**The faecal metabolome and mycobiome in Parkinson’s disease**

**Authors:**

Eduardo De Pablo-Fernandez (MD, PhD);a\* Gerum Gashaw Gebeyehu (MBChB, MRes);b\* Luke Flain (MBChB, MRes);b Rachael Slater (BSc, MSc, PhD);b Alessandra Frau (BSc, MSc, PhD);b Umer Zeeshan Ijaz (BScEng, MScEng, PhDEng);c Thomas Warner (MD, PhD, FRCP);a Chris Probert (MD FRCP).b

\*Eduardo De Pablo-Fernandez and Gerum Gashaw Gebeyehu contributed equally to this manuscript.

**Affiliations:**

a Reta Lila Weston Institute of Neurological Studies and Queen Square Brain Bank for Neurological Disorders, UCL Queen Square Institute of Neurology. 1 Wakefield Street. WC1N 1PJ. London, United Kingdom.

b Institute of Systems, Molecular and Integrative Biology, University of Liverpool, The Henry Wellcome Laboratory. Nuffield Building, Crown Street. L69 3GE. Liverpool, United Kingdom.

c James Watt School of Engineering, University of Glasgow. Rankine building, Oakfield Avenue. G12 8QQ. Glasgow, United Kingdom.

**Corresponding authors:**

Dr. Eduardo de Pablo-Fernandez; Reta Lila Weston Institute of Neurological Studies, UCL Queen Square Institute of Neurology. 1 Wakefield Street. London. WC1N 1PJ. Telephone: +44 (0)2076794025. E-mail: eduardo.fernandez.13@ucl.ac.uk

Dr. Gerum Gashaw Gebeyehu; Institute of Systems, Molecular and Integrative Biology, University of Liverpool, The Henry Wellcome Laboratory, Nuffield Building, Crown Street, Liverpool, United Kingdom. L69 3GE; Telephone: +447752170009; E-mail: hlggasha@doctors.org.uk

**Key words:** Parkinson’s disease; metabolomics; mycobiome; fungal dysbiosis; volatile organic compounds.

**Manuscript word count:** 1991 words

**Declaration of interest:** None reported.

**Funding for the study:** This research was partially supported by The Reta Lila Weston Trust for Medical Research. The funders had no role in the study design; in the collection, analysis and interpretation of the data; in the writing of the report; and in the decision to submit the paper for publication.

ABSTRACT

**Background:** Gut fungal composition and its metabolites have not been assessed simultaneously in Parkinson’s disease (PD) despite their potential pathogenic contribution*.*

**Objective:**To evaluate the faecal metabolome and mycobiome in PD by assessing volatile organic compounds (VOCs) and fungal rRNA.

**Methods:** Faecal VOCs from 35 PD patients and two control groups (n=35; n=15) were assessed using gas chromatography and mass spectrometry. DNA was extracted from 44 samples: 18S rRNA gene amplicons were prepared and sequenced. Metabolomics, mycobiome and integrated analyses were performed.

**Results:** Several VOCs were more abundant and short chain fatty acids were less abundant in PD. *Hanseniaspora*, *Kazachstania,* uncultured *Tremellaceae* and *Penicillium* genera were more abundant, and *Saccharomyces* less abundant in PD (FDR<0.0007). *Torulaspora was* associated with PD and two VOCs.

**Conclusion:** PD patients had a distinct metabolome and mycobiome suggesting that fungal dysbiosis may contribute to PD pathogenesis.

INTRODUCTION

The aetiology of Parkinson’s disease (PD) is likely to be the result of a complex interplay between multiple environmental and genetic factors although the exact mechanisms are unknown [1]. It has been suggested that the enteral mucosa could be the point of entry of a neurotropic pathogen and growing evidence suggests that the connection between the gastrointestinal tract and central nervous system (gut-brain axis) may play an important pathogenic role in PD given its importance in immune response, local and systemic inflammation, barrier function against potential external pathogens and modulation of neurotransmission. Changes in the gut microbiota in PD have been proposed as a contributing factor and they have been evaluated in several studies [2]. Although results are highly heterogeneous and influenced by many confounding factors, a recent meta-analysis showed a pattern of an increase in intestinal mucin layer-degrading *Akkermansia* and reduction in short-chain fatty acid-producing *Roseburia* and *Faecalibacterium* [3]. The functional consequences of these gut microbiome abnormalities have not been systematically evaluated although changes in faecal metabolites with neuroprotective/ neurodegenerative function have been identified [4]. The contribution of non-bacterial members of the microbiome to the gut metabolome is unclear. Fungi are ubiquitous and they may play a similar role to bacteria. Inhalational exposure to fungal volatile organic compounds, including 1-octen-3-ol, induced dopaminergic cell loss and levodopa-responsive parkinsonism in animal models [5] suggesting that exposure to these metabolites may contribute to PD in humans. The discovery of 1-octen-3-ol in human faeces led us to investigate fungal metabolites in PD [6]. Here, we report the faecal mycobiome and metabolite alterations in PD patients after analysing volatile organic compounds (VOC) and highly-conserved fungal rRNA in faecal samples.

METHODS

**Participants and study design**

35 patients with PD diagnosed according to clinical criteria [7] were prospectively recruited from a specialist clinic at the National Hospital for Neurology in London between 2016-2018. Severity of parkinsonism and constipation were evaluated using validated clinical scales (Supplementary Table 1) [8]. There were two control groups with no known neurological or gastrointestinal pathology. The primary control group were healthy individuals from the bowel cancer screening programme who had normal colonoscopies: it was used for the main comparisons. Samples were taken prior to a bowel preparation regimen. As the sample storage temperature differed between PD and primary controls, an secondary control group of healthy volunteers from dietary study was included. Ethical approval was granted by the relevant Research Ethics Committees and informed consent was given by all participants.

**Sample collection**

Methods protocols are summarized in Supplementary Figure 1. Stool was collected at home by participants and delivered within 12 hours. If not produced on the day of collection, participants froze samples at home for 24 hours and kept them cool during transportation to the hospital. On collection by the research team, samples were stored as follows: PD samples -80oC; primary controls -20oC (VOC studies) and -80oC (genomics); and secondary controls -70oC, but -20oC for <48h (n=7) or 6 days (n=8) before VOC analysis.

**Metabolome and mycobiome analysis**

We have reported our optimised protocol [9]. 450-500mg of stool were analysed by headspace gas chromatography and mass spectrometry. Data were processed using Automated Mass Spectral Deconvolution and Identification System software (v2.71, 2012), the National Institute of Standards and Technology mass spectral library (v2.0, 2011) and the R package Metab (v1.8.0). Compounds were named using IUPAC nomenclature.DNA extraction, amplicon production and analysis were performed as described previously [10]. Both PSP Spin Stool DNA (Stratec) and QIAamp Fast DNA Stool Mini Kits (Qiagen) were used, with an additional bead-beating step before PSP extraction [11, 12]. Extracted DNA was quantified, amplified, bar-coded, and sequenced using 18S rRNA primers (listed in Supplementary Table 4) on an Illumina MiSeq platform in two batches [13]. Details of bioinformatic analysis can be found in the supplementary material.

**Statistical analysis**

Metaboanalyst v.5.0 was used for metabolomics statistical analysis [14] as reported previously [15]. Statistical comparisons between groups were performed using Chi-square test for categorical and t-test and analysis of variance (ANOVA) for continuous variables as appropriate; p values were reported and multiple comparisons were adjusted using the false discovery rate (FDR). Using R (v.3.3.2, 2016), a .BIOM file was generated from sequencing data and gut mycobiomes of PD and control patients summarised using taxa abundance plots and compared using measures of alpha and beta diversity. Samples with VOCs and mycobiome data (PD, n=24, Control, n=20) were integrated using the mixOmics R package, specifically the Data Integration Analysis for Biomarker discovery (DIABLO) framework as reported previously [10, 13, 16].

RESULTS

**Participants**

PD cases and primary controls were age-matched. Patient and control characteristics are shown (Supplementary Table 1).

**Metabolome**

After correction for multiple comparisons, 18 faecal VOCs were significantly associated with either primary control or PD groups (Table 1). Of these, only two (butan-2-one and 3-methylsulfanylpropanal) were significantly different when primary and secondary control groups were compared (FDR=0.0036 and FDR=0.016 respectively), suggesting their association with PD may be the result of batch effect (Supplementary Table 2 and Supplementary Figure 2).

There was no correlation between faecal VOC profiles of PD patients with severity of motor symptoms (measured by the MDS-UPDRS III), disease duration, or constipation severity.

1,3-ditert-butylbenzene was strongly associated with PD. We repeated the comparison between PD and secondary controls including and excluding this compound: the PCA showed a similar group separation (Supplementary Figure 3).

**Mycobiome**

After DNA extraction and quantification, 24 PD samples were included in the final analysis: 11 others were discarded due to low / poor yield. Mycobiome data on the primary control group was available for 20 subjects. The composition of the mycobiome was different in PD patients and controls (Figure 1A). The most dominant genus in PD and control samples was *Saccharomyces*. The alpha Shannon index of diversity was greater in PD samples than controls (Figure 1B). The Shannon diversity indices were not influenced by constipation or parkinsonism severity (data not shown).

Beta-diversity measures found a significant difference in the composition of the mycobiome of individuals with and without PD. NMDS plots of beta-diversity using Bray Curtis index (P=0.001; R2=0.15475) and unweighted UniFrac distance matrix (P=0.005; R2=0.07481) showed significant separation between PD and control samples (data not shown).

Taxa differential analysis identified the following genera were more abundant in PD: *Hanseniaspora*, *Kazachstania*, uncultured *Tremellaceae* and *Penicillium* (Figure 1D).

**Integrated Analysis using MixOmics**

The DIABLO model produced a BER (balanced error rate) of <15%; BER was used to ascertain the quality of the fitting of the model: the low value highlights that significant discriminants were found to separate the samples into multiple regions. There were strong overall correlations between VOCs and fungi in PD and in controls (Figure 1C). As PD and control samples were processed separately, a batch effect is possible and storage differences may be relevant. The positive correlations between PD fungi and VOCs were the main focus of the outcome of this analysis. *Torulaspora* (OTU-314) (more abundant in PD) had a strong positive correlation (r=0.86) with two VOCs: 6,6-dimethyl-2-methylene-bicyclo[3.1.1]heptane and 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene (β-pinene). There were negative correlations between VOCs and fungi in the control group. The one positive correlation was between indole and *Saccharomyces*.

DISCUSSION

This is the first study to assess the faecal volatile metabolome and mycobiome in PD patients simultaneously, and in combination. The metabolic analysis revealed compounds with different abundance in PD patients and controls. The mycobiome study showed differences at the genus and species level. These results suggest a distinct gut fungal profile and metabolic function in PD with potential pathogenic implications.

6 VOCs were significantly different when PD samples were compared with both control groups: 1,3-ditert-butylbenzene and 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene were associated with PD; 4 were associated with controls: of these, 3 were short chain fatty acids (SCFA) including butanoic acid. SCFA have a putative neuroprotective function and a relative deficiency has previously been reported in PD [17, 18]. Walker *et al*. demonstrated an inverse relationship between protein misfolding and aggregation and colonization with butanoic acid producing *Enterobacteriaceae* in *C. elegans* [19].

1,3-ditert-butylbenzene was found in 97% of PD patients but only in 11.4% of primary controls (and none of the secondary controls). It has been reported in faeces with coeliac disease, saliva, and sample catheters [20, 21]. Tisch *et al.* identified butylated hydroxytoluene (IUPAC 2,6-ditert-butyl-4-methylphenol) in PD patients [22]: it is similar to 1,3-ditert-butylbenzene. The significance of 1,3-ditert-butylbenzene in PD is unknown and requires further investigation.

1-methyl-2-(1-methylethyl)-benzene was associated with PD samples. Tisch *et al*. reported 1-methyl-2-(1-methylethyl)-benzene in association with Alzheimer’s and1-methyl-3-(1-methylethyl)benzene with PD [22]. These two VOCs are structurally similar and could be hard todistinguish using GCMS alone.

γ-terpinene and α-terpinene are human xenobiotic metabolites, usually derived from aromatic dietary plants rather than fungi, although in our study they were associated with *Torulaspora delbrueckii* in PD. PD patients may have adjusted their diet to manage disease-related constipation, but this is speculative. These compounds are derivatives of terpentine which can have anticholinesterase activity [23, 24].

We considered it unlikely that the changes in VOCs are secondary to different temperature storage conditions as comparison with secondary controls showed similar key findings.

*Hanseniaspora*, *Kazachstania*, *Penicillium* and uncultured *Tremellaceae* were associated with PD samples. *Saccharomyces* was significantly associated with control samples. There is no literature to explain a protective association between *Saccharomyces* and PD. Integrated analysis found Torulaspora was associated with PD. Mihaila *et al.* identified Torulaspora delbrueckii [25] in the oral mucosae of patients with early PD . There was a positive correlation between the presence of Torulaspora and α-pinene and β-pinene.

Only one study has evaluated the gut mycobiome in PD using fungal-specific internal transcribed spacer (ITS) amplicon sequencing, the authors did not combine the data with the metabolome changes[26]: the mycobiome composition was similar that in our study, although they found a significantly lower fungal to bacterial DNA ratio and genera did not significantly differ compared to controls. Whether these conflicting results may be explained by differences in study participants (particularly in control groups), methodological differences between studies or additional confounders that may affect the sensitive gut mycobiome (constipation, diet) is unclear. Both studies performed a cross-sectional evaluation of the mycobiome in PD populations with similar age and disease severity so further studies are warranted to evaluate any potential longitudinal changes in the mycobiome at different stages of the disease.

Ours was a pilot exploratory study and as such it had several limitations. No data on dietary habits, bacterial microbiome and additional comorbidities were available which could potentially have an impact on mycobiome and VOC composition. Similarly, controls were not matched by severity of constipation which may act as a confounder. Samples were extracted and analysed at different times: we acknowledge that results may have been influenced by a batch effect. The sample size was small which makes it difficult to make any generalizable conclusions. Although rDNA for fungi appeared to be associated with PD, it is possible that some DNA was arose from food; further work should investigate RNA, as a template instead of DNA, to determine whether these fungi are alive and not coming from participants’ diet. Whether external or colonizing pathogens, these taxa could potentially trigger or contribute to the cascade of pathogenic changes in the enteric mucosa of PD patients*.* Finally, while our study identified faecal metabolites that were significantly associated with PD, the scope of our study and its cross-sectional design do not establish the nature of these associations; further work is required to determine whether the named fungi synthesise the associated VOCs. Future studies should aim to replicate our results in greater samples whilst controlling for potential confounders and further investigate the causal relationship between identified VOCs and PD through in vivo and in vitro methods.

In this pragmatic, pilot study, there appear to be alterations in the faecal metabolome and mycobiome in patients with PD. More work is required to determine whether the changes are causal or associated with constipation, microbiome or dietary changes, and whether mycobiome composition changes at different disease stages in order to elucidate any potential pathogenic implications.

**Authors’ roles and approval:**

EDP-F: study conception and design, acquisition of data, analysis and interpretation of data, writing of first draft.

GGG: acquisition of data, analysis and interpretation of data, writing of first draft.

LF: acquisition of data, analysis and interpretation of data, critical revision of manuscript for intellectual content.

RS: acquisition of data, analysis and interpretation of data, critical revision of manuscript for intellectual content.

AF: acquisition of data, analysis and interpretation of data, critical revision of manuscript for intellectual content.

UZI: acquisition of data, analysis and interpretation of data, critical revision of manuscript for intellectual content.

TW: study concept and design, study supervision, critical revision of manuscript for intellectual content.

CP: study concept and design, study supervision, critical revision of manuscript for intellectual content.

All authors have read and approved the final version of the manuscript.

REFERENCES

[1] L.V. Kalia, A.E. Lang, Parkinson's disease, Lancet 386(9996) (2015) 896-912.

[2] Q. Wang, Y. Luo, K.R. Chaudhuri, R. Reynolds, E.K. Tan, S. Pettersson, The role of gut dysbiosis in Parkinson's disease: mechanistic insights andtherapeutic options, Brain (2021).

[3] H. Nishiwaki, M. Ito, T. Ishida, T. Hamaguchi, T. Maeda, K. Kashihara, Y. Tsuboi, J. Ueyama, T. Shimamura, H. Mori, K. Kurokawa, M. Katsuno, M. Hirayama, K. Ohno, Meta-Analysis of Gut Dysbiosis in Parkinson's Disease, Mov Disord 35(9) (2020) 1626-1635.

[4] A.H. Tan, C.W. Chong, S.Y. Lim, I.K.S. Yap, C.S.J. Teh, M.F. Loke, S.L. Song, J.Y. Tan, B.H. Ang, Y.Q. Tan, M.T. Kho, J. Bowman, S. Mahadeva, H.S. Yong, A.E. Lang, Gut Microbial Ecosystem in Parkinson Disease: New Clinicobiological Insights from Multi-Omics, Ann Neurol 89(3) (2021) 546-559.

[5] A.A. Inamdar, M.M. Hossain, A.I. Bernstein, G.W. Miller, J.R. Richardson, J.W. Bennett, Fungal-derived semiochemical 1-octen-3-ol disrupts dopamine packaging and causes neurodegeneration, Proc Natl Acad Sci U S A 110(48) (2013) 19561-6.

[6] I. Ahmed, R. Greenwood, B. Costello, N. Ratcliffe, C.S. Probert, Investigation of faecal volatile organic metabolites as novel diagnostic biomarkers in inflammatory bowel disease, Aliment Pharmacol Ther 43(5) (2016) 596-611.

[7] A.J. Hughes, S.E. Daniel, A.J. Lees, Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease, Neurology 57(8) (2001) 1497-9.

[8] L. Frank, L. Kleinman, C. Farup, L. Taylor, P. Miner, Jr., Psychometric validation of a constipation symptom assessment questionnaire, Scand J Gastroenterol 34(9) (1999) 870-7.

[9] S. Reade, A. Mayor, R. Aggio, T. Khalid, D. Pritchard, A. Ewer, C. Probert, Optimisation of Sample Preparation for Direct SPME-GC-MS Analysis of Murine and Human Faecal Volatile Organic Compounds for Metabolomic Studies. , Journal of Analytical & Bioanalytical Techniques 5(2) (2014) 9.

[10] A. Frau, J.G. Kenny, L. Lenzi, B.J. Campbell, U.Z. Ijaz, C.A. Duckworth, M.D. Burkitt, N. Hall, J. Anson, A.C. Darby, C.S.J. Probert, DNA extraction and amplicon production strategies deeply inf luence the outcome of gut mycobiome studies, Sci Rep 9(1) (2019) 9328.

[11] STRATEC Molecular GmbH, User manual PSP Spin Stool DNA Kit / PSP Spin Stool DNA Plus Kit, 2013. <http://www.negev-bioproducts.com/Content/Images/CatalogImages/ProductVariations/PV_ID_51/PSPSpinStool_StoolPlusKit_23.pdf>. 2021).

[12] Qiagen, QIAamp® Fast DNA Stool Mini Handbook, 2020. <https://www.qiagen.com/gb/resources/resourcedetail?id=2a3f2c0b-2e8a-49fd-b442-829108ae1a4a&lang=en>. 2021).

[13] A. Frau, U.Z. Ijaz, R. Slater, D. Jonkers, J. Penders, B.J. Campbell, J.G. Kenny, N. Hall, L. Lenzi, M.D. Burkitt, M. Pierik, A.C. Darby, C.S.J. Probert, Inter-kingdom relationships in Crohn's disease explored using a multi-omics approach, Gut Microbes 13(1) (2021) 1930871.

[14] J. Xia, N. Psychogios, N. Young, D.S. Wishart, MetaboAnalyst: a web server for metabolomic data analysis and interpretation, Nucleic Acids Res 37(Web Server issue) (2009) W652-60.

[15] R. Hough, D. Archer, C. Probert, A comparison of sample preparation methods for extracting volatile organic compounds (VOCs) from equine faeces using HS-SPME, Metabolomics 14(2) (2018) 19.

[16] F. Rohart, B. Gautier, A. Singh, L.C. KA, mixOmics: An R package for 'omics feature selection and multiple data integration, PLoS Comput Biol 13(11) (2017) e1005752.

[17] M.M. Unger, J. Spiegel, K.U. Dillmann, D. Grundmann, H. Philippeit, J. Bürmann, K. Faßbender, A. Schwiertz, K.H. Schäfer, Short chain fatty acids and gut microbiota differ between patients with Parkinson's disease and age-matched controls, Parkinsonism Relat Disord 32 (2016) 66-72.

[18] T.R. Sampson, J.W. Debelius, T. Thron, S. Janssen, G.G. Shastri, Z.E. Ilhan, C. Challis, C.E. Schretter, S. Rocha, V. Gradinaru, M.F. Chesselet, A. Keshavarzian, K.M. Shannon, R. Krajmalnik-Brown, P. Wittung-Stafshede, R. Knight, S.K. Mazmanian, Gut Microbiota Regulate Motor Deficits and Neuroinflammation in a Model of Parkinson's Disease, Cell 167(6) (2016) 1469-1480.e12.

[19] A.C. Walker, R. Bhargava, A.S. Vaziriyan-Sani, C. Pourciau, E.T. Donahue, A.S. Dove, M.J. Gebhardt, G.L. Ellward, T. Romeo, D.M. Czyż, Colonization of the Caenorhabditis elegans gut with human enteric bacterial pathogens leads to proteostasis disruption that is rescued by butyrate, PLoS Pathog 17(5) (2021) e1009510.

[20] R. Di Cagno, M. De Angelis, I. De Pasquale, M. Ndagijimana, P. Vernocchi, P. Ricciuti, F. Gagliardi, L. Laghi, C. Crecchio, M.E. Guerzoni, M. Gobbetti, R. Francavilla, Duodenal and faecal microbiota of celiac children: molecular, phenotype and metabolome characterization, BMC Microbiol 11 (2011) 219.

[21] Z. Dame, F. Aziat, R. Mandal, R. Krishnamurthy, S. Bouatra, S. Borzouie, A. Guo, T. Sajed, L. Deng, H. Lin, P. Liu, E. Dong, D.S. Wishart, The human saliva metabolome, Metabolomics 11 (2015).

[22] U. Tisch, I. Schlesinger, R. Ionescu, M. Nassar, N. Axelrod, D. Robertman, Y. Tessler, F. Azar, A. Marmur, J. Aharon-Peretz, H. Haick, Detection of Alzheimer's and Parkinson's disease from exhaled breath using nanomaterial-based sensors, Nanomedicine (Lond) 8(1) (2013) 43-56.

[23] M. Miyazawa, C. Yamafuji, Inhibition of acetylcholinesterase activity by bicyclic monoterpenoids, J Agric Food Chem 53(5) (2005) 1765-8.

[24] M. Bonesi, F. Menichini, R. Tundis, M.R. Loizzo, F. Conforti, N.G. Passalacqua, G.A. Statti, F. Menichini, Acetylcholinesterase and butyrylcholinesterase inhibitory activity of Pinus species essential oils and their constituents, J Enzyme Inhib Med Chem 25(5) (2010) 622-8.

[25] D. Mihaila, J. Donegan, S. Barns, D. LaRocca, Q. Du, D. Zheng, M. Vidal, C. Neville, R. Uhlig, F.A. Middleton, The oral microbiome of early stage Parkinson's disease and its relationship with functional measures of motor and non-motor function, PLoS One 14(6) (2019) e0218252.

[26] M.S. Cirstea, K. Sundvick, E. Golz, A.C. Yu, R.C.T. Boutin, D. Kliger, B.B. Finlay, S. Appel-Cresswell, The Gut Mycobiome in Parkinson's Disease, J Parkinsons Dis 11(1) (2021) 153-158.

FIGURE LEGEND

**Figure 1.** (A) Taxa summaries of PD and control groups at genus level. (B) Box plot showing gut mycobiome Shannon diversity of PD and healthy controls. (C) Heatmap illustrating identified correlations on multi-omics analysis. Correlation measured using Pearson’s correlation coefficient. (D) Bar chart comparing the normalized abundances of genera significantly associated with PD and control.

TABLE LEGEND

**Table 1.** Summary of volatile organic compounds with significantly difference abundance in Parkinson’s disease and control groups.