

Longitudinal salivary microbial signatures of exacerbations in COPD

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Abstract

Background: Exacerbations of chronic obstructive pulmonary disease (ECOPDs) are frequently triggered by infections and represent major drivers of disease progression. The salivary microbiota may reflect microbial changes relevant to the respiratory tract and serve as a key reservoir for lower airway colonization, but its temporal dynamics during ECOPD remain poorly characterized. We hypothesized that ECOPD occurrence could be accompanied by coordinated temporal changes in the salivary microbiota and in the local inflammatory profile.

Methods:

Thirty-three patients with COPD were followed over a 6-month period with monthly saliva sampling. Of these, patients who experienced an ECOPD underwent additional dense sampling every two days during the exacerbation. In total, 251 saliva samples were collected. Participants were characterized using clinical data and salivary microbiota profiling.

Results:

ECOPDs were associated with microbiota shifts of greater magnitude than steady-state variation (LMER on weighted UniFrac, group $\times$ time-point interaction: $F = 23$, $p < 0.0001$). These shifts were characterized by transient pathobiont blooms, including *Haemophilus*, *Pseudomonas* and *Escherichia*, and an early reduction in microbial diversity in approximately half of the patients. Microbial changes further coincided with enrichment of inferred functional pathways previously linked to pathogenesis. Following ECOPDs, the salivary microbiota of most patients more closely resembled the pre-exacerbation state than the exacerbation peak.

Conclusions: Despite patient-specific variability, ECOPDs were associated with recurring patterns of salivary microbiota perturbation. These findings suggest that saliva may capture ECOPD-associated microbial dynamics and warrant further investigation in larger cohorts.

Background

Chronic obstructive pulmonary disease (COPD) is frequently punctuated by exacerbations (ECOPD), defined as acute events characterised by symptom worsening over ≤ 14 days (1, 2). Between 45–69% of patients have ≥ 1 ECOPD per year (3). Their occurrence is the main driver for individuals' clinical decline, worsening lung function, exercise capacity and health-related quality of life, increasing hospitalizations and mortality rates (4).

ECOPDs are often associated with increased local and systemic inflammation (1, 2). The burst in airway inflammation occurs in response to viral and bacterial infections, exposure to air pollutants or other stimuli (1, 2).

Evidence suggests that around 50–70% (5) of all ECOPDs are triggered by an infectious agent. From those, approximately 50% are caused by bacterial pathogens, while ~ 30–50% are caused by viruses (6). Despite being common inhabitants of the respiratory tract, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Moraxella catarrhalis*, *Streptococcus pneumoniae* and *Staphylococcus aureus* are the typically identified pathogens in “bacterial-ECOPDs” (7). Nevertheless, whether ECOPDs are caused by the acquisition of new strains or by the outgrowth of resident members of the microbiota still remains underexplored (6–8).

Large longitudinal cohort studies using sputum samples collected during both clinical stability and ECOPD have shown that exacerbations are associated with greater beta-diversity than stable periods, with marked deviations of the microbiota from the stable state (9, 10). Specifically, during ECOPDs the lung microbiota deviates from a balanced composition toward a more uneven composition with enrichment of one or a few taxa (9, 10). Common changes include blooms in Proteobacteria, mostly mediated by *Moraxella* and/or *Haemophilus*, and a decline in Bacteroidetes (*Prevotella*) and Firmicutes (*Veillonella*) (9–13). However, no consensus exists regarding changes in diversity during ECOPD, as a few studies report no differences in diversity during ECOPDs (9, 13), while the majority observed a decrease in diversity at onset of ECOPDs (10, 12, 14). Recent studies have also identified shifts in sputum bacterial communities’ functional profiles during ECOPDs (14, 15). Nevertheless, the contribution of microbiota dysbiosis to the onset of ECOPDs or its temporal dynamics during ECOPDs, remain largely underexplored. For example, even large cohorts (9–11, 16), typically collected only one or two samples per patient during ECOPDs over a 1–2-year period, thereby limiting detailed characterization of microbiota dynamics throughout the exacerbation.

Here, we used a dense sampling strategy to track microbiota dynamics with high temporal resolution throughout the entire ECOPD event. This is critical given the rapid and unpredictable nature of microbial community changes (17). Using this strategy, we examined whether ECOPDs coincided with measurable changes in the upper airway microbiota and characterized the nature of these changes, assessing the extent to which the microbiota recovers following the acute episode.

Methods

A prospective longitudinal cohort study was conducted. All methods were performed in accordance with the relevant guidelines and regulations. Ethical approvals were obtained from Administração Regional de Saúde Centro (64/2016), Centro Hospitalar do Baixo Vouga (08-03-17). Written informed consent was obtained from all participants. The study was reported following the “Strengthening the Reporting of Observational Studies in Epidemiology” statement (18).

Participants were identified and referenced by clinicians who briefly explained the purposes of the study. Participants were eligible if diagnosed with COPD according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) criteria (1) and stable with no acute exacerbations in the month prior

to enrolment. Exclusion criteria were presence of severe cardiac, musculoskeletal, or neuromuscular diseases, signs of cognitive impairment, active neoplasia or immune diseases.

At least one baseline assessment prior to an ECOPD was required for inclusion in the study. Participants were followed and monthly assessed until the occurrence of an exacerbation (ECOPD) confirmed by the clinician. This period varied between 30 to 150 days, in a patient dependent manner. In each assessment sociodemographic (age, sex), anthropometric (height and weight to compute body mass index) and general clinical (smoking habits; medication, long-term oxygen use; number of ECOPD and hospitalizations in the past year and comorbidities - Charlson Comorbidity Index (19)) data and saliva samples (passive drool method) were collected with a structured protocol adapted from the team published work (20). Lung function was assessed with spirometry and the forced expiratory volume in the first second (FEV₁) percentage predicted for each individual was extracted to define GOLD grades, as recommended (1, 21). Impact of the disease was assessed with the COPD assessment test (CAT), a 8-item questionnaire, which ranges from 0 to 40 points, with higher scores indicating higher impact (22). GOLD groups were defined combining the number of exacerbations and hospital admissions of each patient in the year before enrolment with their CAT scores. Dyspnoea in activities of daily living was assessed with the modified Medical Research Council Dyspnoea Scale (mMRC), a 5-item scale, which ranges from 0 to 4 and higher scores indicate higher levels of perceived breathlessness in activity (23). Dyspnoea at rest was assessed with the modified Borg Scale (mBorg), a 10-item scale which ranges from 0 to 10 with higher scores indicating higher levels of perceived dyspnoea (24). Functional capacity were assessed with the 1 minute-sit-to-stand (1min-STs) (25).

Day 1 of the exacerbation was defined as the day on which the clinician diagnosed an ECOPD. At this time, the physician contacted the research team, who then met the patient, applied the clinical data collection protocol and collected the first saliva sample (before any medication changes). Subsequent samples were self-collected by the patient every other day until day 21, which was defined as the end of the ECOPD follow-up period (< 14 days of worsening of respiratory symptoms (1) plus additional 7 days for recovery).

At day 21, a post-ECOPD clinical assessment was performed. Aggravation of respiratory symptoms during ECOPD was determined with the published minimal clinical importance differences (MCIDs): -100mL for the FEV₁ (L) (26), + 1 point for the mBorg (27), + 1 point for the mMRC (28) and + 2 points for the CAT (29). Some participants agreed to continue to be clinically monitored and provide saliva samples monthly after the ECOPD, following a similar assessment than before the ECOPD onset.

Prior to sample collection, patients were advised to drink a glass of water (especially if they had recently drunk coffee or citrus juice) and to provide 3–4 mL of saliva using a labelled sample collection cup. Subsequently, the sample was transported in a cooler to the lab as quickly as possible and preserved at -80°C until DNA extraction and/or inflammatory markers' quantification (stored at -80°C for 1 to 6 months).

Deep sequencing of the V4 hypervariable region of 16S rRNA gene (F515/R806 primer pair) was performed for all samples. QIIME2 2020.8 (30) was used to perform microbiota analyses. All statistical analyses were performed in GraphPad Prism 8 and R software v 4.2.1(31). Longitudinal models (*lme4* package (32)) were performed in R software v 4.2.1. Picrust2 was used to predict metagenome functional profiles based on 16S data (33).

Shotgun metagenomics was performed for the 15 samples collected during the 6-month follow-up of patient-1. After all steps of quality control and host DNA removal, Kaiju (34) was used to perform taxonomic assignment of reads with nr_EUK database. Functional profiles were inferred with Superfocus (35).

The bead assay LEGENDplex™ Human Inflammation Panel 1 (13-plex) (BioLegend, San Diego, CA, USA) was used to quantify inflammatory markers in 6 samples from patient-1 comprising stability and ECOPD states.

The full description of the methodology used, including all steps of data collection, processing, and data analyses, are available in Supplementary material.

Results

Cohort characterisation and clinical changes during ECOPD

Thirty-three individuals with COPD were included in this study; 7 (6 male, 68 ± 12 years old, BMI: $26 \pm 4 \text{ kg/m}^2$, FEV₁ $56 \pm 21\%$ predicted, GOLD A-0, B-3, E-4) in the ECOPD group and the remaining 26 patients (21 male, 69 ± 7 years old, BMI: $29 \pm 8 \text{ kg/m}^2$, FEV₁ $56 \pm 19\%$ predicted, GOLD A-12, B-10, C-1, D-3) in the stable COPD group. Table 1 summarizes the baseline characteristics of the two groups.

Table 1

– Sociodemographic, anthropometric, and clinical characteristics of individuals with chronic obstructive pulmonary disease at beginning of the study (n = 33).

Characteristics	ECOPD Group baseline (st) (n = 7)	Stable COPD group baseline (n = 26)	p-value
Age (years), mean $\pm$ SD	68 $\pm$ 12	69 $\pm$ 7	0.7 ¹
Sex, n (%)			
Male	6 (86%)	21 (86%)	> 0.99 ⁴
Female	1 (14%)	5 (19%)	
BMI (kg/m ²), mean $\pm$ SD	26 $\pm$ 4	29 $\pm$ 4	0.2 ²
FEV _{1%} predicted, mean $\pm$ SD	56 $\pm$ 21	56 $\pm$ 19	0.99 ²
Pack-years, mean $\pm$ SD	85 $\pm$ 60	60 $\pm$ 53	0.3 ¹
Comorbidities (CCI), mean $\pm$ SD	4 $\pm$ 1	4 $\pm$ 1	0.2 ¹
GOLD grade, n (%)			
1	2 (29%)	5 (19%)	0.1 ³
2	0	9 (34%)	
3	5 (71%)	9 (34%)	
4	0	3 (12%)	
GOLD group, n (%)			
A	0	12 (46%)	0.02 ³
B	3 (43%)	10 (38%)	
E	4 (57%)	4 (15%)	
Medication, n (%)			

SD: standard deviation, n (%): absolute and relative frequency, respectively; BMI: body mass index, FEV_{1pp}: forced expiratory volume in 1 second percentage predicted, CCI: charlson comorbidity index, GOLD: global initiative for chronic obstructive lung disease, ICS: inhaled corticosteroids, LABA: long-acting beta agonists, LAMA: long-acting muscarinic agonists, LTRA: leukotriene receptor antagonists, SABA: short-acting beta agonists, SAMA: short-acting muscarinic agonists, mMRC: modified medical research council dyspnoea scale, CAT: COPD assessment test; ¹ p-value obtained with Mann Whitney U-test, ² p-value obtained with unpaired t-test with Welch's correction, ³ p-value obtained with Chi-square test, ⁴ p-value obtained with Fisher's exact test.

Characteristics	ECOPD Group baseline (st) (n = 7)	Stable COPD group baseline (n = 26)	p-value
ICS	1 (14%)	12 (46%)	0.06 ³
LABA	3 (43%)	20 (77%)	
LAMA	3 (43%)3	18 (69%)	
LTRA	1 (14%)	1 (4%)	
SABA	1 (14%)	1 (4%)	
SAMA	1 (14%)	0	
Methylxanthines	1 (14%)	2 (8%)	
Antibiotics	0	0	
Mucolytic Agents	2 (29%)	1 (4%)	
Number of exacerbations in the previous year, n (%)			
0 or 1	4 (57%)	22 (85%)	0.1 ⁴
≥2 or 1 with a hospitalisation	3 (43%)	4 (15%)	
Number of hospitalisations in the previous year due to COPD, n (%)			
0	6 (86%)	24 (92%)	0.5 ⁴
1	1 (14%)	2 (8%)	
mMRC, mean ± SD score	3 ± 0.5	1 ± 0.9	0.002 ¹
CAT, mean ± SD SCORE	20 ± 8	10 ± 6	0.002 ²
1-minute sit-to-stand, mean ± SD number of repetitions	22 ± 9	28 ± 8	0.2 ¹
SD: standard deviation, n (%): absolute and relative frequency, respectively; BMI: body mass index, FEV _{1pp} : forced expiratory volume in 1 second percentage predicted, CCI: charlson comorbidity index, GOLD: global initiative for chronic obstructive lung disease, ICS: inhaled corticosteroids, LABA: long-acting beta agonists, LAMA: long-acting muscarinic agonists, LTRA: leukotriene receptor antagonists, SABA: short-acting beta agonists, SAMA: short-acting muscarinic agonists, mMRC: modified medical research council dyspnoea scale, CAT: COPD assessment test; ¹ p-value obtained with Mann Whitney U-test, ² p-value obtained with unpaired t-test with Welch's correction, ³ p-value obtained with Chi-square test, ⁴ p-value obtained with Fisher's exact test.			

Tables S1 and S2 in Supplementary material summarize changes in clinical data associated with the ECOPD. FEV₁ (L) and CAT showed the most significant changes during the onset of ECOPD (Table S1 in

Supplementary material), both exceeding their respective minimal clinically important differences (MCID) (26, 29). FEV₁ decreased by 140 mL, while the CAT total score increased by 5 points, corresponding to an increase 2.5-fold higher than the reported MCID, before returning to baseline values by day 21 of ECOPD.

Regarding alterations in medication as a consequence of ECOPD, antibiotics were prescribed to three patients, acetylcysteine was prescribed to one patient, and bacterial lysates were prescribed to another patient (Table S2 in Supplementary material).

ECOPDs are associated with salivary microbiota restructuring

Temporal changes in salivary microbiota composition were assessed by comparing beta-diversity trajectories between patients experiencing ECOPD and those who remained stable over an equivalent 3-month period (Fig. 1).

ECOPDs were associated with significant changes in salivary microbiota composition and dynamics (linear mixed-effects regression on weighted UniFrac distance, group×time-point interaction: $F = 23$, $p < 0.0001$; Fig. 1A), with a 17% increase in beta diversity at the ECOPD peak, indicating marked microbiota restructuring.

In three out of seven patients, individual within-ECOPD microbiota variation exceeded fluctuations observed in patients who remained stable (Kruskal–Wallis, $H = 70.6$, $p < 0.0001$; Fig. 1B), consistent with increased microbiota instability during exacerbations.

Principal coordinate analysis revealed significant separation between pre- and post ECOPD samples in two patients (P1, PERMANOVA, $F = 3.9$, $p = 0.03$ and P2, PERMANOVA, $F = 3.2$, $p = 0.03$; Fig. 2A and B), indicating pronounced within-patient microbiota shifts during exacerbations.

Among patients with post-ECOPD available data (P4, P5-P7), the distance between pre-and ECOPD-peak exceeded the distance between pre- and post-ECOPD (signalled by black and white arrows, respectively. Figure 2H and L-N), indicating a peak in dysbiosis during ECOPDs followed by partial recovery.

Transient diversity loss and patient-specific pathobiont blooms characterize ECOPDs

During the first week of ECOPD, alpha diversity declined across patients compared with the period pre-ECOPD and with that of patients who remained stable ((One-way ANOVA with Holm-Sidak's correction, $H = 4.2$, $p = 0.017$; ECOPD vs pre-ECOPD: $p = 0.018$; ECOPD vs stable: $p = 0.009$; Figure S1 in Supplementary material) but largely recovered till the end of the ECOPD (Fig. 3).

At the individual-patient level (Fig. 3), marked reductions in alpha diversity were observed only in a subset of ECOPD patients, most notably P1–P3 (Fig. 3B-D). These reductions coincided with blooms of specific pathobiont genera, including *Haemophilus* in P1 and *Pseudomonas* in P2 and P3. By contrast, patients P4–P7 (Figure E-H) showed no major diversity loss during ECOPD, although compositional changes were still observed in some cases. These patient-specific trajectories were further supported by the biplot analyses, which highlighted distinct genera contributing to temporal microbiota shifts within individual patients (Fig. 2A–D and I–K). In P1, early ECOPD samples were enriched in *Haemophilus*, whereas later ECOPD samples showed greater contribution from *Streptococcus*. P2 showed an early increase in *Pseudomonas*, while P3 displayed an early increase in *Streptococcus*. P4 was characterized mainly by depletion of *Prevotella* and *Neisseria*. For P5–P7, the available data were insufficient to support robust patient-specific taxonomic conclusions.

Together, these findings indicate that ECOPDs are frequently accompanied by salivary microbiota restructuring, with pathobiont-dominated configurations and reduced diversity in some individuals, but without overt dominance in others.

To facilitate comparison with previous studies, ECOPD samples were pooled for differential abundance analyses. Comparison of pre-ECOPD samples with samples collected during the first week of ECOPD identified significant enrichment of *Haemophilus* ($p_{\text{adj}} = 0.04$), *Pseudomonas* ($p_{\text{adj}} = 0.02$), and *Escherichia* ($p_{\text{adj}} < 0.0001$) at ECOPD onset (DESeq2; Figure S2 in Supplementary material). Depletion of several commensal genera, including *Neisseria*, was also observed. Similar results were obtained when analysing the complete ECOPD time course (Figure S3 in Supplementary material).

Consistent with previous reports, five of the seven ECOPD patients showed low *Prevotella* abundance before exacerbation, whereas *Prevotella* levels in patients who remained stable (Figure S4 in Supplementary material) were comparable to published values for stable COPD salivary microbiota (36).

To explore the potential functional implications of the observed shifts in salivary microbial composition, we used PICRUSt2 (32) to infer predicted microbial metabolic pathways that were differentially abundant during ECOPD relative to pre-ECOPD. During early ECOPD (days 1–9), pathways related to bacterial energy and carbon metabolism, including glycogen degradation II ($p_{\text{adj}} = 0.029$) and the reductive acetyl-CoA pathway ($p_{\text{adj}} = 0.045$), were predicted to be enriched.

When the complete ECOPD time course was considered, ten pathways were significantly enriched, including multiple pathways previously associated with bacterial pathogenesis, particularly in *Pseudomonas* (according to MetaCyc (v28.5) pathway definitions; Figure S5 in Supplementary material). These findings suggest that ECOPD-associated shifts in salivary microbial composition may be accompanied by changes in the predicted differences in microbial functional potential.

To illustrate how dense longitudinal sampling can capture patient-specific microbial and inflammatory dynamics during ECOPD, we next examined patient P1, in whom a marked but transient loss of diversity coincided with a pathobiont bloom.

Shotgun metagenomics and inflammatory profiling was applied to the same salivary samples previously examined by 16S rRNA target metagenomics. Most reads (~ 97%) were assigned to bacteria, followed by DNA viruses and unicellular eukaryotes, including fungi and protozoa, whereas archaea were detected at low relative abundance (< 3%; Fig. 4A). Within the bacterial fraction, marked blooms of *Haemophilus* and *Pseudomonas* were observed during ECOPD, peaking around days 5–7 and day 15, respectively (Fig. 4A). The *Haemophilus* bloom was mainly assigned to a strain of *Haemophilus influenzae*, though it was not possible to be sure whether this was a novel strain or a previous colonizer, due to insufficient sequence coverage.

Shotgun-based beta-diversity analysis confirmed a clear separation between pre-ECOPD and ECOPD samples and revealed larger compositional fluctuations during ECOPD than during stability (Fig. 4B and C). By one month after ECOPD, the microbiota had shifted back toward a composition more closely resembling the pre-ECOPD state (Fig. 4A-C).

Overall, the main taxa involved, and the magnitude of the changes observed at key time points were broadly consistent with inferences derived from 16S rRNA gene sequencing data, except for Actinobacteria, whose relative abundance differed substantially between the two datasets (Fig. 3B and Fig. 4A).

Owing to substantial differences in sequencing depth between metagenomic samples obtained during stability and ECOPD, no formal differential abundance analyses were performed to minimize false discoveries.

Longitudinal cytokine profiling in saliva revealed a pronounced inflammatory response during ECOPD (Fig. 4D-F). Early in ECOPD (day 5; Fig. 4D), marked increases in TNF- α , IL-10, IL-1 β , and IL-6 were observed relative to stability, coinciding temporally with shifts in salivary microbiota composition, including increases in *Haemophilus* (Fig. 3B and Fig. 4A). Cytokine levels declined thereafter and approached baseline by day 21. At day 15, a distinct inflammatory profile emerged, characterized by increased IL-33, IFN- γ , IL-17A, IL-23, IL-12p70, and IFN- α 2 (Fig. 4E). This later cytokine peak coincided with increased *Pseudomonas* abundance (Fig. 3B and Fig. 4A). IL-8 levels increased early and remained elevated throughout the ECOPD time course, whereas MCP-1 and IL-18 showed minimal variation during the analysed period (Fig. 4F).

Discussion

Exacerbations of COPD were associated with a detectable imprint on the salivary microbiota, which was evident in approximately half of the examined ECOPDs, a frequency consistent with reported rates of bacterial exacerbations (37, 38). Specifically, during the first week of ECOPD, a subset of patients exhibited marked microbiota restructuring, including increased temporal variability, reduced within-individual diversity, and blooms of pathobionts such as *Haemophilus*, *Pseudomonas*, and *Escherichia*. These changes coincided with enrichment of predicted functional pathways associated with bacterial

virulence and were followed by partial recovery of the microbiota toward a stable state after ECOPD resolution.

By following patients over six months, encompassing both clinical stability and ECOPD episodes, we were able to distinguish steady-state microbiota variation from ECOPD-associated perturbations. Moreover, dense every-other-day sampling during ECOPDs enabled detection of transient events, such as diversity loss and pathobiont blooms, that would likely be missed by single-time point sampling designs used in previous studies (9, 10, 12, 13, 15), due to the temporal unpredictability of these events.

The observed pathobiont blooms support the hypothesis that some ECOPDs may be dominated by overgrowth of a single pathogen, although changes in taxa with less clearly defined roles were also detected. Notably, *Prevotella*, which strongly contributed to microbiota variation, was depleted prior to and during ECOPDs in several patients, consistent with previous reports linking low *Prevotella* abundance to severe exacerbations (36).

Differential abundance methods identified significant enrichments in *Haemophilus*, *Pseudomonas* and *Escherichia*, both when considering the first week or the complete ECOPD time-course in pooled patients. These results are in agreement with the most frequent bacterial species identified in sputum of patients during ECOPD: *Haemophilus influenzae*, *Pseudomonas Aeruginosa* and *Streptococcus pneumoniae* (7, 39, 40), further validating the association of salivary microbiota with respiratory conditions even in acute episodes.

Moraxella catarrhalis and *Staphylococcus aureus* have been frequently isolated from sputum samples collected at the onset of ECOPDs (7), and alterations in the frequency and dynamics of *Moraxella catarrhalis* have been largely associated with ECOPD occurrence and severity (9–11, 14). In the present study, no association was observed between ECOPD occurrence and changes in *Moraxella* or *Staphylococcus* dynamics. This likely reflects either the limited number of ECOPD cases analysed or the low representation of these genera in the salivary microbiota, as *Staphylococcus* was not detected and *Moraxella* was present in only eight samples at very low relative abundance (< 0.1%).

Alterations of microbiota functional profiles conserved among patients were inferred. From which, several biosynthesis pathways likely associated with bacterial pathogenesis, were increased during ECOPDs. These findings are in contrast with those previously reported on sputum microbiota, where in comparison with stability, most pathways were decreased and only pathways related with amino acid's metabolism, membrane transport and signal transduction tended to be increased during the ECOPD (14, 15).

A burst of airway inflammation is a well-recognized hallmark of ECOPD onset (1). To explore how rapid microbial changes may align with local inflammatory dynamics, we performed deep longitudinal profiling of the patient who showed the most pronounced microbiota perturbation. Although this single-patient analysis should be interpreted as illustrative, it revealed a temporally structured salivary inflammatory response during ECOPD.

An early increase in inflammatory markers associated with innate immune activation, including TNF- α , IL-1 β , IL-6, and IL-10, was observed at day 5, followed by a second increase in mediators more commonly linked to adaptive and type 17 immune responses, including IL-33, IFN- γ , IL-17A, IL-23, IL-12p70, and IFN- α 2, approximately two weeks into the exacerbation (41, 42). IL-8 increased early and remained elevated throughout the ECOPD time course, consistent with previous reports linking sustained IL-8 upregulation to bacterially associated exacerbations (41, 42).

The early increase in TNF- α , IL-1 β , IL-6, and IL-10, cytokines largely regulated through NF- κ B-associated inflammatory pathways (43), coincided temporally with a marked bloom of *Haemophilus influenzae*. This temporal overlap is compatible with a local inflammatory response occurring alongside expansion of this strain, although causality cannot be inferred. In contrast, the later increase in adaptive immune mediators, including IL-17A, coincided with increased *Pseudomonas* abundance. Given the established role of IL-17-mediated responses in the control of *Pseudomonas* airway infection (44), this observation provides a biologically plausible link between patient-specific microbial blooms and salivary inflammatory dynamics during ECOPD, despite needing validation in larger cohorts.

This study has several limitations that need to be acknowledged. Only 7 patients with ECOPD were followed and sampling was uneven in pre- and post-ECOPDs. Salivary inflammatory markers were determined from only one patient. Therefore, larger cohorts are essential to validate the observed findings and clarify ECOPDs' inflammatory signatures in saliva.

Conclusions

Taken together, these findings indicate that longitudinal characterization of the salivary microbiota during clinical stability and exacerbation can provide useful insight into ECOPD-associated microbial dynamics. Although exploratory, our results suggest that saliva may capture transient microbiota perturbations occurring during ECOPD, including diversity loss, pathobiont blooms, and partial recovery after the acute episode. Future studies in larger cohorts, ideally integrating salivary microbiota, inflammatory markers, clinical outcomes, and paired lower-airway samples, are needed to validate these observations and clarify the role of the salivary microbiota in ECOPD pathophysiology.

Declarations

Ethics approval and consent to participate

Ethical approvals were obtained from Administração Regional de Saúde Centro (64/2016) and from Centro Hospitalar do Baixo Vouga (08-03-17). Written informed consent was obtained from all participants.

Consent for publication

Not applicable.

Competing interests

None declared.

Artificial Intelligence (AI)-Assisted Technology disclosure:

During the preparation of this work the authors used ChatGPT-4o to improve readability and language. After using this tool/service, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Author Contribution

S. Melo-Dias and A. Sousa had full access to all data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. A. Marques was responsible for ethical approval. A. Marques and A. Sousa were responsible for obtaining the funding, conceiving and designing the study. S. Melo-Dias conducted sample processing, DNA extraction procedures and inflammatory markers quantification. S. Melo-Dias, C. R. Almeida and A. Sousa conducted bioinformatics and statistical analyses. S. Melo-Dias and A. Sousa drafted the manuscript. All authors critically revised the manuscript, ensured accuracy and integrity of the work and approved the final version to be published.

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Data Availability

The dataset supporting the conclusions of this article is included within the article (and its additional files). Furthermore, raw sequencing data was deposited in National Centre for Biotechnology Information's (NCBI) Sequence Read Archive (SRA), BioProject PRJNA872131 for participants included in the control group and PRJNA1484334 for participants included in the exacerbation group. Scripts for data analyses are provided in Supplementary material. Please use the link below to access read-only metadata from: BioProject: PRJNA1484334 <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1484334?reviewer=v9n5kkni5qgbtek5b7boefm7ak>

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Figures

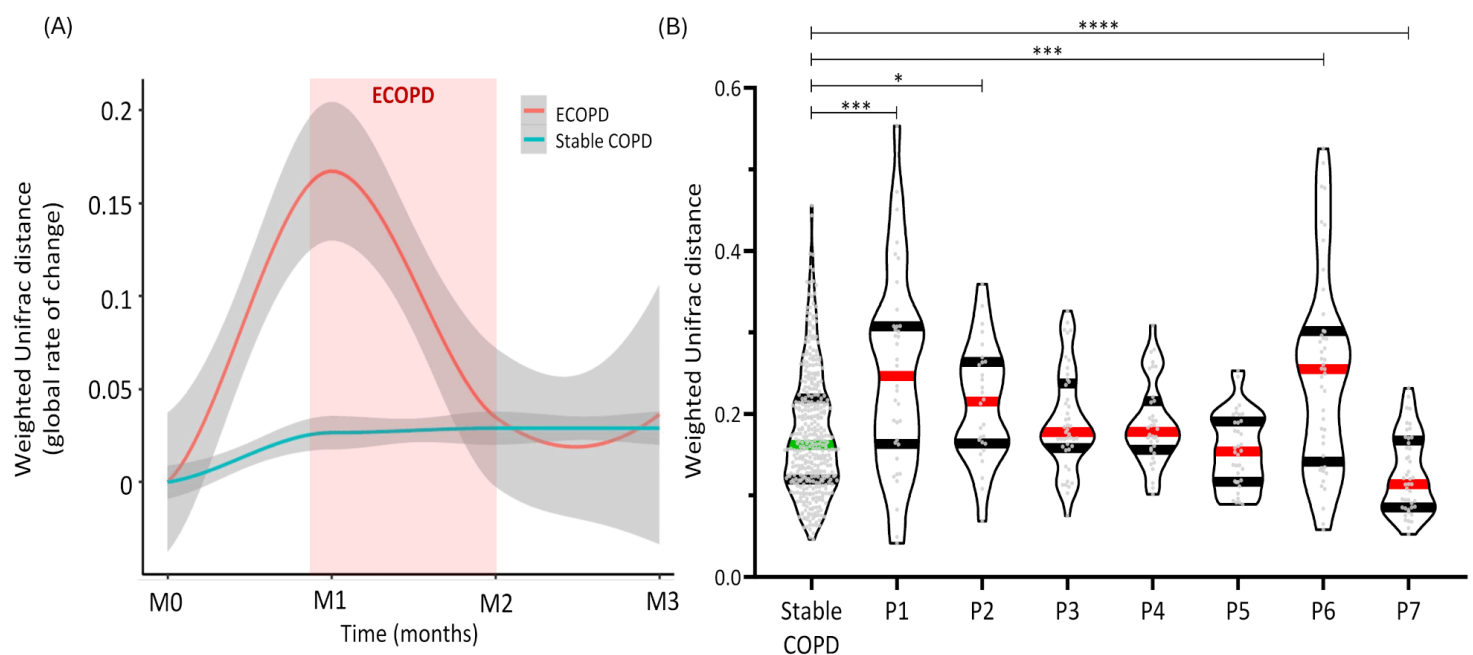


Figure 1

Temporal changes in salivary microbiota composition during ECOPD.

(A) Global rate of change of beta diversity was estimated by subtraction of baseline values at all timepoints. Blue and red loess lines were fitted over the data points representing beta-diversity relative to baseline along time in the ECOPD and stable COPD groups, respectively. The grey areas represent the 0.95 confidence intervals.

For ECOPD patients, four time points were defined: (M0, d-30) one month before ECOPD onset; (M1, ECOPD peak, labelled with black arrows in Figure 2 the most divergent sample within the first 10 days of ECOPD; (M2, d21) the last ECOPD sample; and (M3, d60) one-month post-ECOPD, when available. The stable COPD group was surveyed over an equivalent period using four monthly time points.

ECOPD and stable COPD group presented different microbiota dynamics over the 3-month follow-up (LMER on Weighted Unifrac distance matrix, Group:Time-Point, $F = 23$, $p < 0.0001$). The red shading indicates the time interval spanning ECOPD.

(B) Weighted UniFrac distances were calculated among salivary microbiota samples collected during clinical stability (patients who remained stable; samples obtained over a three-month period) and among samples collected longitudinally from patients during ECOPD episodes (21-day time course). Distances are shown separately for the group of patients who remained stable and for each patient (P1–P7). Violin plots depict the distribution of distances; horizontal green (stable patients) and red (ECOPD) lines indicate median values. Differences between groups were assessed using a Kruskal–Wallis’s test with Dunn’s multiple-comparison correction ($H = 70.6$, $p < 0.0001$). Pairwise comparisons versus the stable control group yielded: P1, $p = 0.0004$; P2, $p = 0.04$; P3, $p = 0.5$; P4, $p = 0.8$; P5, $p > 0.999$; P6, $p = 0.0002$; P7, $p < 0.0001$.

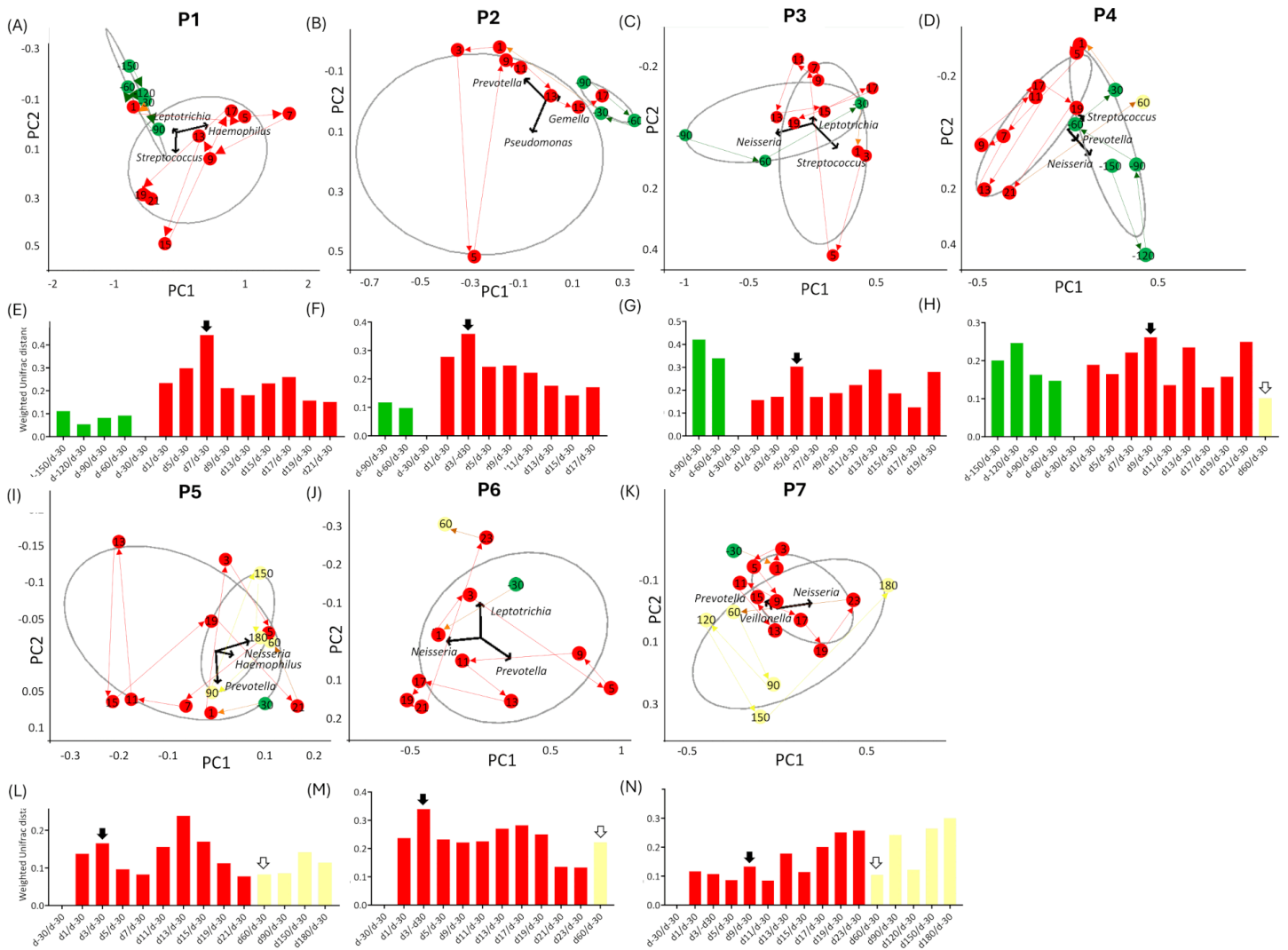


Figure 2

Shifts in microbiota composition during the follow-up period of patients who went through an ECOPD.

(A) – (D) and (I) – (K) Principal coordinate analyses (PCoA) of Weighted Unifrac distance between disease states (pre-ECOPD – green dots; ECOPD – red dots; post-ECOPD – yellow dots). Group segregation was assessed with PERMANOVA pairwise test. Samples were numbered according to the day of collection, and centred at day 1 of exacerbation, i.e. negative numbers represent samples collected pre-ECOPD (monthly) while positive numbers represent samples collected during- (every 2 days) and post ECOPD (monthly); green, orange, and red arrows represent the microbiota trajectory between samples, where green represents variation within stability, orange represents status exchange and red represents the shifts during ECOPD. The biplot (black arrows) show the top genera contributing for sample segregation within each PCoA.

(E) – (H) and (L) – (N) Bar-plots showing distances between each sample and the last point pre-ECOPD (day -30). These graphs quantify the shifts during ECOPD relative to stability. The black arrows represent the more distant sample from stability within the first 10 days of ECOPD, which was selected to conduct

the longitudinal model present in Figure 1 (A). This time-point was considered as the ECOPD peak, as the first 10 days are generally the most symptomatic [2]. White arrows indicate the distance between the last sample collected before ECOPD and the first sample collected one month after ECOPD, representing the shortest temporal distance between stable time points flanking the exacerbation. P1-P7 represent the seven patients who had an ECOPD during the follow-up period. (A), (E) - P1; (B), (F) – P2; (C), (G) – P3; (D), (H) – P4; (I), (L) – P5; (J), (M) – P6; (K), (N) – P7.

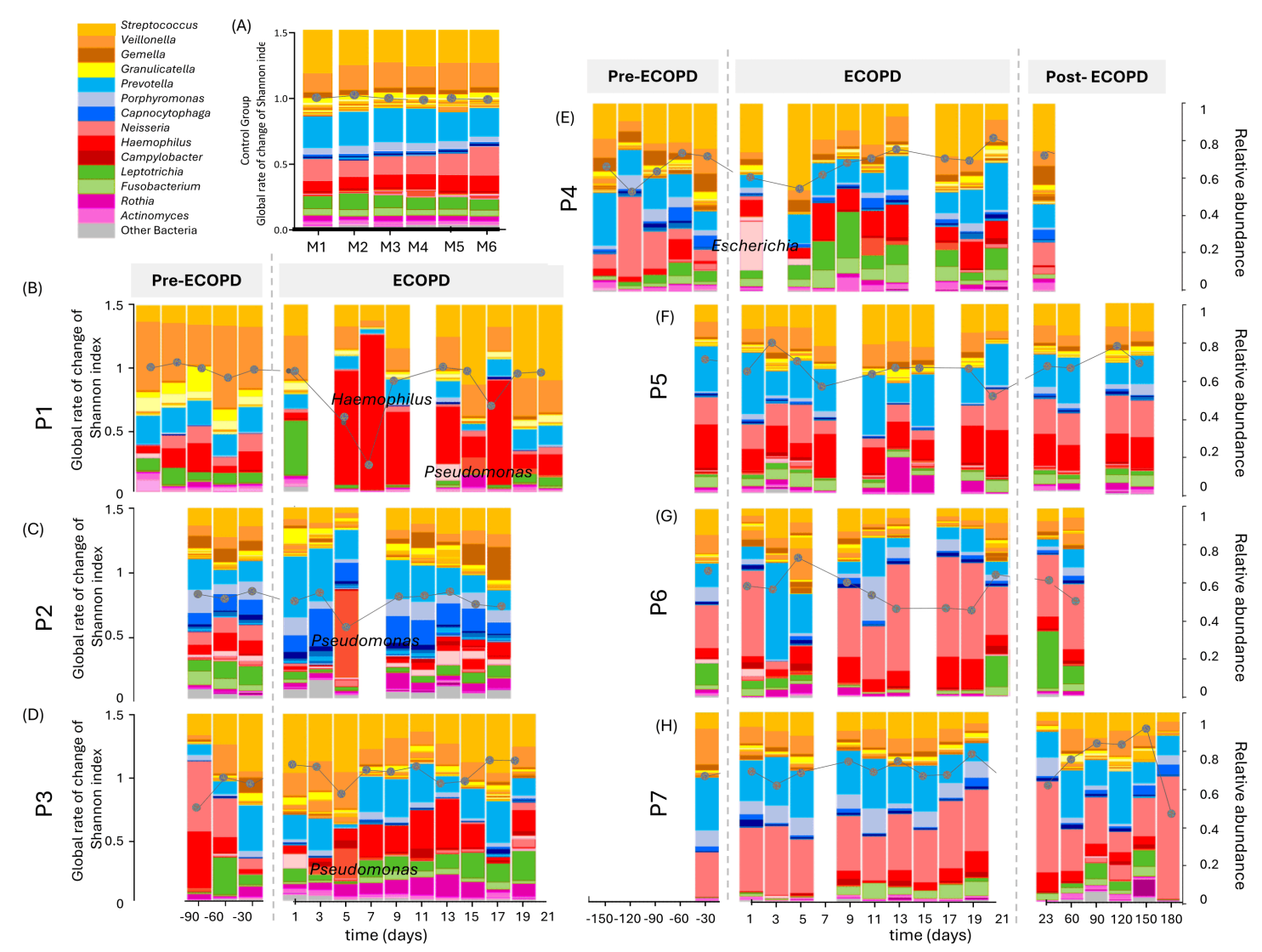


Figure 3

Longitudinal dynamics of salivary microbiota composition across pre-ECOPD, ECOPD, and post-ECOPD periods.

Stacked bar plots depict the mean relative abundance of bacterial genera (present at >1% relative abundance in the control group) over time for the group of patients who remained stable (A) and for individual patients (B–H, Patients 1–7). Bacterial genera belonging to Firmicutes are represented in shades of yellow/orange, Bacteroidetes in blue, Proteobacteria in red, Fusobacteria in green, and Actinobacteria in pink.

Samples from patients experiencing an ECOPD were temporally aligned to the onset of exacerbation (defined as day 1). The ECOPD period (days 1–21) is delimited by vertical dashed lines.

The grey dots connected by a line indicate the global rate of change in α -diversity (Shannon index), normalized to the first available sample for each subject (left y-axis). Pathobionts whose transient expansion coincided with a marked decline in microbial diversity during ECOPD are indicated above the stacked bar plots.

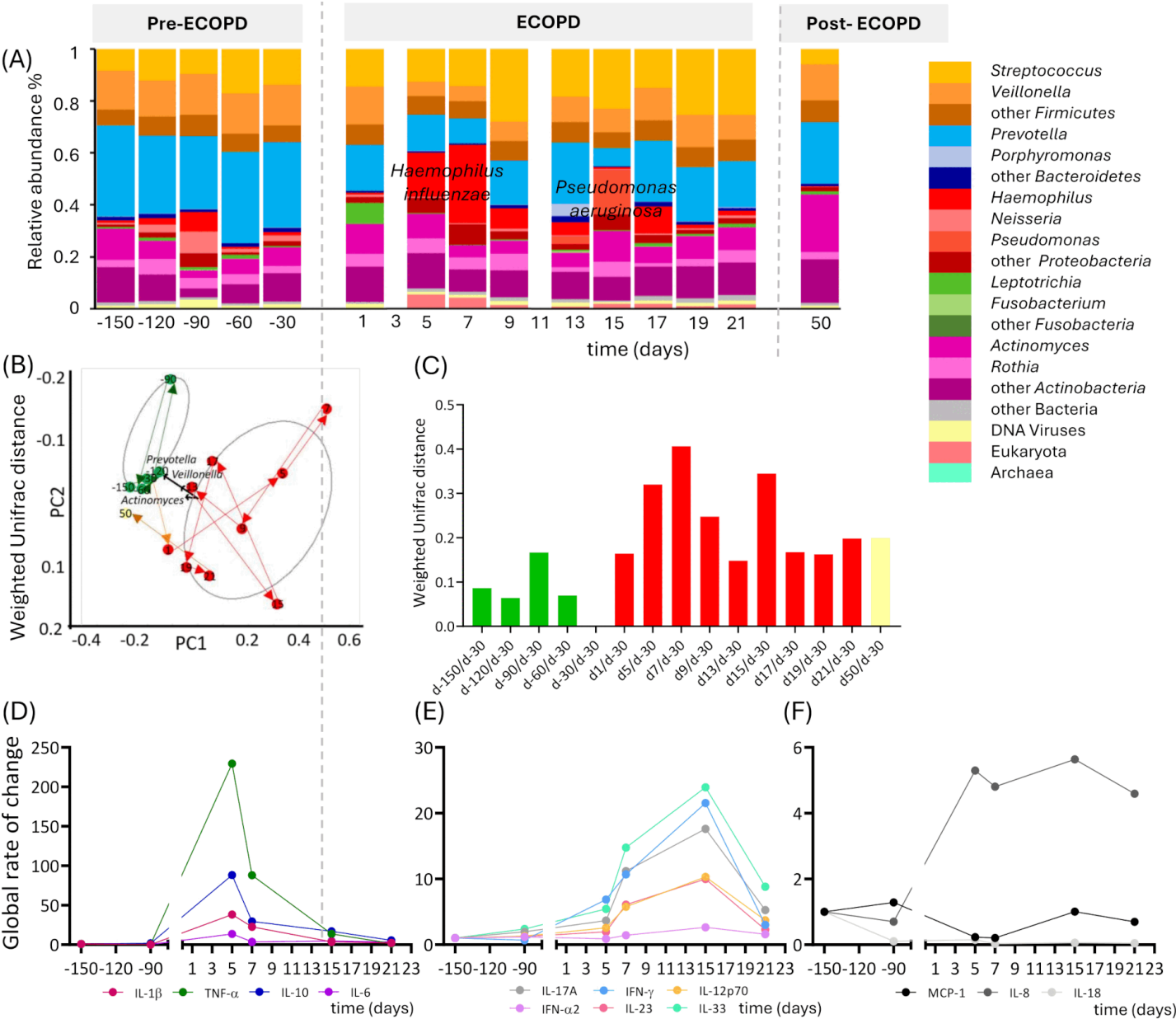


Figure 4

Longitudinal shotgun metagenomic and salivary inflammatory profiling during ECOPD in Patient 1.

(A) Relative abundance of eukaryotes, archaea, DNA viruses, and the dominant bacterial genera detected by shotgun metagenomics in saliva samples collected pre-, during-, and post-ECOPD follow-up. (B)

Principal coordinate analysis based on weighted UniFrac distances showing separation of pre-ECOPD, ECOPD, and post-ECOPD salivary microbiota profiles. Arrows indicate the temporal trajectory of microbiota composition across sampling points.

(B) Principal coordinate analysis (PCoA) based on weighted UniFrac distances showing microbiota composition across pre-ECOPD, ECOPD, and post-ECOPD time points. Samples are coloured according to clinical status: pre-ECOPD stability in green, ECOPD in red, and post-ECOPD in yellow. Sample labels indicate the day of collection relative to ECOPD onset, defined as day 1; negative values correspond to samples collected before ECOPD, whereas positive values correspond to samples collected during or after the exacerbation. Arrows indicate the temporal trajectory of the microbiota, with green arrows representing variation during stability, orange arrows indicating transitions between clinical states, and red arrows representing shifts during ECOPD. Black biplot arrows indicate the main genera contributing to sample separation.

(C) Weighted UniFrac distance between each sample and the last pre-ECOPD sample, day -30, showing the magnitude of microbiota divergence from the pre-exacerbation state over time. Bars are coloured according to clinical status as in panel A.

(D-F) Temporal changes in oral cytokine levels across stability and ECOPD are shown for Patient 1. Cytokine values are expressed as the global rate of change, calculated as the ratio between concentrations measured at baseline during stability (day -150) and those measured at subsequent time points (day -90 during stability, and days 5, 7, 15, and 21 during ECOPD). Panels group cytokines according to their temporal response patterns: (D) cytokines peaking at ECOPD onset, (E) cytokines peaking at day 15, and (F) cytokines displaying alternative patterns of variation during ECOPD.

Supplementary Files

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