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Original Article

Impact of Esophageal Motility on Microbiome Alterations in Symptomatic Gastroesophageal Reflux Disease Patients With Negative Endoscopy: Exploring the Role of Ineffective Esophageal Motility and Contraction Reserve

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Background/Aims
Ineffective esophageal motility (IEM) is common in patients with gastroesophageal reflux disease (GERD) and can be associated with poor esophageal contraction reserve on multiple rapid swallows. Alterations in the esophageal microbiome have been reported in GERD, but the relationship to presence or absence of contraction reserve in IEM patients has not been evaluated. We aim to investigate whether contraction reserve influences esophageal microbiome alterations in patients with GERD and IEM.

Methods
We prospectively enrolled GERD patients with normal endoscopy and evaluated esophageal motility and contraction reserve with multiple rapid swallows during high-resolution manometry. The esophageal mucosa was biopsied for DNA extraction and 16S ribosomal RNA gene V3-V4 (Illumina)/full-length (Pacbio) amplicon sequencing analysis.

Results
Among the 56 recruited patients, 20 had normal motility (NM), 19 had IEM with contraction reserve (IEM-R), and 17 had IEM without contraction reserve (IEM-NR). Esophageal microbiome analysis showed a significant decrease in microbial richness in patients with IEM-NR when compared to NM. The beta diversity revealed different microbiome profiles between patients with NM or IEM-R and IEM-NR ($P = 0.037$). Several esophageal bacterial taxa were characteristic in patients with IEM-NR, including reduced *Prevotella* spp. and *Veillonella dispar*, and enriched *Fusobacterium nucleatum*. In a microbiome-based random forest model for predicting IEM-NR, an area under the receiver operating characteristic curve of 0.81 was yielded.

Conclusions
In symptomatic GERD patients with normal endoscopic findings, the esophageal microbiome differs based on contraction reserve among IEM. Absent contraction reserve appears to alter the physiology and microbiota of the esophagus.
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Key Words
Contraction reserve; Esophageal motility disorders; High-resolution manometry; Ineffective esophageal motility; Microbiota

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Introduction

Esophageal motor function contributes to the pathophysiology of gastroesophageal reflux disease (GERD).[1] Ineffective esophageal motility (IEM), identified using high-resolution manometry (HRM), may contribute to impaired clearance of refluxate from the esophagus.[1-3] Although the prevalence of IEM increases with the severity of GERD,[4] causal relationships are complicated since IEM can occur in asymptomatic healthy individuals.[5] For further characterization of IEM, a provocative test termed multiple rapid swallows (MRS) is used to assess esophageal contraction reserve during HRM.[6] The vigor of the contraction reserve induced by MRS inversely correlates with esophageal acid exposure, especially in patients with IEM.[7,8]

The esophagus is hosted by microbial species similar to that seen in the oral cavity because of its anatomic continuity to the mouth, and its role in transferring food and saliva from the mouth to the stomach.[9] Changes in the esophageal microbiome have been reported with gastric reflux.[10] In the healthy esophagus, *Streptococcus* is consistently reported to be a dominant genus, together with *Prevotella, Veillonella*, and *Neisseria*.[9] Y.T.Y. et al analyzed 34 esophageal biopsy samples and classified the esophageal microbiome into 2 types using the 16S ribosomal RNA (rRNA) gene sequencing. Type 1 was dominated by the genus *Streptococcus* and was deemed phenotypically normal. By contrast, type 2 included a greater proportion of gram-negative bacteria and was correlated with severer GERD phenotypes, such as erosive esophagitis and Barrett's esophagus.[11] In addition, patients with esophageal adenocarcinoma were also reported to have esophageal microbiota different from healthy controls.[12,13]

Esophageal motor function and esophageal microbiome alteration have each been linked to GERD,[1] but their relationship in patients with reflux symptoms has not been studied in detail.[9,13] In this study, we tested the hypothesis that abnormal esophageal motor function contributes to the altered esophageal microbiome in patients with reflux symptoms. Specifically, by comparing the esophageal microbiome among GERD patients with normal motility (NM), IEM with contraction reserve (IEM-R), and IEM without contraction reserve (IEM-NR), we aim to explore the role of esophageal microbiota in esophagus hypomotility, especially in the presence or absence of contraction reserve.

Materials and Methods

Participants

Adult patients (age 18-70 years) having a history of exclusively typical GERD symptoms (heartburn and/or acid regurgitation) for at least 6 months were prospectively enrolled from the Gastrointestinal Outpatient Department of Hualien Tzu Chi Hospital. Exclusion criteria consisted of recent use of acid-suppressive therapy, antibiotics, histamine-2 receptor antagonists (H2RAs), or nonsteroidal anti-inflammatory drug (NSAID) in the past 6 months; any major organ disease (including scleroderma), systemic disease (including diabetes and hypertension), irritable bowel syndrome, functional dyspepsia, or cancer. The study was approved by the Research Ethics Committee of Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Hualien, Taiwan (IRB109-210-A). Written informed consent was obtained from each participant. The study was performed in accordance with the principles of the Declaration of Helsinki.

Protocol

All participants underwent esophagogastroduodenoscopy (EGD) prior to initiation of acid-suppressive therapy, and those who had erosive esophagitis, Barrett's esophagus, esophageal neoplasm, or hiatal hernia were excluded for preventing their influences on esophageal microbiota.[12-15] Microbiome samples were obtained by biopsies at the distal esophagus 5 cm above the esophagogastric junction during EGD. We performed esophageal biopsies solely at the mucosal layer, which theoretically should not affect esophageal motility. However, to minimize the possibility of any impact and to ensure consistency, we obtained biopsy samples from each patient at the same anatomical height, thereby maintaining a uniform effect across all participants. We performed HRM 1 day after EGD, par-

ticipants were categorized into study groups based on esophageal motility on HRM: NM, IEM-R, and IEM-NR.

High-resolution Manometry

HRM was performed by using a catheter with 22 water-perfused sensors (MMS, HRM, Enschede, the Netherlands) in patients under supine position after an overnight fast. The catheter was zeroed to atmospheric pressure, inserted transnasally into the esophagogastric lumen and then positioned with at least 3 distal sensors in the stomach.

Primary peristalsis was assessed using 10 swallows of 5 mL water at 30-second intervals. Esophageal smooth muscle contractility was measured using the distal contractile integral (DCI) which consists of the amplitude, duration, and length of distal esophageal contraction extending from the proximal pressure trough to the lower esophageal sphincter (LES). Esophageal contractility was characterized using DCI value as hypercontractile (> 8000 mmHg·s·cm), normal (450-8000 mmHg·s·cm), and ineffective (< 450 mmHg·s·cm).[16] Provocative testing with 5 MRS sequences was undertaken by steadily injecting water into the mouth through a syringe. Each successful MRS sequence requires at least 4 rapid 2 mL water swallows with an interval ≤ 4 seconds between swallows. Intact contraction reserve was defined as a ratio greater than 1 between the post-MRS DCI and the average DCI for 10 standard single swallows (SS) (ie, MRS/SS DCI > 1).[8]

Multichannel Intraluminal Impedance pH

Following the HRIM studies, part of study patients underwent multichannel intraluminal impedance pH (MII-pH) testing as per standard clinical protocol. A multichannel intraluminal impedance pH catheter (MMS, HRM) was placed transnasally, with positioning based on the manometrically identified LES. The catheter consisted of 6 impedance sensors positioned 3, 5, 7, 9, 15, and 17 cm from the LES and pH sensors positioned 5 cm above and 10 cm below the LES. MII-pH testing was used to assess mean nocturnal baseline impedance (MNBI) and acid exposure time (AET) in this study. MNBI was calculated by averaging baseline impedance values at 3 cm above the upper border of the LES during stable nocturnal 10-minute periods at 1 AM, 2 AM, and 3 AM.[17] Total AET, defined as the fraction of time the distal esophagus is exposed to a pH < 4.0, was extracted from pH-impedance studies and considered pathological when > 6%. A positive symptom association probability (SAP) was defined as > 95%, corresponding to $P < 0.05$. Based on AET and SAP, patients were stratified into non-erosive reflux disease (NERD) (AET > 6%), reflux hypersensitivity (AET < 4%, positive SAP), and functional heartburn (AET < 4%, negative SAP).[2]

Microbial Profiling

The genomic DNA from the biopsied specimen was extracted using the column-based method (QIAamp PowerFecal DNA Kit; Qiagen, Hilden, Germany). DNA concentration was determined by Qubit 4.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and adjusted to 1 ng/uL for the following process. The genomic DNA of all 56 samples were used for V3-V4 amplicon sequencing, and the remaining genomic DNA from 52 samples were used for full-length amplicon sequencing.

16S ribosomal RNA gene V3-V4 amplicon sequencing

Polymerase chain reaction (PCR) targeting the 16S rRNA V3-V4 region was performed with the primer pair 341F/805R, using KAPA HiFi HotStart ReadyMix (Roche, Munich, Germany) with Illumina adaptor sequences on their 5' end. DNA was recovered from excised gels after electrophoresis of the PCR products using an EasyPure PCR/Gel extraction kit (Bioman Scientific Co, New Taipei City, Taiwan).

The DNA library was constructed from extracted DNA using a Nextera XT index kit (Illumina, San Diego, CA, USA). The resulting DNA was then purified using AMPure XP beads (Beckman Coulter, Brea, CA, USA). A Quant-Ti dsDNA Assay Kit (Thermo Fisher Scientific Inc, Waltham, MA, USA) and an Agilent Technologies 2100 Bioanalyzer (Santa Clara, CA, USA) were used to quantify DNA concentration (adjusted to 10 nM for sequencing). The samples were sequenced in MiSeq (Illumina), and the obtained reads were processed with Quantitative Insights Into Microbial Ecology 2 (QIIME2).[18] The DADA2' plugin was used to identify amplicon sequence variants (ASVs) from demultiplexed sequence files. The Human Oral Microbiome Database (eHOMD) was used for taxonomy assignment.[19] Read count of each sample was rarefied to the minimal counts among all samples (15 928 reads). The ASVs were collapsed into the phylogenetic levels for downstream analyses.

16S ribosomal RNA gene full-length amplicon sequencing

The full-length 16S rRNA genes (V1-V9 regions) were amplified according to the Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell Library Preparation and Sequencing Procedure (Pacbio, Menlo Park, CA, USA). Each primer is designed to contain a 5' buffer sequence (GCATC) with a 5' phosphate modification, a 16-base barcode, and the degenerate

16S gene-specific forward or reverse primer sequences (Forward: 5'-Phos/GCATC-16-base barcode–AGRGTTYGATYMTGGCTCAG-3', Reverse: 5'-Phos/GCATC-16-base barcode–RGYTACCTTGTTACGACTT-3'). In brief, 2 ng of gDNA was used for the PCR reaction carried out with KAPA HiFi HotStart ReadyMix (Roche). The PCR products were monitored on 1% agarose gel. Samples with a bright main strip around 1500 base pairs were chosen and purified by using the AMPure PB Beads for the following library preparation.

The library preparation was performed according to the Amplification of Full-Length 16S Gene with Barcoded Primers for Multiplexed SMRTbell Library Preparation and Sequencing Procedure (Pacbio). The SMRTbell library was incubated with sequencing primer v4 and sequel II Binding Kit 2.1 for the primer annealing and polymerase binding. At last, sequencing was performed in the circular consensus sequence mode on a PacBio Sequel IIe instrument to generate the HiFi reads with Predicted Accuracy (Phred scale) = 30. After demultiplexing, the circular consensus sequence were further processed with DADA2 to identify ASVs. The QIIME2 algorithm was employed for taxonomy assignment by alignment against the eHOMD. Read count of each sample was rarefied to the minimal counts among all samples (1278 reads). The ASVs were collapsed into phylogenetic levels for downstream analyses.

Re-analysis of Published Microbiota Data

To verify the esophageal microbiota in our study is representative of esophageal microbiome profile, we compared our sequences with datasets by Li et al[20] (BioProject accession number PRJNA628659) and Lopetuso et al[21] (PRJNA553177). Li et al[20] included patients with esophageal squamous cell carcinoma and esophagogastric junction cancer from China, whereas Lopetuso et al[21] included patients with esophageal adenocarcinoma and Barrett esophagus from Italy; both included healthy controls. The downloaded 16S rDNA sequences were trimmed to fragments from bases 341 to 805 to be comparable with the sequences in the present study.

Statistical Methods

The richness of microbiota species (alpha diversity) was assessed by observed species, Shannon index, Simpson index, and Chao1 index. Compositional differences (beta diversity) were compared by Bray-Curtis dissimilarity and plotted with principal coordinate analysis and nonparametric multidimensional scaling. An analysis of similarities (ANOSIM) test was used to test heterogeneity between groups. All numerical analyses were tested using two-tailed Student's *t* tests, Mann-Whitney *U* tests, or ANOVA tests as deemed appropriate under a significance level of $P < 0.05$. All statistics were analyzed using R software version 3.6.3 or GraphPad Prism (version 9). Finally, a microbiome-based random forest classifier was built with random Forest R package.

Data Availability

16S rRNA gene V3-V4 amplicon sequencing data have been deposited on NCBI public repository (BioProject #PRJNA746612) and 16S rRNA gene full-length amplicon sequencing data have been deposited on NCBI public repository (BioProject #PRJNA831571).

Results

Clinical Characteristics of the Enrolled Gastroesophageal Reflux Disease Patients

A total of 78 participants met the criteria of chronic typical GERD symptoms after excluding recent use of proton pump inhibitor (n = 27), H2RA (n = 32), antibiotics (n = 4), or NSAID (n = 7) were enrolled. After receiving EGD, 10 patients with erosive esophagitis, 5 patients with hiatal hernia, and 7 patients with erosive esophagitis as well as hiatal hernia were excluded. Finally, 56 patients with reflux symptoms and normal EGD were included (mean age 48.6 ± 12.0 years, 30 females). Characterized by HRM metrics and MRS response, 20 patients had NM, 19 patients were grouped as IEM-R, and 17 patients were grouped as IEM-NR (Fig. 1A). Patients with IEM-NR exhibited higher body mass index compared to patients with IEM-R ($P = 0.032$). Furthermore, patients with NR demonstrated greater DCI values than both patients with IEM-R and those with IEM-NR ($P < 0.001$). There were no significant differences in age, gender distribution, gastroesophageal reflux disease questionnaire scores, and 4-second integrated relaxation pressure among the 3 patient groups ($P > 0.05$) (Supplementary Table).

Esophageal Microbiome Pattern in Gastroesophageal Reflux Disease Patients

Among the 56 esophageal biopsy samples, 4668 amplicon sequence variants were identified and grouped into 212 species, 136 genera, 94 families, and 14 phyla. *Streptococcus, Prevotella, Neisseria, Veillonella, Haemophilus, Alloprevotella, Porphyromonas*, and

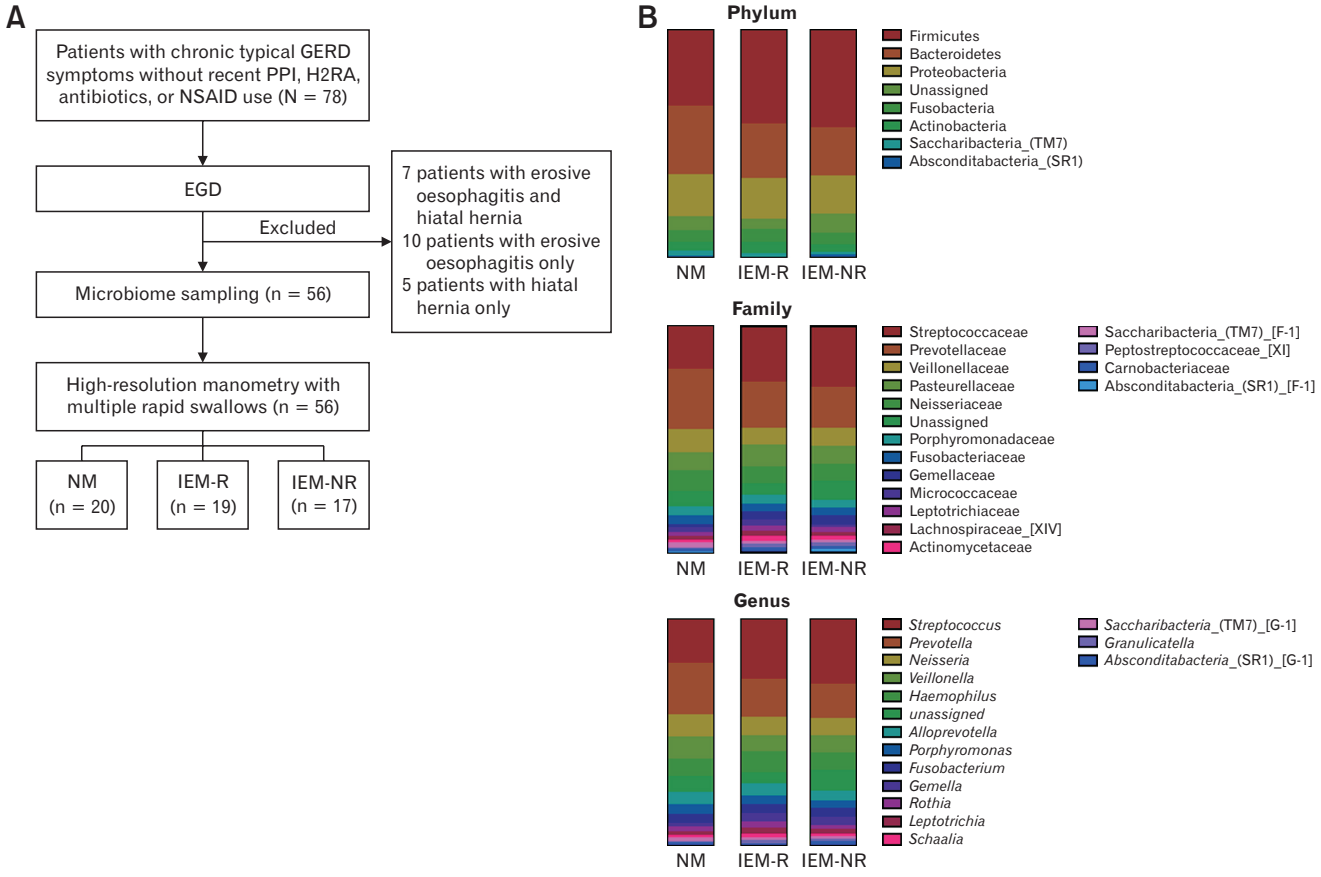

Figure 1. Study flow chart and comparative analysis of esophageal microbiota in normal and ineffective esophageal motility groups. (A) Study flow chart. (B) The distribution of phylum, family, and genus of the esophageal microbiota among normal motility (NM), ineffective esophageal motility with contraction reserve (IEM-R), and ineffective esophageal motility without contraction reserve (IEM-NR) groups. GERD, gastroesophageal reflux disease; PPI, proton pump inhibitor; H2RA, histamine-2 receptor antagonist; HRM, high-resolution manometry; EGD, esophagogastroduodenoscopy.

Fusobacterium were the most abundant genera (Fig. 1B). *Streptococcus* constituted a predominant proportion of the esophageal microbiota in the NM, IEM-R, and IEM-NR groups, which were consistent with the type 1 microbiome of the normal esophageal mucosa. We then explored the similarity between the esophageal microbiome in this study with 2 previously published datasets,[14,21] and found our samples were within the spectrum of microbiota-derived from healthy esophageal tissues of these 2 studies (Supplementary Fig. 1). Of note, a trend of increased *Streptococcus*-to-*Prevotella* ratio was observed in the IEM groups despite not reaching statistical significance.

Microbiome in Different Esophageal Motility Phenotypes

Microbiota species richness (alpha diversity) and compositional differences (beta diversity) were compared among the NM, IEM-R, and IEM-NR groups. The observed species and Chao1 index representing the richness of microbial community were significantly decreased in the IEM-NR group as compared with the NM group ($P = 0.040$, ANNOVA + Tukey test) (Fig. 2). On the other hand, the evenness-weighted Shannon and Simpson index showed a decreased trend in the IEM-NR group.

For the comparison of beta diversity, the principal coordinate analysis showed extensive overlapping of microbial composition and no significant difference among the 3 groups (ANOSIM, $P = 0.196$) (Fig. 3A). The microbiome compositions were also not different between the NM and IEM (IEM-R and IEM-NR) groups (ANOSIM, $P = 0.771$) (Fig. 3B). Of note, a significant difference in microbiome profile was observed between patients with NM or IEM-R and those with IEM-NR (ANOSIM, $P = 0.037$) (Fig. 3C).

We then performed multivariate sparse partial least-squares

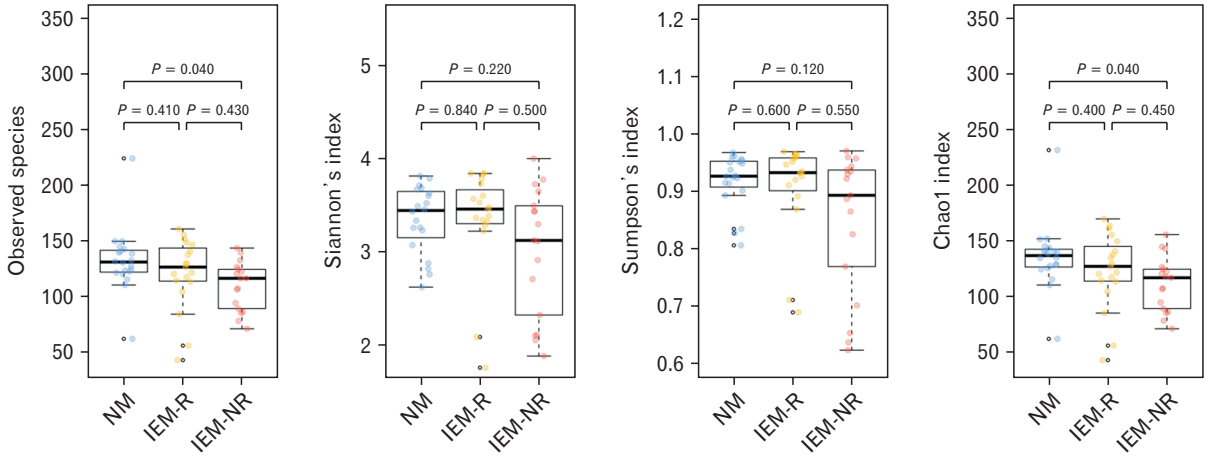

Figure 2. Biodiversity analysis for the esophageal microbiome of patients with normal motility (NM), ineffective esophageal motility with contraction reserve (IEM-R), and ineffective esophageal motility without contraction reserve (IEM-NR). Alpha diversity indexes indicating microbial richness, such as observed species and Chao1 index, were significantly decreased in the IEM-NR groups when compared with the NM group ($P = 0.040$, Tukey test).

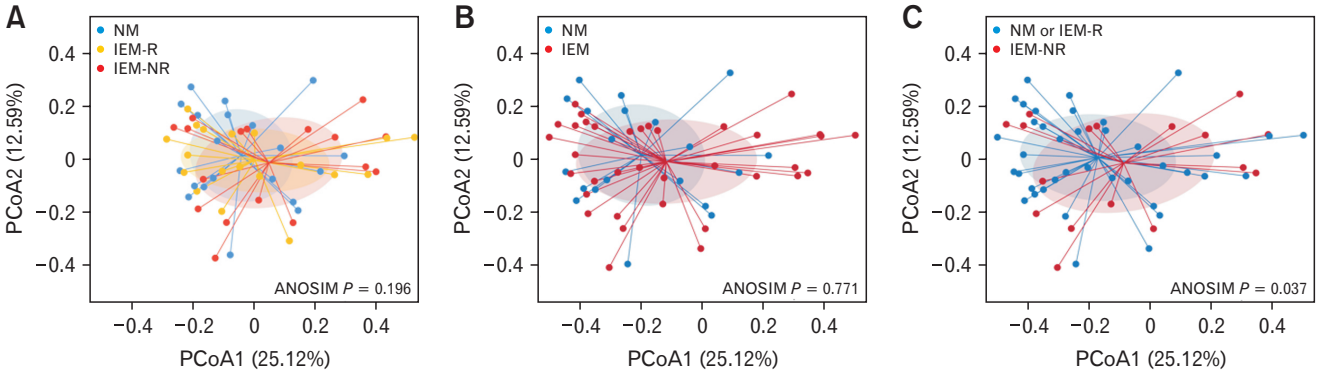

Figure 3. Esophageal microbiome profile comparison between the normal motility (NM), ineffective esophageal motility with contraction reserve (IEM-R), and ineffective esophageal motility without contraction reserve (IEM-NR) groups by using principal coordinate analysis (PCoA). (A) No significant difference in esophageal microbiome profiles was found among the 3 groups. (B) Unremarkable difference in esophageal microbiome profiles between patients with NM and patients with IEM. (C) A significant microbiome compositional difference was observed between patients with NM or IEM-R and patients with IEM-NR (analysis of similarities [ANOSIM] $P = 0.037$).

discriminant analysis (sPLS-DA) to identify bacterial species contributing to the distinction between the study groups (Fig. 4A). The abundance of microbial taxa decreased in patients with IEM-NR, including *Actinomyces* spp., *Peptidiphaga* spp., *Rothia mucilaginosa*, *Prevotella* spp., and *Veillonella dispar*, which contributed to the separation along with component 1 (Fig. 4B). A heatmap plotted by significantly different species observed between NM or IEM-R and IEM-NR groups (Mann-Whitney *U* test, $P < 0.05$) showed multiple esophageal bacterial species decreased markedly in the IEM-NR group (Fig. 5). Notably, several differential taxa, including *Actinomyces* spp._HMT_169, *Peptidiphaga* spp._HMT_183, *R. mucilaginosa*, *Prevotella denticola*, *Kingella* spp._HMT_932, and *Prevotella pallens* were markedly reduced in patients without a contraction reserve and were consistent with the result of sPLS-DA.

Moreover, 27 patients of this study received MII-pH testing and parameters such as AET and MNBI were obtained. The MNBI values for patients with NM and those with IEM-R were both higher than the values observed in patients with IEM-NR ($P = 0.015$). The proportions of GERD phenotypes and AET values showed no significant differences among the 3 patient groups ($P > 0.05$) (Supplementary Table). Regarding

the phenotypes, 5 patients were NERD, 12 patients were reflux hypersensitivity, and 10 patients were functional heartburn. There were no significant difference of microbiota species richness and compositional differences among phenotypes. Among the identified 212 esophageal bacterial species, 3 taxa including *Parvimonas micra, Gemella morbillorum*, and *Schaalia* spp._HMT_172 were identified to be correlated positively with AET and negatively with MNBI (Supplementary Fig. 2).

Microbiome-based Predicting Model for the Absence of Contractile Reserve

Finally, a microbiome-based random forest model was built to determine if the distinctive esophageal bacterial taxa could be used to predict patients with IEM-NR. The model yielded an area under the receiver operating characteristic curve (AUROC) of 0.81 (95% CI, 0.68-0.93) in differentiating GERD patients with and without a contraction reserve (Fig. 6A). Moreover, we further

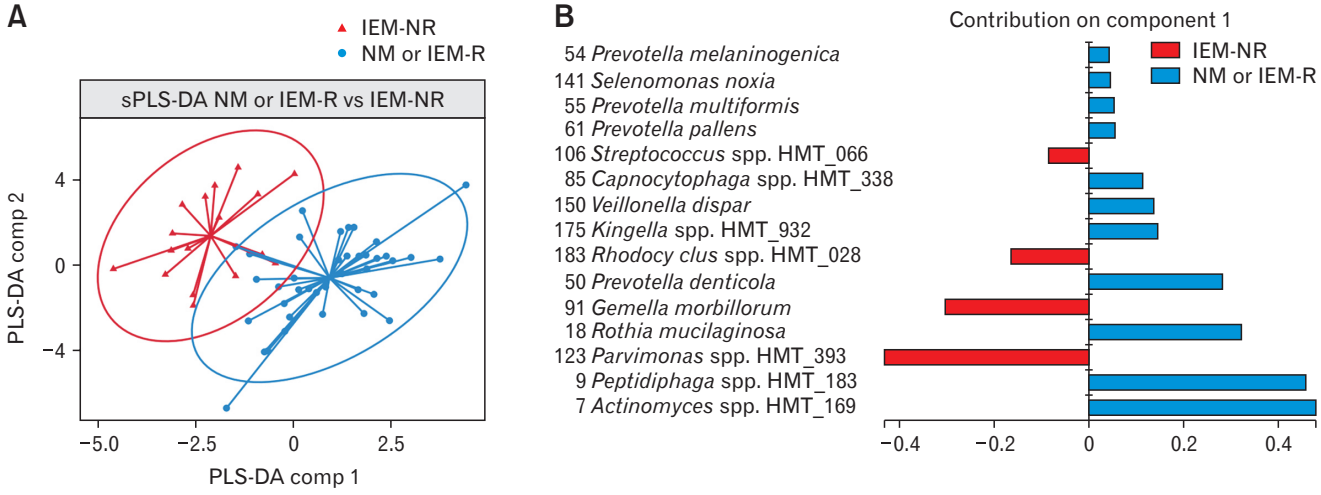

Figure 4. Multivariate sparse partial least-squares discriminant analysis (sPLS-DA) and bacterial species contributions in esophageal motility groups. (A) sPLS-DA for identifying variances responsible for the distinction between the study groups. (B) Specific bacterial species contribute to the separation along with component 1. NM, normal motility; IEM-R, ineffective esophageal motility with contraction reserve; IEM-NR, ineffective esophageal motility without contraction reserve.

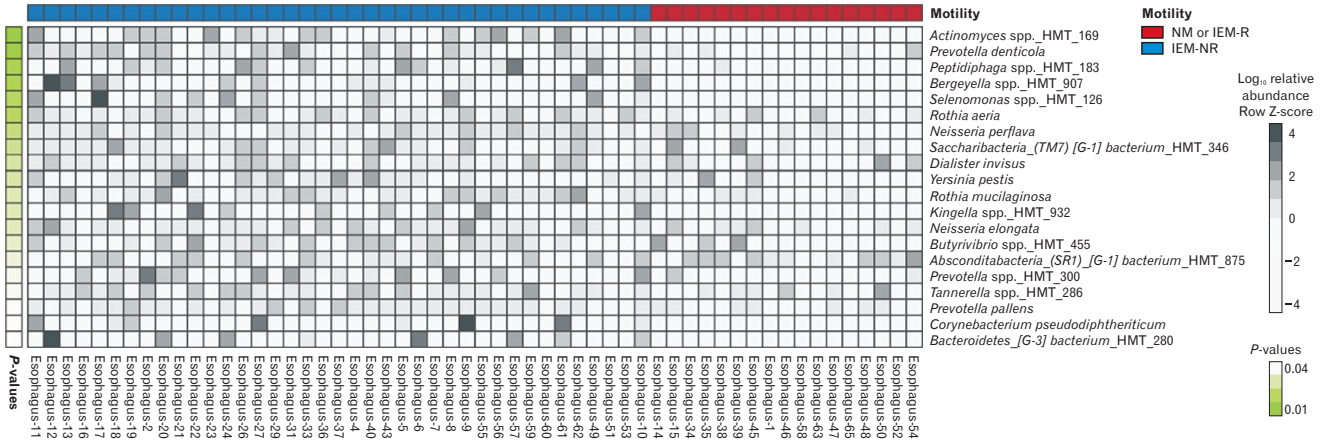

Figure 5. A heatmap diagram showing the abundance of esophageal bacterial species differs significantly between patients with normal motility (NM) or ineffective esophageal motility with contraction reserve (IEM-R) and patients with ineffective esophageal motility without contraction reserve (IEM-NR). Gray scale represented log-transformed relative abundance normalized into Z-scores by row. The *P*-values indicate the significance of differential abundance of microbial taxa between patients with NM or IEM-R and patients with IEM-NR by using Mann-Whitney *U* test.

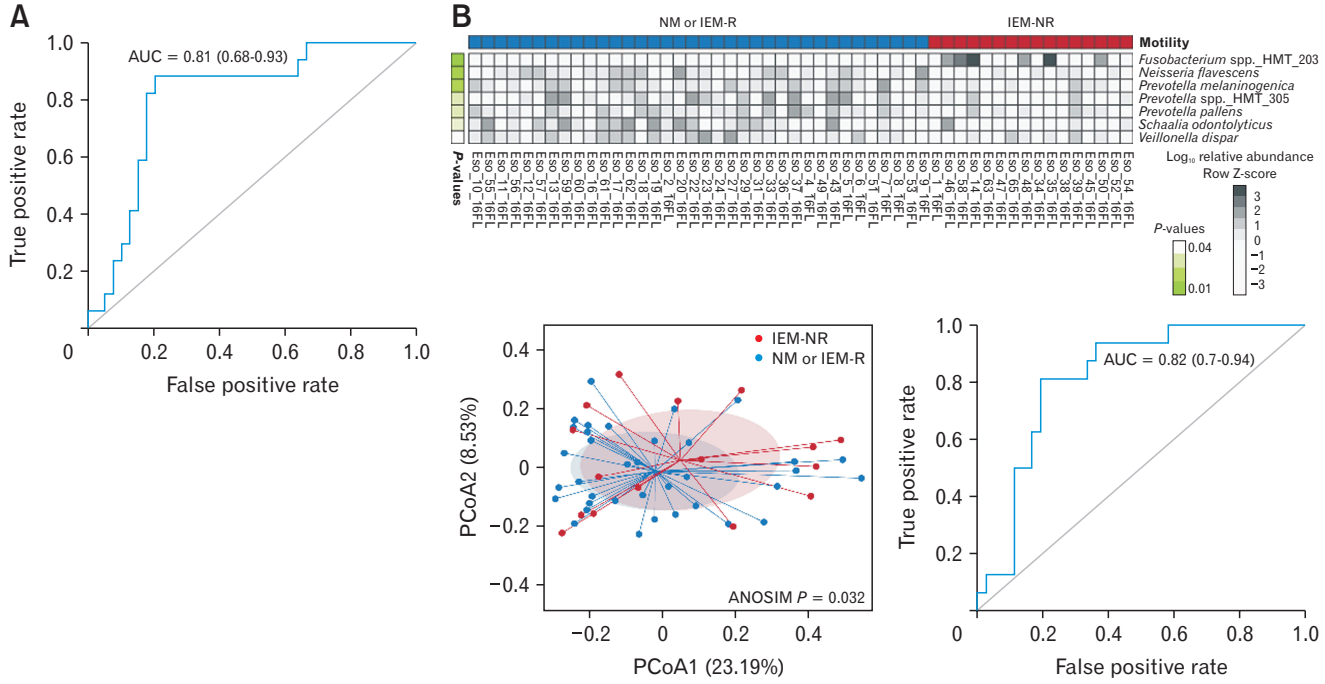

Figure 6. Microbiome-based random forest model and differential bacterial species in esophageal motility groups. (A) A microbiome-based random forest model was built with 20 selected 16S V3-V4 microbial features significantly different between normal motility (NM) or ineffective esophageal motility with contraction reserve (IEM-R) and ineffective esophageal motility without contraction reserve (IEM-NR), and an area under the receiver operating characteristic curve (AUROC) of 0.81 (95% CI, 0.68-0.93) for predicting IEM-NR was obtained. (B) Differential bacterial species between NM or IEM-R and IEM-NR identified by using 16S full-length (FL) sequencing showed consistent results with that found by using 16S V3-V4 sequencing. Gray scale represented log-transformed relative abundance normalized into Z-scores by row. The *P*-values indicate the significance of differential abundance of microbial taxa between patients with NM or IEM-R and patients with IEM-NR by using Mann-Whitney *U* test. A microbiome-based random forest model was built with seven selected 16S FL microbial features yielded an AUROC of 0.82 (95% CI, 0.7-0.94) for predicting IEM-NR. AUC, area under the curve; PCoA, principal coordinate analysis.

performed 16S rRNA full-length (FL) amplicon sequencing (Pacbio HiFi reads) by using residual extracted DNA from the biopsied esophagus specimen (n = 52; NM:19, IEM-R:17, IEM-NR:16) to verify the results observed in the 16S rRNA V3-V4 amplicon sequencing (Illumina Miseq). In brief, the results of 16S FL sequencing were compatible with the findings of 16S V3-V4 sequencing. Some distinctive microbial features decreased in IEM-NR were noted, including *Prevotella pallens, Prevotella melaninogenica, V. dispar*, and *Neisseria* spp. Notably, *Fusobacterium* spp._HMT_203 was significantly enriched in the IEM-NR group (false discovery rate [FDR] < 0.01), which was not detected by the 16S V3-V4 sequencing. We then BLAST the ASVs of *Fusobacterium* spp._HMT_203 against the NCBI database and found the sequences belonged to *F. nucleatum* (99% identity with the ATCC 25 586). With the distinctive esophageal bacterial taxa identified by the 16S FL sequencing, a FL-based random forest classifier was established for predicting an absent contraction reserve and an AUROC of 0.82 (95% CI, 0.7-0.94) was obtained (Fig. 6B).

Discussion

This study demonstrated that impairment of esophageal contraction reserve contributes to esophageal microbiome alterations in patients with reflux symptoms and IEM in the setting of normal endoscopy. Patients who had IEM with absent contraction reserve also exhibited a marked decrease of observed bacterial species (alpha diversity), which included several taxa of *Prevotella* spp. and *V. dispar*. Based on these findings, we developed a potential microbiome-based random forest model for predicting the absence of esophageal contraction reserve. In addition, we used the state-of-the-art long-read sequencing technique for 16S FL esophageal microbiome profiling and further verified the results found in 16S V3-V4 sequencing. Of note, *F. nucleatum* was observed to be significantly increased in patients without a contraction reserve (FDR < 0.01).

Streptococcus, Prevotella, Veillonella, Neisseria, and *Hemophilus* have been consistently reported to colonize the healthy esophageal mucosa in both culture-dependent and culture-independent studies,[9] which was also evident in the current study. Reflux-related disease, such as esophagitis, Barrett's esophagus, and esophageal adenocarcinoma, was also known to be an important cause of altered esophageal microbiota.[9] To date, however, the microbiota had not been explored in nonerosive reflux disease, reflux hypersensitivity, or functional heartburn, which may range in severity from a healthy esophagus to esophageal mucosal injury at endoscopy. The results of the present study demonstrate that the esophageal microbiota in patients with chronic reflux symptoms and normal endoscopy findings are similar to that of healthy controls (*Streptococcus*-predominant type 1 microbiota).[11] In turn, this suggests that a shift in esophageal microbiota colonization may reflect GERD-related mucosal injury seen at endoscopy.

In the present study, a significant decrease in the richness of esophageal microbiota was observed in the IEM-NR group, rather than the IEM-R group, when compared with patients having normal esophageal motility. Since the contraction reserve is the compulsive compensatory function of esophagus with ineffective motility, it plays a critical role in protecting the esophageal mucosa from prolonged exposure to acid reflux in the IEM patients. The impaired contraction reserve may inhibit the growth of acid-sensitive esophageal microbes by increasing esophageal acid exposure time. Besides, the impaired compression forces along the esophagus in IEM patients without contraction reserve may reduce the adhesion of saliva microbes to the esophageal mucosa. Thus, the presence or absence of esophageal contraction reserve could be a key environmental factor that affects the biodiversity of esophageal microbial community.

Among the 27 patients who received MII-pH testing, *P. micra* and *G. morbillorum* were strongly associated with increased esophageal acid exposure and decreased mucosal integrity. Interestingly, fecal *P. micra* and *G. morbillorum* had been reliably associated with human colorectal cancer[22] and saliva *P. micra* was also reported significantly increased with pathologic stage of oral squamous cell carcinoma.[23] Whether these microbes are linked to the pathogenesis of esophageal disorders requires further investigation.

For the microbial composition, our analysis demonstrated different microbiome profiles between those with and without contraction reserve rather than those with and without IEM. These results further provide evidence that esophageal contraction reserve during MRS may uncover important pathophysiological differences relative to GERD in patients with IEM.[5] Among the various bacterial species decreased in patients without esophageal contraction reserve, the decrease in *P. pallens, P. melaninogenica*, and *V. dispar* were constantly observed with different analytical approaches, including the 16S FL sequencing, and might become potential biomarkers in those who are absent in esophageal contraction reserve. Interestingly, *F. nucleatum* was identified to be significantly enriched in the IEM-NR group (FDR < 0.01) by analyzing the 16S FL sequences. As one of the periodontal bacteria, *F. nucleatum* was considered as an opportunistic pathogen and has been causally correlated with an increased risk of colorectal cancer and esophageal cancer through its oncogenic and pro-inflammatory properties.[24,25] Despite an increased abundance of *F. nucleatum* in patients with IEM-NR, however, whether there is a causal role of *F. nucleatum* for an absent esophageal contraction reserve still requires to be investigated by further study.

Furthermore, we established a microbiome-based random forest model (AUROC $= 0.81$) that could predict the absence of esophageal contraction reserve based on the changes in the microbiota. These findings reinforce the view that a distinct pattern of esophageal microbiota is present in patients with reflux symptoms who have normal endoscopic findings and that these changes are significantly associated with a change in the esophageal contraction reserve.

The novelty and significance of this study is that it is the first to explore the impact of esophageal motor function on the esophageal microbiota. Specifically, esophageal contraction reserve was shown to exert more contribution to esophageal microbiome alteration than IEM per se. This supports the notion that IEM is a manometric rather than a clinical diagnosis and that evaluating esophageal contraction reserve using MRS further identifies the severity of esophageal hypomotility and IEM, which are part of GERD related pathophysiology.[5,8] Besides, this is the first study that applies the technique of 16S FL sequencing in esophageal microbiota research. The FL sequence-based random forest modeling by 7 selected features shows a comparable prediction accuracy (AUROC $= 0.82$) with the V3-V4 sequence-based modeling by 20 selected features. This is probably because the 16S FL sequences have a higher resolution in assigning bacterial taxa. Besides, the differential taxa at species level obtained from LEfSe were highly consistent with differential species mentioned above in both 16S V3-V4 and 16S FL amplicon sequencing datasets (Supplementary Fig. 3). These consistent findings observed in both 16S V3-V4 and 16S FL microbiome analysis further strengthen the significance and robustness of this study.

While our study primarily focused on the relationship between esophageal motility and the esophageal microbiome in patients

with GERD symptoms, it is important to consider the possibility of a bidirectional relationship between these 2 factors. The findings may have implications for the treatment of GERD. A deeper understanding of the esophageal microbiome and its association with esophageal motility dysfunction could potentially help in developing targeted therapeutic strategies. However, it is crucial to first establish the causality between these factors. For instance, if alterations in the esophageal microbiome are found to play a significant role in GERD pathogenesis and a causal relationship is established, probiotics or other microbiome-modifying therapies could be considered as a part of a comprehensive treatment plan. Additionally, identifying specific motility patterns that contribute to microbiome alterations may guide the development of tailored therapies to address underlying motility issues in GERD patients. The concept of treating GERD symptoms by adjusting the microbiota to further improve esophageal motility function could be an innovative approach to managing this condition. It is essential to note that further research is necessary to confirm these associations and establish the efficacy of such therapeutic approaches. Future studies could explore the direct impact of esophageal motility-targeted interventions on the esophageal microbiome and GERD symptom improvement, while also focusing on elucidating the causal relationships involved and investigating the potential benefits of microbiota modulation in improving esophageal motility. In particular, understanding the potential bidirectional relationship between esophageal motility and the esophageal microbiome will be crucial to advancing our knowledge of these interactions and their implications for GERD treatment.

This study has several limitations that we acknowledge. First, we did not perform MII-pH testing to further phenotype all patients with reflux symptoms, which may have a risk of type II error to conclude no significant difference of microbiota species richness and compositional differences among phenotypes. However, the primary aim of this exploratory research was to determine relationships, if any, between esophageal motor function and the microbiota in symptomatic patients with normal endoscopy. Now that we have established the presence of such relationships, future studies are warranted to evaluate esophageal microbiota patterns in GERD phenotypes determined by reflux monitoring. Second, we did not evaluate gene expression in the esophageal epithelium for host-microbe interactions, and there was no further causal interpretation between patients with and without esophageal contraction reserve. Third, the altered esophageal microbiome could be induced by liquid retention rather than esophageal hypomotility; however, since there is no available data from patients with achalasia or scleroderma for comparison, it remains uncertain whether the microbiome changes in patients with IEM-NR is due to lack of motility or liquid retention. Finally, this work did not enroll patients with absent contractility, which may represent more severe hypomotility. If microbiota patterns between patients with absent contractility and IEM patients with absent contraction reserve are similar, it may provide additional support to current findings.[26,27]

In conclusion, esophageal contraction reserve rather than IEM appears to be significantly associated with altered richness and composition of esophageal microbiome in patients with chronic reflux symptoms and normal endoscopy findings. Specific distinctive bacteria taxa include reduced *Prevotella* spp. and *V. dispar*, and enriched *F. nucleatum* in patients with absent esophageal contraction reserve. The microbiome-based random forest models established by both 16S V3-V4 and 16S FL sequences can predict absent contraction reserve. Further studies are needed to determine the interactions among esophageal motility, mucosa integrity, reflux burden, and microbiota colonization.

Supplementary Materials

Note: To access the supplementary table and figures mentioned in this article, visit the online version of *Journal of Neurogastroenterology and Motility* at http://www.jnmjournal.org/, and at https://doi.org/10.5056/jnm22191.

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