Characterising the association between gut microbiome composition and Alzheimer's Disease

Introduction

Alzheimer's Disease (AD) is a progressive neurodegenerative disorder characterised by the presence of extracellular amyloid-β plaques and intracellular neurofibrillary tangles of tau protein (1, 2). Disease classification is determined by age, with the vast majority of cases being considered late-onset (3). While age, gender, and genetics, in particular the presence of the APOE-ε4 allele, can function as non-modifiable risk factors in late-onset cases, the exact mechanisms which contribute to pathogenesis in this disease are poorly understood and highlight the potential role of environmental factors in driving AD development (4, 5).

Current treatments for AD are symptomatic, able to alleviate symptoms of cognitive impairment by altering either cholinergic or glutamatergic signalling in the brain, but do not directly prevent or cure the disease itself (1). As such, early-stage interventions are required to delay disease progression to improve the duration at which current treatments will remain effective for patients, given that prevalence of AD is expected to increase dramatically by 2050 due to population aging (6). In order to reduce the growing burden of this disease in the coming years, identification of modifiable environmental risk factors and non-invasive clinical biomarkers is critical going forward.

In recent years, increasing evidence has highlighted the potential role of the gut microbiome as both an environmental marker and driver of many neurodegenerative diseases, including Parkinson's disease, multiple sclerosis, and amyotrophic lateral sclerosis (7-9). These interactions between the microbiome and the brain are mediated through the actions of the microbiota-gut-brain axis, involving immune, metabolic, and neuronal pathways (10). This axis may also play a key role in AD, as alterations in composition and diversity of the gut microbiome have been reported in both animal models and human patients with AD. Preclinical evidence from mouse models of AD has shown that faecal microbiota transfer from healthy controls can reduce formation of amyloid-β plaques, although the specific bacterial species that drive this preventative effect are currently unclear (11).

While alterations in the composition and diversity of the gut microbiome have been reported in both AD animal models and human patients with AD, these findings are inconsistent across studies (12). This is due to both the influence of other variables, such as country, presence of comorbidities or diet, which can alter microbiome composition independent of AD, as well as technical limitations (2). The majority of studies to date which have examined gut microbiome composition in AD have utilised 16s rRNA sequencing, which cannot discriminate bacteria to a species or strain level and does not provide similar sensitivity or specificity as shotgun metagenomic sequencing (13). As such, there is a critical need to provide a clearer definition of gut microbiome composition in AD before novel biomarkers or therapeutics can be developed.

This study aimed to determine whether AD is associated with an altered gut microbiome composition. Whole metagenome shotgun sequencing was used to characterise gut microbiome diversity and composition down to a species level in individuals with AD and cognitively normal controls (NC). In addition, the impact of age, gender and presence of comorbidities were investigated as potential confounding variables.

Methods

Participant Characteristics and Data Pre-processing

A total of 49 subjects (AD, n=26; NC, n=23) were recruited in this study. Figure 1 shows patient characteristics. Whole-metagenome shotgun sequencing was performed on stool samples collected from subjects. Raw metagenomic data was analysed using the MetaPhlAn3 tool. Taxonomic abundance profiles from each sample and associated subject metadata was provided for further downstream analysis in R (R Version 4.2.1). Unclassified and duplicated OTUs were removed prior to statistical analysis. Data was merged and analysed using *phyloseq, microbiome* and *vegan* R packages. All statistical analysis was performed using the Wilcoxon rank-sum test to compare differences between groups unless stated otherwise. Visualisation of subject characteristics was preformed using the *table1* package, while all other graphics were created using the *ggplot2* package.

	Control (N=23)	Disease (N=26)
Gender		
Female	12 (52.2%)	16 (61.5%)
Male	11 (47.8%)	10 (38.5%)
Age (years)		
Mean (SD)	83.0 (10.0)	82.2 (8.50)
Median [Min, Max]	88.0 [65.0, 93.0]	83.5 [65.0, 95.0]
Comorbidity		
Absent	12 (52.2%)	12 (46.2%)
Present	11 (47.8%)	14 (53.8%)

Figure 1 - Participant Characteristics

Diversity Analysis

Bacterial diversity was determined using α-diversity measures to determine richness and evenness within each sample and β-diversity measures to quantify overall differences between groups. Absolute abundance values were used to determine α-diversity across three metrics; Chao1, Shannon index and Simpson index. Relative abundance values were used to determine β-diversity using Bray-Curtis dissimilarity. Results were plotted using PCoA, with statistical differences in β-diversity between groups compared using PERMANOVA. Bacterial diversity was determined using absolute abundance values for α-diversity metrics (Chao 1, Shannon index and Simpson index) and relative abundance values for β-diversity (Bray-Curtis dissimilarity).

Differential Abundance Analysis

Differential abundance of taxa between groups was determined at a species level by comparing mean relative abundance of each species between AD and control groups. Absolute abundance values were filtered to remove any species with mean abundance less than $1x10^{-5}$. False discovery rate correction was applied to account for any false positive significant differences between groups. For analysis at the phylum and genus level, data was agglomerated and similar analysis was then performed. Results were expressed as mean difference in relative abundance in AD relative to the control group. Species showing greatest significant changes in relative abundance between groups were determined and comparison of both relative and absolute abundances across groups was calculated.

Analysis of Potential Confounding Variables

In order to confirm that any alterations in the gut microbiome are due to disease and not any other confounding variables, analysis was preformed examining the impact of age, gender, and presence of comorbidities on gut microbiome diversity. The impact of age was calculated using correlation analysis. Correlation analysis was preformed to determine the impact of age. Spearman's correlation coefficients were calculated between age and each α-diversity metric (Chao 1, Shannon index and Simpson index). Gender and comorbidity were both analysed by subsetting α-diversity results using the *facet_grid* function in the *ggplot2* package. Differences for each variable were compared separately within disease and control groups.

Results

Gut microbiome diversity is altered in AD

The diversity of the gut microbiome between AD and control groups was determined using measures comparing richness (Chao1); the number of species present in a sample, α-diversity (Shannon and Simpson indexes); the combination of richness and evenness within a sample, and β-diversity (Bray-Curtis Dissimilarity); overall differences in microbiome composition. The microbiome of the AD group showed reduced α-diversity across all measures, with Chao1 (AD vs NC: 83.16 vs 98.22, p = 0.0037), Shannon index (AD vs NC: 2.47 vs 3.27, p < 0.0001) and Simpson index (AD vs NC: 0.76 vs 0.93, p < 0.0001) significantly decreased in AD compared to controls. Additionally, calculation of β-diversity showed significant compositorial differences were evident between AD and control groups (F = 6.45, p = 0.0009).

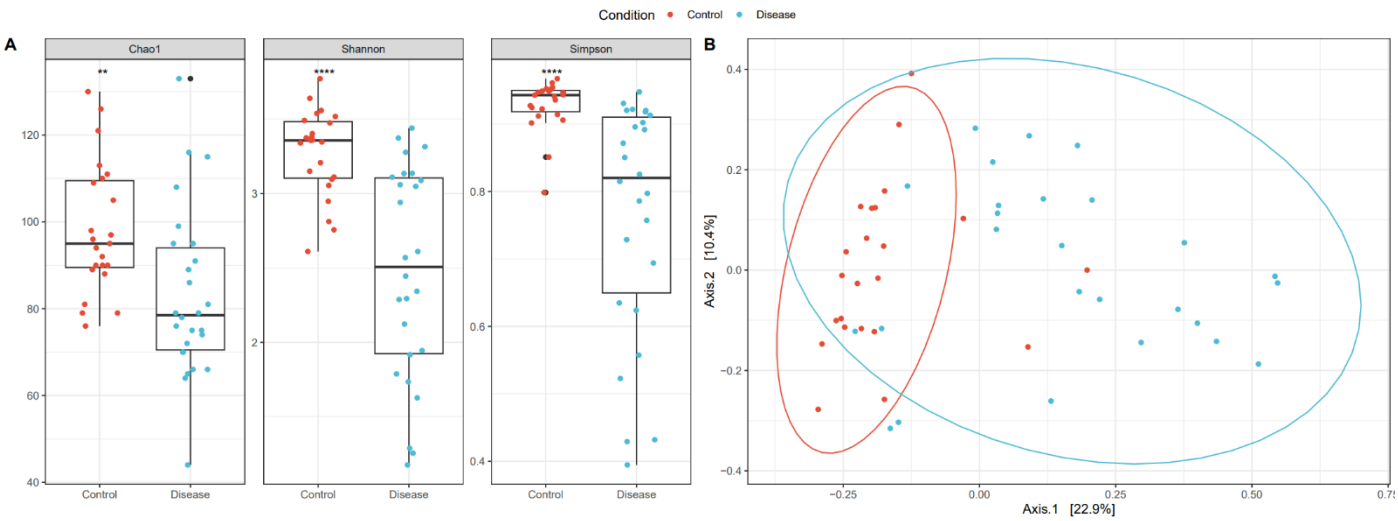

Figure 2 - Alzheimer's Disease is associated with alterations in gut microbiome diversity

*A) Boxplots representing the α-diversity of the gut microbiome across AD (Blue) and NC (Red) groups according to Chao1, Shannon and Simpson indexes. P values were determined using the Wilcoxon rank-sum test. B) PCoA plot of Bray-Curtis Dissimilarity of AD (Blue) and NC (Red) groups. Each dot represents a measure of microbiome composition of a given sample. Ellipses represent 95% confidence interval for each group. **p < 0.01, ****p < 0.0001.*

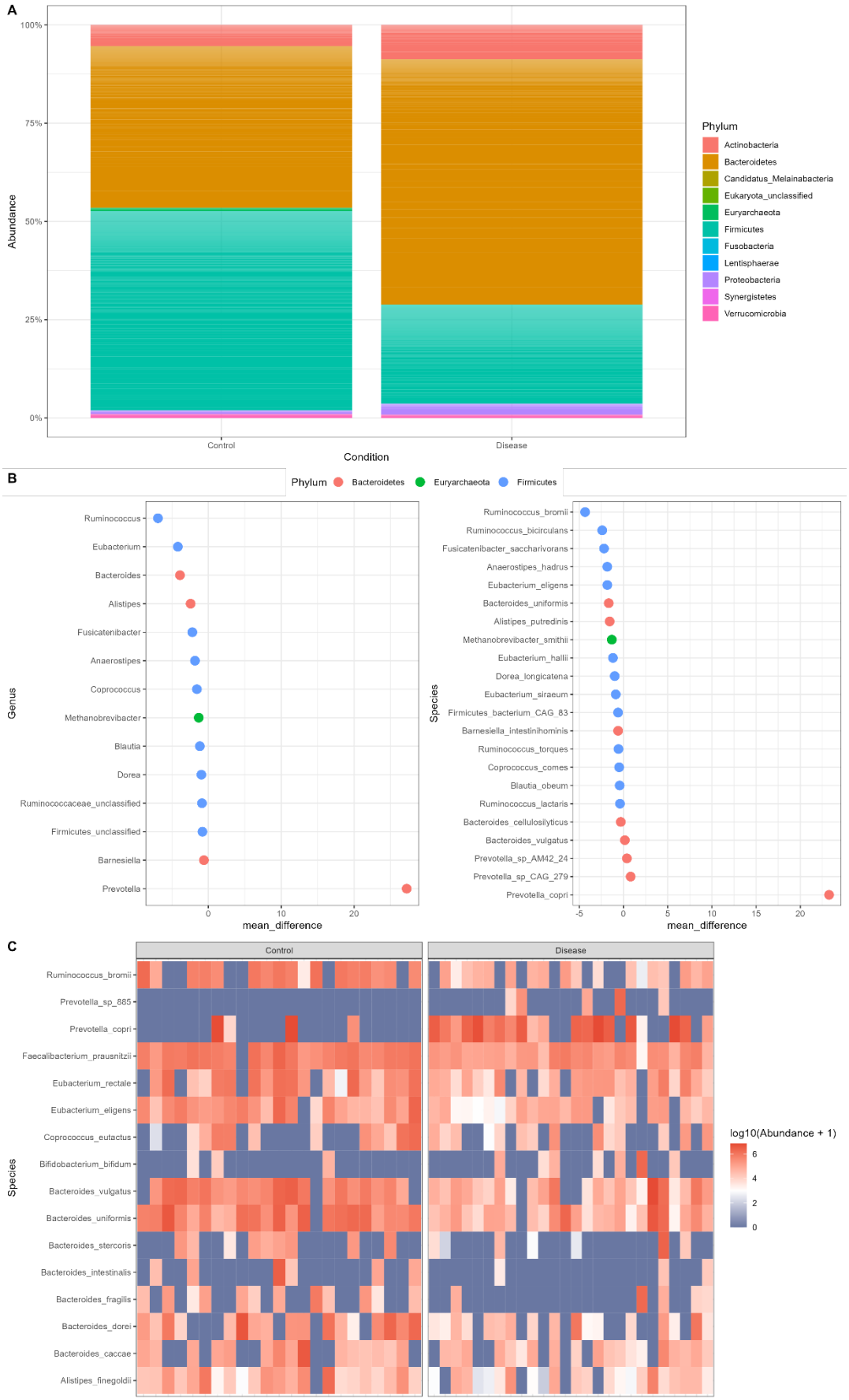

Figure 3 - Differential abundance of gut microbiome taxa in Alzheimer's Disease

A) Stacked barplots showing mean relative abundance of phyla present in AD and NC samples. B) Mean differences of significantly altered genera (left) and species (right) determined by Wilcoxon rank-sum test and false discovery rate correction. Each dot represents the difference in mean abundance in the AD group compared to the control group and is coloured according to phylum. C) Heatmap representing most abundant species across all samples, subset into AD (right) and control groups (left).

Changes in microbiome composition in AD are driven by *Prevotella copri* and *Ruminococcus bromii*

OTUs (Observable taxonomic units) were grouped and differential abundance was analysed at phylum, genus, and species levels (Figure 3). At a phylum level, patients with AD demonstrated significant decrease in *Firmicutes* (AD vs NC: 20.81% vs 43.39%, $p < 0.0001$) and *Euryarchaeota* (AD vs NC: 0.16% vs 1.43%, $p = 0.0121$). Conversely, *Proteobacteria* (AD vs NC: 1.81% vs 0.26%, $p = 0.0377$) and *Bacteroidetes* (AD vs NC: 43.73% vs 23.26%, $p = 0.0081$) were enriched in AD patients compared to controls (Figure 3A).

Similar significant alterations between AD and control groups were found at the genus and species level. The decrease in *Firmicutes* was mostly due to decreased abundance of the *Ruminococcus* genus (AD vs NC: 1.38% vs 8.30%, $p < 0.0001$)(Figure 3B, left). Notably, one of the most abundant species across all samples (Figure 3C), *Ruminococcus bromii*, showed a similar significant decrease in AD compared to controls (AD vs NC: 0.96% vs 5.30%, $p = 0.0027$)(Figure 3B, right). Increased abundance of *Bacteroidetes* in patients with AD was mostly due to a dramatic increase in abundance of *Prevotella* (AD vs NC: 30.62% vs 3.39%, $p = 0.0001$)(Figure 3B, left), in turn this increase at a genus level was explained by marked increased abundance of the species *Prevotella copri* (AD vs NC: 26.38% vs 3.13%, $p = 0.0002$)(Figure 3B, right), another abundant species across all samples (Figure 3C). As the species showing the largest mean differences in relative abundance between AD and control groups, *P.copri* and *R.bromii* showed similar changes across absolute abundance (Figure 4).

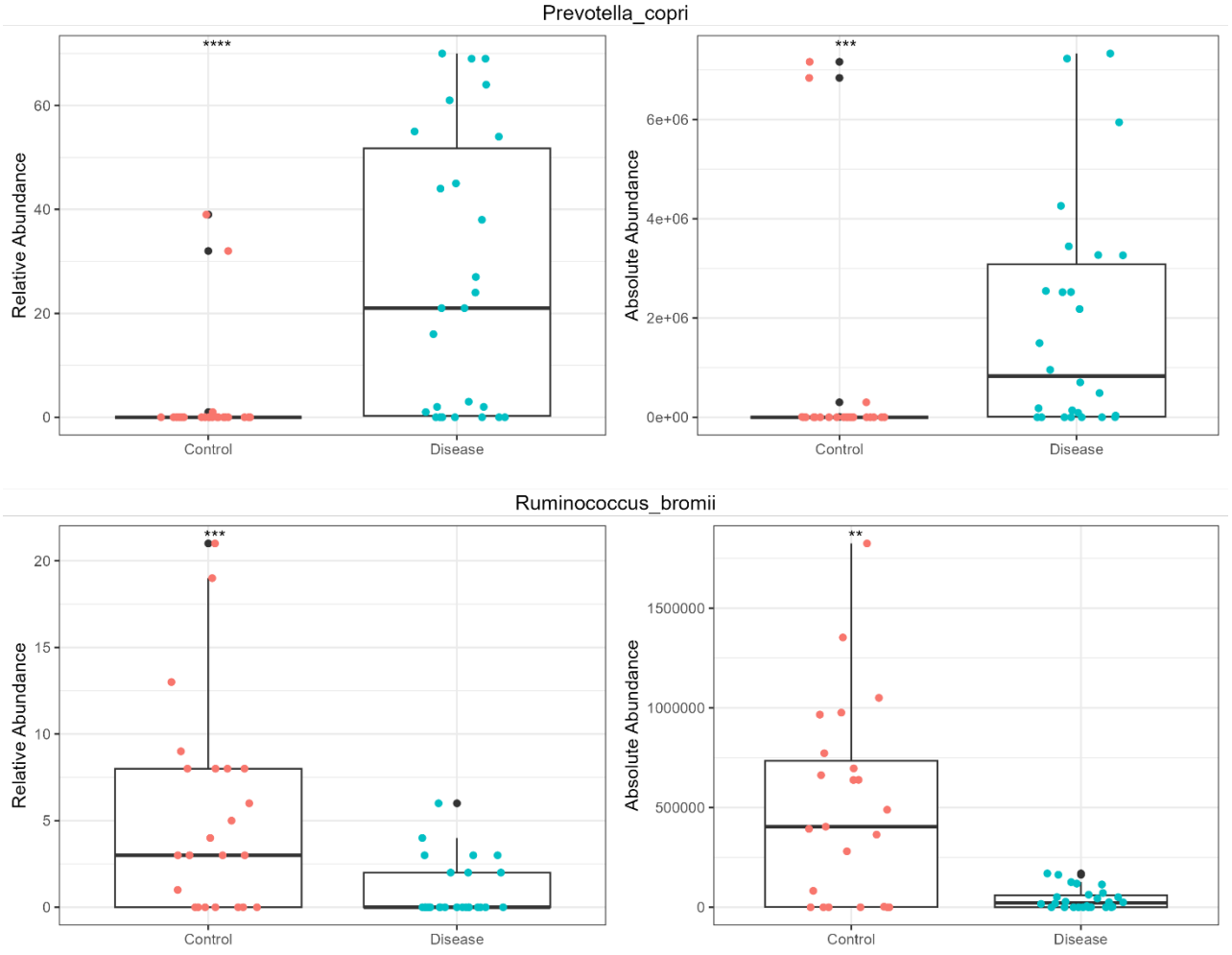

Figure 4 – Differential abundance of key species Prevotella copri and Ruminococcus bromii

*Boxplots representing relative and absolute abundance values for Prevotella copri (Top) and Ruminococcus bromii (Bottom) species. P values were determined using the Wilcoxon rank-sum test. **p < 0.01, ***p < 0.001, ****p < 0.0001.*

Age, gender, and presence of comorbidities do not significantly impact gut microbiome diversity in AD

In order to validate that our findings were due to changes in the gut microbiome in AD and not merely result of other confounders, analysis was preformed using three variables which were included in participant metadata; age, gender, and presence of comorbidities. Across all participants, there were no correlation between any α-diversity measures and age (Figure 5A), which was likely to be due to all participants being over the age of 65+. Similarly, comparison of gender (Figure 5B) and comorbidities (Figure 5C) within AD and control groups did not reveal any significant differences across any of the three α-diversity measures. However, disease groups did show a wider range of diversity scores, particularly notable in female AD patients.

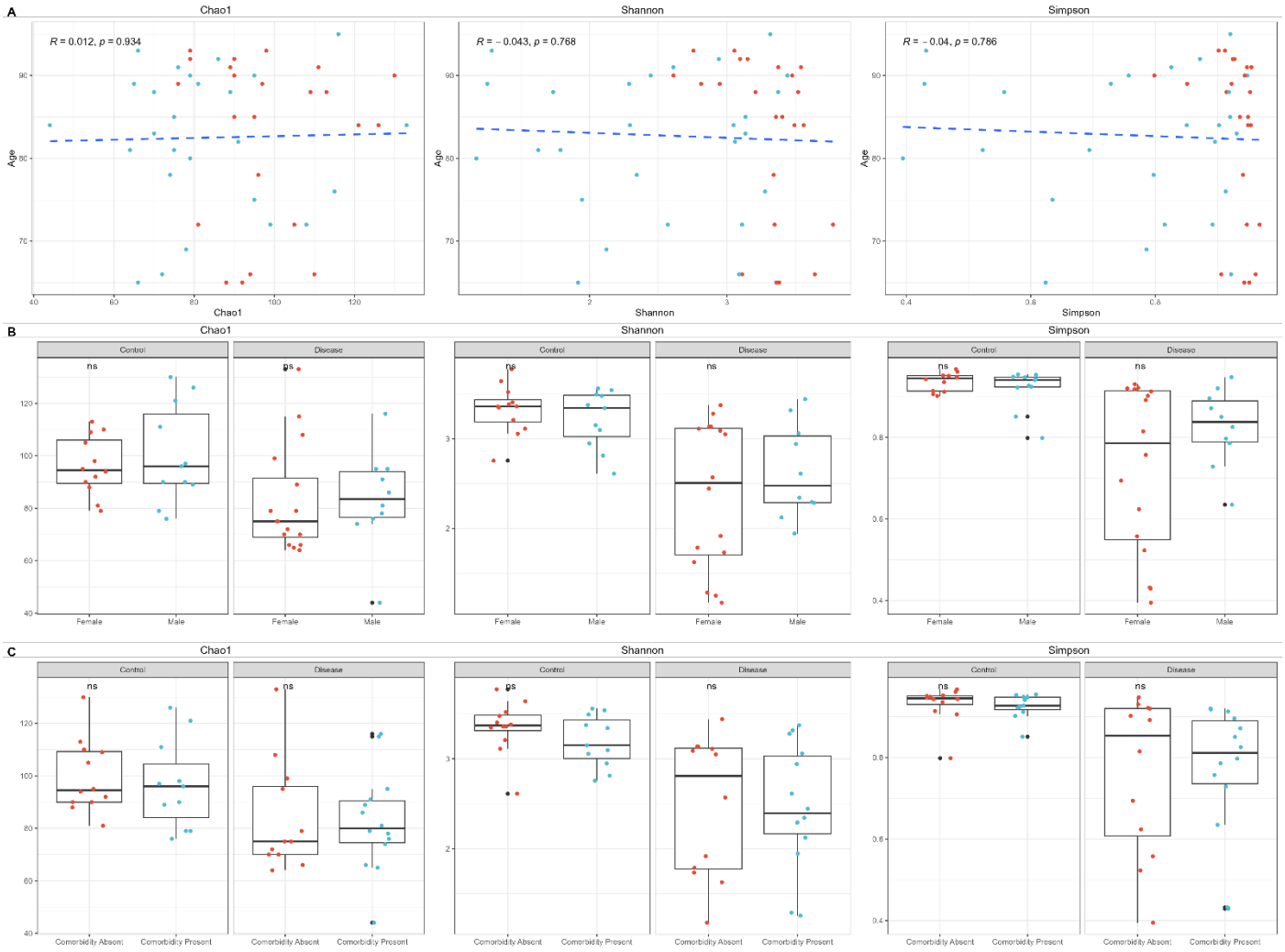

Figure 5 – Age, gender and presence of comorbidities do not alter gut microbiome α-diversity

A) Scatter plots showing correlation of α-diversity measures with age in AD (Blue) and control (Red) groups using Spearman's rank correlation coefficients. B) Boxplots representing the α-diversity of the gut microbiome across female (Red) and male (Blue) groups according to Chao1, Shannon and Simpson indexes. Data was subset by condition and p values were determined using the Wilcoxon rank-sum test. C) Boxplots representing the α-diversity of the gut microbiome across absence (Red) and presence (Blue) of comorbidities according to Chao1, Shannon and Simpson indexes. Data was subset by condition and p values were determined using the Wilcoxon rank-sum test. Non-significant results were assigned as 'ns,' where p > 0.05.

Discussion

The gut microbiome has the potential to act as both a novel biomarker and therapeutic target for Alzheimer's disease. However, inconsistent results from profiling and analysing microbiome composition in AD patients has limited this potential (12). In this study, whole-metagenome shotgun sequencing was used to characterise the diversity and composition of the gut microbiome in AD patients and cognitively normal controls. Our findings suggest that alterations in the gut microbiome in AD are primarily driven by an increase in *P. copri* and a subsequent decrease in *R. bromii*. Furthermore, we showed that these alterations are associated with AD and not factors such as age, gender, and comorbidities.

Gut microbiome diversity, measured across Chao 1, Shannon, and Simpson indexes, showed significant decreases in AD compared to controls. These findings match those seen in a recent systematic review of gut microbiome composition in AD (12), as well as reported decreases in diversity in other conditions, including Parkinson's disease and Irritable Bowel Syndrome (IBS) (7, 14). Given the variation observed in identification of specific species altered in AD, understanding the impact of dysbiosis, combining overall changes in composition and diversity, may highlight a clearer relationship between the gut microbiome as a whole and AD pathology. Indeed, recent preclinical evidence has shown that targeting dysbiosis with faecal matter transplantation in a mouse model of AD was sufficient to delay disease progression (11).

In this study, the phylum *Firmicutes*, as well as genera and species classified within *Firmicutes*, were decreased in AD. In parallel, the phylum *Bacteroidetes* showed a significant increase compared to controls, which was reflected by increased *Prevotella* at the genus level. These two phyla comprise the majority of bacteria characterised in the human gut microbiome (15). A decrease in the ratio of *Firmicutes* to *Bacteroidetes*, which was observed here, has also been reported in neurodegenerative disease such as ALS and Parkinson's disease (7, 8). At a genus level, AD was associated with a decrease in *Ruminococcus*. These findings are consistent with analysis of the gut microbiome in a Chinese cohort (16), but have not been observed across other studies (17, 18). Similarly, in line with our findings, the genus *Prevotella* has been reported to be increased in AD (19). However, these results were not observed in a recent systematic review, which examined gut microbiome composition in AD across American and Chinese studies, highlighting the impact of country on studies of the gut microbiome (12).

At a species level, *P. copri* showed a dramatic increase in relative abundance in AD. This species has not been previously associated with AD, however, this may be due to the majority of studies to date using 16s rRNA sequencing to analyse the gut microbiome in AD, which does not allow for classification at a species level (13). Despite a lack of correlation with AD, *P. copri* has been reported to increase in Rheumatoid Arthritis and is thought to act as a driver of pro-inflammatory immune responses in the disease (20-22). Given the increasing evidence supporting the role of inflammation in AD, understanding the relationship of this species with inflammatory markers in AD may be promising going forward (23, 24). The short-chain fatty acid (SCFA) producer *R. bromii* showed the largest decrease in relative abundance in AD compared to controls. Similar to *P. copri*, this species has not been previously linked with AD, however, a decrease in *R. bromii* has been reported in IBS (14). Evidence has highlighted the ability of *R. bromii* to breakdown resistant starches into SCFAs (25), which are essential metabolites for both maintenance of intestinal permeability and microglial function (26). Dysfunction of either of these features has been observed in AD (26, 27) and as such, our findings support a mechanism of AD pathogenesis, mediated by a decrease in SCFA-producing species, including *R. bromii*.

Analysis of age, gender and presence of comorbidities did not reveal any association of such variables on gut microbiome diversity in AD, suggesting that these variables are not influencing our results. However, the impact of such factors on gut microbiome composition and relative abundance of species cannot be completely ruled out.

As the prevalence of Alzheimer's disease continues to climb, identification of modifiable risk factors and non-invasive biomarkers is crucial going forward. This study provides evidence that AD is associated with an altered gut microbiome composition. Identification of key species showing altered abundances in AD suggests a potential role of *P. copri* and *R. bromii* as novel microbial biomarkers of AD. Future research should prioritise the use of whole-metagenome shotgun sequencing to provide species-level analysis in order to validate the findings reported here. Furthermore, analysis of this taxonomic data in combination with immune and metabolic factors is needed to understand the mechanisms which could link gut microbiome species, inflammation, and metabolites in AD. Overall, while these findings provide a basic groundwork for more comprehensive analysis of the gut microbiome in Alzheimer's disease, more work is needed to understand the mechanisms and microbial signatures that underlie this fascinating connection.

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