ORIGINAL RESEARCH

A decreased abundance of clostridia characterizes the gut microbiota in eosinophilic esophagitis

Purna C. Kashyap[1,2], Stephen Johnson[3], Debra M. Geno[1], Heather R. Lekatz[1], Crystal Lavey[1], Jeffrey A. Alexander[1], Jun Chen[2] & David A. Katzka[1] (iD)

1 Gastroenterology and Hepatology, Mayo Clinic, Rochester, Minnesota
2 Physiology and Biomedical Engineering, Mayo Clinic, Rochester, Minnesota
3 Biomedical Statistics and Informatics, Mayo Clinic, Rochester, Minnesota

Abstract

Abnormalities in the gut microbiome are associated with suppressed Th2 response (Belizario et al., 2018 *Mediators Inflamm.* 2018:2037838) and predisposition to atopic disease such as asthma and eczema. We investigated if this applies to eosinophilic esophagitis (EoE). Stool bacterial DNA was extracted and followed by 16S rRNA amplification from 12 patients with eosinophilic esophagitis and 12 controls. Alpha- and beta-diversity were analyzed. Only two patients had asthma or atopy and one patient was on budesonide. No patients were on PPIs. Patients with EoE had lower gut microbiota alpha diversity (species richness, $P = 0.09$; Shannon index, $P = 0.01$). The microbial composition was distinct as evidenced by significantly different beta diversity ($P = 0.03$) when compared to healthy controls. There were also significant differences in relative abundance at multiple taxonomic levels when comparing the two communities; at the phylum level, we observed a marked decrease in Firmicutes and increase in Bacteroidetes and at the order and family level there were significant decreases in *Clostridia* and *Clostridiales* in patients with EoE ($q \leq 0.1$). We conclude that there are significant differences in microbial community structure, microbial richness, and evenness and a significant decrease in taxa within the *Clostridia* in patients with EoE. Our data suggest that *Clostridia* based interventions could be tested as adjuncts to current therapeutic strategies in EoE.

Introduction

Abnormalities in the gut microbiome are associated with perturbations in systemic immune reaction (Belizario et al., 2018) and predisposition to atopic disease such as asthma and eczema (Zimmermann et al., 2019). While several genetic (Sherrill and Rothenberg, 2011), environmental (Hill and Spergel, 2018) and esophageal microbiome (Benitez et al., 2015; Harris et al., 2015; Norder Grusell et al., 2018) factors have been implicated in EoE, there is evidence that supports a role for the gut microbiome. For example, factors associated with altered gut microbiome such as lack of breast feeding and performance of Cesarean section(Jensen et al., 2018) or early life antibiotic use (Radano et al., 2014) have been associated with EoE. Multiple studies in human subjects have found an association between altered gut microbiota and atopic disorders (Nimwegen et al., 2011; Ismail et al., 2012; Lau et al., 2012) and impaired mucosal barrier function (Katzka et al., 2014; Sherrill et al., 2014) in response to circulating cytokines (Sherrill et al., 2014). The sensitization to food antigens triggering an immune response and driving esophageal inflammation in EoE (Noti et al., (2013)) also suggests a role for gut microbes as

commensal bacteria within *Clostridium* clusters XIVa, XIVb, and IV have previously been described to protect against development of food allergies in rodent studies by driving expansion of FoxP3 + regulatory T cells (Tregs) and improving barrier function (Stefka et al., 2014). Finally the positive response to elemental diet seen in EoE (Kelly et al., 1995; Markowitz et al., 2003; Warners et al., 2017) with its potential effects on stool microbiota{Kajiura, 2009 #17869}points to role for gut microbiome. Hence in this study we evaluated the gut microbiome represented in the stool from patients with EoE and compared it with matched healthy controls.

Methods

Ethical approval of human studies

All human studies were approved by the Mayo Clinic IRB [EoE (16-001097), healthy controls (14-003005, 16-006388)].

Patients

Adult patients with eosinophilic esophagitis defined by consensus criteria (Dellon et al., 2013) were sequentially recruited from our eosinophilic esophagitis outpatient clinic. Patients were excluded if they were on proton pump inhibitors, had antibiotic exposure or systemic steroids within the past 2 months. Patients mailed the stool sample in a cold pack within 1 week of endoscopy which was then stored in −80°C till further processing. Healthy adult control patients were recruited as part of different studies and matched with the EoE patients for age, sex and BMI. None of the healthy controls had history of asthma, antibiotic use within past 4 weeks or PPI use. Only one control was delivered by C-section and no controls had early life antibiotic exposure. All were on a regular diet.

Microbiome analysis

Stool bacterial DNA was extracted using MoBio fecal DNA extraction kit, followed by 16S rRNA amplification using Nextera library compatible primers flanking the V4 hypervariable region ([forward overhang] + 515F: [TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG]GT GCCAGCMGCCGCGGTAA; and [reverse overhang] + 806R: [GTCTCGTGGGCTCGGAGATGTGTATAAGAGA-CAG]GGACTACHVGGGTWTCTAAT) and prepared for sequencing using a dual-indexing protocol. All samples were sequenced together in 2x300 paired-end mode on an Illumina MiSeq instrument using v3 reagents by the University of Minnesota Genomics Center. Paired R1

Table 1. Clinical and demographic characteristics of study patients.

|	Age	Gender	Allergies	Asthma	atopic dermatitis	Delivered by C-section	Hx of antibiotics < 1 year old	Breast feed	Meds	EoE Diet therapy
Active EoE	34	F	Seasonal,Dogs/cats	Yes	No	Yes	No	No	albuterol	Yes
	42	M	Seasonal	No	No	No	Yes	No	AlbuterolFlonase	No
	44	F	None	No	No	No	No	No	Multivitamin	No
	49	F	Seasonal	No	No	No	No	Yes	Synthroid, Lisinophril	Yes
	53	F	None	No	No	No	No	UNK	AdderallFlonase	No
	54	F	None	No	No	No	No	UNK	NorvascNeurontin, Wellbutrin-XL	No
	58	M	Cat dander	No	No	No	No	UNK	AfrinLipitor	No
	64	M	MSG, bee stings	No	No	No	No	No	FloventZantac	No
Inactive EoE	37	F	None	No	No	No	No	Yes	Allegra, montelukastZantac	Yes
	46	F	None	No	No	No	No	No	Zoloft	Yes
	46	F	Animals/dust	No	No	No	No	No	Budesonide	No
	52	M	Seasonal/animal	No	Yes	UNK	UNK	UNK	SidenafilSynthroid	Yes
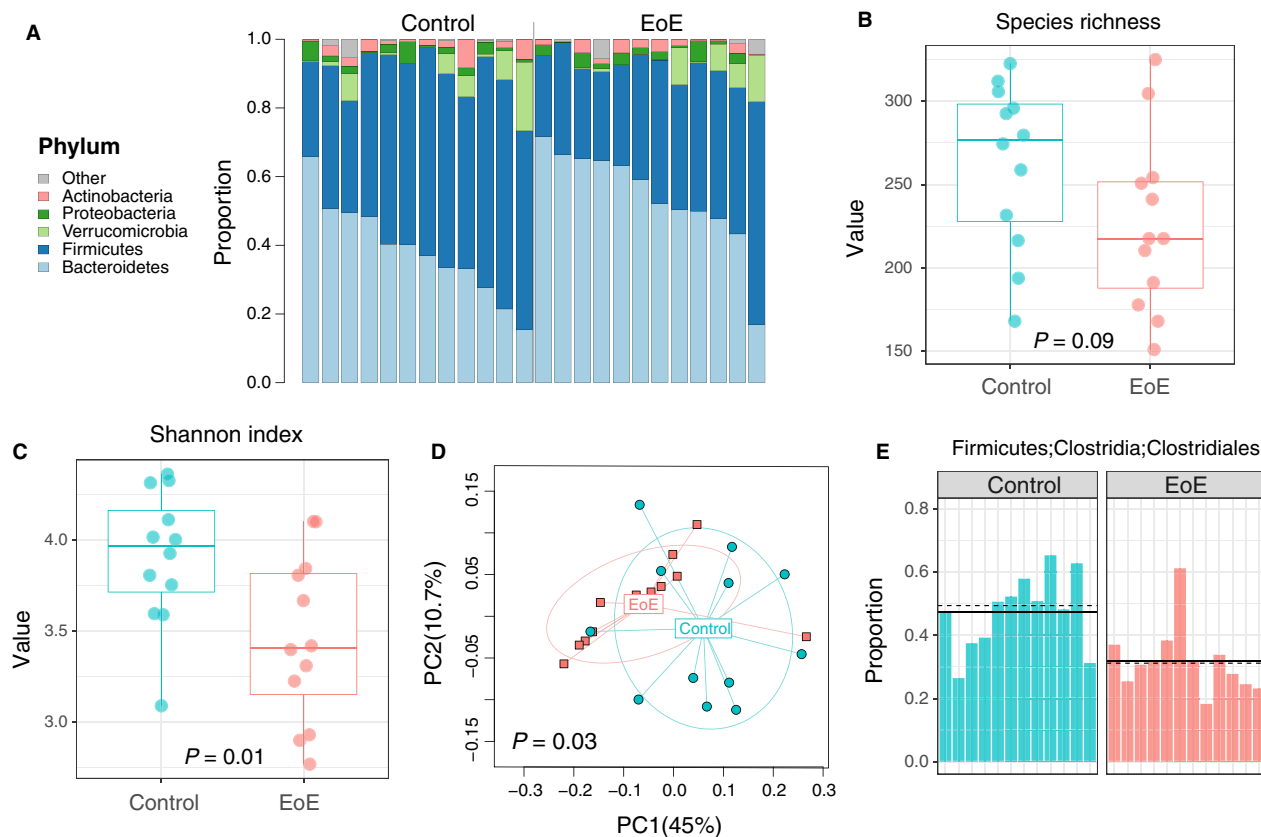
Figure 1. The phylum-level profiles (A), alpha diversity (within subject) based on species richness (B) and Shannon index (C), principal coordinate analysis (PCoA) plot showing beta diversity based on weighted UniFrac (D), and barplot comparing the abundance of Clostridia/ *Clostridiales* (E) among healthy controls (n = 12) and EoE patients (n = 12).

and R2 sequence reads were processed via the *hybrid-de-novo* bioinformatics pipeline (Chen et al., 2018), which clustered good-quality paired and single-end reads into operational taxonomic units (OTUs) at 97% similarity level. In total, 989,199 reads (median: 41,727 reads per sample, range: 21,861 to 53,839 reads per sample) were included. OTUs were assigned taxonomy using RDP classifier trained on the Silva database (v132). These OTUs belong to 15 phyla, 84 families, and 248 genera.

Alpha-diversity (species richness (observed number of OTU) and Shannon index) and beta-diversity (unweighted, generalized (α = 0.5), and weighted UniFrac and Bray-Curtis distance) were analyzed for the OTU data (R "GUni-Frac" package v1.1; Chen et al., 2012). Rarefaction was performed on the OTU table for the diversity analyses (rarefied to 20,000 reads per sample). A linear regression model (t-test) was used for testing the association with alpha-diversity. Beta-diversity association was tested using PERMA-NOVA based on the aforementioned distance measures. Taxa-level association analyses were performed at multiple taxonomic levels. The count data were normalized by GMPR size factor to address potential compositional

effects. Taxa with prevalence less than 10% or with a maximum proportion less than 0.2% were excluded. A permutation test (1000 permutations) was used to identify differentially abundant taxa based on the t-statistic of a linear regression model with the square-root transformed taxa relative abundance as the response variable. Permutation-based false discovery rate (FDR) control was used to correct for multiple testing on each taxonomic level, and FDR-adjusted p-values or q-values ≤ 0.1 were considered significant. All statistical analyses were performed in R 3.4.2.

Results

Twelve patients with eosinophilic esophagitis (eight active) had a median age of 48 (34-64), body mass index (BMI) was 29.51 (22.40–36.49), four were male and all were Caucasian (Table 1). Only one patient had asthma and one had atopic dermatitis. Six patients were on a regular diet. No patients were on PPIs. The healthy control patients had a median age 51 (33–61), BMI 24.75 (19.73–41.39), three were male and all were Caucasian. None had a history of asthma, atopic dermatitis, or PPI use.

We compared the 16S rRNA-based microbial community composition in stool samples from the patients with EoE ($n = 12$) and healthy control subjects ($n = 12$). At the phylum level, we observed a marked decrease in Firmicutes and increase in Bacteroidetes in patients with EoE (Fig. 1A). The gut microbial communities from patients with EoE were characterized by a lower alpha diversity (species richness, *P = 0.09*; Shannon index, *P = 0.01*; linear regression; Fig. 1B, 1). The beta-diversity was also significantly different between patients with EoE and healthy controls (*P* = 0.03, PERMANOVA based on weighted UniFrac; *P* = 0.04, omninbus test combining multiple beta diversity measures; Fig. 1D). There were significant differences in relative abundance at multiple taxonomic levels when comparing the two communities, which included significant decreases in *Clostridia* and *Clostridiales* in patients with EoE (*q* ≤ 0.1, permutation test with t-statistic; Fig. 1E).

Discussion

In our pilot study we found significant differences in microbial community structure and a decrease in microbial richness and evenness in patients with EoE. Additionally we found a significant decrease in taxa within the Clostridia in patients with EoE. In the differential abundance analysis, we used a permutation test based on the normalized and transformed data, thus, the observed difference in Clostridia was less likely to be driven by sequence depth variation and outliers. In fact, similar *P*-values were obtained even if we rarefied the data to 20,000 reads per sample. This finding is particularly important as previous studies show that members of *Clostridia* group are protective against the development of food allergies in a rodent model. *Clostridia*-containing microbiota was associated with the expansion of intestinal regulatory T cells (Treg), induction of IL-22 production by RORγt$^+$ ILCs and class switching to IgA promoting an immune environment conducive for tolerance to dietary antigens (Stefka et al., 2014). The abundance of *Clostridiales* correlated with the expression of antimicrobial peptide *Reg3b* suggesting *Reg3b* may play a role in modulating the abundance of C*lostridiales* in the colon (Stefka et al., 2014). In addition to rodent studies, *Clostridia* strains isolated from healthy human feces have also been shown to induce Tregs in colonic lamina propria when transferred to germ-free mice (Nagano et al., 2012).

Our study is limited by the small sample size although the ability to show significant differences given the number of variables in study of the microbiome is noteworthy. Strengths of the study are a lack of medications, such as proton pump inhibitors (Bajaj et al., 2018), or other atopic illnesses in most patients that have been associated with altered stool microbiome. This is difficult to achieve as most patients with atopic illness associated with microbiome differences commonly have other atopic conditions and/or usage of medications for these diseases. Furthermore, we limited our analyses to stool samples as we were interested in the association of an altered gut microbiome rather than the esophageal microbiome as a change in the former has been reported to predispose to organ specific atopic disorders, such as asthma, later in life. On the other hand, we cannot precisely identify the relationship of early life changes in stool microbiome to what is found in these patients as adults. Finally, whether our data support the use of Clostridia based interventions as adjuncts to current therapeutic strategies in EoE needs to be further assessed.

Acknowledgments

Conflict of Interest

Purna C Kashyap is on the advisory board of uBiome and as an ad hoc advisory board member for Salix pharmaceuticals. David A. Katzka is on the advisory board for Shire and Celgene.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.:

Figure S1: Proposed pathogenesis of microbiome in eosinophilic esophagitis.