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Metagenomics study reveals altered composition of *Avispirillum, Phocaeicola, Bacteroides*, and *Faecalibacterium* in the gut of patients suffering with Alzheimer's disease

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ABSTRACT

Alzheimer's disease (AD) is a chronic neurodegenerative disorder that is characterized by memory loss and changes in behaviours, associated with the presence of amyloid-beta and tau proteins in the brain, which interferes with the normal functioning of the brain. Recent studies have tried to establish the structural relationship between the gut microbiota and the brain referred to as the Microbiota–Gut–Brain Axis. The present study aims to investigate a 16S rRNA gene sequencing sample, to analyze the differences in gut microbiota between 116 AD patients and 60 healthy controls retrieved from NCBI ((PRJNA770746, PRJNA533610, and PRJNA811324). Each sample was retrieved, demuxed and denoised to remove low-quality and chimeric sequences. The feature table was then constructed to determine the alpha diversity. The Kruskal-Wallis test done for prediction of alpha diversity calculated in patients with AD had a p-value of 0.0592. The bacterial features calculated through the Adonis test had a f-test value of 2.724 indicating huge microbial dysbiosis in the patient sample. Further ANCOM statistical test identified increased composition of Phocaeicola (clr 3.585), Bacteroides (clr 3.411) and Faecalibacterium (clr 3.3165) while Avispirillum (clr −1.0804) were found in reduced composition in patients with AD. The increasing microbes in Alzheimer's disease patients could be attributed to alterations in diet, immune system changes, and metabolic disturbances that create a gut environment conducive to the growth of these specific bacterial communities. Therefore, it can be an essential research area for neurodegenerative diseases, advancing our knowledge of potential biomarkers and therapeutic targets for minimizing the burden of AD.

1. Introduction

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by progressive cognitive decline, memory loss, communication problem, daily life challenges and behavioral changes. In the early stages of Alzheimer's disease, individuals experience mild symptoms and can live independently. However, in the moderate and late stages, symptoms worsen, and they require full-time care and support. This is the most common type of the dementia and can be characterized by neurofibrillary tangles[1] where certain proteins (amyloid-beta and tau) build up abnormally in the brain, disrupting communication leading to cell death. Amyloid-beta (Aβ) forms clumps called Amyloid plaques outside the cells, while tau forms twisted fibres called neurofibrillary tangles inside the cells. These problems are especially common in areas like the medial temporal lobe (important for memory) and other parts of the brain's cortex that are involved in complex thinking processes as shown in Fig. 1. This buildup of proteins disrupts normal brain function and leads to the development of Alzheimer's disease where these proteins are secreted by gut. The intestine, also known as the gastrointestinal tract, is a complex system that regulates the digestion, absorption, and elimination of microorganisms that inhabit the human digestive tract, including bacteria, archaea, fungi, and viruses[17,25,28]. These play an important role in the regulation of mood, appetite and digestion, among other functions [54]. Effects of the gut on mood and emotions are important in the gut microbiome, which produces metabolites that affect mood, cognitive function, and behavior This intimate relationship gives rise to the gut-brain area. Growing evidence indicates that there is a bidirectional connection between the gut microbiota and the brain, which is called the Microbiota–Gut–Brain Axis [3]. The gut, often referred to as the second brain which produces hormones and neurotransmitters that send signals to brain, influencing mood, cognitive function and behavior[27]. The bidirectional communication allows the

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gut and brain to work together, sharing information and resources to maintain overall health and well-being [3,4]. Gut bacteria produce substances like amyloids and lipopolysaccharides, which change communication in the cell and cause inflammation in the body. These buildups of amyloid-beta (Aβ) in the brain [5, [24][26] disrupt its functioning and increase the chances of occurrence of the disease in the human body. This also leads to age-related memory problems and dementia. Changes in the gut microbiota can be one of the major reasons people develop such diseases, which means keeping our gut bacteria healthy can slow down the memory loss process [6] (see Table 5, Fig. 6 and 7a and 12).

Employing high-throughput sequencing technologies, researchers can analyze vast amounts of genetic information, identify previously unknown or uncultured microbial species, and uncover the functional roles these microbes play in health and disease. Metagenomic studies have revealed altered functional pathways in the gut microbiome of Alzheimer's patients, including changes in carbohydrate metabolism, amino acid synthesis, and detoxification processes. These findings underscore the potential of the gut microbiome as a critical factor in Alzheimer's disease progression and highlight its importance as a target for therapeutic interventions [52]. To gain a comprehensive understanding of microbial communities in the context of Alzheimer's disease, researchers often analyze both alpha and beta diversity. Alpha diversity measures the richness and evenness of species within a single sample, providing insights into the overall microbial diversity in each environment [13,56]. Metrics such as the Shannon index, Simpson index, and evenness are commonly used to quantify alpha diversity, with higher values indicating a more balanced and diverse microbial community. Beta diversity, on the other hand, compares the microbial diversity between different samples, helping researchers assess how distinct microbial communities are from one another. In the QIIME 2 pipeline, beta diversity is evaluated using metrics like Bray-Curtis dissimilarity, Jaccard index, weighted UniFrac, and unweighted UniFrac, which consider both the abundance and phylogenetic relationships of microbial species [14] as shown in Fig. 2.

The gut-brain axis is a well-documented pathway by which gut microbiota can influence brain health and function (see Fig. 3). The relationship between gut microbiota and neurological conditions, particularly Alzheimer's disease (AD), has gained significant attention in recent years. Studies have shown that individuals with Alzheimer's disease exhibit an altered gut microbiome. Alterations in gut microbiota composition have been linked to systemic inflammation, immune dysregulation, and the production of neuroactive compounds, all of which are implicated in the development and progression of Alzheimer's disease, exhibiting unique microbiota signatures with observed differences in gut microbiota composition and diversity which suggest a possible link between microbiota diversity and Alzheimer's disease [35]. Gut microbiotas are known to be influenced by numerous factors, including diet, lifestyle, and health status. Their composition can fluctuate with age, health conditions, and even within a short timeframe due to dietary changes[2,32]. This variability emphasizes the need for longitudinal studies to track microbial changes over time and their correlation with disease progression. Hence the altered gut microbiome of people suffering Alzheimer's is analyzed in order to search for genes in gut bacteria and identify specific bacterial signatures associated with Alzheimer's disease [55]. Although metagenomics studies have revealed altered functional pathways in the gut microbiome of Alzheimer's patients, including changes in carbohydrate metabolism, amino acid synthesis, and detoxification processes [53], these findings suggest that the gut microbiome plays an important role in Alzheimer's disease progression and it helps in identifying therapeutic targets and biomarkers of neurological disorders.

2. Methods

Microbiome sequencing data can be analyzed and processed using the QIIME platform, the workflow for amplicon sequencing data in QIIME 2 is as follows.

2.1. Raw sequence retrieval

Raw sequence data required for hypothesis testing in this study were obtained from pooled biological samples using sequencing technology such as Illumina. These sequences, including nucleotide sequences including DNA, were obtained from BioProjects, Sequence Read Archive (SRA), and the PubMed database at the National Center for Biological Sciences (NCBI)[11]. Etc. Were obtained from large repositories The Data were primarily in paired-end format (where DNA is read from both ends) and single-end format (where DNA is read from one end), some being demultiplexed or multiplexed. To facilitate further analysis, the raw sequences were converted into. qza format, compatible with the QIIME 2 workflow[44].

2.2. Demultiplexing and denoising sequences

The first step involves multiplexing the sequence, which separates sequences based on sample-specific barcodes and outputs the front-end in paired-end sequencing, reading it combines from both ends to increase the accuracy. The QIIME 2 demux summarize tool was used to search and control sample attribution, ensuring accurate sample identification and data preparation for microbiome analysis. After demultiplexing, the denoised sequence [53] was performed using the QIIME 2 DADA2 method. This process had four steps: trimming to remove inferior bases from read-ends, similarity distortion to assemble similar sequences and reduce complexity [53], filtering to remove biased or erroneous sequences, remove chimeras to identify and exclude artifacts

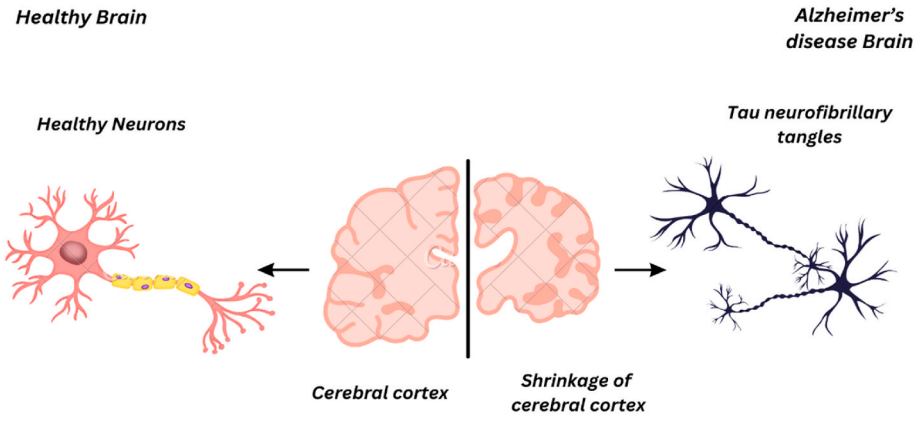


Fig. 1. Difference between the healthy brain and Alzheimer's disease brain representing the shrinkage of the cerebral cortex due to tau neurofibrillary tangles.

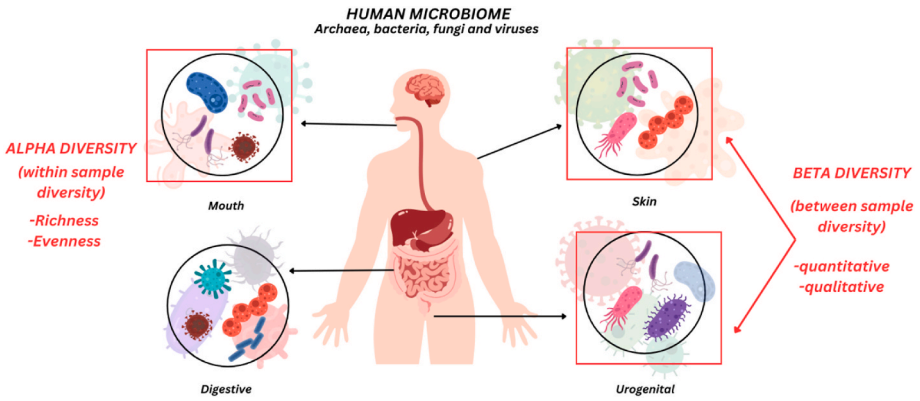


Fig. 2. Alpha and beta diversity representing the human microbes within sample and between sample depicting the intrinsic and comparative characteristics.

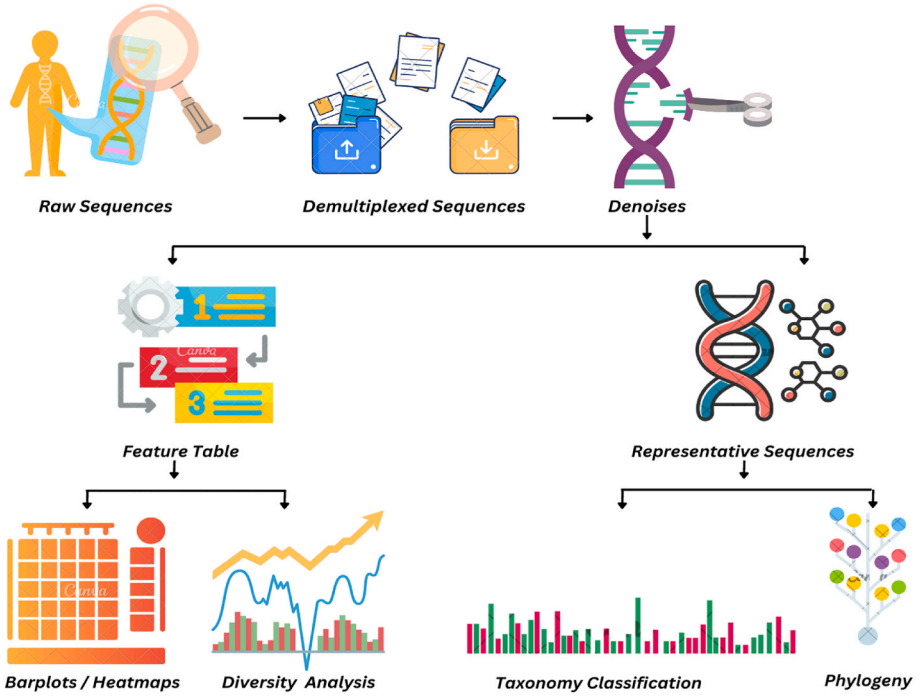


Fig. 3. Flowchart representing the complete study of the methodology for Identification, Isolation, and Analysis of Microorganisms.

from PCR amplification and then visualize the noise-free sequences of the physical characteristics were generated for selection, followed by microbial community analysis was very accurate [15,16].

2.3. Feature table

The feature table in QIIME 2 contains the counts for each Amplicon Sequence Variant (ASV) in all samples. This table counts each unique ASV occurrence, facilitating in-depth analysis and classification. The correlational pattern annotation feature allows for the evaluation of sequence depth and rarity, which affects the retention or loss of patterns. Providing detailed information on ASV distribution, the feature table supports the analysis of microbial range and community structure.

2.4. Diversity analysis

Diversity analysis in microbiological studies determine the number and structure of bacteria in samples and compare these values between samples Alpha-species coefficients, such as Shannon index, Simpson index, and evenness, measure support species richness and evenness of a sample [49]. Beta diversity compares bacterial species between samples using disparity measure of distance core metrics phylogenetic analysis in QIIME 2 provides key metrics including Bray-Curtis dissimilarity, Jaccard index, weighted UniFrac, and unweighted UniFrac, which provide insight into differences in microbial communities and their responses to environmental factors.

2.5. Visualization of microbial community in gut

Bar plots and heatmaps are used in QIIME 2 to visualize microbial community data. Bar plots show the number of classes in each sample, facilitating comparative analysis across groups such as patients and controls. Heat maps represent quantitative data in matrix form, where color intensity indicates the relative abundance of classes, thus revealing patterns and relationships. These visual tools are important for understanding microbial diversity and under the change in circumstances.

2.6. Representative sequence retrieval

Representative sequences were selected to identify groups of similar

sequences derived from amplicon sequence data. Clustering is based on sequence similarity, followed by representative sequences for taxonomic classification and phylogenetic analysis. This approach simplifies data manipulation by focusing on a limited number of sequences, enabling accurate assessment of microbial community structure and diversity while preserving biodiversity on important information.

2.7. Taxonomy classification and phylogenetic analysis

Taxonomy involves the identification and classification of microorganisms based on their genetic structure. Using 16S rRNA sequences, sequences are compared to reference databases[29,45] to assign taxonomic labels from broad groups (e.g., phylum) to more specific ones (e.g., genus) The QIIME 2 pipeline generates microbial-descriptive taxonomic profiles composition of samples in detail. Manufacturing processes are visualized using stacks of wooden blocks, providing insight into microbial diversity and their role in the environment in conditions, such as Alzheimer's between disease and healthy controls. Phylogenetic analysis is used to understand the evolutionary relationships among microorganisms [20]. Sequences and MAFFT alignments were generated using the qiime phylogeny align-to-tree-mafft-fasttree pipeline from the q2-phylogeny plugin and a phylogenetic tree was constructed using FastTree. This de novo method, which does not rely on reference databases, enables detection of evolution. A phylogenetic tree that also supports subsequent research on beta diversity helps to understand the evolutionary relationships and microbial communities associated with Alzheimer's disease.

2.8. Statistical analysis

The ADONIS (Analysis of Similarities) test, a component of PERMANOVA and a permutation-based method, was conducted to further confirm the importance of different categories of beta variables, these statistical methods are used to determine if there are significant differences in microbiome composition between groups, such as control and diseased patients. It works by calculating distances between samples based on their microbial composition, using distance metrics like Bray-Curtis or UniFrac. The test then assesses whether the differences between groups are greater than the variation within each group. The results of Adonis include a p-value, which indicates whether the differences are statistically significant (with a p-value less than 0.05 suggesting a significant difference), and an R-squared value, which shows how much of the variation in the data can be explained by the group differences. If the test shows a significant difference, it suggests that the disease may cause noticeable changes in the microbiome. On the other hand, if no significant difference is found, it means the microbiomes of control and diseased patients are quite similar. This method helps researchers understand whether diseases like Alzheimer's might be linked to specific changes in gut microbial communities. ANCOM test stands for Analysis of Composition of Microbiomes and is used to differentially abundant features in a microbial dataset.

In the context of a microbiome study, QIIME2 pipeline with ANCOM (Analysis of Composition of Microbiomes) was used to compare groups of control and disease Alzheimer's individuals and to identify abundant features such as differential bacteria[21]. ANCOM is a tool that defines the number or ratios represented in the form of a volcano plot.

3. Result

3.1. Study participants

The 16S rRNA sequencing data of Alzheimer's patient was assessed through the Gene expression omnibus (GEO) database of NCBI. Only studies with patients between the ages of 40 and 80 were included. From the seven studies, three met this age criterion for a total of 176 samples obtained information from on an Alzheimer's patient gut microbiome from Vancouver (Canada), Norwich, (UK) and Kazakhstan [7–9]. All three projects were chosen for their relevance to the analysis of gut microbiota in the context of Alzheimer's disease (AD). These Bioprojects (PRJNA770746, PRJNA533610, and PRJNA811324), use Illumina NovaSeq 6000 platform and Amplicon assay type [48] to sequence stool or faecal samples. Of these, 116 samples came from those with clinically diagnosed AD and 62 control samples. These categorizations are detailed in Fig. 4 subjects with mental disorders not related to AD, or those not meeting our specified criteria, were excluded from the analysis. The age difference shown in Table 1 highlights that AD is more prevalent in the older population. People with AD had a lower body mass index (BMI) [19], meaning they were lighter than the control group.

3.2. Demultiplexing and denoising samples

The final set of 176 samples from individuals suffering from Alzheimer's and healthy individuals were divided into two subgroups, i.e., single-ended data (involves reading only one end of a DNA fragment) and paired-ended data (involves reading both ends of a DNA fragment, producing two reads per fragment), according to 16S rRNA sequencing data[51]. The quality check of raw data was performed using FastQ programmer (the software tool used for this purpose). Sequence data were then processed by the QIIME 2 [10] pipeline. The initial step involved demultiplexing sequencing data obtained from NCBI to separate reads belonging to different samples taken with the following parameters: sequence length = 250, Truculent = 0, trim left = 0, maximum expected error = 2.0, pooling method = independent, chimaera method = consensus, minimum fold parent in abundance = 1.0 in single-ended data and Truncate Sequence Length = 450, truncate length forward = 224, truncate length reverse = 184, trim left forward = 0, trim left back = 0, maximum expected error a forward = 2.0, maximum expected backward error = 2.0, minimum overlap = 12, pooling method = independent, chimera method = consensus, minimum fold parent in number = 1.0 for paired-end data. The forward end (reads from one end of the data) and the reverse end (reads from the opposite end of the data) are both equally important because when combined they complete the DNA sequence, the quality of each base in the sequence such scored, which say accuracy of the reads That means errors can occur in the readings, especially near the end of the series. Truncating the minimum baseline ensures that only the most reliable information is retained for analysis. This improves the overall accuracy of the results by removing negative parts of the sequence.(see Fig. 5a, 5b and 5c

3.3. Alpha diversity analysis

Shannon and Simpson metrics were used to compare bacterial diversity in individual samples between control and patient groups. These indices provide insight into microbial community richness and evenness and reflect species richness and diversity, which are shown through the boxplots.

These differences in alphas between control and patient groups were determined by the Kruskal-Wallis test. Statistical analysis of alphas between control Alzheimer's disease groups of patients reveals significant differences. Alpha rarefaction curves showed a 9.5 plateau at 6000 sequences in (A) and 3.4 plateau at 2 sequences in (B), indicating sufficient sampling depth. Kruskal- Wallis tests yielded $p = 0.05927$ in (A) and $p = 0.065477$ in (B), suggesting borderline significant differences in diversity between groupsFig. 6.

3.4. Beta diversity analysis

Beta diversity measures the differences in microbial community structure between different tissue samples. This compares diversity across multiple samples, providing insight into how microbial communities vary across sites or conditions. Features of beta diversity include

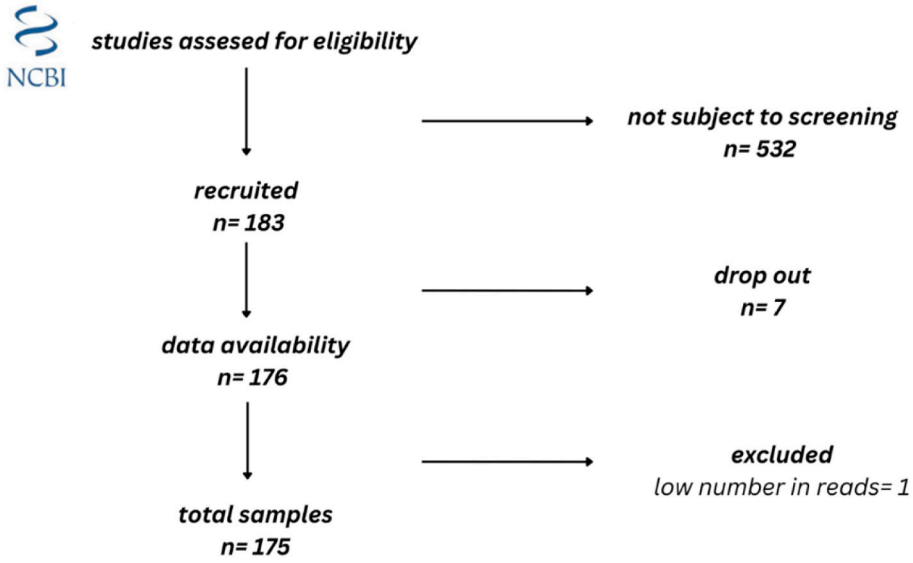


Fig. 4. Flowchart depicting the sample extraction process, where "n" represents the total number of samples. The total number of samples obtained was 175 while 7 were dropped out and 532 were not subjected to screening.

Table 1
Study subjects of diseased and healthy controls.

Characteristics	Control (n = 65)	Patient (n = 118)
Age, median	*42*	*70*
BMI, median	*25*	*23*
Female %	*44 %*	*55 %*
Male %	*22 %*	*77 %*

unweighted: Jaccard index, unweighted UniFarc and weighted: Bray-Curtis distance, Weighted UniFarc. These helps in understanding the similarities or dissimilarities among the samples based on the presence, absence or abundance of different microbial taxa [47][50].

3.5. Emperor PCoA plot - Bray-Curtis

These tools highlight differences in community composition between groups. In plots (A) and (B), the clustering of groups shows that similar samples are grouped together, while the dispersion reflects the diversity within each group. Overlapping areas indicate similarity, and outliers represent unique community compositions. This analysis revealed significant differences in community compositions among groups, explaining 28.445 % in (A) and 12.62 % in (B) of variations which suggest disease associated changes in microbial communityFig. 7a.

3.6. Unweighted UniFrac PCoA

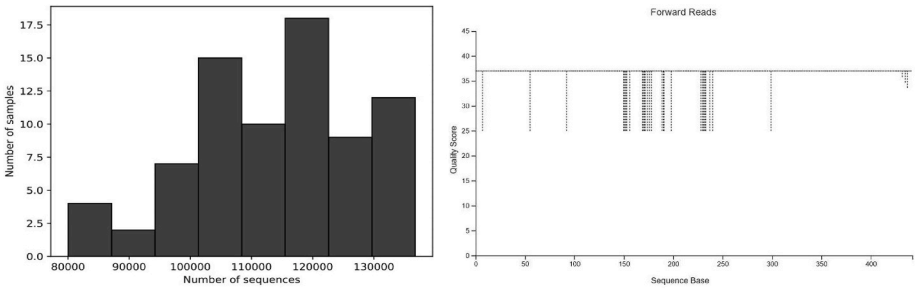
The Unweighted UniFrac PCoA plot provides insight into the microbial species present in control patients and their relationships. In plots (A) and (B), the clustering indicates that similar samples are grouped closely, while the dispersion highlights the diversity within each group. Overlapping regions suggest similarities, and outliers indicate unique community compositions. Plot (A) accounts for 35.484 % of the variation, while plot (B) shows 33.981 %, indicating that the samples are relatively similar (see Fig. 7b).

3.7. Weighted UniFrac PCoA

Weighted UniFrac PCoA suggests both the abundance of species and their evolutionary relationships between samples. In plots (A) and (B), the clustering indicates that similar samples are grouped closely, while the dispersion highlights the diversity within each group. Overlapping regions suggest similarities, and outliers indicate unique community compositions. Plot (A) revealed 49.891 % and (B) revealed 61.05 %, variations among groups indicating significant microbiome differences, suggesting distinct evolutionary relationships (see Fig. 7c).

3.8. Jaccard PCoA

These plots illustrate how the microbial composition varies between



(A) (B)

Fig. 5a. (A) Shows the histogram distribution of forward reads frequencies across samples in single-end data and (B) Shows the quality score distribution of forward reads. Truncation and trimming positions indicated in the single-end data.

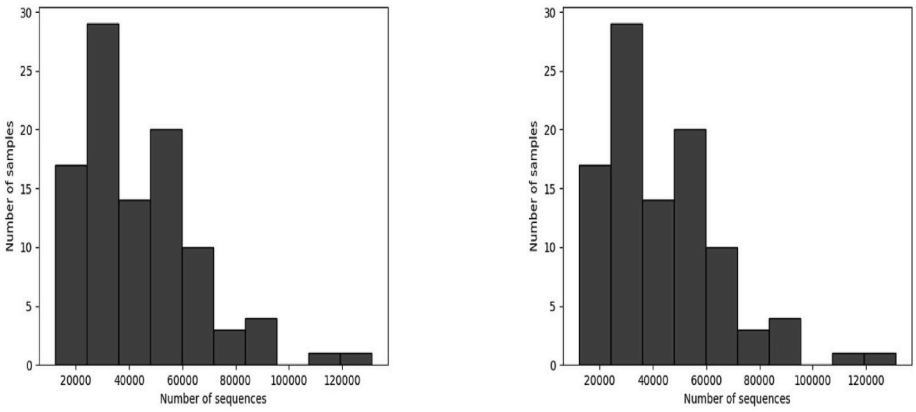


Fig. 5b. Histogram showing the distribution of forward reads (A) and reverse reads (B) frequencies across samples in paired-end data.

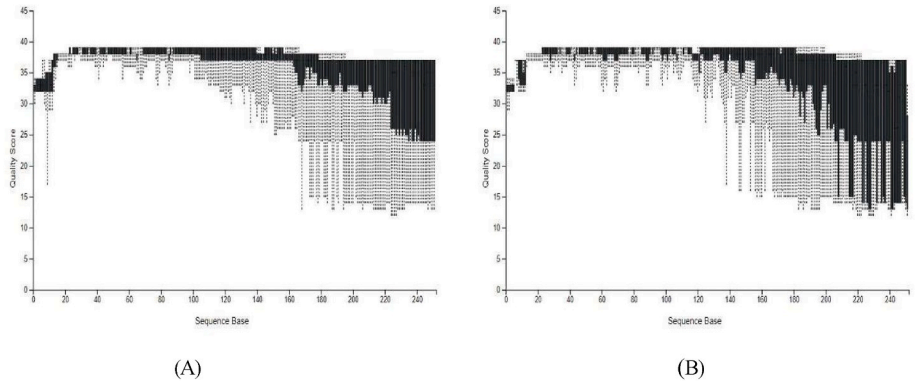


Fig. 5c. Quality score distribution of forward reads (A) and reverse reads (B) Truncation and trimming positions indicated in paired-end data.

controls and patients based on the presence or absence of certain species. In plots (A) and (B), clustering shows that similar samples are closely grouped, while dispersion highlights the diversity within each group. Overlapping areas indicate similarities, and outliers represent unique community compositions. In plot (A), the analysis identified moderate differences, accounting for 15.261 % of the variation, whereas plot (B) revealed smaller differences in species, explaining 10.948 % of the variation. This suggests that disease-associated changes in microbial community composition exist, with a shared core of microbes among the groups (seeFig. 7d).

3.9. Visualization of gut diversity

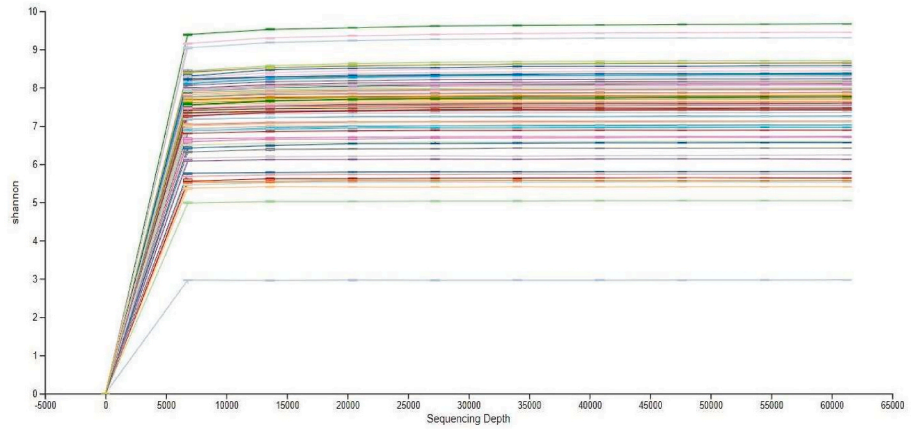
Heatmap was constructed using QIIME 2 feature-table heatmap tool, which was accessed through Galaxy Server Europe platform. Heatmaps are graphical representations of data where individual values are represented as colors. In the context of microbial community analysis, heatmaps are used to visualize the abundance or presence of different microbial taxa across multiple samples darker colors for higher abundance and lighter colors for lower abundance in taxa abundance. Species shows a high abundance (dark color) in diseased patients but a low abundance (light color) in healthy control, it indicates that species are more in the diseased group. The reverse pattern (higher in controls, lower in cases) suggests that species may be more common in healthy populations. Control patients may have different coloration compared to diseased patients, indicating different microorganisms. These differences can help researchers identify species linked to disease or health. In heatmap matrix (A), OTUs 4 to 8 are found to be highly abundant in certain samples, while OTUs 9 to 14 are less abundant in most samples. OTUs 1 to 3 show moderate abundance across the samples. In heatmap matrix (B), OTUs 21 and onwards are associated with high abundance, while OTUs 1 to 20 are less abundant. The dominant families, *Firmicutes* and *Bacteroidota*, have been associated with various diseases, including inflammatory bowel disease and obesity, particularly when their balance is disrupted (see Fig. 8).

3.10. ADONIS test results

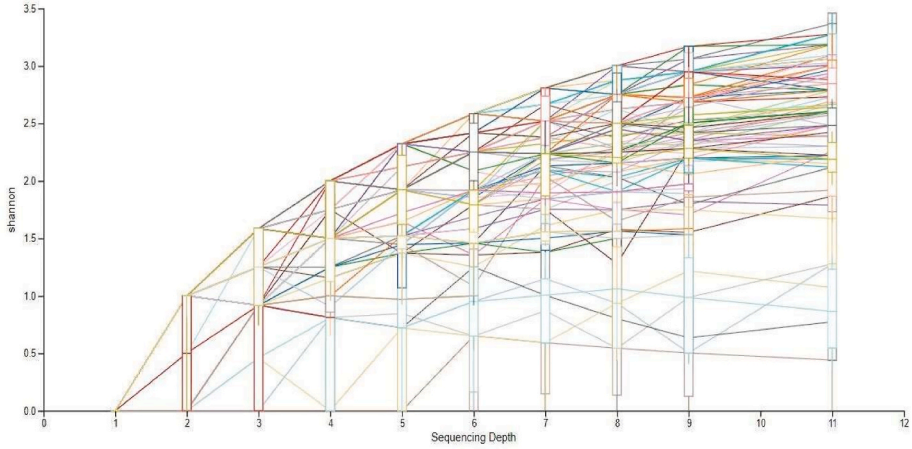
The Adonis test results confirmed the significant difference in beta variables between control and patient groups, with a p-value of 0.013 indicating a statistically significant difference R-squared value of 0.0182 indicating the variability of microbial community structure within 3.5 % results indicate that there is a statistically significant difference in the microbial community composition between the groups analyzed, with the grouping variable explaining a small but significant portion of the total variance in single-end data and for paired-end data the p-value (0.968) indicates that any observed differences between groups are not statistically significant. The R-squared value is negative (−0.001108), which is not interpretable in the usual sense. Statistical differences between the healthy and disorganized conditions were converted to statistics using the Adonis test for beta and measures of variability[17,18.]

3.11. Faecal microbiota analysis

To characterise the gut microbiome in Alzheimer's disease, we examined the composition and diversity of bacterial communities in patients with confirmed disease compared to controls at different phylogenetic and phylogenetic levels. The X-axis represents the

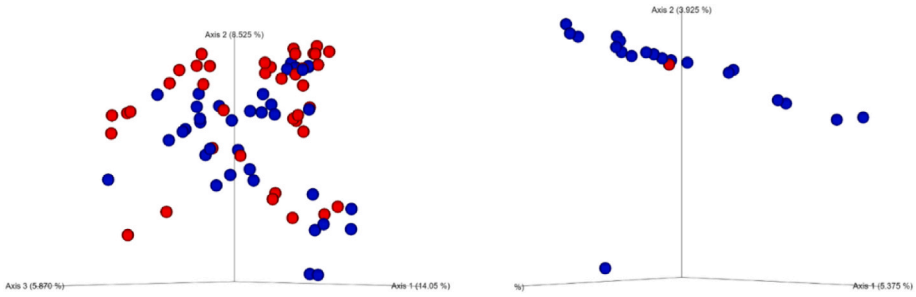


(A)



(B)

Fig. 6. (A) Shows alpha rarefaction curves depicting Shannon diversity across different sequencing depths for each sample. Plateaus indicate sufficient sampling depth in single-end data and (B) showing Alpha rarefaction curves depicting Shannon diversity across different sequencing depths for each sample. Plateaus indicate sufficient sampling depth in paired-end data.



(A) (B)

Fig. 7a. Emperor plot of PCoA matrix computed from Bray-Curtis dissimilarities, highlighting abundance-based compositional differences between AD patient samples (blue dots) and control samples (red dots) in (A) single-end data and (B) paired-end data.

frequency of each taxon, while the Y-axis groups the samples into control patients. The analysis was conducted at two taxonomic levels: genus and species, which means that classes were observed at both the broad bacterial level (phyla) and the specific level (genus).

Phylum-level analysis: This is one of the most extensive taxonomic levels and helps to identify major bacterial groups. In one-ended pairwise conclusions, *Bacteroidota* and *Firmicutes* predominated in AD patients and controls. This suggests that these two phyla are key players in the gut microbiome irrespective of disease status.

3.12. Genus-level analysis

At the genus level, the analysis shows more specific bacterial groups, which is important for understanding how particular bacteria are associated with disease or health as shown in Fig. 9. High relative abundance indicates that a particular taxon is more prevalent in that group,

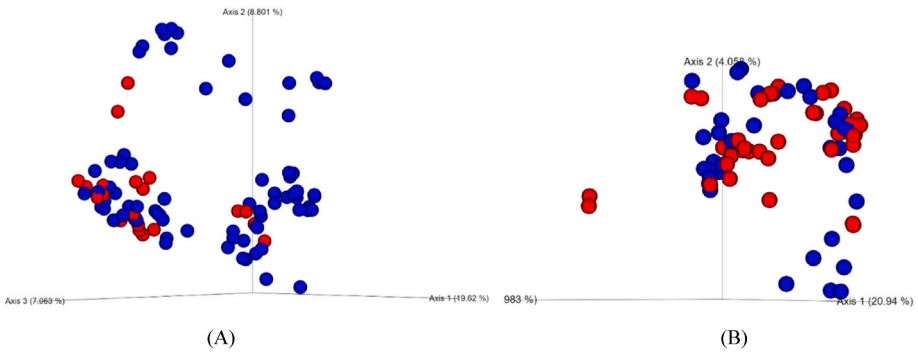


Fig. 7b. Emperor plot of PCoA matrix computed from unweighted UniFrac, illustrating microbial community composition differences between AD patient samples (blue dots) and control samples (red dots) in (A) paired-end data and (B) single-end data.

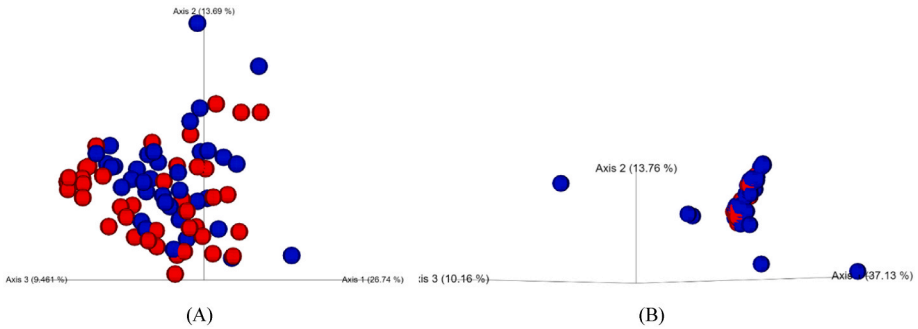


Fig. 7c. Emperor plot of PCoA matrix computed from weighted UniFrac, showing compositional differences Influenced by abundances between AD patient samples (blue dots) and control samples (red dots) in (A) single-end data and (B) paired-end data.

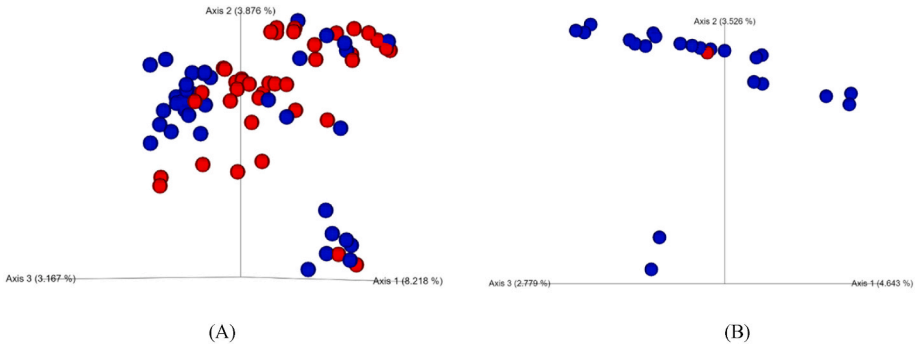


Fig. 7d. Emperor plot of PCoA matrix computed from Jaccard distances, depicting presence-absence dissimilarities between AD patient samples (blue dots) and control samples (red dots) in (A) single-end data and (B) paired-end data.

providing a detail view of community composition at this taxonomic level.

3.13. Differential microbes abundance comparison within the groups

In the ANCOM analysis, the clr (centred log ratio) transformed OTU table was used at the genus level to adjust zero values to one. There are different bacterial taxa whose relative abundances in the gut microbiome are measured and compared between the control and disease groups. In Fig. 10, each point represents a bacterial taxon [23]. In single-end data, the figure indicates *d Bacteria; p Firmicutes_A; c Clostridia_258,483; o Oscillospirales; f CAG272; g Avispirillum* as the bacterial feature of two groups control and disease group with clr −1.0804. The abundance of the bacterial feature *Avispirillum* is lower in the diseased group compared to control group which indicates more variability in bacterial abundance among control individuals. In paired-end data it indicates three bacterial features i.e., *d Bacteria;p Bacteroidota;c Bacteroidia;o Bacteroidales;f Bacteroidaceae;g Phoca eicola_A_858,004, d Bacteria;p Bacteroidota;c Bacteroidia;o Bacteroidales;f_Bacteroidaceae; g_Bacteroides_h,d_Bacteria;p_Firmicutes_A;c_Clostridia_258,483: o _Oscillospirales;f_Ruminococcaceae;g Faecalibacterium*. With clr 3.585, clr 3.411, clr 3.3165 respectively. All three bacterial features show significantly higher abundance in the diseased group in comparison to the control group (see Fig. 10 , Fig. 11).

3.14. Statistical significance

ANCOM analysis revealed several features with significant differences in abundance between the control and patient groups [39]. Tables 2 and 3provide a summary of these features, including their W-statistics and the outcome of the null hypothesis test (see Table 4). Table 5 provides differentially abundant features identified through ANCOM, including W-statistics and results of null hypothesis test in paired-end data (see Table 5).

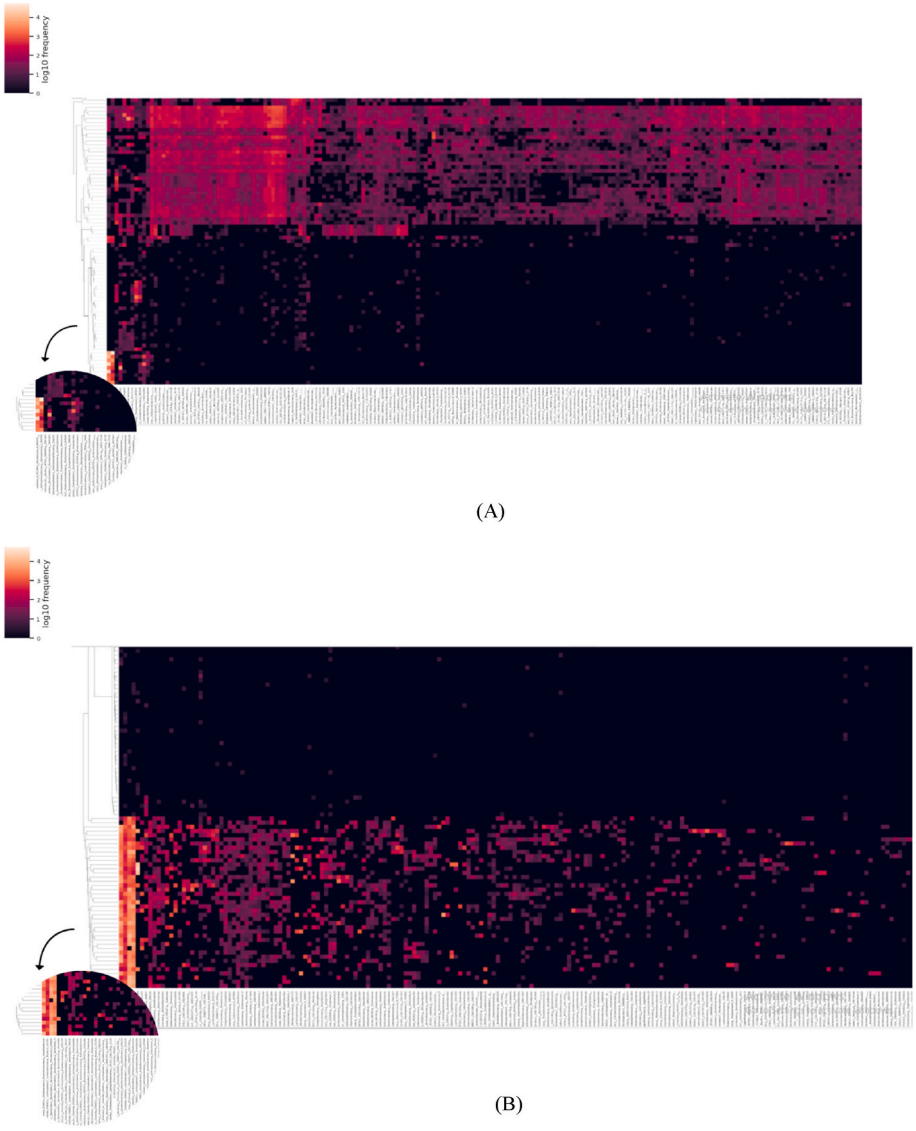


Fig. 8. Heatmap matrix, illustrating correlations between abundance in microbial taxa in (A) single-end data and (B) paired-end data.

Avispirillum: These are member of the *Firmicutes* phylum and is associated with the gut microbiome. It has been shown to play a role in maintaining gut health and can modulate inflammation. Although we found no significant differences in levels, any changes in gut microbiota may affect systemic inflammation, which is known to affect neurological diseases, but the specific role of *Avispirilm* in Alzheimer's disease remains unclear is elusive because there is no information linking these viruses.

Phocaeicola: They are the part of the *Bacteroidetes* phylum, *Phocaeicola* is involved in breaking down complex carbohydrates in the gut, changes in the abundance of Bacteroidetes have been linked to various health conditions, including inflammatory diseases. While our study didn't find a significant difference, shifts in this genus might still influence gut health and, indirectly, brain health through gut-brain axis interactions [37,40].

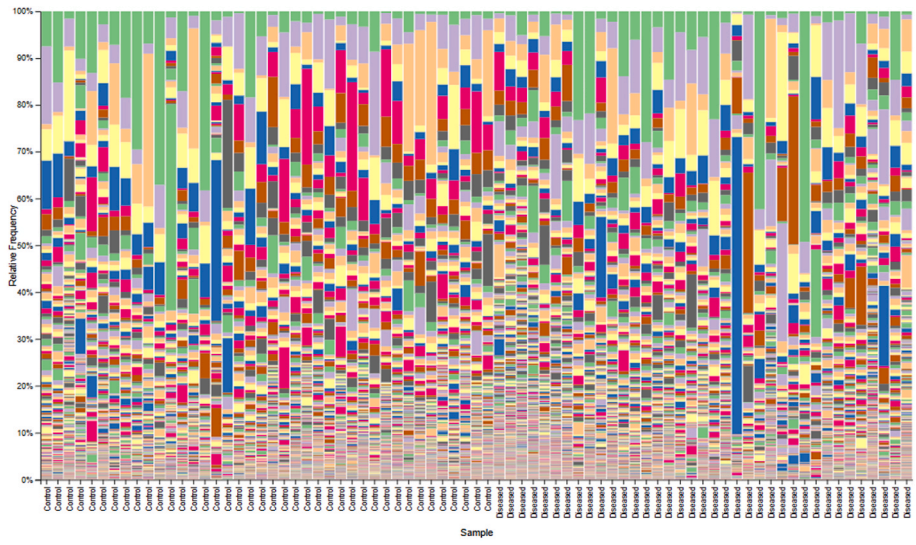
Bacteroides: Also, a member of the *Bacteroidetes* phylum, *Bacteroides* species are known for their role in digestion and modulation of immune responses. *Bacteroides* have been implicated in several diseases due to their role in gut health and systemic inflammation [40]. Although no significant differences were found in our analysis, fluctuations in this genus could potentially impact brain function through inflammatory pathways.

Faecalibacterium: This genus, belonging to the *Firmicutes* phylum, is considered beneficial for gut health. It is known for its anti-inflammatory properties. These has been associated with reduced inflammation and improved gut barrier function. In Alzheimer's disease, a reduction in beneficial bacteria like *Faecalibacterium* could potentially contribute to increased inflammation and neurodegeneration [41,42].

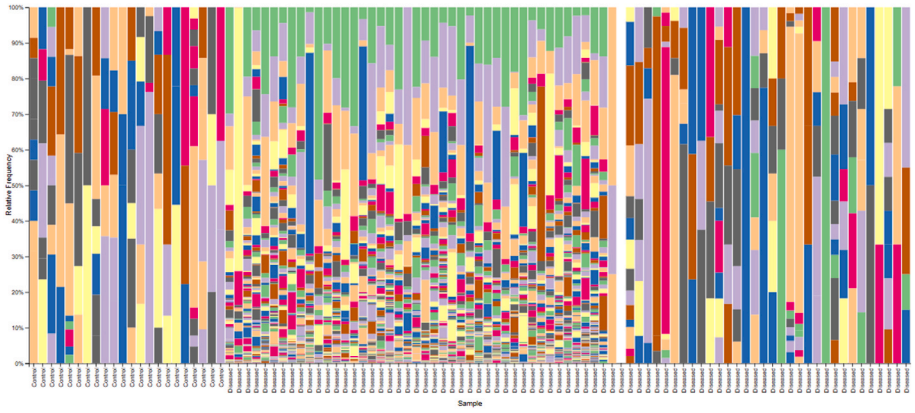
4. Discussion

This study presents research on the gut microbiota of individuals suffering with Alzheimer's disease. Certain bacterial populations may become imbalanced, leading to dysbiosis. Dysbiosis can contribute to increased intestinal permeability, often referred to as "leaky gut," allowing microbial metabolites and toxins to enter systemic circulation. This, in turn, can lead to neuroinflammation and other pathological processes associated with Alzheimer's, such as the accumulation of tau proteins and amyloid beta.

The findings suggest that 16S rRNA sequencing and bioinformatics analysis using QIIME2 analyzed regions of the gut microbiota of individuals with Alzheimer's disease compared to healthy controls. The findings revealed marked differences in the number of bacterial communities between the AD diseased and control groups, shedding light on



(A)



(B)

Fig. 9. Bar plot of taxonomic composition at the Genus level, highlighting the distribution of bacterial genera in control and diseased patient groups in (A) single-end data and (B) paired-end data.

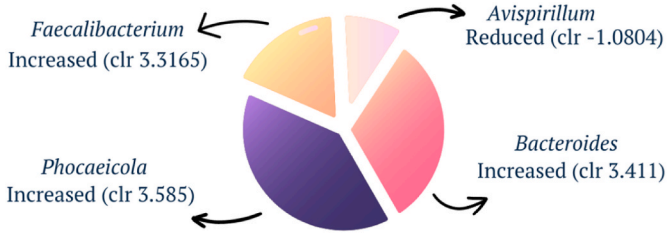


Fig. 10. Composition of the bacteria present in the AD group representing their clr values, Positive values indicate higher abundance in the disease group, while negative values indicate higher abundance in the control group.

potential pathological implications. The application of ANCOM (Analysis of Composition of Microbiomes) to our dataset identified several microbial taxa with differences in abundance between Alzheimer's patients and healthy controls. These findings suggest that gut microbes are dynamic and change over time, especially in response to dietary and health-related factors. This plasticity indicates that while gut microbes may not be the primary cause of Alzheimer's, they are undoubtedly one of the contributing factors influencing disease progression among interactions between gut microbiota and brain health [36]. However, the taxa investigated, *Avispirillum* (classified within '*d_Bacteria;p_Firmicutes_A;c_Clostridia_258,483;o_Oscillospirales;f_CAG-272*') was found to have a W statistic of 1381. This indicates that while '*Avispirillum*' showed some differential abundance between groups. Therefore, our study suggests that '*Avispirillum*' does not have a significant impact on the differences observed between Alzheimer's patients and controls. However, it is important to recognize that the absence of significant differential abundance does not imply a lack of relevance [38]. Similarly, the taxa *Phocaeicola* and '*Bacteroides* (both within the '*Bacteroidaceae*' family, '*Bacteroidota*' phylum) exhibited W statistics of 357. These bacteria are integral to the breakdown of complex carbohydrates in the gut and have been associated with inflammatory and metabolic processes [39]. This group of bacteria can influence the metabolism of neurotransmitters and modulate the gut-brain axis, impacting mood and cognitive functions. Although our results do not indicate a significant difference in their abundance, changes in the composition of these bacteria may still influence the gut-brain axis, potentially affecting the pathophysiology of Alzheimer's disease through indirect mechanisms. In particular, *Bacteroides fragilis* strain of *Bacteriodes* spp and their metabolites 12-hydroxy-heptadecatrienoic acid (12-HHTrE) and Prostaglandin E2 (PGE2) activate microglia and induce AD pathogenesis in neuronal C/EBPβ transgenic mice models [55]. Elevated levels of 12-HHTrE and PGE2 corresponds to elevated levels of aggregated amyloid beta plaques. As amyloid beta plaques accumulate, harmful cytokine levels rise, resulting in a detrimental feedback cycle. To further comprehend the microglial stimulatory action, bioactive metabolites involved in the activation process were found. Metabolite identification

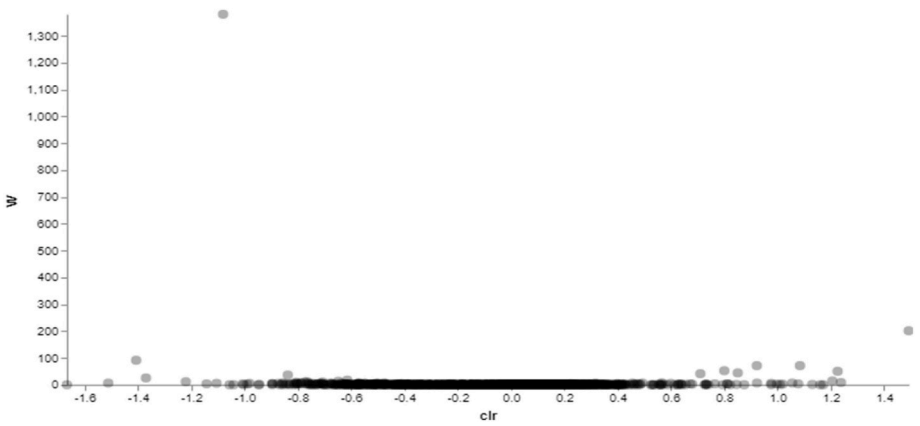


Fig. 11. ANCOM Volcano Plot showing differentially abundant features between control and patient groups. W statistic is plotted against clr-transformed abundances in single-end data. Points far to the right or left (high effect size) and high up (high significance) indicate taxa that are both significantly and substantially different in abundance between the two groups [22]. A point far to the right and high up might represent a bacterium that is significantly more abundant in the AD group compared to the control group and a point far to the left and high up might represent a bacterium that is significantly more abundant in the control group compared to the AD group.

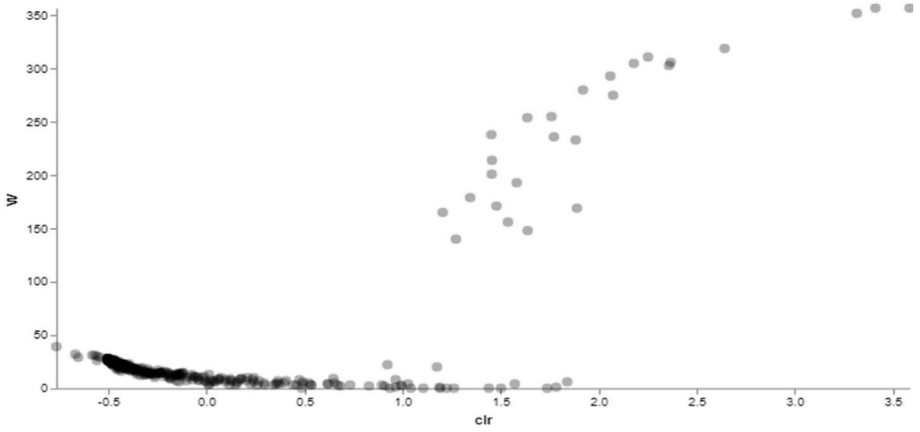


Fig. 12. ANCOM Volcano Plot showing different features between control and patient groups. W statistic is plotted against clr transformed abundances in paired-end data. Points far to the right or left (high effect size) and high up (high significance) indicate taxa that are both significantly and substantially different in abundance between the two groups [22]. A point far to the right and high up might represent a bacterium that is significantly more abundant in the AD group compared to the control group, and a point far to the left and high up might represent a bacterium that is significantly more abundant in the control group compared to the AD group.

Table 2
This table displays the phyla that were more abundant in AD patients as observed from Single-End and Paired-End data.

Phylum	Single-End Data (%)	Paired-End Data (%)	Condition
Verrucomicrobiota	*25.249*	*73.981*	*AD*
Methanobacteriota_A_1229	*17.289*	-	*AD*
Proteobacteria	*71.306*	-	*AD*
Firmicutes_D	-	*80.597*	*AD*
Actinobacteriota	-	*33.333*	*AD*

Table 3
This table shows the relative abundance of various genera in AD patients and controls as observed from Single-End and Paired-End data.

Genus	Single-End Data (%)	Paired-End Data (%)	Condition
Bifidobacterium_388,775	*17.806*	*33.333*	*Control*
Ruminococcus_E	*22.857*	-	*Control*
CAG-27	*43.990*	-	*Control*
Blautia_A_141,781	-	*44.444*	*Control*
Parabacteroides_B_862,066	-	*22.222*	*Control*
Fimenecus	-	*35.714*	*Control*
Prevotella	*47.140*	*100.000*	*AD*
Phocaeicola_A_858,004	*23.033*	*100.000*	*AD*
Akkermansia	*25.238*	*73.981*	*AD*
Sphingomonas_L_486,704	*63.464*	-	*AD*
Methanobrevibacter_A	*17.284*	-	*AD*
Bacteroides_H	-	*50.000*	*AD*

was conducted using germ-free 3xTg mice and Abx-treated C/EBPβ transgenic mouse models. After then, HHTrE and PGE2 were identified. Both of these chemicals were discovered to be raised in the brain and faeces of mice. The two identified bioactive compounds were administered to Thy1-C/EBPβ transgenic mice, resulting in the same stimulation of microglia and neuroinflammation observed in Germ-free 3xTg mice and Abx-treated C/EBPβ transgenic mouse models [55]. Bacteroides modulate microglia eventually altering the levels of amyloid beta and tau levels in the body. To better understand this, young APP/PS1 mouse models were considered. B. fragilis inhibited the production of proteins involved in receptor-mediated phagocytosis, phagosome formation and maturation, and engulfed protein degradation in microglia from young APP/PS1 mice. Stimulating microglia with TLR4-dependent lipopolysaccharide (LPS) can decrease autophagy, impairing its ability to breakdown phagocytised Aβ. Blocking TLR2 signaling has been demonstrated to enhance microglial lysosomal Aβ breakdown. Thus the

Table 4
Differentially abundant features identified by ANCOM, including W-statistics and results of null hypothesis test in single-end data.

Feature	W	Reject Null Hypothesis
d_Bacteria;p_Firmicutes_A;c_Clostridia_258,483; o_Oscillospirales;f_CAG-272;g_Avispirillum	*1381*	*No*

Table 5
Differentially abundant features identified by ANCOM, including W-statistics and results of null hypothesis test in paired-end data.

Feature	W	Reject Null Hypothesis
d_Bacteria;p_Bacteroidota;c_Bacteroidia;o_Bacteroidales; f_Bacteroidaceae;g_Phocaeicola_A_858,004	*357*	*No*
d_Bacteria;p_Bacteroidota;c_Bacteroidia;o_Bacteroidales; f_Bacteroidaceae;g_Bacteroides_H	*357*	*No*
d_Bacteria;p_Firmicutes_A;c_Clostridia_258,483; o_Oscillospirales;f_Ruminococcaceae; g_Faecalibacterium	*357*	*No*

mechanism of action by which B. fragilis inhibits Aβ clearance was understood [56]. The genus *Faecalibacterium* (within '*d_Bacteria;p_Firmicutes_A;c_Clostridia_258,483;o_Oscillospirales;f_Ruminococcaceae*'), which is known for its anti-inflammatory properties, also did not exhibit significant differences in abundance between the patient and control groups, as indicated by a W statistic of 352. '*Faecalibacterium*' is a critical component of a healthy gut microbiome, and its presence is often associated with reduced inflammation [41] and a more robust gut barrier. It produces short-chain fatty acids (SCFAs) that help modulate immune responses. A decrease in *Faecalibacterium* levels may exacerbate inflammatory processes in the brain. To gain a better insight, Fp14 strain of *Faecalibacterium prausnitzii* was selected. This selected strain was pasteurised and this pasteurization Fp14 strain yielded better results than live Fp14 strain in PA test for cognitive memory impairment. To understand efficacy of Fp14 metabolome and RNA-seq analyses of the hippocampus was done. The results of metabolome research revealed that Fp14 drastically reduced thymine and 6 mA while also reducing suberic acid. Thymine glycol, an oxidised form of thymine, has been found to accumulate in the brains of AD patients. Second, 6 mA was found to increase with prolonged restraint stress in mouse brains, which was linked to an increase in reactive oxygen species (ROS) generation in mitochondria. Third, dicarboxylic acids (DCAs), such as suberic acid and azelaic acid, are produced by the oxidative degradation of unsaturated fatty acids and are known to affect mitochondrial activity. This evidence revealed a link between the efficiency of pasteurised Fp14 against oxidative stress and mitochondrial activity. Results from RNA-seq analysis revealed that pasteurised Fp14 drastically lowered PACS-2 transcript levels. PACS-2 has been linked to a variety of disorders, including Alzheimer's disease, and is thought to play key roles in mitochondrial dynamics. This evidence reinforced the link between pasteurised Fp14 and mitochondrial activity in the brain [57]. Although these bacteria may not be present in high abundance, their mere presence suggests they could play a role in the development of Alzheimer's disease. They may either contribute directly to disease mechanisms or influence other physiological activities in the body that exacerbate the condition. Their effects, even in low quantities, could be significant enough to impact gut health and, subsequently, brain function.

Alzheimer's disease is a multifactorial disease with various genetic, environmental, and lifestyle influences. The contribution of the gut microbiome to Alzheimer's disease can vary greatly among individuals, making it difficult to identify a single microbial signature associated with the disease. Moreover, the cross-sectional nature of the study might not capture the dynamic changes in gut microbiota over time, which could be more relevant to the disease's progression[18,43][30,31,34]. Future research should consider these interactions and use holistic approaches such as metagenomics and metabolomics to gain a deeper understanding of the role of gut microbiota. The findings from this study suggest several avenues for further investigation. First, longitudinal studies that track changes in gut microbiota composition over time in Alzheimer's patients could provide more insight into how these changes correlate with disease progression. Additionally, larger sample sizes may help to detect more subtle differences in microbial abundance that were not captured in this analysis. Functional studies are also needed to explore the specific roles that these bacteria play in the gut-brain axis. For example, research into the metabolic pathways influenced by *'Avispirillum', 'Phocaeicola', 'Bacteroides',* and *'Faecalibacterium*' could reveal how changes in these bacteria affect the production of neuroactive compounds, inflammatory mediators, or other factors that could influence brain health. Moreover, understanding how these bacterial changes impact the host's immune response and gut barrier integrity could provide insights into their potential role in neurodegenerative diseases. For instance, does a reduction in beneficial bacteria like '*Faecalibacterium*' lead to increased gut permeability, systemic inflammation, and subsequent neuroinflammation? Or do changes in 'Bacteroidota' species alter the immune system in ways that could exacerbate or mitigate Alzheimer's disease?

5. Conclusion

The link between gut microbiota and Alzheimer's disease underscores the importance of a holistic approach to health, emphasizing diet, lifestyle, and microbial balance. While gut microbes are not the sole cause of Alzheimer's, they play a significant role in its progression. By focusing on maintaining a healthy gut microbiome through dietary and lifestyle choices, we may be able to influence the course of Alzheimer's disease and improve overall brain health. Further research is essential to fully understand the complexities of this relationship and to develop effective interventions. This conclusion is supported by the understanding that Alzheimer's disease is a multifactorial disorder, resulting from the interplay of various disease pathways. The complexity of this disease suggests that multiple factors, including gut dysbiosis, contribute to its pathogenesis, ultimately leading to the manifestation of this chronic condition.

CRediT authorship contribution statement

Jahvnavi: Writing – original draft, Formal analysis, Data curation, Writing – original draft, Formal analysis, Data curation. **Prekshi Garg:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Prachi Srivastava:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data details are shared within the manuscript

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