

The esophageal microbiota in health and disease

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The esophageal mucosa is among the sites colonized by human microbiota, the complex microbial ecosystem that colonizes various body surfaces and is increasingly recognized to play roles in several physiological and pathological processes. Our understanding of the composition of the esophageal microbiota in health and disease is challenged by the need for invasive sampling procedures and by the dynamic nature of the esophageal environment and remains limited in comparison with the information available for other body sites. Members of the genus *Streptococcus* appear to be the major components of the microbiota of the healthy esophagus, although the presence of several other taxa has also been reported. Dysbiosis, consisting of enrichment in some Gram-negative taxa (including *Veillonella*, *Prevotella*, *Haemophilus*, *Neisseria*, *Campylobacter*, and *Fusobacterium*), has been reported in association with gastroesophageal reflux disease and is hypothesized to contribute to the evolution of this condition toward Barrett's esophagus (which is the most common esophageal precancerous lesion) and, eventually, adenocarcinoma. Some *Campylobacter* species (mostly *C. concisus*) are also putatively involved in the progression of disease toward adenocarcinoma. However, variable findings have recently been reported in additional studies. Causative relationships between dysbiosis or specific bacterial species and esophageal diseases remain controversial and warrant further investigations.

Keywords: esophageal diseases; microbiota; esophagitis; Barrett's esophagus; esophageal adenocarcinoma; esophageal squamous cell carcinoma

Introduction

The human body has symbiotic relationships with a complex and diverse array of microbial communities at various nonsterile body sites, which overall constitute the human microbiota (HM). The sites colonized by the HM include skin and various mucous membranes (e.g., mouth, upper respiratory tract, lower genitourinary tract, gastrointestinal tract). The composition of the HM exhibits a remarkable variability at various sites, among different individuals, and at different times, depending on several factors that are only partially understood.^{1–3}

Until recently, knowledge of the HM composition was largely hampered by the limited resolution allowed by cultural methods, which cannot recover a large number of components of the

HM. The advent of culture-independent metagenomic methods capable of also characterizing the nonculturable fraction of complex microbial communities, in combination with high-throughput next-generation sequencing (NGS) technologies, has provided a fundamental breakthrough in studying the composition of the HM.^{4–6}

The HM is now considered an additional apparatus of the human body, capable of modulating several developmental, metabolic, and immunological pathways and playing a fundamental role in defense against colonization by microbial pathogens.^{1,7–10} It is also clear that HM alterations (a condition termed dysbiosis) can be associated with disease.¹¹ The most paradigmatic example is represented by the association between dysbiosis of the intestinal microbiota, promoted by antibiotic treatments, and

Clostridium difficile infection (CDI),^{12–14} which is the leading cause of diarrhea among hospitalized patients and has become a major public health issue in several settings.¹⁵ Under these circumstances, the conventional treatment of CDI, based on antibiotics active against *C. difficile*, may sustain a vicious cycle wherein the dysbiotic status persists and favors recurrences of CDI. In this context, reconstitution of the intestinal microbiota by fecal microbiota transplantation has proved extremely successful and has definitively confirmed the role of dysbiosis in the pathogenesis of CDI.^{16–18} An association with altered composition of the HM has now also been reported for several other diseases, including inflammatory bowel diseases,¹⁹ colorectal cancer,²⁰ obesity,²¹ diabetes,²² nonalcoholic steatohepatitis,²³ bacterial vaginosis,²⁴ atopic dermatitis, and psoriasis,^{9,25} although in these cases a causative relationship remains to be definitively established.

The esophageal mucosa is one of the sites colonized by HM, and consequently there is an interest in understanding a possible role of HM alterations in esophageal diseases. Some articles have recently reviewed various aspects of this topic.^{26–31} Here, we provide an updated review of current knowledge on the esophageal microbiota in health and in different esophageal diseases and discuss the major challenges encountered in investigation of this topic.

The healthy esophagus and its microbiota

Structure and physiology of the healthy esophagus

The esophagus is a muscular conduit of approximately 20–27 cm in length, with an internal mucosa carrying a stratified squamous epithelial layer. It plays the fundamental role of transferring the alimentary bolus from the pharynx to the stomach. At its distal end, a complex valve mechanism allows passage of the bolus into the stomach and restricts reflux of the gastric contents back into the esophagus. The esophagus has a virtual narrow cavity that is empty and collapsed, except during swallowing. The epithelial layer of the esophageal mucosa is normally wet with saliva for the whole length and, as a result, the pH is usually around 7. However, reflux of gastric material may occur and cause a sudden lowering of pH values (down to 2). This is mostly limited to the distal third of the organ, and, normally, acidification is rapidly buffered by esophageal

motility. The potentially harmful effect of reflux on the esophageal mucosa depends on the composition of the refluxate (commonly gastric acid and ingesta, less frequently bile and pancreatic enzymes) and the time of contact. In addition to mucosal damage, reflux is capable of inducing microenvironmental variations. Therefore, whereas the environment of the proximal esophageal mucosa is similar to the oral one, an environment that is intermediate between the oral-like one of the proximal esophagus and the gastric one has to be expected in the distal esophageal mucosa.

The microbiota of the healthy esophagus

The esophageal mucosa hosts a resident microbiota, although in a smaller population as compared with other districts of the gastrointestinal tract. In fact, the HM of the gastrointestinal tract exhibits substantial qualitative and quantitative differences, with populations ranging from 10 cells per g/mL of sampled material in the esophagus and stomach to 10¹² per g/mL of sampled material in the large intestine.^{32,33}

Considering the anatomic structure and function, it was originally unclear whether the esophagus was associated with a defined microbiota. The first studies on the esophageal microbiota, dating back to the early 1980s and based on cultural methods, demonstrated that the esophagus was not a sterile site and did not simply contain a transient microbial population originating from the oral cavity by swallowing or from the stomach by gastroesophageal reflux (GER).^{34–36} Consistently, it was later observed that bacteria were closely associated with the esophageal mucosal surface, confirming the presence of a resident microbiota at this site.³⁷ More recently, knowledge regarding the composition of the microbiota of healthy individuals has been expanded through the use of investigations based on metagenomic approaches.^{4–6} However, the difficulties in obtaining samples at this body site, compared with other sites, have limited the number of studies on the esophageal microbiota.

Few studies have focused on the composition of the microbiota of the normal esophagus alone (Table 1),^{37–40} but additional information has come from studies investigating the esophageal microbiota in disease and including healthy patients as controls for comparison (Tables 2^{6,41–47} and 3^{48–50}). The available information on the microbiota of the

Table 1. Studies reporting the characterization of esophageal microbiota in the healthy individuals

Year	No. of studied subjects	Exclusion criteria	Method	Samples	Sampled esophageal tract	Main findings	Reference
1998	20	Esophageal abnormalities	Culture-based	Aspirate	Middle third	<i>Streptococcus</i> , <i>Enterococcus</i> , <i>Staphylococcus</i> , <i>Klebsiella</i>	38
2004	4	Recent antibiotic use Previous gastric/esophageal surgery Active oral cavity infection	Culture-independent (sequencing of 16S rRNA gene clones, 25–30 clones/sample analyzed)	Biopsy	2 cm above the squamocolumnar junction	<i>Streptococcus</i> , <i>Prevotella</i> , <i>Veillonella</i>	37
2012	15	Esophageal abnormalities Increased risk for endoscopic complications	Culture-independent (NGS, V2–V3 region of 16S rRNA genes)	Biopsy and esophageal string test	Mid to distal	<i>Streptococcus</i> , <i>Prevotella</i> , <i>Veillonella</i>	39
2013	40	Age <20 or >75 years Recent use of antibiotics or PPIs Gastroesophageal diseases Ongoing infections	Culture-based	Biopsy and brush	5 cm below the upper esophageal sphincter, 2 cm above the squamocolumnar junction	<i>Streptococcus</i> , <i>Neisseria</i> , <i>Haemophilus</i> , <i>Prevotella</i>	40

NGS, next-generation sequencing; PPIs, proton pump inhibitors.

healthy esophageal mucosa, in terms of prevalence and proportions of bacterial taxa, is summarized in Figure 1 and briefly discussed below, with the caveat that different methodological approaches, differences in the sampled areas within the esophagus, and the heterogeneity of inclusion/exclusion criteria used in the various studies do not allow fine-tuned comparisons and make it difficult to reach a consensus on the comprehensive microbiota composition of the healthy esophagus.

The presence of *Streptococcus* spp. was constantly reported, and in high proportions, by all studies: therefore, members of this genus appear to be a dominant taxon in the microbiota of the normal esophagus. Other bacterial genera frequently detected (at least in half of the studies) in association with streptococci, although usually in lower proportions, include *Prevotella*, *Fusobacterium*, and *Veillonella*. The presence of other genera (e.g., *Haemophilus*, *Neisseria*, *Gemella*, *Granulicatella*, *Lactobacillus*, *Actinomyces*, *Staphylococcus*, *Bacteroides*, and *Porphyromonas*) was less frequently reported, and some genera were only reported by single studies (Fig. 1).

Notably, the prevalence of *Streptococcus*, *Fusobacterium*, *Veillonella*, and *Prevotella* has consistently been reported in several studies based on either culture-dependent or culture-independent approaches and also on different specimens (biopsies, brushes, aspirates) (Tables 1–3), thus providing a strong indication of their contribution to the composition of the core microbiota that colonizes the healthy esophageal mucosa. The dominance of streptococci and the frequent presence of other taxa

typical of the oropharyngeal microbiota have been related to the composition of the microbial communities of the oropharyngeal cavity, where a high prevalence of streptococci is found, together with *Veillonella*, *Fusobacterium*, *Gemella*, *Granulicatella*, and *Rothia*,^{51,52} and have supported the notion that the esophageal microbiota is primarily of oral origin. However, not all oral bacteria are apparently able to colonize the esophageal mucosa, while several members of the esophageal microbiota are not present or are underrepresented in the oral cavity, pointing to a diverse microbiota adaptation in the two body sites.^{37,39,40,49}

Major esophageal diseases

The environment of the esophagus and its pathologic states are closely connected. There is no doubt that, apart from esophageal squamous cell cancer (ESCC), whose pathogenesis remains largely unknown (although related to cigarette smoking and alcohol consumption), all the most important esophageal diseases are associated with GER and GER-related environmental changes. These are represented by gastroesophageal reflux disease (GERD) with or without esophagitis, Barrett's esophagus (BE), and esophageal adenocarcinoma (EAC), with GERD generally representing the first step in the evolution of disease toward BE and EAC.

The prevalence of GERD has been rapidly increasing in Western countries, where ≈20% of adults today may be affected by this disease.^{53,54} The presence of short-duration reflux episodes is considered physiologic, but GERD occurs when reflux becomes symptomatic and/or causes esophageal mucosal

Table 2. Studies reporting the characterization of esophageal microbiota in major esophageal diseases (E, BE, EAC, ESCC)

Year	No. of studied subjects	Disease group (No.)	Control group (No.)	Exclusion criteria	Method	Samples	Sampled esophageal tract	Main findings	Reference
1981	79	EC (79)	–	–	Culture-based	Aspirate	–	Detected bacteria included <i>Bacteroidetes</i> (39%), <i>Streptococcus</i> (10%), and coliforms (8%)	34
1982	12	EAC (7) ESCC (5)	–	–	Culture-based	Biopsy	Proximal resection line after cancer excision	<i>Streptococcus</i> and <i>Bacteroidetes</i> most prevalent taxa	35
1983	101	EC (50)	NE (51)	Use of antibiotics	Culture-based	Aspirate	–	No differences in the presence/absence of specific taxa in EC vs. controls. <i>Streptococcus</i> and <i>Peptococcus</i> the most prevalent taxa	36
2004	20	EC (20)	–	–	Culture independent (sequencing of 16S rRNA gene clones of most members of the oral microbiota, except <i>Neisseria</i> , >10 clones/sample analyzed)	Biopsy	Cancerous and noncancerous tissues from each patient	<i>Treponema denticola</i> , <i>Streptococcus mitis</i> , and <i>Streptococcus anginosus</i> prevalent in both cancerous and noncancerous tissues and suggested to have roles in carcinogenic process	68
2005	24	E (12) BE (3)	NE (9)	Recent antibiotic use Previous gastric/esophageal surgery Active oral cavity infection	Culture-independent (sequencing of 16S rRNA gene clones, two clones/sample analyzed)	Biopsy	2 cm above the squamocolumnar junction (NE and E), 2 cm above the gastroesophageal junction (BE)	Proof-of-concept study demonstrating the presence of a nontransient microbiota in the reflux-exposed esophageal mucosa and the importance of culture-independent approaches	41
2007	14	BE (7)	NE (6) E (1)	Recent antibiotic use	Culture-based	Biopsy and aspirate	Middle to distal	High-level colonization by <i>Campylobacter</i> (mostly <i>C. concisus</i>) in BE, absence in controls	42
2009	34	E (12) BE (10)	NE (12)	Recent antibiotic use Previous gastric/esophageal surgery Active oral cavity infection HIV infection	Culture-independent (sequencing of 16S rRNA gene clones, 200 clones/sample analyzed)	Biopsy	2 cm above the squamocolumnar junction	Type I microbiota (dominated by Gram-positive taxa of the phylum Firmicutes) in normal esophagus, type II microbiota (enriched in Gram-negative taxa) in E and BE	43
2013	18	E (6) BE (6)	NE (6)	Recent use of antibiotics or PPIs Gastric/esophageal surgery Active oral cavity infection HIV, HBV, or HCV infection	Culture-independent (sequencing of 16S rRNA gene clones, about 24 clones/sample analyzed)	Biopsy	1 cm above the gastroesophageal junction	Enrichment of <i>Prevotella</i> , <i>Fusobacterium</i> , <i>Veillonella</i> , and <i>Neisseria</i> in BE	44
2013	34	E (8) BE (8) EAC (10)	NE (8)	Recent use of antibiotics or PPIs	Culture-based plus qRT-PCR for detection of specific taxa in a larger cohort	Biopsy	5 cm above the esophagogastric junction, or at the upper limit of BE or at site of pathology	<i>Campylobacter</i> (mostly <i>C. concisus</i>) expansion in E and BE compared to AD and controls (suggested to be specific to reflux-exposed mucosa)	45
2014	34	E (13) BE (6)	NE in GERD (15)	Recent use of antibiotics or PPIs	Culture-independent (NGS, V6–V7 region of 16S rRNA genes)	Biopsy	Normal-appearing mucosa above the inflammation site or BE	No significant difference between microbiota of normal and abnormal esophagus. Relevant effect of PPI treatment	46

Continued

Table 2. *Continued*

Year	No. of studied subjects	Disease group (No.)	Control group (No.)	Exclusion criteria	Method	Samples	Sampled esophageal tract	Main findings	Reference
2015	12	BE (12)	–	EAC	Culture-independent (NGS, V3–V4 region of 16S rRNA genes)	Biopsy and brush	Normal-appearing and BE mucosa	<i>Streptococcus</i> and <i>Prevotella</i> dominant species. No substantial intraindividual difference between squamous and BE mucosal microbiota	59
2016	26 ^a	BE (13) EAC (5)	NE in GERD (8)	–	Culture-independent (PCR–ESI–MS–TOF)	Biopsy	In some cases, both normal and diseased mucosa	Higher prevalence of <i>Streptococcus pneumoniae</i> in GERD and BE, compared to EAC	47

–, absent or not specified.

^aThe authors reported 28 patients, which possibly included two patients with dysplasia, in addition to those indicated in the table. E, reflux-related esophagitis; EC, esophageal carcinoma (not further specified); EAC, esophageal adenocarcinoma; ESCC, esophageal squamous cell carcinoma; GERD, gastroesophageal reflux disease; NE, normal esophagus; BE, Barrett's esophagus; NGS, next-generation sequencing; PCR–ESI–MS–TOF, PCR-based electron spray ionization–time-of-flight mass spectrometry; PPIs, proton pump inhibitors; qRT-PCR, quantitative real-time PCR.

damage associated with inflammation (esophagitis). This seems to be attributable to an increased GER/mucosa contact time, which overwhelms the protective mechanisms of the esophageal mucosa, including esophageal clearing, salivation, bicarbonate secretion by esophageal submucosal glands, and the histologic/functional endothelial configuration. Salivation may play a major role in the protective effect of esophageal mucosa.⁵⁵ In fact, it does not appear incidental that esophagitis, BE, and EAC are generally limited to the lower third of the esophagus, where the salivation effect is lower and the environment is intermediate between the esophageal and the gastric environments.

Although the role of the individual components of GER has been debated,⁵⁶ there is no doubt that chronic exposure to reflux may determine the onset of esophagitis and its progression, although not frequently, to BE and EAC. BE is represented by the replacement of the stratified squamous epithelium of the distal esophagus with an intestinal columnar metaplastic epithelium. It is the most common esophageal precancerous lesion and an intermediate step in the sequence GER–esophagitis–intestinal metaplasia–dysplasia–EAC. EAC, therefore, like colon cancer, identifies a multistep cancer model that is being thoroughly studied. The evolution of the sequence has been shown to largely depend upon inflammation and its mediators, such as cyclooxygenase-2 (COX-2).⁵⁷ However, although mucosal inflammation can be directly caused

by GER, it could also be caused by esophageal microbiome variations induced by GER (see below).

The microbiota in diseased esophagus and its potential role in diseases

Microbiota in GERD, BE, and esophageal cancers

As previously mentioned, GERD-related esophagitis and Barrett's metaplasia are the major conditions associated with chronic reflux of acidic material from the stomach into the lower part of the esophagus. Under these circumstances, a modification of the esophageal microbiota is expected to occur owing to the altered environment, and it has been hypothesized that the modification of the microbiota could contribute to disease persistence and/or progression.^{26–31} The relevant literature available on this subject is summarized in Table 2 and discussed below.

A positive association between the load of bacterial colonization and severity was first reported in 2004 by a study based on Gram staining of esophageal biopsies in reflux-related esophagitis and BE.⁵⁸ Shortly thereafter, Pei *et al.*⁴¹ demonstrated the presence of a nontransient microbial population in GER-exposed esophageal mucosa and, at the same time, established the importance and feasibility of culture-independent approaches for investigating the complexity of the esophageal microbiota. A subsequent culture-based characterization of microbial communities in patients affected by BE compared to controls was carried out by Macfarlane

Table 3. Studies reporting the characterization of esophageal microbiota associated with some uncommon esophageal diseases

Year	No. of studied subjects	Disease group (No.)	Control group (No.)	Exclusion criteria	Method	Samples	Sampled esophageal tract	Main findings	Reference
2002	25	CME (15)	NE (10)	Grade IV megaesophagus Recent antibiotic use GERD (for controls)	Culture-based	Aspirate	Distal	Higher bacterial load and greater variability in CME (e.g., enrichment in <i>Veillonella</i>) compared to controls	48
2015	68	EoE (35)	NE and mild non-EoE (33)	Use of antibiotics or oral steroids Immune or other inflammatory GI disorders	Culture-independent (NGS, V1–V2 region of 16S rRNA genes)	Biopsy	Distal	Enrichment in Proteobacteria (<i>Neisseria</i> and <i>Corynebacterium</i>) and decrease of Firmicutes (<i>Streptococcus</i> and <i>Atopobium</i>) in EoE	49
2015	70	EoE (37) E (8)	NE (25)	Age <7 years Esophageal abnormalities Antibiotic use Increased risk for endoscopic complications	Culture-independent (NGS, V1–V2 region of 16S rRNA genes)	Esophageal string test	Middle to distal	Enrichment in Proteobacteria (<i>Haemophilus</i>) and decrease of Firmicutes in active EoE	50

CME, chagasic megaesophagus; E, reflux-related esophagitis; EoE, eosinophilic esophagitis; GI, gastrointestinal; NE, normal esophagus; NGS, next-generation sequencing; GERD, gastroesophageal reflux disease.

*et al.*⁴² In that study, although microbial growth was observed only from a small subset of samples (i.e., four and three samples from BE and controls, respectively), the authors observed a uniquely high level of colonization by *Campylobacter* spp. (namely *C. concisus* and *C. rectus*) in BE and hypothesized a potential role of this pathogen in disease progression to EAC.

In 2009, following a large-scale, culture-independent study (i.e., 6800 16S bacterial rRNA gene clones sequenced from 34 individuals), Yang *et al.*⁴³ first identified two distinct patterns of esophageal microbiota associated with normal and abnormal esophageal mucosa. In particular, a microbiota dominated by Gram-positive taxa of the *Streptococcus* genus (phylum Firmicutes), named type I microbiota, was found to characterize the normal esophageal mucosa, while a shift toward a microbiota enriched in Gram-negative taxa (e.g., *Veillonella*, phylum Firmicutes; *Prevotella*, phylum Bacteroidetes; *Haemophilus*, *Neisseria*, and *Campylobacter*, phylum Proteobacteria; *Fusobacterium*, phylum Fusobacteria), named type II microbiota, was found to be associated with the esophageal mucosa in cases of GERD-related esophagitis and BE. The presence of distinct microbiota compositions in normal esophagus, GERD-related esophagitis, and BE was also confirmed in a subsequent study, performed with a similar approach, although with a lower discrimination power (i.e.,

424 bacterial 16S rRNA gene clones sequenced from 18 individuals).⁴⁴ Indeed, in that study, Liu *et al.* observed that, compared with healthy controls, the esophageal microbiota in GERD-related esophagitis and BE was enriched in *Prevotella* (phylum Bacteroidetes) and *Fusobacterium* (phylum Fusobacteria), and showed differences in the relative abundance of Firmicutes (a decrease of *Streptococcus* and an enrichment in *Veillonella*, mostly in BE) and of Proteobacteria (an overall decrease, but with enrichment in *Neisseria*).⁴⁴

On the other hand, the first study that used NGS technologies for characterization of the esophageal microbiota failed to detect significant differences between patients with normal mucosa and patients with reflux-related esophagitis or BE.⁴⁶ These authors hypothesized that the lack of identification of the previously described type I and type II esophageal microbiota in healthy and diseased esophagus could be due to issues related to the experimental design and the methodological approach (e.g., in this study, all controls had GERD symptoms, and samples were obtained from normal-appearing mucosa above the diseased tissue,⁴⁶ while in the study by Yang *et al.*,⁴³ only one-third of controls had GERD symptoms, and samples were obtained from diseased mucosa). Moreover, this study first demonstrated a remarkable effect of proton pump inhibitor (PPI) treatment on the microbiota composition in the esophageal

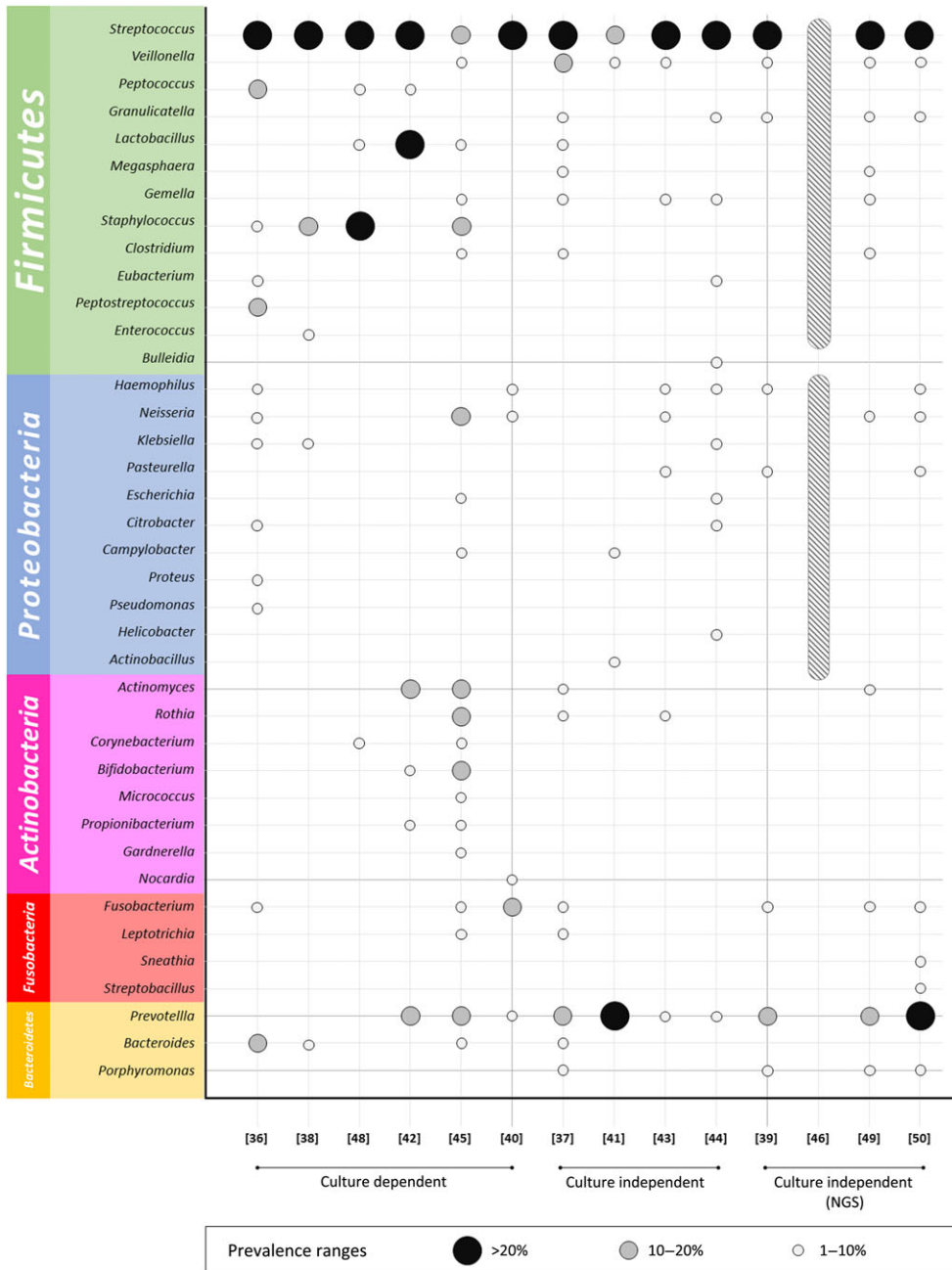


Figure 1. Overview of the frequency and relative abundance of bacterial taxa (at the genus level) associated with the healthy esophageal mucosa reported by studies investigating the composition of the esophageal microbiota in healthy individuals. The presence of each bacterial genus is indicated by a circle whose size and color indicate the relative abundance: black-filled larger circles represent bacterial genera with a relative abundance $\geq 20\%$, gray-filled intermediate circles represent genera with a relative abundance $\geq 10\%$ and $< 20\%$, and white-filled smaller circles represent genera with a relative abundance $\geq 1\%$ and $< 10\%$. For Ref. 49, the reported frequencies were calculated on rarefied OTUs provided as supplemental material. Bacterial genera with an abundance $< 1\%$ and cases where classification at the genus level was not available (e.g., the unidentified oral bacteria in Ref. 41) are not reported. Clustering of genera in different phyla are also shown. For Ref. 46, data at the genus level were not available, and the predominant phyla are indicated by striped boxes. When data for different types of esophageal specimens were available (e.g., in Refs. 39, 42, and 40), only those related to biopsy specimens were considered and included for comparison. For Ref. 40, the reported data refer to lower esophageal samples.

mucosa. In particular, PPI treatment was found to be associated with an enrichment in several members of the phylum Firmicutes (e.g., *Clostridiaceae* and *Lachnospiraceae*) and with a decrease in members of the phylum Proteobacteria (e.g., *Comamonadaceae*). Since, in the study by Yang *et al.*,⁴³ the information on PPI treatment was not available, it is possible that some of the observed shifts might be associated with PPI treatment, and this could represent an additional factor contributing to the lack of consistency in the results of the two studies.

More recently, Gall *et al.*⁵⁹ observed that *Streptococcus* and *Prevotella* dominated the esophageal microbiota of a cohort of BE patients, with no substantial intraindividual differences between normal and metaplastic esophageal mucosa. In that study, the authors also pointed out that the *Streptococcus* to *Prevotella* ratio was significantly associated with some important risk factors for BE and EAC (e.g., waist-to-hip ratio, hiatal hernia length) and encouraged further studies to investigate the clinical significance of this finding.

The microbiota composition in esophageal cancers has been investigated since the early 1980s,³⁴ and during the last three decades it has been studied using diverse culture-dependent and culture-independent approaches (Table 2). The interest in such studies arises from the recognition that gastrointestinal tract dysbiosis may play a role in disease initiation and perpetuation.^{20,26–31,60–62} The mechanisms accounting for dysbiosis-mediated carcinogenesis could involve both the induction of a cancer-promoting inflammatory response (mainly mediated by innate immunity) and the production of procarcinogenic compounds by the bacterial taxa enriched in dysbiosis.^{20,26,28–31,60,61,63} In fact, innate immunity receptors, such as Toll-like receptors and Nod-like receptors, are major effectors in the maintenance of the gastrointestinal microbiota equilibrium, and their activation in the presence of dysbiosis has been demonstrated to trigger an inflammatory response promoting carcinogenesis.^{20,26,28–31,60,61} Inflammation, in turn, is known to contribute to dysbiosis by several still incompletely understood mechanisms (e.g., inflammation-derived increased availability of nitrates may promote enrichment of facultative anaerobes, such as *Enterobacteriaceae*, which are able to use nitrates as electron acceptors), giving

rise to the vicious cycle of dysbiosis–inflammation–dysbiosis.^{20,26,28–31,60,61} Besides the activation of innate immunity and the possible direct proinflammatory potential, certain bacteria are also capable of modulating carcinogenesis through the production of genotoxic compounds (e.g., cytotoxic distending toxin and colibactin, produced by members of *Enterobacteriaceae*, *Campylobacter* spp., and *Helicobacter* spp.)^{8,64,65} or other procarcinogenic metabolites (e.g., superoxide and hydrogen sulfide, produced by *Enterococcus faecalis* and *Fusobacterium*, respectively).^{66,67}

The earlier studies on the correlation between microbiota and esophageal cancers suffered from the intrinsic limitations of culture-dependent approaches, the absence of control groups, and/or the lack of histological differentiation between ESCC and EAC, which are known to have distinct risk profiles (Table 2).^{34–36} In 2004, Narikiyo *et al.*⁶⁸ first used a culture-independent approach for the characterization of the microbiota in esophageal cancers, even though it is not clear if the study included ESCC, EAC, or both. In particular, Narikiyo *et al.* characterized the microbiota of normal and cancerous esophageal tissue in 20 patients undergoing surgery for esophageal carcinoma and observed that both were consistently dominated by *Treponema denticola* and streptococci, including *S. mitis* and *S. anginosus*. On the basis of the detection of those three species in a number of additional biopsies of esophageal cancer from different countries (by polymerase chain reaction amplification with species-specific primers), and considering their proinflammatory potential, the authors suggested that these three species could play roles in the carcinogenic progress.

More recently, Blackett *et al.*,⁴⁵ using a mixed culture-dependent and culture-independent approach, investigated the role of microbiota in the progression of EAC through the GER–esophagitis–intestinal metaplasia–dysplasia–adenocarcinoma cascade and observed a significant enrichment in *Campylobacter* (mostly *C. concisus*) in GERD and BE, compared with controls and esophageal EAC. On the basis of the observation that *Campylobacter* spp. (mostly *C. concisus*) are among the Gram-negative species consistently enriched in type II esophageal microbiota^{42,43,45} and on their overall higher prevalence in early stages of the EAC cascade (i.e., higher prevalence in GERD–esophagitis and

BE compared to EAC and controls),⁴⁵ it has been proposed that this pathogen could play a role in EAC progression similar to that of *Helicobacter pylori* in gastric cancer.⁶⁹ *C. concisus* can colonize the oral cavity and has recently been recognized as a potential human pathogen, with diverse pathotypes being identified in addition to commensal strains.^{70,71} Chronic esophageal colonization by virulent *C. concisus* strains, favored by the inflammation and dysbiosis associated with GER, could induce augmentation of the inflammatory and metaplastic responses, favoring progression to EAC. Indeed, virulent *C. concisus* strains have been shown to induce the expression of proinflammatory cytokines associated with carcinogenesis.⁶⁹ On the other hand, the lower prevalence of this pathogen in EAC would be consistent with the “driver–passenger” theory (i.e., a “driver” pathogen would be responsible for the initial steps in carcinogenesis, in turn favoring the proliferation of “passenger” bacteria with a competitive advantage in the tumor microenvironment and involved in disease progression)⁷² and would mirror the lower prevalence of *H. pylori* in gastric cancers.⁶⁹

Besides the specific role of *Campylobacter* spp., the GERD-related dysbiosis, characterized by the shift from type I to type II esophageal microbiota, has been suggested to contribute to EAC development.^{26,28–31} In the proposed pathogenetic scheme, a refluxated esophageal mucosa would initially favor the switch between a Gram positive-dominated microbiota (i.e., type I microbiota) to a Gram negative-enriched one (i.e., type II microbiota), owing to the lower susceptibility of many Gram-negative species to acidic pH and bile salts. In turn, the increased abundance of Gram-negative bacteria would induce a strong inflammatory response through lipopolysaccharide (LPS, a component of Gram-negative outer membranes)-mediated activation of innate immunity, initiating the vicious cycle of dysbiosis–inflammation–dysbiosis.^{26,28–31,60,61} Exacerbated innate immunity signaling has been demonstrated for GERD—esophagitis, BE, and EAC,^{26,28–31} and also in a recent animal model of EAC.⁴⁷ In addition, LPS has been associated with a dose-dependent decrease of the basal tone of the lower esophageal sphincter and with a delay in gastric emptying, both factors contributing to the maintenance and exacerbation of GER.^{26,28–31}

Concerning ESCC, a negative correlation between esophageal microbial richness and esophageal squamous dysplasia (ESD) has recently been observed using a culture-independent approach,⁷³ suggesting that individuals with a lower esophageal microbial complexity could be more prone to develop ESD. An additional study performed using an NGS approach demonstrated an enrichment in *Clostridiales* and *Erysipelotrichales* (phylum Firmicutes) in the gastric corpus microbiota of patients affected by ESD and ESCC compared with controls, pointing to a possible involvement of gastric dysbiosis in ESD–ESCC progression.⁷⁴ Recently, a possible role for specific bacteria in the pathogenesis of ESCC has also been investigated by Gao *et al.*,⁷⁵ who focused on the possible relationships between *Porphyromonas gingivalis*, an oral pathogen thought to be involved in oral squamous cell carcinoma tumorigenesis, and ESCC, given the histological similarity of the two cancers. The authors reported that *P. gingivalis* could selectively infect the cancerous and adjacent esophageal mucosa of ESCC subjects but not the healthy mucosa of controls. Moreover, the presence of *P. gingivalis* was positively correlated with ESCC severity (i.e., cancer cell differentiation, metastasis) and poor prognosis (i.e., ESCC survival rate) and, therefore, it has been suggested that *P. gingivalis* may serve as biomarker of ESCC.

Microbiota in uncommon esophageal diseases

Two recent studies (Table 3)^{49,50} investigated, using NGS approaches, the esophageal microbiota in eosinophilic esophagitis (EoE), an allergic disease characterized by a dense eosinophilic infiltrate within the esophageal mucosa.⁷⁶ Despite differences in the study design and experimental procedures, both studies identified a distinct esophageal microbiota in active EoE (i.e., with ≥ 15 eosinophils/high-power field) compared to controls.

Benitez *et al.*⁴⁹ observed a shift in the relative abundance of specific taxa in active EoE compared with controls. In particular, *Neisseria* and *Corynebacterium* were significantly more abundant in the active EoE cohort, while Firmicutes (including *Streptococcus* and *Atopobium*) were consistently enriched among controls. Interestingly, no significant differences were observed between inactive EoE (i.e., < 15 eosinophils/high-power field) and controls, suggesting a major role of the esophageal

inflammatory state in modulating the local microbiota composition. In support of this finding, the authors also observed an increase in the relative abundance of *Granulicatella* (phylum Firmicutes) and *Campylobacter* (phylum Proteobacteria) following the reintroduction of highly allergenic foods in EoE patients previously treated with an empiric six food–elimination diet, a situation that leads to an increased esophageal inflammatory state. Overall concordant results, in terms of inflammation-mediated modulation of microbiota composition, were obtained in the study by Harris *et al.*,⁵⁰ who observed an enrichment in Proteobacteria (mostly *Haemophilus*) and a decrease in Firmicutes in patients with active EoE compared with inactive EoE and controls. In the same study, a modulatory effect of PPI treatment was also observed in a small number of GERD patients (i.e., decrease of Firmicutes and increase in Proteobacteria), which did not confirm what was previously observed by Amir *et al.*⁴⁶ and points out the need to further investigate the effects of PPIs on the esophageal microbiota.

A single study has investigated the esophageal microbiota composition in patients affected by chagasic megaesophagus (a sequela of *Trypanosoma cruzi* infection), using a culture-dependent approach (Table 3).⁴⁸ In addition to a higher bacterial load related to chronic stasis, a greater variability and an apparent enrichment in *Veillonella* were observed, compared with controls. Acknowledging that chagasic megaesophagus represents an important risk factor for ESCC, the authors encouraged further studies to assess the potential role of a procarcinogenic dysbiosis in disease progression.

Conclusions and perspectives

An increasing number of studies published during the last decades have significantly advanced our knowledge on the composition of esophageal microbiota in healthy subjects (Table 1) and have underscored a possible role of dysbiotic conditions in the pathogenesis of some esophageal diseases, especially those related to GER (Tables 2 and 3). However, experimental findings have been variable, and a current understanding of these aspects remains limited. Therefore, additional studies are warranted to confirm causative relationships between dysbiosis or specific bacterial species and GERD-related or other esophageal diseases and to establish the underlying molecular mechanisms.

When planning similar studies, it must be considered that investigation of the esophageal microbiota in health and disease poses a number of methodological and technological challenges. First, obtaining samples requires invasive procedures that are not always acceptable, and taking longitudinal samples from the same subject can be difficult or impossible. These aspects have negatively affected the performance of long-term longitudinal studies and limited the recruitment of large human cohorts, as demonstrated by the limited number of enrolled subjects in various studies (Tables 1–3). Second, the interactions within the esophageal environment are difficult to reproduce *in vitro*, which limits the possibility of studying the esophageal ecosystem using *in vitro* models,^{77–79} while the available animal models to study esophageal diseases (e.g., the BE animal model based on a surgical anastomosis in the rat) are complex and have several shortcomings, including species differences, the requirement of surgical expertise, duration, and costs.⁸⁰ Third, culture-dependent methods can contribute only partially to depicting the population structure of the resident microbiota, since several taxa are difficult or impossible to cultivate. In this context, the use of culture-independent approaches, based on molecular analysis of metagenomic libraries, has greatly helped in overcoming the limitations of conventional culture-dependent approaches, allowing for a higher analytical resolution and providing for a more comprehensive picture of the microbial communities associated with the esophageal mucosa.^{37,39,41,43–47,49,50,59,68} However, metagenomic data alone cannot determine whether an organism is alive or whether only DNA traces are present. Moreover, in culture-independent approaches, significant biases could be introduced during the sample-processing steps, from the DNA extraction to the amplification and sequencing of 16S rRNA genes, which can be responsible for artifactual diversity in the microbiota composition.⁸¹ Fourth, the study design must be carefully considered in terms of patient characteristics, inclusion and exclusion criteria, and sampling criteria, which can be critical for the comparative analysis of results. The presence or absence of GERD symptoms, for example, has been indicated among the causes responsible for data discrepancies.⁴⁶ Moreover, exclusion criteria were highly heterogeneous or not always clearly specified, particularly concerning the use

of some drugs that can influence the composition of the microbiota (e.g., antibiotics, PPIs, non-steroidal anti-inflammatory drugs, probiotics)^{82–84} (Tables 1–3). Another variable aspect related to the use of drugs is the time between the last drug administration and endoscopy considered at risk for alterations in esophageal microbiota (from a minimum of 2 to a maximum of 8 weeks), but in fact no information is currently available on how long the resident microbiota could take to completely restore its original composition and structure (resilience)¹⁰ within the esophagus. Another source of variability may be the sampling methodology, which can differ in terms of the type of sample (biopsy, brush, aspirate) (Tables 1–3). Most studies have investigated the esophageal microbiota using tissue biopsies, which would be the most suitable specimen, considering the presence of a mucosal adherent microbiota at this body site. However, sampling by brushes or aspirates was adopted by some authors, and a less invasive sampling method has been recently proposed and used as an alternative to esophageal biopsies.^{39,50} However, despite the choice of the sampling method, esophageal biopsies are always necessary for a direct histological examination of the diseased tissues in order to avoid misclassification and require additional invasive procedures. Moreover, taking esophageal samples is often associated with the risk of contamination by oropharyngeal or gastric secretions.

In this scenario, the following aspects would seem important for future investigations: (1) enrollment of larger cohorts of homogeneous categories of subjects and inclusion of longitudinal follow-ups; (2) expanding the studies addressed to investigate the transcriptomic and metabolomic aspects of the esophageal microbiota; and (3) association of microbiota investigations with immunological investigations to clarify the potential role of microbial components in induction/persistence of the inflammatory status that characterizes the evolution of GERD to BE and EAC. In particular, it would be important to confirm and better clarify the potential role played by dysbiosis of the esophageal microbiota or of specific pathogens in the multistep evolution model of GERD toward EAC, which still remains unclear.

Conflicts of interest

The authors declare no conflicts of interest.

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