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# Alterations to the Esophageal Microbiome Associated with Progression from Barrett's Esophagus to Esophageal Adenocarcinoma

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## Abstract

**Background:** The incidence of esophageal adenocarcinoma (EAC) has risen dramatically over the past half century, and the underlying reasons are incompletely understood. Broad shifts to the upper gastrointestinal microbiome may be partly responsible. The goal of this study was to describe alterations in the esophageal microbiome that occur with progression from Barrett's esophagus (BE) to EAC.

**Methods:** A case-control study of patients with and without BE who were scheduled to undergo upper endoscopy. Demographic, clinical, and dietary intake data were collected, and esophageal brushings were collected during the endoscopy. 16S rRNA gene sequencing was performed to characterize the microbiome.

**Results:** A total of 45 patients were enrolled and included in the analyses (16 controls; 14 BE without dysplasia (NDBE); 6 low grade dysplasia (LGD); 5 high grade dysplasia (HGD); and 4 EAC). There was no difference in alpha diversity between non-BE and BE, but there was evidence of decreased diversity in patients with EAC as assessed by Simpson index. There was an apparent

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SPECIFIC AUTHOR CONTRIBUTIONS

EJS: planning of the study, conducting the study, data collection, data analysis, data interpretation, drafting the manuscript. EJS approved the final version of the submitted manuscript.

GC: conducting the study, data collection. GC approved the final version of the submitted manuscript.

DEF: data interpretation, critical revision of the manuscript. DEF approved the final version of the submitted manuscript.

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shift in composition at the transition from LGD to HGD, and patients with HGD and EAC had decreased Firmicutes and increased Proteobacteria. Additionally, patients with HGD or EAC had increased *Enterobacteriaceae* and *Akkermansia muciniphila* and reduced *Veillonella*. In the study population, patients taking proton pump inhibitors had increased *Streptococcus* and decreased Gram-negative bacteria overall.

**Conclusions:** Shifts in the BE-associated microbiome were observed in patients with HGD and EAC, with increases in certain potentially pathogenic bacteria.

**Impact:** The microbiome may play a role in esophageal carcinogenesis.

## INTRODUCTION

The incidence of esophageal adenocarcinoma (EAC) has increased 10-fold since the late 1960s (1), and Barrett's esophagus (BE) incidence likely began to rise as early as the 1950s. Known modifiable risk factors for EAC do not adequately explain these incidence trends. GERD prevalence began to rise in the 1970s (2,3), and modeling studies suggest that only a minority of EAC cases are attributable to GERD (4). The obesity epidemic did not begin until 1980, and obesity may only account for a small fraction of the rise in EAC (5).

*Helicobacter pylori* infection is associated with a 30-40% reduced risk of BE and EAC (6), and *H. pylori* prevalence has plummeted since the mid-20th century (7). When present, *H. pylori* dominates the gastric microbiome, and its absence results in major shifts to gastric microbiome composition (8,9). Thus, dramatic changes in the upper GI microbiome in western populations likely occurred at the same time that BE and subsequently EAC began to rise in incidence. Any role of the microbiome the development of EAC is likely complex and multi-factorial, and may represent a co-factor in the development of BE, the progression from BE to EAC, or both.

There is ample evidence that elements of the microbiome can directly contribute to the development of colon cancer (10). However, the role of the microbiome in the progression of Barrett's esophagus (BE) to EAC has not been well described. In health, the esophageal microbiome is broadly similar in composition to the oral microbiome, with a high relative abundance of the phylum Firmicutes (11). Previously published data suggest that the esophageal microbiome in patients with reflux esophagitis or BE is heavily populated with Gram-negative bacteria, which may contribute to a chronic inflammatory, pro-neoplastic state (12,13). More recent analyses of EAC surgical resections have shown that the tumor-associated microbiome demonstrates decreased microbial richness and diversity compared to non-dysplastic BE and normal squamous tissue (14).

In order to understand the potential role of the microbiome in esophageal carcinogenesis, knowledge of microbiome alterations that occur along the neoplastic pathway from BE to EAC is needed. The current study aimed to elucidate shifts in the esophageal microbiome that occur in the setting of progression from Barrett's esophagus to associated dysplasia and adenocarcinoma.

# MATERIALS AND METHODS

## Study Population

This was a case-control study of patients ≥18 years old, enrolling subjects without or with a diagnosis of Barrett's esophagus who were scheduled to undergo upper endoscopy for clinical indications. Analysis of the salivary microbiome in these patients has been previously reported (15). Subjects were prospectively enrolled over 18 months at a single academic medical center (Columbia University Medical Center, New York, NY). Barrett's esophagus subjects had histologically confirmed BE measuring ≥2 cm, had never received endoscopic therapy, and were taking at least once daily proton pump inhibitors (PPI) for the prior month. BE subjects were categorized based on worst prior or current confirmed pathology: no dysplasia (NDBE), low grade dysplasia (LGD), high grade dysplasia (HGD), or adenocarcinoma (EAC). Controls were patients with no prior history of BE and were included if taking at least once daily PPI or no acid suppression (PPIs or H2-receptor antagonists) for the prior month. Other details of the exclusion criteria have been described previously. (15)

Demographics, clinical data, and anthropometric measures were collected. History of reflux symptoms was assessed using by questionnaire (16), and dietary fat and fiber intake over the preceding 4 weeks was used using a food frequency questionnaire (17,18). All participants provided written informed consent. The Institutional Review Board of Columbia University approved the study on February 25, 2015.

## Sample Collection

Details of the sample collections have been described previously. (15,19) The microbiome was sampled by brushing the squamous esophagus as well as BE tissue (BE patients) or gastric cardia, within 1 cm of the squamo-columnar junction (controls). Sampling of any nodules, masses, or other focal lesions was avoided, in case grossly altered topography affected bacterial colonization. Biopsies were also taken from the mid-BE segment or gastric cardia for subsequent gene expression analyses.

## Microbiome Characterization

After DNA extraction from esophageal brushings, the V4 hypervariable ribosomal RNA region was amplified using primers 515F and 806R (20). Sequencing of the 16S rRNA gene V4 region was performed, and sequence data were uploaded to the NCBI Sequence Read Archive (BioProjectID PRJNA517734). Greengenes was used as reference database (21). Clustering of taxonomic units was made at 97% sequence similarity using USEARCH. The functions classify.seqs and classify.otu (both with default settings) from the mothur project (22) were used to make taxonomic assignments to OTUs. FastTree version 2.1.7 was used to generate a phylogenetic tree of the contigs (23). Using mothur and the phylogenetic tree, weighted and unweighted UniFrac distances as well as diversity indices were calculated (24).

Semi-quantitative PCR (SsoAdvanced Universal SYBR Green Supermix, Bio-Rad, Hercules, CA) was also performed from esophageal brushing DNA for *Enterobacteriaceae* to further assess key findings from 16S rRNA gene sequencing analyses using previously published primer pairs (25). ΔΔCt values were calculated, using as a reference the Ct value for Eubacteria for the corresponding sample. qPCR for Eubacteria represents the entire bacterial DNA in the sample; thus, the ΔΔCt values were analogous to relative abundance data from 16S rRNA gene sequencing.

## Statistical Analyses

Continuous variables were analyzed using t-tests and rank sum tests, and categorical variables were analyzed using Fisher's exact tests. ANOVA or Kruskal-Wallis tests were used to compare continuous variables across multiple categories. The main analyses for this study were of brushings from Barrett's mucosa (BE patients) or gastric cardia (controls). Within-individual correlations were assessed between paired swabs from esophageal squamous lining and from paired swabs from BE or cardia by calculating Spearman rank correlation coefficients at the genus level for all genera with non-zero read counts in both of the paired swabs. There were high correlations between paired swabs from the same site within the same individual (esophageal squamous, mean rho 0.85, SD 0.15; BE or cardia, mean rho 0.86, SD 0.12). For the purpose of these analyses, the mean relative abundance for each taxon from paired swabs was calculated from each sampling site. Of note, there was also high within-individual correlation between esophageal squamous and BE or cardia brushings (mean rho 0.82, SD 0.13).

Alpha diversity was assessed by observed OTUs and Shannon and Simpson indices. Pair-wise weighted and unweighted UniFrac beta diversity was calculated using functions implemented in QIIME. Non-parametric permutational MANOVA, as implemented in the FATHOM Toolbox for MATLAB, was used to compare beta diversity measures between BE vs. controls and between NDBE/LGD vs. HGD/EAC groups. Principal coordinate analyses for these tests were also performed using functions implemented in the FATHOM Toolbox for MATLAB. Differentially abundant taxa between groups were identified using linear discriminant analysis effect size (LEfSe) (https://huttenhower.sph.harvard.edu/galaxy/). Functional composition of the esophageal microbiome was assessed using predicted metabolic pathways derived by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis (26). Analyses were performed focused on the relative abundance of Gram-negative bacteria; Gram-negative genera and species were identified using a reference list assembled by our group (Supplementary Table S1), and the relative abundances of these taxa were summed for each sample. Additional analyses were performed on relative abundance of *Streptococcus*, the most abundant genus in the esophagus; alterations in the relative abundance of this genus have been associated with a variety of esophageal conditions. (13,27,28)

Upon visual observation of relative abundance of phyla across levels of BE and associated neoplasia, it appeared that there were shifts in relative abundance of Firmicutes and Proteobacteria, the two most abundant phyla in the esophageal samples, with the transition from low- to high-grade dysplasia. (Supplementary Figure S1) Thus, additional analyses were performed with BE subjects categorized as NDBE/LGD or HGD/EAC. Multivariable linear regression analyses were performed to assess for covariates independently associated with relative abundance of differentially abundant phyla and other select taxa. Full models were created including all covariates with a univariate p-value <0.10. Variables with the highest p-value and >0.15 were then sequentially removed to generate a final reduced model. Statistical significance was defined as p<0.05. Analyses were performed using Stata 14.1 (StataCorp) and MATLAB (The MathWorks, Inc.).

# RESULTS

A total of 45 subjects were enrolled and had brushings collected for analysis. The characteristics of the subjects are shown in Table 1. There were 16 non-BE subjects and 29 subjects with BE (14 without dysplasia, 6 LGD, 5 HGD, and 4 intramucosal EAC).

## Microbiome Analyses

There were no significant differences in alpha diversity comparing BE to non-BE patients, both in terms of richness and evenness. (Supplementary Figure S2) There was decreased diversity assessed by Simpson index, but not by Shannon index or observed OTUs, across levels of BE-associated neoplasia (NDBE, LGD, HGD, and EAC). (Supplementary Figure S3) In *post hoc* pairwise comparisons, the Simpson index in EAC was significantly reduced compared to NDBE (p=0.006), LGD (p=0.01), and HGD (p=0.01). None of the other pairwise comparisons were significant. On beta-diversity analyses there was no evidence of significant clustering comparing BE vs. controls. (Supplementary Figure S4)

The most abundant phyla in the samples from BE and gastric cardia were Firmicutes (46.2%), Proteobacteria (22.9%), Bacteroidetes (19.6%), Actinobacteria (5.6%), and Fusobacteria (5.1%). BE subjects had significantly reduced relative abundance of Bacteroidetes compared to controls (16.3% vs. 25.5%, p=0.04), although there was no association after adjusting for patient characteristics. (Supplementary Table S2)

There were no overall differences in relative abundance of phyla across levels of BE-associated neoplasia. However, upon visual inspection of the results, it appeared that there was a shift in composition with regard to Firmicutes and Proteobacteria, the two predominant phyla, with the transition from LGD to HGD. (Supplementary Figure S1) Thus, subsequent analyses were performed with BE subjects categorized as (NDBE or LGD) and (HGD or EAC). Compared to NDBE/LGD, subjects with HGD/EAC had decreased relative abundance of Firmicutes (38.3% vs. 55.0%, p=0.04) and increased relative abundance of Proteobacteria (32.1% vs. 17.7%, p=0.04). (Figure 1) In multivariable analyses, HGD/EAC remained independently associated both with increased Firmicutes (p=0.03) and decreased Proteobacteria (p=0.01). (Supplementary Table S2) On beta-diversity analyses there was no evidence of significant clustering comparing HGD/EAC vs. NDBE/LGD. (Supplementary Figure S4)

## Taxonomic Differences

As compared to controls, subjects with BE had increased relative abundance of *Sphingomonas* and an unclassified species of *Campylobacter*. Non-BE subjects had increased relative abundance of various taxa including *Prevotella pallens, Porphyormonas endodontalis*, and *Aggregatibacter segnis*. (Supplementary Table S3) Based on the observations that there was a shift with transition from LGD to HGD at the phylum level, additional differences in relative abundance of taxa were assessed by LEfSe with subjects again categorized as NDBE/LGD and HGD/EAC. (Figure 2A) Patients with NDBE/LGD had significantly increased *Veillonella*. Several taxa were increased in the HGD/EAC subjects, notably in *Enterobacteriaceae* and *Verrucomicrobiaceae*, specifically *Akkermansia muciniphila*. (Figure 2B)

As members of *Enterobacteriaceae* can promote gut inflammation and neoplasia, the data on this family was examined in greater detail. Compared to NDBE/LGD, patients with HGD/EAC were more likely to be smokers (p=0.03) and had higher dietary fat intake (p=0.05). After adjusting for these two factors, HGD/EAC remained significantly associated with the relative abundance of *Enterobacteriaceae* (p=0.02). (Supplementary Table S2) Two subjects had very high relative abundance of *Enterobacteriaceae*; one of these had HGD and a relative abundance of 38.3%, and one had intramucosal EAC and a relative abundance of 30.4%. These findings were replicated in the esophageal squamous brushings, where these two subjects again had the highest relative abundance of *Enterobacteriacea* in the study population. For each of these subjects, a single distinct OTU drove the high relative abundance. On further evaluation of these OTUs using NCBI BLAST, one matched predominantly to species in the genera *Klebsiella* and *Enterobacter*, and the other matched to species in genera including *Escherichia* and *Shigella*.

Esophageal and cardia biopsies were then analyzed by qPCR to assess whether they harbored differences compared to brushings in relative abundance of *Enterobacteriaceae*. There was no significant difference by qPCR comparing patients with NDBE/LGD and HGD/EAC (median ΔΔCt 12.5 vs. 12.8, respectively; p=0.57).

## Gram-Negative Bacteria

In brushings the mean relative abundance of Gram-negative bacteria in all of the subjects was 54.7% (SD 23.0). There was no significant difference in the relative abundance of Gram-negative bacteria comparing non-BE controls to BE subjects (61.6% vs. 50.9%, p=0.14). There were also no significant alterations in the relative abundance of Gram-negative bacteria across levels of BE-associated neoplasia (ANOVA p=0.66). In the entire study population (BE and non-BE), PPI users had decreased relative abundance of Gram-negative bacteria compared to PPI non-users (51.1% vs. 67.3%; p=0.05). (Figure 3A)

## Streptococcus

The mean relative abundance of *Streptococcus* in the study population was 32.6% (SD 20.9%). There was no significant difference in the relative abundance of *Streptococcus* comparing BE patients to non-BE controls (35.7% vs. 26.9%, p=0.18) and no significant overall alteration in the relative abundance of *Streptococcus* across levels of BE-related neoplasia (ANOVA p=0.51). With regard to PPI use, all subjects (BE and non-BE) on PPIs had greater relative abundance of *Streptococcus* compared to controls not on PPIs (36.2% vs. 19.9%, p=0.03). (Figure 3B)

### Functional Profiling

PICRUSt analyses were performed to assess for functional alterations to the esophageal microbiome. Several gene pathways were significantly altered comparing patients with BE to non-BE controls. (Supplementary Figure S5A) Controls had increased RNA degradation and vitamin B6 metabolism, whereas BE patients had increased glycerolipid metabolism. Compared to patients with NDBE or LGD, those with HGD or EAC exhibited increased glycerophospholipid metabolism and decreased other glycan degradation. (Supplementary Figure S5B)

## DISCUSSION

In the current study, we assessed the Barrett's esophagus microbiome with progression to dysplasia and adenocarcinoma. We observed a shift in composition with progression, notably at the transition from LGD to HGD. This was manifested by significant clustering in beta diversity analyses, as well as alterations to the two predominant phyla, with reductions in Firmicutes and increases in Proteobacteria.

There is little previous data describing esophageal microbiome changes that occur in the development of EAC. Elliott et al. reported microbiome alterations comparing esophageal squamous samples from non-BE controls, Barrett's samples from patients without dysplasia, and tumor tissue from patients with EAC (14). The authors noted that EAC tumors had decreased alpha diversity compared to BE, and in the present study there was some evidence of a decline in diversity with progression. However, many of the specific taxonomic alterations were distinct. This may be explained in part by the fact that the EAC tumor-microbiome was analyzed in this prior study (14), whereas in the current study sampling were performed only of normal appearing Barrett's mucosa, avoiding any nodules or lesions, in patients with EAC. Also in the current study, there were high within-individual correlations between squamous and BE or cardia brushings, but the across-group alterations were less marked in squamous as compared to BE or cardia (data not shown). Finally, the EAC subjects in the current study all had very early lesions (T1a), and thus microbiome alterations in these patients would not have been caused by stasis due to tumor obstruction.

The increased relative abundance of *Enterobacteriaceae* in esophageal brushings from patients with HGD and EAC has potential biological significance. Certain species within *Enterobacteraiceae* harbor the *pks* genomic island and can produce colibactin, a genotoxin that induces DNA damage (29). Colibactin-producing *E. coli* promote tumor growth in xenograft mouse models (30), modify the tumor microenvironment (31), and have been found in high abundance in colonic biofilms in patients with familial adenomatous polyposis (32). Members of the family *Enterobacteriaceae* have also been implicated in gut inflammation in inflammatory bowel disease (33–35). Thus, it is plausible that increased levels of *Enterobacteriaceae* in Barrett's esophagus may promote progression to EAC, either directly via colibactin or other bacterial products or indirectly by triggering an immune response and local inflammation.

Interestingly, the *Enterobacteriaceae* findings from 16S analyses of esophageal brushings were not replicated by qPCR of esophageal biopsies. However, the two subjects with high relative abundance of *Enterobacteriaceae* had similar findings in the squamous esophagus, in line with prior work demonstrating that there is little within-individual variability in the microbiome in the squamous and Barrett's lining in patients with BE (36). Further, our group previously showed that patients with HGD or EAC have increased *Enterobacteriaceae* in saliva, and that there is strong within-individual correlation between the salivary and esophageal microbiome (15). Thus, possible explanations for the discrepant findings are that esophageal brushings are superior to biopsies for microbiome assessment, as previously reported by Gall et al (36), and that *Enterobacteriaceae* may reside predominantly within the esophageal biofilm rather than within the mucosa (37).

The increased relative abundance of *Akkermansia muciniphila* in subjects with HGD or EAC was also notable. In the colon, *A. muciniphila* has been associated with many beneficial effects related to obesity and metabolic syndrome (38). However, depending on the context, this species also can degrade mucins and thin the mucus layer (39), potentially leading to increased interaction between pathobionts and the underlying epithelium. In this fashion, the presence of *A. muciniphila* could conceivably lead to increased Barrett's tissue inflammation and promote progression to EAC.

Yang et al previously described a microbiome associated with reflux esophagitis and BE that was characterized by decreased relative abundance of *Streptococcus* and increased relative abundance of Gram-negative bacteria (13). In the current study, there were no differences in relative abundance of *Streptococcus* or in overall Gram-negative bacteria comparing non-dysplastic BE to controls (data not shown) or with progression from BE to EAC. However, controls not taking PPIs had increased Gram-negative bacteria and decreased *Streptococcus* compared to subjects on PPIs, and our group has previously demonstrated that PPIs cause significant increases in *Streptococcus* in the distal gut (40). If Gram-negative bacteria in the esophagus promote chronic inflammation and increase the risk of BE and EAC (12), then PPIs may provide a chemoprotective effect by reducing overall levels of Gram-negative bacteria. However, the PPI results from the current study should be interpreted with caution, as the PPI users were a mix of BE and non-BE patients.

The current study has several strengths. There were patients from all stages of BE-associated neoplasia, which permitted the ascertainment of microbiome shifts prior to the development of EAC. During the endoscopy only flat BE tissue was sampled, avoiding lesions so as to minimize confounding by the presence of bacteria that may have been mere colonizers due to an altered tumor macro- and micro-environment. Care was taken with regard to exclusion criteria to minimize the effects of certain factors on the microbiome such as antibiotics and immunosuppressants. Detailed clinical information and dietary intake data were recorded and assessed in the analyses.

There were also certain limitations. The sample size was relatively small, and the study may have been underpowered to detect additional important microbiome alterations associated with neoplastic progression in BE. The current study describes associations with various stages of BE neoplasia but no information on causative effects on progression. However, the findings provide key hypothesis-generating data for follow-up functional studies.

In conclusion, there were pronounced shifts in the microbiome in Barrett's esophagus associated with progression to EAC, particularly at the transition from low- to high- grade dysplasia. Notably, patients with HGD and EAC had increased relative abundance of *Enterobacteriaceae*, and members of this family have been implicated in gut inflammation and carcinogenesis. Further studies are indicated to identify specific bacteria that may promote the development of EAC, and also whether therapies targeting the microbiome can be developed to modify the risk of EAC.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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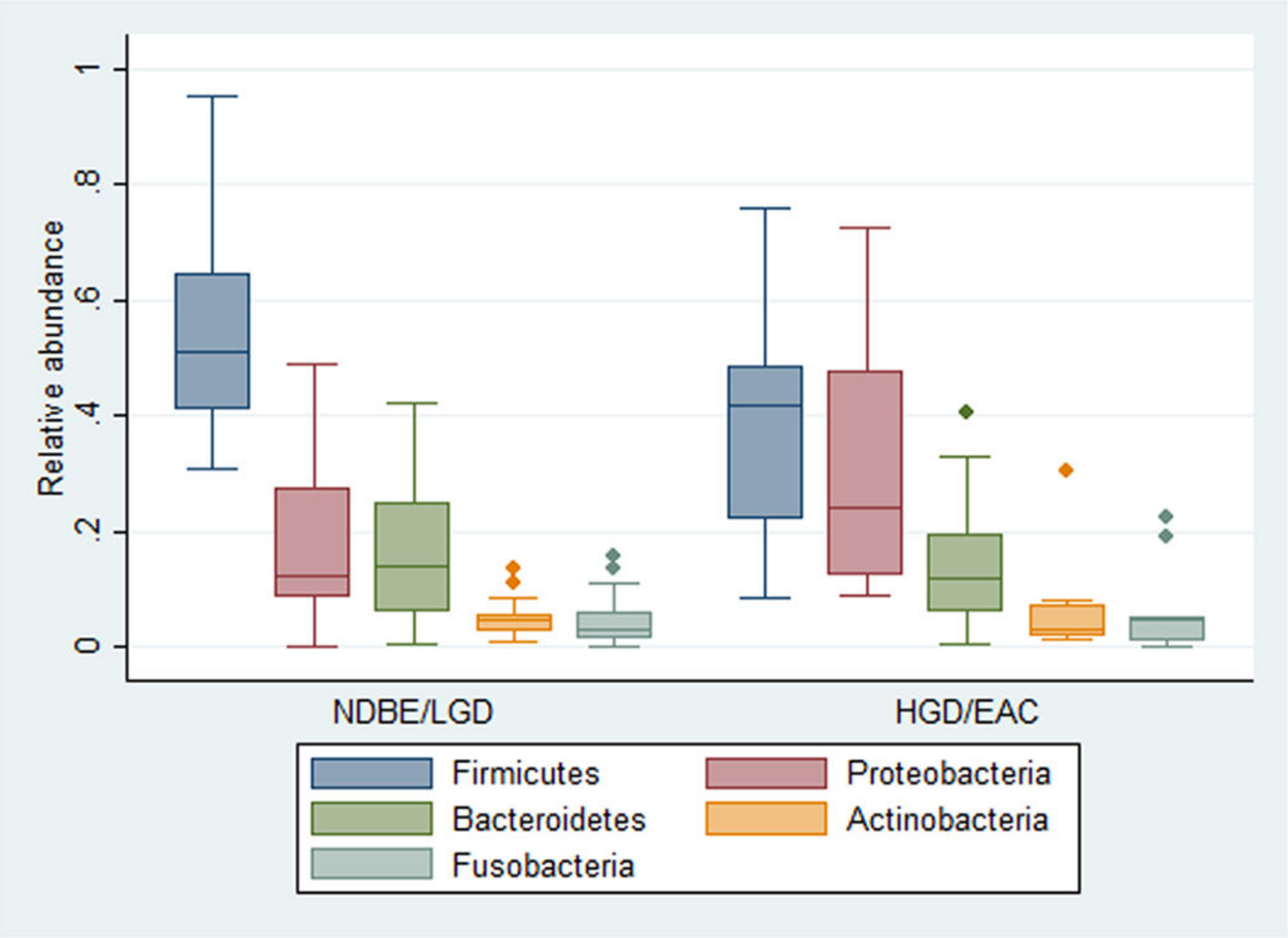

**Figure 1.**

Relative abundance of the major phyla comparing subjects with no dysplasia or LGD to those with HGD or EAC. Compared to NDBE/LGD subjects, those with HGD or EAC had decreased Firmicutes (p=0.04) and increased Proteobacteria (p=0.04).

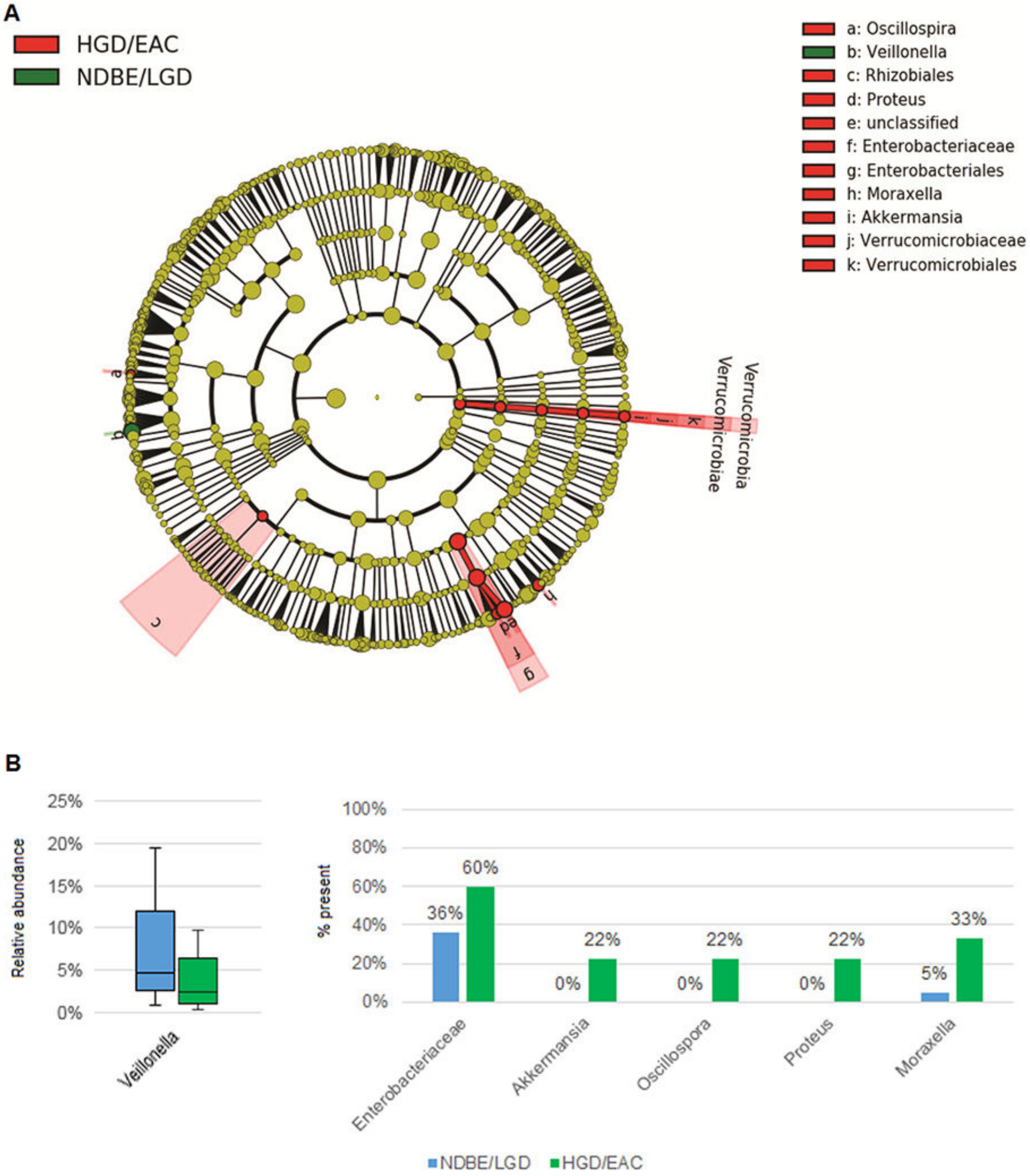

**Figure 2.**

**(A)** Cladogram from LEfSe analyses of differentially abundant taxa comparing BE patients without dysplasia (NDBE) or LGD vs. HGD or EAC. **(B)** Subjects with HGD or EAC had reduced relative abundance of *Veillonalla* (left), and had increased proportion of samples with presence of the other differentially abundant taxa (right), which were relatively rare. (Presence defined as having any reads, except for *Enterobacteriaceae*, which was defined as relative abundance >0.1%.)

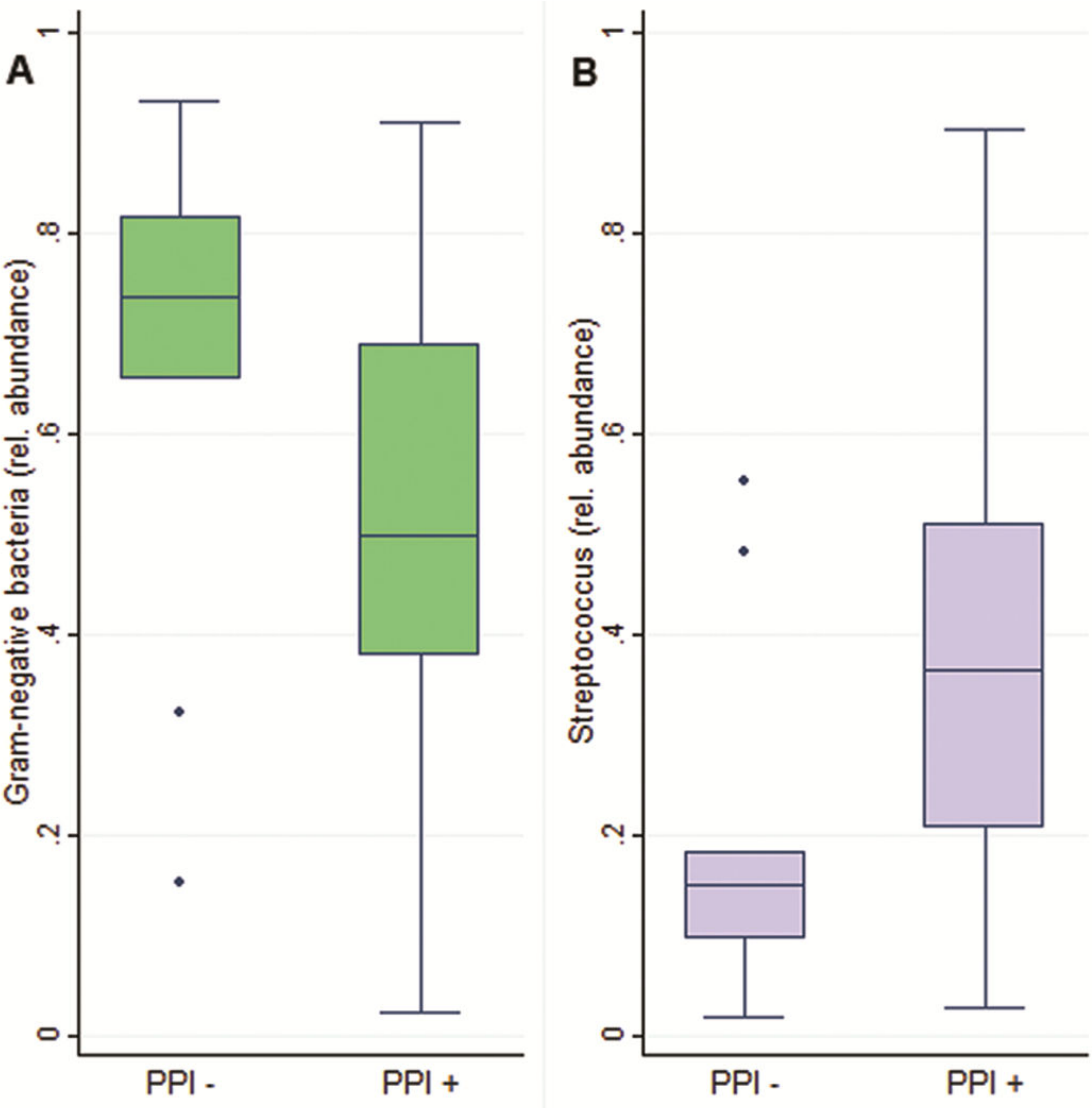

**Figure 3.**

Compared to controls not taking PPIs, patients taking PPIs had: (**A**) reduced relative abundance of Gram-negative bacteria (p=0.05), and (**B**) increased relative abundance of *Streptococcus* (p=0.03).

## Table 1.

Characteristics of patients who underwent upper endoscopy and had microbiome analyses, comparing those without to those with Barrett's esophagus (BE).

| | Non-BE (n=16) | BE (n=29) | p |
|---|---|---|---|
| Age, mean (SD) | 60.1 (14.9) | 63.6 (11.7) | 0.39 |
| Sex, male | 9 (56%) | 25 (86%) | 0.04 |
| WHR, mean (SD) | 0.95 (0.08) | 0.97 (0.05) | 0.37 |
| GERD | 10 (63%) | 27 (93%) | 0.02 |
| Ever smoker | 7 (44%) | 19 (66%) | 0.21 |
| PPI use | 6 (38%) | 29 (100%) | <0.001 |
| Aspirin use | 3 (19%) | 11 (38%) | 0.31 |
| Dietary fiber [*], grams per day; mean (SD) | 15.2 (5.3) | 16.5 (4.5) | 0.42 |
| Dietary fat [*], % daily calories; mean (SD) | 33.6 (2.3) | 34.3 (3.2) | 0.46 |

SD: standard deviation; WHR: waist-to-hip ratio; GERD: gastro-esophageal reflux disease; PPI: proton pump inhibitor

[*] Dietary data missing in 1 subject.