negative bacteria. The link between *H. pylori* and GERD, however, remains controversial. A recent meta-analysis in Indonesia reported a high prevalence of GERD, whereas the prevalence of *H. pylori* infection was comparatively low (9). This phenomenon can further be justified with a detailed microbial perspective. The gastric biopsy specimens were exploited using the 16S rRNA approach for the evaluation of differences in the gastric microflora between ERD or NERD patients. The abundance of *H. pylori* in the disease groups was inferred and the shift in the microflora was considered for the functional analysis in the disease groups. Our objective was to analyze the alterations in the gastric microbial consortium of ERD and NERD groups which could give insight into the progression of GI diseases.

MATERIALS AND METHODS

Collection and screening of samples

This study was conducted to evaluate the microbial dysbiosis in ERD and NERD samples in comparison to control samples. Patients (n = 50) visiting the Department of Gastroenterology, Max Super Specialty Hospital, Vaishali, Ghaziabad, Uttar Pradesh, for their upper gastroesophageal symptoms were included in this study and were evaluated by a gastroenterologist. The patients underwent upper gastrointestinal endoscopic screening for their evaluation and treatment protocol. Three biopsy samples of each patient were collected from their stomach antrum as per inclusion and exclusion criteria. One sample was used for a rapid urease test (RUT) and the second sample was collected in phosphate buffer saline for DNA isolation and sequencing and the third sample was collected in 10% buffered formalin for sequencing. Age 15 to 90 years old, upper gastrointestinal endoscopic consent and clinical indications of gastro-duodenal disease associated with *H. pylori* were among the inclusion criteria. On the other hand, as an exclusion criterion, the use of proton pump inhibitors and antimicrobial medications was prohibited for three months before the sample collection. Based on a questionnaire, demographic details were collected, consisting of gender, smoking, and alcohol-drinking habits as risk factors for GERD and dyspepsia (Table 1). On the basis of our study design, subjects with incomplete specimens or with GC, gastric or duodenal ulcers, and intestinal metaplasia were excluded. The symptoms and upper-endoscopy evaluations by the gastroenterologist were considered the basis of disease characterization. The patients who were diagnosed with both reflux symptoms and mucosal lesions (esophageal erosions) and were also fulfilling the Los Angeles classification were concluded with ERD, whereas the patients diagnosed with only reflux symptoms, heartburn and chest pain were concluded with NERD. Random sampling was performed to obtain 50 samples of ERD, NERD, and Control for sequencing and post-filtering based on the questionnaire and clinical diagnosis, a total of 27 samples were selected for this study.

Ethics approval

All the procedures performed in the study were in accordance with the ethical standards of the institution. This study was approved by the institutional ethical committee of Amity Institute of Biotechnology, Noida, U.P, dated 2-12-2015, and Max Hospital, Vaishali, U.P, India (RS/MSSH/VSH/CRL/IEC/ GASTRO/17-19).

Consent to participate and publish

Written informed consent was taken from all participants. The authors affirm that human research participants provided informed consent for publication.

Data availability

The datasets generated and analysed during the current study are available in the NCBI Sequence Read Archive under Bio Project accession no PRJNA904926 with the accession numbers SRR22426106 to SRR22426144 and SRR22859444 to SRR22859448

Table 1: Demographic characteristics of the samples

Parameters		Control (%)	ERD (%)	p-value[a]	NERD (%)	p-value[b]
Gender	Male	2 (40%)	12 (66.66%)	0.3428	1 (25%)	1.00
	Female	3 (60%)	6 (33.33%)		3 (75%)	
Alcohol status	Yes	1 (20%)	8 (44.44%)	0.6106	1 (25%)	1.00
	No	4 (80%)	10 (55.55%)		3 (75%)	
Smoking status	Yes	-	8 (44.44%)	0.1221	1 (25%)	0.444
	No	5 (100%)	10 (55.55%)		3 (75%)	

[a]p-value: calculated using the Fisher Exact test between ERD and control groups.
[b]p-value: calculated using the Fisher Exact test between NERD and control groups.

DNA extraction and metagenomic profiling of 16S rRNA sequences

Genomic DNA from human tissue biopsy samples was extracted using the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany). It was quantified and assessed for quality using agarose gel electrophoresis. A specific primer with a sequencing linker was created for the 16S rRNA V3 and V4 region using the KAPA HiFi HotStart PCR) Kit (R&D Cape Town, South Africa) for 26 cycles. Amplified signals were checked on a 1.2 percent agarose gel. Round 1 PCR amplicons were further amplified (10 cycles) to include Illumina sequencing barcoded adaptors. Round-2 PCR amplicons (the sequencing libraries) were examined

on a gel. Further for multiplex sequencing, the libraries were additionally normalized and pooled. For 16S rRNA gene amplification, the illumina adapter sequences listed below were utilized.

5' AATGATACGGCGACCACCGAGATCTACAC[i5]TCGT CGGCAGCGTC 3'
5' CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGG GCTCGG 3'

Samples underwent paired-end sequencing on the Illumina MiSeq v3 600-cycle cartridge, focusing on the V3-V4 primer sequences' quality bases. Reads were stitched using Fastq-join3 and used for microbiome analysis in the QIIME pipeline. The UCLUST5 method clustered query sequences against the Greengenes 16S rRNA database (v13.8), generating a biome file with taxonomies assigned by the RDP7 classifier at ≥97 percent sequence similarity. Operational taxonomic units (OTUs) were identified using acquired reads and QIIME scripts. Software like MicrobiomeAnalyst and STAMP were used to conduct statistical analysis and functional prediction.

Statistical analysis

The microbial alpha diversity analysis of human tissue biopsy samples was determined by using QIIME (Version 1.7.0) and represented by various indices, using MicrobiomeAnalyst. The relative abundance of the bacterial diversity among the ERD, NERD, and control samples was determined by analysis of variance (ANOVA) and represented using a stacked bar plot chart. Beta diversity was analysed using the Bray-Curtis dissimilarity index with Permutational Multivariate Analysis of Variance (PERMANOVA), represented by the PCA plot. The correlation analysis was performed using Spearman Rank Correlation as the distance measure. Using the Shotgun Data Profiling feature of MicrobiomeAnalyst, Linear discriminant analysis effective size (LEfSe) was performed to determine the significant Kyoto Encyclopaedia of Genes and Genomes (KEGG) IDs (p-values < 0.05) that explain the involvement of the microbial flora in the functional differences between the ERD, NERD, and control samples. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to estimate the functional content of a metagenome using marker gene data and a database of reference genomes. STAMP was used to graphically represent the results. The p-value was determined using the Kruskal-Wallis H-test and Welch's t-tests; differences were deemed significant for p-values less than 0.05.

RESULTS

Patients' characteristics

The samples were divided into three study groups: 18 ERD, 4 NERD, and 5 Control. Compared to the control samples, alcohol, and smoking status did not significantly correlate with study groups. The rates of alcohol consumption and smoking were 44.44% in the ERD group (Table 1).

Gastric microbiota diversity comparison among study groups

In this dataset, 1144 taxonomic units were detected and assigned to respective phyla, among them 7 phyla were found to be significantly abundant among the study groups. The relative abundance of the phyla in each sample across the diseased groups is illustrated below (Fig. 1). At the phylum level, the gastric microbiota was majorly composed of *Proteobacteria, Firmicutes, Bacteroidetes, Campylobacteria, Actinobacteria,* and *Fusobacteria*, with varied percentages of relative abundance, in both *H. pylori*-positive and -negative individuals. The gastric microbiome profiles in the three groups of subjects were inhabited by *Bacteroidetes* (ERD 10.42%, NERD 1.87%, and control 14.7%), *Firmicutes* (ERD 21.5%, NERD 19.08%, and control 68.5%), *Proteobacteria* (ERD 5.29%, NERD 5.09%, and control 2.95%) (Fig. 1). The relative abundance between the study groups at the genus level showed an increase in the abundance of *Helicobacter*, *Corynebacterium*, *Staphylococcus*, and *Veillonella* in the disease groups; whereas the abundance of *Streptococcus* decreased in the diseased groups as compared to the control group (Fig. 1).

Additionally, the alpha-diversity (within-sample diversity) indices of the bacteriomes of the research groups—Chao1, ACE, Fisher, Shannon, and Simpson—were used to estimate the OTU richness. A significant difference in alpha diversity was found in the ACE index, at the order ($p = 0.0192$) (Fig. 2a) and class ($p = 0.0239$) (Fig. 2b) taxonomic levels. Using the Bray-Curtis dissimilarity metric, the beta-diversity reflecting differences in the bacterial community profile between the samples was analyzed and utilized in the principal component analysis (PCA) plot. This showed that there was no significant difference between the bacteriome of either group ($F=0.92976$, $R^2 = 0.071908$, $p > 0.05$) (Fig. 2c).

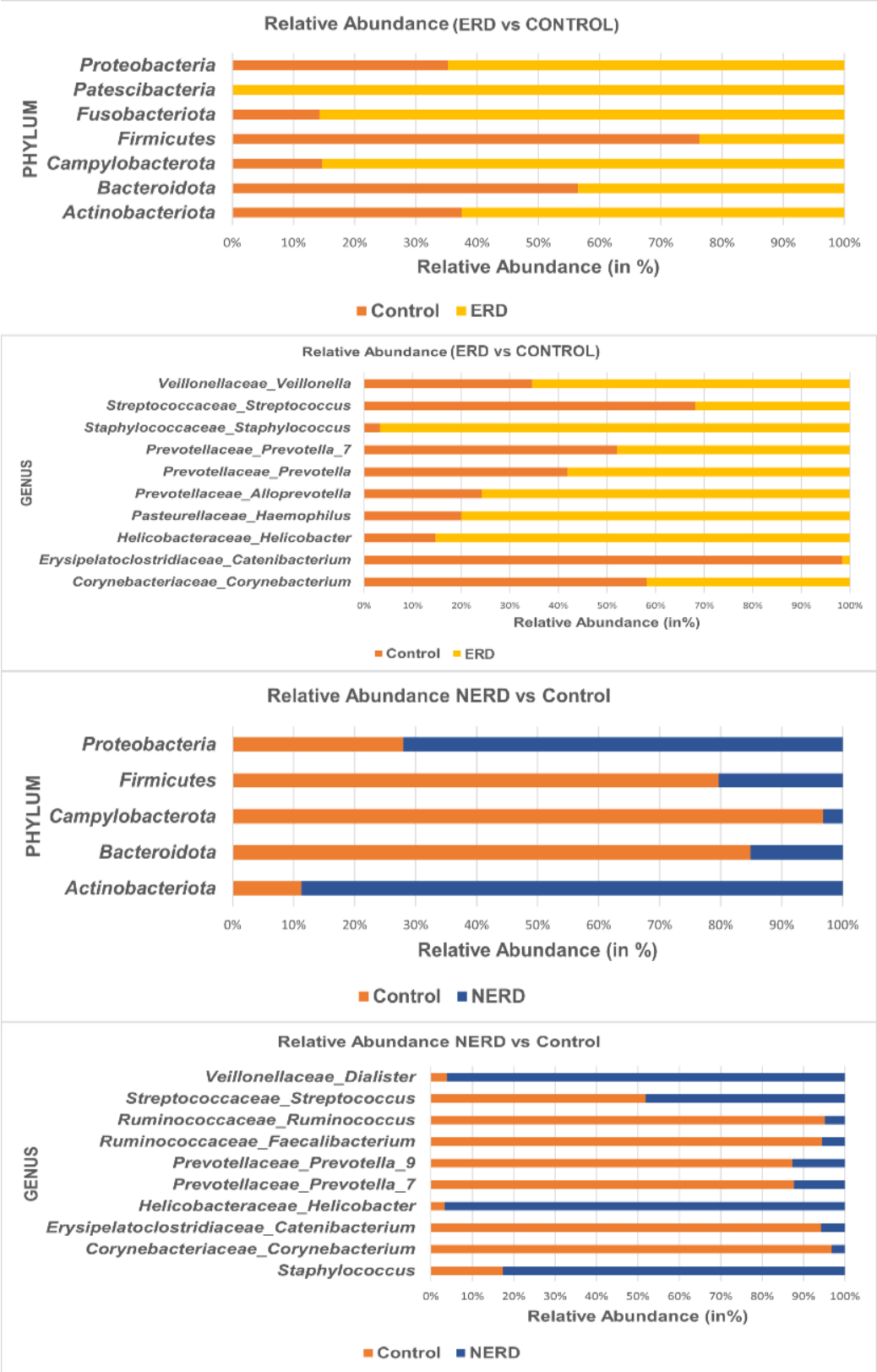


Fig. 1: To compare the relative taxa abundance among control, ERD, and NERD samples at the (a) phylum level and (b) genus level

Core microbiome in disease categories

Despite the differences in the disease outcomes, a diverse core microbiome was observed at the genus level which remains unaltered in its composition across the study groups. Core microbiome analysis was performed with sample prevalence ≥ 20% and relative abundance ≥ 0.01% (Fig. 3). The 21 core bacterial genera were identified amongst which, *Streptococcus*, *Veillonella*, *Helicobacter*, and *Prevotella_7*, *Staphylococcus* genera were observed to be prevalent among the disease categories (Fig. 3).

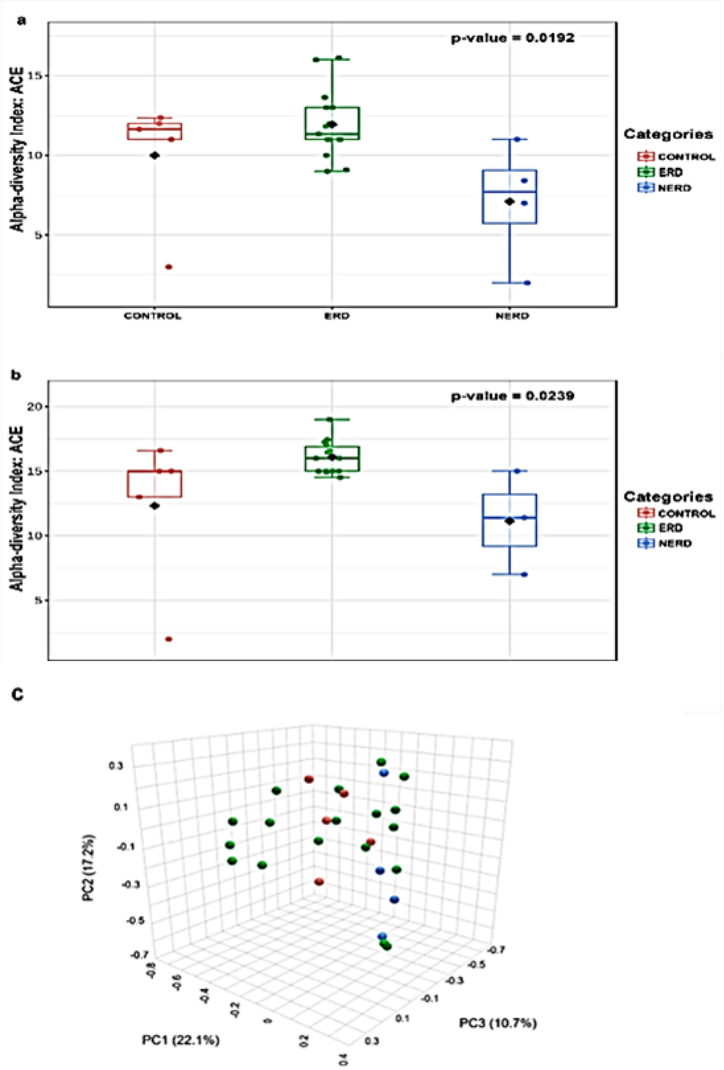


Fig. 2: Alpha diversity between the study groups was found statistically significant at the ACE index. The p-value was determined using T-test/ANOVA. Control, ERD, and NERD are represented by red, green, and blue bars, respectively. (a) Class Level and (b) Order Level. (c) Beta diversity analysis was performed using the Bray-Curtis dissimilarity metric and represented using PCoA for bacterial community comparison in ERD, NERD, and control samples

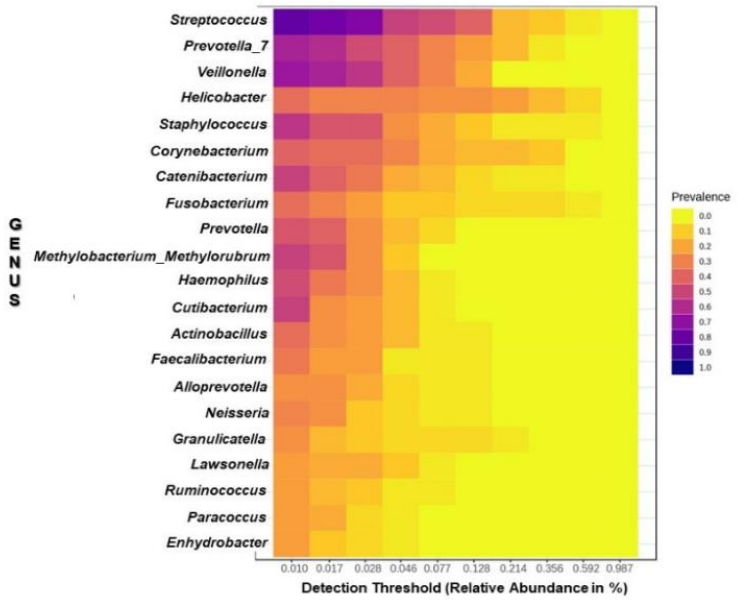
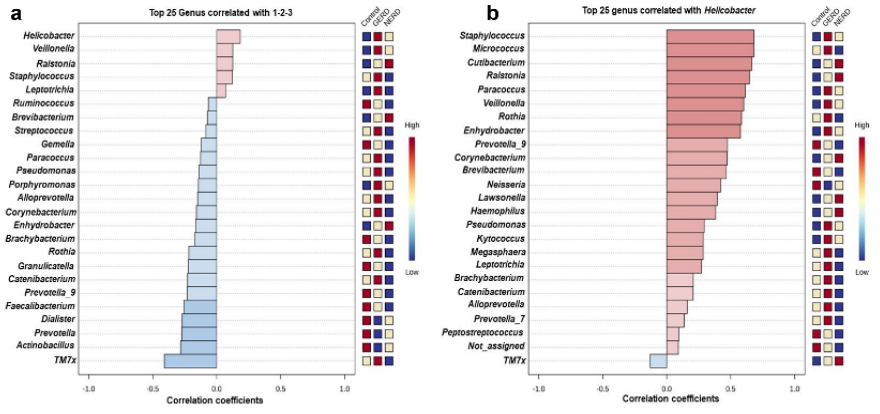


Fig. 3: Core bacteriome analysis of the samples as a whole using the default criteria. The heatmap shows the relative abundances of the most prevalent bacterial genera in the disease groups as well as the detection thresholds for each. The colour key displays a range of threshold relative abundance of individual values.



c

Bacterial Genera	Disease Categories (↑- High, ↓- Low)		Disease Categories (↑- High, ↓- Low)	
	Control	ERD	Control	NERD
Helicobacter	↓	↑	↑	↓
Ralstonia	↓	↑	↓	↑
Neisseria	↓	↑	↓	↑
Veillonella	↓	↑	↑	↓
Haemophilus	↓	↑	↑	↓
Gemella	↑	↓	↑	↓

Fig. 4: To identify the correlation of different taxa along with the sample categories, we performed a pattern search with Spearman rank correlation as a distance measure. Red bars represented a positive correlation and blue bars represented a negative correlation, (a) correlation of top 25 genera with disease categories, (b) correlation of top 25 genera with Helicobacter, and (c) relative change in abundance of important genera

Alteration pattern in study groups

We performed a pattern search at the genus level to identify the correlation between the disease categories using Spearman Rank Correlation as the distance measure. At the genus level, *Helicobacter*, *Ralstonia*, *Veillonella*, and *Staphylococcus* were positively correlated with the disease categories of patients (Fig. 4a). Since *Helicobacter* showed maximum positive correlation, another pattern search was performed along with *Helicobacter* as a feature (Fig. 4b). *Staphylococcus*, *Micrococcus*, *Neisseria*, *Haemophilus*, and *Veillonella* were found to be positively correlated and these genera were found to be prevalent in ERD and NERD samples. According to the heat map generated, we observed that the abundance of these bacterial taxa was maximum in the ERD group, followed by the NERD group and control (Fig. 4b, 4c). *Gemella, Paracoccus, Porphyromonas, Alloprevotela*, and *Enhydrobacter* showed a negative correlation with the disease categories. The abundance of these bacterial taxa was found to be decreasing in NERD, followed by a further decrease in ERD patients.

Co-occurrence network of bacterial genera associated with study groups

A co-occurrence plot at the genus level was generated to determine plausible associations within the bacterial communities of the study groups, using MicrobiomeAnalyst software. Overall, it was discovered that there were significant differences in the prevalence of 289 genera throughout the study groups (Fig. 5). Of them, 283 significant positive correlations and 6 negative correlations (p-value < 0.05 and coefficient correlation > 0.3) were found. An interactive correlation network was generated and as *Helicobacter* showed the highest abundance in ERD and NERD group and control, its correlation was further analysed with other bacterial genera. *Helicobacter* was found to be positively correlated with *Lawsonella* (p=0.0374), *Methylobacterium methylorubrum* (p=0.0012), *Micrococcus* (p= 0.0245), *Ralstonia* (p = 0.0024), *Staphylococcus* (p = 0.0007), and *Paracoccus* (p = 0.0036) (Fig. 5).

Predictive function of the gastric microbiome in study groups

Using the PICRUSt algorithm, we investigated the probable interactions between the gastric microbiome and the functional pathways based on inferred metagenomes and contrasted the variations of these differences between the ERD, NERD, and control groups. Of the 116 associated KEGG pathways, nine were statistically significant (P< 0.05) and distinct among the ERD and control groups, whereas two pathways were statistically significant (P <0.05) and distinct among the NERD and control groups. Interestingly, pathways related to genetic information (Purine metabolism,Thymine metabolism,

and Nucleotide metabolism), pathways related to human diseases (Benign chronic pemphigus), along with other major metabolic pathways (carbon metabolism, methane metabolism, lipid metabolism, and pyruvate metabolism) were found to be enriched in ERD group as compared to the control **(**Fig. 6a). Three KEGG pathways were statistically significant (P<0.05) and different amongst NERD and control groups, pathways related to human diseases (Benign chronic pemphigus), and metabolism (nitrogen metabolism) were enriched in contrast to the control group (Fig. 6b). These findings might correlate the effects of the gastric microbiome on both ERD and NERD groups, leading to plausible changes in the body's disease susceptibility and basal metabolic functions. The role of the gastric microbiome in influencing gastric inflammation can also be concluded from this analysis, which in turn indicates the relation between *H. pylori* infection and ERD resulting in a progression toward Barette's oesophagus, oesophageal adenocarcinoma (EAC) and GC.

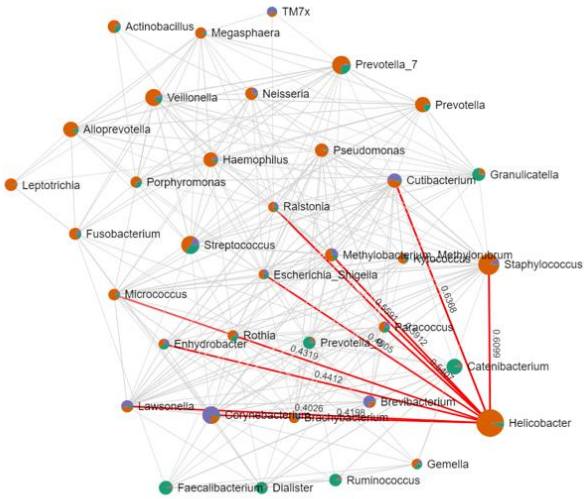


Fig. 5: Correlation network of *Helicobacter* with other significant genera

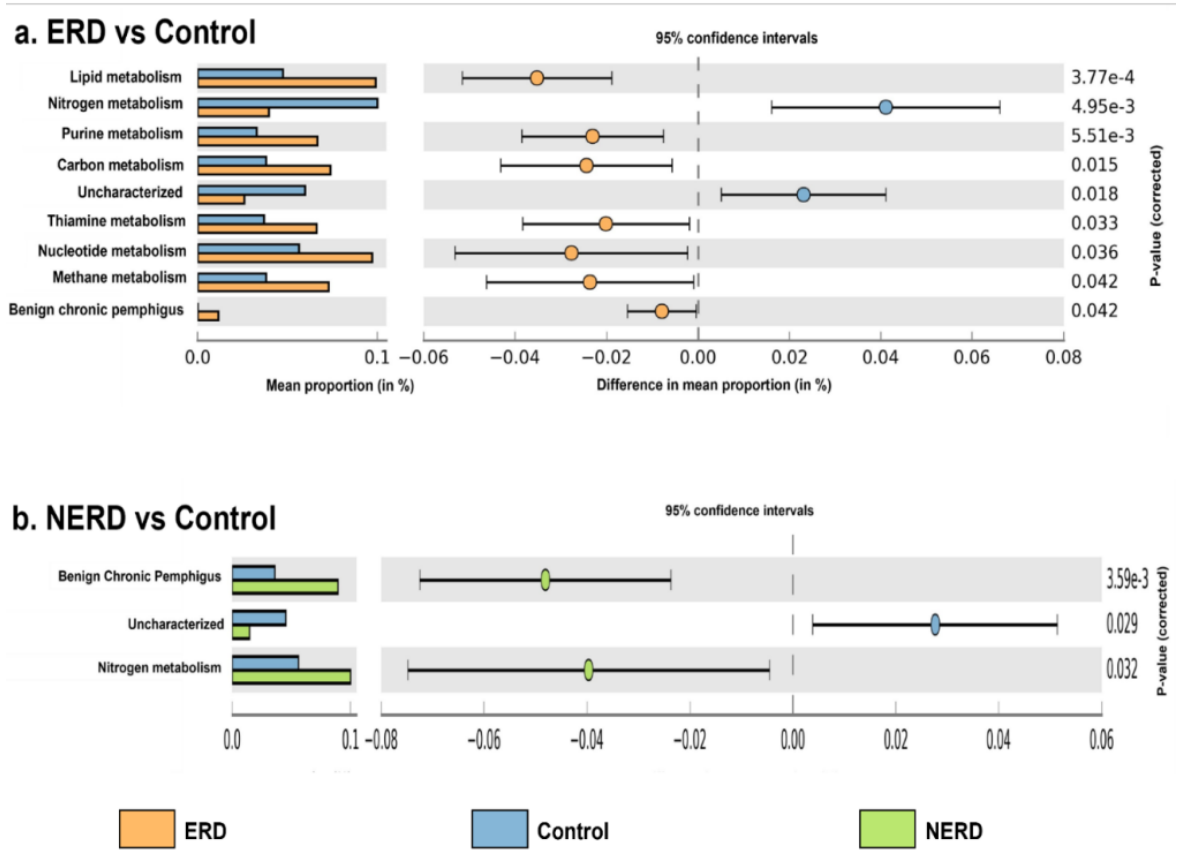


Fig. 6: Based on KEGG pathway analysis, predicted metagenome function was predicted, and the abundance of significantly different KEGG pathways between (a) ERD v/s control and (b) NERD v/s control, is illustrated by the extended error bar graphs. The proportion (left side) shows the variation in proportions for each attribute as well as the potential abundance of microorganisms with each functional characteristic. The difference between the mean proportion of bacteria (the effect size) and its corresponding confidence interval (error bars) are represented by circles on the right side.

DISCUSSION

A prior study found a correlation between specific microbial changes in ERD and NERD, conditions that may not be fatal but can significantly lower patients' quality of life (10). The metaplastic columnar epithelium that results from the esophageal inflammation caused by ERD at the gastroesophageal junction (GEJ) might spread into the esophageal cavity (ECC) by transforming the native squamous epithelium (11). Along with the presence of *H. pylori*, EAC has been found to host unique microbiomes which could be a side effect of the highly acidic environment created by ERD (12,13). This study was done to understand the dysbiosis of the microbiome in the case of ERD and NERD patients when compared to control patients. We observed a possible increased influence of *H. pylori* on the microbial diversity in both groups. Our results were found to be in line with the study of Serrano *et al.,* in which they reported that the predominant phyla in the gastric microbiota were *Firmicutes*, *Proteobacteria* and *Bacteroidetes* in the absence of *H. pylori* infection, whereas in the case of dominance of *H. pylori*, the gastric microbiota showed a reduced bacterial diversity (14).

In the present study, an evaluation of the relative abundances of different bacterial taxa inhabiting the gastric environment of the three study groups was performed which showed that microbiota composition among ERD and NERD groups differ from the control group. In ERD and NERD groups, the microbial consortium was dominated by: *Firmicutes*, *Proteobacteriota*, *Actinobacteriota*, *Campylobacterota*, and *Bacteroidota*. Our results are consistent with research by Nardone *et al.,* that found that the lower esophageal tract microbiome is specific to patients with either ERD or NERD and contains high concentrations of Gram-negative species such as *Proteobacteria*, *Fusobacteria*, and *Campylobacter* (15). The gastric microbiome composition at the genus level of the study groups, as represented in (Fig. 1) showed a decrease in the abundance of Gram-positive bacterial species such as *Streptococcus*, *Corynebacterium*, and *Granulicatella* along with an increase in the abundance of Gram-negative species such as *Helicobacter* and *Veillonella*. In another study, Macfarlane *et al.,* reported a gradual shift from *Streptococcus*-dominated Gram-positive bacterial species in normal patients to Gram-negative colonisation in patients with either ERD or NERD, along with the appearance of genera not usually found in the distal oesophagus, like *Campylobacter*. Our study is also in line with this study where we found the richness of *Campylobacter* in both disease groups (16).

To evaluate the species richness, the α–diversity of bacteria present between the ERD, NERD, and control patients was observed, which was more in the ERD as compared to that in the control, whereas was less in the NERD group. Findings in the case of the NERD group were in contrast with the study conducted by Yang *et al.*, wherein they reported that the gastric microflora of the NERD patients had much more genus-level diversity than the control group (17). Similarly, in the case of ERD, our findings were in contrast to the study conducted by Shi *et al.,* which reported that bacterial diversity is decreased in the ERD groups in contrast to the control group (18).

To evaluate the correlation, a pattern search was conducted, which illustrated an increase in the abundance of different bacterial genera in the case of ERD and NERD groups as compared to the control group. *Veillonella*, *Haemophilus*, *Ralstonia*, *Neisseria*, and *Helicobacter* showed increased abundance in the ERD group; whereas the abundance of *TM7x*, and *Neisseria* was increased in case of NERD group as compared to control. Our findings were in accordance with Hao *et al.,* which reported that *Veillonella* and *Ralstonia* show increased abundances in the ERD group (19). Another study conducted by Yang *et al.*, reported an increase in the abundance of *Haemophilus*, *Neisseria*, and *Veillonella* in the ERD group (16). According to the findings of Sugihartono *et al.*, *Helicobacter* is a bacterium able to dominate gastric microbiota and induce significant inflammation in the case of the ERD group (20). In a different study, Samolka *et al.,* described a condition called hypochlorhydria, which is elevated stomach pH brought on by gastric atrophy or *H. pylori*-induced suppression of the gastric proton pump. However, this condition is only seen in a small percentage of patients who also have elevated gastrin levels and antral-predominant gastritis (21). Another study by Pero *et al.*, found that *H. pylori* infection increases β-Defensin 2, a type of innate antimicrobial factor produced by host epithelia, which likely affects the proliferation of other gastric microorganisms and suggests that the host immune response may affect the gastric microbiota (22). Nevertheless, the relationship between ERD and *H. pylori* infection still remains controversial, and to draw conclusions needs further research. In accordance with our findings of the NERD group, Zhou *et al.*, reported an increased abundance of *TM7x* and *Neisseria* (23). In light of the above findings, the alteration of gastric microflora is evident in both groups, which could in turn promote the alteration of various pathways. Thus, it becomes essential to analyse the possible pathways enriched with the abundance of Gram-negative bacterial genera in both ERD and NERD groups.

To identify the altered KEGG pathways, a metagenome-wide association study was performed which showed that pathways related to purine metabolism, carbon metabolism, and lipid metabolism were enriched in the ERD group, whereas the nitrogen metabolism was enriched in the NERD group. The end

product of purine metabolism is uric acid, an enrichment in this metabolism in the ERD group might result in the conditions of hyperuricemia further leading to GOUT; this observation is in accordance with the research by Yuan *et al.*,(24). The association of polymorphisms in the one-carbon metabolism (OCM) pathway with GC has been reported by Zhao *et al.*, (25). Another study by Haber et. al., reported the association between GERD and gastritis, hence it can be concluded that enrichment in the carbon metabolism might result in the progression of ERD to gastritis and further to gastric carcinoma. Bi *et al.*, reported the dependence of *Helicobacter* on unsaturated fatty acids for the maintenance of its membrane structure and functions (26). Ktsoyan *et al.*, stated a significant elevation in the levels of unsaturated fatty acids in the blood of patients with *H. pylori* infection-induced ulcerations (27), suggesting an association between *H. pylori* infection and higher lipid metabolism, our analysis also revealed the enrichment of lipid metabolism in ERD patients. Liu *et al.*, reported that nitrate reduction by bacteria that produce nitrite is thought to be the reason for the damage associated with NERD; in acidic environments, nitrate reduction can result in the production of cancer-causing N-nitroso compounds and nitrous oxide, which inhibits DNA repair enzymes thereby enhancing the mutagenesis (28). Our analysis also revealed the enrichment of nitrogen metabolism in the case of the NERD group. These alterations in KEGG pathways suggest that gastric microflora dysbiosis might influence the progression of ERD and NERD, towards gastritis and GC. Consequently, additional analysis of the alteration of the gastric microbiome is required to confirm the actual association between *H. pylori*, ERD, and NERD. However, the limitations of our study include a small sample size, the nature of the self-disclosure questionnaire and the existence of partial recall bias due to which the symptoms reported might not be exact. Similar research with a larger sample size might overcome these limitations, yielding helpful insights into clinical therapeutics.

CONCLUSION

Conclusively, this study showed that the gastric microflora in the ERD and NERD groups had compositional and functional dysbiosis, which was further aggravated by *H. pylori* infection. This study indicates that a combination of certain specific bacterial genera could modulate the gastric microbiota, reversing the shift of the microbiome to improve the clinical outcome in ERD and NERD patients. The findings of this study might pave the way for the initiation of larger-cohort clinical validations and the development of guidance for therapeutic strategies with probiotics.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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