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Pulmonary rehabilitation: impact on salivary microbiota and immune response of people with chronic obstructive pulmonary disease

Andreia Sofia da Silva Furtado

DISSERTAÇÃO PARA A OBTENÇÃO DO GRAU DE MESTRE

(DEZEMBRO, 2021)

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Pulmonary rehabilitation: impact on salivary microbiota and immune response of people with chronic obstructive pulmonary disease

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Dissertação apresentada para cumprimento dos requisitos necessários à obtenção do grau de mestre em Ciências Biomédicas

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Andreia Furtado Pulmonary rehabilitation: impact on salivary microbiota and immune response of people with chronic obstructive disease

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Resumo

Enquadramento: A doença pulmonar obstrutiva crónica (DPOC) é a terceira causa de morte mundial. A reabilitação respiratória (RR) é a terapia mais custo-efetiva para esta doença com benefícios físicos, psicológicos e socias amplamente demonstrados. Contudo, o seu impacto na microbiota não está estabelecido. Este estudo teve como objetivo investigar o efeito da RR na microbiota salivar e no perfil inflamatório de indivíduos com DPOC.

Métodos: Recrutaram-se dois grupos de participantes, o grupo experimental-GE (n=38) que realizou RR comunitária durante 12 semanas e o grupo controlo-GC (n=38) que não foi submetido a nenhuma intervenção adicional. Foram recolhidos dados clínicos (e.g., impacto da doença e dispneia) antes e imediatamente após RR e amostras mensais de saliva durante 6 meses. Os 2 grupos foram emparelhados para idade, género, índice de massa corporal, volume expiratório máximo num segundo percentagem do previsto (FEV_{1pp}) e hábitos tabágicos. Os perfis da microbiota e inflamatórios das amostras de saliva foram determinados através da sequenciação do gene 16S rRNA e através da quantificação de citocinas inflamatórias, IgA secretado (SIgA) e proteína total, respetivamente. Modelos Lineares Mistos foram usados para avaliar as dinâmicas da microbiota ao longo do tempo. As análises de abundância diferencial foram conduzidas de forma direcionada para Proteobacteria, Bacteroidetes, *Haemophilus* e *Prevotella*. Alfa e beta diversidades foram estimadas com os índices filogenético e Shannon e a distância Weighted Unifrac, respetivamente. Regressões logísticas foram aplicadas para determinar a associação entre a microbiota antes da RR e a resposta dos doentes aos domínios clínicos. A variação da concentração das citocinas e SIgA ao longo do tempo foram avaliadas com o teste de Friedman.

Resultados: Participaram setenta e seis indivíduos com DPOC (grupo controlo: 31 homens, 70±7.6 anos, FEV_{1pp} 52.3±19.8 e grupo experimental: 29 homens, 72±9 anos, FEV_{1pp} 49.2±16). A microbiota do grupo experimental mudou significativamente face ao grupo controlo ($p<0.0001$). A RR pareceu reduzir a heterogeneidade da composição da microbiota. A maioria dos doentes submetidos à RR apresentou um aumento significativo de Proteobacteria ($p=0.00070$) e *Haemophilus* ($p=0.019$), bem como, diminuição de Bacteroidetes ($p=0.030$) e *Prevotella* ($p=0.013$). Não foram observadas alterações significativas no grupo controlo. A diversidade da microbiota em resposta à RR não se alterou significativamente. Verificou-se uma associação entre a composição da microbiota antes da RR e o impacto da doença e a dispneia. Durante e imediatamente após a RR, os doentes apresentaram um aumento significativo de IL-1β ($p=0.041$) e TNF-α ($p=0.041$), respetivamente; contudo, não foram observadas diferenças na SIgA.

Conclusões: O nosso estudo sugere que a RR modula a microbiota salivar e o perfil inflamatório de pessoas com DPOC. A curto prazo, a microbiota parece aproximar-se do perfil característico da DPOC grave devido ao aumento de Proteobacteria e diminuição de Bacteroidetes. Uma resposta pro-inflamatória à RR parece ocorrer devido ao aumento de IL-1β e TNF-α. Novos estudos são necessários para melhor caracterizar as alterações na microbiota salivar e resposta inflamatória induzidas pela RR.

Palavras-Chave: Doença Pulmonar Obstrutiva Crónica, Reabilitação Respiratória, microbiota salivar, resposta inflamatória

Abstract

Background: Chronic obstructive pulmonary disease (COPD) is the third world leading cause of death. Pulmonary rehabilitation (PR) is the most cost-effective therapy for this disease with physical, psychological, and social benefits widely demonstrated. Nevertheless, its impact on the microbiota has not been established. This study aimed to investigate the effect of PR in the salivary microbiota and inflammatory profile of people with COPD.

Methods: Two groups of participants were recruited, the experimental group-EG (n=38) that performed community-based PR for 12-weeks and the control group-CG (n=38) that was not submitted to any additional intervention. Clinical data were collected (e.g., the impact of disease and dyspnoea) prior and immediately after PR, and monthly saliva samples for 6 months. The 2 groups were matched for age, sex, body mass index, forced expiratory volume in 1 second (FEV_{1pp}), and pack-years. Microbiota and inflammatory profiles of saliva samples were determined through 16S rRNA gene sequencing and quantification inflammatory cytokines, secretory IgA (SIgA) and total protein, respectively. Liner Mixed Models were used to evaluate microbiota dynamics over time. Differential abundance analyses were targeted to Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella*. Alpha and beta diversities were estimated with phylogenetic and Shannon indexes and Weighted Unifrac distance, respectively. Logistic regressions were applied to explore the association among microbiota before PR and patients' response to clinical domains. Variation of cytokines and SIgA concentrations over time were assessed with Friedman test.

Results: Seventy-six individuals with COPD participated (control group: 31 male, 70±7.6y, FEV_{1pp} 52.3±19.8 and experimental group: 29 male, 72±9y, FEV_{1pp} 49.2±16). The microbiota of the experimental group changed significantly over time compared to the control group (p<0.0001). PR seemed to reduce the heterogeneity of the microbiota composition. Most patients submitted to PR presented a significant increase in Proteobacteria (p=0.00070) and *Haemophilus* (p=0.019) as well as a decrease in Bacteroidetes (p=0.030) and *Prevotella* (p=0.013). No significant differences were observed in the control group. The microbiota diversity in response to PR did not change significantly. There was an association between the microbiota composition before PR and the impact of the disease and dyspnoea. During and immediately after PR, patients presented a significant increase in IL-1β (p=0.041) and TNF-α (p=0.041), respectively; however, no significant differences were observed regarding SIgA.

Conclusions: Our study suggests that PR modulates the salivary microbiota and the inflammatory profile of people with COPD. In short term, the microbiota composition seems to become closer to the profile characteristic of severe COPD due to the increase in Proteobacteria and the decrease in Bacteroidetes. A pro-inflammatory response to PR also seems to occur due to the increase in IL-1β and TNF-α. Further studies are needed to further explore the effects of PR in microbiota composition and inflammatory response in COPD context.

Keywords: Chronic Obstructive Pulmonary Disease, Pulmonary Rehabilitation, salivary microbiota, inflammatory response

Index

List of abbreviations and acronyms

6MWT	Six-Minute Walk Test
95% CI	95% Confidence Interval
ASVs	Amplicon Sequence Variants
BAL	Bronchoalveolar Lavage
BCA	Bicinchoninic Acid
BMI	Body Mass Index
CAT	COPD Assessment Test
CCI	Charlson Comorbidity Index
COPD	Chronic Obstructive Pulmonary Disease
DALYs	Disability-Adjusted Life Years
DCs	Dendritic Cells
FEV_1	Forced Expiratory Volume in 1 second
FEV_{1pp}	Forced Expiratory Volume in 1 second Percent Predicted
FVC	Forced Vital Capacity
GOLD	Global Initiative for Chronic Obstructive Lung Disease
IFN	Interferon
IL	Interleukin
mBS	modified Borg Scale
MCIDs	Minimal Clinically Important Differences
MCP-1	Monocyte Chemoattractant Protein-1

mMRC	modified British Medical Research Council
NR	Non-Responder
OTUs	Operational Taxonomic Units
PCoA	Principal Coordinate Analyses
PERMANOVA	Permutational multivariate analysis of variance
pIgR	Polymeric Immunoglobulin Receptor
PR	Pulmonary Rehabilitation
PRR	Pattern Recognition Receptor
R	Responder
ROS	Reactive Oxygen Species
SGRQ	Saint George’s Respiratory Questionnaire
SIgA	Secretory Immunoglobulin A
Th17	T helper 17 cells
TLR	Toll-Like Receptors
TNF	Tumor Necrosis Factor
WHO	World Health Organisation

1 Introduction

1.1 Definition of chronic obstructive pulmonary disease

According to the 2022 Global Initiative for Chronic Obstructive Lung Disease (GOLD) report (1), Chronic Obstructive Pulmonary Disease (COPD) is defined as:

‘a common, preventable, and treatable disease that is characterised by persistent respiratory symptoms and airflow limitation that is due to airway and/or alveolar abnormalities usually caused by significant exposure to noxious particles or gases and influenced by host factors including abnormal lung development’ (p.4).

All these risk factors influence the prevalence, mortality, and morbidity of COPD around the world.

1.2 Epidemiology and impact worldwide of chronic obstructive pulmonary disease

The accurate prevalence of COPD worldwide is still unknown (1,2). It has been estimated to affect 384 million people (1,2); however, this number is likely to be underestimated as this population is highly under and late diagnosed. This occurs mainly due to the lack of knowledge about the disease, both by patients and healthcare professionals; frequent unavailability of spirometry, which is critical for the diagnosis; and misinterpretation of symptoms (dyspnoea, chronic cough, or sputum production) often associated with the aging process (1,2). Nevertheless, several efforts have been conducted to estimate the prevalence of this disease at a global level (3,4). A global COPD prevalence of 13.1% (95% confident interval (CI) 10.2% - 15.6%) has been estimated recently (5). Regarding gender, COPD is usually more prevalent in men than in women in several regions of the world; however, those differences among men and women’s prevalence of COPD are not significant (6). Therefore, the prevalence of COPD among men and women is considered similar nowadays (6). Besides that, several studies (3,4,7) agree that the prevalence of COPD is higher in elderly people, and smokers and ex-smokers in comparison with never-smokers; nevertheless, in women, COPD seem to appear at an early age than in men as well as in individuals that smoke less and present lower economic status in comparison with men (8). Projections indicate a continuous growth of COPD prevalence due to the long-term exposure of people to the risk factors of COPD and the aging of the worldwide population (4,5).

Nowadays, the World Health Organisation (WHO) (9–11) considers COPD the third leading cause of death globally. In 2019, approximately 3.23 million people died due to COPD, corresponding to nearly 6% of the worldwide deaths (10,11).

COPD is also a leading cause of morbidity (1). According to the few data available (1), it seems that the morbidity caused by COPD increases with age. Furthermore, a significant proportion of people with COPD suffer simultaneously from other chronic diseases (over 80% of patients), such as cardiac diseases and diabetes mellitus, which can worsen the patient’s health, and affect the morbidity caused by COPD (1,12).

Given that COPD is one of the top causes of death and morbidity worldwide, it is also responsible for a significant increase in the economic and social burden (1).

In 2019, COPD was in the top 10 causes of disability-adjusted life years (DALYs) at a global level occupying the seventh place with 74 million healthy years of life lost (9,13). This measure allows us to estimate the social burden of major health conditions and injuries and gives us the respective number of years of full health lost (e.g., one DALY corresponds to the loss of one year of full health) (1,14).

1.3 Epidemiology and impact of chronic obstructive pulmonary disease in Portugal

According to the 13º report of the “Observatório Nacional de Doenças Respiratórias” (15), the prevalence of COPD in Portuguese people over 40 years of age is about 14.2%. At a global level, the highest incidence of COPD was noted in male subjects (15). In 2016, approximately 66% of patients hospitalised in Portugal due to COPD were males (15).

According to WHO, in 2019, COPD was reported the seventh leading cause of death in Portugal (9). Similarly to what happened worldwide in 2019, COPD occupied the eighth position in the top 10 causes of DALYs with 848.5 healthy years of life lost per 100 000 population in Portugal (9).

1.4 Risk factors for chronic obstructive pulmonary disease

COPD results from the interaction of several risk factors, such as the prolonged exposure to tobacco smoke and other toxic particles and the existence of host characteristics that may induce the development of this chronic disease (1). Currently, tobacco smoking is the major cause of COPD (1). However, we need to keep in mind that not all smokers develop COPD during their lifetime and that non-smokers may also develop this disease due to passive exposure to tobacco smoke or other risk factors (1).

Genetic predisposition to COPD is also considered a risk factor for this disease. The most well-established COPD genetic risk factor is the lack of alpha-1-antitrypsin in the body; however, it only affects 1-2% of people with COPD (1,16).

In addition, age can also influence the development of COPD (1). Some features observed in the lungs of elderly people are also observed in people with COPD, such as the increased oxidative stress and cellular senescence (1). For a long time, gender was considered a risk factor of COPD (1). Nevertheless, nowadays, it is recognised that the prevalence of this disease is similar among men and women (1,6).

Furthermore, impaired development of the lungs, recurrent lungs infections in the early stages of life, asthma, airway hyper-reactivity, and occupational exposure (i.e., exposure to dust and chemical compounds, such as fuels used for cooking and heating) can also increase the risk of developing COPD in adult life (1).

1.5 Pathology, pathogenesis, and pathophysiology of chronic obstructive pulmonary disease

In COPD, patients' lungs are characterised by a remarkable chronic inflammation along with high levels of inflammatory cells due to the body's response to several toxic particles such as tobacco smoke (1). Patients' lungs can also present higher levels of oxidative stress and proteases which might induce multiple irreversible structure modifications in the small airways, lung parenchyma, and pulmonary vasculatures, such as airway narrowing, peribronchiolar and interstitial fibrosis, and destruction of the alveolar attachments (Figure 1) (1,17). These COPD pathological features contribute to the airflow limitation and gas trapping, inducing hyperinflation and leading to dyspnoea and lower tolerance to exercise (1,18). A decrease in the gas transfer may also happen due to alveolar attachments disruption as well as the occurrence of mucous hypersecretion (1). This hypersecretion is caused by several mediators and proteases released by the goblet cells present in higher levels in patients' lungs (1). People with COPD also have difficulty clearing the mucous produced due to the impairment of the mucociliary clearance (19), allowing the establishment of a resident microbiota community.

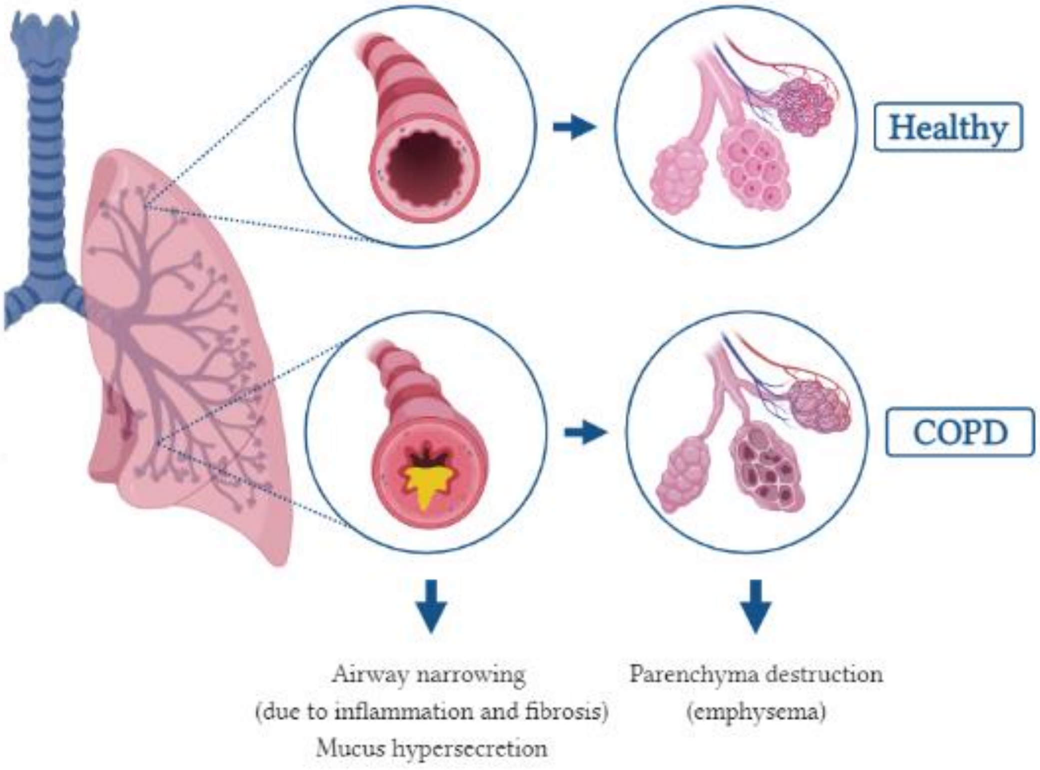


Figure 1 – Healthy subjects present non-inflamed lungs with wide-open small airways due to the normal alveolar attachments. In chronic obstructive pulmonary disease (COPD), patients' lungs are characterised by chronic inflammation. At the small airways, this inflammation along with the development of fibrosis and destruction of the alveolar attachments leads to the narrowing of these small airways. In this chronic disease, some patients may also present mucus hypersecretion. Created with BioRender.com

1.6 Symptoms and diagnosis of chronic obstructive pulmonary disease

Clinical manifestations of people with COPD commonly include persistent dyspnoea and/or cough, along with sputum production or not (1). Some patients may also present wheezing and chest tightness (1). These patients often have acute exacerbations, defined as episodes of acute worsening of respiratory symptoms that result in additional therapy, throughout their disease trajectory (20).

Diagnosis should be performed in patients with persistent and progressive dyspnoea and/or with productive or unproductive chronic cough (1). However, clinical manifestation by themselves cannot be used to determine if someone has or not COPD (1). The medical and family history of the patient (e.g., recurrent infections in the lower respiratory tract during adulthood and/or childhood as well as family members with COPD), historic exposure to risk factors (e.g., tobacco smoke or occupational exposures), and spirometry after bronchodilator inhalation also needs to be considered to establish the COPD diagnosis (1). Spirometry is used to determine the airflow limitation of patients by estimating the FEV_1/FVC ratio (forced expiratory volume in 1 second/forced vital capacity) (1). A ratio of 0.70 or lower indicates airflow limitation (Figure 2) (1) and confirms a diagnosis of COPD.

Once a COPD diagnosis is established, the degree of airflow limitation can be determined based on forced expiratory volume in 1 second percent predicted, and the impact of the disease by the refined ABCD assessment tool (Figure 2) (1). This method of COPD assessment is based on the risk of exacerbation and symptoms intensity considering the modified British Medical Research Council (mMRC) questionnaire, which evaluates the degree of activity-related dyspnoea, or the COPD assessment test (CAT), which assesses the health status/impact of the disease (1).

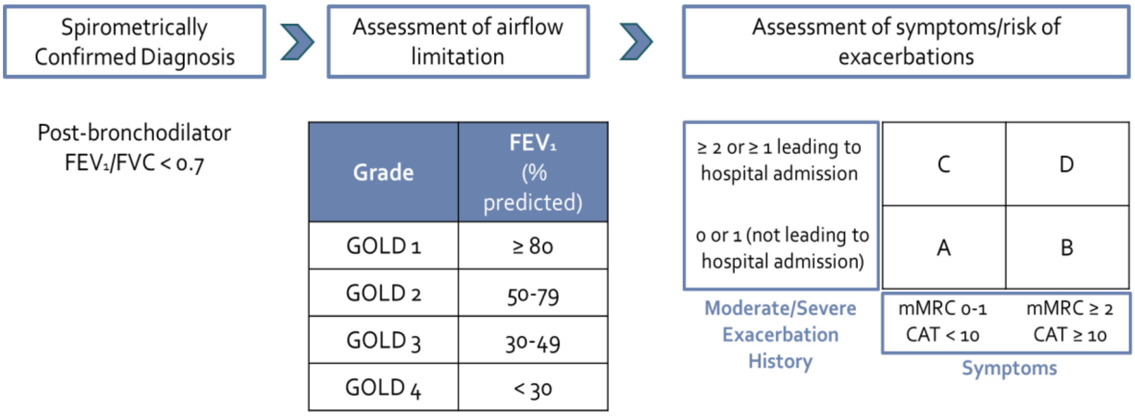


Figure 2 – The combined and refined COPD assessment tool. The COPD diagnosis is confirmed with spirometry when a FEV_1/FVC ratio of 0.7 or lower is obtained, meaning the presence of airflow limitation. Then, the severity of COPD can be assessed based on the airflow limitation, where patients are classified as belonging to GOLD grade 1 to 4, i.e., mild to very severe airflow limitation. Additionally, patients can also be assessed concerning their exacerbations' severity (patients with more severe exacerbations are included in GOLD C and D) and the symptoms presented (GOLD B and D include patients with more COPD symptoms). Adapted from GOLD 2022 (1).

1.7 Treatment of chronic obstructive pulmonary disease

In people with COPD, pharmacological therapy mainly composed of bronchodilators is beneficial, improving patients' periods of worsening condition, tolerance to exercise, and reducing hospitalisations caused by exacerbations (1). However, the use of $beta_2$-agonists in people with COPD seem to have some adverse cardiovascular effects, such as tachycardia (1). Pulmonary rehabilitation (PR) is a grade A non-pharmacological therapy and the most cost-effective to manage COPD (21). Several physical, psychological, and social benefits of PR have been widely demonstrated, such as improvements in health status, dyspnoea, tolerance to exercise, and anxiety/depression symptoms as well as reduction of hospitalisations due to severe exacerbations (1). Although both therapies benefits these patients, improvements are three to five times higher in those who undertake PR than under long-acting bronchodilators (Figure 3) (22). Therefore, PR is considered fundamental for the management of COPD (1,22).

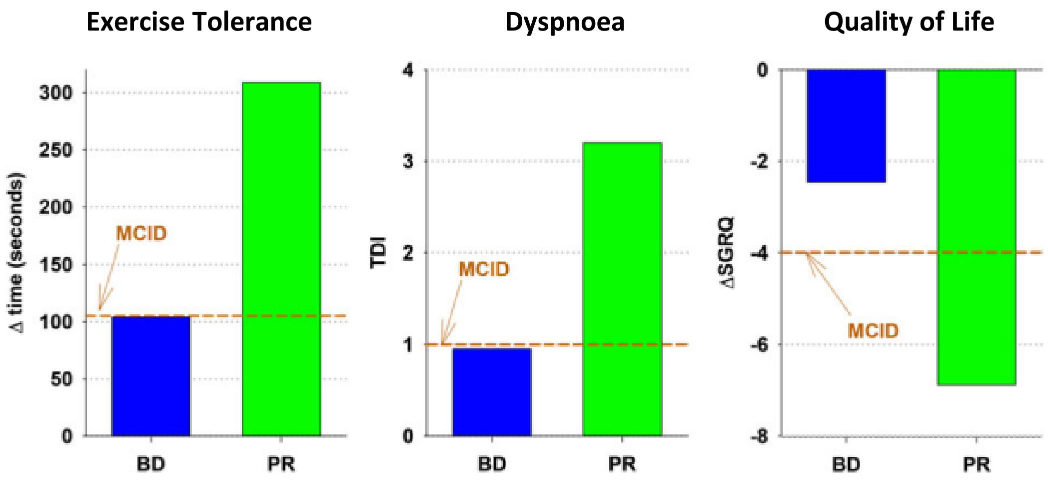


Figure 3 – Comparison of the improvement of people with COPD under long-acting bronchodilators (BD - blue) and under PR (green) regarding exercise tolerance, dyspnoea, and quality of life. Patients had an improvement in the tolerance to exercise when the duration of the exercise performed during the cycle endurance test increased. For dyspnoea, an increase in the score obtained from Mahler's Transitional Dyspnoea Index (TDI) was considered an improvement in this domain. When assessing patients' quality of life with the St. George's Respiratory Questionnaire (SGRQ), a decrease in the score was determined as beneficial for individuals. In all domains, a clinically significant improvement was established when the time and scores obtained in individuals were equal or higher than the minimal clinically important difference (MCID – orange line) recognised for each domain. Adapted from Casaburi (22).

According to the last official American Thoracic Society/European Respiratory Society statement regarding essential concepts and advances in PR (23), PR is defined as:

'a comprehensive intervention based on thorough patient assessment followed by patient-tailored therapies, which include, but are not limited to, exercise training, education, and behaviour change, designed to improve the physical and psychological condition of people with chronic respiratory disease and to promote the long-term adherence to health-enhancing behaviours' (p.e16).

Pulmonary rehabilitation is a multidisciplinary and multicomponent intervention, delivered in a personalised manner, given the heterogeneity of the disease and patients' different goals and preferences (1). Thus, before enrolling in a PR programme, each patient is comprehensively assessed (1). Then, typically exercise training sessions, twice a week; and education and psychosocial support sessions, once every other week, follows for 8 to 12 weeks (23).

After PR, responders (R) and non-responders (NR) can be defined according to the minimal clinically important differences (MCIDs) established for each clinical variable assessed since a global criterion to determine patients' response to PR is not available. Some of these variables are the 6-minute walk test (6MWT) for exercise tolerance (24), modified Borg Scale for Dyspnoea (mBS) (25), and CAT for the impact of the disease (26). The 6MWT, this is a test used to assesses the maximum walking distance that a person achieves during six minutes (24). The mBS is a scale to assess the level of dyspnoea during exercise (25), usually the 6MWT. The highest the score obtained, the greater is the level of patients' perceived dyspnoea (25). Finally, the CAT corresponds to an 8-questions questionnaire assessing the COPD impact on patients' life (the greater the score, the greater the impact in patients) (26).

1.8 Microbiota in health and chronic obstructive pulmonary disease

1.8.1 Main source of lungs microbiota

Until the last decade, lungs were considered sterile organs, *i.e.*, free of microorganisms. However, through the advent of next-generation sequencing, this assumption was proven wrong (27–29) and instead, lungs were shown to harbour a diverse and interacting microbial community (27).

In healthy individuals, the flow of fluids and saliva from the upper respiratory tract (environment enriched in microbes) can generate significant amounts of aerosols that can be microaspirated into the lungs (27,28). During breathing, it has already been reported that up to 10% of healthy subjects present aspiration of saliva (30). Therefore, it is believed that the main source of healthy lungs' microbiota is the oral microbiota (28). In agreement, the oral microbiota was described to be more similar to the lung than to the nasal microbiota (28). Additionally, in healthy individuals, the elimination of microorganisms from the lungs occurs through mucociliary movements and host mucosal defence, making less likely the establishment of a resident microbial community (27,28).

In COPD and elderly people, the normal swallowing process is impaired (30).

People with COPD present a higher risk of aspiration due to impaired upper airway-protective mechanisms, such as dysfunctional laryngeal–pharyngeal musculature, decreased coordination between swallowing and breathing, and alterations in patients' habits of breathing due to the disease (30). Furthermore, in COPD, the mucociliary clearance is impaired (19), and microorganisms' elimination from the lungs becomes harder. Therefore, the microorganisms microaspirated from the upper airway, such as the oral cavity, might remain in the individuals' lungs.

1.8.2 Airway microbiota composition in healthy and chronic obstructive pulmonary disease

Several studies using invasive biological samples, such as bronchoalveolar lavages (BAL), protective specimen brushings, and sputum samples, have consistently shown that airway microbiota composition of healthy individuals is mainly characterised, at the phylum level, by Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria (29,31). At the genus level, *Streptococcus*, *Veillonella*, *Prevotella*, *Haemophilus*, *Fusobacterium*, and *Actinomyces* seem to be the most abundant genera in healthy individuals (28,29,31–33), when using BAL, protective specimen brushings, sputum, and oral wash samples. Concerning individuals with COPD, their airway microbiota is similar to the healthy subjects, i.e., the same phyla are characteristic of those patients, but the relative abundance of each one is different. In patients with this chronic disease, the airway microbiota is usually characterised by a decrease in Firmicutes (31), mainly *Veillonella* genus (31,33), when using sputum and BAL samples, and a decrease in Bacteroidetes (33,34), mainly *Prevotella* genus (31–34) in sputum, BAL, and cytologic brushings within the lungs. Moreover, a significant increase in Proteobacteria (31,34) has been detected in sputum and cytological brushing within the lungs from patients with a strong association between the high relative abundance of *Haemophilus* genus (31,34) and COPD.

A recent study (unpublished (35)) from the team, using saliva samples, showed that these are suitable to assess COPD since the differences among the airway microbiota of healthy and COPD using saliva and invasive samples were very similar. Melo-Dias *et* *al.* observed that the salivary microbiota profile of people with COPD was enriched in Proteobacteria (*Haemophilus*) as well as depleted in Firmicutes and Bacteroidetes (*Prevotella*). The fact that saliva seems to be suitable for the study of COPD is a major discovery (35), since it could replace, at least in part, samples collected by invasive procedures, such as bronchoscopies. Therefore, an increase in adherence of healthy individuals to studies comparing the airway microbiota of healthy and COPD subjects would probably occur. In addition, COPD studies could include higher sample sizes and balanced numbers of patients and healthy individuals to accelerate knowledge about this highly heterogeneous and complex disease.

Most studies exploring the airway microbiota of healthy subjects and people with COPD usually use BAL and sputum samples instead of upper respiratory tract samples. Although the upper respiratory samples (oropharyngeal swabs and oral washes), in health (36) and COPD (37), present greater diversity than samples from the lower respiratory tract (protected specimen brushing, BAL, and sputum), the microbiota profile among those samples is indistinguishable. Figure 4 also evidence an overlap of most microorganisms (taxa 6 to 15) from oral wash and lung tissue samples from people with COPD, according to the neutral model theory (38). This theory suggests that the lung microbiota is acquired due to the immigration of bacteria from the oral microbiota, their reproduction in the lung, and clearance of lung bacteria; thus, lung microbiota might be a representation of the oral microbiota (38). The remain taxa, 1 to 5 and 6 do not follow the neutral model theory (38). The greater representation of taxa 1 to 5 and less representation of taxa 6 in the lung tissue might be due to the environmental selection of these taxa, mainly increase of taxa 1 to 5 and depletion of taxa 6, in the lungs caused by changes regarding the oxygen tension and impaired mucociliary clearance, for example (38).

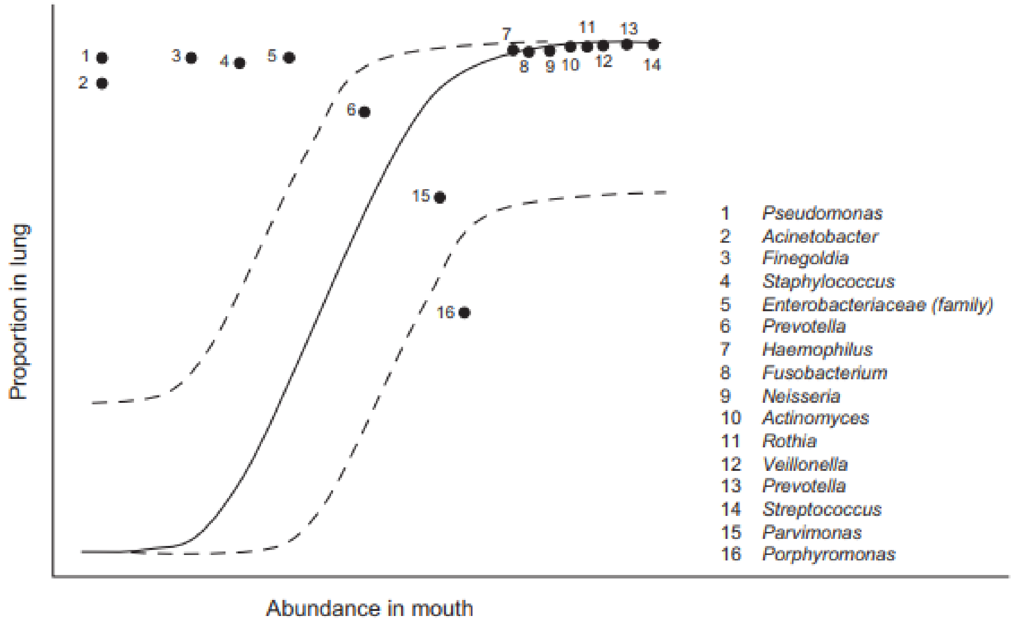


Figure 4 – Neutral theory model based on oral wash and lung tissue samples from people with COPD. The y-axis represents the proportion of the sixteen taxa detected in the lung, and the x-axis represents the abundance of the same taxa in the mouth of people with COPD. The dark black line represents the best fit line, and the dashed lines correspond to the 95% CIs. Numbers from 1 to 16 represent the taxa observed in both oral wash and lung tissue samples from patients with COPD, and the black dots represent their abundance in both types of samples. Adapted from Gaeckle et. al (38).

1.9 Microbiota dysbiosis in chronic obstructive pulmonary disease

Dysbiosis is a term that describes changes in the resident commensal microbiota composition, in our case of the lungs, relative to the microbiota composition observed in healthy individuals (39).

According to the “vicious circle hypothesis” (Figure 5) (40), dysbiosis in COPD (changes in microbiota composition, relative abundance, and diversity) arises as a consequence of modifications in the innate lung defence that are enhanced by patients’ continuous exposure to tobacco smoke and other toxic particles.

This theory proposes that when harmful bacteria reach the lung, an inflammatory response is triggered due to its detection by pathogen recognition receptors on the airway epithelial cells and on cells from the immune system (40). This inflammation increases airway vessels permeability, allowing the translocation of important nutrients for bacteria and immune cells (28). Damage of the lung epithelial cells can also occur with inflammation, stimulating the secretion of innate cytokines against bacteria, such as interleukin (IL)-33 (28). Thus, those cytokines might stimulate the production of more inflammatory cytokines, further increasing inflammation (28). All this inflammation contributes to the continuous damage of the innate lung defence mechanisms, allowing bacteria to survive and multiply in the lungs (40). This translates in a repetitive cycle of lung inflammation resulting in further damage and loss of function (40). In sum, the “vicious circle hypothesis” proposes an explanation for how COPD origins and progresses and how the lung dysbiosis and inflammatory response are related.

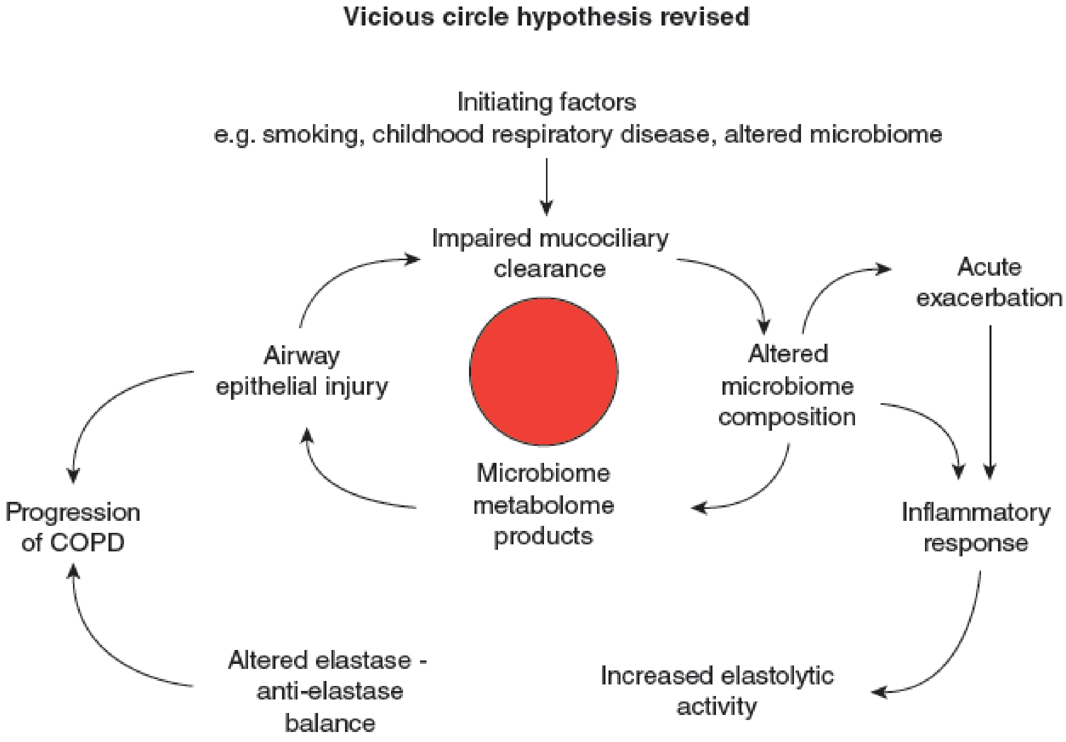


Figure 5 – The vicious circle hypothesis of COPD progression and association between lungs’ dysbiosis and inflammatory response. An initial insult, such as tobacco smoke, might lead to alterations in the innate immune defence of people with COPD, modifying the lung microbiota composition and, therefore, contributing directly or indirectly (through worsening periods of COPD symptoms) to inflammation. In addition, the altered microbiota and increased inflammation might cause physic alterations in the lungs, allowing the progression of COPD. Adapted from Mammen et. al (40).

Another model (called “Life in Antarctica” (28)), which does not exclude the “vicious circle hypothesis”, postulates that the lung microbiota is determined by the balance of three factors, i.e., i) immigration of bacteria into the airways by microaspiration and inhalation, ii) clearance of bacteria from airways through mucociliary elimination, cough, and host immune defences, and iii) the reproductive rate of bacteria in the airways. This last factor can be affected by inflammation since it alters the growth conditions in the lungs (nutrient availability, pH, temperature, oxygen tension), favouring the growth of some bacteria over others (28). An unbalance between these factors can result in dysbiosis.

Although the “vicious circle hypothesis” and the “Life in Antarctica” models try to explain how dysbiosis might occur in the lung/airway microbiota of people with COPD, it is still unknown whether this dysbiosis is a cause or effect of COPD.

1.10 Inflammatory response in chronic obstructive pulmonary disease

A common trait of COPD is the chronic inflammation of the lungs mediated by immune cells from the innate and adaptive immune system (41).

In this chronic disease, the innate immune response (Figure 6) begins when smoke particles or pathogen-associated molecular patterns directly interact with pattern recognition receptors (PRRs) in the surface of alveolar macrophages, dendritic cells (DCs), and epithelial cells (41). The activation of PRRs may also occur indirectly through damaged-associated molecular patterns released by damaged cells (41). As a result of this interaction among PRRs and pathogen- or damaged-associated molecular patterns, epithelial cells and alveolar macrophages release proinflammatory mediators, such as tumor necrosis factor (TNF)-α, IL-8, and IL-1β (41). Then, those mediators allow the recruitment of other immune cells to the lungs (neutrophils and inflammatory monocytes), which, when activated, release more proinflammatory cytokines, reactive oxygen species (ROS), and proteolytic enzymes (41). All these mediators will contribute to more inflammation, disruption of the alveolar walls, and mucus hypersecretion (41). IL-6, IL-23, IL-33, and monocyte chemoattractant protein-1 (MCP-1) are other proinflammatory mediators released by immune cells during the innate response (42). During the innate immune response, natural killer cells play an essential role in the maturation of DCs through the production of interferon (IFN)-γ and TNF-α (41). Once DCs are matured, the adaptative immune response can rise (41).

During the adaptative immune response (Figure 6), the mature DCs interact with both naïve $CD8^+$ and $CD4^+$ T cells through antigen presentation, leading to $CD8^+$ cytotoxic T cells and $CD4^+$ T helper (Th) cells production (41). The activation of $CD8^+$ cytotoxic T cells allows the release of proteolytic enzymes, able to destroy lung structural cells (41). In COPD, $CD4^+$ Th cells are mostly differentiated into Th1 cells (due to the presence of IL-12, also known as IL-12p70, and IL-18 in the environment) and into Th17 cells (due to the presence of IL-1β, IL-6, TGF-β, IL-23 in the lungs) (41). Then, Th1 cells can release IFN-γ and TNF-α, increasing the innate immune responses, while Th17 cells can produce other proinflammatory mediators, such as IL-17A, leading to the production of substances by the epithelial cells that are important for the accumulation of neutrophils in the injured lung and, thus, its persistent inflammation (41). Regulatory T cells are another lineage of Th cells, produced at lower levels in patients' lungs, that regulates the inflammatory response through the production of IL-10 and TGF-β (41). Moreover, people with severe COPD may also present, in the lungs, lymphoid follicles, a structure that incorporates DCs, T and B lymphocytes (41). When those lymphocytes are activated by inflammation, for example, the recruitment of more lymphocytes occurs as well as the arrangement of the follicles (41).

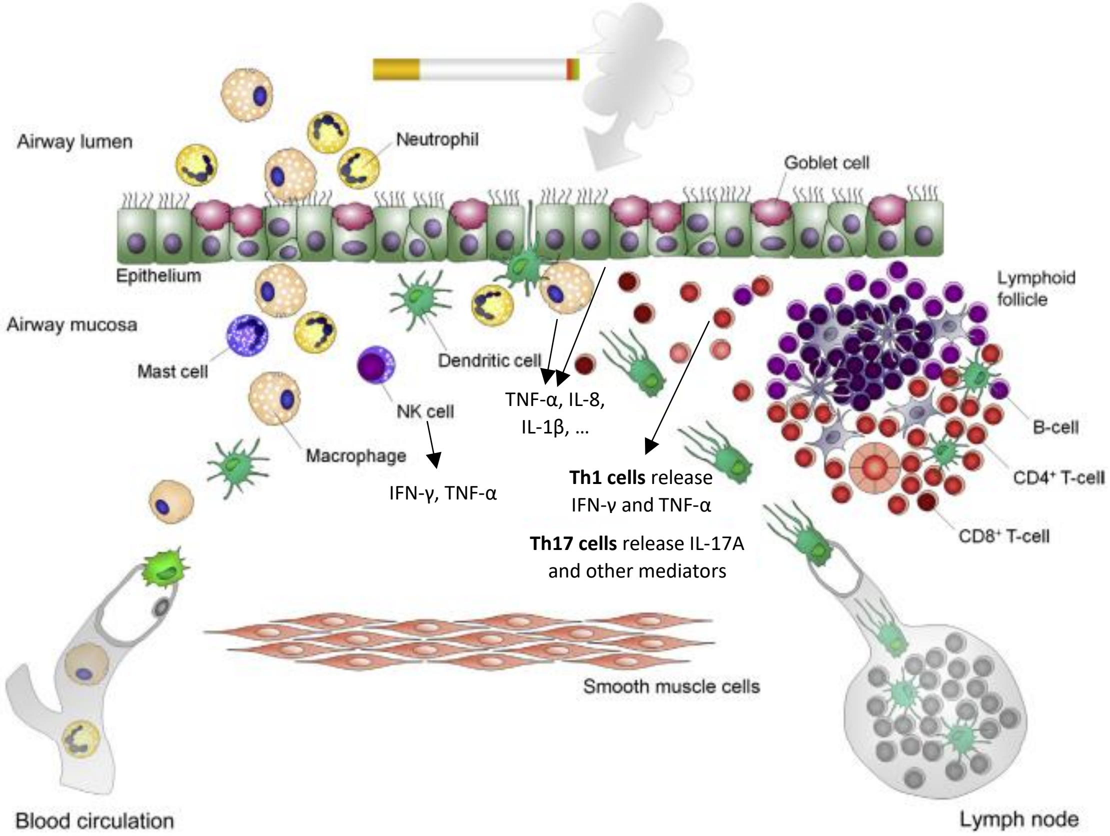


Figure 6 – The innate and adaptive immune response in the lungs of people with COPD. At the beginning of the inflammatory response, innate immune cells are activated mainly by particles from tobacco smoke. Then, dendritic cells suffer maturation and initiate the adaptive immune response. In this response, CD8$^+$ cytotoxic T cells and CD4$^+$ T helper cells are produced, and CD4$^+$ Th cells can be differentiated into several lineages. When individuals suffer from severe COPD, lymphoid follicles (B and T lymphocytes strictly organised) can be detected in their lungs. Adapted from Bracke et. al (41).

The previous inflammatory mediators involved in the lungs' immune response of people with COPD can be assessed in circulation (blood, plasma, and serum samples) and samples with direct contact with the airway epithelial cells, such as BAL and sputum samples. Since saliva is produced in glands highly vascularized, the transport of the inflammatory mediators from the blood to the saliva might happen, potentially allowing the use of these samples to assess inflammation in patients with COPD (43). However, the knowledge about the inflammatory response in COPD has been mostly obtained using blood, plasma, serum, BAL, and sputum samples.

Most of the proinflammatory mediators already mentioned (MCP-1, IL-1β, IFN-γ, TNF-α, IL-6, IL-8, IL-12p70, IL-17A, IL-18, IL-23, and IL-33) have been detected as increased in the blood, plasma, and/or serum of people with COPD in comparison to healthy individuals, non-smokers, and/or smokers without COPD (44–53). There is also evidence that IL-1β, TNF-α, and IL-6 is increased in serum from patients with higher grades of COPD severity (45). Moreover, an increase in IL-1β and IL-18 in sputum samples (54,55) and TNF-α, IL-6, and IL-8 in BAL samples (47,48) from people with COPD in comparison to healthy individuals has been observed. Additionally, IL-33 has also been reported as increased in lung lysates and exhaled breath condensate samples from people with COPD in comparison to healthy controls (52,56). These results evidence that people with COPD present chronic inflammation detectable in the lungs and at a systemic level. The lower levels of IL-10, an anti-inflammatory cytokine described as resisted by the human body in some diseases (57), have been reported in serum samples from people with COPD in comparison with healthy individuals (58), which corroborates the incapacity of patients' bodies to regulate the chronic inflammation.

Production of secretory immunoglobulin A (SIgA) also occurs in the mucosa surface (59). SIgA is the main immunoglobulin present in mucous secretions from the salivary glands and respiratory system that combats infections inhibiting bacteria or viruses' adhesion to the epithelial cells from the mucosa surface (immune exclusion), neutralizing bacterial toxins or viruses, and/or secreting antigens that entered the mucosa epithelial cells and airway mucosa (Figure 7) (59–62).

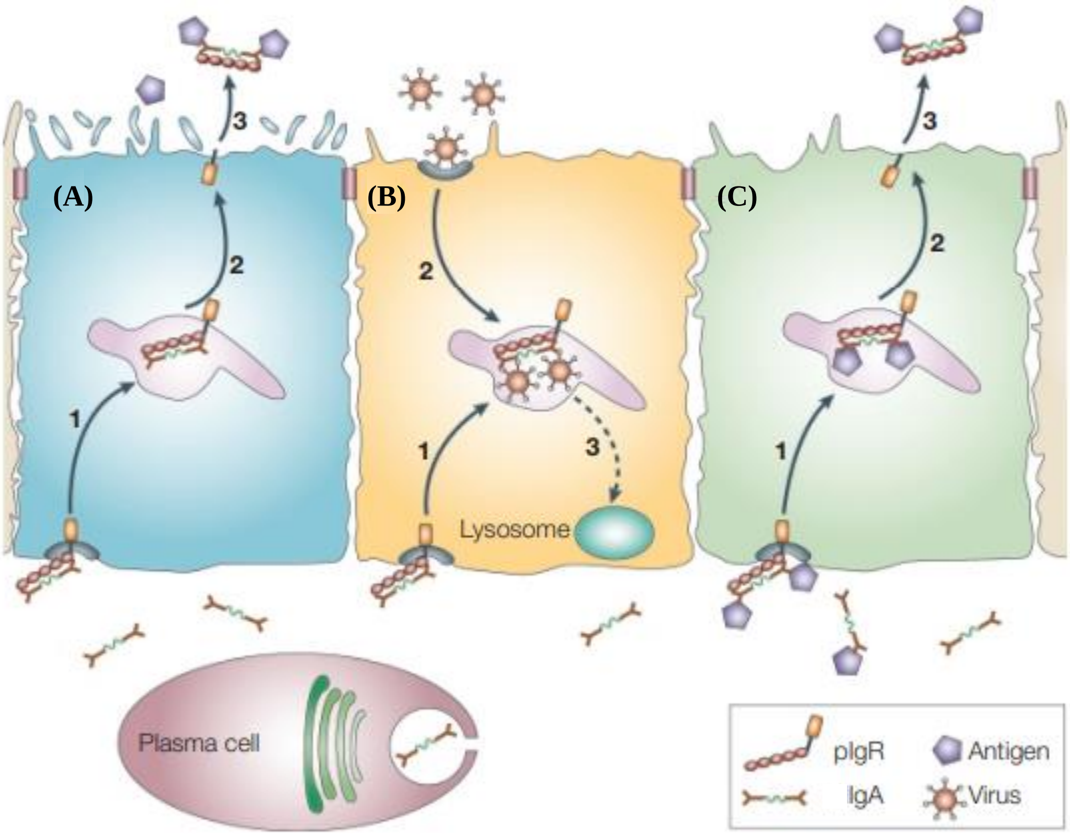


Figure 7 – Role of SIgA in the mucosa surface. **(A)** Production of SIgA and immune exclusion. After the activation of plasma cells, immunoglobulin A is produced. Then, IgA binds to the pIgR in the basolateral region of the mucosa epithelial cell, allowing (1) the transport of IgA to the apical region (2). After that, pIgR is cleaved, enabling the formation and release of SIgA (3) as well as avoiding the adherence of pathogens to the mucosa epithelial cells due to the interaction of pathogens with SIgA. **(B)** Intracellular neutralisation. In the presence of viruses on the mucosa surface, the IgA-pIgR complex is transported into the cell via endocytic vesicles (1). Then, this complex interacts with the viruses, forming the pIgR-IgA-virus complex (2), consequently destroyed by lysosomes (3). **(C)** Secretion of pathogenic antigens. Once the antigens invade the mucosa epithelial cells and enter the lamina propria, the IgA-pIgR complex interacts with those antigens (1), allowing their secretion in the apical region of the mucosa epithelial cells (3) through their transport across the cell in vesicles (2). Adapted from Rojas et. al (59).

People with COPD have been found to have lower levels of SIgA than healthy individuals in BAL samples (63). These low levels of SIgA might happen due to the reduced expression of pIgR in the lung epithelial cells (64), suggesting less transport of IgA through the cells, an increase of IgA accumulation in the basolateral region and an almost absence of SIgA in the mucous surface (63,65).

Most information regarding SIgA and COPD is obtained with BAL and lung tissue samples instead of saliva samples. Since saliva seems to be the main contributor to the airway microbiota composition, the levels of salivary SIgA might partially represent the levels of the SIgA detected in BAL and lung tissue since the salivary microbiota is more diverse than the lung microbiota. However, this suggestion needs to be directly tested to confirm the relationship between the SIgA detected in saliva and the lungs.

1.11 Pulmonary rehabilitation and microbial and inflammatory response in chronic obstructive pulmonary disease

Exercise training is one of the main components of PR. During exercise, an increase in the uptake of oxygen occurs as well as an increase in the blood flow, allowing the delivery of oxygen to several organs (66,67). Given that modifications in lungs' conditions such as oxygen tension, blood flow, pH, and temperature can interfere in bacterial proliferation (27), exercise training may also alter the airways microbiota. Thus, it would be interesting to study the impact of PR on the airway microbiota of people with COPD to understand whether its beneficial impact could be, at least in part, mediated by the microbiota. We would like to highlight that our study is pioneer in tackling this question since this has not yet been established.

Regarding the inflammatory response, some studies have already tried to understand the effect of PR, mainly exercise training, in people with COPD. Nevertheless, the conflicting results are yet found in the literature.

An increase in TNF-α levels in people with COPD compared with healthy individuals, has been reported after 8 weeks of moderate-intensity exercise training and in plasma samples collected 11 minutes after the exercise session, whereas IL-6 levels remained the same (68). Nevertheless, an increase of IL-6 levels in plasma of people with COPD in comparison with healthy individuals and similar levels of TNF-α have also been reported immediately after exercise (69). In addition, a different study (70), including a programme of 12 weeks of training, showed an increase of IL-6 and a decrease of TNF-α in plasma of people with COPD. No significant differences were observed in plasma samples of people with COPD for IL-10 over time (70). To our knowledge, there are not yet studies assessing the inflammatory mediators in saliva samples after 4-weeks of PR in people with COPD.

Effects of PR in salivary SIgA levels of people with COPD are still not very well established. Levels of salivary SIgA have been found increase significantly in elderly people after performing exercise training for 12 months (71) as well as immediately after exercise (72).

Effects of exercise training in the inflammatory response of people with COPD are still not well established due to the controversy observed in the previous studies regarding the inflammatory mediators and lack of investigation concerning the effect of PR in salivary SIgA levels in COPD subjects.

1.12 Aims

1) Explore if PR impacts the salivary microbiota and inflammatory response of people with COPD.
2) Explore if the salivary microbiota alterations are related to the beneficial effect of PR.
3) Assess if the salivary microbiota composition of responders and non-responders differs after PR.

2 Material and Methods

A longitudinal study was conducted. Ethical approvals were obtained within the "PRIME – Pulmonary Rehabilitation and microbiota in exacerbations of COPD" project, from Administração Regional de Saúde Centro (64/2016) and Centro Hospitalar do Baixo Vouga (08-03-17). Approval from the National Data Protection Committee was also obtained. Written informed consent was obtained from all participants prior to any data collection.

2.1 Subjects and sample collection

Participants were identified and referenced by clinicians collaborating with the team, who briefly explained the purposes of the study. Participants were eligible if diagnosed with COPD according to the GOLD criteria (1) and were clinically stable, i.e., no acute exacerbations or significant adjustments to medication in the month prior to enrolment. Exclusion criteria were presence of severe or unstable cardiac, musculoskeletal, or neuromuscular diseases, signs of cognitive impairment, active neoplasia, or immune diseases, that could impair participant's participation in PR. Patients included in the present study were recruited from March 2018 to March 2020.

All participants were offered the opportunity to integrate a PR programme. Those who were unable or were not interested to participate but still accepted to be assessed, composed the control group. The experimental group (intervention group) was composed of those participating in a 12-week community-based PR. The PR program was composed by 60-minute sessions of moderate to high intensity exercise training twice a week and psychoeducational sessions once every other week. The PR program was delivered by a multidisciplinary team of healthcare professionals. Detail description of the intervention delivered can be found elsewhere (73). All patients (intervention and control groups) were followed for 6 consecutive months, month 1 (M1) to month 6 (M6). In the intervention group, the first month of the follow-up correspond to 1 month before PR (M1) and, thus, the period before PR (Pre period). The following 3 months correspond to the months of PR participation, i.e., month 2 (M2), month 3 (M3), and month 4 (M4); therefore, the period during PR (PR period). The following months correspond to the 2 months after PR, i.e., month 5 (M5) and month 6 (M6).

Sociodemographic and anthropometric data were collected at baseline. Clinical data (monthly collected in the control group, and collected immediately before and after PR in the intervention group) and saliva samples (monthly, passive drool method) were collected using a structured protocol (73).

Sociodemographic (age, sex, educational level), anthropometric (weight and height to compute body mass index), and clinical data such as smoking habits, medication used, long-term oxygen, comorbidities - Charlson Comorbidity Index (CCI) (74), and level of airway obstruction-spirometry (FEV_1, FVC, FEV_{1pp}) (75) were collected with a structured protocol adapted from the team published work (73) to characterise our samples in the baseline. Other clinical data such as number of exacerbations and hospitalisations in the past year (collected in the baseline) and past 3 months (collected in M4), self-perceived dyspnoea during activities - mMRC (76), self-perceived dyspnoea after exercise – mBS (77), impact of the disease – CAT (78), quality of life – Saint George's Respiratory Questionnaire (SGRQ) (79), and exercise tolerance – 6MWT (80) were mostly collected in the baseline and in M4 according to the protocol previously mentioned (73). Saliva samples, passive drool method, were collected monthly in both groups and with the mentioned structured protocol adapted from the team published work (73). GOLD grades were defined according to FEV_{1pp} for each individual. GOLD groups were defined combining the number of exacerbations and hospital admissions of each patient in the year before enrolment with their CAT scores (1). Before saliva sample collection, each participant was advised to drink a glass of water (especially if he 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 -80ºC until DNA extraction.

2.2 DNA extraction

Prior to DNA extraction, samples were thawed at room temperature and centrifuged at 10,000xg and 4 ºC for 10 minutes. Supernatants were discarded and DNA extraction from cell pellets followed QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) protocol with minor modifications: initial sample volume was set to 400µL and the volumes of buffers and Qiagen protease were adjusted. Elution volume was reduced to a quarter of the recommended. Thirty-eight negative controls where saliva was replaced by phosphate-buffered saline were performed in order to control for background bacterial contamination. Quality and quantity of the extracted DNA was assessed in Denovix DS-11 spectrophotometer, with OD260/280 and OD260/230 ratios.

2.3 16S rRNA gene amplification and sequencing

16S rRNA gene amplification and sequencing was carried out at the Gene Expression Unit from Instituto Gulbenkian de Ciência following the implemented protocol. Briefly, for each sample, the hypervariable V4 region of 16S rRNA gene was amplified, using universal pair of primers F515 (5′-CACGGTCGKCGGCGCCATT-3′) / R806 (5′-GGACTACHVGGGTWTCTAAT-3′). Samples were then pair-end-sequenced on an Illumina MiSeq Benchtop Sequencer, following Illumina recommendations.

2.4 Microbiota and statistical analyses

For the purpose of this study, participants of both groups were matched for sex, age, body mass index (BMI), FEV_{1pp}, and pack-years.

2.4.1 Sample characterisation

Descriptive statistics was used to describe the sample at baseline, normality of data was assessed with Shapiro-Wilk and D’Agostino-Pearson omnibus normality tests, ensuring the assumptions of parametric statistics approach. Comparisons between intervention and control group were conducted with unpaired t-test with Welch’s correction, when quantitative data followed normal distribution, Mann-Whitney U-test, when quantitative data violated the assumptions of parametric tests and Fisher exact test as well as Chi-square test, when categorical data was considered (statistical analyses were performed in IBM SPSS Statistics version 27, GraphPad Prism 8, and R).

2.4.2 Analysis of illumina paired-end reads

Demultiplexed 16S paired-end sequences were imported and q2-vsearch plugin was applied to join forward and reverse reads. Quality control was assessed via q-score base filtering, chimera removing and 16S-denoising with Deblur. Next, to exclude bacterial contaminations, we used the DECONTAM package in R with threshold set at 0.5, which allowed us to identify 235 contaminant amplicon sequence variants (ASVs) by evaluating the prevalence (presence/absence across samples) of each ASV in the saliva samples compared to the prevalence in the negative controls. These ASVs together with ASVs from mitochondria, chloroplasts, and cyanobacteria were removed from the dataset prior to conducting subsequent analyses. Results from previous steps were summarized in a feature table. q2-phylogeny plugin was next employed to produce a MAFFT alignment (81) of ASVs which was consequently used to construct a rooted phylogeny with FastTree2 (82) for subsequent applications.

Taxonomy assignment of ASVs was performed with q2-feature-classifier plugin (83), through classify-sklearn method with pre-trained Naïve Bayes classifier against 99%-eHOMD_v15.1 reference sequences (84) (sequences trimmed to only include 250bp of V4 region, bound by the F515/R806 primer pair).

All the subsequent analyses were performed with data upon ASVs. Differential abundance analyses were conducted with both data upon ASVs and operational taxonomic units (OTUs) at taxonomic level 6.

2.4.3 Diversity analyses

Alpha and beta diversities were estimated with q2-diversity plugin after rarefaction of samples (subsample without replacement) to 8000 sequences per sample (see annex I – Figure S1). Alpha diversity metrics used to explore the microbiota diversity within a sample (85) were Shannon and Faith's phylogenetic indexes. While Shannon index (86) is calculated based on richness (presence or absence of ASVs in participants' microbiota) and evenness (uniformity of the microbiota community based on the relative abundance of each ASV), Faith's phylogenetic index (86) is based on the richness and the phylogenetic relationship among all ASVs present in the microbiota of people with COPD. Spatial dissimilarities between bacterial communities of different time points and periods were assessed with principal coordinate analyses (PCoA) and biplots based on Weighted Unifrac distance. The Weighted Unifrac is a quantitative and phylogenetic beta diversity metric, allowing the calculation of distances among groups based on the relative abundance of each ASV and their phylogenetic relationship (87).

Therefore, the branches of a phylogenetic tree are weighted based on the relative abundance of each ASV (87).

Wilcoxon test and Friedman with Dunn’s correction were employed to compare alpha diversity among time points, periods, and groups (statistical analyses were performed in GraphPad Prism 8 and R). Differences in beta diversity between time points, periods, and groups were quantified by permutational multivariate analysis of variance (PERMANOVA).

2.4.4 Differential abundance analyses of OTUs

Targeted statistical analyses were performed to identify significant changes in the microbiota composition of patients. These analyses consisted in comparing the relative abundance of several taxa (Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella*) between time points and groups as well as in comparing the magnitude of change of the previous taxa among patients with an increase and decrease of each taxon. The relative abundant data was obtained by the feature table collapsed at genus taxonomic level (L6). To perform the mentioned analyses, we applied the Wilcoxon test and Mann-Whitney U-test (statistical analyses were performed in GraphPad Prism 8 and R).

2.4.5 Microbiota composition and clinical variables analyses

Patients from the intervention group were separated into responders and non-responders to exercise tolerance (6MWT), dyspnoea (mBS), and impact of the disease (CAT) according to the established MCIDs reported in the literature for each variable. A MCID is defined as the smallest change in a score/outcome obtained by patients after an intervention (88). The MCID usually includes patients' perception, but, in the literature, this term is also used without their perception (88).

For the 6MWT, the MCID used was 25 m (24), i.e., when performing the difference between the meters walked by each participant immediately after PR (M4) and at baseline (M1), responders were those who walked at least 25 meters or more, while the remaining participants were classified as non-responders. For the mBS, we used a MCID of 1 point (25), i.e., those who decreased at least 1 point in their level of dyspnoea in the mBS after PR compared to baseline values were classified as responders to this clinical variable, whereas maintenance or an increase of more than 1 point meant that participants did not respond to mBS. Finally, for the CAT, we used a MCID of 2 points (26), meaning that responders were classified based on a decrease of 2 or more points from baseline to immediately after PR and non-responders with maintenance or increase of more than 2 points.

The analyses with responders and non-responders were conducted to explore if the microbiota composition (Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella*) of these two groups before PR was significantly different, and, if so, explore if those differences were associated with participants' responses. Therefore, we firstly performed a Mann-Whitney U-test to execute the targeted statistical analyses (statistical analyses were performed in GraphPad Prism 8).

Univariate logistic regressions were performed to explore if the microbiota profile of patients before PR could be associated with their response to 6MWT, mBS, and CAT (statistical analyses were performed in R). These analyses were performed with the relative abundance of Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella* transformed with the base two logarithm to deal with our skewed data.

Targeted statistical analyses were also performed to compare the microbiota profile of responders and non-responders to the previous clinical variables between M1 and M4 and to compare the magnitude of changes among responders and non-responders. Thus, we performed a Wilcoxon test and Mann-Whitney U-test, respectively (statistical analyses were performed in GraphPad Prism 8).

2.4.6 Longitudinal analysis with Linear Mixed Effect models

Weighted Unifrac distances in each timepoint of both groups were subtracted to the baseline values. Loess regressions were fitted over the resulting datapoints and then plotted (ggplot2 package from R software) to observe dissimilarity tendencies during the 6 consecutive months, M1 to M6.

To assess the effect of PR on beta diversity (Weighted Unifrac distance), a linear mixed model was performed over the same datapoints obtained to fit the loess lines. Independent variables included the timepoints (6 months) and the experimental design groups. Subjects were incorporated in the model as random factors, using lmer function of the lme4 package (89) in R software. P values were gathered using the ANOVA function. Furthermore, a contrast analysis (lsmeans function from R software) with Bonferroni correction was conducted to determine differences in Weighted Unifrac means between groups in each timepoint. The same analysis was carried out to evaluate the effect of PR on alpha diversity (Shannon and Faith’s Phylogenetic indexes).

A smoothing spline analysis of variance (fitTimeSeries function from metagenomeSeq package (90) in R software) was performed to identify ASVs and OTUs that had significantly different abundances between PR and control patients, as well as the specific intervals in which those differences occurred. Raw data was filtered so that the analysis included only ASVs/OTUs that were present in ≥20% of the samples.

2.5 Inflammatory response and statistical analyses

A bead-based multiplex assay using fluorescence–encoded beads was performed to quantify cytokines in the supernatants of saliva samples from twenty-six patients from the intervention group, mainly in M1, M2, and M4. For four patients, we did not have samples available in M4, so we quantified the cytokines in the previous month (M3). The LEGENDplex™ Human Inflammation Panel 1 (13-plex) with V-bottom Plate (BioLegend, San Diego, CA, USA) was used to detect thirteen cytokines (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, and IL-33) in the supernatants. The experimental activity was conducted accordingly to the procedures provided by the manufacturer. Succinctly, 25 µL of our samples were incubated for 2 hours with the beads’ mixture. Next, a washing step was performed with wash buffer and followed by centrifugation at 1050 rpm for 5 min. The supernatant obtained was rejected and incubation of 1 hour with the detected antibodies was accomplished. Then, our samples were under another incubation for 30 min with streptavidin-phycoerythrin, a fluorescent tag. After that, a spin down of the beads and removal of the supernatant was performed. Finally, samples were resuspended in wash buffer, and we proceed to data acquisition with a flow cytometer (BD AccuriTM C6 Plus) and the respective analysis using the LEGENDplex™ Data Analysis Software

(BioLegend, San Diego, CA, USA). In this assay sixteen wells were allocated for the standards.

For some samples, it was not possible to acquire the concentrations of some cytokines since they were below the detection limit (see annex I - Table S1). Therefore, in these situations, we used the concentration obtained by the equation of the straight line. For only two samples and considering IL-1β, this cytokine concentration was above the limit of detection (see annex I - Table S1). In these cases, we used the maximum concentration detected.

To compare the levels of each cytokine (not normalised and normalised by baseline) among more than two time points, we applied the Friedman test with Dunn's correction (statistical analyses were performed in GraphPad Prism 8). Additionally, to investigate if responders had on average higher concentrations of each cytokine in M2 and M4 than non-responders, we performed a Mann-Whitney U-test to our data after logarithm transformation (statistical analyses were performed in GraphPad Prism 8). Finally, to explore potential associations between the increase of a cytokine and being a responder to 6MWT, mBS, and CAT, we applied a Fisher test and used our data normalised by the baseline (statistical analyses were performed in R).

The concentrations of SIgA and total protein present in saliva samples from eighteen patients belonging to the intervention group were also quantified. For SIgA quantification, an ELISA sandwich method was performed using the Secretory IgA (Human) ELISA kit from Abnova, while the total protein was quantified with the Pierce™ BCA Protein Assay Kit from ThermoFisher, a kit based on the bicinchoninic acid (BCA) method. For both quantifications, the experimental procedures were followed according to the manufacture guidelines.

Briefly and regarding SIgA quantification, saliva samples were firstly diluted with red EIA buffer. Then, to each well allocated for saliva, the addition of 190 µl of the previous buffer occurred. Next, 10 µl of each diluted saliva was added to each well, and all the contents were mixed and incubated for 90 minutes at 37 ºC. Afterward, three washes were performed with the washing buffer solution, and 100 µl of the conjugate was added to each saliva well. Another incubation for 30 minutes at 37 ºC was executed and followed by five washes. 100 µl of the substrate was then added, and an incubation (10 to 20 minutes at 18 to 25 ºC) was performed. At the end, 100 µl of stop solution was also added. When performing this assay, fourteen wells were reserved for the duplicates of the six calibrators and control serum. Finally, using a microplate reader (TECAN) and an appropriated software (“i-Control 1.9”), the absorbance at 450 nm on each well was measured.

Summarily and concerning total protein quantification, 20 µl of the albumin standards, saliva samples, and blanks were added to the 96-well plate. Afterward, 200 µl of Working Reagent (200 µl of BCA Reagent A and 4 µl of BCA Reagent B) were added to each well. Saliva samples were allocated to the 96-well plate randomly. After all, the 96-well plate was covered with foil paper, carefully submitted to a 30-second shake, and incubated for 30 minutes at 37 ºC. Finally, the absorbance on each well was measured at 562 nm using a microplate reader (TECAN) and an appropriated software (“i-Control 1.9”).

For 2 out of 18 patients, for which we had SIgA concentrations, we did not have baseline data, so those patients were excluded from the statistical analysis. When analysing data normalised by total protein concentration with the Friedman test, we could only include 7 out of 18 patients, given that some samples no longer existed, and the Friedman test does not handle missing values.

To compare the levels of SIgA over time we applied the Friedman test with Dunn’s correction (statistical analyses were performed in GraphPad Prism 8).

3. Results

3 Results

3.1 Cohort characterisation

Seventy-six subjects with COPD were included in this study, 38 in each group (experimental group: 29 male, 72±9y, FEV_{1pp} 49.15±16 and control group: 31 male, 70±7.6y, FEV_{1pp} 52.34±19.76). Table 1 presents in detail the general characterisation of the cohort.

Table 1 – Sociodemographic, anthropometric, and clinical characteristics of participants with chronic obstructive pulmonary disease at beginning of the study.

Participant's Characteristics	Intervention Group (n=38)	Control Group (n=38)	*P* value
Age (years), mean±SD	72±9	70±7.60	0.19[1]
Gender, n (%)			0.57[2]
Male	29 (76%)	31 (82%)	
Female	9 (24%)	7 (18%)	
BMI (Kg/m^2), mean±SD	26.03±4	26.41±4.78	0.72[3]
FEV_{1pp}, mean±SD	49.15±16	52.34±19.76	0.44[3]
Pack Years, mean±SD	42.43±43	53.01±51.15	0.27[1]
Comorbidities (CCI), mean±SD	4.24±1.36	3.76±1.26	0,10[1]
GOLD Grades, n (%)			0.22[4]
1	3 (8%)	6 (16%)	
2	14 (37%)	12 (32%)	
3	20 (53%)	15 (39%)	
4	1 (3%)	5 (13%)	
GOLD ABCD Assessment, n (%)			0.069[4]
A	8 (21%)	17 (45%)	
B	20 (53%)	12 (32%)	
C	0 (0%)	1 (3%)	
D	10 (26%)	8 (21%)	
Number of exacerbations in the last year, n (%)			0.60[2]
0 or 1	27 (71%)	29 (76%)	

≥2 or 1 with a hospitalisation	11 (29%)	9 (24%)	
Number of hospitalisations in the last year due to COPD, n (%)			0.67[4]
0	32 (84%)	34 (89%)	
1	2 (5%)	3 (8%)	
2	3 (8%)	1 (3%)	
Missing values	1 (3%)	0 (0%)	

SD: standard deviation, n (%): absolute and relative frequency, respectively; BMI: body mass index, FEV_{1pp}: forced expiratory volume in 1 second percent predicted, CCI: Charlson comorbidity index, GOLD: global initiative for chronic obstructive lung disease, [1] *p* value obtained with Mann-Whitney U-test, [2] *p* value obtained with Chi-square test, [3] *p* value obtained with Unpaired t-test with Welch's correction, [4] *p* value obtained with Fisher exact test.

3.2 Pulmonary rehabilitation modifies the salivary microbiota of people with chronic obstructive pulmonary disease

It has been extensively demonstrated that PR has multiple benefits regarding patients' symptoms and quality of life (1). However, its impact on the microbiota of people with COPD was not yet established. Therefore, the first and major question addressed in this study was if PR could modulate the salivary microbiota of patients with this chronic disease.

In order to answer the previous question, a longitudinal analysis was performed applying a linear mixed model to our data, Weighted Unifrac distances normalised to the baseline. This model was fitted as follows: WUdistances ~ time + group + time*group + (1|Subject). The results showed that the two groups varied significantly regarding beta diversity (measure of similarity or dissimilarity among groups) over time and depending on presence or absence of PR (ANOVA test, $F(5,4401.9)=17.58$, $p<0.001$). These differences were significant for all time points after the beginning of rehabilitation (Table 2).

Weighted Unifrac distances normalised to the baseline were represented with loess lines (Figure 8) to describe the dynamic of the microbiota composition in the intervention and control groups over the 6-months follow-up. The groups present different trajectories over time, which was confirmed with the previous longitudinal analysis. Regarding microbiota composition, patients undergoing PR became more homogeneous over time (decreasing tendency of Weighted Unifrac distances) (Figure 8). However, the magnitude of the observed changes was low (*η^2*=0.02). Concerning the control group, patients appeared to remain stable during the 6-months follow-up (Figure 8).

It should be noted that Weighted Unifrac distances were used in these analyses since this is the most robust measure of diversity to investigate whether two or more groups are more similar or dissimilar from each other based on their microbiota composition, including the phylogenetic relationship among ASVs (87). Besides that, Weighted Unifrac was the beta diversity metric that explained the biggest percentage of diversity of the data.

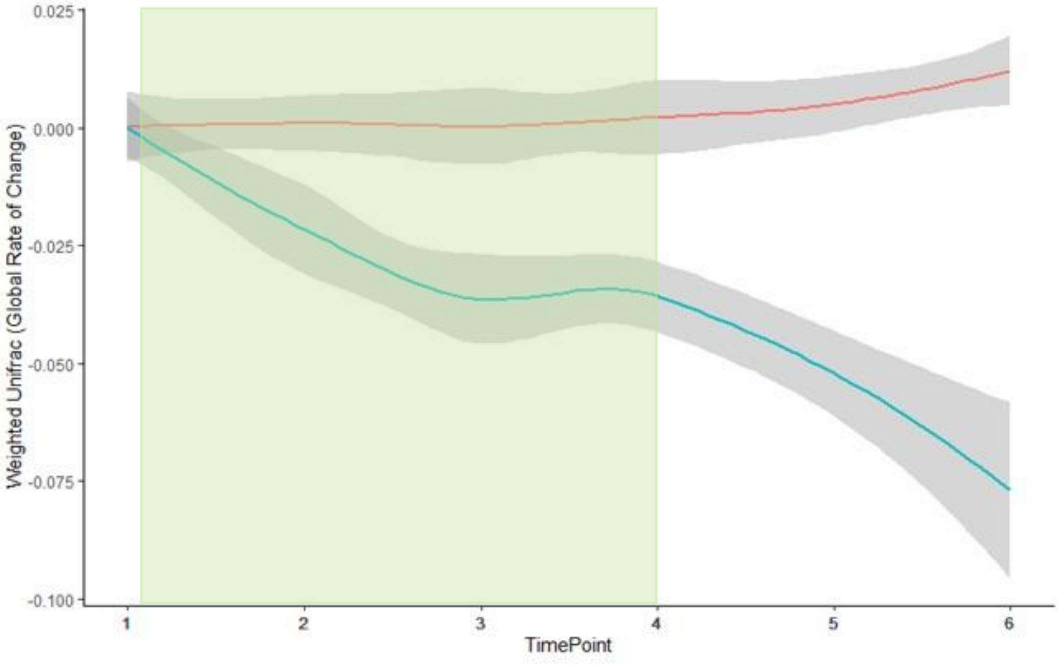


Figure 8 – Dynamics of the microbiota composition in the intervention and control groups over the 6-months follow-up. The red line represents the control group while the blue line represents the intervention group. The green rectangle includes all the time points where patients were under PR. Both groups presented significantly different trajectories (ANOVA test, F(5,4401.9)=17.58, *p*<0.001).

Table 2 – Time points where significant differences between the intervention and control groups were observed after contrast analysis.

Contrasts between Intervention and Control groups *	**Estimate**	***P* value**
M2	0.030	<0.001
M3	0.039	<0.001
M4	0.031	<0.001
M5	0.071	<0.001
M6	0.072	<0.001

*The contrasts analysis was displayed with t tests along with Bonferroni corrections.

Since the microbiota of the intervention group changed significantly with PR, we asked whether PR had induced similar changes among patients. For this we compared different time points (M1 vs M4, M1 vs M2, M1 vs M3, and M1 vs M2+M3+M4) using PERMANOVA tests based on Weighted Unifrac distances.

Firstly, we generate a PCoA plot only with data regarding M1 (immediately before PR) and M4 (immediately after PR), but no segregation of two clusters was observed (one representing the microbiota composition of patients in M1 and another in M4, as we were expecting), meaning that distances between patients in the same time point were greater than distances within patients regarding two different time points (PERMANOVA test, test statistic =1.19, p=0.30) (Figure 9). Analyses were also conducted considering M1 and M2 (after one month of PR) as well as M1 and M3 (in the middle of PR), and similar results were obtained, i.e., no clustering per timepoint (see annex I - Table S2). A PCoA including all samples from Pre and PR (M2+M3+M4) periods was also generated, but no significant differences concerning patients' microbiota composition were observed among both periods (see annex I - Table S2).

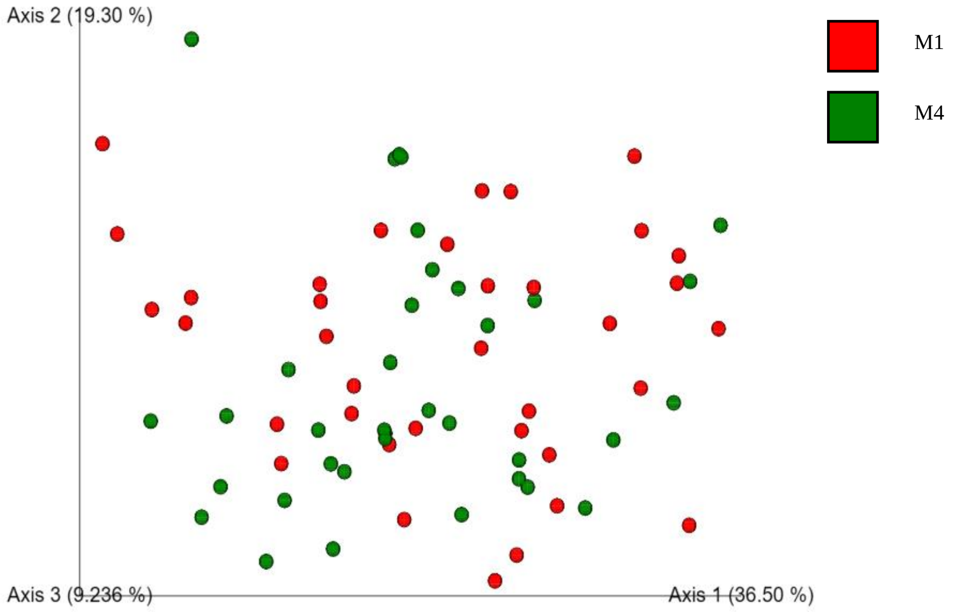


Figure 9 – Principal coordinate analysis plot based on a Weighted Unifrac distance matrix representing beta diversity of patients from the intervention group immediately before (M1) and after (M4) PR. The patients' microbiota composition in M4 was not significant different from the microbiota composition of in M1 (PERMANOVA test, test statistic=1.19, *p*=0.30).

3.3 After pulmonary rehabilitation, microbiota composition of people with chronic obstructive pulmonary disease approaches the profile of severe chronic obstructive pulmonary disease

According to the microbiota profile from healthy individuals and people with COPD using several biological samples, including saliva, are significantly different (28,29,31–35,91). While healthy individuals are characterised by a microbiota enriched in Firmicutes and Bacteroidetes, people with COPD present a microbiota enriched in Proteobacteria (28,29,31–35,91).

Since PR is beneficial to people with COPD, we hypothesised that the salivary microbiota of people with COPD could change towards the microbiota composition of healthy individuals, i.e., increasing in Bacteroidetes and decreasing in Proteobacteria as consequence of PR.

To explore the changes that might have happened in the microbiota profiles of patients under PR, we calculated the mean relative abundance of all genera for each time point, M1 to M6 (Figure 10A). The microbiota profile of people with COPD presents five predominant phyla corresponding to Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria, and Actinobacteria. The most abundant genera of each phylum were *Streptococcus*, *Prevotella*, *Haemophilus*, *Leptotrichia*, and *Rothia*, respectively. When comparing the first month immediately before PR (M1) with the three months of PR (M2, M3, M4), it seems that Firmicutes, specifically *Streptococcus*, remain stable while a decrease in Bacteroidetes, mainly *Prevotella*, and an increase in Proteobacteria, particularly in *Haemophilus,* occurred.

Since a PERMANOVA failed to detect differences between the global microbiota composition of patients before and after PR (M1 vs M4) (PERMANOVA test, test statistic=1.19, *p*=0.30), we decided to perform a targeted statistical analysis to query for the differential abundance of Proteobacteria, *Haemophilus*, Bacteroidetes, and *Prevotella*, since these were previously found to distinguish patients from healthy and different severity grades (Melo-Dias et al. (35)) and were also varying in patients during PR (Figure 10A).

Firstly, we compared the relative abundance of each phylum and genus before PR (M1) with each month representing the period of rehabilitation (M2, M3, M4). From M1 to M2, a significant increase in *Haemophilus* genus was detected (Wilcoxon test, W=321, *p*=0.015). When comparing M1 with M3, a significant increase in Proteobacteria (Wilcoxon test, W=290, *p*=0.036) and *Haemophilus* (Wilcoxon test, W=313, *p*=0.023) was found. Finally, from M1 to M4, only a significant decrease in Bacteroidetes phylum (Wilcoxon test, W=-273, *p*=0.039) was observed in patients under PR. Significant differences in relative abundance are shown in Figure 10B, non-significant results are presented in annex (see annex I - Tables S3, S4, and S5).

Then, we assessed if the changes previously observed in the intervention group could be a normal variation of patients’ microbiota or a consequence of PR. Therefore, we performed a similar analysis to the one conducted in the intervention group. Firstly, we considered all the possibilities of 1-month intervals in the control group (M1 vs M2, M2 vs M3, M3 vs M4, M4 vs M5, M5 vs M6) and tried to see if the increase in *Haemophilus* in the intervention group from M1 to M2 was a normal variation of patients’ microbiota composition. No significant differences regarding the *Haemophilus* genus were found (see annex I - Table S6). Next, we considered all the possibilities of

2-months intervals in the control group (M1 vs M3, M2 vs M4, M3 vs M5, M4 vs M6) and assessed if the increase in Proteobacteria and *Haemophilus*, from M1 to M3, were a normal variation of patients' microbiota or not. Once more, no significant increases in Proteobacteria and *Haemophilus* relative abundance were found in the control group (see annex I - Table S7). Finally, we considered all the possibilities of 3-months intervals (M1 vs M4, M2 vs M5, M3 vs M6) in the control group and investigate if the significant decrease of Bacteroidetes observed in the intervention group, from M1 to M4, was also occurring in patients from the control. According to the results obtained, no significant differences in Bacteroidetes relative abundance were observed in the control group and for all the 3-months intervals (see annex I - Table S8).

Furthermore, we have also explored if the magnitude of change of Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella* relative abundances between each time point of PR (M2, M3, M4) and the baseline (M1) differed between patients with an increase and decrease of the mentioned phyla and genera. For these analyses, we calculated the module of the difference of the relative abundance of Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella* from each time point of PR and baseline. Then, we compared the groups with an increase and decrease of each taxon. When normalising the relative abundance of all taxa from M2 with the baseline (|M2-M1|), no significant differences were observed (see annex I - Table S9). However, when we normalised the relative abundance of *Haemophilus* from M3 with M1, we found that the magnitude of change was significantly higher in patients with an increase in this genus. (Mann-Whitney U-test, U=94, *p*=0.019). Besides that, when we performed the same analysis but considering the relative abundance of Proteobacteria in M4 and normalised by M1, we observed that the magnitude of change was significantly higher in patients with an increase in this phylum (Mann-Whitney U-test, U=63, *p*=0.00070). Considering the same time interval, it was also found that the magnitude of change in patients with a decrease in Bacteroidetes and *Prevotella* was significantly higher than the observed in patients with a decrease of these phylum and genus (Mann-Whitney U-test, Bacteroidetes: U=95, *p*=0.030, *Prevotella*: U=84, *p*=0.013). All significant results are presented in Figure 10C, while the remaining can be observed in the annexes (see annex I - Tables S9, S10, and S11).

With the purpose of assessing if the magnitudes of change previously observed in the intervention group could be a normal variation of patients' microbiota, we performed a similar analysis but with patients from the control group and all possible time intervals where the significant differences were detected in the intervention group. Firstly, we considered all possibilities of 2-months intervals (|M3-M1|, |M4-M2|, |M5-M3|, |M6-M4|) and evaluated if a higher and significant difference in the magnitude of change of *Haemophilus* in control patients with an increase of this genus was observed as it happens in the intervention group. No significant differences regarding the *Haemophilus* genus were found (see annex I - Table S12). Then, we considered all possibilities of 3-months intervals (|M4-M1|, |M5-M2|, |M6-M3|) and attempted to see if Proteobacteria, Bacteroidetes, and *Prevotella* magnitude of change observed in the intervention group among patients with an increase and decrease of those taxa was also seen in the control patients. No significant differences regarding these taxa were found (see annex I - Table S13).

The previous results suggest that with PR, people with COPD acquire a microbiota profile more similar to the profile characteristic of more severe COPD, i.e., microbiota enriched in Proteobacteria, mainly *Haemophilus*, and depleted in Bacteroidetes, specifically, *Prevotella*.

3. Results

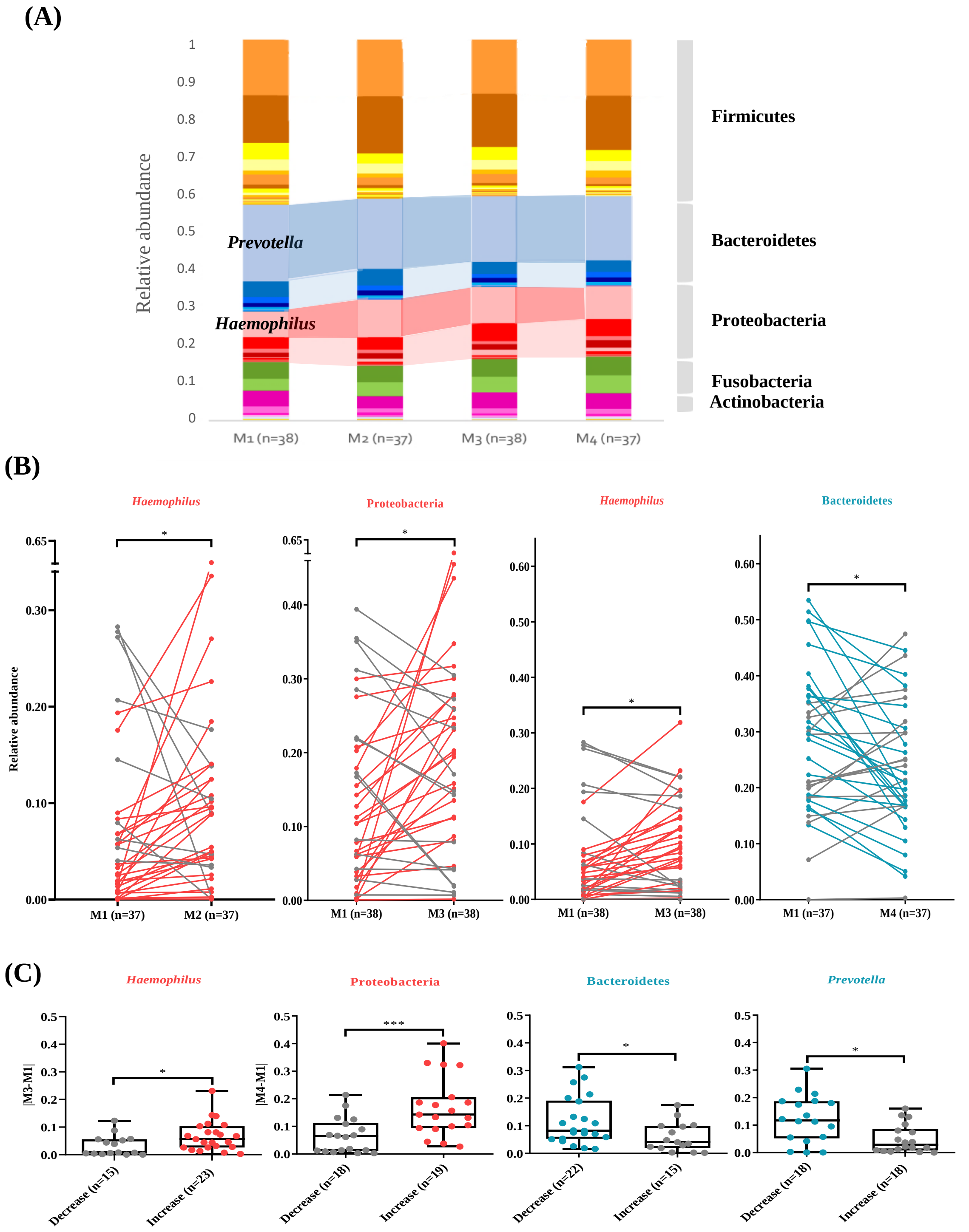


Figure 10 – Microbiota composition of patients from the intervention group. **(A)** Microbiota profile of patients over the first four months of follow-up. **(B)** Significant changes detected in patients' microbiota composition during PR. A significant increase in *Haemophilus* during M2 was observed (Wilcoxon test, W=321, p=0.015) as well as a significant increase of this genus and Proteobacteria phylum during M3 (Wilcoxon test, *Haemophilus*: W=313, p=0.023; Proteobacteria: W=290, p=0.036). Immediately after PR (M4), a significant decrease in

Bacteroidetes was also found (Wilcoxon test, W=-273, p=0.039). **(C)** Significant results obtained with the module of the magnitude of change in the relative frequency of *Haemophilus*, Proteobacteria, Bacteroidetes, and *Prevotella*. A higher and significant magnitude of change regarding *Haemophilus* (Mann-Whitney U-test, U=94, *p*=0.019), between M3 and M1, and Proteobacteria (Mann-Whitney U-test, U=63, *p*=0.00070), between M4 and M1, was detected. Moreover, a lower and significant magnitude of change between M4 and M1 and concerning Bacteroidetes (Mann-Whitney U-test, U=95, *p*=0.030) and *Prevotella* (Mann-Whitney U-test, U=84, *p*=0.013) was observed. **p*<0.05, ***p*<0.01, ****p*<0.001

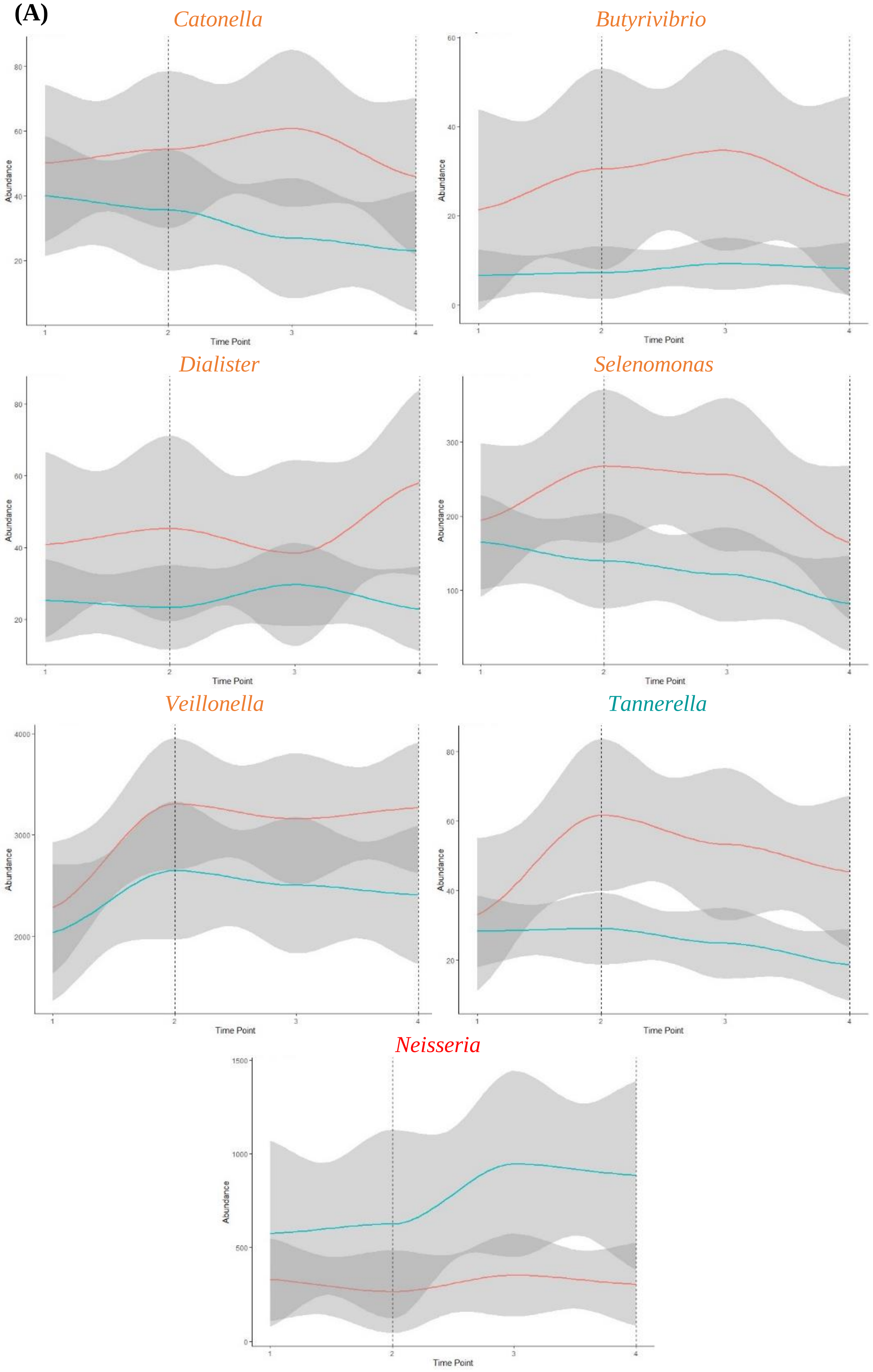
Furthermore, we also performed a smoothing spline analysis of variance (longitudinal) to explore which OTUs and ASVs were differentially abundant between the intervention and control group during PR.

Regarding the OTUs (Figure 11), we found nine differentially abundant OTUs between the two groups, seven of them from M2 to M4 comparison and two from M3 to M4 comparison. Regarding the seven differentially abundant OTUs between M2 to M4, we observed that the majority (*Catonella*, *Butyrivibrio*, *Dialister*, *Selenomonas*, *Veillonella*, *Tannerella*) was significantly abundant in the control group whereas *Neisseria* genus was significantly abundant in the intervention group. These results suggest that the control seemed to have a microbiota significantly enriched in genera from Firmicutes and Bacteroidetes phyla in comparison to the intervention group which presented a significant enrichment of a genus belonging to the Proteobacteria phylum. Moreover, it was also observed that, from M3 to M4, patients from the control were enriched in *Porphyromonas*, a genus from the Bacteroidetes phylum, and in [*Prevotella*].

Concerning the AVSs (Figure 12), we observed that six ASVs from Firmicutes and Bacteroidetes phyla (*Mogibacterium*, two ASVs of *Prevotella*, *Leptotrichia*, *Actinomyces*, and *Corynebacterium Durum*) were significantly enriched in the control group from M2 to M4 and an additional ASV of *Prevotella* was also enriched in the control group from M3 to M4. In the intervention group, we were only able to detect an ASV of *Prevotella* (different from the above mentioned) increased in M3 to M4 comparison. See annex I – Table S14 to observe the taxonomic annotation of all ASVs.

3. Results

(A)


Catonella
Butyrivibrio
Dialister
Selenomonas
Veillonella
Tannerella
Neisseria
Abundance
Time Point
1
2
3
4
20
40
60
80
0
100
200
300
2000
3000
4000
500
1000
1500

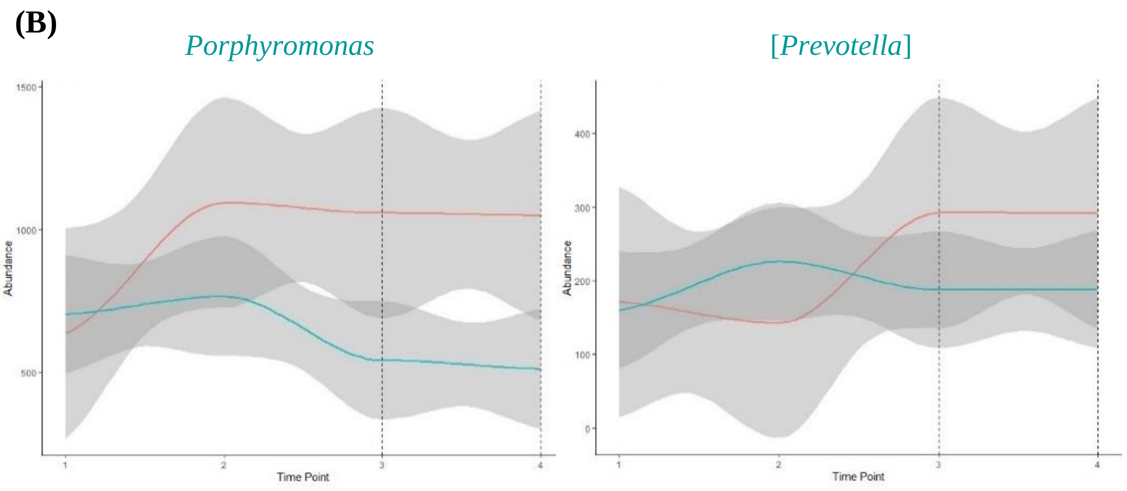
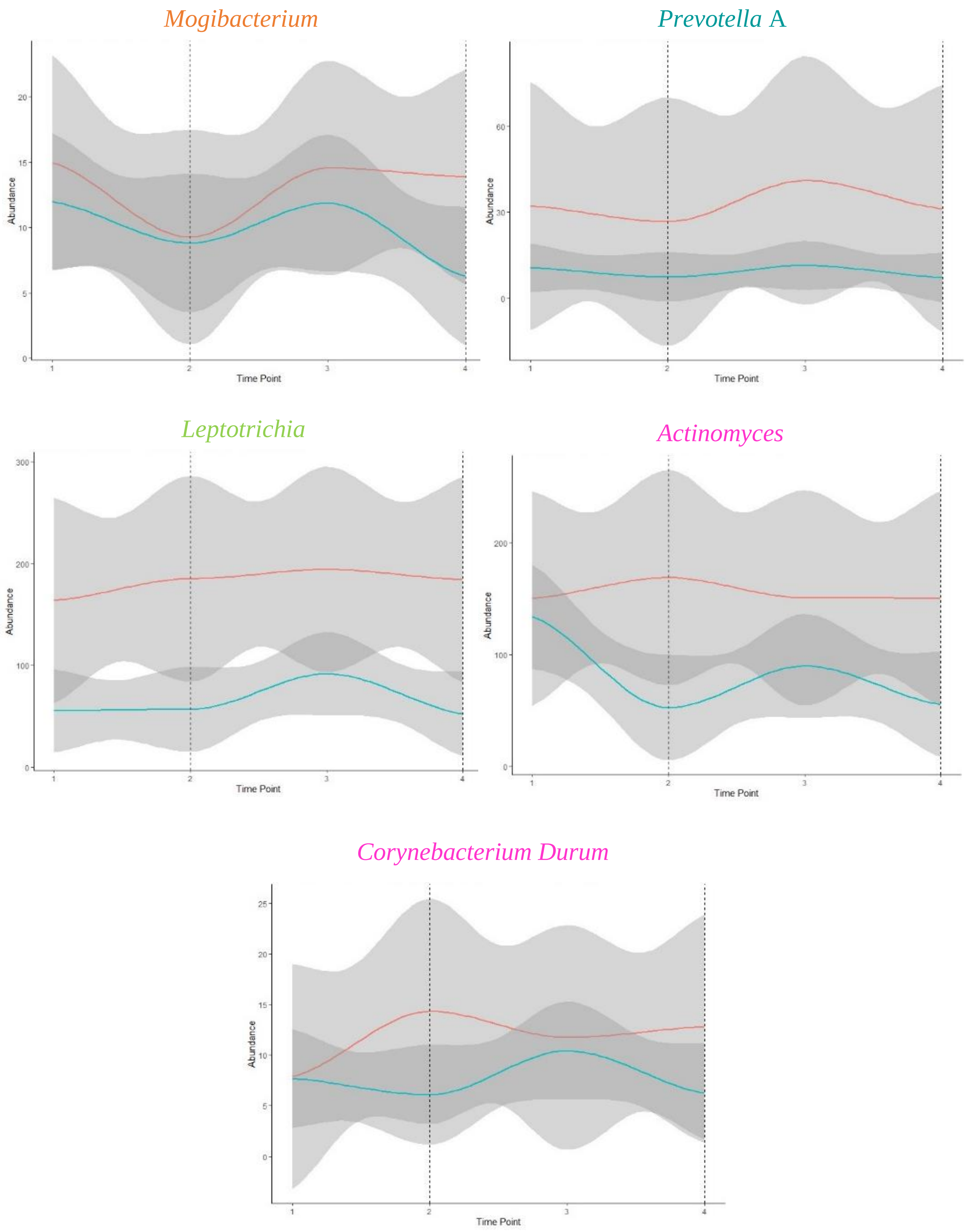


Figure 11 – Differentially abundant OTUs among the intervention and control groups during the PR period, **(A)** M2 to M4 comparison and **(B)** M3 to M4 comparison. The red line and dots represent the control group, while the light blue line and dots represent the intervention group. The genera in orange belong to the Firmicutes phylum, while the genera in blue belong to the Bacteroidetes phylum and in red to the Proteobacteria phylum. The grey areas correspond to the 95% confidence interval.

3. Results

(A)


Mogibacterium
Prevotella A
Leptotrichia
Actinomyces
Corynebacterium Durum
Abundance
Time Point
0
5
10
15
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30
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200
300
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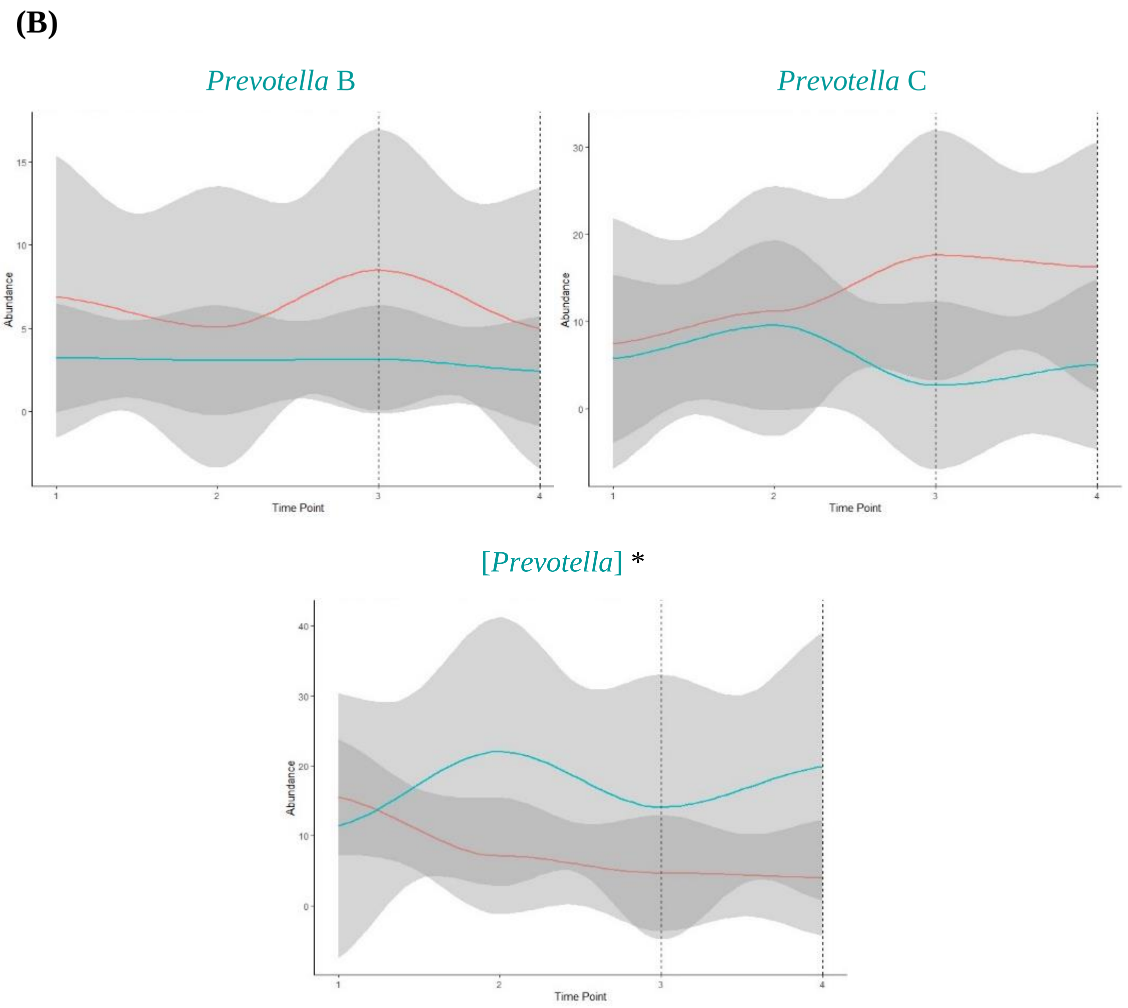

Figure 12 – Differentially abundant ASVs among the intervention and control groups during the PR period. **(A)** The differentially abundant ASVs from M2 to M4 are: *Mogibacterium*, *Prevotella* A, *Leptotrichia*, *Actinomyces*, and *Corynebacterium Durum*. **(B)** The differentially abundant ASVs from M3 to M4 are: *Prevotella* B and C, and [*Prevotella*]. The red line and dots represent the control group, while the light blue line and dots represent the intervention group. The genera in orange, blue, green, pink, and red belong, respectively, to the Firmicutes, Bacteroidetes, Fusobacteria, Actinobacteria, and Proteobacteria phyla. The grey areas correspond to the 95% confidence interval. * The square brackets indicate that the taxonomic annotation of the ASV in question has not yet been revised and confirmed as a *Prevotella* ASV.

Results obtained with the longitudinal analyses mainly suggest that, during PR, patients from the intervention group are depleted in several OTUs and ASVs from the Firmicutes and Bacteroidetes phyla in comparison with the control group. Besides that, the intervention group is enriched in *Neisseria*, a genus from Proteobacteria, as well as in an ASV suggested has being *Prevotella*.

3.4 Pulmonary rehabilitation does not seem to change microbiota diversity of people with chronic obstructive pulmonary disease

According to previous results from the team (35), people with COPD seem to have a lower salivary microbiota diversity in comparison with healthy individuals. Besides, during exercise, some studies (92–94) have shown that gut microbiota diversity increase in athletes. Therefore, we hypothesised that, with PR, an increase in alpha diversity might occur.

To explore whether alpha diversity was being affected by PR, we performed two different approaches: longitudinal and cross-sectional analyses (by time point).

Similar to what was done in section 3.2., a linear mixed model was applied to alpha diversity metrics normalised to baseline. The models were fitted as follows for Shannon and Faith's phylogenetic index: Shannon_diversity ~ time + group + time*group + (1|Subject) and Faith_diversity ~ time + group + time*group + (1|Subject), respectively. Loess lines plots were also produced for each metric (Figure 13A). No significant differences were observed between intervention and control group (ANOVA test, Shannon index: F(5,308.20)=0.84, *p*=0.52; Faith's phylogenetic index: F(5,307.97=0.13, *p*=0.99), suggesting that PR does not have an effect in alpha diversity.

For the cross-sectional analyses, we calculated the mean rank of alpha diversity values for M1 to M4, and then compared them with each other to see if significant differences were detected. No significant differences were found in the microbiota diversity during PR (Friedman test, Shannon index: Friedman statistic=0.091, *p*=0.99; Faith's phylogenetic index: Friedman statistic=1.4, *p*=0.71) (Figure 13B). Besides that, the mean rank of alpha diversity values for the Pre and PR periods was also calculated and compared with each other, but no significant differences were detected (see annex I - Table S15). Overall, the results suggest that alpha diversity does not seem to be modulated by PR.

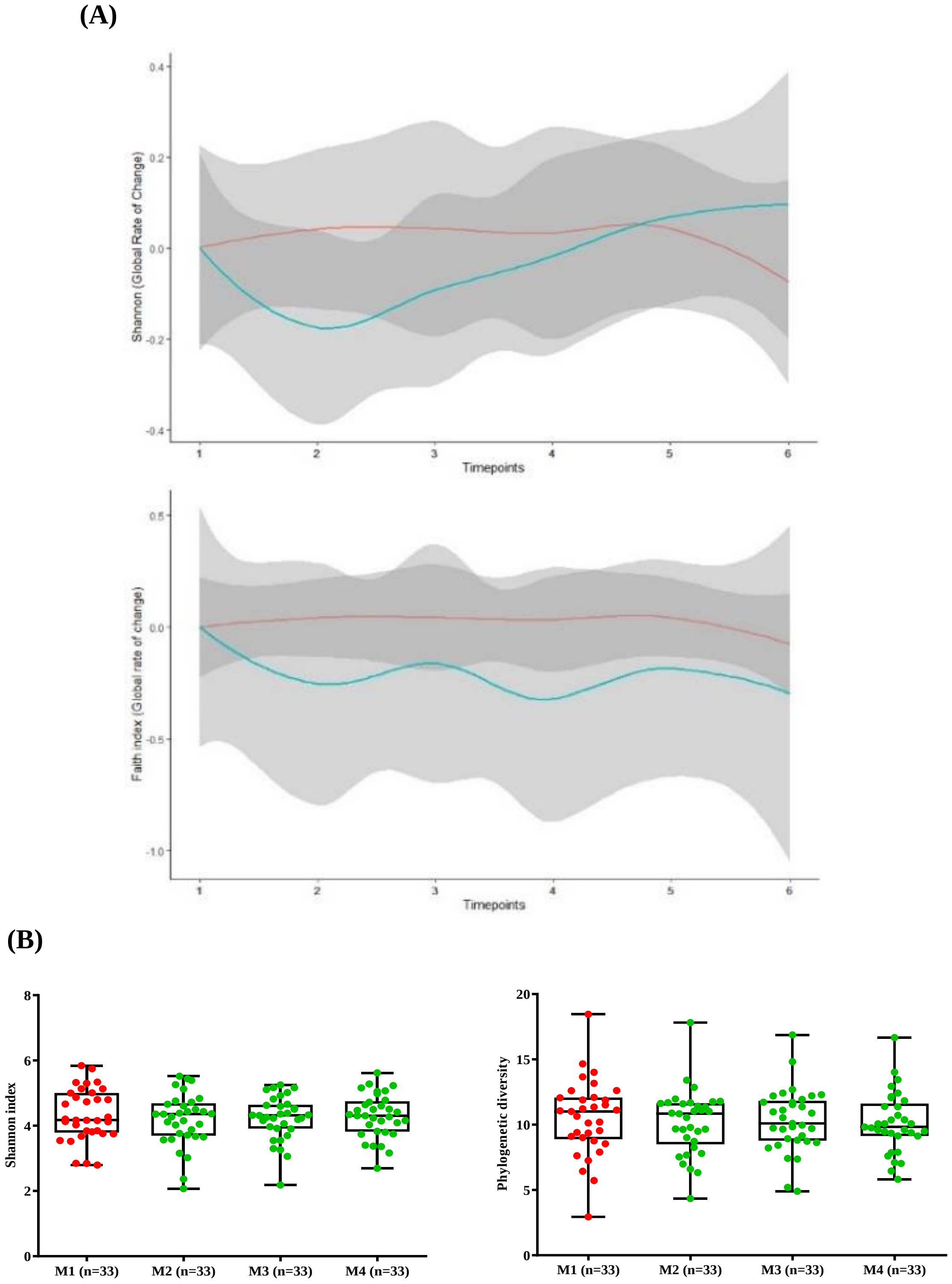

Figure 13 – Microbiota diversity within people with COPD. **(A)** Variation of alpha diversity in the intervention and control groups over the 6-months follow-up. No significant differences among both groups were observed (ANOVA test, Shannon index: F(5,308.20)=0.84, *p*=0.52; Faith's phylogenetic index: F(5,307.97)=0.13, *p*=0.99). **(B)** Behaviour of alpha diversity in the

intervention group from M1 to M4. No significant differences regarding alpha diversity were observed during PR (Friedman test, Shannon index: Friedman statistic=0.091, *p*=0.99; Faith's phylogenetic index: Friedman statistic=1.4, *p*=0.71).

3.5 Differences in microbiota profiles of responders and non-responders before pulmonary rehabilitation

Not all people with COPD respond to PR. Besides that, there is not a global criterion to determine if a patient respond or not to rehabilitation; therefore, the same patient can be classified either as responder and non-responder depending on a specific clinical variable, such as the 6MWT, the mBS, and the CAT, accordingly to the MCID established in the literature for each domain. After studying the effect of PR in patients' microbiota, we wanted to know whether microbiota composition could influence the success of PR. Therefore, we started by characterising differences in microbiota between responders and non-responders immediately before engaging in PR.

Number of participants who responded to the 6MWT and to the CAT was similar, nevertheless these were not all the same individuals. The same applies to the non-responders for these two clinical variables. This situation is considered normal since COPD is a heterogeneous disease and, therefore, is manifest in different ways among patients. Only 9 patients responded to mBS.

To have an idea of responders and non-responders' microbiota profiles in M1, we calculated, for both groups and each clinical variable, the mean relative abundance of all genera presented in patients' microbiota (Figure 14).

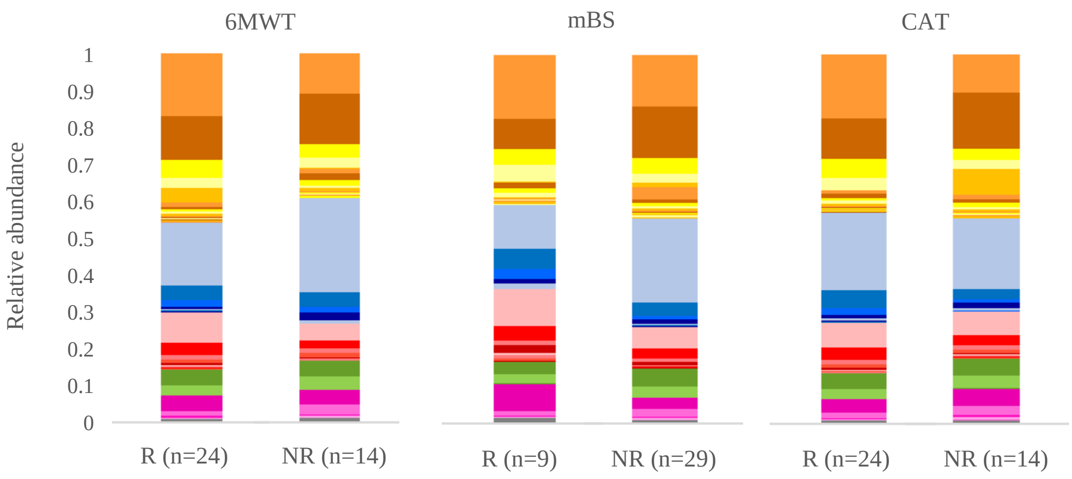

Figure 14 – Microbiota profiles of responders and non-responders before PR and considering the 6MWT, the mBS, and the CAT.

In terms of the global microbiota composition, we only observed a significant difference between responders and non-responders for mBS (PERMANOVA test, test statistic=2.62, *p*=0.037) (Figure 15). Results obtained with the 6MWT and the CAT are reported in annex I - Table S16. Since this approach evaluates only the global microbiota, changes regarding a few OTUs might not be seen. Therefore, we proceed with a targeted approach similar to the one described in section 3.3, but comparing the microbiota composition of responders and non-responders before PR.

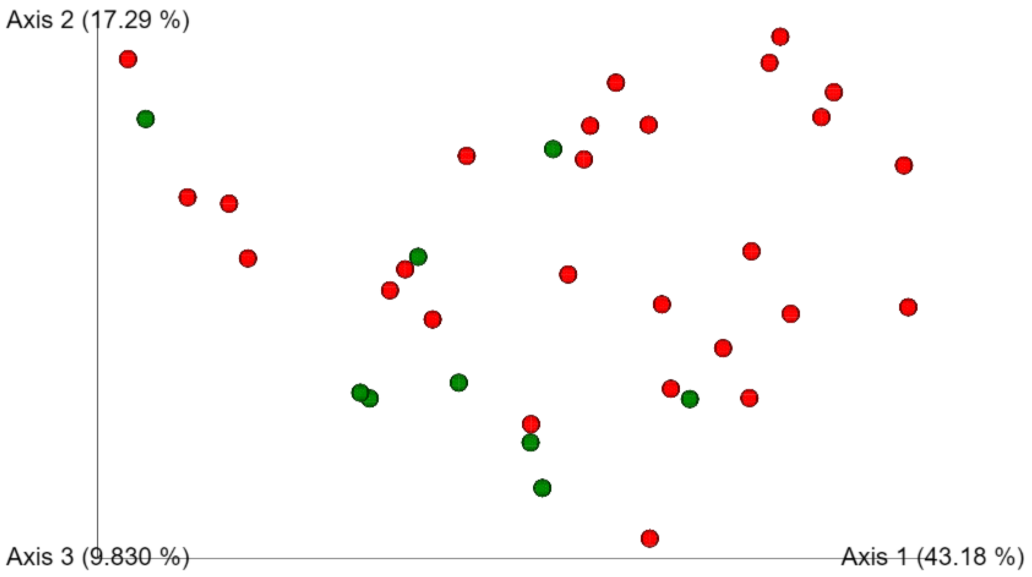

Figure 15 – Principal coordinate analysis plot based on a Weighted Unifrac distance matrix representing beta diversity of responders (green dots) and non-responders (red dots) to mBS before PR. Patients' microbiota composition in M1 was significantly different among responders and non-responders to mBS (PERMANOVA test, test statistic=2.62, *p*=0.037).

For the 6MWT and the mBS, before PR, responders present a microbiota profile enriched in Proteobacteria, mainly *Haemophilus*, and depleted in Bacteroidetes, mainly *Prevotella*, in comparison with the microbiota profile of non-responders (Figure 14). From these, only Proteobacteria (Mann-Whitney U-test, U=68, *p*=0.040) and *Haemophilus* (Mann-Whitney U-test, U=66, *p*=0.033) were significantly enriched and *Prevotella* (Mann-Whitney U-test, U=69, *p*=0.044) was significantly depleted in responders to mBS (Figure 16).

The same was not observed regarding responders and non-responders to the CAT, where the differences between responders and non-responders are slight and follow the opposite trend. Responders to the CAT were significantly enriched in *Prevotella* (Mann-Whitney U-test, U=96, *p*=0.042) (Figure 16). Table S17, S18, and S19 in annex I summarise the non-significant results obtained for 6MWT, mBS, and CAT.

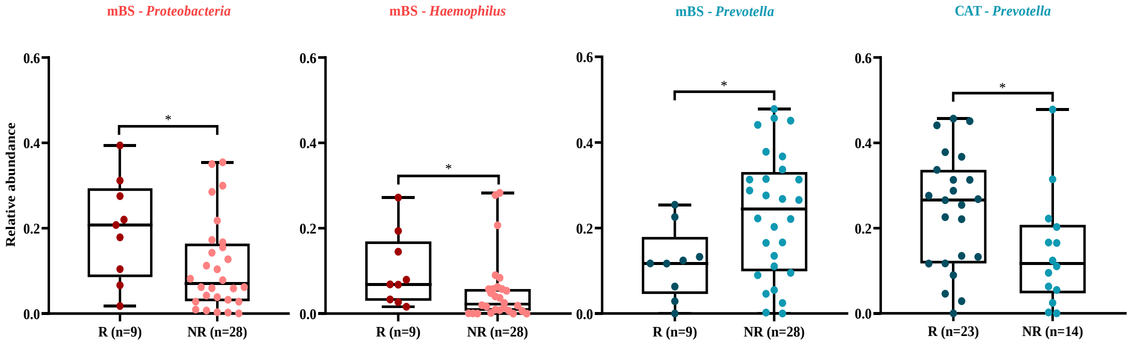

Figure 16 – Significant changes detected in the microbiota composition of patients when comparing responders with non-responders. Regarding mBS, responders' microbiota composition is enriched in Proteobacteria and *Haemophilus* and depleted in *Prevotella* (Mann-Whitney U-test, Proteobacteria: U=68, *p*=0.040, *Haemophilus*: U=66, *p*=0.033, *Prevotella*: U=69, *p*=0.044). *Prevotella* is also significantly increased in responders when considering CAT (Mann-Whitney U-test, U=96, *p*=0.042). *p<0.05, **p<0.01, ***p<0.001

3.5.1 The response to pulmonary rehabilitation is associated with the microbiota profile at baseline

Since responders and non-responders seem to be significantly different before PR regarding their microbiota composition, we then explored if there was an association between patients' microbiota profile (Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella*) in M1 and their response to the clinical domains with univariate logistic regressions.

Proteobacteria and *Haemophilus* relative abundances were identified as good predictors of mBS response (Proteobacteria: AUC=84%; *Haemophilus*: AUC=86%) while the relative frequency of *Prevotella* was pointed as a good predictor of response to CAT (AUC=76%) (Tables 3 and 4, Figure 17). Meaning that an enrichment in Proteobacteria and *Haemophilus* before PR was associated with a positive response to dyspnoea during exercise (Proteobacteria: odd ratio (OR) = 3.6, IC 95% 1.3–17.6; *Haemophilus*: OR = 2.5, IC 95% 1.2–7.6) whereas an enrichment of *Prevotella* was associated with a positive response to CAT (OR = 1.9, IC 95% 1.2–4.2).

Tables 3 and 4 – Results obtained for logistic regression models for mBS and CAT based on Proteobacteria, *Haemophilus*, and *Prevotella*.

Model	Coefficients	*p* value	x^2	df	*p* value	AIC	OR (IC 95%)
A	Proteobacteria	0.0488	7.9	1	0.005	28.1	3.6 (1.3; 17.6)
B	*Haemophilus*	0.0457	7.5	1	0.006	24.9	2.5 (1.2; 7.6)
C	*Prevotella*	0.0497	7.6	1	0.006	42.3	1.9 (1.2; 4.2)

Model	Coefficients	AUC	ROC cut-off	Specificity	Sensitivity
A	Proteobacteria	84%	0.23	79%	83%
B	*Haemophilus*	86%	0.14	79%	100%
C	*Prevotella*	76%	0.77	58%	92%

A: Model of response to mBS and $\log_2$ of Proteobacteria relative frequency; B: Model of response to mBS and $\log_2$ of *Haemophilus* relative frequency; C: Model of response to CAT and $\log_2$ of *Prevotella* relative frequency; AIC: Akaike Information Criterion; OR: Odds Ratio; IC 95%: 95% Interval of Confidence; AUC: Area Under the ROC curve; ROC: Receiver Operating Characteristic Curve.

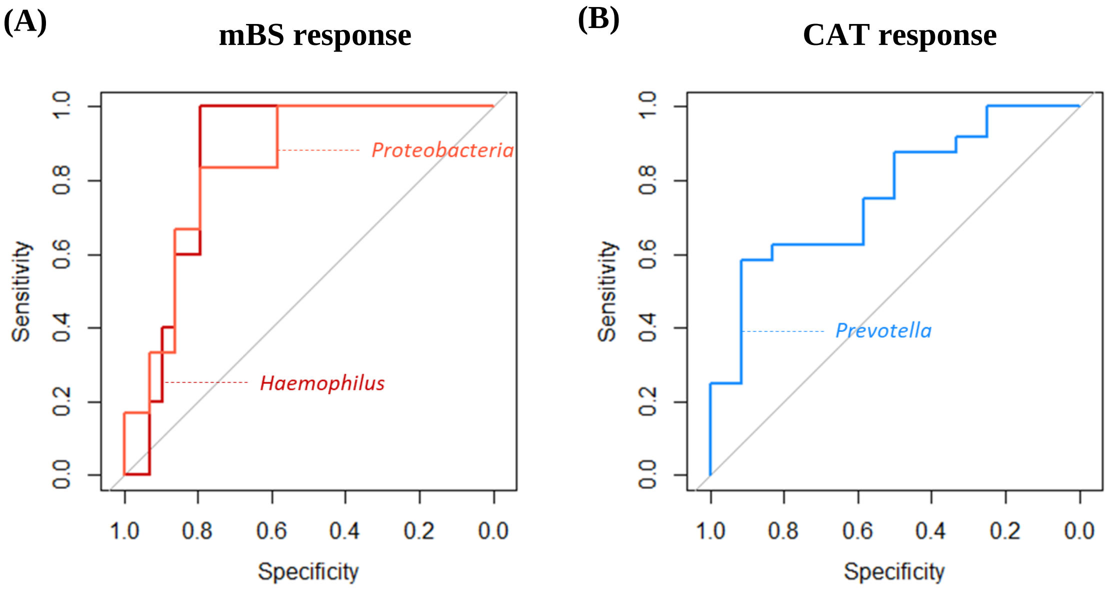

Figure 17 – ROC analysis of response prediction based on logistic regression models. **(A)** ROC curves of mBS response prediction based on Proteobacteria and *Haemophilus* relative frequencies. The light red curve corresponds to the prediction based on Proteobacteria relative frequency (AUC = 84%), while the dark red curve represents the prediction based on *Haemophilus* relative frequency (AUC = 86%). **(B)** ROC curves of CAT response prediction based on *Prevotella* relative frequency. The light blue curve corresponds to the prediction based on *Prevotella* relative frequency (AUC = 76%).

3.5.2 Pulmonary rehabilitation changes differentially the microbiota of responders and non-responders to the modified Borg scale and to the COPD assessment test

Next, we investigated if the microbiota (particularly, Proteobacteria, Bacteroidetes, *Haemophilus*, and *Prevotella*) of responders and non-responders to the 6MWT, the mBS, and the CAT changed significantly with PR (from M1 to M4) and if those changes differed among responders and non-responders. For the 6MWT, no significant differences from M1 to M4 were observed (see annex I – Table S20). For the mBS, we detected in non-responders an increase in *Haemophilus* (Wilcoxon test, W=210, *p*=0.016) and a decrease in Bacteroidetes (Wilcoxon test, W=-178, *p*=0.043) immediately after PR, whereas responders did not change significantly (Figure 18A and see annex I – Table S21). Finally, we observed an increase in *Haemophilus* (Wilcoxon test, W=138, *p*=0.035) and a decrease in Bacteroidetes (Wilcoxon test, W=-164, *p*=0.011) in responders to CAT while non-responders did not change significantly (Figure 18B and see annex I – Table S22).

Additionally, we also explored if the magnitude of change among responders and non-responders to each clinical variable differed significantly. No significant differences were observed (see annex I – Table S23), suggesting that the magnitude of change among responders and non-responders is similar.

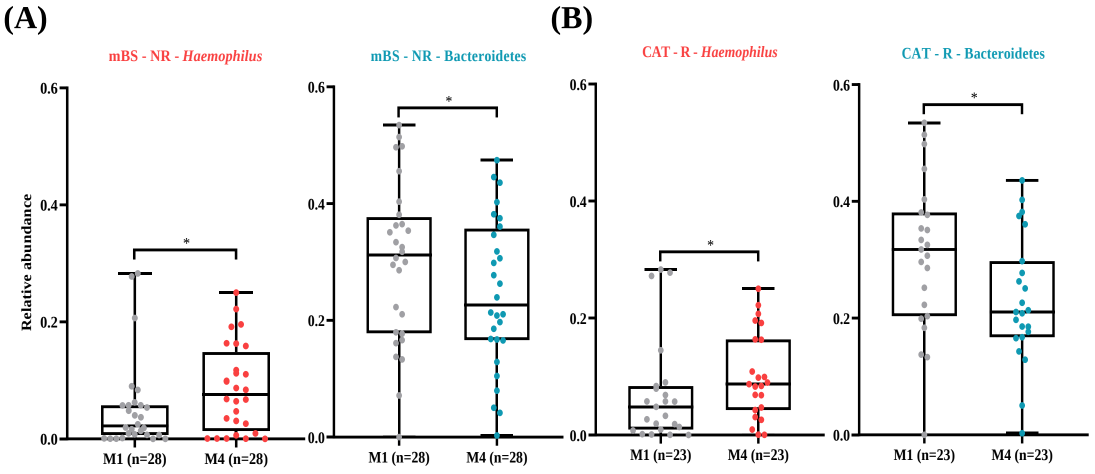

Figure 18 – Variation of relative abundance of *Haemophilus* and Bacteroidetes from M1 to M4 in non-responders to mBS **(A)** and in responders to CAT **(B)**. An increase in *Haemophilus* was observed in non-responders to mBS (Wilcoxon test, W=210, *p*=0.016) and in responders to CAT (Wilcoxon test, W=138, *p*=0.035) as well as a decrease in Bacteroidetes (Wilcoxon test – non-responders to mBS: W=-178, *p*=0.043 and R to CAT: W=-164, *p*=0.011).

3.6 Alterations in inflammatory profile in response to pulmonary rehabilitation

To study if PR altered the inflammatory profile of people with COPD and assess potential acute (after 4 weeks, M2) and chronic (after 12 weeks, M4) effects of exercise training, we quantified fourteen inflammatory mediators (IL-1β, IFN-α2, IFN-γ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33, and SIgA).

3. Results

We started by observing the tendencies of each inflammatory mediator over time. Overall, 10 of the 13 cytokines tend to increase in M2 and decrease from M2 to M4 but remain (in M4) slightly higher than M1 (see annex I – Figure S2).

From these, statistically significant differences were an increase of IL-1β at M2 when compared to the baseline (Friedman test, Friedman statistic=6.38, p=0.041; Dunn's multiple comparisons, M1 vs M2, p=0.041) and an increase of TNF-α at M4 (Friedman test, Friedman statistic=6.38, p=0.041; Dunn's multiple comparisons, M1 vs M4, p=0.041) (Figure 19A). Regarding IL-10, the Friedman test suggest that this cytokine varied significantly over time (Friedman test, Friedman statistic=7.14, *p*=0.028) with a significant increase from M1 to M2 and M1 to M4 (Dunn's multiple comparisons, M1 vs M2 and M1 vs M4, *p*=0.041). Non-significant results obtained for the remain cytokines can be seen in annex I – Table S24.

When normalising the concentration of cytokines by the baseline (ratio), the same significant differences were detected (Friedman test, IL-1β: Friedman statistic=6.38, *p*=0.041; Dunn's multiple comparisons, M1 vs M2, *p*=0.041; TNF-α: Friedman statistic=6.38, *p*=0.041; Dunn's multiple comparisons, M1 vs M4, *p*=0.041; IL-10: Friedman statistic=7.14, *p*=0.028; Dunn's multiple comparisons, M1 vs M2 and M1 vs M4, *p*=0.041) (Figure 19B). Non-significant results obtained for the remain cytokines can be seen in annex I – Table S24.

These results suggest that an inflammatory response to PR seems to occur. This response is firstly characterised by an increase in IL-1β (a pro-inflammatory cytokine) and then an increase in TNF-α (also a pro-inflammatory cytokine).

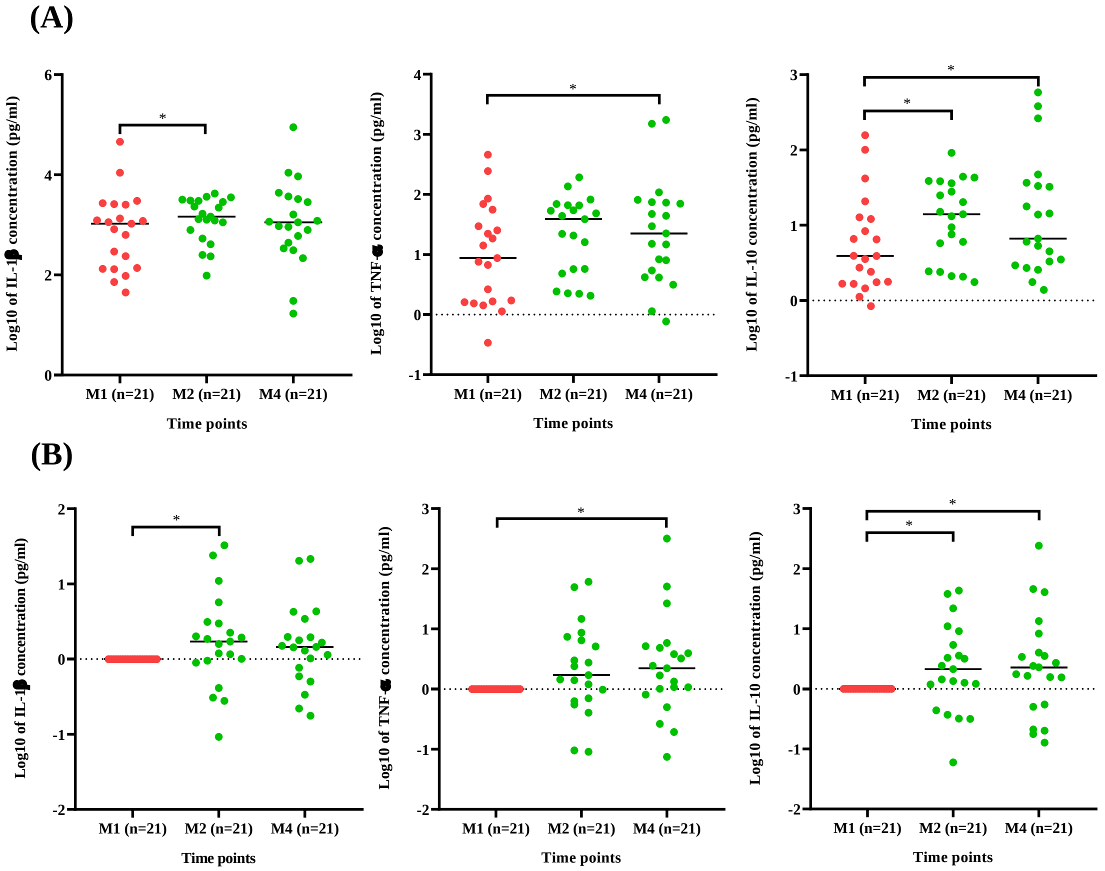

Figure 19 – Variation of IL-1β, TNF-α, and IL-10 concentrations (pg/ml) over time. **(A)** Concentration of each cytokine not normalised. A significant increase, from M1 to M2, was observed in IL-1β (Friedman test, Friedman statistic=6.38, *p*=0.041; Dunn's multiple comparisons, M1 vs M2, *p*=0.041). Further, a significant increase, from M1 to M4, was detected in TNF-α (Friedman test, Friedman statistic=6.38, *p*=0.041; Dunn's multiple comparisons, M1 vs M4, *p*=0.041). A significant increase in IL-10 was also detected from M1 to M2 and M1 to M4 (Friedman test, Friedman statistic=7.14, *p*=0.028; Dunn's multiple comparisons, M1 vs M2 and M1 vs M4, *p*=0.041). **(B)** Concentration of each cytokine normalised by the baseline concentration (ratio). A significant increase, from M1 to M2, was observed in IL-1β (Friedman test, Friedman statistic=6.38, *p*=0.041; Dunn's multiple comparisons, M1 vs M2, *p*=0.041). Additionally, a significant increase, from M1 to M4, was detected in TNF-α (Friedman test, Friedman statistic=6.38, *p*=0.041; Dunn's multiple comparisons, M1 vs M4, *p*=0.041). A significant increase in IL-10 was also detected from M1 to M2 and M1 to M4 (Friedman test, Friedman statistic=7.14, *p*=0.028; Dunn's multiple comparisons, M1 vs M2 and M1 vs M4, *p*=0.041). *p<0.05, **p<0.01, ***p<0.001

Regarding SIgA, there is evidence (71,72) that an increase in SIgA in elderly people occurs after exercise training. Therefore, we explored the effect of PR in the levels

SIgA.

To assess the behaviour of SIgA before and during PR, we calculated and compared the mean rank of SIgA concentration in all four-time points, M1 to M4. In this analysis we normalised the values by total protein concentration detected in each saliva sample since other salivary proteins might influence SIgA concentration and those concentrations differ among patients. Only 18 out of 38 patients from the intervention group were analysed.

No significant differences were observed in the levels of SIgA not normalised (Figure 20A) and normalised by total protein (Figure 20B) during and immediately after PR (Friedman test: Not normalised – Friedman statistic=0.83, *p*=0.84, and Normalised – Friedman statistic=1.80, *p*=0.61). Therefore, in our data the levels of SIgA were not modulated by PR.

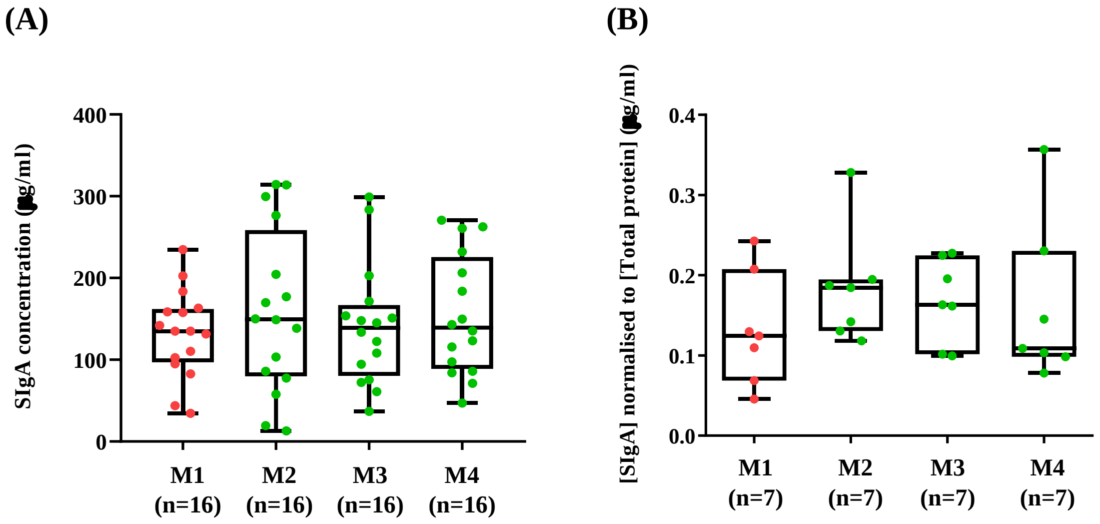

Figure 20 – Variation in SIgA concentration before (M1) and during (M2, M3, M4) PR when non-normalising SIgA concentrations **(A)** and when normalising SIgA concentrations by total protein concentrations **(B)**. No significant differences were detected (Friedman test: (A) – Friedman statistic=0.83, *p*=0.84 and (B) – Friedman statistic=1.80, *p*=0.61).

4 Discussion and Conclusions

In the current study, we observed that PR alters the microbiota composition of people with COPD, towards a microbiota profile of more severe patients, with increased Proteobacteria and decreased Bacteroidetes. Besides that, we did not observe significant differences in alpha diversity with PR. Furthermore, the microbiota dynamics of responders and non-responders to the mBS and the CAT, when considering each domain or the same response among both domains, is different. Moreover, we observed that, prior to PR, patients' microbiota composition was significantly associated with the response to mBS and CAT. Higher frequencies of Proteobacteria and *Haemophilus* in M1 were associated with response to the mBS while higher frequencies of *Prevotella* were associated with response to the CAT. Finally, regarding the inflammatory response, our results suggest that during PR an increase of some inflammatory mediators occurs.

The main goal of the present study was to explore if PR had an impact on patients' microbiota and inflammatory profiles. Our results suggest that PR modulates the microbiota composition of people with COPD, although the effects were of low magnitude. We observed a decrease in microbiota beta diversity of the intervention group, which suggests the group tends to become more homogeneous regarding the microbiota composition. Although we were not able to observe significant differences in cross-sectional comparisons per time point/period with PERMANOVA, this might be due to the high correlation between samples from the same patient (higher than the effect caused by PR), which is the major confounding variable in this study.

It is known that PR has several clinical benefits in people with COPD (1). Additionally, it is also known that the airway microbiota composition of people with COPD and healthy individuals is significantly different (31–33,35). Several studies (31–35,95) reported that the airway microbiota of healthy individuals is enriched in Firmicutes and Bacteroidetes, mainly *Streptococcus* and *Prevotella* genera, whereas the airway microbiota of subjects with COPD is characterised by the enrichment in Proteobacteria, essentially *Haemophilus* and *Neisseria* as well (96). Besides that, *Veillonella* and *Actinomyces* genera have also been described (31,33) as some of the several genera, mostly found in the airway microbiota of healthy subjects, which could be markers of microbiota's normal state. Therefore, the expectation was that PR could modulate patients' microbiota towards the typical profile of healthy subjects.

Our results refuted our primary hypothesis, given that, during PR, we observed an increase in *Haemophilus* and Proteobacteria and a decrease in Bacteroidetes. Although we did not observe a significant decrease in *Prevotella* in most patients immediately after PR, we observed that the magnitude of change of *Prevotella*, during the three months of PR, was significantly higher in patients with a decrease of this genus. Similar changes in microbiota composition were not observed in the control group (for the same time points intervals), suggesting that these alterations might have been caused by PR. Furthermore, the longitudinal analyses with OTUs and ASVs also refuted the hypothesis and supported the previous results.

During PR, we observed a decrease of genera from Bacteroidetes and Firmicutes and enrichment in *Neisseria*, a genus from the Proteobacteria phylum. Additionally, we observed a decrease of multiple ASVs from the Bacteroidetes phylum, mainly *Prevotella*, and *Actinomyces*. Surprisingly, an ASV from a genus suggested as *Prevotella* was also found increased in the intervention group. Given that we only sequenced one gene (16S rRNA gene) to acquire the salivary microbiota profile of people with COPD, we were unable to perform functional characterisation of these differentially abundant bacteria identified among the intervention and control groups, as it is possible with shotgun metagenomics (97). Thus, we were unable to figure out if they are beneficial bacteria. However, bacteria increased in patients under PR are usually characteristic of people with severe COPD, whereas the decreased bacteria are typically associated with the microbiota of healthy individuals. Together, these results also showed that during exercise training, the salivary microbiota of people with COPD mostly changes towards a decrease in obligate anaerobes, favouring aerobes and facultative anaerobes. Therefore, the environmental changes in the airway, such as greater availability of oxygen, potentially caused by PR, could translate in salivary microbiota modulation towards a consistent enrichment in “bad” bacteria and depletion in “good” bacteria.

During exercise, the demand for oxygen to the entire body, mainly to the skeletal muscle, increases, thus the blood flow changes to allow a greater distribution of oxygen to several organs (66,98,99). At the same time, after a single session of exercise, the skeletal muscle suffers damage and the ROS production by neutrophils and macrophages recruited to the damaged area occurs (66). It is also known that the skeletal muscle is not the only source of ROS in people under exercise, these oxidants can also be generated by the lungs, heart, and blood (98). Several factors might affect the production of ROS (66) and, therefore, the degree of inflammation, i.e., the higher the intensity of exercise, the greater the production of ROS as well as the inflammatory response. There are also evidence that regular and moderate-intensity exercise, the same intensity prescribed to our patients, can lead to an adaptation process in the skeletal muscle of healthy individuals and, thus, an increase of the antioxidant defence mechanisms and a decrease in the oxidative stress (66). However, and to our knowledge, there are no studies assessing the oxidative stress in the lungs of people with COPD under a 12-week PR programme including moderate-intensity exercise. Therefore, we do not know if the effect of regular and moderate-intensity training is the same in the lungs and skeletal muscle. As we know, COPD is characterised by chronic inflammation of the lungs and increased oxidative stress (1) due to the production of endogenous oxidants, such as ROS and nitric oxide, a reactive nitrogen specie, (17) in the lungs. Therefore, the moderate-intensity exercise performed by our patients might be stimulating an even higher production of ROS and nitric oxide in their airways. It is known that in gastrointestinal microbiota (100) members from the Proteobacteria phylum are able to strive during inflammation due to their metabolic capacity of handling metabolites produced during inflammation. For example, the reaction among reactive oxygen and nitrogen species can lead to the production of peroxynitrite and, thus the production of nitrate (101). Nitrate can be, then, used by Betaproteobacteria (e.g. *Neisseria*) and Gammaproteobacteria (e.g. *Haemophilus*) to allow their anaerobic respiration (101), an ability likely absent in obligate anaerobes, such as Bacteroidia and Clostridia classes (Bacteroidetes and Firmicutes phyla, respectively). For that reason, Proteobacteria are more prone to survive and compete against other bacteria. So, similarly to what happens in gut microbiota (100), the increase in Proteobacteria and a decrease in Bacteroidetes that we observed in patients microbiota during PR might be a consequence of increased oxidative stress, ROS and nitric oxide in the airways, as consequence to exercise training. Bacteroidia and Clostridia classes were the main classes composing the Bacteroidetes and Firmicutes phyla of patients' microbiota. Strikingly, all OTUs and ASVs that were significantly depleted in the intervention group during PR belong to both Bacteroidia and Clostridia classes. Regarding *Actinomyces* ASV, it is known that most of these species are facultative anaerobes; however, some important *Actinomyces* are obligate anaerobes (102), which can be the case of the ASV that we observed as depleted in the intervention group. Unfortunately, we were not able to have greater resolution than genus (taxonomic level 6) regarding *Actinomyces* due to limitations intrinsic to the use of 16S rRNA sequencing (97).

In inflammatory bowel diseases, it has been hypothesised that oxygen might have a role in intestinal dysbiosis of patients with these diseases (103). Briefly, the oxygen hypothesis suggests that, under inflammation, the increase of oxygen in the gut might be an advantage to facultative anaerobes and aerobes, leading to their overgrowth (103). On the other hand, this increase of oxygen might confer a disadvantage to the obligate anaerobes due to the harmful effect of oxygen on them (103). This hypothesis may also explain what we observed during PR. With PR, lungs likely become an environment enriched in oxygen (104), and as a consequence, facultative anaerobes (Proteobacteria) in the lungs bloom. On the other hand, obligate anaerobes (some genera from Bacteroidetes and Firmicutes phylum) cannot tolerate the high oxygen levels present in the lungs, and as consequence decrease in number.

Overall, the increase in Proteobacteria (particularly *Haemophilus*) and decrease in Bacteroidetes (particularly *Prevotella*) that occurred in patients undergoing PR was coherent with physiological alterations (increase of oxygen availability and production of reactive oxygen and nitrogen species) that might be induced by moderate-intensity exercise at short-term, but not at long-term in the skeletal muscle. Nevertheless, the effect of regular and moderate-intensity exercise in patients' lungs during PR programmes might not be the same as the one observed at the skeletal muscles. Therefore, more studies approaching the effect of regular and moderate-intensity exercise in the lungs are needed.

Another surprising result was no alteration of alpha diversity in response to PR. In the literature (32,33,35), it has been reported that microbiota diversity within individuals differs between healthy and people with COPD, being increased in the healthy subjects. Additionally, there is also evidence that exercise increases gut microbiota diversity of athletes (92–94,105). So that, since PR has beneficial effects to people with COPD, we expected that alpha diversity could increase in response to PR. Nevertheless, a study (93) with a 6-week programme of physical activity to unfit individuals did not detect differences in alpha diversity during this period, which is in accordance with the results obtained.

Moreover, another interesting finding was that responders and non-responders to different PR domains, exercise tolerance – 6MWT, dyspnoea during exercise – mBS and symptomatology in daily life – CAT, present different microbiota profiles prior PR, and different microbiota dynamics in response to PR.

Regarding mBS, responders seem to be in a worse status (prior to PR) concerning the microbiota composition than non-responders, since they presented an increased relative abundance of Proteobacteria and a decreased relative abundance of Bacteroidetes. Furthermore, the association obtained among the increased abundance of Proteobacteria and *Haemophilus* (in M1) with responding to the mBS also corroborates the previous result (responders in a worse status in M1). One possible explanation for what we observed might be the possibility of patients in a worse condition for that domain being more prone to respond to it. This suggestion is in accordance with a recent study (106) where it was found that dyspnoea scores (measured with the medical research council dyspnoea scale) before two approaches of following patients (one with a multidisciplinary team that, for example, encouraged patients to become more active, and another only composed by a nurse that executed regular visits to patients, including the execution of spirometry, as well as additionally visits if an exacerbation occurred) was very important for patients' clinical improvement. Regardless of previous approaches of following people with COPD, patients with worse dyspnoea scores in the baseline were the ones with a greater room for improvement in their quality of life after 12-months (106). Therefore, the same might be applied to microbiota, specifically for this clinical variable, but more studies approaching this theme with larger sample sizes are needed to confirm this suggestion. Furthermore, another interesting finding was that non-responders to the mBS experienced greater alterations in microbiota during PR. The non-significant differences in responders to the mBS, from M1 to M4, might suggest that, prior to PR, their dysbiotic microbiota (enriched in *Haemophilus* and Proteobacteria and depleted in *Prevotella*) is somehow more adapted to the alterations in the airway environment induced by PR (mainly increase of oxidative stress).

When considering responders and non-responders to the CAT, we observed the opposite trend in terms of microbiota composition at M1. The only significant difference observed among the groups was an enrichment in *Prevotella* in responders prior to PR. This result suggests that higher frequencies of *Prevotella* (prior to PR) could be associated with better responses to the CAT, which was confirmed with the logistic regression performed (AUC=76%). Nevertheless, the group of responders to the CAT were the patients in worse clinical condition (according to this parameter) and, in M4, they presented an increase in *Haemophilus* and a decrease in Bacteroidetes, which was concordant with the global effects of PR when considering the whole intervention group. Contrary to what was observed in mBS, regarding the CAT domain, the microbiota of responders was more prone to be altered by PR. Opron et al. (107) has reported significant associations between CAT scores and lung microbiota signatures in mild to moderate COPD. Moreover, a positive correlation between the number of neutrophilic extracellular traps (part of neutrophilic response to presence of harmful bacteria in the lungs) and CAT scores, but also with severity of COPD, severity of exacerbations and frequency of *Haemophilus* in sputum was reported (108). Nevertheless, the association between CAT (patient related measure of symptoms burden) and airway microbiota needs further clarification in order to better understand the beneficial effects, in microbiota terms, of the reduction in CAT scores.

With the present study, we also observed an increase of the inflammation in response to PR. In healthy individuals, there is evidence that short periods of exercise can lead to the release of some inflammatory cytokines such as TNF-α, IL-6, IL-8, and IL-1β, all pro-inflammatory cytokines, and IL-10, an anti-inflammatory cytokine (109). We observed a significant increase in IL-1β (pro-inflammatory cytokine) immediately after the first month of PR, suggesting an increase in inflammation. This result has not been reported in any of the few studies focused on the effect of exercise on the immune response of people with COPD, considering a based-community PR programme and assessing inflammation after one month of exercise. However, some evidence suggest an increase of IL-1β in healthy subjects with physical training (109). Moreover, it has been demonstrated (68) that people with COPD under moderate-intensity exercise can suffer an increase in TNF-α and no changes in IL-6 plasma levels, which corroborates what we have seen in our patients immediately after PR. Another study (69) with the same purpose observed the opposite. The discrepancy of results regarding the effect of exercise in people with COPD might be due to small sample sizes and different time frames used. Therefore, more studies approaching the effect of PR in people with COPD, carefully considering the previous topics, are needed. Our results also suggest that immediately after the first month and 12 weeks of PR, the levels of IL-10 tend to be higher than in M1; however, we were not able to see significant differences among pairs of time points. This result might propose an attempt of patients' immune system to reduce other pro-inflammatory cytokines, such as IL-6, IL-1β, and TNF-α (110). Our results also did not corroborate the results obtained by Silva et al. (70) when people with COPD were under a 12-week PR programme. This might have happened due to the sample size, the type of exercise, and the use of different samples, i.e., we used saliva samples, while Silva et al. (70) used plasma samples.

The results regarding the inflammatory mediators suggest that, during PR, there is an increase in inflammation, firstly characterised by IL-1β and then by TNF-α. Both cytokines have been reported as cytokines released during the COPD innate immune response, and this response can be stimulated by microbe-associates molecular patterns, such as lipopolysaccharide (28,41). Therefore, the Proteobacteria increased in our patients during PR might be inducing the release of the previous inflammatory mediators. Gammaproteobacteria, such as *Haemophilus*, can present hexa-acylated lipopolysaccharide (28,111). This lipopolysaccharide structure can bind to TLR4 and lead to greater immune stimulation (28,111) and, therefore, an increase of inflammatory mediators released during the innate immune response, such as IL-1β and TNF-α.

Regarding SIgA levels, a study (71) with elderly people observed a significant increase in salivary SIgA levels after 12 months of exercise training. Therefore, we were expecting that in our study patients with COPD, mostly considered elderly people, could also present an increase in the salivary SIgA levels after the 12-week PR programme. However, no significant differences were observed after PR. Therefore, the period encompassing exercise training might be the reason we do not see changes in salivary SIgA. A similar study to ours with more patients and a longer period of PR should be performed to confirm if SIgA levels in saliva increase with long-term exercise.

This study presents several limitations, first the sample size is small to study a disease as heterogeneous as COPD. Nevertheless, a study regarding PR is very demanding in terms of patient recruitment and enrolment during the longitudinal follow-ups, human resources required and budget. Ideally, more studies with similar study design should be carried out with larger longitudinal cohorts to validate the identified impacts of PR in terms of microbiota and inflammatory profiles of people with COPD.

Second, and as previously mentioned in this thesis, the changes observed in patients' microbiota composition during PR were small. However, this does not mean that alterations in bacteria functions were not occurring during PR. Unfortunately, with the 16S rRNA gene sequencing, we are not able to assess bacteria functions, which is another limitation of the present study. In the future, methods that allow the prediction of bacteria functions, such as shotgun metagenomics sequencing should be used.

Third, all the results obtained with responders and non-responders to mBS should be carefully interpreted since the two groups are not balanced. It is very hard or even impossible to predict patients' response to PR domains at the time of the recruitment, and for that reason very hard to balance the groups of responders and non-responders.

Fourth, due to exposure to pharmacological therapy (mainly bronchodilators), it is very hard for some patients to produce saliva. So, during sample collection, drinking water was allowed to stimulate saliva production and so help in the sample collection. Nevertheless, we are aware that drinking water prior to saliva collection might have diluted cytokines present in the samples, and difficulted the quantification of them.

In the future, similar studies to this one should include larger sample sizes and other clinical variables assessing different domains of PR, such as functionality (using the 1-minute sit-to-stand test), muscle strength (using dynamometer or repetition maximum tests), and exacerbations (measured monthly). It is also important to acquire absolute values regarding bacteria abundance in the salivary microbiota to have a great understanding of their variation during PR. In the future, shotgun metagenomic sequencing should be performed to assess bacteria functional profiles. Besides that, it would be interesting to study if *Haemophilus* and *Prevotella* interact with each other since these two genera varied significantly over time (during PR) and contrarily (increase in *Haemophilus* and decrease in *Prevotella*). Additionally, the oxidative status of our patients, using saliva samples, should be assessed to determine if the oxidative stress increased with PR. If so, the next step would be to investigate if an increase of oxidants interferes with the abundance of bacteria in saliva (increase in facultative anaerobes and aerobes, and decrease in obligate anaerobes). The execution of predictive models to determine if an individual would respond to dyspnoea and CAT would be interesting and helpful to health professionals to determine if an individual is a good candidate for PR, i.e., will benefit from this therapy.

In conclusion, although PR has proven clinical beneficial effects for people with COPD, it seems to alter salivary microbiota composition to ensembles closer to the ones presented by more severe patients with COPD in comparison to healthy subjects, with higher relative frequencies of *Haemophilus* and depleted in *Prevotella*. Additionally, we have also observed an increase of inflammation in response to PR, with an increase of IL-1β after 4 weeks of PR and an increase of TNF-α after 12 weeks of PR. Therefore, physiological alterations induced by exercise might allow the survival of bacteria able to thrive in high oxygen levels as well as to stimulate patients’ immune response. Further understanding of the stability of microbiota and inflammatory alterations, and the long-term clinical implications of those alterations, for example, in the prevention of future exacerbations, need to be investigated.

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6 Annexes

Annex I – Additional results obtained in the present study

Table S1 – Concentrations of the thirteen cytokines obtained for the twenty-six patients over time. The blue cell indicates concentrations that were below the detection limit, while the red cells correspond to the concentrations that were above the detection limit.

Sample	[IL-1β]	[IFN-α2]	[IFN-γ]	[TNF-α]	[MCP-1]	[IL-6]	[IL-8]	[IL-10]	[IL-12p70]	[IL-17A]	[IL-18]	[IL-23]	[IL-33]
3R443 M1	1195.51	11.03	7.487	22.13	3038.07	1201.96	6677.459	12.08	2.32	0.965	548.18	12.76	36.0
3R443 M2	2213.13	10.90	9.193	66.21	3045.78	183.43	5238.619	38.35	4.94	1.310	2137.18	14.97	103.4
3R443 M4	601.57	2.14	1.212	29.52	1172.75	64.49	1257.108	6.63	0.90	0.383	1063.29	3.23	15.7
3R478 M1	629.71	4.90	2.762	6.70	1155.48	44.32	3885.822	3.92	2.33	0.798	217.42	11.82	40.2
3R478 M2	1264.15	10.15	4.951	16.03	3154.61	52.73	5218.301	14.02	1.75	0.674	684.41	10.96	28.7
3R478 M4	1121.37	14.31	2.812	5.42	6147.76	85.56	4681.519	13.83	2.33	0.725	404.78	8.08	42.5
3R479 M1	2344.55	3.36	13.837	51.66	243.67	4.66	10031.808	0.96	1.79	5.023	481.22	232.37	18.6
3R479 M4	1159.62	6.68	13.014	46.66	554.62	14.14	10363.495	3.03	2.26	3.144	331.68	114.97	39.2
3R481 M1	236.50	7.98	1.036	1.72	3113.56	77.53	3449.849	1.75	1.29	0.825	34.62	2.74	10.3
3R481 M2	530.89	8.32	3.534	2.06	2674.53	76.04	6843.397	5.75	4.48	1.009	314.30	10.04	48.2
3R481 M4	339.11	9.18	3.849	4.20	3288.29	58.85	4203.193	2.71	2.15	0.973	1280.91	10.74	33.8
3R482 M1	2588.52	6.50	3.907	29.64	2954.68	82.36	6560.973	8.35	2.49	2.352	1681.78	92.43	52.7
3R482 M2	2320.14	9.08	8.036	191.63	1728.07	240.29	6900.444	20.26	2.97	1.981	1214.45	17.79	50.8
3R482 M4	11000	57.28	75.870	1497.55	12515.65	2737.94	12222.151	381.96	14.38	25.369	4899.07	3709.51	197.2
3R488 M1	11000	16.39	14.660	244.11	12694.60	1089.01	18170.545	156.96	3.81	4.969	8137.24	29.30	102.8
3R488 M2	3063.10	4.37	3.112	22.08	724.35	9.46	4988.422	9.37	1.62	0.595	2514.59	7.12	21.5
3R488 M4	3700.47	10.65	11.855	47.13	1508.77	33.87	7589.764	33.18	2.29	1.165	3154.39	18.86	52.6
3R492 M1	1051.66	3.82	3.944	2.12	525.41	8.68	4518.510	2.24	1.95	7.375	2942.83	11.78	22.4
3R492 M4	6349.55	13.57	133.977	34.16	2559.01	130.19	15119.379	8.92	5.21	62.893	3088.40	242.38	90.3
3R495 M1	3019.42	7.10	7.723	69.13	4051.11	71.12	5473.421	20.73	1.53	0.468	5031.73	6.85	16.1
3R495 M2	2874.29	2.45	1.664	48.50	1569.65	43.74	3210.973	27.85	0.81	0.146	2566.71	2.64	12.5
3R495 M4	4386.07	7.07	5.453	74.34	3885.68	142.65	4479.332	47.23	2.05	0.526	6473.80	7.50	30.4
3R496 M1	1055.74	9.03	1.667	18.49	2658.93	128.75	2951.102	12.71	1.30	0.480	2090.12	8.29	26.3
3R496 M2	1226.13	12.79	35.694	136.26	2872.35	132.10	7176.272	15.05	3.35	2.086	7356.55	26.31	51.2
3R496 M4	1203.78	12.42	3.932	72.79	4221.91	95.67	4367.269	2.56	1.47	0.614	7378.89	14.56	22.5
863 M1	2526.89	60.73	55.523	85.20	3058.97	383.25	8389.730	100.59	49.65	6.338	2203.58	98.34	271.7
863 M2	3015.33	14.27	10.327	53.59	4588.49	165.80	8611.675	44.18	2.60	2.303	3783.37	13.16	37.8
863 M3	3282.39	24.86	9.755	22.41	4189.52	126.29	7981.589	17.79	2.45	4.858	2622.23	17.22	39.2
P1 M1	45638.86	6.22	2.272	25.22	2797.30	25.45	10771.913	3.91	1.46	3.885	501.64	101.84	30.1
P1 M2	4206.15	16.37	8.224	69.31	948.04	753.73	14266.536	43.03	2.85	5.913	3461.32	430.20	55.1
P1 M4	89184.31	17.65	19.398	81.23	1048.78	296.87	13281.589	32.47	4.26	9.918	2564.73	306.41	125.5
P115 M1	132.36	1.36	1.624	7.56	4.67	4.34	25.280	3.56	1.49	1.425	2.27	4.03	17.6
P115 M2	3176.12	4.41	7.834	65.57	844.31	745.23	5488.052	7.56	2.01	1.844	1849.53	13.62	53.0
P115 M4	2847.63	3.86	4.053	44.05	1862.34	34.51	4113.719	14.29	1.79	0.704	944.38	5.72	26.4
P121 M1	820.06	18.27	10.779	14.08	3123.76	82.26	8028.279	2.73	1.98	7.294	344.47	17.05	28.7
P121 M2	1303.43	26.15	15.495	5.71	9110.66	771.26	6540.462	24.88	1.22	4.320	82.82	17.50	49.5
P121 M4	1612.32	22.82	16.633	15.06	7721.08	367.17	8578.366	36.65	1.11	4.599	140.91	58.24	36.4
P127 M1	72.00	1.61	1.247	1.61	567.03	4.89	415.790	1.67	1.27	0.239	39.74	2.78	16.9
P127 M2	790.54	2.14	0.819	2.26	413.24	17.45	1448.283	2.11	1.15	0.158	439.68	2.58	22.4
P127 M4	310.81	3.90	2.892	8.30	1502.06	16.02	1581.210	2.93	1.92	1.012	245.78	5.77	21.4
P129 M1	342.34	6.84	2.309	3.28	1337.29	53.89	3843.182	0.60	0.80	1.131	207.99	4.57	13.9
P129 M4	1686.09	6.70	6.230	14.87	1906.70	41.58	4550.105	5.30	5.61	3.762	2302.60	23.77	68.8
P13 M1	130.04	30.66	3.093	2.64	11042.85	1041.81	5631.771	1.77	1.85	1.496	236.67	4.96	24.0
P13 M2	251.04	12.83	15.239	38.80	3196.06	288.72	4681.094	38.75	2.27	1.273	1332.92	9.88	29.5
P13 M4	215.68	22.00	27.268	70.22	9309.50	177.06	4192.284	6.03	0.61	0.202	1057.03	4.52	16.6
P134 M1	293.28	0.61	1.000	1.13	291.94	0.98	2230.490	1.12	0.58	0.618	23.45	1.80	7.6
P134 M2	1668.35	4.10	3.747	5.76	948.68	16.28	6473.803	6.02	1.08	1.121	415.04	6.57	14.7
P134 M4	440.39	1.84	0.783	1.14	682.77	12.89	1702.698	1.76	0.62	0.456	103.27	2.25	5.8
P140 M1	137.80	13.40	2.740	1.54	5993.17	36.60	1507.729	6.57	5.30	0.650	449.80	11.84	55.8
P140 M2	235.80	4.39	0.676	2.22	1140.51	15.42	1126.754	2.44	1.60	0.838	211.45	5.05	21.5
P140 M3	30.32	5.27	1.106	0.77	1921.23	2.91	715.547	3.30	2.24	0.398	99.90	6.63	28.4
P143 M1	1227.50	39.60	22.484	55.62	11007.96	732.63	15758.254	41.85	12.35	3.238	9705.29	17.93	151.8
P143 M2	3650.09	19.22	14.401	54.38	8756.63	248.41	13111.087	13.19	6.88	9.029	5548.66	28.22	82.7
P143 M4	940.11	5.73	3.330	4.14	388.57	33.07	1197.777	5.33	4.80	1.635	145.82	6.50	22.7
P145 M1	44.58	0.29	0.395	0.34	218.58	1.12	590.425	2.41	0.36	0.224	3.42	1.61	6.3
P145 M2	1456.99	18.28	18.320	20.67	3090.17	63.22	10694.235	91.51	1.88	3.964	553.88	111.92	42.9
P145 M3	906.14	6.45	6.778	108.16	1415.48	164.44	7698.060	580.87	2.31	5.711	1049.83	39.32	27.7
P147 M1	95.72	2.31	3.100	1.42	576.66	5.60	3529.928	1.45	0.69	0.626	43.68	6.75	9.8
P147 M2	96.55	4.33	2.200	2.42	1409.05	10.18	4132.790	1.76	1.21	0.690	242.33	5.37	13.4
P147 M4	16.93	1.94	2.246	3.14	277.79	5.30	823.305	3.50	2.42	0.385	9.26	7.66	34.0
P150 M1	1778.24	5.10	4.798	2.86	484.06	23.68	6408.488	2.79	2.75	1.166	159.84	8.69	21.2
P150 M4	1843.79	1.61	5.267	4.13	365.35	20.68	4800.498	2.60	0.88	0.514	750.79	2.50	9.4
P16 M1	1339.09	7.26	1.504	8.73	2509.70	14.85	3805.575	1.66	0.89	0.424	849.36	4.67	15.4
P16 M2	411.52	5.33	1.879	4.81	1868.54	15.07	1620.661	2.39	1.03	0.397	316.22	3.76	20.3
P16 M4	788.40	5.88	1.373	14.70	2141.16	48.20	2609.893	4.50	8.07	0.236	376.85	4.47	28.5
P18 M1	2716.67	32.40	80.567	457.99	4701.20	386.43	15564.415	6.47	2.88	11.857	1961.39	107.95	86.7
P18 M2	1119.92	11.74	21.668	43.78	4335.24	50.52	5028.749	2.07	1.03	2.170	709.39	19.44	13.8
P18 M4	9307.73	67.94	727.074	1739.11	16411.13	5119.24	16690.052	263.00	5.21	37.916	3924.51	261.97	82.1
P2 M1	1131.60	2.47	1.101	1.66	944.20	18.50	4831.345	0.84	0.43	0.217	420.96	3.71	10.8
P2 M2	3538.76	7.62	4.133	81.89	4167.49	212.97	5838.339	36.17	3.71	1.349	1020.77	6.63	59.9
P2 M3	1158.08	2.35	1.559	8.05	596.77	148.66	5527.031	1.38	0.77	0.392	418.65	3.68	9.9
P7 M1	459.91	3.52	1.043	4.94	993.72	37.88	547.850	1.11	1.34	0.637	79.76	4.69	17.2
P7 M2	0.02	0.16	0.407	0.60	3.41	1.96	55.862	0.61	0.69	0.139	10.80	1.79	7.6
P7 M4	1474.91	3.26	0.955	22.18	1577.35	5.23	4205.544	5.56	0.75	0.348	730.86	3.64	9.5

Table S2 – PERMANOVA results obtained when comparing beta diversity values among patients from the intervention group in M1 and M2, M1 and M3, and Pre and PR period.

	Test statistic	***p* value**
M1 vs M2	0.58	0.75
M1 vs M3	1.32	0.23
Pre vs PR	1.54	0.17

Table S3 – No significant changes in the relative abundance of Proteobacteria, Bacteroidetes, and *Prevotella* in the intervention group from M1 to M2.

M1 to M2	**Test statistic (W)**	***p* value**
Proteobacteria	169	0.21
Bacteroidetes	-45	0.74
Prevotella	-71	0.60

Table S4 – No significant changes in the relative abundance of Bacteroidetes and *Prevotella* in the intervention group from M1 to M3.

M1 to M3	**Test statistic (W)**	***p* value**
Bacteroidetes	-245	0.77
Prevotella	-195	0.16

Table S5 – No significant changes in the relative abundance of Proteobacteria, *Haemophilus*, and *Prevotella* in the intervention group from M1 to M4.

M1 to M4	**Test statistic (W)**	***p* value**
Proteobacteria	235	0.077
Haemophilus	215	0.11
Prevotella	-156	0.23

Table S6 – No significant changes in the relative abundance of *Haemophilus* in the control group and considering all the possible 1-month intervals.

1-months intervals	**M1 vs M2**	**M2 vs M3**	**M3 vs M4**	**M4 vs M5**	**M5 vs M6**
Haemophilus	W=-3 *p*=0.99	W=205 *p*=0.14	W=-77 *p*=0.59	W=87 *p*=0.54	W=49 *p*=0.73

Table S7 – No significant changes in the relative abundance of Proteobacteria and *Haemophilus* in the control group and considering all the possible 2-months intervals.

2-months intervals	**M1 vs M3**	**M2 vs M4**	**M3 vs M5**	**M4 vs M6**
Proteobacteria	W=219 *p*=0.11	W=147 *p*=0.29	W=165 *p*=0.24	W=201 *p*=0.15
Haemophilus	W=153 *p*=0.27	W=79 *p*=0.58	W=11 *p*=0.94	W=173 *p*=0.22

Table S8 – No significant changes in the relative abundance of Bacteroidetes in the control group and considering all the possible 3-months intervals.

3-months intervals	**M1 vs M4**	**M2 vs M5**	**M3 vs M6**
Bacteroidetes	W=121 *p*=0.39	W=-97 *p*=0.49	W=-243 *p*=0.079

Table S9 – No significant module of changes in the relative abundance of Proteobacteria, *Haemophilus*, Bacteroidetes, and *Prevotella* in the intervention group from M1 to M2.

|M2-M1|	**Test statistic (U)**	***p* value**
Proteobacteria	160	0.99
Haemophilus	106	0.50
Bacteroidetes	139	0.34
Prevotella	126	0.18

Table S10 – No significant module of changes in the relative abundance of Proteobacteria, Bacteroidetes, and *Prevotella* in the intervention group from M1 to M3.

|M3-M1|	**Test statistic (U)**	***p* value**
Proteobacteria	121	0.16
Bacteroidetes	128	0.19
Prevotella	137	0.26

Table S11 – No significant module of changes in the relative abundance of *Haemophilus* in the intervention group from M1 to M4.

|M4-M1|	**Test statistic (U)**	***p* value**
Haemophilus	139	0.91

Table S12 – No significant module of changes in the relative abundance of *Haemophilus* in the control group and considering all the possible 2-months intervals.

2-months intervals	**|M3-M1|**	**|M4-M2|**	**|M5-M3|**	**|M6-M4|**
Haemophilus	U=141 *p*=0.28	U=178 *p*>1	U=175 *p*=0.89	U=106 *p*=0.12

Table S13 – No significant module of changes in the relative abundance of Proteobacteria, Bacteroidetes, and *Prevotella* in the control group and considering all the possible 2-months intervals.

3-months intervals	**|M4-M1|**	**|M5-M2|**	**|M6-M3|**
Proteobacteria	U=166 *p*=0.78	U=114 *p*=0.59	U=144 *p*=0.48
Bacteroidetes	U=139 *p*=0.24	U=16 *p*=0.78	U=144 *p*=0.48
Prevotella	U=145 *p*=0.32	U=126 *p*=0.13	U=137 *p*=0.21

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Table S14 – Taxonomic annotation of the amplicon sequence variants identified as differentially abundant among patients from the intervention and control groups from M2 to M4 and M3 to M4.

Time interval	**Amplicon sequence variant (ASV)**	**Taxonomic annotation**
M2 to M4	fd75fae7f9721be5ba0e4a3704a1dedf	***Mogibacterium***
	fe2f04ee87e40784f5104a15bec2058f	***Prevotella* A**
	dfc9a1ff9b06e08d2132da15e61b3919	***Leptotrichia***
	608e6548b1b4cbb6176c8fce090991a5	***Actinomyces***
	d32274e6eeffddfd841ee35b5ba8ea1f	***Corynebacterium Durum***
M3 to M4	e3beabd067252bb387cfe12787744633	***Prevotella* B**
	c17057f15de467ed9c2c7a57817aa221	***Prevotella* C**
	6d43cac09b0febf5f95abf46b0080739	**[*Prevotella*]**

Table S15 – No significant differences in alpha diversity between Pre and PR period considering Shannon and Faith's phylogenetic indexes.

	Shannon index	**Faith's phylogenetic index**
Pre vs PR	W=-24 *p*=0.86	W=-148 *p*=0.25

Table S16 – No significant differences in beta diversity among responders and non-responders to the 6MWT and the CAT before PR.

	Test statistic	***p* value**
6MWT – R vs NR	0.59	0.67
CAT – R vs NR	2.07	0.088

Table S17 – No significant changes in relative abundance of Proteobacteria, *Haemophilus*, Bacteroidetes, and *Prevotella* among responders and non-responders to the 6MWT before PR.

	Test statistic (U)	***p* value**
Proteobacteria	129	0.40
Haemophilus	119	0.25

Bacteroidetes	124	0.32
Prevotella	130	0.42

Table S18 – No significant changes in relative abundance of Bacteroidetes among responders and non-responders to the mBS before PR.

	Test statistic (U)	***p* value**
Bacteroidetes	87	0.18

Table S19 – No significant changes in relative abundance of Proteobacteria, *Haemophilus*, and Bacteroidetes among responders and non-responders to the CAT before PR.

	Test statistic (U)	***p* value**
Proteobacteria	154	0.84
Haemophilus	136	0.45
Bacteroidetes	103	0.071

Table S20 – No significant changes in relative abundance of Proteobacteria, *Haemophilus*, and Bacteroidetes among responders and non-responders to the 6MWT from M1 to M4.

M1 vs M4	**Responders**	**Non-responders**
Proteobacteria	W=60 *p*=0.41	W=45 *p*=0.13
Haemophilus	W=92 *p*=0.20	W=51 *p*=0.080
Bacteroidetes	W=-90 *p*=0.21	W=-51 *p*=0.080
Prevotella	W=-18 *p*=0.81	W=-42 *p*=0.11

Table S21 – No significant changes in relative abundance of Proteobacteria, *Haemophilus*, and Bacteroidetes among responders and non-responders to the mBS from M1 to M4.

M1 vs M4	**Responders**	**Non-responders**
Proteobacteria	W=15 *p*=0.43	W=136 *p*=0.13
Haemophilus	W=-5 *p*=0.82	-
Bacteroidetes	W=-5 *p*=0.82	-
Prevotella	W=15 *p*=0.43	W=-142 *p*=0.091

Table S22 – No significant changes in relative abundance of Proteobacteria, *Haemophilus*, and Bacteroidetes among responders and non-responders to the CAT from M1 to M4.

M1 vs M4	**Responders**	**Non-responders**
Proteobacteria	W=120 *p*=0.070	W=21 *p*=0.54
Haemophilus	-	W=21 *p*=0.54
Bacteroidetes	-	W=3 *p*=0.95
Prevotella	W=-119 *p*=0.054	W=31 *p*=0.36

Table S23 – No significant changes in relative abundance of Proteobacteria, *Haemophilus*, Bacteroidetes, and *Prevotella* among responders and non-responders to the 6MWT, the mBS, and the CAT.

ΔR vs ΔNR	**6MWT**	**mBS**	**CAT**
Proteobacteria	U=126 *p*=0.35	U=126 *p*>1	U=149 *p*=0.72
Haemophilus	U=136 *p*=0.54	U=78 *p*=0.093	U=139 *p*=0.51
Bacteroidetes	U=132	U=102	U=101

	p=0.46	*p*=0.41	*p*=0.062
Prevotella	U=118 *p*=0.24	U=86 *p*=0.16	U=103 *p*=0.071

Table S24 – Results from the comparison of the ten cytokines concentration over time and for which no significant differences were observed.

		IFN-α2	**IFN-γ**	**MCP-1**	**IL-6**	**IL-8**	**IL-12p70**	**IL-17A**	**IL-18**	**IL-23**	**IL-33**
Not normalised	p value	0.83	0.56	0.65	0.40	0.40	0.45	0.87	0.26	0.83	0.65
	Statistic	0.38	1.14	0.86	1.81	1.81	1.61	0.29	2.67	0.38	0.86
Normalised (ratio)	p value	0.83	0.56	0.65	0.40	0.40	0.45	0.87	0.26	0.83	0.65
	Statistic	0.38	1.14	0.86	1.81	1.81	1.61	0.29	2.67	0.38	0.86

6. Annexes

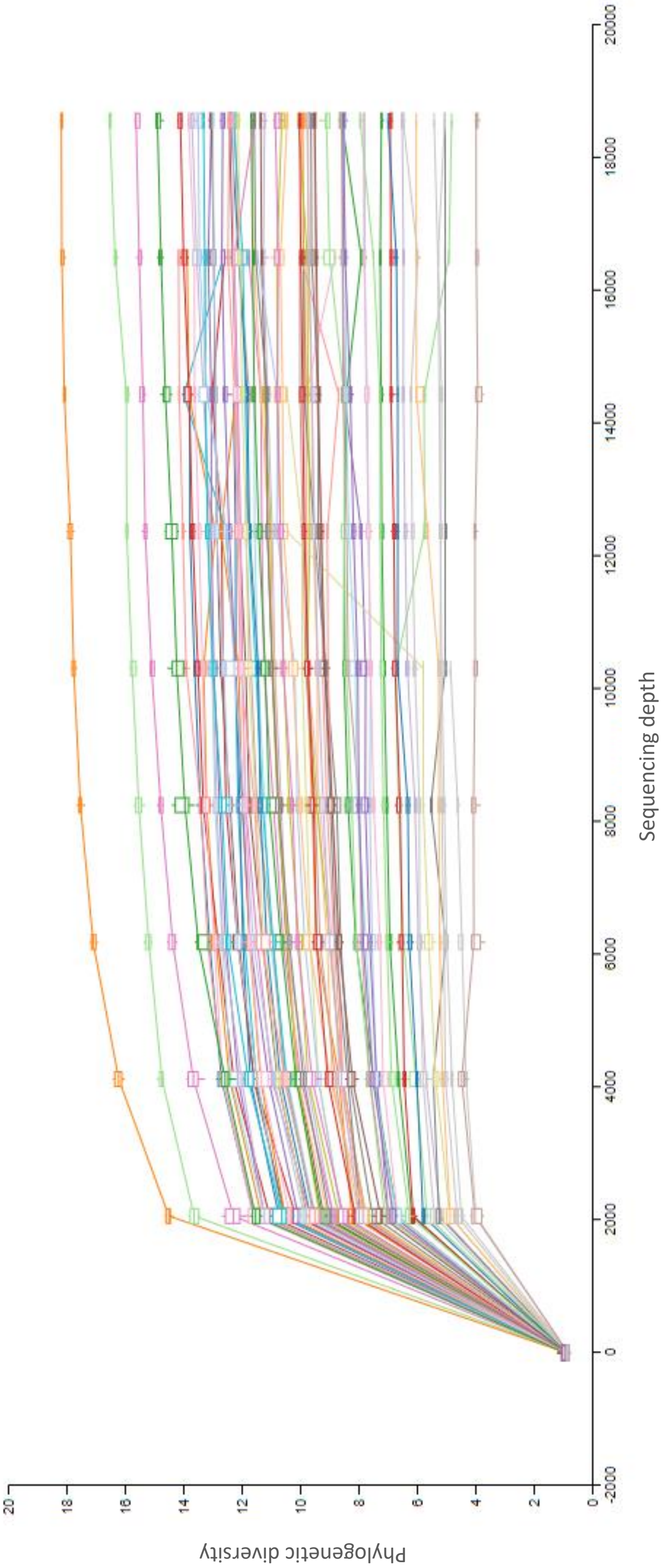

Figure S1 – Rarefaction curve of alpha diversity using Faith's phylogenetic diversity. Each coloured line represents each sample included in the present study.

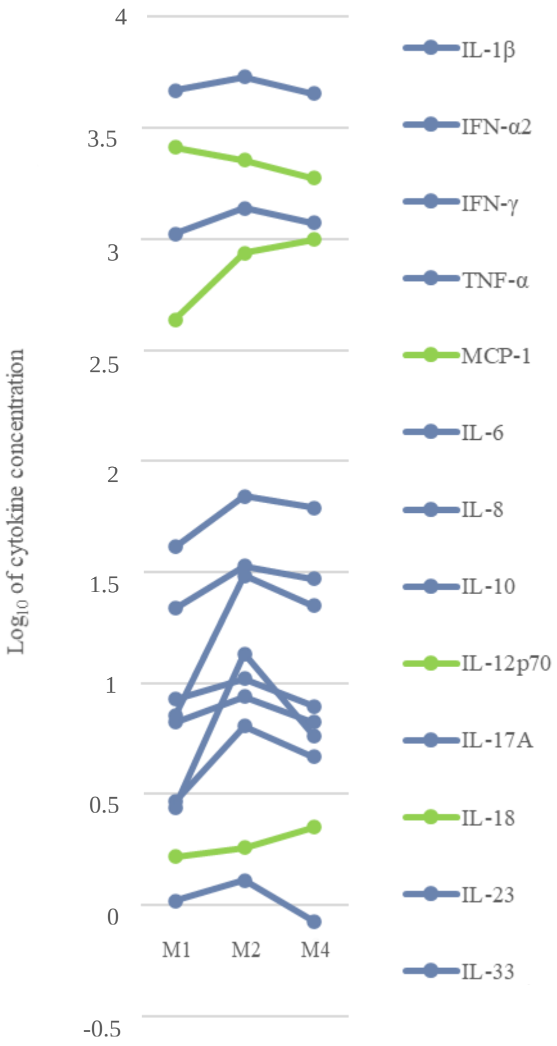

Figure S2 – Log$_{10}$ of each thirteen cytokines mean concentration over time. The blue lines correspond to cytokines that increase in M2 and decrease in M4, while the green lines correspond to cytokines with a different behaviour over time.