Investigation of oral, gastric, and duodenal microbiota in patients with upper gastrointestinal symptoms

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ABSTRACT

Disease-associated alterations of the intestinal microbiota composition, known as dysbiosis, have been well described in several functional gastrointestinal (GI) disorders. Several studies have described alterations in the gastric microbiota in functional dyspepsia, but very few have looked at the duodenum.

Here, we explored the upper GI tract microbiota of inpatients with upper GI dyspeptic symptoms, and compared them to achalasia controls, as there is no indication for an esophagogastroduodenoscopy in healthy individuals.

We found differences in the microbiota composition at the three sites evaluated (ie, saliva, stomach and duodenum). Changes observed in patients with dyspepsia included an increase in *Veillonella* in saliva, an oral shift in the composition of the gastric microbiota, and to some degree in the duodenum as well, where an important abundance of anaerobes was observed. Metabolic function prediction identified greater anaerobic metabolism in the stomach microbial community of patients with dyspepsia. Proton pump inhibitor use was not associated with any particular genus. Co-abundance analysis revealed *Rothia* as the main hub in the duodenum, a genus that significantly correlated with the relative abundance of *Clostridium, Haemophilus*, and *Actinobacillus*.

We conclude that patients with upper GI symptoms consistent with dyspepsia have alterations in the microbiota of saliva, the stomach, and duodenum, which could contribute to symptoms of functional GI disorders.

INTRODUCTION

Dyspepsia is one of the most prevalent functional gastrointestinal (GI) disorders.[1] Although considered a multifactorial disorder without a definitive organic cause, low-grade duodenal inflammation has been proposed in the etiopathogenesis of functional dyspepsia.[2] Such low-grade inflammation may affect barrier function, leading to important alterations of host-microbial interactions.[3 4]

Significance of this study

What is known about this subject?
► An increase in the relative abundance of *Streptococcus*, while at the same time presenting a significant decrease in *Prevotella, Veillonella, Actinomyces, Aptopobium*, and *Leptotrichia* have been shown in the duodenal mucosa of patients with functional dyspepsia compared with controls.

What are the new findings?
► We did not find such differences in our study, but similar to what has been reported in the duodenum of patients with GI symptoms, we observed the occurrence of an oral shift, with presence of various genera that are commonly found at the oral and gastric level, such as *Scardovia, Bifidobacterium*, and *Lactobacillus*.
► Changes observed in patients with dyspepsia included an increase in *Veillonella* in saliva, an oral shift in the composition of the gastric microbiota, and to some degree in the duodenum, where an important abundance of anaerobes was observed.

How might these results change the focus of research or clinical practice?
► This research sets the stage for further research using larger patient populations to categorize important subsets of the dyspepsia population, including those receiving a proton pump inhibitor, *Helicobacter pylori* related gastritis, and delayed gastric emptying to overcome the obstacles of heterogeneity in these patients.

The intestinal microbiota is a key player in intestinal health and function.[5] Disease-related alterations in the intestinal microbiota composition, known as dysbiosis, have been well described in several functional GI disorders.[6] Several studies have described alterations in the gastric microbiota in functional dyspepsia.[7–9]

Recent studies have started to show that duodenal dysbiosis underlies symptoms associated with functional GI disorders,[10–12] suggesting that particular microbial activity inappropriately triggers sensory and inflammatory pathways, with consequent dysregulation of gastric secretions and gut motility.[2]

In this study we aimed to investigate the microbiota of the duodenum and compare it with the stomach and saliva in patients with upper GI symptoms of dyspepsia versus patients with achalasia used as controls. Our goal was to explore the upper gut microbiota to assess any possible correlations in different clinical settings.

MATERIAL AND METHODS
Study population and sample collection procedure
Patients were enrolled in the study from December 2018 to April 2019, after obtaining informed consent. Patients' age ranged from 17 years to 82 years, and (24/36) 66% were women. Routine esophagogastroduodenoscopy (EGD) was performed in 25 patients with symptomatology, consistent with gastroesophageal reflux disease, dyspepsia, and dysphagia. Eleven patients with achalasia were regarded as 'controls', based on the evidence that achalasia occurs due to the loss of the nerve function in the esophageal smooth muscles with no mucosal pathology beyond the gastroesophageal junction. Four patients had gastroparesis documented by gastric emptying study. Four patients had diabetes mellitus. Eighteen patients were receiving a proton pump inhibitor (PPI) for their suspected acid-peptic mediated symptoms prior to the endoscopy. Saliva samples were obtained immediately prior to endoscopy. Mucosal bacteria were collected using a standard cytology brush (Olympus) by gently rubbing the mucosa of the second part of the duodenum and the gastric antrum (online supplemental figure 1). Gastric and duodenal biopsies were obtained for conventional pathological examination specifically to exclude gluten enteropathy in the duodenum and assess for *Helicobacter pylori* infection in the stomach.

Endoscopic finding for the control group included dilated esophagus and absent peristalsis. In the study group the endoscopic findings were: mild to moderate gastritis 6/25, gastric mucosal atrophy 2/25, Los Angeles grade A esophagitis 2/25, hiatal hernia 2/25, lower esophageal stenosis 2/25, salmon colored mucosa in the lower esophagus 1/25, and a Schatzki ring 1/25. Gastric biopsies revealed *H. pylori* in five patients (one in the control group, four in the study group). Duodenal biopsies did not reveal any evidence of sprue or villous blunting.

Bacterial DNA extraction
DNA purification used the QIAamp UCP (ultraclean production) Pathogen Mini Kit from Qiagen. For saliva, the stored sample was thawed, precipitated with an equal volume of isopropanol, and centrifuged at 4000 rpm. The pellet was resuspended in 1 mL of the guanidine-containing QIAamp lysis buffer in a 1.5 mL microfuge screw-cap tube containing glass beads, as described in the kit. For the brushes, the QIAamp lysis buffer was added directly to the brush and incubated at 50°C for 15 min, then vortexed to disperse the mucosal tissue and bacteria. The lysate was pipetted into a fresh screw cap tube with glass beads. After

this point all samples received identical treatment. Agitation of the glass beads used a 24-tube Mo Bio Adaptor platform disk attached to a Fisher vortexer. Agitation was at top vortexer speed for 30 min at room temperature. Subsequent steps were as described in the kit. Purified human and microbial DNA was eluted from the glass fiber filter spin column in a final volume of 50 μL 0.2 mM EDTA pH 8.0, and stored at −20°C.

Illumina library preparation and 16S rRNA sequencing
Relatively small amounts of mucosal bacteria were recovered in brush samples. To optimize libraries, 5 μL of all purified DNA samples were subjected to high-stringency preamplification as follows. High-fidelity PCR used universal primers to produce roughly 930 bp amplimers which contained variable regions V1 through V5 of the 16S rRNA gene. V1a-for: AGAGTTTGATCATGGCTCAGATTGAACGCT, V1b-for: AGAGTTTGATCCTGGCTCAGGATGAACGCT, V5-rev: TTGTGCGGGCCCCCGTCAATTCHTTTGAGTTT.

Each 50 μL reaction mixture contained 0.5 μM of V1a-for, 0.5 μM of V1b-for, and 1.0 μM of V5-rev reverse primer, in a standard 50 μL reaction with Phusion Hot-Start DNA Polymerase (New England Biolabs, Ipswich, Massachusetts, USA). Temperature cycling: 8 min at 96°C to activate the polymerase, followed by 25 cycles of 20 s at 96°C, annealing at 65°C for 20 s, and extension at 72°C for 1 min, terminated by 5 min 72°C, finally holding at 4°C. DNA for each completed PCR reaction was purified using the WizardDNA Clean-Up System (Promega, Madison Wisconsin, USA), and eluted from the spin column in a final volume of 50 μL of 0.2 mM EDTA. Subsequent library preparation followed standard Illumina procedures based on amplification of a~460 bp V3-V4 16S rRNA gene interval within the ~930 bp preamplified V1-V5 sequences. The Illumina amplicon V3-V4 primers were: TCGTCGGC AGCGTCAG ATGT GTATAAGA GACA GCCTACGG GNGGCWGCAG, and GTCTCGTGGGCTCGGAGATG TGTATAAGAGACAGGACTACHVGGGTATCTAATCC. The 3' end 16S universal homologous sequences are in bold. The 5' end of these amplicon primers contained the standard adaptors for MiSeq sequencing. Library construction was carried out exactly as described previously,[13] following the Illumina 16S sequencing preparation instructions . Briefly, 15 cycles of PCR amplification with 16S rRNA gene V3-V4 Amplicon primers at an annealing temperature of 55°C using KAPA HiFi HotStart ReadyMix (Sigma), were followed by 8 cycles of PCR with unique forward-reverse pairs of 96-sample Illumina Nextera bar-coding primers. After measuring ds-DNA yields with a Qubit 2 fluorometer (Thermo-Fisher), 0.25 μg of DNA from each sample was pooled to produce a 24 μg library, which was purified with WizardDNA Clean-Up. The pooled library was sequenced by SeqMatic, Fremont California, USA, for a minimum of 300 bp on both forward and reverse strands using MiSeq paired-end sequencing. Each sample, including the three controls, yielded high-quality sequence.

16S rRNA sequence processing
Raw sequences were processed using mothur[14] standard operating procedure with modifications. Data were trimmed and chimera removed. Each sequence was classified using

RDP (Ribosomal Database Project) classifier against RDP database. All analysis was performed in phylum-level and genus-level classification. Shannon Diversity Index was calculated for each sample and pairwise ThetaYC distance was measured for community diversity evaluation. Alpha-diversity and beta-diversity were visualized using R. Relative abundances of each sample were visualized in the bar chart using Phinch.[15] Differential abundance was analyzed via linear effect size measurements (LEfSe).[16] Co-occurrence analysis was performed using MicrobiomeAnalyst.[17] Predictive functional profiling was conducted using a phylogenetic investigation of communities by reconstruction of unobserved states,[18] with details described by Hong *et al*.[19]

Statistical analysis

Difference in alpha diversity was tested using Wilcoxon rank sum test. Significant clustering in principal coordinated analysis (PCoA) plot was tested using analysis of molecular variance.[20] Co-occurrence analysis was performed based on Spearman Rank correlation.

Bacterial cultures

Duodenal brush samples from 17 of the 25 patients, including 6 of the achalasia controls, were plated on Centers for Disease Control and Prevention, KV (kanamycin vancomycin), and PEA (phenylethyl alcohol agar) blood agar (anerobic), as well as on TSA (trypticase soy agar) with 5% sheep blood and Levine EMB (eosin methylene blue) (aerobic). Colonies were counted post incubation, and hemolytic reactions were observed. Gram stain was performed on all colonies from all plates. Catalase test was run on colonies of Gram positive cocci. The API Staph, API Coryne, API 20E, API 20NE, Rapid ID32 (BioMerieux, Durham, North Carolina, USA) test strips were used.

RESULTS

Diversity of the microbiota is similar between dyspepsia and achalasia cases

To estimate the diversity of the microbial communities in saliva, stomach, and duodenum from patients with dyspepsia and achalasia, we calculated the Shannon's Diversity Indices
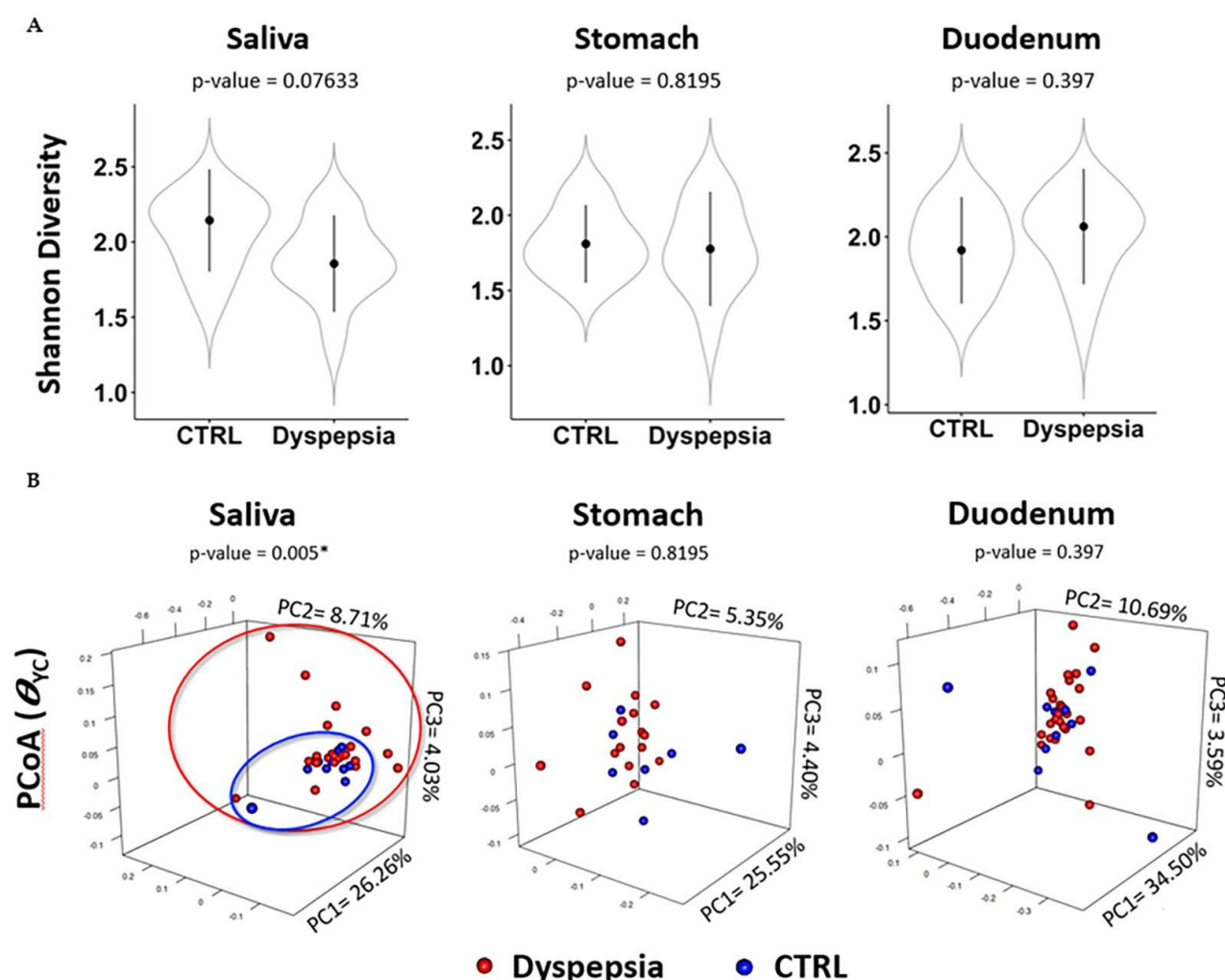

Figure 1 Overall community microbiota diversity of patients with dyspepsia and achalasia at three sites. (A) Shannon Diversity Indices for dyspepsia microbiota and controls (CTRL) in saliva, and from stomach and duodenal samples. (B) PCoA clustering observed in saliva, but not in stomach or duodenal samples. CTRL, control; PCoA, principal coordinated analysis.
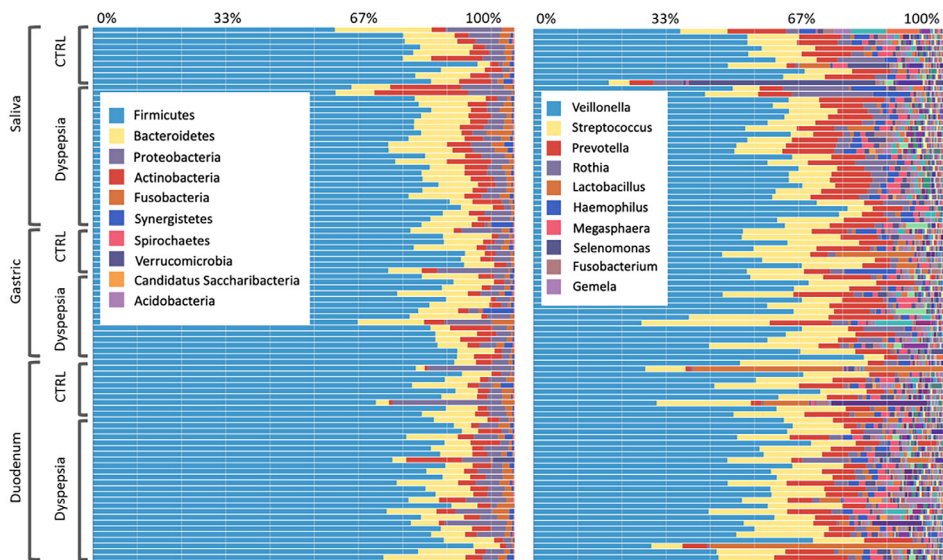
Figure 2 Relative abundance of individual samples. taxonomy bar chart showing taxonomic composition of microbial communities for each individual sample at the level of phylum (left) and by genus (right). CTRL, control.

at the three different sites. No difference in this parameter was observed between both groups (figure 1A).

The data were then analyzed for clustering using PCoA, comparing patients with dyspepsia versus achalasia controls. The PCoA showed a distinct separation between the patients with dyspepsia versus patients with achalasia in saliva, which was statistically significant (p=0.005) (figure 1B). These results suggest that despite bearing similar diversity, the microbiota composition of saliva in patients with dyspepsia is different from that of achalasia controls.

The upper GI microbiota is different between dyspepsia and achalasia cases

We then analyzed the composition of the microbiota in dyspeptic cases vs achalasia. No visual grouping was observed in the relative abundance of the main phyla or genera when the individual samples are displayed altogether

(figure 2). No evident overall difference was apparent at the phylum level or genus level at the three sites (online supplemental figure 2).

We used LEfSe to determine genera over-represented in our two groups. In saliva, patients with dyspepsia exhibited increased presence of *Veilonella*, *Cohnella*, *Sporolactobacillus*, *Propionigenium*, and *Anoxybacter*, while achalasia controls showed increased presence of *Scardovia*, *Brevibacillus*, *Kinecoccus*, *Phascolarbacterium*, *Peptostreptococcus*, *Dethiosulfatibacter*, *Bulleida*, *Prolixibacter*, *Pullulanibacillus*, *Mycobacterium*, *Lactonifactor*, *Trabulsiella*, *Edwardsiella*, *Acetobacterium*, *Parvimonas*, *Ruminococcus*, *Aurebacter*, and *Enterorhabdus* (figure 3A).

When looking at the gastric microbiota, two genera, *Pseudoclavibacter* and *Tannerella*, appeared to be differentially represented in dyspeptic samples, while *Clostridium*, *Actinobaculum*, *Deinococcus*, *Citricoccus*, *Pediococcus*,
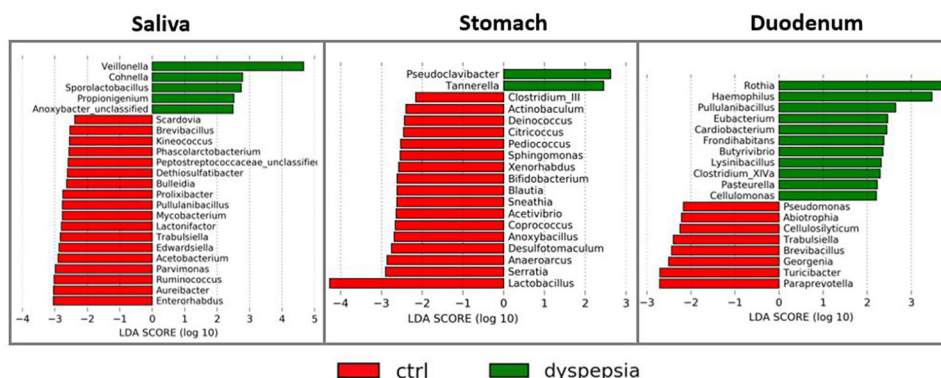
Differentially abundant genera

Figure 3 Differentially abundant genera in (A) saliva, (B) stomach, and (C) duodenum. Linear discriminant analysis (LDA) coupled with effect size measurements (LEfSe) analysis showing the most differentially abundant genera in dyspepsia samples (green) and achalasia controls (red), at the three sites: saliva, gastric, and duodenal mucosa.
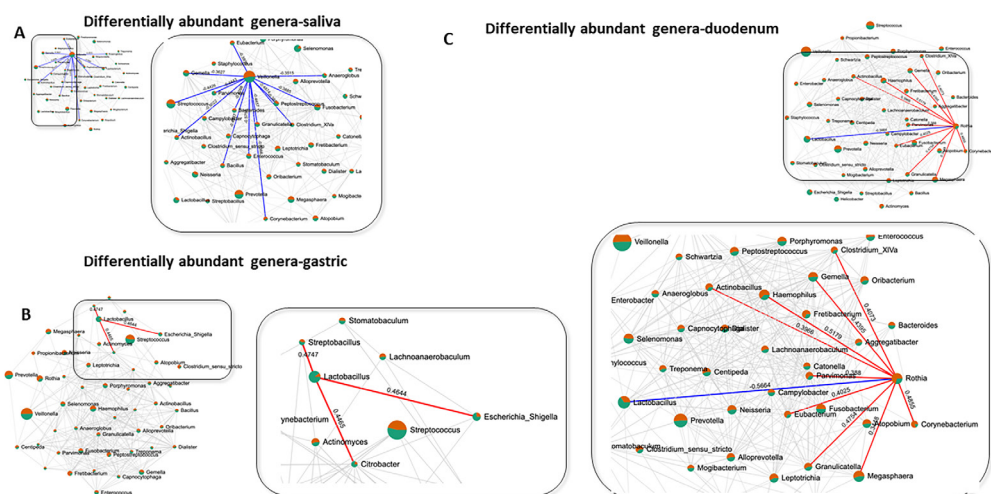
Figure 4 Significant genera association at each site. Network analysis of bacterial co-occurrence. Moderate-to-high correlated genera at each site are depicted as connected nodes. The color of edges in the enlarged section represent the correlation coefficient from the Spearman's rank correlation test with red as positive correlation and blue as negative correlation. Each node represents taxon. Node size indicates the relative abundance in dyspepsia (orange) and control groups (green).

Sphingomonas, Xenorhabdus, Bifidobacterium, Blautia, Sneathia, Acetivibrio, Coprococcus, Anoxybacillus, Desulfotomaculum, Anaeroarcus, Serratia, and *Lactobacillus* were increased in patients with achalasia (figure 3B).

The duodenum of patients with dyspepsia showed a higher presence of *Rothia, Haemophilus, Eubacterium, Clostridium, Pululanibacillus, Frondihabitans, Cellumonas, Butyvibrio*, and *Pasteurella*, while patients with achalasia showed more *Brevibacillus, Georgenia, Paraprevotella*, and *Turicibacter* (figure 3C).

No particular genus was found to be associated with PPI use, gender, or diabetes as comorbidity (data not shown).

Significant genera associations revealed by network analysis

We then performed a network analysis of bacterial co-occurrence, for the most abundant genera at each site. We analyzed the top genera in saliva and observed that *Veillonella* acts as a main hub that downregulates the abundance of various other anaerobic bacteria (figure 4A). At the gastric site, *Lactobacillus*, which was over-represented in achalasia controls, co-occurred with *Escherichia/Shigella*, as well as with *Lachnospiracea*, but downregulated bacteria form the genus *Bacillus* (figure 4B). *Rothia* was the main hub in the duodenum, and significantly co-occurred with *Clostridium, Haemophilus*, and *Actinobacillus* (figure 4C).

Metabolic functional prediction of microbiota changes

In order to estimate the potential impact of the observed dysbiosis in microbial metabolic pathways, we examined the predicted metagenomes of the microbial communities using LEfSe analysis of metabolic pathways of patients with dyspepsia versus patients with achalasia. We identified a number of pathways that were significantly differentially represented in each group, except for the duodenum where no significant difference was observed.

The over-represented predicted pathways in saliva of patients with dyspepsia included purine, cysteine-methionine, thiamine, and nitrogen metabolism, biosynthesis of ubiquinone, lipopolyshaccaride, tRNA, and terpenoid, as well as amino acid related enzymes (figure 5). The main Kyoto Encyclopedia of Genes and Genomes categories for metabolic pathways in saliva of patients with achalasia, on the other hand, showed significant enrichment of functions related to the metabolism of various saccharides, as well as proteins involved in bacterial cell motility and chemotaxis. At the gastric level, patients with dyspepsia predicted pathways involved the tricarboxylic acid cycle, peptidases, protein folding and associated processing. Pathways identified for achalasia gastric microbial communities identified transporters, and proteins involved in replication, recombination and repair.

Culture-based microbial composition in duodenal samples

To characterize the presence of live bacteria in the duodenum of both groups, a subset of duodenum brush samples was cultured for aerobic and anaerobic bacteria. Although not statistically significant, bacterial culturing showed a higher amount of anaerobic and aerobic bacteria in achalasia samples compared with dyspepsia samples (figure 6A). The ratio of anaerobic/aerobic bacteria in both groups showed no statistically significant difference. Microbial composition in the duodenal samples for each group, according to our culture-based approach is presented in figure 6B.

DISCUSSION

Understanding functional GI disorders such as dyspepsia has always been challenging for clinicians, as they are characterized by non-structural symptomatology. Small intestinal microbiota has been shown to be a key player
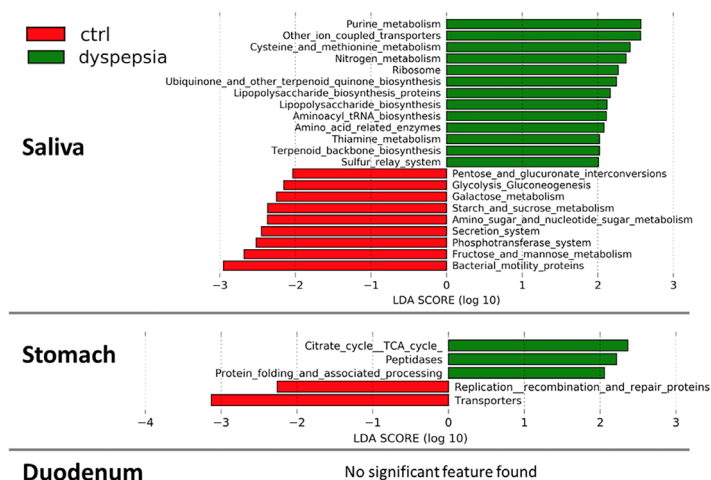
Figure 5 Metabolic potential between dyspepsia and achalasia controls. Metabolic function prediction associated to the microbiota profiles. Linear effect size measurements (LEfSe) analysis, performed on metabolic functions inferred by phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt) analysis shows statistically significant enrichment of KEGG (Kyoto Encyclopedia of Genes and Genomes categories in saliva, stomach, and duodenum. LEfSe results indicate significant ranking among groups (alpha value=0.05 for the factorial Kruskal–Wallis test among classes). The threshold for the logarithmic Linear Discriminant Analysis (LDA) Score was 2.0. CTRL, control.

in GI function. We aimed to explore changes in bacterial communities of patients with upper GI tract symptoms, looking at three different sites: the oral cavity, stomach, and duodenum, as the esophagus has already been shown to harbor a transitional microbiota between the mouth and the stomach.[21]

The microbiota of patients with idiopathic achalasia has not been characterized.[22] Studies of the microbiota of patients with megaesophagus of Chaga's disease have demonstrated an overgrowth of *Streptococcus*.[23] It is unknown if this is also the case with achalasia. We chose patients with this condition as surrogates for healthy controls, as achalasia is an esophageal motility disorder explained by loss of enteric neurons in the smooth muscle leading to aperistalsis and impaired relaxation of the lower

esophageal sphincter. Since there are no indications to perform an EGD in healthy individuals, there is less opportunity for performing invasive microbe collection methods to obtain normal control data.

We did not observe any significant difference in the microbial diversity of the upper GI tract, as a whole, between dyspepsia and achalasia cases. This contrasts very much with what happens at the end of the GI tract, moving from the small intestine towards the rectum.[24]

Saliva samples of patients with dyspepsia showed a marked relative abundance of *Veillonella* compared with patients with achalasia. From our co-abundance network analysis, we found that *Veillonella* downregulated the abundance of various other bacteria at this site. *Veillonella* and *Peptostreptococcus* are part of the oral core microbiome[25] present
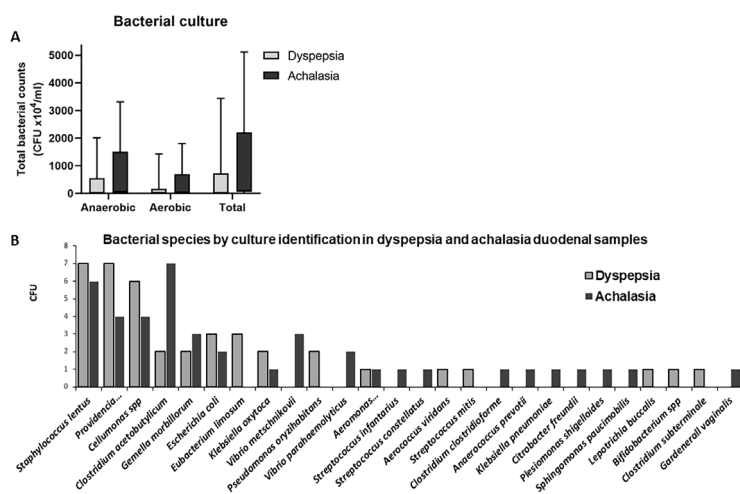

Figure 6 Live duodenal bacterial culture from dyspepsia and achalasia samples. (A) Mean and SE of anaerobic, aerobic, and total bacterial counts from eight dyspepsia, and six acahalasia samples. (B) Distribution of species among bacterial cultures, by API system.

in saliva of normal individuals,[21] and along with *Ruminococcus* also found in the esophagus.[26] *Scardovia, Bulleida, Mycobacterium,* and *Parvimonas* are also frequently present in the oral cavity.[27] *Cohnella* has been isolated from hemodialysis patients or patients with neutropenia, and other bacteria such as *Sporolactobacillus* may be normal inhabitants of the GI tract. The role of clostridiales such as *Propionigenium* or *Anoxybacter* in humans is unknown.

The two bacterial genera found differentially represented in the gastric mucosa of patients with dyspepsia, *Pseudoclavibacter* and *Tannerella,* also have a niche in the pharynx. Genera such as *Sphingomonas* present in achalasia controls, are negatively correlated with gastritis.[28] An increase in *Lactobacillus* and *Bifidobacterium* in our achalasia controls is in line with a lower abundance in the stomach of patients with functional dyspepsia, compared with healthy controls.[7 8]

We found an over-representation of *Clostridium* in the gastric microbiota of our achalasia controls which has been previously reported in healthy individuals.[21] *Clostridium* can also be normally present in the duodenum,[21 29] however, we also found *Clostridium* at higher levels in the duodenum of patients with dyspepsia.

Most of the genera found in the duodenal samples are consistent with other reports,[10 11 21 24 29] with high presence of *Streptococcus, Prevotella, Veillonela,* and to a lesser extent *Haemophilus, Fusobacterium, Lactobacillus, Gemela,* and *Butyrivibrio.* The duodenum of patients with dyspepsia also showed a higher presence of *Haemophilus,* which has been observed in the duodenum of patients with celiac disease,[29] and along with *Butyrivibrio,* also increased in individuals with various GI symptoms.[10] A higher presence of *Rothia,* which has been positively correlated with upper abdominal pain,[28] was also observed. The high presence of *Rothia* in the duodenum of patients with dyspepsia appears to, in turn, increase the abundance of other bacteria such as *Clostridium, Haemophilus,* and *Actinobacillus* at this site. *Rothia,* and other bacteria seen over-represented in the duodenum of our patients with dyspepsia such as *Pasteurella,* and *Butyrivibrio,* may be reaching this site from the oral cavity.

An increase in the relative abundance of *Streptococcus,* while at the same time presenting a significant decrease in *Prevotella, Veillonella, Actinomyces, Aptopobium,* and *Leptotrichia* have been shown in the duodenal mucosa of patients with functional dyspepsia compared with controls.[11 12] We did not find such differences in our study, but similar to what has been reported in the duodenum of patients with GI symptoms, we observed the occurrence of an oral shift,[10] with presence of various genera that are commonly found at the oral and gastric level, such as *Scardovia, Bifidobacterium,* and *Lactobacillus.*

The microbiota affects host physiology and metabolic functions, contributing to normal development and homeostasis of the immune system in the intestine, modulating epithelial cell proliferation, and protecting against pathogenic bacteria.[30] Different bacteria are enriched with operons containing genes for different metabolic functions.[31] Regarding the functional contribution of microbiota profiles, we observed distinctive functional acquisitions between patients with dyspepsia and patients with achalasia. Metabolic pathways for amino acid and nucleotide metabolism, and biosynthesis of Gram-negative toxin were enriched in the saliva of patients with dyspepsia, while achalasia controls showed a core of sugar-related metabolic capabilities, along with proteins related to bacterial cell motility and chemotaxis. Although the latter could possibly be representing traits of virulence, motile commensal intestinal bacteria that are non-invasive do exist.[32]

The composition of the bacterial communities in the duodenum is affected by the pH. The higher concentration of *Streptococcus* in the duodenum, observed in dyspepsia[11] positively correlates with a higher pH, while anaerobes like *Prevotella* and *Pasteurellaceae* have shown a negative correlation.[33] Although the metabolic functional prediction did not find any difference between duodenal samples of patients with dyspepsia and achalasia controls, anaerobic metabolism was distinctive in the patients with stomach dyspepsia. The large presence of *Lactobacillus, Clostridium, Bifidobacterium,* and *Coprococcus* in our achalasia controls, may suggest an under-representation of these genera in dyspepsia.[34 35] Whether these bacteria have some effect on the gastric emptying time to the duodenum, something that has been observed with other genus like *Veillonella,* is unknown.[36] We did not find any difference regarding the use of PPI between dyspepsia samples and controls for any specific genus (data not shown).

In summary, we have explored the upper GI tract microbiota of patients with dyspepsia and compared them to achalasia controls, and found differences in their composition, at the three sites evaluated (ie, saliva, stomach and duodenum). Changes observed in patients with dyspepsia included an increase in *Veillonella* in saliva, an oral shift in the composition of the gastric microbiota, and to some degree in the duodenum, where an important abundance of anaerobes was observed.

This research sets the stage for further research using larger patient populations to categorize important subsets of the dyspepsia population, including those receiving a PPI, *H. pylori* related gastritis, and delayed gastric emptying to overcome the obstacles of heterogeneity in these patients.

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