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Assessment of the intestinal microbiota in adults with erosive esophagitis

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ABSTRACT – **Background** – The intestinal microbiota influences the appropriate function of the gastrointestinal tract. Intestinal dysbiosis may be associated with a higher risk of esophageal lesions, mainly due to changes in gastroesophageal motility patterns, elevation of intra-abdominal pressure, and increased frequency of transient relaxation of the lower esophageal sphincter. **Objective** – The aim of this study was to evaluate the intestinal microbiota in individuals with erosive esophagitis and in healthy individuals using metagenomics. **Methods** – A total of 22 fecal samples from adults aged between 18 and 60 years were included. Eleven individuals had esophagitis (eight men and three women) and 11 were healthy controls (10 men and one woman). The individuals were instructed to collect and store fecal material into a tube containing guanidine solution. The DNA of the microbiota was extracted from each fecal samples and PCR amplification was performed using primers for the V4 region of the 16S rRNA gene. The amplicons were sequenced using the Ion Torrent PGM platform and the data were analyzed using the QIIME™ software version 1.8. Statistical analyses were performed using the Mann-Whitney non-parametric test and the ANOSIM non-parametric method based on distance matrix. **Results** – The alpha-diversity and beta-diversity indices were similar between the two groups, without statistically significant differences. There was no statistically significant difference in the phylum level. However, a statistically significant difference was observed in the abundance of the family *Clostridiaceae* (0.3% vs 2.0%, *P*=0.032) and in the genus *Faecaliumbacterium* (10.5% vs 4.5%, *P*=0.045) between healthy controls and esophagitis patients. **Conclusion** – The findings suggest that reduced abundance of the genus *Faecaliumbacterium* and greater abundance of the family *Clostridiaceae* may be risk factors for the development of erosive esophagitis. Intervention in the composition of the intestinal microbiota should be considered as an adjunct to current therapeutic strategies for this clinical condition.

Keywords – Erosive esophagitis; intestinal microbiota; metagenomic; *Faecalibacterium*; *Clostridiaceae.*

INTRODUCTION

The human intestinal microbiota has recently become the subject of extensive research and knowledge about the resident species and their influence is growing rapidly. The human digestive system houses a complex community of microbial cells that influence human physiology, metabolism, nutrition and immune function[1-4]. The imbalance of this microbiota, which is termed dysbiosis, may be involved in the pathogenesis of various digestive and extra-digestive diseases that include irritable bowel syndrome, inflammatory bowel disease, celiac disease, diverticulitis, gastric cancer, obesity, asthma, diabetes mellitus, coronary disease, atopy, autism, autoimmune diseases, and others[5-7].

The frequency of esophageal pathologies has increased in recent decades. Although the majority of solid organ tumors has decreased in the last 40 years, esophageal adenocarcinoma (EAC) has become more prevalent over time[8]. This increase in the number of EAC cases has been especially apparent in western countries and Asia. In the United States, the incidence of EAC is increasing faster than any other cancer[9]. In addition, the prevalence of gastroesophageal reflux disease (GERD) in North America, Europe, and Southeast Asia increased by approximately 50% in relation to the baseline in the early and mid-1990s, and subsequently stabilized[10]. It is important to adequately identify the risk factors associated with these conditions to initiate more effective preventive measures and reduce health care costs.

Dysbiosis influences sensory and motor mechanisms of the upper digestive tract[11-13]. This imbalance in the microbiota can increase the production of intraluminal gases, leading to gastric distension, increased intra-abdominal pressure, and an increase in the frequency of transient lower esophageal sphincter (LES) relaxations[14-17]. A higher exposure of the esophageal epithelium to reflux of gastric and duodenal material occurs, which increases the risks of erosive esophagitis and Barrett's esophagus[18,19].

The impact of intestinal dysbiosis in the upper gastrointestinal tract is still unclear. The objective of our study was to evaluate and compare the intestinal microbiota in patients with erosive esophagitis and in healthy volunteers. This comparison involved a detailed taxonomic description using 16s ribosomal RNA (rRNA) metagenomic analysis.

METHODS

Approval

This study was approved by the Research Ethics Committee of the Clinical Hospital of the Faculty of Medicine, University of São

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Paulo (HC-FMUSP; Approval No. 1.463.131). Every patient signed an Informed Consent Form prior to the collection of samples. The study was conducted with patients registered at the HC-FMUSP. The experimental analysis was carried out at the Laboratory of Medical Research – LIM 46, sector of Parasitology of the Institute of Tropical Medicine of the University of São Paulo.

Fecal samples

Fecal samples were collected from 22 Brazilian male and female adults aged between 24 and 55 years, from March 2017 to February 2018. Of the 22 individuals, 11 had been diagnosed as erosive esophagitis (EE) (mean age 38.8 years, eight men and three women) and 11 were asymptomatic healthy adults (mean age 34.9 years, 10 men and one woman). Body mass index ranged from 22 to 28 kg/m^2. Further details of the participants is provided in TABLE 1.

TABLE 1. Sociodemographic data of the study participants.

	Erosive esophagitis	Control
Sex		
Female	27.3%	9.1%
Male	72.7%	90.9%
Age (years)	39 (±11)	34 (±6)
Weight (kg)	73 (±6)	74 (±8)
Height (meters)	1.7 (±0.08)	1.7 (0.06)
Body mass index (kg/m^2)	25.5 (±1.9)	24.6 (±1.8)

Data are provided as a percentage (95% confidence interval) or mean (± standard deviation)

Examinations

This study recruited patients with dyspeptic symptoms (heartburn, epigastric pain, fullness, bloating) and asymptomatic controls. The symptomatic patients performed an upper gastrointestinal endoscopy. Those who presented erosive esophagitis were included in this study and collected stool samples. To assess erosive esophagitis, we used Los Angeles Classification[20]. During the examination, biopsies of the gastric body and antrum were performed, using the Operative Link for Gastric Assessment system[21] to rule out severe or extensive gastric atrophy. The control group consisted of asymptomatic volunteers who collected feces for the same analysis. All participants underwent an anamnesis, physical examination, and anthropometry examinations.

Inclusion and exclusion criteria

The male and female patients were 18 to 60 years of age with confirmed diagnosis of erosive esophagitis (Los Angeles grades A and B) through clinical and endoscopic criteria. The exclusion criteria included usage of proton pump inhibitors (PPIs), H_2 antagonists, or antacids in the prior 30 days; usage of antimicrobials or probiotics in the preceding 3 months; presence of histologically confirmed severe and/or extensive atrophic gastritis; comorbidities that may interfere in the motility of the gastrointestinal tract, including diabetes mellitus, previous cerebrovascular accident, neurological diseases, autoimmune diseases, gastrinoma, hyperparathyroidism, and mastocytosis; usage of drugs that can interfere in the motility of the gastrointestinal tract or in salivation, such as calcium channel blockers, nitrates, anticholinergics, and estrogens; bulky hiatal hernia ≥5 cm; long-distance journeys in the last 3 months outside the southeastern region of the country; pregnancy or breastfeeding patients; previous history of surgery of the upper GI tract; obesity defined as a body mass index ≥30 kg/m^2; and consumptive syndrome or malnutrition.

Sample collection and DNA extraction

Fecal samples were collected by the participants, who were instructed to store the stool in a sterile falcon tube containing 12 mL of guanidine 6M/EDTA 200 nM solution to maintain the integrity of the genetic material of the samples. Samples were immediately delivered to the laboratory, where they were stored at -20°C until DNA extraction[22,23]. The extraction and purification of microbial DNA was carried out in the Parasitology sector of the Institute of Tropical Medicine, University of São Paulo. Accordingly, 0.25 g of each fecal sample was processed with the DNA Power Soil™ kit (QIAGEN, Carlsbad, CA, USA) according to the manufacturer's instructions.

Microbiome analysis

The microbiome was characterized by amplifying the V4 domain of the bacterial ribosomal 16S segment using the primers F515 (5'-CACGGTCGKCGGCGCCATT-3') and R806 (5'-GGACTACHVGGGTWTCTAAT-3'). The bacterial amplicons were sequenced using the Ion PGM Torrent™ platform (Invitrogen). The readings obtained after sequencing were processed using QIIME™ version 1.8 pipeline and assigned to taxonomic units. Alpha-diversity (taxonomic diversity within the same population) analysis was done using the Shannon, Simpson, Chao1 indices and number of species observed. To measure beta-diversity (diversity between populations), principal coordinate analysis (PCoA) was performed based on the UniFrac distance matrix, to demonstrate similarities or dissimilarities between the samples analyzed. All sequencing raw reads have been deposited in the National Center for Biotechnology Information (NCBI) under the project accession number PRJNA656138.

Statistical analyses

To determine the statistically significant differences in the microbial populations that occurred between the two groups studied, rarefaction was first performed for the same number of sequences between all samples (59,300 when comparing the EE patients and the healthy volunteers). After rarefaction, the relative abundance was calculated using the Mann-Whitney non-parametric test. The statistical analysis of alpha-diversity was performed using the Mann-Whitney U test. The beta-diversity analysis was performed using the ANOSIM test, a non-parametric method based on a distance matrix, using the QIIMETM software, with $P<0.05$ considered statistically significant. Boxplot graphs and tables were generated to study the diversity according to Simpson, Shannon, Chao1, and Observed Species values. Bar graphs and tables were generated with means, standard deviation (SD), median, and the 25th (P25) and 75th (P75) percentiles for the taxonomic organization (phylum, class, order, family, and genus). The Kolmogorov-Smirnov test was used to assess the normality of the variables analyzed. All tests performed took into account a bidirectional α of 0.05 and a 95% confidence interval, and were performed with SPSS 25 software (IBM, Armonk, NY, USA) and Excel 2010® software (Microsoft, Redmond, WA, USA).

RESULTS

There were no statistical differences in gender, age, or BMI between the two groups (*P*>0.05 for all). The severity of the erosive esophagitis assessed was grade A 54.5% (6/11) and grande B 45.5% (5/11). A rarefaction curve was generated to determine whether all operational taxonomic units (OTUs) in the data sets were sufficiently evaluated. Each rarefaction curve showed a similar pattern, reaching a plateau and a saturation stage, which indicated that the majority of species present in each sample of the two groups were observed (FIGURE 1). The basis of the rarefaction process was the sample with the fewest sequences (n=59,300). The sequences were grouped into OTUs based on 97% similarity using the QIIME™ program, with the Greengenes database (version 13.8) as a source. Only groups with an average frequency >0.1% were analyzed.

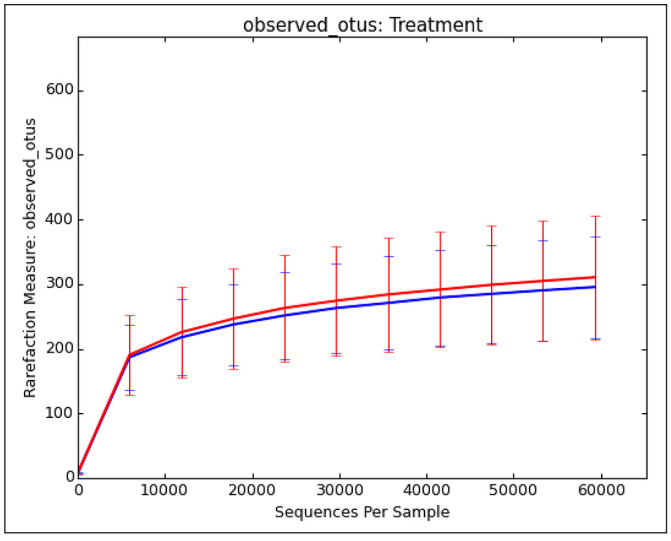

FIGURE 1. Rarefaction curve showing the estimated number of operational taxonomic units (OTUs) in the control group (blue) and the erosive esophagitis group (red), as a function of the sampling sequencing generated using QIIME™ software.

The Shannon and Simpson Diversity indices, the estimated richness by Chao1, and number of species observed indicate no difference in the alpha-diversity in the two groups (TABLE 2, FIGURE 2).

There was no difference in the beta-diversity analyzed using PCoA, based on the weighted and unweighted UniFrac distance matrix (FIGURE 3). No differences were found in the phylum level when the two groups were compared. However, at the genus level, a statistically significant difference was observed in the abundance of the genus *Faecaliumbacterium* between healthy controls and EE patients (FIGURE 4; 10.5% versus 4.5%, *P*=0.045, FIGURE 5).

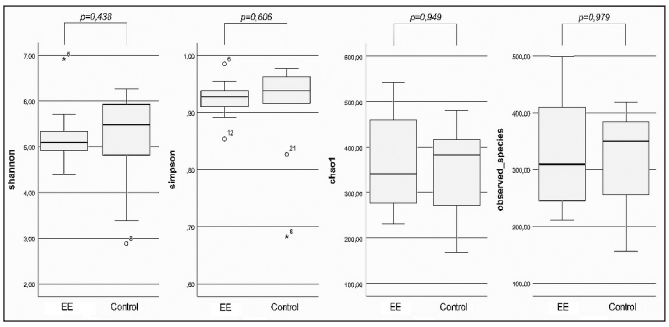

FIGURE 2. Boxplot graphs of Alpha-diversity indices. No statistically significant difference in alpha-diversity was evident between the erosive esophagitis (EE) and the control groups.

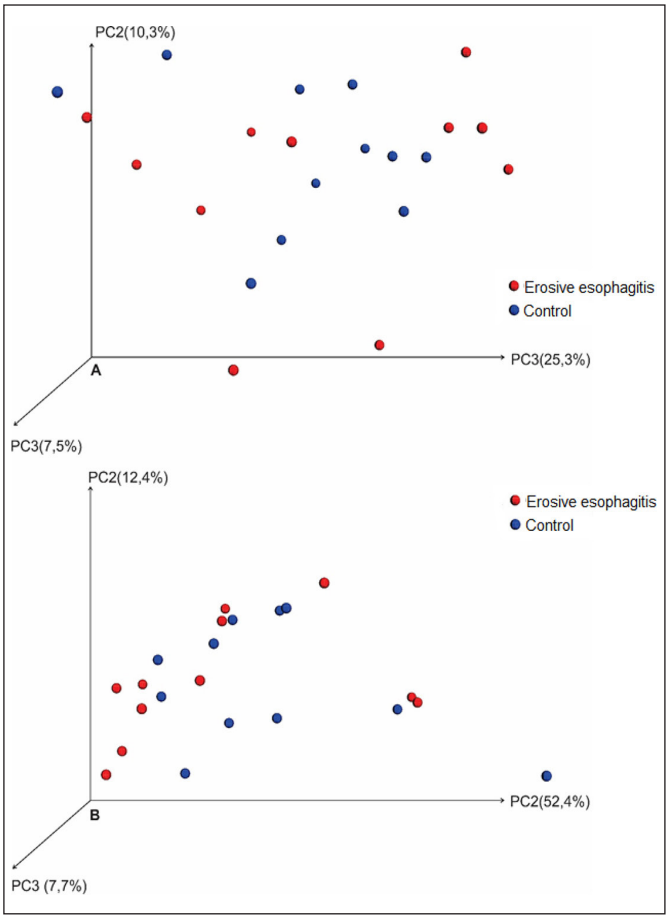

FIGURE 3. Unifrac analysis of the bacterial community, in erosive esophagitis (red) and controls (blue), by the unweighted (A) and weighted (B) principal coordinates analysis (PCoA) method. **A** *P*-value (ANOSIM) =0.870; **B** *P*-value (ANOSIM) =0.430.

TABLE 2. Alpha-diversity index.

	Erosive esophagitis					Control				
	Mean	SD	Median	P25	P75	Mean	SD	Median	P25	P75
Simpson	0.93	0.03	0.93	0.9	0.94	0.91	0.09	0.94	0.92	0.97
Chao1	368.41	112.14	342.97	276	515.17	350.57	98.9	384.16	265	421.49
Observed species	330.18	100.73	310	240	456	319.82	89.15	351	243	389

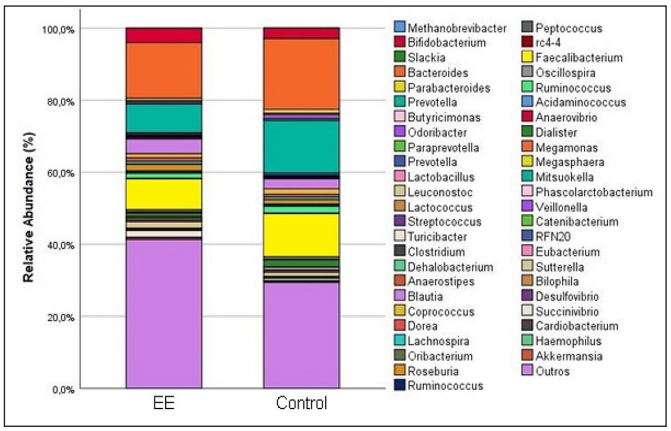

FIGURE 4. Relative abundance and taxonomic classification, at the level of genus, in fecal samples obtained from patients in the control group and the erosive esophagitis (EE).

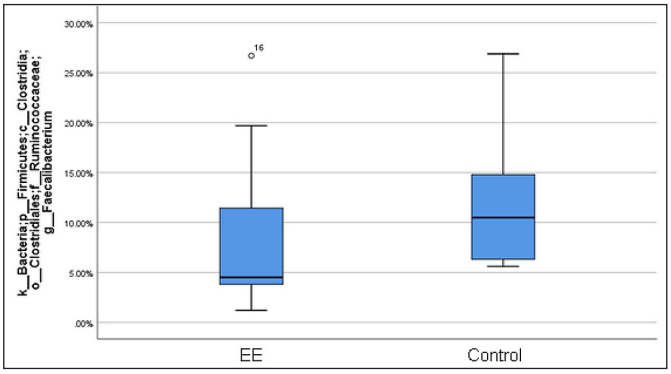

FIGURE 5. Boxplot graph with relative abundance of the genus *Faecalibacterium* in the control group and the esophagitis group (EE).

At the family level, there was a statistically significant difference in the abundance of the family *Clostridiaceae* between healthy controls and EE patients (FIGURE 6; 0.3% versus 2.0%, P=0.032, FIGURE 7).

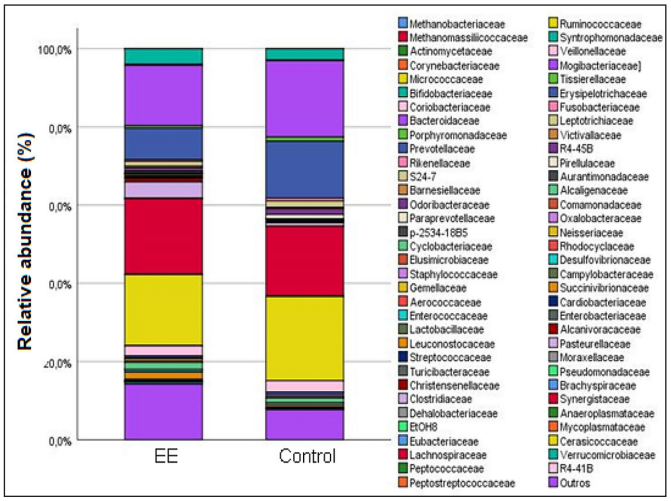

FIGURE 6. Relative abundance and taxonomic classification of bacteria at the family level, in fecal samples obtained from healthy controls and erosive esophagitis (EE) patients.

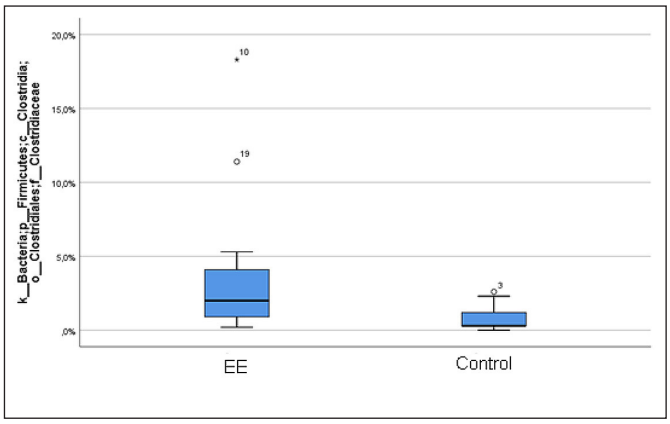

FIGURE 7. Boxplot graph with relative abundance of the family *Clostridiaceae* from the control group and the erosive esophagitis (EE) group.

DISCUSSION

Erosive esophagitis onset depends on many factors, such as the anti-reflux barrier (lower esophageal sphincter and intact crural diaphragm), adequate esophageal clearance (action of gravity, peristalsis, and salivation), esophageal mucosa resistance, and intragastric emptying and pressure[24].

In addition, some authors observed a higher frequency of bacterial overgrowth of the small intestine in patients with esophagitis[25]. Other study observed that the colonic fermentation of non-digestible carbohydrates by intestinal microbiota caused a higher rate of transient relaxation of the LES, a larger number of episodes of acid reflux, and symptoms of GERD[17]. Conversely, others found higher levels of gases in the stomach and duodenum of patients with reflux esophagitis[26]. Bacterial dysbiosis can lead to deconjugation of bile acids, which may have implications on the pathophysiology of gastroesophageal mucosal lesions[27]. Finally, a recent study reported the benefit of probiotics along with PPI in the treatment of reflux esophagitis, with a reduction of relapses[28]. The collective findings indicate that alterations of the intestinal microbiota are related to the increased production of intraluminal gases and a greater risk of developing esophageal lesions.

However, the association between intestinal dysbiosis and esophageal involvement is poorly documented. Thus, we performed this study to evaluate the intestinal microbiota in EE patients and healthy individuals. During the selection of participants, we excluded several situations that may influence the composition of the intestinal microbiome. These included recent use of PPIs, antibiotics, or probiotics; severe or extensive atrophic gastritis; GI tract surgeries, comorbidities, or medications that interfere with the motility of the GI tract; and recent long-distance journeys, among others. The latter criterion reflected the recent reports that the intestinal microbiome is sensitive to changes in climate and diet[29-32]. These exclusion criteria were rigorously applied to reduce possible bias.

Our results agree with the literature, revealing a greater abundance of four phyla in the intestinal microbiota of patients in both groups (GERD and normal individuals). More than 50 phyla have been identified in the environment. However, the characterization of the human microbiota identifies only four as dominant phyla – *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria*[33]. We observed no difference in beta-diversity between the two groups,

without any clusters in the PCoA data (FIGURE 3). There was also no difference in alpha-diversity (FIGURE 2). Thus, considering global biodiversity, the two groups exhibited similar intestinal microbiota in the total number, composition, and relative abundance of species.

The taxonomic level assessment revealed a lower relative abundance of the genus *Faecalibacterium* and a higher relative abundance of the family *Clostridiaceae* in the EE patients (FIGURES 5 and 7).

Faecalibacterium prausnitzii is the only species that has been identified in the genus *Faecalibacterium*. *F. prausnitzii* is the main representative of the phylum *Firmicutes*, class *Clostridium*, family *Ruminococcaceae*. In humans, the genus *Faecalibacterium* is divided into two different phylogroups, although whether they have different physiological functions is unknown[34]. *F. prausnitzii* is extremely sensitive to oxygen and is difficult to cultivate, even in anaerobic conditions[34]. *F. prausnitzii* represents approximately 5% of the total fecal microbiota in healthy adults, and may reach 15% in some individuals[35]. The abundance and ubiquity of *F. prausnitzii* suggest that this is a functionally important member of the microbiota, with a possible impact on the physiology and health of the host. Changes in the abundance of this bacterium have already been widely described in different intestinal and metabolic diseases in humans[34]. The beneficial effects of *F. prausnitzii* reflect its ability to produce butyrate, which positively modulates the intestinal immune system, oxidative stress, and the metabolism of colonocytes[36-38]. *F. prausnitzii* was reported to secrete anti-inflammatory compounds, such as salicylic acid[39]. In a recent study, seven peptides present in the supernatant from *F. prausnitzii* cultures were derived from a single anti-microbial inflammatory molecule, a 15 kDa protein, capable of blocking the nuclear factor-kappa B pathway in intestinal epithelial cells[39]. Another study described that patients with reduced abundance of *F. prausnitzii* displayed higher serum levels of interleukin 8. The authors concluded that alterations in the microbial composition are associated with an increase in intestinal permeability and increased plasma levels of pro-inflammatory cytokines[40]. Others reported that administration of *F. prausnitzii* restored serotonin levels in the colon of rats with low-grade chronic inflammation[41]. Although serotonin is not a direct marker of motility, it stimulates peristalsis, secretion, vasodilation, and sensory signaling in the intestine, and directly and indirectly regulates intestinal motility[42].

Finally, we observed increased abundance of the *Clostridiaceae*. Greater abundance of *Clostridiaceae* has already been described in other pro-inflammatory pathological contexts. In one study, a greater abundance of *Clostridiaceae* was observed in patients with inflammatory bowel disease and in patients with rheumatoid arthritis[43]. An increase in the abundance of *Clostridiaceae* was also observed in infants with food allergies[44].

The collective results of our study indicate differences in the microbiota associated with the generation of a pro-inflammatory bowel environment, with direct and indirect effects on the function of the digestive tract, including its upper segment. An important consideration is the possibility that the reduction in the abundance of *Faecalibacterium* may be due to a possible prior use of PPIs by patients with EE. The use of PPIs can be associated with the reduced abundance of *Faecalibacterium* in the intestinal microbiome. Another study observed a reduction in the abundance of *Faecalibacterium* in patients with prolonged use of PPI[45], with a lower the abundance of *Faecalibacterium* in PPI users compared to non-users reported elsewhere[46]. A study conducted using healthy male dogs demonstrated that omeprazole decreased the *Faecalibacterium* count in healthy male dogs[47]. In order to reduce the influence of PPI in the intestinal microbiota, subjects included in the present study had purportedly not used PPIs for at least 30 days preceding their participation. A recovery of intestinal microbiome 30 after suspending the use of PPI has been described[48]. However, further studies are still needed to better evaluate the changes caused by PPI in the intestinal microbiome.

This study has some limitations. Firstly, based on the Lyon Consensus, erosive esophagitis grade A and B are less accurate in the diagnosis of gastroesophageal reflux disease. Similar studies may be performed in non-erosive reflux disease and compared with the erosive form. Secondly, dietary surveys were not performed. Such surveys can be useful in evaluating the intestinal microbiota, as the diet has an influence on bacterial composition[49,50]. The study participants lived in the same region, which may have reduced the variation in the diets. Besides, the frequency and consistency of stools of the participants, which may have an impact in the composition of the intestinal microbiota, were not evaluated[51]. There was also no metabolomics assessment of the samples. In addition, the sample size was small, which prevented extrapolation of the findings to other populations.

CONCLUSION

In conclusion, reduced abundance of the genus *Faecalibacterium* and greater abundance of the family *Clostridiaceae* may contribute to the development of erosive esophagitis. Further studies are needed to confirm these findings and the importance of using a therapeutic strategy for this clinical condition.

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Authors' contribution

Baima DC: conceptualization, methodology, validation, investigation, data curation, writing – original draft. Carvalho NS: writing – review & editing, visualization. Barbuti RC: conceptualization, supervision, writing – review & editing, project administration. Navarro-Rodriguez T: conceptualization, methodology, validation, writing – original draft, writing – review & editing, supervision, project administration. All authors approved the final version of this manuscript

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RESUMO – **Contexto** – A doença do refluxo gastroesofágico (DRGE) é uma das enfermidades mais comuns na prática clínica e possui fisiopatologia multifatorial. Disbiose da microbiota intestinal pode ter influência em mecanismos envolvidos nesta doença, como mudanças nos padrões motores gastrointestinais, elevação da pressão intra-abdominal e aumento da frequência de relaxamentos transitórios do esfíncter esofágico inferior. Contudo, a avaliação da microbiota intestinal, neste contexto, ainda é pouco documentada. **Objetivo** – Este estudo avaliou a microbiota bacteriana intestinal, em indivíduos com doença do refluxo gastroesofágico erosivo e em indivíduos saudáveis, utilizando técnicas de metagenômica. **Métodos** – Estudo incluiu amostras fecais de 22 adultos, com idades entre 18 e 60 anos: 11 com esofagite erosiva (oito homens e três mulheres) e 11 controles saudáveis (dez homens e uma mulher). Os pacientes foram orientados a coletar e armazenar o material fecal em tubo contendo solução de guanidina. O DNA da microbiota foi extraído das amostras de fezes e amplificação por PCR foi realizada usando iniciadores para a região V4 do gene 16S rRNA. Os *amplicons* foram seqüenciados usando a plataforma Ion PGM Torrent e os dados foram analisados usando o software QIIME™ versão 1.8 (*Quantitative Insights Into Microbial Ecology*). Análise de estatística foi realizada utilizando-se o teste não paramétrico de Mann-Whitney e o teste ANOSIM, método não paramétrico baseado em matriz de distância. **Resultados** – Os índices de alfa-diversidade e beta-diversidade foram semelhantes entre os dois grupos, sem diferença estatisticamente significante. Não houve diferença estatisticamente significante no nível de filo, classe e ordem. Entretanto, observou-se diferença estatisticamente significante na abundância da família *Clostridiaceae* (0,3% vs 2,0%, *P*=0,032) e no gênero *Faecaliumbacterium* (10,5% vs 4,5%, *P*=0,045) entre controles saudáveis e pacientes com DRGE erosiva, respectivamente. **Conclusão** – Os achados sugerem que menor abundância do gênero *Faecaliumbacterium* e maior abundância da família *Clostridiaceae*, nos pacientes com DRGE, podem influenciar na fisiopatologia desta doença.

Palavras-chave – Esofagite erosiva; microbiota intestinal; metagenômica; *Faecalibacterium*; *Clostridiaceae*.

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