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Original Article

Functional Pathways of the Gut Microbiome Associated with SCFA Profiles in Preclinical Alzheimer's Disease

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ABSTRACT: Functional activities of the gut microbiome, particularly those contributing to short-chain fatty acid (SCFA) metabolism, play a central role in host-microbe interactions and are linked to neuroinflammatory mechanisms underlying Alzheimer's disease (AD). How microbial metabolic functions relate to SCFA concentrations and cerebral amyloid-β (Aβ) burden during the preclinical stage of AD remains poorly understood. In this study, faecal metagenomes from 87 cognitively unimpaired adults were profiled using HUMAnN3 to generate MetaCyc pathway abundance data, normalised and filtered to retain pathways present in at least 30% of participants. A keyword-based search identified 362 SCFA-related pathways spanning acetate, propionate, butyrate, isobutyrate, valerate and isovalerate metabolism. Associations between microbial functions, SCFA concentrations and Aβ status were evaluated using Spearman correlations, Kruskal-Wallis tests across SCFA quartiles, and multivariable linear regression with false discovery rate correction, supported by canonical correspondence analysis and network modelling. A total of 38 significant SCFA pathway correlations were identified. Acetate, butyrate and total SCFA levels showed positive associations with biosynthetic pathways, including L-arginine biosynthesis II, peptidoglycan biosynthesis and flavin biosynthesis, whereas fermentative pathways such as pyruvate fermentation to acetone and lysine fermentation to butanoate were negatively correlated. Butyrate quartiles demonstrated dose-dependent increases in biosynthetic functions and declines in fermentative routes. Canonical Correspondence Analysis (CCA) confirmed a significant multivariate association, and network analysis revealed enhanced fermentative and methanogenic connectivity among Aβ High participants. These findings indicate that amyloid burden is associated with a shift from anabolic to fermentative microbial metabolism and may inform future studies examining potential mechanistic links in preclinical AD.

Keywords: Gut microbiome, Short-chain fatty acids (SCFAs), Amyloid-β, Alzheimer's disease, Metagenomic pathways

INTRODUCTION

The gut microbiome exerts profound effects on host health through its functional capacity to generate metabolites that regulate immune, metabolic, and neural processes. Among these metabolites, short-chain fatty acids (SCFAs) primarily acetate, propionate, and butyrate are central fermentation products that maintain intestinal

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barrier integrity, modulate inflammation, and influence brain function via the microbiota-gut-brain axis [1, 2]. The production and utilisation of SCFAs depend on distinct microbial functional pathways involving carbohydrate fermentation, amino-acid metabolism, and biosynthesis. Thus, alterations in these pathways can provide insight into associations between microbial metabolism and systemic and neural health [3, 4].

In Alzheimer's disease (AD), accumulating evidence links gut microbial dysbiosis to neuroinflammation and amyloid pathology through disrupted SCFAs metabolism [5-7]. Reduced butyrate and propionate levels have been associated with increased intestinal permeability, microglial activation, and oxidative stress processes known to contribute to early amyloid-β (Aβ) deposition [5, 8]. However, most studies have focused on taxonomic composition or SCFAs concentrations alone, offering limited understanding of which microbial functions drive these metabolic alterations or how they differ across stages of amyloid pathology. The preclinical phase of AD when Aβ accumulation occurs before cognitive decline represents a critical but understudied window for detecting functional microbial changes that may signal early disease risk [9-11].

A major gap in the current literature lies in the lack of integrated functional analyses that connect microbial gene pathways to SCFAs output and host pathology. Previous research has identified reduced abundances of SCFAs-producing taxa (e.g., *Faecalibacterium, Blautia*), but few studies have examined the metabolic pathways underpinning these changes [12-14]. Moreover, the functional balance between biosynthetic pathways (which generate anti-inflammatory and neuroprotective metabolites) and fermentative or methanogenic pathways (which promote energy extraction and oxidative stress) remains poorly understood in the context of amyloid pathology [15-17]. Clarifying how these functional processes are reorganised in preclinical AD could reveal early mechanistic shifts in gut metabolism linked to neurodegeneration.

Technological advances in shotgun metagenomics and computational tools such as HUMAnN3 now allow pathway level resolution of microbial function. When coupled with quantitative SCFAs profiling, these methods enable a systems level examination of how microbial metabolism interacts with host factors [18, 19] . Despite this capability, no previous study has comprehensively integrated metagenomic functional pathways, SCFAs concentrations, and amyloid status in cognitively unimpaired individuals. Addressing this gap can reveal how specific microbial functions rather than taxa alone relate to metabolic signatures of early AD risk.

Current study, therefore, investigates functional pathways of the gut microbiome associated with SCFAs profiles in individuals stratified by cerebral Aβ burden. Using HUMAnN3-derived MetaCyc pathway data integrated with faecal SCFAs quantification, we applied correlation, regression, quartile, co-occurrence, canonical correlation, and network-based approaches to characterise both direct and higher order functional relationships. By linking microbial biosynthetic and fermentative capacities to SCFAs production across amyloid strata, this work provides the first detailed map of how gut microbial functional organisation may shift during the preclinical phase of AD. These analyses aim to bridge the current gap between composition and function, offering novel insight into early metabolic pathways that could serve as non-invasive biomarkers or therapeutic targets in AD prevention.

MATERIALS AND METHODS

Participants

Participants were drawn from two established Australian ageing cohorts: the Australian Imaging, Biomarkers and Lifestyle (AIBL) Study and the Western Australian Memory Study (WAMS) [20, 21]. Eligible individuals provided written informed consent and contributed faecal samples. Amyloid status was determined using amyloid positron emission tomography (PET) imaging and quantified on the Centiloid (CL) scale. Cognitively unimpaired participants were classified as Aβ Low (CL ≤ 15) or Aβ High (CL > 15) [22, 23].

This study was cross-sectional in design and aimed to explore microbial signatures associated with early and symptomatic stages of AD. For the present analyses, only participants with complete SCFAs and metagenomic data were included (n=87), comprising cognitively unimpaired individuals with low amyloid-beta (Aβ Low, n = 68) and cognitively unimpaired individuals with high amyloid-beta (Aβ High, n = 19) [20].

Ethics

Faecal sample collection was approved by the Ramsay Health Care WA/SA Human Research Ethics Committee (AIBL: 2006/ETH/0215; approval date: 06 December 2022; WAMS: 2003/ETH/0139; approval date: 23 July 2023). Data management was approved by the Edith Cowan University Human Research Ethics Committee (REMS 2023-04565-DISSANYAKA).

SCFA Quantification

Faecal CFAs (acetic, propionic, butyric, isobutyric, valeric, and isovaleric acids) were quantified using gas chromatography-mass spectrometry (GC-MS). SCFAs

were extracted, derivatised, and measured relative to internal standards following an optimised laboratory protocol. Concentrations were expressed as μmol/g of wet faeces [24].

DNA Extraction and Metagenomic Sequencing

Genomic DNA was extracted from faecal samples using the DNeasy PowerSoil Pro Kit (QIAGEN) with mechanical lysis. Shotgun metagenomic libraries were prepared using the Illumina DNA Prep (M) tagmentation workflow and sequenced on the NovaSeq X Plus platform (2 × 150 bp). Sequencing targeted ~10 Gbp per sample, with an observed mean depth of ~21 million reads [25].

Bioinformatics Processing

Reads were quality-filtered using Trim Galore, and human reads were removed by alignment to GRCh38. Taxonomic profiling was performed using Kraken2 and Bracken, generating species-level relative abundances [26]. Microbial species were retained if detected in ≥10% of participants and with ≥100 total reads to minimise noise from rare taxa.

Functional Pathway Profiling

Functional pathways were profiled using HUMAnN3 with alignment to the MetaCyc database. Pathway tables were normalised using total-sum scaling. Non-informative entries (UNMAPPED, UNINTEGRATED) and zero-abundance features were removed. Pathways were retained if present in ≥30% of participants and with ≤70% zero values. A keyword and ID-based search identified 362 SCFA-related pathways spanning acetate, propionate, butyrate, isobutyrate, valerate, and isovalerate metabolism. Pathway abundances were log-transformed with a pseudocount for statistical analyses.

Statistical Analysis

All analyses were performed in R v4.4.1 (version 4.4.1; R Foundation for Statistical Computing, Vienna, Austria) [27]. Continuous variables were compared using t-tests or Wilcoxon rank-sum tests, and categorical variables using chi-square tests. Associations between SCFA concentrations and microbial pathways were assessed using Spearman correlations with Benjamini-Hochberg false discovery rate (FDR) correction. Multivariable linear regression models were fitted for each pathway to evaluate SCFA function relationships adjusted for age, sex, APOE ε4 status, and Aβ group. Non-linear associations across SCFA quartiles were examined using Kruskal-Wallis tests with FDR correction. Pathway differences between Aβ High and Aβ Low groups were assessed using Wilcoxon tests. Functional co-occurrence patterns were evaluated using pairwise Spearman correlations followed by hierarchical clustering (Ward.D2). Canonical correspondence analysis (CCA) with 999 permutations assessed multivariate links between SCFAs and functional composition. A multi-layered network integrating SCFAs, microbial species, pathways, and host metadata (age, sex, APOE ε4, Aβ) was constructed using correlation thresholds of $|\rho| > 0.3$ and FDR < 0.05. Networks were visualised using igraph and visNetwork. Statistical significance was defined as $p < 0.05$ or FDR < 0.05. Analyses were conducted on a well-characterised. SCFA-pathway-matched subset of participants.

Table 1. Participant characteristics and faecal SCFAs concentrations by Aβ status.

Characteristic	CU Aβ Low (n = 68)	CU Aβ High (n = 19)	p-value
Age, median (IQR)	76 (67-80)	81 (76-83)	0.019
Gender (Female), n (%)	48 (70.6%)	9 (47.4%)	0.107
Education, mean ± SD	14.43 ± 3.00	13.00 ± 2.47	0.057
APOE4 Positivity, n (%)	9 (13.2%)	7 (58.3%)	0.026
BMI, mean ± SD	25.41 ± 3.52	26.27 ± 4.37	0.467
Total SCFAs (μmol/g), median (IQR)	56.0 (42.8-68.3)	60.0 (36.9-77.0)	0.90
Acetic acid (μmol/g), median (IQR)	35.3 (26.3-43.3)	32.6 (24.5-47.5)	0.89
Propionic acid (μmol/g), median (IQR)	8.9 (6.1-11.7)	9.7 (5.9-13.5)	0.62
Isobutyric acid (μmol/g), median (IQR)	1.2 (0.84-1.60)	1.2 (0.94-1.45)	0.89
Butyric acid (μmol/g), median (IQR)	6.8 (2.5-9.2)	8.1 (3.0-13.0)	0.61
Isovaleric acid (μmol/g), median (IQR)	1.9 (1.2-2.5)	1.7 (1.45-2.35)	0.996
Valeric acid (μmol/g), median (IQR)	1.3 (0.82-1.63)	1.4 (1.05-2.05)	0.41

Data are presented as mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normally distributed variables (e.g., SCFAs concentrations). Group comparisons for continuous variables were performed using independent samples t-tests or Wilcoxon rank-sum tests as appropriate, and categorical variables were compared using chi-square tests. Abbreviations: Aβ, amyloid-beta; APOE, Apolipoprotein E; CU, Cognitively Unimpaired; SCFAs, short-chain fatty acid

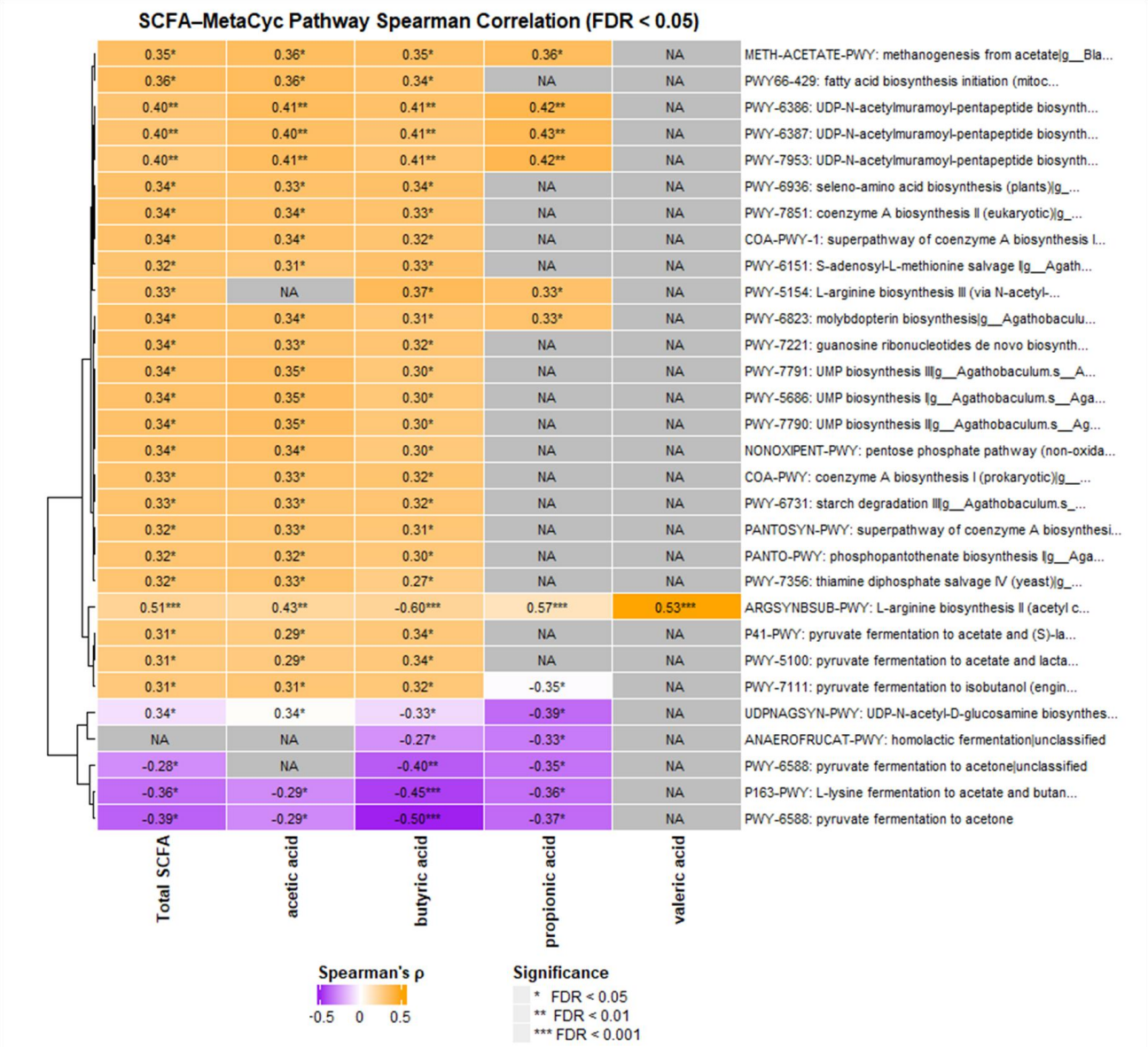

Figure 1. Heatmap of significant Spearman correlations (FDR < 0.05) between short-chain fatty acid (SCFA) concentrations and microbial metabolic pathways (MetaCyc). Correlation coefficients (ρ) are color-coded: orange indicates positive, and purple indicates negative associations. Asterisks denote significance levels: *FDR < 0.05, **FDR < 0.01, ***FDR < 0.001. Only pathways with at least one significant SCFA association are shown. Pathways were clustered by correlation profile. NA = not significant after FDR correction.

RESULTS

Descriptive Overview and Data Inclusion

Descriptive statistics summarised participant demographics and faecal SCFAs concentrations for individuals included in the pathway level analyses (n = 87) (Table 1). CU Aβ High participants were significantly older than those in the CU Aβ Low group (81 vs 76 years, p = 0.019) and had a higher frequency of APOE ε4 carriage (58.3 % vs 13.2 %, p = 0.026). Sex distribution, education, and BMI did not differ significantly between groups. All seven SCFAs showed comparable concentrations between Aβ groups, with no statistically significant differences (all p > 0.40). These demographic and biochemical profiles confirm that subsequent pathway-based analyses were conducted on a well-characterised, SCFAs-pathway-matched subset of participants.

Correlation Between SCFA Concentrations and Functional Microbial Pathways (whole data set)

A total of 38 SCFAs-pathway pairs showed statistically significant correlations (FDR < 0.05), revealing distinct yet overlapping trends across individual SCFAs (Fig. 1, (Supplementary Table 2). Correlation coefficients (ρ) ranged from -0.55 to +0.57, indicating both positive and negative associations between faecal SCFAs concentrations and microbial functional pathways.

Acetic and propionic acids exhibited similar profiles, showing consistent positive correlations (ρ ≈ 0.31–0.42) with multiple biosynthetic and anabolic pathways, including L-arginine biosynthesis I (PWY-5154), UDP-N-acetylmuramoyl-pentapeptide biosynthesis I–III (PWY-6385 – 6387), selenium-amino-acid biosynthesis (PWY-6935), coenzyme A biosynthesis (PWY-5505), and nucleotide-related pathways such as ribonucleotide de novo synthesis (PWY-7221) and UMP biosynthesis I–II (PWY-7790, PWY-7791). Negative correlations were primarily observed for fermentation-related pathways, including pyruvate fermentation to acetone (PWY-6588) and pyruvate fermentation to acetate and lactate (PWY-5100).

Butyric acid displayed both positive and negative associations. Positive correlations were observed with biosynthetic pathways such as L-arginine biosynthesis (PWY-5154, ρ = 0.57, FDR < 0.001), UDP-N-acetylmuramoyl-pentapeptide biosynthesis (PWY-6385, ρ = 0.40), coenzyme A biosynthesis (PWY-5505, ρ = 0.33), and selenium-amino-acid biosynthesis (PWY-6935, ρ = 0.39–0.41). Additional positive correlations were detected for nucleotide biosynthetic pathways, including ribonucleotide de novo synthesis (PWY-7221, ρ = 0.35), UMP biosynthesis I (PWY-7790, ρ = 0.34), and UMP biosynthesis II (PWY-7791, ρ = 0.33). Strong negative correlations were identified for fermentation-related pathways such as pyruvate fermentation to acetone (PWY-6588, ρ = –0.55, FDR < 0.001), L-lysine fermentation to acetate and butanoate (P163-PWY, ρ = –0.50), and pyruvate fermentation to acetate and lactate (PWY-5100, ρ = –0.39).

Valeric acid demonstrated the strongest positive correlations overall, particularly with L-arginine biosynthesis (PWY-5154, ρ = 0.53), UDP-N-acetyl-muramoyl-pentapeptide biosynthesis (PWY-6385, ρ = 0.44), selenium-amino-acid biosynthesis (PWY-6935, ρ = 0.40–0.46), coenzyme A biosynthesis (PWY-5505, ρ = 0.34), and nucleotide biosynthesis (PWY-7221, PWY-7790, PWY-7791; ρ = 0.35–0.38). Negative associations mirrored those of other SCFAs, including L-lysine fermentation to acetate and butanoate (P163-PWY) and pyruvate fermentation to acetone or lactate (PWY-6588, PWY-5100; ρ = –0.35 to –0.37).

Total SCFA concentrations exhibited a pattern similar to individual acids, showing positive correlations with biosynthetic pathways (PWY-5154, PWY-6385–6387, PWY-6935, PWY-5505, PWY-7221, PWY-7790, PWY-7791) and negative correlations with fermentative pathways (PWY-6588, P163-PWY, PWY-5100).

Adjusted Regression Models of SCFA-Pathway Associations (Whole Dataset; Covariate-Adjusted)

Adjusted linear regression models were applied to assess associations between faecal SCFA concentrations and microbial functional pathways after controlling for age, sex, APOE ε4 status, and cerebral Aβ group to ensure observed effects were microbiome driven. Statistically significant associations after false-discovery-rate correction (FDR < 0.05) were predominantly observed for acetic acid, butyric acid, and total SCFA concentrations. Positive associations were consistently observed for multiple biosynthetic pathways, including L-arginine biosynthesis I (PWY-5154), which correlated positively with both acetic acid and total SCFA levels, representing the strongest relationship across the dataset. Several additional anabolic pathways also displayed significant or near-significant positive relationships: flavin biosynthesis I (RIBOSYN2-PWY), phosphopantothenate biosynthesis I (PANTO-PWY), ribonucleotide de novo biosynthesis (PWY-7221), and UMP biosynthesis I and II (PWY-7790, PWY-7791). These pathways, primarily involved in nucleotide and coenzyme A synthesis, were positively associated with acetic acid and total SCFAs. Several additional pathways showed emerging positive trends (FDR ≈ 0.05-0.10), including coenzyme A biosynthesis I (PWY-5505), selenium-amino-acid biosynthesis I (PWY-6935), UDP-N-acetylmuramoyl-pentapeptide bio-synthesis I-III (PWY-6385-6387), thiamine and molybdopterin biosynthesis (PWY-7799, PWY-7800), and guanosine biosynthesis I (PWY-6700). These relationships suggest that higher SCFA concentrations are generally linked with enhanced microbial anabolic capacity.

In contrast, negative associations were observed for fermentation-related pathways, including pyruvate fermentation to acetate and lactate (PWY-5100) and L-lysine fermentation to acetate and butanoate (P163-PWY), which showed significant inverse relationships with butyric acid and total SCFA levels. A weaker but directionally similar negative trend was also seen for pyruvate fermentation to acetone (PWY-6588). These adjusted relationships are summarised in Figure 2, where each dot represents an individual SCFA-MetaCyc pathway pair. The x-axis indicates regression estimates, dot size reflects $-\log_{10}$(FDR), and red points denote statistically significant associations (FDR < 0.05). Only pathways with at least one significant relationship are displayed. Additional volcano plots for each SCFA are

provided in Supplementary Figures 2 and 3 (Supplementary Table 3).

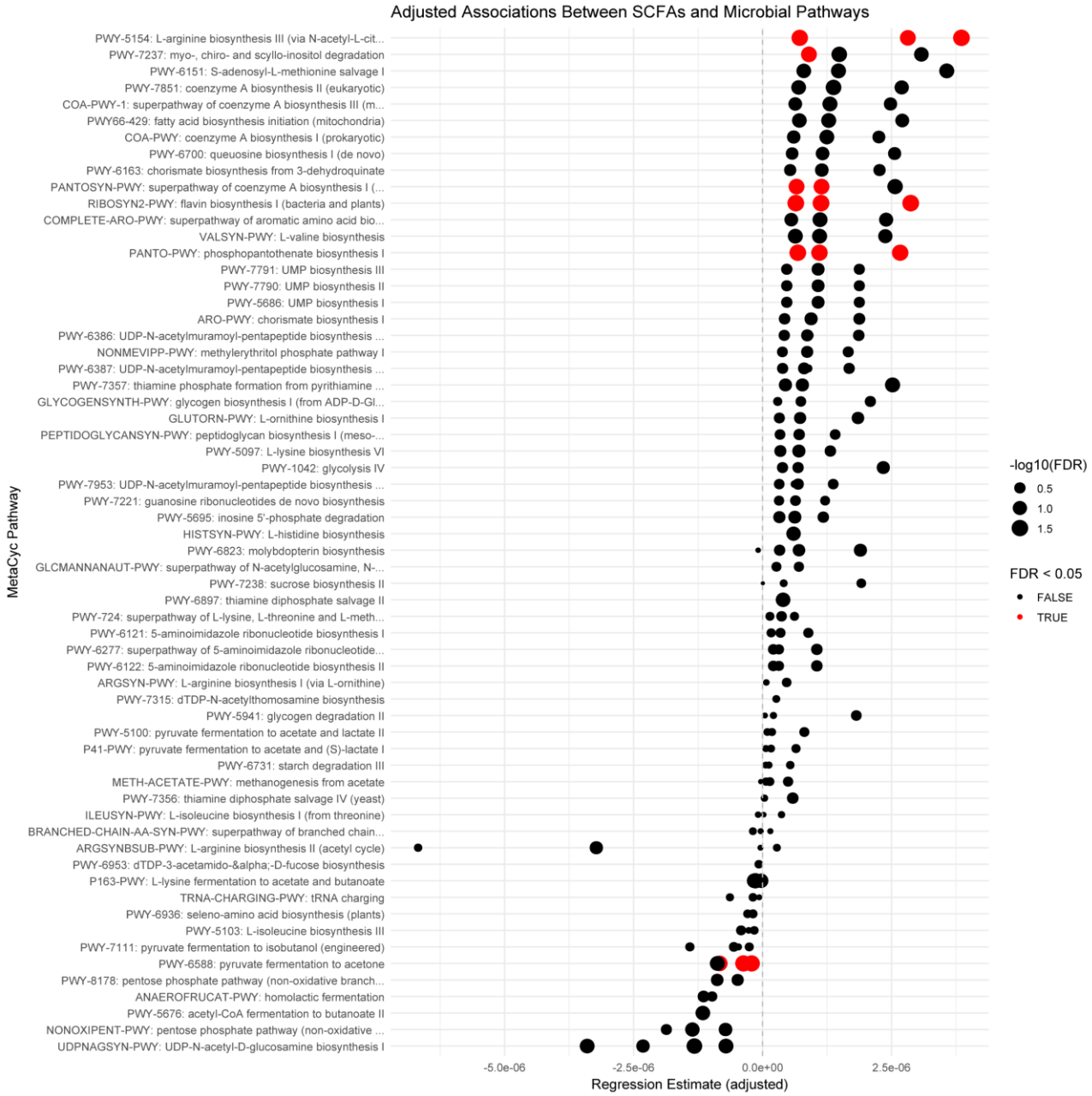

Figure 2. Dot Plot of Adjusted SCFA-Pathway Associations. A dot plot displaying adjusted regression estimates (x-axis) for each SCFAs-MetaCyc pathway pair (y-axis). Dot size corresponds to the -$\log_{10}$(FDR), and color indicates statistical significance (red = FDR < 0.05). Only pathways with at least one significant association are displayed. Regression models were adjusted for age, sex, APOE4 status, and Aβ group.

Pathway Trends Across SCFA Quartiles (Whole Dataset)

Several microbial pathways demonstrated significant variation across butyric acid quartiles (FDR < 0.05, Kruskal-Wallis test*) (Fig. 3, Supplementary Table 4). Biosynthetic pathways including three variants of L-arginine biosynthesis II (ARGSYNSUB-PWY) attributed to *Blautia wexlerae, Clostridium sp.*, and *Methanobrevibacter smithii*, showed progressive increases in relative abundance from the lowest to highest butyrate quartile. Similarly, UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (PWY-6386) demonstrated an upward trend, suggesting enhanced anabolic activity at

higher butyrate concentrations. In contrast, several fermentation-related pathways displayed declining abundance with increasing butyrate levels. These included L-lysine fermentation to acetate and butanoate (P163-PWY), pyruvate fermentation to acetone (PWY-6588), and an unclassified pyruvate fermentation variant (PWY-6588 unclassified), all of which showed stepwise reductions from Q1 to Q4. None of the other SCFAs showed pathways that met the FDR significance threshold. Detailed quartile counts for Aβ High and Aβ Low groups for all significant pathways are summarised in Supplementary Table 1.

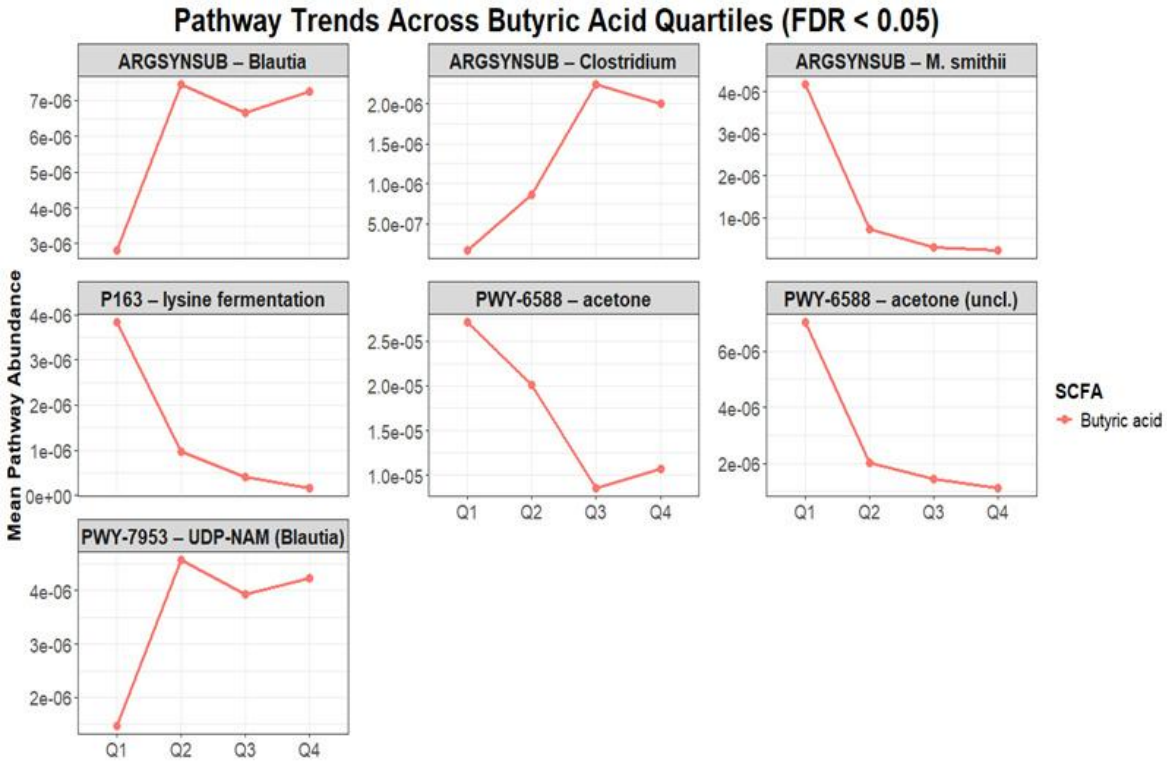

Figure 3. Significant microbial pathway trends across butyric acid quartiles. Only butyric acid demonstrated FDR-significant pathway variation. Line plots show changes in mean pathway abundance (FDR < 0.05) across quartiles, highlighting increasing trends in biosynthetic pathways and decreasing trends in fermentative pathways consistent with higher butyrate concentration.

SCFA Associated Functional Shifts Between Aβ High and Low Groups

Thirteen SCFAs relevant microbial pathways exhibited significant group level differences in relative abundance between the Aβ High (Aβ H) and Aβ Low (Aβ L) groups (unadjusted $p < 0.05$; Wilcoxon rank sum test; Fig. 4). The most distinct differences were observed for L-arginine biosynthesis II (ARGSYNSUB-PWY) represented by three taxon specific variants (acetyl cycle in *Blautia wexlerae, Clostridium sp.*, and *Methano-brevibacter smithii*) which showed consistently higher abundance in the Aβ Low group. Similarly, multiple variants of UDP-N-acetylmuramoyl-pentapeptide biosynthesis (lysine and meso-diaminopimelate-containing forms; PWY-6385, PWY-6386, PWY-6387) were enriched among Aβ Low participants, indicating greater prevalence of peptidoglycan related biosynthetic activity.

In contrast, pathways associated with fermentative metabolism including methanogenesis from acetate (METH-ACETATE-PWY), pyruvate fermentation to acetate and lactate II (PWY-5100), and pyruvate fermentation to isobutanol (PWY-7111) were more abundant in the Aβ High group. Although these pathway differences did not remain significant after FDR correction, they reveal consistent directional trends that suggest biologically meaningful variation between amyloid groups. Given the limited sample size, these findings should be considered exploratory and validated in larger, longitudinal studies to confirm their relevance to early Alzheimer's pathology. (Pathways were ranked by ascending p-values based on Wilcoxon rank-sum tests (Supplementary Fig. 4), allowing identification of the most differentially abundant functions between the amyloid groups, with smaller p-values indicating stronger evidence of group-level separation).

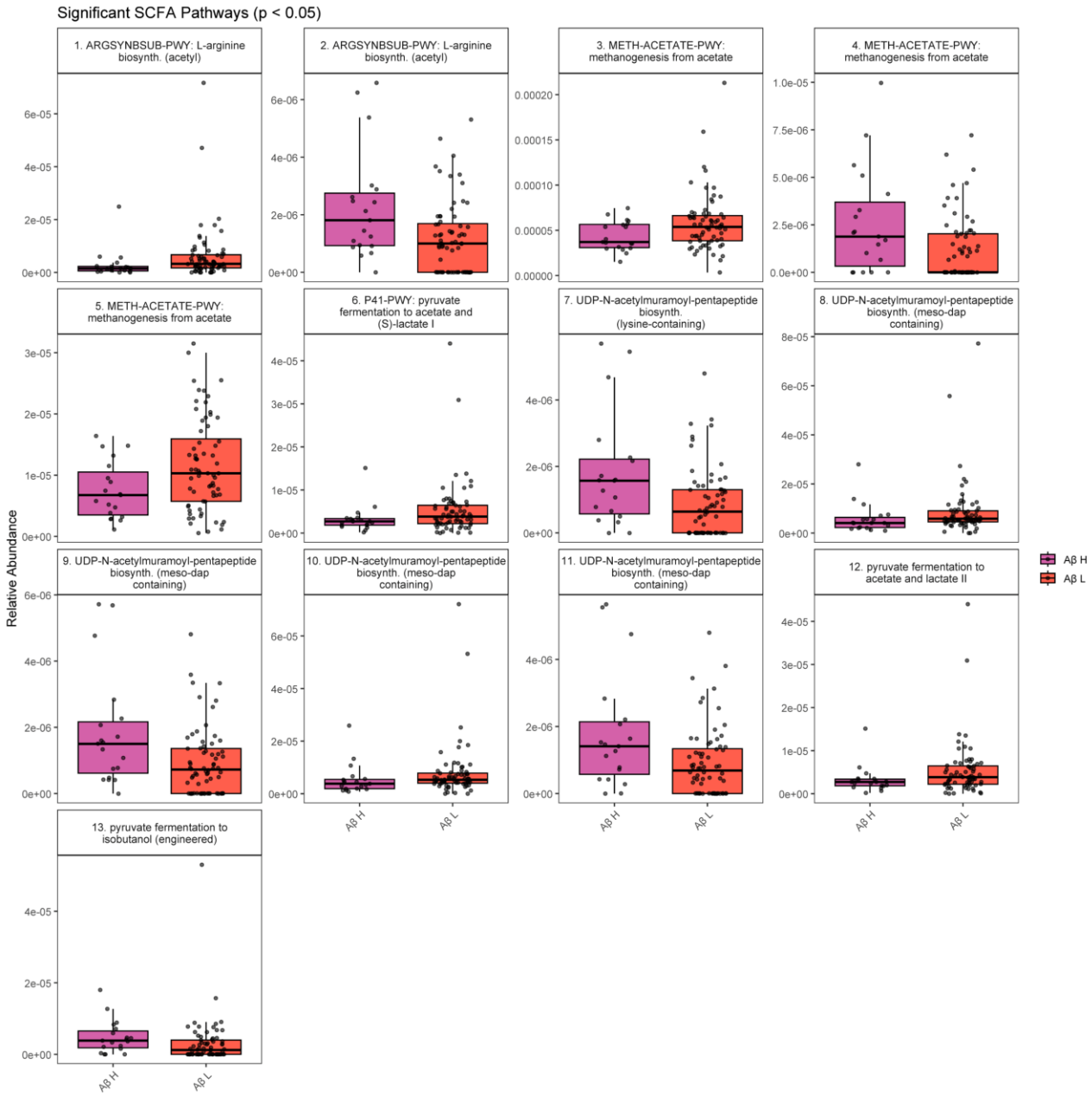

Figure 4. Relative abundances of the most statistically distinct SCFA-relevant microbial pathways (ranked by p-value) between Aβ High and Aβ Low groups. Each boxplot shows the interquartile range and median, with points representing individual participants. Pathways elevated in the Aβ Low group reflect biosynthetic functions (e.g., L-arginine biosynthesis), whereas those enriched in Aβ High suggest increased fermentation (e.g., methanogenesis).

Pathway Co-occurrence and Functional Module Analysis

Three distinct co-occurrence modules were identified among SCFAs related microbial pathways (Fig. 5). Pairwise Spearman correlations (ρ) were calculated between all 362 pathways, and hierarchical clustering was performed using (Ward.D2) with a distance metric of 1 – ρ. Pathways with $|\rho| > 0.2$ were retained to emphasise meaningful co fluctuations. The final number of clusters ($k = 3$) was determined by dendrogram inspection and average silhouette width, delineating three coherent functional modules.

The biosynthetic module (green) contained pathways mainly involved in amino acid and nucleotide biosynthesis (e.g., L-arginine, flavin, and selenium-amino-acid biosynthetic routes). This cluster displayed strong positive intra-module correlations ($\rho > 0.6$), reflecting tightly coordinated anabolic activity within the gut microbiome. The fermentative module (orange)

encompassed pathways linked to carbohydrate breakdown, pyruvate conversion, and SCFAs formation (e.g., pyruvate fermentation to acetone, lysine fermentation to acetate and butanoate). Intra-module correlations were moderately positive ($\rho \approx 0.3–0.6$), consistent with metabolic redundancy and cross feeding among fermentative taxa.

The amino acid and mixed metabolism module (blue) comprised pathways related to amino-acid degradation, cofactor recycling, and intermediary metabolism. This cluster exhibited both positive and negative correlations ($\rho \approx –0.4$ to $+0.4$), indicating variable or competing metabolic activities. Across modules, weak negative correlations were observed between the biosynthetic and fermentative clusters, suggesting partial functional segregation between energy generating and anabolic pathways. Collectively, these modules highlight the structured yet interdependent organisation of microbial metabolic networks supporting SCFAs production in the gut. The clear separation between biosynthetic and fermentative clusters suggests functional compartmentalisation that may reflect early metabolic differentiation associated with preclinical AD.

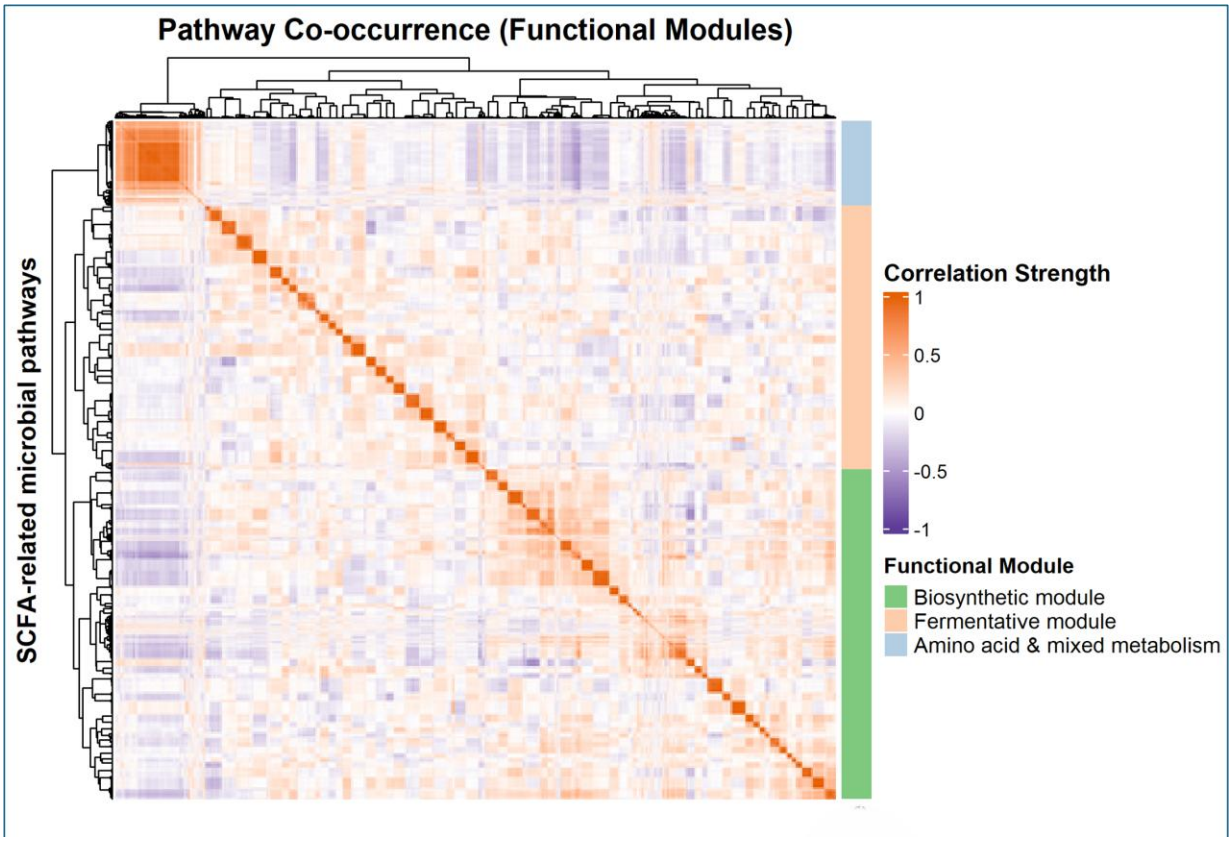

Figure 5. Pathway co-occurrence heatmap of SCFA-related microbial functions. Pairwise Spearman correlation coefficients (ρ) were computed among SCFAs-associated microbial metabolic pathways to assess inter-pathway connectivity and functional clustering within the gut microbiome. Hierarchical clustering (Ward.D2) revealed three major functional modules corresponding to biosynthetic (green), fermentative (orange), and amino acid & mixed metabolism (blue) processes. The heatmap displays correlation strength, where positive correlations are shown in shades of orange to red and negative correlations in shades of blue to purple, with colour intensity reflecting the magnitude of association (|ρ|). Rows and columns represent individual MetaCyc pathways, ordered according to hierarchical relationships.

Partial Canonical Correspondence Analysis (CCA) of SCFAs and Microbial Functional Pathways (Whole Dataset; Amyloid Included as Covariate)

Partial Canonical Correspondence Analysis (CCA) was performed to examine how overall microbial functional composition corresponded with combined SCFA profiles, providing a systems-level view of microbiome-metabolite interactions. Unlike univariate tests, this multivariate approach considered all SCFAs and pathways simultaneously, enabling detection of coordinated functional patterns rather than isolated associations. The analysis was conducted on Hellinger-transformed pathway abundances and adjusted for age, sex, APOE ε4 status, and Aβ group to isolate the portion of microbial functional variation specifically explained by SCFA

concentrations. The model revealed a significant overall association between SCFA profiles and microbial functional composition ($p = 0.041$).

The first canonical axis (CCA1) explained 7.8 % of the constrained variance and was predominantly influenced by acetic and butyric acids, whereas CCA2 accounted for 4.6%, reflecting variation linked mainly to propionic and total SCFA concentrations. Together, these two axes captured 12.4% of the total constrained variance, indicating moderate but biologically meaningful covariation between microbial functions and SCFA profiles. In the ordination space, Aβ High participants aligned more closely with the acetic and butyric vectors, while Aβ Low individuals were distributed along the propionic gradient, suggesting distinct functional configurations in relation to amyloid status. Biosynthetic pathways such as L-arginine biosynthesis II (ARGSY NSUB-PWY) and UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (PWY-6386) contributed positively along the butyrate axis, whereas fermentative pathways (pyruvate fermentation to acetone, L-lysine fermentation to butanoate) loaded in the opposite direction. This separation reflects a metabolic divergence between biosynthetic and fermentative functional modules potentially linked to differences in microbial SCFA production and energy utilization (Fig. 6, Supplementary Table 5).

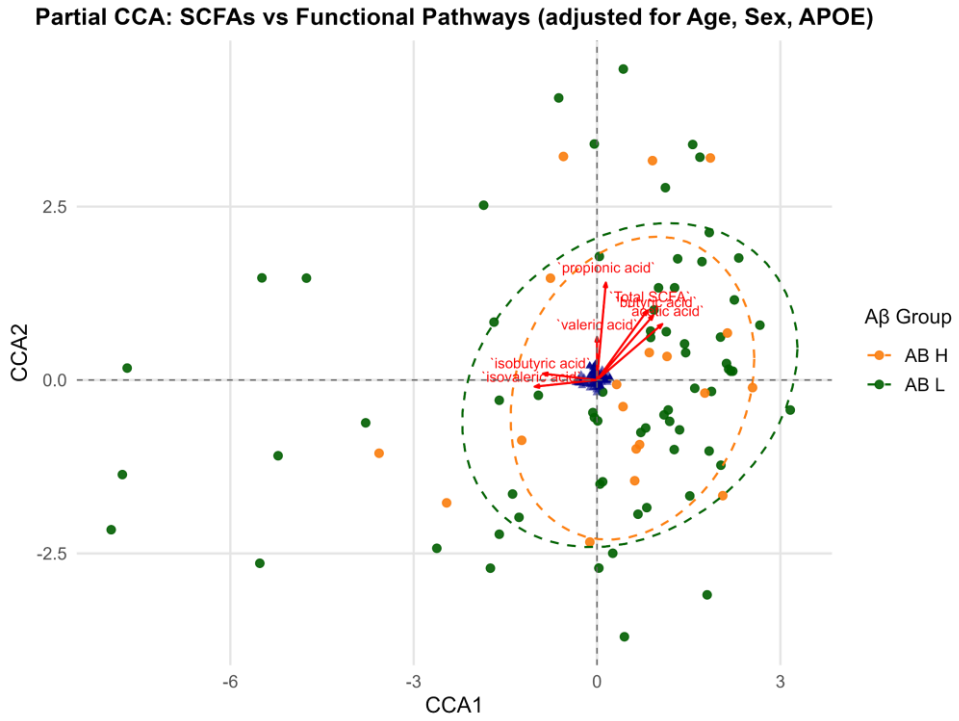

Figure 6. Partial Canonical Correspondence Analysis (CCA) of SCFAs and Microbial Functional Pathways. Ordination biplot showing multivariate associations between faecal SCFA concentrations and microbial functional pathways, adjusted for age, sex, APOE ε4 status, and Aβ status. Each point represents a participant coloured by Aβ group (Aβ High = orange; Aβ Low = green). Red arrows denote SCFA vectors (length proportional to canonical correlation strength). Purple triangles represent key MetaCyc functional pathways contributing to the ordination. Dashed ellipses show 68% confidence intervals for each group. CCA1 = 7.8 %, CCA2 = 4.6 %, model p = 0.041.

Integrated SCFA-Species-Pathway-Metadata (Amyloid-Stratified)

A multi-layered correlation network was constructed to explore functional relationships linking the microbiome, metabolites, and host factors (Supplementary Figure 1). Pairwise Spearman's rank correlations were computed to assess cross-domain relationships among different layers of biological data, including metagenomic features (microbial species and functional pathways), metabolomic profiles (faecal SCFA concentrations), and host metadata (age, sex, APOE ε4 status, and Aβ status). This approach characterised inter-omics connectivity within the amyloid-stratified cohort, revealing how microbial, metabolic, and host variables co-varied across individuals.

A total of 142 significant edges were based on FDR-adjusted significance ($q < 0.05$) and correlation strength

($|\rho| > 0.3$). The resulting network comprised 10 microbial species, 7 SCFAs, 362 SCFA-relevant functional pathways, and 4 host metadata nodes. Distinct structural clusters emerged around acetic and butyric acids, which served as major metabolic hubs. These SCFAs displayed dense positive associations with both microbial species and biosynthetic pathways such as L-arginine biosynthesis II (ARGSYNSUB-PWY) and UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (PWY-6386). Conversely, butyric acid also connected to fermentative routes including pyruvate → acetate/lactate (PWY-5100) and L-lysine → butanoate (P163-PWY), indicating functional interplay between anabolic and catabolic microbial processes. (Fig. 5.7). Host metadata contributed additional structure to the network. Aβ status and APOE ε4 formed key bridge nodes linking microbial composition and metabolic activity. Aβ High participants exhibited stronger connections with fermentative and methanogenic pathways, whereas Aβ Low individuals were more closely linked to biosynthetic modules patterns consistent with canonical correlation and regression findings.

Topological analysis revealed that SCFAs and functional pathways possessed the highest node degree and betweenness centrality, functioning as key mediators of cross-domain communication. The clustering coefficient indicated modular organisation, with three sub-networks corresponding to biosynthetic, fermentative, and amino-acid-related metabolism. Positive correlations (red edges) reflected synergistic or co-occurring interactions, while negative correlations (blue edges) indicated competitive or inverse relationships among variables. Collectively, this integrated network underscores the multiscale interdependence between microbial species, functional metabolism, SCFA production, and host amyloid status, highlighting coordinated microbial–metabolite–host interactions that may shape early metabolic signatures in preclinical AD.

DISCUSSION

Functional alterations in the gut microbiome are increasingly recognised as pivotal modulators of host physiology, particularly through the production and regulation of SCFAs. In this study, functional pathway analysis revealed distinct patterns of SCFA related metabolic potential between individuals with low and high cerebral Aβ burden, highlighting a functional divergence between biosynthetic and fermentative microbial processes. These findings extend prior taxonomic-level observations and highlight the potential relevance of microbial metabolic function in early AD pathophysiology.

In participants with low Aβ burden, microbial pathways related to biosynthesis particularly L-arginine biosynthesis, peptidoglycan biosynthesis, and selenium-containing amino acid metabolism were positively associated with SCFA concentrations, especially acetic and butyric acid. These pathways suggest enhanced microbial anabolic activity supporting mucosal immunity, oxidative-stress regulation, and epithelial integrity [28, 29]. L-arginine biosynthesis promote neuroprotective nitric oxide signalling [29], while selenium-associated metabolism contributes to antioxidant defence mechanisms relevant to early neurodegeneration [30, 31]. This suggests that, even at the preclinical stage, differences in amyloid burden are associated with differences in gut metabolic equilibrium, with lower amyloid levels linked to a more balanced and stable microbial environment [2, 32].

Importantly, these associations between SCFA concentrations and microbial functional pathways remained significant after controlling for potential confounding factors, including age, sex, APOE ε4 status, and amyloid group [10, 33-35]. The persistence of these associations indicates that the observed functional relationships are intrinsic to microbial metabolism rather than explained by demographic or genetic variation. This strengthens the interpretation that SCFA-linked microbial activity reflects genuine biological differences associated with amyloid status.

Conversely, individuals with a high Aβ burden showed increased activity in fermentative and methanogenic pathways, including pyruvate fermentation to acetone, lactate, and acetate, as well as lysine fermentation and methanogenesis from acetate. These pathways indicate a shift toward an anaerobic gut environment that produces more secondary metabolites such as ammonia, hydrogen, and reactive oxygen species. Such by products can disrupt mucosal barrier integrity, promote endotoxaemia, and induce systemic inflammation[36] . Elevated methanogenic activity may further exacerbate redox imbalance and alter gut motility, features that could influence gut-brain communication and proinflammation [36-38]. Collectively, findings indicate that amyloid burden is associated with a metabolic shift in the gut microbiota toward increased fermentative activity.

Quartile based analyses offered deeper insight into how metabolite levels relate to microbial function. Participants with higher butyric acid levels predominantly observed with low Aβ burden, showed increased abundance of biosynthetic pathways such as L-arginine biosynthesis and reduced abundance of fermentation pathways, including pyruvate fermentation to acetone. This pattern is consistent with an association between higher butyrate levels and a more metabolically balanced

gut microbiome in the Aβ Low group. Similar trends were observed for total SCFA concentrations, suggesting that the combined action of these metabolites may work together to maintain both gut and brain homeostasis [39]. These findings further indicate that even during the preclinical stage of AD, variations in amyloid burden are linked to functional metabolic differences in the gut microbiome that may influence early disease mechanisms through the microbiota-gut-brain axis [40, 41].

Building on these findings, the pathway co-occurrence analysis provided a broader, systems-level view of how microbial functions are organised within the gut microbiome. Unlike the previous analyses that focused on direct associations between SCFAs and individual pathways, this approach explored how pathways interact with one another, revealing the internal structure of the microbiome's metabolic network. Three major functional modules were identified biosynthetic, fermentative, and amino acid/mixed metabolism with clear differences between amyloid groups [42]. In the Aβ Low group, biosynthetic pathways were closely linked, suggesting coordinated metabolic activity and a stable gut environment. In contrast, the Aβ High group showed stronger correlations among fermentative pathways, reflecting a shift toward energy extraction and reduced biosynthetic balance. This shift indicates that as amyloid burden increases, the functional organisation of microbes becomes reorganised, mirroring the metabolic and inflammatory changes characteristic of early AD [43, 44].

The canonical correspondence analysis showed that variations in amyloid levels were linked with distinct relationships between microbial functional pathways and SCFA profiles. Participants with higher amyloid burden tended to cluster with pathways related to acetic and butyric acid, suggesting greater fermentative and energy-yielding activity. In contrast, those with lower amyloid levels were more closely associated with propionate-related biosynthetic functions, indicating a more balanced and stable metabolic environment. These findings suggest that as amyloid accumulation increases, the gut microbiome undergoes a coordinated shift in its functional profile rather than isolated pathway changes [45-47]. This broader reorganisation of microbial metabolism may reflect an early adaptive response in the gut, where fermentative activity becomes more dominant and biosynthetic capacity is reduced changes that could contribute to early inflammatory and metabolic disturbances seen in AD [48, 49].

The integrated SCFA species pathway metadata network provided a holistic view of interactions across microbial, metabolic, and host domains. Central nodes particularly acetic and butyric acid acted as key metabolic hubs linking multiple microbial species and functional pathways. Biosynthetic processes such as L-arginine and peptidoglycan biosynthesis clustered closely with these SCFAs, whereas fermentative and methanogenic pathways showed stronger associations with Aβ High and APOE ε4 nodes. This network pattern suggests that increasing amyloid burden alters the organisation of microbial metabolism, strengthening fermentation related connections while weakening biosynthetic coupling. Negative correlations reflected competition between microbial processes, while positive correlations indicated cooperative SCFA pathway relationships, supporting the existence of functionally distinct microbial communities. Importantly, the disruption of biosynthetic SCFA clustering and the dominance of fermentation linked associations in Aβ High individuals may represent an early microbial signature associated with metabolic imbalance. These network level changes highlight the potential of gut functional markers particularly butyrate and acetate related pathways as predictive indicators of preclinical AD [11, 50-52].

A key finding from this work is that microbial function does not necessarily reflect taxonomic composition. Species level analysis presented in previous studies showed that Bacteroides were present in both Aβ Low and Aβ High groups; however, the functional analyses in this study revealed that their associated metabolic pathways differed markedly, suggesting that the same taxa may perform different metabolic roles under varying host conditions. This highlights the importance of pathway-based approaches, which capture functional differences that may remain undetected at the taxonomic level. While other research studies described a reduction in several beneficial species within the Aβ High group, the current findings extend this by demonstrating how such compositional changes correspond to reduced SCFA biosynthesis and increased fermentation and methanogenesis [53, 54].

These results should, however, be interpreted with caution. Functional pathway profiles derived from metagenomic data represent predicted gene potential rather than active expression or metabolite output. Although the observed associations align with known SCFA metabolic pathways, validation using metatranscriptomic or metabolomic approaches is needed to confirm biological activity [54]. In addition, pathway annotations may be incomplete or redundant, limiting fine scale mechanistic interpretation.

Despite these limitations, the findings provide evidence that alterations in SCFA related microbial functions are associated with amyloid burden even before cognitive decline occurs. The loss of biosynthetic capacity and the shift toward fermentative metabolism observed in individuals with higher amyloid levels suggest that early amyloid pathology is accompanied by systemic metabolic imbalance within the gut microbiome. Given that SCFAs

regulate blood-brain barrier permeability, microglial activation, and epigenetic processes, such functional changes may represent an upstream signal contributing to neuroinflammation and early neurodegenerative risk [55, 56].

Together, these findings support the growing view that SCFA metabolism is integral to brain health. Previous studies have linked lower butyrate levels and reduced expression of SCFA-producing pathways to poorer memory and increased neuroinflammation [56-59]. Extending this evidence, the present study shows that even at the preclinical stage of AD, distinct functional imbalances in the gut microbiome are detectable and may help identify individuals at greater metabolic risk for disease progression.

Limitations and Future Directions

Despite its strengths, including multi-omics integration and stringent pathway-level filtering, this study has several limitations. The cross-sectional design precludes causal inference, making it unclear whether microbial functional alterations precede or follow amyloid accumulation. In addition, host-level mechanisms such as SCFA receptor expression (e.g., GPR41, GPR43, GPR109A), blood-brain barrier integrity, and inflammatory mediators were not directly assessed in this study. Longitudinal studies incorporating repeated sampling are required to determine the temporal sequence of microbiome-metabolite-amyloid interactions. Furthermore, pathway abundances derived from metagenomic data represent potential gene functions rather than active biochemical processes. Integration of complementary metatranscriptomic, metaproteomic, and targeted metabolomic approaches would help validate the transcriptional and metabolic activity of identified pathways.

Dietary intake, a known modifier of SCFA profiles and microbial metabolism, was not quantitatively assessed in the present analysis and represents a potential confounding factor when interpreting faecal SCFA concentrations. Although faecal SCFA concentrations provide an indirect marker of fermentation activity, detailed dietary profiling or controlled feeding interventions are needed to disentangle the influence of diet from amyloid related effects. The modest sample size also limits generalisability, and replication in larger, ethnically and geographically diverse cohorts is warranted. Finally, while faecal SCFAs reflect luminal metabolic output, concurrent measurement of circulating SCFAs, intestinal permeability markers, and systemic inflammatory mediators would provide a more comprehensive picture of gut-systemic interactions.

Future work should prioritise longitudinal, multi time point studies linking microbial functional shifts with neuroimaging, cognitive, and biochemical markers of AD. Experimental models could further test causality by modulating key biosynthetic or fermentative pathways through dietary fibres, prebiotics, or defined microbial consortia. Future studies examining host SCFA receptors, including GPR41, GPR43, and GPR109A, may help clarify potential mechanistic links between microbial metabolites and AD pathology. Such studies may clarify whether restoring biosynthetic balance and reducing fermentative overactivity can mitigate amyloid pathology or neuroinflammatory processes. Ultimately, delineating how microbial metabolism interfaces with host physiology may inform microbiome-based biomarkers and preventive strategies for early AD.

Conclusion

Through a series of complementary analyses, this study demonstrates functional alterations in the gut microbiome are associated with early amyloid burden in cognitively unimpaired adults. Correlation and regression analyses revealed consistent associations between SCFA concentrations and microbial pathways, linking gut metabolic activity to amyloid status independent of age, sex, and APOE ε4 genotype. Quartile-based analyses further showed that higher butyrate and total SCFA levels corresponded with increased biosynthetic activity and reduced fermentation, suggesting a metabolically balanced gut environment among individuals with low amyloid burden.

Pathway co-occurrence and multivariate analyses identified distinct functional clusters biosynthetic, fermentative, and amino acid/mixed metabolism whose organisation differed by amyloid status. Aβ Low individuals exhibited coordinated biosynthetic activity, whereas Aβ High individuals showed enhanced fermentative interactions. Integrated network analysis positioned acetate and butyrate as central metabolites linking microbial pathways, with rising amyloid levels associated with weakened biosynthetic coupling and strengthened fermentation-related connectivity.

Collectively, these findings indicate that increasing amyloid burden is accompanied by a shift from biosynthetic to fermentative microbial functions, while lower amyloid levels reflect greater metabolic stability and SCFA-driven homeostasis. Such early gut functional reorganisation may represent a non-invasive feature associated with preclinical AD and highlights dietary, prebiotic, and microbial-based strategies as areas for future investigation.

Author Contributions

Conceptualization, D.M.S.D.; Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, W.M.A.D.B.F.; Writing – review & editing, Methodology, supervision, T.N.J.; Methodology, supervision, H.R.S.; supervision, Data curation, S.R.R.S.; Data curation, supervision, K.T.; Data curation, C.L.M.; Data curation, R.N.M.; supervision.

Institutional Review Board Statement

Not applicable.

Declaration of interest

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Originality

This manuscript is an original work and has not been published previously nor is it under consideration for publication elsewhere.

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Data Availability Statement

The data underlying this article were accessed from the Australian Imaging, Biomarkers, and Lifestyle (AIBL) study and the Western Australian Memory Study (WAMS) and are not publicly available due to ethical and privacy restrictions (including raw sequencing data and all derived metagenomic outputs) Access to AIBL data can be requested through the Expression of Interest (EOI) process outlined on the AIBL study website (https://aibl.csiro.au) in accordance with their data access procedures. Additional information regarding data access for AIBL and WAMS can be obtained by contacting the corresponding author.

Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.1539.

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