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Identification of a species-specific bacterial signature in Parkinson's and modification with a probiotic intervention

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Abstract:

The gut-brain axis is a bi-directional signalling system that includes the enteric system, the gut microbiome (GM) and its metabolites. An unhealthy (dysbiotic) gut is associated with deleterious perturbation to the GM resulting in systemic inflammation thought to contribute to a range of conditions including Parkinson's. Most studies investigating the human gut GM do not focus on species-level analyses needed to establish prevalence and abundance of commensal and pathobiont bacteria, and whether therapeutic interventions alter the GM composition. In this study we conducted an RCT where one group of people with Parkinson's (PwP) was assigned to a probiotic arm and the other to placebo arm. Stool samples were collected sequenced and analysed at week 0 and week 12. Conventional bioinformatic analyses suggested changes to the GM of the placebo group at week 12 compared to placebo but lacked specificity and were uninformative. Through careful analyses of species-level data we established a species-specific-signature for PwP in our study and a positive effect of the probiotic (Symprove™) on the GM in those assigned to the probiotic arm. To conclude – bioinformatic analyses lack specificity when used with small samples, we detected a species-specific signature in our group of PwP by analysing species prevalence across individuals, and the therapeutic use of a 12-week probiotic improved the overall composition of the GM in probiotic compared to placebo group.

Introduction:

Gut-brain axis and gut microbiome (GM) in humans:

The gut-brain axis is a bi-directional signalling system that involves enteric, autonomic, endocrine, and immune systems. Vagal cranial nerves also form part of the gut-brain axis providing a sensorimotor and physical pathway from viscera to brain (Barker, 2024). The gut-brain axis enables the enteric system and brain to engage in a constant communicative loop,

modulating digestion, hunger, satiety, and endocrine and immune functions (Barker, 2024). The enteric system comprises sensory, motor and interneurons of the gastrointestinal tract, together considered the 'second brain' (Gershon, 1999). This system also includes the GM *and* metabolites of these micro-organisms. Recently the proposed functions of the axis have expanded, and its disruption is thought to contribute to many pathological conditions (Loh et al., 2024).

The human GM is comprised of millions of diverse bacterial species, viruses, fungi, parasites and archaea (evolutionary ancient micro-organisms). We have gained considerable insight into the bacterial composition of the GM at phylum and genus levels over recent decades thanks to advancements in microbial DNA sequencing pipelines, but far less is known about the role of microbial *species* to the ecosystem (Yarza et al., 2014). Presently there is an imperative to investigate and identify bacterial species of the GM rather than focus on other phylogenetic taxa. Only at species level of analyses can species-specific signatures (SSS) of a particular condition be identified informing understanding of potential therapeutic targets.

The GM plays a pivotal role in a range of host metabolic and homeostatic functions important for host health including metabolism of essential short chain fatty acids (SCFA's) and other key metabolites, synthesizing neurotransmitters and neural proteins, contributing to neural signalling pathways, and regulating immune function by responding to environmental stressors (Zapanta, Schroeder & Fisher, 2022). However, when the gut milieu becomes unbalanced or *dysbiotic* the gut-brain axis becomes a potential route for microbiota-derived pathogenesis in several conditions including Parkinson's (Vizcarra, Wilson-Perez & Espay, 2015).

One possible origin of dysbiosis is breakdown of the gut wall epithelium, a functional mucus lined barrier. Integrity of the gut wall requires the production of short-chain fatty acids (SCFA's) by gut microbes through the metabolism of indigestible fibre along with other processes carried out by host cells. Humans cannot manufacture SCFA's and depend upon commensal microbes for contribution to the maintenance of the gut wall. Environmental factors such as antibiotic use, poor diet, illness, injury, disease, and stress can adversely affect gut permeability leading to a 'leaky gut' and dysbiosis (Camilleri, & Vella, 2022). Degeneration of the gut wall increases production of inflammatory cytokines leaching potentially harmful microbes and their metabolites into systemic blood supply. One theory is that microbes/microbial metabolites travel to the brain and other organs through the blood supply or access the brain by exploiting vagal nerve structures, in the same way that the herpes virus exploits the exoskeleton of cranial nerves to access the brain.

GM in people with Parkinson's (PwP):

Parkinson's is one of the most common neurodegenerative conditions, yet after decades of research little is known about causative factors. Parkinson's is primarily considered a motor disorder caused by diminution of both dopamine production and dopamine metabolism in the brain. More recently, there have been revisions to this narrow conceptualisation of the condition because it does not capture the whole-body symptomatology of Parkinson's. The condition also adversely impacts sleep, smell, taste, cognition, mood, digestion, and motivation. It is now accepted that the condition is systemic as opposed to brain localised, a theory known as the *body-first* model (Horsager & Borghammer, 2024).

Importantly, there is growing recognition that the pathophysiology of Parkinson's might originate outside of the brain (Borghammer, 2023). Crucially it is estimated that more than 50%

of dopamine (the neurotransmitter diminished in Parkinson's), is produced by the GM (Dinan & Cryan, 2017). Indeed, the eponymous James Parkinson, in his original treatise, recognised that gut problems, particularly constipation, were an early and reliable feature of the 'shaking palsy,' (Keshavarzian et al., 2015).

The pathophysiological hallmark of Parkinson's is dopamine depletion and aggregation of misfolded alpha synuclein proteins (α-Syn) forming Lewy bodies within the neurons of the central, autonomic, and enteric nervous systems (Kalia, & Lang, 2015). There is evidence that α-syn misfolding may be found very early on in intestinal neurons, almost a decade before the appearance of Parkinsonian motor symptoms (Sanchez-Ferro, Rabano, Catalan et al., 2015; Hilton, Stephens & Kirk, et al., 2014). From this location the proteins translocate from gut to brain where deposits follow a precise trajectory from brainstem to limbic structures and finally cortices (Braak et al., 2003; Braak et al., 2006). Importantly, α-Syn possesses anti-microbial properties, therefore new approaches propose that it may be generated as a response to microbial presence rather than a causative factor and there is a correlation between α-Syn over-expression and upregulation of immune response (Park et al., 2016).

Wallen et al., (2020) identified 15 genera associated with Parkinson's disease. These were Porphyromonas, Prevotella, Corynebacterium 1, Bifidobacterium, and Lactobacillus. Ten of these genera had lower abundances compared to controls including - Faecalibacterium, Agathobacter, Blautia, Roseburia, Fusicatenibacter, Lachnospira, Butyricicoccus, Lachnospiraceae ND3007 group, Lachnospiraceae UCG-004, and Oscillospira. The authors distinguished three specific clusters (i), increased levels of opportunistic pathogens (ii), reduction of SCFA-producing microbes (iii), and elevation of microbes that metabolise carbohydrates. They also found several significant predictors for GM dysbiosis in PwP including sex, age, BMI, gastrointestinal issues, and diet.

Petrov et al., (2017) used 16S rRNA sequencing in 89 PwP and a healthy control group (n = 66) and found differences in 9 genera and 15 species in those with Parkinson's. These data show a striking difference in microbial abundance compared to controls at genus and species level. Interestingly, the Parkinson's group showed high levels of Lactobacillus and Bifidobacterium, probiotic commensals, compared to controls. Conversely, controls had higher levels of beneficial genera and greater abundance of Bacteroides strains than the Parkinson's group. This contrasting composition of the microbiome between PwP and controls might provide clues about what constitutes a healthy gut milieu compared to one susceptible to dysbiosis. The authors concluded that this microbial profile contributes to inflammatory processes and ultimately α-Syn misfolding. However, these analyses only partly focussed on species level data and there has been no attempt, to date, to investigate whether there is a species-specific signature (SSS) across PwP.

It is axiomatic that when the gut microbiome becomes dysbiotic a shift in the microbial ecosystem has occurred and that this arguably precedes negative consequences for the host that may be cumulative, worsening over time. Thus, the mechanism that maintained the healthy microbiome in eubiosis no longer prevails as the gut microbiome becomes dysbiotic (Lozupone et al., 2012). The exact cause of dysbiosis is known but disruption to short chain fatty acid (SCFA) supply or metabolism, or breakdown of the protective mucus layer, might trigger breeches in the gut wall epithelium resulting in leaky gut and local and systemic inflammation (Sampson et al., 2016).

Probiotic studies in PwP:

Few studies have examined whether GM composition was altered following probiotic use in PwP (Sun et al., 2022., Du et al., 2022). Studies that have been conducted have focussed on genera rather than species taxa and highlighted increased Lactobacillus and Bifidobacterium genera. Although the number of studies in vivo is limited, there is evidence of compositional shifts to the GM in PwP after probiotic use. Hamilton et al (2024) proposed such findings as significant because if Parkinson's associated taxa are decreased or enriched by probiotic use the GM composition reconfigures to a less Parkinson's-like state.

We collected stool samples from three PwP to create microbiotas in an *in-vitro* gut model in a mechanistic study (SHIME® https://prodigest.eu/). The effect of Symprove™ dosing on bacterial composition and metabolic activity was evaluated over 48 hours and compared to healthy controls. Results showed adaptive change in bacterial composition in the microbiotas of PwP, heightened production of short chain fatty acids (SCFAs) and lactate, increased levels of anti-inflammatory cytokines, and decreased levels of pro-inflammatory cytokines and chemokines (MCP-1 and IL-8). This study provided the rationale for the current in vivo investigation of the microbiome and probiotic use in PwP.

Other research investigated the control of growth of pathogenic gut bacteria using probiotics. *C difficile* is an antibiotic-resistant opportunistic microbe that proliferates in the gut after antibiotic use causing inflammation and diarrhea. Findings showed that Symprove™ had an inhibitory effect against C difficile, possibly due to a change in the PH milieu with the introduction of different strains of beneficial bacteria. Additionally, other results show that Symprove™ has antipathogenic activity against *E. coli*, *S. sonnei* and *MRSA* (Dodoo, 2018) and bacteria survive to the gut (Fredua-Agyeman & Gaisford, 2015).

Overall prebiotic and probiotic intervention and supplementation are each emerging as viable methodologies to augment the gut microbiome and potentially limit Parkinsonian symptoms. It is still early, and a detailed catalogue of bacterial species associated with Parkinson's is needed, alongside knowledge of archae, viruses and fungi that also constitute the human gut microbiome. Symprove™ has a growing empirical base, and it has been established that microbes in the probiotic make it to the gut alive where they form a niche that may positively impact the overall GM composition.

Measurement and analyses of the human GM:

Most microbiome studies codify data at the higher taxonomic levels of phyla, family, or genera but seldom at species level. However, there is contention about the informativeness of such broad-brush strokes at these taxonomic levels (Thomas & Segata, 2019). Reports of genus level GM data are commonplace because short-length 16S rRNA sequence analyses are depth-limited to genus level taxonomy (Yarza et al., 2014; Yang et al., 2020). To identify species full-length 16S rRNA sequences or shotgun metagenomic sequencing are required, which is typically more costly. However, there is an argument that genus level analyses provide inaccurate results so sequencing of species level data remains key for contemporary studies (Lozupone et al., 2012; Yang et al., 2020; Van Treuren, & Dodd, 2020). Patterns of microbiome composition associated with conditions and prevalent pathogenic and commensal species, can only be detected by cataloguing species-level data (Thomas & Segata, 2019).

Bioinformatic analyses of bacterial GM typically depend upon measures of alpha and beta diversity. Alpha diversity is a measure of the number of individual species detected and is

calculated on raw data within samples. Chao1 index and Shannon index represent sample richness and diversity, respectively, at the level of alpha diversity (Li, Wu, Hu et al. 2017). The difference in gut bacterial composition between individuals is measured by beta diversity. These analyses are broadly applied to human GM data because high levels of diversity within an ecosystem are considered a marker of a healthy environment.

Nevertheless, the usefulness of these types of analyses has been questioned because they lack sensitivity when used with individuals with neurological conditions and where sample size is small (Beck, Holloway & Schwanghart, 2013). For example, in a moderate-sized meta-analysis 11 of 26 studies found no difference between PwP and healthy controls in alpha diversity metrics (sample richness - Chao1), and 7 of 26 studies excluded this analysis entirely. Conversely, 8 from 26 studies found a significant difference between the GM of PwP and controls on sample richness (Li, Wu, Hu et al. 2017). There was no difference between PwP and healthy controls for 2 of 26 studies and no data available for 9 from 26 studies on beta diversity (weighted and/ or unweighted Unifrac). In contrast there were significant differences between PwP and controls on the beta diversity metric for 15 of 26 studies, although for most of the studies (n = 13) the direction of the difference was not specified, making it unclear whether PwP had less diversity in their GM's compared to controls. It is a confusing picture and might indicate that this statistical approach conceals more than it reveals when sample sizes are small.

Summary

There is increasing evidence that Parkinson's may not originate in the brain but may be linked to gut dysbiosis (McDonald et al., 2018; Zapanta, Schroeder & Fisher 2022). Gut function problems associated with, and preceding Parkinsonian symptoms, support the proposed link that dysbiosis might contribute to the neurodegeneration of Parkinson's (Fasano et al., 2015). However, research to date has been mostly confined to animal models, or laboratory simulations with artificial digestive systems (Ghyselinck et al., 2021), and there is less data collected from people with Parkinson's and little consensus about what constitutes a healthy GM other than microbial diversity (Rinninella et al., 2019). Indeed, estimates are that approximately 70% of gut microbial species have not been cultured (Almeida et al., 2020).

In the present study we addressed current limitations in GM research by collecting faecal samples in two groups of PwP randomly assigned to probiotic or placebo arm, at week 0 and week 12. We compared data at species level to (i), establish whether there was a species-specific signature associated with Parkinson's and (ii), whether this changed with use of Symprove™ probiotic in the experimental group compared to controls over a 12-week period using standard bioinformatic *and* a unique SSS formulation to evaluate data.

Method

We recruited a cross-sectional sample of 29 people with idiopathic PD who fulfilled the UK PD Society Brain Bank criteria: https://www.parkinsons.org.uk/research/parkinsons-uk-brain-bank and did not meet exclusion criteria.

All experimental protocols were approved by Sheffield Hallam University, NHS IRAS and MHRA regulatory bodies. Informed consent was provided by all participants. Clinical Trial Registration: 04140760 First Registration Date 21st/June/2019.

Using an internet based, random generator app to ensure unbiased randomisation, participants were randomly assigned to one of two groups: one group (n = 16) were the Probiotic group, and the other group (n = 13) were the Placebo group.

The study was a double-blind randomised placebo-controlled pilot study (IRAS Number: 262286). At study start (0 weeks) and end (12 weeks) participants provided a stool sample for extraction and sequencing of microbial DNA to establish whether the probiotic altered bacterial composition of the GM in probiotic compared to placebo groups. Participants consumed one capful of probiotic or placebo each morning on an empty stomach for 12-weeks. At study end, for equipoise, the placebo group was offered the 12-week probiotic for free if they wished to take the product.

This research was conducted with NHS and Faculty ethical approval and in line with the revised Declaration of Helsinki (2024). The datasets generated and/or analysed during the current study are available in the Harvard Dataverse repository https://doi.org/doi:10.7910/DVN/GDA3FL

Table 1: Participant Demographics stratified by group.

	Probiotic (N = 16)	Placebo (N = 13)
Age at time of study (years)		
Range	53-73	54-76
Mean	64.71	67.00
SD	5.48	6.97
Age at diagnosis (years)		
Range	44-71	46-67
Mean	58.14	58.41
SD	7.53	6.64
Gender		
Female	8	6
Male	8	7

All participants were taking Parkinson's medication. Co-beneldopa (Madopar), Co-careldopa (Sinamet), Ropinirole (Requip, Repinex) and Rotigotine (Neupro patches) were the most common.

Symprove™ Probiotic and Placebo:

Symprove™ is a commercial probiotic company with a track record of involvement in research. The company provides the probiotic and placebo product for free, and following the study, the free probiotic for those PwP allocated to the placebo arm of the study. The company does not receive any intellectual property rights or has any claims on the data or study outcomes. Symprove™ has been included in numerous published studies (Fredua-Agyeman & Gaisford, 2015).

The four bacterial strains within the Symprove™ probiotic are: Lactobacillus rhamnosus NCIMB 30174, Enterococcus faecium NCIMB 30176, Lactobacillus acidophilus NCIMB 30175, Lactobacillus plantarum NCIMB 30173. The Symprove™ placebo has been used in all other clinical trials using the probiotic so we decided that the safest option for participants was to use this (already well studied) placebo rather than devising our own. The placebo is

comprised of sterile water, mango and passion fruit flavouring and beta-carotene. It tastes smells and looks the same as Symprove™ and the Quality Assurance is to the same standard as the product according to manufacturers.

Stool sample collection and analysis:

Human stool collection guidelines were followed in accordance with the Human Microbiome Core Sampling Protocols and using Norgen Biotek stool collection system (http://hmpdacc.org/doc/HMP_MOP_Version12_0_072910.pdf). Participants were provided with detailed instructions and the necessary kit for collecting and labelling their sample.

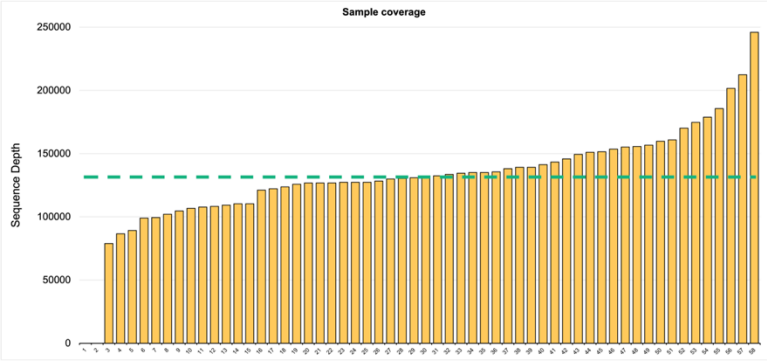
We used Norgen's stabilization kit because it contains a solution that destroys any viruses and stabilizes the sample so that it does not need to be stored at -80 degrees. All samples were coded with a number identifier following the companies detailed protocol for anonymization, coding, and transport. Samples had no information relating to participants. For transport they were ice-packed and transported to Diversigen, USA.

Bacterial DNA was extracted, sequenced, and analysed using the DADA2 16S BenchMark Diversigen pipeline (long-read). Custom bioinformatic analyses were performed to determine if the probiotic caused shifts in the composition of the gut microbiome that differed from the placebo group.

Bacterial microbial analyses: Sequence depth and quality

Raw fastsqs were used to calculate the mean sequencing depth per sample (Sample Coverage), the median quality scores for each sample, and the sequence quality per base for each sample. Note that quality scores above 25 are considered acceptable, and that on Illumina sequencers quality scores always decrease toward the end of the read.

Figure 1. Sequence depth for each participant read.



Method: Amplicon sequence variants (ASVs) were generated via dada2 v1.16.0 using post-QC FASTQ files. Within the workflow, the paired FASTQ reads were trimmed, and then filtered to remove reads containing Ns, or with maximum expected errors >=2. The dada2 learn error rate model was used to estimate the error profile prior to using the core dada2 algorithm for inferring the sample composition. Forward and reverse reads were merged by overlapping sequence, and chimeras were removed before taxonomic assignment. AV taxonomy was assigned up to genus level using the SILVA v.138 database with the method described by Wang and a minimum bootstrapping support of 50%. Species-level taxonomy was assigned to ASVs

only with 100% identity and unambiguous matching to the reference. Samples with fewer than 1,000 sequences were discarded. ASVs accounting for less than one millionth of all strain-level markers were discarded. Data was available as count data or relative abundance. In situations where microbes could not be reliably matched, they were labelled as *other*.

The Chao1 index, Shannon Index and observed ASV count were calculated from a rarefied, filtered ASV table set to the minimum depth allowed for a sample (1,000) using QUIME 1.9.1. Note: The pipeline produced alpha diversity outputs with ASV data, but QUIME 1.9.1 is technically only intended to handle OTU data. Amplicon Sequence Variants (ASVs) are a more accurate alternative to the traditional OTU approach and refer to sequences differing from each other by a single nucleotide. ASV-level analyses provide better resolution of microbial communities and enables accurate measurement of diversity.

Results of bioinformatic analyses:

Alpha diversity measures capture the species diversity (richness) within a functional community. Chao1 is an indicator of species richness (total number of species in a sample) that is sensitive to rare OTUs (singletons and doubletons). Higher values indicate higher diversity. The Shannon diversity measures uncertainty of predicting the species identity of an individual that is taken at random from the dataset. Rarefaction curves illustrate the diversity of microbes sampled across the whole cohort.

Figure 2: Rarefaction curves for the cohort.

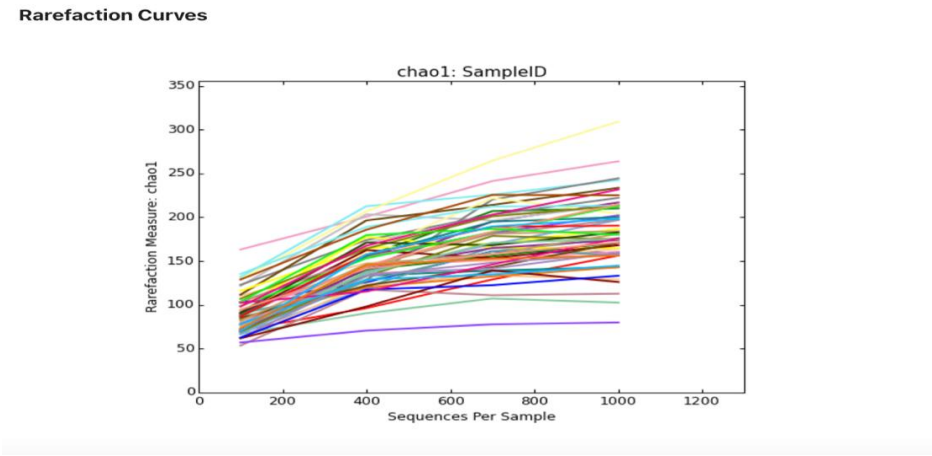


Table 2: Alpha diversity metrics, including ASV count, Chao 1, and Shannon for probiotic and placebo groups.

Metric	Probiotic		Placebo	
	Time 1	Time 2	Time 1	Time 2
OTU (ASV)	1893	1907 ↑	1625	1612 ↓
CHAO 1	2635.96	2640.48 ↑	2159.187	2088.37 ↓
SHANNON	79.18	79.12 ~	68.01	68.39 ~

↓ Reduced
↑ Increased
~ Unchanged

The alpha diversity metrics suggested a different pattern of diversity overall for the two groups, with a trend for increased diversity in the probiotic group at Time 2.

Beta diversity:

Beta diversity is a comparison of the difference of samples to each other. Beta diversity is the ratio between the regional and local species diversity. In other words, it measures the distance or dissimilarity between each sample pair. Weighted and unweighted UniFrac beta diversity metrics were calculated from the filtered ASV table using QIME 1.9.1. Bray-Curtis distances were calculated from the filtered ASV table using QUIME 1.9.1.

Weighted Unifrac distance relies on species abundance data and weights the branch length (greater genetic change or divergence) abundance difference. Hence it is most sensitive to any change in abundant lineages. However, weighted UniFrac distances may not detect change in *moderately* abundant lineages (Chen et al., 2012).

We investigated significant changes in beta diversity, or the diversity between samples by calculating pairwise distance between samples from a rarefied count table using 3 different metrics: Bray-Curtis distance, Unweighted UniFrac, and Weighted UniFrac. Both UniFrac metrics use information from the taxonomic tree. Unweighted UniFrac looks solely at taxa presence/absence, while Weighted UniFrac also includes taxa abundance. To visualize differences between samples we used Principal Coordinates Analysis (PCoA). In these figures, each point represents a sample, and the closer two samples are to one another, the more similar their microbiomes. Samples from the same participant are connected via coloured lines. The percent's listed on each axis are the percent of total variation between samples explained by that axis.

To test whether the probiotic treatment caused significantly larger changes in the microbiome compared to placebo, we compared the distance of each participant at Timepoint 1 and Timepoint 2 based on group. We tested for significant differences using non-parametric Wilcoxon tests, and visualized results as boxplots.

Additionally, we also examined differences in Bray-Curtis distance at the phylum and species levels (figures 3 and 4). Figure 3 shows the difference in beta diversity measured by Weighted UniFrac at the ASV level. Some participants within the probiotic showed large shifts in the microbiome after 12 weeks, the analysis showed no significant difference magnitude of change of the microbiome of probiotic compared to placebo group, as shown by the boxplot on the right.

Figure 3: Weighted UniFrac PCoA and distance boxplot: Placebo and Probiotic data.

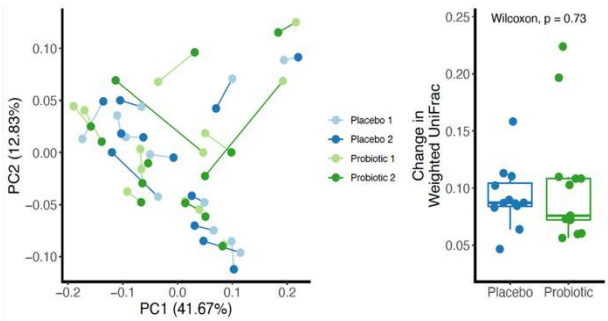
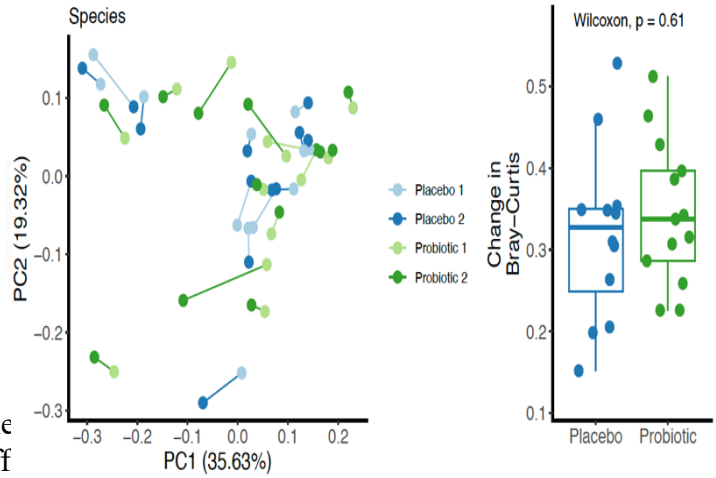


Figure 4: Bray-Curtis analysis of Phylum level data for probiotic and placebo groups.



The species composition between two diff ed non-parametric analysis showed no significant difference across the two groups at phylum level.

Figure 5: Bray-Curtis analysis of Species level data for probiotic and placebo groups.

Bray-Curtis ranked non-parametric analysis showed no significant difference across the two groups at the species level. We explored data at the species level taxa to ensure whether bioinformatic analyses results were not under powered or biased due to small samples.

Species-Specific Signature (SSS) Prevalence data:

Firstly, we catalogued all the species present for each participant that were identified through extraction and sequencing. This was an extremely lengthy process that was completed manually – approximately 166 species were identified per person but also there was a moderately large category of 'other' that were not identifiable.

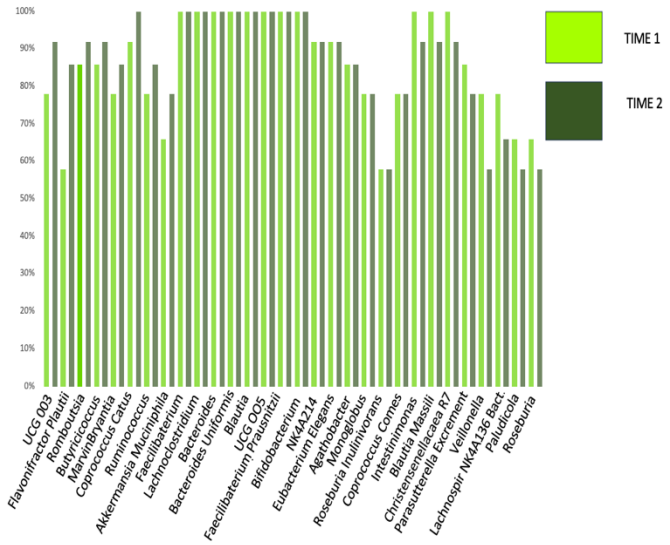
Figures 6 and 7 show the distribution of species across the two groups at baseline and at T2 to determine whether a PD microbial signature could feasibly be identified based on presence of species across all participants.

Percentage ratio of species present was computed for each species identified across the whole cohort and the charts show those present at 100% (present in everyone in the group) through to around 58% (present in above average number of the cohort).

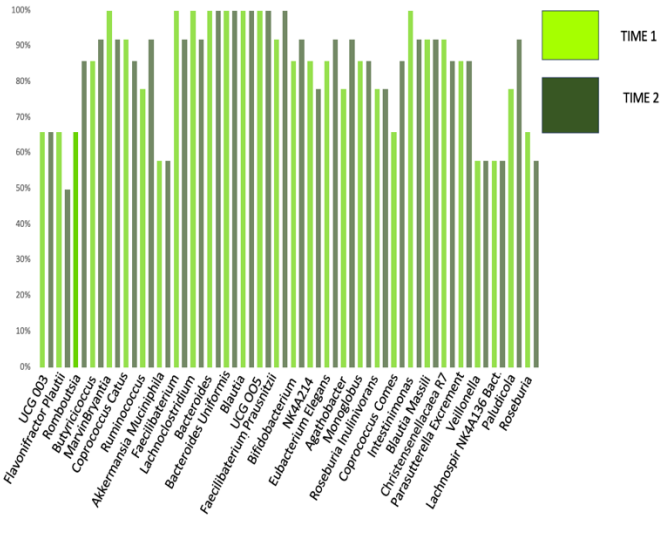
Note: these are the most *prevalent species* not necessarily the most abundant. The following ratio was used X divided by N (where X = number of participants where species has been identified and N = total cohort) x 100 to provide % *ratio* of prevalence figure. Once we had established the prevalent species across the entire group we classified them as commensal, pathobiont, hybrid – based on at least three papers in the literature that described their properties and mode of action, and the consensus of two reviewers. The hybrid group were difficult to classify as they could be either commensal or pathobiont depending on the environment and are not presented here.

Figure 6: Probiotic Group and Placebo Group prevalence at T1 and T2 – Commensal bacteria

Probiotic



Placebo

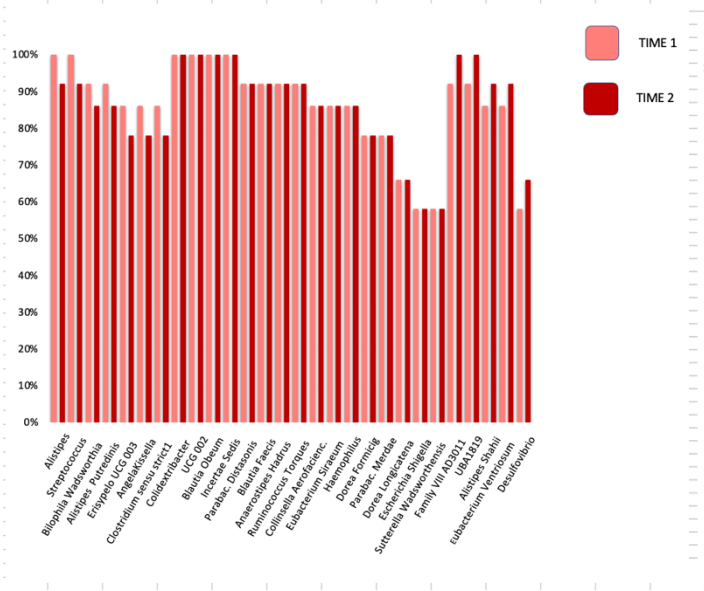


A higher number of commensal species increased in prevalence in the probiotic group compared to the placebo. These were UCG 003, Flavonifractor Plautii, Butyricicoccus, Marvinbryantia, Coprococcus Catus, Akkermansia Muciniphila and Ruminococcus. Romboutsia increased in prevalence across both groups. Additionally, Faecalibacterium Prausnitzii, Bifidobacterium, Agathabacter, Coprococcus Comes, and Eubacterium Elegans increased in prevalence at T2 in the placebo group. Different species-specific change in prevalence across groups at T2 potentially indicates differential effect of the probiotic on the GM in the probiotic group. Changes that occurred in the placebo group can be surmised to reflect the natural trajectory of the GM in PwP – arguably a negative trajectory. Fewer commensal species decreased in prevalence in the probiotic group compared to placebo. These

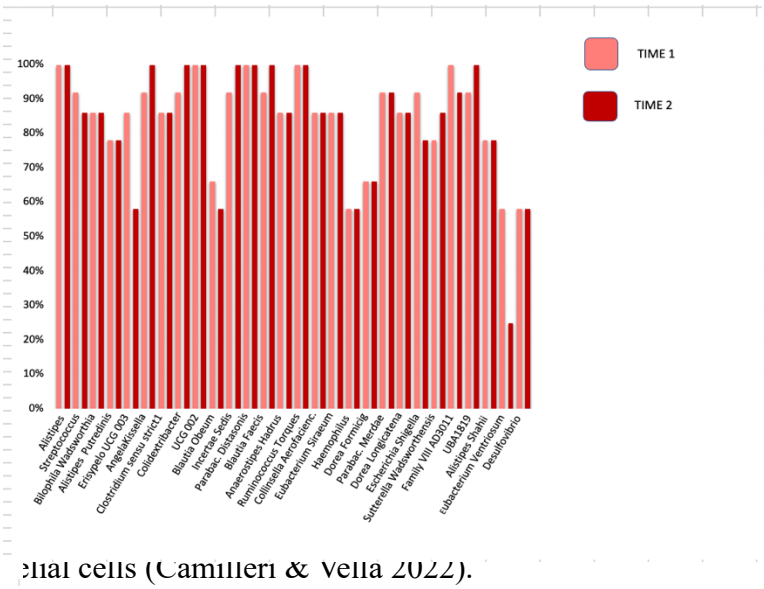
were Blautia Massiliensis, Parasutterella excrementihominis, Lachnospira and Veillonella. Christenellaceae R7, Intestimonas, and Roseburia decreased in prevalence in both groups. Additionally, Faecalibacterium, Lachnoclostridium, Butyricoccus, Flavonifractor Plautii, and NK4A214 were reduced in prevalence in the placebo group.

Figure 7: Probiotic Group and Placebo Group prevalence at T1 and T2 – Pathobiont bacteria

Probiotic



Placebo



ɛlial cells (Camilleri & Vella 2022).

Sixteen other species maintained the same prevalence over time. In the placebo group, species that decreased in prevalence over time were Blautia Obeum, Escherichia Shigella, Sutterella Wadsworthensis and Eubacterium Ventriosum. Fifteen species maintained the same prevalence over time. In both groups Streptococcus, and Erysipelotrichaceae UCG-003 decreased in prevalence over 12 weeks.

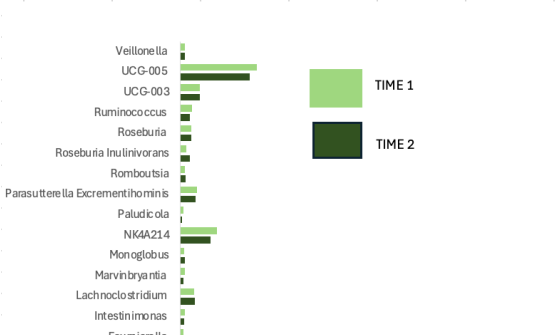
Pathobiont species that increased in prevalence in the probiotic group were Sutterella Wadsworthensis, Family XIII AD3011, Alistipes Shahii and Eubacterium Ventriosum. A different pattern was seen in the placebo group with Angelakissela, Colidextribacter, Blautia Faecis, Bacteria Incertae Sedis (taxa not known). Both groups showed increased prevalence of UBA1819 at T2.

Relative abundance data: signature

To explore potential differences between the groups at T2 this further we computed total relative abundance data (signature species only) for commensal and pathobiont species for both groups at T1 and T2 (see figure 8 and 9)

Figure 8 Commensal signature species probiotic and placebo, total relative abundance.

Probiotic



Placebo

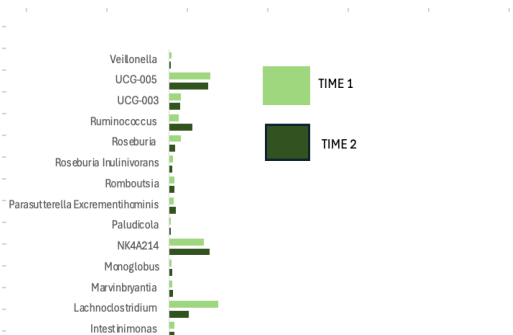


Figure 9 Pathobiont signature species for probiotic and placebo groups, total relative abundance.

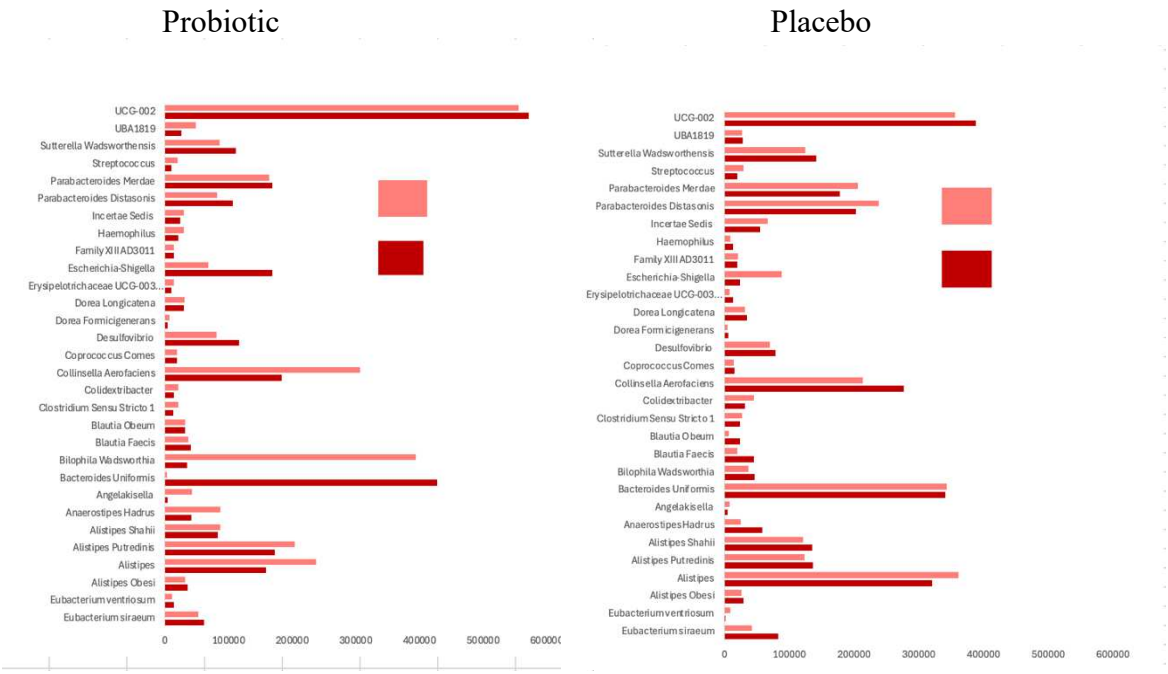


Figure 9 shows that 63% of signature pathobionts reduced in relative abundance at T2 in the and 36% of species increased in relative abundance in the probiotic group. Surprisingly the placebo group species relative abundance fell in the opposite direction 63% of species increased in abundance at T2, whilst 36% decreased in relative abundance. In sum the use of Symprove™ appeared to shift the GM into a more stable state (less dysbiotic) evidenced by reduced prevalence of pathobiont species and decreased relative abundance of pathobionts compared to placebo group GM.

The datasets generated and/or analysed during the current study are available in the Sheffield Hallam University research data repository (https://shurda.shu.ac.uk/id/eprint/288) on request.

Discussion

Identifying microorganisms involved in gut dysbiosis is essential for understanding their role in disease processes. In this study, typical bioinformatic analyses produced equivocal results although they were suggestive of a potential trend for differences between the groups at T2 compared to T1 data. In contrast detailed analyses of species data at the level of each participant identified a species signature for this group of PwP and changes to prevalence and abundance of signature species after 12 weeks of daily use of Symprove™ probiotic. Unexpectedly, there were also evident changes to the placebo group signature species at T2 that included increased prevalence of pathobionts and decrease in prevalence and relative abundance of commensal species different from baseline and from the probiotic group profile. Without this granular species-level analyses changes to GM in Parkinson's after probiotic use and without probiotic use, would not have been identified.

Comprehensive and detailed analyses of species-level data for each participant, although time-consuming, enabled detection of a pattern of species present across all cases at a prevalence ratio of 58% (above average/chance levels) up to 100%. These species were considered representative of PwP potentially forming a species-specific signature. Our cohort was relatively small but the consistent prevalence of signature species at baseline across the total group is a new finding in PwP. Arguably, species not included in the signature might constitute the *unique fingerprint* of each person's microbiota.

Our Parkinson's signature comprised a total of 71 species including 30 pathobionts and 28 commensal species categorised based on published data on the functions of each species. Additionally, there were 12 species categorised as both commensal and pathobionts depending upon the ecological niche based on extant literature, and a large proportion of Other that were unclassified (not reported here).

Our findings align well with the limited data available, Yang et al., (2020) also found a significant proportion of species were shared (90 ± 19 of 116) in their high prevalent bacteria group but they did not categorise these shared species as a signature nor evaluate the frequency of prevalence across participants. Importantly, Wallen et al., (2020) proposed that PD-associated micro-organisms have not been reliably identified due to small samples with low power that missed true associations. Our approach offers a solution to this through detailed species count across each participant including all species present above average (58%), and though the sample is small, this method yields robust and reliable data.

Many potential pathobionts were present in the signature (excluding the composition of the wider microbiome which may include additional pathobionts). This is atypical and indicative of a dysbiotic bacterial milieu when compared to healthy populations. For example, Li et al., (2023) found around 20 potential pathogenic species in the *complete* microbiome of a healthy Chinese cohort. Arguably, the high prevalence of pathobionts across the entire group in our study indicates a microbiome in decline and/or following an unhealthy trajectory. Introduction of the probiotic seemed to stall this process by increasing prevalence of commensals and reducing pathobionts after 12-weeks of dosing. Rather than seeing a specific increase in certain strains the introduction of the probiotic caused a shift in the overall composition of the GM in the probiotic group in a healthier direction than the placebo group.

Li et al., (2023) found that Klebsiella Pneumoniae and Bacteroides Fragilis were the most abundant *and* prevalent pathobionts in their healthy cohort, but neither of these were detected in the Parkinson's *signature* in the present study. It may be plausible that the prevalent pathobionts in healthy cohorts differ from those with illness and/or neurodegenerative conditions. Microbes known to be opportunistic pathogens are a particular focus of clinical interest. It is only by establishing the functions of these pathobionts that we can determine the way in which they may contribute to the development of Parkinson's and other conditions (Wallen et al., 2020).

There is no space here to describe the functions of each signature species based on our current knowledge though some are better documented than others. Briefly Alistipes Indistinctus has been associated with hypertension and high blood pressure (Jama et al., 2019; Kim et al., 2018), and most Alistipes strains are potentially harmful proliferating in the GM of PwP (Ghyselinck et al., 2021). Family XIII AD3011 group is associated with rapid eye movement sleep behaviour disorder and transition to Parkinson's (Nishiwaki et al., 2020), and Veillonella is associated with obesity and has been shown to be increased in PwP and considered a risk factor for developing Parkinson's (Bell et al., 2019).

The optimal microbial composition of the GM required to ensure a stable and healthy microbiome, beyond species diversity, is not known. It remains to be determined if this can be identified at the individual level of analysis at lower taxa than phylum level. Additionally, although species diversity is strongly advocated as a marker of a healthy gut microbiome the underlying mechanism is not known, beyond an association with lower species diversity in people with specific conditions compared to controls.

Species that increased and decreased in prevalence in *both* groups suggest resilience to probiotic effects and instead may represent the unperturbed natural evolution of the GM signature. It may transpire that these species should be prioritised as therapeutic targets, although changes in other species may have driven this recalibration and it is plausible to assume that not all bacterial species are influenced by a probiotic intervention. There is a need for longitudinal data with and without probiotic and a fuller exploration of the GM including viruses, archaea, fungi and parasites to establish a comprehensive understanding.

The emergence of research about condition-specific signatures may hold promise to target microbial ecosystems in vivo where the microbiome is considered dysbiotic. If we train our attention on species-level patterns in microbiome data of individuals with diverse conditions we may shed some light on how gut dysbiosis affects metabolic, immune and endocrinological functions of the host, and understand which microbes to target therapeutically. Condition-specific signatures require lengthy data analyses, and it would be useful to develop new pipelines to achieve these analyses across datasets and conditions through standardised techniques.

Our findings expand the list of PD-associated microbes at species level by identification of a species-specific-signature. The signature could not have been identified through standard bioinformatic approaches and required analyses and coding of each individual's microbiome at species level. Recognising signatures provides a means to condense complex data to evaluate efficacy of therapies/treatments and potentially target microbes. Well-formulated probiotics with an evidence base, hold real potential as a way forward as an adjunct to conventional treatment, but there is still much to learn about how they alter the overall microbiome milieu.

However, they are cost-effective, with few if any side effects, easy to tolerate and may even be a means to target antibiotic resistant strains in the future as our knowledge expands.

Gut microbiota composition is highly variable according to age, ethnicity, lifestyle, diet, metabolism, hormonal and immune processes. That said, a healthy gut microbiome achieves a particular stability and resilience to perturbation through some, as yet, unknown mechanism. Factors that trigger a healthy gut microbiome into dysbiosis have not been mechanistically determined raising the issue of whether microbial composition is cause, consequence or side effect of conditions (Rinninella et al., 2019).

Study limitations

Our study used 16S rRNA gene sequencing (long-read), the most used method of bacterial gene sequencing and future work could improve on this by adopting the shotgun metagenomic method. We had a relatively small sample, and the study needs replication with a much larger sample. Longitudinal data would be useful in PwP taking probiotic and placebo over greater time frame and increased frequency of stool analysis. A healthy control group would also provide more information about a healthy GM. Finally, the field overall needs better methods to capture viruses, fungi and archaea within the GM alongside bacteria so that we have a more comprehensive picture of the composition of the human GM.

Conclusion

Standardisation of the microbiome across conditions and in healthy groups is critical for translational research and development of therapeutic interventions in an environment of growing microbial antibiotic resistance. This must occur at species-level because other taxonomies lack detailed specificity and current bioinformatic analyses do not meet this need. Medicine is moving toward precision health strategies and species level-analyses alongside other metrics will enhance our knowledge of microbial manipulation informing bespoke probiotics and ‘drugging-the-bug’ approaches to promote health allowing us to map the mechanistic pathways that lead to dysbiosis and host ill health (Van Treuran & Dodd, 2020).

Our three main findings were (i) bioinformatic analyses were uninformative with a small sample (ii), there was a species-specific signature across the cohort of PwP that contained many pathobionts (iii), the GM was positively influenced by probiotic consumption compared to placebo.

Revision of the historical view of brain disorders as somatic, whole person conditions including 30 trillion human cells and 38 trillion bacteria is a possible way forward in health research (Sender, Fuchs & Milo, 2016). Precise interventions could lead to innovative, safe, effective treatments, and new ways of enhancing commensals and limiting pathobionts (Morais, Schreiber & Mazmanian, 2021). We may yet find that we can achieve this, at least in part, through prebiotics, probiotics and dietary change targeting specific gut microbial species.

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