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Salivary microbiota composition may discriminate between patients with eosinophilic oesophagitis (EoE) and non-EoE subjects

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Summary

Background: Data on the role of the microbiome in adult patients with eosinophilic oesophagitis (EoE) are limited.

Aims: To prospectively collect and characterise the salivary, oesophageal and gastric microbiome in patients with EoE, further correlating the findings with disease activity.

Methods: Adult patients with symptoms of oesophageal dysfunction undergoing upper endoscopy were consecutively enrolled. Patients were classified as EoE patients, in case of more than 15 eosinophils per high-power field, or non-EoE controls, in case of lack of eosinophilic infiltration. Before and during endoscopy, saliva, oesophageal and gastric fundus biopsies were collected. Microbiota assessment was performed by 16s rRNA analysis. A Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was implemented to identify biomarkers.

Results: Saliva samples were collected from 29 EoE patients and 20 non-EoE controls;, biopsies from 25 EoE and 5 non-EoE controls. In saliva samples, 23 Amplicon Sequence Variants (ASVs) were positively associated with EoE and 27 ASVs with controls, making it possible to discriminate between EoE and non-EoE patients with a classification error (CE) of 24%. In a validation cohort, the accuracy, sensitivity, specificity, positive predictive value and negative predictive value of this model were 78.6%, 80%, 75%, 80% and 60%, respectively. Moreover, the analysis of oesophageal microbiota samples observed a clear microbial pattern able to discriminate between active and inactive EoE (CE = 8%).

Conclusion: Our preliminary data suggest that salivary metabarcoding analysis in combination with machine learning approaches could become a valid, cheap, non-invasive test to segregate between EoE and non-EoE patients.

Sonia Facchin and Matteo Calgaro contributed equally to the article.

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1 | INTRODUCTION

Eosinophilic oesophagitis (EoE) is an allergen/immune-mediated disease characterised by symptoms of oesophageal dysfunction and eosinophilic infiltration of the oesophageal mucosa in the absence of secondary causes of eosinophilia.[1] The prevalence (0.5–1 case per 1000) and the incidence (5–10 cases per 100,000 per year) markedly increased in the last decade and is now considered to be one of the most important causes of dysphagia in children and young adults. The diagnosis is based on suggestive clinical features (e.g. dysphagia and/or bolus impaction), the presence of eosinophilic inflammation (≥15 eosinophils per high-power field [eos/HPF] in at least one of multiple oesophageal biopsies) and exclusion of other causes of eosinophilia.[2,3] EoE affects more males than females (3:1), and the mean age at diagnosis is between 30 and 50 years in adults and 5 and 10 years among children.[4]

The pathogenesis is still uncertain. Among genetic factors, thymic stromal lymphopoietin (TSLP), Calpain 14 (CAPN14), chemokine C-C motif Ligand 26 (STAT6) appear to be involved in the development of EoE.[5,6] Moreover, environmental factors, including aero- and alimentary allergens, and early life conditions (Caesarean section, use of antibiotics, preterm birth) seem to have a predominant role in causing EoE and suggest that alterations in the microbiota may play a role in EoE pathogenesis.[7–9] In this context, the role of oesophageal microbiome has been evaluated in the evolution of this disease. In fact, a change in the composition or in the load of gastrointestinal microbiota has been involved in molecular pathogenic pathways and in promoting diseases.[10–12]

To date, little is known about the possible role of the gut microbiome in EoE, with most of the studies focusing on oesophageal and salivary microbiome.[13-17] These preliminary studies showed that active EoE is associated with an increase in *Haemophilus*, *Neisseria* and *Corynebacterium* in the oesophageal microbiome and, in contrast, inactive EoE patients and healthy controls have a predominance of Gram positive (especially *Streptococcus*) bacteria.[13–15,18] Comparing the salivary microbiome to the oesophageal one in paediatric EoE patients, a study demonstrated that both have an abundance of *Streptococcus*, *Neisseria* and *Prevotella*.[14] Moreover, there are no data on the composition of the gastric microbiome in EoE subjects, whereas in healthy subjects it seems to be composed by Actinobacteria (*Rothia*, *Actinomyces* and *Micrococcus*), Bacteroidetes (*Prevotella*), Firmicutes (*Streptococcus* and *Bacillus*) and Proteobacteria (*H. pylori*, *Haemophilus*, *Actinobacillus* and *Neisseria*).[19–22]

Given the limited knowledge about the characteristics of salivary, oesophageal and gastric microbiome in EoE and its correlation with the progression of the disease, we aimed to prospectively collect and characterise the salivary, gastric and oesophageal microbiome in active and inactive EoE patients, and to correlate these findings with disease activity.

2 | METHODS

2.1 | Study design and case definitions

Adult patients with symptoms of oesophageal dysfunction undergoing oesophagogastroduodenoscopy (OGD) with biopsies at Gastroenterology Unit, Academic Hospital of Padua (Italy), between October 2018 and November 2020 were consecutively and prospectively enrolled. The diagnosis of EoE was established according to international guidelines in case of symptoms of oesophageal dysfunction, the presence of an eosinophilic inflammation (≥15 eos/HPF in at least one of the multiple oesophageal biopsies) and the exclusion of other causes of eosinophilia.[2,3] Active EoE and inactive disease were defined per the 2018 consensus guidelines as a peak eosinophil count of ≥ or <15 eos/HPF in all oesophageal biopsies performed, respectively.[23–25] To compare the gastro-oesophageal microbiome, adult control patients with gastro-oesophageal symptoms but lacking of eosinophilic inflammation were included. Moreover, additional control patients were enrolled to obtain a higher number of saliva samples for in-depth analysis. Some of them underwent endoscopy and biopsies during the same endoscopic sessions for oesophageal symptoms and had a normal upper gastrointestinal endoscopy, while others were EoE patients who underwent follow-up visits to monitor the maintenance of remission and agreed to participate.

The study was approved by the Regional Ethical Committee for Clinical Trials ($n = 3312/AO/14$ and $n = 4204/AO/17$). Written informed consent was obtained from all eligible participants before participation.

2.2 | Clinical, endoscopic and histological data

Clinical data including demographics, coexisting allergic conditions (e.g., allergic rhinitis, asthma, food allergies, environmental allergies, pharmacological allergies), current and recent (within 4 weeks) exposure to medication like proton pump inhibitors (PPIs) and topical corticosteroids, were recorded at the time of the endoscopy. All OGDs were performed by an EoE-trained investigator (ES) and any oesophageal mucosal changes such as oedema (0–2), rings (0–3), exudates (0–2), furrows (0–2) and strictures (0–1) were recorded for the evaluation of EREFS scores (range 0–10; higher scores indicate more severe endoscopic findings).[26]

2.3 | Biopsies sample collection and preprocessing

We obtained from each patient at least six oesophageal biopsies (i.e., from the upper, middle and lower sites) for histology for EoE diagnosis and monitoring (in the case of follow-up endoscopies). For the microbiota analysis, we obtained one biopsy from the upper, middle and lower oesophagus and one from the gastric fundus conserved in a lysis/stabilisation solution until analysis. An expert gastrointestinal pathologist analysed the oesophageal biopsies to determine the EoE HSS score, based on features of: intensity of eosinophilic inflammation, basal zone hyperplasia, dilated intercellular spaces, eosinophilic microabscess, eosinophil surface layering, surface epithelial alterations, dyskeratotic epithelial cells and lamina propria thickness when present.[27] Duodenal and gastric biopsies were also collected for the histopathologic evaluation of gastritis, Hp infection and eosinophilic infiltration, in particular, to exclude cases of concomitant eosinophilic gastritis or enteritis.[27]

2.4 | Saliva sample collection and preprocessing

Saliva samples were collected just before the OGD. Per standard protocol, participants were fasting for at least 6 h before the upper endoscopy. After providing informed consent, between 1 and 2 ml of saliva were collected in Omnigene-oral kit (DNAgenotek). Among the additional EoE cases who did not undergo endoscopic assessment, saliva was collected before outpatient clinics, but they were asked to respect the same conditions of the patients who underwent the upper endoscopy (i.e. fasting for at least 6 h before collection). The samples were stored at −20°C until further analysis.

2.5 | Illumina 16S library construction

Next-generation sequencing (NGS) protocol was performed by BMR genomics (Padua) using standard techniques. Briefly: V3–V4 regions of 16S rRNA gene were amplified using the primers Pro341F: 5′-CCT ACG GGN BGC ASC AG-3′ and Pro805R: Rev 5′-GAC TAC NVG GGT ATC TAA TCC-3′.[28] Primers were modified with forward overhang: 5′-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG [locus-specific sequence]-3′ and with reverse overhang: 5′-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA G [locus-specific sequence]-3′ necessary for dual-index library preparation, following Illumina protocol (REFlink).[29] Samples (saliva and biopsies) were normalised, pooled and run on Illumina MiSeq with a 2 × 300 bp approach.

2.6 | Bioinformatics data analysis

Analysis was performed using R (v4.0.4) (R Core Team, 2019). Primarily, the sequences in fastq format were analysed using DADA2 (v1.18), a tool that implements an error correction model and allows the identification of exact sample sequences that differ as little as a single nucleotide.[30] The final output of DADA2 was an amplicon sequence variant (ASV) table which recorded the number of times each ASV was observed in each sample. DADA2 was run as described in https://benjjneb.github.io/dada2/bigdata.html using default parameters. To improve the overall quality of the sequences, the reads were filtered and trimmed using the filterAndTrim function implemented in DADA2. Consequently, to remove low-quality bases at the end of reads, the truncLen option was set to (290; 250) for the forward and reverse FASTQ files, respectively. Similarly, to remove adapter sequences at the 5′ end, the trimLeft option was set to (17; 21), for forward and reverse reads, respectively. The removeBimeraDenovo function was used to remove chimaeras, via consensus method and then collapseNoMismatch function collapsed together all the reads that are identical up to shifts or length variation. Finally, the taxonomic assignment was performed using the naïve Bayesian classifier method implemented in DADA2 (assignTaxonomy and addSpecies functions) using as reference the Silva 16S database (Version 138), correctly formatted to work with the taxonomic classifier implemented within DADA2 (https://benjjneb.github.io/dada2/assign.html)[31] A phylogenetic tree of the ASVs was obtained using the function AlignSeq implemented in DECIPHER (v2.16.1) an R package to create multiple sequence alignments.[32] FastTree (v2.1.11) was used to create the final tree.[33] The phyloseq package was used to perform all the downstream analysis in the R environment.[34]

2.7 | Data quality assessment and filtering

Rarefaction curves on raw data were evaluated to assess the species richness among samples as a function of the sequencing depth. Data were pre-processed filtering taxa (ASVs) with a low average relative abundance, setting a threshold of 0.005%; furthermore, taxa present in less than two samples were discarded. Phylum members of Chloroflexi (cumulative relative abundance = 0.0001%), Armatimonadota (0.0001%), Acidobacteriota (0.0002%), Abditibacteriota (0.0003%), Verrucomicrobiota (0.0007%) and Desulfobacterota (0.002%) taxa were discarded by this filter. The counts of all the ASVs were collapsed together by genus and by phylum, and the 10 most present genera were plotted to phylum level. Mann–Whitney tests were performed to test relative abundance differences across active disease activity, inactive disease activity and control samples at phylum level and for each of the 10 most abundant genera.

2.8 | Biodiversity measurements

Shannon–Wiener index was used to calculate α-diversity, which was plotted by stratifying the samples according to body site and disease activity. Mann–Whitney tests were performed to verify statistical differences in the α-diversity across active disease activity, inactive disease activity and control samples. To measure β-diversity, data were normalised using the Total Sum Scaling (TSS) normalisation through the phyloseq_standardize_otu_abundance function of the vmikk/metagMisc package (github.com/vmikk/metagMisc). Bray–Curtis distance metrics was used to measure diversity between sample counts and the principal coordinates analysis (PCoA) ordination method was used to ordinate the samples in a reduced dimensional space using the ordinate function of the Vegan package (v2.5–7).[35] To test the multivariate homogeneity of group dispersions, betadisper function of the latter package was used. Finally, the PERMutational ANalysis Of VAriance (PERMANOVA) was performed, using the adonis and the adonis_pairwise functions, in order to investigate disease activity and condition contributions on the beta diversity variability.

2.9 | Biomarkers identification

A discriminant analysis was computed using sPLS-DA (sparse Partial Least Square Discriminant Analysis) methods to identify possible biomarkers associated with the condition (EoE vs non-EoE), disease activity (active based on ≥15eos/HPF vs inactive based on <15eos/

HPF) in the three oesophageal biopsies and the EREFS score. In particular, following the default mixOmics (v6.14) pipeline (http://mixomics.org/case-studies/splsda-srbct/), a pseudo-count value of 1 was added to the raw counts, which were then normalised with TSS and centred log-ratio (CLR) transformed.[36,37] This compositional approach is based on the Centered Log Ratio (CLR) value which is computed through the ratio of an ASV abundance, and the geometric mean of all the other ASV abundances in the sample. A positive (or negative) value of the CLR indicates that the abundance of the considered ASV is CLR-fold bigger (or smaller) than the geometric mean of the abundances of all the ASVs. Consequently, a zero value does not indicate the absence, instead, it indicates that the difference between the ASV's abundance and the geometric mean of the abundances is null.

The sPLS-DA classification performance was measured with a machine learning approach through the function tune.splsda. The tuning was performed with a leave-one-out Cross Validation (CV) process, and a prediction distance (maximal distance) was chosen to predict class membership across all CV runs. The ability of the model to correctly classify samples was summarised by the Classification Error (CE) which is computable by subtracting the classification accuracy to 1.

$$\text{Classification Error} = 1 - \text{Accuracy}$$

In which the accuracy is computable as:

$$\text{Accuracy} = \frac{TP + TN}{TP + TN + FP + FN} \tag{1}$$

where TP, FP, TN and FN are the true positives, false positives, true negatives and false negatives, respectively. For each comparison a summary image was plotted using the HotLoadings function (github.com/mcalgaro93/HotLoadings), displaying the discriminant ASVs loadings and the related heatmap.

Finally, to establish the adequacy of the model, it was tested in a validation set of 14 saliva samples. Accuracy, specificity, sensitivity, positive predictive value and negative predictive value were computed.

2.10 | Statistical analysis

When continuous parameters were compared, a non-parametric test (Mann–Whitney test or Kruskal–Wallis) was used, while the proportions were compared using Fisher's exact test. For the relative abundance analysis, to assess the main microbial differences between EoE and non-EoE patients, Mann–Whitney tests were performed, independently, on the relative abundances of the 10 most abundant genera at phylum level, stratifying the samples by body site. To better characterise and identify potential biomarkers for EoE condition, differences in the microbial compositions between EoE and non-EoE subjects, for each body site we conducted a multivariate analysis based on sPLS-DA data. The sPLS-DA is a variation

of the Partial Least Squares – Discriminant Analysis (PLS-DA) and enables the selection of the most predictive or discriminative features in the data to classify the samples. The sPLS-DA performs variable selection and classification in a one-step procedure. This compositional approach is based on the CLR values that indicate the abundance of a taxa relative to the average (geometric mean) abundance of all the other taxa in the sample. To this respect, when interpreting the results, it is important to remember that we examined ratios between values, that was the change in abundance of a taxon relative to all others in the data set, rather than abundances. Moreover, sPLS-DA analyses in saliva, oesophagus (all segments considered together) and gastric fundus were conducted to investigate whether specific taxa were associated with active or inactive-EoE. Finally, to investigate the differences between the three oesophageal biopsies of each subject and the association with eos/HPF counts, sPLS-DA was performed.

3 | RESULTS

3.1 | Demographics and clinical parameters

Of 49 adults enrolled (mean age 35 years, range 18–76 years), 29 were EoE-patients (16 inactive and 13 active) and 20 were non-EoE controls. Saliva samples were collected from all the subjects, whereas biopsies for microbiome assessment were collected from 25 out of 29 EoE patients and only 5 out of 20 non-EoE controls. The latter five non-EoE controls, they had symptoms of oesophageal dysfunction, lack of eosinophilic inflammation at upper endoscopy and no previous treatment with proton pump inhibitors. Demographic and clinical characteristics of the whole population are detailed in Table 1. The groups were comparable for age (EoE patients' interquartile range, IQR, 25–50 years vs non-EoE patients' IQR 27–48 years, $p = 0.63$), while they differed in terms of sex ($p = 0.01$). At the time of OGD for microbiome samples, 26 out of 29 (90%) EoE patients were taking PPIs and the proportion was comparable in both inactive (88%) and active-EoE (92%) groups ($p = 1$) but not between all the EoE patients and the controls (55%, $p = 0.01$).

3.2 | Microbial composition of the samples according to body sites

The 16S rRNA metabarcoding analysis of saliva samples was performed for a total of 16 inactive-EoE patients, 13 active-EoE patients and 20 non-EoE controls. Moreover, the 16S rRNA metabarcoding analysis of gastro-oesophageal mucosal samples was performed for 15 inactive-EoE patients, 10 active-EoE patients and 5 non-EoE controls. They resulted in 761 ASVs with a median of 62,333 bacterial reads (IQR 46532, 71,358) per sample retained after data processing, quality control and filtering (Figures S1–S4). The most abundant phyla overall were *Firmicutes*, *Bacteroidota* and *Proteobacteria* with more than 86% of the total counts, followed by *Fusobacteriota*, *Actinobacteriota*, *Patescibacteria*, *Campilobacterota* and some other low abundant phyla

TABLE 1 Demographic and clinical characteristics of the whole population

Features	Inactive EoE (N = 16)		Active EoE (N = 13)		Controls (N = 20)		*p*-value
Demographics							
Male, *n* %	14	88%	10	77%	8	40%	0.01[a]
Median age (interquartile range years)	37	25–52	29	21–43	39	27–47	0.65[b]
Clinical symptoms, *n* %							
Dysphagia	3	19%	4	31%	0	0%	0.02[a]
Bolus impaction	2	13%	4	31%	2	10%	0.29
Heartburn/regurgitation	4	25%	4	31%	6	30%	1.00
Chest pain	1	6%	1	8%	1	5%	1.00
Abdominal pain	1	6%	4	31%	7	35%	0.09
Nausea/vomiting	1	6%	0	0%	1	5%	1.00
Allergic comorbidities, *n* %							
Rhino/conjunctivitis	4	25%	6	46%	1	5%	0.02
Asthma	2	13%	3	23%	1	5%	0.35
Food allergies	1	6%	3	23%	1	5%	0.29
Environmental allergies	3	19%	7	54%	2	10%	0.02
Other atopic manifestations (e.g. atopic dermatitis)	1	6%	2	15%	3	15%	0.75
Therapies, *n* %							
Proton pump inhibitors	14	88%	12	92%	11	55%	0.03
Topical steroids	7	44%	9	69%	0	0%	0.00
Endoscopy lesions, *n* %							
Edema	2	13%	2	15%	—		1.00
Rings	3	19%	10	77%	—		0.00
Exudates	7	44%	8	62%	—		0.46
Furrows	4	25%	6	46%	—		0.27
Stricture	2	13%	1	8%	—		1.00
Histology							
Median peak eos/HPF[c], n (interquartile range)	1	(0–3.25)	35	(20–45)	—		0.00[d]
16S Analysis							
Saliva	16	100%	13	100%	20	75%	
Upper oesophagus	15	94%	10	77%	5	25%	
Middle oesophagus	15	94%	10	77%	5	25%	
Lower oesophagus	15	94%	10	77%	5	25%	
Gastric Fundus	15	94%	10	77%	5	25%	

[a]Fisher's exact test.
[b]Kruskal–Wallis rank sum test.
[c]We consider the highest eosinophilic peak in one of the biopsies.
[d]Mann–Whitney test.

(Table S1). At the genus level, the 10 most abundant genera were *Streptococcus, Prevotella, Haemophilus, Veillonella* and *Neisseria* which contributed to the 60% of the total counts, followed by *Fusobacterium, Alloprevotella, Actinobacillus, Porphyromonas* and *Gemella* which contained almost the 25% of the counts (Table S2).

Alpha diversity was different between body sites, displaying a significantly higher Shannon index in saliva and gastric fundus, compared to the three oesophageal segments (Figure 1A). Beta diversity (Figure 1B) showed that the dispersion of the samples was homogeneous between body sites, while it was significantly different between active/inactive EoE patients ($p = 0.026$), active vs non-EoE patients ($p = 0.015$) and tended to be significant between inactive and non-EoE patients ($p = 0.093$). Considering the homogeneity of variances between body sites, PERMANOVA
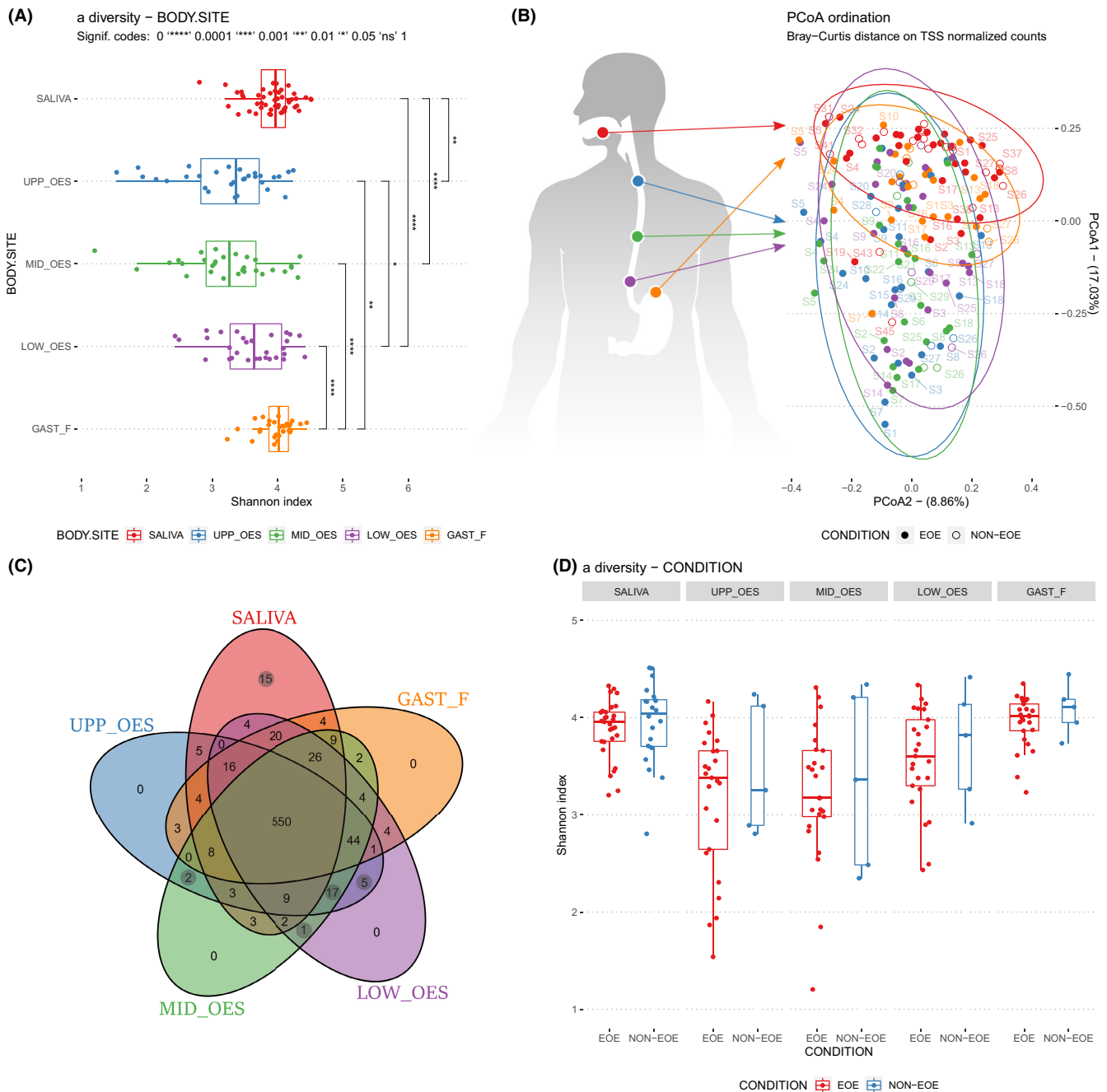
FIGURE 1 (A) Shannon-Wiener α-diversity, over body site. *P*-values signif. codes are reported for the significant comparisons according to the Mann-Whitney tests. (B) Anatomical body sites and bidimensional representation of β-diversity (PCoA ordination method on Bray-Curtis distance matrix of TSS normalised counts). Colored and circled by body site and shaped by case/control groups condition. (C) Venn diagram for the ASVs in each body site. (D) Shannon-Wienerα-diversity, over condition and faceted by body site. (Mann-Whitney tests between EoE and non-EoE status resulted not statistically significant, *p* >0.05)

analysis highlighted that body sites were significantly associated with the beta diversity measurements (*p* = 0.001). Specifically, the pairwise comparisons between body sites displayed non-significant differences only between the three oesophageal segments. Of the total 761 ASVs, 550 were present in all the body sites, while 15 of them were present exclusively in saliva and 25 were present exclusively in the oesophagus (in more than one tract: Figure 1C).

3.3 | Oesophageal, gastric and salivary microbiome composition between EoE and non-EoE patients

Microbial composition by site in active and inactive EoE and non-EoE samples is summarised in Figure 2 where the 10 most abundant genera are reported. No significant differences were found comparing their relative abundances (see Methods and File S2 for details). However, a minor trend was observed for *Bacteroidota*
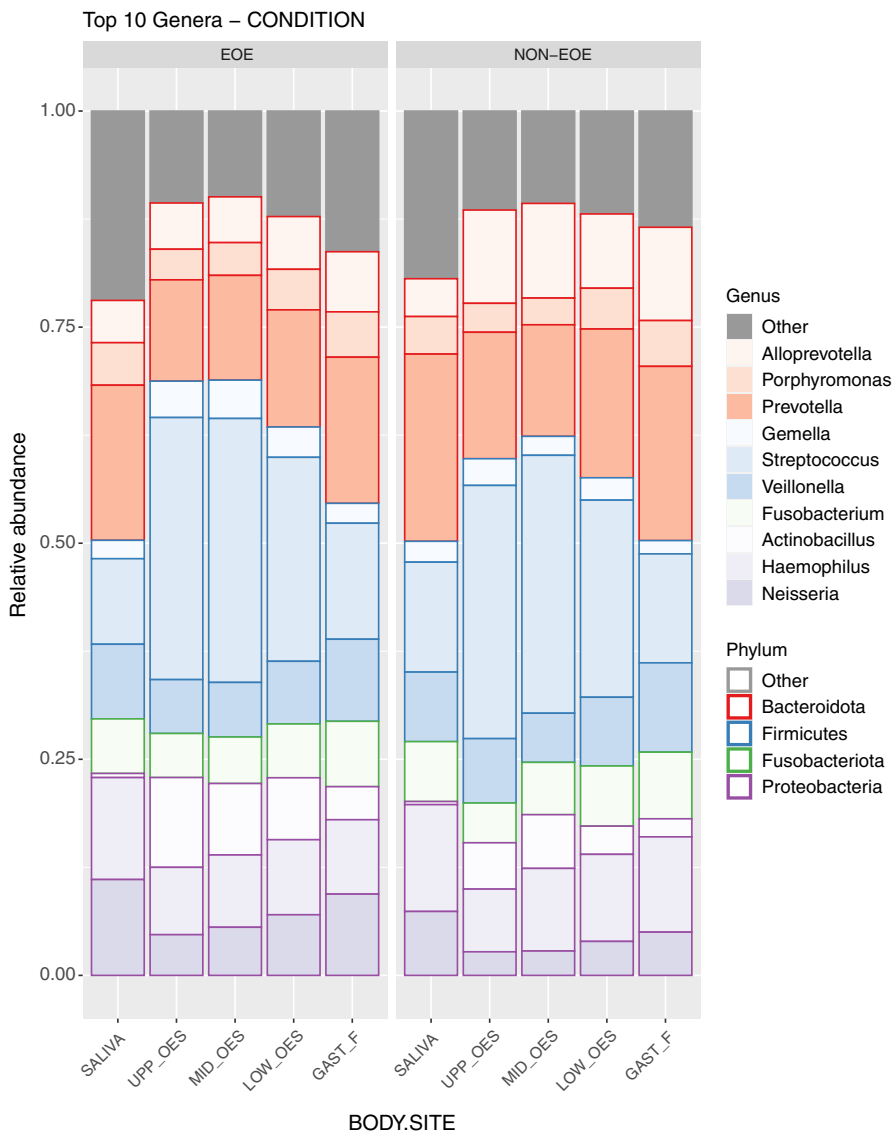
FIGURE 2 The top 10 genera and related phyla are shown; taxa are plotted for their mean relative abundance over body site in case and control groups

phylum that resulted to be less abundant (unadjusted $p = 0.03$) in gastric fundus samples of EoE patients compared to that of non-EoE (30% vs 36.7%; File S2a). Similarly, the *Neisseria* genus was found to be more abundant (unadjusted $p = 0.04$) among the saliva samples of EoE patients compared to that of non-EoE (11.09% vs 7.39%; File S2c).

A similar microbial-richness was shown by the alpha diversity analysis between EoE and non-EoE samples stratified by body sites (Figure 1D), although the alpha-diversity values of non-EoE were slightly higher than those of EoE without reaching statistical significance. Similarly, the first two principal coordinates of beta diversity were unable to show a clear separation between EoE and non-EoE patients (Figure 1B).

To further investigate differences in the microbial composition, we applied a multivariate statistical analysis based on sPLS-DA to identify possible biomarkers associated with EoE and non-EoE patients. The analysis performed on the saliva samples revealed that a group of 50 ASVs were able to discriminate between EoE and non-EoE patients with a classification error of

24%. In particular, 23 of them were positively associated with EoE samples, while the remaining 27 were positively associated with the non-EoE ones. Among the most discriminant ASVs positively associated with EoE samples, we found *Streptococcus cristatus*, *Prevotella oris*, *Veillonella massiliensis* and *Peptostreptococcus stomatis* species, together with ASVs of *[Eubacterium] nodatum group*, *Porphyromonas*, *Alloprevotella*, *Selenomonas* and other *Streptococcus* genera. Conversely, among the ASVs associated with non-EoE patients, we found members belonging to *Prevotella*, *Alloprevotella*, *Porphyromonas*, *Neisseria* and *Streptococcus* genera, along with *Mogibacterium*, *[Eubacterium]* brachy group genera and *Haemophilus pittmaniae* species (Figure 3).

To establish the adequacy of the model, this was validated on a set of 14 saliva samples (10 from EoE patients and 4 from non-EoE controls) which was comparable to the group where the model was estimated in terms of demographics and clinical characteristics of the patients. As shown in Figure S8, 8 out of 10 EoE patients and 3 out of 4 non-EoE patients were classified as true positives and true negatives, respectively. The classification accuracy, sensitivity,
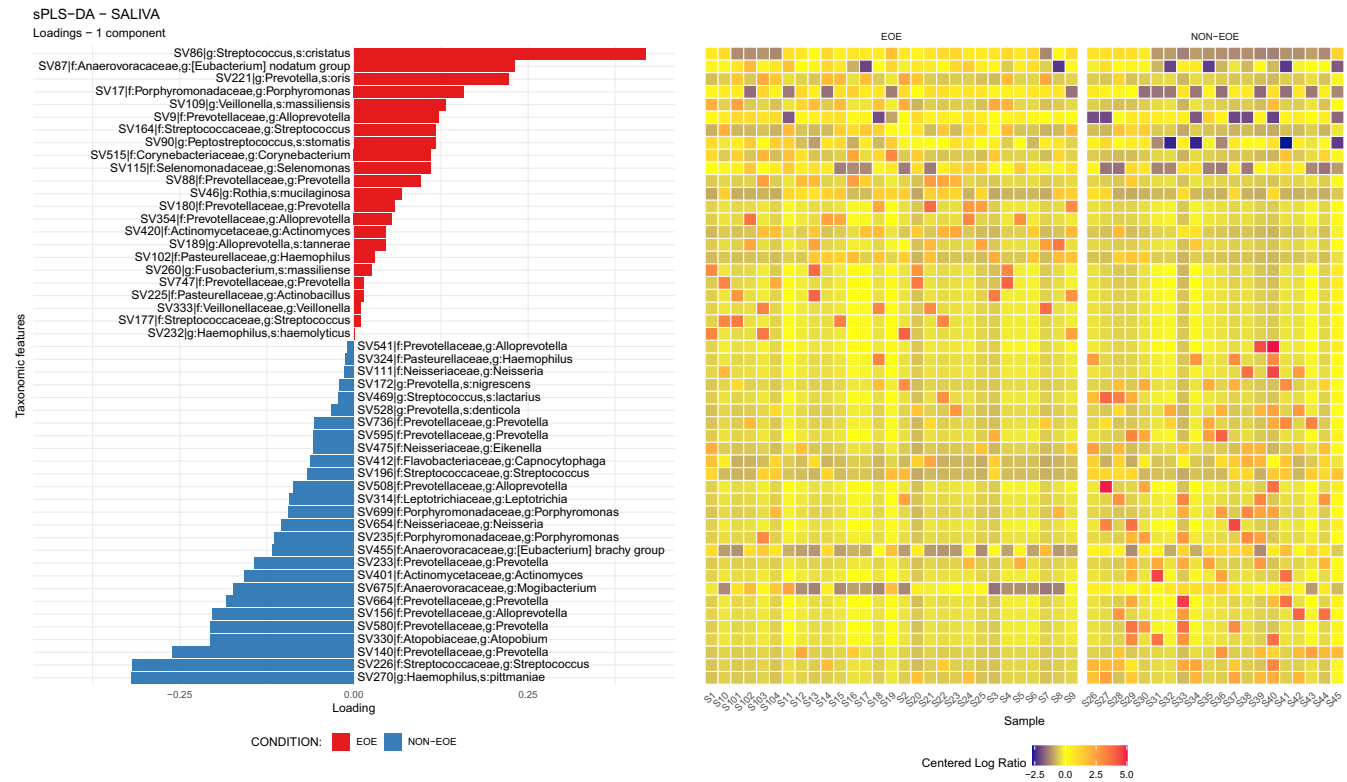
FIGURE 3 sPLS-DA analysis of saliva samples. Loading values (on the left) represent the discriminant taxa of the first component, associated with the condition. Bigger the loading absolute value, stronger the association. Heatmap (on the right) shows the CLR values of the discriminant taxa in all the samples

specificity, positive predictive value and negative predictive value of the above-specified model were 78.6%, 80%, 75%, 80% and 60%, respectively.

The sPLS-DA analysis was also performed on the gastric and oesophageal samples and the results are detailed in the Table S3 and File S3.

3.4 | Oesophageal, gastric and salivary microbiome composition between active- and inactive-EoE

No significant differences were found in terms of relative abundance analysis. However, a minor trend was observed for *Neisseria* genus that resulted to be less abundant (unadjusted p-value = 0.04) in mid oesophagus samples of active-EoE patients compared to that of inactive-EoE (3.02% vs 7.27%; see File S2b). On the contrary, the *Actinobacillus* genus was found to be slightly more abundant (unadjusted p-value = 0.04) among the gastric fundus samples of active-EoE patients compared to that of inactive-EoE (6.48% vs 2.06%; see File S2d).

The alpha diversity analysis performed on active and inactive-EoE, stratified by body sites, showed a similar microbial-richness (Figure S5). The first two principal coordinates in beta diversity did not show any clear difference between active and inactive-EoE patients (Figure S6).

As to the analysis for identifying a potential biomarker of disease activity in EoE, a group of 151 discriminant ASVs was found

in saliva samples between active and inactive-EoE patients, with a classification error of 48%. Among the top 50 ASVs (Figure 4), 22 were associated with active-EoE samples, while the remaining 28 were associated with inactive-EoE. We found, as biomarkers of active disease, *Catonella morbi*, *Haemophilus parainfluenzae* species and various ASVs belonging to *Prevotella*, *Alloprevotella*, *Actinobacillus*, *Treponema* and *Mycoplasma* genera. Instead, other *Prevotella* genera were associated with inactive-EoE samples, together with *gingivalis* and *leadbatteri* species of *Capnocytophaga* genera, *Streptococcus* and *Actinomyces* genera. Moreover, *Oribacterium asaccharolyticum* and *Streptococcus cristatus* species were characterised by some samples with negative CLR values in active-EoE samples. Further information about the biomarkers found in the other body sites are available in File S3.

3.5 | Oesophageal microbiome composition according to the different sites and eosinophil counts in EoE patients

With a classification error of 17%, sPLS-DA revealed that 243 ASVs were associated with the dichotomic separation of the histological values (<15 eos/HPF, and ≥15 eos/HPF). Figure 5 reports the top 50 discriminant ASVs showing a heterogeneous scenario. Members of the *Actinobacillus*, *Bergeyella*, *Porphyromonas* and *Alloprevotella* genera were associated with biological samples with ≥15 eos/

FIGURE 4 sPLS-DA analysis of saliva samples. Loading values (on the left) represent the discriminant taxa of the first component, associated with the clinical status. Bigger the loading absolute value, stronger the association. Heatmap (on the right) shows the CLR values of the discriminant taxa in all the samples
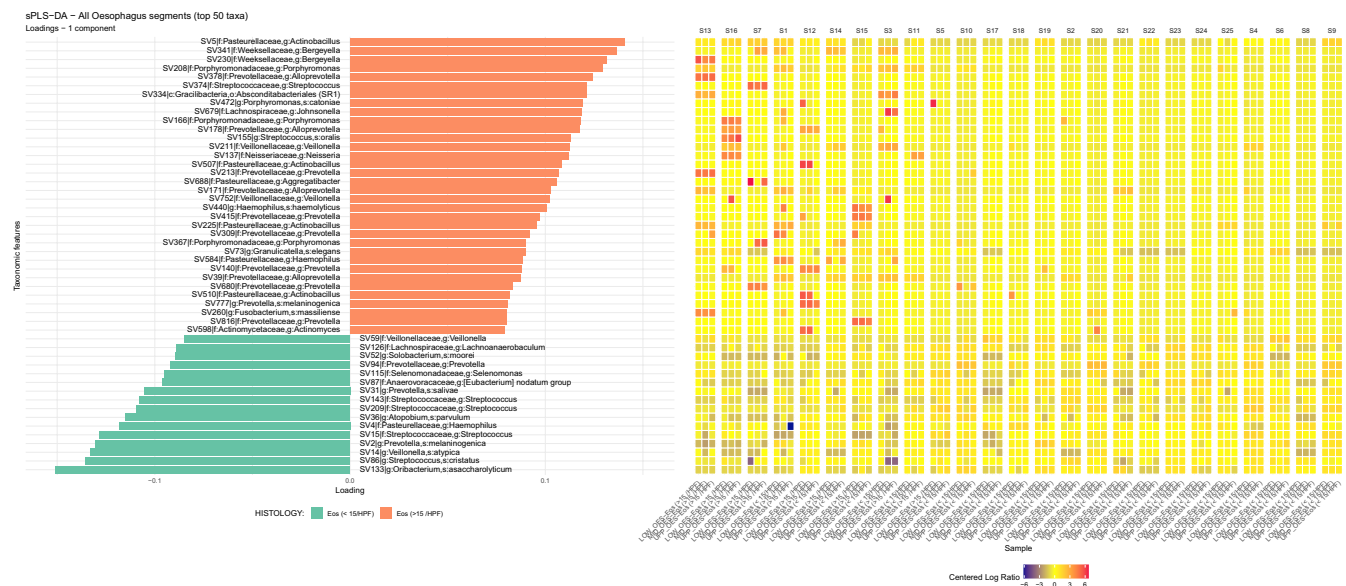
FIGURE 5 sPLS-DA analysis on all oesophagus samples (proximal, mid, and distal). The top 50 loading values (on the left) represent the discriminant taxa of the first component, associated with the histology. Bigger the loading absolute value, stronger the association. Heatmap (on the right) shows the CLR values of the discriminant taxa in all the samples

HPF, while *Oribacterium asaccharolotycum*, *Streptococcus cristatus*, *Veillonella atypica*, *Prevotella melaninogenica* species and others, were associated with <15 eos/HPF. Interestingly, for some patients

and for some ASVs, such as for patients S1, S11 and S3, SV208 - Porphyromonas CLR values were homogeneous across oesophageal segments even if the histological values were different between

biopsies of the same patient. Except for a few rare cases (the top 5 most discriminant taxa), it was difficult to identify a microbial pattern common to multiple samples.

4 | DISCUSSION

The pathogenesis of EoE is still uncertain. Recent studies hypothesized a role of the oesophageal microbiome in both molecular pathogenesis and as a predisposing risk factor for disease development. However, prospective data, including multiple analyses not limited to the saliva or single-site oesophagus are lacking. Thus, we performed this prospective pilot study to characterise mainly the salivary and partially the oesophageal, and gastric microbiome in EoE and to correlate it with disease activity, with the final aim of discriminating a microbial signature (or a complex of signatures) between patients with EoE compared to patients with oesophageal symptoms due to a non-EoE condition.

Using a sPLS-discriminant analysis we observed that in saliva samples, 23 ASVs associated with EoE and 27 ASVs associated with non-EoE were able to discriminate between EoE and non-EoE patients with a reasonably low classification error (CE = 24%). We also validated the model on an additional small sample of patients, observing a 78.6% accuracy, 80% sensitivity and 75% specificity. This represents a promising result considering the ease of collecting salivary samples from our patients as compared to the more cumbersome execution of upper endoscopy and suggests the potential utility of saliva microbiome assessment as a non-invasive disease marker to be confirmed in future larger studies. In contrast, the analysis of oesophageal microbiota samples did not identify a specific microbial pattern that distinguished between the study groups, in agreement with a recent study which observed that there were no significant differences in the oesophageal microbiome between newly diagnosed EoE cases and non-EoE controls in adults, or within EoE cases based on clinical features.[38] However, it is true that the small number of samples available does not allow us to reach conclusive results on this issue.

To the best of our knowledge, this is the first study comparing the salivary microbiome with the oesophageal microbiome examining multiple oesophageal biopsy sites and the gastric microbiome in patients with EoE. From an analysis divided by collection site, we highlighted a substantial difference between salivary and oesophageal microbiota, with greater intra-diversity in saliva and gastric fundus than in the oesophagus (Figure 1A). This microbiological difference may be explained by PPI administration in the majority of our subjects with the consequent increase of intragastric pH and loss of barrier effect of the stomach. Moreover, the same results were observed in both EoE and non-EoE subjects, suggesting that this difference was not influenced by any pathological condition. On the other hand, we cannot exclude that this microbiological difference between saliva and oesophageal microbiome could be due to the presence of atopic pathologies presented by both non-EoE controls and EoE patients. Indeed, it has been reported that both

eosinophils and basophils can kill bacteria, the former through a number of antimicrobial products including granule cationic proteins and defensins, and the latter through extracellular traps. These products could modify the local microbiota in atopic diseases where there is a significant infiltration of these granulocytes.[39]

In this study, we also tried to compare the composition of the salivary, gastric and oesophageal microbiome in active and inactive EoE. The analysis of oesophageal microbiota samples observed a clear microbial pattern able to discriminate between active and inactive EoE (CE = 8%), while the performances in identifying active and inactive-EoE of salivary and gastric fundus microbiota patterns were less precise (CE = 48% and 40%, respectively). Thus, our findings suggest that salivary samples seem less practical to be used for segregating EoE patients according to their disease activity, due to the fact that a large group of 151 discriminant ASVs was found in saliva samples between active and inactive-EoE patients. Considering the top 50, 22 ASVs were associated with active-EoE and 28 were associated with inactive-EoE. Similarly, a recent study has tried to correlate the modification of the salivary microbiome to disease activity, both in terms of endoscopic activity according to the EREFS score and histologic activity according to the Eosinophilic oesophagitis Histologic Scoring System (EoHSS).[15] Hiremath et al. found a higher abundance of *Haemophilus* in patients with active EoE and higher EREFS and EoHSS scores associated with this bacteria.[15] On the other hand, we observed that a microbial signature characterising the salivary microbiota of active patients (*Catonella morbi*) was also abundant in some gastric biopsy samples. *Catonella morbi* is a non-motile, non-spore forming, obligately anaerobic Gram-negative rod that ferments carbohydrates and produces major amounts of acetic acid and smaller amounts of formic and lactic acids. *Catonella morbi* is a normal inhabitant of the oral cavity and has been suggested to be associated with marginal periodontitis. This signature has been also associated with different disorders, including endodontic lesions and coronary heart disease, and oral squamous cell carcinoma.[40] *Catonella morbi* is not the only microbial signature characterising the salivary microbiota in EoE patients to be associated with periodontal diseases. Indeed, in the EoE salivary microbiome, at least two well-characterised signatures (*Prevotella oris* and *Alloprevotella tannerae*) and other genera (*Prevotella*, *Selenomonas* and *Phorphyromonas*) were associated with oral cavity diseases.[41]

At the oesophageal level, we showed a Bacteroidota predominance (Porphyromonas, Alloprevotella and Bergeyella) in active-EoE patients, which is in contrast with other studies, while patients with the inactive disease showed an undifferentiated presence of Firmicutes, Bacteroidota and Proteobacteria (File S1).[13–15,18] An additional sPLS-DA analysis was performed to verify whether different microbial signatures were present on the surface of oesophageal biopsies characterised by <15 eos/HPF compared to biopsies characterised by >15 eos/HPF. We showed members of the *Actinobacillus*, *Bergeyella*, *Porphyromonas* and *Alloprevotella* genera were positively associated with biological samples with eos/HPF >15. These are Gram(−) microbial signatures associated mainly with the oral

cavity (*Porphyromonas* and *Alloprevotella*) or with the respiratory tract (*Actinobacillus*) and sometimes associated with endocarditis (*Actinobacillus*, *Bergeyella*). On the other hand, bacteria associated with eos/HPF < 15 histologies as *Oribacterium asaccharolyticum* and *Streptococcus cristatus* Gram (+) or *Veillonella atypica*, *Prevotella melaninogenica* Gram(−) are species differently associated with the healthy oral microbiota.

A limitation of the study is related to relatively small sample size and the low number of biopsy samples collected from non-EoE controls. This prevented us from clearly evaluating biopsy microbial signatures as possible discriminating signatures. However, we opted for this approach because our initial preliminary analysis on a few EoE subjects and non-EoE controls did not show relevant differences for the oesophageal and gastric microbiome and therefore, we decided to focus more on salivary evaluation. Our decision was also supported by a recent meta-analysis underlining the importance of oral microbiome assessment to predict in the future various oesophageal diseases via oral samples that can be easily obtained as compared to oesophageal samples.[42] Another limitation is represented by the lack of metabolomic analysis, which could have provided more data on the role of the oesophageal microbiome on EoE. A further limitation includes the lack of control of factors that could influence the microbiome composition as well as the demographic differences observed between our EoE patients and non-EoE controls, including diet, drugs and gender. However, previous studies showed that diet, gender and PPI have no or limited effect on the salivary microbiota composition,[43] whereas data on topical steroids are lacking. On the other hand, previous studies suggested that drugs like PPIs and topical steroids may have a role in changing gastro-oesophageal microbiome composition.[44]Then again, some points of strengths should be emphasised. This pilot study had a prospective design, which allowed us to collect all the patients' data and control for confounding factors. Moreover, we collected samples of different types and locations from the same subjects, providing a more clear and comprehensive analysis of the microbiome characteristics of the upper GI tract, both in disease and healthy state, whereas previous studies focused on salivary or oesophageal microbiome only. Finally, we correlated the microbiome characteristics with clinical features to increase our understanding of the complex interaction between the upper GI tract microbiome and EoE. Another point of strength should be emphasised: the ease of saliva sampling. Saliva is easy and non-invasive to collect and offers an attractive biofluid for diagnosis and prognostic value. Alterations in salivary microbial ecology are linked to increasing numbers of oral and systemic disease states.[45] Emergent knowledge of the salivary microbiome alongside that of the gut microbiome may offer significant potential for applications in precision or P4 medicine (*predictive, preventative, personalised, participatory*). The gold standard in the diagnosis of EoE will remain OGDS for many years to come. However, in the near future, our preliminary data suggest that the analysis of the salivary microbiota will help for a better management of patients with oesophageal dysfunction leading to a more rapid and efficient screening of the population to refer for endoscopy in order to confirm the diagnosis of EoE.

In conclusion, our data confirmed that microbial signatures of *Actinobacillus* and *Haemophilus* characterise the salivary microbiota of patients with EoE compared to control patients.[17] Additionally, the discriminant analysis allowed us to characterise a plethora of bacteria in the saliva (as many as 23 positive signatures and 27 negative microbial signatures for EoE patients) whose interaction could be involved in EoE pathogenesis. Moreover, in this pilot study, the validation of our machine learning model, allowed us to reach a sensitivity of 80% and a specificity of 75% for EoE diagnosis. Thus, the metabarcoding analysis of saliva samples in combination with classification methods based on machine learning approaches could become a valid, cheap, non-invasive discriminating test between EoE and non-EoE patients.

AUTHOR CONTRIBUTIONS

Sonia Facchin: Conceptualization (equal); data curation (equal); methodology (equal); writing – original draft (equal). **Matteo Calgaro:** Conceptualization (equal); formal analysis (equal); methodology (equal); writing – original draft (equal). **Mattia Pandolfo:** Data curation (equal); formal analysis (equal); methodology (equal); writing – original draft (equal). **Federico Caldart:** Funding acquisition (equal); methodology (equal); project administration (equal). **Matteo Ghisa:** Data curation (equal); methodology (equal); project administration (equal); resources (equal). **Eliana Greco:** Methodology (supporting); project administration (equal); resources (equal). **Eleonora Sattin:** Formal analysis (supporting); software (equal); validation (equal). **Giorgio Valle:** Software (equal); supervision (supporting); validation (supporting). **Evan S Dellon:** Supervision (equal); writing – original draft (equal). **Nicola Vitulo:** Conceptualization (equal); formal analysis (lead); methodology (equal); software (lead); supervision (equal); validation (lead); writing – original draft (equal). **Edoardo Vincenzo Savarino:** Conceptualization (lead); investigation (lead); project administration (lead); resources (lead); supervision (lead); writing – original draft (lead).

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