑*Prevotella* [64,102] ↑*Streptococcus* [64,102] ↑*Porphyromonas gingivalis* [64,102] ↑*F. nucleatum*[39] ↑Clostridiales phylum [63] ↑Erysipelotrichal phylum [63]

been associated with enhancing the progression of GERD[65–67,69]. Obesity also promotes GERD, indirectly linking it to an increased risk of Barrett's esophagus and progression of EAC[68].

Another potential indirect risk factor that has been of great interest recently is the change in the esophageal microbiome due to GERD (**Table 6**). Multiple studies have confirmed that the healthy esophageal microbiota is composed mainly of Gram-positive bacteria. When acid reflux enters from the stomach into the distal esophagus, it promotes the enrichment of gram-negative bacteria since these are less susceptible to the low pH and the bile salts. Consequently, the esophageal microbiota of GERD and

Table 6. Changes in bacterial abundance found in GERD and BE patients compared to healthy individuals.

	Distal esophagus
General differences in GERD and BE	↑gram-negative [72]
Taxa differences in GERD and BE patients compared to healthy individuals	↑ *Prevotella* [103] ↑*Fusobacterium* [103] ↑*Veilonella* [103] ↑*Neisseria* [103] ↑*Campylobacter* [77,80]

BE patients consists of an enriched population of Gram-negative bacteria[72]. Due to this enrichment, increased inflammatory signaling occurs through LPS[72], potentially driving tumorigenesis.

Microorganisms Thriving in A Harsh, Bile-Rich Environment

The composition of the acid reflux that enters from the stomach into the distal esophagus can include four types of bile salts: primary and secondary, conjugated and non-conjugated[73]. The primary human bile salts are cholate and chenodeoxycholate, which are synthesized in the liver from cholesterol. Before secretion, all primary bile salts are conjugated with either glycine or taurine. This increases their water solubility and fat emulsification abilities[74]. The conjugated products are then secreted into the gastrointestinal tract. Some intestinal bacteria can hydrolyze the amide bond between the glycine or taurine conjugated bile via a deconjugation reaction. This deconjugation reaction is catalyzed by the bacterial enzyme Bile Salt Hydrolase (BSH)[73]. The expression of BSH is seen in all major phyla of the gastrointestinal microbiota (Actinobacteria, Firmicutes, Bacteroidetes, and Proteobacteria), and its enzymatic activity may help to explain why these organisms can thrive in the harsh environment of the gastrointestinal tract[75].

The BSH enzyme provides many advantages to the organisms that utilize it in the gastrointestinal tract. The deconjugation reaction allows a BSH-expressing organism to utilize the amino acid portion of conjugated bile salts, giving that organism a nutritional advantage[76]. It has been proposed that BSH can facilitate the incorporation of bile and cholesterol into the membrane. The incorporation of these molecules into the bacterial membrane most likely enhances defense mechanisms by changing membrane tensile strength and fluidity (**Figure 4**)[76]. It has also been proposed that the deconjugation of bile salts via BSH is a way to detoxify the environment around the organism[76].

In addition to deconjugation, dehydroxylation is another method of bile salt metabolism carried out by some organisms in the gastrointestinal tract[73]. The deconjugation of tauro-conjugated or glycol-conjugated primary bile salts produces their unconjugated counterparts. These unconjugated counterparts can then be further metabolized via a dehydroxylation reaction carried by the enzyme 7-dehydroxylase, ultimately producing secondary bile acids[75]. In humans, 7-dehydroxylase converts cholate and chenodeoxycholate into deoxycholate and lithocholate, respectively[73]. This function may inhibit the growth of other bacteria that are sensitive to secondary bile salts, allowing the 7-dehydroxylase containing organism to successfully compete for its niche (**Figure 4**). Unlike BSH activity, which

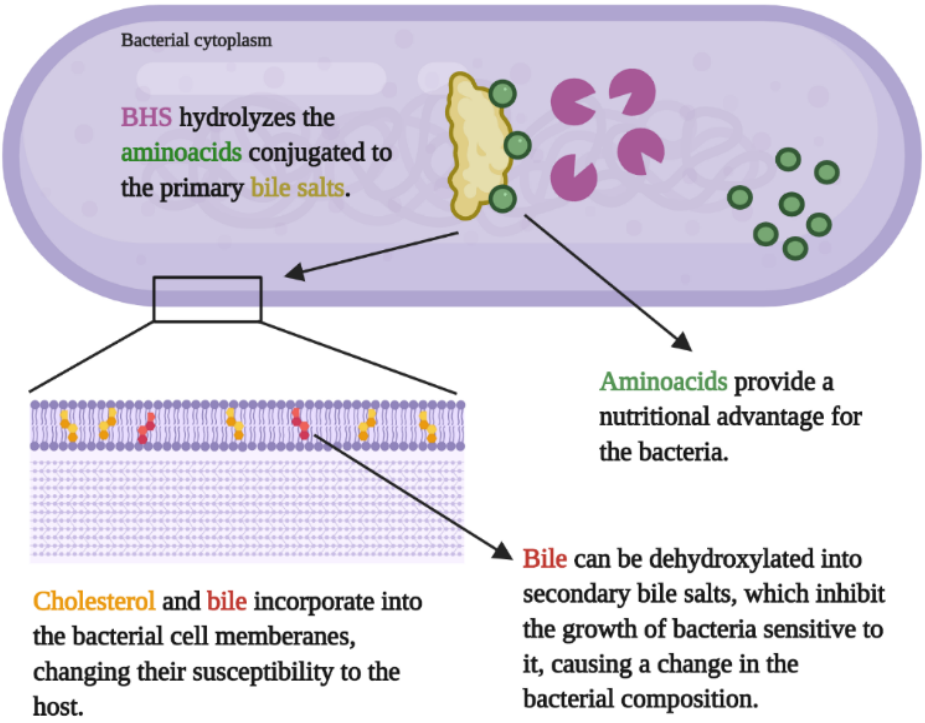

Figure 4. Roles of BSH on bacterial survival and changes in bacterial composition. The BSH enzyme hydrolyzes primary bile salts (deconjugation), producing amino acids that can provide nutrients to the bacteria. [76] Additionally, the bile and cholesterol produced during this hydroxylation can be incorporated into the bacterial membrane and change the bacterial susceptibility to the host, enhancing their defense mechanisms. [76] The unconjugated bile salts can be further metabolized via a dehydroxylation reaction, producing secondary bile acids, which inhibit the growth of bacteria sensitive to it, causing a change in the bacterial composition. [75]

is very common in GI tract microbiota, the 7-dehydroxylase is seen in only a small number of bacterial species[73].

From an evolutionary standpoint, it has been suggested that in the intestinal tract, where bile salt concentration is high, organisms expressing BSH have been selected, while pathogens and transients lacking BSH are disfavored[76]. Interestingly, BSH can also be found in the esophagus microbiome, although the esophagus is not normally exposed to bile. As aforementioned, the healthy esophageal mucosa in healthy individuals is composed mainly of Gram-positive organisms[72]. However, when diseases such as GERD, Barrett's esophagus, and ultimately EAC develop, the distal esophagus transitions to a Gram-negative rich population[72]. During these disease states, microorganisms in the distal esophagus are exposed to higher amounts of reflux from the stomach and may utilize the BSH enzyme to survive.

One genus of particular interest is Lactobacillus, a Gram-positive commensal organism found in healthy esophageal mucosa[72]. This species is also found in patients with the diseased esophagus, despite the overall enrichment of Gram-negative organisms. This can be seen in a study conducted by Macfarland et al., which found that Lactobacilli were present in aspirate specimens taken from healthy subjects as well as subjects who had Barrett's esophagus[77]. Another study conducted by Jing Lv et al. found that *Lactobacillus fermentum* was enriched in EAC compared to healthy individuals[78]. It is proposed that a low pH[78], and the utilization of BSH[76], may help these organisms thrive when the esophagus environment changes to an acidic, bile-rich area, as seen in diseased patients.

Mechanisms Proposed for *Campylobacter concisus* Role in GERD, BE and EAC Tumorigenesis

In EAC patients, *Campylobacter concisus* presence has been shown to correlate with the production of IL-18, a cytokine associated with carcinogenesis[79,80]. *In vitro*, *C. concisus* upregulates the expression of p53, known as the guardian of the genome and an important regulator of the cell cycle, e.g., proliferation and death homeostasis. Inflammatory pathways can also be induced as seen by increased IL-18 and TNF-α upon *C. concisus* exposure of Barrett's and EAC cell lines[79]. The main chemoattractants for *C. concisus* are mucins and glycoproteins, both secreted by the epithelial cells of the gastrointestinal tract[81] and often altered between the esophagus of healthy subjects and BE patients[82]. Since *Campylobacter* is enriched in GERD and BE patients[80], it could be of interest to further study its interactions with specific mucins and determine the consequences in disease.

To aid in the colonization of the epithelium, *Campylobacters* possess adhesion proteins on its surface, including CADF (binds fibronectin) and the protein CapA[81]. Campylobacter also expresses virulence factors for adherence and invasion, including Exotoxin 9/DnaI, which allows the bacteria to survive inside the host cells[5]. It also expresses Zot, which has the potential to affect tight junctions that seal adjacent epithelial cells. By disrupting tight junctions, Zot is causing permeability in the epithelium barrier, leading to further inflammatory responses deeper in the tissue[5]. *C. concisus* upregulates the expression of TLR3, which is a nucleic acid-sensing pattern recognition receptors (PRRs), through which the cells recognize the lysed bacterial DNA, inducing an inflammatory signaling response that upregulates the expression of the inflammasome IFI16 (**Figure 5 right**)[83]. In intestinal epithelial cells, *C. concisus,* binds to TGF-β, activating its pathway via SMADs, RhoA, PI3K, and ILK (integrin-linked kinase), and also through the NOTCH pathway (**Figure 5 left**)[83]. These signaling pathways can induce epithelial-mesenchymal transition (EMT), which is a process by which epithelial cells acquire characteristics such as resistance to apoptosis, invasion into adjacent tissues and metastasis[53].

As presented here, all the characteristics observed in GERD-related bacteria represent two of the mechanisms

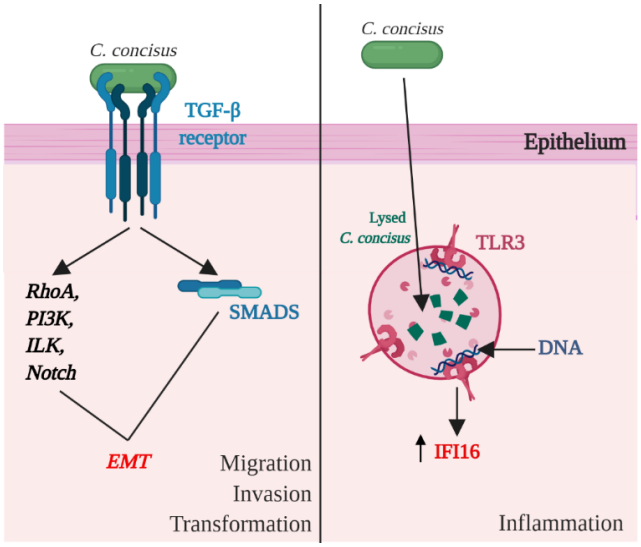

Figure 5. Known interactions of *C. concisus* with host epithelial cells. *C. concisus* has the capacity to adhere to cells through the protein CADF (binds fibronectin) and the protein CapA.[81] *C. concisus* upregulates the expression of TLR3, which is a nucleic acid sensing pattern recognition receptors (PRRs), through which the cells recognize the lysed bacterial DNA, inducing an inflammatory signaling response that upregulates the expression of the inflammasome IFI16 (left panel). [83] *C. concisus* also induces epithelial-mesenchymal transition (EMT), which is a process by which epithelial cells are transformed and acquire characteristics that help them resist apoptosis, invade adjacent tissues and disseminate through the host's body (metastasis). [53] This process occurs in intestinal epithelial cells through the TGF-β pathway, via SMADs, RhoA, PI3K, and ILK (integrin-linked kinase), and also through the NOTCH pathway (right panel). [83]

potentially contributing to tumorigenesis: dysregulation of signaling pathways and chronic inflammation[7]. Identifying the virulence factors expressed during microbial homeostasis and dysbiosis is crucial towards the understanding of *Campylobacter's* role in disease and what triggers its pathogenic behaviors.

Various species from the genus *Campylobacter*, including *C. jejuni*, *C. coli*, and *C. concisus*, have also been associated with periodontitis[81]. Virulent *C. concisus* strains are also enriched in GERD and BE patients[77,80]. While *C. concisus* is found as a commensal in the saliva of healthy controls[5,80,81], it is also found as a pathogen in the saliva of all the IBD patients (epidemiological studies have shown that patients with an acute *Campylobacter* infection have a higher risk of developing IBD)[81]. Therefore, *C. concisus* can be considered a pathobiont, which is a commensal microbe that becomes pathogenic due to the imbalance in the host-microbiome relationship[6]. However, studies to elucidate what triggers commensal bacteria to become pathogenic are still required[3].

Stomach Cancer Epidemiology and Bacteria as an Established Risk Factor

Stomach or gastric cancer has an incidence of 5.5% and a mortality of 78% worldwide. Tobacco smoking, alcohol consumption, and diet are established risk factors for gastric cancer. The main risk factor for 90% of the noncardia gastric cancers is *H. pylori*[16]. *H. pylori* colonize the gastric mucosa of 50% of the population in the world, and it is usually acquired during childhood in developing countries and adulthood in industrialized countries. Once *H. pylori* colonize the gastric mucosa, it expresses pathogenic markers, including cytotoxin-associated gene A, BabA adhesin, and a vacuolating cytotoxin. All of these toxins promote a complex inflammatory response that damages the mucosa[84]. Consequentially, this causes gastritis and peptic ulcers, which are directly associated with the tumorigenesis of gastric carcinoma[85].

The gastric infections with *H. pylori* have seen a steady drop in most populations of the US, Europe, and Australia, although its prevalence remains high (approximately 4.4 billion individuals worldwide) [86,87]. The first line of treatment most commonly followed to eradicate *H. pylori* includes two antimicrobial agents: clarithromycin and metronidazole[88,89]. Different antimicrobial regimens have shown varied responses in different geographical populations[88,89]. Some geographical regions have shown a higher percentage of resistance than others. In most regions, these antimicrobials are eradicating less than 90% of H. pylori[89], which is considered non-effective and should not be utilized as an independent treatment[88]. Currently, if the patient strain shows more than 15% resistance, the second to the fourth line of treatments are put in place, including PPIs and various combinations of other antimicrobials: amoxicillin, bismuth, rifabutin, and quinolone[89]. To decrease the risk of cancer development, the eradication of *H. pylori* needs to occur before the patient develops atrophic gastritis, a premalignant condition that can progress into gastric cancer[88].

Interestingly, along with *H. pylori* eradication, an increased incidence of EAC development has been observed[86]. It has been proposed that *H. pylori* might have a protective effect against GERD and EAC[65,66,68,86]. The present hypothesis is that an *H. pylori* infection causes atrophy of the stomach, which leads to a decrease in the overall acidic environment of the stomach, preventing reflux and lowering risks of EAC[65,66]. Additionally, another hypothesis is that an infection causes a decrease in appetite and weight, along with a decrease in obesity, which indirectly decreases EAC risk[68]. This alleged protective effect and increase in EAC due to the eradication of *H. pylori* in many countries remains controversial[87]. This ensuing dilemma of *H. pylori* eradication is an example of the challenges that arise from therapeutic targeting of one bacteria as the single cause of a disease (following Koch's theory), without considering the entire bacterial community. Targeting specific bacteria, like *H. pylori*, can cause dysbiosis and eventually lead to other diseases[6]. Once the microbial balance is lost, finding ways or treatments (e.g., microbial transplantation, probiotics, and prebiotics[63]) to restore that balance can be challenging.

Protective Roles of Certain Bacterial Genera

The presence or absence of some bacteria in cancer patients suggests a protective role of specific bacterial species. For example, *Prevotella* and *Peptostreptococcus* have been shown to limit the growth of pathogenic bacteria[24]. Consequently, their decreased abundance in cigarette smokers leaves an empty niche for pathogens to colonize[24]. Likewise, a greater abundance of the commensals *Corynebacterium* and *Kingella* is associated with a decreased risk of developing HNSCC, suggesting a protective role of these strains against HNSCC[15]. In ESCC, a decrease in abundance of the genera *Lautropia, Bulleidia, Catonella, Corynebacterium, Moryella, Peptococcus,* and *Cardiobacterium*, has been associated with increased risk of ESCC, suggesting their protective role against ESCC[64].

S. gordonii is an established commensal, promoting homeostasis due to its ability to antagonize the upregulation of ZEB2 and, as a result, prevents *P. gingivalis*-induced EMT[90]. In a similar way, as mentioned before, commensals *Streptococcus spp* have the ability to suppress *F. nucleatum*-induced IL8 and NFkB signaling, suppressing a chronic inflammatory response[43]. The protective role of some bacterial species is a topic that needs to be addressed more thoroughly through *in vivo* and *in vitro* research. A better

understanding of which bacterial species play a protective role would be important to develop preventive measures.

Limitations for Bacterial-Host Relationship Studies

While many patterns of distinctive enrichment and depletion of several bacterial types have been identified, these results have not been consistent among research groups. The approach of most of the studies is determining conserved sequences of the 16S rRNA region, followed by alignments using databases to identify the isolated strains. Still, differences in sampling protocols, localization of collected samples, control tissues selected, exclusion/inclusion criteria for patients, and clinical case definitions and measurement to describe the disease stage exist and are not standardized among studies (**Table 7**). These factors hinder the possibility to do thorough meta-analyses of these studies.

Table 7. Differences in sampling and similarities in analysis techniques.

Sampling collection procedure	Sample Location	Analysis Technique	Reference
Biopsy tissues: tumor and non-tumor from the same patient were collected (5 cm distant from the tumor area).	Oral cavity	Specimens were cultured on nonselective media and isolates were identified with 16S rRNA gene sequencing.	60
Biopsy tissues: tumor and non-tumor from the same patient were collected (5 cm distant from the tumor area).	Oral cavity: tongue and floor of the mouth	Culture independent 16S rRNA approaches: Denaturing gradient gel electrophoresis, and 454 pyrosequencing.	59
Biopsy specimens: collected from distal esophagus, 1cm above the gastroesophageal junction.	Normal/reflux esophagitis/BE patients	Fragments of 16S rDNA genes were amplified by PCR using general bacterial primers.	104
Tissue Biopsy: **Deep-epithelium** with Catch-All Sample Collection swabs (control subjects). First a light swab was utilized to remove surface cells and adherent bacteria, followed by second swab stroked with pressure 10 times in one direction on one side and 10 times in the other direction of the other side.	Oral cavity	Sequencing of V1V3 region. *Analyses performed using QIIME, PICRUSt and LEfSe.*	58
Tissue biopsies	Oral cavity	PathoChip screening and next generation sequencing.	100
Saliva samples: expectoration from OSCC-free and OSCC subjects.	Oral cavity	Checkerboard DNA-DNA hybridization with 40 common oral bacteria.	101
Saliva from overnight fasting patients.	Oral cavity	454 Pyrosequencing of the V3-V4 region of 16S rRNA.	64
Saliva sampled using the Salivette Corticol saliva detection kit, which contains a polyester swab for saliva absorption (patient chew on the swab foor 60s to stimulate salivation), followed by centrifugation for recovery of the saliva.	Oral cavity	Amplification of 16S rRNA gene pool. *The raw reads were processed via QIIME and checked for the presence of chimeras and grouped into OTUs.*	96
Oral wash – *swish vigorously with 10mL Scope mouthwash and expectorate into specimen tube*	Oral cavity	16S rRNA gene sequencing *Sequences clustered into OTUs using QIIME and metagenomic content was inferred using PICRUSt.*	25
Mouthwash samples: swish with 10mL Scope mouthwash and expectorate into tube.	Oral cavity of ESCC patients	16S rRNA gene sequencing. *Metagenome content predicted using PiCUST.*	102
Oral rinse – 50mL of sterile normal saline for 30s, spit into sterile tube	Oral cavity	16S rRNA V3V4 amplicon sequencing.	56
Oral rinse: swish and gargle with 10mL of 0.9% saline solution for 60s, and expectorate into a sterile tube.	Oral cavity	16S rRNA gene amplicon sequencing.	99
With nylon-flocked swabs	Nasopharynx: through the nares Oropharynx: trans-orally adjacent to the tonsillar pillars	454 Pyrosequencing of 16S rRNA.	24
Inflatable Rubber Balloon covered with cotton mech attached to a 0.2 cm diameter single lumen rubber tube Cytomesh esophageal cytology device.	Upper digestive tract	Human Oral Microbe Identification Microarray. The array uses 16S rRNA-based oligonucleotide probes.	98

For the oral cavity, many of the surveys to report gum disease are self-reporting; therefore, these do not include measurements of periodontal pocket depth, attachment loss, and bone loss[91]. The sampling methods range from swabs of surface or deep-epithelium, saliva absorption oral rinse (mouth wash) with Scope or saline solutions for different amounts of time, saliva spitting, and biopsies. None of these techniques has been universally accepted, and some of them may introduce bias during the sampling process. This is an immense limitation in this field of study, as it prevents the data from being successfully reproduced[9]. Oral swabs fail to include bacteria in hidden areas of the tonsillar crypts or the tongue base; this problem can be solved utilizing multiple saliva samples. Mouthwash would help dislodge adherent bacteria from the teeth, gingiva, tongue, and buccal mucosa[27], providing information from many locations of the oral cavity. Nevertheless, saliva samples do not collect bacteria present in oral biofilms, for which swabs or biopsies of specific regions would be a better option[57]. For the esophagus, the sampling methods are all invasive and performed during endoscopic procedures, including biopsies, mucosal brushes[92]. The differences in sampling methods post a challenge in the ability to compare published results[57].

Studies consider different inclusion criteria during patient screenings, including factors influencing the health condition of the patient cohorts[57], like medications used (e.g., PPIs), hygiene habits (e.g., the use of mouthwash, toothbrush frequency, flossing, visits to the dentist, and the presence of caries) and lifestyle factors (e.g., smoking and alcohol consumption). Additionally, antibiotics also alter the normal microbiome, leading to dysbiosis and allowing pathogens to colonize[6,93]. The use of multiple courses of penicillin has been associated with a higher risk of developing esophageal and gastric cancer[93], as well as colorectal cancer[94]. Broad-spectrum antibiotic use has increased at an alarming rate in the USA. The average American consumes low doses of antibiotics in their food and water daily[95], while 15% of the western population is prescribed with at least one antibiotic course each year[93]. In order to make informed decisions towards prevention, there is a need for more studies to focus on antibiotic use as a potential risk factor for gastrointestinal cancer and to understand the mechanisms through which antibiotics could be indirectly driving carcinogenesis. Overall, reporting all these factors during patient screening will provide enough information to adjust for their presence during statistical analysis, allowing scientists to determine when bacteria could be considered an independent risk factor, and when it could be working synergistically with other risk factors.

Some studies include the specific bacterial species being enriched or depleted[56–60], while other studies only mention the genera or the phylum[57,96]. Similarly, while some reports only mention which bacteria are enriched or depleted, other studies specify the percentage of enrichment or depletion. Either way, most studies do not specify the bacterial load found in the samples. More consistency and specificity are required to determine the most significant species and the most physiologically relevant conditions for subsequently improved *in vivo* and *in vitro* studies to be designed. These will be necessary to elucidate the specific role of bacteria in the host, as well as allow for biomarker validation and evaluation of diagnostic sensitivity.

Conclusions

Bacteria have been shown to play significant roles in several types of cancers. While the role of bacteria has been widely explored in colorectal cancer, a lot remains unknown regarding the role of bacterial communities in upper gastrointestinal cancers. We present evidence that bacteria in the upper gastrointestinal tract can support tumorigenesis by working synergistically with other risk factors[23], including tobacco use and alcohol consumption[9]. Based on the data we reviewed, bacteria involved in poor oral hygiene and gum diseases appear to be an independent risk factor for HNSCC[18]. Regardless of being a synergistic or an independent risk factor, pathogenic bacteria have the capacity to affect the host signaling pathways resulting in a chronic inflammatory response. The metabolizations of host and xenobiotic factors is another mechanism through which bacteria can contribute to the tumorigenesis process[7].

While it has been shown that most cancer risk factors (e.g., smoking, alcohol, gum disease, GERD) affect bacterial homeostasis in the upper gastrointestinal tract, there is a need for *in vitro* and *in vivo* studies that help elucidate how these risk factors could be driving certain commensal bacterial species into pathogenic strains. Also, understanding the mechanisms through which bacteria could be working synergistically with risk factors to accelerate the tumorigenesis process could help implement better preventive measures, as well as establishing biomarkers for early detection.

Multiple population-based studies are showing the overall microbial changes induced by the mentioned cancer risk factors. Nevertheless, we discussed various limitations that need to be addressed in order to obtain more consistent results among studies and allow the field to move forward. Further *in vitro* and *in vivo* studies focused on the specific enriched or depleted bacterial species could help elucidate their potential role in promoting cancer or protecting the host against it.

While it is challenging to understand the relationship of bacterial communities with the host, studying the individual pathogen-host and bacteria-bacteria interactions is the

first stepping-stone towards the understanding of the bigger picture. The goal is to understand how bacterial communities interact among them and with the host, what factors affect these bacterial communities, and what are the potential roles of these populations and communities in cancer development, progression, and prevention.

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