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## Microbiome in Reflux Disorders and Esophageal Adenocarcinoma

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### Abstract

The incidence of esophageal adenocarcinoma has increased dramatically in the United States and Europe since the 1970s without apparent cause. Although specific host factors can affect risk of disease, such a rapid increase in incidence must be predominantly environmental. In the stomach, infection with *Helicobacter pylori* has been linked to chronic atrophic gastritis, an inflammatory precursor of gastric adenocarcinoma. However, the role of *H. pylori* in the development of esophageal adenocarcinoma is not well established.

Meanwhile, several studies have established that a complex microbiome in the distal esophagus might play a more direct role. Transformation of the microbiome in precursor states to esophageal adenocarcinoma—reflux esophagitis and Barrett’s metaplasia—from a predominance of gram-positive bacteria to mostly gram-negative bacteria raises the possibility that dysbiosis is contributing to pathogenesis. However, knowledge of the microbiome in esophageal adenocarcinoma itself is lacking. Microbiome studies open a new avenue to the understanding of the etiology and pathogenesis of reflux disorders.

### Keywords

GERD; reflux esophagitis; Barrett’s esophagus; adenocarcinoma; microbiome; inflammation; cancer; 16S rRNA gene; sequencing; risk factors

## Introduction

Squamous cell carcinoma and adenocarcinoma are the two most common types of esophageal cancer. In the United States and Europe, squamous cell carcinoma made up the vast majority of esophageal cancers until the 1970s, when it was surpassed by adenocarcinoma.<sup>1</sup> In recent years, it has become clear that esophageal adenocarcinoma (EA) is a consequence of longstanding reflux esophagitis (RE), an inflammatory condition of the distal esophagus.<sup>2</sup> This sequence often progresses through Barrett's esophagus (BE), a metaplastic malady that may become dysplastic.<sup>3,4</sup>

The pathophysiology of RE is complex and involves diverse factors, including gastroesophageal reflux, gastric acid secretion, dysfunction of the anti-reflux barrier, gastric emptying disturbances, and abnormalities in esophageal defense mechanisms.<sup>5</sup> As with other chronic inflammatory conditions, RE is associated with an increased risk of developing cancer: patients with RE have a 2- to 40-fold increased risk of developing EA. Relative risk increases to 30–400 in patients with BE<sup>3</sup>.

The incidence of EA in the U.S. has increased 6-fold since the 1970s<sup>1</sup>. Although host factors can predispose toward disease, such a rapid shift must be predominantly environmental. The current understanding of the etiology of EA is mainly derived from epidemiological studies of risk factors.<sup>6</sup> Cigarette smoking, obesity, gastroesophageal reflux disorders (GERD), and low fruit and vegetable consumption account for 39.7%, 41.1%, 29.7%, and 15.3% of EA, respectively, with a combined population attributable risk of 78.7%.<sup>7</sup> However, few studies have investigated the factors driving the increase in incidence of EA. Given the advent of widespread antibiotic use preceded the surge of EA, it has been hypothesized that the microbiome in GERD is altered, and that chronic exposure to an abnormal microbiome is carcinogenic.<sup>8,9</sup>

An enormous number of microorganisms, the vast majority of which are bacterial species, are known to colonize and form complex communities, or microbiota, at various sites within the human body. The human microbiota is estimated to be composed of  $\sim 10^{14}$  bacterial cells, which is 10 times more than the total number of human cells. The host relationship with these microorganisms can range from mutualism to pathogenicity.<sup>10,11</sup> In various diseases, the best example being inflammatory bowel disease, the microbiota appears to play a key pathogenic role.<sup>11</sup> On the other hand, bacterial mutualists within the gastrointestinal tract benefit the human host, aiding digestion, assisting in the synthesis of vitamins, promoting the development of the gut immune system, and providing competitive barriers to pathogen invasion. In return, the host provides these bacteria with safe housing and nutrients during lean times. To sustain this symbiotic relationship, the immune system has to balance permissive, tolerogenic responses to food antigens and commensal microbes with potentially damaging inflammatory responses to pathogens.<sup>12</sup>

Two theories can explain bacterial disease. The classic pathogen theory, attributed to Koch, ascribes the cause of disease to specific pathogens—typically, one or several bacteria. The microecologic disease theory, or the “Pathogenic Microbial Community” theory, is a new

model in which the entire community contributes to pathogenicity, with no individual community members being classified as pathogens.<sup>13,14</sup> Dysbiosis is a similar concept that refers to an abnormal state of the microbial ecosystem, dividing commensal bacteria into “protective” and “harmful” species. With dysbiosis, the cause of certain chronic diseases is an upset balance between the two groups.<sup>13</sup>

Recent studies of a small number of hosts have shown that there is a complex microbial community in the distal esophagus.<sup>8,9,13,15,16,17,18</sup> Although the exposure and response of the esophageal epithelium to gastric contents has been extensively investigated, little attention has been given to the microbiome, its effect on the esophageal epithelial layer, and its potential changes in GERD.<sup>13</sup> In this review, we present and summarize the most recent advances in the field of microbiology pertinent to GERD and EA.

### Early studies of the normal esophageal microflora

The first studies of the esophageal biota were in the field of surgery, where bacteria may play a role in post-operative infection.<sup>19,20,21</sup> This work was burdened by the contemporary belief that no indigenous bacteria populated the esophagus. Cultivation of luminal washes was unable to consistently show the presence of bacteria (Table 1). The few microbes isolated by conventional culture were suspected to be transiently deposited, either from the oropharynx by swallowing, or from the stomach by reflux.<sup>22</sup> Only a countable few bacterial species were detected—far fewer than the 280 species that have been directly observed in the oral cavity by isolation from culture.<sup>23</sup>

These data reflect the limitations of cultivation as a technique. Cultivation, when combined with selective media and chemical tests, is useful for isolation and characterization of single bacterial pathogens, but is not suitable for use in defining a complex microbial community. Cultivation is unable to identify bacteria in a viable but nonculturable (VBNC) state.<sup>24</sup> Further, cultivation introduces bias by selecting for bacteria capable of growing on artificial media and overlooking those whose nutrition needs are undetermined. Uncertainty about the esophageal microbiome was not resolved until cultivation-independent technology became widely applied to human microbiome studies in the early 2000s (Table 2).<sup>25</sup>

16S rRNA genes, which are widely-used to estimate the evolutionary history and taxonomic assignment of individual organisms, have become the basis of the most common cultivation-independent technique.<sup>26,27,28</sup> This approach began in the 1980s, when it was observed that all bacteria possess one or more 16S ribosomal RNA genes. These genes are key components of protein synthesis and are essential for life. Since all bacteria are descendants of a common ancestor, the regions of the 16S genes most critical for conformation of ribosomal structure and function have remained conserved over billions of years. PCR based on universal primers to these regions can amplify 16S rRNA genes of nearly all bacterial species. The less conserved regions whose sequences recorded the evolutionary history of individual species, meanwhile, serve as signatures for taxonomic classification. It is the combination of PCR, sequencing technology, and comprehensive 16S rRNA gene databases that has made 16S rRNA gene surveys the mainstay of cultivation-independent studies of simple and complex bacterial communities.

Using broad-range 16S rRNA gene PCR, coupled with cloning and Sanger sequencing and histology technique, Pei and colleagues examined mucosal biopsies from the distal esophagus of four normal human adults.<sup>8</sup> From analysis of 900 recovered clones, they made three key observations. First, an esophageal microbiome is invariably present (Table 2). Second, the esophageal microbiome is comparable to the oral microbiome, as they share the same six major phyla (*Firmicutes*, *Bacteroides*, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *TM7*) and are both uneven communities dominated by the genus *Streptococcus*. Third, bacteria are visible on the mucosal surface by tissue gram-stain. Later, Macfarlane and colleagues demonstrated viable bacteria on normal esophageal mucosa in microcolonies/aggregates (Fig. 1).<sup>15</sup> These observations established the existence of an esophageal microbiome and laid the foundation for later studies of its role in disease.

### Microbiome in GERD

The first study to apply cultivation-independent technique to the microbiome in esophageal disease was reported in 2005, with the goal of demonstrating feasibility.<sup>9</sup> Two 16S rRNA gene clones were recovered and examined from each of the esophageal biopsies taken from 24 subjects (9 with normal mucosa, 12 with GERD, and 3 with BE). As expected, bacterial signals were successfully detected in all biopsies, and the overall diversity and community membership resembled those of the normal esophageal microbiome. A large scale survey to compare the esophageal microbiome among subjects with RE, BE, and healthy esophagus was performed by Yang and coworkers in 2009.<sup>13</sup> It was one of the largest human microbiome studies to date, with a total of 6,800 16S rRNA gene clones from 34 subjects analyzed by Sanger sequencing. Using unsupervised cluster analysis and phenotype-guided analyses, samples were found to contain one of two distinct microbiomes. Microbiome type I was mainly associated with a normal esophagus and was dominated by gram-positive bacteria from the *Firmicutes* phylum, of which *Streptococcus* was the most dominant genus. Microbiome type II had greater proportion of gram-negative anaerobes/microaerophiles (phyla *Bacteroidetes*, *Proteobacteria*, *Fusobacteria*, and *Spirochaetes*) and primarily correlated with RE (odds ratio, 15.4) and BE (odds ratio, 16.5). The microbiome did not differ between GERD and BE patients. Recently, a study in Japan used cloning and Sanger sequencing to analyze the esophageal microbiome of 18 subjects (6 each with normal esophagus, RE, and BE).<sup>18</sup> A unique test performed was quantification of total bacterial loads by quantitative 16S rRNA gene PCR. Notably, the three groups harbored similar numbers of bacteria, equivalent to  $10^6$ – $10^7$  colony forming units per sample. Thus, changes in the relative abundance of taxa, rather than absolute bacterial loads, are likely more relevant to esophageal diseases. Although far from comprehensive (only approximately 24 clones were sequenced per sample), the study found *Veillonella* (19%), *Prevotella* (12%), *Neisseria* (4%), and *Fusobacterium* (9%) to be more prevalent in patients with RE and BE than in controls. These observations support those published by Yang and Pei, confirming that the esophageal microbiome is reliably altered in reflux disorders.<sup>9,13</sup>

Osias et al. quantified bacteria by staining of biopsies from esophageal diseases.<sup>29</sup> Bacteria were detected more often in BE than non-BE, and increasing bacterial stain scores were associated both with metaplasia and increasing dysplasia. Interestingly, Macfarlane and colleagues found *Campylobacter* species (*C. concisus* and *C. rectus*)—which have been

implicated in the pathogenesis of enteritis, periodontal diseases, and tumorigenesis in animal models—in the majority of BE patients but none of the controls (Figure 1).<sup>15</sup> Fluorescence in situ hybridization using specific 16S ribosomal RNA oligonucleotide probes revealed colonization of Barrett's esophageal mucosae by *Campylobacter* species (Fig. 1).

### Microbiome in gastroesophageal adenocarcinoma

Nearly all studies of the local microbiome associated with esophageal cancer do not distinguish between adenocarcinoma and squamous cell carcinoma (Table 1).<sup>17,19,20,21</sup> Because these two cancers have marked differences in epidemiology and etiology, these studies cannot be assumed to be applicable to EA and thus will not be discussed here. To date, there has been only one published study to address the link between the microbiome and EA, in which Blackett and colleagues compared 30 cases of EA with 39 cases of controls using culture analysis.<sup>16</sup> The study recovered more species (n=73) in EA than controls (n=56). However, no statistical difference in specific taxa was reported.

### Role of *Helicobacter pylori*

Case-control studies have suggested that *H. pylori* gastritis may play a protective role in the development of GERD and associated EA. However, eradication of *H. pylori* does not increase new GERD cases or worsen GERD symptoms (except in patients with hiatal hernia and corpus gastritis).<sup>30</sup> The role of *H. pylori* in the pathogenesis of GERD, BE and EA remains an unclear and controversial topic that has been extensively reviewed elsewhere.<sup>31</sup>

**Perspectives**—Esophageal microbiology is an understudied field, especially in EA. Data from the few available studies have established a convincing association between an altered microbiome and the reflux disorders that precede EA. A large scale study of the microbiome in the development of EA has been funded under the NIH Human Microbiome Project and may fill the knowledge gap.<sup>32, 33</sup> Prospective studies to explore whether the microbiome changes before or after onset of disease are the next logical step to evaluate causality. Large cohorts such as the National Cancer Institute-Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial Cohort (NCI-PLCO) and the American Cancer Society Cancer Prevention Study II Cohort (ACS-CPS-II) provide access to tissue samples collected prior to cancer diagnosis and therefore should prove invaluable to further characterize the role of the esophageal microbiome in carcinogenesis.<sup>34,35</sup>

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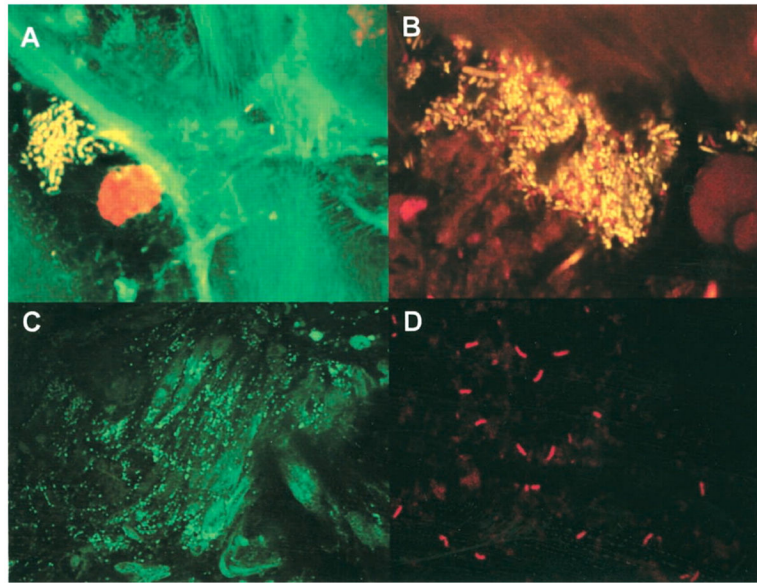
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**Figure 1.**

In situ visualization of bacteria in esophageal mucosal biopsies. A and B: Confocal sections of mucosal biopsy specimens stained for cell viability, containing mixtures of living (yellow) and dead (red) organisms. Microcolony and aggregate formation can be seen in mucosal samples from control subjects (A) and from patients with Barrett's esophagus (B). C and D: Fluorescence light micrographs of transverse sections of Barrett's esophageal mucosae showing colonization by streptococci (C) and *Campylobacter* species (D), using 16S ribosomal RNA oligonucleotide probes labeled with FITC and cy3. Figure originally published in *Journal of Clinical Infectious Diseases*<sup>15</sup> and permission to reuse of the figure is granted by the Journal.



**Table 1**

Summary of culture-based studies on esophageal microbiome (1981–2013)

| Category                                  | Lau <sup>19</sup> 1981 | Finlay <sup>20</sup> 1982 | Mannell <sup>21</sup> 1983 | Gagliardi <sup>22</sup> 1998 | Macfarlane <sup>15</sup> 2007 | Blackett <sup>16</sup> 2013 |
|---|------------------------|---------------------------|----------------------------|------------------------------|-------------------------------|-----------------------------|
| Disease                                   | Cancer                 | Cancer                    | Cancer                     | Normal                       | Normal BE                     | Normal GERD BE, EA          |
| Specimen                                  | Aspirate               | Resection                 | Aspirate                   | Aspirate                     | Biopsy Aspirate               | Biopsy                      |
| Number of cases                           | 79                     | 12                        | 101                        | 30                           | 14                            | 151                         |
| Number of isolates                        | 61                     | 85                        | 377                        | 30                           | ND                            | ND                          |
| Number of species                         | 14                     | 15                        | 32                         | 11                           | 46                            | 111                         |
| Mean species per case                     | 1                      | 6                         | 4                          | 1                            | ND                            | ND                          |
| % cases positive for bacteria             | 64                     | 100                       | 100                        | 67                           | 50–71%                        | 100                         |
| % cases positive for <i>Streptococcus</i> | 10                     | 92                        | ND                         | ND                           | 50                            | ND                          |

ND: not determined or reported.

**Table 2**  
 Summary of cultivation-independent studies on esophageal microbiome (2004–2013)

| Category                                  | Pet <sup>8</sup> 2004 | Narikiyo <sup>17</sup> 2004 | Pet <sup>9</sup> 2005 | Yang <sup>13</sup> 2009 | Liu <sup>18</sup> 2013 |
|---|-----------------------|-----------------------------|-----------------------|-------------------------|------------------------|
| Disease                                   | Normal                | Cancer                      | RE<br>BE              | Normal<br>RE, BE        | Normal<br>RE, BE       |
| Specimen                                  | Biopsy                | Biopsy                      | Biopsy                | Biopsy                  | Biopsy<br>Aspirate     |
| No. cases                                 | 4                     | 20                          | 24                    | 34                      | 18                     |
| No. clones                                | 900                   | 100                         | 147                   | 6800                    | 424                    |
| No. species                               | 95                    | 7                           | 39                    | 166                     | ND                     |
| Mean species per case                     | 43                    | 6                           | ND                    | ND                      | ND                     |
| % cases positive for bacteria             | 100                   | ND                          | 100                   | 100                     | 100                    |
| % cases positive for <i>Streptococcus</i> | 100                   | 87                          | ND                    | 100                     | 83.3                   |