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A prebiotic dietary pilot intervention restores faecal metabolites and may be neuroprotective in Parkinson's Disease



Janis Rebecca Bedarf^{1,2,3}✉, Stefano Romano³, Silke Sophie Heinzmann⁴, Anthony Duncan^{3,5}, Maria H. Traka⁶, Duncan Ng⁶, Daniella Segovia-Lizano⁶, Marie-Christine Simon⁷, Arjan Narbad³, Ullrich Wüllner^{1,2} & Falk Hildebrand^{3,5}✉

Current treatment of Parkinson's Disease (PD) remains symptomatic, and disease-modifying approaches are urgently required. A promising approach is to modify intestinal microbiota and key metabolites of bacterial fermentation: short-chain fatty acids (SCFA), which are decreased in PD. A prospective, controlled pilot study (DRKS00034528) was conducted on 11 couples (PD patient plus healthy spouse as control (CO)). Participants followed a 4-week diet rich in dietary fibre, including intake of the prebiotic Lactulose. Gut metagenomes, faecal and urinary metabolites, and clinical characteristics were assessed. The dietary intervention significantly augmented faecal SCFA and increased *Bifidobacteria* spp., reducing PD-related gastrointestinal symptoms. The pre-existing bacterial dysbiosis in PD (depletion of *Blautia*, *Dorea*, *Erysipelatoclostridium*) persisted. Bacterial metabolite composition in faeces and urine positively changed with the intervention: Brain-relevant gut metabolic functions involved in neuroprotective and antioxidant pathways, including S-adenosyl methionine, glutathione, and inositol, improved in PD. These promising results warrant further investigation in larger cohorts.

The human gut microbiota forms a complex community with high taxonomic diversity and complex metabolic activity. The bi-directional microbiota-related gut-brain signalling has emerged as an important factor in human brain (patho-)physiology¹. The gut microbiome is involved in immune homeostasis and might have a role in the development or progression of neuro-psychiatric diseases, including multiple sclerosis, Alzheimer's and Parkinson's disease (PD)^{2,3}. The early involvement of the gastrointestinal tract in PD – sometimes preceding motor symptoms for years—has been linked to the intestinal dysbiosis in multiple independent cohorts (reviewed in refs. 4,5). Taxonomic alterations hinted at impairments of the intestinal barrier and altered immune function as well as metabolic changes⁶, but it remains unclear, whether these changes are the causes or consequences of disease.

Among the variety of metabolites that have been shown to beneficially impact both intestinal barrier and blood-brain barrier (BBB)-integrity^{7–9}, short-chain fatty acids (SCFA, represented mainly

by butyrate, propionate, and acetate, and fermented from dietary fibres¹⁰) seem to play a pivotal role. For example, SCFAs can directly influence the colonic epithelium, act as major energy substrates for colonocytes and promote tight junction proteins and neuronal activation through stimulation of G protein-coupled receptors^{11,12}. Administration of butyrate in transgenic animal models of PD improved motor deficits, reduced inflammation and alleviated dopamine deficiency^{13,14} and together with a high-fibre diet, reduced markers of cerebral inflammation in aged mice^{15,16}. SCFAs may thus present a link between gut microbiota dysbiosis and neurodegenerative alterations¹². In line with a concept of anti-inflammatory and neuroprotective effects of SCFA, studies in human PD found depleted faecal SCFA concentrations and reduced abundance of SCFA-producing bacteria compared with healthy controls^{17,18}. Moreover, an integrated study examining both faecal and plasma SCFA levels, suggested that SCFAs may even reflect disease severity and could thus

¹Department of Movement Disorders (PSB), Centre of Neurology, University Hospital Bonn, Bonn, Germany. ²German Centre for Neurodegenerative Diseases, DZNE Bonn, Bonn, Germany. ³Food, Microbiome, and Health, Quadram Institute Bioscience, Norfolk, UK. ⁴Research Unit Analytical BioGeoChemistry, Helmholtz Centre Munich, Neuherberg, Munich, Germany. ⁵Decoding Biodiversity, Earlham Institute, Norfolk, UK. ⁶Food & Nutrition National Bioscience Research Infrastructure, Quadram Institute Bioscience, Norfolk, UK. ⁷Institute of Nutritional and Food Sciences (IEL), Nutrition and Health, University of Bonn, Bonn, Germany. ✉e-mail: janis.bedarf@ukbonn.de; falk.hildebrand@quadram.ac.uk



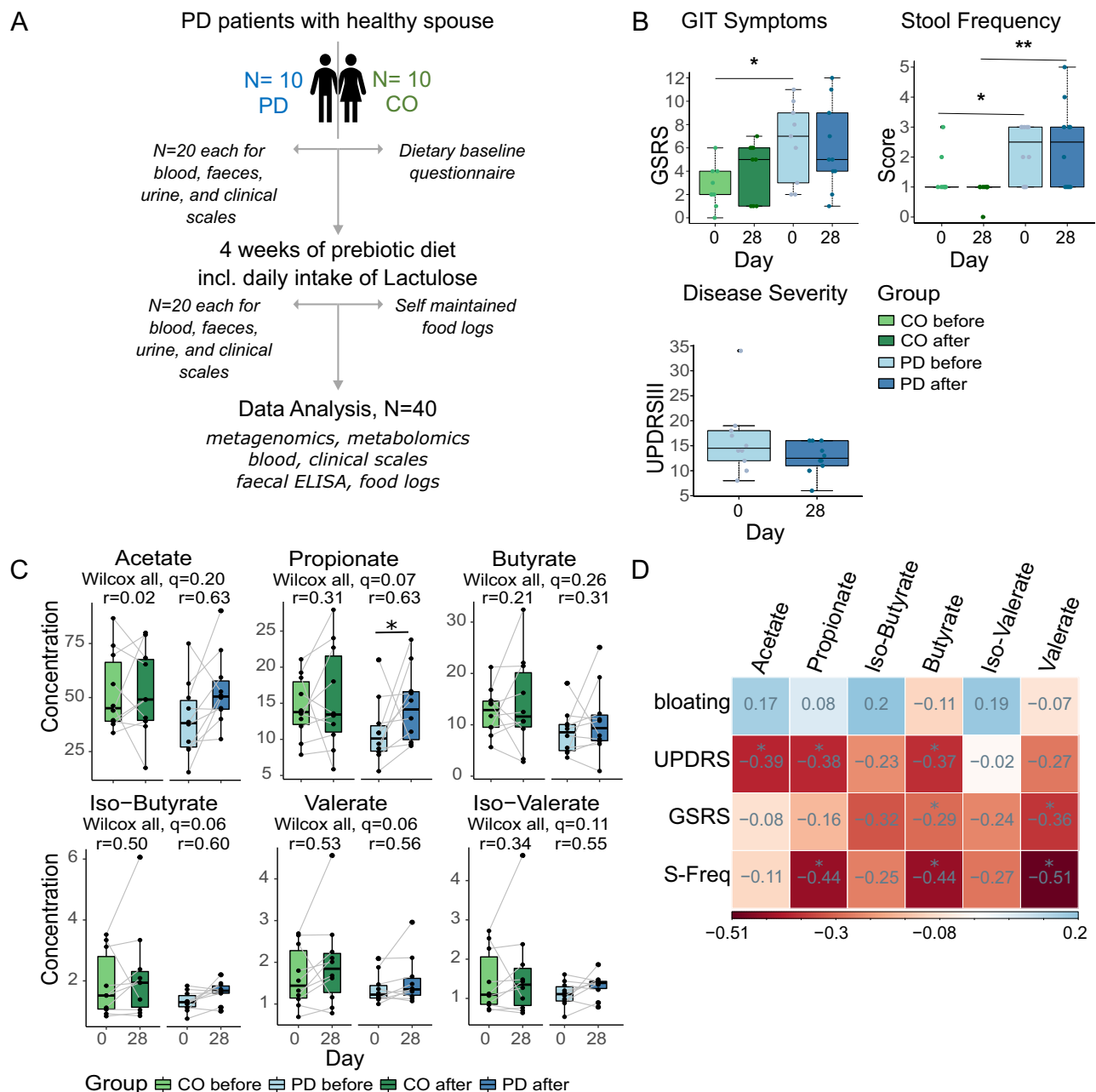


Fig. 1 | SCFA and clinical scales. **A** Study design; **B** clinical measures, disease severity measured with MDS-Unified Parkinson's Disease Rating Scale Part III (UPDRSIII), gastrointestinal symptom scale (GSRs) with improvement in PD after prebiotics, and stool frequency score before and after prebiotics. Asterisk indicates the level of significance (single asterisk = $p < 0.05$, double asterisk = $p < 0.01$); **C** targeted SCFA measurements in faecal samples show increasing SCFA concentrations in both groups after prebiotics (Wilcoxon signed-rank test). "Wilcox all" represents testing of

the entire sample set, and r gives the respective effect size for post-hoc tests; an asterisk indicates a significant difference in post-hoc tests; and **D** partial correlation of SCFA concentrations with clinical measure shows a sign. inverse correlation of acetate, propionate, and butyrate with disease severity measured with UPDRSIII, while stool frequency was inversely correlated to propionate, butyrate, and valerate. GSRs shows a sign. negative correlation with butyrate and valerate, red = positive and blue = negative correlation, numbers indicate the correlation coefficient r .

serve as a surrogate marker for PD¹⁹. In concordance with this data, a high fibre 'Mediterranean' diet was reported to decrease the risk for PD^{20,21}, in comparison to a low fibre, high caloric 'western' diet^{22,23}.

Altering the microbiome composition and metabolism through dietary interventions, particularly targeting SCFA²⁴ might thus evolve as a therapeutic option to protect against or modulate the course of PD and other neurodegenerative diseases^{25,26}. We investigated here whether a dietary intervention designed to prebiotically enrich colonic SCFA production would alter faecal SCFA concentrations and ameliorate gut microbiome dysbiosis as well as gastrointestinal symptoms in PD.

Results

Participants and clinical measures

Ten of eleven enrolled couples (each consisting of 1 PD and 1 spouse as control (CO)) completed the study (clinical characteristics and dietary baseline information in Supplementary Table 1) and performed the dietary intervention for 4 weeks (Fig. 1A). One couple dropped out of the study before the second visit due to non-study related reasons. PD patients were mild to moderately advanced (Hoehn and Yahr 1–2).

No serious adverse events occurred. Some participants ($n = 4$ PD and $n = 4$ CO) had tolerable bloating when starting the dietary

intervention, which ceased during the study (self-reported); no diarrhoea was reported. Safety parameters (sodium, potassium, C-reactive protein, and thyroid-stimulating hormone) were comparable between groups before prebiotics, and there were no changes after prebiotics. All participants followed an omnivorous diet prior to the study with occasional intake of probiotics in 3 subjects ($n = 1$ PD and $n = 2$ CO, Supplementary Table 1).

To measure the participants' compliance with the dietary recommendations, a food adherence score was calculated based on the recommended consumption of raw apples (see methods). This showed overall good adherence throughout the study cohort; four participants ($n = 2$ CO and $n = 2$ PD subjects, of whom two belong to one couple) had less compliance with our dietary intervention. (Supplementary Fig. 1a).

The average fibre intake during the intervention was similar in both CO and PD (median 25.1 g/d and 23.5 g/d, respectively, Supplementary Fig. 1b). Although this is below the recommended daily intake of 30 g/d in Germany, it is still above average reported levels in Germany of 23 g/d and 20 g/d for males and females, respectively²⁷.

UPDRSIII, as a measure of the PD disease severity, was slightly reduced after the prebiotic intervention (Wilcoxon signed-rank test, $p > 0.05$, Fig. 1B). While gastrointestinal symptoms (sum score of the modified GSRS) were significantly higher in PD patients compared to CO before prebiotics, no significant difference could be observed after prebiotics (Wilcoxon rank sum test, $p = 0.025$ and $p = 0.48$, respectively, Fig. 1B); however, this could be related to increasing gastrointestinal symptoms in the CO, mostly related to constipation and bloating. Stool frequency (as a sub-measure of the GSRS) significantly differed between PD and CO, and this did not change during diet intervention (Wilcoxon rank sum test, $p = 0.043$ and $p = 0.007$, respectively, Fig. 1B).

Stool frequency and gastrointestinal symptoms differed in participants with a low (<25 g/d) or high (>25 g/d) fibre intake, with the latter having reduced motor symptoms after prebiotics, compared to the low fibre group (Supplementary Fig. 1d and Supplementary Table. 2). Faecal markers of intestinal inflammation (calprotectin) or intestinal protein loss (alpha-1-antitrypsin, Supplementary Fig. 1c) were comparable between study groups and remained unchanged after prebiotics.

The prebiotic diet increased faecal SCFA levels and improved gastrointestinal functioning in PD

Faecal SCFA concentrations were lower in PD subjects at baseline (Wilcoxon rank sum test, $p < 0.05$, $q > 0.1$), note that baseline SCFA results were comparable between targeted SCFA measures (Fig. 1C) and SCFA based on metabolomics (Supplementary Fig. 2a). The short-term dietary intervention increased concentrations of most SCFAs (targeted measures) in both groups, but this was only significant for propionate in PD (Fig. 1C), as the statistical power was likely limited by cohort size. Considering effect sizes (Fig. 1C), the increase in several SCFA concentrations was markedly greater in PD than in CO, possibly related to an increased Lactulose intake in the PD group.

Multivariate testing of the SCFA composition between PD and CO either before or after the diet intervention revealed greater similarity after prebiotics (perMANOVA before $R^2 = 0.13$, $p = 0.08$, and after $R^2 = 0.01$, $p = 0.8$, respectively, Supplementary Fig. 2b), indicating that the SCFA profile of PD patients adapted to a healthy state after prebiotics. PD participants with lower fibre intake (<25 g/d) seemed to increase relatively more in the faecal SCFA concentrations with the diet intervention (Supplementary Fig. 2c), suggesting that the increase of SCFA might indeed depend on the actual fibre deficit.

Stool frequency, modified GSRS, and PD disease severity were inversely correlated with several SCFA, including butyrate, in PD patients (Fig. 1D), suggesting that increased SCFA concentrations were linked with better gastrointestinal functioning and reduced disease symptoms.

The prebiotic diet enhanced beneficial *Bifidobacteria* in both study groups, yet failed to restore the dysbiosis associated with PD

All 40 faecal samples from 20 participants at two time points each, were sequenced using short-read metagenomics. MGS (metagenomic species) dereplicated from MAGs (metagenomics assembled genomes) were used to obtain the taxonomic composition for all samples.

The genera *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Blautia* A, and *Phocaeicola* dominated the overall taxonomic composition (Supplementary Fig. 3b).

Most of the species level differences in faecal sample composition were driven by household ($R^2 = 0.47$, $p < 0.001$, perMANOVA, dbRDA), i.e. most samples of the same household clustered together (Fig. 2A). Disease status (PD vs CO) and diet intervention (before vs after prebiotics) explained only 9.8% of total variance together (Fig. 2B, C). This "household-effect" was the prevalent source of variation between individual gut microbiomes at all taxonomic levels ($R^2 = 0.41$ – 0.47) as well as functional potential and metabolomics profiles ($R^2 = 0.36$ – 0.47 , Supplementary Table 3) and was retained also when removing intra-individual variation (Supplementary Table 4).

For our further analysis, we therefore used statistics conditioned for household effects. An ordination conditioned by household revealed that the dissimilarity in taxonomic composition was driven by differences between PD and CO subjects ($R^2 = 0.27$, $p < 0.001$, permutation test), or differences between before and after prebiotics ($R^2 = 0.2$, $p < 0.001$, permutation test, Fig. 2B, C):

Several taxa were identified representing the previously documented PD dysbiosis, including *Akkermansia muciniphila* and *Prevotella* spp. (*sp900313215*) enriched in PD subjects, while *Bacteroides fragilis* and *Anaerostipes hadrus* were enriched in CO (Fig. 2C). The strongest signal relating to diet intervention was an enrichment of several *Bifidobacteria* spp. after prebiotics in both CO and PD (multivariate and univariate tests, Figs. 2C and 3C and Supplementary Fig. 3c). In PD patients, we observed a significant enrichment in six different *Bifidobacteria* spp., four of which were also increased in CO after prebiotics (Fig. 3A and Supplementary Fig. 3c Wilcoxon signed-rank test). Similarly, *UMGS1975* (Christensenellales) was enriched after prebiotics in both groups, but more so in PD subjects that were also enriched for *UMGS1975* before the intervention. *Streptococcus thermophilus* was reduced only in the PD group after the diet intervention.

The PD-associated dysbiosis was not compensated by the diet intervention: *Blautia*, *Dorea*, *UBA1191* (Anaerovoracaceae) and *Erysipelatoclostridium* remained depleted in PD and *UMGS1975* (Christensenellales) remained increased in comparison to CO before and after prebiotics (all $p < 0.05$, $q > 0.1$ before prebiotics; $p < 0.05$, $q < 0.1$ after prebiotics, Wilcoxon rank sum test, Fig. 2D, E). This was also reflected in a markedly different species composition between PD and CO after prebiotics (perMANOVA, $R^2 = 0.08$, $p = 0.03$ and $R^2 = 0.05$, $p = 0.16$, after and before, respectively, Fig. 2B). Notably, some subjects had elevated levels of genus *Klebsiella* ($p > 0.05$), which decreased after prebiotics. Since this genus contains several pathobionts, this could be a benefit provided by prebiotics.

Alternatively, we cannot exclude that the high abundance of *Klebsiella* in some samples was due to inadequate storage conditions or a missing stabilizer during sample collection by the participant. To assess the extent of dysbiosis further, we assessed common bacterial guilds of the human gut microbiome (represented through Enterotypes, ES) and their prevalence in our samples²⁸. ES, an extension of the earlier enterotype concept²⁹, were recently introduced by us to capture the generalized global gut microbial variation, relying on a machine learning model trained on >5000 gut microbiomes from the public domain²⁸. The five ES thus describe five typical bacterial guilds found in most human gut microbiomes; deviations from these expected bacterial compositions are captured in the "ES model fit" score.

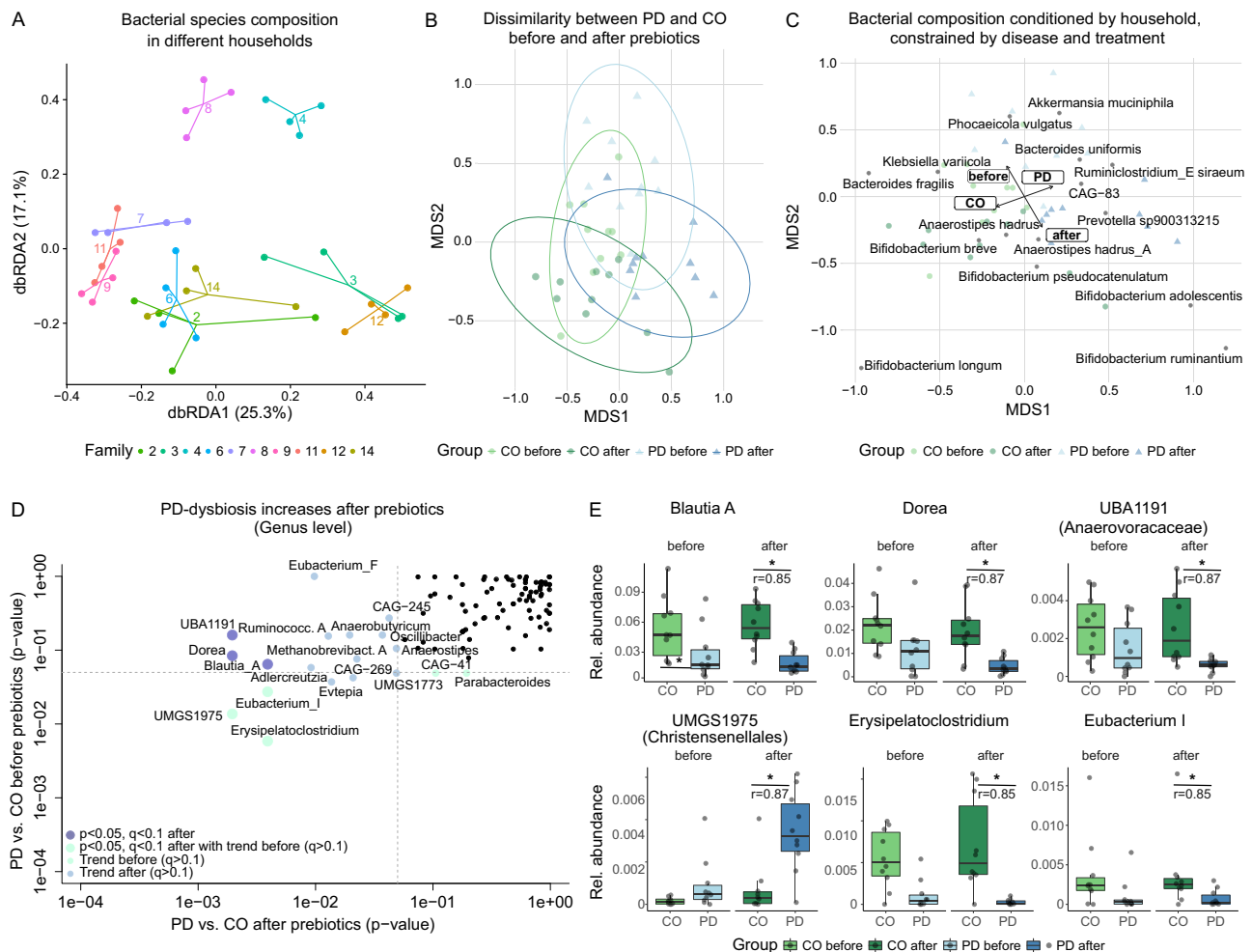


Fig. 2 | Household-effect and taxonomic differences between PD patients and healthy CO. **A** The distance-based redundancy analysis (dbRDA) shows a strong clustering of metagenomes by household, i.e. from PD with their respective healthy spouse/CO, further termed “household-effect”; this effect explained 46% of data variability on MGS species level; **B** dbRDA conditioned for households reveals a sign. difference in taxa dissimilarity between PD and CO after prebiotics, but not before prebiotics; **C** top 15 correlated taxa (MGS species) between the constrained dbRDA (conditioned for households) and study group (PD/CO) or diet intervention (before/after prebiotics), indicating a strong association of

different *Bifidobacteria* spp. with the intervention. **D, E** Several genera were markedly reduced in the PD group after prebiotics, paralleled by several trends, e.g. enrichment in *Eubacterium F* and *Methanobrevibacter_A* in PD; some of these Genera already tended to differ before prebiotics ($p < 0.05$, but $q > 0.1$ before prebiotics; $p < 0.05$, $q < 0.1$ after prebiotics; data in **D** is presented with p -values generated by univariate tests between PD and CO on a log scaled axis, Wilcoxon signed-rank test; data in **E** is presented as relative abundance of the different genera). Asterisk indicates $p < 0.05$, $q < 0.1$, and r gives the respective effect size for Wilcoxon signed-rank tests.

Overall, both our study groups showed a good ES model fit, comparable between both groups (Supplementary Fig. 4a). In the PD group ES model fit increased marginally after prebiotics ($p > 0.05$, Wilcoxon signed-rank test), while this increase was significant in CO ($p = 0.049$, Wilcoxon signed-rank test, Supplementary Fig. 4a), indicating that the gut microbiome after the diet intervention is more similar to an average gut microbiome on which the enterosignature model was trained²⁸.

The dietary intervention had a strong impact on overall ES composition (Supplementary Fig. 4b), explaining 30% of variance amongst samples (after conditioning out the “household-effect”, $R^2 = 0.30$, $p < 0.001$, perMANOVA, Supplementary Fig. 4c). This was probably driven by an increase in ES-Bifi (*Bifidobacterium* dominated guild, $p < 0.01$ in both CO and PD, Wilcoxon signed-rank test) and decrease in ES-Firmi (*Firmicutes* dominated guild, $p = 0.08$ and $p = 0.04$ in CO and PD, respectively, Wilcoxon signed-rank test) after the prebiotic intervention (Fig. 3B). Note that this effect seemed overall stronger in PD than CO microbiomes and is potentially related to a higher intake of Lactulose in this group. In order to capture to what extent baseline conditions in the microbiome composition predict the changes after prebiotics, we correlated the ES model fit of baseline samples (before prebiotics) a measure of “normality” of the gut

microbiome to changes in microbiome composition (MGS Bray–Curtis distance between both time points).

This showed that microbiomes with a higher ES model fit (typical microbiomes) retain a more similar microbiome composition during the diet intervention ($r = -0.42$, $p = 0.07$, Supplementary Fig. 4e), potentially reflecting a greater resilience. These ES typical communities were also able to more profoundly change the SCFA profile they produce ($r = 0.36$, $p = 0.12$, Supplementary Fig. 4e), hinting at greater functional flexibility inherent to such communities.

The prebiotic intervention enhanced the bacterial metabolic potential for fatty acid metabolism in PD and CO and strengthened neuroprotective and antioxidative pathways in PD

Given the apparent consolidation of the PD dysbiosis following prebiotics, we sought to ascertain whether the intervention exerted any influence on the functional potential of the microbiome. For this, we used key metabolic pathway modules relevant for gut bacterial metabolism from the KEGG database³⁰, as well as GBM³¹ modules especially relevant for human brain functions. The functional composition of both, general and brain-relevant pathways, was again dominated by household effects, which accounted for

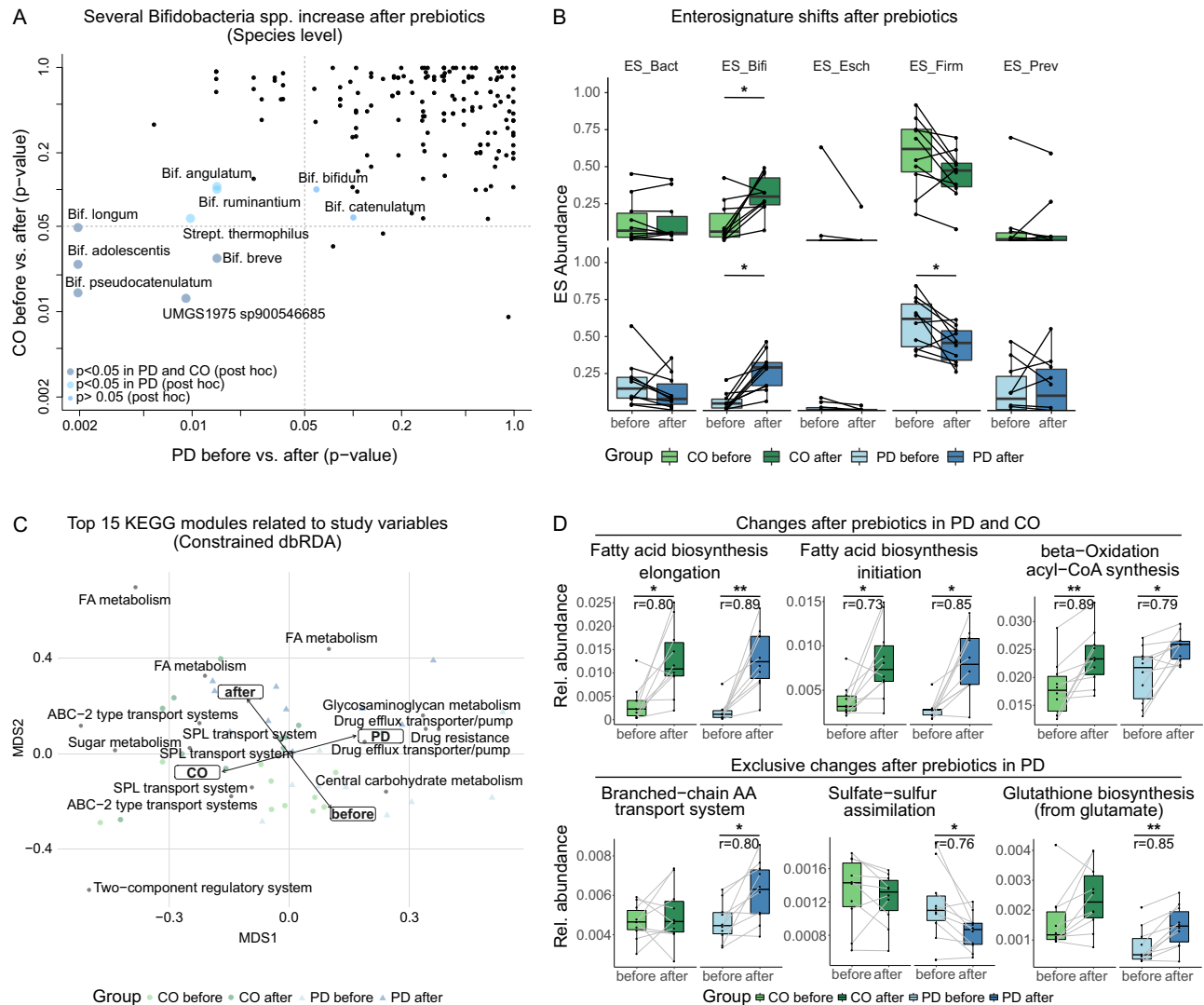


Fig. 3 | Gut bacterial communities affected by the prebiotic diet intervention.

A several taxa were significantly different between before and after prebiotics tests in both groups; $n = 6$ *Bifidobacteria* spp. were enriched after prebiotics in PD patients, of which $n = 4$ were also enriched in healthy CO individuals; $n = 2$ taxa are only changed in PD (*S. Thermophilus*, *UMGS1975*, data is presented as p -values generated with univariate tests/Wilcoxon signed-rank test between before and after prebiotics on log scaled axes); B Enterosignatures (ES) ES-*Bifidobacterium* (ES-Bifi) in both groups while ES-Firmicutes (ES-Firmi) decreased (sign. only in PD group, Wilcoxon rank sum test; C top 15 Kyoto Encyclopaedia of Genes and Genomes (KEGG) modules in relation to the study group (PD/CO and diet intervention (before/after

prebiotics), constrained dbRDA, conditioned for household), shows a strong correlation of fatty acid (FA) metabolism with the intervention (permutation test, $R^2 = 0.3$, $p < 0.001$) as well as several functions correlated to either PD or CO (permutation test, $R^2 = 0.16$, $p < 0.001$, SPL saccharide, polyol and lipid transport system); and D Differences in KEGG Modules before vs after prebiotics (relative abundance, Wilcoxon signed-rank test), increased microbial genes related to fatty acid metabolism after prebiotics were similarly observed in PD and CO subjects (upper panel), on the other hand several genes were only changed in PD (lower panel). Single asterisk = $p < 0.05$, double asterisk = $p < 0.01$ (AA amino acid), r gives the respective effect size for post-hoc tests.

42.1% and 49.1% of the total variance. In contrast, differences between PD and CO, explained 7.7% and 8.8% of variance in KEGG modules, while 7% and 6% of variance were explained through GBM modules, respectively (all $p < 0.001$, perMANOVA). Interestingly, the similarity for KEGG modules between PD and CO before prebiotics increased after prebiotics, while GBM differences between PD and CO became more pronounced after prebiotics (perMANOVA conditioned for household, KEGG before: $R^2 = 0.10$, $p = 0.028$, after prebiotics: $R^2 = 0.08$, $p = 0.048$, GBM before: $R^2 = 0.07$, $p = 0.025$, after prebiotics: $R^2 = 0.10$, $p = 0.003$).

Investigating the modules driving these differences between PD and CO, we find the PD microbiome enriched in bacterial functions related to drug efflux and drug resistance, similar to our findings in 2017⁶. CO microbiomes were instead enriched in genes related to saccharide, polyol, and lipid transport systems (Fig. 3C). PD patients had increased abundance of genes related to butyrate synthesis before and after prebiotics, and

decreased S-adenosylmethionine (SAM) synthesis ($p < 0.05$, $q > 0.1$, Wilcoxon rank sum test) compared to CO, but the latter was normalized after prebiotics (n.s. difference to CO, Supplementary Table 6 and Supplementary Fig. 5).

After the prebiotic intervention, other functions differed between PD and CO, including dihydroxyphenylacetic acid (DOPAC) synthesis and GABAII synthesis (decreased abundance in PD likely as a lack of increase compared to the CO group after prebiotics, $p < 0.05$, $q < 0.1$, Supplementary Table 6 and Supplementary Fig. 5) and Tryptophan degradation (persistently increased abundance in PD compared to CO, Wilcoxon rank sum test, $p < 0.05$, $q > 0.1$, Supplementary Table 6 and Supplementary Fig. 5). In summary, the latter pathways (DOPAC, GABA, Tryptophan) seem to be less responsive to prebiotics in PD relative to the CO group.

We next determined bacterial functions changed with the prebiotic diet (before vs after prebiotics comparisons). In both CO and PD, multiple fatty

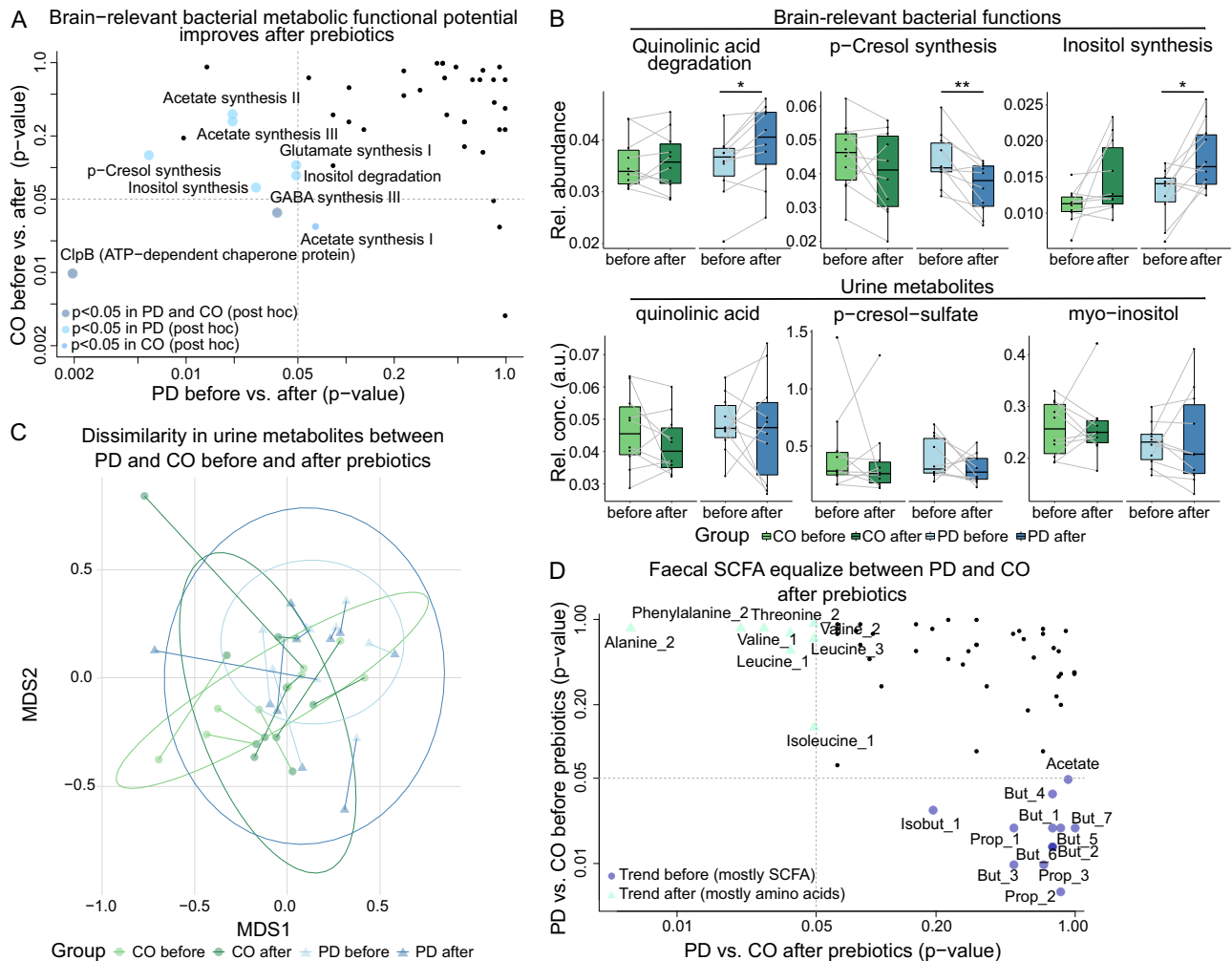


Fig. 4 | Functional metabolic potential and metabolite levels. **A** Brain-relevant functional metabolic potential (gut-brain modules (GBM)) improves after prebiotics in PD in a potentially neuroprotective manner, e.g. p-cresol synthesis decreased and quinolinic acid degradation increased, both leading to neurotoxic compounds. The x- and y-axis correspond to p-values of the Wilcoxon signed-rank test for changes due to the prebiotic intervention of healthy CO (x-axis) and PD (y-axis) patients. **B** The relative abundance of bacterial functional GBM modules improved only in PD patients after the prebiotic intervention compared to baseline before prebiotics (upper panel, $p < 0.05$, $q < 0.1$, Wilcoxon signed-rank test). This was also reflected in corresponding urinary metabolites (bottom panel, $p > 0.05$, Wilcoxon signed-rank

test, relative concentrations, a.u., arbitrary units). **C** Urine metabolite composition differed between PD and CO before prebiotics, but normalized after prebiotics (permutation test, before prebiotics: $R^2 = 0.08$, $p = 0.045$; after prebiotics: $R^2 = 0.05$, $p = 0.36$). Single asterisk $p < 0.05$, double asterisk $p < 0.01$; **D** faecal metabolite profiles before prebiotics were different in SCFA between PD and CO. After prebiotics these differences are no longer observed, instead amino acid concentrations were different between PD and CO (all $q > 0.1$, Wilcoxon rank sum test); But butyrate, Isobut isobutyrate, Prop propionate. The numerical suffix (e.g. _2) associated with each metabolite represents a distinct nuclear magnetic resonance (NMR) peak for that particular metabolite.

acid metabolism-related genes increased in relative abundance after prebiotics, involved in the synthesis of medium- and long-chain fatty acids (Fig. 3C, D).

In addition, 19 KEGG modules related to nucleotide, amino acid, carbohydrate and lipid metabolism were significantly changed in both PD and CO after prebiotics (Supplementary Table 6). Notably, the prebiotic diet decreased formaldehyde assimilation, which is identified as increased in the PD dysbiosis³².

Compared to baseline values before prebiotics, several alterations in KEGG modules ($n = 20$) were only different in PD patients (but not in CO) after prebiotics, e.g. Sulphate-sulphur-assimilation pathway associated genes decreased, genes related to glutathione (GSH), serine synthesis as well as branched chain amino acid transport increased (Wilcoxon signed-rank test, Supplementary Table 6 and Fig. 3D), the latter matching our metabolomic observations (see below). Notably, several bacterial pathways leading to brain-relevant metabolites changed after prebiotics ($p < 0.05$, Wilcoxon signed-rank test, Fig. 4A, B). These could have potentially positive implications for PD patients, e.g. the changes in genes related to both the

neurotoxic p-cresol and quinolinic acid (decreased synthesis and increased degradation, respectively). Inositol synthesis genes increased, and inositol degradation genes decreased (a neuroprotective metabolite).

Bacteria driving changes in functional potential

To better understand the contribution of taxonomic changes to the observed changes in bacterial pathways, we correlated KEGG and GBM modules to MGS species clusters (partial correlation to correct for the time series). These functional metabolic changes could be correlated to different microbes, which were all previously implicated in PD^{4,6} (Supplementary Table 7). Fatty acid synthesis (initiation and elongation) and beta-oxidation/acyl-CoA-synthesis correlated positively to several *Bifidobacteria* species, but negatively to *Alistipes* spp. and *Barnesiella* spp. The former species contributes to the dysbiosis associated with PD, suggesting that an induced depletion of these taxa could benefit PD patients.

Several members of *Bifidobacteria* and *Oscillospiraceae* correlated to branched chain amino acid transport pathways (changed only in the PD group in before vs after prebiotics comparisons), while, e.g. *Blautia A* spp.

showed a strong negative correlation. Further, *Eubacterium I* spp. were the strongest sign. positively correlating taxa with Sulphate-sulphur-assimilation (Supplementary Table 7). Regarding brain-relevant GBM functions, a strong positive correlation was observed for different *Alistipes* spp. with inositol degradation as well as with p-cresol-synthesis, again suggesting that a diet-depleted *Alistipes* spp. would be of benefit in PD due to potentially reduced levels of neurotoxic compounds.

PD faecal and urine metabolites profiles normalized with the diet intervention

Faecal metabolites³³ approximate the bacterial metabolism in the gut, while urine metabolites³⁴ can reflect both human and bacterial metabolism. We therefore analysed both faecal and urine metabolomes (NMR) of our cohort ($n = 80$, coinciding with faecal sample collections). Variance in both urine and faecal metabolites was again mostly driven by differences between households (perMANOVA, $R^2 = 0.36$, $p < 0.001$, and $R^2 = 0.45$, $p < 0.001$; respectively, Supplementary Table 3), but study groups (PD vs CO) had a sign. different urine metabolites composition ($R^2 = 0.04$, $p = 0.03$).

Testing dissimilarities in urine metabolites for both timepoints separately (conditioning for households) revealed that the PD urinary metabolite profiles after the prebiotic intervention resembled the CO profiles (Fig. 4C), although in both CO and PD patients the composition shifted after the diet intervention (Supplementary Table 5). However, no clear causative biological signal was derived from the single metabolite composition to better explain this improvement based on differential abundance testing of all metabolites in urine between PD and CO. Comparing faecal metabolites between PD and CO before and after prebiotics revealed that several faecal SCFA normalized after prebiotics in the PD group (e.g. Iso-/butyrate and propionate); instead, amino acids concentrations differed, such as a decrease in alanine and iso-/leucine (Wilcoxon Rank Sum test, $p < 0.05$, $q > 0.1$, Fig. 4D), or neurotoxic metabolites (but $p > 0.05$, Fig. 4B), corresponding to the observed changes in the gut metabolic functional potential. Although limited by our cohort size, this highlights a plausible biological relation and potentially important result for PD patients.

Discussion

The short-term prebiotic diet intervention was safe and well tolerated in all subjects (mild bloating was the only complaint) and resulted in increased SCFA concentrations and improved gastrointestinal symptoms in PD. We observed a trend towards reduced disease severity (UPDRSIII), which was inversely correlated with SCFA concentrations, indicating the importance of SCFAs for gastrointestinal functioning and possibly also motor symptoms in PD. Interestingly, in a recent study of faecal microbiota transplant³⁵, the improvement in motor symptoms became pronounced between the sixth and twelfth month after the transplant, suggesting that the effects of a dietary intervention might also occur at a later point than assessed here.

We observed several gut microbes that were sign different before and after the intervention between PD and CO metagenomes, and these coincided with several well-known “dysbiotic” PD gut microbes. However, on a functional and metabolic level, the prebiotic diet appeared to remove most of the aberrations observed between PD and CO patients, presumably improving gut microbial metabolism in PD patients: Apart from normalized SCFA concentrations in the faecal metabolite profile, we also found decreased branched chain amino acids (BCAA), potentially harmful for neurons by oxidative stress³⁶, mitochondrial dysfunction³⁶, and a decrease in dopamine synthesis³⁷, while presumably neurotoxic compounds associated with PD^{38,39} were reduced in the urine metabolites after prebiotics (the aromatic amino acid p-cresol-sulfate, and the tryptophan/kynurenine downstream metabolite quinolinic acid). This coincided with changes in corresponding bacterial pathways derived from the metagenome. The prebiotic intervention seemed to normalize metabolite profiles between both groups, as PD urinary metabolomic profiles were no longer distinct from CO after the prebiotic intervention. Based on our patient stratification by fibre intake, prebiotics should be considered, especially in subjects with a fibre deficit.

Our analyses identified and had to be statistically corrected for a pre-vailing “household effect”. The importance of family shared microbes (possibly arising from social interactions, shared environments or diet) has been described before^{40,41}, but the scale accounting for >36% (mean 44%) of explained variance at all data levels (Supplementary Table 3) was surprising to us.

Prebiotics are defined to be “a substrate that is selectively utilized by host microorganisms conferring a health benefit”⁴² and specific criteria⁴³ are employed to determine whether a compound can be classified as a prebiotic. Lactulose has been widely researched for use in chronic constipation. We argue that it also fulfils the function of an efficient representative prebiotic, a role that is perhaps not as widely recognized as it should be. Unlike traditional plant-derived prebiotics, Lactulose is an artificial polysaccharide composed of galactose and fructose⁴⁴ and was observed to increase the number of culturable *Bifidobacteria*, *Lactobacillus*, and *Streptococci* in healthy humans, while simultaneously reducing *Bacteroides*, *Clostridium*, and *Eubacterium*^{45,46} with presumably dose-dependent effects⁴⁷. Moreover, lactulose has been demonstrated to possess the potential to alleviate intestinal inflammation in mice⁴⁸. However, there is little data on its effects on the gut microbiota in other disorders, such as in PD.

It could be argued that the *Bifidobacterium* increase often found in PD patients^{4,49}, could be a side-effect of the often-prescribed Lactulose in this patient group. This would be consistent with our data, observing a strong increase in *Bifidobacteria* upon Lactulose intake in both CO and PD (participants did not take Lactulose prior to the study). Future meta-analysis studies should therefore explicitly include whether a patient was taking Lactulose, as this could be a medication-specific effect that is not directly related to PD.

SCFAs, increased due to the prebiotic intervention in our cohort, have a range of presumed beneficial effects on gut- and blood-brain-barrier (BBB) integrity^{9,50}. Translocating from the intestines to the systemic circulation, SCFA can reduce neuroinflammation¹⁵ and contribute to microglia maturation⁵¹ in murine models. Propionate, enriched in PD patients after prebiotics, has anti-inflammatory and BBB permeability-reducing effects in human brain endothelial cells⁵². Dietary fibre and SCFA can suppress intestinal inflammation, thereby improving the gut barrier function based on animal and cell culture studies^{53–55}. Since our cohort did not have observable intestinal inflammation (low faecal calprotectin concentrations), we would not expect to see further changes in inflammation status with the increased SCFA concentrations; however, in other patient groups that have an active gastrointestinal inflammation, our prebiotic intervention could potentially have a much greater effect.

In addition to the observed changes in SCFA concentrations, we identified enriched bacterial pathways for fatty acid metabolism (middle-chain fatty acids, MCFAs, and long-chain fatty acids, LCFAs). MCFAs have been shown to have beneficial effects, particularly on metabolic features and insulin sensitivity⁵⁶, and LCFAs like palmitic acid (pathways enriched in our study), can lead to enhanced IgA antibody production in vitro and in mice⁵⁷, contributing to host defence against pathogenic microorganisms.

To date, only a few studies have investigated the effects of dietary interventions on PD gut microbiome dysbiosis, focusing on constipation^{58,59} and/or pharmacokinetics⁵⁸. The effects of dietary mixed fibres were investigated in a cohort²⁴ of $n = 10$ PD subjects over 10 days resulting in increased plasma SCFA, gut integrity markers and reduction in potentially pathogenic family (*Enterobacter*), while potentially SCFA-producing species (*Bifidobacterium*, *Faecalibacterium*) were increased, in accordance with our results after a 1-month intervention. With the inclusion of a CO group, we could also characterize and monitor the PD-associated dysbiosis, corroborating our earlier work^{4,6}.

The PD dysbiosis persisted during the diet intervention. Whether extended prebiotic use or targeted prebiotic mixtures based on their functional and structural characteristics could restore the PD microbiome in the long-term, or if other, as-yet-unknown host-microbe or microbe-microbe mechanisms are responsible, remains a subject for further research. The microbiome is highly individual (or family) specific^{40,41} and distinct

microbial communities might show divergent responses to prebiotics, indicating a hierarchical specificity towards gut microbes⁶⁰, potentially limited by physico-chemical fibre characteristics. These mechanisms might be strain-specific. Dysbiosis-associated taxa (e.g. *Akkermansia*) in PD patients might stem from specific strains, which are particularly successful in outcompeting other microbiome taxa but with detrimental effects for the host. The removal of a persistent (dysbiosis-associated) member of the microbiota might require much stronger remedies than a prebiotic, as most bacterial strains persist in the gut microbiome for extended periods⁴¹. Other factors that favour dysbiotic taxa in PD patients include constipation and increased transit time, as discussed in ref. 4. Although it is tempting to speculate, that the PD microbiota is less adaptable and remains, therefore, trapped in a dysfunctional state, we cannot exclude that the persistent dysbiosis might be a less favourable effect of the SCFA-enhancing diet in PD. Moreover, it remains unclear whether the observed lack of microbiome normalisation (i.e. the inability to converge to a “healthy” microbiome) is a universal phenomenon in PD associated with all prebiotics/SCFA-increasing diets or if it is specific to our approach that included Lactulose, in addition to a diet intervention.

This could be further tested using prebiotics that affect different gut microbial groups involved in SCFA production: dairy-based substrates promote the growth of acetate and propionate producers (e.g. *Bifidobacterium* and *Lactobacillus*, “child-like” guilds^{28,61}). Vegetable fibres stimulate the growth of butyrate-producing bacteria (e.g. *Eubacterium*, *Roseburia*, and *Faecalibacterium*, “adult-like” guilds^{28,62}), but cross-feeding⁶³ between bacteria complicates this distinction.

Following prebiotic intervention, alterations in gene abundance were identified in multiple bacterial metabolic pathways, particularly within the PD cohort with potential pathophysiological implications. These findings offer a potential pathophysiological relevance to PD. This included a normalized SAM synthesis, enhanced GSH metabolism, decreased DOPAC synthesis, and an increased Tryptophan degradation along with the above-mentioned reductions in pathways resulting in neurotoxic metabolites (p-cresol sulphate and quinolinic acid) and an increase in a pathway resulting in the neuroprotective metabolite myo-inositol.

SAM, an endogenous amino acid metabolite involved in the methylation and trans-sulfuration processes, critically modulates autophagy⁶⁴, a process also relevant for PD, but excessive SAM, on the other hand, has been shown to cause PD-like symptoms in rats⁶⁵. It has been suggested that PD severity is related to a disturbed host-trans-sulphuration, a pathway central to antioxidant response, along with an increased bacterial Sulphur-metabolism⁶⁶, related potentially to PD medication⁶⁷. GSH (a SAM-derived metabolite) is involved in neuro-immune and neuro-oxidative processes and in the regulation of cell death. Reduced GSH levels are also a key finding in PD brain tissue⁶⁸, associated with mitochondrial dysfunction, constituting a critical factor in the neuroinflammatory and degenerative processes in PD⁶⁹. The major dopamine metabolite, dihydroxyphenylacetic (DOPAC), seems to play a role in PD pathophysiology associated with cell death⁷⁰ and mitochondrial inhibition. Prebiotically decreased bacterial DOPAC concentrations in parallel with elevated antioxidative GSH concentrations in the gut lumen could, in turn, be advantageous for the human host with PD.

Future studies focused on the interconnectedness³³ of metabolite exchanges could also measure blood metabolites and use multi-matrix approaches⁷¹ with advanced metabolomics fingerprinting strategies to cover a wider range of metabolites, but this analysis would go beyond the interpretability of our currently available data. Therefore, it remains to be seen whether the aforementioned changes in bacterial metabolism or respective metabolite changes within the gut lumen will reach the human host in a manner that beneficially influences the PD process.

The prebiotic intervention led to a substantial increase in *Bifidobacteria* spp. *Bifidobacteria* were among the taxa consistently increased in PD patients^{4,72}, albeit several studies controlled for laxatives such as the *Bifidogenic* Lactulose used here.

These *Bifidobacteria* were most likely already colonizing the host's gut and might therefore be more desirable for the PD patient, as seeding the gut microbiome with newly colonizing *Bifidobacteria* (or any other probiotic) could inadvertently negatively impact the gut microbiome, e.g. elicit an immune response to so-far-unknown taxa and/or disrupt the established and stabilized microbial ecosystem through the new strains. Prebiotics may represent a more suitable option for chronic conditions such as PD than probiotics, because of their longer-lasting effects^{73,74}.

Bifidobacteria have the potential to benefit the PD patient, and they are sometimes used as *Psychobiotics*⁷⁵ (a subclass of probiotics), given their ability to stimulate neurotransmitters, SCFA, anti-inflammatory cytokines, production of γ -aminobutyric acid (GABA) or tryptophan^{66,76} or enteroendocrine hormones with potentially brain-protective functions. However, *Bifidobacteria* could also be harmful, as their metabolism of tryptophan can produce potentially harmful metabolites (i.e. the neurotoxic quinolinic acid⁷⁷) and certain *Bifidobacteria* strains can in vitro metabolize L-Dopa via deamination⁷⁸, potentially interfering with standard PD pharmacotherapy. Thus, despite many potential benefits that *Bifidobacteria* could provide, the actual species and strains already residing in a microbiome should be assessed before manipulating their abundances through prebiotic interventions.

Due to the limited number of subjects involved in this pilot study, the resulting data may be subject to bias and therefore require further corroboration. Lactulose was prescribed to PD patients as a basic medication to treat constipation at the start of the diet intervention, matching the start of Lactulose intake in the CO group. While the diet intervention was completely matched between the PD and CO group, the Lactulose dosage in the PD group was higher and might therefore limit comparability. However, given our intention to investigate potential disease-modifying strategies that are particularly focused on PD patients, this approach may be deemed justifiable.

In conclusion, the systematic investigation of metagenomics and metabolomics in both faecal and urine samples demonstrated that a SCFA-promoting diet modulated the metabolism of the intestinal microbiota in PD patients, along with improved gastro-intestinal symptoms. Hence, the potential of a microbiota-directed prebiotic intervention as a modifying therapeutic approach in PD appears promising. To safely exploit the therapeutic potential of *Bifidobacteria* in PD, we suggest using strain-resolved metagenomics to guide prebiotic regimes to control for potentially harmful effects (such as L-Dopa deamination). It remains to be determined whether the dysbiosis associated with PD can be restored with longer-term prebiotic interventions or may require a probiotic approach or a (faecal) microbiome transfer. Larger placebo-controlled trials with longer-lasting dietary interventions, including different types of fibres and probiotic interventions, are required and justified given the beneficial metabolic changes observed in our data.

Methods

Study design and participants

This prospective controlled clinical pilot study was approved by the local ethics committee of the University of Bonn, Germany, and all participants gave written informed consent (internal ethics vote 145/17).

The study was registered in the German Clinical Trials Register (DRKS under the number DRKS00034528, Date of Registration 2024-06-27). Inclusion criteria were: (1) idiopathic PD (mild to moderately advanced, i.e. Hoehn and Yahr 1-2) with (2) stable medication within the previous three months, and (3) willingness for their healthy non-PD spouses to participate; (4) both at ages ≤ 75 years. Exclusion criteria were (1) atypical parkinsonism; (2) PD patients with deep brain stimulation or continuous intestinal levodopa infusion; (3) chronic and inflammatory gastrointestinal diseases including severe chronic constipation; (4) the use of laxatives, antibiotics, or immunosuppressive agents in the past three months (note that $n = 1$ PD took 1 day of antibiotics 4 weeks before study); (5) lactose intolerance; and (6) veganism. Participating PD patients were recruited from the Department of Neurology, University of Bonn, Germany, and underwent the

dietary intervention together with their healthy spouses. The CO group was selected to ensure a certain degree of standardisation and comparability with regard to the dietary recommendations provided throughout the course of the study. Furthermore, it was hypothesised that habitual diets would prove more comparable to those of age-matched healthy controls, thus enhancing the reliability of the baseline conditions. The recruitment of participants was conducted between October 2017 and July 2019, with the relevant study data collected concurrently. $N = 11$ PD patients and their $n = 11$ healthy spouses (CO group) were included in the study. One couple (1 PD, 1 CO) withdrew from the study before the final visit for reasons not related to the study.

PD was clinically diagnosed according to the UK Brain Bank criteria. Disease severity was measured using the Movement Disorders Society Unified PD Rating Scale (MDS-UPDRS) Part III. Gastrointestinal symptoms and constipation presence were evaluated through a Gastrointestinal Symptom Rating Scale (GSRS⁷⁹), selected items: borborygmus, abdominal distension, increased flatus, decreased passage of stools, increased passage of stools, loose stools, hard stools, urgent need for defecation and feeling of incomplete evacuation, rated 0–3 based on intensity, frequency, duration, or social impact) and a post-prebiotic interview for adverse effects.

Dietary intervention

All participants agreed to follow a recommended diet, a combination of nutrients rich in fibres, fruits, and vegetables for 4 weeks: a combination of (i) 2 apples/10 apple rings a day (rich in pectin, a polysaccharidic prebiotic) and (ii) 5 portions per week (portion = the size of the own hand) of foods rich in resistant starch, lignin, and anthocyanins (lentils, potatoes, green beans, onions, oat bran, lettuce, olive oil, and bananas as well as strawberries). PD patients started taking a prebiotic Lactulose syrup to treat constipation as a basic medication at the time of inclusion in the study. The spouses thus were ingesting Lactulose in a prebiotic dosage. Lactulose, a non-digestible synthetic disaccharide, is used for different purposes, such as a prebiotic, for constipation and hepatic encephalopathy treatment (ranging from 10 g to 100 g, respectively). PD patients took 2×15 ml (2×10 g) daily for treatment of constipation, while controls took 1×15 ml (1×10 g, i.e. prebiotic dosage). Lactulose is anaerobically fermented in the colon by microbiota, enhancing *Bifidobacteria*, *Lactobacillus* (and SCFA^{47,80}). Dietary baseline information was assessed with a questionnaire (Supplementary Table 8). Diet compliance was verified through self-maintained food logs over the 4 weeks, reviewed after completion of the study ($n = 7$ representative diet days per subject, i.e. $n = 5$ weekdays and $n = 2$ weekend days across the 4 weeks). Diet diaries were analysed using the Nutritics dietary analysis software (Nutritics Ltd), based on the German Food Composition Database available within Nutritics. To establish a comparable and scalable measure for assessing dietary adherence, we focused on measuring the consumption of raw apples, as they are easily quantifiable, as well as total fibre intake. It should be noted, however, that apple/fibre consumption alone does not necessarily indicate full adherence to our recommendations. Due to the nature of our recommendation as a portion (resembling the size of the own hand) and because we did not systematically quantify dietary habits over a longer period before intervention, application of absolute values of other nutrients was considered inappropriate. The food score was calculated as follows: the consumption of raw apples based on our given recommendations (2 Apples = 365 g/d or 10 Apple rings dried = 65 g/d) was considered 100% adherent. Then the fraction of the participants' diet days with an apple consumption below the mean apple consumption of all participants was computed (mean = 70%/d of our recommendation). A fraction of more than 0.75 days was considered less adherent. Additionally, dietary analysis, including estimating the average fibre intake of every representative day, was calculated and used for basic statistical testing (see below), using the Nutritics dietary analysis software.

Sample collection

Urinary and faecal samples were non-invasively collected at baseline and 4 weeks after diet. Faecal samples were put in a specimen collector (Sarstedt) and stored in a plastic bag under anaerobic conditions (Anaerocult® P,

Merck Millipore). Participants were advised to collect samples the latest the evening prior to or on the day of the study visit. All samples were cooled in a refrigerator (5–7 °C) at the patients' home and transported to the study site with a cool pack. Samples were immediately stored at –80 °C upon arrival at the study site. Additionally, routine laboratory parameters were assessed at baseline and 4 weeks after diet (focusing on serum sodium and potassium levels as safety measures due to the ingestion of Lactulose syrup).

Outcome

Primary: (i) modification of SCFA in faeces, (ii) changes in gut microbial composition including key species for SCFA production, and (iii) changes in bacterial metabolism based on bacterial genomes and metabolomics analysis in urine and faeces.

Secondary: (i) changes in motor symptoms (UPDRSIII), (ii) modification of gastrointestinal symptoms (modified GSRS, stool frequency, side effects).

Faecal DNA extraction and sample preparation

DNA extraction was performed with the Maxwell® RSC PureFood GMO and Authentication Kit (Cat. #AS1600) according to the manufacturer's recommendations. In brief, 200 mg of faeces were placed into a 2 ml microcentrifuge tube and 1 ml of CTAB Buffer was added. Samples were heated at 95 °C for 5 min and allowed to cool down for 2 min. Manually homogenisation was performed with bead beating in the 2 ml Lysing Matrix E tubes (containing 1.4 ceramic spheres, 0.1 silica spheres, and one 4 mm glass bead) using a Homogenizer (FastPrep, Setting/Speed 6.0, three times for 1.0 min). Samples were then mixed with 40 µl of proteinase K and 20 µl of RNase A and incubated at 70 °C for 10 min. Cartridges were prepared according to the manufacturer's recommendations (300 µl lysis buffer). The Maxwell RSC was run with the PureFood Protocol, automatically purifying and eluting DNA in 100 µl.

Sequencing

Genomic DNA was normalised to 5 ng/µl with EB (10 mM Tris-HCl). A miniaturised reaction was set up using the Illumina Nextera DNA Flex Library Prep Kit (Illumina Catalogue No 20018704). zero and five tenth µl tagmentation buffer 1 (TB1) was mixed with 0.5 µl bead-linked transposomes (BLT) and 4.0 µl PCR grade water in a master mix and 5 µl was added to a chilled 96-well plate. 2 µl of normalised DNA (10 ng total) was pipette mixed with the 5 µl of the tagmentation mix and heated to 55 °C for 15 min in a PCR block. A PCR master mix was made up using 4 µl kapa2G buffer, 0.4 µl dNTP's, 0.08 µl Polymerase and 4.52 µl PCR grade water, contained in the Kap2G Robust PCR kit (Sigma Catalogue No. KK5005) per sample and 9 µl added to each well need to be used in a 96-well plate. Two microliters of each P7 and P5 of Nextera XT Index Kit v2 index primers (Illumina Catalogue No. FC-131-2001 to 2004) were added to each well. Finally, the 7 µl of Tagmentation mix was added and mixed. The PCR was run with 72 °C for 3 min, 95 °C for 1 min, 14 cycles of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 3 min. Following the PCR reaction, the libraries were quantified using the Quant-iT dsDNA Assay Kit, high sensitivity kit (Catalogue No. 10164582) and run on a FLUOstar Optima plate reader. Libraries were pooled following quantification in equal quantities. The final pool was double-SPRI size selected between 0.5 and 0.7X bead volumes using KAPA Pure Beads (Roche Catalogue No. 07983298001) and quantified on a Qubit 3.0 instrument and run on a D5000 ScreenTape (Agilent Catalogue No. 5067-5588 & 5067-5589) using the Agilent TapeStation 4200 to calculate the final library pool molarity.

Samples were sent to Novogene (Novogene (UK) Company Limited, 25 Cambridge Science Park, Milton Road, Cambridge, CB4 0FW, United Kingdom) to be run along with sample names and index combinations used. Faecal samples were shotgun sequenced (paired end) using an Illumina Novaseq 6000 at Novogene. Demultiplexed fastq's were returned on a hard drive, and further analysed with the Mafiler Pipeline⁴¹.

Targeted SCFA measurements in faeces

Frozen aliquots of raw faeces were sent to an external laboratory (GAN-ZIMMUN Diagnostics GmbH, Hans-Böckler-Str. 109, 55128 Mainz, Germany) to measure SCFA levels via GC-MS (GC; Perkin Elmer Clarus 680, MS; Perkin Elmer Clarus SQ8). In brief, 1 g of raw faeces was suspended and homogenized in 26% NaCl solution (ACROS - 387640025). Then, 900 ml of 6 M HCl were added for stabilization. 3 ml of the former were transferred into a plastic tube together with 30 µl of Istd-solution (100 µl 2-ethylbutyric acid in 9.9 ml 0.6 M HCl-solution). Samples were thoroughly mixed and 50 µl, placed into a Wheaton-Vial, and closed with a crimp-cap. Calibration standards of the respective SCFA were similarly prepared in 26% NaCl, stabilized with 6 M HCl, and treated analogously as the faecal samples. Then levels of SCFA were measured (Headspace-System: Perkin Elmer TurboMatrix 40, Column: Phenomenex Zebron ZB-FFAP, 30 m length, 0.25 mm ID, 0.25 µm width, gas: Helium, 80 kPa, injection-volume: 1 µl, temperature-program: 2 min at 45 °C, heating (15 °C/min) to reach 250 °C, heat 250 °C for a duration of 10 min, Headspace-temperatures: vial-oven 60 °C, transfer line 110 °C, injection-pin 95 °C). The 2-ethylbutyric acid was employed as an internal standard, whereby a defined quantity of 2-ethylbutyric acid was introduced to each sample, and the signal intensity of the analytes was normalised to the intensity of 2-ethylbutyric acid at the end of the process. Data was sent electronically with SCFA concentrations given in µmol per 1 g faeces.

Non-targeted metabolomics analysis in urine and faeces

Nuclear magnetic resonance (NMR) spectroscopy was used for non-targeted metabolomics. A detailed description of the metabolomics method, including urine sample preparation, NMR analysis and data processing, is outlined in ref. 34. In brief, 150 µl of urine is mixed with 50 µl PO₄-buffer (100% D₂O) and 10 µl 4.5 M KF in D₂O. To extract aqueous faecal metabolites, we homogenized 50 mg faeces in 1 ml H₂O using ceramic beads (NucleoSpin, Macherey-Nagel, Dueren, Germany) and a TissueLyser (Qiagen, Hilden, Germany) mixing for 3 × 20 s at 4500 rpm with a 10 s cooling break (<0 °C). Subsequently, the homogenate was centrifuged (13,000 rpm for 10 min at 4 °C), the supernatant evaporated with a SpeedVac, the dried extract reconstituted in 150 µl H₂O and mixed with 50 µl NMR buffer and 10 µl 4.5 M KF. Samples were immediately submitted to NMR analysis, and the same workflow protocols were used for urine and faecal extracts³⁴. For metabolite identification, we analysed the quality CO sample (i.e. a mixture of all samples in the study) as a representative sample of urine and faecal water extract, respectively, with a series of 2-dimensional NMR analyses as specified in ref. 81. Data were imported into Matlab software R2011b (Mathworks, Natick, MA, USA) and the water region removed, and spectra normalised⁸². Relative quantification of metabolites was done using the peak height of selected peaks, as identified by a peak picking algorithm⁸³. As the urine metabolites were commonly characterized by multiple peaks, we chose the best representative peaks for data reduction prior to statistical testing.

ELISA (faecal calprotectin and alpha-1-antitrypsin

Quantitative determination of calprotectin and alpha-1-antitrypsin in stool each was performed with an enzyme linked immunoassay from Immunodiagnostik AG (Stubenwald-Allee 8a, 64625 Bensheim, Germany; calprotectin: IDK® Calprotectin ELISA, K 6927, K 6927.20, MRP (8/14, S100A8/A9); alpha-1-antitrypsin: IDK® α1-antitrypsin ELISA, K 6760) following the manufacturer's instructions.

Genome reconstruction, taxonomic, and functional profiling of metagenomes

MATAFILER^{41,84} (<https://github.com/hildebra/MATAFILER>) was used to process raw reads, assemble metagenomes, reconstruct metagenomics assembled genomes (MAGs) and dereplicate these to MGS. Briefly, raw shotgun metagenomes were quality filtered using sdm v1.63 with default parameters⁸⁵.

Kraken2⁸⁶ was used to remove human reads. Host-filtered metagenome reads, were assembled using MEGAHIT v 1.2.9⁸⁷, and reads were backmapped onto the assembly using Bowtie2 v2.3.4.1⁸⁸, genes predicted with Prodigal v2.6.1 with parameters “-p meta” and a gene catalogue clustered at 95% nt identity using MMseqs2⁸⁹. Matrix operations on the gene catalogue were carried out using rtk2⁹⁰.

MAGs were calculated using SemiBin2⁹¹, and their completeness and contamination were estimated using checkM2⁹². Using a combination of SemiBin2 MAGs and canopy clusters⁹³ (<https://github.com/hildebra/canopy2>), high-quality reference genomes (>80% completeness, <5% contamination) were dereplicated into MGSs using clusterMAGs (<https://github.com/hildebra/clusterMAGs>) in MATAFILER. Abundances of MGS in different samples were estimated based on conserved marker genes and their median abundances within the gene catalogue.

Enterosignature calculation

The reapplication of ES was generated at <https://enterosignatures.quadram.ac.uk/>. The Genus abundance table was fitted to the five ES model²⁸, and taxa names were matched between the abundance table (H) and the five ES W matrices, which gives the weight of each taxon in each signature. The W matrix and H have been modified so that taxon names and orders in each match. ES are a set of five signatures that represent common microbial guilds complementary in their metabolism and found in all gut samples. The ES model allows for defining a normal/homoeostatic microbiome, i.e. a low model fit = <0.4 could be suggestive of a microbiome in an atypical state (or at least in a state that was not well represented amongst the >5000 gut microbiomes the model was originally learnt from). Brain-related metabolic functional potential was assessed with a previously published database of manually curated gut-brain modules³¹ (GBMs), with each corresponding to a single neuroactive compound production or degradation process.

Statistical analyses

Statistical analysis was conducted in R 4.1.3. Alpha-diversity and Beta-diversity indices and compositional analyses were calculated using the R-packages rtk⁹⁰, phyloseq⁹⁴, microbiome⁹⁵, and vegan⁹⁶, and data were visualized with ggplot2 and custom R scripts. For Alpha-diversity measures, i.e. indices of diversity and evenness (Shannon, Simpson) and richness (observed, Chao1, and ACE), sample count matrices were rarefied to 90% of the minimal sample sum to ensure even sampling depth. For Beta-diversity, i.e. the quantification of sample dissimilarity (between PD and CO or between before and after prebiotics based on Bray-Curtis or Euclidean distance, see below) and taxonomic composition, taxa count matrices were normalized by dividing each feature by the respective total sample sum (TSS). Metabolomics data (faecal and urine metabolomics) were normalized with a log-transformation (log $x + 1$), and faecal SCFA were used as normalized by the external laboratory.

For univariate tests, features from the abundance matrix were removed that were present in less than four samples or had less than 0.001% relative abundance. To account for the “household-effect”, significance between PD and CO was tested with a paired samples Wilcoxon rank-sum test (Wilcoxon signed-rank test, function “wilcox.test (... , paired = TRUE)”) between couples for each time point separately (PD vs CO before and PD vs CO after prebiotics) followed by a multiple testing correction (Benjamini-Hochberg). For within-patient comparisons, a Wilcoxon signed-rank test (see above) was performed on all data (before prebiotics vs after prebiotics), then multiple testing corrected (Benjamini-Hochberg). Post-hoc testing of the former results ($p < 0.05$ and $q < 0.1$) was performed using a Wilcoxon signed-rank test (PD before vs PD after and CO before vs CO after). All p -values were corrected for multiple testing using the Benjamini-Hochberg false discovery rate⁹⁷ (referred to as q -value) using the R function “p.adjust”. Differences in categorical metadata between PD and CO were tested with a Fisher's exact test as implemented in base R (function “fisher.test”). For all univariate tests, a p -value < 0.05 and a q -value < 0.1 were considered statistically significant. Effect sizes were calculated using the R-package coin⁹⁸ and rstatix, using the function “wilcox_effsize” and

“coin::wilcoxsign_test”. The effect size r was calculated as the Z statistic divided by the square root of the sample size (N): $r = (Z/\sqrt{N})$. The Z -value is extracted from `coin::wilcoxsign_test` (paired-samples test). Note that N corresponds to the total number of pairs. The effect size r varies from 0 to close to 1, and we considered 0.10 to <0.3 (small effect), 0.30 to <0.5 (moderate effect) and ≥ 0.5 (large effect).

For estimation of dissimilarity between study variables, we used (un) constrained and conditioned distance-based redundancy analysis (dbRDA, function “`dbRDA`” in `vegan`⁹⁶). As this function does not allow missing data values, we imputed NAs in metadata with the mean value of the respective variable (this was the case for $n = 3$ GSRS and $n = 1$ stool frequency values missing), otherwise, all $n = 40$ samples were included in the analyses. Estimation of dissimilarity was based on Bray–Curtis distance for taxonomic and function tables (composition of TSS normalized data) or based on Euclidean distance for faecal SCFA, faecal and urine metabolomics on log-transformed data.

A perMANOVA (function “`adonis2`” in `vegan`) was used to test the significance of the constraints in the conditioned dbRDA distances. As the household effect had a major impact on data variability (see also “Results” section), we blocked the perMANOVA test for the variable household (i.e. the pairs PD + healthy spouses) with the function “`setBlocks`” in `permute`⁹⁹ and tested the dissimilarity either for the total data or the different time-points separately. Dissimilarity matrices were then associated with different variables (study group, time point, metabolites, taxa) with the function “`envfit`” (with a permutation test as implemented in `vegan`).

For composition plots, taxa were summarized into a higher phylogenetic level (i.e. *Genus* level) and rare taxa were combined (with the function “`microbiome::aggregate_taxa`” or “`microbiome::aggregate_rare`”, respectively, as implemented in `microbiome`⁹⁵/phyloseq⁹⁴).

Partial correlation analyses between taxa and KEGG/GBM modules were conducted with `ppcor`¹⁰⁰ with spearman correlation corrected for autocorrelation (with the function “`pcor.test`” the pairwise partial correlations between two variables (x, y) can be controlled for a third variable (z , the individual participant)) and the correlation matrix was visualized with `corrplot`¹⁰¹.

Data availability

The metagenomic sequencing data generated and/ analysed during the current study are available in the European Nucleotide Archive, ENA repository, PRJEB57228. Further data types are available upon request from the corresponding authors.

Code availability

The underlying code for this study is available on GitHub and can be accessed via this link <https://github.com/PDMicro/PDD-scripts>.

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Author contributions

J.R.B. conceptualised and performed the study, acquired funding, wrote the analytical code and performed the formal analysis and drafted the manuscript. S.R. performed the calprotectin and alpha 1 antitrypsin ELISA, helped in formal analysis and critically revised the manuscript. S.S.H.

performed the NMR metabolomics in faeces and urine and helped in formal analysis of the respective data. A.D. conducted the analysis of the Enterosignatures. D.N. helped in the formal analysis of the household effect. M.H.T. and D.S.-L. formally analysed the food logs with the Nutritics software. M.-C.S. helped in interpreting the SCFA and nutrition data and critically revised the manuscript. A.N. funded the calprotectin and Alpha 1 Antitrypsin ELISA and critically revised the manuscript. U.W. helped in recruiting the study participants and in interpreting the data and critically revised the manuscript. F.H. performed the bioinformatic analyses of the sequencing data, supervised the statistical code writing, interpreted all data and wrote the manuscript with J.R.B. All authors read and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Janis Rebecca Bedarf or Falk Hildebrand.

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