

Investigating *APOE* Genotype, Sex, and Age on Flavonoid Metabolism

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Abstract

Alzheimer's disease (AD) is influenced by several non-modifiable risk factors, notably age, *APOE* genotype, and sex, with risk increasing with advancing age, the presence of *APOE* $\epsilon 4$ alleles, and in females. These factors also shape gut microbiota composition. Dietary flavonoids, plant-based bioactives known to support cognitive health, require microbial metabolism in the colon, as humans lack the necessary enzymes for their breakdown. Although substantial interindividual variation in flavonoid metabolism has been documented, the specific effects of *APOE* genotype, sex, and age remain unexplored.

This thesis employed targeted metabolomics to investigate the metabolism of epicatechin (EC), both as a pure compound and within meals, using a human colon model, *APOE*-targeted replacement (TR) mouse models, and reanalysis of clinical data. 16S rRNA sequencing and untargeted metabolomics were applied to link microbial community patterns with metabolic outcomes, while EC supplementation studies in *APOE*-TR mice assessed physiological impacts.

Results revealed that *APOE* genotype significantly modulated EC metabolism, influencing concentrations of both microbially derived valerolactones and valeric acids, as well as non-microbially derived EC metabolites. These effects were context-dependent, varying with age, sex, and food matrix. In *APOE*-TR mice, sex and genotype shaped gut microbiota composition, while dietary effects were observed only with flavan-3-ol-rich cocoa powder, not purified EC. Human data showed greater intragroup variability. Cognitive improvements in spatial working memory were limited to *APOE* $\epsilon 4/\epsilon 4$ -TR males, underscoring genotype- and sex-dependent effects. *APOE* genotype and sex were also associated with hepatic inflammation and sulfotransferase pathway activity.

Collectively, these findings demonstrate that *APOE* genotype, sex, and age critically influence EC metabolism, gut microbiota structure and physiological responses, emphasising the necessity of considering genetic and biological context when evaluating flavonoid-related metabolism and health outcomes.

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List of Abbreviations and Acronyms

μSPE	Microelution solid-phase extraction
¹ H NMR	Proton nuclear magnetic resonance
3,4-DiHPP-2-ol	1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol
3,4-DiHPVA	5-(3',4'-dihydroxyphenyl)valeric acid
3,4-DiHPVL	5-(3',4'-dihydroxyphenyl)-γ-valerolactone
3-O-MeEC	3'-O-methoxyepicatechin
4-O-MeEC	4'-O-methoxyepicatechin
ABCA1	ATP binding cassette transporter A1
AD	Alzheimer's Disease
ADME	Absorption distribution metabolism and excretion
AOE	Antioxidant enzymes
APOE -/-	APOE knockout
APOE	Apolipoprotein E
APOE2	apolipoprotein E ε2 allele
APOE2/2	Apolipoprotein E ε2 /ε2
APOE2/3	Apolipoprotein E ε2 /ε3
APOE2/4	Apolipoprotein E ε2 /ε4
APOE3	apolipoprotein E ε3 allele
APOE3/3	Apolipoprotein E ε3 /ε3
APOE3/4	Apolipoprotein E ε3 /ε4
APOE4	apolipoprotein E ε4 allele
ARE	Antioxidant response element
AWERB	Animal welfare and the ethical review body
Aβ	Amyloid Beta
BDNF	Brain derived neurotrophic factor
C	Cytosine
CBF	Cerebral blood flow
CID	Collision induced dissociation
CLR	Centred log-ratio transformation
COX-2	Cyclooxygenase 2

CREB	cyclic AMP response element-binding protein
CV	Coefficient of variance
CYP7A1	Cholesterol 7 α hydroxylase
D ₂ O	Deuterated water
DIAMOND	
.....	Dietary pLAnt extracts, colonic MicroBes and apoe geNetics interactions stuDy
EC	Epicatechin
EC-3-S	Epicatechin-3'-sulfate
EC-4/5/7-S	Epicatechin-4'/5/7-sulfate
EC-S-GluAC	Epicatechin-sulfate-glucuronide
eNOS	Endothelial nitric oxide synthase
ERK1/2	extracellular signal-regulated kinase 1/2
ESI	Electrospray ionisation source
FA	Formic acid
FDR	False discovery rate
FFQ	Food frequency questionnaire
GBA	Gut-brain axis
GI	Gastrointestinal
HDL	High-density lipoproteins
H-ESI-II	Heated ESI
HF	High-fat
HFEC	High-fat with epicatechin
IL-1 β	Interleukin 1 β
iNOS	Inducible nitric oxide synthase
JNK	Jun amino-terminal kinase
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LDL	Low-density lipoproteins
LIT-MS	Linear ion trap mass spectrometer
LXR α	Liver X receptor α
MCI	Mild cognitive impairment
MeO-EC-S	Methoxyepicatechin sulfate
MMSE	Mini-mental state examination

MRM	Multiple reaction monitoring
ND	Not detected
NF- κ B	Nuclear factor κ B
NO	Nitric oxide
Nrf2	Nuclear factor erythroid 2-related factor 2
OTU	Operational taxonomic unit
p38 MAPK.....	p38 mitogen activated protein kinase
PA	Phosphoric acid
PCoA	Principal Coordinate Analysis
PDE	Phosphodiesterase
PERMANOVA	PERmutational Multivariate ANalysis Of VAriance
PICRUST 2	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States modules 2
PKC	Protein kinase C
PVA	Phenyl-valeric acids
PVA-3-S	5-(Phenyl)valeric acid-3'-sulfate
PVL	Phenyl- γ -valerolactones
PVL-3-S	5-(phenyl- γ -valerolactone-3'-sulfate
QIB	Quadram institute bioscience
RCT	Randomised control trial
RT	Retention time
SCFA	Short chain fatty acid
SCG-FFQ	Scottish Collaborative Group Food Frequency Questionnaire
-SH	Thiol
SIRT1	Sirtuin 1
sPLS-DA	Sparse partial least squares discriminant analysis
SREC	structurally related epicatechin
T	Thymine
TNF- α	Tumor necrosis factor α
TR	Targeted replacement

UPLC-MS/MS
 .Ultra performance liquid chromatography coupled with tandem mass spectrometry
VLDL Very low-density lipoproteins

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Chapter 1: General introduction

The global population is undergoing a demographic shift towards older age, with current estimates indicating that approximately 10% of individuals worldwide are over the age of 65 [1]. This proportion is projected to increase to 16% by 2050, with estimates suggesting that up to one in four individuals in Europe and North America will fall within this age group [1]. As the population ages, the prevalence of age-related disorders increases [2], and it is anticipated that 150 million people globally will be living with dementia by 2050 [3]. Dementia encompasses a group of progressive, currently irreversible, neurodegenerative disorders, characterised by a decline in two or more higher-order cognitive functions beyond what is expected from ageing and comorbidities, thereby impairing daily functioning [4, 5]. Alzheimer's disease (AD) is the most common form of dementia, accounting for 60-70% of cases worldwide [6]. Pathologically, AD is marked by the accumulation of amyloid-beta ($A\beta$) plaques and neurofibrillary tangles composed of hyperphosphorylated tau (HT) protein [7], which initially appear in the hippocampus and subsequently spread across the cerebral cortex [8-10]. The increasing prevalence of dementia presents substantial medical, financial, and emotional challenges, not only for affected individuals but also for their caregivers.

Despite extensive research efforts, there is currently no cure for AD, with the available pharmacological treatments primarily aimed at slowing disease progression and/or alleviating the symptoms, rather than halting or reversing the condition [11]. A complementary strategy involves identifying and modifying risk factors to prevent or delay disease onset. However, the development of personalised interventions requires a deeper understanding of how these risk factors interact. This thesis investigates the interplay between three non-modifiable risk factors: *apolipoprotein E (APOE)* genotype; sex; and age; along with modifiable factors, specifically the gut microbiota and its metabolism of dietary compounds, such as dietary bioactive (poly)phenols.

1.1. *APOE* Genotype

In addition to ageing, the *APOE* genotype represents the most significant genetic risk factor for the development of AD [12]. The *APOE* gene exists in three allelic forms: $\epsilon 2$, $\epsilon 3$, and $\epsilon 4$, with global frequencies of approximately 8.4%, 77.9%, and 13.7% respectively [13]. These alleles combine to form six possible genotypes: *APOE* $\epsilon 2/\epsilon 2$, $\epsilon 2/\epsilon 3$, $\epsilon 2/\epsilon 4$, $\epsilon 3/\epsilon 3$, $\epsilon 3/\epsilon 4$, and $\epsilon 4/\epsilon 4$ (*APOE2/2*, *APOE2/3*, *APOE2/4*, *APOE3/3*, *APOE3/4*, *APOE4/4*) [14]. Each confers varying levels of risk for AD. Although the prevalence of the apolipoprotein E $\epsilon 4$ allele (*APOE4*) in the global population is relatively infrequent, its distribution varies significantly by geographic region. For instance, its prevalence is reported to be as low as 10% in Southern European populations, while reaching up to 40% in certain sub-Saharan African populations [15, 16] (Figure 1.1).

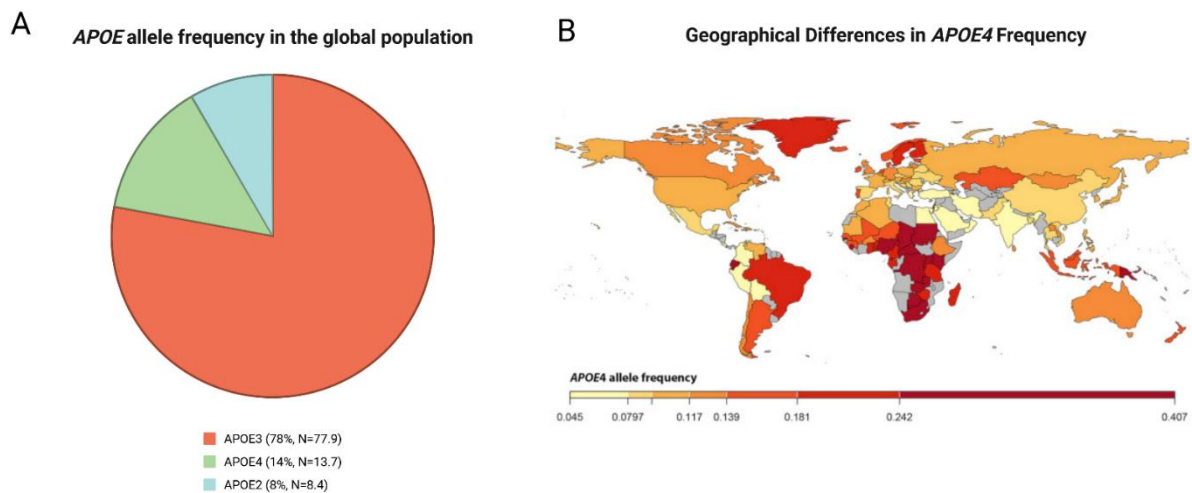


Figure 1.1: (A) *APOE* allele frequency in the global population; (B) Geographical Differences in the *APOE4* frequency. Image B licensed for use in thesis by agreement from Elsevier: Belloy ME, Napolioni V, Greicius MD. A Quarter Century of *APOE* and Alzheimer's Disease: Progress to Date and the Path Forward. *Neuron*. 2019;101(5):820-38.

Approximately 40–60% of individuals diagnosed with AD carry at least one copy of the *APOE4* allele [12]. The presence of *APOE4* is associated with increased neuronal degeneration [17] and a heightened risk of developing cardiovascular disease (CVD) in later life, which is an established comorbid risk factor for AD. The risk of developing AD is dose-dependent with respect to *APOE4* alleles; individuals homozygous for *APOE4* (*APOE4/4*) exhibit up to a 15-fold increased risk compared to non-carriers [18]. In contrast, apolipoprotein E $\epsilon 3$ allele (*APOE3*) is the most prevalent isoform in Western populations and is considered neutral with regard to AD susceptibility. The less common apolipoprotein E $\epsilon 2$ allele (*APOE2*) has been associated with increased longevity [19] and

may exert protective effects against AD, potentially through mechanisms involving reduced A β accumulation [20, 21].

1.1.1. APOE structure

The three common alleles of the *APOE* gene ($\epsilon 2$, $\epsilon 3$, and $\epsilon 4$) are defined by specific single-nucleotide polymorphisms (SNPs) at loci rs429358 and rs7412 [22]. The allelic variants differ based on the presence of cytosine (C) or thymine (T) at these positions: $\epsilon 2$ corresponds to T at both rs429358 and rs7412, $\epsilon 3$ to T at rs429358 and C at rs7412, and $\epsilon 4$ to C at both positions [23]. The resulting APOE protein comprises 299 amino acids and consists of a structured N-terminal domain characterised by a four-helix bundle, a flexible hinge region, and an unstructured C-terminal domain [24, 25]. The different *APOE* alleles give rise to single amino acid substitutions at residues 112 and 158 [26, 27] (Table 1.1).

Table 1.1: APOE polyforms amino acid structures. Summarising the findings of Mahley, R.W., Apolipoprotein E: Cholesterol Transport Protein with Expanding Role in Cell Biology. Science, 1988. 240(4852): p. 622-630. and Rall, S.C., K.H. Weisgraber, and R.W. Mahley, Human apolipoprotein E. The complete amino acid sequence. Journal of Biological Chemistry, 1982. 257(8): p. 4171-4178.

APOE Polyform	Amino Acid at Position 112	Amino Acid at Position 158
APOE2	Cysteine	Cysteine
APOE3	Cysteine	Arginine
APOE4	Arginine	Arginine

APOE4 is considered the ancestral isoform and features arginine both at positions 112 and 158. In *APOE3*, a cysteine replaces arginine at position 112, while *APOE2* contains cysteine substitutions at both sites [24]. These amino acid changes introduce thiol (-SH) groups, resulting in significant alterations in protein conformation. In *APOE3*, these substitutions enhance pairing between N-terminal helices 1 and 4, increasing their separation from helices 2 and 3. *APOE2* exhibits even greater separation between all helices within the N-terminal region [28]. In contrast, *APOE4* adopts a more compact conformation, characterised by stronger interdomain interactions between the N- and C-terminal regions, and a structurally unstable intermediate, wherein helix 1 detaches from the remaining three helices [28]. These isoform-specific structural variations influence ligand-binding affinities and functional properties of the APOE protein, contributing to differential risk profiles in AD pathogenesis [29] (Figure 1.2).

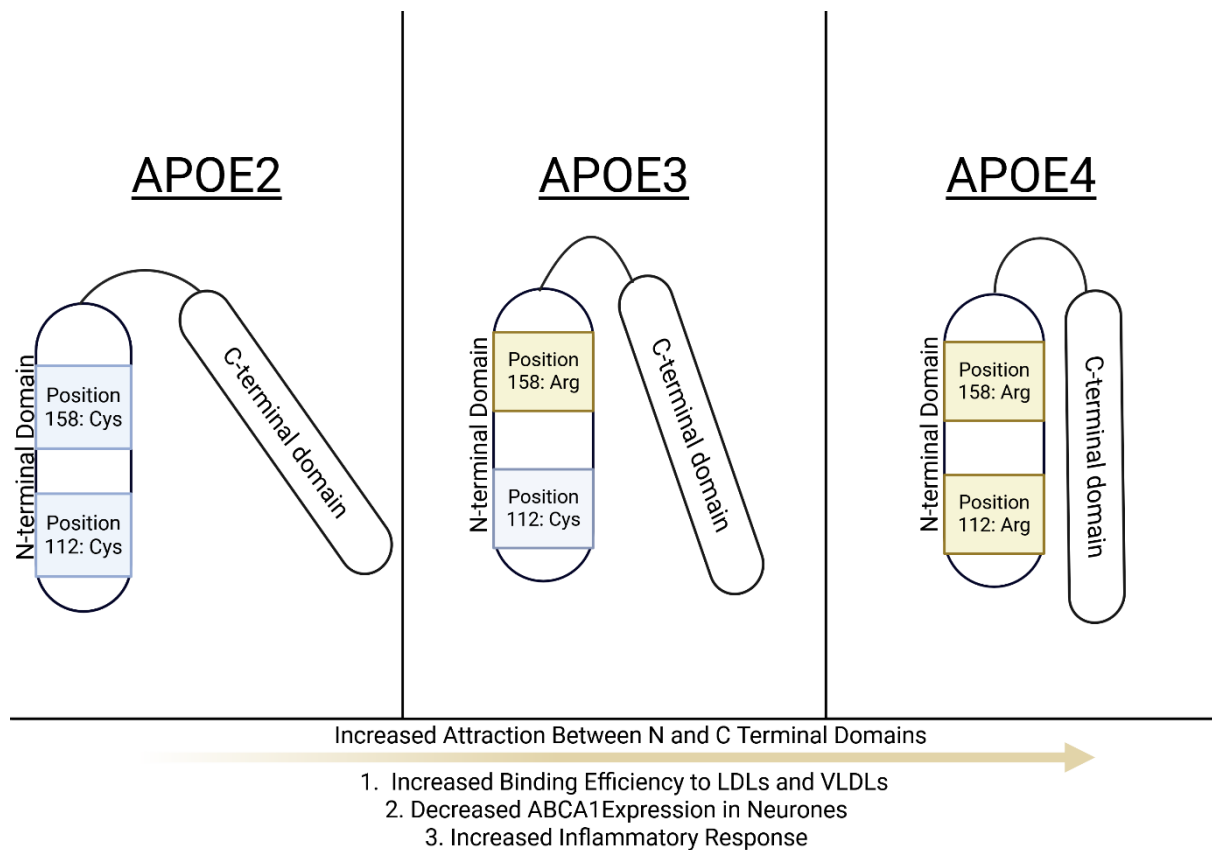


Figure 1.2: Structure and Function of Each APOE polymorph

1.1.2. APOE Role in Lipid Homeostasis and Cognitive Function

APOE is ubiquitously expressed throughout the body, with predominant expression by hepatocytes and macrophages in the liver [26], as well as by microglia and astrocytes within the brain and the central nervous system (CNS) [30]. APOE plays a critical role in plasma lipid transportation and homeostasis [31].

APOE is present on the surface of all four major classes of lipoproteins: chylomicrons, very low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs), where it not only stabilises lipid cargo but also serves as a ligand for cell surface receptors, facilitating lipoprotein clearance from plasma [32]. This clearance pathway directs chylomicron remnants and HDLs to the liver, while VLDLs and LDLs are predominantly taken up by peripheral tissues [33]. The essential role of APOE in lipid clearance is evidenced in *APOE*-knockout (*APOE* $-/-$) murine models, which exhibit severe hypercholesterolemia and spontaneous atherosclerosis [34, 35]. In humans, the APOE4 isoform exhibits increased affinity for VLDLs and LDLs, elevating their plasma

levels and promoting lipid deposition in peripheral tissues, thereby contributing to adiposity and heightened cardiovascular risk [36, 37]. In contrast, the APOE2 isoform preferentially associates with HDLs, enhancing hepatic uptake and clearance of plasma lipids [38]. Altered lipid metabolism, transport, and heightened cardiovascular risk increase the risk of neurocognitive decline, as the cardiovascular-brain axis plays a key role in maintaining neuronal function [39]. The conversion of cholesterol to primary bile acids, essential for dietary lipid absorption and excretion, has been shown to be *APOE*-dependent; however, current findings are primarily derived from *APOE*^{-/-} models, and the specific influence of *APOE* genotype on this pathway remains insufficiently characterised [40, 41].

Beyond its peripheral functions, APOE plays a pivotal role in CNS physiology and cognitive function. The brain is enriched in cholesterol, and APOE is fundamental to the intercellular transport of cholesterol and other lipids, which are required for neuronal development, synaptic remodelling, and membrane maintenance [12]. APOE-lipoprotein complexes activate the ATP-binding cassette transporter A1 (ABCA1) pathway, facilitating cholesterol efflux from neural cells [42]. Notably, the *APOE4* allele is associated with reduced ABCA1 expression and impaired cholesterol efflux in neurons [43], a condition linked to increased A β accumulation [44].

In addition to lipid transport, the *APOE* genotype also modulates several other key metabolic steps linked to neurodegeneration. In patients with AD, individuals carrying the *APOE4/4* genotype exhibit greater cerebral A β deposition compared to those with the *APOE3/3* genotype [45]. Furthermore, *APOE4/4* mouse models have demonstrated increased HT deposition compared to APOE3 animals [46-48]; both A β and HT are biomarkers for AD. As well as pathological cascades linked directly to AD, *APOE4* also promotes several other independent pathways that are also injurious to brain health.

The *APOE* genotype influences the blood-brain barrier (BBB), the semipermeable membranes separating blood from cerebrospinal fluid, with the *APOE4* increasing barrier dysfunction in the hippocampus and medial temporal lobe compared to the *APOE3/3* genotype, irrespective of their A β and HT status [49]. Indicating that APOE mediates BBB permeability before AD pathological onset. BBB dysfunction is associated with greater neurocognitive decline, as the membrane is unable to prevent harmful substances,

pathogens, and immunoregulatory cells from entering cerebral tissue from the bloodstream [50, 51]. Impaired regulation across the BBB can lead to neuroinflammation, with *APOE4* inducing a more pronounced pro-inflammatory profile relative to *APOE3* and *APOE2* [52]. Chronic neuroinflammation is a known contributor to neurodegenerative processes and is implicated in the pathogenesis of AD [53]. As well as the effects on neuroinflammation, the *APOE4* genotype is known to affect synaptic plasticity by inhibiting the recycling of neurotransmitters within the synapse [54]. Synaptic plasticity is key to the generation of new neuronal links during learning, and reduced efficiency can interfere with memory formation [55]. As well as its role in neuronal functioning, the *APOE* genotype alters the brain's energy metabolism. Neuroimaging evidence suggests that *APOE4* carriers are associated with reduced cerebral glucose utilisation, and that these metabolic reductions can be detected in ageing and early disease stages [56]. Mechanistically, disturbances in cerebral glucose metabolism are closely linked to mitochondrial impairment, given the mitochondria's role in the generation of ATP in the final steps of respiration [57]. Consistent with this, evidence is accumulating implicating the *APOE4* allele in mitochondrial dysfunction, increasing oxidative stress, and impairing mitochondrial pathways such as mitophagy [58, 59]. Collectively, the literature highlights several *APOE*-mediated mechanisms that contribute to neurodegeneration; the impact of the *APOE4* allele on cognitive performance remains complex and partially unresolved.

1.1.3. *APOE* and Alzheimer's Disease Risk

Following age, the *APOE4* allele is recognised as the most significant genetic risk factor for the development of AD. Large cohort studies have demonstrated that both the relative and absolute risks increase in an allele-dependent manner. For instance, individuals heterozygous for *APOE4* demonstrate a threefold increase in AD risk, while homozygous individuals may experience between ten- to fifteenfold elevation relative to non-carriers [13, 18]. Importantly, however, *APOE4* is not a deterministic mutation; rather, it is a susceptibility allele, as many *APOE4* carriers remain neurocognitively intact as age advances.

Prospective cohort analyses provide valuable insight into the absolute risk associated with *APOE* genotypes. Data from the Copenhagen general population study indicate that

the 10-year risk of developing AD among *APOE4* homozygotes increases substantially with age, rising from 6-7% in individuals aged 60-69 years old, to 19-24% in those aged 80+ years old [60]. Comparable gradients are observed for all-cause dementia, supporting a strong relationship between allele dose and disease penetrance.

Nevertheless, the observation that a substantial proportion of *APOE4* carriers do not develop dementia highlights the incomplete penetrance characteristics of complex polygenic diseases, suggesting that additional biological and environmental factors influence the transition from genetic susceptibility to clinical disease pathology. Emerging evidence suggests that the magnitude of *APOE4*-associated risk may differ by biological sex.

Beyond increasing disease probability, *APOE4* is associated with poorer overall cognitive outcomes [50, 51]. However, the relationship between *APOE* genotype and specific cognitive domains remains inconsistent; for example, a meta-analysis examining *APOE* and spatial memory reported only weak associations between *APOE4* and impairments in spatial cognition [61, 62]. Thus, while *APOE4* is clearly implicated in accelerated cognitive decline, findings across specific cognitive domains remain mixed.

Nevertheless, *APOE4* is also associated with an earlier age of onset of neurocognitive decline, with several longitudinal studies reporting that *APOE4* carriers develop cognitive impairment approximately 5-10 years earlier than non-carriers [13], with a dose-dependent effect observed as the number of *APOE4* alleles increases [52]. Recent work has further proposed that homozygote carriers may represent a distinct genetic form of AD, given the high prevalence of AD-related biomarker abnormalities observed in these individuals by their mid-sixties [63]. While this interpretation remains an area of active investigation, it reinforces the substantial pathological burden associated with this allele. Collectively, these findings position *APOE4* as a major genetic determinant of Alzheimer's disease risk; however, the clinical expression of this risk is shaped by interacting factors, most notably age and biological sex.

1.1.4. *APOE*, Age, and Sex

Advancing age remains the most significant risk factor for the development of dementia [2]. However, the influence of the *APOE4* allele on cognitive decline is not consistent

across the lifespan. A meta-analysis of 20 studies reported no significant effect of *APOE* genotype on cognitive performance in individuals aged 5-35 years [64]. In middle age, the impact of *APOE4* appears modest; for instance, one study observed that only Caucasian males carrying *APOE4* demonstrated reduced verbal memory performance [50]. It is predominantly in older adulthood that the cognitive effects of *APOE4* become more pronounced [52, 65]. Although many studies dichotomise participants into *APOE4* carriers and non-carriers, evidence also indicates that carriers of the *APOE2* allele may exhibit enhanced cognitive function in later life compared to other genotypes [66]. While specific cognitive domains have shown differential performance across genotypes, further investigation is required to elucidate the interactive effects of age and *APOE* status on cognitive trajectories.

In addition to age, sex also contributes to AD risk, with females demonstrating a higher incidence of AD compared to age-matched males [67]. While this difference has historically been attributed to females' longer average lifespan, emerging evidence suggests that both biological and socio-environmental factors may also contribute. For example, oestrogen has neuroprotective properties in adulthood [68], yet its decline during the menopausal transition has been linked to an elevated risk of dementia [69, 70].

Although *APOE4* carrier status is independent of sex, research indicates that the allele's effect on AD risk may be modulated by sex. Meta-analytic evidence further supports sex-specific effects of the *APOE4* allele on AD risk. For example, in females aged 65-75 years old, the addition of one *APOE4* allele results in an approximately fourfold risk elevation of AD against *APOE3/3*, compared to an estimated threefold increase in males, highlighting a potentially greater genotype penetrance within this subgroup [71]. Interestingly, the sex difference in risk between the genotypes was not consistent throughout the life course. When looking more broadly across the lifespan, the sex differences are lost, indicating that an interaction between age, sex and *APOE* genotype [71].

Female *APOE4* carriers generally perform worse on memory assessments compared to male carriers [72]. This sex-specific vulnerability may be partially explained by differential neuroimmune responses. For instance, in targeted replacement (TR) mouse models, male *APOE3/3*-TR mice exhibit greater astrocyte coverage than female *APOE4/4*-TR mice, with the latter showing increased neuronal injury and astrocyte activation [73]. Moreover,

female *APOE4/4*-TR mice exhibit more frequent interactions between astrocytes and neutrophils, contributing to heightened cognitive decline [74]. These findings suggest that enhanced astrocytic activation, increased immune cell interactions, and exacerbated neuronal damage in female *APOE4* carriers may compromise their ability to mitigate A β plaque formation and neurodegeneration, thereby amplifying cognitive impairment. While the *APOE* genotype plays a direct role in AD pathogenesis, accumulating evidence indicates it may also modulate disease risk via indirect mechanisms, including interactions with the gut microbiota.

1.2. The Gut Microbiota

The gut microbiota is a symbiotic community of microbes that inhabit the gastrointestinal (GI) tract, composed of bacteria, fungi, archaea, and viruses that play a role in aiding digestion, and modulating and regulating the immune system [75]. The wide variety of microbes can produce enzymes not native to the host, which further metabolise the gut contents, producing smaller molecules. These gut-derived molecules are then used as substrates for further microbial metabolism [76] or, with a greater bioavailability to the host, pass through the gut epithelium and then into systemic circulation [77]. Throughout life, the composition of the colonic microbiota continuously changes. A baby is essentially sterile while in the mother's amniotic sac [78], but colonisation by microbes begins at birth, with the mode of delivery influencing which microbes are first introduced to the newborn [79]. From that point onward, the microbiota and its associated genome, the microbiome, begin to develop in accordance with an individual's specific niche. Each microbial species and subspecies contributes its unique genes and, consequently, distinct functions to the intestinal ecosystem [80]. This niche is shaped by biological factors such as age, sex, and genetics [81], as well as environmental and lifestyle factors, such as diet, sleep, exercise, and medication [80]. While the microbiota's composition is not fixed, healthy individuals generally maintain a balanced community of commensal bacteria within the digestive system. However, this balance is delicate and it shifts, driven by the same biological and environmental factors mentioned previously, which can disrupt the GI ecosystem and alter which bacteria are able to thrive.

1.2.1. Age and the Gut Microbiota

The gut microbiota undergoes dynamic changes throughout the lifespan, progressively adapting to the individual's colonic environment. Lifelong lifestyle behaviours play a pivotal role in modulating the microbial ecosystem in older adults [82]. In addition to cumulative life-history influences, ageing itself, characterised by progressive senescence, contributes to microbiota alterations. Immunosenescence, a hallmark of ageing, compromises host immune responses and is associated with an increased propensity for chronic low-grade inflammation [83]. This persistent inflammatory state may disrupt gut barrier integrity, thereby altering microbial colonisation patterns and facilitating the overgrowth of certain taxa [84].

Both the overall structure of the microbiota and individual groups of organisms are affected during ageing. Evidence indicates a general decline in alpha diversity with advancing age, irrespective of overall health status [85]. This decline encompasses reductions in both species' richness (total number of microbial taxa) and evenness (relative abundance distribution), ultimately leading to a microbiota dominated by a limited number of taxa; a hallmark of dysbiosis. Furthermore, ageing is associated with the depletion of specific health-associated genera such as *Bifidobacterium*, *Faecalibacterium*, *Blautia*, *Clostridium VI*, and *Clostridium XIVa* [86-88]. Concurrently, there is an expansion of facultative aerobic pathogens and potentially pro-inflammatory taxa, including *Staphylococcus*, *Streptococcus*, and *Enterobacteriaceae* [87] as well as other organisms such as *Prevotella*, *Roseburia*, and *Coprococcus* [88]. The reduction in beneficial short-chain fatty acid (SCFA) producing bacteria, coupled with an increase in opportunistic pathogenic organisms, reflects a shift toward an imbalanced and potentially unstable gut ecosystem in elderly individuals. While chronological age is a critical intrinsic factor influencing gut microbial diversity and composition, it does not act in isolation. Biological sex also exerts a significant influence, interacting with hormonal, immunological, and metabolic factors to shape the microbial ecosystem.

1.2.2. Sex and the Gut Microbiota

In recent years, biological sex has gained increasing interest as a determinant of gut microbiota composition. Both oestrogens and androgens, along with their intestinal metabolism, play a role in shaping the microbes within the colon [89]. However, sex hormones are not the sole drivers of this interaction. Studies employing mouse models have demonstrated that in the absence of sex hormones, chromosomal differences (XX or XY) alone are also able to change the host microbial interactions [90]. Furthermore, sex-based variations in immune function are well documented, with males exhibiting increased susceptibility to infections, whereas females have a higher predisposition to autoimmune disorders [91, 92]. Similar to the mechanism of ageing, the sex-specific differences in the immune response may modulate host microbiota interactions and potentially alter the microbiota's composition.

Sex-related differences in immune and inflammatory functions significantly influence the colonic environment, thereby affecting the composition of the gut microbiota within. Females typically exhibit stronger innate and adaptive immune responses than males, characterised by heightened antibody production and greater T-cell activation [93, 94]. The heightened immune vigilance in females is associated with a greater protection against certain infectious agents but is also associated with greater susceptibility to chronic inflammatory and autoimmune conditions [95]. These differences in immune response alter the intestinal niche, including epithelial permeability, mucosal immune signalling, and cytokine production, altering which microbes reside within the colon.

Conversely, the microbiota contributes to immunoregulation, suggesting a bidirectional relationship between sex-specific immune traits and the microbiota composition. For example, studies have demonstrated that sex hormones modulate the gut microbiota, which in turn modulate the immune responses [96]. Furthermore, the use of microbiota transplants in germ-free mice demonstrated that the transfer of female microbiota to male mice can alter both the gut microbiota composition and immune response [97]. Together, these interactions underscore the immune function as a plausible pathway linking sex to differences in microbiota composition.

Although the mechanisms underlying related differences in the gut microbiota are well explored, the functional and compositional outcomes remain incompletely understood. Most of the current evidence derives from animal studies, where female mice generally exhibit greater alpha diversity than their male counterparts [98, 99]. However, in humans, the research is less consistent, as most studies focus on sex differences in relation to diseases, which independently affect microbiota composition. Investigations involving healthy participants have yielded conflicting results, with some reporting differences in either microbial diversity or taxa abundance, while others observed no sex-based differences [100]. These discrepancies may be attributed to dietary differences, which impact the microbiota to a greater extent than sex alone. Therefore, further research is warranted to identify the extent to which an individual's sex and age influence microbial diversity and abundance. Alongside an individual's age and sex, the genetic makeup of the host is increasingly recognised as influencing the microbiota.

1.2.3. *APOE* and the Gut Microbiota

The *APOE* genotype is a notable genetic factor that influences the composition of the colonic microbiota. Individuals carrying the *APOE2* allele have been shown to exhibit increased relative abundances of *Ruminococcaceae*, *Lactobacilli* and *Clostridiales* compared to *APOE4* carriers in mouse models [101-103], with the *Ruminococcaceae* and *Clostridiales* results from preclinical models corroborated by human clinical studies [104]. Among these, *Clostridiales* demonstrated the highest prevalence in *APOE2* carriers, while *Prevotellaceae* levels were the lowest in individuals with the *APOE2/3* genotype [104]. A similar, albeit less pronounced, microbial profile was observed in *APOE3* homozygotes, who also harboured higher levels of *Roseburia* and *Clostridiales* than *APOE4* carriers [104]. Notably, elevated levels of the order *Clostridiales* in *APOE2* and *APOE3/3*, the family *Ruminococaceae* in *APOE2*, and the genus *Roseburia* *APOE3/3* genotypes may confer potential microbial benefits, as they are associated with better cognitive outcomes [105-108]. In contrast, preclinical models have found that *APOE4* genotypes are associated with increased abundances of *Deferribacter* and *Anaeroplasm* [103, 104], although these findings remain to be validated in human cohorts. *APOE4* carriers also exhibited the lowest levels of *Clostridiales*. Additionally,

sex-specific differences in the microbiota response to *APOE* genotype have been reported [103]. These overlapping patterns underscore the multifactorial influence of AD risk alleles on the gut microbiota. However, current understanding is limited, highlighting the need for further research in this area.

Beyond influencing individual microbial taxa, the *APOE* genotype also modulates the overall composition and structure of the microbiota. Mouse model studies have demonstrated a stepwise decline in alpha diversity across genotypes, from *APOE2/2* to *APOE3/3* to *APOE4/4*, indicative of a gradient-like phenotype. Heterozygous genotypes exhibit intermediate diversity levels, suggesting a codominant effect of each allele on microbial diversity [101]. Furthermore, significant differences in Unifrac distances have been observed between *APOE3/3* and *APOE4/4* genotypes [104]. These results suggest that *APOE*-associated alterations in the microbiota are not limited to changes in specific microbial populations, but rather involve broader shifts in the overall microbial community structure (Table 1.2).

Table 1.2: Summary findings from [104] Tran TTT, Corsini S, Kellingray L, Hegarty C, Le Gall G, Narbad A, et al. *APOE* genotype influences the gut microbiome structure and function in humans and mice: relevance for Alzheimer's disease pathophysiology. *FASEB J.* 2019;33(7):8221-31.[101] Zajac DJ, Green SJ, Johnson LA, Estus S. *APOE* genetics influence murine gut microbiome. *Scientific Reports.* 2022;12(1). [102] Parikh IJ, Estus JL, Zajac DJ, Malik M, Maldonado Weng J, Tai LM, et al. Murine Gut Microbiome Association With *APOE* Alleles. *Front Immunol.* 2020;11:200.[109] Cammann D, Lu Y, Cummings MJ, Zhang ML, Cue JM, Do J, et al. Genetic correlations between Alzheimer's disease and gut microbiome genera. *Scientific Reports.* 2023;13(1).

Model	Observation
<i>APOE</i>-TR Mouse model findings	- Differences in the UniFrac distance measurements between the <i>APOE3/3</i> and <i>APOE4/4</i> mice [104]. <i>Defferibacter</i> is notably higher in <i>APOE4/4</i> mice [104]. - Stepwise decrease in alpha diversity of the microbiota of male mice <i>APOE4/4</i> → <i>APOE3/4</i> → <i>APOE3/3</i> → <i>APOE2/3</i> → <i>APOE2/2</i> [101]. <i>Clostridiales</i> are higher in <i>APOE2</i> carrier mice [101].
EFAD (<i>APOE</i> x 5x<i>FAD</i>) mice	- Increased <i>Ruminococcaceae</i> and <i>Lactobacilli</i> in <i>APOE2/2</i> mice [102]. - Increased <i>Anaeroplasm</i> in <i>APOE4/4</i> [103]. - Increased <i>Prevotella</i>, <i>Sutterella</i>, and <i>Lactobaccillus</i> in <i>APOE3/3</i> Males than in <i>APOE4/4</i> Males [103]. <i>Ruminococcus</i> higher in <i>APOE3/3</i>-TR females than the <i>APOE4/4</i> females [103].
Healthy humans	- The phyla Firmicutes and order Clostridiales and <i>Ruminococcaceae</i> were significantly higher in <i>E2/E3</i> heterozygotes than in <i>E3</i> homozygotes and <i>E4</i> carriers [104]. <i>Prevotella</i> has a lower abundance in <i>APOE2/3</i> than any other group [104].

While it is well-established that the *APOE* genotype influences gut microbiota composition (Table 1.2), the underlying mechanisms remain poorly understood. Several hypotheses have been proposed to explain how *APOE* polymorphisms may mediate

these effects. One proposed mechanism involves bile acid metabolism. The synthesis of bile acids and the regulation of cholesterol homeostasis are known to depend on functional *APOE* (See Chapter 1.2. *APOE*) [41]. In particular, the hepatic enzyme cholesterol 7 α -hydroxylase (CYP7A1) plays a central role in converting cholesterol into primary bile acids, which are subsequently secreted into the duodenum [110]. It is hypothesised that genotype-dependent variations in *APOE* function may alter this biosynthetic pathway, thereby influencing bile acid profiles. Bile acids are essential not only for lipid absorption but also for shaping and maintaining a diverse gut microbiota [111, 112]. Bile acids not reabsorbed in the upper GI tract can reach the colon, where they undergo deconjugation by the resident microbiota, primarily members of the Firmicutes, Bacteroidetes, and Actinobacteria phyla, into secondary bile acids [113]. These bile salts serve dual roles as metabolic substrates and amphipathic detergents, significantly affecting microbial community structure. Thus, *APOE*-mediated alterations in bile acid synthesis may partially account for the observed microbiota differences across *APOE* genotypes. Furthermore, there is a sex-specific effect on bile acid metabolism. There are sex differences between males and females in the composition, circulation, and microbial metabolism, largely due to the interactions with sex hormones and the gut microbiota [114]. For example, oestrogens influence bile acid transporters and the bile acid synthesis enzymes [115]. Modulating the substrate bile acids released into the GI tract for metabolism by microbes. Again, these recurring patterns between *APOE* and sex indicate that AD risk factors have a multifaceted influence on the microbiota and provide a potential mechanistic route, too.

Another plausible mechanism involves modulation of the immune response. *APOE* genotype has been shown to influence inflammatory pathways, with the *APOE4* allele associated with a heightened pro-inflammatory state [46-48]. This genotype-specific inflammatory profile is further characterised by a reduction in anti-inflammatory SCFAs, likely due to lower abundances of SCFA-producing taxa such as *Roseburia* and *Clostridium* cluster IV [104]. Inflammation itself is a key determinant of gut microbiota composition, as it compromises gut barrier integrity and alters microbial viability and colonisation patterns [116, 117]. While these hypotheses offer compelling explanations, they remain speculative, and further studies are necessary to elucidate the extent to

which either mechanism drives *APOE*-associated microbiota variation. Nevertheless, consistent shifts in microbial composition have been reported across *APOE* genotypes, potentially indicating a transition toward dysbiosis. Such microbial imbalances have been implicated in a range of non-communicable diseases, including neurocognitive decline and AD [109, 118, 119].

1.2.4. The Gut Microbiota and Cognition

A well-established bidirectional relationship exists between the intestinal microbiota and the central nervous system, commonly referred to as the gut-brain axis (GBA). This intricate communication network integrates signals from the colon, liver, brain, and diet, and is mediated through diverse pathways, including hormonal, neuronal, immunological, and microbial metabolite-mediated mechanisms [106]. Notably, the bidirectional nature of the GBA enables mutual influence: physiological processes can alter gut microbiota composition, while microbial populations can, in turn, modulate systemic functions and health outcomes.

Emerging evidence indicates that cognitive function correlates with gut microbiota composition. In a cohort of Chinese adults, the genera *Odoribacter*, *Butyricimonas*, and *Bacteroides* were inversely associated with cognitive decline. Furthermore, individuals exhibiting preclinical markers of AD demonstrated distinct microbial profiles compared to cognitively healthy controls [120]. However, these differences were linked to biomarker expression (e.g., A β and HT pathology) rather than cognitive status per se [120]. AD patients also showed increased relative abundances of *Proteobacteria*, *Firmicutes*, and the family *Enterococcaceae* [121]. Beyond disease status, interactions between the *APOE* genotype, gut microbiota, and cognitive function have garnered attention. In an *APOE4/4*-TR tauopathy mouse model, animals with a depleted microbiota exhibited enhanced cognitive performance relative to those with an intact microbiota, a genotype-specific effect not observed in other *APOE* backgrounds [122]. These findings underscore both the essential role of the gut microbiota in neurocognitive maintenance and the genotype-dependent nature of this relationship. Specifically, *Clostridiales* were found to be significantly reduced in *APOE4* carriers [101], a microbial pattern also noted in

individuals with various psychiatric disorders and in AD patients [123, 124]. Such data suggest that the microbiota of *APOE4* carriers may shift toward a neurodegeneration-associated profile prior to clinical diagnosis. Additionally, the *APOE* genotype appears to influence microbiota composition in AD. Genera such as *Aldercreutzia*, *Eubacterium nodatum*, and *Prevotella* 9 have been associated with *APOE4* carriers [109], further highlighting the role of the host genotype in shaping gut microbial ecology, even during advanced stages of disease. Given the complexity of the GBA, it is crucial to consider the interplay of microbial, genetic, and systemic factors when investigating brain health.

1.3. Flavonoids

This section has been adapted from the review paper: Hunt T, Pontifex MG, Vauzour D. (Poly)phenols and brain health - beyond their antioxidant capacity. FEBS Lett. 2024;598(24):2949-62.[125]

As the primary source of essential macro- and micronutrients, diet plays a fundamental role in maintaining overall health. Emerging evidence indicates that dietary patterns can significantly influence brain health, thereby affecting cognitive function across the lifespan [126-128]. Numerous clinical trials, epidemiological studies, and meta-analyses have demonstrated that the consumption of (poly)phenols, particularly those derived from plant-based foods, is associated with positive outcomes for general health [129], as well as improved memory [130], reduced cognitive decline [131], and a lower risk of developing AD [132]. (Poly)phenols constitute a diverse class of secondary plant metabolites abundant in fruits, vegetables and a variety of plant-derived beverages, including tea, coffee, cocoa, and wine. In European populations, estimated daily (poly)phenol intake ranges from approximately 584 mg to 1786 mg per capita [133]. These compounds are broadly categorised into two main classes: flavonoids and non-flavonoids [134, 135]. Given the emphasis of this research on dietary flavonoids, the following review will primarily focus on this subclass of (poly)phenols.

1.3.1. Flavonoids Structures

Flavonoids contain a C6-C3-C6 structure and are subdivided based on their complexity into six further subcategories: flavan-3-ols, flavonols, isoflavones, anthocyanins, flavones and flavanones (Figure 1.3).

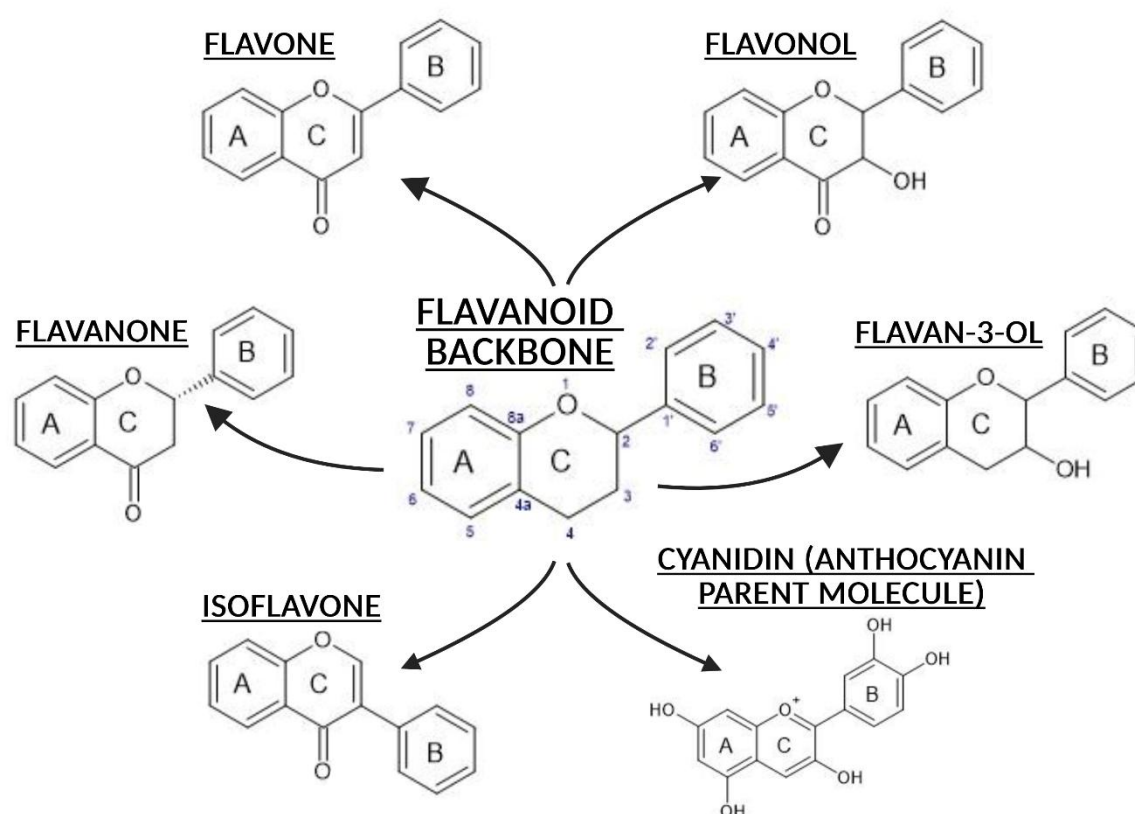


Figure 1.3 Breakdown of the members of the flavonoid family of molecules

The majority of flavonoids are conjugated to other molecular side chains, such as glycosides or glucose esters, tartaric acid and quinic acid [136]. There are, however, a few flavonoids which exist in their free form, such as epicatechin (EC) [137]. Before flavonoids are able to exert any bioactive effects, their bioavailability needs to be increased through catabolic metabolism.

1.3.2. Flavonoids and Brain Health

1.3.2.1. *Epidemiological evidence*

Amongst the most established mechanistic actions, flavonoids have been reported to reduce neuroinflammation, improve synaptic plasticity, increase cerebral blood flow (CBF), counteract mitochondrial dysfunction, and mitigate proteinopathies/excitotoxicity [138]. However, such processes vary depending on the flavonoid, dosage, and bioavailability [138]. Epidemiological studies investigating the impact of flavonoids on brain function have gained significant attention due to the growing interest in the potential benefits of these dietary bioactives for cognitive health and neuroprotection. These studies have utilised a variety of methodologies, including dietary assessment, cognitive tests, and longitudinal follow-ups. Cognitive benefits have been attributed to both specific flavonoids [139], as well as flavonoid-rich foods and beverages [140, 141]. Increasing the consumption of total flavonoids has been positively correlated with episodic memory in middle-aged adults [142], and an increased berry intake was reported to reduce cognitive decline in older adults aged 70 years and over [143]. Furthermore, cognitive decline was assessed using the mini-mental state examination (MMSE), and the clinical dementia rating significantly improved over a 4-5 year period, with daily consumption of green tea, but not those who consumed black tea or coffee [144]. Similarly, a cross-sectional study demonstrated that consumption of flavan-3-ol-rich foods, tea, chocolate and wine significantly improved cognitive performance in a dose-dependent manner in older people [145]. Furthermore, a study of 1,329 participants across three French cities through a twelve-year follow-up demonstrated that participants in the top 20% of polyphenol intake had a 50% lower risk of dementia (95% CI 20%; 68%, p -trend < 0.01) compared to lower consumers [146]. The Framingham Heart Study Offspring Cohort reported similar results, with higher chronic intake of flavonols, anthocyanins and flavonoid polymers associated with lower risks of AD and AD-related dementias in US adults [147]. Finally, a recent meta-analysis and systematic review of the field of observational studies reported that consumption of total flavonoids was inversely associated with cognitive impairment, the incidence of dementia, and related disorders (Relative Risk (RR) = 0.83, 95% CI: 0.76, 0.89). The most

benefit appears to come from anthocyanins (RR = 0.73, 95% CI: 0.60, 0.89), flavones (RR = 0.77, 95% CI: 0.63, 0.94), flavan-3-ols (RR = 0.86, 95% CI: 0.82, 0.91), and flavonols (RR = 0.88, 95% CI: 0.80, 0.96) [148], and these results mirror the findings of other meta-analyses [149].

Despite these cohorts providing an initial indication of cognitive benefits associated with the intake of a flavonoid-rich diet, their use of MMSE [150-152] and/or the AD Assessment Scale-Cognitive Subscale or Clinical Dementia Ratings [153] means that they only assess global cognitive status rather than a whole battery of cognitive tests, which could provide insight into the effects on specific aspects of memory. Additionally, epidemiological studies provide information on the potential benefits of flavonoid consumption on brain health; we must account for the limitations of these studies, such as the dietary assessment method used, other confounding factors, and retro-causality.

It is often the case that (poly)phenol intakes are self-reported and strongly influenced by the number and types of food included in food frequency questionnaires (FFQs). This is further confounded by the use of different reference databases by each FFQ, which may be limited in their estimation of (poly)phenol content in foods, affecting the estimates for participants' (poly)phenol consumption [154]. Further to this, the diversity of flavonoid structures (Figure 1.3) affects their bioavailability and metabolism, making it a challenge to establish causal relationships based solely on observational studies [134]. Notwithstanding these difficulties, the epidemiological evidence suggests an association between flavonoid intake, better cognitive outcomes, and a reduced risk of AD and other neurodegenerative conditions.

1.3.2.2. Clinical evidence

There has been significant interest in the outcomes of clinical intervention studies investigating the impacts of flavonoids on cognition. Many of these studies measure cognitive outcomes through various methods, both before and after the participants have taken a flavonoid-rich food, extract, or supplement. Numerous studies yielded promising results, suggesting the beneficial effects of flavonoid intake on cognition. For example, recent meta-analysis of randomised controlled trials (RCTs) investigating the impact of flavonoid consumption on cognition and comprising of 5,519 participants has

reported that foods rich in flavonoids, and in particular cocoa ($g = 0.224$, $p = 0.036$), ginkgo ($g = 0.187$, $p \leq 0.001$), and berries ($g = 0.149$, $p = 0.009$), significantly improve the effects on long-term memory, processing speeds, and mood in middle-aged and older adults [149]. Amongst these flavonoids, doses of flavan-3-ol greater than 520 mg/day over a period longer than eight weeks have yielded improvements in cognitive tasks. However, these studies only targeted a few cognitive domains [150, 155-157]. Conversely, a dose lower than 500 mg/day has been less effective at achieving neurocognitive benefits [158-162], although a study of young adults (22-24 years old) noted improvements in working memory and executive function at an intake of 500 mg/day [163]. Such results may be attributed to the longer supplement dose time of 2 months [163], whereas those unable to demonstrate such benefits had shortened supplementation times, between 5 and 30 days [158-162]. Although some of these studies were unable to demonstrate an effect on cognition, certain studies did demonstrate improved CBF parameters [160] or increased activation of the brain regions when performing the associated cognitive tasks (but no improvement in said task) using either steady-state probe topography [159] or functional magnetic resonance imaging [158, 164, 165], following flavan-3-ol supplementation.

It is essential to maintain the CBF to allow for the supply of nutrients and oxygen to the cells and for the removal of toxic waste products from cell metabolism. CBF has been demonstrated to gradually decline throughout ageing, and this decline is linked to neurocognitive decline in individuals with dementia [166]. Varying sources of flavonoids (cocoa, cranberry and epigallocatechin gallate) have been previously demonstrated to improve CBF [160, 167], and in turn ameliorate cognitive outcomes. For example, acute flavan-3-ol-rich cocoa intake (8.3 g per individual dose) reportedly enhanced cognitive function and increased blood oxygenation in the frontal cortical areas of young healthy subjects during hypercapnia [168]. Additionally, the consumption of high-flavan-3-ol chocolate (635 mg) was reported to enhance the efficiency of cognitive resource use and reduce the demands of brain activity during effortful tasks [169]. Furthermore, chronic consumption of flavan-3-ol-rich cranberries (*Vaccinium macrocarpon*) over a twelve-week period improved both episodic memory and regional brain perfusion in healthy older adults (50-80 years old) [167]. It is also worth noting that in a recent study of older

adults, a poorer-quality diet and lower habitual flavan-3-ol intake were associated with lower hippocampal-dependent memory, suggesting that low flavan-3-ol intake may be a driving force of cognitive ageing [170].

Higher doses (approximately 900 mg/day) for shorter time spans (6 weeks) improved neither episodic or recognition memory, nor executive function [171]. This indicates that a dose > 520 mg/day over a period of at least two months is needed to exhibit the beneficial cognitive effects in human trials [150, 155, 156, 163]. On the other hand, caution must be taken when extrapolating and interpreting the data from these clinical trials in the scientific literature as, to date, they mainly focus on object recognition memory, semantic memory, and executive function. Other hippocampal-dependent domains, such as short-term working memory and episodic memory, are known to be affected by age as well as neurodegenerative disease, and are rarely investigated.

These apparent inconsistencies in the benefits of flavonoid interventions may be due to differences in the baseline measurement across studies, with the majority conducted in healthy adults. Participants suffering from mild cognitive impairments (MCI), vascular dementia, or mild to moderate AD appear to show more pronounced benefits in their cognitive status following a flavonoid intervention for twelve weeks or longer (up to twenty-four weeks) [172-174]. Therefore, when conducting RCTs using flavonoids, the participants' cognitive status and overall phenotype, along with their dietary and lifestyle habits, must be considered. Consideration must be given to the use of high flavonoid intake as an exclusion criterion, as such individuals are not representative of the general population; a high baseline status is likely to result in a reduced response to the intervention. Finally, inconsistent and often imprecise descriptions of the types of memory assessed, as well as a general preference to test them individually rather than as a panel of cognitive tasks, are likely to add to potential inaccuracies in the interpretation of study results and to inter-study inconsistencies. Some key aspects of memory, which are particularly impaired by ageing and in pathological conditions such as AD and MCI, are particularly underrepresented or remain completely unexplored [175]. Results which use terms such as “neurocognitive benefits”, “improves memory function”, or “rescues cognitive benefits” are often based on too few cognitive tests. A battery of tests would provide a better understanding of general cognitive functioning in

response to intervention. Overall, human intervention studies provide strong evidence for the potential benefits of dietary flavonoid consumption. Nevertheless, it is crucial to bear in mind that further research is needed to fully understand the mechanisms underlying these effects and to determine the optimal doses and sources of flavonoids for better cognitive health.

1.3.3. Flavonoids Metabolism: Focus on Flavan-3-ols

As this study primarily focuses on flavan-3-ols, particular attention is given here to their metabolic pathways. The biotransformation of monomeric flavan-3-ols has been extensively characterised, with EC serving as a representative compound to illustrate the nature, timing, and progression of metabolite production. Following oral ingestion, EC exhibits relatively low absorption in the upper GI tract, with approximately 30% taken up in the small intestine [176]. The absorbed fraction is transported to the liver, where it undergoes Phase II metabolism, resulting in the formation of bioavailable derivatives of EC modified through sulfation, methylation, and glucuronidation [177]. Studies employing radiolabelled (^{14}C) EC have demonstrated that these conjugates appear within the first 4 hours post-consumption [177].

The remaining unabsorbed EC reaches the colon, where it undergoes microbial catabolism [178]. Microbial enzymes initiate the degradation by reducing the C-ring of the flavan-3-ol structure [179], leading to the cleavage of the central 3-carbon ring and formation of (hydroxyphenyl)-propan-2-ol intermediates [180]. The initial metabolite formed is 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (3,4-DiHPP-2-ol), which can subsequently undergo site-specific dehydroxylation at either the 3' or 4' positions to yield various mono-hydroxyphenyl analogues [180-182]. These intermediates undergo further microbial transformations, including A-ring cleavage and lactone formation, producing γ -valerolactones, primarily 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (3,4-DiHPVL) and their mono-hydroxyphenyl counterparts [180-182]. These γ -valerolactones may subsequently undergo ring-opening to generate 5-(3',4'-dihydroxyphenyl)valeric acid (3,4-DiHPVA) and their respective 3' and 4' mono-hydroxyphenyl valeric acid derivatives. Further oxidation of these valeric acids yields a

variety of phenyl-organic and benzoic acids [177, 180, 181, 183] (Figure 1.4). Radiolabelled tracer studies indicate that the formation of γ -valerolactones and valeric acids occurs predominantly within 4–12 hours after EC ingestion [177]. In contrast, polymeric flavan-3-ols undergo depolymerisation prior to C-ring cleavage, and their metabolic pathways display variable hydroxylation patterns at each stage of degradation [184]. The formation of γ -valerolactones and valeric acids is characteristic of flavan-3-ol microbial metabolism. However, due to structural heterogeneity across flavonoid subclasses, the profile of resulting metabolites is specific to the parent compound; for instance, anthocyanins are predominantly degraded to protocatechuic acid [185, 186]. These lower-molecular-weight catabolites serve as substrates for subsequent bacterial metabolism and exhibit enhanced bioactivity, with microbially derived metabolites appearing in the plasma at much higher concentrations than their parent compounds [137], potentially influencing host physiology both locally and systemically. Crucially, before exerting their bioactive effects, flavonoids must first undergo catabolism to increase their bioavailability.

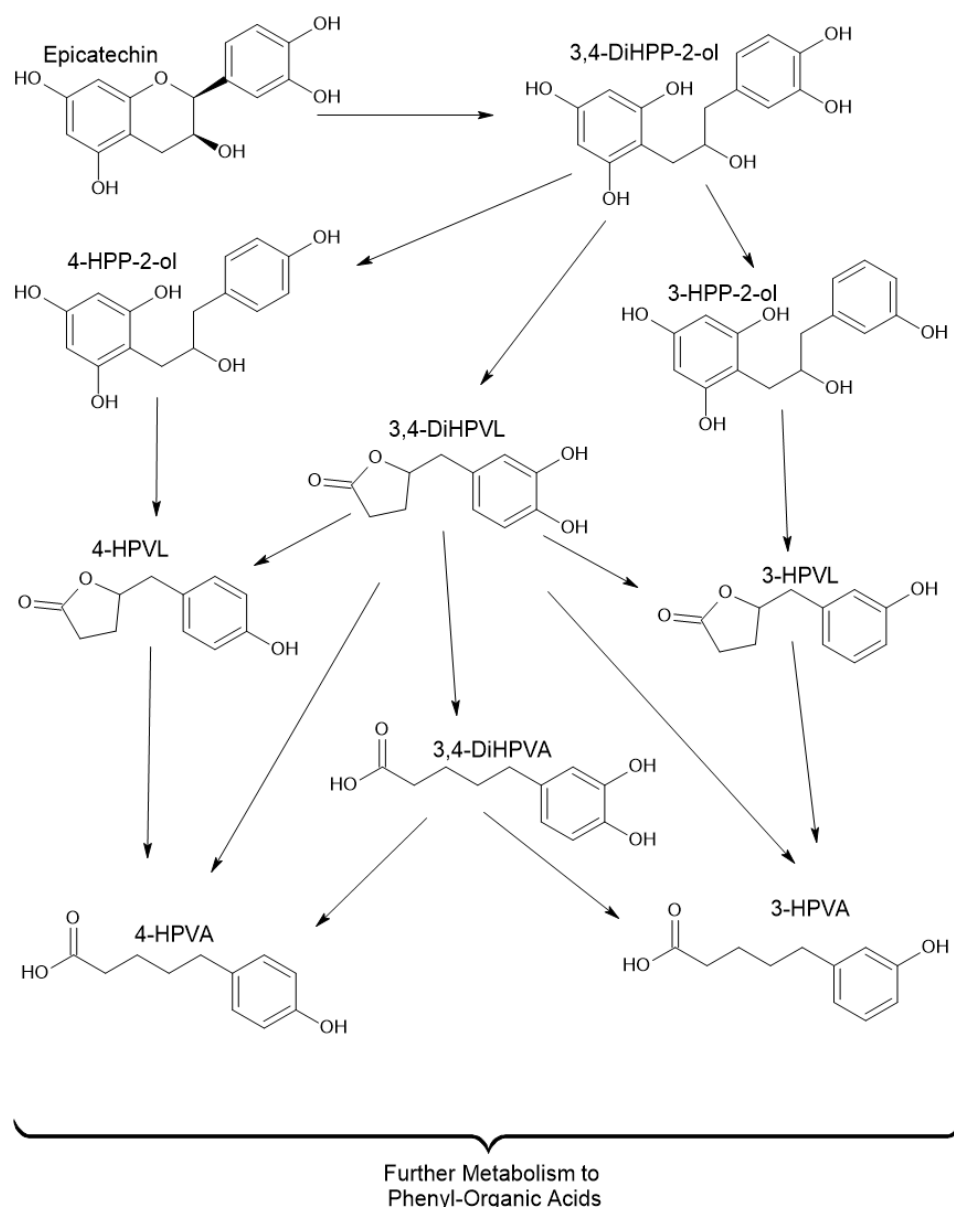


Figure 1.4 Microbial metabolic pathway of Epicatechin, to 1-(3',4'-dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (3,4-DiHPP-2-ol), 1-(3'-hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (3-HPP-2-ol), 1-(4'-hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol (4-HPP-2-ol) through opening of the C Carbon ring, then to 5-(3',4'-dihydroxyphenyl)- γ -valerolactone (3,4-DiHVL), 5-(3'-hydroxyphenyl)- γ -valerolactone (3-HPVL), or 5-(4'-hydroxyphenyl)- γ -valerolactone (4-HPVL) through the opening of the A Carbon ring as well as the attachment of the lactone group, these are then oxidated to form the 5-(3',4'-dihydroxyphenyl)valeric acid (3,4-DiHPVA), 5-(3'-hydroxyphenyl)valeric acid (3-HPVA) or 5-(4'-hydroxyphenyl)valeric acid (4-HPVA), which are then further metabolized to produce other phenyl-organic acids.

1.3.4. Flavan-3-ols and the Gut Microbiota

The presence of flavan-3-ols in the colon exerts several modulatory effects on the gut microbiota. Due to the lack of endogenous enzymatic capacity in humans to degrade dietary flavonoids, their catabolism is entirely dependent on microbial metabolism.

Distinct microbial species and subspecies are responsible for specific stages of flavan-3-ol biotransformation, utilising these compounds as carbon sources (Table 1.3). However, not all the microbial taxa involved in this metabolic pathway have yet been fully elucidated. Given the structural diversity of flavonoids, the microbial consortia involved in their metabolism vary across flavonoid subclasses; here, the focus is restricted to those mediating flavan-3-ol degradation. The availability of a compound that serves as a utilisable carbon source confers a selective growth advantage to microbes capable of its metabolism, promoting their proliferation relative to non-utilising taxa.

Table 1.3 A summary of the microbes involved in each step of microbial flavan-3-ol metabolism in the colon from the papers [181] Lessard-Lord J, Roussel C, Guay V, Desjardins Y. Characterisation of the Interindividual Variability Associated with the Microbial Metabolism of (-)-Epicatechin. *J Agric Food Chem.* 2023;71(37):13814-27.165), [187] Takagaki A, Nanjo F. Bioconversion of (-)-Epicatechin, (+)-Epicatechin, (-)-Catechin, and (+)-Catechin by (-)-Epigallocatechin-Metabolizing Bacteria. *Biological and Pharmaceutical Bulletin.* 2015;38(5):789-94., and [188] Kutschera M, Engst W, Blaut M, Braune A. Isolation of catechin-converting human intestinal bacteria. *Journal of Applied Microbiology.* 2011;111(1):165-75.

Flavan-3-ol Metabolism Step	Bacteria Associated with Metabolic Step
C-ring Cleavage	<i>Eggerthella lenta</i> , <i>Adlcreutzia equolifaciens</i> , <i>Lactiplantibacillus plantarum</i> , <i>Slackia equolifaciens</i> , <i>Slackia isoflavoniconvertens</i> [181, 187, 188]
Dehydroxylation of Hydroxyphenylpropan-2-ols	<i>Eggerthella lenta</i> , <i>Lachnospiraceae</i> UCG-010 spp. [181]
B-ring Cleavage and lactone addition	<i>Flavonifractor plautii</i> , <i>Aldercreutzia equolifaciens</i> , <i>Anaerovoracaceae</i> sp., <i>Eubacterium brachy</i> group spp., <i>Anaerotruncus</i> spp. [181, 187, 188]
Production of the phenyl-organic acids	<i>Flavonifractor plautii</i> [188]

Beyond serving as substrates, flavan-3-ols also modulate gut microbiota composition through additional mechanisms. For example, these compounds have been shown to enhance the colonisation of beneficial microbial genera such as *Lactobacillus*, *Bifidobacterium*, *Faecalibacterium*, and *Akkermansia*, while concurrently suppressing the growth of potentially pathogenic taxa [189, 190]. These microbial shifts are mediated by the bioactivity of flavan-3-ols and their derived metabolites, which exert direct antimicrobial effects. Flavonoid compounds are known to disrupt microbial viability

through interference with nucleic acid and protein synthesis, enzymatic activity, cell wall integrity, biofilm formation, and nutrient acquisition [191-193].

In addition to these direct antimicrobial actions, flavan-3-ol metabolites can also indirectly influence gut microbiota dynamics. Low-molecular-weight metabolites generated via microbial catabolism can traverse the colonic epithelium and elicit local physiological responses, including enhanced barrier integrity and attenuation of intestinal inflammation. For instance, phenylpropanoic acid derivatives have been shown to modulate proinflammatory cytokine expression [185, 194]. These alterations in inflammatory status can affect colonic environmental parameters such as oxygen tension, nutrient gradients, and pH, which subsequently shape microbial ecological niches [195].

Moreover, flavonoid metabolites can enter the systemic circulation and impact hepatic function. In the liver, they have been shown to modulate key signalling pathways, including Liver X receptor alpha (LXR α), nuclear factor kappa B (NF- κ B), and extracellular signal-regulated kinase 1/2 (ERK1/2), thereby influencing the expression of CYP7A1, a rate-limiting enzyme in bile acid synthesis [196]. Changes in bile acid composition alter the chemical environment of the colon by modifying the metabolism of primary to secondary bile acids and influencing microbial community structure through detergent-like effects. This bile acid-mediated modulation of the gut microbiota mirrors one of the proposed mechanisms by which *APOE* genotype also influences microbial composition. While both flavonoid intake and *APOE* genotype have independently been shown to affect the gut microbiota, there is currently a lack of research exploring their potential interactive effects.

In addition to modulating gut microbial communities, dietary flavonoid metabolites are increasingly recognised for their systemic effects, particularly in relation to brain health. Growing evidence over recent decades has highlighted the potential neuroprotective role of flavonoids in the context of neurodegenerative diseases, including AD.

1.3.5. Flavonoids as antioxidants or signalling molecules?

Flavonoids have been posited as antioxidant agents due to their electron-donor capacity from the phenolic groups [197, 198]. However, their plasma concentration is far lower

than endogenous antioxidant molecules (e.g. glutathione, vitamin C and E, etc.). Instead, it has been demonstrated that their protective activities could derive from an indirect modulation of cellular signalling pathways that mediate cell function in both physiological and pathological conditions [135, 199, 200]. For example, flavonoids and their metabolites can stimulate the antioxidant response element (ARE) that encodes cytoprotective proteins such as antioxidant enzymes (AOE). The activation of ARE is induced by the binding with Nuclear factor erythroid 2-related factor 2 (Nrf2), which is promoted by (poly)phenols and their stimulation of the ERK 1/2 and protein kinase C (PKC) [201]. In addition, flavonoids have also been associated with the activation of ERK 1/2 and subsequent cyclic AMP response element-binding protein (CREB) activation, which induce the nuclear transcription of brain-derived neurotrophic factor (BDNF) [202-204]. BDNF is an important factor that stimulates protein synthesis in the brain and synaptic plasticity, thus influencing learning and memory formation [205]. Flavonoids not only induce the expression of AOE and neurotrophic factors but also reduce the synthesis of pro-inflammatory cytokines through the inhibition of NF- κ B [206]. NF- κ B can also be inhibited via the indirect activation of the protein sirtuin-1 (SIRT1) by flavonoids through phosphodiesterase (PDE) enzymes. Furthermore, flavonoids are able to modulate the p38 mitogen-activated protein kinase (p38 MAPK) and Jun amino-terminal kinases (JNK), two key protein kinases which regulate the expression of the tumour necrosis factor α (TNF- α), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) [207-209]. With these mechanisms, flavonoids block the production of reactive oxygen species (ROS), interleukin 1 β (IL-1 β), and nitric oxide (NO), thereby reducing the neuroinflammatory pathways by modulating the inflammatory response of microglia within the brain (Figure 1.5).

Intriguingly, as well as downregulating iNOS, flavonoids also upregulate the expression of endothelial nitric oxide synthase (eNOS), causing a local increase in NO within the endothelial tissue of the brain's vasculature. The local increase in NO leads to increased vasodilation and, thus, improved CBF within the brain [210]. Maintaining CBF is essential to allow the supply of nutrients and oxygen to cells and remove toxic waste products produced during cell metabolism; CBF gradually declines as an individual ages and is linked to neurocognitive decline and a marked decline in dementia patients [166]. Varying

flavonoid sources (cocoa, cranberry and epigallocatechin gallate) have been demonstrated to increase CBF [130, 160], indicating why many dietary flavonoids are associated with improved cognitive outcomes. The simultaneous increased NO synthesis from eNOS and downregulation of NO synthesis from iNOS suggest an ideal concentration or location for the NO to improve cognition. It also demonstrates the multifaceted and complex mechanisms of flavonoids and their associated metabolites on cognition (Figure 1.5).

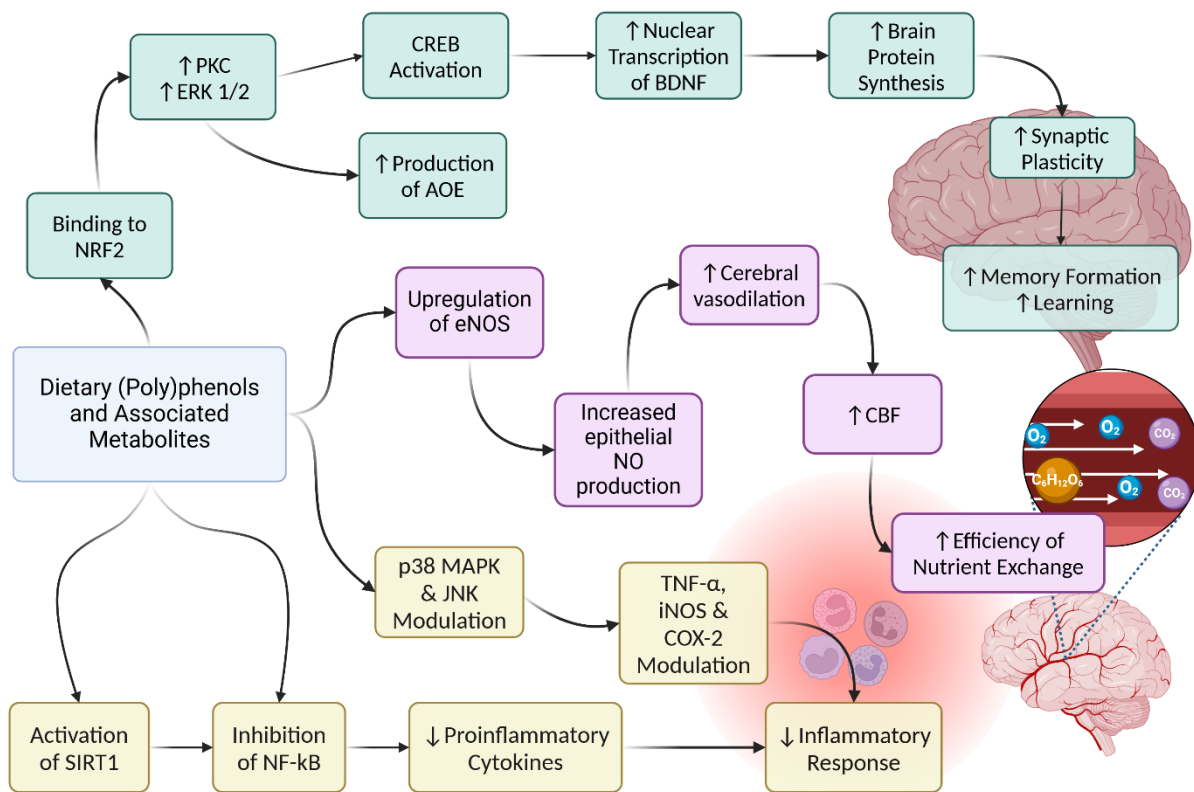


Figure 1.5 Figure licensed from Hunt T, Pontifex MG, Vauzour D. (Poly)phenols and brain health - beyond their antioxidant capacity. *FEBS Lett.* 2024;598(24):2949-62. Mechanisms of dietary (poly)phenols and their metabolites on the brain and the immune response proposed for their observed effects on systemic health. The antioxidant response mechanism shows increased protein kinase C (PKC) and extracellular signal-regulated kinase 1/2 (ERK 1/2) stimulation by binding to NRF2 and subsequent activation of antioxidant enzymes (AOE). Increased ERK 1/2 activation and CREB activation induce the nuclear transcription of the brain-derived neurotrophic factor (BDNF), increasing protein synthesis in the brain and synaptic plasticity, improving learning and memory formation. (Poly)phenols also reduce the synthesis of pro-inflammatory cytokines by inhibiting nuclear factor κ B (NF- κ B) directly and indirectly by inhibiting protein sirtuin-1 (SIRT1). (Poly)phenols also modulate p38 mitogen-activated protein kinase (p38 MAPK) and Jun amino-terminal kinases (JNK). These two kinases regulate the expression of tumour necrosis factor α (TNF- α), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2).

1.4. Interindividual variability in flavonoid metabolism

One of the major challenges in elucidating and harnessing the neuroprotective effects of flavonoids lies in the substantial interindividual variability in response to flavonoid interventions. This variability is largely attributed to differences in their bioavailability and metabolism [211, 212], which are influenced by a range of factors including intrinsic characteristics (such as sex, age, and ethnicity), lifestyle behaviours (diet, smoking, physical activity), (patho)physiological status, genetic background (particularly the *APOE* genotype), and the composition and metabolic activity of the gut microbiota [213, 214]. These variables influence key biological processes governing the absorption, distribution, metabolism, and excretion (ADME) of flavonoids, ultimately affecting their systemic levels and profiles of metabolites. Recent research has identified distinct metabolic phenotypes, or “metabotypes”, characterised by individual differences in

polyphenol-related ADME patterns, as reflected in urinary metabolite profiles [215-217]. Such metabotyping strategies offer a promising approach for accounting for ADME variability, thereby advancing our understanding of the mechanisms underlying individual differences in flavonoid responsiveness and informing the development of stratified or personalised interventions aimed at mitigating neurocognitive decline [218].

1.4.1. Age and Sex Differences in Flavonoid Metabolism

Age is the greatest known risk factor for the development of AD and plays a significant role in shaping the composition of the gut microbiota; however, its impact on flavonoid metabolism remains underexplored. While the metabolism of cocoa-derived flavan-3-ols in the upper gastrointestinal tract has been studied, age appears to exert minimal influence on the production of structurally related epicatechin metabolites in Caucasian males [219]. Notably, these metabolites are formed prior to colonic microbial involvement, leaving the role of age in gut microbiota-mediated metabolism unexamined. To date, no studies have assessed how age influences the metabolism or excretion of any gut-derived flavonoid metabolites, a clear gap that warrants further investigation.

Sex also influences microbiota composition, and existing studies have demonstrated sex-based differences in microbial production of SCFAs, with males typically exhibiting higher production than females [220]. However, the role of sex in the gut microbial metabolism of dietary flavonoids remains unexplored, representing another important area for future research.

1.4.2. *APOE* Genotype in Flavonoid Metabolism

APOE is the most significant genetic risk factor for the development of AD and has also been shown to influence the composition and structure of the gut microbiota. *APOE* function plays a critical role in modulating the gut-derived metabolome. For instance, *APOE* $-/-$ mice exhibit reduced production of gut-derived amino acids compared to mice expressing murine *APOE* [221]. Despite these findings, there is currently no research

investigating how *APOE* affects the metabolism of dietary flavonoids, nor whether different *APOE* genotypes lead to variations in flavonoid metabolism by the gut microbiota, representing a key knowledge gap.

In addition to the *APOE* genotype, other established AD risk factors, namely age and sex, also shape the composition of the gut microbiota. Given that flavonoid metabolism largely depends on colonic microbial populations, understanding how each of these factors (age, sex, and *APOE* genotype) independently and interactively influences flavonoid metabolism is essential. However, no studies to date have explored the relative contributions of these variables to the microbiota-mediated metabolism of flavonoids. Furthermore, it remains unclear at which stage of ADME these factors exert their influence. These gaps underscore the need for comprehensive research to delineate both individual and combined effects of these risk factors on flavonoid metabolism.

1.5. Conclusion and Research Gaps

As the global population continues to expand and age, there is an increasing need to address age-related disorders, including AD and associated dementias. Three primary risk factors for AD have been identified: age, *APOE* genotype, and sex. Following age, the *APOE* genotype represents the most significant non-modifiable risk factor for AD. Polymorphisms in *APOE* are implicated in key physiological pathways associated with AD pathogenesis, including lipid transport and inflammation, with differential effects observed across age and sex groups, particularly increasing risk among women and older individuals.

Beyond its systemic physiological roles, *APOE* has also been identified as a modulator of gut microbial composition, although the mechanisms underlying this interaction remain poorly understood. Age and sex likewise influence the structure and function of the gut microbiota, yet the specific contributions of sex to these changes in humans remain underexplored. Moreover, the individual and combined effects of age, sex, and *APOE* genotype on the gut microbiota and their potential roles in AD pathogenesis have not been systematically investigated.

One component of the GBA receiving increasing attention is the influence of dietary flavonoids, a diverse group of plant-derived (poly)phenols linked to improved cognitive function. Flavonoid metabolism is largely dependent on microbial activity in the colon, with distinct microbial taxa responsible for sequential catabolic steps. While interindividual variability in flavonoid metabolism is recognised, the extent to which age, sex, and *APOE* genotype, via their effects on the gut microbiota, modulate their metabolism and related health outcomes remains unknown.

While purified flavonoids provide an essential mechanistic framework for investigating metabolic pathways, they do not fully reflect the habitual dietary intake, as these compounds are rarely consumed in isolation. Instead, flavonoids are typically ingested as parts of complex food matrices containing macronutrients, dietary fibres, and other (poly)phenolic compounds. All of these factors may also influence the bioaccessibility, microbial fermentation and subsequent physiological effects. Investigation of both isolated EC as well as flavonoid-rich whole foods is therefore necessary to distinguish controlled mechanisms from those arising within realistic dietary contexts, and to better understand how host factors such as *APOE* genotype, sex, and age modulate these processes.

This thesis was developed to address the following key gaps:

1. To what extent do age, sex, and *APOE* genotype influence the colonic metabolism of the dietary flavan-3-ol (–)-epicatechin?
2. Are observed changes in EC metabolism attributable to differences in gut microbial composition?
3. Does *APOE* genotype modulate the health outcomes associated with dietary EC?
4. Does *APOE* genotype alter the metabolism of flavonoid-rich foods, and is this effect microbiota-mediated?

Collectively, these research questions aim to elucidate how non-modifiable AD risk factors interact with the gut microbiota to influence the metabolism and efficacy of dietary flavonoids. Insights gained will advance understanding of the GBA and inform the

development of personalised nutrition strategies for the prevention or mitigation of cognitive decline, particularly in high-risk populations.

Chapter 2: The Role of *APOE* Genotype, Age, and Sex in the Metabolism of Dietary Epicatechin

2.1. Introduction

The *APOE* genotype is the second greatest risk factor for the development of AD, after ageing [12]. It has been implicated in modulating the gut microbiota composition, with evidence showing that microbiota beta diversity varies according to *APOE* genotype [101]. Specifically, the *APOE4* allele is associated with reduced abundance of certain taxa, including *Clostridiales* and *Ruminococcaceae* [104]. Dietary flavonoids are bioactive compounds that have been shown to improve cognitive outcomes under both acute and chronic conditions [157, 167]. The bioavailability of flavonoids depends on their metabolism by the gut microbiota, which produces exogenous enzymes necessary for their transformation and subsequent absorption across the colonic epithelium [178].

Given the observed influence of *APOE* genotype on gut microbiota structure, it is hypothesised that the microbial metabolism of dietary flavonoids, such as EC, is also genotype dependent. Furthermore, since sex and age are well-documented modulators of the gut microbiota composition and function [222, 223], this study additionally aims to investigate their roles in EC metabolism. Accordingly, this chapter will test the hypothesis that the *APOE* genotype significantly affects the microbial metabolism of EC, and that this process is further modulated by biological sex and age.

To address this, two complementary studies were employed. The first employs an *in vitro* human colon model, using faecal samples obtained from prospectively *APOE* genotyped individuals, to examine EC fermentation under controlled conditions. The second study utilises an *APOE*-TR mouse model to trace EC metabolism from colonic biotransformation through systemic circulation to urinary excretion. EC was selected as the model flavonoid due to its partial absorption, with approximately 70% reaching the colon, where it undergoes microbial metabolism before being absorbed in the large bowel. The metabolic fate of EC is well established and studied [177], facilitating the identification of its major metabolites at various stages of the metabolism.

Interindividual variability in the metabolism of dietary flavonoids is influenced by numerous host and microbial factors [224]. Therefore, to comprehensively evaluate the impact of *APOE* genotype on EC metabolism, the contributions of age and sex, both of which are linked to AD risk, as well as their effects on the microbiota, must be considered. Together, these studies aim to elucidate how *APOE* genotype, sex, and age collectively shape the microbial metabolism of EC.

2.2. Materials and Methods

2.2.1. Human Colon Model Development

2.2.1.1. *Human Stool Collection*

Human stool sample donors were recruited from the Quadram Institute Bioscience (QIB) colon model study. Adult men and women who work/live within ten miles of Norwich Research Park were recruited to this study if they fulfilled the following criteria: not diagnosed with chronic GI health conditions, such as inflammatory bowel disease, irritable bowel syndrome or coeliac disease; regularly defecated between thrice daily and thrice weekly and produced an average stool between 3 and 5 on the Bristol Stool Chart. Prior to donation, participants were requested to confirm that they had not taken antibiotics within the last 4 weeks, had not experienced gastrointestinal complaints (such as vomiting or diarrhoea) within the 72 hours preceding donation, were not currently breastfeeding, or had not recently undergone an operation under general anaesthesia. QIB Human Research Governance Committee (IFR01/2025) and London Westminster Research Ethics Committee (15/LO/2169) approved the study. Informed consent was obtained from all participating subjects, and the trial is registered at <http://www.clinicaltrials.gov> (NCT02653001). Due to the ethical setup of this study, aside from the participants' study numbers, no further information on the participants was available to the researchers.

2.2.1.2. *Human Stool Processing*

Upon the receipt of the samples, the stools were processed within three hours to preserve the microbial communities. Stools were mixed 1:1 with deoxygenated phosphate-buffered saline (PBS) (0.01 M deoxygenated with sterile N₂ overnight). The faeces and PBS mixture were homogenised for 45 seconds at 230 rpm in a Stomacher

400 (Seward, UK). The homogenised faecal slurry was then mixed 3:1 with sterile glycerol to produce a 25% glycerol faecal stock. To produce a sterile control, an aliquot of the faecal stock was autoclaved to kill microbes within the sample. Both the faecal glycerol stock and the sterile faecal glycerol stock were frozen at -80 °C until use.

2.2.1.3. Colon Model

An *in vitro* high-throughput colon model was utilised to measure the EC metabolism. All reagents in the media generation were purchased from Sigma (Sigma, Gillingham, UK) unless otherwise stated. Media was made up in two parts: Part A) 2g peptone water, 2g yeast extract, 0.1 g NaCl, 0.04 g Na₂HPO₄, 3.978 g K₂HPO₄, 3.697 g KH₂PO₄, 0.01 g MgSO₄·7H₂O, 0.01 g CaCl₂·6H₂O, 2 g NaHCO₃, 0.05 g hemin, 0.5 g bile salts, 2 mL Tween80, 10 µL Vitamin K1. This was diluted to 500 mL with 18 MΩ cm⁻¹ Milli-Q water. Part B) was made up in the same way as Part A) with the addition of 0.001 g resazurin and 0.5g L-cysteine; this was then autoclaved to sterilise. Once sterilised, the two parts were combined, and the pH was adjusted to 6.8 before one final filter sterilisation. EC (BLD Pharm, Shanghai) was diluted to 9 mM in a 1:1 mixture of methanol and MilliQ water. This was aliquoted and stored at -80 °C until use.

The night before inoculation, the media was deoxygenated with sterile N₂ overnight. All inoculations occurred in a deep 1.1 mL 96-well plate (Bel-Art, USA) and took place in an anaerobic cabinet (CO₂:H₂:N₂) with the temperature maintained at 37 °C. All wells had 794 µL of deoxygenated media combined with 10 µL of the 9 mM EC stock. This was then inoculated with 96 µL of the glycerol faecal stock or the sterile glycerol faecal stock. Each well was homogenised by pipetting three times before sealing the plate with a foil plate seal to prevent media evaporation and contamination from within the anaerobic cabinet. This produced a final volume of 900 µL in each well, a final EC concentration of 100 µM and a final faecal concentration of 4%. This faecal concentration was higher than previously published literature, with the aim of increasing microbe concentration and thereby the rate of EC metabolism. Each participant's faecal glycerol stock and sterile faecal glycerol stock were inoculated into wells in triplicate.

Wells were mixed by repeatedly pipetting the well three times before 50 µL of the sample was taken at the following hourly time points between 0 and 6 hours, and then a final time

point at 24 hours. Each time the fermentation wells were opened, a fresh foil seal was placed over the wells to prevent cross-contamination. Once collected, all samples were immediately transferred to -80 °C to halt the fermentation.

2.2.1.4. pH Change Measurement

The colon model was repeated as above in parallel with the samples for metabolite analysis, using only the bacterial samples, which were measured for changes in pH. The donor's samples were repeated in triplicate as above. Wells were mixed by repeatedly pipetting the well three times before 75 µL of sample was collected at each of the time points outlined above. Wells were resealed between the sampling periods. A larger volume was taken to ensure there was enough sample to use with the microprobe. Samples were transferred into sterile Eppendorf tubes and removed from the anaerobic cabinet, and the pH was measured immediately.

2.2.1.5. Metabolite Extraction and UPLC-MS

Samples were prepared following the method outlined by Di Pede et al. [182], with slight modifications. Due to the smaller sample volume compared to the Di Pede method, the volumes of acidified methanol (0.1% formic acid (FA)) were adjusted to maintain the same solvent-to-sample ratio as in the published protocol. The final volume of the diluted sample was 330 µL. The initial volume of methanol was added to the sample immediately from the -80 °C freezer, before thawing, to kill microbes and prevent further metabolic activity. Once the initial volume of acidified methanol was added, the sample was allowed to thaw before further processing. All subsequent vortexing, sonication, and centrifugation steps were followed as per the published protocol. Following extraction, the samples were returned to -80 °C for storage before analysis.

Calibration curves were generated and matrix-matched to the colon model samples. Media and faecal samples were prepared as outlined above. Instead of inoculating the samples with a 9 mM solution of EC (1:1 methanol:water), a blank solution of 1:1 methanol and water was added to ensure the same final volume as the samples. This matrix was then aliquoted into the same volumes as the samples and frozen at -80 °C before the calibration curves were generated.

Metabolite extraction from the calibration curves was processed in the same way as the samples above. However, the first methanol that was added contained a master mix of EC, 3,4-DiHPVL, and 5-(3'-hydroxyphenyl)- γ -valerolactone (3-HPVL). These were then further processed as per the adapted Di Pede method above. This produced a calibration range of 0 - 15 μ M, matching the maximum EC concentration observed in the samples. Calibration curves were frozen at -80 °C prior to analysis. An internal standard (IS) of 10 μ L of a 10 μ g/mL solution of taxifolin was added to all samples and calibration curve samples prior to analysis. This internal standard was selected due to its molecular similarity to EC and its subsequent metabolites. Samples were vortexed and transferred to Whatman uniprep syringeless (Whatman, UK) 0.45 μ m HDPE filter tubes. Before injection, samples were retained at 4 °C.

Chromatographic separation was performed using a Waters Acquity ultra-performance liquid chromatography coupled with tandem mass spectrometry (UPLC-MS/MS) system equipped with a Waters HSS T3 column (Waters, UK) with the following dimensions 100 mm x 1.2 mm, 1.7 μ m particle size. The mobile phase consisted of two parts A) LCMS grade water (0.1% FA) and B) LCMS grade acetonitrile (0.1% FA), delivered at a flow rate of 0.4 mL/min. The column temperature was maintained at 35 °C. The following gradient programme was used (Table 2.1).

Table 2.1: Solvent gradient for UPLC-MS analysis. Solvent A (H₂O 0.1% formic acid) and Solvent B (Acetonitrile 0.1% formic acid)

Time (min)	%A	%B
0.00	97	3
12.00	80	20
16.00	5	95
16.10	97	3

The total run time was twenty minutes. The injection volume was 5 μ L, and samples were kept at 10 °C during the analysis. The autosampler was operated in partial-loop mode with needle overfill, and the needle washes were performed using 600 μ L of weak wash and 200 μ L of strong wash solvents.

Mass Spectrometry was performed on a Waters Xevo TQ mass spectrometer operated in both positive and negative electrospray ionisation source (ESI) modes using multiple reaction monitoring (MRM). Nitrogen was used as the desolvation and cone gas. Dwell

time for each transition was 0.003 s, with inter-channel and inter-scan delays of 0.02 s. Transitions were optimised for the EC, 3-HPVL and 3,4-DiHPVL in the calibration curves (Table 2.2).

Table 2.2: Chromatographic and spectrometric properties of parent Epicatechin (EC) and the monitored gut microbiota-derived catabolites 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (3,4-DiHPVL) and 5-(3'-hydroxyphenyl)- γ -valerolactone (3-HPVL) measured against standards. RT (Retention Time), m/z (mass to charge ratio), CE (Collision Energy), CV (Collision Voltage).

Name	Ion Mode	Precursor (m/z)	Product (m/z)	CV	CE	RT	Range
EC	ES+	290.877	139.05	20	16	9.18	8.93 - 9.43
3,4-DiHPVL	ES+	207.060	58.880	40	24	9.38	9.13 - 9.63
3-HPVL	ES-	191.000	106.000	20	30	12.75	12.50 - 13.00

Chromatograms, integration of mass-spectral data, and data processing were performed using MassLynx and TargetLynx software (Waters, UK). Metabolites were manually identified and quantified based on the retention time (RT) of the standards in the calibration curves, accounting for the area of the internal controls. Quality controls of data removed peaks that had an RT outside of the predicted range for the standards, a peak area < 1000 and a signal-to-noise ratio < 3.

2.2.2. Dietary pAnt extracts, colonic MicrObes, and apoe geNetics interactions stuDy (DIAMOND)

The Dietary pAnt extracts, colonic MicrObes, and apoe geNetics interactions stuDy (DIAMOND) was a pilot study that grouped participants based on their *APOE* genotype, biological sex, and age group to investigate differences in colonic metabolism of EC. The DIAMOND study (IRAS number 319832) received ethical approval from the Human Research Authority East Midlands - Nottingham 2 Research Ethics Committee (REC reference: 23/EM/0049). The study was registered on ClinicalTrials.gov (NCT05905822). The protocol for the DIAMOND study is summarised below (Figure 2.1).

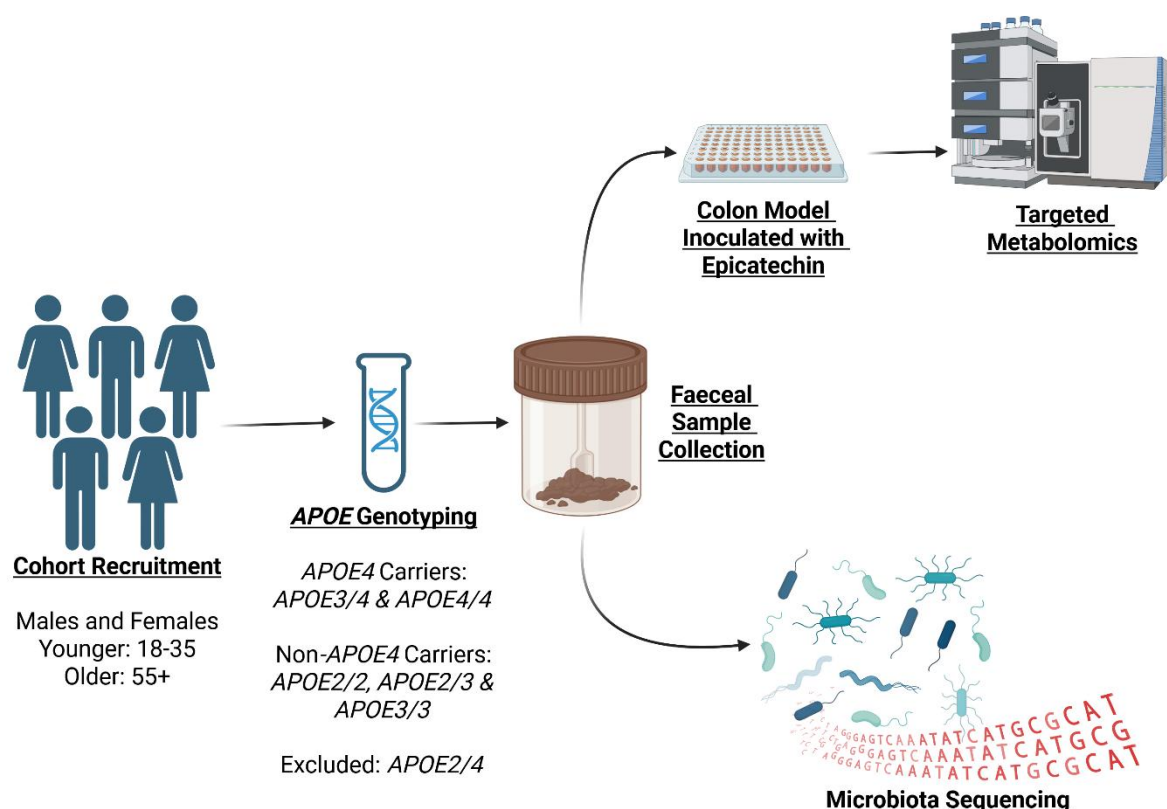


Figure 2.1: Visualised study plan for the Dietary pAnt extracts, colonic Microbes, and apoe geNetics interactions stuDy (DIAMOND)

2.2.2.1. Inclusion Criteria

All participants were recruited if they met the following criteria: living in the UK; aged 18 - 35 years old (younger cohort) or 55+ years old (older cohort); fluency in written and spoken English, and the ability to consent and be available to participate in the study; free from any GI conditions, any forms of dementia or severe cognitive impairment, history of brain damage, chronic fatigue syndrome, liver disease, diabetes mellitus, gall bladder abnormalities; and no history of drug and alcohol conditions. Medical conditions were self-reported. Participants must have either never smoked or ceased smoking more than six months prior to consenting and had not received a COVID-19 diagnosis within the last 30 days. Participants were either recruited *de novo* or from a database of pre-genotyped participants held following the “Early sleep and circadian markers of Alzheimer’s disease: the impact of *APOE*- ϵ 4 on circadian rhythm and sleep-wake homeostasis in humans” (UEA Human Research Governance Committee Reference: 2017/18-135).

2.2.2.2. Genotyping

De novo participants produced a salivary sample. Salivary samples were collected and preserved at ambient temperature (SpeciMAX Saliva Collection Kit, Thermofisher) before the genomic DNA was extracted, and the *APOE* genotype was determined (LGC Genomics, United Kingdom). Participants who carried the *APOE2/4* genotype were excluded due to the contradictory nature of these two alleles. The remaining genotypes were then grouped into *APOE4* non-carriers (*APOE2/2*, *APOE2/3*, and *APOE3/3*) and *APOE4* carriers (*APOE3/4* and *APOE4/4*), which allows us to account for the relative rarity of some of these genotypes. Participants from the local area donated samples in person at the University of East Anglia, and those from elsewhere sent them by post using 24-hour postage, following the protocol for biological category B UN3373. This allowed us to search for participants from across the country, increasing the likelihood of finding those with less common *APOE* genotypes.

2.2.2.3. Food Frequency Questionnaire

Before participants could donate a stool sample, an FFQ was used to assess their habitual dietary patterns over the previous 3 months. This was to quantify dietary intake, paying particular attention to the intake of (poly)phenols and fibres, given their known effects on the structure of the microbiota [189, 225]. Participants completed a Scottish Collaborative Group Food Frequency Questionnaire (SCG-FFQ) (University of Aberdeen, Scotland). The frequency of intake of 169 different food products was measured [226]. SCG-FFQ group collected all the results and quantified the previous three months intake of each participant of the following macronutrients, micronutrients, and plant-based bioactives: energy, total fatty acid, saturated fatty acid, mono-unsaturated fatty acids, poly-unsaturated fatty acids, cholesterol, total carbohydrates, total sugars, total starches, total oligosaccharides, total protein, total fibre, water, alcohol, calcium, copper, iron, iodine, potassium, sodium, phosphorus, zinc, retinol, vitamin B1, vitamin B2, vitamin B3, vitamin B5, vitamin B6, vitamin B9, vitamin B12, vitamin C, vitamin D, vitamin E, vitamin K, carotenes, total flavan-3-ols (epigallocatechin, catechin, EC, epigallocatechingallate, gallic catechin, epicatechingallate), total flavanones (hesperitin

and naringenin), total flavonols (quercetin, kaempferol, and myricetin), and total flavones (apigenin, and luteolin).

2.2.2.4. Stool Sample Collection

Participants who had completed all the above were requested to donate a stool sample. Participants had to refrain from eating the following (poly)phenol-rich foods for 48 hours prior to donating the stool sample: tea, coffee, cocoa, and berries. This washout period allowed time for the dietary (poly)phenols and their subsequent metabolites to leave the participants' digestive systems prior to stool sample donation. Stool samples were collected and transported in Gut Alive Kits (Microviable, Spain) to the QIB, Norwich. If the participants were local, samples were delivered in person; if the study was conducted remotely, samples were sent via post, following the tracked 24-hour delivery protocol for biological category B, UN3373. The Gut Alive Kit was chosen as it had been demonstrated to maintain the microbial community stable for 72 hours in an ambient anaerobic environment [227], permitting us to receive stool samples from across the United Kingdom, and thus widening our search for the less common genotypes.

Once the samples were received at QIB, a small portion of raw stool was frozen at -80 °C for the extraction of microbial DNA. The remainder of each sample was then diluted 1:1 with sterile PBS (0.01 M). This was then homogenised in a stomacher (Seward, UK) for forty-five seconds at 230 rpm. The slurry was then diluted 3:1 with sterile glycerol and vortexed to produce a 37.5% faecal glycerol stock, and frozen at -80 °C to maintain the functionality of the microbiota until further use.

2.2.2.5. Colon Model

The methodology for this colon model was the same as Chapter 2.2.1.3, with the following adjustments. Following the previous colon model work and literature searches. It was decided that the time points would be as follows: 0, 4, 8, and 24 hours. The 4-hour time point was midway between the time points reported in previously published literature [182, 228, 229], while still demonstrating metabolism as observed in Chapter 2.2.1.3's colon model; these studies showed some EC retention, as well as production of gut-derived metabolites. The 8-hour time point was used to further investigate the gap

between the 6- and 24-hour time points, which were previously missing in the Chapter 2.2.1.3 Colon model. This time point was aligned with previous literature to determine whether other metabolites were produced during this time. Each stool sample was run in triplicate through the colon model. As it was not possible to run sterilised controls for every single stool sample, stools from each group were pooled together, equal volumes of the 25% glycerol stock were combined and autoclaved to sterility, and each pool was run in triplicate as a sterile control

2.2.2.6. Metabolite Extraction and UPLC-MS

Metabolite extraction followed the same method as mentioned in Chapter 2.2.1.5, with the following modification: after extraction, the samples were put on dry ice and transferred to the University of Parma, Italy, for subsequent LC-MS analysis. Samples were kept on dry ice until their arrival in Parma. Once in Parma, they were returned to -80 °C prior to analysis. UPLC-MS/MS method is as follows. Before transfer to the LC-MS vials, the samples were filtered through a 0.45 µm filter. Samples were then returned to -80 °C for storage before analysis. Samples were analysed with UPLC-MS/MS to identify and quantify phenolic compounds. In detail, samples were analysed through an Acquity UPLC™ system coupled with an Xevo TQ-S mass spectrometer (Waters, Milford, USA), equipped with an ESI source. Chromatographic separation was performed using a Waters Acquity UPLC system equipped with a Waters HSS T3 VanGuard FIT column (Waters, Milford, USA) with the following dimensions: 100 mm x 1.2 mm, 1.8 µm particle size. The mobile phase consisted of two parts: A) LC-MS grade water (0.01% FA) and B) LC-MS grade acetonitrile (0.01% FA), delivered at a flow rate of 0.4 mL/min. The column temperature was maintained at 40 °C. The following gradient program was used Table 2.3.

Table 2.3: Solvent gradient for UPLC-MS analysis of DIAMOND samples. Solvent A (H₂O 0.1% formic acid) and Solvent B (Acetonitrile 0.1% formic acid)

Time (min)	%A	%B
0.00	93	7
0.50	85	15
3.00	70	30
7.50	60	40
8.50	5	95
9.00	5	95
10.50	93	7
10.70	93	7

The total run time was twenty minutes. The injection volume was 5 μ L, and samples were maintained at 10 °C during analysis. A total of 23 compounds were monitored in MRM mode. Two or three molecular transitions were used to identify and quantify the phenolic compounds (Table 2.4). The system was controlled by MassLynx 4.2 software, and data processed by TargetLynx XS software (Waters, Milford, USA). Catabolite identification was carried out by comparison of the RT with in-house synthesised standards and/or MS/MS fragmentation patterns. Quantification was performed with calibration curves of standards, when available; when unavailable, compounds were quantified with the most structurally similar compound. The nomenclature used for (poly)phenol catabolites follows the current recommendations from Kay et al. [230]. Chromatograms, integration of mass-spectral data and data processing were performed using MassLynx and TargetLynx software (Waters, UK). Metabolites were automatically identified and quantified based on the RT of the standards in the calibration curves. Quality controls of data removed peaks that had an RT outside of the predicted range for the standards, a peak area < 1000 and a signal-to-noise ratio < 3. If a compound was not identified in any sample or at any time point, this was noted, and further analysis was halted.

2.2.2.7. Calibration curves and quantification

Calibration curve standards were generated in-house at the University of Parma. All metabolites were pre-prepared in methanol without matrix-matching standards synthesised in-house. The UPLC-MS/MS calibration curve chromatograms were subject to the same quality control tests as the DIAMOND samples listed above. The concentrations used in the calibration curves ranged from 0.01 to 10 μ M. Calibration curves were produced, where possible, using the same compound as the one to be quantified. In cases where no standard was available, an alternative calibration curve

with a similar molecular mass was used to semi-quantify the standards (Table 2.4). Concentrations of the samples were calculated from the peak area against the gradient of the calibration curves.

Table 2.4: Chromatographic and spectrographic properties of the compounds monitored by UPLC-MS/MS in the Dietary pAnt extracts, colonic MicroObes, and apoe geNetics interactions stuDy (DIAMOND). The predicted RT (Pred RT) was the RT obtained from the standard (STD) or the literature, in cases where the STD was not the same compound as the one being quantified. Observed RT (Obs RT) was the RT observed in the samples. An Obs RT of NA indicates that the compound was not observed in the samples. Mass to charge ratio (m/z), collision energy (CE). Epicatechin (EC), 1-(3',4'-Dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol (3,4-DiHPPol), 1-(4'-Hydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol (4-HPPol), 1-(3'-Hydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol (3-HPP-ol), 5-(3',4'-Dimethoxyphenyl)-γ-valerolactone (3,4-DiMPVL), 5-(Hydroxy-methoxyphenyl)-γ-valerolactone (3'/4') (HMPVL), 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3,4-DiHPVL), 5-(4'-Methoxyphenyl)-γ-valerolactone (4-MPVL), 5-(3'-Methoxyphenyl)-γ-valerolactone (3-MPVL), 5-(4'-Hydroxyphenyl)-γ-valerolactone (4-HPVL), 5-(3'-Hydroxyphenyl)-γ-valerolactone (3-HPVL), 5-(Phenyl)-γ-valerolactone (PVL), 4-Hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid ion I + II (4-H-3,4-DiHPVA ion I + II), 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid (4-H-4-HPVA), 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid (4-H-3-HPVA), 5-(3',4'-Dihydroxyphenyl)valeric acid (3,4-DiHPVA), 5-(4'-Hydroxyphenyl)valeric acid (4-HPVA), 5-(3'-Hydroxyphenyl)valeric acid (3-HPVA), 5-(Phenyl)valeric acid (PVA).

Name	Pred RT (mins)	Obs RT (mins)	Parent Ion (M-H) ⁺ (m/z)	CV	Quantifier Ion		Qualifier Ion1		Qualifier Ion 2		STD	Observed in Samples
					Ion (m/z)	CE (V)	Ion (m/z)	CE (V)	Ion (m/z)	CE (V)		
Flavan-3-ols												
EC	4.27	4.28	289	44	245	14	109	24	NA	NA	EC	Yes
Catechin	3.45	3.47	289	46	245	14	205	18	NA	NA	Catechin	Yes
Procyanidin dimer B2	3.90	3.86	577	42	425	16	289	24	NA	NA	Procyanidin dimer B2	No
Diphenylpropan-2-ols												
3,4-DiHPPol	4.46	4.43	291	40	123	20	147	20	167	20	Catechin	Yes
4-HPPol	5.43	5.41	275	40	231	20	191	20	NA	NA	Catechin	Yes
3-HPPol	5.85	5.84	275	40	191	20	231	20	NA	NA	Catechin	Yes
Phenyl-γ-valerolactones												
3,4-DiMPVL	7.0-10.0	7.58	235	20	220	20	206	30	NA	NA	3,4-DiHPVL	No
HMPVL	5.0-8.0	7.05	221	20	206	20	163	30	NA	NA	3,4-DiHPVL	No
3,4-DiHPVL	4.84	4.83	207	2	163	18	122	20	NA	NA	3,4-DiHPVL	Yes
4-MPVL	7.0-10.0	NA	205	20	190	20	106	30	147	30	NA	No
3-MPVL	7.0-10.0	NA	205	20	190	20	106	30	147	30	NA	No
4-HPVL	6.20	6.21	191	20	93	18	147	12	NA	NA	4-HPVL	Yes
3-HPVL	6.75	6.74	191	20	147	12	93	18	199	24	3-HPVL	Yes
PVL	7.0-10.0	8.34	175	20	175	10	157	20	131	20	3-HPVL	No
Phenylvaleric acids												
4-H-3,4-DiHPVA ion I +II	3.15	3.15	225	6	123	20	101	16	163	16	3,4-DiHPVA	Yes
4-H-4-HPVA	4.04	4.07	209	6	101	14	147	14	NA	NA	4-HPVA	Yes
4-H-3-HPVA	4.62	4.62	209	8	101	14	191	10	147	14	3-HPVA	Yes
3,4-DiHPVA	6.48	6.49	209	4	135	16	122	18	147	18	3,4-DiHPVA	Yes
4-HPVA	7.97	7.98	193	10	175	20	119	18	NA	NA	4-HPVA	No
3-HPVA	8.34	8.35	193	22	149	16	106	22	157	22	3-HPVA	Yes
PVA	9.29	9.2	177	20	177	10	133	20	159	20	3-HPVA	No

After the quantification steps, to ensure reproducibility between the triplicates of each colon model, the following processing steps were followed. In each sample, if a compound failed any of the quality control steps, its concentration was set to not detected (ND). If there were two or more triplicates that had a compound ND, that compound was ND for that participant. Following this, the remaining samples for each compound had their coefficient of variance (CV) calculated. If a sample triplicate of a compound had a CV greater than 45%, they were then subject to outlier removal. This was achieved by removing the compounds that were more than one standard deviation away from the mean of the triplicates. The mean value for the new number of replicates was calculated. If, following quality control steps, the number of replicates was two, values were retained for analysis, as it is not possible to accurately calculate the standard deviation and thus the CV of these samples. Following these steps, the mean concentration of each compound in each triplicate was calculated. If the compound was ND in the sample, the concentration was set to 0 μ M.

The final concentration in the sample was calculated by applying a dilution factor of 6.667, accounting for the dilution of the samples during the metabolite extraction, as per Di Pede's method [182]. As the calibration curves are not matrix-matched to the samples, to account for this, the concentration of each compound was calculated as a percentage of the EC detected at time point 0.

2.2.3. Metabolism of Epicatechin in *APOE-TR* mice

All experimental procedures and protocols used in this section were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB) and were conducted within the provisions of the Animals (Scientific Procedures) Act 1986 (Amendment Regulations 2012). Animal ethics approval reference PPL 79/6503. Reporting of the study outcomes complies with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines [231]. The study design is summarised in Figure 2.2 and then in full below.

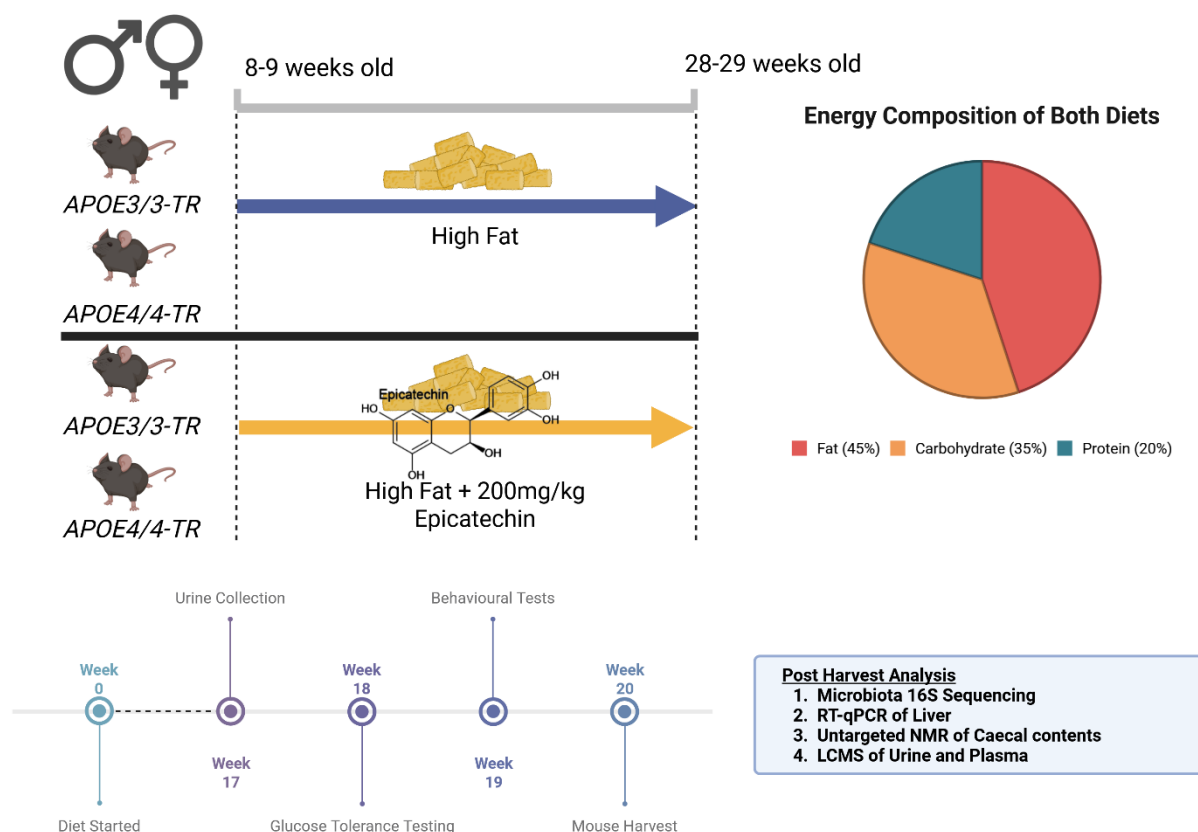


Figure 2.2: Study Design for the *APOE-TR* mice showing the mice split by *APOE3/3* and *APOE4/4* genotype, by sex and then by diet High fat (HF) and High fat and 20 mg/kg body weight epicatechin (HFEC)

Forty-eight human *APOE-TR* mice, *APOE3/3-TR* and *APOE4/4-TR*, were purchased (Taconic, USA). These mice were split evenly between male and female mice, as it is well established that the sex of an individual also plays a role in the structure of the microbiota [222]. Therefore, we hypothesised that both the *APOE* genotype and the sex of the mice play a role in the metabolism of the dietary EC. These mice were aged between 8 and 9 weeks when they started the diet and were on the diet for 20 weeks before they were sacrificed. The mice were fed either a control high-fat (HF) diet or the intervention diet of high-fat supplemented with epicatechin (HFEC) diet. The HF diet was constituted in a way that 45% kcal of the dietary energy came from saturated fats; the HFEC diet had the same 45% kcal energy from saturated fats and was also supplemented with 14.5 mg/kg body weight EC, which provides a human-equivalent dose of ~100 mg/day for an 85 kg adult, a dose that could be achievable through a flavonoid-rich diet [232, 233]. See Supplementary Table 1 for full dietary composition. This dietary model was chosen as the high-fat concentration mimics the key features of the ‘Western Diet’ [234], which is known to accelerate neuroinflammation and cognitive decline [235]. The 45% kcal

saturated fat exceeds that of the typical Western Diet but was deliberately chosen to accelerate the pathophysiological processes of neurocognitive decline within the timeframes of the study. Both the HF and HFEC diets were supplied by Research Diets (New Brunswick, NJ, USA). At the start of the experiment, there were six mice in each genotype, sex, and diet group, which were split between two cages per group. However, one male *APOE3/3-TR* mouse fed the HFEC diet died during the experiment before any samples or experimental results could be collected, resulting in a total of 47 mice (Table 2.5).

Table 2.5: Number of mice in each group in the animal study.

APOE genotype	Sex	Diet	Number of mice
<i>APOE3/3-TR</i>	Male	High Fat	6
<i>APOE3/3-TR</i>	Female	High Fat	6
<i>APOE3/3-TR</i>	Male	High Fat with Epicatechin	5
<i>APOE3/3-TR</i>	Female	High Fat with Epicatechin	6
<i>APOE4/4-TR</i>	Male	High Fat	6
<i>APOE4/4-TR</i>	Female	High Fat	6
<i>APOE4/4-TR</i>	Male	High Fat with Epicatechin	6
<i>APOE4/4-TR</i>	Female	High Fat with Epicatechin	6

2.2.3.1. Urine and serum collection

In the seventeenth week of the diet, urine was collected from the mice. The decision was made to collect urine prior to sacrifice, as it is not always present in the bladder of animals at sacrifice. Urine was also collected at week seventeen, as it was prior to the glucose tolerance testing, which may interfere with the metabolic processes of the animals, which is discussed in Chapter 4. Urine was collected from the mice following the method of Chew and Chua (2003) [236].

On the twentieth week of the diet, the mice were sacrificed by cardiac puncture and perfusion. Blood was collected and kept on ice for 30 minutes prior to centrifugation at 15,000 rpm for 10 minutes. The serum supernatant was separated from the pellet. Samples were stored at -80 °C before further processing.

2.2.3.2. Metabolite Extraction and UPLC-MS

Urine Sample Dilution

When preparing the urine samples for mass spectrometry, the volume of urine for each mouse was too low. Therefore, the decision was made to pool the mice samples based on their cages. Equal volumes of each mouse's urine were pooled and then diluted 1:1 with acidified water (H_2O 0.1% FA). The urine and water mixture was then centrifuged at 14,000 rpm for ten minutes at 4 °C. The supernatant was extracted, and 150 μL was transferred to LC-MS vials to measure the excreted urinary (poly)phenols. To facilitate comparison between the mice, the creatinine concentration in each urine pool was also measured. The remainder of the diluted urine pool was further diluted to produce a 1:1000 dilution with acidified water (H_2O 0.1% FA). The further diluted urine pool was then transferred to vials for creatinine measurements. All samples were then refrozen at -80 °C until ready for analysis. On the day of analysis, all diluted urinary samples were filtered through a 0.45 μm filter before transfer to the chromatography vials.

Microelution Solid Phase Extraction of Mouse Plasma Samples

There was a suitable volume of mouse serum collected from forty-five of the forty-seven mice that were harvested. Unfortunately, two mice did not have a sufficient volume of serum and had to be discarded: one male *APOE4/4*-TR mouse on the HFEC diet and one female *APOE4/4*-TR on the HFEC diet. The remaining serum samples were thawed and centrifuged at 15,000 rpm for fifteen minutes at 4 °C. The supernatant was diluted 1:2 in a phosphoric acid (PA) solution (H_2O 4% PA). This mixture was then vortexed. Each well of the microelution solid-phase extraction (μSPE) plate was activated with 250 μL of methanol, which was vacuumed off for 25 seconds. The plate was then further activated with 250 μL of acidified water (H_2O 0.2% acetic acid), and this was vacuumed through for 25 seconds. 300 μL of each serum sample mixture was then loaded into separate wells, which were subsequently vacuumed through the plate for thirty seconds. The plate was first washed with 200 μL of water, then with 200 μL of acidified water 2O, 0.2% acetic acid). Each liquid was removed by vacuuming through the plate for 20 seconds. All liquids collected to this point were placed into a waste plate and discarded. The waste plates were then swapped for a collection plate. Each well of the collection plate was filled with 20 μL of water to reduce methanol evaporation from the final sample. The wells of the

μSPE plate were then filled with 60 μL of methanol preparation (Methanol 0.1% FA and 10 mM ammonium formate solution). This was vacuumed through to the collection wells until all the volume had passed through the filter. 80 μL of the final sample was transferred to the LC-MS vials for analysis. The final samples were then refrozen at -80 °C until ready for analysis.

Chromatography and Spectroscopy of (Poly)phenolic Compounds

Samples were analysed with UPLC-MS/MS to identify and quantify phenolic compounds and their conjugates. In detail, samples were analysed through an Acquity UPLC™ system coupled with an Xevo TQ-S mass spectrometer (Waters, Milford, USA), equipped with an ESI. Chromatographic separation was performed using a Waters Acquity UPLC system equipped with an *Acquity Premier HSS T3 VanGuard FIT column (Waters, Milford, USA) with the following dimensions: 100 mm x 1.2 mm, 1.8 μm particle size. The mobile phase consisted of two parts: A) LC-MS grade water (0.01% FA) and B) LC-MS grade acetonitrile (0.01% FA), delivered at a flow rate of 0.4 mL/min; the injection volume was 2 μL. The column temperature was maintained at 40 °C. The same gradient program was used as for the fermented samples Chapter 2.2.2.6. A total of 83 compounds were monitored in MRM mode. Two or three molecular transitions were used to identify and quantify phenolic compounds. The system was controlled by MassLynx 4.2 software, and data were processed by TargetLynx XS software (Waters). Metabolite identification was carried out by comparison of the retention time with in-house synthesised standards and/or MS/MS fragmentation patterns. Quantification was performed using calibration curves for standards when available; when unavailable, compounds were quantified using the most structurally similar compound. For full chromatographic and spectroscopic methodology, see Supplementary Table 2.

Urine Sample Analysis

After the quantification steps, to ensure reproducibility across the triplicate measurements for each pooled urine sample, the following processing steps were performed. In each sample, if a compound failed any of the quality control steps, the concentration of that compound was set as ND. If there were two or more triplicates that had a compound ND, that compound was ND for that sample. Following this, the remaining samples for each compound had their CV calculated. If a compound had a CV

greater than 45%, they were then subject to outlier removal. This was carried out by removing the compounds that were greater than one standard deviation away from the mean of the triplicates. The mean value for the new number of replicates was calculated. If, following quality control steps, the number of replicates was two, values were retained for analysis, as it is not possible to accurately calculate the standard deviation and thus the CV of these samples. Following these steps, the mean concentration of each compound in each triplicate was calculated. If the compound was ND in the sample, the concentration was set to 0 μ M. The final concentration in the sample was calculated using a dilution factor of 2 to account for the dilution during metabolite extraction. Final concentrations of (poly)phenol metabolites were normalised to the creatinine concentration.

Plasma Samples

In the case that the compound was ND within the samples, the concentration was set to 0 μ M. To account for the concentration during metabolite extraction from the plasma, all samples were multiplied by the concentration factor of 0.8.

2.2.3.3. Chromatographic and Spectroscopic Methodology of Creatinine Measurement

Urinary creatinine analysis was performed using UHPLC-MS/MS after dilution of samples with water (0.1% FA). Samples were analysed through an Accela uHPLC 1250 apparatus equipped with a Linear Ion Trap Mass Spectrometer (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc.), fitted with a heated-ESI (H-ESI-II). Separation was carried out by means of an XSelect HSS T3 column (50 x 2.1 mm; 2.5 μ m particle size; Waters, Ireland) installed with a precolumn cartridge (Phenomenex, CA, USA). For UHPLC, mobile phase A was acetonitrile containing 0.1% FA, and mobile phase B was water containing 0.1% FA. Chromatographic separation was performed in isocratic mode, maintaining A and B at 50% for 2 minutes (the duration of a single run). The flow rate was set at 0.2 mL/min, the injection volume was 5 μ L, and the column was thermostated at 40°C. The H-ESI-II interface was set to a capillary temperature of 275 °C, and the source heater temperature was 200 °C. The sheath gas (N₂) flow rate was set at 40 (arbitrary units) and the auxiliary gas (N₂) flow rate at 5. The source voltage was 5 kV, and the capillary voltage and tube lens voltage were +3 and +45 V, respectively. Ultra-high purity helium gas and a Collision-

Induced Dissociation (CID) equal to 55 were used to obtain MS/MS fragmentation. Creatinine was analysed with a full-scan MS/MS analysis in a positive ionisation mode, monitoring all the ions with a m/z value between 50 and 130 Da. For identification and quantification, the molecular ion with $m/z = 114$ and the fragment ion with $m/z = 86$ was considered. Identification and quantification were performed by comparison with the standard. Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (ThermoFisher scientific, USA). Creatinine concentration in urine samples was expressed as mM. These results were used to calculate the final concentration of each compound, which was then calculated by normalising the creatinine in the urine pools.

2.2.4. Statistical Analysis

All statistical analyses took place using R 4.4.1 [237]. The Shapiro-Wilk test for normality (using packages in base R) and the Levene Test for homogeneity of variance (using the package car [238]) were carried out on the data.

2.2.4.1. Human Colon Model Development

Both pH and Concentration measurements were compared using the following mixed linear model, which compared the outcomes of each metabolite or pH value to Donor, Stool Type (Microbial or Sterile), and Time, as well as their associated interactions. To account for intra-donor variance, donor ID was used as a random effect in the model. This produced the following formula:

$$\text{Concentration or pH} \sim \text{Donor} * \text{Stool} * \text{Timepoint} + (1|\text{DonorID}).$$

The fixed effects of the mixed linear model were then analysed by ANOVA, and any significance ($p\text{-value} < 0.05$) was further investigated using the least squares means method for pairwise comparisons. The Benjamini-Hochberg (BH) method was utilised to adjust for false discovery rate (FDR).

2.2.4.2. DIAMOND Study

As the DIAMOND study was a pilot study, no sample size estimates were calculated prior to participant recruitment. It is established from the development of the Human colon

model that there are significant differences between time points; the analysis for the DIAMOND study was conducted separately at each time point. Due to the pooling of the sterile stool samples, there was only one datapoint per group; therefore, these were further pooled by *APOE4* carrier status only. Due to the sterile sample pooling, it is not possible to include these samples in the model, and they were excluded from analysis. To account for the abnormal distribution and heteroscedasticity, the following model was run through a 3-way permutational ANOVA (10,000 permutational bootstraps, package permuco [239]), and repeated at each of the four time points measured, comparing the effect of the *APOE4* carrier status, age, and sex as well as their interactions:

*% of Compound compared to T0 Epicatechin ~ APOE4 Carrier Status * Age group * Sex.*

Any significance (p-value < 0.05) was further investigated using a permutational t-test (10,000 permutational bootstraps) for pairwise comparisons, in the package RVAideMemoire [240]. The BH method was utilised to adjust for the FDR.

2.2.4.3. *APOE-TR Mouse Model*

Zero flavonoids were detected in the plasma and the urine of HF samples, and a t-test between the groups highlighted that in all groups the concentration of the metabolites was significantly higher in the HFEC-fed mice than their HF-fed counterparts (p-values < 0.05 urinary metabolite and p-values < 0.01 plasma samples). Due to the absence of flavonoid metabolites in the urine and plasma of HF samples, these samples were removed from subsequent analysis.

Plasma Samples

To account for the abnormal distribution and heterogeneous variances in the data, whilst maintaining the cage effect on these samples, a permutational ANOVA was applied to the following model (10,000 permutation bootstraps) using the package afex [241]:

*Compound Concentration ~ APOE genotype * Sex + (1|Cage).*

Any significance (p-value < 0.05) was further investigated using the least square means method for pairwise comparisons. The BH method was utilised to adjust for the FDR.

Urine Samples

Due to pooling of urine samples, there were only 2 data points per group (sex, genotype, and diet), making it unfeasible to perform a two-way permutational ANOVA. Therefore, the following analyses were carried out. To control for the abnormal distribution and heterogeneous variances in the data (multiple Shapiro-Wilk and Levene $p < 0.05$), Kruskal-Wallis was used for the analysis, using the package from base R. Firstly, the mice were grouped by their genotype only, and the following model was carried out between the mice:

$$\text{Compound Concentration} \sim \text{APOE genotype.}$$

Secondly, the mice were grouped according to their sex only, and the following model was carried out:

$$\text{Compound Concentration} \sim \text{Sex}$$

2.3. Results

2.3.1. Human Colon Model Development

To determine whether the *APOE* genotype, sex, and age of an individual influence the metabolism of dietary flavan-3-ols, it is first necessary to establish a robust *in vitro* colon model capable of metabolising EC using human faecal microbiota. Although several protocols for the *in vitro* fermentation of EC have been reported [182, 229], existing methods have not systematically evaluated a range of experimental variables [224]. Consequently, the pilot study method required adaptation to accommodate a larger participant cohort. Additionally, optimal sampling time points needed to be identified to capture EC catabolism and the production of its metabolites with sufficient temporal resolution, while maintaining a manageable number of samples for downstream analysis. To address these needs, a high-throughput colon model was developed, building upon previously established methodologies, with slight modifications [182, 229]. Notably, prior studies employed PBS or did not report pH stability of their media. As microbial metabolism in faecal samples generates organic acids that decrease the pH, a

process tightly regulated *in vivo* by colonic homeostasis, continuous pH monitoring was implemented. This ensured that the buffering capacity of the selected media maintained pH within a physiologically relevant range of 6.0-7.0 for the human colon [242].

2.3.1.1. pH results

Throughout the experiment, the pH was tightly maintained, fluctuating between pH 6.77 and 6.56 for donor 011 and 6.84 and 6.53 for donor 053. The minimum pH is reached at the 5-hour time point (Figure 2.3). There is neither significance between the donors, nor the time points, nor the interaction between the two (all $p > 0.05$), indicating that the buffering capacity of the media maintained the pH.

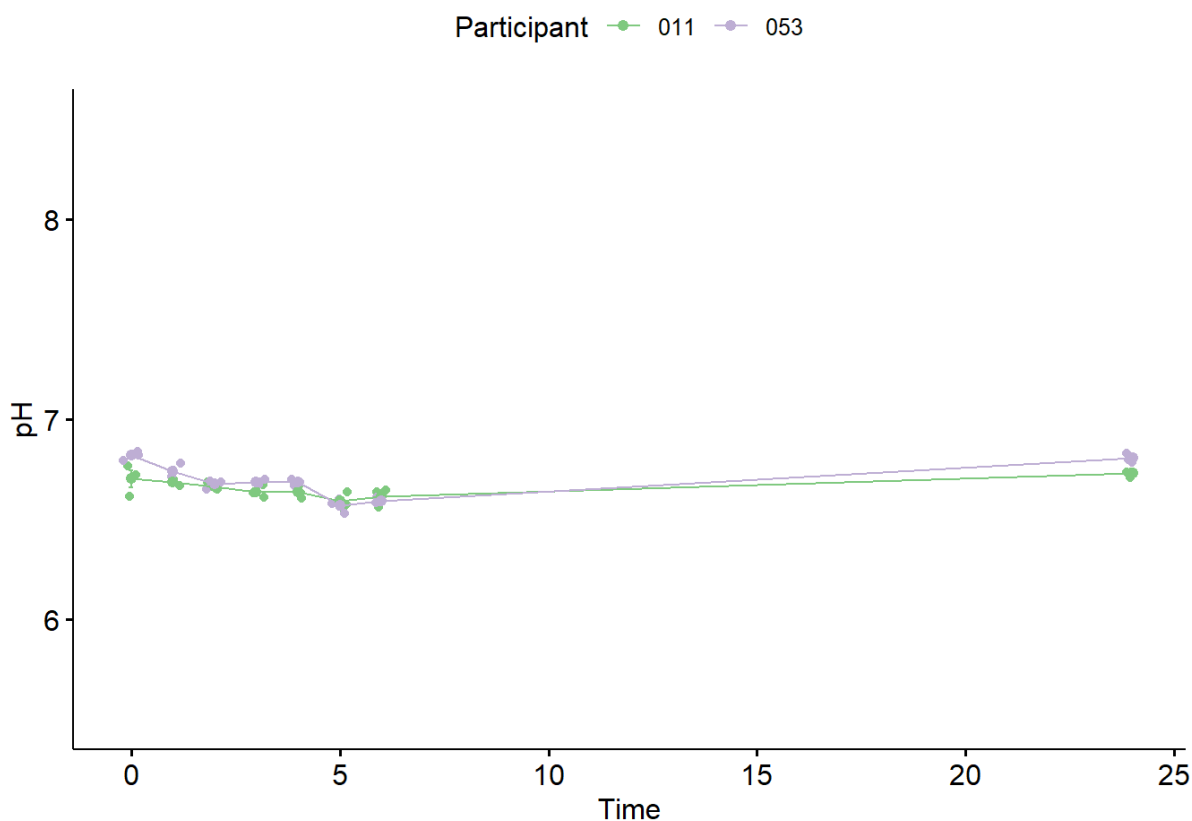


Figure 2.3: pH measurements of the colon model media over a 24-hour colon model metabolising 100 μM of epicatechin. Presenting the pH of colon model media for two separate donors, 011 (green) and 053 (purple)

2.3.1.2. Epicatechin Metabolism and gamma-valerolactones formation

The colon model demonstrated efficient metabolism of 100 μM EC, along with the successful formation of γ -valerolactones. In both the bacterial and sterile stool models from donors 011 and 053, EC levels declined similarly over the initial 2 hours. No

significant differences were observed between donors or between sterile controls and bacterial conditions at these early time points (all $p > 0.05$). However, beyond 2 hours, the rate of EC loss began to diverge between bacterial stool and sterile conditions. At 6 hours, EC concentrations were 72.7 μM and 65.2 μM in sterile samples from donors 011 and 053, respectively, with no significant difference between them ($p = 0.4$). By 24 hours, EC levels further declined to 55.7 μM and 70.9 μM , again with no significant difference between these two sterile stool samples ($p = 0.09$).

In contrast, EC metabolism in bacterial stool models followed a distinct pattern. At 3 hours, significant inter-donor variation was observed ($p = 0.001$), with EC concentrations of 66.4 μM for donor 011 and 99.2 μM for donor 053. Both bacterial models continued to metabolise EC over time. At 4 hours, EC levels had decreased to 54.9 μM and 66.0 μM for donors 011 and 053, respectively, with no significance between donors ($p = 0.1$) nor between bacterial and sterile conditions ($p > 0.05$). By 5 hours, EC concentrations further declined to 23.0 μM and 6.2 μM for donors 011 and 053, respectively, although this donor difference did not reach statistical significance ($p = 0.06$); a significant difference was observed between bacterial and sterile conditions (both $p < 0.001$).

This significant disparity in EC degradation between bacterial and sterile models persisted throughout the remainder of the 24-hour time course. At 6 hours, the EC concentration in bacterial models was 6.4 μM and 1.1 μM for donors 011 and 053, respectively, with no significant inter-donor difference ($p > 0.05$). By 24 hours, EC was fully degraded in both bacterial stool models (Figure 2.4A).

No production of either γ -valerolactones: 3,4-DiHVL or 3-HPVL was observed during the first 6 hours post-inoculation. Additionally, no valerolactone production was detected in sterile stool samples at the final time point. A statistically significant difference in metabolite levels between sterile and bacterial wells ($p < 0.001$) confirms that microbial activity is necessary for γ -valerolactone biosynthesis. At 12 hours post-inoculation, a significant interindividual variation in 3,4-DiHVL production was observed among donors ($p < 0.001$), with donor 011 producing 67.3 μM , whereas donor 053 produced only 7.2 μM . Similar variability was noted for 3-HPVL production ($p < 0.001$), with donor 053 showing no detectable 3-HPVL and donor 011 producing 10.5 μM . These findings indicate that the microbial generation of both 3-HPVL and 3,4-DiHPVL is subject to substantial donor-

dependent variability (Figure 2.4B and C). In the subsequent genotyped study, it would be ideal to use the same time points to investigate EC metabolism by bacteria in stool samples; however, this would yield an unrealistic number of samples to process within the timeframe of the investigation. From these results, it is possible to explore some time points to focus the investigation on. Both the baseline and final 24-hour time points should remain at 0 hours, demonstrating the presence of initial EC and the absence of gut-derived metabolites, and vice versa for the 24-hour time point (Figure 2.4). Conversely, these results are not particularly suitable for determining which time points should be used in the time interval between baseline and the final time point. The initial time points reveal inter-donor significance at 3 hours; however, this is subsequently lost. There is also an 18-hour gap between the 6-hour time point and the final 24-hour time point. As these results cannot identify which time points should be used for the interstitial time points, the scientific literature should be consulted before starting the *in vitro* colon model work on the *APOE* genotyped participants.

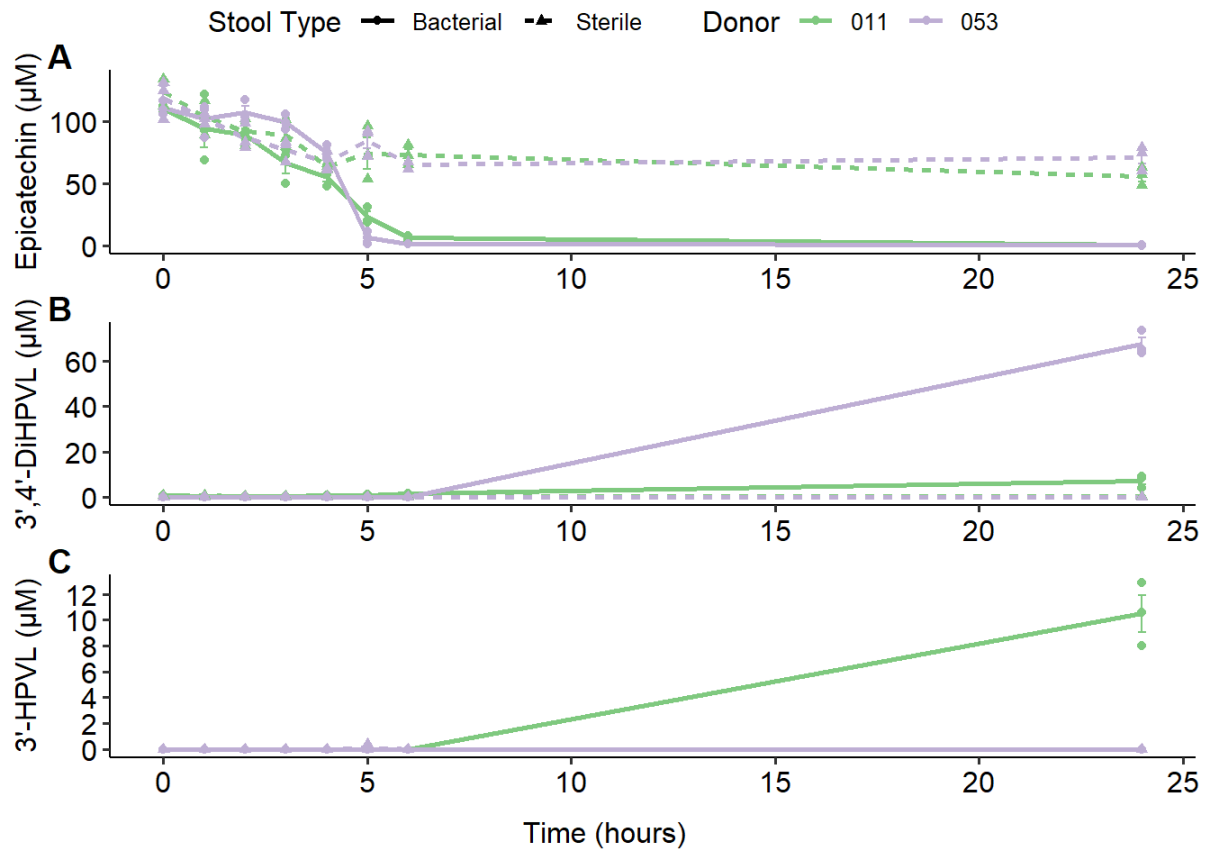


Figure 2.4: Concentration of A) Epicatechin B) 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (3',4'-DiHPVL) and 5-(3'-Monohydroxyphenyl)- γ -valerolactone (3'-HPVL) over a 24-hour colon model. Showing the interindividual response of the metabolism between participants 011 (green) and 053 (purple), as well as the differences between the bacterial stools (solid line) and their sterile stool control (dashed line).

2.3.2. Investigating the role of *APOE* genotype, sex, and age on EC Metabolism – Results from the DIAMOND study

Previous research has demonstrated that an *in vitro* colon model can be employed to metabolise EC, generating several gut-derived metabolites, and enabling the assessment of interindividual variability. However, these earlier studies lacked information regarding participants' demographics or dietary habits. To address these limitations, a larger study was designed to investigate how sex, *APOE* genotype, and age influence the composition of the microbiota and the subsequent metabolism of EC. This led to the design of the DIAMOND study, which aimed to comprehensively explore these factors.

2.3.2.1. *Participants' characteristics*

From a cohort of 168 participants, 49 successfully completed the study. Several reasons were given for exclusion, primarily an overabundance of participants from certain age, sex, or genotype groups, as well as participants failing to meet the inclusion criteria. Participants were stratified by sex, age group and *APOE4* carrier status (Figure 2.5). Among 28 female participants, 17 were aged 55 years and older, and 11 were aged 18-35 years. In the younger female group, 7 were *APOE4* carriers, and 4 were *APOE4* non-carriers. In contrast, the older female group included 11 *APOE4* carriers and 6 *APOE4* non-carriers. Of the 21 male participants, 9 were aged 18-35 years, and 12 were aged 55 years and older. Within the younger male group, 3 were *APOE4* carriers and 6 were *APOE4* non-carriers; the older male group comprised 5 *APOE4* carriers and 7 *APOE4* non-carriers (Figure 2.5).

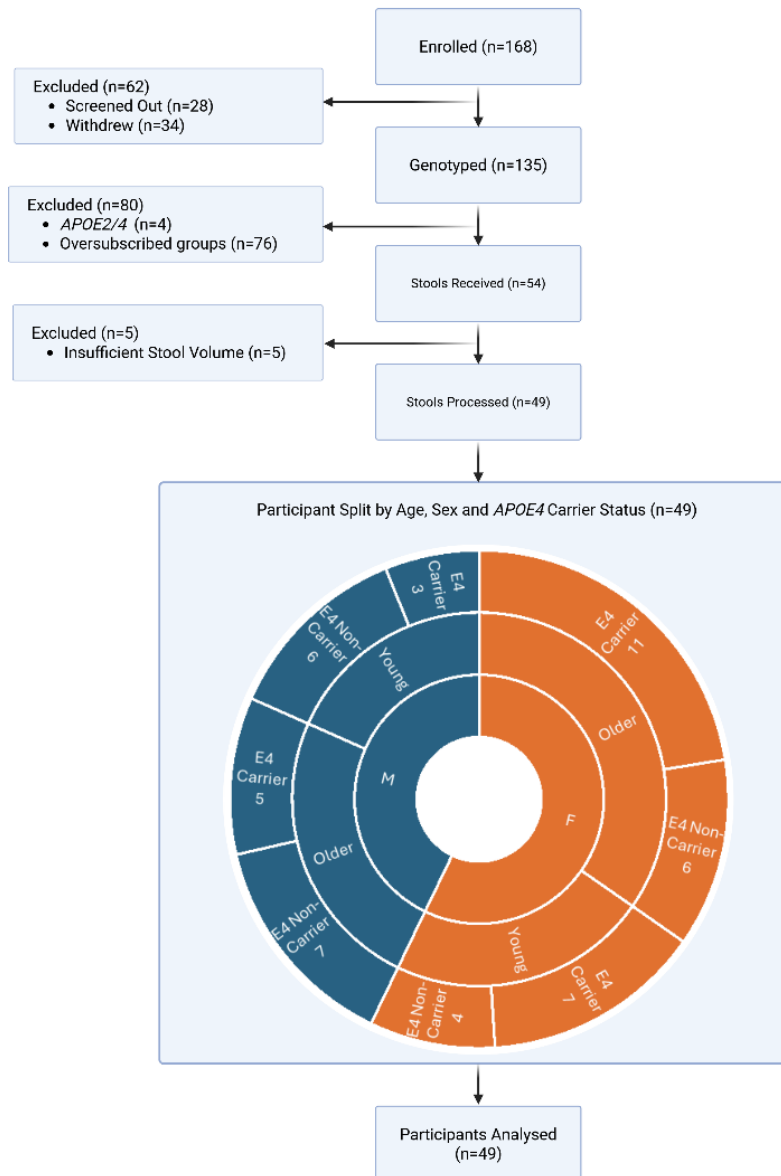


Figure 2.5: Breakdown of the Dietary pAnt extracts, colonic MicroObes, and apoe geNetics interactions stuDy (DIAMOND) participant recruitment and the numbers of each sex, age, and APOE4 carrier status of colon model participants. APOE2/3 and APOE3/3 (E4 Non-Carrier), APOE3/4 and APOE4/4 (E4 Carrier). The APOE2/4 genotype was excluded from the study due to the conflicting natures of carrying both the protective E2 allele, which decreases dementia risk and the E4 allele, which increases dementia risk.

2.3.2.2. Dietary patterns of the participants using Food Frequency Questionnaires

Analysis of habitual dietary intakes revealed several significant differences across age, sex, and genotype groups (Table 2.6). Among macronutrients, trans-unsaturated fatty acids (TUFA) differed significantly by sex ($p = 0.025$), while total sugar intake varied according to genotype ($p = 0.007$). Fibre intake differed significantly across age groups (p

= 0.003). Regarding micronutrients, calcium, potassium, and vitamins B2 (riboflavin), B3 (niacin), B6 (pyridoxine), Vitamin B7 (biotin) and B9 (folate) showed significant variation by genotype, with vitamin B2 (Riboflavin) also influenced by age (all $p < 0.05$). For bioactive compounds, carotene intake differed by both sex and age (both $p = 0.026$). Whereas total flavan-3-ol intake (e.g. epigallocatechin, catechin, EC, epigallocatechin gallate, gallic catechin, EC gallate) and total flavonoid intake varied significantly with age ($p = 0.012$ and $p = 0.023$, respectively). Notably, the observed differences in fibre, carotene, flavan-3-ol, and flavonoid intake are of particular interest, as these compounds are known to reach the colon intact and are recognised for their interactions with the gut microbiota. For the full FFQ dataset, see Supplementary Table 3

Table 2.6: Results from the Food Frequency Questionnaire prior to stool sample donation. Participants were grouped according to their Sex, Age Group, and APOE4 Carrier Status (Gen). Intakes were used in a linear model to calculate the 3-way ANOVA p-values for each variable and their interactions (p-values <0.05 are highlighted in bold). APOE genotype 2/3 and 3/3 (Non E4) and APOE genotype 3/4 and 4/4 (E4). Ages 18- 35 years old: Younger, and 55+ years old: Older. P-values <0.05 are in bold. Total carbohydrates, total fat, and total flavonoids. Significant Macronutrients are as follows Trans unsaturated fatty acid (TUF), Total Sugars (Sugars), Total Protein (Protein), Total Fibre According to Association of Official Analytical Chemists International (AOAC fibre). Significant Minerals: Calcium (Ca) and Potassium (K). Significant Vitamins: Riboflavin, Niacin, Pyridoxine, Biotin, Folic Acid. Plant Bioactives: Carotenes. Total flavan-3-ols: Epigallocatechin, Catechin, epicatechin, epigallocatechin gallate, galocatechin, epicatechin gallate and Total (poly)phenols. Mean value ($\bar{x}$) and standard deviation (SD) reported for each group. Group sizes: male younger E4 carrier n = 3, male younger non-E4 carrier n = 6, male older E4 carrier n = 5, male older non-E4 carrier n = 7, female younger E4 carrier n = 7, female younger non-E4 carrier n = 4, female older E4 carrier n = 11, female older non-E4 carrier n = 6

Sex:	Females				Males				3 way ANOVA p-value						
Age Group	Older		Younger		Older		Younger								
APOE4 Gen	E4	Non E4	E4	Non E4	E4	Non E4	E4	Non E4							
Nutrient	$\bar{x}$ intake (SD)	$\bar{x}$ intake (SD)	$\bar{x}$ intake (SD)	$\bar{x}$ intake (SD)	$\bar{x}$ intake (SD)	$\bar{x}$ intake (SD)	$\bar{x}$ intake (SD)	$\bar{x}$ intake (SD)	Sex	Gen	Age	Sex: Gen	Sex: Age	Gen: Age	Sex: Gen: Age
Total Values															
Total	233.0	206.4	268.1	198.2	273.3	224.7	280.2	229.4							
Carbs (g/day)	(68.0)	(48.0)	(71.4)	(65.4)	(87.2)	(54.1)	(66.5)	(148.5)	0.540	0.063	0.574	0.894	0.772	0.607	0.680
Total Fat (g/day)	83.8	73.0	95.8	75.2	94.3	98.2	103.1	100.8	0.125	0.467	0.476	0.443	0.858	0.693	0.931
	(35.5)	(21.7)	(27.7)	(37.8)	(41.2)	(27.4)	(9.8)	(50.5)							
Macro Nutrients															
TUFA (g/day)	2.1	1.8	2.0	1.4	2.1	2.6	3.4	2.6	0.025	0.403	0.668	0.462	0.282	0.202	0.449
	(1.0)	(0.7)	(1.0)	(1.0)	(1.3)	(0.7)	(0.9)	(1.3)							
Sugars (g/day)	120.1	100.1	133.6	80.4	146.9	111.9	111.3	86.4	0.953	0.007	0.311	0.980	0.193	0.552	0.374
	(44.9)	(24.1)	(47.5)	(24.7)	(36.6)	(37.0)	(11.2)	(48.0)							
Protein (g/day)	84.4	70.0	91.6	71.2	100.8	92.1	124.1	99.8	0.046	0.124	0.375	0.912	0.669	0.617	0.815
	(32.7)	(20.3)	(21.0)	(29.5)	(54.0)	(24.0)	(36.2)	(43.6)							
AOAC fibre (g/day)	6.6	6.1	4.7	2.2	5.6	5.2	3.4	3.8	0.305	0.227	0.003	0.392	0.490	0.597	0.319
	(2.1)	(2.5)	(2.2)	(1.5)	(2.6)	(2.1)	(2.5)	(3.2)							
Minerals															
Ca (mg/day)	1277.1	1035.6	1350.1	860.9	1466.0	1298.2	1151.9	1035.6	0.643	0.043	0.314	0.493	0.300	0.655	0.573
	(557.5)	(147.3)	(483.6)	(178.0)	(499.3)	(152.9)	(391.7)	(537.4)							
K (mg/day)	4040.4	3207.7	4343.9	3396.1	4835.9	3514.5	3533.2	3438.3	0.912	0.012	0.809	0.984	0.238	0.493	0.333
	(1155.4)	(614.5)	(1124.0)	(314.0)	(1523.6)	(841.1)	(942.3)	(1612.6)							
Vitamins															
Riboflavin (mg/day)	2.5	1.8	1.9	1.5	2.8	2.2	2.1	1.7	0.519	0.016	0.027	0.808	0.793	0.684	0.991
	(1.0)	(0.5)	(0.6)	(0.3)	(1.1)	(0.4)	(0.4)	(0.8)							
Niacin (mg/day)	21.4	17.6	20.9	17.6	26.5	20.6	31.1	22.4	0.091	0.045	0.681	0.507	0.517	0.864	0.738
	(7.1)	(6.9)	(5.9)	(5.6)	(15.7)	(5.8)	(6.6)	(9.0)							
Pyroxidine (mg/day)	2.5	1.8	2.4	1.9	2.6	2.1	2.7	2.1	0.673	0.020	0.965	0.928	0.945	0.884	0.777
	(0.9)	(0.5)	(0.6)	(0.5)	(1.1)	(0.5)	(0.5)	(1.1)							
Biotin (ug/day)	59.6	46.5	58.2	41.7	70.0	58.1	47.9	49.2	0.543	0.029	0.154	0.479	0.277	0.712	0.435
	(22.6)	(9.2)	(14.3)	(6.6)	(19.6)	(12.9)	(20.7)	(19.6)							
Folic acid (ug/day)	363.1	282.7	388.1	276.7	403.3	305.6	235.8	278.4	0.415	0.048	0.474	0.518	0.199	0.555	0.252
	(131.3)	(79.8)	(124.4)	(57.7)	(196.1)	(70.0)	(84.2)	(136.4)							
Phytochemicals															

Carotenes (ug/day)	4710.4 (2084.7)	4627.1 (3415.6)	8623.7 (5453.7)	7981.8 (4671.2)	4820.5 (3743.4)	3854.2 (2503.6)	1966.9 (898.5)	4151.6 (2043.5)	0.026	0.965	0.083	0.853	0.026	0.606	0.371
Total flavan-3-ols (mg/day)	61.4 (41.6)	47.3 (21.9)	39.2 (37.0)	32.4 (36.4)	67.1 (54.0)	63.3 (57.5)	19.1 (12.3)	20.0 (9.6)	0.785	0.447	0.012	0.740	0.280	0.799	0.959
Total Flavonoids (mg/day)	171.7 (85.0)	143.1 (35.5)	135.2 (64.9)	116.2 (73.0)	193.4 (182.5)	174.4 (115.6)	52.7 (28.2)	92.4 (47.6)	0.697	0.561	0.023	0.641	0.199	0.580	0.664

2.3.2.3. LC-MS analysis of EC metabolism following faecal fermentation

Of the 23 compounds analysed, 8 were not detected in any sample at any time point, including proanthocyanidin type A (Table 2.4). In contrast, catechin was consistently detected. Both compounds were included as markers of compliance with the prescribed washout diet. The inconsistent detection suggests variable adherence to the washout diet among participants. In sterile control samples, EC degradation was observed; however, gut-derived metabolites such as (phenyl)propanols, valerolactones or valeric acids were either absent or present at extremely low concentrations with high variability. In bacterial fermentation samples, most metabolites emerged after the 4-hour time point (See Supplementary Table 4), indicating that they are products of microbial metabolism of the inoculated EC.

The three-way permutational ANOVA identifies several significant interactions between compound concentrations and participant variables (Table 2.7). At 4 hours, EC levels were significantly affected by sex ($p = 0.015$). By 24 hours, significant effects were observed for all variables as well as all their interactions (all $p < 0.05$). 3,4-DiHPVL showed significant *APOE4*-related effects at both 4 ($p = 0.049$) and 8 hours ($p = 0.036$). 3-HPVL also demonstrated significant *APOE4*-dependent effects at 24 hours ($p = 0.049$). The compound 3,4-DiHPVA at 8 hours was influenced by sex and age interaction ($p = 0.024$). Lastly, catechin concentrations were influenced by sex at 4 and 8 hours ($p = 0.016$ and 0.040 , respectively) and genotype ($p = 0.023$), age ($p = 0.027$), genotype and age interaction ($p = 0.035$), age and sex interaction ($p = 0.047$), and the three-way interaction between genotype, age, and sex ($p = 0.028$) at 24 hours.

Pairwise comparisons following the 3-way permutational ANOVA revealed that at 4 hours, older non-*APOE4* carrier males had degraded significantly more EC than their female counterparts (FDR $q < 0.001$). Indicating an age and genotype-specific, sex-dependent metabolic pattern. By 24 hours, most of the EC has been metabolised; despite this, there is considerable variability among young *E4* males, which led to higher residual EC compared to older *E4* males and females, driven largely by a single slow-metabolising participant; however, no significance was detected (Figure 2.6A). While genotype effects were statistically significant for both 3,4-DiHPVL and 3-HPVL, pairwise group differences were only detected in the metabolism of 3-HPVL in older males, where

the *APOE4* carriers produced significantly more than the non-*APOE4* carriers (FDR $q < 0.05$), indicating both a sex and age modulation of the genotype effect (Figure 2.6B-C). The permutational t-tests highlighted significant differences in the production of 3,4-DiHPVA between the sexes of the younger non-*APOE4* and an age difference between the non-*APOE4* females. However, inspection of the raw values highlights that each of these groups did not produce the compound detected ($0 \mu\text{M}$ and $\text{SD} = 0$). It appears that the significance detected is an artefact of the permutational t-test method. Interestingly, EC degradation occurred even in sterile controls, with complete loss observed in some *APOE4* carriers. However, gut-derived fermentation metabolites were not detected in these sterile samples, confirming their microbial origin. Overall, these findings demonstrate that both EC degradation and the production of downstream gut-derived metabolites are influenced by multiple interacting factors that may vary by compound and time point.

Table 2.7: Significant (3-way permutational ANOVA $p < 0.05$) LC-MS results from the Dietary pAnt extracts, colonic MicroObes, and apoe geNetics interactions stuDy (DIAMOND) colon model. Measuring the concentration of each compound as a percentage of the baseline detected epicatechin. Participants were grouped according to their Sex, Age Group, and APOE4 Carrier Status (Gen). These intakes were used in a linear model to calculate the 3-way permutational ANOVA p -values for each variable and their interactions (p -values < 0.05 are highlighted in bold). APOE genotype 2/3 and 3/3 (Non E4) and APOE genotype 3/4 and 4/4 (E4). Ages 18- 35 years old: Younger, and 55+ years old: Older. P -values < 0.05 are in bold. Compounds producing significance are as follows Epicatechin (EC), 1-(3',4'-Dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)propan-2-ol (3,4-DiHPPol), 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (3,4-DiHPVL), 5-(3'-Hydroxyphenyl)- γ -valerolactone (3-HPVL), 5-(3',4'-Dihydroxyphenyl)valeric acid (3,4-DiHPVA), and Catechin. Group sizes: male younger E4 carrier $n = 3$, male younger non-E4 carrier $n = 6$, male older E4 carrier $n = 5$, male older non-E4 carrier $n = 7$, female younger E4 carrier $n = 7$, female younger non-E4 carrier $n = 4$, female older E4 carrier $n = 11$, female older non-E4 carrier $n = 6$

Sex Age Genotype		Female				Male				Sterile		3 way permuatational ANOVA p-values						
		Older	Younger		Older		Younger											
		E4	Non E4	E4	Non E4	E4	Non E4	E4	Non E4	E4	Non E4							
Compound	Time (hrs)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	Gen	Sex	Age	Gen: Sex	Gen: Age	Sex: Age	Gen: Sex: Age
Flavan-3-ols																		
EC	4	65.234 (23.124)	93.524 (16.623)	66.414 (47.795)	43.201 (32.242)	54.118 (34.522)	20.681 (23.325)	62.739 (23.332)	33.866 (38.479)	94.950 (11.758)	83.419 (9.472)	0.145	0.015	0.481	0.089	0.232	0.072	0.155
EC	24	0.016 (0.054)	0.306 (0.750)	0.000 (0.000)	0.134 (0.267)	0.096 (0.215)	0.022 (0.059)	22.033 (38.162)	0.014 (0.033)	70.589 (13.539)	31.761 (36.911)	0.026	0.025	0.023	0.019	0.023	0.024	0.028
Valerolactones																		
34-DiHPVL	4	0.096 (0.149)	0.018 (0.045)	0.889 (1.540)	4.419 (8.837)	0.220 (0.400)	3.683 (6.802)	0.253 (0.438)	2.626 (3.375)	0.000 (0.000)	0.098 (0.135)	0.049	0.786	0.385	0.628	0.599	0.194	0.335
34-DiHPVL	8	1.084 (2.046)	0.193 (0.238)	12.446 (19.373)	31.361 (54.254)	8.765 (18.065)	33.643 (44.278)	3.832 (6.446)	40.914 (50.343)	0.000 (0.000)	0.110 (0.153)	0.031	0.276	0.238	0.241	0.398	0.282	0.849
3-HPVL	24	68.863 (84.930)	29.362 (29.959)	47.946 (57.642)	40.194 (69.890)	43.648 (24.568)	11.898 (13.223)	119.708 (200.913)	28.491 (27.897)	0.000 (0.000)	1.538 (3.077)	0.049	0.837	0.356	0.384	0.739	0.236	0.298
Valeric Acids																		
34-DiHPVA	8	0.000 (0.000)	0.000 (0.000)	0.043 (0.114)	0.000 (0.000)	0.114 (0.256)	0.246 (0.295)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.021 (0.042)	0.624	0.077	0.080	0.333	0.337	0.024	0.636
Other flavan-3-ols																		
Catechin	4	1.241 (0.450)	1.568 (0.097)	1.446 (0.682)	1.415 (0.469)	0.994 (0.332)	1.137 (0.480)	1.232 (0.528)	0.967 (0.188)	1.871 (0.896)	1.459 (0.181)	0.763	0.016	0.827	0.445	0.166	0.980	0.931
Catechin	8	1.443 (1.258)	2.164 (0.550)	1.720 (1.455)	1.483 (1.577)	1.155 (0.988)	1.034 (0.819)	0.665 (1.053)	1.063 (0.847)	3.128 (0.897)	2.671 (0.356)	0.574	0.040	0.525	0.884	0.751	0.971	0.287
Catechin	24	0.041 (0.137)	0.000 (0.000)	0.064 (0.168)	0.034 (0.068)	0.000 (0.000)	0.017 (0.044)	1.058 (1.832)	0.000 (0.000)	9.083 (1.302)	3.673 (4.103)	0.023	0.059	0.027	0.050	0.035	0.047	0.028

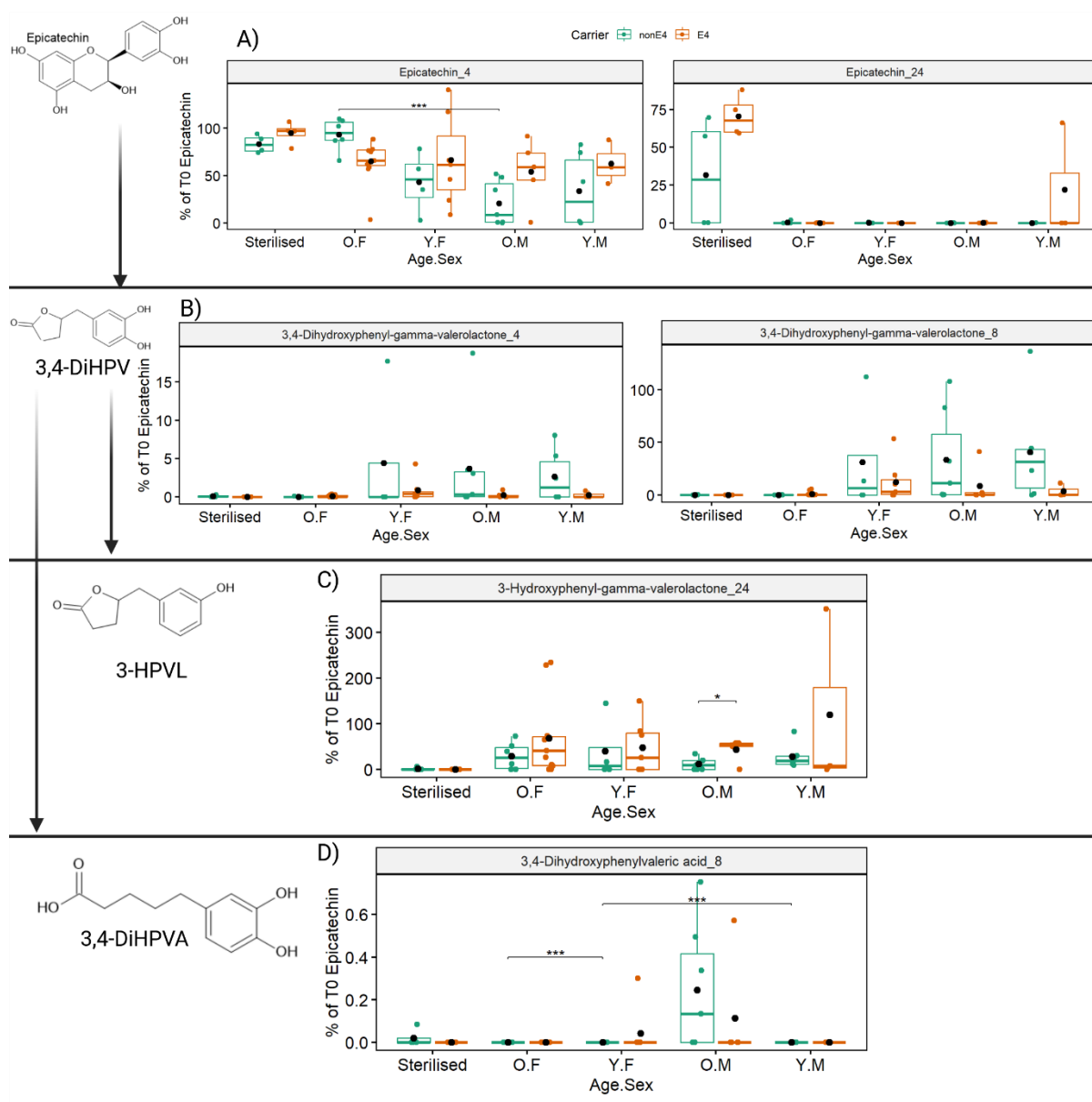


Figure 2.6: Significant (3-way permutational ANOVA $p < 0.05$) Epicatechin and its metabolites detected from the Dietary pAnt extracts, colonic MicroObes, and apoe geNetics interactions study (DIAMOND) colon model measured as a percentage of the epicatechin that was detected at baseline. A) Epicatechin (EC) at $T = 0$ hrs and $T = 24$ hrs, B) 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (3,4-DiHPVL) $T = 4$ hrs and $T = 8$ hrs, C) 5-(3'-hydroxyphenyl)- γ -valerolactone (3-HPVL) $T = 24$ hrs and D) 5-(3',4'-Dihydroxyphenyl)-valeric acid (3,4-DiHPVA) at $T = 8$ hrs. Participants were grouped according to their Sex, Age Group, and APOE Carrier Status. These intakes were used in a linear model to calculate the 3-way permutational ANOVA p -values for each variable and their interactions, and then pairwise interactions were calculated using the permutational t -test method for pairwise comparisons. The Benjamini-Hochberg method was utilised to adjust for the false discovery rate. Significant pairwise interactions are highlighted as follows: * FDR q -value < 0.05 and ≥ 0.01 , ** q -value < 0.01 and ≥ 0.001 , *** q -value < 0.001 . APOE genotype 2/3 and 3/3 (Non E4) and APOE genotype 3/4 and 4/4 (E4). Ages 18-35 years old (Y), and 55+ years old (O). Males (M) and Females (F). Sterilised stools were generated by pooling participants' samples and autoclaving to sterility. The mean value for each group is indicated with the black point. Group sizes: male younger E4 carrier $n = 3$, male younger non-E4 carrier $n = 6$, male older E4 carrier $n = 5$, male older non-E4 carrier $n = 7$, female younger E4 carrier $n = 7$, female younger non-E4 carrier $n = 4$, female older E4 carrier $n = 11$, female older non-E4 carrier $n = 6$

2.3.3. Investigation of EC metabolism in *APOE-TR* mice

To gain further insight into the systemic metabolism of EC, a complementary animal study was initiated. This study focused on examining the influence of *APOE* genotype on EC metabolism in serum and urine following long-term intake, thereby elucidating genotype-specific metabolic responses. The results show that the flavonoid EC and its subsequent metabolites were only observed in the mice on the HFEC diets (Figure 2.7). Therefore, the HF-diet mice were excluded from subsequent analysis.

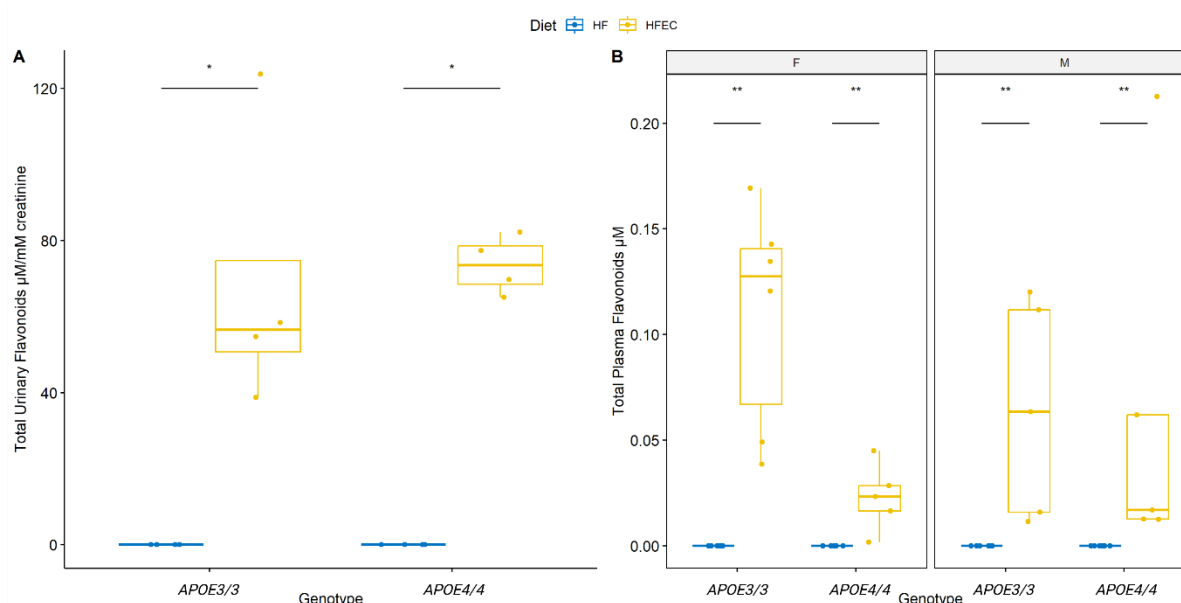


Figure 2.7: Total flavonoids and metabolites detected from *APOE3/3-TR* (*APOE3/3*) and *APOE4/4-TR* (*APOE4/4*) mice fed a high fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC). A) Urine concentration of the dietary flavonoids and metabolites normalised to urinary creatinine concentration. Urinary samples were pooled by cage, this resulted in the animals and by sex to produce the groups. Due to pooling all groups $n = 4$ B) Plasma concentration of flavonoids and metabolites. FDR q -values: * $0.05 > q\text{-value} \geq 0.01$, ** $0.01 > q\text{-value} \geq 0.001$, *** $0.0001 > q\text{-value}$. Group sizes: Female *APOE3/3-TR* HF $n = 6$, female *APOE3/3-TR* HFEC $n = 5$, female *APOE4/4-TR* HF $n = 6$, female *APOE4/4-TR* HFEC $n = 5$, male *APOE3/3-TR* HF $n = 6$, male *APOE3/3-TR* HFEC $n = 5$, male *APOE4/4-TR* HF $n = 6$, male *APOE4/4-TR* HFEC $n = 5$

2.3.3.1. Metabolites in Blood

Of the metabolites in the blood, two compounds demonstrated significance (Table 2.8). 5-(phenyl)- γ -valerolactone-3'-sulfate (PVL-3-S) demonstrated independent genotype and sex effects, as well as a significant interaction between the two (all $p < 0.050$). 5-(phenyl)valeric acid-3'-sulfate (PVA-3-S) demonstrated a genotype effect ($p = 0.030$). The genotype effect indicates that, irrespective of sex, the *APOE3/3-TR* animals had a higher urinary concentration than the *APOE4/4-TR* animals (Table 2.8). Full results in Supplementary Table 5.

Table 2.8: Significant (2-way permutational ANOVA $p < 0.05$) flavonoid metabolites present in the plasma samples of the *APOE3/3-TR* (*APOE3/3*) and *APOE4/4-TR* (*APOE4/4*) mice fed a High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC) following a two way ANOVA investigating the effects of the *APOE* genotype (Gen), Sex, and its interaction on the presence of these metabolites. Compounds are: Phenyl- γ -valerolactone-3-sulfate (PVL-3-S), Phenylvaleric acid-3-sulfate (PVA-3-S). Significant values ($p < 0.05$) are highlighted in bold. Group sizes: Female *APOE3/3-TR* HF $n = 6$, female *APOE3/3-TR* HFEC $n = 5$, female *APOE4/4-TR* HF $n = 6$, female *APOE4/4-TR* HFEC $n = 5$, male *APOE3/3-TR* HF $n = 6$, male *APOE3/3-TR* $n = 5$, male *APOE4/4-TR* HF $n = 6$, male *APOE4/4-TR* HFEC $n = 5$

Sex Gen Diet Compound	Females		Males		Permutational 2 way ANOVA p.values		
	<i>APOE3/3</i>	<i>APOE4/4</i>	<i>APOE3/3</i>	<i>APOE4/4</i>	Gen	Sex	Gen:Sex
	HFEC $\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	HFEC $\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	HFEC $\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	HFEC $\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)			
PVL-3-S	0.001 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.003	0.005	0.003
PVA-3-S	0.024 (0.017)	0.012 (0.009)	0.027 (0.017)	0.008 (0.008)	0.030	0.933	0.587

Running the pairwise analysis on these compounds reveals that both genotype and sex effects influence the metabolism of PVL-3-S. In females, there is a significant (FDR $q < 0.01$) genotype effect, with the *APOE3/3-TR* having higher excretion than their *APOE4/4-TR* counterparts. There is a sex effect with the female *APOE3/3-TR* mice having higher concentrations than their male equivalents (FDR $q < 0.01$). There is also a genotype and sex effect, with the female *APOE3/3-TR* mice having significantly higher excretion than the *APOE4/4-TR* males (Figure 2.8A). Pairwise analysis of the PVA-3-S highlights no pairwise differences (FDR $q > 0.05$); however, the permutational ANOVA indicates that, irrespective of sex, the *APOE3/3* animals have higher excretion than the *APOE4/4* (Figure 2.8B).

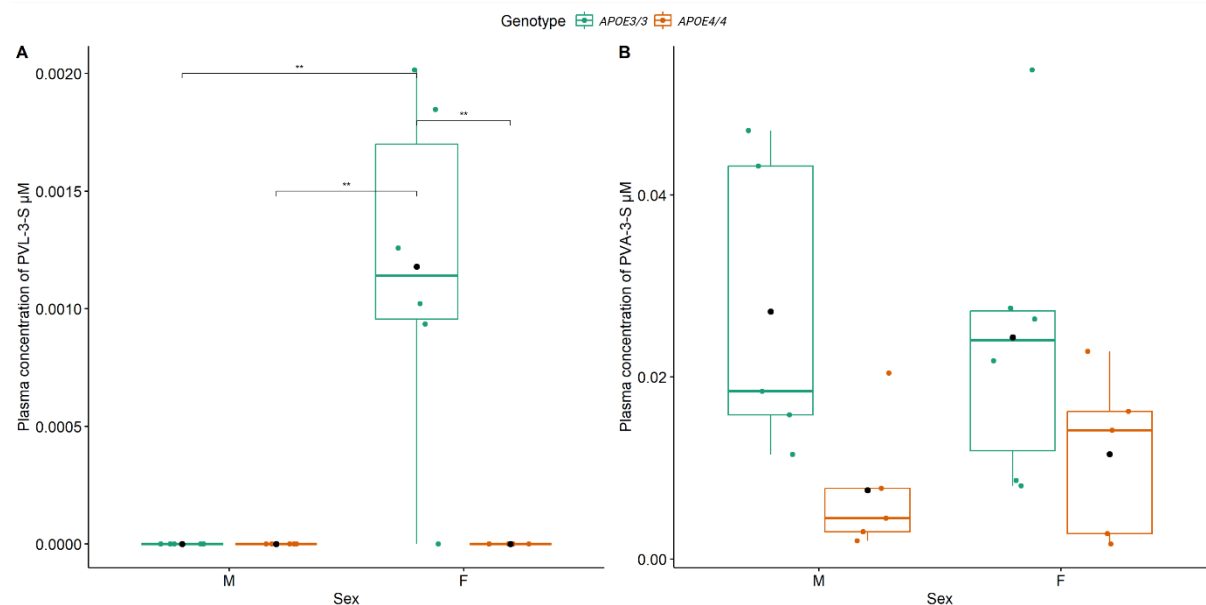


Figure 2.8 Concentration of A) Phenyl- γ -valerolactone-3'-sulfate (PVL-3-S) and B) Phenyl-valeric acid-3'-sulfate (PVA-3-S) present in the plasma samples of the *APOE3/3-TR* (*APOE3/3*) and *APOE4/4-TR* (*APOE4/4*) mice fed a High Fat (45% kcal from saturated fat) supplemented with 14.5 mg/kg body weight epicatechin (HFEC) following a two way ANOVA and pairwise comparison tests, investigating the effects of the *APOE* genotype, Sex, and its interaction on the presence of these metabolites. FDR q -values: * $0.05 > q\text{-value} \geq 0.01$, ** $0.01 > q\text{-value} \geq 0.001$, *** $0.0001 > q\text{-value}$. The mean of each group is

indicated with a black point. Group sizes: Female *APOE3/3-TR HF* n = 6, female *APOE3/3-TR HFEC* n = 5, female *APOE4/4-TR HF* n = 6, female *APOE4/4-TR HFEC* n = 5, male *APOE3/3-TR HF* n = 6, male *APOE3/3-TR* n = 5, male *APOE4/4-TR HF* n = 6, male *APOE4/4-TR HFEC* n = 5

2.3.3.2. *Metabolites in Urine*

The metabolites in the urine indicate that the *APOE* genotype and sex of a mouse can influence the compounds present in the urine of these animals. The following compounds are significantly different based on the sex: 3'-O-methoxyepicatechin (3-O-MeEC), 4-O-Methoxyepicatechin (4-O-MeEC), EC, Epicatechin-3'-S (EC-3-S), Epicatechin-4'/5/7-sulfate (EC-4/5/7-S), Methoxy-epicatechin-sulfate (MeO-EC-S) ion I, MeO-EC-S ion II, MeO-EC-S ion III, PVA-3-S (all p = 0.021). It is the case that for all the non-gut-derived metabolites, females have higher excretion of these compounds than males. However, the gut-derived valeric acid is significantly higher in males. The following compounds exhibit significant differences between the *APOE* genotypes: Epicatechin-diglucuronide (EC-DiGluAC) ion I (p = 0.021), EC-DiGluAC ion III (p = 0.021), Epicatechin-sulfate-glucuronide (EC-S-GluAC) ion I (p = 0.021), EC-S-GlcUA ion II (p = 0.021) and EC-S-GlcUA ion III (p = 0.043). It is the case that for all these compounds, the *APOE3/3* genotype exhibits significantly higher excretion than its *APOE4/4* counterparts. For full results of all metabolites, see Supplementary Table 6.

Table 2.9: Significant flavonoid metabolites present in the urine samples of the APOE3/3-TR (APOE3) and APOE4/4-TR (APOE4) mice fed a High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC). Animals were further grouped together by their sex to facilitate the Genotype Kruskal Wallis, or by their genotype to allow the Sex Kruskal. P-values <0.05 highlighted in bold. Compounds are: 3-O-Methoxy-epicatechin (3-O-MeEC), 4-O-Methoxy-epicatechin (4-O-MeEC), Epicatechin (EC), Epicatechin-3-sulfate (EC-3-S), Epicatechin-4'/5/7-sulfate (EC-4,5,7-S), Epicatechin-diglucuronide ion I (EC-DiGluAC I), Epicatechin-diglucuronide ion III (EC-DiGluAC III), Epicatechin-diglucuronide ion III (EC-DiGluAC III), Epicatechin-sulfate-glucuronide ion I (EC-S-GluAC I), Epicatechin-sulfate-glucuronide ion II (EC-S-GluAC II), Epicatechin-sulfate-glucuronide ion III (EC-S-GluAC III), Methoxy-epicatechin-sulfate ion I (MeO-EC I), Methoxy-epicatechin-sulfate ion II (MeO-EC II), Methoxy-epicatechin-sulfate ion III (MeO-EC III), Phenylvaleric acid-3-sulfate (PVA-3-S). Due to the urinary pooling by cage group size for all groups, n = 2

Sex	Female		Male		Kruskal Wallis p.value	
Genotype	APOE3/3	APOE4/4	APOE3/3	APOE4/4		
Diet	HFEC	HFEC	HFEC	HFEC		
Compound	$\bar{x}$ μ M/mmol creatine (SD)	$\bar{x}$ μ M/mmol creatine (SD)	$\bar{x}$ μ M/mmol creatine (SD)	$\bar{x}$ μ M/mmol creatine (SD)	Gen	Sex
3-O-MeEC	0.004 (0.000)	0.002 (0.003)	0.047 (0.030)	0.013 (0.008)	0.248	0.021
4-O-MeEC	0.007 (0.000)	0.004 (0.002)	0.042 (0.000)	0.025 (0.011)	0.248	0.021
EC	0.029 (0.002)	0.022 (0.007)	0.194 (0.090)	0.048 (0.003)	0.248	0.021
EC-3-S	11.993 (5.299)	7.756 (2.288)	3.632 (2.166)	3.638 (1.336)	0.773	0.021
EC-4,5,7-S	12.098 (5.150)	8.702 (0.954)	4.963 (0.186)	3.637 (1.334)	0.386	0.021
EC-DiGluAC I	0.103 (0.031)	0.054 (0.005)	0.075 (0.014)	0.048 (0.003)	0.021	0.564
EC-DiGluAC III	0.011 (0.000)	0.006 (0.001)	0.016 (0.009)	0.004 (0.005)	0.021	1.000
EC-S-GluAC I	0.656 (0.044)	0.488 (0.028)	0.877 (0.387)	0.194 (0.274)	0.021	0.564
EC-S-GluAC II	0.669 (0.019)	0.507 (0.039)	0.898 (0.420)	0.195 (0.275)	0.021	0.564
EC-S-GluAC III	2.930 (0.322)	1.888 (0.303)	2.968 (1.329)	1.250 (0.134)	0.043	0.386
MeO-EC I	5.357 (2.414)	3.521 (0.425)	1.364 (0.686)	1.412 (1.101)	0.773	0.021
MeO-EC II	0.403 (0.025)	0.305 (0.023)	0.231 (0.001)	0.185 (0.054)	0.248	0.021
MeO-EC III	0.325 (0.058)	0.224 (0.020)	0.173 (0.037)	0.112 (0.026)	0.248	0.021
PVA-3-S	0.031 (0.001)	0.035 (0.006)	0.074 (0.040)	0.086 (0.025)	0.564	0.021

2.4. Discussion

2.4.1. Discussion of the Human Colon Model

2.4.1.1. *pH Control*

The pH of an *in vitro* colon model must be tightly controlled. As microbes within the system ferment the compounds, they produce organic acids. For example, the microbial metabolism of EC leads to the production of valeric acids, and the degradation of dietary fibres within the stool's matrix leads to the production of SCFAs [177, 243]. These organic acids lead to a drop in environmental pH. *In vivo* and in specific batch and continuous *in vitro* colon models, the pH is controlled homeostatically [242, 244]. However, in this simplified batch *in vitro* colon model, the control relies solely on the media's buffering capacity. A tight control of the pH of the model is required, as a change in pH greater than one pH unit alters the structure of the microbiota that inhabits the model, and in turn, alters the associated metabolism [245]. The primary aim of the subsequent study is to investigate interindividual metabolism of EC in relation to *APOE* genotype, sex, and age groups. Therefore, any significant changes in pH introduce another factor that influences this metabolism. The selected model used replicates and tightly maintains a change in pH to within 0.3 pH units; the full range is within physiologically relevant boundaries, and there are no significant differences between the donors or between the time points; the model used is suitable for use in the subsequent metabolism to control the pH of the media during metabolism.

2.4.1.2. *Colon Model Metabolism*

The model also successfully metabolises the EC present in the wells. All wells demonstrate a loss of EC over the first six hours following inoculation. Over the first three time points (0-2 hours), the metabolism of both the bacterial and sterile wells mirrors one another. The reasons for this mirroring between the bacterial and sterile wells could be twofold. Firstly, as the drop occurs in both the bacterial and sterile wells, this could be spontaneous chemical degradation of EC. Spontaneous degradation of flavonoids has been previously reported in *in vitro* colon models; the antioxidant capacity of the flavonoid backbone, paired with the close to neutral pH of the colon model media, leads

to an unstable molecular structure [246]. This spontaneous oxidation of EC leads to its spontaneous degradation, changing its molecular structure and leading to this observed loss within the system [247, 248]. Secondly, spontaneous loss occurs in all samples in the first three hours, not just the sterile controls, indicating that there is no microbial fermentation at the beginning of the fermentation, which could be due to the microbes within the bacterial wells being in a lag phase. The microbiotas within these wells adapt to their new environment. EC has been shown to suppress the growth of some microbes and promote the growth of others [189, 190]. On top of these population shifts, the microbes that can metabolise EC need time to synthesise the cell machinery and subsequent enzymes used in metabolism in the new environment [249]. A lag phase in flavonoid metabolism has previously been reported, and it appears extended by the presence of alternative carbon sources available to the microbes [250]. Several alternative carbon sources were present within the wells, including dietary residues in the stool samples and peptone water in the media. These carbon sources were potentially used in preference to the EC, increasing the lag time in its catabolism, until they were depleted.

At the three-hour time point, significance does emerge between the wells. There is a significant difference between the two donors' bacterial wells and between each donor's respective bacterial and sterile wells. This indicates that at this point, for the donors, the bacteria within the wells are starting to metabolise EC at a faster rate than the spontaneous degradation of EC, and that at this point, more EC has been metabolised by donor 011 than by donor 053. There is, however, no significant difference observed between any of the data points at time points 4-24 hours. There is almost an interindividual difference ($p = 0.06$) between the two donors' bacterial wells at the time point of five hours. However, we can see from the trends that the two donors' bacterial wells follow a different rate of EC metabolism. Although 053 has a slower start, after the rate of EC loss increases, and the majority of EC metabolism is completed. However, donor 011 continues to decline constantly, and all EC has been metabolised within six hours. Although there is no significant difference between them, the two donors do follow different pathways for the rate of EC metabolism, mirroring previous work in human colon models, which shows an interindividual difference in EC loss [229].

However, at the 24-hour time point, there are significant differences between the participants and between the bacterial wells and their sterile controls. This firstly indicates that we can successfully produce some of the products of metabolism that have been previously reported in other *in vitro* colon models. Secondly, there is a significant difference between the two donors; both donors can produce 3,4-DiHPVL, with 011 producing significantly more of this metabolite than 053. On the other hand, the 3-HPVL is only produced by donor 053. Again, these findings mirror the findings of Liu et al. and of Di Pede et al., who saw interindividual differences between the loss of EC and the production of the gut-derived valerolactones [182, 229]. Liu et al. had 24 participants, and at the two-hour time point, they showed a significant difference in the EC present, although not all participants had significant differences, mirroring the EC metabolism in this study. Liu et al. demonstrated that the significance that they saw in the metabolism was mainly due to the gut microbiota composition. As there was no microbiota analysis in this study, it may be the case that there wasn't enough significance between the microbes that drive the loss of EC, but there was enough in other microbes linked to the generation of the valerolactones. However, it is not possible to draw any conclusions at this point.

These results primarily show that this colon model successfully replicates similar findings of other colon models in the literature and produces the previously observed interindividual differences. It also indicates some suitable time points for subsequent sample collection, with a larger dataset. The baseline time point is essential for collecting the concentration of EC. As we saw the most metabolite production at the twenty-four-hour time point, and there were significant differences between the groups, it is essential to collect this time point. Due to the limited significance between the groups, there are limited conclusions that can be drawn for time points in between. There was an interindividual difference seen in the EC concentration at three hours, but the significance was subsequently lost, and subsequent research was needed to determine the suitability of the time points to be collected.

As was seen in the first colon model experiment, the breakdown of EC to its metabolites is dependent on the presence of live bacterial cells. There is still a loss of EC in the sterile controls, however, as was established with the first experiment, this breakdown is most

likely due to spontaneous environmental degradation [246], as there is still a relatively higher percentage of EC remaining at the end of the experiment in the sterile wells compared to the wells with live faecal samples. There is also little production of bacterially derived metabolites in the sterile wells. When detected, the mean is very low and the standard deviation is very high, indicating that this is likely an artefact of the LC-MS detection in random samples.

The human colon model work indicates that the *APOE* genotype may play a role in regulating the metabolism of EC and the production of the gut-derived phenyl-γ-valerolactones (PVLs) and phenyl-valeric acids (PVAs). In particular, the production of the 3',4' dihydroxy and 3' hydroxy forms of these compounds. It is of note that the differences observed follow this pathway, and not the 4' monohydroxy forms of the compounds. It may be the case that bacterial taxa that are responsible for the metabolism of these compounds are different from those which produce the 4' monohydroxy form, and the bacterial taxa responsible for these metabolites are sensitive to the *APOE* genotype of an individual. However, the bacteria which are responsible for these transformations are not well-characterised in the current scientific literature [251].

However, the *APOE* genotype may not have a monopoly on regulating this metabolic pathway; age and sex have also been shown to influence it. It is possible that the metabolism of EC and the production of gut-derived metabolites are generally higher in non-*APOE4* carriers than in their *APOE4* carrier counterparts; this trend is observed across all groups except the older female participants, where it is reversed. These differences could be attributed to variations in the microbiota of these individuals, which in turn alter the microbial metabolism of EC. Previous research into the effect of the *APOE* genotype on microbiota structure has only stratified by the genotype [104]. Prior to this study, there has been no further age or sex stratification. It may be the case that the differences observed between the genotypes are altered in older females compared to other sex and age groups, resulting in the differences demonstrated in the DIAMOND samples. Although the genotype often produced significance in the 3-way permutational ANOVA of the results, the effect on its own was not strong enough to produce pairwise interactions between the groups, indicating that the effect of an individual's *APOE*

genotype is not as strong as age and sex in influencing the metabolic breakdown of EC and production of metabolites.

Age, biological sex, and *APOE* genotype may also not be the only factors that modify the metabolic pathway of EC. Firstly, the results from the FFQ indicate that the intake of some nutrients varies significantly between the groups. In particular, the intake of dietary fibre, carotene and flavan-3-ols varies between the groups. Although the FFQ is a semi-quantitative measure of dietary intake, its limitations include misestimating portion sizes and relying on participants' honesty and recall. These results provide an estimate of participants' dietary intake. The majority of the macro and micronutrients that exhibit significant differences, such as TUFAs, total sugars, protein, and vitamins, are absorbed in the upper GI tract and may have a limited effect on the gut microbiota. However, the differences in the intake of dietary fibres, flavan-3-ols and carotene are all known to impact the structure of the colonic microbiota and thus could interfere with the metabolism of flavan-3-ols [252-254].

Secondly, within the groups, there are also large variations in the metabolism of EC and the production of microbially derived metabolites. This indicates that even within the groups, there are other factors that influence the metabolism of EC. This may be a result of other non-modifiable factors, such as diet, influencing the metabolism of EC. The FFQ results show that there is a considerable variation within the groups for the intake of many food constituents, in turn shaping the microbiota. To try to counter the effects of diet, participants followed a washout diet free from tea, coffee, cocoa, and berries before donation. The results show that adherence, particularly to avoiding catechins, was mixed, as catechins were detected in the samples. This may be because of several reasons, including participants failing to avoid the selected items, or the list being too narrow, paired with the ubiquitous nature of flavonoids. For example, several other non-excluded plant-based foods are known to contain catechin [255, 256]. These could still permit catechin to enter the diet and interfere with the microbiota and the subsequent metabolism of EC. Other modifiable factors, such as medication, could also impact the microbiota [257], although we excluded individuals who had taken antibiotics in the last 90 days, due to its known effects on the microbiota, many other medications that we did not control for could also impact on the microbiota [258], leading to the wide variations

in some of the responses. These other factors could have a greater impact than the age, sex, and *APOE* genotype of an individual and mask some of the observed results. Although non-modifiable factors such as age, sex, and *APOE* genotype can shape the microbiota, other modifiable risk factors, such as diet and medication, may have an effect within these groups and lead to the wide variation observed, which in turn may mask some of the pairwise differences.

2.4.2. Discussion of *APOE-TR* animals

When moving from the *in vitro* human colon model to the *in vivo* animal model, there are differences in the metabolism of both the non-gut-derived EC metabolites and some of the gut-derived metabolites. In the plasma, the concentration of compounds is quite low; this is expected, as many studies have reported low concentrations of plasma flavonoids, even in individuals with high flavonoid intake [259, 260], and it may be the case that many of the compounds in the plasma have concentrations below the limit of detection. However, many of these compounds are concentrated within the urine. It is, however, the case that the most abundant compounds reach a concentration which is detectable in the plasma. There are two compounds that interact with the *APOE* genotype and the sex of the individuals. The two gut-derived compounds show an effect of the *APOE* genotype and sex on their metabolism. It is the case where the PVL-3-S is only detected in the female *APOE3/3-TR* mice and has significantly higher concentrations than the other genotypes and sexes, pairing this result with the subsequent PVA-3-S, which shows that this compound is detected in all groups, but has higher concentrations in the *APOE3/3-TR* than the *APOE4/4-TR* mice, and this reaches significance in the male mice only. The PVA is a microbially derived daughter compound of the PVL [177], and its detection in the plasma indicates that the microbiota of the other groups may have continued to metabolise the PVL before it was absorbed, whereas the female *APOE3/3-TR* mice absorbed the PVL. PVA-3-S has higher plasma concentrations in the *APOE3/3-TR* than their *APOE4/4-TR* counterparts, and this difference reaches significance in the male mice only, highlighting that there may be differences in both sex and genotype in the microbial fermentation of the EC to the PVA and/or subsequent absorption across the colonic epithelium and/or phase II metabolism of this compound. In the urine results,

there is also a sex difference in the concentration of the PVA-3-S, with males reflecting the sex differences, with significantly higher urinary concentrations in the male mice than in the females. There were, however, no genotype differences, and due to the pooling of the samples, it was not possible to differentiate the effect of the genotype on the metabolism simultaneously to see whether this sex difference was also *APOE* genotype dependent, as was seen in the plasma samples.

In urinary samples, there were also differences highlighted between the genotypes and sex of many of the non-gut-derived EC metabolites. These differences were not expected, as these compounds are absorbed in the upper GI tract prior to exposure and metabolism by the colonic microbiota. Ottaviani et al. examined the absorption of radio-labelled EC, identifying that approximately 30% of EC is absorbed in the upper GI tract [177]. However, this work did not incorporate the EC into a food matrix; instead, the participants were fed the ¹⁴C EC in an aqueous solution. It may be the case that the high-fat content of the HF diet increases the uptake of the EC in the upper GI tract. It has been identified that the presence of fat in the diet alters the uptake and metabolism of quercetin and some anthocyanins [261], and thus, the fat content of the diet may promote the uptake of EC in the mice. To see whether an HF diet influences the metabolism of EC, it would be beneficial in future to use low-fat study arms to control for the effects of dietary fat content on altering EC metabolism. The permeability of the gut will also interfere with the uptake of dietary compounds. It may also be the case that the *APOE* genotype and sex of an individual play a role in the permeability of the intestinal epithelium, thereby altering the uptake between the groups. Previous work has identified that *APOE4* carriers and females have higher inflammation [46-48, 96], and increased intestinal inflammation is linked to increased gut permeability. In the observed results, females had higher levels of the EC metabolites, whereas the *APOE3/3-TR* mice had higher concentrations of the intestinal metabolites, not the *APOE4/4-TR*. Therefore, further work should be carried out in future to investigate the mechanisms behind the *APOE* genotype and sex effects on the uptake of EC in the GI tract. It is of note that there are differences observed between the human colon model samples and the *APOE-TR* mouse samples. There is also the possibility that other mechanisms are driving the differences observed. Many of the non-gut-derived metabolites are either sulfated or glucuronidated forms, and they are

conjugated in the liver as part of the phase II metabolism. It may also be the case that sex and *APOE* genotypes alter the hepatic phase II metabolism, as the body conjugates the EC and its metabolites before excretion. This could demonstrate some of the differences observed between the DIAMOND samples and the *APOE*-TR mouse samples. On one hand, it was not possible to detect phase I and II metabolism in the *in vitro* colon model as there were no host cells present in the model; on the other hand, in the *APOE*-TR model, the concentrations of non-conjugated forms of the EC metabolites would have been low, as the mice's liver and kidneys preferentially excreted the hydrophilic conjugates. The effects of the *APOE* genotype and sex on the liver's phase II metabolism are explored in Chapter 4. Additionally, differences were observed in the metabolite backbone produced by each model. For example, the DIAMOND study results produced differences between the 3,4-DiHPVL, 3-HPVL, and 3,4-DiHPVA; the *APOE*-TR mice produced differences in the PVL and PVA. This difference may be due to species differences between the human and murine microbiota, which led to differences in the metabolites produced. The microbiota composition and function of each study are explored in Chapter 3. Nevertheless, both models demonstrate the effect of the *APOE* genotype on EC metabolism, and that it is also altered by other factors such as sex and age.

Due to the volumes of urine extracted from the mice, many of the urinary results had to be pooled. This limited the number of comparisons. There is the possibility that there are interactions between the *APOE* genotype and sex; however, it was not possible to view these interactions in these urine samples. Alternate methodologies should be used in future to collect the urine samples, ensuring that there are sufficient volumes collected for analysis without pooling, which would result in greater resolution in the statistical tests.

From these results, we can conclude that an individual's *APOE* genotype may play a role in modifying the metabolism of EC. The *APOE4* allele may reduce the loss of EC and subsequent metabolite production when examining colonic metabolism alone. However, the *APOE* genotype does not act independently, and sex can often interact with this effect, producing greater differences than what is observed between the genotypes. It is also the case that in moving from an *in vitro* model to an *in vivo* mouse model, other

factors need to be considered before a conclusion can be fully drawn, such as the role of the absorption of EC in the upper GI tract and the role of hepatic phase II conjugation, stages of metabolism which were not possible to observe in the DIAMOND samples. Although this chapter has explored the effects of the *APOE* genotype, sex, and age on the metabolism of EC, the next steps are to investigate the mechanisms driving the observed differences. The first step is to investigate whether there are *APOE* genotype, sex, and age differences in the makeup of the colonic microbiota, which could provide light to the differences between the gut-derived metabolites.

2.5. Conclusion and Next Steps

These results indicate that the interindividual metabolites produced from the breakdown of EC may be influenced by an individual's *APOE* genotype, as well as their sex and age. It is often the case that these factors do not act independently of one another; instead, they can collaborate to alter the metabolism. Although these results indicate differences in the production of colonic metabolites, it is currently assumed that these differences are driven by variations in the microbiota. Further work is required to identify the microbes present in both these models, to see whether there are any patterns that can be drawn between the age, sex, and *APOE* genotype groups. The metabolism of EC is potentially altered by the microbiota of these individuals; however, as it is part of the GBA, the effect is bidirectional, and further work is required to understand the role that EC plays in the overall metabolism of the microbiota. Further experimental work is needed to examine the entire metabolome of the colon comprehensively. The microbiota of the human and animal participants were sequenced, and the next steps are to investigate the genomes of these microbiotas, as well as their overall functionality. The structure and functionality of the microbiota from both the DIAMOND participants and *APOE-TR* mice will be explored in Chapter 3.

In addition to the differences in the concentration of gut-derived metabolites, the *in vivo* work also highlighted differences in the concentration of structurally related EC (SREC) metabolites. These SREC metabolites are absorbed in the upper GI tract before exposure to the colonic microbiota [177]. However, the genotype and sex differences were

observed in the plasma and the urine of the animals. There are several metabolic steps that these compounds undergo between their absorption across the intestinal epithelium, their circulation in the blood, and excretion into the urine by the kidneys. One of these steps is the phase II metabolism in the liver; thus, further work is needed to address whether there are differences in the phase II metabolism of these compounds in the liver. The roles that the *APOE* genotype and sex play in phase II metabolism are explored in Chapter 4.

Chapter 3: *APOE* genotype, sex, and diet effects on the gut microbiota speciation and function

3.1. Introduction:

The flavan-3-ol EC possesses a structurally complex molecular configuration that cannot be metabolised by endogenous enzymes [262]. Consequently, only approximately 30% of ingested EC is absorbed in the upper GI tract, while the remaining 70% proceeds to the colon, where it undergoes extensive biotransformation by the colonic microbiota into various metabolites, including valerolactones and valeric acids [177]. Previous studies have established that host factors such as age, sex, and *APOE* genotype significantly influence the composition and function of the gut microbiota. These host-specific variables are associated with physiological differences, such as chromosomal [90], hormonal [89], and inflammatory profiles [83] that can alter the colonic environment and consequently, the microbial communities capable of colonising it. These inter-individual differences modulate both the diversity and the abundance of specific microbial taxa residing in the colon [101, 102, 104]. The previous chapter demonstrated that the catabolism of EC and the resultant metabolite profile are modulated by *APOE* genotype, sex, and age. These findings suggest that the interaction of these three factors contributes to variability in the production of EC-derived microbial metabolites (See Chapter 2.3). However, the mechanisms underlying these observed differences remain to be fully elucidated. Building upon these findings, the current chapter tests the hypothesis that inter-individual differences in EC metabolism are attributable to underlying variations in gut microbiota composition.

In addition to the *APOE* genotype, numerous factors influence gut microbiota composition. Given that EC metabolism is microbiota-dependent, the presence of EC in the gut exerts a selective pressure favouring microbial taxa capable of utilising EC as a carbon source, over non-utilisers [181]. Beyond serving as a substrate, EC actively shapes microbial composition through additional mechanisms, including the promotion of several beneficial genera and suppression of potentially pathogenic taxa [189, 190]. These modulatory effects are likely mediated by the bioactivity of EC and its metabolites,

which can exert antimicrobial activity by disrupting microbial biosynthetic pathways [191-193] and altering gut physiological conditions, thereby reshaping the colonic environment and the resident microbiota [185, 194]. Given that baseline microbiota composition varies with age, sex, and *APOE* genotype, this chapter further investigates whether the microbial response to EC supplementation is contingent on these host variables.

As the gut microbiota plays a crucial role in the GBA, it is essential to not only assess its taxonomic composition but also its functional potential. This includes the microbial metabolism of EC and other compounds that collectively shape the microbial metabolome, which in turn influences host neurophysiology and systemic health [106]. Accordingly, this chapter will also explore the hypothesis that age, sex, *APOE* genotype, and EC supplementation interact to affect the overall metabolic functionality of the gut microbiota.

3.2. Materials and Methods:

3.2.1. Study designs:

The mouse study described in this section was part of the same experiment framework outlined in Chapter 2. A total of 47 mice were used, equally divided between *APOE3/3-TR* and *APOE4/4-TR* genotypes. Mice were further stratified by sex and dietary intervention- See Chapter 2.2.3 for full study details. Mice were assigned to either an HF diet or an HFEC (200 mg/Kg; EC) diet for 20 weeks. At the end of the experiments, the colon and caeca were collected and immediately placed on dry ice and subsequently stored at -80 °C.

Human faecal samples were collected concurrently with those used for metabolic analysis in the colon model, in compliance with the DIAMOND study protocol (IRAS number 319832): see Chapter 2.2.2 for full study details. Samples were transported using the Gut Alive Kit (Microviable, Spain), which maintains anaerobic and ambient conditions necessary to preserve microbiota viability for up to 72 hours [227]. All samples were processed within 48 hours of collection. Following preparation of the 25% faecal glycerol

stock, any remaining unprocessed stool was frozen at -80 °C for subsequent microbial DNA extraction.

3.2.2. Microbial DNA extraction and 16S rRNA amplicon sequencing

Microbial DNA was isolated from approximately 200 mg of faecal samples using the Invitrogen™ PureLink™ Microbiome DNA Purification Kit (Thermo Fisher Scientific, USA) as per the manufacturer's instructions. In the rodent study, colon contents were thawed prior to DNA isolation using the same kit. A total of 41 mice out of 47 were included in the downstream analysis due to insufficient sample material from six animals. For the *in vitro* study, 45 of the 49 stool samples yielded sufficient material for both 16S rRNA sequencing and colonic fermentation experiments.

DNA concentration was measured using a Nanodrop 2000 Spectrophotometer (Fisher Scientific, UK), while quality and integrity were assessed via agarose gel electrophoresis. The V3-V4 hypervariable regions of the 16S rRNA gene were sequenced using the Illumina NovaSeq 6000 PE250. Sequencing was conducted by BGI Tech Solutions (BG ITech Solutions, Poland) for the animal samples and Novogene (UK) for the DIAMOND cohort.

Raw sequence data were processed using Uparse software (Uparse v7.0.1001) [19], and operational taxonomic units (OTUs) were assigned at a $\geq 97\%$ similarity threshold. Representative sequences were assigned to the same OTU. A representative sequence for each OTU was annotated using Mothur software against the SILVA SSU rRNA database (release 138) [263].

3.2.3. ¹H NMR Caecal Metabolomic Analysis

Metabolic profiling of caecal samples from *APOE-TR* mice was conducted using proton nuclear magnetic resonance (¹H NMR) spectroscopy. Sample preparation followed a protocol previously described [104], with minor modifications. Briefly, 50mg of murine caecal sample was mixed with 800 μ L of NMR buffer [0.26 g Na₂HPO₄, 1.44 g K₂HPO₄, 17 mg TSP (sodium 3-(trimethylsilyl)-propionate-d₄ (3-Trimethylsilyl) propionic-2,2,3,3-d₄ acid sodium salt) in 100 mL deuterated water (D₂O)]. After vortexing to achieve

complete homogenisation, samples were centrifuged at 15,000 g for 5 minutes. A 550 μ L aliquot of the supernatant was transferred to a 5-mm NMR tube for spectral acquisition.

Spectra were acquired using a Bruker Avance 600-MHz spectrometer equipped with a 5-mm TCI proton-optimised triple-resonance NMR inverse cryoprobe and a 24-slot autosampler (Bruker, Coventry, England). All measures were performed at 300 K (27 °C). Each spectrum consisted of 128 scans of 65,536 complex data points with a spectral width of 20 ppm (acquisition time 2.6 s). Water signal suppression was achieved using a standard NOESY-PR1D pre-saturation pulse sequence, with low-power selective irradiation at the water frequency during the recycle delay ($D1 = 4$ s) and mixing time ($D8 = 0.01$ s). A 90 ° pulse length of 8.1 μ s was set for all samples.

Spectral processing included exponential multiplication with 0.3-Hz line broadening, zero-filling, manual phasing, baseline correction, and chemical shift referencing to the TSP methyl signal at 0 ppm. Metabolites were identified and quantified using the Chenomx software (version 8.6).

3.2.4. Inference of metabolic pathways

Due to the limited volume of human faecal samples acquired in the DIAMOND study, ^1H NMR-based metabolomics analysis could not be performed. Instead, the metabolic potential of the participants' gut microbiota was inferred through bioinformatic approaches. Microbial DNA was extracted from the samples, sequenced, and OTU tables were generated as previously described. These OTU tables were analysed using the PICRUSt 2 (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States modules 2) pipeline to predict microbial functional profiles based on the Kyoto Encyclopaedia of Genes and Genomes (KEGG). Functional differences in KEGG orthologous pathways were evaluated at level 3 using classical univariate statistical methods. Specifically, Kruskal-Wallis tests were applied, and multiple testing was accounted for using the BH method for FDR. Given the large number of pairwise comparisons, a statistical significance cut-off was defined as an adjusted $q < 0.2$.

3.2.5. Statistical analysis

For the microbiota analysis, the analysis was carried out by microbiomenalyst.ca [264]. OTU abundance data were normalised to the lowest sequencing depth across all samples (e.g. rarefaction). Alpha-diversity was assessed using Chao1, Simpson and Shannon H diversity index, whilst beta diversity was assessed using Aitchison distances following centred log-ratio transformation (CLR). Diversity metrics are plotted as box and whisker plots with individual data points overlaid and the mean represented by a black dot. In the animal study, alpha diversity was analysed using a mixed linear model:

$$\text{alpha diversity score} \sim \text{APOE genotype} * \text{Sex} * \text{Diet} + (1|\text{Cage})$$

where the animal's cage was included as a random effect. For the DIAMOND cohort, the model used was:

$$\text{alpha diversity score} \sim \text{APOE4 carrier status} * \text{Sex} * \text{Age group}.$$

Both models were subjected to 3-way ANOVA, and statistically significant results ($p < 0.05$) were further examined via pairwise comparison using the least square means method. Multiple testing correction was applied using the BH method for FDR. Beta diversity significance was determined using Kruskal–Wallis or Permutational Multivariate Analysis of Variance (PERMANOVA). Principal Coordinate Analysis (PCoA) was used for visualisation, with significance defined as $p < 0.05$. Taxonomic comparisons at the Phylum, Family and Genus levels were conducted using classical univariate analysis via the Kruskal–Wallis test, coupled with the BH method for FDR correction. Given the large number of pairwise comparisons, an adjusted $q < 0.2$ was considered significant. All results were visualised using box and whisker plots.

For metabolomics analysis, metabolomic analysis was carried out through metaboanalyst.ca [265]. Data were stratified by sex prior to normalisation, with each sex normalised to its respective APOE3/3-TR HF control group. Sparse Partial Least Squares-Discriminant Analysis (sPLS-DA) was employed to visualise the contribution of metabolites' variance to group separation, and corresponding loading plots were used to identify key discriminative metabolites. Metabolite comparisons were conducted using univariate analysis via the Kruskal–Wallis test with BH FDR correction for multiple testing.

Given the extensive number of pairwise comparisons, statistical significance was defined as an adjusted $q < 0.2$. Results are visualised using box-and-whisker plots.

Correlation analysis between metabolomics data and microbiota data was performed using the M2IA platform [266]. Features with missing values in over 80% of samples or with a relative standard deviation $< 30\%$ were excluded [267]. The remaining missing values were imputed using the Random Forest method. Data were normalised using total sum scaling. All correlation analyses were conducted using Spearman's rank-order correlation [268].

3.3. Results:

3.3.1. EC supplementation affects microbiota structure and metabolism in *APOE-TR* mice

3.3.1.1. *Relative abundance of microbiota*

The relative abundance profiles provide a concise visual representation of the colonic microbiota composition in mice. At the Phylum level, Firmicutes were the most predominant across all groups; however, both inter- and intra-group variations were observed. Notably, a substantial proportion of microbiotas consisted of bacteria of unknown phyla. Female mice exhibited a higher abundance of Bacteroidetes, and those receiving EC supplementation showed an increased presence of Actinobacteria, an observation not mirrored in the male groups (**Error! Reference source not found.A**). At the family levels, differences related to sex and genotype were evident, particularly in the abundance of Lactobacillaceae and Lachnospiraceae. In female mice, these families were relatively balanced. In contrast, male *APOE3/3-TR* mice, regardless of diet, displayed a higher abundance of Lactobacillaceae, with minimal Lachnospiraceae. Male *APOE4/4-TR* mice, however, showed a more balanced composition, resembling that of the females (**Error! Reference source not found.B**). At the genus level, *Lactobacillus* (family Lactobacillaceae) was the most abundant and consistently present across all groups, in line with family-level observations. More pronounced differences emerged within the Lachnospiraceae family at the genus level, further emphasising the influence of sex and *APOE* genotype. Genera such as *Blautia*, *Roseburia*, *Lachnoclostridium*, and the *NK4A136* group (all within the Lachnospiraceae) were consistently present in all female groups and in male *APOE4/4-TR* mice, irrespective of diet. In contrast, only *Lachnoclostridium* was detected in the microbiota of male *APOE3/3-TR* mice (**Error! Reference source not found.C**).

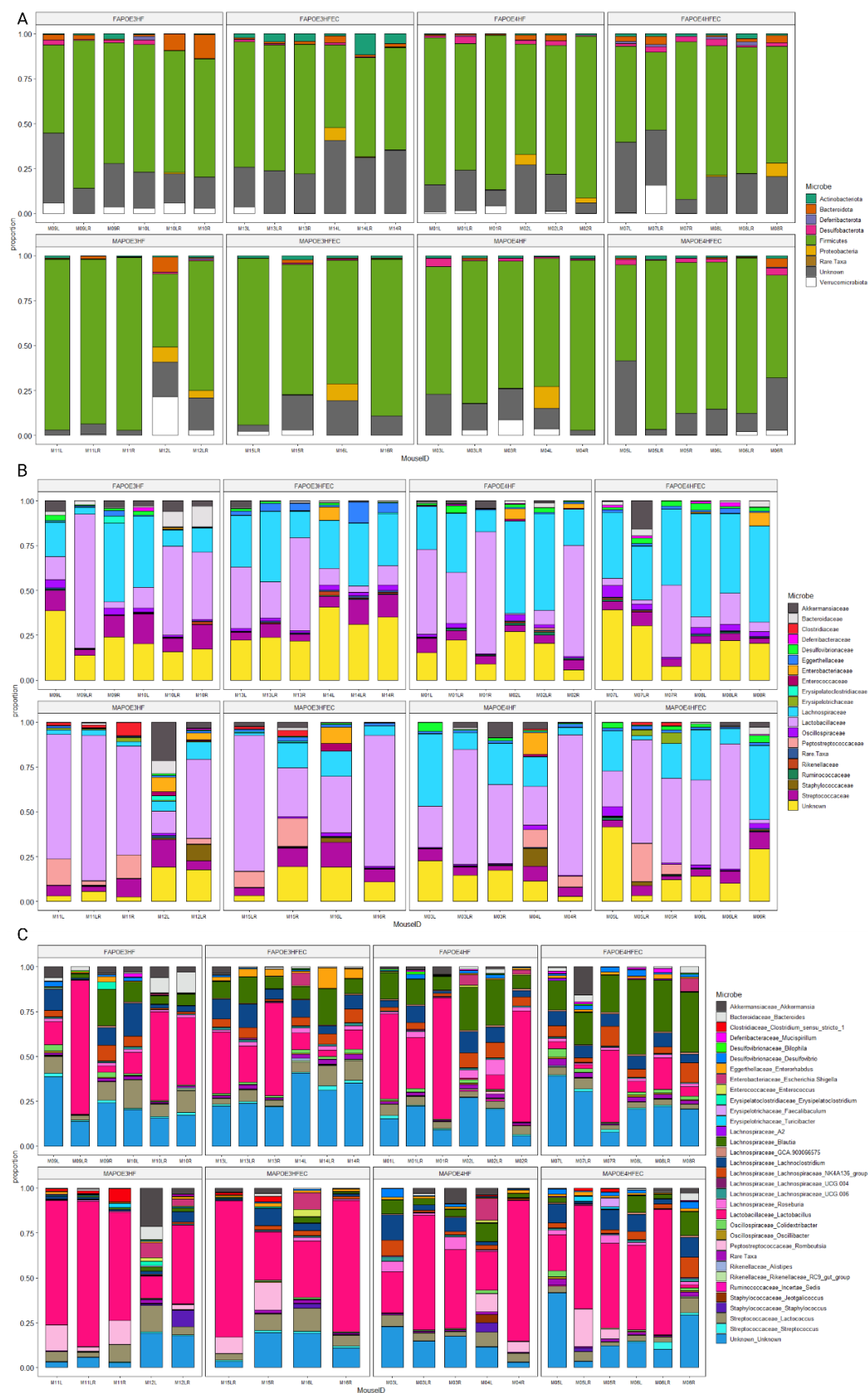


Figure 3.1 Relative abundance of colonic microbiota of male (M) and female (F) APOE3/3-TR (APOE3) and APOE4/4-TR (APOE4) mice fed a high fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 14.5 mg/kg body weight epicatechin (HFEC) presented at the A) Phylum B) Family and C) Genus levels. Group sizes: FAPOE3HF n = 6, FAPOE3HFEC n = 6, FAPOE4HF n = 6, FAPOE4HFEC n = 6, MAPOE3HF n = 5, MAPOE3HFEC n = 4, MAPOE4HF n = 5, MAPOE4HFEC n = 6

3.3.1.2. Alpha and beta diversity metrics

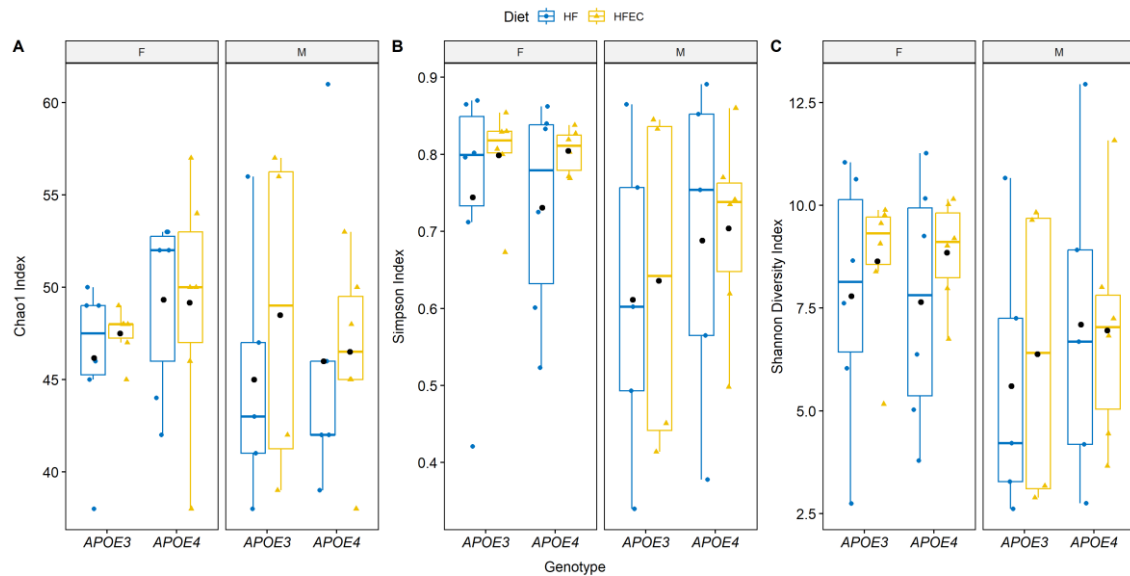


Figure 3.2 Alpha Diversity of the Colonic Microbiota of Male (M) and Female (F) APOE3/3-TR (APOE3) and APOE4/4-TR (APOE4) mice fed a high-fat (HF) 45% kcal from saturated fat or High-fat (45% kcal from saturated fat) supplemented with 14.5 mg/kg body weight epicatechin (HFEC). A) Chao1 Index for species richness. B) Simpson Index for species evenness. C) Shannon Diversity Index for both genus richness and evenness. The black point in each boxplot represents the group's mean. Group sizes: FAPOE3HF $n = 6$, FAPOE3HFEC $n = 6$, FAPOE4HF $n = 6$, FAPOE4HFEC $n = 6$, MAPOE3HF $n = 5$, MAPOE3HFEC $n = 4$, MAPOE4HF $n = 5$, MAPOE4HFEC $n = 6$

While relative abundance plots offer a visual representation of intra- and inter-group variation in microbiota, quantitative analyses of overall microbial structure are essential to substantiate these observations. Alpha diversity metrics assess within-sample diversity, encompassing both genus richness and evenness. Genus' richness, reflecting the number of distinct genera, was estimated using the Chao1 index, where higher values indicate greater microbial diversity (Figure 3.2A). Analysis of Chao1 scores across all mouse groups revealed that male mice exhibited significantly lower species richness than females ($p = 0.03$). However, no statistically significant differences were detected in any pairwise group comparisons. Among female mice, those with the *APOE4/4* genotype appeared to have greater species richness than those with the *APOE3/3* genotype, although this difference was not statistically significant ($p > 0.05$). Epicatechin supplementation was associated with an increase in species richness in *APOE3/3*-TR females, but not in *APOE4/4*-TR females; however, this difference did not reach significance ($p > 0.05$). In male mice, genus-level richness showed higher variability overall, with no consistent genotype-related differences observed under either the HF or

HFEC diet ($p > 0.05$). While epicatechin supplementation tended to increase richness in both *APOE* genotypes in males, no significant differences were observed between dietary groups ($p > 0.05$).

Alpha diversity includes a measure of genera evenness, which reflects how uniformly individual microbes are distributed across the genera present within a sample. The Simpson index quantifies this evenness, with higher values indicating a more balanced microbial community where no single genus dominates. As shown in Figure 3.2B, no statistically significant differences in species evenness were observed between experimental groups (all $p > 0.05$). However, biological trends suggest that male mice exhibit lower species evenness than females across all genotype and diet combinations. In female mice, genotype appears to have minimal impact on species evenness under either HF or HFEC diets. Notably, the HFEC diet was associated with higher average evenness compared to the HF diet, irrespective of *APOE* genotype, suggesting a potential role of EC in promoting microbial evenness. In contrast, *APOE4/4*-TR mice under both HF and HFEC conditions tended to show greater species evenness than their *APOE3/3*-TR counterparts. Across both genotypes, EC supplementation consistently increased species evenness relative to the HF diet alone.

Microbial diversity, encompassing both richness and evenness, was assessed using the Shannon diversity index, with higher values indicating greater within-sample microbial diversity. As shown in Figure 3.2C, Shannon index results closely mirrored those obtained with the Simpson index, and no statistically significant differences were observed across groups (all $p > 0.05$). However, biological trends suggest that male mice exhibited lower diversity scores compared to females. Among females, no significant genotype-related differences were detected under either dietary condition, although mice on the HFEC diet showed a tendency toward higher diversity relative to HF controls. In males, *APOE4/4*-TR mice demonstrated higher average microbial diversity than *APOE3/3*-TR mice across both diets. Notably, EC supplementation increased microbial diversity in *APOE3/3*-TR males, whereas it exerted a minimal effect in *APOE4/4*-TR mice counterparts.

Beta diversity, which quantifies differences in microbial composition between samples, revealed significant separation between groups ($p = 0.001$). While not clearly distinguishable in 2D principal coordinates analysis (Figure 3.3A), the addition of a third

dimension enabled visualisation of distinct clustering by *APOE* genotype (Figure 3.3B), with *APOE3/3*-TR mice grouping to the left of the plot and the *APOE4/4*-TR mice towards the right-hand side of the plot. Sex-based separation was also evident within each genotype group (Figure 3.3C), with female mice clustering on the left and the males on the right. EC supplementation did not produce discernible effects on beta diversity as dietary groups showed substantial overlap. These findings indicate that sex and *APOE* genotype are the primary determinants of microbiota composition in this model.

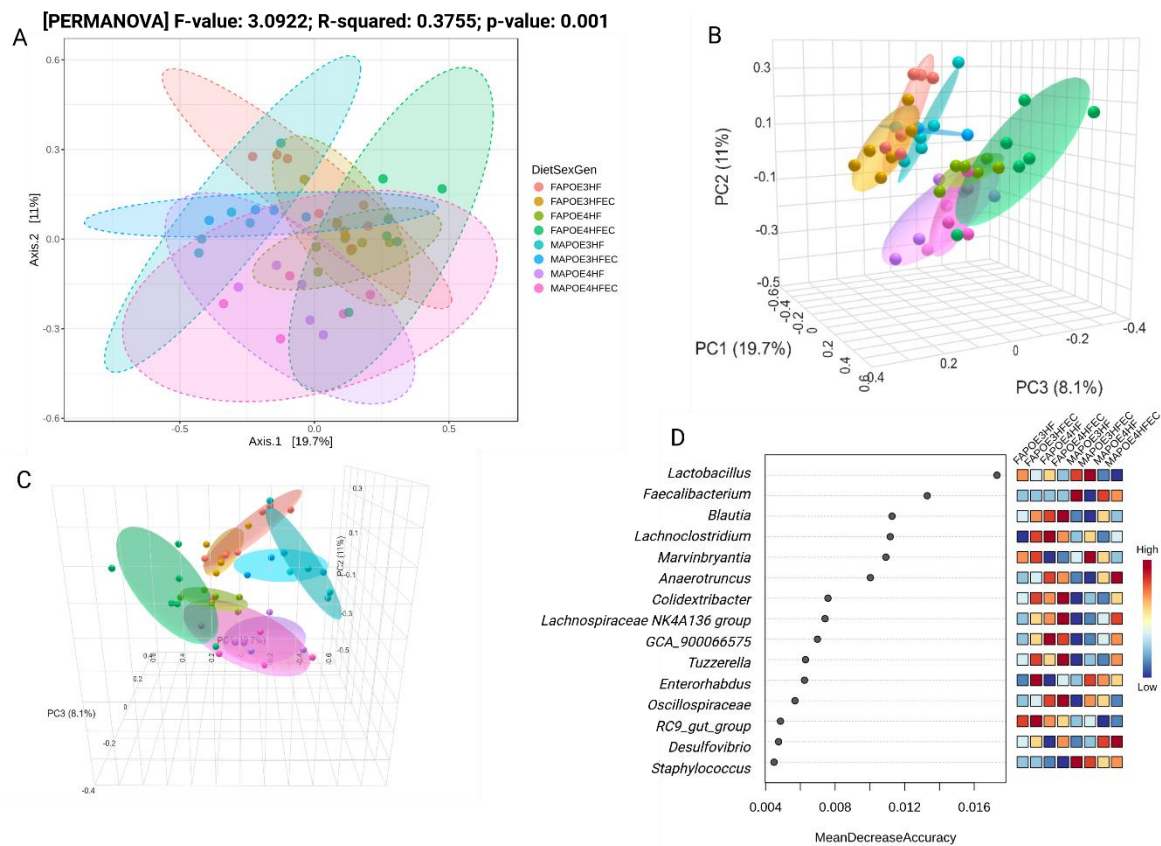


Figure 3.3 PCoA Beta Diversity Plot of Mouse Colonic Microbiota. A) 2D PCoA Plot B) & C) Differing Angles of the 3D PCoA plot visually showing the differences between *APOE* genotype and sex differences in the microbiota. D) Important Features Characteristics Random Forest Plot, listing the top 15 most influential microbial genera and their effect on the colonic microbiota. Mice were either fed High-fat (HF) 45% kcal saturated fat or high-fat with 200 mg/kg. Female *APOE 3/3*-TR Mice fed HF (FAPOE3HF), Female *APOE 3/3*-TR Mice fed HFEC (FAPOE3HFEC), Female *APOE 4/4*-TR Mice fed HF (FAPOE4HF), Female *APOE 4/4*-TR mice fed HFEC (FAPOE4HFEC), Male *APOE 3/3*-TR Mice fed HF (MAPOE3HF), Male *APOE 3/3*-TR Mice fed HFEC (MAPOE3HFEC), Male *APOE 4/4*-TR Mice fed HF (MAPOE4HF), Male *APOE 4/4*-TR Mice fed HFEC (MAPOE4HFEC). Group sizes: FAPOE3HF *n* = 6, FAPOE3HFEC *n* = 6, FAPOE4HF *n* = 6, FAPOE4HFEC *n* = 6, MAPOE3HF *n* = 5, MAPOE3HFEC *n* = 4, MAPOE4HF *n* = 5, MAPOE4HFEC *n* = 6

Analysis of microbial genera contributing to the observed differences in beta diversity revealed that *Lactobacillus* had the most substantial impact. Variations in the abundance of this genus were associated with both genotype and dietary conditions (Figure 3.3D), consistent with trends observed in relative abundance profiles. However, these patterns were not consistent across all groups. In males, a stepwise decrease in *Lactobacillus* abundance was observed across the following sequence: *APOE3/3-TR HFEC* > *APOE3/3-TR HF* > *APOE4/4-TR HFEC* $\approx$ *APOE4/4-TR HF*. In contrast, females exhibited a different pattern with relative abundances ordered as follows: *APOE3/3-TR HF* > *APOE4/4-TR HF* > *APOE3/3-TR HFEC* > *APOE4/4-TR HFEC*. These findings indicate that the effects of EC supplementation are genotype-specific and sex-dependent. The genus *Faecalibacterium* was the second most influential contributor to microbiota structure. A pronounced sex effect was evident, with male mice generally exhibiting a higher prevalence of this microbial genus than their female counterparts, except for *APOE3/3-TR HFEC* male mice, which showed the lowest abundance across all cohorts. These observations suggest that complex interactions between sex, *APOE* genotype, and dietary intervention shape microbial composition.

3.3.1.3. Differential Abundance of Microbes

The overall microbial composition provides insight into ecosystem-wide differences across experimental groups. However, it is equally important to assess whether specific genera of microorganisms colonising the colon differ between groups. Such differences may not be fully captured by relative abundance metrics alone. The microbial communities associated with each group may harbour distinct genetic features, potentially contributing to the metabolic variations observed in EC. More broadly, these microbial differences could influence the production of other metabolites, which may exert health-related effects, either locally within the gut or systemically throughout the host.

In female mice, differential abundance analysis identified significant differences in only two bacterial genera across groups. Specifically, a genotype-related variation was observed among HF diet females: *Staphylococcus* was significantly more abundant in

APOE3/3-TR females (FDR $q = 0.132$), whereas *Lachnoclostridium* showed significantly reduced abundance in the *APOE4/4-TR* females (FDR $q = 0.132$) (Figure 3.4). No additional significant differences were detected among the remaining female groups.

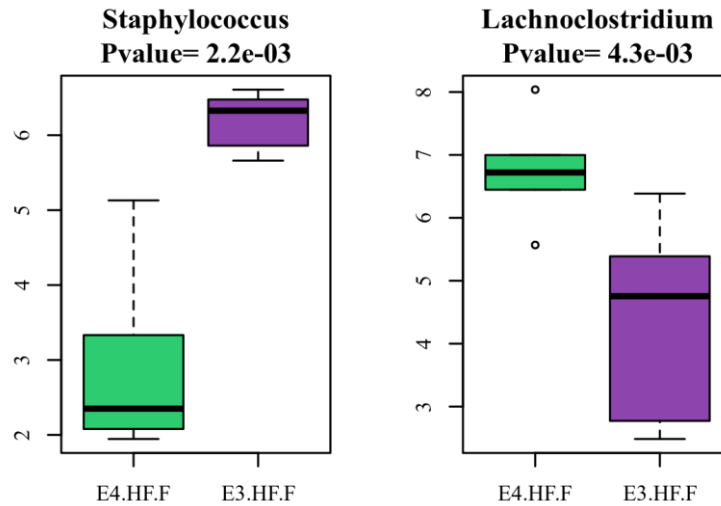


Figure 3.4 Significant (Benjamini-Hochberg $q < 0.2$) differential abundance of bacterial genera from the colonic microbiota of female (F) *APOE3/3-TR* (E3), and *APOE4/4-TR* (E4) High fat 45% kcal saturated fats (HF) mice. Raw p-value are depicted on the plot. FDR adjusted q values of 0.132 for both *Staphylococcus* and *Lachnoclostridium*. Group sizes: E4.HF.F $n = 6$, E3.HF.F $n = 6$

Comparisons among male mice revealed no significant differences in microbial composition across *APOE* genotypes or dietary conditions (all FDR $q > 0.2$). In contrast, sex-based comparisons within the *APOE4/4-TR* group on HF diet demonstrated notable differences in the relative abundance of several bacterial genera. Specifically, *Ligilactobacillus* (FDR $q = 0.176$), *Enterorhabdus* (FDR $q = 0.185$) (Figure 3.5), and *[Eubacterium]_xylanophilum_group* (FDR $q = 0.185$) (See Supplementary Table 7) were significantly more abundant in male mice compared to females. Conversely, *Ruminococcaceae_UBA1819* (FDR $q = 0.176$), *Lachnosporidium* (FDR $q = 0.176$), *Rikenellaceae_RC9_gut_group* (FDR $q = 0.185$), *Anaerofustis* (FDR $q = 0.185$), *Bacteroides* (FDR $q = 0.185$), *GCA-900066575* (FDR $q = 0.185$), and *Streptococcus* (FDR $q = 0.185$), were significantly more abundant in female *APOE4/4-TR* HF mice than in their male counterparts (Figure 3.5).

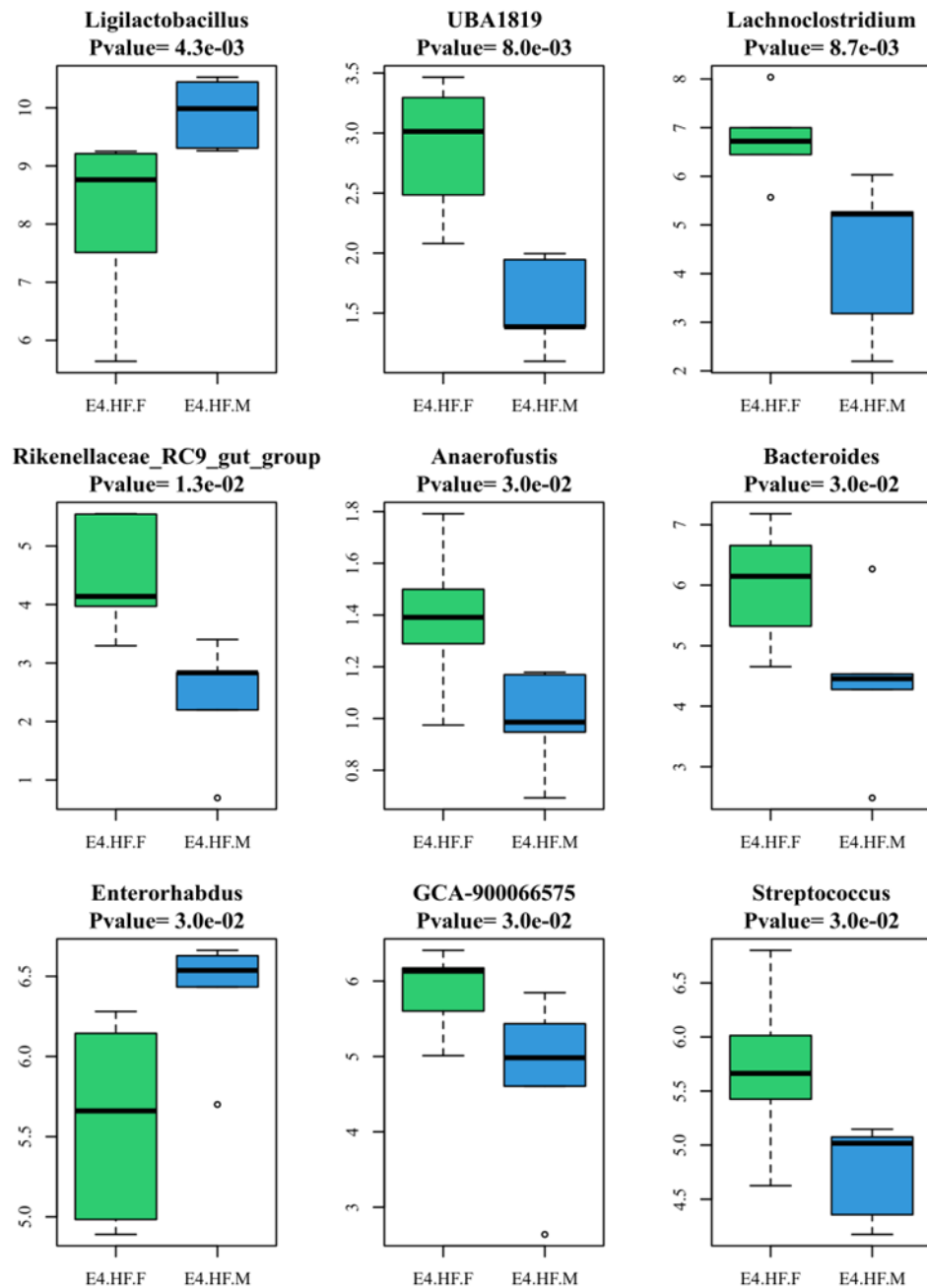


Figure 3.5: The 9 most significant (Benjamini-Hochberg FDR adjusted $q < 0.2$) differential abundance of bacterial genera from the colonic microbiota of female (F) and male (M), APOE4/4-TR (E4) High fat 45% kcal saturated fats (HF) mice. Raw p-value printed above each plot and FDR adjusted q-values as follows: Ligilactobacillus (FDR adjusted $q = 0.176$), Ruminococcaceae UBA1819 (FDR adjusted $q = 0.176$), Lachnoclostridium (FDR adjusted $q = 0.176$), Rikenellaceae_RC9_gut_group (FDR adjusted $q = 0.185$), Anaerofustis (FDR adjusted $q = 0.185$), Bacteroides (FDR adjusted $q = 0.185$), Enterorhabdus (FDR adjusted $q = 0.185$), GCA-900066575 (FDR adjusted $q = 0.185$), and Streptococcus (FDR adjusted $q = 0.185$). Group sizes: E4.HF.F $n = 6$, E4.HF.M $n = 5$

Sex-specific differences were also observed in the gut microbiota composition of *APOE4/4-TR* mice on the HFEC diet. Notably, the relative abundances of *Blautia*, *unidentified Oscillospiraceae*, and *Lachnospiridium* were significantly higher in *APOE4/4-TR* HFEC females compared to their male counterparts (FDR $q = 0.132$, 0.132 , and 0.176 , respectively) (Figure 3.6).

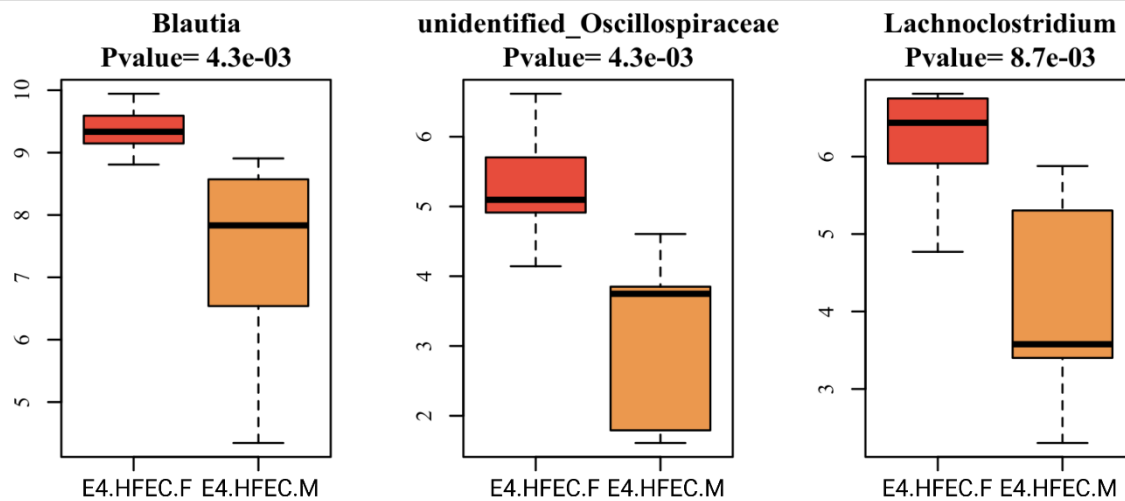


Figure 3.6 Significant (Benjamini-Hochberg $q < 0.2$) differential abundance of bacterial genera from the colonic microbiota of female (F) and male (M) *APOE4/4-TR* (E4) High fat 45% kcal saturated fats supplemented with 200 mg/kg epicatechin (HFEC) mice. Raw p-value are depicted on the plot. FDR adjusted q values: *Blautia* (FDR adjusted $q = 0.132$), *unidentified Oscillospiraceae* (FDR adjusted $q = 0.132$), and *Lachnospiridium* (FDR adjusted $q = 0.176$) Group sizes: E4.HFEC.F $n = 6$, E4.HFEC.M $n = 6$

3.3.2. EC supplementation affects the gut metabolome

sPLS-DA analysis revealed that under an HF diet, metabolomic profiles differ between *APOE* genotypes, irrespective of their sex. In female mice, EC supplementation reduced this genotype-associated divergence, aligning the metabolomic profiles of *APOE*3/3-TR and *APOE*4/4-TR groups (Figure 3.7A&C). In contrast, male mice continued to exhibit genotype-related differences despite EC supplementation, with inosine and tauroconjugated bile acids emerging as key metabolites associated with these shifts (Figure 3.7B&D).

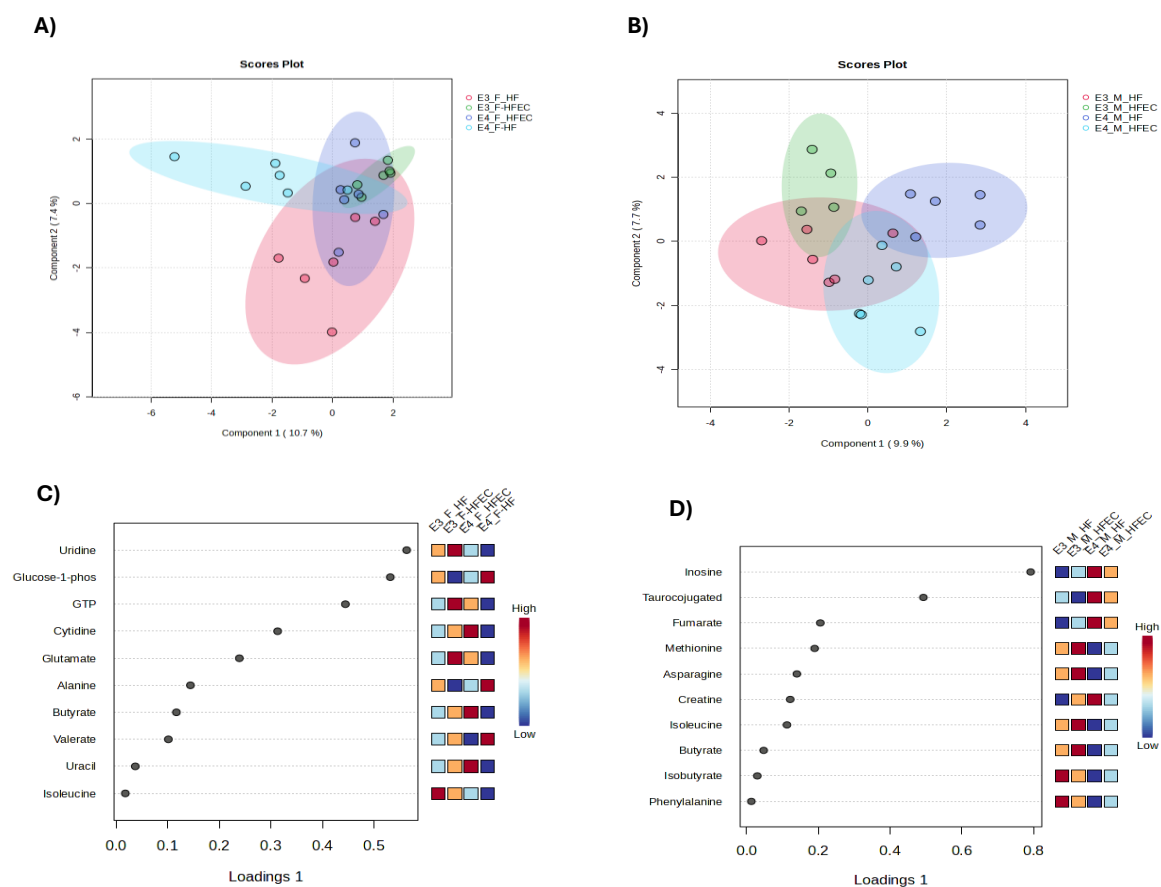


Figure 3.7 Sparse Partial Least Squares-Discriminant Analysis (sPLS-DA) and important features characteristics of metabolomic data separated by sex. A&C) Females B&D) Males. Mice were fed either a high-fat 45% kcal saturated fat (HF) or high-fat 45% kcal saturated fat supplemented with 200 mg/kg epicatechin (HFEC). Female *APOE* 3/3-TR Mice fed HF (E3_F_HF), Female *APOE* 3/3-TR Mice fed HFEC (E3_F_HFEC), Female *APOE* 4/4-TR Mice fed HF (E4_F_HF), Female *APOE* 4/4-TR mice fed HFEC (E4_F_HFEC), Male *APOE* 3/3-TR Mice fed HF (E3_M_HF), Male *APOE* 3/3-TR Mice fed HFEC (E3_M_HFEC), Male *APOE* 4/4-TR Mice fed HF (E4_M_HF), Male *APOE* 4/4-TR Mice fed HFEC (E4_M_HFEC). Group sizes: E3_F_HF n = 6, E3_F_HFEC n = 6, E4_F_HF n = 6, E4_F_HFEC n = 5, E3_M_HF n = 6, E3_M_HFEC n = 4, E4_M_HF n = 5, E4_M_HFEC n = 6

Analysis of individual metabolites revealed differences in concentrations that may underlie the separation observed in the sPLS-DA plot. In female mice, no significant differences in individual metabolites were detected across groups (FDR $q > 0.2$). In contrast, male mice on the HFEC diet exhibited genotype-specific differences: levels of aspartate (FDR $q = 0.079$), uracil and ornithine (FDR $q = 0.109$) were higher in *APOE3/3*-TR mice compared to their *APOE4/4*-TR counterparts (Figure 3.8A). Additionally, a sex-specific difference was observed within the *APOE4/4*-TR group on the HFEC diet, where fumarate levels were significantly higher in females than in males (FDR $q = 0.019$) (Figure 3.8B).

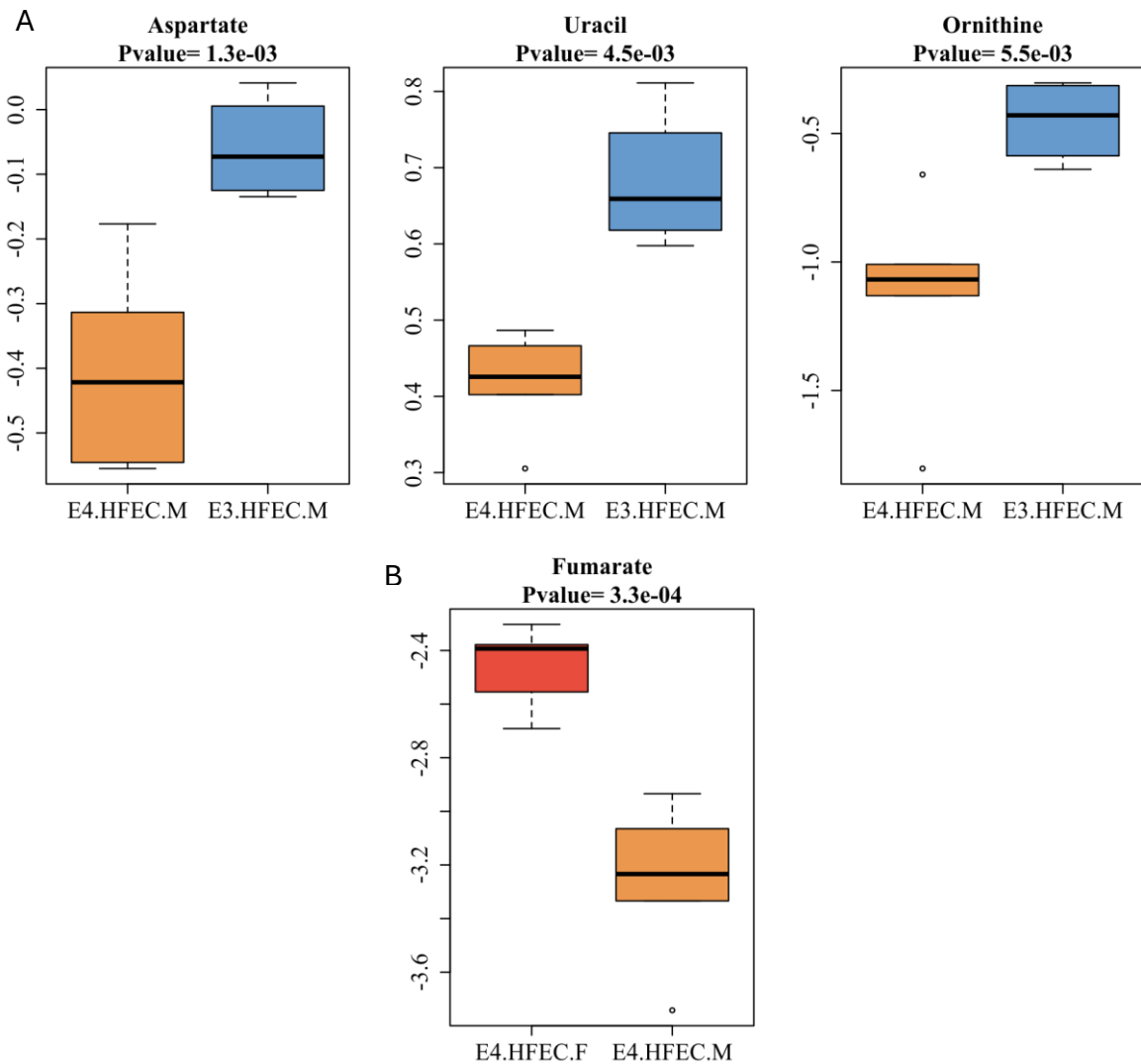


Figure 3.8 The significant (Benjamini-Hochberg adjusted $q < 0.2$) differential abundance of metabolites from the colonic microbiota of A) male (M), APOE4/4-TR (E4) and APOE3/3-TR (E3) fed a high fat 45% kcal saturated fats supplemented with 200mg/kg epicatechin (HFEC) mice. Raw p-values are displayed above each plot, and the FDR p-values are as follows Aspartate (FDR adjusted $q = 0.079$), Uracil (FDR adjusted $q = 0.109$), and Ornithine (FDR adjusted $q = 0.109$) B) of male (M), and female (F) APOE4/4-TR (E4) fed a high fat 45% kcal saturated fats supplemented with 200 mg/kg epicatechin (HFEC) mice. Raw p-values are displayed above each plot, and the FDR p-values are as follows: Aspartate (FDR adjusted $q = 0.079$), Uracil (FDR adjusted $q = 0.109$), and Ornithine (FDR adjusted $q = 0.109$)

3.3.3. Microbiota-metabolome congruence analysis

To gain a deeper insight into the health implications of the microbiota, it is essential to link the microbial function with the metabolome that it influences. Genotype-dependent differences were observed in females fed an HF diet. For instance, *Staphylococcus*, which was more abundant in the APOE3/3-TR HF females, showed a significant negative correlation with tauro-conjugated bile acids. Conversely, *Lachnoclostridium*, enriched in APOE4/4-TR HF females, was positively correlated with AMP, 3-phenylpropionate, and tauro-conjugated bile acids (Figure 3.9A).

Sex-specific associations were also detected among *APOE4/4*-TR HF mice. In males, *Enterobacteriaceae* and *[Eubacterium]_xylanophilum_group*, which were more abundant than in females, negatively correlated with ornithine levels. In contrast, *Streptococcus*, enriched in females, showed a positive correlation with ornithine (Figure 3.9B).

Further sex differences were also evident in *APOE4/4*-TR HFEC mice. Several Firmicutes, including *Blautia*, *unidentified Oscillospiraceae* and *Lachnospiridium*, were more abundant in female and positively correlated with a broad spectrum of metabolites, including: 1,3-Dihydroxyacetone, choline, N-Acetylglucosamine, galactose, glucose-6-phosphate, fumarate, glutamate, serine, histidine, glycine, glutamine, alanine, urocanate, threonine, leucine, guanine tri phosphate, valine, lysine, isoleucine, methionine, tyrosine, phenylalanine, aspartate, cytidine, glucose, ribose, ornithine, and xanthine (Figure 3.9C). Fumarate, notably elevated in the metabolic profile, showed strong positive correlations with *Bifidobacteriaceae*, *Rikenellaceae RC9 group 9*, *Lachnoclostridium*, *unknown Oscillospiraceae*, *Eubacterium brachy group*, *Blautia*, and *Intestimonas*, negative correlations with *Ligilactobacillus*, *ASF356*, and *Turibacter* (Figure 3.9C).

Finally, aspartate, ornithine, and uracil were elevated in *APOE3/3*-TR males relative to their *APOE4/4*-TR counterparts, with corresponding microbial associations. Negative correlations were observed between these metabolites and *Anaerococcus*, *Lachnospiraceae FCS020 group*, *ASF356*, *Intestimonas*, and *Desulfovibrio*. Positive correlations included *Lactococcus* and *Marvinbryantia* with ornithine, *Staphylococcus* with aspartate, and *Lactobacillus* with aspartate and uracil (Figure 3.9D).

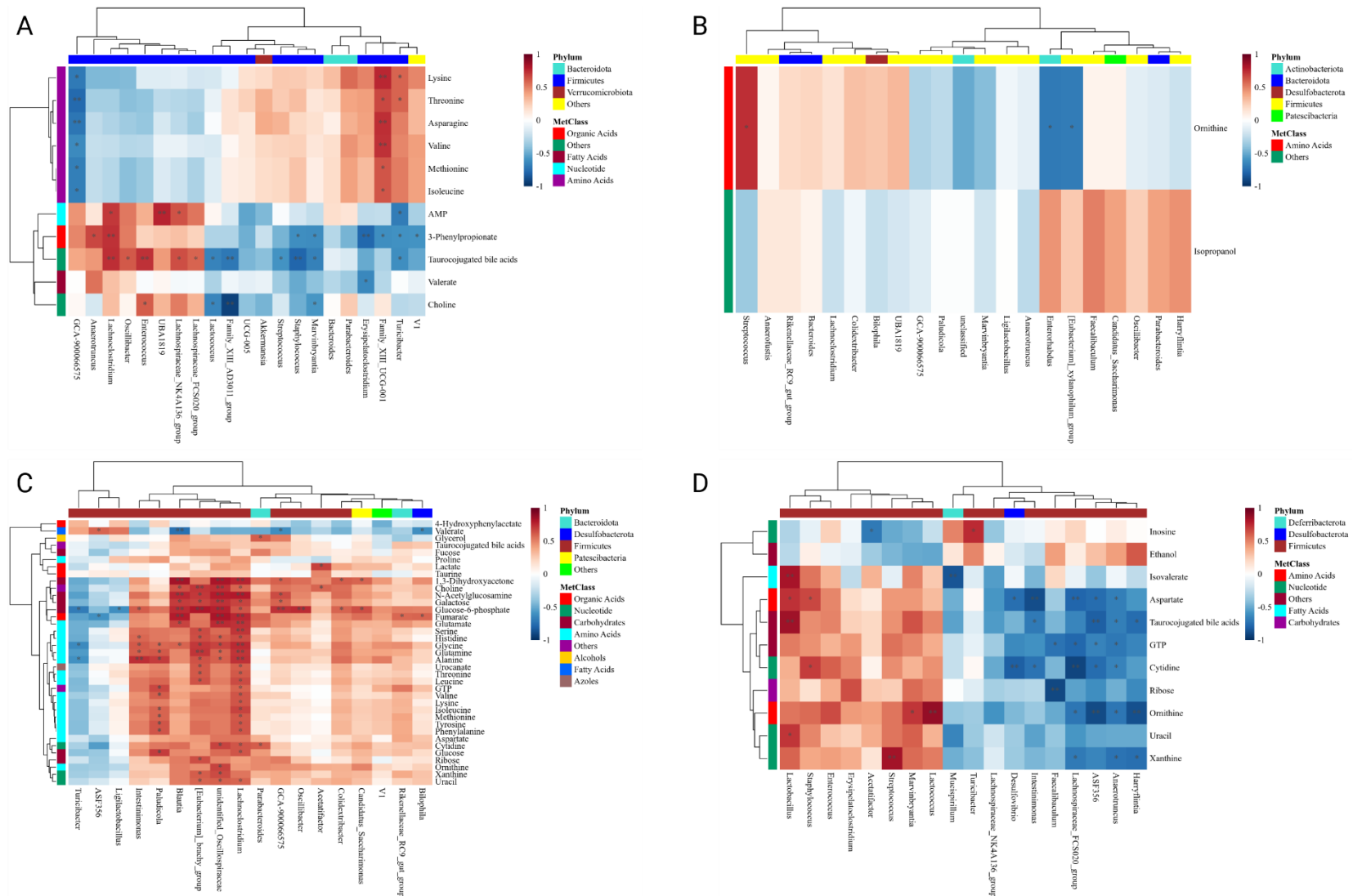


Figure 3.9: Correlation heatmaps between the metabolome and microbiota of APOE-TR mice separated into the following groups. A) Female APOE4/4-TR and APOE3/3-TR mice fed a high-fat 45% kcal saturated fat diet (HF). B) Male and Female APOE4/4-TR mice fed HF diet C) Male and Female APOE4-TR mice fed 45% kcal saturated fat with 200 mg/kg (HFEC) diet D) APOE3/3-TR and APOE4/4-TR male mice fed the HFEC diet. Significance in the correlation is highlighted * $0.05 > p\text{-value} \geq 0.01$, ** $0.01 > p\text{-value}$. Positive correlations highlighted in red, negative correlations highlighted in blue.

3.3.4. DIAMOND Results

3.3.4.1. *Relative abundance of microbiota*

The DIAMOND data revealed notable differences compared to the mouse model described above. At the Phylum level, Actinomycetota and Firmicutes were the most abundant across all groups and exhibited relatively consistent representation. However, Actinomycetota appear in greater numbers overall. More striking differences were observed in the presence of the other phyla. In female participants, *APOE4* carriers exhibited a greater abundance of additional phyla, particularly Bacteroidota and the Verrumicrobiota, which are largely absent in *APOE4* non-carriers. This pattern was not observed in males, where Bacteroidota were infrequently detected in both *APOE4* carriers and non-carrier groups (Figure 3.10A).

Age-related differences were apparent at the family level, with older individuals exhibiting a more diverse representation within the Actinomycetota phylum compared to younger participants, a trend consistent across all groups. Within Actinomycetota, the *Bifidobacteriaceae* family was predominant. In the Firmicutes phylum, *Lachnospiraceae* and *Oscillospiraceae* were the most frequently observed families, with no visually discernible differences among groups (Figure 3.10B).

At the genus level, the DIAMOND dataset revealed substantial inter- and intra-group variability. The most prevalent genera were *Bifidobacterium* (from Bifidobacteriaceae) and *Vescimonas* (from Oscillospiraceae). Notably, *Bifidobacterium* appeared more abundant in younger participants than in the older ones. Although many other genera did not show clear visual group distinctions, subsequent analyses revealed statistically significant differences between groups (Figure 3.10C).

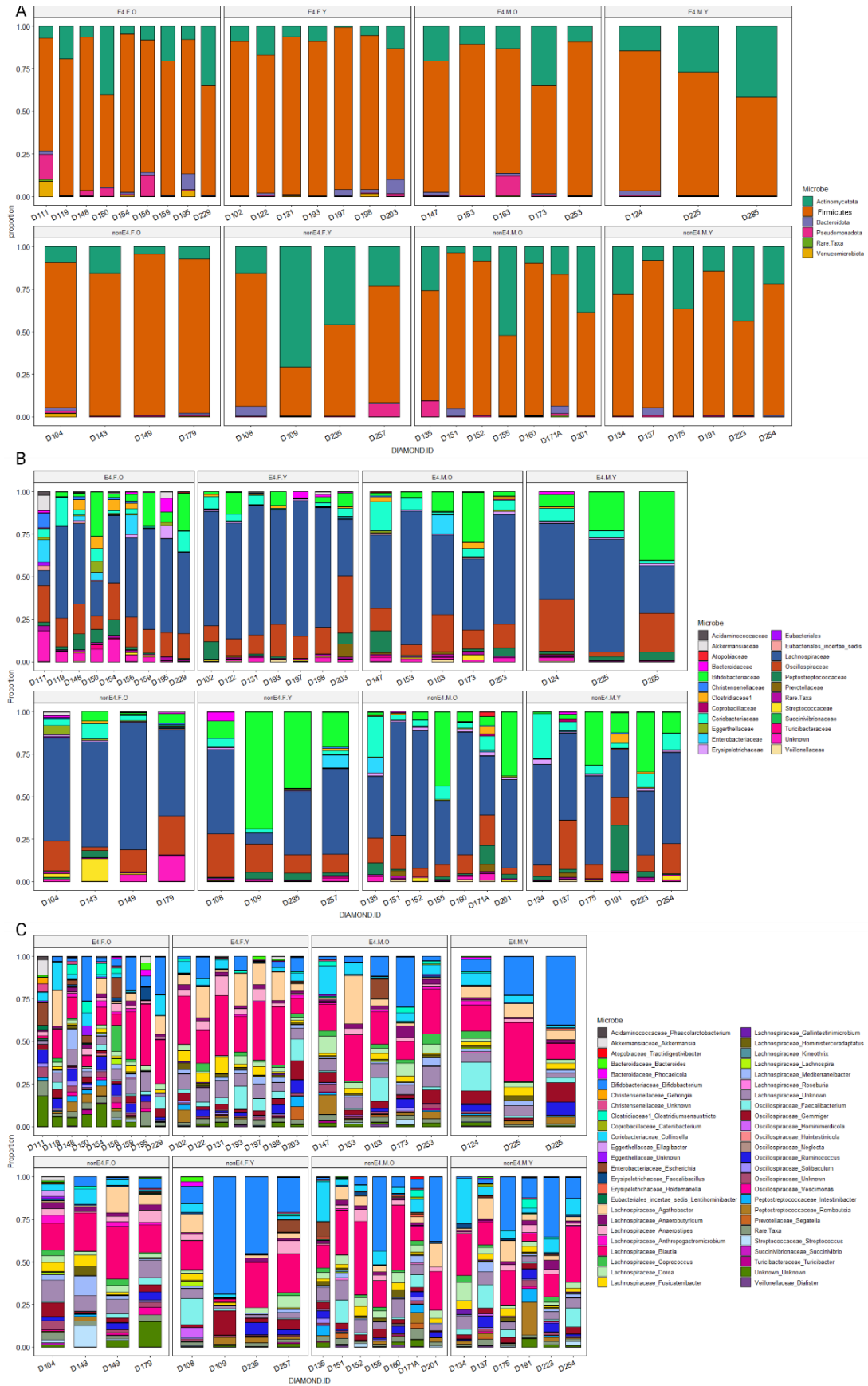


Figure 3.10 Relative abundance of microbiota of participants from the Dietary pInt extracts, colonic Microbes, and apoe geNetics interactions study (DIAMOND). Participants were stratified by sex: females (F), Males (M), and by genotype: APOE4 carrier status: APOE2/3, APOE2/2, and APOE3/3 (NonE4) and APOE3/4, and APOE4/4 (E4), and age: 18-35years old (Younger), 55+ years old (Older). A) Phylum level, B) Family level, C) Genus level. Group sizes: E4.M.Y n = 3, NonE4.M.Y n = 6, E4.M.O n = 5, nonE4.M.O n = 7, E4.F.Y n = 7, nonE4.F.Y n = 4, E4.F.O n = 9, nonE4.F.O n = 4

3.3.4.2. Alpha and Beta Diversity Metrics

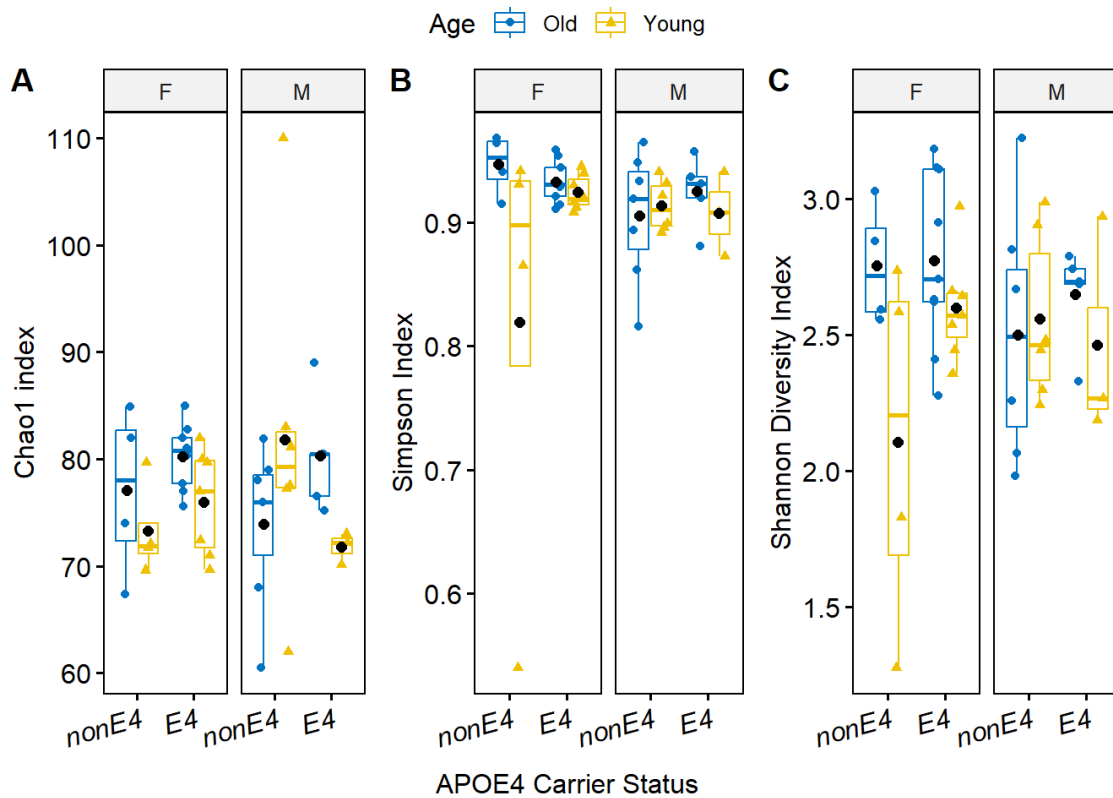


Figure 3.11: Alpha diversity of the microbiota of participants from the Dietary pAnt extracts, colonic Microbes, and apoe geNetics interactions study (DIAMOND). Participants were stratified by sex: females (F), Males (M), APOE genotype: APOE4 carrier status: APOE2/3, APOE2/2, and APOE3/3 (NonE4) and APOE3/4, and APOE4/4 (E4), and age: 18-35years old (Younger), 55+ years old (Older). A) Chao1 Index for species richness. B) Simpson Index for species evenness. C) Shannon Diversity Index for both species' richness and evenness. The black point in each boxplot represents the group's mean. Group sizes: male younger E4 carrier $n = 3$, male younger non-E4 carrier $n = 6$, male older E4 carrier $n = 5$, male older non-E4 carrier $n = 7$, female younger E4 carrier $n = 7$, female younger non-E4 carrier $n = 4$, female older E4 carrier $n = 9$, female older non-E4 carrier $n = 4$

Analysis of the overall microbiota composition using the DIAMOND dataset revealed no statistically significant differences in alpha diversity metrics (Chao1 index, Simpson index, and Shannon diversity index) across the experimental groups (all $p > 0.05$). However, a trend toward significance was observed in species evenness, with a three-way ANOVA, indicating a potential interaction between age, sex, and genotype ($p = 0.06$) (Figure 3.11B). Additionally, the Shannon diversity index exhibited a marginal association with age ($p = 0.08$), suggesting that younger individuals may exhibit lower evenness and richness in microbial diversity (Figure 3.11C). This age-related trend appeared consistent across groups, except for male non-E4 carriers, in whom the effect was less pronounced. Notably greater variability in diversity was observed among young female non-E4 carriers, primarily due to an outlier contributing substantial within-group variance. These findings

suggest that microbial diversity may be modulated by the interplay between sex, age, and *APOE* carrier status genotype.

[PERMANOVA] F-value: 1.0392; R-squared: 0.1643; p-value: 0.34

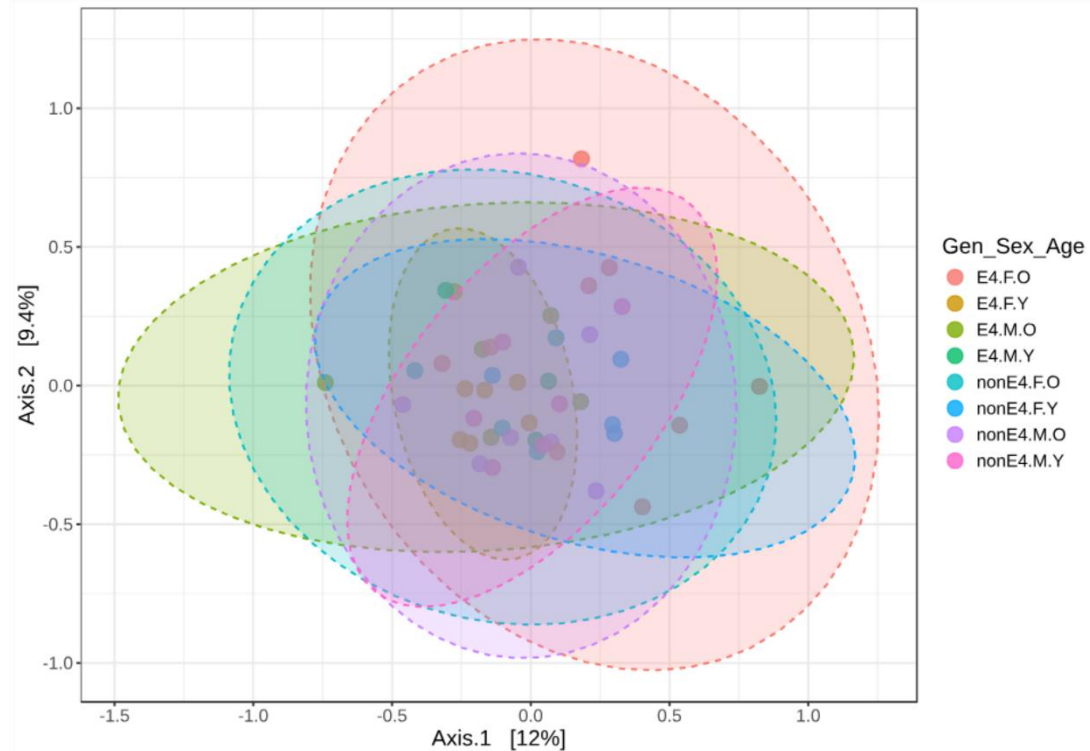


Figure 3.12 PCoA plot for beta diversity of the microbiota of participants from the Dietary pAnt extracts, colonic Microbes, and apoe geNetics interactions study (DIAMOND). Participants are grouped by their *APOE4* Carrier Status (E4 Carrier: genotypes *APOE3/4* and *APOE4/4* (E4) and Non-E4 Carrier: genotypes *APOE2/2*, *APOE2/3* and *APOE3/3* (nonE4)), Sex and Age (Younger: 18-35 years old (Y) and Older: 55+ years old (O)) to produce the following groups. E4 Females O (E4.F.O), E4 Females Y (E4.F.Y), E4 Male O (E4.M.O), E4 Male Y (E4.M.Y), nonE4 Female O (nonE4.F.O), nonE4 Female Y (nonE4.F.Y), NonE4 Male O (nonE4.M.O), and nonE4 Male Y (nonE4.M.Y). Group sizes: E4.M.Y n = 3, NonE4.M.Y n = 6, E4.M.O n = 5, nonE4.M.O n = 7, E4.F.Y n = 7, nonE4.F.Y n = 4, E4.F.O n = 9, nonE4.F.O n = 4

The high p-value ($p = 0.34$) indicates the absence of statistically significant differences between the groups. This finding is further supported by the plots, which show that most datapoints are clustered near the origin, suggesting minimal variation between groups. These results imply that age, sex, and *APOE4* carrier status did not influence the overall structural differences between the groups (Figure 3.13).

3.3.4.3. Differential Abundance of Microbes

Analysis of differential microbial abundances revealed no significant differences in any pairwise group comparisons ($\text{FDR } q > 0.2$). This result suggests that age, sex, and *APOE*

genotype of the participants are not major contributors to the observed variation in microbiota within this sample set; full dataset in Supplementary Table 9.

3.3.4.4. *Inference of metabolic pathways in the DIAMOND dataset*

The functional potential of the microbiota was assessed using KEGG pathway analysis. Kruskal-Wallis tests revealed that several microbial pathways are significantly influenced by age, *APOE* genotype, and sex. Notably, many of these differences are concentrated in female *APOE4* carriers. In this sub-group, age is associated with the upregulation of pathways including glycine, serine, and threonine metabolism; ubiquinone and other terpenoid-quinone biosynthesis; epithelial cell signalling in *Helicobacter pylori* infection; carbon metabolism; D-glutamine and D-glutamate metabolism; Carbon fixation pathways; non-homologous end-joining; *Staphylococcus aureus* infection; and taurine and hypotaurine metabolism. Conversely, these individuals show downregulation in the zeatin biosynthesis, fluorobenzoate degradation, photosynthesis, and histidine metabolism (Figure 3.A).

Genotypic comparisons among older females further indicate that *APOE4* carriers exhibit distinct functional profiles relative to non-carriers. Younger *APOE4* females also show significant upregulation in histidine metabolism, ribosome biogenesis, glutathione metabolism, tyrosine metabolism, RNA degradation, NO signalling pathways, fatty acid biosynthesis, and protein digestion and absorption, compared to age-matched non-*APOE4* counterparts. At the same time, they display significant downregulation in zeatin biosynthesis, bacterial chemotaxis, porphyrin and chlorophyll metabolism, NOD-like receptor signalling pathways, ribosome metabolism, and one-carbon pool by folate (Figure 3.B).

Lastly, sex-specific differences were observed, with older female *APOE4* carriers exhibiting a significant upregulation of atrazine degradation relative to their male counterparts (Figure 3.C).

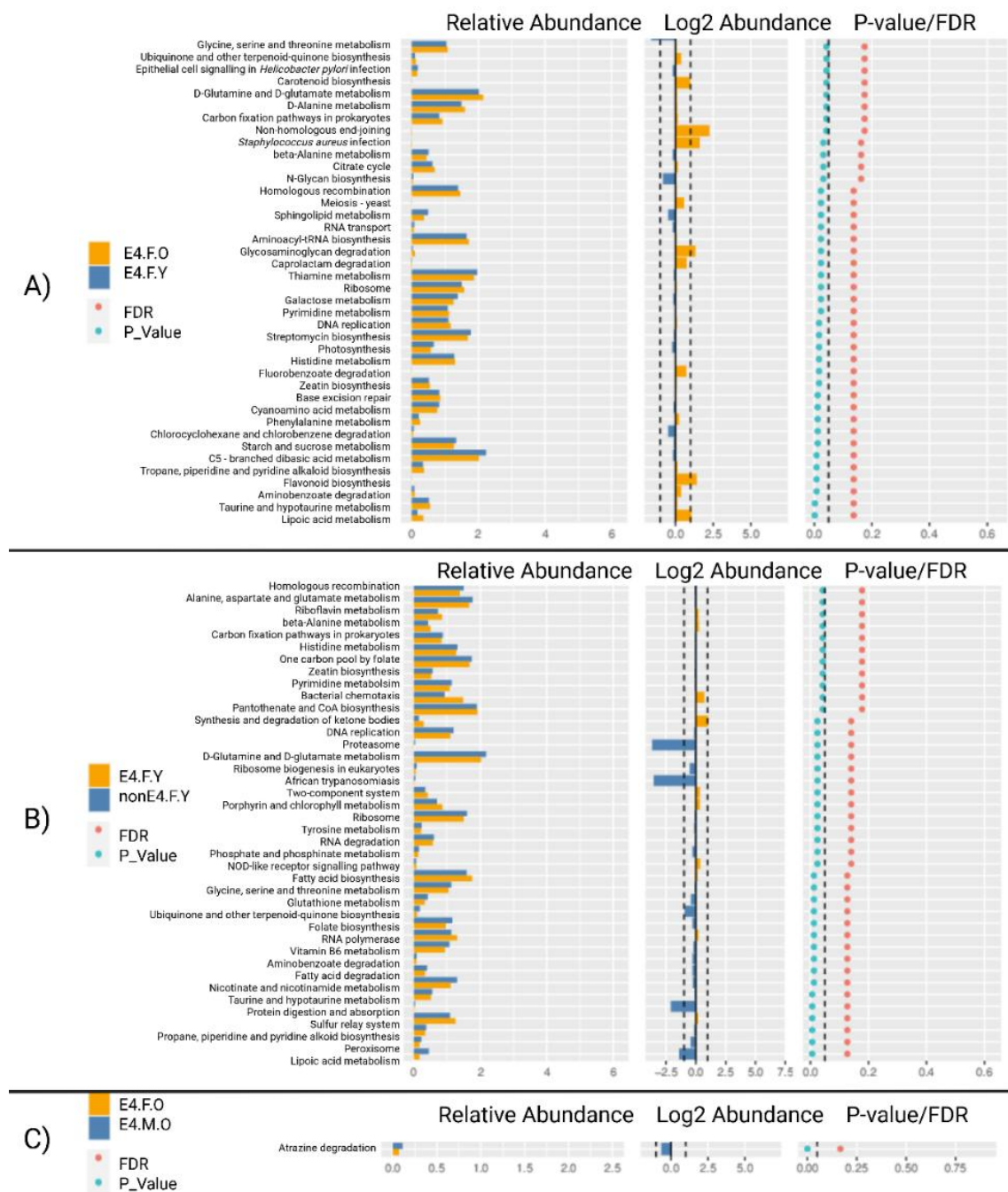


Figure 3.14 The significant (Benjamini Hochberg $p_{adj} < 0.2$) differences in the theoretical functionality of the DIAMOND study microbiota inferred from Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathways from the Dietary plant extracts, colonic Microbes, and apoe geNetics interactions study (DIAMOND). Participants are grouped by their APOE4 Carrier Status (E4 Carrier: genotypes APOE3/4 and APOE4/4 (E4) and Non-E4 Carrier: genotypes APOE2/2, APOE2/3 and APOE3/3 (nonE4)), Sex, and Age (Younger: 18-35 years old (Y) and Older: 55+ years old (O)) to produce the following groups E4 Female O (E4.F.O), E4 Female Y (E4.F.Y), E4 Male O (E4.M.O), E4 Male Y (E4.M.Y), nonE4 Female O (nonE4.F.O), nonE4 Female Y (nonE4.F.Y), nonE4 Male O (nonE4.M.O), and nonE4 Male Y (nonE4.M.Y). A) E4.F.O compared to E4.F.Y B) E4.F.Y compared to nonE4.F.Y C) E4.F.O compared to E4.M.O. Group sizes: E4.M.Y $n = 3$, NonE4.M.Y $n = 6$, E4.M.O $n = 5$, nonE4.M.O $n = 7$, E4.F.Y $n = 7$, nonE4.F.Y $n = 4$, E4.F.O $n = 9$, nonE4.F.O $n = 4$

3.4. Discussion

These findings suggest that sex, age, and *APOE* genotype may influence the structure and functionality of the microbiota. However, the magnitude and underlying mechanisms of these effects require further investigation. In both murine and human studies, a high abundance of Firmicutes was observed, consistent with the dietary patterns of DIAMOND participants and indicating that the *APOE-TR* mouse model effectively replicated a ‘Western’ diet [269]. In the murine model, female mice exhibited significantly higher species richness, regardless of diet or genotype. Notably, overall microbiota composition was primarily affected by the sex and the *APOE* genotype, with no significant effect of EC supplementation on alpha or beta diversity. This contrasts with previous research demonstrating that flavan-3-ols, such as EC, can modulate microbial composition, though their effects on diversity have been inconsistent [270, 271]. Importantly, the current study administered EC as a pure compound, independent of its natural food matrix (e.g. cocoa or tea), potentially limiting its influence on microbial diversity.

Differential abundance analyses further demonstrated that microbial shifts were more strongly associated with *APOE* genotype and sex rather than with EC supplementation. For instance, in HF female mice, *Staphylococcus* and *Lachnoclostridium* exhibited genotype-specific patterns, with elevated levels in *APOE3/3* and *APOE4/4* mice, respectively. While prior research has not linked *Staphylococcus* abundance to *APOE* genotype, *Lachnoclostridium* has previously been reported at higher levels in *APOE2* carriers compared to *APOE4* [101, 104]. However, those studies did not involve HF diets, suggesting dietary content may modulate this relationship. Interestingly, EC supplementation attenuated the effects of genotype on specific bacterial genera, suggesting that while EC does not significantly alter global microbial structure, it may influence the abundance of specific taxa.

Sex-dependent differences in microbial composition were also observed, particularly for *Lachnoclostridium*, which was more abundant in female *APOE4/4-TR* HF than in males, a finding that contradicts existing literature, which reports greater abundance in males [272]. This discrepancy may be due to differences in sampling sites, as prior studies often

sequenced the small intestine, whereas the current study focused on colonic microbiota, which is shaped by distinct environmental factors (e.g. oxygen tension, pH and nutrient availability) [273]. Additional genera such as *Bacteroides* and *Enterobacter* were enriched in both females and males *APOE4/4*-TR HF mice. However, following EC supplementation, only three sex-specific differences remained: *Lachnospirillum*, *Blautia*, and an unidentified genus of *Oscillospiraceae*. As expected, *Blautia* was enriched in females and is known to be modulated by oestrogens [274]. Interestingly, while EC supplementation modified some sex-dependent microbial differences, the sex effect on *Lachnospirillum* remained consistent. These sex- and diet-associated differences were exclusive to *APOE4/4*-TR mice, suggesting that the *APOE* genotype may modulate susceptibility to dietary and sex-dependent microbial shifts. In contrast, *APOE3/3*-TR mice appeared more resistant to such changes.

Metabolomic analysis revealed that only *Bacteroides* were directly associated with flavan-3-ol metabolism, particularly the degradation of EC via C-ring cleavage [181], as demonstrated in *in vitro* colon models. Broadly, both sex and *APOE* genotype influenced the metabolomic profiles of mice, with notable genotype-related differences emerging in response to the HF diet. However, EC supplementation in female mice diminished these genotype-related differences, resulting in convergent metabolomic profiles between genotypes. Despite the absence of a dietary effect on microbiota beta diversity, EC supplementation appeared to influence microbiota functionality, potentially altering metabolic activity without restructuring community composition; a novel finding requiring further investigation.

At the levels of individual metabolites, only sex differences were observed. In *APOE3/3*-TR HFEC mice, the metabolism of aspartate, uracil, and ornithine was increased in the males over the female counterparts. Each of these three metabolites plays a role in the GBA, modulating the neuronal metabolism and/or inflammation [181, 275]. Such findings may help to explain why females are at a greater risk of developing cognitive decline than males. In contrast, in the *APOE4/4*-TR HFEC animals, fumarate, a key intermediate in the tricarboxylic acid cycle, was more abundant in females than males, suggesting EC supplementation may preferentially modulate energy metabolism in females [276].

The correlation analyses between the microbial taxa and metabolites provided further insight. In the *APOE3/3*-TR HF females, *Staphylococcus* abundance negatively correlated with the production of tauroconjugated bile acids. Conversely, in *APOE4/4*-TR HF females, *Lachnoclostridium* was positively correlated with tauroconjugated bile acids. These results suggest that the *APOE4/4* genotype may shape a microbiota more conducive to tauroconjugated bile acid production. Previous research has demonstrated that these bile acids are key players in the GBA, where tauroconjugated bile acids can cross the intestinal barrier and modulate brain inflammation by downregulating NF- κ B expression [277]. Thus, these findings imply that *APOE4/4*-TR females may experience heightened neuroinflammation due to impaired deconjugation of bile acids or altered hepatic cholesterol metabolism associated with *APOE* polymorphism. However, these hypotheses will require further investigation before conclusions can be drawn about the mechanism for this difference. Despite this, it is worth noting that this genotype difference is observed only in female mice, suggesting an interplay between sex and *APOE* genotype. Additionally, this genotype-dependent effect was absent following EC supplementation, suggesting a modulatory role of EC on bile acid metabolism.

In male HFEC mice, *APOE3/3*-TR animals exhibited higher production of aspartate, ornithine and uracil, relative to their *APOE4/4*-TR counterparts. Aspartate and uracil were positively correlated with *Lactobacillus* abundance. *Lactobacillus* has been previously linked to modulation of the GBA and improved cognitive outcomes [278]. Conversely, the production of ornithine positively correlated with the production of *Marvinbryantia*, a genus linked to reduced brain white matter health [279]. Both aspartate and ornithine also correlated with the abundance of *Lachnospiracea FCS020 group*, a taxon with conflicting associations with brain health across studies, with a Mendelian-randomisation study linking its abundance to AD development [280], while other studies also reported positive associations between this genus and better cognitive scores [279]. These findings suggest that EC supplementation may influence colonic metabolism through shifts in specific microbial taxa, though whether these changes differentially benefit *APOE3/3* versus *APOE4/4* remains to be determined. Whether the behavioural/cognitive outcomes of these mice are influenced by their diet, *APOE* genotype status, or sex is explored in Chapter 4.

Translating these effects to the human cohort revealed less pronounced differences in microbiota composition. In DIAMOND participants, neither *APOE4* carrier status, age, nor sex significantly influenced overall microbial structure or the abundance of individual genera. It is likely that other factors, including modifiable elements such as diet, medications, and hormonal changes [281] none of which were controlled in this pilot study, may have obscured genotype or sex-related differences; see Chapter 2.3.2. The small sample size relative to the number of study arms may also have reduced statistical power. Additionally, microbiota sequencing post-fermentation of EC in the *ex vivo* colon model was not feasible due to limited sample volumes. Future studies should assess microbiota and metabolomic responses to EC across sex, age, and *APOE* genotype in both controlled and real-world settings.

KEGG pathway analysis in the human cohort revealed significant increases in taurine and hypotaurine metabolism in the older female *APOE4* carriers compared to their male counterparts, as well as in the younger female *APOE4* carriers in comparison to their non-*APOE4* carrier equivalents. Given that taurine is released through microbial deconjugation of tauroconjugated bile acids [282], this may indicate elevated taurine availability in these individuals. Reduced taurine metabolism could imply greater NF- κ B activity and increased inflammation, though this hypothesis requires further validation.

In summary, these findings highlight complex interactions between *APOE* genotype, sex, and EC supplementation in shaping both the microbiota and its metabolic outputs. While these effects are more evident in tightly controlled murine models, some parallels were observed in human data. For instance, these effects were more pronounced in the *APOE-TR* mouse models, where the animals' diets were tightly controlled. In the DIAMOND study, considerable intra-group variations were observed, which may have been driven by other modifiable factors, such as participant diet and medication, that were not controlled for in this study and potentially masked any differences in the microbiota. However, some of the metabolomic patterns observed in the animal data were reflected in the KEGG pathway analysis of the DIAMOND participants' microbiotas. Future research should investigate the role of tauroconjugated bile acid metabolism in the context of neuroinflammation and cognitive health, as well as how it may be influenced by age, sex, *APOE* genotype, and dietary flavan-3-ol intake.

Chapter 4: Does *APOE* influence the health outcomes of dietary epicatechin?

4.1. Introduction

EC and other flavonoids have been shown to exert a range of cognitive benefits, with both acute and chronic intake associated with enhanced cognitive health outcomes [130, 144, 157]. These improvements in cognitive function are mediated through multiple mechanisms, including increased CBF, enhanced synaptic plasticity and suppression of inflammatory processes [130]. Beyond its neurocognitive effects, EC has been reported to improve glucose sensitivity [283, 284], and to modulate weight gain [285]. As obesity is a well-established risk factor for AD, and a key driver of type II diabetes (another major risk factor for AD and related dementias [286]), these metabolic effects are of particular relevance. The bioavailability of EC in the upper GI tract is limited due to the absence of endogenous mammalian enzymes required for its degradation. Consequently, approximately 70% of ingested EC undergoes microbial metabolism in the colon, yielding bioactive valerolactones and valeric acids [176, 177].

Both *APOE* genotype and sex are established risk factors for AD, and each has been shown to influence the gut microbiota composition [89, 104]. Previous chapters have examined whether EC metabolism and microbiota composition are influenced by *APOE* genotype, sex, and age (Chapter 2.3 & Chapter 3.3). However, the extent to which these factors modulate the health benefits of EC consumption remains unclear.

Given the association between energy dysregulation and AD risk [287], the *APOE* genotype and metabolic dysfunction [56, 58, 59], and EC supplementation and changes in glucose homeostasis and regulation [309, 329], it is plausible that the *APOE* genotype and sex may influence the physiological response to EC beyond microbial metabolism alone. This chapter will investigate whether *APOE* genotype and sex modulate EC-associated improvements in a) biometric measurements, b) energy and glucose metabolism, c) inflammatory status, and d) cognitive performance.

Prior findings in this thesis demonstrated sex and *APOE* genotype-dependent differences in the urinary profile of conjugated structurally derived EC metabolites. These conjugates arise from hepatic metabolism of the fraction of EC absorbed in the upper GI tract, before exposure to the colonic microbiota [177] (See Chapter 2). Specifically, phase II metabolism, mediated by transferase enzymes, introduces conjugate groups, such as sulfate or glucuronic acid, to the parent molecule, enhancing water solubility and promoting urinary excretion as part of the detoxification pathways [288]. Variability in phase II metabolism between sex or *APOE* genotypes may contribute to differential physiological responses to EC supplementation, providing a mechanistic explanation for earlier observations. Accordingly, this chapter will also evaluate the hypothesis that hepatic phase II metabolism is influenced by sex and *APOE* genotype.

4.2. Materials and Methods

4.2.1. Study Design

This mouse study was conducted as part of the experiments described in previous chapters. A total of forty-seven mice were used, distributed between the *APOE3/3*-TR and *APOE4/4*-TR genotypes and further stratified by sex and dietary intervention (See Chapter 2.2.3 for full study details). Animals were maintained on either an HF diet or an HF diet supplemented with EC (15mg/kg body weight) for 20 weeks. At the end of the intervention, animals were sacrificed, and colon and caeca were excised, immediately placed on dry ice, and subsequently stored at -80 °C.

4.2.2. Biometric measurements

To assess the food consumption of the mice over the 20-week intervention, food was checked twice a week. To prevent the degradation of EC, all leftover food was discarded each time, and food was replaced with new pellets, which were kept at -20 °C. The weight of food scattered in the bedding was not assessed. Average daily food consumption per mouse was calculated as follows:

$$\text{Average daily food consumption per mouse} = \frac{\text{Refill food weight} - \text{Final food weight}}{\text{Number of Days} * \text{Number of Mice}}$$

This daily consumption was then averaged across each cage for the duration of the experiment. As there were only two cages per group, it was not possible to complete a statistical assessment of the food weights; however, the mean daily consumption per mouse in each group was reported along with the standard deviation. To assess whether the *APOE* genotype and sex influence the weight-modulating effects of EC, the final body weight of the mice was taken on the day of sacrifice. After sacrifice, the visceral adipose fat pads and liver were removed and weighed. The liver was then transferred to dry ice and then to -80 °C for long-term storage. Changes in body weight between baseline and the final week were reported, as were the percentage changes. EC intake was calculated based on the 200 mg EC / kg diet. As the weight of the mice increased throughout the diet, the median body weight of all the mice on the HFEC diet was used to calculate daily EC intake using the following values:

$$\text{Daily EC intake/kg mouse body weight} = \frac{200\text{mg/kgEC} * \text{mean daily intake of food}}{\text{Median HFEC body weight}}$$

Subsequently, the human equivalent dose (HED) was calculated using the human equivalent body surface area conversion factor of 0.081 [289], which was then multiplied by the average weight for UK males (85 kg) and females (72 kg) for the equivalent daily intake for a human [290].

4.2.3. Behavioural Analysis

Cognitive and behavioural performance in mice was assessed using three tests. All procedures were conducted according to the methods outlined below, adapted from Pontifex et al. [291]. Animals' movements were tracked, recorded and analysed using video-tracking software (Ethovision XT 17.5, Noldus, The Netherlands). To minimise the order effect, cages were selected for behavioural testing in a random sequence.

4.2.3.1. Open field and novel object recognition tests

The open field (OF) and novel object recognition (NOR) tests were conducted as follows: The experimental apparatus consisted of a grey maze measuring 50 cm x 50 cm x 50 cm illuminated with 100 lux lighting. On day one, the OF was performed as a measure of

anxiety-like behaviour and locomotor activity in mice and simultaneously served as the habituation phase for the NOR task. Mice were placed in the centre of the maze and allowed to freely explore for 10 minutes. Anxiety-like behaviour was quantified using a tracking software, which calculated a score as the ratio of time spent in the central 25cm x 25cm zone to the time spent in the peripheral zone. Higher ratios indicate reduced anxiety-like behaviour, reflecting a greater tendency of the animals to explore the centre rather than remain in the periphery. Distance travelled during the 10-minute assessment was also recorded.

On day 2 of the trials, animals were conditioned to two identical objects for a 15-minute period, after which they were returned to their cages. This was followed by a 60-minute interval, during which one of the familiar objects was exchanged for a novel object. After the inter-trial period, the mice were reintroduced to the maze for a subsequent 10 minutes. Videos were analysed for a 5-minute period. If the total object exploration (either novel or familiar) did not reach a cumulative 10 seconds, video analysis was continued until the 10-second mark was reached. Animals that failed to reach a total of 10 seconds were excluded from the analysis. NOR testing is a measure of recognition memory. Discrimination index (DI) was calculated as follows:

$$DI = \frac{\text{Time at Novel Object} - \text{Time at Familiar Object}}{\text{Total time at Both Objects}}$$

4.2.3.2. *Y-maze*

The Y-maze spontaneous alternation test is a measure of an animal's spatial working memory and was performed at the end of day 1 of testing for the OF and NOR. Briefly, the maze apparatus consisted of a white plastic maze with dimensions of 38.5 × 8 × 13 cm, spaced 120° apart, and illuminated with 100 lux of lighting. Mice were introduced to the maze and allowed to freely explore for 7 minutes. Analysis measured zone tracking and locomotor activity. Spontaneous alternations were calculated as follows:

$$\text{Spontaneous alternation} = \frac{\text{Number of alternations}}{\text{Total arm enteries} - 2} * 100$$

4.2.4. Intraperitoneal glucose tolerance test

To evaluate differences in energy metabolism, an intraperitoneal glucose tolerance test (IPGTT) was performed on week 17 of the dietary intervention. Cage selection for behavioural testing was randomised to control for the order effect. To minimise the influence of dietary carbohydrates on glucose response, mice were subjected to a 16-hour overnight fast with *ad libitum* access to water. Animals were transferred to clean cages with fresh bedding to prevent ingestion of residual food. Blood samples were obtained via tail microvessel sampling, and the procedure was performed under local anaesthesia (Intubease 20mg/ml, Dechra, UK) to reduce stress-related effects on glucose metabolism.

Baseline fasting glucose and ketone (beta-hydroxybutyrate (BHB)) levels were measured using the CareSens Dual (i-Sens Inc, South Korea) with corresponding glucose (CareSens Pro) and ketone (KetoSens) test strips. Glucose (2 mg/g body weight) was administered intraperitoneally, and blood glucose concentrations were measured at 15-, 30-, 60- and 120-minutes post-injection. The IPGTT was performed across four days, with cages randomly assigned to minimise cage effects. Individual glucose profiles were generated for each mouse, and overall glucose responses were quantified as the area under the curve (AUC) using the trapezoidal method.

4.2.5. RNA sequencing of hippocampal tissue

Total RNA was isolated from hippocampi (n = 3 randomly selected per group, sex, and genotype) using the Qiazol reagent (Qiagen, UK). Messenger RNA was then purified from total RNA using poly-T oligo-attached magnetic beads. After fragmentation, the first strand complementary DNA (cDNA) was synthesised using random hexamer primers, followed by the second strand cDNA synthesis using either deoxyuridine triphosphate for a directional library or deoxythymidine triphosphate for a non-directional library [292]. The libraries were checked with Qubit and real-time PCR for quantification and bioanalyser for size distribution detection. Quantified libraries were pooled and sequenced on Illumina NovaSeq 6000 platform using a paired-end 150 bp (BGI, China), according to effective library concentration and data amount. Raw data (raw reads) of

FASTQ format were first processed through in-house Perl scripts. The index of the reference genome was built using Hisat2 v2.0.5 [293], and paired-end clean 1 reads were aligned to the reference genome using Hisat2 v2.0.5. FeatureCounts v1.5.0-p3 was used to count the read numbers mapped to each gene [294]. FPKM of each gene was calculated based on the length of the gene and the read count mapped to this gene.

4.2.6. Real-time quantitative polymerase chain reaction of liver inflammation and polyphenol metabolism

The following experiments were performed in collaboration with Oona Hinshelwood, a visiting PhD student in the lab.

Total RNA was extracted from the livers, and real-time quantitative polymerase chain reaction (qRT-PCR) was conducted to measure differential expression of inflammatory marker genes and the genes associated with phase I and II metabolism of dietary polyphenols in the liver.

RNA extraction, cDNA synthesis, and qRT-PCR were carried out using methods described previously by Pontifex et al. [295]. Briefly, 100 mg of liver tissue was homogenised in QIAzol (Qiagen, UK) lysis reagent at 6,000 rpm in a Precellys R24 (Bertin Technologies, France). Following chloroform extraction, isopropanol precipitation, and ethanol washing, RNA concentration and purity were analysed using a 2000 nanodrop spectrophotometer (Fisher Scientific, UK). cDNA synthesis was carried out by transcribing 2 µg of RNA. This involved the use of DNase and thermal cycling (25 °C for 30 mins, 75 °C for 5 mins) to remove trace amounts of contaminating DNA. RNA was added to a master mix of the following reagents: buffer (5X), deoxynucleoside triphosphates, dithiothreitol, random primers, Moloney murine leukaemia virus reverse transcriptase, and RNase-free water. Thermocycling (37°C for 90 minutes and 72°C for 10 minutes) was carried out to reverse transcribe RNA into cDNA. QRT-PCR was performed using a SYBR green master mix, assessing the relative gene expression as previously described by Pontifex et al. [295]. Each sample was tested in duplicate using four housekeeping genes (*Gapd*, *Hprt*, *B2m*, and *Tbp*). *Tbp* was selected as the internal gene control using RefFinder software [296]. Gene relative expression was calculated using the $2^{-\Delta\Delta C_t}$

method. Primer sequences are provided (Table 4.1). Due to the normalisation steps, the mice were split by sex and normalised to their respective HF *APOE3/3*-TR mice.

Table 4.1: Forward and reverse sequences for PCR primers used, along with their accession numbers.

Full gene name	Gene name	Accession number	Forward sequence (5'→3')	Reverse sequence (5'→3')
Beta-2-microglobulin	<i>B2m</i>	NM_009735	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCCGGCTTCCCATTG
Catechol-OMethyltransferase	<i>Comt</i>	NM_001111062	CCAGCAAATGCTGGACTTCGCA	AAGGTAGCGGTCTTCCAGTGG
Cytochrome P450, family 3, subfamily, polypeptide 41A	<i>Cyp3a41a</i>	NM_017396	TGCTCTTCACCATGACCCACAG	CCTCATGCCAATGCAGTTCCTG
Glyceraldehyde-3-phosphate dehydrogenase	<i>Gapdh</i>	NM_008084	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Glucosylceramidase beta 2	<i>Gba2</i>	NM_172692	TCAAGGCTTCTGGAGGACATG	TGGTCTGCGTAGCCTCCATTCT
Hypoxanthine guanine phosphoribosyl transferase 1	<i>Hprt</i>	NM_013556	CTGGTGAAAAGGACCTCTCGAAG	CCAGTTTCACTAATGACACAAACG
Interferon-gamma	<i>IFN-γ</i>	NM_008337	GCCACGGCACAGTCATTGA	TGCTGATGGCCTGATTGTCTT
Interleukin-10	<i>IL-10</i>	NM_010548	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Interleukin-1-beta	<i>IL-1β</i>	NM_008361	TGGACCTTCCAGGATGAGGACA	GTTTCATCTCGGAGCCTGTAGTG
Nuclear factor NFkappa-B p105 subunit	<i>Nfkb1</i>	NM_008689	ATGGCAGACGATGATCCCTAC	TGTTGACAGTGGTATTTCTGGTGATGGCAGACGATGATCCCTAC
NOD-like receptor family pyrin domain containing 6	<i>Nlrp6</i>	NM_001081389	CTGGCGTCATTGTGGAACCTCT	TCTCACTCAGCTCCACAGAGGT
Nitric oxide synthase 2 (inducible)	<i>Nos2 (iNos)</i>	NM_010927	GAGACAGGGAAGTCTGAAGCAC	CCAGCAGTAGTTGCTCCTCTTC
Prostaglandin endoperoxide synthase 2 (Cyclooxygenase-2)	<i>Ptgs2 (Cox2)</i>	NM_011198	GCGACATACTCAAGCAGGAGCA	AGTGGTAACCGCTCAGGTGTTG
Sulfotransferase 1C1	<i>Sult1c1</i>	NM_018751	GCCAATACCTATGACCGACACC	GGTGAAGCATTTGAACAGGCAAG
Sulfotransferase 1D1	<i>Sult1d1</i>	NM_016771	CACACCTTCTCTGTTGAGCTGCT	TGCCAGGCTCTGGGTGGATTTT
Sulfotransferase 5A1	<i>Sult5a1</i>	NM_020564	TTCTTCGGCTCCTGGTTTGACC	ACTTGCGGATAGTAGAGCGAGG
TATA box-binding protein	<i>Tbp</i>	NM_013684	CTACCGTGAATCTTGGCTGTAAAC	AATCAACGCAGTTGTCCGTGGC
Tumour necrosis factor alpha	<i>Tnf-α</i>	NM_013693	GGTGCCCTATGTCTCAGCCTCTT	GCCATAGAACTGATGAGAGGGAG
UDP glucuronosyltransferase family 1 member A	<i>Ugt1a</i>	NM_201645	ATGCCAAGCGCATGGAACTCG	ATAGGACGGTCTTGTGAAGGC

4.2.7. Statistical Analysis

Statistical analysis was conducted in R 4.4.1 [237]. The Shapiro-Wilk test for normal distribution was carried out in base R. The Levene test for homogeneity of variance was carried out in the package car [238]. For the change in body weight measurements, the Shapiro-Wilk result was insignificant ($p > 0.05$); however, the Levene test result was

significant ($p < 0.05$). To account for this, the following mixed linear model was fitted with the package lme4 [297]:

$$\Delta \text{Body Weight} \sim (\text{APOE genotype} * \text{Sex} * \text{Diet}) + (1|\text{Cage})$$

To account for the unequal variances while maintaining the mixed-model structure, cluster-robust standard errors were applied using the clubSandwich package [298]. Fixed effects, including the three-way interaction, were evaluated using with CR2 covariance estimator and clustering by Cage. This approach provides robust t-values and p-values for the fixed effects while accounting for both random effects and heteroscedasticity.

Liver weights, fat pad weights, IPGTT results, Y-maze results, and all distances travelled yielded both insignificant Shapiro-Wilk and Levene Tests (all $p > 0.05$). Because of this, they were analysed by the following mixed linear model fitted with the package lme4 [297]:

$$\text{Independent Variable measurement} \sim (\text{APOE genotype} * \text{Sex} * \text{Diet}) + (1|\text{Cage})$$

Fixed effects and their interactions were assessed via Type III ANOVA. Pairwise comparisons of least-squares followed significant effects ($p < 0.05$) means with BH FDR correction [299].

The OF study results produced significant Shapiro-Wilk ($p < 0.05$) and Levene ($p < 0.05$) results. To account for the repeated measures within cages, a linear mixed-effects model was fitted using the lme4 package [297]:

$$\% \text{ time in centre} \sim \text{APOE genotype} * \text{sex} * \text{diet} + (1|\text{Cage})$$

To account for unequal variances and non-parametric data while maintaining the mixed-model structure, cluster-robust standard errors were calculated using the clubSandwich package [298]. For the NOR test, only 3 out of the 47 mice interacted with the objects in the maze, producing a score. Therefore, statistical analysis was not possible for this test.

Analysis of the RT-qPCR results was performed using GraphPad Prism version 10 (San Diego, USA). Levene and Shapiro-Wilks Tests for normality were run prior to analysis. Due to the sex split caused by the normalisation, the following mixed linear model was carried out to analyse the mice's gene expression:

$$\text{Relative gene expression} \sim \text{APOE genotype} * \text{Diet} + (1|\text{Cage})$$

This was subsequently run through a 2-way ANOVA. If p-values were identified as significant ($p < 0.05$), pairwise comparisons were carried out using the least-squares means method. The BH method was utilised to account for FDR. Data was plotted as a bar plot, with the raw data points displayed on top, and error bars show the standard deviation. Outliers were identified using the ROUT test ($Q = 1\%$) and removed before statistical analysis. Outlier data points are identified in red in the graphs.

Analysis of the RNA sequencing data was carried out by Dr Tom visualisation software (BGI, China). Data was analysed using the DESeq2 package in R (version 4.3.2) to investigate the effects of diet, sex, and genotype on gene expression. Raw count data were filtered to remove low-abundance genes, retaining those with a minimum count of 5 in at least 15% of the samples. Genes with less than 15% variance were excluded. A generalised linear model (GLM) assessed the main effects of diet and sex. Differential expression was tested for each factor, which captures differential responses to diet between sexes. P-values were adjusted using the BH method to control the FDR. Enrichr was used to perform KEGG analysis of differentially expressed genes (DEGs) [300, 301].

4.3. Results

4.3.1. Biometric measurements

Examination of the average daily food consumption per mouse revealed that in the *APOE4/4*-TR mice, the consumption was 2.7 g for females HF-fed, and 2.9 g for all the other sex and diet groups. For the *APOE3/3*-TR mice, the average daily consumption per mouse was 2.7 g for HF-fed females and 2.8 g for HF-fed males. For all HFEC-fed mice, the average daily consumption was 2.9 g. As there were only two cages per group, it was not possible to run a statistical analysis. However, when accounting for variation in standard deviation, the assumption can be made that there were no significant differences in dietary intake between the groups. With the average daily consumption of the HFEC diet at 2.9 g/mouse, it was possible to estimate the EC dose of the diet. Using the median body weight across the experiment for HFEC diet mice of 39.90 g, the HED of

EC was calculated at an intake of 86 mg/day for a 75 kg adult female and 100 mg/day for an adult 85 kg male [290].

The biometric analyses highlight the influence of *APOE* genotype, sex, and EC supplementation on weight gain and body fat distribution in mice. Overall weight gain exhibited biological variation, with females on the HFEC (*APOE3/3*-TR HFEC females: 26.0 g (SD: 3.4) and *APOE4/4*-TR HFEC females: 25.7 g (SD: 8.0)) diet showing greater increases compared to those on the HF diet (*APOE3/3*-TR HF females: 20.1 g (SD: 5.2) and *APOE4/4*-TR HF females: 22.8 g (SD: 7.1)). In males, *APOE4/4*-TR mice on the HFEC diet (*APOE3/3*-TR HFEC males: 21.2 g (SD: 2.4) and *APOE4/4*-TR HFEC males: 21.6 g (SD: 1.2)) displayed reduced weight gain relative to their HF-fed (*APOE3/3*-TR HF males: 21.6 g (SD: 2.2) and *APOE4/4*-TR HF males: 24.2 g (SD: 2.6)) counterparts. Additionally, *APOE4/4*-TR mice of both sexes tended to gain more weight on the HF diet than *APOE3/3*-TR mice; however, these differences did not reach statistical significance ($p > 0.05$) (Figure 4.1A).

Fat pad weights revealed a pronounced effect of sex ($p < 0.001$), with pairwise comparison confirming that females had significantly heavier fat pads than males across all *APOE* genotype and diet groups (all FDR $q < 0.05$) (*APOE3/3*-TR HF: females 3.2 g (SD: 0.9), males 1.8 g (SD: 0.4)) (*APOE3/3*-TR HFEC: females 3.4 g (SD: 1.1), males 2.0 g (SD: 0.3)) (*APOE4/4*-TR HF: females 3.7 g (SD: 1.2), males 1.9 g (SD: 0.4)) (*APOE4/4*-TR HFEC: females 3.7g (SD: 1.5), males 1.8 g (SD:0.4))(Figure 4.1B).

Liver weights also differed significantly by sex ($p < 0.001$), with males exhibiting heavier livers than females across all diet and genotype groups (all FDR $q < 0.001$) (*APOE3/3*-TR HF: females 1.4 g (SD: 0.2), males 2.7 g (SD: 0.8)) (*APOE3/3*-TR HFEC: females 1.3 g (SD: 0.3), males 2.8 g (SD: 0.9)) (*APOE4/4*-TR HF: females 1.4 g (SD: 0.3), males 3.1 g (SD: 0.8)) (*APOE4/4*-TR HFEC: females 1.6 g (SD: 0.4), males 2.8 g (SD: 0.5)) (Figure 4.1C).

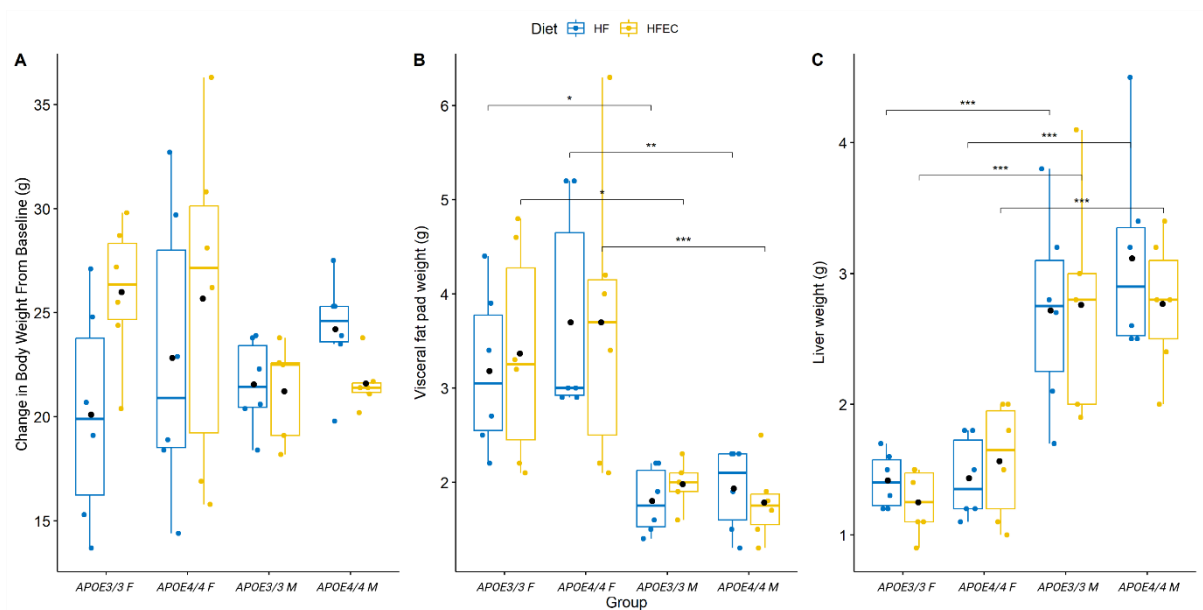


Figure 4.1: Biometric measurements from male (M) and female (F) APOE3/3-TR (APOE3) and APOE4/4-TR (APOE4) mice fed a high-fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 1520 mg/kg body weight epicatechin (HFEC). A) Change in body weight of the mice throughout the experiment B) Weight of the Visceral Fat Pads after sacrifice C) Liver weights at sacrifice. Significant pairwise (Benjamini Hochberg corrected q -value <0.05) indicated with stars: * $0.05 < q\text{-value} \leq 0.01$, ** $0.01 < q\text{-value} \leq 0.001$, *** $0.001 < q\text{-value}$. Mean value for each group plotted as the black point. Group sizes: APOE3/3 F HF $n = 6$, APOE3/3 F HFEC $n = 6$, APOE4/4 F HF $n = 6$, APOE4/4 F HFEC $n = 6$, APOE3/3 M HF $n = 6$, APOE3/3 M HFEC $n = 5$, APOE4/4 M HF $n = 6$, APOE4/4 M HFEC $n = 6$

4.3.2. Behavioural Results

The behavioural assessment revealed differences in certain aspects of mouse behaviour, while others remained unaffected. Y-maze testing showed a significant interaction between sex and diet on spontaneous alternations performance ($p = 0.017$). Specifically, in male mice, both genotypes demonstrated increased spontaneous alternations when supplemented with EC, although pairwise comparisons only showed significance in APOE4/4-TR mice (FDR $q = 0.035$). Interestingly, APOE3/3-TR females on the HFEC diet displayed lower alternation scores compared to their HF controls, whereas no effect was observed in APOE4/4-TR female mice; however, these differences did not reach statistical significance (all $p > 0.05$) (Figure 4.2A). In contrast, in the OF test, all but one mouse exhibited scores below 0.5, indicating that the majority of animals spent the trial duration along the periphery of the arena due to increased anxiety-related behaviours. No significant effects of sex, genotype, or diet were observed (all FDR $q > 0.05$) (Figure 4.2B). This behavioural pattern also impacted the NOR task, which was conducted in the same arena. Only 3 out of 47 mice engaged with the object for more than the required 10

seconds, precluding statistical analysis on these results. Heat maps from the second phase of the NOR illustrate that mice predominantly explored the arena edges, rather than interacting with the objects (Figure 4.2C).

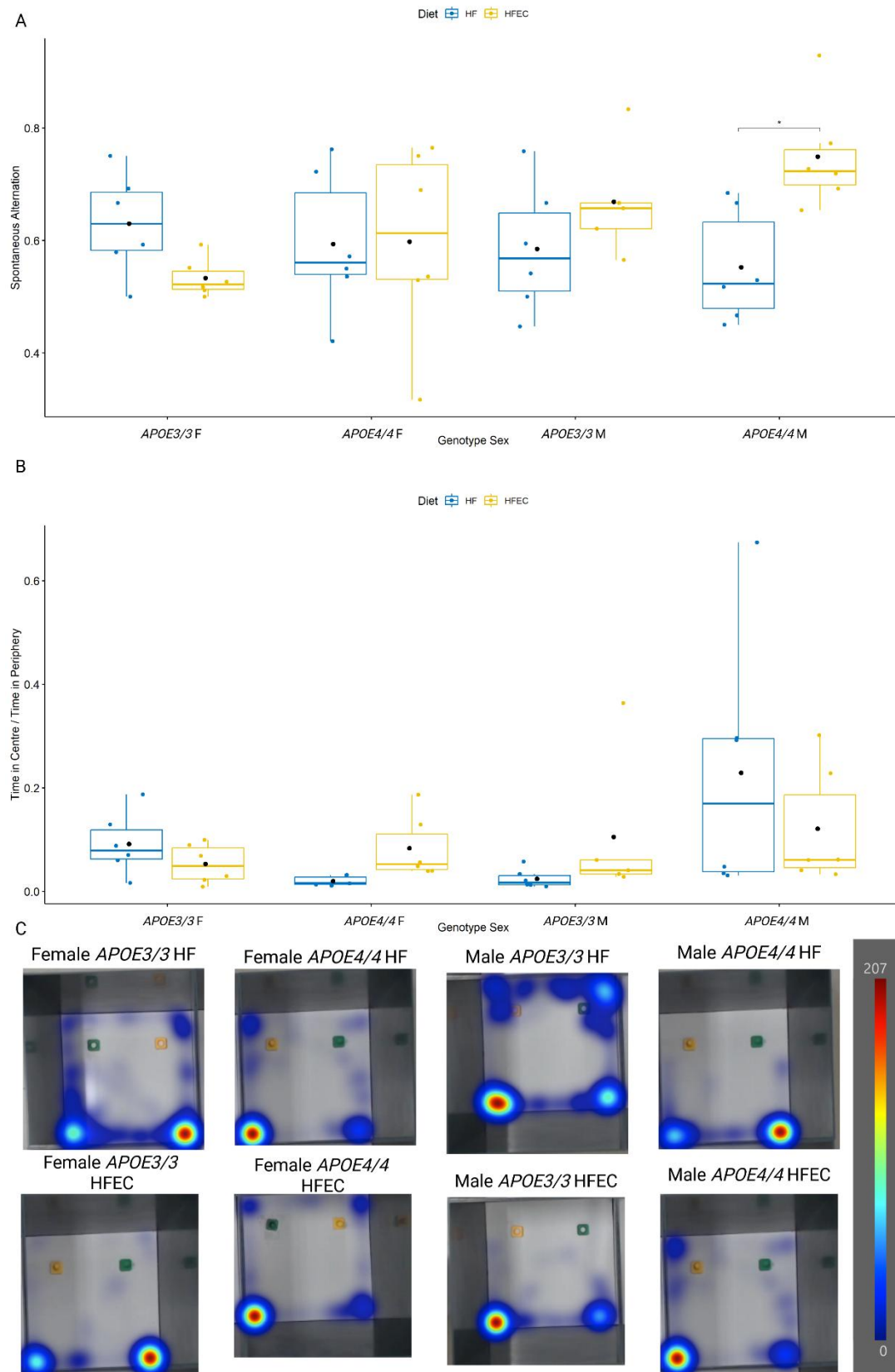


Figure 4.2: Results from the behaviour trial results from male (M) and female (F) APOE3/3-TR (APOE3/3) and APOE4/4-TR (APOE4/4-TR) mice fed a high-fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC). A) Y-maze results. 3-way ANOVA results for each measurement in table in the corner of plot, significance p -value < 0.05 highlighted in bold. Significant pairwise (Benjamini Hochberg corrected q -value < 0.05) indicated with stars: * $0.05 > q$ -value ≥ 0.01 . Mean value for each group plotted as the black point. The higher the spontaneous alternation, the better the working memory. B) Open field result. The greater the score, the greater the time in the centre and the lower the anxious behaviour. C) Representative heatmaps for each group during the second day of the novel object recognition. Group sizes: APOE3/3 F HF $n = 6$, APOE3/3 F HFEC $n = 6$, APOE4/4 F HF $n = 6$, APOE4/4 F HFEC $n = 6$, APOE3/3 M HF $n = 6$, APOE3/3 M HFEC $n = 5$, APOE4/4 M HF $n = 6$, APOE4/4 M HFEC $n = 6$

The total distance travelled by each mouse was also measured throughout each of the behavioural tests. During the OF and the NOR, the *APOE* genotype had a significant effect on travelled distance (both $p < 0.05$), with the *APOE3/3-TR* mice often travelling further than their *APOE4/4-TR* counterparts (Table 4.2). Pairwise analysis of the distance travelled indicated that in the OF, significance was only reached between the HF-fed female mice (FDR $q = 0.033$); in the NOR, significance was not observed between any pairwise genotype groups (all FDR $q > 0.05$). However, in the NOR, the sex of the mice also had a significant effect on the distance travelled ($p < 0.001$), with females often travelling a greater total distance than their male equivalents, pairwise analysis of this highlighting significance in the HF-fed mice of both genotypes (*APOE3/3-TR* FDR $q = 0.029$ and *APOE4/4-TR* FDR $q = 0.041$) There was no significant effect of any of the variables in the Y-maze results (Table 4.2).

Table 4.2: Results from the total distance travelled (cm) from male (M) and female (F) APOE3/3-TR (APOE3/3) and APOE4/4-TR (APOE4/4) mice fed a high-fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC) in each of the behaviour tests. Open field (OF), Novel object recognition (NOR) and Y-maze. Significant ($p < 0.05$) 3-way ANOVA results highlighted in bold. Group sizes: APOE3/3 F HF $n = 6$, APOE3/3 F HFEC $n = 6$, APOE4/4 F HF $n = 6$, APOE4/4 F HFEC $n = 6$, APOE3/3 M HF $n = 6$, APOE3/3 M HFEC $n = 5$, APOE4/4 M HF $n = 6$, APOE4/4 HFEC $n = 6$. The greater the distance travelled the more active the mice.

Sex	Female				Male				3-way ANOVA p-value						
Diet	HF		HFEC		HF		HFEC								
Gen	APOE3/3	APOE4/4	APOE3/3	APOE4/4	APOE3/3	APOE4/4	APOE3/3	APOE4/4							
Test	$\bar{x}$ cm (SD)	$\bar{x}$ cm (SD)	$\bar{x}$ cm (SD)	$\bar{x}$ cm (SD)	$\bar{x}$ cm (SD)	$\bar{x}$ cm (SD)	$\bar{x}$ cm (SD)	$\bar{x}$ cm (SD)	Gen	Diet	Sex	Gen: Diet	Gen: Sex	Diet: Sex	Gen: Diet: Sex
OF	2053 (327)	1501 (319)	1750 (410)	1369 (246)	1480 (372)	1187 (298)	1547 (406)	1625 (123)	0.029	0.857	0.095	0.256	0.141	0.060	0.671
NOR	1637 (595)	1275 (444)	1531 (585)	1089 (329)	982 (336)	672 (174)	968 (477)	874 (274)	0.020	0.835	<0.001	0.786	0.422	0.339	0.553
Y-maze	2482 (56)5	2229 (486)	2382 (551)	2296 (347)	2288 (277)	1796 (363)	2083 (348)	2247 (315)	0.184	0.667	0.055	0.102	0.983	0.574	0.326

4.3.3. Intraperitoneal Glucose Tolerance Test Results

The three different measurements collectively highlighted sex-, genotype-, and diet-dependent differences in the metabolism of both BHB and glucose in these mice. In the fasted state, ketone levels were significantly influenced by sex ($p = 0.008$), *APOE* genotype ($p = 0.004$), and EC supplementation ($p = 0.028$). Specifically, females, *APOE4/4*-TR mice, and those maintained on an HF diet exhibited higher BHB concentrations relative to their respective counterparts. Pairwise comparisons further revealed that male *APOE4/4*-TR mice on a high-fat diet had significantly higher BHB levels than the *APOE3/3*-TR equivalents (FDR $q = 0.03$) (Figure 4.3A). For fasted glucose concentrations, only sex main effects were observed ($p = 0.015$), with males displaying significantly higher levels than females, independently of genotype or diet. Pairwise analysis indicated that male *APOE4/4*-TR on HFEC had significantly higher glucose concentrations compared to their female counterparts (FDR $q = 0.04$) (Figure 4.3B). Finally, the glucose tolerance test demonstrated a sex effect in the total glucose response over 2 hours, with males exhibiting a significantly greater response than females ($p = 0.007$), however, there was no pairwise significance observed (FDR $q > 0.05$), suggesting that all males irrespective of diet and genotype, have higher glucose response than all females (Figure 4.3C).

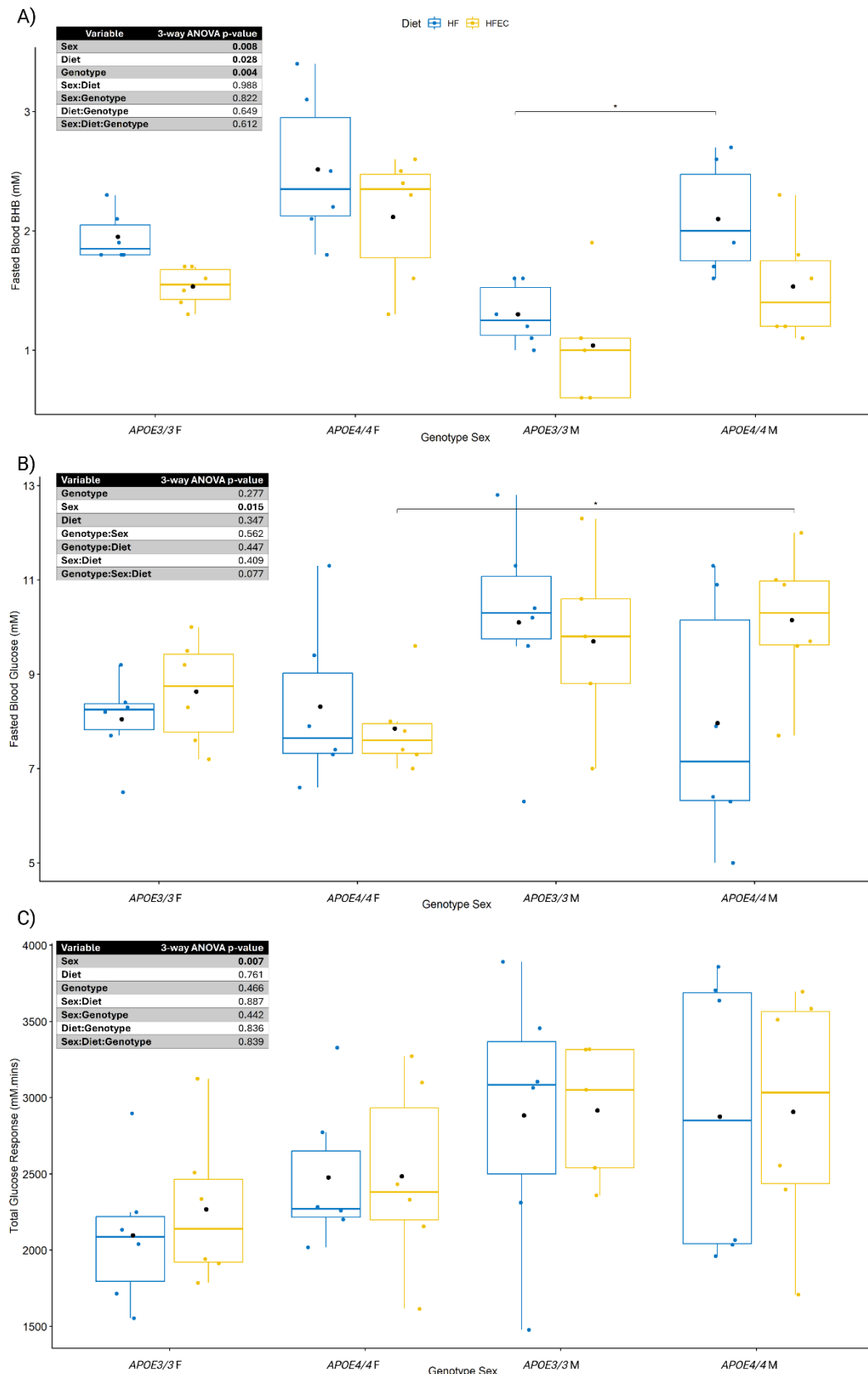


Figure 4.3: Results from the intraperitoneal glucose tolerance test results from male (M) and female (F) APOE3/3-TR (APOE3/3) and APOE4/4-TR (APOE4/4) mice fed a high-fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC). A) Fasted blood β -hydroxybutyrate (BHB) concentration. B) Fasted blood glucose concentration C) Total blood glucose response after 120 mins after 2mg/g body weight injection of glucose. 3-way ANOVA results for each measurement in table in the corner of plot significance p-value <0.05 highlighted in bold. Significant pairwise (Benjamini Hochberg corrected q-value <0.05) indicated with stars: * 0.05>q-value $\geq$ 0.01. Group sizes: APOE3/3 F HF n = 6, APOE3/3 F HFEC n = 6, APOE4/4 F HF n = 6, APOE4/4 F HFEC n = 6, APOE3/3 M HF n = 6, APOE3/3 M HFEC n = 5, APOE4/4 M HF n = 6, APOE4/4 HFEC n = 6

4.3.4. Quantitative real-time polymerase chain reaction

4.3.4.1. Immunomodulatory genes

The study demonstrated sex- and genotype-specific differences in the hepatic inflammatory response to EC supplementation. In HFEC-fed *APOE4/4*-TR females, the expression of pro-inflammatory genes *Nos2* (FDR $q = 0.038$), *TNF- α* (FDR $q = 0.010$), and *Ptgs2* (FDR $q < 0.001$) was significantly up-regulated, as well as anti-inflammatory genes *IL-10* (FDR $q = 0.046$) and *Nlrp6* (FDR $q < 0.001$) when compared to their HF counterparts (Figure 4.4A-E). In contrast, EC supplementation did not alter the expression of either pro- or anti-inflammatory genes in *APOE3/3*-TR mice of either sex or in *APOE4/4*-TR males (all FDR $q > 0.05$). Genotype-related differences were also observed. Under the HF diet, *APOE3/3*-TR males exhibited higher expression of *Nlrp6* and *TNF- α* compared to *APOE4/4*-TR equivalents (both FDR $q = 0.004$). Under the HFEC diet, female *APOE4/4*-TR mice showed significantly greater expression than their *APOE3/3*-TR counterparts for the proinflammatory *Ptgs2* (FDR $q = 0.040$) and the anti-inflammatory *IL-10* (FDR $q = 0.001$) (Figure 4.4F-J).

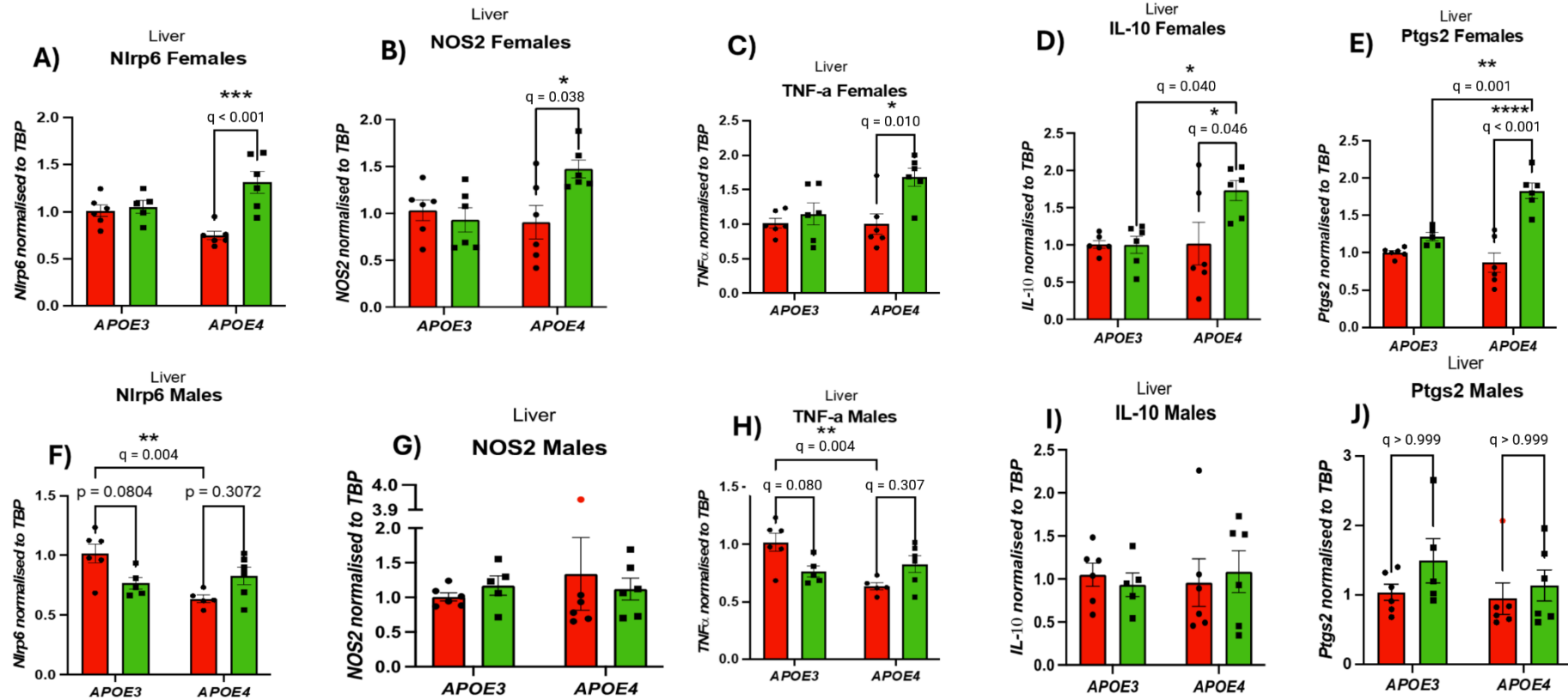


Figure 4.4: Expression of pro- and anti-inflammatory genes in response to EC supplementation in A-E) females and F-J) males, respectively. Mice were fed either a high fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC). Significant pairwise (Benjamini Hochberg corrected q -value < 0.05) indicated with stars: * $0.05 > q$ -value ≥ 0.01 , ** $0.01 > q$ -value ≥ 0.001 , *** $0.001 > q$ -value. Group sizes: APOE3/3 F HF $n = 6$, APOE3/3 F HFEC $n = 6$, APOE4/4 F HF $n = 6$, APOE4/4 F HFEC $n = 6$, APOE3/3 M HF $n = 6$, APOE3/3 M HFEC $n = 5$, APOE4/4 M HF $n = 6$, APOE4/4 HFEC $n = 6$

4.3.4.2. Phase II metabolic genes

The hepatic expression of sulfotransferase genes was significantly modulated in response to dietary EC. In male *APOE3/3*-TR mice, *Sult1d1* expression was upregulated relative to their HF-fed controls (FDR $q = 0.030$). Moreover, a genotype-dependent difference was observed in both sexes, with *APOE3/3*-TR mice showing a significantly higher *Sult1d1* expression compared to *APOE4/4*-TR counterparts (Female FDR $q = 0.006$, Male FDR $q = 0.010$) (Figure 4.5A, B). Notably, expression of the isoform *Sult1c1* was significantly increased in response to the EC supplementation in female *APOE4/4*-TR mice only (female FDR $q = 0.025$) (Figure 4.5C, D).

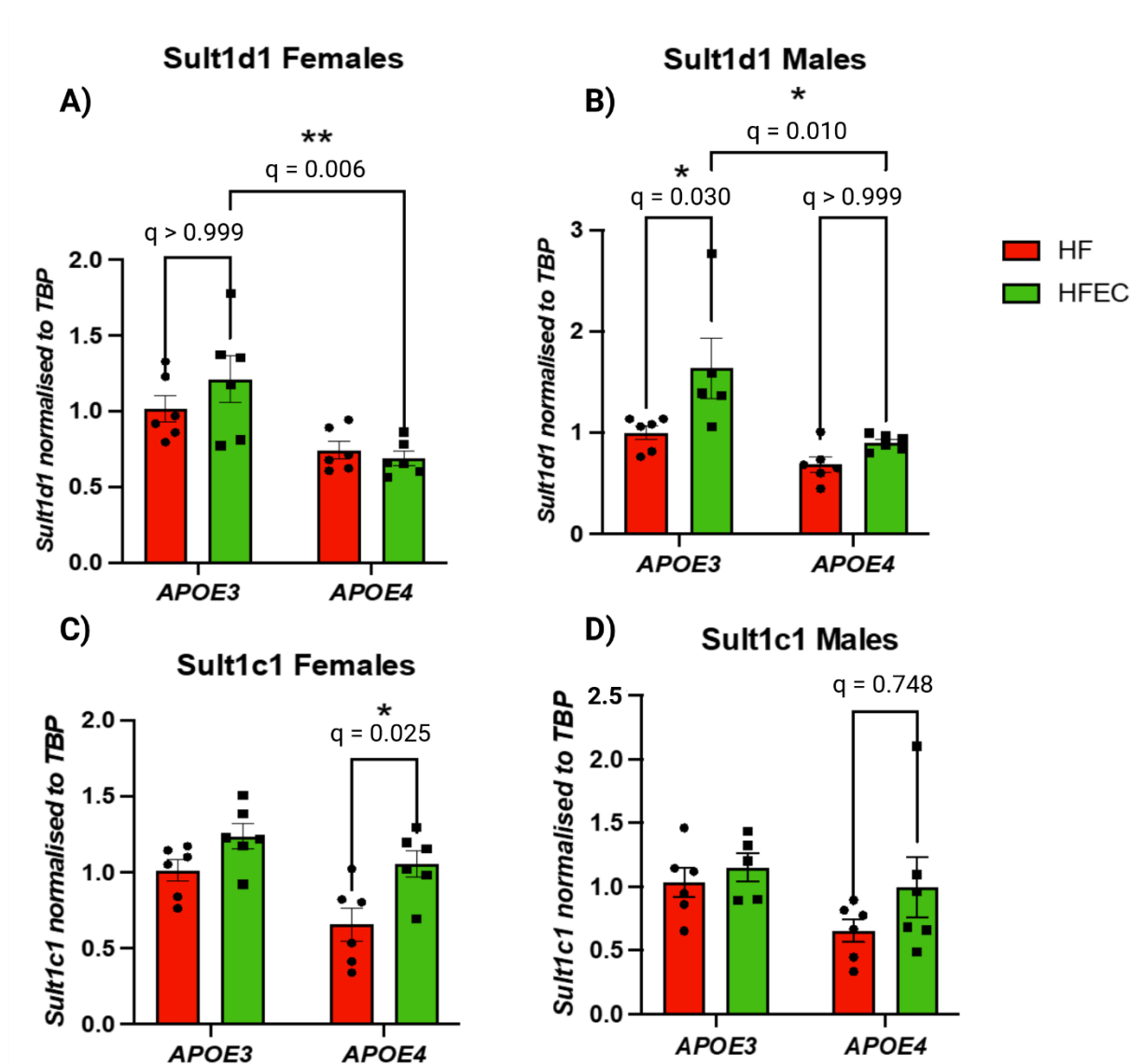


Figure 4.5: Differential gene expression of sulfotransferase genes in the liver. A) and B) show *Sult1d1* in females and males, respectively. C) and D) Show *Sult1c1* in females and males, respectively. Significant pairwise (Benjamini Hochberg corrected q -value < 0.05) indicated with stars: * $0.05 > q$ -value ≥ 0.01 , ** $0.01 < q$ -value ≥ 0.001 , *** $0.001 < q$ -value. Group sizes: *APOE3/3* F HF $n = 6$, *APOE3/3* F HFEC $n = 6$, *APOE4/4* F HF $n = 6$, *APOE4/4* F HFEC $n = 6$, *APOE3/3* M HF $n = 6$, *APOE3/3* M HFEC $n = 5$, *APOE4/4* M HF $n = 6$, *APOE4/4* M HFEC $n = 6$

4.3.5. Hippocampal RNA sequencing data

A total of 24 samples were tested using the DNBSEQ platform, with an average yield of 6.70 G data per sample. The average alignment ratio of the sample comparison genome was 97.86%, which was comparable. The average alignment of the gene set was 79.78% and a total of 18,954 genes were detected. Hippocampal RNA sequencing identified 18,954 genes. Initial visualisation of the full transcriptome revealed the majority of datapoints clustered in the bottom right corner of the plot, indicating the similarity in the transcriptome between these groups. A few individual datapoints in each group deviated from this cluster and are plotted further away, illustrating the occasional intragroup variation in the *APOE*-TR mice's transcriptome (Figure 4.6A). Following the differential expression, a limited number of genes were identified as significant (FDR $q < 0.2$). The greatest number of significant differences between groups was identified between the *APOE4/4*-TR and *APOE3/3*-TR HF-fed females, where 200 genes were upregulated and 40 were downregulated. Many of the other groups also identified significant (FDR $q < 0.2$) differences in the gene expression between groups; however, the number of significant genes was much lower, with the maximum being 22 upregulated between the *APOE4/4*-TR and *APOE3/3*-TR HFEC-fed males (Figure 4.6B).

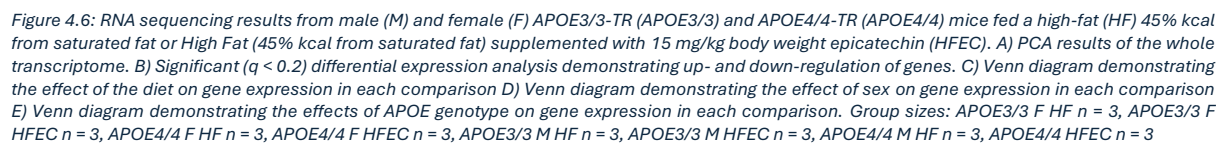
Venn diagrams identified shared modulation in the gene expression between comparison groups, highlighting that the majority of gene modulation was unique to each comparison. However, there were some interesting trends which emerged in the differential abundance. Examination of the comparisons within the same genotype and sex, but different diets, highlighted that the gene regulation was limited and highly specific to the conditions; only one significant gene regulation was modulated in several groups, *Fam220a*. This gene's regulation is shared between the following comparisons: female *APOE3/3*-TR HFEC and HF mice, male *APOE4/4*-TR HFEC and HF mice, and female *APOE4/4*-TR HFEC and HF mice. KEGG analysis of this gene failed to yield any association of *Fam220a* with any metabolic or disease pathways. As this is shared across multiple groups, it is likely that the EC in the diet plays a role in modulating this gene. However, as it is not common to all groups, excluding male *APOE3/3*-TR mice, the modulation by EC is both sex and *APOE*-specific. There was also a single common gene modulation shared between the female *APOE3/3*-TR HF and HFEC mice, and between

the male *APOE3/3*-TR HFEC and HF mice: *Trt*, which indicates a diet effect that only affects the *APOE3/3*-TR mice (Figure 4.6C). However, KEGG correlation analysis identified a significant link to thyroid hormone metabolism (FDR $q = 0.009$).

Examination of the same diet and *APOE* genotype, but in different sexes, revealed that there was again high specificity of the gene regulation to the individual groups. However, there were 6 regulated genes which were common to all comparisons: *Ddx3x*, *Eif2s3x*, *Kdm5d*, *Uty*, *Ddx3y*, and *Eif2s3y*. KEGG analysis of these genes revealed that they have a significant correlation (FDR $q < 0.2$) with the following pathways: RIG-I receptor signalling pathways, Hepatitis B, transcriptional misregulation in cancer, and viral carcinogenesis. Each of these genes, although regulated by sex, unfortunately does not link to the topics of this thesis. There were also significant modulations of individual genes between the following groups. Female and male *APOE3/3*-TR HFEC-fed mice, and female and male *APOE4/4*-TR HFEC-fed mice: *Capn11*. This comparison demonstrated that there was a sex effect occurring in both genotypes, but only in the presence of EC in the diet. However, KEGG analysis failed to find any correlation with metabolism or disease. There was also a single gene modulated in female and male *APOE3/3*-TR HFEC-fed mice, female and male *APOE4/4*-TR HFEC-fed mice, and female and male *APOE3/3*-TR HF-fed mice: *Kdm6a*. Again, KEGG analysis significantly correlated this gene's role in transcriptional misregulation in cancer. Additionally, there was another single gene modulation between the female and male *APOE4/4*-TR HF-fed mice, female and male *APOE3/3*-TR HF-fed mice, and the female and male *APOE3/3*-TR HFEC-fed mice: *Kdm5c*. KEGG analysis failed to reveal any correlation with metabolism or disease. As they were not common to all groups, they demonstrated an effect of sex according to *APOE* genotype and diet (Figure 4.6D).

Final comparisons were between groups with the same sex and diet but different genotypes. 8 genes were modulated in common across all groups: *Eps8l1*, *Rdh13*, *Tmc14*, *Zfp14*, *Oscar*, *Zfp772*, *Inafm1*, and *Gm45844*. KEGG analysis significantly (all FDR $q < 0.06$) correlated these genes to Herpes simplex virus 1 infection, retinol metabolism, osteoclast differentiation, and biosynthesis of cofactors. These genes demonstrated an *APOE* genotype effect on genetic expression irrespective of diet and sex. There were two genes which were modulated in both male *APOE4/4*-TR and *APOE3/3*-TR HF mice, and

the female *APOE4/4*-TR and *APOE3/3*-TR HF mice: *Sae1* and *Arhgap35*. KEGG analysis revealed a significant correlation (FDR $q = 0.050$) between these genes and the regulation of the actin cytoskeleton, focal adhesion, ubiquitin-mediated proteolysis, platelet activation, and leukocyte trans-endothelial migration. These findings demonstrate a genotype effect in expression that is present in HF mice only, and this effect is lost in HFEC mice. There were also 2 genes modulated between the male *APOE4/4*-TR and *APOE3/3*-TR HF mice, the female *APOE4/4*-TR and *APOE3/3*-TR HF mice, and the male *APOE4/4*-TR and *APOE3/3*-TR HFEC mice: *Ptprh* and *Gm167*. This demonstrates that these genes were affected by the *APOE* genotype, and EC silenced these differences in females only; however, there was no correlation with any KEGG pathways. There was also an effect on two genes in the female *APOE4/4*-TR and *APOE3/3*-TR HFEC-fed mice, and the male *APOE4/4*-TR and *APOE3/3*-TR HF-fed mice, demonstrating genotype dependence with modulation by sex and diet: *Mboat7* and *Myadm*. These genes have a significant (FDR $q = 0.011$) correlation with the KEGG pathway for glycerophospholipid metabolism. There was a single common gene modulation between the male *APOE4/4*-TR and *APOE3/3*-TR HFEC-fed mice, as well as the female *APOE4/4*-TR and *APOE3/3*-TR HF- and HFEC-fed animals: *Mid1*. This gene correlated with the KEGG pathway for ubiquitin-mediated proteolysis (FDR $q = 0.017$). This demonstrates a genotype effect that is mediated by both sex and diet. Finally, there was a single shared gene mediated between the male *APOE4/4*-TR and *APOE3/3*-TR HFEC-fed mice, as well as the female *APOE4/4*-TR and *APOE3/3*-TR HFEC-fed mice: *Vav1*, which indicated that this gene was impacted by *APOE* genotype, but only in the HFEC-fed mice (Figure 4.6E). KEGG pathways analysis of this gene uncovers significant (FDR $q = 0.025$) correlations with the following pathways: FC epsilon R1 signalling pathways, B cell receptor signalling pathways, FC gamma R-mediated phagocytosis, T cell receptor signalling pathways, natural killer cell mediated cytotoxicity, leukocyte transendothelial migration, *Yersinia* infection, chemokine signalling pathways, Focal adhesion, proteoglycans in cancer, Rap1 signalling pathways, Lipid metabolism and atherosclerosis, cAMP signalling pathways, and regulation of actin cytoskeleton.



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mediated proteolysis are downregulated in the *APOE3/3*-TR animals; again, this is dependent on the sex and the diet. Downregulation occurs in all female diet groups but only in the HFEC-fed males (Female HF FDR $q < 0.01$, female HFEC FDR $q < 0.01$, male HFEC FDR $q < 0.01$). Finally, *Vav1* has strong associations with several immunological pathways; it is upregulated in *APOE3/3*-TR in the HFEC diet in both sexes (Males FDR $q < 0.01$, females FDR $q = 0.04$) (Table 4.3).

Table 4.3: Differential expression of hippocampal genes with significant ($q < 0.2$) correlations to KEGG pathways related to the gut-brain axis from male (M) and female (F) *APOE3/3*-TR (*APOE3/3*) and *APOE4/4*-TR (*APOE4/4*) mice fed a high-fat (HF) 45% kcal from saturated fat or High Fat (45% kcal from saturated fat) supplemented with 15 mg/kg body weight epicatechin (HFEC). Gene expression is expressed as Log2(Fold Change) with supporting FDR-adjusted q values. Group sizes: *APOE3/3* F HF $n = 3$, *APOE3/3* F HFEC $n = 3$, *APOE4/4* F HF $n = 3$, *APOE4/4* F HFEC $n = 3$, *APOE3/3* M HF $n = 3$, *APOE3/3* M HFEC $n = 3$, *APOE4/4* M HF $n = 3$, *APOE4/4* HFEC $n = 3$

Gene Symbol	Log2(Fold Change)	FDR adjusted q value
Male <i>APOE4/4</i> HF – Male <i>APOE3/3</i> HF		
<i>Sae1</i>	-0.34	0.06
<i>Arhgap35</i>	-0.25	0.12
Female <i>APOE4/4</i> HF – Female <i>APOE3/3</i> HF		
<i>Sae1</i>	-0.32	<0.01
<i>Arhgap35</i>	-0.26	0.01
<i>Mboat7</i>	0.34	0.09
<i>Myadm</i>	0.37	0.01
<i>Mid1</i>	-3.60	<0.01
Male <i>APOE4/4</i> HFEC – Male <i>APOE3/3</i> HFEC		
<i>Mboat7</i>	0.36	0.02
<i>Myadm</i>	0.47	0.10
<i>Mid1</i>	-3.66	<0.01
<i>Vav1</i>	1.16	<0.01
Female <i>APOE4/4</i> HFEC – Female <i>APOE3/3</i> HFEC		
<i>Mid1</i>	-3.41	<0.01
<i>Vav1</i>	0.91	0.04

4.4. Discussion

The health outcomes of the *APOE*-TR mouse study provide insight into the interplay between sex, *APOE* genotype, and EC supplementation. The findings suggest sex- and genotype-specific responses in weight regulation, fat distribution, hepatic metabolism, and cognitive performance.

4.4.1. Weight gain and adiposity

EC supplementation elicited divergent effects between sexes. It increased weight gain in females irrespective of genotype, whereas it reduced weight gain only in *APOE4/4*-TR males. However, variability within groups, particularly in females, prevented statistical significance in total weight gain. These results contrast with published reports showing greater weight gain in male than in female mice on HF diets [288] and increased

susceptibility in *APOE3/3-TR* mice regardless of diet type [302]. Importantly, no prior studies have assessed the combined influence of *APOE* genotype and sex on weight gain, raising the possibility of interactive effects that may obscure expected differences. As a pilot study, with limited sample sizes, the current findings may also be underpowered to detect significant and meaningful differences.

Although overall body weight gain was not significantly affected, adipose tissue distribution differed by sex. Females exhibited greater visceral adipose mass, whereas males had higher liver weights, independent of genotype or diet. The female phenotype diverged from established pre-clinical and clinical patterns [303, 304], while the male phenotype may be consistent with increased hepatic lipid deposition (e.g. steatosis), although this would need to be assessed in our samples [305]. Increased visceral fat in females has previously been linked to a greater risk of CVD [306], another risk factor for cognitive decline. Current knowledge on the weight management benefits of EC is mixed, with preclinical high-fat models presenting significant benefits of EC supplementation, while others are not reporting any [307, 308]. It is therefore possible that EC metabolism is altered either by the microbiota, which was explored in the previous chapters (Chapter 2 and Chapter 3), and/or by phase II conjugation in the liver. Given that the body's distribution of adipose tissue is determined by the sex hormones, with oestrogen decreasing the accumulation of visceral fat, it can also be hypothesised that EC may act as an antagonist of adipocyte oestrogen receptor activity, in turn increasing lipogenesis [309], although further experiments would be needed to confirm this.

4.4.2. Hepatic metabolism

Gene expression analysis revealed that *APOE* genotype influences hepatic sulfotransferase (SULT) isoform expression, with both *Sult1d1* and *Sult1c1* upregulated under EC supplementation. While *Sult1c1* is functional in humans, *Sult1d1* activity is rodent-specific [310, 311]. These findings support previous evidence that flavonoids modulate SULT activity in a tissue- and substrate-dependent manner [312, 313]. Notably, the increase in SULT expression was not uniform between the *APOE* genotypes or sexes. Indeed, a marked sex difference in hepatic enzyme function has been previously

reported, particularly focusing on sulfation, with androgens regulating the sulfotransferase isoform predominance in preclinical rodent models [314, 315].

The alterations in both SULT enzymes may help explain the sex and *APOE* genotype-dependent differences observed in Chapter 2. The observation in this chapter that *Sult1d1* expression was significantly higher in the *APOE3/3-TR* mice relative to their *APOE4/4-TR* counterparts, in both males and females, provides a potential mechanism for the metabolite differences. Increased hepatic SULT activity may promote phase II sulfation pathways, leading to greater sulphated metabolite formation, such as the observed EC-3-S, PVL-3-S, and PVA-3-S, which were significantly higher in the *APOE3/3* mice compared to their *APEO4/4* counterparts in a sex-dependent context. EC-induced upregulation of *Sult1c1* in female *APOE4/4* mice may represent a compensatory pathway to altered EC handling in this group. Although *Sult1d1* is a rodent-specific gene [316], collectively the effects of these two genes support the concept that the *APOE* genotype and sex influence not only microbial metabolism, but also the hepatic phase II SULTS, thereby shaping the circulating metabolic profile. No prior studies have linked *APOE* genotype to phase II metabolism. However, *APOE4/4* carriers have been associated with reduced phase I CYP2D6 activity [317], further supporting the possibility of downstream interactions between the *APOE* genotype and host metabolic pathways that warrant future investigation.

4.4.3. Hepatic inflammatory response

In addition to addressing hepatic metabolism, our study also examined immunomodulatory gene expression in the liver. Although EC is widely accepted as a protective molecule with anti-inflammatory effects [318, 319], the present study found no suppression of inflammatory gene expression in *APOE3/3-TR* females or males. Notably, EC supplementation increased both pro- and anti-inflammatory gene expression in *APOE4/4-TR* females, suggesting a complex, sex-dependent immune modulation. While the mechanisms underpinning the increased inflammatory response in the *APOE4/4-TR* females remain unclear, sex hormones are likely to have played a role. Indeed, oestrogens exert antioxidant effects and provide protection against a range of

diseases, including CVD, osteoporosis and AD [69, 320-322]. In addition, the sulfation of oestrogens is controlled by the action of phase II metabolic enzyme *SULT1E1* and results in the deactivation of the immunoregulatory hormones [323]. It is therefore possible that the EC metabolites and other flavonoids may act as substrates for the oestrogen-metabolising enzymes [324]. However, the role of the catechins and other flavonoid metabolites on the expression and activity of *Sult1e1* remains underexplored in the current scientific literature.

An alternative mechanism for the genotype difference in the immune activation could be related to the increased oxidative stress triggered by the *APOE4/4* genotype [325]. As EC is able to scavenge ROS, oxidation of the B-ring catechol moiety results in the production of epicatechin-*o*-quinone, which has been reported to exert a spectrum of bioactivities, ranging from beneficial to toxic effects [326, 327]. The oxidative environment of the *APOE4/4* genotype, paired with the enhanced estrogenic sulfation in females, may shift the functionality of the EC-*O*-quinone towards toxicity and induce the immune response observed in the animals. However, these mechanisms remain speculative and require further validation.

4.4.4. Energy metabolism

Beyond its effects on hepatic metabolism and immune response, EC also influenced systemic metabolism in a genotype- and sex-dependent manner. Previous literature has reported mixed benefits from EC and EC-rich foods in preclinical models on energy metabolism, with some findings indicating that EC may ameliorate glucose sensitivity in animals fed HF diets [307, 328]. However, these benefits have only been observed in studies of a single sex, generally using wild-type mouse models. Only one study looked into sex differences in animals fed a high-fat diet and supplemented with EC; however, no benefits from the EC supplementation were reported, and only sex differences in glucose response were noted [308]. Our study mirrors some of the previously reported findings, with males displaying higher glucose responses than females, regardless of genotype or diet and consistent with oestrogen's protective role in insulin sensitivity [329]. *APOE4/4*-TR males exhibited particularly elevated fasting glucose under HFEC

supplementation, suggesting additive deleterious effects of sex and genotype. Previous studies similarly reported impaired glucose in *APOE4/4-TR* mice and sex differences in glucose handling [330]. Of note, these metabolic disturbances were not observed to the same extent in the low-fat control arms of the study [330]. The lack of low-fat control arms in our intervention limits interpretation of whether EC's effects depend on dietary fat content; a question for future research.

Glucose is not the body's sole energy source, and in this study, the effects of EC supplementation, sex, and *APOE* genotype on energy metabolism were also demonstrated on fasted blood BHB concentrations, with each variable independently producing a significant effect on fasted BHB. For instance, female mice exhibited higher fasting BHB concentrations than males; *APOE4/4-TR* mice had higher BHB concentrations than *APOE3/3-TR*; and EC supplementation reduced BHB concentrations. Pairwise comparisons revealed genotype differences in males on HF diets that were abolished with EC supplementation, suggesting EC may alter fuel utilisation during fasting. Previous murine models have reported that mice on a high-fat ketogenic diet display the same sex differences in their blood ketone concentrations [331]. On the other hand, although these animals were not fasted prior to ketone body testing, the results indicate that sex does change the fat β -oxidation. Sex hormones may also have influenced the metabolism of energy sources; however, this mechanism still needs further exploration before it can be confirmed. The *APOE* genotype also influenced the liberation of ketone bodies, with the *APOE4/4-TR* genotype having a greater concentration than the *APOE3/3-TR* genotype. Previous literature has demonstrated that *APOE4/4-TR* mice exhibit increased expression of proteins involved in fatty acid β -oxidation [302], a key metabolic pathway that liberates ketones for energy metabolism. Furthermore, *APOE* allele structure plays a key role in the uptake of lipids from the blood into the liver, one of the key sites for energy metabolism, with the *APOE4* allele having an increased affinity for LDLs and VLDLs [36, 37]. Such genotype differences in the mice's lipid transportation and subsequent oxidative metabolism may be driving the differences in BHB concentrations observed in our study. However, further work is needed to explore this.

In addition to the genotype effects, EC supplementation can alter the animals' BHB profile, with the mice on the HFEC diet having lower fasted blood BHB than those on the HF diet. Although there is limited literature on the effect of EC on ketogenesis, previous studies have demonstrated that EC can increase the β -oxidation pathways in obese human adults [332]. It is therefore possible that this effect may be reflected in the *APOE*-TR animal model, impacting the BHB blood concentrations and leading to the results that were observed in the animal study. It is also interesting to examine the significant pairwise results between the animals' fasted ketone results. There was a significant difference between the male *APOE* genotypes on the HF diet. However, when examining the HFEC-fed mice from the same groups, the effect was lost, indicating that EC supplementation may offset any genotype differences in the male mice by shifting the energy sources used in the fasted state away from ketones. However, such results were observed only for the ketones, not for baseline glucose concentrations, thus, further work is needed to explore the mechanisms underlying this interaction. It is also important to highlight that this study of the *APOE*-TR mice only examined the concentration of BHB; however, BHB is not the only ketone that is produced by the body. Further work could investigate the production of other ketone bodies to examine how sex, *APOE* genotype, and EC supplementation affect the entire ketogenic profile in the fasted state.

4.4.5. Hippocampal Gene Expression

RNA-seq analysis revealed distinct patterns in the hippocampal transcriptome of the *APOE*-TR mice. Differential expression analysis identified a limited number of significantly altered genes. Examination of the Venn diagrams revealed that transcriptional changes were specific primarily to particular combinations of *APOE* genotype, sex, and diet. Yet, there were limited numbers of shared modulations across age, sex, and *APOE* genotype combinations, with the *APOE* genotype being the most significant factor, followed by sex, diet, and then interactions between these factors. Functional analysis of the modulated genes revealed *APOE* genotype-driven associations with cognitive health and the GBA. However, these changes do not provide a clear link to neuroinflammation or cognitive impairment pathways.

Two genes, *Sae1* and *Arhgap35*, were upregulated in *APOE4/4*-TR mice of both sexes fed the HF diet. KEGG pathways analysis associated these genes with several pathways, including leukocyte transendothelial migration, suggesting increased movement of white blood cells across the BBB into the hippocampus, potentially indicating chronic inflammation [333]. EC supplementation eliminated the genotype differences in the expression of these genes. Given that EC is known to modulate BBB function and immune response [334], these findings suggest that EC may help restore BBB integrity in *APOE4/4*-TR mice.

The effects of EC, however, were not consistent across all groups. For example, *Vav1*, which was linked by KEGG analysis to several pro-inflammatory pathways, was upregulated in *APOE3/3*-TR HFEC-fed mice in both sexes. While flavonoids have been associated with anti-inflammatory effects [196], further investigation is needed to understand why EC appeared to enhance, rather than suppress, this response.

A further two genes, *Mboat7* and *Myadm*, involved in glycerophospholipid metabolism, were upregulated in *APOE3/3*-TR mice. These genes play roles in maintaining cell membrane integrity [335]. Previous studies have shown that the *APOE* genotype influences the hepatic lipidome [336] and cerebral cholesterol efflux [43], so its role in modulating glycerophospholipid metabolism is plausible. However, these effects were also shaped by sex and diet (e.g. transcription was increased in HF-fed females and HFEC-fed males). Since both sex hormones and EC metabolites can cross the BBB [337, 338], they may contribute to this modulation, although further work is required to confirm it.

Another gene of interest, *Mid1*, was upregulated in *APOE4/4*-TR mice across all female groups and in HFEC-fed males. Upregulation of this gene was linked via KEGG to ubiquitin-mediated proteolysis, a key process for degrading misfolded proteins in the brain. Impairment in this pathway contributes to HT and A β accumulation, hallmark features of AD [339]. However, to date, no studies have explored how *APOE* genotype, sex, or EC supplementation influences this pathway.

These findings contrast with previous preclinical studies that demonstrated that *APOE* genotype modulates hippocampal gene expression and responses to environmental

stimuli, particularly in pathways related to synaptic function, neuroinflammation, and neuronal signalling [340]. EC was also reported to ameliorate brain inflammation induced by an HF diet [341], and that sex may influence transcriptional response to immune challenges in the hippocampus [342]. Notably, no previous study has examined the combined effects of age, sex, and *APOE* genotype on hippocampal transcription.

The PCA revealed that the overall transcriptomic profiles were broadly similar across groups. This suggests the inflammatory and BBB-related changes may be subtle, with some intra-group variability. It is possible that the present pilot study lacked sufficient statistical power to detect complex interactions among these variables. Future work should include power calculations to determine the appropriate sample size for adequately resolving the effects of *APOE* genotype, sex, and diet on hippocampal transcription.

4.4.6. Cognition and behaviour

While the influence of EC, sex, and *APOE* genotype on metabolic outcomes such as glucose and ketone handling highlights important physiological differences, these factors may also affect neurocognitive and behavioural outcomes. Previous work has highlighted the important role of chronic exposure to flavonoids in modulating cognition and behaviour [130, 149]. However, the benefits of EC on cognition in this study were mixed. The principal result was that *APOE-TR* mice, irrespective of genotype, sex, or diet, showed similar exploratory behaviour. The OF test results indicated that the animals preferred to remain at the edge of the trial arena. This result was further evidenced by the NOR heatmaps, which indicated that although the mice did explore the arena, they often remained around the edge or in one corner, a response known as thigmotaxis.

Although the preference index and time in the centre were unaffected by the controlled variables, it is interesting to note that the total distance travelled in each test did produce significant differences. The *APOE* genotype of the mice influenced the distance travelled in the OF arena, with the *APOE3/3-TR* mice travelling further than the *APOE4/4-TR* mice, particularly in HF-fed females, where significance was reached. Sex and genotype influenced the distance travelled in the NOR test, with HF-fed females travelling farther than the males. This result highlights that, although the mice's cognition was ultimately

not affected, their motility was. Several other factors may have influenced the animal's motility, including its exploratory drive. This has been noted to be suppressed in an anxious state [343]. Firstly, it is important to note that, from the OF results, the HF-fed *APOE4/4*-TR mice and the HF-fed males in the NOR task travelled shorter distances. These animals may have exhibited a reduced exploratory drive, consistent with the trends reported in the literature [344]. Although these factors do not directly influence the quantitative behavioural scores assessed in this thesis, they do affect the quantitative measure of locomotor activity. This reduction in locomotor function could indirectly limit the animals' opportunity to explore, potentially affecting the extent of their interaction with the objects and arena zones, and consequently the sensitivity of these behavioural measures.

Secondly, it is interesting that effects on distance travelled were observed only in HF-fed animals. It appears that the HFEC diet reduced any significant differences between the groups. EC may not be able to improve the OF and NOR behavioural scores, but it may reduce the anxious phenotype by improving the exploratory drive of the groups. Kang et al. observed an improvement in anxiety-related behaviour with EC supplementation [345]; however, their mice were given a slightly higher dose of EC than the *APOE-TR* mice (e.g. 20 mg/kg body weight), which was continually adjusted for weight gain. Initially, the *APOE-TR* mice were calculated to receive the same, albeit unadjusted, daily dose of 20 mg/kg body weight. However, they consumed a smaller mean mass of food per day (2.9 g instead of the estimated 3.5 g) and had a higher median body weight, resulting in a lower average daily dose of 14.5 mg/kg body, which also fluctuated with body weight gain. As noted by Kang, EC may be acting in a dose-dependent manner [345], which may explain the discrepancies in our findings. This study also had fewer study arms, only investigating diet differences between male wild-type mice [345]. It is therefore possible that, as a pilot study, the diet, genotype, and sex arms of the *APOE-TR* study were underpowered, masking any improvements of EC in the diet. A diet rich in saturated fat is known to increase the risk of anxious behaviour in preclinical models, and is associated with anxiety in clinical studies [345-347]; however, without the introduction of low-fat control arms, it was not possible to confirm whether the high-fat diet worsened anxious behaviour in the *APOE-TR* mice. Future study designs should include low-fat control arms

and power calculations to ensure suitable comparisons can be made with sufficient statistical power to assess anxious behaviour.

Although the OF and NOR tests failed to observe improvements in behaviour, the Y-maze yielded some interesting results regarding spatial working memory. There was a significant sex and diet interaction in the study, with male mice demonstrating improvements in spatial working memory following EC supplementation; however, this effect reached significance only in the *APOE4/4*-TR mice. Conversely, the females presented no improvement with supplementation, and *APOE3/3*-TR EC supplementation reduced the number of spontaneous alternations completed, although this did not reach statistical significance. These results suggest that EC's cognitive effects are neither uniform nor universally beneficial, and may depend on sex, genotype, and potentially gut microbial metabolism of flavonoids as reported in Chapter 2. Previous literature has reported that EC can improve spatial working memory by increasing hippocampal angiogenesis [348]; however, such mechanisms would need further investigation. In our study, only *APOE4/4*-TR HFEC male mice scored significantly higher, suggesting that EC benefits on spatial working memory are sex- and genotype-dependent. Since EC and its metabolites have been demonstrated to cross the BBB and enter the cerebral tissue from the circulatory system [349], and both sex hormones and the *APOE4* allele have been reported to alter BBB integrity [350, 351], differences in cerebral bioavailability may underlie the observed behavioural divergence.

4.5. Conclusions and Next Steps

This *APOE*-TR animal study demonstrates that the effect of EC supplementation is highly context-dependent, varying by sex and *APOE* genotype across metabolic, inflammatory, and cognitive domains. Importantly, EC was not universally beneficial and, in some instances, exacerbated deleterious outcomes. These findings highlight the potential importance of sex- and genotype-specific dietary recommendations in the context of personalised nutrition. However, as a pilot study, limited sample sizes and group variability constrain statistical power. Future studies should employ adequately powered designs, include low-fat dietary controls, and extend analyses to whole-food flavonoid

sources, which better reflect dietary exposure. Clarifying the interactions between *APOE* genotype, sex, diet, and EC metabolism will be essential for understanding both risks and therapeutic opportunities. To this end, the next chapter will address the effects of the *APOE* genotype on the metabolism of flavonoids within plant food matrices.

Chapter 5: Impact of *APOE* genotype, Sex, and Diet in the metabolism of dietary flavan-3-ols when present in food matrices.

5.1. Introduction

Flavonoids are a class of bioactive phytochemicals present in many plant-derived foods and beverages and are associated with health benefits relevant to healthy ageing, including reduced neuroinflammation, enhanced synaptic plasticity, increased cerebral blood flow, mitigation of mitochondrial dysfunction, and attenuation of proteinopathies and excitotoxicity [138]. Because mammals lack the endogenous enzymes required for flavonoid digestion, their absorption primarily depends on the uptake of microbial metabolites generated by colonic fermentation [177, 178]. Consequently, flavonoid metabolism is strongly influenced by the composition and activity of the gut microbiota, which in turn are shaped by microbial host factors. Among these, age, sex, and *APOE* genotype are particularly relevant, as they not only modulate the colon's microbial ecosystem [89, 104, 352] but also influence susceptibility to age-related diseases such as AD [2, 12, 67].

The preceding chapters of this thesis have examined how *APOE* genotype, sex, and age affect gut microbiota composition and the subsequent metabolism of EC (Chapter 2 and Chapter 3) as well as the related health outcomes (Chapter 4). Purified EC was used to allow precise tracking of a single flavonoid metabolic fate. However, in the human diet, flavonoids are rarely consumed in isolation but rather occur within complex food matrices alongside macro and micronutrients. The physicochemical interactions among these food components, collectively termed the food matrix, can substantially affect flavonoid bioaccessibility and bioavailability [353]. Interactions between flavonoids and dietary lipids, proteins, carbohydrates, and fibres have been reported across a variety of food matrices [261, 354, 355]. Such interactions may alter flavonoid absorption in the upper GI tract, thereby affecting the fraction that reaches the colon for microbial catabolism.

Additionally, and of particular interest to this study, constituents of the food matrix can independently modulate the gut microbiota. For example, dietary fibres resist host digestion and reach the colon, where they serve as a prebiotic substrate that shapes microbial composition [356]. Whole foods also contain multiple (poly)phenolic compounds, each with distinct metabolic pathways and microbiota-modulating effects [357], which may in turn influence the microbial populations responsible for metabolising flavan-3-ols, such as those investigated in this chapter. Thus, the food matrix can simultaneously affect flavonoid bioaccessibility/availability and reshape the microbial community that carries out their catabolism, ultimately altering the spectrum of microbial metabolites produced and interacting with host variables. This chapter will therefore test the hypothesis that *APOE* genotype, sex, and age modulate the microbial metabolism of dietary flavan-3-ols when consumed as part of whole foods.

The three studies presented in this chapter were chosen to address complementary but distinct translational questions regarding the effects of *APOE* genotype, sex, and age on the metabolism of flavonoids within food matrices. The Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) study provides a tightly controlled preclinical model in which flavan-3-ol-rich cocoa was administered, allowing isolation of the *APOE* genotype effect independent of environmental variability over chronic consumption. In contrast, the chocolate, orange juice and blackberry (COB) study examines whether the *APOE* genotype, age, and sex dependent effects on flavonoid metabolism are observable in humans following acute consumption of a flavonoid-rich meal within the real-world dietary context. Finally, the Oral (Poly)Phenol Challenge Test (OPCT) was used to further investigate the role of the *APOE* genotype, age, and sex in acute dietary flavonoid metabolism in a larger cohort of participants, using a wider range of flavonoid-rich extracts designed to replicate the Mediterranean diet.

Collectively, these studies span preclinical and acute clinical challenges, enabling assessment of whether *APOE*-, age-, and sex-dependent metabolic signatures are preserved when flavonoids are consumed as part of complex food matrices. Together, they build on the translational relevance of the findings presented in previous chapters, putting the effects into real-world dietary settings.

5.2. Materials and Methods

5.2.1. Cognitive Ageing Nutrition and Neurogenesis Animal study: The (CANN-AN) Study

The following animal study was carried out by Dr Anneloes Martinsen (UEA) as part of a larger UKRI BBSRC-funded project entitled “Enhanced cognition through dietary modulation of neuroinflammation in high-risk APOE4 carriers” (BB/M004449/1). Analysis of urinary (poly)phenols was carried out by Dr Pedro Mena (Parma University). The author of this thesis analysed and interpreted the data related to the impact of APOE genotype on flavan-3-ols metabolism as a secondary outcome of this project.

5.2.1.1. Ethical approval and Study Design

All experimental procedures and protocols used in this section were reviewed and approved by the AWERB and were conducted within the provisions of the Animals (Scientific Procedures) Act 1986 (Amendment Regulations 2012), under the PPL 70/8710. Reporting of the study outcomes complies with the ARRIVE guidelines (*Animal Research: Reporting of In Vivo Experiments*) [231].

Thirty-six male human *APOE3/3* (B6.129P2-Apo^{tm2(APOE*3)Mae} N8) and *APOE4/4* (B6.129P2-Apo^{tm2(APOE*4)Mae} N8) TR mice (Taconic, Germantown, NY, US) were used in these experiments. Mice were maintained in a controlled environment (21°C; 12-h light–dark cycle; light from 07:00 hours) and fed *ad libitum* on a standard diet (RM3-P, Special Diet Services, Essex, UK) until the age of 6 months after which mice were switched to one of three diets (Research Diets, New Brunswick, NJ, USA) for 24 weeks. The three diets were as follows: (1) low-fat diet (10 kCal% from fat; LF) (2) HF diet (45 kCal% from fat) and (3) HF diet (45 kCal% from fat) with the addition of 6% of flavan-3-ols rich cocoa powder (Acticoa, Barry Callebaut) delivering 100 mg/Kg body weight epicatechin/catechin per day (FLAV). The LF and HF diets were also supplemented with a low flavan-3-ol cocoa (Royal Cocoa Drops, Barry Callebaut, Belgium), ensuring that they were matched to the FLAV diet along with other bioactives within the cocoa matrix, such as theobromine and caffeine. For full dietary composition, see Supplementary Table 8. An HF diet was chosen to mimic a human “Western-style” diet [234], a risk factor prevalent in modern society

that exacerbates AD-like age-related cognitive decline and neuroinflammation [235]. At the end of the experiments, mice were sedated with a mixture of isoflurane (1.5%) in nitrous oxide (70%) and oxygen (30%) and transcardially perfused with an ice-cold PBS. Urine and faecal samples were collected and stored at -80 °C until analysis. The study design is summarised in Figure5.1 and in full below.

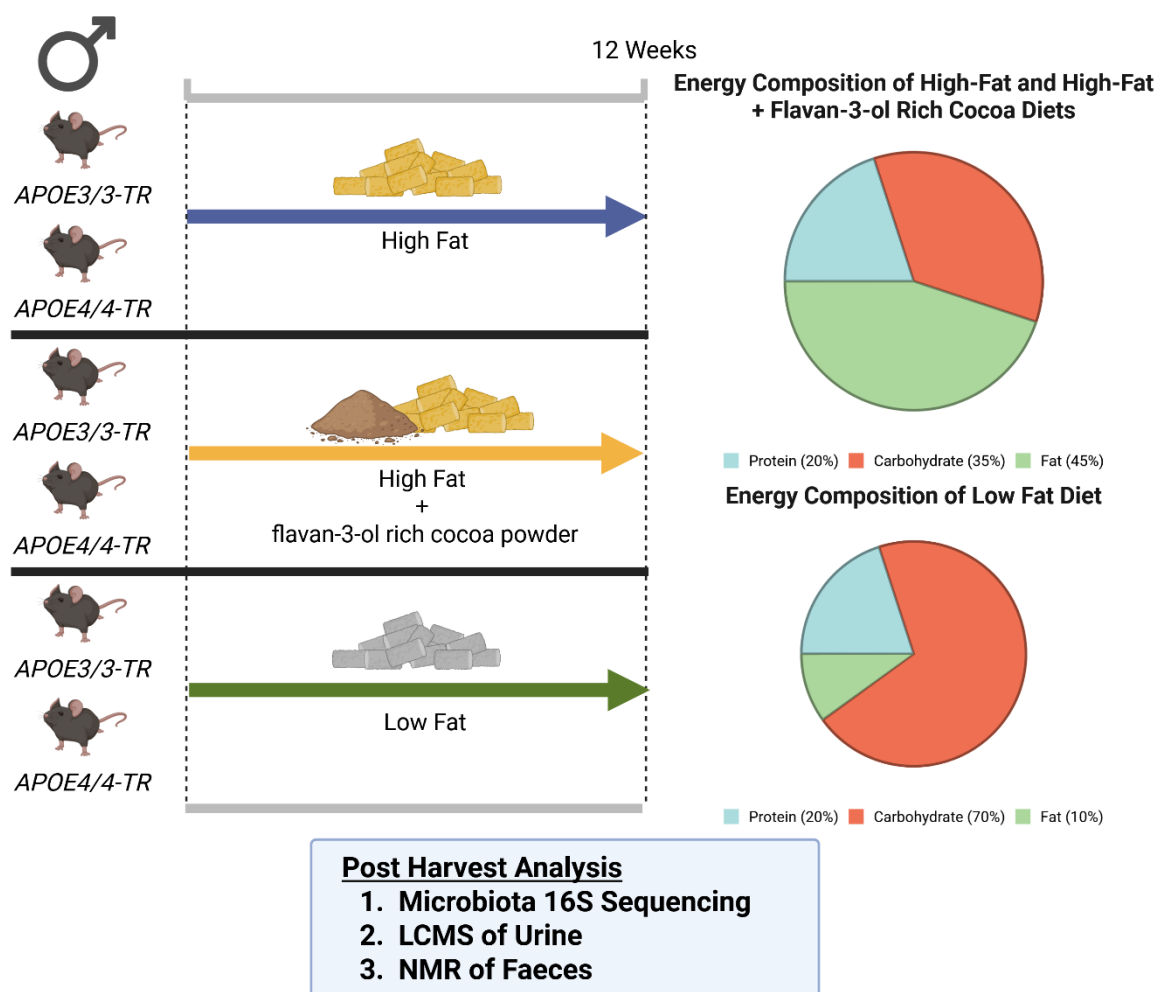


Figure5.1: Study Design for the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study showing the mice split by APOE3/3-TR and APOE4/4-TR genotype and then by diet: High fat (HF) and High fat supplemented with flavan-3-ol rich cocoa powder (FLAV) and Low-fat (LF)

5.2.1.2. Urine analysis of phenolic metabolites by UHPLC-ESI-MS/MS

Urine samples were analysed as previously reported [216]. Briefly, urine samples were diluted 1/4 with water acidified with 0.1% FA. The samples were then vortexed vigorously for 5 minutes and centrifuged (8,765 x g for 5 minutes). The solution was filtered (0.22 µm nylon filter) directly in vials before ultra-high performance liquid chromatography coupled with mass spectrometry (UHPLC-ESI-MS/MS) analysis. Phenolic metabolites in

urine samples were analysed using a UHPLC DIONEX Ultimate 3000 equipped with a TSQ Vantage triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionisation source (H-ESI; Thermo Fisher Scientific Inc.). UHPLC mobile phase A was 0.2% FA in water, and mobile phase B was acetonitrile containing 0.2% FA. Separations were performed with a Kinetex EVO C18 (100×2.1 mm) column, 2.6 µm particle size (Phenomenex, Torrance, CA, USA). The gradient started with 5% B, keeping isocratic conditions for 0.5 min, reaching 95% B at 7 min, maintained for 1 minute and then 5 min at the start conditions to re-equilibrate the column. The flow rate was set at 0.4 mL/min, the injection volume was 5 µL, and the column was thermostated at 40°C. The applied MS method was also carried out in SRM mode, with negative ionisation. The MS worked with a capillary temperature of 270 °C while the source was 200 °C. The sheath gas flow was 50 units, while the auxiliary gas pressure was set to 10 units. The source voltage was 3 kV. Ultra-high-purity argon gas was used for collision-induced dissociation (CID). Data processing was performed using Xcalibur software from Thermo Scientific. Quantification was performed with calibration curves of standards when available. When unavailable, the conjugated metabolites were quantified with the most structurally similar compound [358]. To adjust the concentration of each (poly)phenol metabolite to the volume and concentration of the urine, samples were normalised to the urinary concentration of creatinine using a creatinine assay kit (Biotechnne, R&D systems, Minnesota, USA) according to the manufacturer's recommended procedures. The nomenclature used for (poly)phenol catabolites follows the current recommendations from Kay et al. [230]. The following compounds reanalysed for this thesis are presented in Table 5.1.

Table 5.1 A list of the compounds that are analysed in the Cognitive Ageing Nutrition and Neurogenesis Animal study: The (CANN-AN) Study

Compounds Analysed in the Cognitive Ageing Nutrition and Neurogenesis Animal study: The (CANN-AN) Study	
Flavan-3-ols	
3-O-Methylepicatechin (3-O-MeEC)	
4-O-Methylepicatechin (4-O-MeEC)	
Epicatechin (EC)	
Epicatechin-3'-glucuronide (EC-3-GluAC)	
Epicatechin-3'-sulfate (EC-3-S)	
Epicatechin-4'/5'/7'-sulfate (EC-4/5/7-S)	
Epicatechin-4'-glucuronide (EC-4-GluAC)	
Epicatechin-5/7-glucuronide (EC-5/7-GluAC)	
Epicatechin-DiGlucuronide (EC-DiGluAC) ion I	
EC-DiGluAC ion II	
EC-DiGluAC ion III	
Epicatechin-Sulfate-Glucuronide (EC-S-GluAC) ion I	
EC-S-GluAC ion II	
EC-S-GluAC ion III	
Methoxy-epicatechin-glucuronide (MeEC-GluAC) ion I	
MeEC-GluAC ion II	
MeEC-GluAC ion III	
Methoxy-epicatechin-sulfate (MeEC-S) ion I	
MeEC-S ion II	
MeEC-S ion III	
Methoxy-epicatechin-sulfate-glucuronide (MeEC-S-GluAC) ion I	
MeEC-S-GluAC ion II	
Hydroxyphenyl-propan-2-ols	
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol (3,4-DiHPP-2-ol)	
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol-methoxy-glucuronide (3,4-DiHPP-2-ol-MeO-GluAC)	
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol-methoxy-sulfate (3,4-DiHPP-2-ol-MeO-S)	
1-(3',4'-Dihydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)propan-2-ol-sulfate (3,4-DiHPP-2-ol-S) ion I	
3,4-DiHPP-2-ol-S ion II	
1-(Hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol-glucuronide (HPP-2-ol-GluAC)	
1-(Hydroxyphenyl)-3-(2'',4'',6''-trihydroxyphenyl)-propan-2-ol-sulfate (HPP-2-ol-S)	
γ-valerolactones	
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3,4-DiHPVL)	
5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3-HPVL-4-GluAC)	
5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate (3-HPVL-4-S)	
5-(3'-methoxyphenyl)-γ-valerolactone-4'-glucuronide (3-MPVL-4-GluAC)	
5-(3'-methoxyphenyl)-γ-valerolactone-4'-sulfate (3-MPVL-4-S)	
5-(4'-hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4-HPVL-3-GluAC),	
5-(phenyl)-γ-valerolactone-3',4'-diglucuronide (PVL-3,4-DiGluAC)	
5-(Phenyl)-γ-valerolactone-3',4'-disulfate (PVL-3,4-DiS)	
5-(phenyl)-γ-valerolactone-3'-glucuronide (PVL-3-GluAC)	
5-(Phenyl)-γ-valerolactone-3'-sulfate (PVL-3-S)	
5-(Phenyl)-γ-valerolactone-4'-sulfate (PVL-4-S)	

5-(phenyl)-γ-valerolactone-sulfate-glucuronide (PVL-S-GluAC)
Valeric Acids
4-hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid (4-H-3,4-DiHPVA)
4-hydroxy-5-(3'-hydroxyphenyl)valeric acid-4'-glucuronide (4-H-3-HPVA-4-GluAC)
4-hydroxy-5-(3'-hydroxyphenyl)valeric acid-4'-sulfate (4-H-3-HPVA-4-S)
4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-4HPVA-3-GluAC)
4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-sulfate (4-H-4-HPVA-3-S)
4-Hydroxy-5-(phenyl)valeric acid-3'-glucuronide (4-H-PVA-3-GluAC)
4-Hydroxy-5-(phenyl)valeric acid-3'-sulfate (4-H-PVA-3-S)
4-Hydroxy-5-(phenyl)valeric acid-4'-glucuronide (4-H-PVA-4-GluAC)
4-Hydroxy-5-(phenyl)valeric acid-4'-sulfate (4-H-PVA-4-S)
5-(Hydroxyphenyl)valeric acid-glucuronide (HPVA-GluAC)
3-(phenyl)valeric acid-4'-sulfate (PVA-3-S)
3-(Phenyl)valeric acid-4'-glucuronide (PVA-4-GluAC)

5.2.1.3. Microbial DNA extraction and 16S rRNA amplicon sequencing

Microbial DNA was isolated from approximately 50 mg of caecal content using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Manchester, UK) as per the manufacturer's instructions. DNA quantity was assessed using a Nanodrop 2000 Spectrophotometer (Fisher Scientific, UK). Quality assessment was performed by agarose gel electrophoresis to detect DNA integrity, purity, fragment size and concentration. The 16S rRNA amplicon sequencing of the V3-V4 hypervariable region was performed with an Illumina NovaSeq 6000 PE250. Sequence analysis was performed by Uparse software (Uparse v7.0.1001) [359], using all the effective tags. Sequences with $\geq 97\%$ similarity were assigned to the same OTUs. A representative sequence for each OTU was screened for further annotation. For each representative sequence, the Mothur software was used against the SSUrRNA database of SILVA Database 138 [263]. OTUs' abundance information was normalised using a standard of sequence number corresponding to the sample with the least sequences. Alpha-diversity was assessed using both Chao1 and Shannon H diversity index whilst, beta diversity was assessed using Aitchinson distances. Statistical significance was determined by Kruskal-Wallis or PERMANOVA. Comparisons at the Phylum, Family, and Genus level were made using classical univariate analysis using Kruskal-Wallis combined with an FDR approach used to correct for multiple testing.

5.2.1.4. *¹H NMR Metabolomics*

Caecal metabolites were analysed and quantified by ¹H NMR analysis from the same n = 6 randomly chosen animals utilised in the 16S analysis. The preparation method was as previously described [104]. Briefly, frozen caecal contents were thoroughly mixed at 5,000 rpm in a Precellys 24 (Bertin Technologies, France) and diluted to a faeces-to-buffer ratio of 13 (e.g., 50 mg in 750 µL) by adding deuterated phosphate buffer (1.9 mM Na₂HPO₄, 8.1 mM NaH₂PO₄, and 1 mM sodium 3-(trimethylsilyl)-propionate-d₄ in deuterated water (Goss Scientifics, Crewe, United Kingdom)). After mixing and centrifugation, 500 µl was transferred into a 5-mm NMR tube for spectral acquisition. High-resolution ¹H NMR spectra were recorded on a 600-MHz Bruker Avance spectrometer fitted with a 5-mm TCI proton-optimised triple resonance NMR inverse cryoprobe and a 24-slot autosampler (Bruker, Rheinstetten, Germany). Sample temperature was controlled at 300 K. Each spectrum consisted of 128 scans of 65,536 complex data points with a spectral width of 20 ppm (acquisition time 2.6 s). The noesypr1d presaturation sequence was used to suppress the residual water signal with low-power selective irradiation at the water frequency during the recycle delay (D1 = 2 s) and mixing time (D8 = 0.01 s). A 90° pulse length of 11.4 µs was set for all samples. Spectra were transformed with 0.1-Hz line broadening and zero-filling, manually phased, baseline corrected and referenced by setting the trimethylsilylpropanoic acid methyl signal to 0 ppm. Metabolites were identified using information found in the literature or on the web (Human Metabolome Database, <http://www.hmdb.ca/>) and quantified using the software Chenomx® NMR Suite 8.6™.

5.2.1.5. *Statistical analyses*

Statistical analysis for the concentrations of each of the metabolites was calculated as follows and carried out on R 4.4.1 [237]. Shapiro-Wilk tests for normal distribution were carried out in base R. Due to significant (p < 0.05) results in all cases, permutational ANOVA (10,000 permutations) was carried out through the package permuco [239], which accounts for the differences in variance as well as any differences in homogeneity. For the microbiota analysis, the analysis was carried out by MicrobiomAnalyst 2.0 [264]. OTU abundance data were normalised to the lowest sequencing depth across all

samples (e.g. rarefaction). Alpha-diversity was assessed using Chao1, Simpson and Shannon H diversity index, whilst beta diversity was assessed using Aitchison distances following CLR transformation. Diversity metrics are plotted as box and whisker plots with individual data points overlaid and the mean represented by a black dot. Alpha diversity was analysed using a mixed linear model. Any significance ($p < 0.05$) in the ANOVA results was further investigated using the least square means method for pairwise comparisons. The BH method was used to adjust for the FDR. Beta diversity significance was determined using Kruskal–Wallis or PERMANOVA. PCoA was used for visualisation, with significance defined as $p < 0.05$. Taxonomic comparisons at the Phylum, Family and Genus levels were conducted using classical univariate analysis via the Kruskal–Wallis test, coupled with BH correction. Given the large number of pairwise comparisons, adjusted $q < 0.2$ were considered significant. All results were visualised using box-and-whisker plots.

Metabolomic analysis was performed using MetaboAnalyst 6.0 [265]. Data were stratified by sex prior to normalisation, with each sex normalised to its respective *APOE3/3*-TR HF control group. sPLS-DA was employed to visualise the contribution of metabolites' variance to group separation, and corresponding loading plots were used to identify key discriminative metabolites. Metabolite comparisons were conducted using univariate analysis via the Kruskal–Wallis test with BH FDR correction for multiple testing. Given the extensive number of pairwise comparisons, statistical significance was defined as an adjusted $q < 0.2$. Results are visualised using box-and-whisker plots.

Correlation analysis between metabolomics data and microbiota data was performed using the M2IA platform [266]. Features with missing values in over 80% of samples or with a relative standard deviation $< 30\%$ were excluded [267]. The remaining missing values were imputed using the Random Forest method. Data were normalised using total sum scaling. All correlation analyses were conducted using Spearman's rank-order correlation [268].

5.2.2. Effect of APOE genotype, sex, and age on metabolism of flavonoids - The Chocolate, Orange Juice and Blackberry (COB) Study

The following study is adapted from the chocolate, orange juice and blackberry (COB) study funded by the UKRI ISP [360]. Statistical analysis and interpretation of the data, split by APOE genotype, sex, and age, was carried out by the author of this thesis.

5.2.2.1. Ethical approval Study Design

The chocolate, orange juice and blackberry (COB) study grouped participants based on their APOE genotype, biological sex, and age group to investigate differences in the metabolism of flavonoids from a chocolate bar and orange and freeze-dried blackberry juice. The COB study (ClinicalTrials.gov number NCT01922869) received ethical approval from the East of England, Norfolk, Research Ethics Committee (REC reference: 13/EE/0066).

The study was a non-randomised acute nutritional intervention study. For 3 days prior to the administration of the test meal, participants followed a washout diet free from fruits, vegetables, wholegrain foods, teas, coffee, and fruit juice. To eliminate variation in results from participants' diet, they were provided with standardised flavonoid-free meals (Brakes, Kent, UK) throughout the experiment at the following timepoints: Standardised evening meals - the night before the test meal (T = -10hrs), the day of the test meal (T = 10hrs) and the day following the test meal (T = 34hrs). Standardised lunch - the day of the test meal (T = 3.5hrs), the day following the test meal (T = 27.5 hrs). A standardised breakfast - the day following the test meal (T = 24hrs). Full details of the standardised meals are published in the scientific report [360]. In the 10 hours prior to the test meal, participants collected a spot urine sample, which served as the baseline for subsequent measurements. On the test day, participants received a test meal of a 50 g dark chocolate bar (Barry Callebaut, Belgium) and a drink containing 37.5 g freeze-dried blackberries (Nutra Ingredients Ltd, UK) and 362 mg orange extract (Monteloeder, Spain) dissolved in water. Following the test meal, urine was collected for the subsequent 48 hours and pooled into the following time points: 0-7 hrs, 7-24 hrs, 24-48 hrs. All urine samples were preserved at -80 °C prior to subsequent analysis.

All participants were recruited if they met the following criteria: White, Caucasian Males and females aged 18-35 years (younger cohort) and 65-77 years (older cohort) of European origin; able to provide written consent to participate in the study; generally healthy, with no history of medical conditions such as type 2 diabetes, cardiovascular, renal, liver, or gastrointestinal disease; not taking medication likely to influence study outcomes; and with no allergies to chocolate, orange, or blackberries. Participants must have been non-smokers and not taken any antibiotics or vaccinations within the last 3 months, or take dietary supplements or herbal remedies, and be unwilling to stop taking them. They must not have taken part in any other research study within the last 3 months. Participants must not have donated blood within 16 weeks of starting or finishing the study. Participants must have a Body mass index (BMI) and blood pressure of $18.5 < \text{BMI kg/m}^2 < 30$ and $90/60 < \text{blood pressure mmHg} < 140/90$. The protocol for the COB study is summarised in Figure 5.2 and in full below.

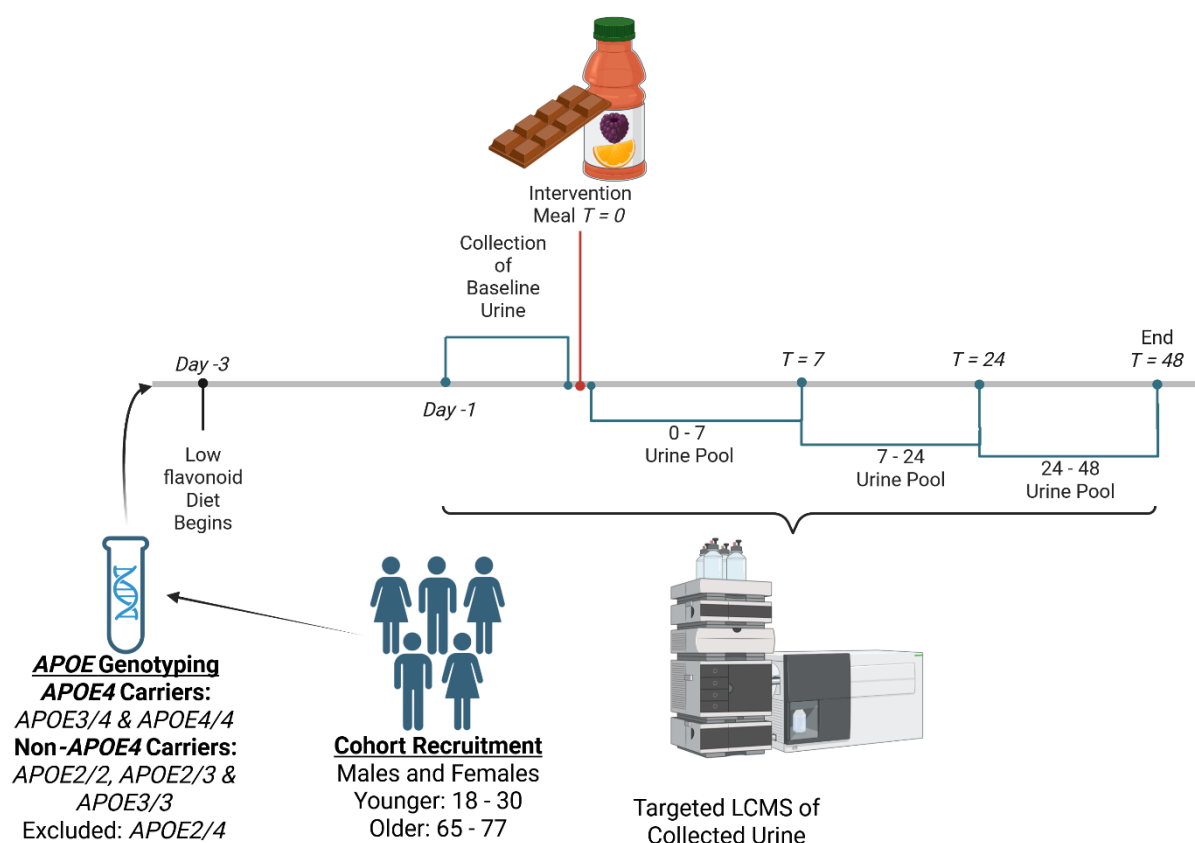


Figure 5.2: Visualised Study Plan for the Chocolate, Orange, and Blackberry (COB) study

5.2.2.2. APOE genotyping

A blood sample was collected from each participant, and DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Manchester, UK). Genotyping of *APOE* SNPs rs429358 and rs7412 was performed with the MassARRAY and iPLEX systems of the Sequenom genotyping platform (Sequenom, San Diego, CA), which uses the MALDI-TOF primer extension assay.

5.2.2.3. Urine analysis of phenolic metabolites by UHPLC-ESI-MS/MS

Metabolites were extracted from 50 μ L human urine by 96-well plate solid phase extraction (SPE; Strata™-X Polymeric Reversed Phase, microelution 2 mg/well) prior to analysis. Samples were analysed with ultra-performance liquid chromatography coupled with tandem mass spectrometry to identify and quantify the phenolic compounds. In detail, samples were analysed using UHPLC-ESI-MS/MS SCIEX QTRAP 6500+ enhanced ultrahigh performance hybrid triple quadrupole-linear ion trap mass spectrometer with

electrospray IonDrive Turbo-V Source. Samples were injected through a Kinetex PFP UPLC column (1.7 μm particle size, 100 Å pore size, 100 mm length, 2.1 mm internal diameter; Phenomenex®). The mobile phase consisted of two parts: A) 0.1% v/v. FA in water (Optima grade, Fisher Scientific) and B) 0.1% v/v. FA in LC-MS grade acetonitrile (Honeywell Burdick and Jackson, Muskegon, MI, USA), delivered at a flow rate ranging from 0.55 - 0.75 mL/min. The column temperature was maintained at 37 °C. The phase gradient ranged from 2% B to 90% B over 30 min. MS/MS scanning was accomplished by advanced scheduled multiple reaction monitoring (ADsMRM) using polarity switching between positive and negative ionisation modes in Analyst (v.1.6.3, SCIEX) and with quantitation conducted using MultiQuant (v.3.0.2, SCIEX) software platforms.

Calibration curves were generated as follows. Internal standards included L-tyrosine- $^{13}\text{C}_9,^{15}\text{N}$, resveratrol-(4-hydroxyphenyl- $^{13}\text{C}_6$), and phlorizin dehydrate (Sigma-Aldrich), and 11-point calibration curves (1 nM to 10 μM) were established for 10 compounds, while 12-point calibration curves (1 nM to 25 μM) were required for 2 compounds. Calibration curves were made by spiking reference standards in matrix-matched SPE extracted Surine, which is an alternative to human urine (synthetic urine negative control; S-020, Sigma-Aldrich Corporation, St. Louis, MO, USA). Source parameters included: curtain gas 35, ion-spray voltage 4000, temperature 550, nebuliser gas 70, and heater gas 70. The optimised analyte-specific quadrupole voltages (mean $\pm$ SD) for negative mode were 50 ± 19 for declustering potential, 9 ± 2 for entrance potential, 26 ± 05 for collision energy, and 11 ± 6 for collision cell exit potential. Similarly, for positive mode, the optimised analyte-specific quadrupole voltages (mean $\pm$ SD) were 29 ± 0 for declustering potential, 6 ± 0 for entrance potential, 29 ± 12 for collision energy, and 12 ± 4 for collision cell exit potential.

The targeted metabolite analysis protocol was optimised and validated to detect 43 analytes, which were quantified relative to 12 authentic commercial and synthetic standards. Where reference standards for metabolites (including flavanols Phase II conjugates) were not available (31 analytes), identification was based on fragmentation profiling involving the precursor structure and 3–5 product transitions and confirmed in pooled samples. These metabolites were then quantified relative to their closest structural reference standard with similar ionisation intensities, which are available in

the published paper [360]. Finally, all metabolites were confirmed based on established retention times (using authentic and synthesised standards where possible) and three or more precursor-to-product ion transitions.

To adjust the concentration of each (poly)phenol metabolite to the volume and concentration of the urine, samples were normalised to the urinary concentration of creatinine using a creatinine assay kit (Biotechnie, R&D Systems, Minnesota, USA) according to the manufacturer's recommended procedures. The nomenclature used for (poly)phenol catabolites follows the current recommendations from Kay et al. [230]. For the purposes of this thesis, the analysis focused on the following flavan-3-ols and their metabolites: EC, catechin, 3,4-DiHPVL, 4-valerolactone (4-VL), 5-HPVL, 5-(Hydroxyphenyl)- γ -valerolactone-O-sulphate (5-HPVL-O-S)

5.2.2.4. Statistical analysis

Study samples' concentrations were pooled for each metabolite across all time points after the test meal and then calculated as a change from baseline for each metabolite. Due to the large number of participants in this study, the results were checked for anomalous values. The results for each metabolite were grouped by age, sex, and carrier, and then the results were removed by Tukey's interquartile range method. The remaining results were then run through the following linear model

$$\Delta \text{Concentration} \sim \text{APOE4 Carrier Status} * \text{Age} * \text{Sex}$$

The intervention meals contained a mixture of many distinct (poly)phenol classes. However, for the purposes of this thesis, the analysis focused on flavan-3-ols and their subsequent metabolites. If significance ($p < 0.05$) was detected in the PERMUTATIONAL ANOVA, permutation t-tests were then carried out through the package RVAideMemoire (10,000 permutations) between the groups, and the BH method was used to adjust for FDR of the multiple testing [240]. Participants who carried the *APOE2/4* genotype were excluded from analysis due to the contradictory nature of these two alleles. The remaining genotypes were then grouped into *APOE4* non-carriers (*APOE2/2*, *APOE2/3*, and *APOE3/3*) and *APOE4* carriers (*APOE3/4* and *APOE4/4*), which allowed the study to account for the relative rarity of some genotypes.

5.2.3. The impact of the APOE genotype, sex, and age on the Oral polyphenol Challenge Test (OPCT)

The laboratory work was carried out by Mena, P., et al., Development of an Oral (Poly)Phenol Challenge Test (OPCT) to Identify Aggregate Metabotypes for Dietary (Poly)Phenols and Their Drivers: A Study Protocol. Current Developments in Nutrition, 2022. 6: p. 1148. [361]. Reanalysis and interpretation of the data from this study, splitting by age, sex, and APOE genotype and focusing on flavan-3-ols, was carried out by the author of this thesis.

5.2.3.1. Ethical approval Study Design

The OPCT was developed to investigate the role of several health variables in the metabolism of a wide range of (poly)phenols. The study was a single-dose, one-arm, acute clinical trial carried out on 300 volunteers recruited in Parma, Italy. Volunteers provided written informed consent, prepared in accordance with the guidelines of the Oviedo Convention. The study was conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice Guidelines of the International Conference on Harmonisation. Once approved by the Ethics Committee, the trial was registered on ClinicalTrials.gov (registration number: NCT05414084).

The study design is summarised in Figure 5.3 and in full below. Volunteers underwent a screening visit (visit 1) to determine eligibility. During the visit, participants were asked to provide personal information and undergo anthropometric measurements. Participants were requested to avoid the consumption of (poly)phenol-rich food and beverages in the afternoon and dinner the day before the OPCT challenge (since 15:00), during and after the challenge. To facilitate this, a list of permitted and forbidden foods and beverages, along with examples of meals and recipes, was supplied. However, to check dietary compliance, a 24-hour dietary recall was conducted at visit 2 and the following day, when volunteers provided the collected urine (visit 3). At baseline (in the morning of visit 2), participants arrived following an overnight fast and provided a spot urine (t0) sample and a blood sample for subsequent genotyping. The participants then underwent the OPCT, consisting of 3 tablets rich in several classes of (poly)phenols, by using 14 different plant-

based food extracts (Figure 5.3). The tablets were consumed alongside a standardised breakfast consisting of a (poly)phenol-free, nutritionally balanced solid meal. At visit 3, all participants were asked to collect a spot urine sample 24 hours after the challenge test.

Participants were eligible to participate in the study if they met the following criteria: An adult aged 18-74 years old, have a body mass index between 18.5 and 25.0 kg/m²; no medical history of past cardiovascular events or metabolic diseases, including diabetes, inflammatory bowel disease or GI surgery, renal diseases, immunodeficiency and autoimmune disorders, mental disorders; no antibiotic therapy within the last month; food allergies; are not currently pregnant or lactating. These criteria were set to avoid likely confounding factors and in line with similar work in the field.

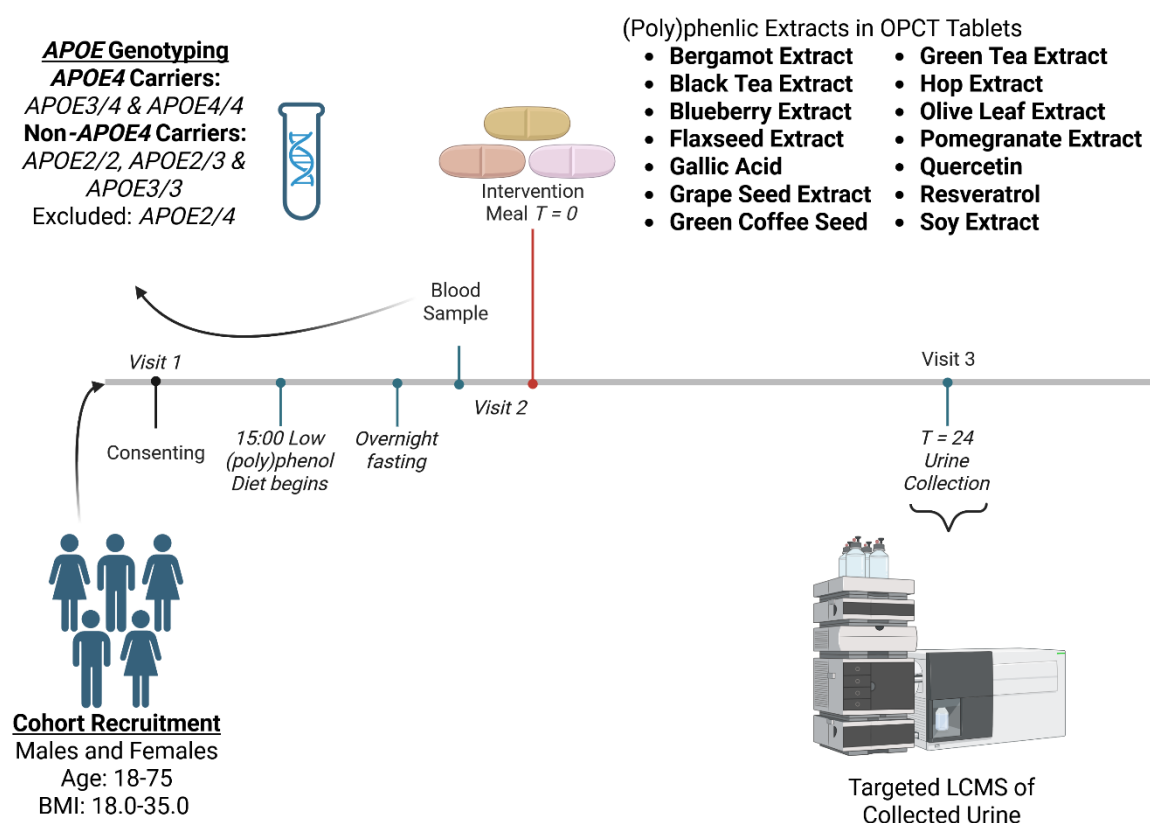


Figure 5.3: Summary Study Design of the Oral (Poly)phenol Challenge Test (OPCT) Study.

5.2.3.2. APOE genotyping

A total of 300 whole blood samples underwent DNA extraction and genotyping. APOE genotyping was performed by RT-qPCR using TaqMan probes targeting the two characterising SNPs, namely rs429358 (TaqMan C___3084793_20 assay) and rs7412 (TaqMan C___904973_10 assay).

5.2.3.3. *Urine analysis of phenolic metabolites by UHPLC-ESI-MS/MS*

Water, acetonitrile, methanol, and FA utilised to prepare solvents for the analysis were bought from Sigma-Aldrich (Schnelldorf, Germany). Standard materials for (poly)phenol aglycones and phase II metabolites were purchased from different companies such as Biosynth (Staad, Switzerland), TRC (Toronto, Canada), Sigma-Aldrich (Schnelldorf, Germany), Santa Cruz Biotechnology (Dallas, Texas, USA), Cayman Chemical Company (Ann Arbor, Michigan, USA), Extrasynthese (Genay, France). ISs such as hippuric acid D5, ferulic acid D3, were bought from Merck (Darmstadt, Germany), and dihydroferulic acid sulfate D3 was provided by Santa Cruz Biotechnology (Dallas, Texas, USA). 100 μ L of urine was diluted with 395 μ L of water, 0.1% of FA, and fortified with 5 μ L of a mix of different deuterated ISs (hippuric acid, ferulic acid, 3,4-dihydroferulic-sulphate acid and caffeic acid). Diluted samples were vortexed and filtered with 0.22 μ m nylon filters in a vial and stored at -80 °C.

An UHPLC Acquity UHPLC I-Class Plus system (Waters, Milford, MA, USA) equipped with a binary pump, autosampler and oven with an Acquity HSS T3 premier column with 1.8 μ m, 2.1 x 100 mm i.d. (Waters) was used. The injection volume was 5 μ L, the samples were maintained at 10 °C and the column at 40 °C. The mobile phase consists of water with 0.01% FA (A) and acetonitrile with 0.01% FA (B). A gradient elution was applied as follows: 0 min, 99% (A); 0.5 min, 99% (A); 3.0 min, 85% (A); 6.0 min, 50% (A), 9.0 min, 5% (A), 10.0 min, 5% (A), 11.0 min, 99% (A); 14.0 min, 99% (A). A flow rate of 400 μ L/min was applied.

A Xevo (Waters) triple-quadrupole mass spectrometer (UHPLC-ESI-QqQ-MS/MS) using an electrospray ionisation source operating in negative and positive mode was used. The capillary voltage was set at 2.3 kV. The source temperature was set to 150 °C, and the desolvation gas to 600 °C. The gas flows were set to cone gas 150 L/h, desolvation gas 800 L/h and nebuliser at 7.0 L/h. The mass spectrometer was operated in MRM, and the cone and collision voltage were optimised for each compound by infusion experiments with standards. When not available, the energies were optimised in chromatography or used for molecules with similar structures. Equipment control and data acquisition were

performed using MassLynx 4.2 software (Waters, Milford, MA, USA) and data processing by TargetLynx XS 4.1.1.0 software (Waters, Milford, MA, USA).

Quality controls were performed using one point of the calibration curve during the batch to control possible chromatography drift and instrument sensitivity. Calibration curves ranging from 0.05 to 2000 pM were used to quantify the (poly)phenols metabolites applying the internal standard method. Ferulic acid D3 was used as an IS for correcting instrument variations and extraction issues. The samples were quantified and expressed in mmol of analyte/mol of creatinine.

Urine samples were also analysed at “Struttura semplice dipartimentale - Biochimica ad elevata automazione” of the Parma University Hospital for creatinine determination. Samples were tested on the AU5800 Beckman Coulter AU analyser (Beckman Coulter, Inc., 250 S. Kraemer Blvd., Brea, CA 92821 U.S.A.) by using creatinine reagent OSR6178 according to standardised hospital routine procedures. Methodology used was a kinetic modification of the Jaffe procedure, traceable with the IDMS reference method. Reference intervals were 0.5-1.4 mg/dL, linearity: (0.06-25 mg/dL), sensitivity: 0.011 mg/dL. Full chromatographic and spectral methodology is available in Mignogna, 2025 [362]. The metabolites reanalysed in this thesis are listed in Table 5.2.

Table 5.2 Compounds analysed in the Oral (Poly)phenol Challenge Test study

Compounds Analysed in the Oral (Poly)phenol Challenge Test (OPCT) study	
Flavan-3-ols	
Epicatechin-3-glucuronide (EC-3-GluAC)	
Epicatechin-3-sulfate (EC-3-S)	
Epicatechin-sulfate-glucuronide (EC-S-GluAC) ion I	
EC-S-GluAC ion II	
EC-S-GluAC ion III	
Epicatechin-glucuronide (EC-GluAC)	
γ-valerolactones	
5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone (3,4,5-TriPHVL)	
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3,4-DiPHVL)	
5-(3',5'-Dihydroxyphenyl)-γ-valerolactone (3,5-DiHPVL)	
5-(3',5'-Dihydroxyphenyl)-γ-valerolactone-4'-glucuronide (3,5-DiHPVL-4-GluAC)	
5-(3'-hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3-HPVL-4-GluAC)	
5-(3'-hydroxyphenyl)-γ-valerolactone-4'-sulfate (3-HPVL-4-S)	
5-(3'-Methoxyphenyl)-γ-valerolactone-4'-glucuronide (3-MPVL-4-GluAC)	
5-(4',5'-Dihydroxyphenyl)-γ-valerolactone-3'-glucuronide (4,5-DiHPVL-3-GluAC)	
5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (5-HPVL-3-GluAC)	
5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate (5-HPVL-3-S)	
5-(Dihydroxyphenyl)-γ-valerolactone-sulfate (DiHPVL-S)	
5-(Hydroxy-methoxyphenyl)-γ-valerolactone-sulfate (HMPVL-S) ion I	
HMPVL-S ion II	
5-(Methoxyphenyl)-γ-valerolactone-glucuronide (MPVL-GluAC)	
5-phenyl-γ-valerolactone-3'-glucuronide (PVL-3-GluAC)	
5-phenyl-γ-valerolactone-3'-sulfate (PVL-3-S)	
Valeric acids	
4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid-3'-sulfate (4-H-3-HPVA-3-S)	
4-Hydroxy-3-hydroxyphenylvaleric acid-4-glucuronide (4-H-3-HPVA-4-GluAC)	
4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-4HPVA-3-GluAC),	
4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-4'-sulfate (4-H-4-HPVA-4-S)	
4-Hydroxy-5-(5'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-5-HPVA-3-GluAC)	
4-Hydroxy-5-(dihydroxyphenyl)valeric acid-sulfate (4-H-DiHPVA-S)	
4-Hydroxy-5-(hydroxy-methoxyphenyl)valeric acid-sulfate (4-H-HMPVA-S) ion II	
4-Hydroxy-5-(methoxyphenyl)valeric acid-glucuronide (3',4') (4-H-MPVA-GluAC (3,4))	
4-Hydroxy-phenylvaleric acid-3-glucuronide (4-H-PVA-3-GluAC)	
4-Hydroxy-5-(phenyl)valeric acid-4'-glucuronide (4-H-PVA-4-GluAC)	
4-Hydroxy-5-(phenyl)valeric acid-sulfate (4-H-PVA-S)	
Phenylvaleric acid-4-glucuronide (PVA-4-GluAC)	

5.2.3.4. Statistical Analysis

To ensure that the variables between the OPCT study and the other studies in this thesis (Chapter 2.2.2 (DIAMOND study) and Chapter 5.2.2 (COB study)) were matched, the

following exclusionary criteria were applied to the samples. Firstly, current smokers were excluded. Secondly, participants were grouped by age (younger: 18-35 and older: 55+); participants between the ages of 35 and 55 were removed from the analysis. Participants were then subsequently grouped by age, sex, and *APOE4* carrier status. As some groups in the OCPT study were small, they were not subject to anomaly removal; the following model was used for each metabolite concentration:

$$\text{Concentration} \sim \text{APOE4 Carrier Status} * \text{Age} * \text{Sex}$$

As for the COB study, the OPCT study intervention meals contained a mixture of many distinct (poly)phenol classes. However, for the purposes of this study, the analysis focused on flavan-3-ols and their subsequent metabolites. If significance ($p < 0.05$) was detected in the PERMUTATIONAL ANOVA, permutation t-tests were carried out through the package RVAideMemoire (10,000 permutations) between the groups, and the BH method was used to adjust for FDR of the multiple testing [240].

5.3. Results

5.3.1. Investigating the effects of the *APOE* genotype on the murine metabolism of flavan-3-ol-rich cocoa – Results from the CANN-AN Study.

5.3.1.1. HPLC measurement of urinary flavan-3-ol metabolites.

Urinary metabolite analysis of the CANN-AN *APOE*-TR mice revealed key findings. First, flavan-3-ol metabolites were detected across all diets, confirming that both LF and HF diets were matrix-matched and contained small amounts of cocoa and, consequently, flavan-3-ols. However, the concentration of all measured compounds was significantly higher in the flavan-3-ol-enriched FLAV diet compared with both HF and LF diets (all $p > 0.05$), irrespective of whether these metabolites originated from the gut-microbiota or not (Table 5.3). This demonstrated that the FLAV diet, enriched with flavan-3-ol-rich cocoa powder, led to markedly increased urinary levels of downstream flavan-3-ol metabolites.

Secondly, significant effects of genotype and/or diet and genotype interactions were observed for specific gut-derived flavan-3-ols metabolites: 3-HPVL-4-S (interaction $p = 0.036$), 4-HPVL-3-GluAC (genotype $p = 0.016$ and interaction $p = 0.007$), 4-H4-HPVA-S (interaction $p = 0.026$), and HPVA-4-S (genotype $p = 0.031$ and interaction $p = 0.014$) (Table 5.3).

Pairwise comparisons focusing on genotype revealed that these metabolites were consistently more excreted in *APOE4/4*-TR mice than their *APOE3/3*-TR counterparts when fed the FLAV diet (all FDR $q > 0.01$; Figure 5.4A-D). No significant genotype-related differences were detected in the LF or HF diet groups, indicating that these effects were specific to flavan-3-ol-rich dietary intake.

Table 5.3: Urinary flavan-3-ols and associated metabolites present in the urine of the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Mice were split by the APOE genotype (APOE3/3-TR and APOE4/4-TR) and then diet: Low fat (10 kcal% energy from fat) (LF), High fat (HF) (45 kcal% from fat), High fat (45kcal % from fat) supplemented with 6% flavan-3-ol rich cocoa (HF FLAV). Values are normalised to urinary creatinine concentrations and expressed in $\mu\text{M}/\text{M}$ creatinine. Significant (p-values <0.05) 2-way ANOVA results are highlighted in bold. Compounds are Epicatechin-glucuronide-sulfate (EC-GluAC-S), Epicatechin-glucuronide (EC-GluAC), Epicatechin (EC), 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (34-DiHPVL), 5-(3'-Hydroxyphenyl)- γ -valerolactone-4'-sulfate (3HPVL-4-S), 5-(4'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide (5-HPVL-3-GluAC), 5-phenyl- γ -valerolactone-3'-glucuronide (PVL-3-GluAC), 5-phenyl- γ -valerolactone-3'-sulfate (PVL-3-S), 5-phenyl- γ -valerolactone-sulfate-glucuronide (PVL-S-GluAC), 4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (4-H-PVA-S), 5-(Hydroxyphenyl)valeric acid-4-sulfate (HPVA-4-S). Group size: APOE3 LF n = 6, APOE3 HF n = 4, APOE3 FLAV n = 6, APOE4 LF n = 6, APOE4 HF n = 4, APOE4 FLAV n = 3

Gen	APOE3/3-TR			APOE4/4-TR			2-way PERMUTATIONAL ANOVA p-value		
Diet	LF	HF	HF FLAV	LF	HF	HF FLAV			
Compound	$\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	$\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	$\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	$\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	$\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	$\bar{x}$ $\mu\text{M}/\text{mmol}$ creatinine (SD)	Gen	Diet	Gen: Diet
EC-GluAC-S	0.010 (0.013)	0.002 (0.004)	0.548 (0.243)	0.011 (0.008)	0.007 (0.009)	0.531 (0.482)	0.965	<0.001	0.995
EC-GluAC	0.282 (0.051)	0.373 (0.267)	13.970 (5.738)	0.365 (0.422)	0.486 (0.348)	8.754 (8.594)	0.353	<0.001	0.387
EC	0.082 (0.164)	0.095 (0.190)	0.605 (0.173)	0.000 (0.000)	0.091 (0.114)	0.251 (0.299)	0.059	0.001	0.158
3,4-DiHPVL	0.051 (0.102)	0.122 (0.243)	0.336 (0.232)	0.026 (0.052)	0.000 (0.000)	0.414 (0.395)	0.801	0.010	0.663
3-HPVL-4-S	6.309 (4.545)	6.180 (2.435)	94.076 (14.607)	8.191 (3.271)	3.519 (1.574)	181.384 (90.284)	0.071	<0.001	0.036
4-HPVL-3-GluAC	0.054 (0.063)	0.073 (0.026)	1.244 (0.371)	0.115 (0.135)	0.050 (0.067)	3.305 (1.533)	0.012	<0.001	0.007
PVL-3-GluAC	0.096 (0.070)	0.137 (0.159)	0.487 (0.133)	0.164 (0.224)	0.272 (0.432)	0.448 (0.362)	0.633	0.048	0.815
PVL-3-S	0.068 (0.083)	0.009 (0.019)	2.796 (2.580)	0.011 (0.022)	0.006 (0.011)	2.925 (1.517)	0.973	0.001	0.991
PVL-S-GluAC	0.003 (0.005)	0.000 (0.001)	0.055 (0.037)	0.000 (0.000)	0.000 (0.000)	0.105 (0.067)	0.255	0.001	0.191
4-H-HPVA-S	0.000 (0.000)	0.006 (0.013)	0.649 (0.173)	0.000 (0.000)	0.007 (0.008)	1.351 (0.688)	0.048	<0.001	0.023
HPVA-4-S	0.010 (0.010)	0.003 (0.006)	0.033 (0.024)	0.009 (0.011)	0.007 (0.013)	0.140 (0.089)	0.031	0.001	0.014

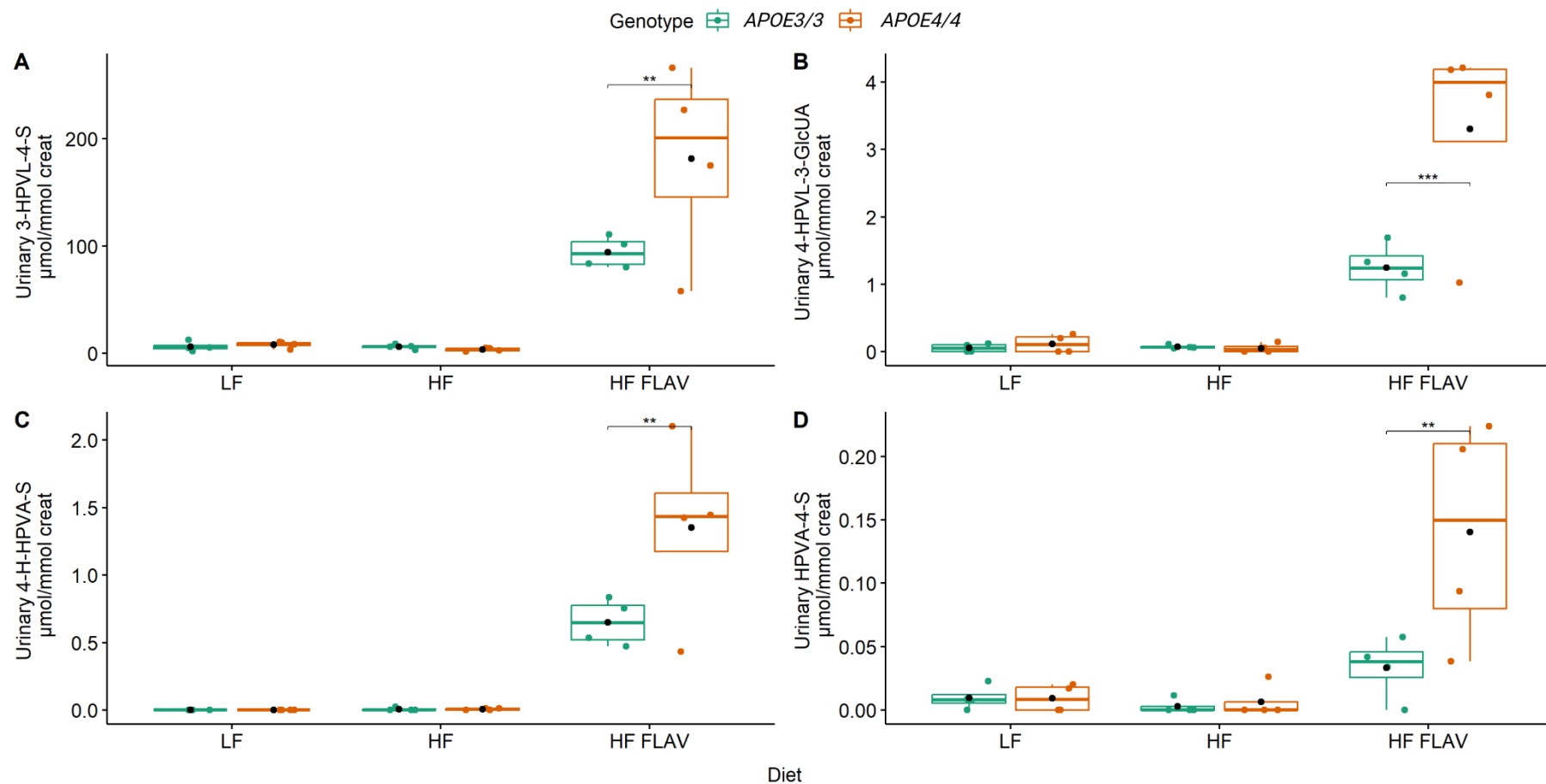


Figure 5.4: Significant Genotype Differences of urinary metabolites of Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Mice were split by the APOE genotype (APOE3/3-TR and APOE4/4-TR) and then diet: Low fat (10 kcal% energy from fat; LF), High fat (45 kcal% from fat; HF), High fat (45 kcal% from fat) supplemented with 6% flavan-3-ol rich cocoa (HF FLAV). A) 5-(3'-Hydroxyphenyl)- γ -valerolactone-4'-sulfate (3-HPVL-4-S). B) 5-(4'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide (4-HPVL-3-GlcUA). C) 4-Hydroxy-5-(hydroxyphenyl)valeric acid-sulfate (4-H-HPVA-S) D) 5-(Hydroxyphenyl)valeric acid-4-sulfate (HPVA-4-S). P-values: ** 0.01>p-value $\geq$ 0.001, *** 0.0001>p-value. The black point is the mean value for each group. Group size: APOE3 LF n = 6, APOE3 HF n = 4, APOE3 FLAV n = 6, APOE4 LF n = 6, APOE4 HF n = 4, APOE4 FLAV n = 3

5.3.1.2. *Relative abundance of microbiota*

This method provides a concise visual overview of the murine microbiota composition. At the Phylum level, Firmicutes were the most predominant, although both interindividual and intergroup variability were evident. A substantial proportion of the microbiota consisted of Bacteroidota, with *APOE3/3*-TR mice exhibiting a higher relative abundance of this phylum. In contrast, Deferribacterota were detectable only in *APOE4/4*-TR animals (Figure 5.5A). At the Family level, genotype-associated differences were more pronounced, with *APOE4/4*-TR animals harbouring greater proportions of Lachnospiraceae than their *APOE3/3*-TR counterparts. Among the *APOE3/3*-TR animals, dietary effects were apparent as Lactobacillaceae abundance increased with the higher fat content of the HF and FLAV diets, a trend not observed in *APOE4/4*-TR mice (Figure 5.5B).

At the genus level, the dominant taxa largely comprised uncultured, or otherwise unclassified, genera across all groups, regardless of *APOE* genotype or diet. Nonetheless, specific genus-level differences emerged. *Bacteroides* were more abundant in *APOE3/3*-TR mice than in *APOE4/4*-TR mice, while the pattern observed for Lachnospiraceae family was mirrored within their genera, with the genus *Lachnospiraceae NK4A136* being the most abundant in the *APOE4/4*-TR mice relative to their *APOE3/3*-TR counterparts (Figure 5.5C).

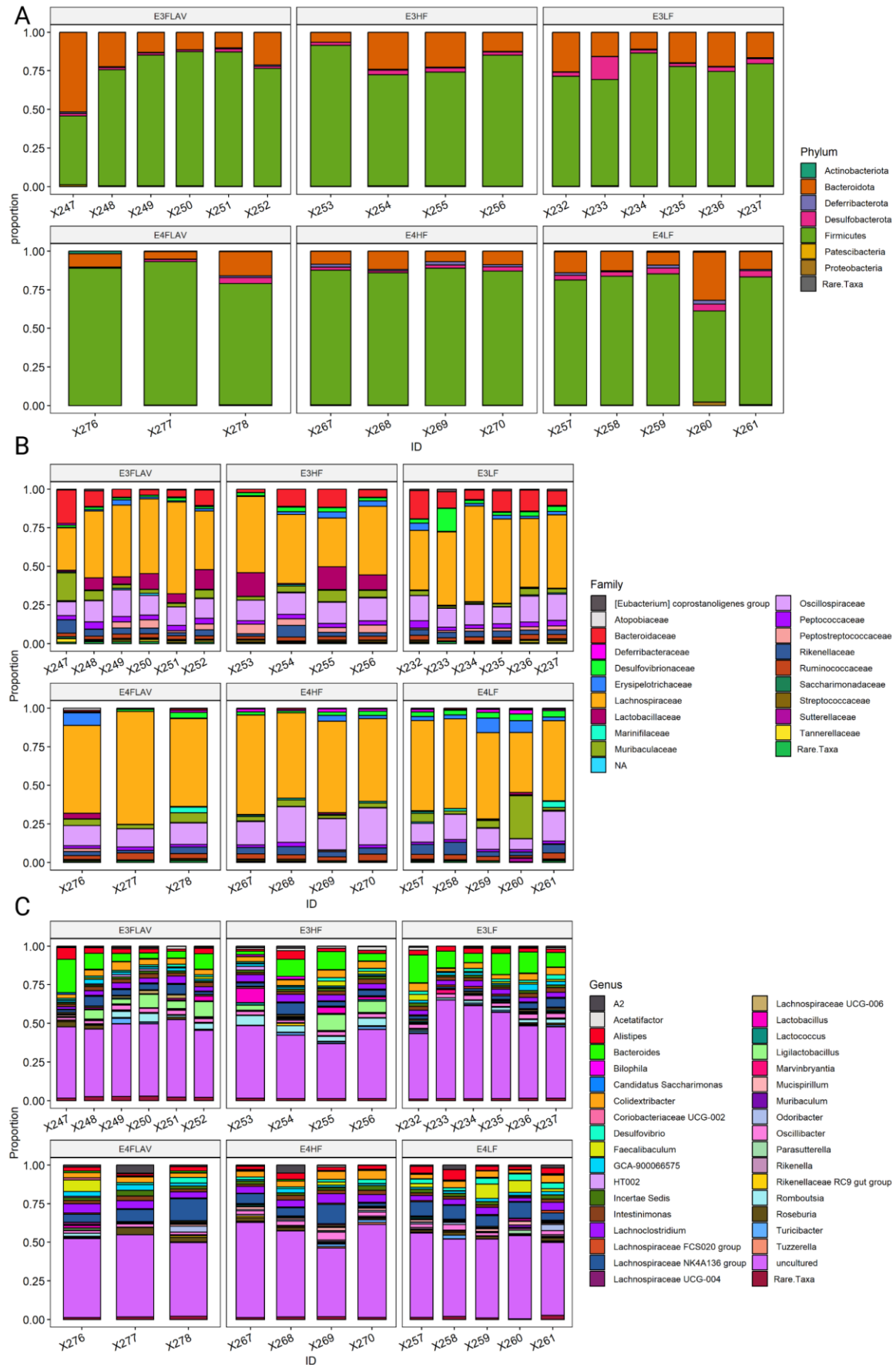


Figure 5.5: Relative abundance of colonic microbiota of mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Mice were split by the APOE genotype (APOE3/3-TR and APOE4/4-TR) and then diet: Low fat (10 kcal% energy from fat; LF), High fat (45kcal% l from fat; HF), High fat (45 kcal% from fat) supplemented with 6% flavan-3-ol rich cocoa (FLAV). A) Phylum relative abundance B) Family relative abundance C) Genus relative abundance. Group sizes: APOE3 LF n = 6, APOE3 HF n = 4, APOE3 FLAV n = 6, APOE4 LF n = 5, APOE4 HF n = 4, APOE4 FLAV n = 3

5.3.1.3. *Alpha and beta diversity metrics*

While relative abundance provides a rapid visual insight into the inter- and intra-group variation in the microbiota, quantitative analysis of community structure is necessary to substantiate these observations. Alpha diversity metrics, which reflect within-sample diversity, were assessed in terms of both genus richness and evenness. Genus richness, representing the number of distinct genera, was estimated using the Chao1 index. Higher Chao1 values indicate a greater number of unique genera present in the sample. Analysis of the Chao1 scores revealed no significant differences between the groups (all $p > 0.05$). Nevertheless, biological trends were apparent. In *APOE4/4*-TR mice, richness tended to decrease from the LF to the HF, to FLAV diets. Furthermore, *APOE4/4*-TR mice generally exhibited lower mean richness than *APOE3/3*-TR mice across all diets, except HF, where means were similar between the genotypes. However, wide within-group variation precluded statistical significance (all $p > 0.05$; Figure 5.6A).

Community evenness was evaluated using the Simpson index, which measures the uniform distribution of genera within a sample. Higher Simpson values indicate a balanced spread of microbes in a community. No significant differences were detected among all groups (all $p > 0.05$). However, in *APOE3/3*-TR mice, evenness showed an upward trend from LF to HF and FLAV diets, whereas *APOE4/4*-TR mice exhibited a stepwise decrease across these diets, again without statistical significance ($p > 0.05$; Figure 5.6B).

Overall microbial diversity, encompassing both richness and evenness, was assessed using the Shannon diversity index. Shannon diversity patterns paralleled those of the Simpson index; again, no significant differences were observed between the groups (all $p > 0.05$). Biological trends, however, suggested that in the *APOE3/3*-TR animals, the diversity increased from LF to HF and to FLAV. This trend was reversed in the *APOE4/4*-TR mice, with the LF having the highest diversity score, followed by the HF and the FLAV. These findings imply potential genotype-specific responses of the gut microbiota to dietary composition, despite the absence of statistical significance (Figure 5.6C).

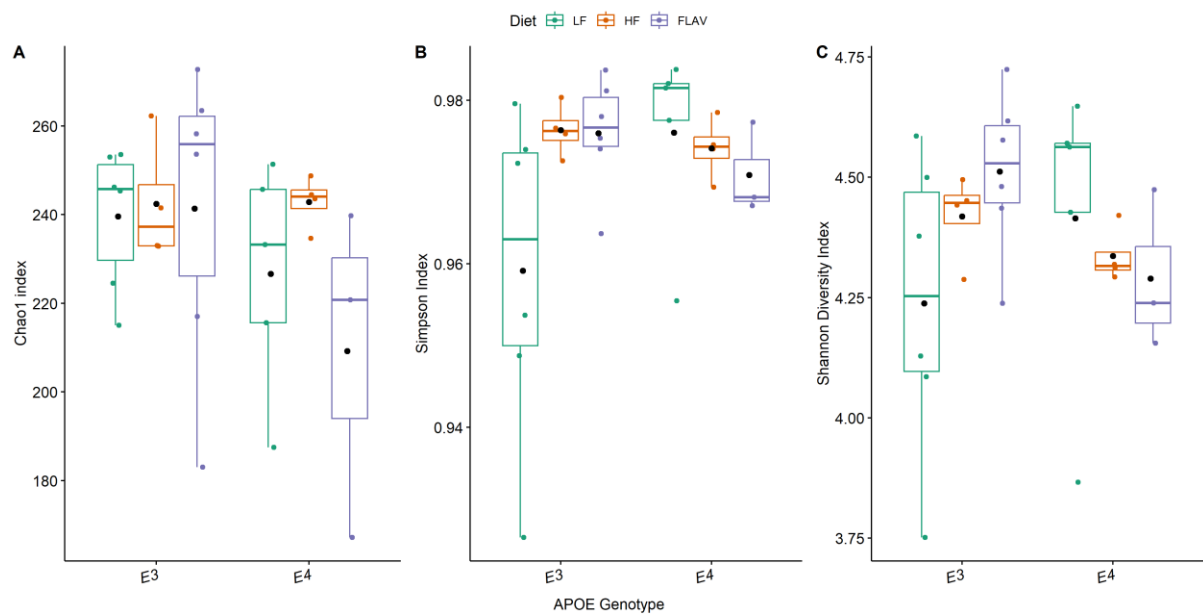


Figure 5.6: Alpha Diversity of the colonic microbiota of mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Mice were split by the APOE genotype (*APOE3/3-TR* and *APOE4/4-TR*) and then diet: Low fat (10 kcal% energy from fat; LF), High fat (45 kcal% from fat; HF), High fat (45 kcal% from fat) supplemented with 6% flavan-3-ol rich cocoa (FLAV). A) Chao1 Index for species richness. B) Simpson Index for species evenness. C) Shannon Diversity Index for both genus richness and evenness. The black point in each boxplot represents the group's mean. Group sizes: *APOE3/3 LF* $n = 6$, *APOE3/3 HF* $n = 4$, *APOE3/3 FLAV* $n = 6$, *APOE4/4 LF* $n = 5$, *APOE4/4 HF* $n = 4$, *APOE4/4 FLAV* $n = 3$

While alpha diversity reflects within-sample microbial variation, beta diversity captures differences in microbial composition between samples. PERMANOVA revealed significant separations among groups (p -value = 0.001). In the 2D ordination plot, *APOE* genotype clearly stratified the samples, with all *APOE3/3-TR* mice clustering to the left and all *APOE4/4-TR* mice to the right of the origin. Within the *APOE3/3* genotype, diet exerted a marked effect: with *APOE3/3-TR LF* mice clustering in the lower left quadrant, whereas HF-fed mice occupied the upper left quadrant, with non-overlapping confidence ellipses. This indicates that dietary fat content strongly influenced gut microbiota composition in *APOE3/3-TR* animals. In contrast, supplementation with flavan-3-ol-rich cocoa attenuated the effect of dietary fat. FLAV samples shifted relative to LF, points intersect with the HF. However, there is also an overlap with LF points, but their ellipses overlapped with both HF and LF groups, suggesting a partial protective effect of flavan-3-ols on microbial structure. *APOE4/4-TR* mice showed no discernible diet-related clustering, with samples distributed across both upper and lower right-hand quadrants. It is worth noting that there were only three samples of the *APOE4/4-TR FLAV* mice, and therefore it was not possible to calculate the ellipses for this group; however, the three datapoints overlapped the HF and LF clusters (Figure 5.7A).

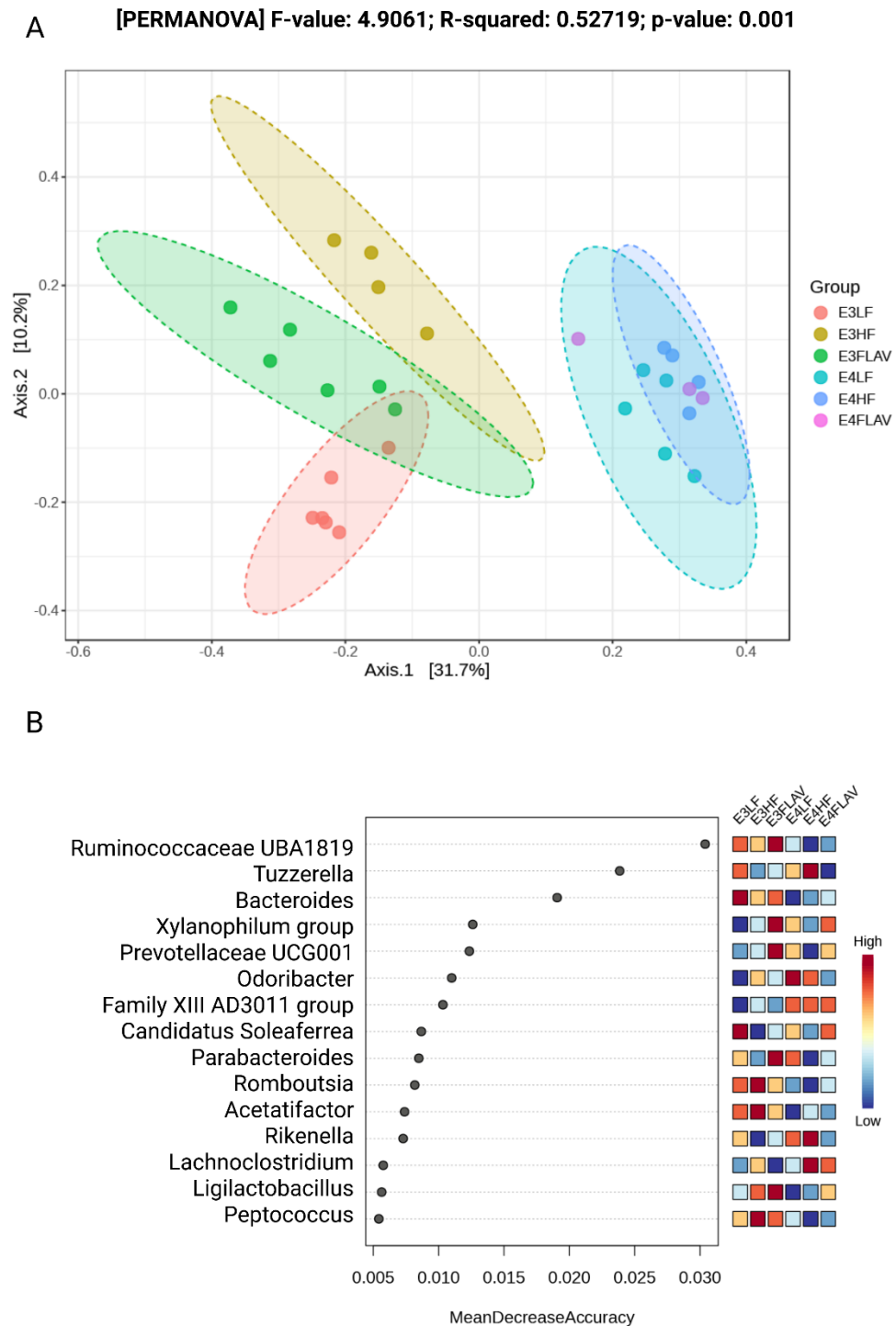


Figure 5.7: Figure 7 : PCoA Beta Diversity Plot of Mouse Colonic Microbiota of mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Mice were split by the APOE genotype (APOE3/3-TR and APOE4/4-TR) and then diet: Low fat (10 kcal% energy from fat; LF), High fat (45 kcal% from fat; HF), High fat (45 kcal% from fat) supplemented with 6% flavan-3-ol rich cocoa (FLAV). A) 2D PCoA with PERMANOVA results; B) Important Features Characteristics Random Forest Plot, listing the top 15 most influential microbial genera and their effect on the colonic microbiota. Group size: E3 LF n = 6, E3 HF n = 4, E3 FLAV n = 6, E4 LF n = 5, E4 HF n = 4, E4 FLAV n = 3

Analysis of the microbial genera most contributing to the overall observed differences revealed that *Ruminococcaceae UBA1819* had the most substantial effect. Variations in

this genus were associated with both *APOE* genotype as well as the diet. This finding is noteworthy because this genus occurs at very low levels in the relative abundance making the family Ruminococcaceae difficult to visualise. In fact, the genus *Ruminococcaceae UBA1819* is sufficiently rare that it appears within the ‘Rare Taxa’ category, defined as taxa with an abundance below 1% in every sample. Moreover, its distribution was not uniform across experimental groups. In *APOE3/3-TR* animals, abundance follows a stepwise trend (*APOE3/3-TR* HF < *APOE3/3-TR* LF < *APOE3/3-TR* FLAV), whereas in *APOE4/4-TR* animals the pattern is reversed (*APOE4/4-TR* HF < *APOE4/4-TR* FLAV < *APOE4/4-TR* LF), indicating that the response to flavan-3-ol supplementation was genotype dependent. The second most influential taxon, *Tuzzerella*, also showed a complex interaction between *APOE* genotype and diet, with the following pattern: *APOE4/4-TR* FLAV < *APOE3/3-TR* HF < *APOE3/3-TR* FLAV < *APOE4/4-TR* LF < *APOE3/3-TR* LF < *APOE4/4-TR* HF. Together, these results suggest intricate genotype-diet interactions in shaping the gut microbiota structure (Figure 5.7B).

5.3.1.4. Differential abundance of microbes

While overall microbiota structure provides insight into community-wide differences among experimental groups, it is equally important to determine whether specific microbial genera colonising the colon differ across treatments. Such genus-level variations may not be captured by the relative abundance metrics alone. Distinct microbial communities may harbour unique genetic features, potentially contributing to the metabolic variations observed in the metabolism of the flavan-3-ols. Moreover, alterations in individual microbial genera can influence the production of other metabolites, with potential health effects, either locally within the GI tract and/or systemically throughout the host.

Clear diet-driven effects were evident in *APOE3/3-TR* mice. Comparison of LF and HF diets revealed significantly greater abundance of *Blautia* (FDR $q = 2.10E-03$) and *Defluviitalea* (FDR $q = 0.123$), in LF-fed mice, whereas *Clostridiaceae SMB53* (FDR $q = 0.052$), *Ruminococcus* (FDR $q = 0.123$), and *Lactobacillus* (FDR $q = 0.149$) were more abundant under HF feeding (Figure 5.8A). Within the same genotype, *Blautia* and *Parabacteroides* (both FDR $q = 0.159$) were significantly enriched in mice receiving the

FLAV diet compared to their HF counterparts (Figure 8 B). Because the LF and the FLAV diets differ in both fat and flavan-3-ol content, direct comparisons between these groups were not performed.

In contrast, *APOE4/4*-TR mice exhibited minimal genus-level changes, mirroring the limited shifts observed in beta diversity. No significant differences were detected between LF and HF-fed *APOE4/4*-TR mice (all FDR $q > 0.2$). However, comparisons of HF and the FLAV diets identified *Adlercreutzia* as significantly more abundant in the FLAV group (FDR $q = 0.027$; Figure 8 C). As with *APOE3/3*-TR mice, LF-FLAV comparisons were not conducted due to confounding differences in both fat and flavan-3-ol contents.

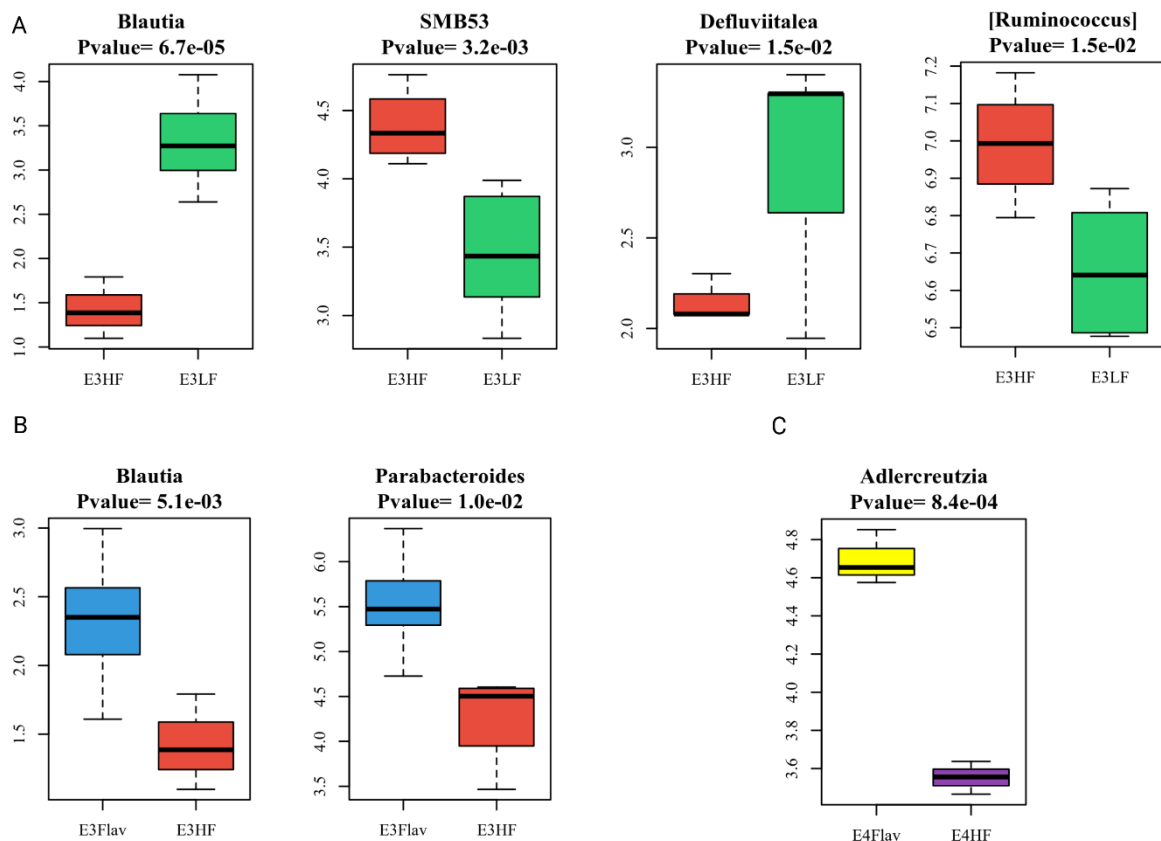


Figure 5.8: The most significant (Benjamini Hochberg FDR adjusted q values < 0.2) differential abundance for *APOE3/3*-TR (E3) and *APOE4/4*-TR (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. A) Comparing the differential abundances between the E3 mice on the high fat (HF) (45kcal% from fat) diet and the low fat (LF) (10 kcal% from fat) diet. FDR adjusted values are as follows: *Blautia* (FDR adjusted $q = 2.10E-03$), *Clostridiales* SMB53 (FDR adjusted $q = 0.052$), *Defluviitalea* (FDR adjusted $q = 0.123$), *Ruminococcus* (FDR adjusted $q = 0.149$) B) Comparing the differential abundances between the E3 mice on the HF diet and the high fat (45 kcal% from fat) supplemented with flavan-3-ol rich cocoa powder (6%) (FLAV), FDR adjusted values are as follows: *Blautia* (FDR adjusted $q = 0.159$), *Parabacteroides* (FDR adjusted $q = 0.159$). C) Comparing the differential abundances between the E4 mice on the HF and FLAV diets, FDR adjusted values are as follows: *Adlercreutzia* (FDR adjusted $q = 2.70E-02$). Raw p-values are printed on the plots. Group sizes: *APOE3* LF $n = 6$, *APOE3* HF $n = 4$, *APOE3* FLAV $n = 6$, *APOE4* LF $n = 5$, *APOE4* HF $n = 4$, *APOE4* FLAV $n = 3$

Consistent with the beta-diversity analysis, marked differences in microbial composition were observed between *APOE* genotypes across all diets. Within the LF diet, the *APOE3/3*-TR genotype exhibited a greater abundance of *Bacteroides* (FDR $q = 0.025$),

Dehalobacterium (FDR q = 0.025), *Peptococcaceae rc4-4* (FDR q = 0.043), *Clostridiaceae SMB53* (FDR q = 0.066), *Dorea* (FDR q = 0.076), *Anaerotruncus* (FDR q = 0.152), *Parabacteroides* (FDR q = 0.189), and *Faecalibacterium* (FDR q = 0.198) (Figure 5.9 & Supplementary Table 10). In contrast, the *APOE4/4*-TR genotype displayed significantly higher abundances of *Mucispirillum* (FDR q = 0.025), *Odoribacter* (FDR q = 0.043), *Desulfovibrio* (FDR q = 0.152), *Butyricicoccus* (FDR q = 0.152), and *Ruminococcus* (FDR q = 0.189).

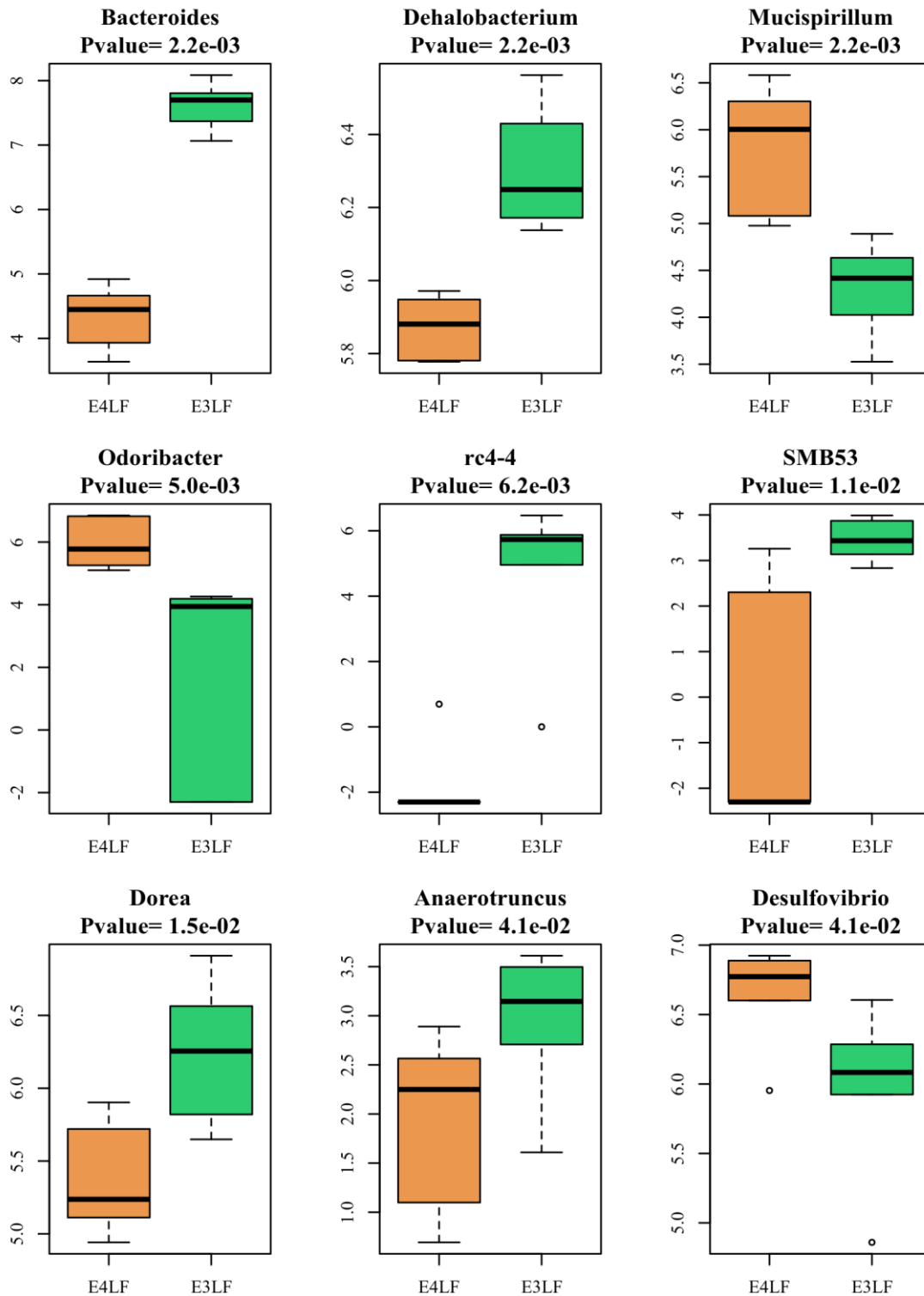


Figure 5.9: The top 9 most significant (Benjamini Hochberg FDR adjusted q values < 0.2) differential abundance for APOE3/3-TR (E3) and APOE4/4-TR (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing mice on the low fat (10 kcal% fat; LF) diet. Raw p -values are printed on the plot and the FDR adjusted values are as follows: Bacteroides (FDR adjusted $q = 0.025$), Dehalobacterium (FDR adjusted $q = 0.025$), Mucispirillum (FDR adjusted $q = 0.025$), Odoribacter (FDR adjusted $q = 0.043$), rc4-4 (FDR adjusted $q = 0.043$), SMB53 (FDR adjusted $q = 0.066$), Dorea (FDR adjusted $q = 0.076$), Anaerotruncus (FDR adjusted $q = 0.152$), Desulfovibrio (FDR adjusted $q = 0.152$). Group sizes: E3 LF $n = 6$, E4 LF $n = 5$

Examination of the mice on the HF diet revealed that there were genotype differences between genera. The *APOE3/3-TR* genotype had a greater abundance of *Bacteroides* (FDR $q = 0.169$), *Lactobacillus* (FDR $q = 0.169$), and *Faecalibacterium* (FDR $q = 0.169$). In contrast, the *APOE4/4-TR* mice displayed greater abundances of *Ruminococcus* (FDR $q = 0.169$), *AF12* (FDR $q = 0.169$), and *Mucispirillum* (FDR $q = 0.169$)(Figure 5.10).

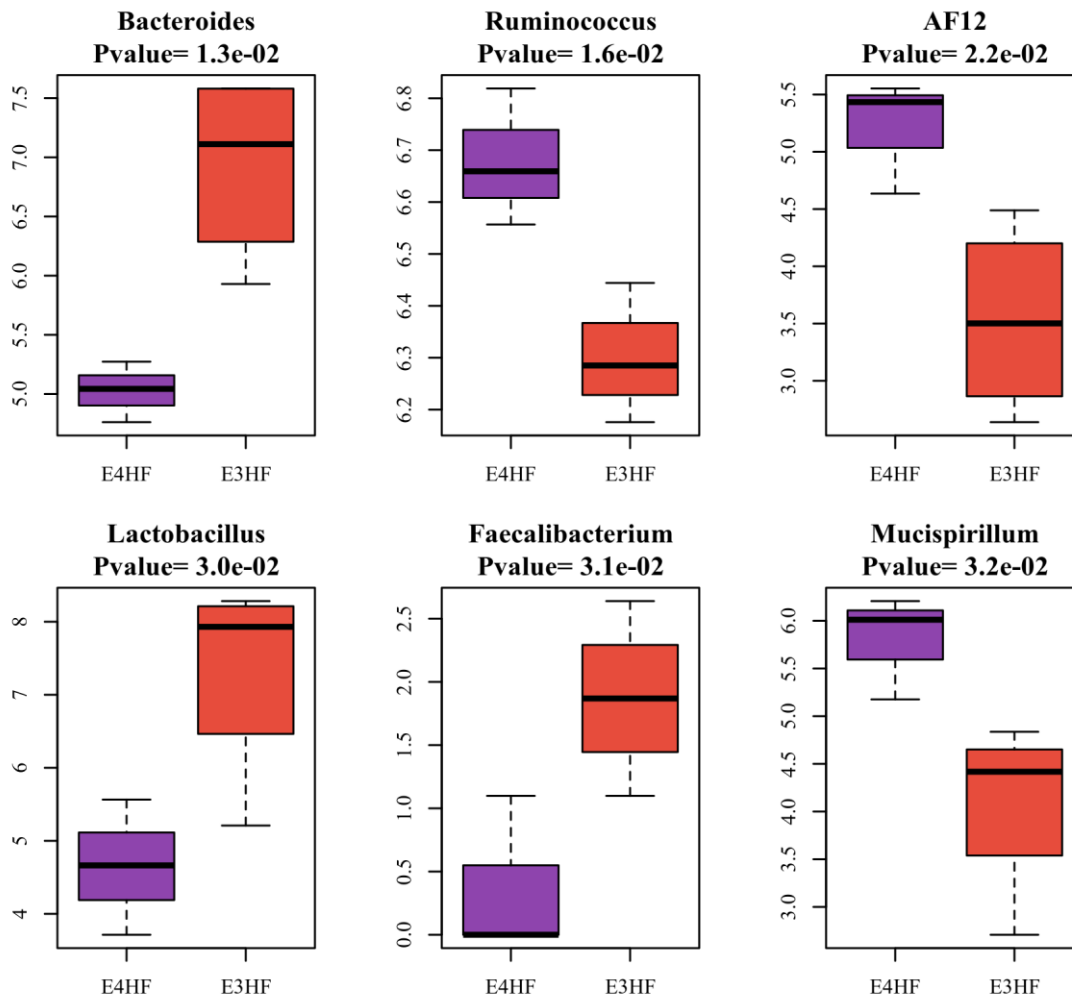


Figure 5.10: The significant (Benjamini Hochberg FDR adjusted q values <0.2) differential abundance for *APOE3/3-TR* (E3) and *APOE4/4-TR* (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing mice on the high-fat (HF) (45% kcal fat) diet. Raw p -values are printed on the plot and the FDR adjusted values are as follows: *Bacteroides* (FDR adjusted $q = 0.169$), *Ruminococcus* (FDR adjusted $q = 0.169$), *AF12* (FDR adjusted $q = 0.169$), *Lactobacillus* (FDR adjusted $q = 0.169$), *Faecalibacterium* (FDR adjusted $q = 0.169$), *Mucispirillum* (FDR adjusted $q = 0.169$). Group sizes: E3 HF $n = 4$, E4 HF $n = 4$

Comparative analysis of the microbiota in animals fed the FLAV diet identified several genera with significant genotype-associated differences. Specifically, the *APOE3/3-TR* genotype exhibited a significantly higher relative abundance of *Bacteroides* (FDR $q = 0.086$), whereas the *APOE4/4-TR* genotype showed increased abundance of unclassified/other taxa (FDR $q = 0.009$), *Ruminococcus* (FDR $q = 0.181$), and *Aldercreutzia* (FDR $q = 0.197$) (Figure 5.11). These findings indicate a complex interplay

between *APOE* genotype and dietary composition, whereby their combined effects create distinct ecological niches that facilitate the proliferation of specific bacterial genera.

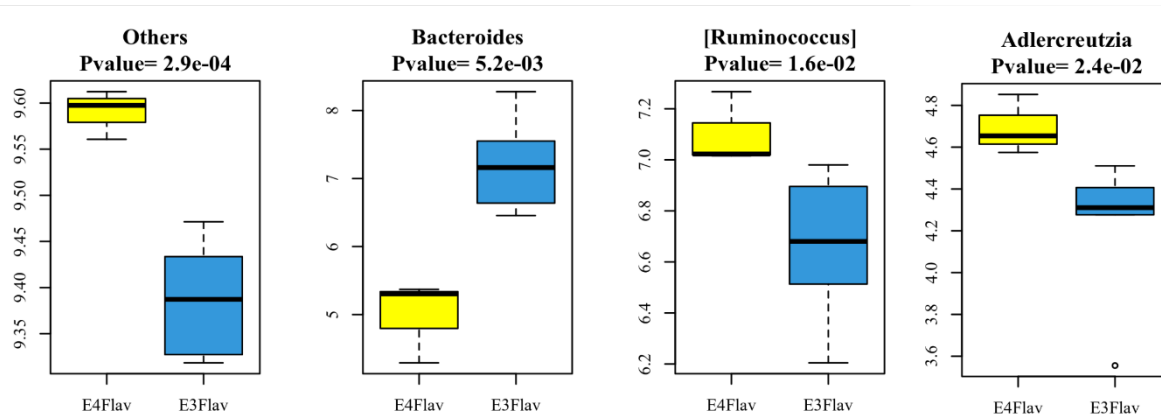


Figure 5.11: The significant (Benjamini Hochberg FDR adjusted q values <0.2) differential abundance for *APOE3/3-TR* (E3) and *APOE4/4-TR* (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing mice on the high-fat (45 kcal% fat) diet supplemented with flavan-3-ol rich cocoa powder (6%)(FLAV). Raw p values are printed on the plots and the FDR adjusted values are as follows: Other genera (FDR adjusted $q = 0.009$), *Bacteroides* (FDR adjusted $q = 0.086$), *Ruminococcus* (FDR adjusted $q = 0.181$) and *Adlercreutzia* (FDR adjusted $q = 0.197$). Group sizes: E3 FLAV $n = 6$, E4 FLAV $n = 3$

5.3.1.5. Flavan-3-ol-rich cocoa supplementation affects microbial metabolism

sPLS-DA analysis indicated subtle but distinct differences in metabolomic profiles associated with both diet and the *APOE* genotype in mice. In *APOE3/3-TR* animals, the LF and the FLAV diets clustered together, whereas the HF diet separated clearly from the other dietary groups. In contrast, *APOE4/4-TR* mice exhibited a different pattern where LF and HF diets showed substantial overlap, while the FLAV diet shifted modestly towards the *APOE3/3-TR* mice LF and FLAV cluster, but still overlapped with the other *APOE4/4-TR* diets (Figure 5.12A). Lactate metabolism emerged as the primary contributor to these global differences, displaying clear genotype and diet-dependent variations in production (Figure 5.12B).

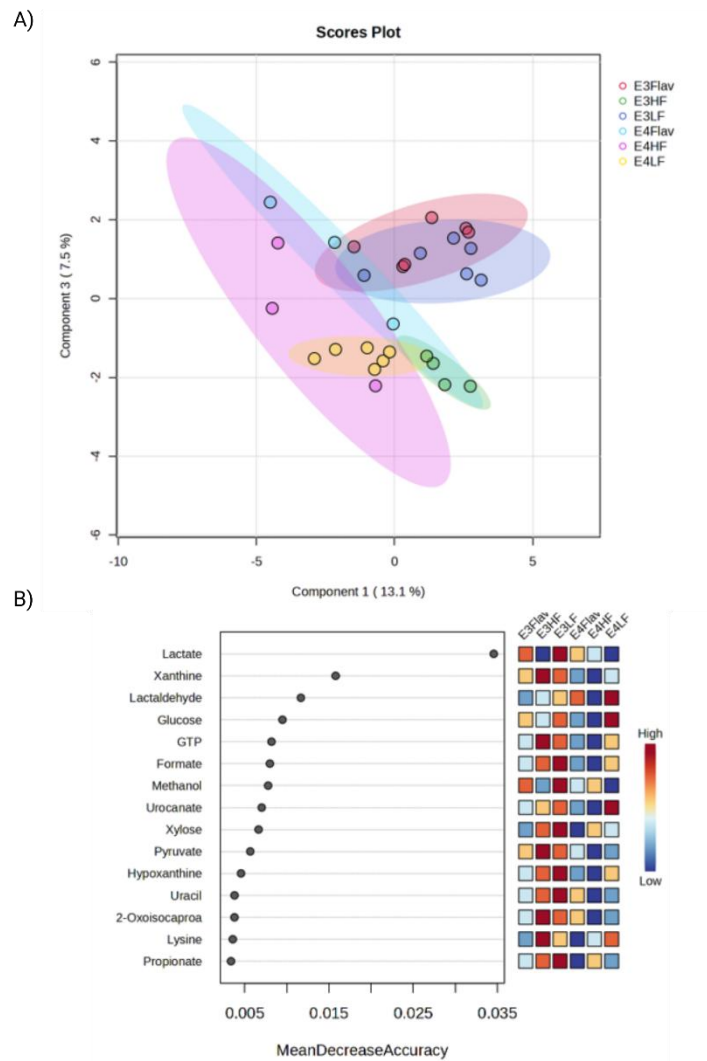


Figure 5.12: A) Sparse Partial Least Squares-Discriminant Analysis (sPLS-DA) and B) important features and characteristics of metabolomic data of faecal metabolome of mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Mice were split by the APOE genotype (APOE3/3-TR and APOE4/4-TR) and then diet: Low fat (10 kcal% energy from fat; LF), High fat (45 kcal% from fat; HF), High fat (45kcal% from fat) supplemented with 6% flavan-3-ol rich cocoa (FLAV). Group sizes: E3 LF n = 6, E3 HF n = 4, E3 FLAV n = 6, E4 LF n = 6, E4 HF n = 3, E4 FLAV n = 3

While global metabolomic analyses captured overall structural shifts, examination of individual metabolites revealed additional group-specific effects. In *APOE3/3-TR* mice, significant differences were detected between the HF and the FLAV diets. Methanol was significantly elevated under the FLAV diet (FDR $q = 0.032$), whereas, xylose (FDR $q = 0.146$), lysine (FDR $q = 0.150$), hypoxanthine, valerate (FDR $q = 0.150$), 2-methylbutyric acid (FDR $q = 0.150$), xanthine (FDR $q = 0.150$), GTP (FDR $q = 0.183$), 1,3-Dihydroxyacetone (FDR $q = 0.183$), and 3-Methyl-2-oxovalerate (FDR $q = 0.183$) were all significantly higher in the HF diet (Figure 5.13 and Supplementary Table 11). No

significant differences were observed between LF and HF diets in this genotype (all FDR $q > 0.2$).

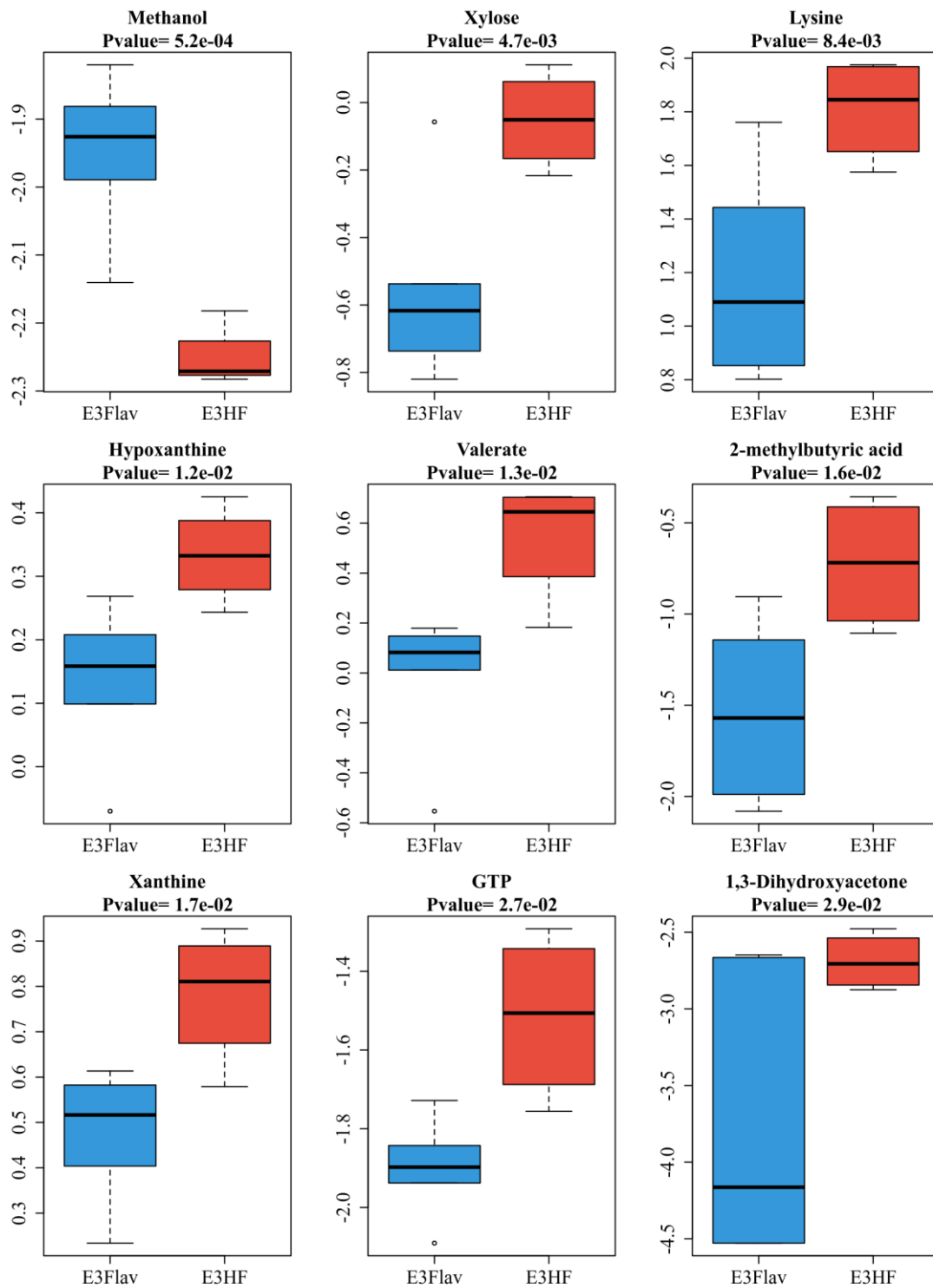


Figure 5.13: The top 9 most significant (Benjamini Hochberg FDR adjusted q values < 0.2) differential abundance of metabolites for APOE3/3-TR (E3) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing the differential abundances between the E3 mice on the HF diet and the high fat (45 kcal% from fat) supplemented with flavan-3-ol rich cocoa powder (6%) (FLAV), FDR adjusted values are as follows: methanol (FDR adjusted $q = 0.032$), xylose (FDR adjusted $q = 0.146$), Lysine (FDR adjusted $q = 0.150$), hypoxanthine (FDR adjusted $q = 0.150$), valerate (FDR adjusted $q = 0.150$), 2-methylbutyrate (FDR adjusted $q = 0.150$), xanthine (FDR adjusted $q = 0.150$), GTP (FDR adjusted $q = 0.183$), and 1,3-dihydroxyacetone (FDR adjusted $q = 0.183$). Raw p -values are printed on the plots. Group sizes: E3 HF $n = 4$, E3 FLAV $n = 6$

In *APOE4/4*-TR mice, dietary contrasts were more pronounced. Comparison of LF and HF diets showed that 3-methyl-2-oxovalerate, 3-phenylpropionate, GTP, methionine, ornithine, threonine, trimethylamine, and fumarate were significantly more abundant in the LF diet group (all FDR $q = 0.190$), whereas methanol was significantly higher in the HF group (FDR $q = 0.190$) (Figure 5.14). However, no metabolites differed significantly between the HF and FLAV (all FDR $q > 0.2$).

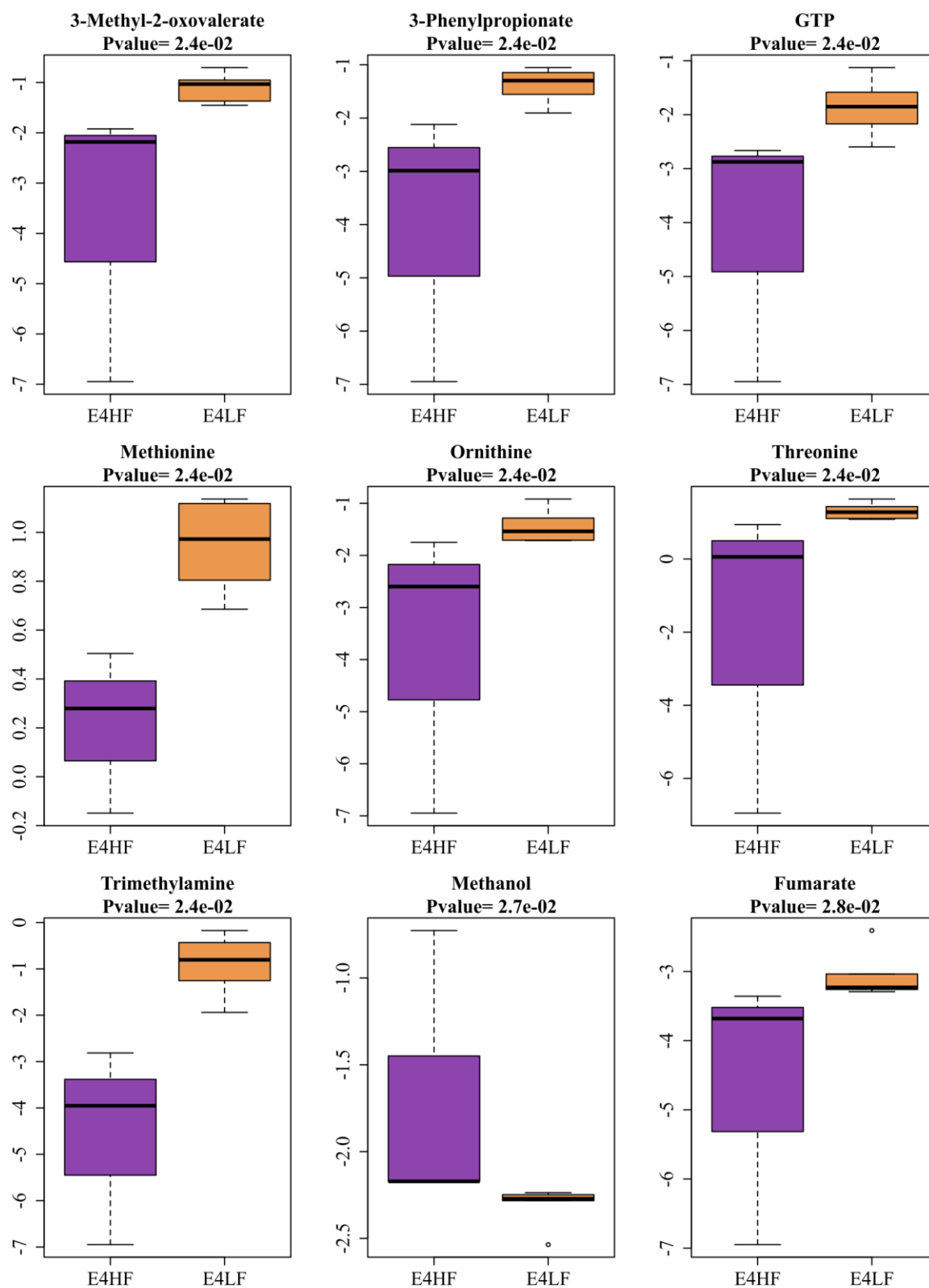


Figure 5.14: The most significant (Benjamini Hochberg FDR adjusted q values < 0.2) differential abundance of metabolites for APOE4/4-TR (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing the differential abundances between mice on the Low Fat (LF) diet (10% kcal fat) and the High Fat (HF) diet. Raw p -values are printed on the plot, and the FDR-adjusted values are 0.190 for all metabolites. Group sizes: E4 LF $n = 6$, E4 HF $n = 3$

Direct comparisons between *APOE* genotypes further highlighted metabolite-specific effects. Under the LF diet, 18 metabolites differed significantly. *APOE3/3*-TR mice displayed higher concentrations of xanthine (FDR $q = 0.047$), methanol (FDR $q = 0.176$), propionate (FDR $q = 0.176$), and uracil (FDR $q = 0.176$) (Figure 5.15). Conversely, the *APOE4/4*-TR mice had a greater abundance of aspartate (FDR $q = 0.065$), AMP (FDR $q = 0.176$), glucose (FDR $q = 0.176$), nicotinate (FDR $q = 0.176$), cytidine monophosphate (FDR $q = 0.181$), alanine (FDR $q = 0.181$), ferulate (FDR $q = 0.181$), ethanol (FDR $q = 0.181$), proline (FDR $q = 0.181$), 3-phenylpropionate (FDR $q = 0.181$), asparagine (FDR $q = 0.181$), urocanate (FDR $q = 0.196$), and lactaldehyde (FDR $q = 0.196$) (Figure 5.15 and Supplementary Table 12). No genotype-dependent differences were observed under HF or FLAV diets (all FDR $q > 0.2$).

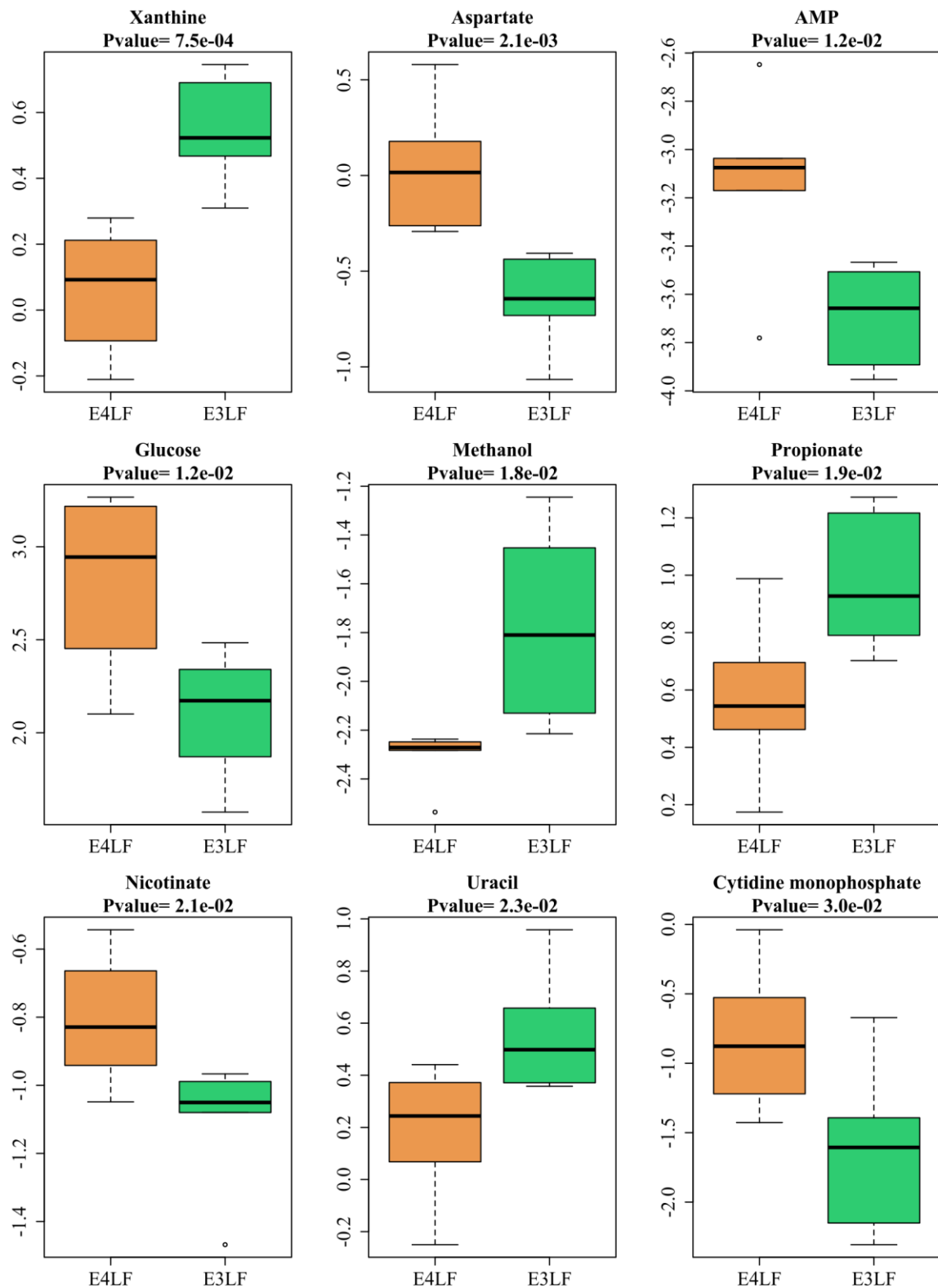


Figure 5.15: The top 9 most significant (Benjamini Hochberg FDR adjusted q values <0.2) differential abundance for APOE3/3-TR (E3) and APOE4/4-TR (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing mice on the low-fat (LF) (10% kcal fat) diet. Raw p -values are printed on the plot and the FDR adjusted values are as follows: Xanthine (FDR adjusted $q = 0.047$), aspartate (FDR adjusted $q = 0.065$), AMP (FDR adjusted $q = 0.176$), glucose (FDR adjusted $q = 0.176$), methanol (FDR adjusted $q = 0.176$), propionate (FDR adjusted $q = 0.176$), nicotinate (FDR adjusted $q = 0.176$), uracil (FDR adjusted $q = 0.176$), and cytidine monophosphate (FDR adjusted $q = 0.181$). Group sizes: E3 LF $n = 6$, E4 LF $n = 6$

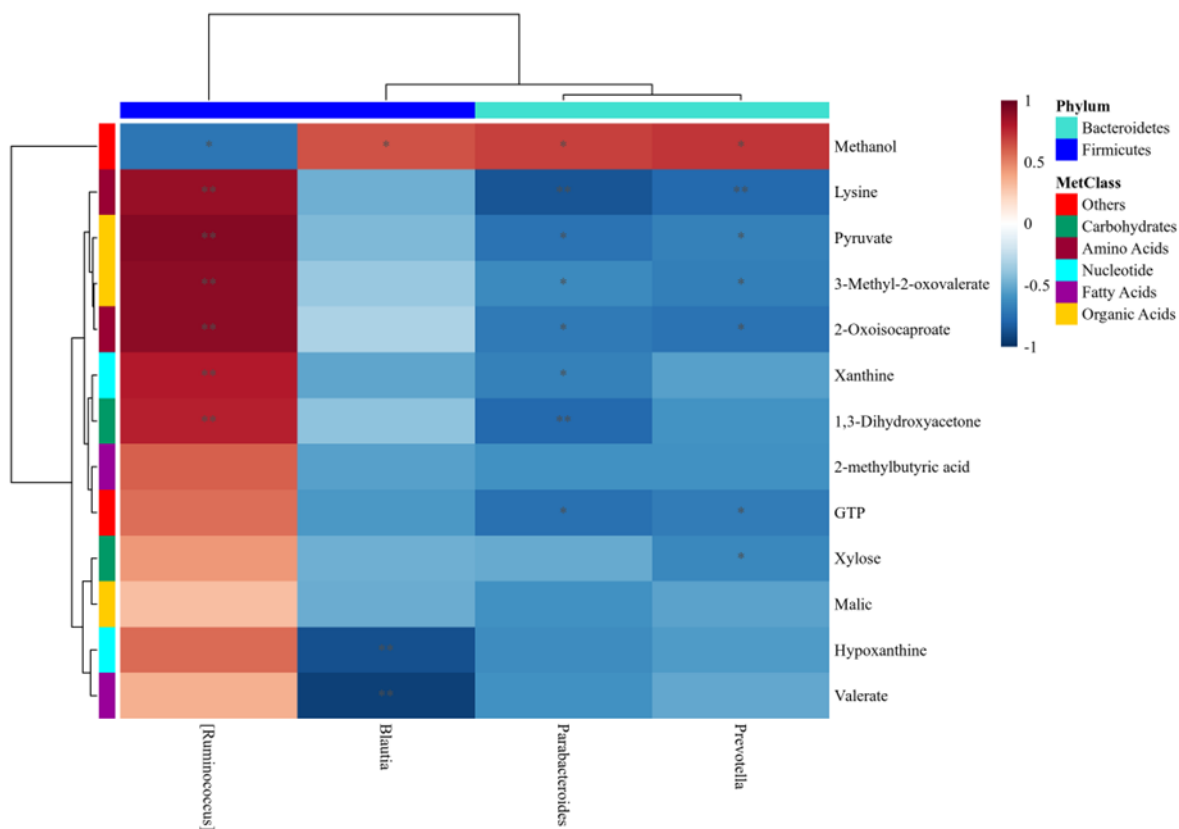
5.3.1.6. Microbiota-metabolome correlation analysis

Examination of the correlations between the microbiota and the subsequent metabolome it produces helps to identify links between the two. Spearman's correlation of each group reveals that both diet and *APOE* genotype have effects on metabolome and microbiota interactions. In the *APOE3/3*-TR mice, comparing the HF and FLAV diets, there was an association between the microbes [*Ruminococcus*], *Blautia*, *Parabacteroides* and *Prevotella*. [*Ruminococcus*] had significant positive correlations with the following compounds: lysine, pyruvate, 3-methyl-2-oxovalerate, 2-oxoisocaproate, xanthine, and 1,3-dihydroxyacetone (all $p < 0.01$). It is significantly negatively correlated with methanol ($p < 0.05$). *Blautia* positively correlated with methanol production ($p < 0.05$) and negatively correlated with the following lysine, 1,3-dihydroxyacetone (both $p < 0.01$), pyruvate, 3-methyl-2-oxovalerate, 2-oxoisocaproate, xanthine, and GTP (all $p < 0.05$). The genus *Prevotella* had a significant positive correlation with methanol ($p < 0.05$), and negative correlations with lysine ($p < 0.01$), pyruvate, 3-methyl-2-oxovalerate, 2-oxoisocaproate, GTP and xylose ($p < 0.05$). Trends demonstrate that the correlations between [*Ruminococcus*] and all of the metabolites are reversed in all the other microbes listed (Figure 16A). In the *APOE4/4*-TR animals, there is a diet difference between the LF-fed and HF-fed mice. Associations were identified between the following microbes and the metabolome: *Desulfovibrio*, *Bacteroides*, and [*Ruminococcus*]. *Desulfovibrio* has a significant negative correlation with methanol ($p < 0.01$) and significant positive correlations with fumarate and ribose ($p < 0.05$). *Bacteroides* has a significant positive correlation with methanol ($p < 0.01$) and significant negative correlations with fumarate and ribose ($p < 0.05$). [*Ruminococcus*] significantly correlates positively with methanol ($p < 0.05$) and negatively with fumarate, ornithine (both $p < 0.01$), and GTP ($p < 0.05$). Additionally, the trends in the correlations between *Desulfovibrio* and the metabolome are reversed in the other microbes (Figure 16B). There are also *APOE* genotype differences between the *APOE3/3*-TR and *APOE4/4*-TR animals, with significant correlations observed in both the LF-fed and FLAV-fed animals. For the LF-fed animals, there are correlations between the microbes: *Bacteroides*, *Dehalobacterium*, *rc4-4*, *Dorea*, *SMB53*, *Anaerotruncus*, *Desulfovibrio*, *Butyricoccus*, *Odobacter*, and *Mucispirillum*. Observing the overall trends in correlations between the microbes and the metabolome indicates that the trends in *Bacteroides*, *Dehalobacterium*, *rc4-4*, *Dorea*,

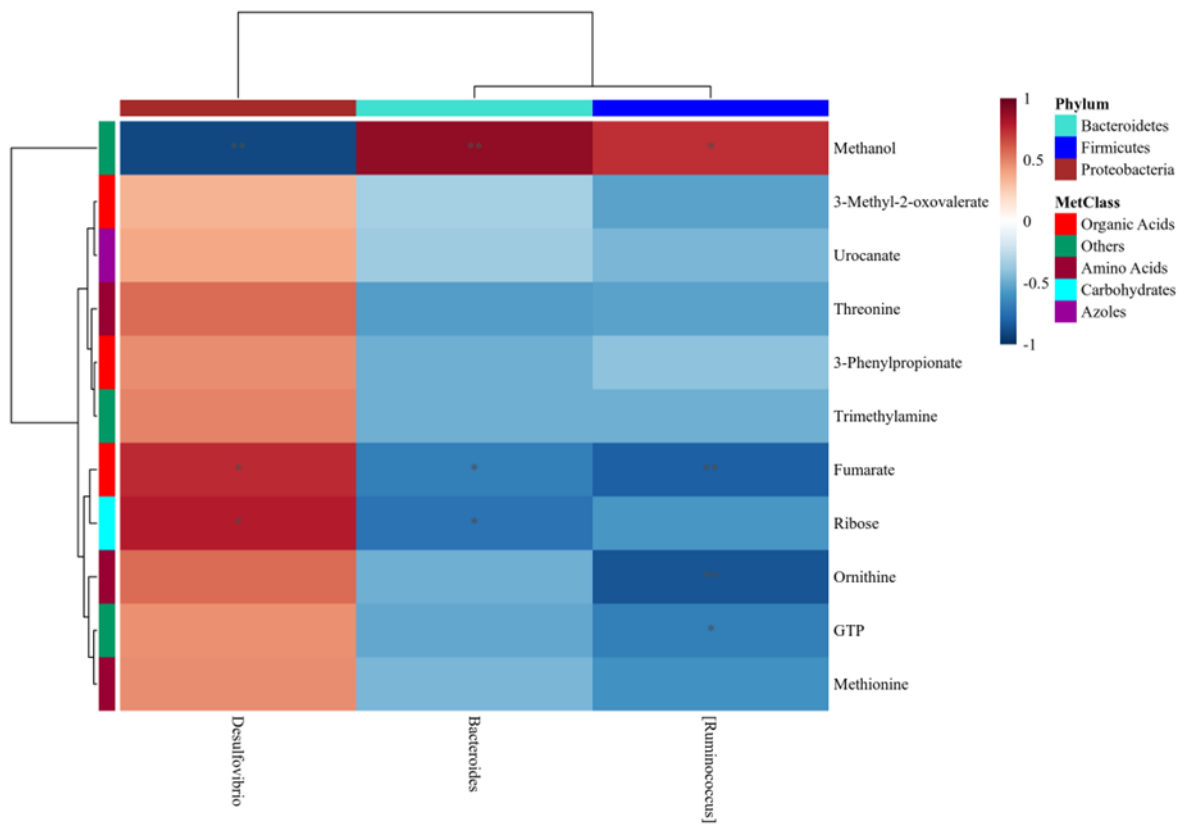
SMB53, and *Anaerotruncus* are reversed in the other microbes. *Bacteroides* significantly positively correlated with uracil, xanthine (both $p < 0.05$) and methanol ($p < 0.01$). It negatively correlated with ethanol, AMP, cytidine monophosphate, alanine (all $p < 0.05$), aspartate, asparagine, and glucose (all $p < 0.01$). *Dehalobacterium* positively correlated with uracil ($p < 0.05$), xanthine, and methanol (both $p < 0.01$) and negatively correlated with aspartate, asparagine, nicotinate (all $p < 0.01$), glucose, and alanine (both $p < 0.05$). *rc44* had positive correlations with xanthine ($p < 0.05$) and methanol ($p < 0.01$). This genus negatively correlated with AMP, glucose, alanine (all $p < 0.05$), cytidine monophosphate, aspartate, asparagine, and nicotinate (all $p < 0.01$). *Dorea* negatively correlated with cytidine monophosphate, aspartate (both $p < 0.05$), asparagine, and nicotinate (both $p < 0.01$). *SMB53* positively correlated with xanthine and methanol (both $p < 0.05$). It also negatively correlated with ethanol, aspartate (both $p < 0.05$), AMP, cytidine monophosphate, and asparagine (all $p < 0.01$). The genus *Anaerotruncus* negatively correlated with ethanol, AMP, asparagine (all $p < 0.05$) and aspartate ($p < 0.01$). The genus *Desulfovibrio* has significant negative correlations with methanol ($p < 0.01$) and positive correlations with ethanol, aspartate and glucose (all $p < 0.05$). *Butyricoccus* has significant negative correlations with propionate ($p < 0.05$) and positive correlations with ferulate, nicotinate (both $p < 0.05$), and 3-phenyl propionate ($p < 0.01$). The genus *Odoribacter* exhibited significant negative correlations with propionate, xylose (both $p < 0.05$), xanthine, and methanol (both $p < 0.01$). This genus positively correlated with proline, ferulate, ethanol, asparagine, nicotinate (all $p < 0.05$), 3-phenyl propionate, and aspartate (both $p < 0.01$). Finally, the genus *Mucispirillum* had significant negative associations with uracil ($p < 0.05$), xanthine, and methanol (both $p < 0.01$). *Mucispirillum* had significant positive associations with aspartate ($p < 0.01$), asparagine, nicotinate, and glucose (all $p < 0.05$) (Figure 16C). Comparing the genotypes of the FLAV-fed mice, there is a positive correlation between the metabolome and the genera *Aldercreutzia*, *[Ruminococcus]*, *Bacteroides*, *Dorea*, and *Dehalobacterium*. *Aldercreutzia* and *[Ruminococcus]* have significant negative correlations with glucose (*Aldercreutzia* $p < 0.05$, *[Ruminococcus]* $p < 0.01$) and hypoxanthine (both genera $p < 0.05$). *Bacteroides* had a significant positive association with pyruvate, glucose (both $p < 0.05$), and hypoxanthine ($p < 0.01$). *Desulfovibrio* exhibits a significant positive association with pyruvate ($p < 0.05$). *Dorea* exhibited no significant correlations; however, the patterns of this microbe

followed those of *Bacteroides* and *Dehalobacterium* (Figure 16D). There were no other significant correlations between microbial genera and metabolites in any of the other groups ($p > 0.05$).

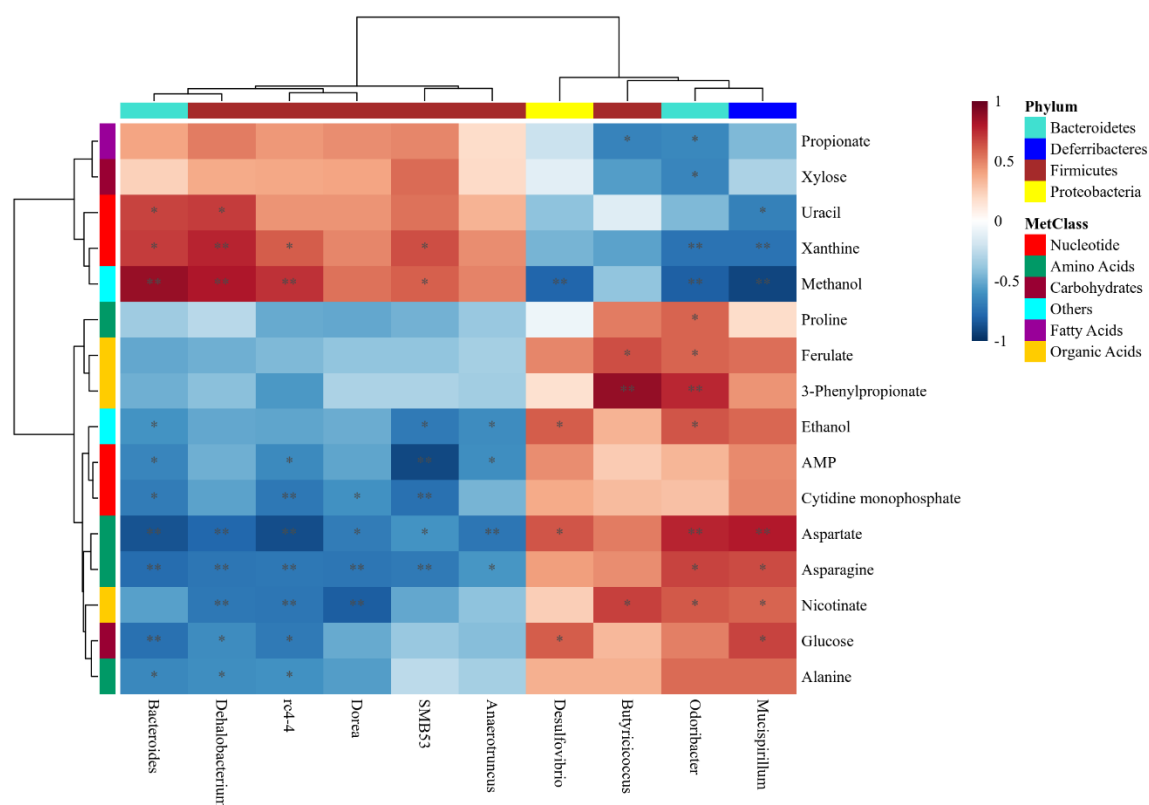
A



B



C



D

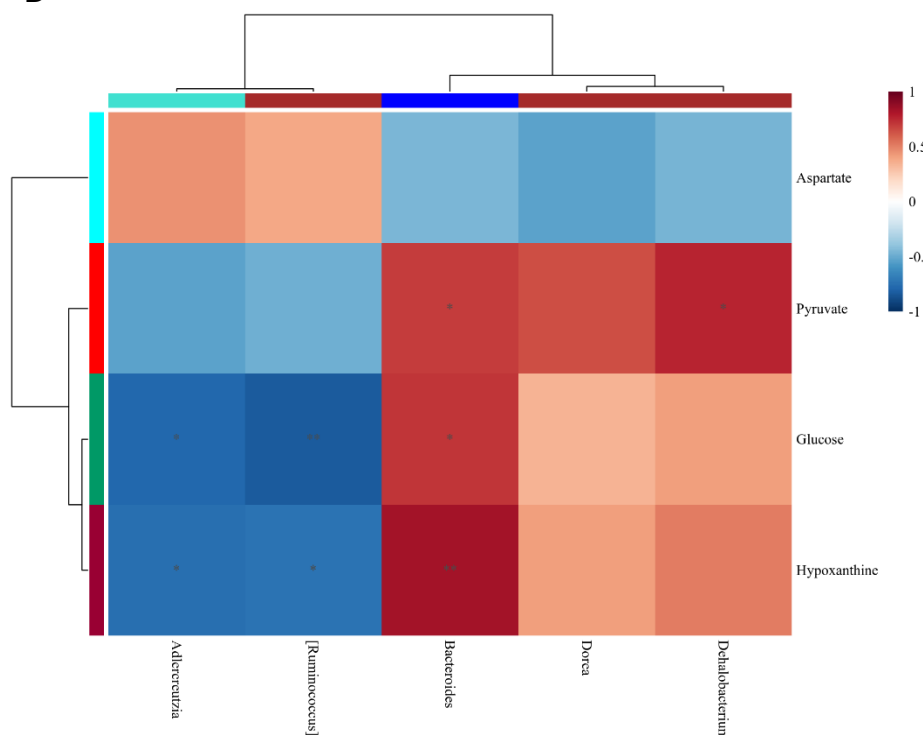


Figure 5.16: Figure 16: Significant metabolome and microbiota correlations in mice of the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Mice were split by the APOE genotype (APOE3/3-TR and APOE4/4-TR) and then diet: Low fat (10 kcal% from fat; LF), High fat (45 kcal% from fat; HF), High fat (45kcal% from fat) supplemented with 6% flavan-3-ol-rich cocoa (FLAV). A) Correlations in the APOE3/3-TR mice on the HF and FLAV diets. B) Correlations in APOE4/4-TR mice fed the LF and HF diets. C) Correlations between APOE3/3-TR and APOE4/4-TR mice fed the LF diet. D) Correlations between the APOE3/3-TR and APOE4/4-TR mice fed the FLAV diet. Group sizes: E3 LF n = 6, E3 HF n = 4, E3 FLAV n = 6, E4 LF n = 6, E4 HF n = 3, E4 FLAV n = 3

5.3.2. Investigating Age, Sex, and *APOE* genotype influence on the metabolism of a flavan-3-ol-rich meal: Results from the COB study

5.3.2.1. *Participant Characteristics*

From a cohort of 273 participants, 163 completed the 4-day study protocol. Assigning the *APOE* genotype data to the participants and removing those carrying the *APOE2/4* genotype produced a total of 157 participants. These participants were subsequently split by age, sex, and *APOE4* carrier status. Of the 74 women analysed from the study, 44 were from the older age group (14 *APOE4* carriers and 30 *APOE4* non-carriers), and 30 were from the younger age group (8 *APOE4* Carriers and 22 *APOE4* non-carriers). Of the 83 males analysed from the study, 43 were from the older age group (11 *APOE4* carriers and 32 *APOE4* non-carriers) and 40 were from the younger age group (10 *APOE4* carriers and 30 *APOE4* non-carriers) (Figure 5.17).

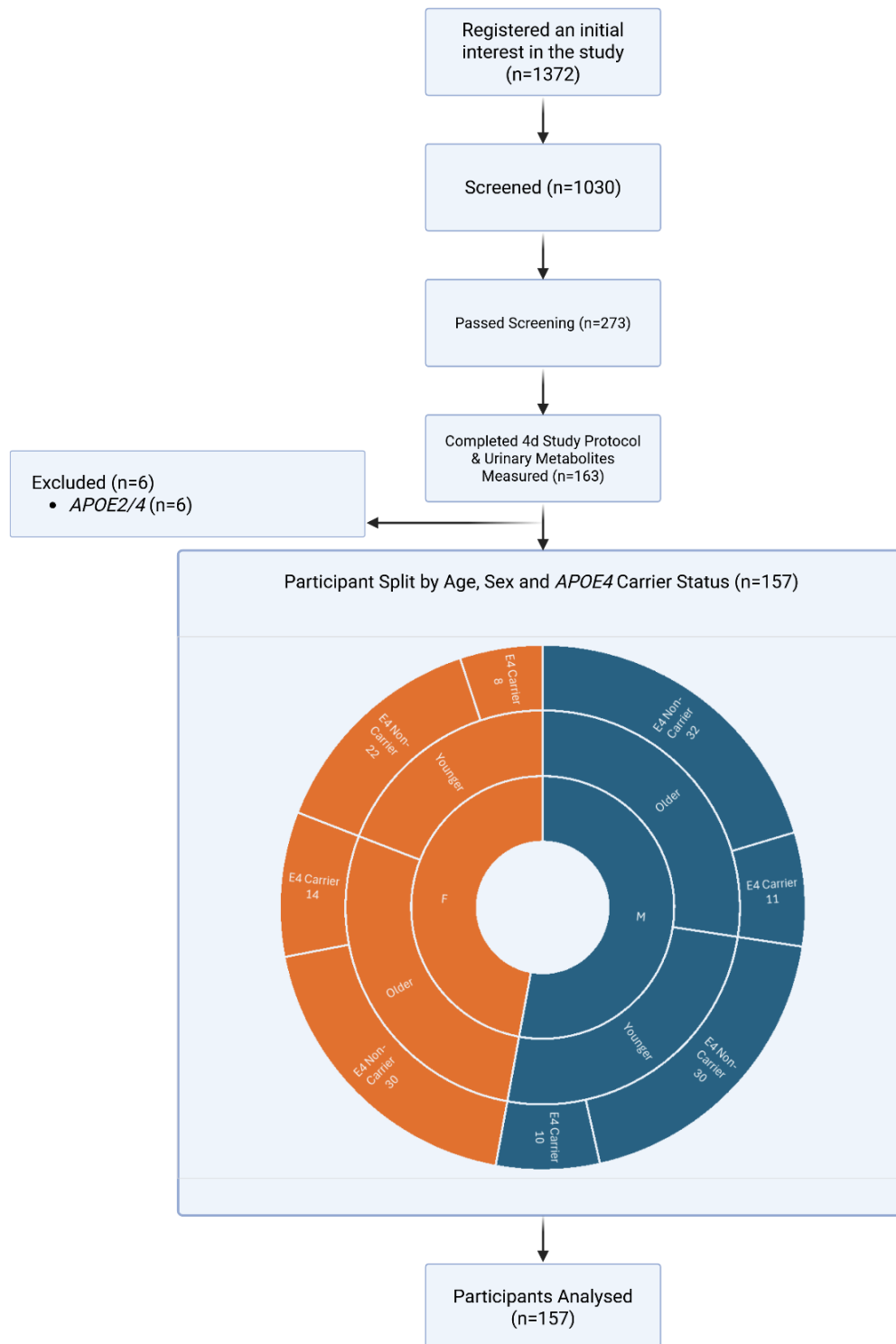


Figure 5.17: Breakdown of the Cocoa, Orange and Blackberry (COB) study. Participant recruitment and the numbers of each sex, age, and APOE4 carrier status of colon model participants. APOE2/3 and APOE3/3 (E4 Non-Carrier), APOE3/4 and APOE4/4 (E4 Carrier). The APOE2/4 genotype was excluded from the study due to the conflicting nature of carrying both the protective E2 allele, which decreases dementia risk and the E4 allele, which increases dementia risk. Figure is adapted from Kay, C.D., et al., Effect of age and sex on the urinary elimination of a single dose of mixed flavonoids: results from a single-arm intervention in healthy United Kingdom adults. *The American Journal of Clinical Nutrition*, 2025. 122(1): p. 101-111

5.3.2.2. HPLC measurement of urinary flavan-3-ol metabolites.

Analysis of urinary samples from the COB study demonstrated significant differences in flavan-3-ols and their metabolites across genotype, sex, and age. Catechin concentrations differed significantly between genotypes ($p = 0.042$), while EC showed a sex-related difference ($p = 0.037$). Several gut-derived valerolactones were also affected. For 4-VL, significant effects were observed for sex ($p = 0.049$), genotype and age ($p = 0.015$), genotype*sex ($p = 0.033$), sex*age ($p = 0.017$) interactions, and a three-way interaction genotype*sex*age ($p = 0.023$). 5-HPVL exhibited a strong sex*age interaction ($p < 0.001$) and a three-way interaction (genotype*sex*age; $p = 0.004$). Its sulfate conjugate, 5-HPVL-O-S showed significant genotype*age interaction ($p = 0.015$) (Table 5.4).

Pairwise comparisons revealed specific group differences among these metabolites. For 4-VL, *APOE4* females excreted significantly higher urinary concentrations than older non-*APOE4* females (FDR $q < 0.05$). Among non-*APOE4* females, younger participants had significantly higher urinary 4-VL concentrations than older counterparts (FDR $q < 0.05$). For 5-HPVL, older *APOE4* males excreted more than older non-*APOE4* males (FDR $q < 0.05$; Figure 5.18A). Sex-related differences were evident with older *APOE4* females having significantly higher concentrations than males (FDR $q < 0.01$) and younger non-*APOE4* females exceeding younger non-*APOE4* males (FDR $q < 0.05$). Age-related differences between the *APOE4* females were also observed, with younger *APOE4* females having significantly higher concentrations than older females (FDR $q < 0.05$), whereas *APOE4* males showed a reverse pattern, with higher urinary concentrations in older participants compared with younger males (FDR $q < 0.05$; Figure 5.18B).

For 5-HPVL-O-S genotype-specific differences were observed in males. Older *APOE4* males had significantly higher urinary concentrations than the non-*APOE4* carriers (FDR $q < 0.05$), whereas younger non-*APOE4* males excreted more than the *APOE4* carriers (FDR $q < 0.05$). Age effects were also evident, with older *APOE4* males having higher concentrations than younger *APOE4* males (FDR $q < 0.05$), while the opposite trend occurred among non-*APOE4* male carriers, with younger male participants excreting more than older participants (FDR $q < 0.05$; Figure 5.18C).

Table 5.4: Change from baseline in the metabolism of dietary flavan-3-ols and their associated metabolites present in the urine of participants of the chocolate orange and blackberry (COB) study. Participants were split by their sex, Age (18-35years old – younger (Y) and 65-77 years old – older (O)), and their APOE4 Carrier (Gen): genotypes APOE2/2, APOE2/3 and APOE3/3 (nonE4), APOE3/4 and APOE4/4 (E4)). Values in table are the mean value for each group as well as the standard deviation. Significant 3-way ANOVA ($p < 0.05$) values are highlighted in bold. Compounds listed are as follows: Epicatechin (EC), Catechin, 4-valerolactone (4-VL), 5-(3',4'-Dihydroxyphenyl)- γ -valerolactone (3,4-DiHPVL), 5-(hydroxyphenyl)- γ -valerolactone (3'&4') (HPVL), 5-(Hydroxyphenyl)- γ -valerolactone-O-sulfate (HPVL-O-S). Group sizes: F Y E4 $n = 8$, F Y non-E4 $n = 22$, F O E4 $n = 14$, F O non-E4 $n = 30$, M Y E4 $n = 10$, M Y non-E4 $n = 30$, M O E4 $n = 11$, M O non-E4 $n = 32$

Sex	F				M				3-way PERM ANOVA p-values						
Age	O		Y		O		Y								
Gen	E4	NonE4	E4	Non E4	E4	Non E4	E4	Non E4							
Compound	$\bar{x}$ μ M/mol	$\bar{x}$ μ M/mol	$\bar{x}$ μ M/mol	$\bar{x}$ μ M/mol	$\bar{x}$ μ M/mol	$\bar{x}$ μ M/mol	$\bar{x}$ μ M/mol	$\bar{x}$ μ M/mol	Gen	Sex	Age	Gen: Sex	Gen: Age	Sex: Age	Gen: Sex: Age
	Creatine (SD)	Creatine (SD)	Creatine (SD)	Creatine (SD)	Creatine (SD)	Creatine (SD)	Creatine (SD)	Creatine (SD)							
EC	115.795 (179.923)	51.290 (62.202)	79.241 (83.330)	60.468 (76.157)	53.297 (41.785)	40.151 (38.867)	32.750 (36.750)	55.845 (65.670)	0.223	0.037	0.585	0.111	0.169	0.702	0.871
Catechin	7.842 (24.755)	64.549 (106.627)	16.286 (21.999)	109.857 (212.512)	34.089 (57.340)	131.145 (191.043)	30.825 (32.481)	31.072 (53.000)	0.042	0.813	0.688	0.658	0.622	0.196	0.273
4-VL	262.159 (384.739)	4.611 (27.137)	29.541 (61.978)	53.222 (98.229)	1.657 (32.751)	7.118 (27.897)	45.408 (301.499)	60.955 (134.002)	0.072	0.049	0.476	0.033	0.015	0.017	0.023
3,4-DiHPVL	5062.191 (3953.829)	4654.683 (3714.717)	4291.998 (2005.937)	4495.175 (2971.020)	1908.047 (1830.535)	4469.447 (3504.537)	4704.488 (3480.565)	3627.515 (1738.381)	0.596	0.112	0.663	0.469	0.210	0.222	0.076
5-HPVL	154.320 (185.144)	494.209 (586.007)	1348.008 (1777.952)	658.899 (781.747)	761.431 (659.490)	347.517 (464.006)	185.544 (162.664)	262.210 (221.078)	0.202	0.041	0.186	0.982	0.313	<0.001	0.004
5-HPVL-O-S	4651.524 (4322.701)	4913.173 (4587.782)	2662.563 (1854.219)	4439.816 (3976.816)	4533.085 (3870.024)	2195.106 (2058.102)	1424.818 (1285.802)	4373.316 (3741.058)	0.331	0.129	0.208	0.596	0.015	0.576	0.165

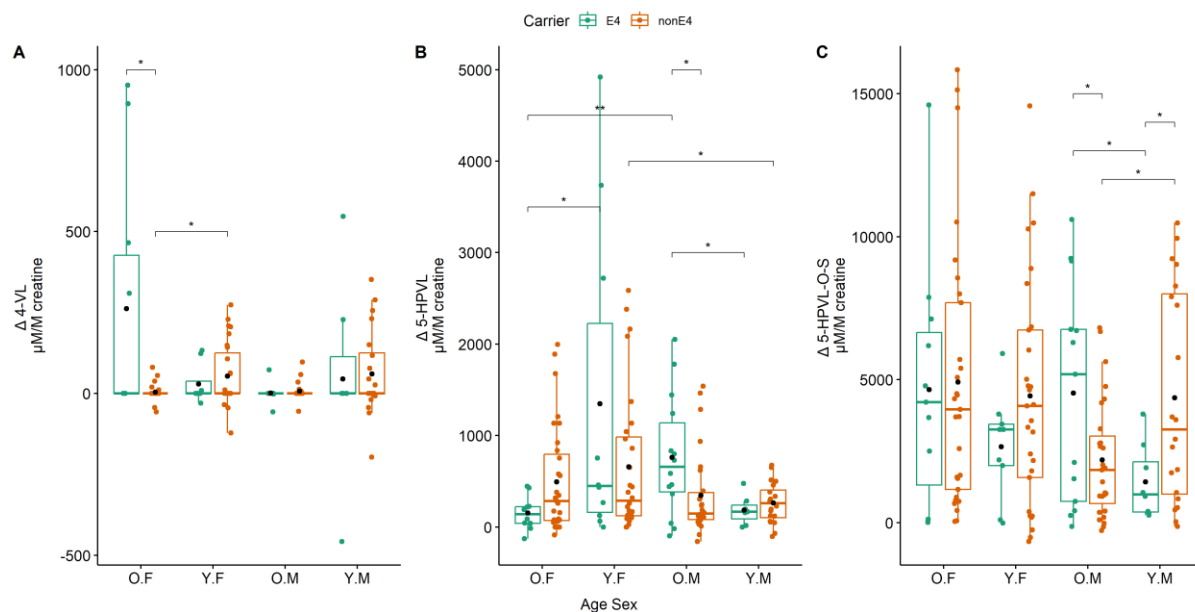


Figure 5.18: Significant (3-way permutational ANOVA p -value $< .05$) difference in total metabolism of A) 4-valerolactone (4-VL) B) 5-(hydroxyphenyl)- γ -valerolactone (5-HPVL) C) 5-(hydroxyphenyl)- γ -valerolactone-O-sulfate (5-HPVL-O-S) between the baseline concentration and total concentration 24hrs following the consumption of the flavan-3-ol rich meal as part of the Chocolate, Orange Juice and Blackberry (COB) Study. Participants were separated by sex, age (Younger (Y) 18-35 years old and Older (O) 65-77 years old), and their APOE4 Carrier status: genotypes APOE2/2, APOE2/3 and APOE3/3 (nonE4), APOE3/4 and APOE4/4 (E4). The mean value of each group is plotted in black. FDR q -values: ** $0.01 > q\text{-value} \geq 0.001$, *** $0.0001 > q\text{-value}$. Group sizes: F Y E4 $n = 8$, F Y non-E4 $n = 22$, F O E4 $n = 14$, F O non-E4 $n = 30$, M Y E4 $n = 10$, M Y non-E4 $n = 30$, M O E4 $n = 11$, M O non-E4 $n = 32$

5.3.3. Investigating Age, Sex, and *APOE* genotype influence on the metabolism of a flavan-3-ol-rich meals: Results from the OPCT Study

5.3.3.1. *Participant Characteristics*

Of a total of 300 participants, 297 participants' samples were measured. Applying the inclusion and exclusion criteria, which mirrored the previous COB study in this thesis (e.g. non-smokers and age groups (18-35 and 55+)), 120 participants were deemed suitable for analysis. These participants were then split by age groups and *APOE4* carrier status (Figure 5.19). 109 females took part in the study, which were split into 46 older (10 *APOE4* carriers, 36 non-*APOE4* carriers) and 63 younger (10 *APOE4* carriers and 53 non-*APOE4* carriers). 68 males took part in the study, consisting of 24 older (2 *APOE4* carriers and 22 non-*APOE4* carriers) and 44 younger (11 *APOE4* carriers and 33 non-*APOE4* carriers). Due to the exploratory nature of this pilot study, even though the number of participants in the older male *APOE4* were less than 3, all groups were maintained in the analysis of the samples.

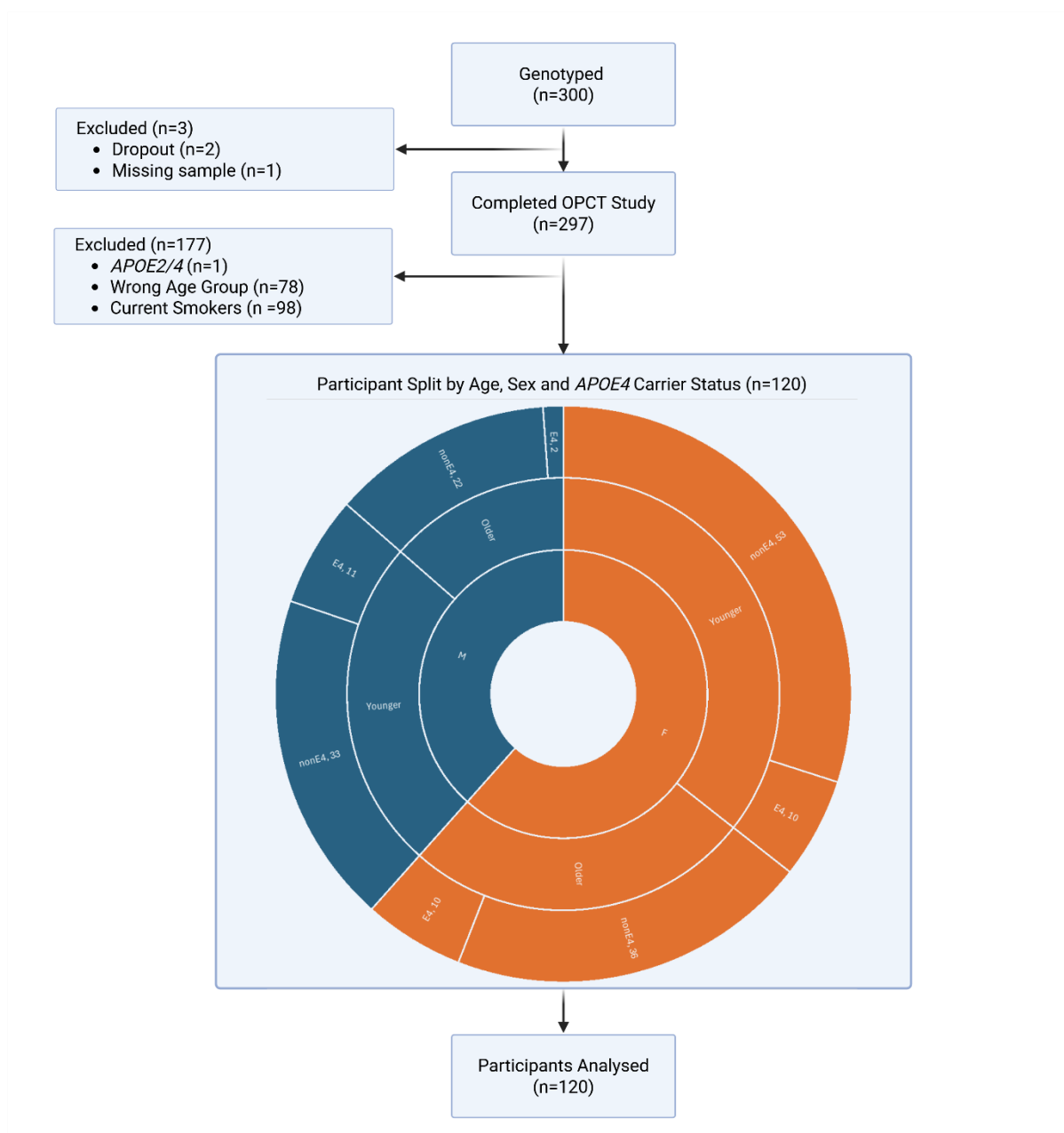


Figure 5.19: Breakdown of the Oral (Poly)phenol Challenge Test (OPCT) study. Participant recruitment and the numbers of each sex, age, and APOE4 carrier status of colon model participants. APOE2/3 and APOE3/3 (E4 Non-Carrier), APOE3/4 and APOE4/4 (E4 Carrier). The APOE2/4 genotype was excluded from the study due to the conflicting natures of carrying both the protective E2 allele, which decreases dementia risk and the E4 allele, which increases dementia risk.

5.3.3.2. HPLC measurements of urinary flavan-3-ol metabolites

Results from the OPCT study indicate that multiple factors influence the metabolism of a (poly)phenol-rich dietary supplement. Among the 34 compounds analysed, 12 exhibited significant differences in metabolic outcomes. Metabolites generated in the upper GI tract prior to exposure to the intestinal microbiota were notably affected by participants' characteristics. Specifically, the upper gut-derived EC-S-GluAC III was

significantly modulated by age ($p = 0.035$), while EC-GluAC was modulated by sex ($p = 0.025$). All gut microbiota-derived metabolites including 3,4,5-TriHPVL, 3,5-DiHPVL-4-GluAC, 3-HPVL-4-GluAC, 3-MPVL-4-GluAC, 5-HPVL-3-GluAC, 5-MPVL-GluAC, 4-H-3-HPVA-4-GluAC, 4-H-4-HPVA-3-GluAC, 4-H-5-HPVA-3-GluAC, 4-H-MPVA-GluAC (3,4), 4-H-PVA-4-GluAC were all significantly influenced by sex (all $p < 0.05$). In addition, 5-MPVL-GluAC demonstrated a significant association with age ($p = 0.007$) (Table 5.5). All other compounds analysed failed to reach significance (all $p > 0.05$), see Supplementary Table 13.

Pairwise permutational t-tests reveal that the sex and age effects are limited to specific groups and compound-specific. Age effects were restricted to non-*APOE4* carriers: EC-S-GluAC III and 5-MPVL-GluAC exhibited higher concentrations in older participants of both sexes (EC-S-GluAC III all FDR $q < 0.01$ and 5-MPVL-GluAC FDR $q < 0.001$) (Figure 5.20A&H). Sex differences were most evident in the younger groups, with females consistently producing higher concentrations than their male counterparts. This pattern was observed in EC-3-GluAC in younger non-*APOE4* carriers (FDR $q < 0.01$) (Figure 5.20B), 3,5-DiHPVL-4-GluAC in younger *APOE4* carriers (FDR $q < 0.05$) (Figure 5.20D), and for several valerolactones glucuronides including 3-HPVL-4-GluAC (non-*APOE4* FDR $q < 0.01$ and *APOE4* FDR $q < 0.05$) (Figure 5.20E), 3-HMVL-4-GluAC (both carrier groups FDR $q < 0.05$) (Figure 5.20F), 5-HMVL-3-GluAC (younger non-*APOE4* carriers FDR $q < 0.05$) (Figure 5.20G), and 4-H-3-HPVA-4-GluAC (*APOE4* carrier FDR $q < 0.01$, non-*APOE4* carriers FDR $q < 0.05$) (Figure 5.20I). The same trends are also demonstrated for 4-H-4-HPVA-3-GluAC, 4-H-5-HPVA-3-GluAC and 4-H-MPVA-GluAC (3,4) (all FDR $q < 0.05$), which all exhibit sex differences in the younger non-*APOE4* carriers (Figure 5.20J-L). Among these, 5-MPVL-GluAC was the only metabolite to exhibit both age and sex effects: concentrations were higher in older non-E4 participants and in females across multiple subgroups (younger E4 FDR $q < 0.05$, older non-E4 FDR $q < 0.05$, younger non-E4 FDR $q < 0.001$) (Figure 5.20H). Despite highlighting a significant permutational ANOVA score, 3,4,5-TriHPVL did not yield any significant pairwise interactions (Figure 5.20C).

Table 5.5: Significant urinary flavan-3-ol metabolites of the oral (poly)phenol challenge test (OPCT). Participants were split by age: younger (Y) 18-35 years old and older (O) 55+ years old. Sex and APOE4 Genotype (Gen) E4: APOE3/4 and APOE4/4 and Non E4: APOE2/2, APOE2/3, and APOE3/3. Compounds measured were (-)-Epicatechin-sulfate-glucuronide III (EC-S-GluAC III), Epicatechin-glucuronide (EC-GluAC), 5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone (3,4,5-TriHPVL), 5-(3',5'-Dihydroxyphenyl)-γ-valerolactone-4'-glucuronide (3,5-DiHPVL), 5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3HPVL-4-GluAC), 5-(3'-Methoxyphenyl)-γ-valerolactone-4'-glucuronide (3-MPVL-4-GluAC), 5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (5-HPVL-3-GluAC), 5-(Methoxyphenyl)-γ-valerolactone-glucuronide (5-MPVL-GluAC), 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid-4'-glucuronide (4-H-3HPVA-4-GluAC), 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-4HPVA-3-GluAC), 4-Hydroxy-5-(5'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-5HPVA-3-GluAC), 4-Hydroxy-5-(methoxyphenyl)valeric acid-glucuronide (3',4') (4-H-MPVA-GluAC), 4-Hydroxy-5-(phenyl)valeric acid-4'-glucuronide (4-H-PVA-4-GluAC). Significant 3-way PERMUTATIONAL ANOVA ($p < 0.05$) values are highlighted in bold. Group sizes: M O E4 $n = 2$, M O non-E4 $n = 22$, M Y E4 $n = 11$, M Y non-E4 $n = 33$, F O E4 $n = 10$, F O non-E4 $n = 36$, F Y E4 $n = 10$, F Y non-E4 $n = 53$

Sex Age Gen	F				M				3-way PERMUTATIONAL ANOVA p-values						
	O	Y	O	Y	O	Y	O	Y	Gen	Sex	Age	Gen: Sex	Gen: Ag	Sex: Age	Gen: Sex: Age
Compound	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)	Gen	Sex	Age	Gen: Sex	Gen: Ag	Sex: Age	Gen: Sex: Age
EC-S-GluAC III	0.021 (0.016)	0.026 (0.042)	0.023 (0.016)	0.011 (0.015)	0.027 (0.038)	0.024 (0.023)	0.010 (0.011)	0.009 (0.011)	0.608	0.595	0.035	0.862	0.437	0.366	0.346
EC-GluAC	0.015 (0.011)	0.021 (0.023)	0.011 (0.009)	0.011 (0.015)	0.000 (0.000)	0.013 (0.010)	0.006 (0.010)	0.004 (0.004)	0.184	0.025	0.178	0.706	0.102	0.391	0.508
3,4,5-TriHPVL	0.130 (0.261)	0.090 (0.121)	0.056 (0.102)	0.043 (0.069)	0.020 (0.016)	0.040 (0.062)	0.019 (0.047)	0.027 (0.042)	0.769	0.029	0.119	0.338	0.861	0.217	0.631
3,5-DiHPVL-4-GluAC	1.288 (1.525)	1.374 (1.680)	1.056 (0.885)	0.996 (1.380)	0.721 (0.934)	0.656 (0.959)	0.298 (0.550)	0.531 (0.800)	0.870	0.041	0.321	0.904	0.896	0.958	0.701
3-HPVL-4-GluAC	0.249 (0.134)	0.332 (0.244)	0.350 (0.213)	0.289 (0.259)	0.205 (0.216)	0.226 (0.160)	0.162 (0.163)	0.148 (0.146)	0.886	0.022	0.752	0.937	0.373	0.362	0.578
3-MPVL-4-GluAC	0.224 (0.111)	0.240 (0.134)	0.219 (0.123)	0.168 (0.112)	0.191 (0.173)	0.181 (0.119)	0.112 (0.066)	0.113 (0.073)	0.677	0.018	0.036	0.815	0.594	0.504	0.446
5-HPVL-3-GluAC	1.284 (0.712)	1.423 (1.097)	1.216 (0.669)	1.168 (0.832)	0.753 (0.539)	1.175 (0.820)	0.748 (0.663)	0.720 (0.551)	0.533	0.036	0.323	0.698	0.425	0.861	0.742
5-MPVL-GluAC	0.020 (0.010)	0.018 (0.009)	0.014 (0.008)	0.013 (0.008)	0.010 (0.007)	0.013 (0.007)	0.007 (0.005)	0.007 (0.004)	0.877	0.001	0.007	0.420	0.813	0.905	0.676
4-H-3HPVA-4-GluAC	0.043 (0.022)	0.054 (0.038)	0.048 (0.037)	0.038 (0.039)	0.024 (0.029)	0.038 (0.026)	0.019 (0.021)	0.023 (0.023)	0.539	0.013	0.314	0.589	0.335	0.794	0.720
4-H-4HPVA-3-GluAC	0.068 (0.037)	0.066 (0.038)	0.052 (0.031)	0.049 (0.045)	0.026 (0.025)	0.045 (0.027)	0.023 (0.024)	0.027 (0.023)	0.572	0.002	0.114	0.375	0.596	0.729	0.681
4-H-5HPVA-3-GluAC	0.057 (0.041)	0.061 (0.063)	0.049 (0.033)	0.049 (0.041)	0.026 (0.015)	0.049 (0.036)	0.028 (0.029)	0.029 (0.028)	0.493	0.043	0.339	0.611	0.535	0.949	0.676
4-H-MPVA-GluAC (3',4)	0.122 (0.065)	0.125 (0.082)	0.106 (0.096)	0.088 (0.066)	0.099 (0.078)	0.105 (0.071)	0.049 (0.042)	0.056 (0.042)	0.973	0.042	0.021	0.678	0.746	0.478	0.734

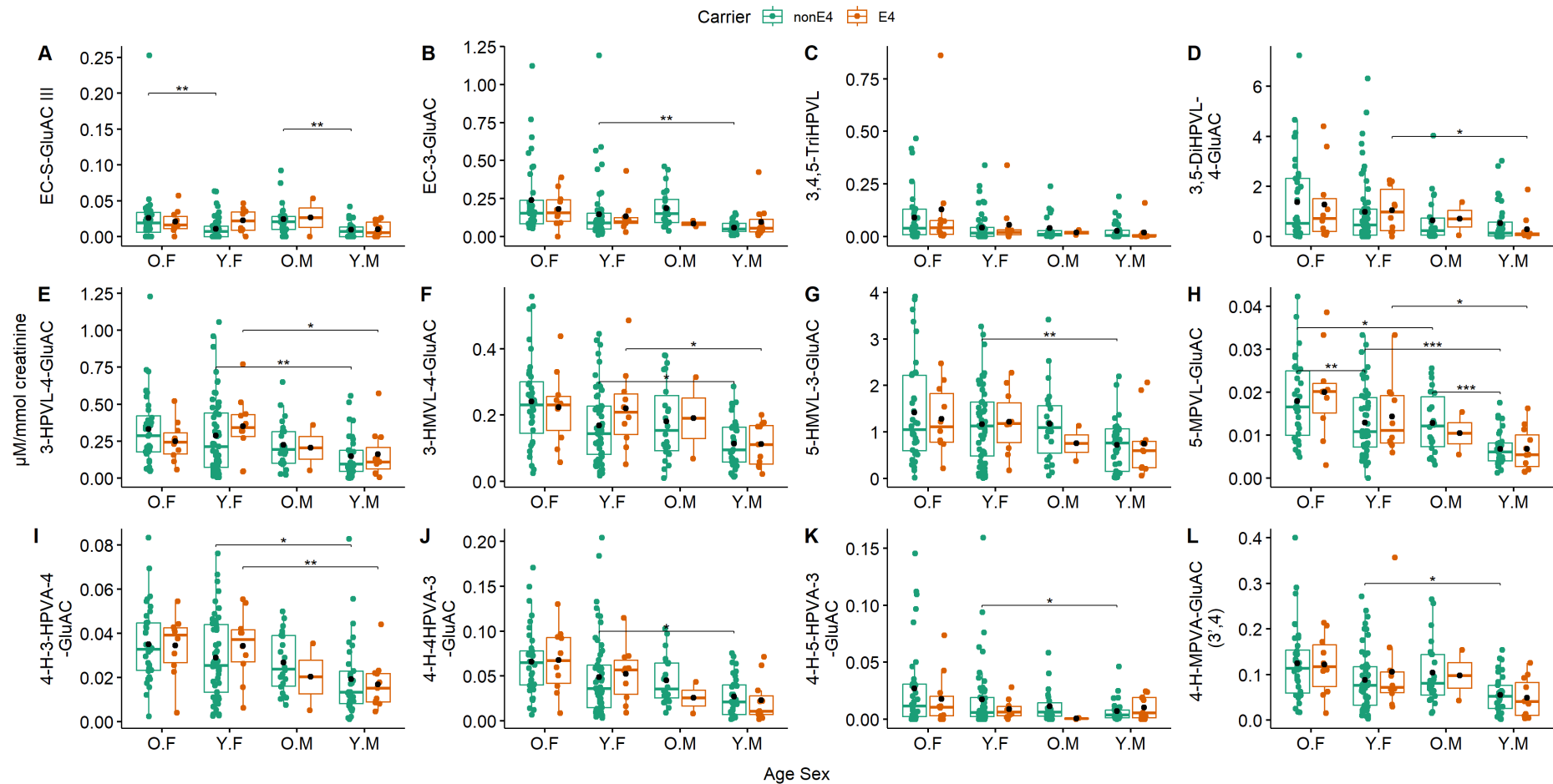


Figure 5.20: Pairwise analysis of the significant ($p < 0.05$) compounds in the Oral (Poly)phenol Challenge Test (OPCT) following 3-way permutational ANOVA. Participants are split by their age: Younger (Y) 18-35 years old and Older (O) 55+ years old. Sex: male (M) and female (F), and APOE4 carrier status: non E4 (APOE2/2, APOE2/3, APOE3/3) and E4 (APOE3/4 and APOE4/4). Significant pairwise values from permutational t-test and Benjamini-Hochberg adjustment are highlighted as follows. * $0.05 > q\text{-value} \geq 0.01$, ** $0.01 > q\text{-value} \geq 0.001$, *** $q\text{-value} \leq 0.0001$. A) (-)-Epicatechin-sulfate-glucuronide III (EC-S-GluAC III), B) Epicatechin-glucuronide (EC-GluAC), C) 5-(3',4',5'-Trihydroxyphenyl)- γ -valerolactone (3,4,5-TriHPVL), D) 5-(3',5'-Dihydroxyphenyl)- γ -valerolactone-4'-glucuronide (3,5-DiHPVL), E) 5-(3'-Hydroxyphenyl)- γ -valerolactone-4'-glucuronide (3HPVL-4-GluAC), F) 5-(3'-Methoxyphenyl)- γ -valerolactone-4'-glucuronide (3-MPVL-4-GluAC), G) 5-(5'-Hydroxyphenyl)- γ -valerolactone-3'-glucuronide (HPVL-3-GluAC), H) 5-(Methoxyphenyl)- γ -valerolactone-glucuronide (5-MPVL-GluAC), I) 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid-4'-glucuronide (4-H-3HPVA-4-GluAC), J) 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-4HPVA-3-GluAC), K) 4-Hydroxy-5-(5'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-5HPVA-3-GluAC), L) 4-Hydroxy-5-(methoxyphenyl)valeric acid-glucuronide (3',4') (4-H-MPVA-GluAC). Group sizes: M O E4 n = 2, M O non-E4 n = 22, M Y E4 n = 11, M Y non-E4 n = 33, F O E4 n = 10, F O non-E4 n = 36, F Y E4 n = 10, F Y non-E4 n = 53

5.4. Discussion

This chapter investigated how *APOE* genotype, sex, and age influence the metabolism of flavan-3-ols derived from flavonoid-rich meals, revealing pronounced genotype- and diet-specific effects on both microbial composition and metabolite production.

5.4.1. Preclinical Findings: Microbiota and Metabolite Profiles

In the preclinical CANN-AN study, urinary concentrations of gut microbiota-derived flavan-3-ol metabolites, notably valerolactones and their downstream products such as valeric acids, varied significantly between groups, whereas no genotype effect was observed for flavan-3-ols absorbed in the upper GI tract. *APOE4/4*-TR mice consistently excreted higher levels of these conjugates than *APOE3/3*-TR mice, and this effect was dose dependent, despite the HF and LF control diets being matched to the FLAV diet by the addition of low-flavanol royal cocoa. This highlights the dependence of valerolactone formation on colonic microbiota, which is mediated by both C-ring cleavage and subsequent dihydroxylation [178].

Microbiota profiling revealed marked differences in both community structure and individual taxa driven by *APOE* genotype and diet. Within each diet, beta-diversity analyses separated *APOE3/3*-TR and *APOE4/4*-TR mice along the vertical axis, consistent with previous reports in *APOE*-TR models [102]. Differential abundance testing identified significantly higher *Bacteroides* in *APOE3/3*-TR HF and FLAV groups compared with *APOE4/4*-TR equivalents, whereas *Ruminococcus* was enriched in *APOE4/4*-TR mice across HF and FLAV diets. Similar patterns were observed for *Adlercreutzia* (FLAV diet only) and *Mucispirillum* (LF and HF diets). These taxa have been previously linked to *APOE* genotype [104, 109], although *Ruminococcus* showed an inverse association in previously published studies, with greater abundance in *APOE3/3*-TR animals [103]. Maldonado et al. reported such an effect in EFAD mice (a cross between *APOE*-TR and familial AD models), suggesting that FAD genes may also influence microbiota structure, consistent with known microbiota differences in AD patients [121].

Dietary interventions reshaped the microbiota within genotypes. In *APOE3/3*-TR mice, LF and HF diets produced distinct beta-diversity clusters and genus-level differences.

Blautia and *Defluviitalea* were more abundant in LF-fed animals, whereas *Ruminococcus* and *SMB53* were enriched in HF-fed animals. *Blautia* has mixed associations with HF diets [363], while *Defluviitalea* has no published dietary links. *Ruminococcus* is often associated with lower dietary fat [364], although some species of *Ruminococci* were linked to HF diets. On the other hand, the FLAV diet occupied an intermediate position, aligning with the evidence that EC supplementation maintains gut permeability and counteracts HF diet-induced dysbiosis [365]. This may be the mechanism by which the stability of the microbiota is maintained in these mice.

Whilst there were differences observed between the dietary groups of the *APOE3/3-TR* mice, the *APOE4/4-TR* animals' beta diversity remained unaffected by diet, indicating a greater similarity between these groups than between each of the diet groups in the *APOE3/3-TR* mice. This contrasts with the prevailing view that diet exerts a stronger influence on microbiota structure than the host genotype [366], suggesting that the *APOE4* allele may buffer against dietary interventions, including changes in dietary fat and flavan-3-ol content. This could be of interest in the development of personalised nutritional strategies in the future, and further work would be required to identify whether these patterns replicate in clinical settings. Within the *APOE4/4* genotype, only *Aldercreutzia* differed significantly between HF and FLAV diets, consistent with its reported association with flavonoid ring cleavage and metabolism [187, 367]. This may indicate that the flavan-3-ols in the diet are acting as potential prebiotics to individual genera, but significant differences in one microbial genus are not significant enough to cause overall structural changes to the β -diversity metrics.

5.4.2. Metabolic signatures

Metabolomic profiles mirrored microbial trends. In *APOE3/3-TR* mice, colonic metabolomes were more sensitive to dietary manipulation, with significant differences observed between the HF and FLAV groups, driven by the SCFA valerate and branched SCFA 2-methylbutyrate, both linked to immune modulation [368, 369]. Unexpectedly, these SCFAs were higher in the HF-fed than the FLAV-fed animals, suggesting that flavan-3-ols may suppress SCFA production. Correlations with the microbiota reveal that this

may be modulated by the lack of the genus [*Ruminococcus*]; several species of this genus have been linked to SCFA production [370]. By contrast, *APOE4/4*-TR mice exhibited metabolomic changes primarily in response to dietary fat, consistent with altered lipid transport in this genotype.

When genotypes were compared under LF feeding, propionate levels were significantly higher in *APOE3/3*-TR than in *APOE4/4*-TR mice. Propionate is known to support colonic health, modulate intestinal immunity, and help maintain gut–brain axis integrity [371, 372]. Several microbial genera, specifically *Mucisprillum*, *Odorbacter*, and *Butyricoccus*, were negatively correlated with propionate, uracil, and xanthine, and positively correlated with glucose and the organic acid nicotinate. These genera were more abundant in *APOE4/4*-TR animals. Certain *Mucispirillum* species are associated with large-bowel inflammation [373], suggesting a possible link to *APOE4*-related disease risk. However, only genus-level associations could be assessed, and further work would be required to identify species-level effects. These metabolite trends are also reversed for the genus *Bacteroides*, *Dehalobacterium*, *rc4-4*, *SMB53*, *Dorea*, and *Anaerotruncus*, which were elevated in the *APOE3/3*-TR mice, suggesting that potential genotype-dependent microbes may be required for this wider metabolome response and may induce the protective effect of SCFA in the *APOE3/3*-TR mice. Comparing the genotypes on the FLAV diets revealed a genotype-dependent split in the microbiota and metabolome. Many of the same microbes highlighted were also found in the differential abundance analyses. However, there were no significant differences between the metabolites. Further metabolomic work is necessary to identify whether there is an effect on these metabolites. Additionally, bile acid metabolism, known to depend on the microbiota and to be influenced by the *APOE* genotype [22, 36, 37, 40, 41, 374], was not captured by NMR profiling and warrants targeted investigation.

5.4.3. Translation to human studies

The clinical COB and OPCT trials extended these prior findings. In the COB study, permutational ANOVA revealed significant differences in change-from-baseline of urinary concentrations of both microbially derived and non-microbially derived

metabolites. Catechin showed sex-related differences, and epicatechin exhibited *APOE* genotype effects not observed in the CANN-AN mice. These discrepancies likely reflect differences in food matrix: the COB meal (dark chocolate plus fruit extract drink) differed in fat and sugar content from the cocoa powder used in CANN-AN, and food matrix is known to influence (poly)phenol absorption and GI metabolism [137]. Nevertheless, gut-derived valerolactones (4-VL, 5-HPVL, 5-HPVL-O-S) were consistently higher in *APOE4* carriers, in line with preclinical data [104]. Therefore, it may be possible that microbes in *APOE4* carriers are prepared to metabolise EC and to produce a greater concentration of metabolites. The 5-HPVL aglycone and its 5-HPVL-O-S conjugate exhibited sex and age differences between groups. Each pathway in the metabolism of EC to its subsequent valerolactones is known to be altered by specific microbes [181, 187, 188]. As each step is governed by different microbes, this could also explain why the patterns of results for 4-VL and 5-HPVL differ, and are affected by age, sex, and *APOE* genotype groups differently. Differences between the 5-HPVL aglycone and its sulfated conjugate indicate modulation by phase II metabolism, which is influenced by age, sex, and *APOE* genotype [288]. The role of *APOE* genotype and sex on interindividual phase II metabolism in the livers of mice was explored in Chapter 4. However, whether such an effect is also translated to humans is currently unclear, and future research is needed to identify whether an individual's age, sex, and *APOE* genotype alter the phase II conjugations of flavan-3-ol aglycones in humans.

In the OPCT trial, which included 16 polyphenol-rich foods, sex, and age were the primary determinants of urinary metabolite profiles, while *APOE* genotype had a lesser effect, with most differences observed in the *APOE4* non-carriers. This likely reflects the complexity of the dietary (poly)phenol mixture, which included monomeric flavan-3-ols (e.g., catechin, EC and their gallate derivatives from tea [375]) and polymeric proanthocyanidins (from sources such as blueberries and grape seeds [376]). Different (poly)phenols follow distinct microbial pathways, including aglycone formation and proanthocyanidin depolymerisation [66]. Competition for microbial enzymes or cross-inhibition between (poly)phenols may attenuate genotype-specific effects, including the removal of conjugates to produce the aglycones or the depolymerisation of the proanthocyanidins by the gut microbiota [253]. In addition, sex is known to alter the

biological handling of bioactive molecules, particularly in renal filtration [377]. Renal function is also decreased with age [378]. Such effects may have altered the urinary concentrations of both bacterially and non-bacterially derived metabolites. However, before any conclusion may be drawn, future work is needed to explore the extent to which these factors influence renal excretion of (poly)phenol metabolites.

The food matrix may also have had an important effect on these parameters. Previous literature has shown that the addition of sucrose and lipids in the processing of cocoa to chocolate alters the bioavailability and subsequent metabolism of catechin and EC [379]. In addition to the differences between cocoa and chocolate, there were also differences in the bioavailability of flavan-3-ols between tea and cocoa products [380]. These differences could lead to the microbes within the GI tract being exposed to different starting concentrations and thus affecting the metabolites produced.

Regional and methodological factors may also have contributed to these differences. The COB and the OPCT studies recruited cohorts from the United Kingdom and Italy, respectively. Although the age, sex, and *APOE* genotype are known to alter the microbiota, modifiable factors also play a significant role in shaping the microbiota [366]. A prime example of this could be the participants' diets. In the Mediterranean basin, the typical diet is known to be richer in plant-based foods, and subsequently in bioactive (poly)phenols [381]. The prebiotic effects of the (poly)phenols in this diet may have primed the microbiotas of one cohort, ready to metabolise subsequent dietary (poly)phenols [382]. This readiness to catabolise flavonoids could have masked minor variations in metabolism from other variables, such as the *APOE* genotype. Furthermore, the OPCT study had a relatively small washout window before the intervention; it can take up to 24 hours for (poly)phenols and their metabolites to leave the system [383], but the OPCT protocol only had an 18-hour washout. This could have meant that some metabolites from the participants' regular diet were still being metabolised and excreted when the tablets were taken and could subsequently influence the results of their metabolism. Thus, to avoid such artefacts, future studies should include a minimum 24-hour washout period before dietary interventions to prevent potential interactions.

Finally, differences observed may be due to the exploratory nature of both studies; the findings are pilot reanalyses of previously carried out studies. It is therefore possible that

some groups may be underpowered; in particular, the OPCT study, which included only 2 older male *APOE4* carriers. This could lead to discrepancies and incorrect assumptions when running statistical models on the data, particularly when examining multiple interactions in a single model. In addition to addressing the points previously made in this chapter, any future clinical study must conduct appropriate power calculations to ensure that each group is balanced to accommodate the number of interactions among the variables.

5.4.4. Limitations and future directions

Species-level resolution was restricted by 16S rRNA amplicon sequencing. Shotgun metagenomics or long-read sequencing would enable finer taxonomic and functional characterisation. Larger, adequately powered clinical cohorts are needed to examine interactions among diet, age, sex, and genotype. Inclusion of both sexes in preclinical models is essential, as sex differences in gut microbiota and flavonoid metabolism have been reported [89]. Targeted metabolomics focusing on SCFAs, bile acids, and other microbially mediated metabolites could clarify mechanistic links between microbial changes and host physiology.

Furthermore, it would also be beneficial to investigate the health outcomes, identifying, for example, whether cognition and behaviour, as well as the inflammatory responses, were affected. The previous chapter explored these intergroup differences with EC supplementation (Chapter 4). However, future work is needed to see whether the food matrix that encapsulates the EC influences any of this flavan-3-ol's health benefits.

5.5. Conclusion

Collectively, these findings demonstrate that *APOE* genotype modulates the metabolism of dietary flavan-3-ols, but this effect is strongly influenced by sex, age, and the food matrix. *APOE4* carriers exhibit higher urinary excretion of gut-derived flavan-3-ol metabolites, yet the magnitude of this effect varies with dietary complexity and (poly)phenol composition. Interactions among (poly)phenols, host factors, and microbial

communities can obscure genotype-specific responses in complex dietary settings. Future work using sex-balanced animal models, high-resolution sequencing, and targeted metabolomics will be critical to unravel the interplay between *APOE* genotype, gut microbiota, and polyphenol metabolism, and to inform personalised nutritional strategies.

Chapter 6: General Discussion

6.1. Introduction

AD is a complex, multifactorial disorder, with neuropathological changes occurring decades before the onset of clinical symptoms [384]. Several well-established, non-modifiable risk factors contribute to AD development, including age, sex, and *APOE* genotype [385]. Epidemiological and mechanistic studies confirmed that increased age, female sex, and carrying the *APOE4* allele are each associated with increased risk of developing the condition [2, 12, 67]. These factors act through multiple biological pathways, such as lipid transport modulation, immune system regulation, and sex hormone fluctuations, that influence both central and peripheral processes [43, 68, 73]. Their effects extend beyond direct brain pathology and exert systemic consequences, including alterations in gut microbial ecology. Indeed, mounting evidence suggests that age, sex, and *APOE* genotype can shape the composition and diversity of the microbiota, influencing not only community structure, but also the abundance of key taxa [82, 89, 104]. This is particularly relevant given the growing recognition of the microbiota within the GBA as a mediator of cognitive health [106].

In addition to these non-modifiable risk factors, modifiable lifestyle and dietary factors play a crucial role in neurological decline and resilience. Among them, diet, specifically flavonoid consumption, has received considerable attention. Flavonoids are a class of (poly)phenols that have been linked to improved cognitive function and reduced risk of neurocognitive decline, both in acute and chronic studies [126-128]. The mechanisms underlying the cognitive benefits of flavonoids are diverse, including the modulation of immune responses, enhancement of CBF, and improvement of synaptic plasticity [125]. Importantly, mammals lack the endogenous enzymes required for the metabolism of flavonoids, thus the majority of their catabolism occurs in the large intestine, where each step in the breakdown of the compound is modulated by individual taxa within the microbiota [181, 187, 188]. The resulting microbial metabolites are not only potential bioactive compounds influencing the GBA, but also may act as prebiotics, reshaping microbial communities in ways that they may sustain long-term benefits [191-193].

The interdependence between host factors (age, sex, and *APOE* genotype), diet (flavonoid intake) and the microbiota, underpins the central challenge of interindividual variability and potential health benefits of flavonoids. Since microbial composition shapes flavonoid metabolism, and host genetics and physiology shape microbial ecology, understanding how these elements interact is essential. This thesis sought to investigate these interactions using the well-studied flavan-3-ol, EC, as a model compound [177, 180, 181, 183]. Specifically, four interrelated questions were addressed: (1) To what extent do age, sex, and *APOE* genotype influence the colonic metabolism of EC? (2) Are differences in EC metabolism attributable to alterations in gut microbial composition? (3) Does the *APOE* genotype modulate the health outcomes associated with EC consumption? (4) Does *APOE* genotype alter the metabolism of flavonoid-rich foods, in a microbiota-dependant manner? These questions were examined through an approach combining *in vitro* human colon models, *APOE-TR* animal studies, and reanalysis of human clinical data. To facilitate synthesis of these findings, Table 6.1 summarises the principal outcomes across Chapters 2-5 and highlights the key genotype-, sex-, and age-dependent effects observed at each biological level.

Table 6.1 Summary of the key findings from each chapter of this thesis. Results answer the following Q1) To what extent do age, sex, and APOE genotype influence the colonic metabolism of dietary Epicatechin (EC)? Q2) Are the observed changes in EC metabolism attributable to differences in gut microbiota composition? Q3) Does APOE genotype modulate the health outcomes of dietary EC? Q4) Does APOE genotype alter the metabolism of flavonoid-rich foods, and is this effect microbiota-related? Study acronyms: Dietary Plant Extracts, Colonic Microbes, and APOE Genetics Interactions Study (DIAMOND), Cognitive Ageing Nutrition and Neurogenesis Animal study (CANN-AN), Chocolate Orange and Blackberry (COB), Oral (Poly)phenol Challenge Test (OPCT)

Study	Question	Key Findings	Translational Implication
EC Metabolism			
DIAMOND - colon model	Q1	EC degradation and 3,4-DiHPVL and 3-HPVL production are <i>APOE</i> dependent, also showing sex and age effects	Colonic EC metabolism is multifactorial; <i>APOE</i> acts with sex and age. Extent of the effect varies by metabolite observed and time.
<i>APOE</i>-TR model	Q1	Sulfated metabolites dominate circulation. PVL-3-S, PVA-3-S and non-microbially-derived metabolites altered by <i>APOE</i> and sex. Sulfated metabolites often present at higher concentrations in <i>APOE3/3</i> -TR mice, with sex-dependent interactions.	Host genotype and sex alter the systemic EC metabolite exposure.
Gut microbiota composition			
<i>APOE</i>-TR model	Q2	<i>APOE</i> and sex drive microbiome structure and taxa abundance. Tauroconjugated bile acid formation genotype linked.	<i>APOE</i> interacts with sex to shape the murine microbiota. EC supplementation less effective. Bile acids potential mechanism for mediation of microbiota.
DIAMOND - microbiota	Q2	High interindividual variability. No consistent age/sex/ <i>APOE</i> differences.	Human microbial variability obscures the effects of age, sex, and <i>APOE</i> genotype on human microbiota.
Hepatic phase II metabolism			
<i>APOE</i>-TR model	Q3	<i>Sult1d1</i> and <i>Sult1c1</i> were upregulated by EC. <i>APOE3/3</i> regulated <i>Sult1d1</i> transcription.	<i>APOE</i> influences hepatic sulfation; consistent with sulfated metabolite differences in <i>APOE</i> -TR mice.
Biometric, metabolic, & Inflammatory outcomes			
<i>APOE</i>-TR model	Q3	<i>APOE</i> -, sex-, and diet-dependent changes in weight gain, adiposity, energy metabolism, and inflammatory response.	The functional benefit of EC on metabolic and inflammatory outcomes is sex- and <i>APOE</i> -dependent and not uniformly beneficial.
Cognitive performance			
<i>APOE</i>-TR model	Q3	EC improves spatial working memory in <i>APOE4/4</i> males, detrimental to <i>APOE3/3</i> females.	The functional benefit of EC appears most consistent in the at-risk male <i>APOE4/4</i> subgroup; effects are context dependent.
Food matrix and human translation			
CANN-AN, COB, & OPCT	Q4	Mouse model clear genotype effect on colonic metabolites and microbiota. Increased variability in human cohorts with weaker genotype effect signals.	Food matrices and background diet variability attenuate and obscure the genotype-dependent metabolic signatures observed in preclinical models, highlighting further context dependence.

6.2. The role of *APOE* genotype in the metabolism of dietary flavan-3-ols

The *in vitro* findings presented in Chapter 2 demonstrate that *APOE* genotype influences EC metabolism, particularly in relation to the production of microbially derived 3-HPVL and 3,4-DiHPVL. However, the *APOE* genotype does not exert complete control, as age and sex also modulate metabolic patterns. For example, trends in the data reveal that non-*APOE4* carriers consistently produced higher levels of 3,4-DiHPVL, except in older females, where the trend was not observed. These results extend previous evidence of interindividual variability in flavonoid metabolism [181, 229], but uniquely reveal the layered influence of genotype, sex, and age when considered together. Animal models confirmed these observations, although the metabolite families that reflected the genotype effect varied between organisms. In the *APOE-TR* mouse models, higher concentrations of conjugated EC metabolites were observed in *APOE3/3-TR* compared to *APOE4/4-TR* animals. Furthermore, urinary metabolite profiles differed between groups, suggesting genotype-linked differences not only in microbial metabolism but also in host uptake and excretion pathways. These differences may be modulated by dietary fat, as high-fat diets are known to enhance flavonoid absorption in the upper gastrointestinal tract [261], highlighting the importance of considering the food matrix in flavonoid studies.

6.3. Flavan-3-ols within food matrices

Building on previous findings from the purified compound studies, Chapter 5 examined EC metabolism within whole-food contexts, including cocoa and mixed flavonoid-rich foods. Although genotype-dependent effects remained evident, diet composition and food matrix interactions markedly influenced metabolic outcomes. Indeed, unlike in purified EC studies, *APOE4/4-TR* mice showed higher urinary excretion of several gut-derived metabolites, including 3-HPVL-4-S and 4-HPVL-3-GluAC, suggesting that whole-food matrices may alter microbial or host metabolism in genotype-specific ways. This observation was consistent with evidence that fibre and macronutrient composition can modulate flavonoid bioavailability. In addition, the *APOE-TR* mouse model also revealed

a clear genotype effect on gut-derived EC metabolites, with *APOE4/4*-TR animals excreting higher levels than *APOE3/3*-TR counterparts. Yet, because this model included only male mice, the addition of human data showed that both age and sex further modulated these outcomes. This finding corroborated the DIAMOND *in vitro* results, which indicated that *APOE* genotype alone does not fully determine EC metabolism. Interestingly, the CANN-AN data displayed opposing trends to those of the initial *APOE*-TR mouse study (Chapter 2), with *APOE4/4*-TR mice exhibiting higher concentrations of valerolactones and valeric acids than *APOE3/3*-TR animals. No genotype effect was observed for upper GI-derived metabolites, unlike in Chapter 2. This may reflect differences in macronutrient composition, as the fibre in cocoa could act as a physical barrier or increase intestinal viscosity, reducing EC absorption in the upper GI tract [386], thereby decreasing genotype-related differences. Subsequently, more EC metabolites may have reached the colon, where microbial metabolism predominates.

Furthermore, when participants consumed complex flavonoid-rich foods, such as those used in the COB and OPCT studies (e.g., cocoa beverages and fruit juices), additional variability was observed. This likely arose from interactions between different flavonoids and population-specific dietary backgrounds. Differences in upper gut-derived catechin and EC metabolites were observed across both studies, potentially driven by variations in matrix composition, including higher lipid and sugar contents from chocolate and juice. Similarly, the lipid content in the HFEC-fed *APOE*-TR mice influenced upper GI metabolite production, suggesting a comparable mechanism. The presence of multiple flavonoids, each with distinct metabolic pathways and prebiotic effects, may have also interfered with EC metabolism, contributing to the sex- and age-dependent effects observed in the OPCT study. As in the DIAMOND study, uncontrolled factors in both COB and OPCT trials (e.g. differences in the food matrix, consumption patterns, and participant nationality) may have further contributed to interindividual variability. Collectively, these findings underscore the difficulty of translating outcomes from purified compound studies to complex dietary interventions and highlight the need to account for food matrix effects in future nutritional research.

6.4. The role of the microbiota in the observed changes.

As the metabolic transformation of dietary flavonoids relies on colonic microbial activity, the contribution of the microbiota to the observed interindividual differences was also investigated. Chapter 3 explored the effects of *APOE* genotype, age, sex, and EC supplementation on the structural and functional characteristics of the microbiota. In the *APOE*-TR mouse model, significant alterations in microbial composition and functionality were identified, primarily driven by genotype, sex, and dietary intervention. Among these, β -diversity was strongly influenced by the animals' sex and *APOE* genotype, while dietary factors exerted a comparatively smaller effect. EC supplementation modulated specific microbial taxa, leading to the attenuation of sex-related differences, notably in the *APOE4/4*-TR mice, suggesting that this genotype exhibited greater susceptibility to microbiota fluctuations, whereas the *APOE3/3*-TR mice appeared comparatively resilient. However, no direct associations were identified between the microbial taxa examined and EC metabolism or the generation of the key metabolites reported in the preceding chapters. This limitation may stem from the use of 16S rRNA sequencing, which provides limited taxonomic resolution and may have precluded identification at the species or subspecies level, thereby restricting the interpretation of EC-microbiota interactions.

Although these microbial patterns were not mirrored in the human samples, several noteworthy relationships between the metabolome and microbiota emerged. In particular, genotype-dependent differences were observed in the tauroconjugation of bile acids across both species, suggesting a possible mechanistic link. This finding warrants further investigation, given the established association between *APOE* genotype and hepatic lipid transport [31], a process closely linked to bile salt synthesis [387]. Such a connection may represent a plausible pathway underlying the genotype-mediated microbial differences observed in the dataset [101, 104].

Building upon these findings, the CANN-AN mouse model further elucidated the role of *APOE* genotype in shaping microbiota composition. Consistent with earlier results, genotype significantly influenced overall microbial diversity. Additionally, a diet effect was apparent, but only in the *APOE3/3*-TR mice. The absence of this effect in the

APOE4/4-TR group suggests that dietary modulation of the microbiota is more pronounced when EC is consumed as part of whole cocoa, rather than as a purified compound, implying a synergistic interaction between flavan-3-ols and other food constituents that amplifies microbial responses. Differential abundance analysis identified notable genotype-related variations in specific taxa, particularly the genus *Aldercreutzia*, which was more abundant in *APOE4/4-TR* mice fed with FLAV compared with *APOE3/3-TR* counterparts. As *Aldercreutzia* is known to participate in flavan-3-ol metabolism, its increased abundance may partially account for the genotype-specific differences observed. However, given the multistep nature of EC catabolism leading to valerolactone and valeric acid formation, it is unlikely to be the sole contributor to these effects.

6.5. *APOE* genotype, sex, and age modulation of EC health benefits

Numerous health benefits have been associated with the consumption of EC, however, interindividual variability in these responses has not previously been examined. Chapter 4, therefore, aimed to determine whether differences in metabolism, hepatic function, and cognitive performance exist between individuals. Distinct sex- and genotype-specific patterns were observed in weight gain, adipose tissue distribution, hepatic activity, and cognitive outcomes, although only a subset of these effects could be directly attributed to variations in dietary EC metabolism. For instance, EC supplementation induced the expression of both pro- and anti-inflammatory hepatic genes exclusively in female *APOE4/4-TR* mice. The reduced production of valerolactones observed in this genotype may be linked to these responses, although additional research is required to confirm this association. These effects could not be validated in the context of the food matrix, as corresponding experiments were not conducted; thus, further investigation is warranted.

qPCR analyses of hepatic tissue also revealed genotype-dependent differences in the transcription of the sulfotransferase *Sult1d1*, with higher expression levels detected in *APOE3/3-TR* animals. This may contribute to increased urinary excretion of valerolactones and valeric acids. In addition to the genotype-dependent microbial

metabolism of EC in the colon, differential expression of hepatic sulfotransferases suggests genotype variation in phase II metabolism of flavan-3-ol metabolites. As phase II conjugation enhances compound hydrophilicity to facilitate excretion [288], such differences may underpin the observed genotype-specific urinary concentrations. This mechanism may also explain variations in urinary levels of non-gut-derived metabolites, which similarly undergo hepatic phase II metabolism.

An important translational question which arises from this work is whether specific population subgroups may derive greater benefit from EC supplementation. Across each study, *APOE* genotype, sex, and age consistently modulated the metabolism of flavan-3-ols. However, when exploring functional outcomes, the benefits didn't always directly align with the specific metabolic groups. For example, cognitive improvements in spatial working memory were most evident in the *APOE4/4*-TR males, despite the *APOE3/3*-TR mice often exhibiting higher circulating and urinary concentrations of certain metabolites than their *APOE4/4*-TR counterparts. This result suggests that systemic flavan-3-ol metabolite concentrations may not singlehandedly determine physiological efficiency. From these results, it is plausible to suggest that EC supplementation may benefit the *APOE4/4* genotype, particularly in the male subgroup, warranting further investigation. However, given the complex genotype, sex, and age interactions, the reversal of metabolic profiles in the presence of the food matrix, and the limited sample sizes within certain experimental arms, it remains premature to definitively conclude that specific subgroups could benefit from EC supplementation more than other populations. Thus, the present findings support the concept that EC supplementation may exert differential effects depending on genetic and biological context, reinforcing the need for stratified clinical trials incorporating *APOE* genotype, sex, and age as key modifiers.

6.6. Strengths and weaknesses

This thesis employed a combination of pilot preclinical *in vitro* and *in vivo* experiments, alongside the reanalysis of existing clinical trials data, to investigate the influence of *APOE* genotype, sex, and age on the metabolism of EC. This integrated design provided new insight into interindividual variation in EC metabolism at a level of resolution not

previously explored. Each experimental model and its corresponding analysis contributed to a framework that addressed distinct aspects of the overarching research questions. While each approach enabled the independent observation of specific effects, their collective integration facilitated a more comprehensive understanding of the potential mechanisms underlying these interactions. The investigation of both purified EC extracts and EC within a whole-food matrix further strengthened this approach, allowing the effects of the isolated compound to be examined in parallel with those observed within a complex dietary context. This dual strategy enabled the research to directly characterise the metabolic and physiological impacts of EC, while maintaining a broader perspective on how these effects manifest within realistic nutritional environments.

Nevertheless, several limitations must also be considered when interpreting the findings and designing future investigations. Beyond those discussed in the individual chapters, all data originated from pilot studies or from clinical datasets in which *APOE* genotype stratification was not a primary objective. Consequently, these studies may have been underpowered, as no prior statistical power calculations were performed. Although many findings were validated across both animal and human models, it was not feasible to replicate this for all investigations. For instance, the health outcomes described in Chapter 4 and the metabolomic profiling of flavan-3-ol-rich cocoa detailed in Chapter 5 were conducted exclusively in *APOE*-TR mouse models. As such, species-specific metabolic differences may limit the direct extrapolation of these results to human settings. These methodological and translational limitations must be acknowledged and carefully addressed in the design of subsequent studies to refine our understanding and enhance reproducibility.

6.7. Future Considerations

Flavan-3-ols have been consistently demonstrated to exert beneficial effects on human health. To enhance the bioavailability of these compounds, and thereby maximise their physiological impact, they must undergo metabolism by the colonic microbiota. This thesis has identified notable differences in the metabolism of flavan-3-ols according to

APOE genotype, age, and sex. The findings suggest that such variations have the potential to modulate the health outcomes associated with flavan-3-ol supplementation. These observations may ultimately contribute to the development of personalised nutritional strategies, although further research is required before these findings can be translated into practical dietary guidelines.

A novel clinical study is therefore warranted, in which participants are stratified according to *APOE* genotype, sex, and age. The inclusion of all *APOE* genotypes, rather than grouping participants simply as *APOE4* carriers or non-carriers, could provide greater insight into within-group variability. For instance, the *APOE2* allele has been reported to exert a distinct effect on the microbiota [104], which could result in different outcomes among *APOE2/2*, *APOE2/3*, and *APOE3/3* individuals. A similar effect may also exist within the *APOE4* allele group. It is essential that appropriate power calculations are undertaken to ensure adequate participant numbers within each genotype-sex-age subgroup.

Attention should also be given to the washout period and dietary restrictions imposed on participants. A sufficient washout duration (≥ 24 hours) is required to allow for the clearance of flavonoids and their metabolites, while dietary control should minimise residual intake of flavonoid-rich foods. Providing standardised meals during this period could improve adherence and consistency across participants. A chronic intervention design may be particularly valuable, as it would more accurately reflect realistic dietary intake patterns of EC and other flavonoids. The primary outcomes should assess the influence of *APOE* genotype, sex, and age on the metabolism of flavan-3-ol-rich cocoa, with metabolite concentrations quantified in both urine and plasma. Secondary endpoints, including untargeted metabolomic profiling, shotgun metagenomic sequencing of faecal microbiota pre- and post-intervention, and liver and kidney function assessments, would provide invaluable mechanistic insight into the observed effects. Moreover, chronic exposure would allow for the evaluation of broader health outcomes that are unlikely to manifest acutely. These may include anthropometric measures such as body weight, BMI variation, and fat distribution, alongside glucose tolerance, cognitive performance, and hepatic function (through blood tests or imaging). Such measures

would help elucidate whether *APOE* genotype, sex, and age influence the health effects of EC, as suggested by the mouse model results presented in Chapter 4.

Flavan-3-ol-rich cocoa currently remains the only food for which the European Food Safety Authority (EFSA) has approved a health claim relating to endothelium-dependent vasodilation [388]. It would therefore be of interest to determine whether this vascular effect is modulated by age, sex, or *APOE* genotype, given that arterial stiffness represents a key biomarker of cardiovascular health, a significant risk factor for AD, and that the *APOE4* allele is independently associated with cardiovascular disease [389].

Furthermore, this thesis demonstrated that the food matrix in which EC is consumed significantly influences metabolic and physiological outcomes. Consequently, future clinical trials should compare the effects of EC administered as a purified extract with those of EC delivered within a complex food matrix, such as cocoa. Finally, differences observed between British and Italian participants highlight the importance of cultural and dietary context. Multi-centre trials conducted across diverse populations could therefore enhance understanding of interindividual and interpopulation variability in response to flavan-3-ol interventions, ultimately advancing global insights into personalised nutrition.

6.8. Contribution to the field

This thesis makes several novel contributions to the understanding of diet-gene-microbiota interactions. First, it is the first study to integrate *APOE* genotype, sex, and age as potential determinants of flavan-3-ol metabolism, linking these variables to both microbial and host metabolic pathways. Second, it identifies distinct roles of the food matrix, demonstrating that bioavailability and metabolism differ substantially between purified and whole-food flavonoid forms. Third, it provides evidence that *APOE* genotype modulates both microbial catabolism and hepatic phase II metabolism, suggesting multi-level host-microbe interactions underlying differential health responses. Together, these findings advance the field of nutritional neuroscience by establishing a foundation for precision nutrition strategies tailored to genetic and physiological risk profiles in cognitive decline and AD.

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Supplementary Data

Supplementary Table 1: Rodent diet used in Chapters 2, 3, and 4

Diet	High Fat Diet Kcal%	High Fat With Epicatechin Diet g%
Protein	20	20
Carbohydrate	35	35
Fat	45	45
Total	100	100
Ingredients	gm	gm
Casein	200.000	200.000
L-Cysteine	3.000	3.000
Corn Starch	72.800	72.800
Maltodextrin 10	100.000	100.000
Sucrose	172.800	172.800
Cellulose, BW200	50.000	50.000
Soybean Oil	25.000	25.000
Lard	177.5000	177.5000
Mineral Mix S10026	10.000	10.000
DiCalcium Phosphate	13.000	13.000
Calcium Carbonate	5.500	5.500
Potassium Citrate, 1 H ₂ O	16.500	16.500
Vitamin mix V10001	10.000	10.000
Choline Bitartrate	2.000	2.000
Epicatechin	0.000	0.172
FD&C Red Dye #40	0.050	0.050
FD&C Blue Dye #1	0.000	0.000
Total	858.15	858.15
Epicatechin (mg/kg)	0.000	200.000

Supplementary Table 2: Full Chromatographic and Spectroscopic Methodology for the APOE-TR Mouse Study. Abbreviations are as follows (–)-Epicatechin (EC), (–)-Epicatechin-3'-glucuronide (EC-3-GlcUA), (–)-Epicatechin-3'-sulfate (EC-3-S), (–)-Epicatechin-4'-glucuronide (EC-4-GlcUA), (–)-Epicatechin-diglucuronide (EC-DiGlcUA), (–)-Epicatechin-disulfate (EC-DiS), (–)-Epicatechin-glucuronide (5/7) (EC-GlcUA (5/7)), (–)-Epicatechin-sulfate (4'/5/7) (EC-S (4/5/7)), (–)-Epicatechin-sulfate-glucuronide (EC-S-GlcUA), 3'/4'-Methoxy-(–)-epicatechin-glucuronide ((3/4) MeO-EC-GlcUA), 3'/4'-Methoxy-(–)-epicatechin-sulfate ((3/4) MeO-EC-S), 3'/4'-Methoxy-(–)-epicatechin-sulfate-glucuronide ((3/4) MeO-EC-GlcUA), 3'-Methoxy-(–)-epicatechin (3-MeO-EC), 4'-Methoxy-(–)-epicatechin (4-MeO-EC), 1-(3',4'-Dihydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol (3,4-DiHPPol), 1-(3',4'-Dihydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol-glucuronide (3,4-DiHPPOL-GlcUA), 1-(3',4'-Dihydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol-methoxy-glucuronide (3,4-DiHPPOL-MeO-GlcUA), 1-(3',4'-Dihydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol-methoxy-sulfate (3,4-DiHPPOL-MeO-S), 1-(3',4'-Dihydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol-sulfate (3,4-DiHPPOL-S), 1-(3'-Hydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol (3-HPOL), 1-(4'-Hydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol (4-HPPOL), 1-(Hydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol-glucuronide (3-HPPOL-GlcUA), 1-(Hydroxyphenyl)-3-(2',4',6"-trihydroxyphenyl)propan-2-ol-sulfate (3-HPPOL-S), (3'-Hydroxyphenyl)-γ-valerolactone-4'-(glucuronide-sulfate) (3-MHPVL-4-(GlcUA-S)), (4'-Hydroxyphenyl)-γ-valerolactone-3'-(glucuronide-sulfate) (4-MHPVL-3-(GlcUA-S)), (Phenyl)-γ-valerolactone-(glucuronide-sulfate) (PVL-(GlcUA-S)), (Phenyl)-γ-valerolactone-3',4'-diglucuronide (PVL-DiGlcUA), (Phenyl)-γ-valerolactone-3',4'-disulfate (PVL-DiS), (Phenyl)-γ-valerolactone-3'-glucuronide (PVL-3-GlcUA), (Phenyl)-γ-valerolactone-3'-sulfate (PVL-3-S), (Phenyl)-γ-valerolactone-4'-glucuronide (PVL-4-GlcUA), (Phenyl)-γ-valerolactone-4'-sulfate (PVL-4-S), (Phenyl)-γ-valerolactone-sulfate-glucuronide (3',4') (PVL-S-GlcUA (3,4)), 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3,4-DiHPVL), 5-(3'-Hydroxyphenyl)-γ-valerolactone (3-MHPVL), 5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3-MHPVL-4-GlcUA), 5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate (3-MHPVL-4-S), 5-(3'-Methoxyphenyl)-γ-valerolactone (3-MePVL), 5-(3'-Methoxyphenyl)-γ-valerolactone-4'-glucuronide (3-Me-PVL-4-GlcUA), 5-(3'-Methoxyphenyl)-γ-valerolactone-4'-sulfate (3-Me-PVL-4-S), 5-(4'-Hydroxyphenyl)-γ-valerolactone (4-MHPVL), 5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (4-MHPVL-3-GlcUA), 5-(4'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate (4-MHPVL-3-S), 5-(4'-Methoxyphenyl)-γ-valerolactone (4-MePVL), 5-(4'-Methoxyphenyl)-γ-valerolactone-3'-glucuronide (4-Me-PVL-3-GlcUA), 5-(4'-Methoxyphenyl)-γ-valerolactone-3'-sulfate (4-Me-PVL-3-S), 5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (5-MHPVL-3-GlcUA), 5-(Hydroxy-methoxyphenyl)-γ-valerolactone (3',4') (MeO-HPVL (3,4)), 4-Hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid (4-OH-3,4-DiHPVA), 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid-4'-glucuronide (4-OH-3-HPVA-4'-GlcUA), 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid-4'-sulfate (4-OH-3-HPVA-4-S), 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid (4-OH-3-HPVA), 4-Hydroxy-5-(3'-methoxyphenyl)valeric acid-4'-glucuronide (4-OH-3-MPVA-4-GlcUA), 4-Hydroxy-5-(3'-methoxyphenyl)valeric acid-4'-sulfate (4-OH-3-MPVA-4-S), 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-glucuronide (4-OH-4-HPVA-3-GlcUA), 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-sulfate (4-OH-4-HPVA-3-S), 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid (4-OH-4-HPVA), 4-Hydroxy-5-(4'-methoxyphenyl)valeric acid-3'-glucuronide (4-OH-4-MPVA-3-GlcUA), 4-Hydroxy-5-(4'-methoxyphenyl)valeric acid-3'-sulfate (4-OH-4-MPVA-3-S), 4-Hydroxy-5-(phenyl)valeric acid-3'-glucuronide (4-OH-PVA-3-GlcUA), 4-Hydroxy-5-(phenyl)valeric acid-3'-sulfate (4-H-PVA-3-S), 4-Hydroxy-5-(phenyl)valeric acid-4'-glucuronide (4-H-PVA-4-GlcUA), 4-Hydroxy-5-(phenyl)valeric acid-4'-sulfate (4-H-PVA-4-S), 5-(3',4'-Dihydroxyphenyl)valeric acid (3,4-DiHPVA), 5-(3'-Hydroxyphenyl)valeric acid (3-HPVA), 5-(4'-Hydroxyphenyl)valeric acid (4-HPVA), 5-(Hydroxyphenyl)valeric acid-glucuronide (3',4') (HPVA-GlcUA (3,4)), 5-(Hydroxyphenyl)valeric acid-sulfate (3',4') (HPVA-S (3,4)), 5-(Methoxyphenyl)valeric acid-glucuronide (3',4') (MPVA-GlcUA (3,4)), 5-(Phenyl)valeric acid-3'-glucuronide (PVA-3-GlcUA), 5-(Phenyl)valeric acid-3'-sulfate (PVA-3-S), 5-(Phenyl)valeric acid-4'-glucuronide (PVA-4-GlcUA), 5-(Phenyl)valeric acid-4'-sulfate (PVA-4-S), Eriodictyol (ED)

Name	Pred RT (mins)	Curve RT (mins)	Sample RT (mins)			Parent Ion (M-H) ⁺ (m/z)	CE (V)	Quantifier Ion		Qualifier Ion1		Qualifier Ion2		Qualifier Ion3		STD
			1	2	3			Ion (m/z)	CE (V)	Ion (m/z)	CE (V)	Ion (m/z)	CE (V)	Ion (m/z)	CE (V)	
Epicatechin derivatives																
EC	4.26	NA	4.26			289	44	245	14	109	24					ED
EC-3-GlcUA	3.72	3.78	3.74			465	12	289	20	245	28	109	28			EC-3-GlcUA
EC-3-S	4.40	4.59	4.57	4.63	4.71	369	34	289	20	245	22	205	22			EC-3-S
EC-4-GlcUA	2.96		2.96			465	12	289	20	245	28	109	28			EC-3-GlcUA
EC-DiGlcUA ion I			2.24	2.31		641	12	465	20	289	20	113	20			EC-3-GlcUA
EC-DiGlcUA ion II	2.40		2.42	2.26		641	12	465	20	289	20	113	20			EC-3-GlcUA
EC-DiGlcUA ion III			2.76	2.80		641	12	465	20	289	20	113	20			EC-3-GlcUA
EC-DiS	5.00		ND			449	12	369	20	289	20					EC-3-S
EC-GlcUA (5/7)	2.66		2.88			465	12	289	20	245	28	109	28			EC-3-GlcUA
EC-S (4/5/7)	3.50-5.50		4.28	4.36	4.70	369	34	289	20	245	22	205	22			EC-3-S
EC-S-GlcUA ion I	1.50-4.00		3.02	3.08	3.12	545	12	369	20	465	20	289	30	113	30	EC-3-GlcUA
EC-S-GlcUA ion II	1.50-4.00		3.18	3.24	3.29	545	12	369	20	465	20	289	30	113	30	EC-3-GlcUA
EC-S-GlcUA ion III	1.50-4.00		3.38			545	12	369	20	465	20	289	30	113	30	EC-3-GlcUA
(3/4) MeO-EC-GlcUA ion I	3.50-6.50		3.41			479	20	303	35	288	32	113	19			EC-3-GlcUA
(3/4) MeO-EC-GlcUA ion II	3.50-6.50		3.83			479	20	303	35	288	32	113	19			EC-3-GlcUA
(3/4) MeO-EC-GlcUA ion III	3.50-6.50		4			479	20	303	35	288	32	113	19			EC-3-GlcUA

(3/4) MeO-EC-S ion I	4.50-7.50	5.07	5.18	383	20	303	20	288	26					EC-3-S	
(3/4) MeO-EC-S ion II	4.50-7.50	5.37	5.44	383	20	303	20	288	26					EC-3-S	
(3/4) MeO-EC-S ion III	4.50-7.50	5.67	5.75	383	20	303	20	288	26					EC-3-S	
(3/4) MeO-EC-S-GlcUA ion I	1.50-4.50	3.15			559	20	383	20	303	30	479	20			EC-3-GlcUA
(3/4) MeO-EC-S-GlcUA ion II	1.50-4.50	3.34			559	20	383	20	303	30	479	20			EC-3-GlcUA
3-MeO-EC	5.20	5.1			303	18	97	24	244	20	259	14			4-MeO-EC
4-MeO-EC	5.91	5.91			303	36	137	16	165	18	109	18			4-MeO-EC
Diphenylpropan-2-ols															
3,4-DiHPPol	4.46	4.41			291	40	123	20	167	20	147	20			EC-3-GlcUA
3,4-DiHPPOL-GlcUA	2.50-4.50	ND			467	40	291	20	123	20	113	20			
3,4-DiHPPOL-MeO-GlcUA	3.50-6.50	3.54			481	40	305	20	291	20	113	20			EC-3-S
3,4-DiHPPOL-MeO-S	4.50-7.50	4.81	4.9		385	40	305	20	291	20					EC-3-S
3,4-DiHPPOL-S ion I	3.50-5.50	4.53	4.61		371	40	291	20	123	20					EC-3-S
3,4-DiHPPOL-S ion II	3.50-5.50	4.67	4.76		371	40	291	20	123	20					ED
3-HPPOL	5.85	ND			275	40	191	20	231	20					
4-HPPOL	5.43	ND			275	40	231	20	191	20					
3-HPPOL-GlcUA	3.00-5.00	3.64			451	40	275	20	113	20	191	20			EC-3-S
3-HPPOL-S	4.00-6.00	5.13	5.18		355	40	275	20	191	20					5-MHPVL-3-GlcUA
Phenyl-γ-valerolactones															
3-HPVL-4-(GlcUA-S)	3.97	ND			463	20	254	20	207	20	175	20			
4-HPVL-3-(GlcUA-S)	4.15	ND			463	20	254	20	207	20	175	20			
PVL-(GlcUA-S)	4.35	ND			447	20	254	20	191	20	175	20			
PVL-DGlcUA	1.50-3.50	2.18	2.24	2.30	559	20	383	20	207	20	175	20	113	20	PVL-3-GlcUA
PVL-DS	4.00-6.0	4.70			367	20	287	20	207	20					PVL-3-S
PVL-3-GlcUA	4.16	4.18	4.16		367	20	191	20	113	20					PVL-3-GlcUA
PVL-3-S	4.89	5.11	4.97	5.03	5.21	271	44	191	18	147	32				PVL-3-S
PVL-4-GlcUA	3.00-4.00	ND			367	20	191	20	113	20					
PVL-4-S	4.53	4.60	4.77	4.90	271	44	191	18	147	32					4-MHPVL
PVL-S-GlcUA (3,4)	2.68	2.67	2.75	2.83	463	20	287	20	207	20	383	20			3-MHPVL
3,4-DiHPVL	4.76	4.75	4.74		207	2	163	18	122	20					5-MHPVL-3-GlcUA
3-HPVL	6.65	6.65	ND		191	20	147	12	93	18	119	24			
3-HPVL-4-GlcUA	3.39	2.57	3.41		383	20	207	20	113	20	163	20			3-MHPVL
3-HPVL-4-S	4.09	4.5	4.42	4.25	287	26	207	20	163	28					5-MHPVL-3-GlcUA
3-MPVL	7.00-10.0	ND			205	20	190	20	106	30	147	30			
3-MeO-PVL-4-GlcUA	3.94	3.94			397	20	221	20	206	20	113	20			4-MHPVL
3-MeO-PVL-4-S	4.00-5.50	4.73	4.85	4.93	301	20	221	20	206	20	145	40			5-MHPVL-3-GlcUA

4-HPVL	6.16	6.16	ND	191	20	93	18	147	12				
4-HPVL-3-GlcUA	3.73	2.57	3.79	383	20	207	20	113	20	163	20		4-MHPVL
4-HPVL-3-S	4.29		ND	287	26	207	20	163	28				5-MHPVL-3-GlcUA
4-MPVL	7.00-10.00		ND	205	20	190	20	106	30	147	30		3-MHPVL-4-S
4-MPVL-3-GlcUA	4.58		ND	397	20	221	20	113	20	206	20		4-MHPVL
4-MPVL-3-S	4.50-6.00		ND	301	20	221	20	206	20	145	40		4-MHPVL
5-HPVL-3-GlcUA	2.53	2.53	ND	383		207		113		163			
MHPVL (3,4)	5.0-8.0		ND	221	20	206	20	163	30				5-MHPVL-3-GlcUA
Phenylvaleric acids													
4-H-3,4-DiHPVA	3.08		3.09	225	6	123	20	101	16	163	16		3-MHPVL
4-H-3-HPVA-4'-GlcUA	2.29		2.32	401	10	225	20	207	20	175	20	101	20 5-MHPVL-3-GlcUA
4-H-3-HPVA-4-S	2.53		3.11	3.11	3.14	305	20	225	20	123	20		3-MHPVL-4-S
4-H-3-HPVA	4.53		ND	209	8	101	14	191	10	147	14		
4-H-3-MPVA-4-GlcUA	2.00-4.00		ND	415	20	113	20	239	20	101	20		
4-H-3-MPVA-4-S	2.50-4.50		ND	319	20	239	20	224	20	150	20		
4-H-4-HPVA-3-GlcUA	2.60		2.64	401	10	225	20	123	20	207	20	101	20 5-MHPVL-3-GlcUA
4-H-4-HPVA-3-S	3.15		3.23	3.33	305	20	123	20	163	20			3-MHPVL-4-S
4-H-4-HPVA	3.99		ND	209	6	101	14	147	14				
4-H-4-MPVA-3-GlcUA	2.00-4.00		ND	415	20	113	20	239	20	101	20		
4-H-4-MPVA-3-S	2.50-4.50		ND	319	20	239	20	224	20	150	20		
4-H-PVA-3-GlcUA	2.95		2.98	385	20	113	20	209	20	101	20		PVL-3-S
4-H-PVA-3-S	2.50-4.50		3.81	3.86	289	20	101	20	209	20	147	20	4-MHPVL
4-H-PVA-4-GlcUA	2.37		2.29	2.38	385	20	113	20	209	20	101	20	5-MHPVL-3-GlcUA
4-H-PVA-4-S	3.59		3.65	3.72	289	20	101	20	209	20	147	20	5-MHPVL-3-GlcUA
3,4-DiHPVA	6.43	6.41	ND	209	4	135	16	122	18	147	18		
3-HPVA	8.30	8.28	ND	193	22	149	16	106	22	157	22		
4-HPVA	7.93	7.91	ND	193	10	175	20	119	18				
MPVA-GlcUA (3,4)	2.50-4.50		3.43	385	20	209	20	113	20	219	20		3-MHPVL-4-S
MPVA-S (3,4)	4.50-6.50		ND	289	20	209	20	168	20	147	20		
MPVA-GlcUA (3,4)	3.00-5.00		ND	399	20	223	20	209	20	113	20	323	20
MPVA-GlcUA (3,4)	5.00-7.00		ND	303	20	223	20	209	20	182	20		
PVA-3-GlcUA	5.23		5.12	369	20	193	20	113	20	149	20		PVL-3-S
PVA-3-S	6.00-8.0		6.84	6.90	6.96	273	20	193	20	149	20	175	20 ED
PVA-4-GlcUA	4.00-6.00		4.82	369	20	193	20	113	20	149	20		EC-3-GlcUA
PVA-4-S	5.00-7.00		6.67	273	20	193	20	149	20	175	20		EC-3-S
Other Compounds													
ED	8.39	8.39	NA	287		151		135					

Supplementary Table 3: Full Food Frequency Questionnaire of participants, prior to stool sample donation. Participants were grouped according to their Sex, Age Group, and APOE4 Carrier Status (Gen). These intakes were used in a linear model to calculate the 3-way ANOVA p-values for each variable and their interactions. APOE genotype 2/3 and 3/3 (Non E4) and APOE genotype 3/4 and 4/4 (E4). Ages 18- 35 years old: Younger, and 55+ years old: Older. P-values <0.05 are in bold. Saturated fatty acid (SFA), Monounsaturated fatty acid (MUFA), Polyunsaturated fatty acids (PUFA), Trans unsaturated fatty acid (TUFA), Total Carbohydrates (Carbs), Total Sugars (Sugars), Total Oligosaccharides (Oligosacchs), Total Fibre According to Association of Official Analytical Chemists International (AOAC fibre), Non-starch polysaccharides fibre definition (NSP fibre). Polyphenol compounds measured: Epigallocatechin, Catechin, epicatechin, epigallocatechingallate, galocatechin, epicatechingallate -the sum of these compounds is total flavan-3-ols. Quercetin, Kaempferol, and Myricetin – total flavonols. Apigenin, and Luteolin – total flavones. Hesperitin and Naringenin-total flavanones. The total flavonoid intake was total of all the flavan-3-ols, flavonols, and flavones

Sex:	Females				Males				3 way ANOVA p.value						
Age Group	Older		Younger		Older		Younger								
APOE4 Gen	E4	Non E4	E4	Non E4	E4	Non E4	E4	Non E4							
Nutrient	$\bar{x}$ intake (σ)	$\bar{x}$ intake (σ)	$\bar{x}$ intake (σ)	$\bar{x}$ intake (σ)	$\bar{x}$ intake (σ)	$\bar{x}$ intake (σ)	$\bar{x}$ intake (σ)	$\bar{x}$ intake (σ)	Sex	Gen	Age	Sex: Gen	Sex: Age	Gen: Age	Sex: Gen: Age
Macro Nutrients															
Energy (kJ/day)	8598.1 (2832.3)	7453.5 (1757.9)	9501.2 (2255.2)	7419.6 (3070.0)	9970.1 (3783.8)	9177.7 (2104.9)	10501.7 (1745.8)	9490.2 (5135.4)	0.166	0.181	0.572	0.732	0.923	0.736	0.847
Fat (g/day)	83.8 (35.5)	73.0 (21.7)	95.8 (27.7)	75.2 (37.8)	94.3 (41.2)	98.2 (27.4)	103.1 (9.8)	100.8 (50.5)	0.125	0.467	0.476	0.443	0.858	0.693	0.931
SFA (g/day)	30.8 (14.7)	26.1 (7.4)	34.1 (13.2)	25.9 (15.2)	33.4 (15.6)	38.3 (10.5)	40.8 (0.6)	37.0 (17.8)	0.070	0.489	0.595	0.362	0.988	0.488	0.758
MUFA (g/day)	30.7 (12.5)	28.0 (9.7)	34.1 (9.9)	27.4 (14.1)	35.4 (16.9)	35.8 (10.5)	38.2 (4.7)	36.6 (17.6)	0.124	0.505	0.614	0.615	0.952	0.683	0.896
PUFA (g/day)	15.4 (6.3)	12.9 (4.6)	17.5 (5.2)	13.4 (5.7)	18.3 (6.5)	16.0 (6.0)	14.9 (3.6)	17.3 (9.4)	0.367	0.300	0.707	0.500	0.611	0.768	0.414
TUFA (g/day)	2.1 (1.0)	1.8 (0.7)	2.0 (1.0)	1.4 (1.0)	2.1 (1.3)	2.6 (0.7)	3.4 (0.9)	2.6 (1.3)	0.025	0.403	0.668	0.462	0.282	0.202	0.449
Cholesterol (mg/day)	271.6 (185.0)	248.4 (71.8)	294.3 (189.4)	329.1 (163.4)	329.0 (204.3)	341.4 (115.3)	493.8 (190.9)	419.2 (247.3)	0.053	0.915	0.171	0.857	0.529	0.962	0.507
Carbs (g/day)	233.0 (68.0)	206.4 (48.0)	268.1 (71.4)	198.2 (65.4)	273.3 (87.2)	224.7 (54.1)	280.2 (66.5)	229.4 (148.5)	0.540	0.063	0.574	0.894	0.772	0.607	0.680
Sugars (g/day)	120.1 (44.9)	100.1 (24.1)	133.6 (47.5)	80.4 (24.7)	146.9 (36.6)	111.9 (37.0)	111.3 (11.2)	86.4 (48.0)	0.953	0.007	0.311	0.980	0.193	0.552	0.374
Starch (g/day)	109.1 (33.2)	103.5 (26.5)	128.5 (38.7)	112.6 (68.9)	122.0 (50.1)	110.6 (26.8)	162.3 (59.9)	138.2 (97.7)	0.305	0.458	0.134	0.846	0.582	0.717	0.969
Oligosacchs (g/day)	0.7 0.5	0.4 0.2	1.0 0.5	0.7 0.5	1.1 0.8	0.5 0.3	0.7 0.6	1.0 1.2	0.554	0.230	0.191	0.975	0.786	0.326	0.284
Protein (g/day)	84.4 (32.7)	70.0 (20.3)	91.6 (21.0)	71.2 (29.5)	100.8 (54.0)	92.1 (24.0)	124.1 (36.2)	99.8 (43.6)	0.046	0.124	0.375	0.912	0.669	0.617	0.815
AOAC fibre (g/day)	6.6 (2.1)	6.1 (2.5)	4.7 (2.2)	2.2 (1.5)	5.6 (2.6)	5.2 (2.1)	3.4 (2.5)	3.8 (3.2)	0.305	0.227	0.003	0.392	0.490	0.597	0.319
Fibre NSP (g/day)	21.8 (7.7)	18.7 (4.3)	27.2 (12.0)	18.4 (4.9)	21.6 (9.1)	17.4 (6.5)	16.9 (6.8)	16.5 (10.4)	0.112	0.093	0.708	0.637	0.255	0.756	0.363
Water (mL/day)	2223.7 (606.7)	1605.4 (247.6)	1975.4 (904.9)	2116.7 (758.8)	2659.8 (1137.6)	2085.7 (600.0)	1423.7 (415.2)	1696.5 (820.7)	0.990	0.164	0.180	0.911	0.098	0.078	0.922

Alcohol (g/day)	11.0 (4.9)	8.7 (9.8)	3.3 (4.4)	8.2 (7.4)	13.3 (13.6)	13.1 (9.0)	3.2 (2.6)	13.5 (19.4)	0.211	0.523	0.147	0.546	0.773	0.162	0.784
Minerals															
Ca (mg/day)	1277.1 (557.5)	1035.6 (147.3)	1350.1 (483.6)	860.9 (178.0)	1466.0 (499.3)	1298.2 (152.9)	1151.9 (391.7)	1035.6 (537.4)	0.643	0.043	0.314	0.493	0.300	0.655	0.573
Cl (mg/day)	4295.1 (1647.0)	3926.5 (1270.3)	4814.3 (1661.7)	3688.8 (1761.1)	4611.8 (2411.4)	4502.9 (1210.5)	4602.5 (949.4)	4324.5 (1979.8)	0.632	0.361	0.841	0.640	0.713	0.624	0.775
Cu (mg/day)	1.6 (0.6)	1.4 (0.3)	1.9 (0.5)	1.3 (0.5)	1.8 (0.5)	1.4 (0.6)	1.6 (0.6)	1.6 (1.0)	0.903	0.075	0.432	0.698	0.865	0.929	0.259
Fe (mg/day)	14.8 (5.8)	12.2 (2.6)	16.9 (5.2)	11.3 (4.0)	14.9 (3.6)	13.0 (3.3)	15.3 (4.2)	13.4 (7.6)	0.801	0.053	0.563	0.544	0.834	0.584	0.626
I (ug/day)	252.9 (133.8)	212.9 (60.3)	200.0 (103.7)	177.6 (88.6)	331.5 (188.0)	238.7 (51.9)	258.3 (118.0)	189.0 (91.2)	0.373	0.091	0.114	0.451	0.858	0.768	0.965
K (mg/day)	4040.4 (1155.4)	3207.7 (614.5)	4343.9 (1124.0)	3396.1 (314.0)	4835.9 (1523.6)	3514.5 (841.1)	3533.2 (942.3)	3438.3 (1612.6)	0.912	0.012	0.809	0.984	0.238	0.493	0.333
Na (mg/day)	2764.8 (1104.0)	2576.0 (820.7)	3184.9 (1076.9)	2482.2 (1072.8)	3032.1 (1533.6)	2935.7 (821.8)	3058.0 (552.5)	3008.9 (1547.5)	0.526	0.455	0.622	0.653	0.788	0.695	0.685
P (mg/day)	1708.9 (645.7)	1378.8 (290.2)	1798.1 (479.3)	1311.0 (429.6)	1986.4 (801.4)	1737.5 (331.9)	1818.9 (552.6)	1626.0 (771.2)	0.300	0.062	0.830	0.647	0.626	0.854	0.762
Zn (mg/day)	10.7 (4.2)	8.5 (1.8)	11.6 (2.5)	8.4 (3.8)	11.2 (4.4)	11.8 (3.8)	15.1 (5.0)	12.8 (5.4)	0.052	0.163	0.284	0.365	0.527	0.467	0.684
Vitamins															
Retinol (ug/day)	457.4 (304.9)	376.8 (191.6)	374.2 (207.5)	296.3 (180.2)	413.2 (216.3)	459.2 (161.9)	357.3 (162.7)	418.5 (211.7)	0.695	0.716	0.334	0.328	0.788	0.952	0.964
Thiamin (mg/day)	1.9 (0.7)	1.5 (0.5)	1.9 (0.5)	1.4 (0.5)	2.0 (1.1)	1.7 (0.5)	2.0 (0.4)	1.7 (0.9)	0.679	0.094	0.845	0.871	0.944	0.835	0.890
Riboflavin (mg/day)	2.5 (1.0)	1.8 (0.5)	1.9 (0.6)	1.5 (0.3)	2.8 (1.1)	2.2 (0.4)	2.1 (0.4)	1.7 (0.8)	0.519	0.016	0.027	0.808	0.793	0.684	0.991
Niacin (mg/day)	21.4 (7.1)	17.6 (6.9)	20.9 (5.9)	17.6 (5.6)	26.5 (15.7)	20.6 (5.8)	31.1 (6.6)	22.4 (9.0)	0.091	0.045	0.681	0.507	0.517	0.864	0.738
Pantothenic acid (mg/day)	6.8 (2.8)	5.3 (0.9)	6.2 (1.5)	5.5 (1.2)	7.4 (2.1)	6.6 (1.3)	7.1 (2.0)	5.8 (2.0)	0.384	0.057	0.452	0.893	0.787	0.896	0.627
Vit B6 (mg/day)	2.5 (0.9)	1.8 (0.5)	2.4 (0.6)	1.9 (0.5)	2.6 (1.1)	2.1 (0.5)	2.7 (0.5)	2.1 (1.1)	0.673	0.020	0.965	0.928	0.945	0.884	0.777
Biotin (ug/day)	59.6 (22.6)	46.5 (9.2)	58.2 (14.3)	41.7 (6.6)	70.0 (19.6)	58.1 (12.9)	47.9 (20.7)	49.2 (19.6)	0.543	0.029	0.154	0.479	0.277	0.712	0.435
Folic acid (ug/day)	363.1 (131.3)	282.7 (79.8)	388.1 (124.4)	276.7 (57.7)	403.3 (196.1)	305.6 (70.0)	235.8 (84.2)	278.4 (136.4)	0.415	0.048	0.474	0.518	0.199	0.555	0.252
Vit B12 (ug/day)	6.7 (4.0)	5.9 (2.3)	5.9 (4.2)	4.5 (3.4)	10.3 (7.6)	6.9 (0.9)	9.8 (5.4)	6.7 (3.2)	0.091	0.098	0.522	0.355	0.766	0.946	0.865
Vit C (mg/day)	120.4 (56.7)	112.5 (40.4)	156.6 (82.9)	142.1 (48.2)	159.4 (74.8)	103.4 (39.7)	76.3 (36.4)	118.9 (74.8)	0.443	0.460	0.573	0.820	0.134	0.293	0.163
Vit D (ug/day)	5.1 (3.7)	5.4 (3.1)	4.9 (3.2)	5.0 (2.4)	7.9 (6.2)	4.4 (1.9)	5.7 (3.2)	4.9 (2.2)	0.645	0.353	0.659	0.197	0.904	0.621	0.503

Vit E (mg/day)	12.6 (4.1)	11.5 (3.9)	14.7 (4.6)	11.3 (4.2)	13.9 (5.8)	11.5 (4.4)	9.7 (3.6)	12.6 (7.2)	0.684	0.386	0.773	0.574	0.468	0.732	0.213
Vit K (mg/day)	103.0 (69.1)	78.5 (42.0)	281.4 (281.7)	64.5 (7.2)	199.6 (264.8)	51.6 (67.4)	29.4 (20.5)	127.4 (62.6)	0.456	0.078	0.188	0.598	0.154	0.987	0.018
Phytochemicals															
Carotenes (ug/day)	4710.4 (2084.7)	4627.1 (3415.6)	8623.7 (5453.7)	7981.8 (4671.2)	4820.5 (3743.4)	3854.2 (2503.6)	1966.9 (898.5)	4151.6 (2043.5)	0.026	0.965	0.083	0.853	0.026	0.606	0.371
Total flavan-3-ols (mg/day)	61.4 (41.6)	47.3 (21.9)	39.2 (37.0)	32.4 (36.4)	67.1 (54.0)	63.3 (57.5)	19.1 (12.3)	20.0 (9.6)	0.785	0.447	0.012	0.740	0.280	0.799	0.959
Total flavanones (mg/day)	26.5 (26.1)	20.4 (20.0)	35.1 (33.0)	22.8 (36.0)	36.5 (48.6)	28.0 (41.0)	6.8 (6.0)	23.6 (16.1)	0.906	0.627	0.814	0.623	0.283	0.692	0.416
Total flavones (mg/day)	4.3 (7.7)	8.0 (5.5)	2.3 (3.1)	2.3 (2.9)	6.1 (7.7)	3.6 (5.9)	0.8 (0.7)	5.8 (11.9)	0.941	0.487	0.297	0.700	0.505	0.782	0.195
Total flavonols (mg/day)	35.7 (16.0)	33.5 (8.6)	32.7 (10.2)	36.0 (13.0)	33.5 (32.6)	33.8 (24.1)	12.7 (5.0)	27.3 (18.5)	0.286	0.681	0.297	0.612	0.325	0.401	0.697
Total Flavonoids (mg/day)	171.7 (85.0)	143.1 (35.5)	135.2 (64.9)	116.2 (73.0)	193.4 (182.5)	174.4 (115.6)	52.7 (28.2)	92.4 (47.6)	0.697	0.561	0.023	0.641	0.199	0.580	0.664

Supplementary Table 4: LC-MS results from the Dietary pAnt extracts, colonic Microbes, and apoe geNetics interactions stuDY (DIAMOND) colon model. Measuring the concentration of each compound as a percentage of the baseline detected epicatechin. Participants were grouped according to their Sex, Age Group, and APOE4 Carrier Status (Gen). These intakes were used in a linear model to calculate the 3-way permutational ANOVA p-values for each variable and their interactions (p-values <0.05 are highlighted in bold). Compound abbreviations are as follows: Epicatechin (EC), 3',4'-Dihydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol (3,4-DiHP-246-TriHPP-2-ol), 3-Hydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol (3-HP-2,4,6-TriHPP-2-ol), 4'-Hydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol (4-HP-2,4,6-TriHPP-2-ol), 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3,4-DiHPVL), 5-(3',4'-Dimethoxyphenyl)-γ-valerolactone (3,4-DiMPVL), 5-(3'-Hydroxyphenyl)-γ-valerolactone (3-HPVL), 5-(4'-Hydroxyphenyl)-γ-valerolactone (4-HPVL), 5-(hydroxymethoxyphenyl)-γ-valerolactone (HMPVL), 3',4'-Dihydroxyphenylvaleric acid (3,4-DiHPVA), 3'-Hydroxyphenylvaleric acid (3-HPVA), 4-Hydroxy-3',4'-dihydroxyphenylvaleric acid ion I + ion II (4-H-3,4-diHPVA I + II), 4-Hydroxy-3-hydroxyphenylvaleric acid (4-H-3HPVA), 4-Hydroxy-4'-hydroxyphenylvaleric acid (4-H-4-HPVA), 4'-Hydroxyphenylvaleric acid (4-HPVA)

Compound	Sex Age Genotype	Female				Male				Sterile		3 way permutational ANOVA p-values						
		Older		Younger		Older		Younger		E4	Non E4	Gen	Sex	Age	Gen: Sex	Gen: Age	Sex: Age	Gen: Sex: Age
		E4	Non E4	E4	Non E4	E4	Non E4	E4	Non E4									
	T hr	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)	$\bar{x}$ μ M (SD)							
Catechin	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.283 (0.565)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
Catechin	4	1.241 (0.450)	1.568 (0.097)	1.446 (0.682)	1.415 (0.469)	0.994 (0.332)	1.137 (0.480)	1.232 (0.528)	0.967 (0.188)	1.871 (0.896)	1.459 (0.181)	0.763	0.016	0.827	0.445	0.166	0.980	0.931
Catechin	8	1.443 (1.258)	2.164 (0.550)	1.720 (1.455)	1.483 (1.577)	1.155 (0.988)	1.034 (0.819)	0.665 (1.053)	1.063 (0.847)	3.128 (0.897)	2.671 (0.356)	0.574	0.040	0.525	0.884	0.751	0.971	0.287
Catechin	24	0.041 (0.137)	0.000 (0.000)	0.064 (0.168)	0.034 (0.068)	0.000 (0.000)	0.017 (0.044)	1.058 (1.832)	0.000 (0.000)	9.083 (1.302)	3.673 (4.103)	0.023	0.059	0.027	0.050	0.035	0.047	0.028
EC	0	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	100.000 (0.000)	0.831	0.824	0.829	0.831	0.837	0.823	0.820
EC	4	65.234 (23.124)	93.524 (16.623)	66.414 (47.795)	43.201 (32.242)	54.118 (34.522)	20.681 (23.325)	62.739 (23.332)	33.866 (38.479)	94.950 (11.758)	83.419 (9.472)	0.145	0.015	0.481	0.089	0.232	0.072	0.155
EC	8	28.554 (31.254)	27.980 (33.581)	9.359 (23.579)	35.041 (42.192)	30.803 (44.779)	0.747 (0.972)	28.349 (48.029)	10.570 (25.621)	76.415 (6.645)	74.562 (3.485)	0.559	0.436	0.900	0.059	0.313	0.615	0.704
EC	24	0.016 (0.054)	0.306 (0.750)	0.000 (0.000)	0.134 (0.267)	0.096 (0.215)	0.022 (0.059)	22.033 (38.162)	0.014 (0.033)	70.589 (13.539)	31.761 (36.911)	0.026	0.025	0.023	0.019	0.023	0.024	0.028
3,4-DiHP- 246-TriHPP- 2-ol	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.069 (0.155)	0.291 (0.632)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.555	0.251	0.250	0.559	0.551	0.249	0.570
3,4-DiHP- 246-TriHPP- 2-ol	4	2.468 (3.750)	29.203 (38.041)	36.043 (45.443)	26.665 (32.762)	4.696 (5.984)	32.637 (44.074)	10.102 (14.060)	8.281 (12.841)	0.030 (0.059)	0.000 (0.000)	0.235	0.289	0.743	0.815	0.075	0.171	0.865
3,4-DiHP- 246-TriHPP- 2-ol	8	35.997 (41.176)	57.537 (33.639)	33.053 (21.112)	18.314 (22.381)	26.365 (24.329)	38.854 (40.031)	22.570 (26.229)	16.125 (23.720)	0.000 (0.000)	0.000 (0.000)	0.745	0.322	0.086	0.984	0.178	0.697	0.673
3,4-DiHP- 246-TriHPP- 2-ol	24	3.308 (10.830)	9.037 (22.137)	11.055 (19.643)	4.441 (8.882)	15.266 (34.136)	0.285 (0.754)	8.065 (13.969)	0.284 (0.695)	0.000 (0.000)	0.000 (0.000)	0.255	0.856	0.841	0.285	0.800	0.613	0.350
3-HP-2,4,6- TriHPP-2-ol	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.061 (0.136)	0.053 (0.140)	0.000 (0.000)	0.000 (0.000)	0.077 (0.154)	0.000 (0.000)	0.933	0.198	0.195	0.938	0.927	0.211	0.927
3-HP-2,4,6- TriHPP-2-ol	4	1.509 (4.489)	0.700 (1.275)	1.297 (1.160)	0.126 (0.252)	2.910 (5.591)	3.876 (7.940)	1.988 (2.165)	1.403 (2.044)	0.118 (0.157)	0.045 (0.090)	0.771	0.229	0.452	0.675	0.726	0.630	0.838

3-HP-2,4,6-TriHPP-2-ol	8	13.141 (24.005)	8.791 (16.719)	21.101 (24.552)	5.464 (8.500)	15.467 (19.824)	13.263 (32.404)	23.629 (35.165)	4.767 (7.465)	0.113 (0.150)	0.066 (0.081)	0.146	0.768	0.880	0.971	0.327	0.865	0.844
3-HP-2,4,6-TriHPP-2-ol	24	4.796 (9.833)	3.089 (7.081)	8.762 (19.679)	0.464 (0.771)	4.937 (10.915)	0.277 (0.601)	0.000 (0.000)	0.339 (0.458)	0.084 (0.169)	0.035 (0.070)	0.261	0.362	0.791	0.662	0.894	0.631	0.361
4-HP-2,4,6-TriHPP-2-ol	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-HP-2,4,6-TriHPP-2-ol	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.029 (0.065)	0.000 (0.000)	0.000 (0.000)	0.038 (0.093)	0.000 (0.000)	0.000 (0.000)	0.852	0.171	0.860	0.875	0.171	0.853	0.172
4-HP-2,4,6-TriHPP-2-ol	8	0.046 (0.104)	0.000 (0.000)	0.028 (0.075)	0.000 (0.000)	0.025 (0.056)	0.097 (0.103)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.983	0.608	0.124	0.106	0.552	0.253	0.326
4-HP-2,4,6-TriHPP-2-ol	24	0.000 (0.000)	0.000 (0.000)	0.027 (0.072)	0.053 (0.106)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.604	0.113	0.105	0.636	0.597	0.112	0.614
34-DiHPVL	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
34-DiHPVL	4	0.096 (0.149)	0.018 (0.045)	0.889 (1.540)	4.419 (8.837)	0.220 (0.400)	3.683 (6.802)	0.253 (0.438)	2.626 (3.375)	0.000 (0.000)	0.098 (0.135)	0.049	0.786	0.385	0.628	0.599	0.194	0.335
34-DiHPVL	8	1.084 (2.046)	0.193 (0.238)	12.446 (19.373)	31.361 (54.254)	8.765 (18.065)	33.643 (44.278)	3.832 (6.446)	40.914 (50.343)	0.000 (0.000)	0.110 (0.153)	0.031	0.276	0.238	0.241	0.398	0.282	0.849
34-DiHPVL	24	12.466 (22.920)	0.769 (0.884)	20.779 (32.053)	38.014 (52.446)	10.947 (13.000)	22.741 (29.825)	10.027 (9.283)	41.012 (38.345)	0.000 (0.000)	0.091 (0.121)	0.170	0.726	0.071	0.293	0.168	0.428	0.780
34-DiMPVL	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
34-DiMPVL	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
34-DiMPVL	8	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
34-DiMPVL	24	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
3-HPVL	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.446 (0.891)	NA	NA	NA	NA	NA	NA	NA
3-HPVL	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.322 (2.643)	NA	NA	NA	NA	NA	NA	NA
3-HPVL	8	0.000 (0.000)	0.000 (0.000)	3.179 (8.412)	1.758 (3.517)	0.000 (0.000)	18.866 (28.076)	4.252 (7.365)	4.186 (10.254)	0.000 (0.000)	1.492 (2.983)	0.250	0.131	0.724	0.181	0.169	0.309	0.254
3-HPVL	24	68.863 (84.930)	29.362 (29.959)	47.946 (57.642)	40.194 (69.890)	43.648 (24.568)	11.898 (13.223)	119.708 (200.913)	28.491 (27.897)	0.000 (0.000)	1.538 (3.077)	0.050	0.837	0.356	0.384	0.739	0.236	0.298
4-HPVL	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-HPVL	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-HPVL	8	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.243 (0.643)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.524	0.527	0.527	0.531	0.519	0.516	0.518
4-HPVL	24	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA

HMPVL	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
HMPVL	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
HMPVL	8	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
HMPVL	24	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
3,4-DiHPVA	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
3,4-DiHPVA	4	0.004 (0.014)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.038 (0.101)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.027 (0.053)	0.608	0.610	0.474	0.472	0.607	0.613	0.459
3,4-DiHPVA	8	0.000 (0.000)	0.000 (0.000)	0.043 (0.114)	0.000 (0.000)	0.114 (0.256)	0.246 (0.295)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.021 (0.042)	0.624	0.077	0.080	0.333	0.337	0.024	0.636
34-DiHPVA	24	1.273 (2.500)	2.137 (4.242)	3.461 (8.661)	7.052 (14.104)	7.205 (11.203)	40.229 (69.625)	7.197 (12.329)	11.233 (26.912)	0.000 (0.000)	0.018 (0.037)	0.279	0.154	0.577	0.407	0.500	0.358	0.426
3-HPVA	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.470 (1.150)	0.000 (0.000)	0.000 (0.000)	0.421	0.416	0.395	0.403	0.421	0.400	0.408
3-HPVA	4	0.273 (0.502)	0.789 (1.458)	0.701 (0.893)	0.213 (0.269)	0.703 (1.401)	1.117 (1.130)	0.285 (0.249)	0.702 (1.109)	0.278 (0.246)	1.523 (1.307)	0.486	0.495	0.420	0.507	0.416	0.576	0.406
3-HPVA	8	0.261 (0.537)	0.751 (1.412)	0.663 (0.805)	0.298 (0.372)	0.798 (1.617)	1.109 (1.068)	0.282 (0.246)	0.693 (1.058)	0.264 (0.242)	1.474 (1.245)	0.487	0.467	0.426	0.625	0.544	0.466	0.438
3-HPVA	24	6.709 (19.967)	54.966 (70.572)	40.312 (93.575)	9.571 (12.549)	36.263 (48.464)	48.242 (58.489)	1.248 (1.731)	0.857 (1.137)	0.236 (0.309)	1.923 (1.271)	0.660	0.704	0.142	0.928	0.168	0.279	0.311
4-H-34-diHPVA I + II	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-H-34-diHPVA I + II	4	0.000 (0.000)	0.000 (0.000)	1.273 (3.369)	0.210 (0.420)	0.000 (0.000)	0.555 (1.056)	0.000 (0.000)	0.172 (0.268)	0.000 (0.000)	0.000 (0.000)	0.897	0.752	0.615	0.329	0.468	0.307	0.776
4-H-34-diHPVA I + II	8	4.113 (9.932)	0.487 (0.925)	27.922 (47.024)	4.000 (4.619)	5.925 (12.057)	20.764 (42.098)	0.443 (0.444)	18.617 (27.501)	0.000 (0.000)	0.000 (0.000)	0.869	0.775	0.557	0.067	0.620	0.295	0.494
4-H-34-diHPVA I + II	24	53.596 (101.526)	2.754 (3.349)	68.315 (99.170)	54.080 (44.242)	66.963 (62.791)	38.143 (52.854)	54.087 (82.120)	57.443 (39.792)	0.000 (0.000)	0.000 (0.000)	0.330	0.684	0.438	0.674	0.473	0.518	0.963
4-H-3HPVA	0	0.000 (0.000)	0.000 (0.000)	0.014 (0.038)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.338 (0.828)	0.000 (0.000)	0.381 (0.761)	0.431	0.441	0.363	0.374	0.440	0.435	0.364
4-H-3HPVA	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.488 (1.195)	0.000 (0.000)	1.182 (2.363)	0.403	0.395	0.398	0.391	0.403	0.395	0.401
4-H-3HPVA	8	0.414 (1.345)	0.000 (0.000)	5.881 (10.070)	2.476 (4.952)	0.563 (1.214)	0.361 (0.667)	0.057 (0.100)	14.832 (32.539)	0.000 (0.000)	1.175 (2.351)	0.553	0.717	0.139	0.233	0.484	0.768	0.258
4-H-3HPVA	24	82.445 (76.706)	79.060 (91.498)	57.192 (63.362)	51.904 (72.228)	76.449 (47.020)	42.410 (64.921)	25.535 (23.962)	53.575 (64.045)	0.000 (0.000)	1.346 (2.692)	0.854	0.401	0.283	0.973	0.486	0.882	0.462
4-H-4HPVA	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-H-4HPVA	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA

4-H-4HPVA	8	0.012 (0.041)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.118 (0.312)	0.000 (0.000)	0.059 (0.144)	0.000 (0.000)	0.000 (0.000)	0.763	0.760	0.585	0.162	0.372	0.382	0.571
4-H-4HPVA	24	0.506 (0.785)	0.000 (0.000)	0.447 (0.692)	0.361 (0.533)	0.601 (0.584)	0.095 (0.251)	0.178 (0.308)	0.443 (0.822)	0.000 (0.000)	0.000 (0.000)	0.279	0.995	0.759	0.633	0.126	0.621	0.635
4-HPVA	0	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-HPVA	4	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-HPVA	8	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA
4-HPVA	24	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	NA	NA	NA	NA

Supplementary Table 5: Flavonoid metabolites present in the plasma samples of the APOE3/3-TR (APOE3/3) and APOE4/4-TR (APOE4/4) mice fed a High Fat (45% kcal from saturated fat) supplemented with 14.5 mg/kg body weight epicatechin (HFE) following a two way ANOVA investigating the effects of the APOE genotype (Gen), Sex and its interaction on the presence of these metabolites. Compounds are: 3-O-Methoxy-epicatechin (3-O-MeEC), 4-O-Methoxy-epicatechin (4-O-MeEC), Epicatechin (EC), Epicatechin-3-glucuronide (EC-3-GluAC), Epicatechin-3-sulfate (EC-3-S), Epicatechin-4-5-7-sulfate (EC-4,5,7-S), Epicatechin-4-glucuronide (EC-4-GluAC), Epicatechin-5-7-glucuronide (EC-5,7-GluAC), Epicatechin-diglucuronide ion I (EC-DiGluAC I), Epicatechin-diglucuronide ion II (EC-DiGluAC II), Epicatechin-diglucuronide ion III (EC-DiGluAC III), Epicatechin-sulfate-glucuronide ion I (EC-S-GluAC I), Epicatechin-sulfate-glucuronide ion II (EC-S-GluAC II), Epicatechin-sulfate-glucuronide ion III (EC-S-GluAC III), Methoxy-epicatechin-glucuronide ion I (MeO-EC-GluAC I), Methoxy-epicatechin-glucuronide ion II (MeO-EC-GluAC II), Methoxy-epicatechin-glucuronide ion III (MeO-EC-GluAC III), Methoxy-epicatechin-sulfate ion I (MeO-EC-S I), Methoxy-epicatechin-sulfate ion II (MeO-EC-S II), Methoxy-epicatechin-sulfate ion III (MeO-EC-S III), Methoxy-epicatechin-sulfate-glucuronide ion I (MeO-EC-S-GluAC I), Methoxy-epicatechin-sulfate-glucuronide ion II (MeO-EC-S-GluAC II), f, 3,4-Dihydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol-methoxy-glucuronide (34-DiHPP-2-ol-MeO-GluAC), 3,4-Dihydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol-methoxy-sulfate (34-DiHPP-2-ol-MeO-S), 3,4-Dihydroxyphenyl-triHPOL-sulfate ion I (3,4-DiHPP-2-ol-S I), 3,4-Dihydroxyphenyl-triHPOL-sulfate ion II (3,4-DiHPP-2-ol-S II), Hydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol-glucuronide (HPP-2-ol-GluAC), Hydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol-sulfate (HPP-2-ol-S), 3,4-Dihydroxyphenyl-γ-valerolactone (3,4-DiHPVL), 3-Hydroxyphenyl-γ-valerolactone-4-glucuronide (3-HPVL-4-GluAC), 3-Hydroxyphenyl-γ-valerolactone-4-sulfate (3HPVL-4-S), 3-Methoxyphenyl-γ-valerolactone-4-glucuronide (3MPVL-4-GluAC), 3-Methoxyphenyl-γ-valerolactone-4-sulfate (3MPVL-4-S), 4-Hydroxyphenyl-γ-valerolactone-3-glucuronide (4HPVL-3-GluAC), Phenyl-γ-valerolactone-3,4-digluconide (PVL-3,4-DiGluAC), Phenyl-γ-valerolactone-3,4-disulfate (PVL-3,4-DiS), Phenyl-γ-valerolactone-3-glucuronide (PVL-3-GluAC), Phenyl-γ-valerolactone-3-sulfate (PVL-3-S), Phenyl-γ-valerolactone-4-sulfate (PVL-4-S), Phenyl-γ-valerolactone-sulfate-glucuronide (PVL-S-GluAC), 4-Hydroxy-3,4-dihydroxyphenylvaleric acid (4-H-3,4DiHPVA), 4-Hydroxy-3-hydroxyphenylvaleric acid-4-glucuronide (4-H-3HPVA-4-GluAC), 4-Hydroxy-3-hydroxyphenylvaleric acid-4-sulfate (4-H-3HPVA-4-S), 4-Hydroxy-4-hydroxyphenylvaleric acid-3-glucuronide (4-H-4HPVA-3-GluAC), 4-Hydroxy-4-hydroxyphenylvaleric acid-3-sulfate (4-H-4HPVA-3-S), 4-Hydroxy-phenylvaleric acid-3-glucuronide (4-H-PVA-3-GluAC), 4-Hydroxy-phenylvaleric acid-3-sulfate (4-H-PVA-3-S), 4-Hydroxy-phenylvaleric acid-4-glucuronide (4-H-PVA-4-GluAC), 4-Hydroxy-phenylvaleric acid-4-sulfate (4-H-PVA-4-S), Hydroxyphenylvaleric acid-glucuronide (HPVA-GluAC), Phenylvaleric acid-3-sulfate (PVA-3-S), Phenylvaleric acid-4-glucuronide (PVA-4-GluAC)

[illegible]

[illegible]

Supplementary Table 6: Flavonoid metabolites present in the urine samples of the APOE3/3-TR (APOE3/3) and APOE4/4-TR (APOE4/4) mice fed a High Fat (45% kcal from saturated fat) supplemented with 14.5 mg/kg body weight epicatechin (HFEC) following a two way ANOVA investigating the effects of the APOE genotype (Gen), Sex and its interaction on the presence of these metabolites. Compounds are: 3-O-Methoxy-epicatechin (3-O-MeEC), 4-O-Methoxy-epicatechin (4-O-MeEC), Epicatechin (EC), Epicatechin-3-glucuronide (EC-3-GluAC), Epicatechin-3-sulfate (EC-3-S), Epicatechin-4-5-7-sulfate (EC-457-S), Epicatechin-4-glucuronide (EC-4-GluAC), Epicatechin-5-7-glucuronide (EC-5,7-GluAC), Epicatechin-diglucuronide ion I (EC-DiGluAC I), Epicatechin-diglucuronide ion II (EC-DiGluAC II), Epicatechin-diglucuronide ion III (EC-DiGluAC III), Epicatechin-sulfate-glucuronide ion I (EC-S-GluAC I), Epicatechin-sulfate-glucuronide ion II (EC-S-GluAC II), Epicatechin-sulfate-glucuronide ion III (EC-S-GluAC III), Methoxy-epicatechin-glucuronide ion I (Me-EC-GluAC I), Methoxy-epicatechin-glucuronide ion II (MeO-EC-GluAC II), Methoxy-epicatechin-glucuronide ion III (Me-EC-GluAC III), Methoxy-epicatechin-sulfate ion I (MeO-EC-S I), Methoxy-epicatechin-sulfate ion II (MeO-EC-S II), Methoxy-epicatechin-sulfate ion III (MeO-EC-S III), Methoxy-epicatechin-sulfate-glucuronide ion I (MeO-EC-S-GluAC I), Methoxy-epicatechin-sulfate-glucuronide ion II (MeO-EC-S-GluAC II), 3,4-Dihydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol (3,4-DiHPP-2-ol), 3,4-Dihydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol-methoxy-glucuronide (3,4-DiHPP-2-ol-MeO-GluAC), 3,4-Dihydroxyphenyl-2,4,6-trihydroxyphenylpropan-2-ol-methoxy-sulfate (3,4-DiHPP-2-ol-MeO-S), 3,4-Dihydroxyphenyl-triHPPOL-sulfate ion I (3,4-DiHPP-2-ol-S I), 3,4-Dihydroxyphenyl-triHPPOL-sulfate ion II (3,4-DiHPP-2-ol S II), Hydroxyphenyl-2,4,6-trihydroxyphenyl-propan-2-ol-glucuronide (HPP-2-ol-GluAC), Hydroxyphenyl-2,4,6-trihydroxyphenyl-propan-2-ol-sulfate (HPP-2-ol-S), 3,4-Dihydroxyphenyl-γ-valerolactone (3,4-DiHPVL), 3-Hydroxyphenyl-γ-valerolactone-4-glucuronide (3-HPVL-4-GluAC), 3-Hydroxyphenyl-γ-valerolactone-4-sulfate (3HPVL-4-S), 3-Methoxyphenyl-γ-valerolactone-4-glucuronide (3-MPVL-4-GluAC), 3-Methoxyphenyl-γ-valerolactone-4-sulfate (3MPVL-4-S), 4-Hydroxyphenyl-γ-valerolactone-3-glucuronide (4HPVL-3-GluAC), Phenyl-γ-valerolactone-3,4-diglucuronide (PVL-3,4-DiGluAC), Phenyl-γ-valerolactone-3,4-disulfate (PVL-3,4-DiS), Phenyl-γ-valerolactone-3-glucuronide (PVL-3-GluAC), Phenyl-γ-valerolactone-3-sulfate (PVL-3-S), Phenyl-γ-valerolactone-4-sulfate (PVL-4-S), Phenyl-γ-valerolactone-sulfate-glucuronide (PVL-S-GluAC), 4-Hydroxy-3,4-dihydroxyphenylvaleric acid (4-H-3,4-DiHPVA), 4-Hydroxy-3-hydroxyphenylvaleric acid-4-glucuronide (4-H-3-HPVA-4-GluAC), 4-Hydroxy-3-hydroxyphenylvaleric acid-4-sulfate (4-H-3-HPVA-4-S), 4-Hydroxy-4-hydroxyphenylvaleric acid-3-glucuronide (4-H-4-HPVA-3-GluAC), 4-Hydroxy-4-hydroxyphenylvaleric acid-3-sulfate (4-H-4-HPVA-3-S), 4-Hydroxy-phenylvaleric acid-3-glucuronide (4-H-PVA-3-GluAC), 4-Hydroxy-phenylvaleric acid-3-sulfate (4-H-PVA-3-S), 4-Hydroxy-phenylvaleric acid-4-glucuronide (4-H-PVA-4-GluAC), 4-Hydroxy-phenylvaleric acid-4-sulfate (4-H-PVA-4-S), Hydroxyphenylvaleric acid-glucuronide (HPVA-GluAC), Phenylvaleric acid-3-sulfate (PVA-3-S), Phenylvaleric acid-4-glucuronide (PVA-4-GluAC)

Sex Genotype Diet Compound	Female				Male				Kruskal Wallis p.value	
	APOE3/3		APOE4/4		APOE3/3		APOE4/4		Genotype	Sex
	HF ̄x μM/mmol creatinine (SD)	HFEC ̄x μM/mmol creatinine (SD)	HF ̄x μM/mmol creatinine (SD)	HFEC ̄x μM/mmol creatinine (SD)	HF ̄x μM/mmol creatinine (SD)	HFEC ̄x μM/mmol creatinine (SD)	HF ̄x μM/mmol creatinine (SD)	HFEC ̄x μM/mmol creatinine (SD)		
3-O-MeEC	0.000 (0.000)	0.004 (0.000)	0.000 (0.000)	0.002 (0.003)	0.000 (0.000)	0.047 (0.030)	0.000 (0.000)	0.013 (0.008)	0.248	0.021
4-O-MeEC	0.000 (0.000)	0.007 (0.000)	0.000 (0.000)	0.004 (0.002)	0.000 (0.000)	0.042 (0.000)	0.000 (0.000)	0.025 (0.011)	0.248	0.021
EC	0.000 (0.000)	0.029 (0.002)	0.000 (0.000)	0.022 (0.007)	0.000 (0.000)	0.194 (0.090)	0.000 (0.000)	0.048 (0.003)	0.248	0.021
EC-3-GluAC	0.000 (0.000)	0.357 (0.396)	0.000 (0.000)	0.288 (0.076)	0.000 (0.000)	0.193 (0.113)	0.000 (0.000)	0.260 (0.112)	0.564	0.564
EC-3-S	0.000 (0.000)	11.993 (5.299)	0.000 (0.000)	7.756 (2.288)	0.000 (0.000)	3.632 (2.166)	0.000 (0.000)	3.638 (1.336)	0.773	0.021
EC-(4/5/7)-S	0.000 (0.000)	12.098 (5.150)	0.000 (0.000)	8.702 (0.954)	0.000 (0.000)	4.963 (0.186)	0.000 (0.000)	3.637 (1.334)	0.386	0.021
EC-4-GluAC	0.000 (0.000)	1.824 (0.676)	0.000 (0.000)	0.847 (0.150)	0.000 (0.000)	0.904 (0.346)	0.000 (0.000)	0.530 (0.139)	0.083	0.083
EC-5,7-GluAC	0.000 (0.000)	1.772 (0.645)	0.000 (0.000)	0.819 (0.142)	0.000 (0.000)	0.928 (0.362)	0.000 (0.000)	0.514 (0.120)	0.083	0.083
EC-DiGluAC I	0.000 (0.000)	0.103 (0.031)	0.000 (0.000)	0.054 (0.005)	0.000 (0.000)	0.075 (0.014)	0.000 (0.000)	0.048 (0.003)	0.021	0.564
EC-DiGluAC II	0.000 (0.000)	0.013 (0.010)	0.000 (0.000)	0.001 (0.001)	0.000 (0.000)	0.010 (0.005)	0.000 (0.000)	0.005 (0.002)	0.083	0.386
EC-DiGluAC III	0.000 (0.000)	0.011 (0.000)	0.000 (0.000)	0.006 (0.001)	0.000 (0.000)	0.016 (0.009)	0.000 (0.000)	0.004 (0.005)	0.021	1.000
EC-S-GluAC I	0.000 (0.000)	0.656 (0.044)	0.000 (0.000)	0.488 (0.028)	0.000 (0.000)	0.877 (0.387)	0.000 (0.000)	0.194 (0.274)	0.021	0.564
EC-S-GluAC II	0.000	0.669	0.000	0.507	0.000	0.898	0.000	0.195	0.021	0.564

	(0.000)	(0.019)	(0.000)	(0.039)	(0.000)	(0.420)	(0.000)	(0.275)		
EC-S-GluAC III	0.000	2.930	0.000	1.888	0.000	2.968	0.000	1.250	0.043	0.386
	(0.000)	(0.322)	(0.000)	(0.303)	(0.000)	(1.329)	(0.000)	(0.134)		
Me-EC-GluAC I	0.000	0.013	0.000	0.006	0.000	0.008	0.000	0.008	0.083	0.773
	(0.000)	(0.001)	(0.000)	(0.000)	(0.000)	(0.002)	(0.000)	(0.004)		
MeO-EC-GluAC II	0.000	0.136	0.000	0.024	0.000	0.034	0.000	0.080	0.149	0.564
	(0.000)	(0.139)	(0.000)	(0.000)	(0.000)	(0.010)	(0.000)	(0.078)		
Me-EC-GluAC III	0.000	0.064	0.000	0.043	0.000	0.043	0.000	0.119	0.564	0.564
	(0.000)	(0.003)	(0.000)	(0.008)	(0.000)	(0.026)	(0.000)	(0.140)		
MeO-EC-S I	0.000	5.357	0.000	3.521	0.000	1.364	0.000	1.412	0.773	0.021
	(0.000)	(2.414)	(0.000)	(0.425)	(0.000)	(0.686)	(0.000)	(1.101)		
MeO-EC-S II	0.000	0.403	0.000	0.305	0.000	0.231	0.000	0.185	0.248	0.021
	(0.000)	(0.025)	(0.000)	(0.023)	(0.000)	(0.001)	(0.000)	0.054		
MeO-EC-S III	0.000	0.325	0.000	0.224	0.000	0.173	0.000	0.112	0.248	0.021
	(0.000)	(0.058)	(0.000)	(0.020)	(0.000)	(0.037)	(0.000)	(0.026)		
MeO-EC-S-GluAC I	0.000	0.079	0.000	0.047	0.000	0.074	0.000	0.122	0.248	0.564
	(0.000)	(0.002)	(0.000)	(0.000)	(0.000)	(0.016)	(0.000)	(0.113)		
MeO-EC-S-GluAC II	0.000	0.088	0.000	0.048	0.000	0.074	0.000	0.108	0.248	0.773
	(0.000)	(0.010)	(0.000)	(0.001)	(0.000)	(0.016)	(0.000)	(0.093)		
3,4-DiHPP-2-ol	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	NA	NA
	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)		
3,4-DiHPP-2-ol-MeO-GluAC	0.000	0.003	0.000	0.003	0.000	0.000	0.000	0.011	0.139	0.767
	(0.000)	(0.000)	(0.000)	(0.004)	(0.000)	(0.000)	(0.000)	(0.002)		
3,4-DiHPP-2-ol-MeO-S	0.000	0.009	0.000	0.019	0.000	0.025	0.000	0.035	0.386	0.149
	(0.000)	(0.012)	(0.000)	(0.019)	(0.000)	(0.012)	(0.000)	(0.021)		
3,4-DiHPPP-2-ol-S I	0.000	0.241	0.000	0.054	0.000	0.092	0.000	0.097	0.386	0.773
	(0.000)	(0.249)	(0.000)	(0.018)	(0.000)	(0.045)	(0.000)	(0.109)		
3,4-DiHPP-2-ol-S II	0.000	0.281	0.000	0.097	0.000	0.085	0.000	0.185	0.773	0.386
	(0.000)	(0.166)	(0.000)	(0.050)	(0.000)	(0.064)	(0.000)	(0.163)		
HPP-2-ol-GluAC	0.000	0.006	0.000	0.005	0.000	0.003	0.000	0.003	0.773	0.248
	(0.000)	(0.003)	(0.000)	(0.000)	(0.000)	(0.001)	(0.000)	(0.004)		
HPP-2-ol-S	0.000	0.009	0.000	0.010	0.000	0.005	0.000	0.005	0.773	0.248
	(0.000)	(0.010)	(0.000)	(0.001)	(0.000)	(0.005)	(0.000)	(0.007)		
3,4-DiHPVL	0.000	25.408	0.000	22.441	0.000	7.603	0.000	23.212	0.248	0.386
	(0.000)	(20.210)	(0.000)	(1.225)	(0.000)	(9.837)	(0.000)	(4.113)		
3-HPVL-4-GluAC	0.000	0.183	0.000	0.111	0.000	0.083	0.000	0.211	0.564	0.386
	(0.000)	(0.027)	(0.000)	(0.026)	(0.000)	(0.011)	(0.000)	(0.124)		
3-HPVL-4-S	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	NA	NA
	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)	(0.000)		
3-MPVL-4-GluAC	0.000	0.018	0.000	0.008	0.000	0.022	0.000	0.049	0.773	0.083
	(0.000)	(0.012)	(0.000)	(0.002)	(0.000)	(0.015)	(0.000)	(0.049)		
3-MPVL-4-S	0.000	0.044	0.000	0.043	0.000	0.054	0.000	0.040	0.564	0.564
	(0.000)	(0.015)	(0.000)	(0.013)	(0.000)	(0.023)	(0.000)	(0.002)		
4-HPVL-3-GluAC	0.000	0.020	0.000	0.016	0.000	0.011	0.000	0.048	0.384	0.468

[illegible]

Supplementary Table 7: Differential abundance of bacterial genera from the colonic microbiota of female (F) and male (M), APOE4/4-TR (E4) High fat 45% kcal saturated fats (HF) mice.

Group Name	HF APOE4/4-TR Females $\bar{x}$ (SD)	HF APOE4/4-TR Males $\bar{x}$ (SD)	Fold Change	P-value	FDR q
<i>Ligilactobacillus</i>	8.19 (1.4)	9.9 (0.6)	1.21	4.30E-03	0.176
<i>UBA1819</i>	2.89 (0.52)	1.56 (0.39)	0.54	8.00E-03	0.176
<i>Lachnoclostridium</i>	6.75 (0.8)	4.38 (1.62)	0.65	8.70E-03	0.176
<i>Rikenellaceae_RC9_gut_group</i>	4.44 (0.92)	2.39 (1.04)	0.54	1.30E-02	0.185
<i>Anaerofustis</i>	1.39 (0.27)	0.99 (0.2)	0.72	3.00E-02	0.185
<i>Bacteroides</i>	6.02 (0.95)	4.4 (1.34)	0.73	3.00E-02	0.185
<i>Enterorhabdus</i>	5.6 (0.61)	6.39 (0.4)	1.14	3.00E-02	0.185
<i>GCA-900066575</i>	5.91 (0.51)	4.7 (1.24)	0.8	3.00E-02	0.185
<i>Streptococcus</i>	5.7 (0.72)	4.75 (0.45)	0.83	3.00E-02	0.185
<i>[Eubacterium]_xylanophilum_group</i>	3.44 (1.55)	5.03 (0.56)	1.46	3.00E-02	0.185
<i>Paludicola</i>	1.87 (0.54)	1.29 (0.4)	0.69	4.40E-02	0.242
<i>Faecalibaculum</i>	4.94 (0.55)	3.98 (0.73)	0.81	0.052	0.264
<i>unclassified</i>	9.57 (0.35)	8.67 (1.07)	0.91	0.126	0.516
<i>Marvinbryantia</i>	2.3 (0.39)	3.12 (0.86)	1.36	0.142	0.516
<i>Bilophila</i>	4.49 (2.02)	2.57 (1.4)	0.57	0.143	0.516
<i>Oscillibacter</i>	4.87 (0.76)	4.31 (0.6)	0.89	0.17	0.516
<i>Anaerotruncus</i>	5 (0.79)	4.22 (0.54)	0.84	0.177	0.516
<i>Candidatus_Saccharimonas</i>	3.84 (0.57)	3.09 (1.01)	0.8	0.177	0.516
<i>Colidextribacter</i>	5.92 (0.83)	4.97 (1.26)	0.84	0.177	0.516
<i>Harryflintia</i>	1.81 (0.77)	2.32 (0.15)	1.28	0.177	0.516
<i>Parabacteroides</i>	3.38 (0.69)	2.76 (0.68)	0.82	0.177	0.516
<i>Blautia</i>	8.89 (0.53)	8.22 (0.8)	0.92	0.247	0.602
<i>Enterococcus</i>	4.86 (1.28)	3.4 (1.94)	0.7	0.247	0.602
<i>Roseburia</i>	4.06 (1.66)	3.18 (0.41)	0.78	0.247	0.602
<i>UCG-003</i>	2.45 (0.27)	2.28 (0.09)	0.93	0.247	0.602
<i>Candidatus_Arthromitus</i>	2.46 (0.2)	2.35 (0.15)	0.95	0.329	0.743
<i>Intestinimonas</i>	3.76 (1.06)	3.19 (0.83)	0.85	0.329	0.743
<i>Negativibacillus</i>	1.7 (0.44)	1.89 (0.46)	1.11	0.407	0.744
<i>Staphylococcus</i>	2.86 (1.21)	3.64 (2.35)	1.27	0.409	0.744
<i>Erysipelatoclostridium</i>	2.86 (0.59)	3.41 (1.31)	1.19	0.41	0.744
<i>A2</i>	4.37 (0.65)	3.35 (1.58)	0.77	0.429	0.744
<i>ASF356</i>	2.97 (1.09)	3.31 (0.31)	1.11	0.429	0.744
<i>Family_XIII_UCG-001</i>	1.62 (0.53)	1.9 (0.46)	1.17	0.429	0.744
<i>Lactobacillus</i>	8.17 (1.77)	7.65 (2.3)	0.94	0.429	0.744
<i>Turicibacter</i>	4.06 (1.68)	4.77 (0.76)	1.17	0.429	0.744
<i>Tuzzerella</i>	3.76 (1.45)	3.15 (1.14)	0.84	0.46	0.744
<i>Alistipes</i>	3.26 (1.49)	2.79 (1.16)	0.86	0.464	0.744
<i>Lachnospiraceae_NK4A136_group</i>	5.16 (0.88)	4.61 (0.75)	0.89	0.464	0.744
<i>UCG-009</i>	1.86 (0.71)	1.63 (0.42)	0.87	0.522	0.744
<i>Akkermansia</i>	5.59 (1.91)	6.34 (1.98)	1.13	0.537	0.744
<i>Clostridium_sensu_stricto_1</i>	5.9 (0.58)	6.04 (0.63)	1.02	0.537	0.744
<i>Escherichia-Shigella</i>	4.65 (3.27)	7.42 (0.72)	1.6	0.537	0.744
<i>Peptococcus</i>	2.07 (1)	2.04 (0.78)	0.99	0.537	0.744
<i>Romboutsia</i>	7.31 (0.55)	5.44 (2.69)	0.74	0.537	0.744
<i>UCG-005</i>	1.27 (0.11)	1.22 (0.3)	0.96	0.647	0.777
<i>Odoribacter</i>	3.21 (1.34)	3.54 (0.22)	1.1	0.647	0.777
<i>V1</i>	7.82 (0.85)	7.5 (0.71)	0.96	0.662	0.777
<i>Acetatifactor</i>	4.7 (0.52)	4.8 (0.59)	1.02	0.662	0.777
<i>Butyricicoccus</i>	2.21 (0.95)	1.81 (0.25)	0.82	0.662	0.777
<i>Lachnospiraceae_FCS020_group</i>	3.17 (0.9)	2.75 (1.05)	0.87	0.662	0.777
<i>Limosilactobacillus</i>	2.66 (0.49)	2.75 (0.14)	1.04	0.662	0.777
<i>Rikenella</i>	2.79 (0.73)	3.01 (0.29)	1.08	0.662	0.777
<i>Family_XIII_AD3011_group</i>	1.46 (0.76)	1.66 (0.33)	1.13	0.714	0.819
<i>[Eubacterium]_brachy_group</i>	3.15 (0.85)	3.01 (0.86)	0.96	0.782	0.819
<i>Desulfovibrio</i>	6.06 (1.01)	6.2 (0.96)	1.02	0.792	0.819
<i>Lachnospiraceae_UCG-006</i>	4.36 (0.61)	4.52 (1.11)	1.04	0.792	0.819
<i>Lactococcus</i>	7.68 (0.21)	7.77 (0.42)	1.01	0.792	0.819
<i>unidentified_Lachnospiraceae</i>	2.88 (0.32)	3.09 (0.72)	1.07	0.792	0.819
<i>unidentified_Oscillospiraceae</i>	4.69 (0.69)	4.55 (0.49)	0.97	0.792	0.819
<i>[Eubacterium]_ventriosum_group</i>	1.69 (0.31)	1.7 (0.39)	1.01	0.855	0.869
<i>Mucispirillum</i>	3.9 (1.73)	4.64 (0.64)	1.19	1	1

Supplementary Table 8: Rodent diets used in Chapter 5

Diet Ingredient	Low Fat Mass (g)	High Fat Mass (g)	High Fat with Flavanols Mass (g)
Casein, 80 Mesh	198.9	199.1	195.9
L-Cysteine	3.000	3.000	3.000
Corn Starch	449.700	70.900	86.900
Maltodextrin 10	75.000	100.000	100.000
Sucrose	130.100	138.000	156.500
Cellulose, BW200	48.400	48.700	45.900
Palm Oil	24.800	152.880	147.280
Corn Oil	6.200	38.220	36.820
Mineral Mix S10026	10.000	10.000	10.000
DiCalcium Phosphate	13.000	13.000	13.000
Calcium Carbonate	5.500	5.500	5.500
Potassium Citrate.1H2O	16.500	16.500	16.500
Vitamin Mix V10001	10.000	10.000	10.000
Choline Bitartrate	2.000	2.000	2.000
Royal Cocoa Drops-2011	61.064	49.671	0.000
CHD-Q65ACTICOA-558	0.000	0.000	50.980
Caffeine	0.0306	0.0249	0.000
Theobromine	0.2165	0.176	0.000
FD&C Yellow Dye #5	0.025	0.000	0.050
FD&C Red Dye #40	0.025	0.025	0.000
FD&C Blue Dye #1	0.000	0.025	0.000
Totals	1054.461	857.722	880.33
Nutritionals	Kcal %	Kcal %	Kcal %
Protein	20	20	20
carbohydrate	70	35	35
Fat	10	45	45

Supplementary Table 9: Differential abundance of the microbiota of participants from the Dietary pLAnt extracts, colonic Microbes, and apoe geNetics interactions study (DIAMOND). Participants were stratified by sex: females (F), Males (M), APOE genotype: APOE4 carrier status: APOE2/3, APOE2/2, and APOE3/3 (NonE4) and APOE3/4, and APOE4/4 (E4), and age: 18-35years old (Younger), 55+ years old (Older). Median value and interquartile range in brackets. Comparisons made through Wilcoxon producing a p-value (p) and then adujusted with Benjamini-Hochburg to adjust for false discovery rate (q)

Age	O	Y	O	Y	O	Y	O	Y	Comparison											
Sex	F	F	M	M	F	F	M	M	E4 F O	E4 F O	E4 F Y	E4 M O	E4 M O	E4 M O	E4 M Y	E4 M Y	nonE4 F O	nonE4 M O	nonE4 M O	nonE4 M Y
Gen	E4	E4	E4	E4	Non E4	Non E4	Non E4	Non E4	E4 F Y	nonE4 F O	nonE4 F Y	E4 F O	E4 M Y	nonE4 M O	E4 F Y	nonE4 M Y	nonE4 F Y	nonE4 F O	nonE4 M Y	nonE4 F Y
Genus	X̄ (IQR)								p (FDR q)											
Acidaminococcus	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.001 (0.004)	0.314 (0.783)	NA	0.571 (0.924)	NA	NA	0.499 (0.946)	0.663 (0.897)	0.220 (0.967)	NA	0.571 (0.852)	0.337 (1.000)	0.149 (0.973)
Acinetobacter	0.019 (0.013)	0.014 (0.015)	0.029 (0.012)	0.006 (0.003)	0.010 (0.010)	0.021 (0.012)	0.024 (0.016)	0.024 (0.014)	0.837 (0.969)	0.414 (0.930)	0.788 (1.000)	0.190 (0.896)	0.036 (0.855)	0.876 (1.000)	0.117 (0.897)	0.048 (0.967)	0.343 (0.794)	0.164 (0.741)	0.836 (1.000)	0.610 (0.973)
Actinomyces	0.019 (0.014)	0.009 (0.005)	0.014 (0.014)	0.005 (0.007)	0.009 (0.019)	0.008 (0.010)	0.013 (0.007)	0.005 (0.004)	0.008 (0.506)	0.330 (0.930)	1.000 (1.000)	0.438 (0.930)	0.571 (0.855)	1.000 (1.000)	1.000 (1.000)	0.714 (0.991)	1.000 (1.000)	0.648 (0.882)	0.014 (1.000)	0.521 (0.973)
Adhaeribacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.641 (0.882)	1.000 (1.000)	0.571 (0.924)	1.000 (1.000)	0.606 (0.855)	0.803 (1.000)	0.663 (0.897)	0.637 (0.967)	0.453 (0.794)	0.674 (0.882)	0.910 (1.000)	0.540 (0.973)
Adlercreutzia	0.094 (0.213)	0.081 (0.045)	0.065 (0.041)	0.075 (0.027)	0.127 (0.144)	0.050 (0.019)	0.066 (0.063)	0.034 (0.100)	0.918 (0.992)	0.503 (0.930)	0.230 (0.924)	0.699 (0.970)	1.000 (1.000)	0.639 (1.000)	0.517 (0.897)	0.548 (0.967)	0.029 (0.794)	0.042 (0.741)	0.945 (1.000)	0.914 (1.000)
Aeromicrobium	0.005 (0.003)	0.002 (0.002)	0.002 (0.000)	0.004 (0.004)	0.003 (0.003)	0.003 (0.002)	0.000 (0.002)	0.003 (0.004)	0.286 (0.783)	0.938 (1.000)	0.924 (1.000)	0.503 (0.930)	0.653 (0.890)	0.178 (0.946)	0.818 (0.926)	1.000 (1.000)	0.772 (1.000)	0.276 (0.741)	0.091 (1.000)	0.392 (0.973)
Aeromonas	0.003 (0.005)	0.002 (0.002)	0.000 (0.004)	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.002 (0.003)	0.001 (0.002)	0.581 (0.868)	0.450 (0.930)	0.536 (0.924)	0.569 (0.937)	0.329 (0.855)	0.931 (1.000)	0.158 (0.897)	0.220 (0.967)	1.000 (1.000)	0.680 (0.882)	0.881 (1.000)	0.810 (1.000)
Aestuariivirga	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.001 (0.002)	0.607 (0.877)	0.251 (0.930)	0.810 (1.000)	0.738 (1.000)	0.606 (0.855)	0.803 (1.000)	0.416 (0.897)	0.220 (0.967)	0.453 (0.794)	0.571 (0.852)	0.257 (1.000)	0.337 (0.973)
Agathobacter	0.980 (8.463)	12.467 (12.226)	1.495 (3.089)	6.298 (1.109)	3.658 (7.675)	2.399 (3.698)	3.884 (4.777)	3.208 (7.766)	0.091 (0.735)	0.604 (0.930)	0.109 (0.924)	0.606 (0.953)	0.250 (0.855)	1.000 (1.000)	0.667 (0.897)	0.548 (0.967)	0.886 (1.000)	0.788 (0.945)	1.000 (1.000)	0.476 (0.973)
Agathobaculum	0.062 (0.081)	0.169 (0.151)	0.126 (0.045)	0.047 (0.054)	0.108 (0.051)	0.004 (0.087)	0.144 (0.171)	0.091 (0.068)	0.210 (0.783)	0.330 (0.930)	0.164 (0.924)	0.240 (0.896)	0.250 (0.855)	0.755 (1.000)	0.117 (0.897)	0.714 (0.991)	0.343 (0.794)	0.927 (1.000)	0.628 (1.000)	0.257 (0.973)
Agrobacterium	0.009 (0.010)	0.002 (0.008)	0.010 (0.005)	0.008 (0.010)	0.004 (0.006)	0.007 (0.011)	0.009 (0.006)	0.012 (0.009)	0.089 (0.735)	0.260 (0.930)	0.294 (0.924)	0.797 (1.000)	0.786 (0.940)	0.343 (0.946)	0.166 (0.897)	1.000 (1.000)	0.486 (0.794)	0.570 (0.852)	0.366 (1.000)	0.476 (0.973)
Agromyces	0.000 (0.000)	0.000 (0.000)	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.232 (0.783)	0.391 (0.930)	NA (0.930)	0.214 (0.896)	0.172 (0.855)	0.033 (0.946)	NA	NA	NA	NA	NA	NA
Akkermansia	0.157 (0.572)	0.009 (0.247)	0.043 (0.021)	0.010 (0.048)	0.360 (0.618)	0.010 (0.058)	0.020 (0.070)	0.004 (0.004)	0.252 (0.783)	0.940 (1.000)	0.927 (1.000)	0.147 (0.896)	0.571 (0.855)	0.745 (1.000)	0.732 (0.926)	0.795 (1.000)	0.343 (0.794)	0.107 (0.741)	0.615 (1.000)	0.114 (0.973)
Alistipes	0.058 (0.286)	0.095 (0.103)	0.021 (0.003)	0.008 (0.023)	0.130 (0.244)	0.031 (0.059)	0.011 (0.023)	0.029 (0.076)	0.758 (0.953)	0.825 (1.000)	0.788 (1.000)	0.019 (0.859)	0.786 (0.940)	0.876 (1.000)	0.267 (0.897)	0.905 (1.000)	0.486 (0.794)	0.164 (0.741)	0.628 (1.000)	0.914 (1.000)
Allisonella	0.000 (0.000)	0.000 (0.000)	0.000 (0.005)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	NA (0.924)	NA (0.930)	0.257 (0.924)	0.063 (0.859)	0.733 (0.938)	0.394 (0.946)	0.190 (0.897)	0.239 (0.967)	0.453 (0.794)	0.571 (0.852)	0.440 (1.000)	0.307 (0.973)
Alloscardovia	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.002 (0.004)	0.000 (0.001)	0.000 (0.000)	0.450 (0.783)	0.540 (0.930)	0.068 (0.924)	0.662 (0.970)	0.606 (0.855)	1.000 (1.000)	NA (0.967)	0.637 (0.794)	0.408 (1.000)	1.000 (1.000)	0.847 (1.000)	0.512 (0.973)
Amedibacillus	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.009)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.854 (0.971)	0.540 (0.930)	0.889 (1.000)	0.551 (0.930)	NA (0.855)	NA (1.000)	0.663 (0.897)	0.637 (0.967)	1.000 (1.000)	0.257 (0.741)	0.355 (1.000)	1.000 (1.000)
Amedibacterium	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.120 (0.757)	0.251 (0.930)	0.257 (0.924)	0.194 (0.896)	NA (0.855)	NA (1.000)	NA (0.897)	NA (0.967)	0.453 (0.794)	NA (1.000)	NA (1.000)	0.307 (0.973)
Aminipila	0.007 (0.047)	0.002 (0.007)	0.007 (0.012)	0.000 (0.000)	0.007 (0.061)	0.001 (0.016)	0.000 (0.002)	0.001 (0.006)	0.439 (0.783)	0.812 (1.000)	0.921 (1.000)	0.892 (1.000)	0.081 (0.855)	0.100 (0.946)	0.158 (0.897)	0.220 (0.967)	0.460 (0.794)	0.149 (0.741)	0.515 (1.000)	1.000 (1.000)
Anaerobutyricum	1.405 (1.603)	1.805 (0.853)	2.583 (1.055)	1.406 (0.356)	2.828 (0.750)	1.228 (0.515)	1.664 (0.851)	1.574 (1.171)	0.351 (0.783)	0.050 (0.930)	0.164 (0.924)	0.147 (0.896)	0.143 (0.855)	0.202 (0.946)	0.183 (0.897)	0.905 (1.000)	0.057 (0.794)	0.042 (0.741)	1.000 (1.000)	0.476 (0.973)
Anaerococcus	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.232 (0.783)	0.391 (0.930)	0.257 (0.924)	0.559 (0.930)	0.329 (0.855)	0.105 (0.946)	NA (0.897)	NA (0.967)	0.453 (0.794)	NA (1.000)	NA (1.000)	0.307 (0.973)
Anaerofilum	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.001 (0.003)	0.000 (0.000)	0.000 (0.000)	0.000 (0.004)	0.520 (0.825)	0.300 (0.930)	0.186 (0.924)	1.000 (1.000)	0.606 (0.855)	0.310 (0.946)	0.262 (0.897)	0.377 (0.967)	0.186 (0.794)	0.068 (0.741)	0.140 (1.000)	0.287 (0.973)
Anaerofustis	0.002 (0.018)	0.000 (0.002)	0.002 (0.002)	0.000 (0.000)	0.000 (0.001)	0.005 (0.013)	0.000 (0.001)	0.000 (0.002)	0.309 (0.783)	0.356 (0.930)	0.471 (0.924)	0.579 (0.948)	0.172 (0.855)	0.469 (0.946)	0.262 (0.897)	0.377 (0.967)	0.408 (0.794)	1.000 (1.000)	0.930 (1.000)	0.401 (0.973)
Anaeromassilibacillus	0.011 (0.008)	0.000 (0.005)	0.005 (0.007)	0.002 (0.001)	0.001 (0.005)	0.014 (0.014)	0.000 (0.004)	0.004 (0.008)	0.014 (0.506)	0.076 (0.930)	0.042 (0.924)	0.699 (0.970)	0.036 (0.855)	0.130 (0.946)	1.000 (1.000)	0.358 (0.967)	0.191 (0.794)	0.743 (0.942)	0.313 (1.000)	0.336 (0.973)
Anaeroplasma	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	NA (0.930)	0.674 (0.973)	NA (1.000)	NA (0.855)	NA (0.946)	0.663 (0.897)	NA (0.967)	0.453 (0.794)	NA (1.000)	NA (1.000)	0.307 (0.973)
Anaerostipes	1.981 (0.909)	3.546 (3.436)	1.630 (1.720)	0.455 (0.873)	3.628 (2.113)	1.871 (4.185)	2.502 (1.577)	1.897 (2.418)	0.114 (0.757)	0.414 (0.930)	0.315 (0.924)	0.797 (1.000)	0.393 (0.855)	0.530 (0.946)	0.067 (0.897)	0.262 (0.967)	0.686 (0.980)	0.315 (0.741)	0.945 (1.000)	1.000 (1.000)

Anaerotignum	0.010 (0.029)	0.023 (0.041)	0.005 (0.010)	0.000 (0.007)	0.022 (0.009)	0.001 (0.006)	0.002 (0.016)	0.000 (0.004)	0.915 (0.992)	0.757 (1.000)	0.209 (0.924)	0.364 (0.896)	0.549 (0.855)	0.744 (1.000)	0.240 (0.897)	1.000 (1.000)	0.183 (0.794)	0.439 (0.852)	0.393 (1.000)	0.904 (1.000)
Anaerotruncus	0.000 (0.002)	0.000 (0.007)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.808 (0.969)	0.777 (1.000)	0.186 (0.924)	0.194 (0.896)	NA (0.897)	NA (0.897)	0.262 (0.967)	0.637 (0.967)	0.453 (0.794)	0.257 (0.741)	0.355 (1.000)	0.540 (0.973)
Anaerovorax	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	0.257 (0.924)	0.551 (0.930)	NA (0.946)	0.499 (0.946)	NA (0.967)	0.637 (0.967)	0.453 (0.794)	0.571 (0.852)	1.000 (1.000)	1.000 (1.000)
Anthropogastromicrobium	0.464 (1.432)	0.498 (0.688)	0.766 (1.140)	0.228 (1.142)	2.113 (1.720)	0.005 (0.098)	0.015 (0.301)	0.336 (0.660)	0.470 (0.799)	0.330 (0.930)	0.042 (0.924)	0.364 (0.896)	0.786 (0.940)	0.268 (0.946)	0.833 (0.926)	0.714 (0.991)	0.057 (0.794)	0.042 (0.741)	0.534 (1.000)	0.171 (0.973)
Aquabacterium	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.440 (0.783)	0.391 (0.930)	0.186 (0.924)	0.325 (0.896)	NA (0.946)	0.261 (0.897)	0.262 (0.967)	0.377 (0.967)	NA (0.741)	0.327 (0.741)	1.000 (1.000)	0.287 (0.973)
Aquariibacter	0.009 (0.019)	0.009 (0.008)	0.014 (0.015)	0.000 (0.001)	0.012 (0.008)	0.007 (0.005)	0.010 (0.017)	0.007 (0.004)	0.681 (0.915)	1.000 (1.000)	0.788 (1.000)	0.699 (0.970)	0.036 (0.855)	0.639 (1.000)	0.022 (0.897)	0.088 (0.967)	0.486 (0.794)	0.788 (0.945)	0.731 (1.000)	0.914 (1.000)
Arboricoccus	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.854 (0.971)	0.540 (0.930)	0.674 (0.973)	0.551 (0.930)	NA (0.930)	NA (0.930)	0.663 (0.897)	NA (1.000)	1.000 (0.741)	0.257 (0.741)	NA (1.000)	0.307 (0.973)
Arenivirga	0.003 (0.005)	0.000 (0.013)	0.007 (0.005)	0.002 (0.004)	0.001 (0.004)	0.003 (0.009)	0.003 (0.005)	0.004 (0.004)	0.334 (0.783)	0.393 (0.930)	1.000 (1.000)	0.841 (1.000)	0.653 (0.890)	0.935 (1.000)	1.000 (0.967)	0.437 (0.967)	0.878 (1.000)	0.215 (0.741)	0.836 (1.000)	0.593 (0.973)
Arthrobacter	0.009 (0.012)	0.012 (0.012)	0.012 (0.002)	0.002 (0.003)	0.004 (0.008)	0.003 (0.007)	0.013 (0.008)	0.007 (0.012)	0.750 (0.953)	0.187 (0.930)	0.287 (0.924)	0.364 (0.896)	0.036 (0.855)	1.000 (1.000)	0.489 (0.897)	0.511 (0.967)	1.000 (1.000)	0.072 (0.741)	0.283 (1.000)	0.441 (0.973)
Azohydromonas	0.000 (0.002)	0.000 (0.001)	0.002 (0.014)	0.000 (0.000)	0.000 (0.001)	0.001 (0.003)	0.002 (0.007)	0.002 (0.004)	0.898 (0.992)	1.000 (1.000)	0.743 (1.000)	0.154 (0.896)	0.081 (0.855)	0.457 (0.946)	0.416 (0.897)	0.220 (0.967)	0.620 (0.942)	0.410 (0.848)	0.881 (1.000)	0.570 (0.973)
Azospirillum	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.120 (0.757)	0.925 (1.000)	NA (1.000)	0.738 (1.000)	0.606 (0.855)	0.310 (0.946)	NA (0.967)	0.637 (0.967)	0.453 (0.794)	0.257 (0.741)	0.355 (1.000)	0.540 (0.973)
Bacillus	0.000 (0.002)	0.000 (0.002)	0.000 (0.000)	0.002 (0.002)	0.000 (0.001)	0.003 (0.020)	0.002 (0.018)	0.002 (0.004)	0.907 (0.992)	0.379 (0.930)	0.606 (0.954)	0.351 (0.896)	0.306 (0.855)	0.119 (0.946)	0.808 (0.926)	0.896 (1.000)	0.408 (0.794)	0.092 (0.741)	0.828 (1.000)	0.912 (1.000)
Bacteroides	0.184 (0.330)	0.175 (0.442)	0.085 (0.073)	0.056 (0.254)	0.405 (0.567)	0.058 (0.625)	0.044 (0.041)	0.112 (0.204)	0.837 (0.969)	1.000 (1.000)	0.927 (1.000)	0.364 (0.896)	1.000 (1.000)	0.432 (0.946)	0.833 (0.926)	1.000 (1.000)	0.886 (1.000)	0.315 (0.741)	0.628 (1.000)	1.000 (1.000)
Barnesiella	0.012 (0.087)	0.030 (0.035)	0.014 (0.012)	0.002 (0.029)	0.015 (0.024)	0.003 (0.013)	0.009 (0.014)	0.008 (0.022)	1.000 (1.000)	0.825 (1.000)	0.636 (0.968)	0.947 (1.000)	0.881 (1.000)	0.870 (1.000)	0.644 (0.897)	0.896 (1.000)	0.343 (0.794)	0.507 (0.852)	0.720 (1.000)	0.914 (1.000)
Barrientosiimonas	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.713 (0.971)	0.889 (1.000)	0.551 (0.930)	NA (0.897)	NA (0.897)	0.663 (0.967)	0.637 (0.967)	1.000 (1.000)	0.257 (0.741)	0.355 (1.000)	0.761 (0.973)
Bdellovibrio	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.713 (0.971)	0.571 (0.924)	0.827 (1.000)	0.606 (0.855)	0.310 (0.946)	0.663 (0.897)	NA (1.000)	0.453 (0.794)	0.257 (0.741)	NA (1.000)	NA (0.973)
Beduinibacterium	0.008 (0.012)	0.000 (0.002)	0.002 (0.002)	0.000 (0.000)	0.003 (0.003)	0.000 (0.001)	0.002 (0.003)	0.001 (0.002)	0.047 (0.707)	0.587 (0.930)	0.810 (1.000)	0.349 (0.886)	0.081 (0.855)	0.457 (0.946)	0.416 (0.897)	0.220 (0.967)	0.356 (0.794)	0.439 (0.852)	0.881 (1.000)	0.631 (0.973)
Bifidobacterium	4.037 (16.968)	3.128 (7.137)	3.346 (8.836)	22.658 (16.685)	3.096 (5.001)	32.550 (32.939)	5.205 (18.533)	11.827 (21.969)	0.536 (0.825)	0.414 (0.930)	0.012 (0.848)	1.000 (1.000)	0.250 (0.855)	0.876 (0.946)	0.117 (0.897)	0.548 (0.967)	0.029 (0.794)	0.788 (0.945)	0.836 (1.000)	0.257 (0.973)
Bilophila	0.009 (0.065)	0.002 (0.010)	0.002 (0.000)	0.000 (0.004)	0.004 (0.004)	0.001 (0.003)	0.002 (0.002)	0.000 (0.003)	0.744 (0.953)	0.579 (0.930)	0.384 (0.924)	0.785 (1.000)	0.541 (0.855)	0.363 (0.946)	0.481 (0.897)	0.878 (1.000)	0.460 (0.794)	0.246 (0.741)	0.938 (1.000)	0.719 (0.973)
Blautia	12.915 (18.974)	28.390 (13.876)	19.058 (10.857)	15.488 (12.709)	19.419 (8.753)	19.864 (10.423)	22.765 (17.460)	16.766 (10.091)	0.114 (0.757)	0.414 (0.930)	0.230 (0.924)	0.438 (0.930)	1.000 (1.000)	0.876 (1.000)	0.667 (0.897)	0.905 (1.000)	1.000 (1.000)	0.927 (1.000)	0.534 (1.000)	1.000 (1.000)
Bosea	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	NA (0.896)	NA (0.855)	NA (1.000)	0.233 (0.896)	0.606 (0.855)	0.831 (1.000)	NA (0.967)	0.637 (0.967)	NA (0.741)	0.327 (0.741)	0.699 (1.000)	0.540 (0.973)
Brachybacterium	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.001 (0.002)	0.000 (0.001)	0.000 (0.000)	0.001 (0.002)	0.835 (0.969)	0.396 (0.930)	0.810 (1.000)	1.000 (1.000)	0.606 (0.855)	0.803 (1.000)	0.416 (0.897)	0.220 (0.967)	0.408 (0.794)	0.279 (0.741)	0.337 (1.000)	0.337 (0.973)
Bradyrhizobium	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.003 (0.001)	0.000 (0.001)	0.002 (0.003)	0.000 (0.000)	0.898 (0.992)	0.111 (0.930)	0.810 (1.000)	0.738 (1.000)	0.696 (0.926)	0.205 (0.946)	1.000 (1.000)	0.596 (0.967)	0.124 (0.794)	0.699 (0.903)	0.143 (1.000)	1.000 (1.000)
Brevibacterium	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.266 (0.783)	0.713 (0.971)	0.444 (0.924)	0.827 (1.000)	0.606 (0.855)	0.310 (0.946)	0.262 (0.897)	NA (1.000)	1.000 (0.741)	0.257 (0.741)	NA (1.000)	0.307 (0.973)
Breznakia	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	0.182 (0.930)	0.571 (0.924)	NA (0.924)	NA (0.855)	NA (0.946)	0.663 (0.897)	NA (1.000)	0.453 (0.794)	0.257 (0.741)	NA (1.000)	NA (0.973)
Brucella	0.005 (0.007)	0.005 (0.002)	0.002 (0.004)	0.006 (0.007)	0.003 (0.003)	0.002 (0.005)	0.002 (0.003)	0.006 (0.012)	0.351 (0.783)	0.199 (0.930)	0.294 (0.924)	0.062 (0.859)	0.368 (0.855)	0.866 (1.000)	0.667 (0.897)	0.897 (1.000)	0.882 (1.000)	0.562 (0.852)	0.419 (1.000)	0.441 (0.973)
Buchnera	0.009 (0.007)	0.005 (0.006)	0.009 (0.010)	0.002 (0.002)	0.013 (0.019)	0.001 (0.002)	0.005 (0.005)	0.009 (0.019)	0.038 (0.707)	0.372 (1.000)	0.372 (0.924)	0.947 (1.000)	0.177 (0.855)	0.326 (0.946)	0.557 (0.897)	0.787 (1.000)	0.183 (0.794)	0.504 (0.852)	1.000 (1.000)	0.570 (0.973)
Burkholderia	0.002 (0.003)	0.002 (0.001)	0.002 (0.002)	0.002 (0.003)	0.003 (0.006)	0.000 (0.001)	0.004 (0.003)	0.003 (0.004)	0.166 (0.783)	0.814 (1.000)	0.092 (0.924)	0.281 (0.896)	1.000 (1.000)	0.408 (0.946)	1.000 (1.000)	1.000 (1.000)	0.408 (0.794)	0.923 (1.000)	0.942 (1.000)	0.139 (0.973)
Butyricoccus	0.000 (0.002)	0.000 (0.000)	0.002 (0.002)	0.002 (0.004)	0.001 (0.003)	0.000 (0.000)	0.034 (0.058)	0.000 (0.000)	0.487 (0.802)	0.792 (1.000)	0.571 (0.924)	0.460 (0.930)	1.000 (1.000)	0.321 (0.946)	0.262 (0.897)	0.357 (0.967)	0.186 (0.794)	0.287 (0.741)	0.052 (1.000)	0.540 (0.973)
Butyricimonas	0.002 (0.002)	0.002 (0.008)	0.002 (0.002)	0.000 (0.002)	0.004 (0.007)	0.000 (0.001)	0.000 (0.001)	0.001 (0.001)	0.385 (0.783)	0.234 (0.930)	0.198 (0.924)	0.677 (0.970)	1.000 (1.000)	0.277 (0.946)	0.347 (0.897)	0.671 (0.991)	0.219 (0.794)	0.099 (0.741)	0.415 (1.000)	0.471 (0.973)
Butyrivibrio	0.002 (0.025)	0.000 (0.000)	0.002 (0.005)	0.000 (0.004)	0.000 (0.001)	0.000 (0.005)	0.000 (0.023)	0.000 (0.000)	0.029 (0.707)	0.208 (0.930)	0.257 (0.924)	1.000 (1.000)	0.751 (0.940)	0.587 (0.998)	0.190 (0.897)	0.860 (1.000)	1.000 (1.000)	0.810 (0.952)	0.699 (1.000)	1.000 (1.000)
Caproicibacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.232 (0.783)	0.391 (0.930)	NA (1.000)	0.802 (1.000)	1.000 (1.000)	0.394 (0.946)	0.190 (0.897)	0.878 (1.000)	NA (0.852)	0.571 (1.000)	0.385 (1.000)	0.287 (0.973)

<i>Carnobacterium</i>	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.118 (0.757)	NA	0.327 (0.924)	0.233 (0.896)	0.696 (0.926)	0.334 (0.946)	1.000 (1.000)	0.596 (0.967)	NA	0.186 (0.741)	0.257 (1.000)	0.540 (0.973)
<i>Catenibacillus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.020)	0.002 (0.001)	0.002 (0.008)	0.000 (0.001)	0.000 (0.000)	0.000 (0.003)	0.232 (0.783)	0.509 (0.930)	0.257 (0.924)	0.452 (0.930)	1.000 (1.000)	0.287 (0.946)	0.034 (0.897)	0.888 (1.000)	0.620 (0.942)	0.279 (0.741)	0.499 (1.000)	0.694 (0.973)
<i>Catenibacterium</i>	0.000 (0.002)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.003 (0.094)	0.002 (0.001)	0.000 (0.001)	0.000 (0.000)	0.607 (0.877)	0.333 (0.930)	0.303 (0.924)	0.877 (1.000)	0.329 (0.855)	0.772 (1.000)	0.416 (0.897)	0.637 (0.967)	0.882 (1.000)	0.584 (0.854)	0.847 (1.000)	0.230 (0.973)
<i>Caulobacter</i>	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.001 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.535 (0.825)	0.251 (0.930)	0.327 (0.924)	0.354 (0.896)	0.329 (0.855)	0.287 (0.946)	0.416 (0.897)	0.637 (0.967)	0.186 (0.794)	0.279 (0.741)	0.910 (1.000)	0.540 (0.973)
<i>Cellulomonas</i>	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.535 (0.825)	0.617 (0.930)	1.000 (1.000)	0.354 (0.896)	0.329 (0.855)	0.772 (1.000)	0.416 (0.897)	0.637 (0.967)	0.453 (0.794)	0.327 (0.741)	0.562 (1.000)	0.761 (0.973)
<i>Cellulosilyticum</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.004)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	NA (0.930)	0.551 (0.930)	NA (0.946)	0.261 (0.946)	NA	NA	NA	0.327 (0.741)	0.212 (1.000)	NA (0.973)
<i>Cellvibrio</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.450 (0.783)	0.713 (0.971)	0.257 (0.924)	0.551 (0.930)	0.302 (0.855)	0.499 (0.946)	0.190 (0.897)	0.239 (0.967)	1.000 (1.000)	0.674 (0.882)	0.440 (1.000)	0.307 (0.973)
<i>Cephalothrix</i>	0.000 (0.002)	0.002 (0.005)	0.002 (0.000)	0.000 (0.000)	0.002 (0.002)	0.000 (0.001)	0.000 (0.001)	0.001 (0.004)	0.351 (0.783)	0.208 (0.930)	0.410 (0.924)	0.096 (0.896)	0.032 (0.855)	0.043 (0.946)	0.158 (0.897)	0.220 (0.967)	0.538 (0.864)	0.216 (0.741)	0.328 (1.000)	0.631 (0.973)
<i>Chitinimonas</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	0.257 (0.924)	0.827 (1.000)	0.606 (0.855)	0.310 (0.946)	NA	NA	0.453 (0.794)	NA	NA	0.307 (0.973)
<i>Christensenella</i>	0.002 (0.003)	0.000 (0.000)	0.002 (0.002)	0.000 (0.000)	0.001 (0.004)	0.000 (0.000)	0.000 (0.001)	0.002 (0.004)	0.013 (0.506)	0.696 (0.971)	0.571 (0.924)	0.138 (0.896)	0.172 (0.855)	0.587 (0.998)	0.663 (0.897)	0.220 (0.967)	0.186 (0.794)	0.325 (0.741)	0.254 (1.000)	0.149 (0.973)
<i>Chryseobacterium</i>	0.002 (0.005)	0.000 (0.004)	0.000 (0.002)	0.002 (0.002)	0.000 (0.001)	0.002 (0.006)	0.000 (0.002)	0.003 (0.783)	0.197 (0.930)	0.060 (1.000)	0.918 (0.896)	0.092 (0.886)	0.634 (0.998)	0.587 (0.998)	0.808 (0.926)	0.604 (0.967)	0.408 (0.794)	0.444 (0.852)	0.826 (1.000)	0.746 (0.973)
<i>Cloacibacillus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	0.257 (0.924)	0.551 (0.930)	NA (0.930)	NA (0.946)	NA	0.637 (0.967)	0.453 (0.794)	NA	0.355 (1.000)	1.000 (1.000)
<i>Clostridiumsensustricto</i>	0.323 (5.712)	0.368 (1.031)	1.588 (2.511)	0.253 (0.472)	0.048 (0.372)	0.259 (0.570)	0.126 (0.566)	0.133 (0.818)	0.408 (0.783)	0.076 (0.930)	1.000 (1.000)	0.606 (0.953)	0.571 (0.855)	0.343 (0.946)	0.833 (0.926)	1.000 (1.000)	0.200 (0.794)	0.927 (1.000)	0.731 (1.000)	0.914 (1.000)
<i>Colidextribacter</i>	0.038 (0.048)	0.002 (0.032)	0.039 (0.010)	0.000 (0.012)	0.057 (0.097)	0.008 (0.003)	0.010 (0.055)	0.028 (0.029)	0.090 (0.735)	0.825 (1.000)	0.776 (1.000)	1.000 (1.000)	0.036 (0.855)	0.755 (1.000)	0.347 (0.897)	0.237 (0.967)	0.343 (0.794)	0.705 (0.906)	0.775 (1.000)	0.476 (0.973)
<i>Collinsella</i>	2.905 (6.179)	3.653 (4.083)	5.695 (1.860)	4.095 (2.902)	3.439 (2.621)	1.923 (1.331)	3.476 (4.411)	6.373 (4.272)	0.536 (0.825)	0.940 (1.000)	0.788 (1.000)	0.518 (0.930)	0.571 (0.855)	0.876 (1.000)	0.833 (0.926)	0.262 (0.967)	0.486 (0.794)	0.788 (0.945)	0.366 (1.000)	0.067 (0.973)
<i>Coprobacillus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.005)	0.000 (0.000)	NA	NA	NA	0.233 (0.896)	0.606 (0.855)	0.831 (1.000)	NA	NA	NA	0.327 (0.741)	0.212 (1.000)	NA
<i>Coprobacter</i>	0.000 (0.016)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.001 (0.003)	0.000 (0.028)	0.000 (0.000)	0.001 (0.002)	0.560 (0.853)	0.801 (1.000)	0.913 (1.000)	0.113 (0.896)	NA (0.946)	0.499 (0.897)	0.262 (0.920)	0.220 (1.000)	0.869 (0.741)	0.279 (1.000)	0.257 (1.000)	0.810 (1.000)
<i>Coprococcus</i>	0.557 (1.552)	1.342 (1.401)	2.373 (1.024)	0.503 (1.892)	2.028 (2.785)	0.334 (0.532)	1.498 (1.583)	1.159 (2.051)	0.351 (0.783)	0.414 (0.930)	0.109 (0.924)	0.060 (0.859)	0.393 (0.855)	0.202 (0.946)	0.833 (0.926)	0.905 (1.000)	0.486 (0.794)	0.648 (0.882)	0.731 (1.000)	0.171 (0.973)
<i>Corynebacterium</i>	0.002 (0.003)	0.002 (0.002)	0.002 (0.005)	0.000 (0.002)	0.001 (0.003)	0.000 (0.001)	0.000 (0.000)	0.000 (0.002)	0.704 (0.934)	0.812 (1.000)	0.216 (0.924)	1.000 (1.000)	0.525 (0.855)	0.176 (0.946)	0.808 (0.926)	0.878 (1.000)	0.408 (0.794)	0.400 (0.848)	0.629 (1.000)	0.694 (0.973)
<i>Croceibacterium</i>	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.835 (0.969)	0.391 (0.930)	0.327 (0.924)	0.676 (0.970)	0.329 (0.855)	0.499 (0.946)	0.416 (0.897)	NA	NA	0.327 (0.741)	0.212 (1.000)	NA
<i>Curvibacter</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.002 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	0.233 (0.896)	0.495 (0.855)	0.310 (0.946)	0.034 (0.897)	0.052 (0.967)	NA	NA	NA	NA
<i>Cutibacterium</i>	0.000 (0.003)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.001 (0.003)	0.000 (0.001)	0.000 (0.000)	0.060 (0.707)	0.379 (0.930)	0.068 (0.924)	0.277 (0.896)	0.606 (0.855)	0.670 (1.000)	NA	0.637 (0.967)	0.408 (0.794)	0.810 (0.952)	0.847 (1.000)	0.512 (0.973)
<i>Daejeonella</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	0.182 (0.930)	0.571 (0.924)	NA	NA	NA	0.663 (0.897)	NA	0.453 (0.794)	0.257 (0.741)	NA	NA
<i>Deinococcus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.002 (0.002)	0.000 (0.000)	1.000 (1.000)	0.617 (0.930)	0.674 (0.973)	0.827 (1.000)	0.606 (0.855)	0.147 (0.946)	0.663 (0.897)	NA	0.453 (0.794)	0.101 (0.741)	0.045 (1.000)	0.307 (0.973)
<i>Delftia</i>	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.330 (0.783)	0.251 (0.930)	0.571 (0.924)	0.867 (1.000)	0.606 (0.855)	0.803 (1.000)	0.663 (0.897)	NA	NA	0.571 (0.852)	0.440 (1.000)	NA
<i>Desertimonas</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.641 (0.882)	1.000 (1.000)	0.889 (1.000)	0.853 (1.000)	0.606 (0.855)	0.440 (0.946)	0.663 (0.897)	NA	1.000 (1.000)	0.913 (1.000)	0.101 (1.000)	0.307 (0.973)
<i>Desulfovibrio</i>	0.000 (0.007)	0.000 (0.006)	0.005 (0.052)	0.000 (0.000)	0.010 (0.016)	0.007 (0.013)	0.004 (0.041)	0.005 (0.008)	0.797 (0.969)	0.450 (0.930)	0.303 (0.924)	0.658 (0.970)	0.172 (0.855)	0.869 (1.000)	0.416 (0.897)	0.120 (0.967)	1.000 (1.000)	1.000 (1.000)	0.717 (1.000)	0.746 (0.973)
<i>Devosia</i>	0.000 (0.000)	0.000 (0.002)	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.002 (0.001)	0.000 (0.002)	0.520 (0.825)	0.391 (0.930)	0.444 (0.924)	0.162 (0.896)	0.172 (0.855)	0.741 (1.000)	0.262 (0.897)	0.377 (0.967)	0.453 (0.794)	0.050 (0.741)	0.202 (1.000)	0.694 (0.973)
<i>Dialister</i>	0.011 (0.072)	0.012 (0.255)	0.006 (0.268)	0.067 (0.129)	0.006 (0.078)	0.121 (0.095)	0.043 (0.801)	0.027 (0.175)	0.750 (0.953)	0.587 (0.930)	0.648 (0.971)	1.000 (1.000)	0.571 (0.855)	1.000 (1.000)	0.667 (0.897)	0.511 (0.967)	0.343 (0.794)	0.527 (0.852)	0.281 (1.000)	0.450 (0.973)
<i>Dielma</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.003)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.756 (0.953)	0.835 (1.000)	0.571 (0.924)	0.325 (0.896)	NA (0.946)	0.499 (0.946)	0.663 (0.897)	NA	0.453 (0.794)	0.674 (0.882)	0.440 (1.000)	NA
<i>Diplocloster</i>	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.699 (0.933)	1.000 (1.000)	0.444 (0.924)	0.325 (0.896)	0.302 (0.855)	NA	0.608 (0.897)	0.239 (0.967)	1.000 (1.000)	0.257 (0.741)	NA	0.307 (0.973)
<i>Dorea</i>	2.213 (0.717)	2.595 (1.466)	5.210 (4.417)	2.086 (0.390)	2.967 (1.583)	2.364 (2.695)	4.869 (1.115)	2.950 (1.337)	0.536 (0.825)	0.260 (0.930)	1.000 (1.000)	0.004 (0.859)	0.071 (0.855)	0.876 (1.000)	1.000 (1.000)	0.381 (0.967)	0.686 (0.980)	0.024 (0.741)	0.051 (1.000)	0.610 (0.973)

Duganella	0.002 (0.002)	0.000 (0.002)	0.000 (0.002)	0.000 (0.000)	0.000 (0.002)	0.002 (0.001)	0.002 (0.003)	0.000 (0.000)	0.726 (0.953)	0.675 (0.971)	0.536 (0.924)	0.393 (0.930)	0.329 (0.855)	0.340 (0.946)	0.416 (0.897)	NA	0.538 (0.864)	0.680 (0.882)	0.045 (1.000)	0.026 (0.973)
Duncaniella	0.002 (0.005)	0.002 (0.001)	0.005 (0.005)	0.004 (0.005)	0.003 (0.005)	0.000 (0.001)	0.000 (0.002)	0.002 (0.002)	0.747 (0.953)	0.485 (0.930)	0.372 (0.924)	0.585 (0.949)	1.000 (1.000)	0.365 (0.946)	0.489 (0.897)	0.358 (0.967)	0.183 (0.794)	0.137 (0.741)	0.587 (1.000)	0.570 (0.973)
Duodenibacillus	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.001 (0.003)	0.000 (0.010)	0.000 (0.000)	0.000 (0.000)	NA (0.930)	0.037 (0.924)	0.257 (0.924)	NA (0.855)	0.302 (0.855)	NA (0.897)	0.190 (0.967)	0.239 (0.967)	0.869 (1.000)	0.068 (0.741)	NA (1.000)	0.307 (0.973)
Dyadobacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.002 (0.001)	0.000 (0.000)	0.001 (0.003)	0.000 (0.001)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	0.068 (0.924)	0.551 (0.930)	0.079 (0.855)	0.261 (0.946)	0.034 (0.897)	0.220 (0.967)	0.186 (0.794)	0.327 (0.741)	0.847 (1.000)	0.238 (0.973)
Dysosmobacter	0.005 (0.004)	0.000 (0.013)	0.000 (0.002)	0.002 (0.001)	0.005 (0.011)	0.001 (0.011)	0.000 (0.001)	0.005 (0.013)	0.088 (0.735)	0.940 (1.000)	0.918 (1.000)	0.023 (0.859)	0.874 (1.000)	0.923 (1.000)	1.000 (1.000)	0.358 (0.967)	0.468 (0.794)	0.037 (0.741)	0.140 (1.000)	0.741 (0.973)
Eggerthella	0.019 (0.034)	0.012 (0.049)	0.005 (0.001)	0.013 (0.006)	0.047 (0.170)	0.002 (0.163)	0.000 (0.012)	0.007 (0.002)	0.832 (0.969)	0.710 (0.971)	0.629 (0.968)	0.029 (0.859)	0.393 (0.855)	0.866 (1.000)	1.000 (1.000)	0.381 (0.967)	0.468 (0.794)	0.287 (0.741)	0.826 (1.000)	0.450 (0.973)
Ellagibacter	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.045)	0.000 (0.827)	0.000 (0.001)	0.037 (0.067)	0.000 (0.003)	0.835 (0.969)	0.835 (1.000)	1.000 (1.000)	1.000 (1.000)	0.696 (0.926)	0.277 (0.946)	0.779 (0.926)	1.000 (1.000)	1.000 (1.000)	0.680 (0.882)	0.393 (1.000)	0.694 (0.973)
Enorma	0.000 (0.000)	0.000 (0.000)	0.000 (0.007)	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.003)	0.000 (0.000)	0.232 (0.783)	1.000 (1.000)	0.257 (0.924)	0.559 (0.930)	0.329 (0.855)	0.772 (1.000)	NA (0.967)	0.637 (1.000)	1.000 (0.952)	0.810 (1.000)	0.562 (1.000)	0.761 (0.973)
Enterobacter	0.019 (0.031)	0.067 (0.044)	0.012 (0.003)	0.030 (0.013)	0.061 (0.064)	0.028 (0.053)	0.028 (0.044)	0.025 (0.050)	0.114 (0.757)	0.106 (0.930)	0.412 (0.924)	0.364 (0.896)	0.393 (0.855)	0.343 (0.946)	0.383 (0.897)	0.905 (1.000)	0.343 (0.794)	0.164 (0.741)	0.731 (1.000)	0.610 (0.973)
Enterocloster	0.007 (0.014)	0.007 (0.014)	0.002 (0.002)	0.004 (0.002)	0.006 (0.009)	0.001 (0.004)	0.002 (0.009)	0.002 (0.006)	0.750 (0.953)	0.938 (1.000)	0.153 (0.924)	0.423 (0.930)	1.000 (1.000)	0.626 (1.000)	0.209 (0.897)	1.000 (1.000)	0.309 (0.794)	0.394 (0.848)	0.713 (1.000)	1.000 (1.000)
Enterococcus	0.000 (0.002)	0.002 (0.002)	0.010 (0.027)	0.000 (0.005)	0.000 (0.001)	0.000 (0.000)	0.002 (0.007)	0.000 (0.002)	0.641 (0.882)	0.925 (1.000)	0.101 (0.924)	0.064 (0.859)	0.222 (0.855)	0.326 (0.946)	1.000 (1.000)	0.878 (1.000)	0.453 (0.794)	0.198 (0.741)	0.202 (1.000)	0.287 (0.973)
Escherichia	0.065 (4.378)	0.028 (0.030)	0.118 (0.086)	0.059 (0.023)	0.021 (0.106)	0.041 (1.879)	0.046 (0.587)	0.038 (0.055)	0.091 (0.735)	0.330 (0.930)	0.927 (1.000)	0.699 (0.970)	0.143 (0.855)	0.639 (1.000)	0.517 (0.897)	1.000 (1.000)	0.886 (1.000)	0.109 (0.741)	0.534 (1.000)	0.914 (1.000)
Eubacterium	0.003 (0.005)	0.000 (0.000)	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.001 (0.003)	0.000 (0.003)	0.002 (0.012)	0.068 (0.711)	0.095 (0.930)	0.279 (0.924)	0.569 (0.937)	0.733 (0.938)	1.000 (1.000)	0.745 (0.926)	0.480 (0.967)	0.186 (0.794)	0.186 (0.741)	0.393 (1.000)	0.570 (0.973)
Exiguobacterium	0.000 (0.000)	0.005 (0.006)	0.002 (0.002)	0.000 (0.004)	0.000 (0.000)	0.001 (0.003)	0.002 (0.001)	0.003 (0.004)	0.036 (0.707)	0.391 (0.930)	0.105 (0.924)	0.351 (0.896)	1.000 (1.000)	0.744 (1.000)	0.249 (0.897)	0.686 (0.991)	0.186 (0.794)	0.023 (0.741)	0.829 (1.000)	0.441 (0.973)
Faecalibacillus	0.364 (0.640)	0.386 (0.365)	0.370 (0.340)	0.597 (0.283)	0.629 (0.354)	0.329 (0.469)	0.802 (0.842)	0.850 (0.830)	1.000 (1.000)	0.825 (1.000)	1.000 (1.000)	1.000 (1.000)	0.786 (0.940)	0.639 (1.000)	0.667 (0.897)	0.714 (0.991)	0.486 (0.794)	0.788 (0.945)	0.945 (1.000)	0.610 (0.973)
Faecalibacterium	1.157 (3.102)	4.192 (6.563)	0.836 (1.219)	2.914 (7.962)	1.508 (2.491)	0.703 (4.424)	0.999 (3.833)	3.907 (8.179)	0.042 (0.707)	0.940 (1.000)	0.230 (0.924)	0.898 (1.000)	0.250 (0.855)	0.876 (1.000)	0.833 (0.926)	0.905 (1.000)	0.886 (1.000)	1.000 (1.000)	0.295 (1.000)	0.476 (0.973)
Faecalibaculum	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.021)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA (0.930)	0.182 (0.930)	NA (0.924)	NA (0.896)	NA (0.855)	NA (1.000)	NA (0.897)	NA (0.967)	0.453 (0.794)	0.257 (0.741)	NA (1.000)	NA (0.973)
Faecalicoccus	0.014 (0.053)	0.000 (0.001)	0.000 (0.007)	0.000 (0.000)	0.013 (0.027)	0.001 (0.019)	0.000 (0.002)	0.000 (0.002)	0.101 (0.757)	0.475 (0.930)	0.584 (0.924)	0.187 (0.896)	0.329 (0.855)	0.717 (1.000)	0.416 (0.897)	0.377 (0.967)	1.000 (1.000)	0.471 (0.852)	0.807 (1.000)	0.719 (0.973)
Faecalitalea	0.002 (0.007)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.006)	0.000 (0.000)	0.000 (0.000)	0.000 (0.016)	0.144 (0.783)	0.450 (0.930)	0.571 (0.924)	0.376 (0.911)	0.606 (0.855)	0.803 (1.000)	0.663 (0.897)	0.377 (0.967)	0.453 (0.794)	0.674 (0.882)	0.385 (1.000)	0.287 (0.973)
Fenollaria	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.835 (0.969)	0.391 (0.930)	0.327 (0.924)	0.559 (0.930)	0.329 (0.855)	0.105 (0.946)	0.416 (0.897)	NA (0.967)	NA (0.794)	NA (0.882)	NA (1.000)	NA (0.973)
Finegoldia	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.607 (0.877)	0.251 (0.930)	1.000 (1.000)	0.616 (0.954)	0.606 (0.855)	0.310 (0.946)	0.416 (0.897)	NA (0.967)	0.453 (0.794)	NA (0.882)	NA (1.000)	0.307 (0.973)
Flavobacterium	0.000 (0.000)	0.002 (0.005)	0.000 (0.002)	0.000 (0.006)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.001 (0.002)	0.072 (0.711)	0.617 (0.930)	0.101 (0.924)	0.266 (0.896)	1.000 (1.000)	0.772 (1.000)	1.000 (1.000)	1.000 (1.000)	NA (0.741)	0.327 (0.741)	0.625 (1.000)	0.149 (0.973)
Flintibacter	0.004 (0.005)	0.002 (0.004)	0.000 (0.000)	0.000 (0.001)	0.005 (0.005)	0.001 (0.010)	0.000 (0.002)	0.000 (0.000)	0.439 (0.783)	0.481 (0.930)	0.921 (1.000)	0.184 (0.896)	1.000 (1.000)	0.562 (0.991)	0.627 (0.897)	0.860 (1.000)	0.468 (0.794)	0.066 (0.741)	0.541 (1.000)	0.359 (0.973)
Fournierella	0.000 (0.003)	0.000 (0.005)	0.000 (0.000)	0.000 (0.000)	0.000 (0.049)	0.000 (0.002)	0.000 (0.002)	0.000 (0.000)	1.000 (1.000)	1.000 (1.000)	0.810 (1.000)	0.867 (1.000)	0.606 (0.855)	1.000 (1.000)	0.416 (0.897)	0.637 (0.967)	1.000 (1.000)	1.000 (1.000)	0.847 (1.000)	1.000 (0.973)
Frisingicoccus	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.154)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.120 (0.757)	1.000 (1.000)	NA (0.954)	0.616 (0.855)	0.606 (0.946)	0.310 (0.946)	NA (0.967)	0.637 (0.794)	0.453 (0.741)	0.257 (1.000)	0.355 (1.000)	0.540 (0.973)
Fumia	0.002 (0.007)	0.000 (0.001)	0.002 (0.000)	0.002 (0.002)	0.004 (0.003)	0.000 (0.000)	0.000 (0.004)	0.000 (0.002)	0.243 (0.783)	0.937 (1.000)	0.327 (0.924)	0.892 (1.000)	0.881 (1.000)	0.501 (0.946)	0.369 (0.897)	0.480 (0.967)	0.069 (0.794)	0.198 (0.741)	0.935 (1.000)	0.287 (0.973)
Fusicatenibacter	1.270 (1.594)	5.130 (4.347)	3.103 (1.759)	3.360 (1.411)	3.545 (4.350)	0.842 (1.628)	2.159 (2.070)	3.439 (1.907)	0.031 (0.707)	0.330 (0.930)	0.073 (0.924)	0.147 (0.896)	0.393 (0.855)	1.000 (1.000)	0.833 (0.926)	1.000 (1.000)	0.486 (0.794)	0.788 (0.945)	0.445 (1.000)	0.171 (0.973)
Fusobacterium	0.000 (0.000)	0.000 (0.001)	0.005 (0.003)	0.000 (0.002)	0.000 (0.000)	0.002 (0.005)	0.000 (0.001)	0.000 (0.000)	0.835 (0.969)	0.391 (0.930)	0.584 (0.924)	0.046 (0.859)	0.222 (0.855)	0.194 (0.946)	1.000 (1.000)	0.				

Gemmiger	2.711 (2.722)	3.105 (1.579)	4.108 (2.755)	7.242 (4.904)	3.385 (2.918)	4.148 (5.891)	3.200 (1.273)	3.070 (0.696)	0.351 (0.783)	0.604 (0.930)	0.927 (1.000)	0.190 (0.896)	0.786 (0.940)	0.343 (0.946)	0.667 (0.897)	0.381 (0.967)	0.886 (1.000)	0.648 (0.882)	0.836 (1.000)	0.762 (0.973)
Gemmobacter	0.002 (0.004)	0.002 (0.002)	0.000 (0.002)	0.000 (0.003)	0.000 (0.001)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.744 (0.953)	0.276 (0.930)	0.050 (0.924)	0.477 (0.930)	1.000 (1.000)	1.000 (1.000)	0.814 (0.926)	0.596 (0.967)	0.453 (0.794)	0.584 (0.854)	0.337 (1.000)	0.540 (0.973)
Gordonia	0.007 (0.002)	0.000 (0.002)	0.002 (0.004)	0.007 (0.002)	0.002 (0.005)	0.001 (0.003)	0.003 (0.004)	0.001 (0.004)	0.053 (0.707)	0.036 (0.930)	1.000 (1.000)	0.161 (0.896)	0.393 (0.855)	0.870 (1.000)	0.100 (0.897)	0.149 (0.967)	0.878 (1.000)	0.629 (0.882)	0.713 (1.000)	0.733 (0.973)
Gordonibacter	0.026 (0.056)	0.028 (0.025)	0.006 (0.007)	0.008 (0.011)	0.008 (0.005)	0.017 (0.022)	0.005 (0.016)	0.012 (0.009)	0.791 (0.969)	0.330 (0.930)	1.000 (1.000)	0.125 (0.896)	0.881 (1.000)	0.755 (1.000)	0.209 (0.897)	0.795 (1.000)	0.686 (0.980)	1.000 (1.000)	0.836 (1.000)	0.521 (0.973)
Gp6	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.450 (0.783)	0.540 (0.930)	0.257 (0.924)	0.827 (1.000)	0.606 (0.855)	1.000 (1.000)	NA (0.967)	0.637 (1.000)	1.000 (1.000)	1.000 (1.000)	0.847 (1.000)	0.761 (0.973)
Granulicatella	0.000 (0.005)	0.000 (0.001)	0.002 (0.002)	0.000 (0.000)	0.001 (0.002)	0.001 (0.003)	0.000 (0.003)	0.001 (0.002)	0.362 (0.783)	0.801 (1.000)	0.584 (0.924)	0.887 (1.000)	0.172 (0.855)	0.862 (1.000)	0.416 (0.897)	0.220 (0.967)	1.000 (1.000)	0.918 (1.000)	0.698 (1.000)	0.909 (1.000)
Guopingia	0.005 (0.017)	0.000 (0.002)	0.004 (0.005)	0.000 (0.007)	0.025 (0.023)	0.001 (0.003)	0.002 (0.006)	0.001 (0.004)	0.214 (0.783)	0.389 (0.930)	1.000 (1.000)	0.677 (0.970)	0.751 (0.940)	0.866 (1.000)	1.000 (1.000)	1.000 (0.794)	0.059 (0.741)	0.045 (0.741)	0.652 (1.000)	0.733 (0.973)
Haemophilus	0.000 (0.016)	0.016 (0.019)	0.002 (0.015)	0.017 (0.028)	0.000 (0.009)	0.001 (0.003)	0.004 (0.022)	0.000 (0.000)	0.309 (0.783)	0.925 (1.000)	0.147 (0.924)	0.319 (0.896)	0.653 (0.890)	0.934 (1.000)	0.818 (0.926)	0.220 (0.967)	0.869 (1.000)	0.536 (0.852)	0.254 (1.000)	0.512 (0.973)
Harryflintia	0.000 (0.005)	0.000 (0.002)	0.002 (0.004)	0.002 (0.001)	0.000 (0.001)	0.001 (0.015)	0.000 (0.000)	0.001 (0.002)	0.797 (0.969)	0.638 (0.942)	0.743 (1.000)	0.768 (1.000)	0.033 (0.940)	0.700 (0.946)	0.787 (0.926)	0.408 (1.000)	0.257 (0.794)	0.053 (0.741)	0.570 (1.000)	0.733 (0.973)
Helimonas	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.118 (0.757)	NA (0.924)	NA (0.924)	NA (0.946)	0.499 (0.897)	0.416 (0.897)	NA (0.897)	NA (0.897)	NA (0.897)	0.571 (0.852)	0.440 (1.000)	NA (1.000)
Herbaspirillum	0.002 (0.005)	0.002 (0.002)	0.002 (0.002)	0.004 (0.004)	0.003 (0.002)	0.001 (0.003)	0.002 (0.003)	0.007 (0.007)	0.667 (0.910)	1.000 (1.000)	0.384 (0.924)	0.503 (0.930)	1.000 (1.000)	0.530 (0.946)	0.493 (0.897)	0.905 (1.000)	0.460 (0.794)	1.000 (1.000)	0.474 (1.000)	0.161 (0.973)
Holdemanela	0.000 (0.002)	0.004 (0.154)	0.002 (0.002)	0.822 (0.443)	0.000 (0.025)	0.004 (0.013)	0.002 (1.016)	0.004 (0.355)	0.070 (0.711)	1.000 (1.000)	0.633 (0.930)	0.555 (0.855)	0.359 (0.946)	0.408 (0.946)	0.356 (0.897)	0.358 (0.967)	0.538 (0.864)	0.198 (0.741)	0.828 (1.000)	0.914 (1.000)
Holdemania	0.000 (0.003)	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.004)	0.000 (0.001)	0.000 (0.001)	0.001 (0.002)	0.825 (0.969)	0.860 (1.000)	0.276 (0.924)	0.437 (0.930)	0.606 (0.855)	0.831 (1.000)	0.089 (0.897)	0.220 (0.967)	1.000 (1.000)	1.000 (1.000)	0.625 (1.000)	0.810 (1.000)
Hominimerdicola	0.067 (0.476)	0.097 (0.063)	0.061 (0.059)	0.020 (0.338)	0.043 (0.051)	0.083 (1.508)	0.029 (0.240)	0.058 (0.319)	0.918 (0.992)	0.940 (1.000)	1.000 (1.000)	0.640 (0.970)	1.000 (1.000)	0.935 (1.000)	0.667 (0.897)	1.000 (1.000)	0.886 (1.000)	0.788 (0.945)	0.775 (1.000)	1.000 (1.000)
Hoministercoradaptatus	0.195 (0.339)	1.076 (0.466)	0.729 (0.258)	0.982 (1.100)	0.386 (1.638)	0.279 (0.372)	1.346 (1.397)	0.739 (0.358)	0.031 (0.707)	0.414 (0.930)	0.012 (0.848)	0.083 (0.896)	0.393 (0.855)	0.343 (0.946)	0.833 (0.926)	0.262 (0.967)	0.686 (0.980)	0.412 (0.848)	0.295 (1.000)	0.114 (0.973)
Howardella	0.000 (0.000)	0.017 (0.056)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.199 (0.783)	1.000 (1.000)	0.092 (0.924)	0.676 (0.970)	0.329 (0.855)	0.629 (1.000)	0.089 (0.897)	NA (1.000)	1.000 (1.000)	1.000 (1.000)	0.212 (1.000)	0.307 (0.973)
Hoytesella	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.713 (0.971)	0.571 (0.924)	0.551 (0.930)	NA (0.897)	NA (0.897)	0.663 (0.897)	NA (0.794)	0.453 (0.741)	0.257 (0.741)	NA (1.000)	NA (1.000)
Huintesticincola	0.000 (0.030)	0.000 (0.015)	0.000 (0.032)	0.000 (0.089)	0.000 (0.004)	0.000 (0.019)	0.002 (0.002)	0.001 (0.002)	0.584 (0.869)	0.482 (0.930)	1.000 (1.000)	0.941 (1.000)	1.000 (1.000)	1.000 (1.000)	0.779 (0.926)	1.000 (1.000)	1.000 (1.000)	0.488 (0.852)	0.508 (1.000)	0.631 (0.973)
Hydrocarboniphaga	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.002)	0.450 (0.783)	0.617 (0.930)	0.257 (0.924)	0.551 (0.930)	NA (0.930)	NA (0.930)	NA (0.930)	0.377 (0.967)	0.453 (0.794)	NA (1.000)	0.140 (1.000)	0.694 (0.973)
Hydrogeniclostridium	0.002 (0.005)	0.002 (0.002)	0.000 (0.002)	0.000 (0.000)	0.004 (0.008)	0.001 (0.003)	0.000 (0.000)	0.001 (0.002)	0.581 (0.868)	1.000 (1.000)	0.766 (1.000)	0.477 (0.930)	0.329 (0.855)	0.394 (0.946)	0.158 (0.897)	0.220 (0.967)	0.645 (0.969)	0.186 (0.741)	0.337 (1.000)	1.000 (1.000)
Hydrotalea	0.005 (0.000)	0.000 (0.002)	0.000 (0.002)	0.000 (0.001)	0.001 (0.003)	0.002 (0.004)	0.000 (0.000)	0.002 (0.000)	0.010 (0.506)	0.313 (0.930)	0.471 (0.924)	0.018 (0.859)	0.733 (0.938)	0.523 (0.946)	0.608 (0.897)	0.028 (0.967)	1.000 (1.000)	0.279 (0.741)	0.029 (1.000)	0.915 (1.000)
Hymenobacter	0.000 (0.002)	0.000 (0.000)	0.002 (0.004)	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.330 (0.783)	0.638 (0.942)	0.571 (0.924)	0.376 (0.911)	0.525 (0.855)	0.365 (0.946)	0.515 (0.897)	0.878 (1.000)	0.453 (0.794)	0.810 (0.952)	0.930 (1.000)	0.287 (0.973)
Hyphomicrobium	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.487 (0.802)	0.251 (0.930)	0.571 (0.924)	0.867 (1.000)	0.606 (0.855)	1.000 (1.000)	0.663 (0.897)	0.637 (0.967)	NA (0.741)	0.327 (1.000)	0.847 (1.000)	0.540 (0.973)
Ihubacter	0.000 (0.020)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.021)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.330 (0.783)	1.000 (1.000)	0.571 (0.924)	0.194 (0.896)	0.302 (0.855)	0.261 (0.946)	0.515 (0.897)	0.596 (0.967)	0.453 (0.794)	1.000 (1.000)	0.699 (1.000)	0.540 (0.973)
Intestinibacter	0.593 (2.248)	0.062 (1.445)	0.196 (0.910)	0.449 (0.287)	0.040 (0.378)	0.146 (0.325)	0.372 (2.524)	0.324 (0.793)	0.142 (0.783)	0.076 (0.930)	0.927 (1.000)	0.240 (0.896)	1.000 (1.000)	1.000 (1.000)	0.833 (0.926)	0.905 (1.000)	0.686 (0.980)	0.527 (0.852)	0.534 (1.000)	0.610 (0.973)
Intestinimonas	0.003 (0.014)	0.000 (0.002)	0.000 (0.002)	0.000 (0.000)	0.007 (0.018)	0.000 (0.005)	0.002 (0.002)	0.002 (0.007)	0.258 (0.783)	0.937 (1.000)	0.913 (1.000)	0.255 (0.896)	0.329 (0.855)	1.000 (1.000)	0.262 (0.897)	0.220 (0.967)	0.356 (0.794)	0.092 (0.741)	0.393 (1.000)	0.810 (1.000)
Jutongia	0.005 (0.003)	0.046 (0.111)	0.007 (0.008)	0.006 (0.067)	0.046 (0.059)	0.019 (0.036)	0.014 (0.042)	0.010 (0.063)	0.005 (0.506)	0.034 (0.930)	0.109 (0.924)	0.898 (1.000)	0.786 (0.940)	0.639 (1.000)	0.267 (0.897)	1.000 (1.000)	0.343 (0.794)	0.230 (0.741)	0.731 (1.000)	0.762 (0.973)
Kineococcus	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.001)	0.000 (0.001)	0.000 (0.002)	0.089 (0.735)	0.675 (0.971)	0.889 (1.000)	0.184 (0.896)	1.000 (1.000)	0.831 (1.000)	0.745 (0.926)	0.878 (1.000)	1.000 (1.000)	1.000 (1.000)	0.930 (1.000)	0.694 (0.973)
Kineothrix	0.072 (0.078)	0.121 (0.039)	0.052 (0.020)	0.055 (0.032)	0.081 (0.047)	0.111 (0.237)	0.053 (0.153)	0.029 (0.013)	0.210 (0.783)	1.000 (1.000)	0.927 (1.000)	0.606 (0.953)	1.000 (1.000)	1.000 (1.000)	0.117 (0.897)	0.548 (1.000)	1.000 (0.882)	0.648 (1.000)	0.731 (1.000)	1.000 (1.000)
Klenkia	0.002 (0.001)	0.000 (0.002)	0.000 (0.004)	0.000 (0.001)	0.001 (0.003)	0.001 (0.002)	0.000 (0.003)	0.001 (0.006)	0.277 (0.783)	0.584 (0.930)	1.000 (1.000)	0.682 (0.970)	0.733 (0.938)	1.000 (1.000)	0.608 (0.897)	0.480 (0.967)	0.878 (1.000)	1.000 (1.000)	0.587 (1.000)	0.733 (0.973)
Labilithrix	0.002 (0.002)	0.002 (0.001)	0.002 (0.002)	0.002 (0.002)	0.000 (0.000)	0.002 (0.004)	0.002 (0.000)	0.000 (0.000)	0.447 (0.783)	0.095 (0.930)	1.000 (1.000)	0.781 (1.000)	1.000 (1.000)	0.870 (1.000)	1.000 (1.000)	0.220 (0.967)	0.186 (0.794)	0.023 (0.741)	0.035 (1.000)	0.238 (0.973)
Lachnospira	0.161 (0.137)	0.234 (0.097)	0.052 (0.117)	0.017 (0.350)	0.136 (0.423)	0.022 (0.622)	0.086 (0.087)	0.086 (0.080)	0.299 (0.783)	0.825 (1.000)	0.230 (0.924)	0.797 (1.000)	0.393 (0.855)	0.639 (1.000)	0.517 (0.897)	1.000 (1.000)	0.343 (0.794)	0.527 (0.852)	0.945 (1.000)	1.000 (1.000)

Lacibacterium	0.000 (0.000)	0.000 (0.000)	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	NA	0.571 (0.924)	0.016 (0.859)	0.172 (0.855)	0.082 (0.946)	0.663 (0.897)	NA	NA	0.571 (0.852)	0.440 (1.000)	NA
Lacrimispora	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.232 (0.783)	1.000 (1.000)	NA	1.000 (1.000)	0.606 (0.855)	1.000 (1.000)	NA	0.637 (0.967)	0.453 (0.794)	1.000 (1.000)	0.699 (1.000)	0.540 (0.973)
Lacticaseibacillus	0.000 (0.002)	0.000 (0.000)	0.007 (0.009)	0.000 (0.000)	0.000 (0.014)	0.000 (0.000)	0.005 (0.008)	0.003 (0.007)	0.487 (0.802)	1.000 (1.000)	0.571 (0.924)	0.237 (0.896)	0.172 (0.855)	0.736 (1.000)	0.663 (0.897)	0.120 (0.967)	0.453 (0.794)	0.680 (0.882)	1.000 (1.000)	0.072 (0.973)
Lactiplantibacillus	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.232 (0.783)	0.835 (1.000)	NA	0.559 (0.930)	0.329 (0.855)	0.105 (0.946)	NA	NA	0.453 (0.794)	0.257 (0.741)	NA	NA
Lactobacillus	0.000 (0.000)	0.000 (0.000)	0.004 (0.002)	0.000 (0.000)	0.000 (0.004)	0.000 (0.000)	0.000 (0.002)	0.000 (0.003)	0.641 (0.882)	0.835 (1.000)	0.571 (0.924)	0.161 (0.896)	0.081 (0.855)	0.238 (0.946)	0.663 (0.897)	0.377 (0.967)	0.453 (0.794)	0.913 (1.000)	1.000 (1.000)	0.287 (0.973)
Lactococcus	0.014 (0.036)	0.002 (0.007)	0.007 (0.009)	0.000 (0.001)	0.109 (0.289)	0.002 (0.002)	0.004 (0.007)	0.002 (0.003)	0.480 (0.802)	0.273 (0.930)	1.000 (1.000)	1.000 (1.000)	0.134 (0.855)	0.416 (0.946)	0.466 (0.897)	0.500 (0.967)	0.245 (0.794)	0.215 (0.741)	0.612 (1.000)	0.746 (0.973)
Laedolimicola	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.004)	0.000 (0.004)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	0.257 (0.924)	0.354 (0.896)	0.329 (0.855)	1.000 (1.000)	NA	NA	0.453 (0.794)	0.327 (0.741)	0.212 (1.000)	0.307 (0.973)
Lancefieldella	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.002)	0.000 (0.001)	0.000 (0.003)	0.002 (0.002)	0.002 (0.002)	0.641 (0.882)	0.835 (1.000)	0.279 (0.924)	0.559 (0.930)	1.000 (1.000)	0.856 (1.000)	0.515 (0.897)	0.893 (1.000)	0.869 (1.000)	0.913 (1.000)	0.260 (1.000)	0.741 (0.973)
Latilactobacillus	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.450 (0.783)	0.713 (0.971)	NA	0.827 (1.000)	0.606 (0.855)	1.000 (1.000)	NA	0.637 (0.967)	0.453 (0.794)	0.674 (0.882)	0.910 (1.000)	0.540 (0.973)
Lawsonella	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.120 (0.757)	0.251 (0.930)	NA	0.194 (0.896)	NA	NA	NA	NA	NA	NA	NA	NA
Lawsonibacter	0.007 (0.019)	0.002 (0.008)	0.002 (0.000)	0.000 (0.001)	0.013 (0.015)	0.003 (0.004)	0.000 (0.002)	0.004 (0.009)	0.455 (0.788)	0.816 (1.000)	0.633 (0.968)	0.284 (0.896)	0.359 (0.855)	0.178 (0.946)	0.347 (0.897)	0.480 (0.967)	0.245 (0.794)	0.092 (0.741)	0.393 (1.000)	1.000 (1.000)
Lentihominibacter	0.137 (0.409)	0.070 (0.052)	0.159 (0.652)	0.044 (0.015)	0.166 (0.241)	0.125 (0.214)	0.144 (0.101)	0.144 (0.122)	0.351 (0.783)	0.940 (1.000)	0.788 (1.000)	0.797 (1.000)	0.250 (0.855)	0.755 (1.000)	0.267 (0.897)	0.262 (0.967)	0.886 (1.000)	0.788 (0.945)	0.836 (1.000)	0.762 (0.973)
Lentzea	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.060 (0.707)	0.158 (0.930)	NA	0.113 (0.896)	NA	NA	NA	NA	NA	NA	NA	NA
Leuconostoc	0.000 (0.000)	0.000 (0.002)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.000 (0.002)	0.266 (0.783)	0.713 (0.971)	0.913 (1.000)	0.354 (0.896)	0.329 (0.855)	0.287 (0.946)	0.262 (0.897)	0.377 (0.967)	1.000 (1.000)	0.674 (0.882)	0.385 (1.000)	1.000 (1.000)
Leyella	0.000 (0.000)	0.002 (0.001)	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.001 (0.010)	0.000 (0.002)	0.000 (0.002)	0.243 (0.783)	0.391 (0.930)	0.629 (0.968)	0.437 (0.930)	0.172 (0.855)	0.488 (0.946)	0.089 (0.897)	0.377 (0.967)	0.186 (0.794)	0.186 (0.741)	0.935 (1.000)	0.719 (0.973)
Ligilactobacillus	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.756 (0.953)	0.391 (0.930)	0.674 (0.973)	0.325 (0.896)	NA	0.261 (0.946)	0.663 (0.897)	0.637 (0.967)	0.453 (0.794)	0.327 (0.741)	0.847 (1.000)	1.000 (1.000)
Limnobacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	0.182 (0.930)	NA	NA	0.302 (0.855)	NA	0.190 (0.897)	0.239 (0.967)	0.453 (0.794)	0.257 (0.741)	NA	NA
Limosilactobacillus	0.000 (0.002)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.000 (0.003)	0.000 (0.001)	0.000 (0.007)	0.000 (0.000)	1.000 (1.000)	1.000 (1.000)	1.000 (1.000)	0.756 (1.000)	0.329 (0.855)	0.587 (0.998)	0.416 (0.897)	0.637 (0.967)	1.000 (1.000)	0.584 (0.854)	0.257 (1.000)	0.761 (0.973)
Longicatena	0.000 (0.014)	0.000 (0.003)	0.000 (0.000)	0.000 (0.000)	0.002 (0.004)	0.002 (0.006)	0.002 (0.007)	0.000 (0.000)	0.484 (0.802)	1.000 (1.000)	0.488 (0.924)	0.113 (0.896)	NA	0.066 (0.946)	0.262 (0.897)	0.637 (0.967)	1.000 (1.000)	0.847 (0.991)	0.143 (1.000)	0.093 (0.973)
Luoshenia	0.003 (0.006)	0.000 (0.002)	0.004 (0.006)	0.000 (0.000)	0.000 (0.005)	0.000 (0.001)	0.000 (0.000)	0.001 (0.002)	0.185 (0.783)	0.330 (0.930)	0.743 (1.000)	1.000 (1.000)	0.172 (0.855)	0.033 (0.946)	0.262 (0.897)	0.220 (0.967)	1.000 (1.000)	0.257 (0.741)	0.053 (1.000)	0.631 (0.973)
Luteolibacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	NA	0.551 (0.930)	0.302 (0.855)	0.499 (0.946)	0.190 (0.897)	0.239 (0.967)	NA	0.571 (0.852)	0.440 (1.000)	NA
Macrococcus	0.000 (0.000)	0.000 (0.002)	0.020 (0.015)	0.000 (0.000)	0.000 (0.000)	0.002 (0.004)	0.005 (0.020)	0.000 (0.007)	0.835 (0.969)	0.391 (0.930)	0.913 (1.000)	0.032 (0.859)	0.081 (0.855)	0.563 (0.991)	0.416 (0.897)	0.377 (0.967)	0.186 (0.794)	0.101 (0.741)	0.485 (1.000)	1.000 (1.000)
Marisediminitalia	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	NA	NA	0.257 (0.924)	NA	NA	NA	NA	NA	0.453 (0.794)	NA	NA	0.307 (0.973)
Marmoricola	0.000 (0.003)	0.000 (0.002)	0.002 (0.003)	0.000 (0.000)	0.001 (0.003)	0.000 (0.001)	0.000 (0.002)	0.000 (0.002)	0.484 (0.802)	0.933 (1.000)	0.444 (0.924)	0.445 (0.930)	0.081 (0.855)	0.260 (0.946)	0.262 (0.897)	0.377 (0.967)	0.408 (0.794)	0.743 (0.942)	1.000 (1.000)	0.694 (0.973)
Massilia	0.009 (0.008)	0.009 (0.007)	0.011 (0.005)	0.012 (0.005)	0.009 (0.005)	0.009 (0.003)	0.007 (0.010)	0.009 (0.008)	0.681 (0.915)	0.940 (1.000)	0.927 (1.000)	0.364 (0.896)	1.000 (1.000)	0.432 (0.946)	0.517 (0.897)	0.905 (1.000)	0.886 (1.000)	0.927 (1.000)	0.836 (1.000)	0.762 (0.973)
Massilimalia	0.002 (0.010)	0.000 (0.006)	0.002 (0.005)	0.000 (0.001)	0.007 (0.007)	0.002 (0.002)	0.002 (0.004)	0.001 (0.004)	0.439 (0.783)	0.393 (0.930)	0.498 (0.924)	0.946 (1.000)	0.222 (0.855)	0.457 (0.946)	0.608 (0.897)	0.671 (0.991)	0.200 (0.794)	0.153 (0.741)	1.000 (1.000)	0.589 (0.973)
Massilimicrobiota	0.000 (0.007)	0.000 (0.007)	0.002 (0.002)	0.000 (0.000)	0.001 (0.003)	0.000 (0.007)	0.000 (0.002)	0.000 (0.005)	0.903 (0.992)	1.000 (1.000)	0.743 (1.000)	0.883 (1.000)	0.172 (0.855)	0.488 (0.946)	0.262 (0.897)	0.377 (0.967)	0.869 (1.000)	0.606 (0.878)	0.935 (1.000)	1.000 (1.000)
Massiliprevotella	0.000 (0.000)	0.000 (0.000)	0.000 (0.009)	0.000 (0.125)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.026)	0.314 (0.783)	NA	0.571 (0.924)	0.063 (0.859)	1.000 (1.000)	0.105 (0.946)	0.745 (0.926)	1.000 (1.000)	NA	NA	0.140 (1.000)	0.287 (0.973)
Mediterranea	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	NA	0.571 (0.924)	0.063 (0.859)	0.329 (0.855)	0.105 (0.946)	0.663 (0.897)	NA	NA	NA	NA	NA
Mediterraneibacter	1.301 (1.577)	1.909 (0.857)	0.941 (0.373)	1.717 (0.659)	2.577 (3.387)	1.053 (0.795)	0.780 (1.250)	1.211 (1.482)	0.210 (0.783)	0.260 (0.930)	0.164 (0.924)	0.797 (1.000)	0.250 (0.855)	0.268 (0.946)	0.667 (0.897)	0.548 (0.967)	0.200 (0.794)	0.230 (0.741)	0.731 (1.000)	0.610 (0.973)
Megamonas	0.003 (0.007)	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.008 (0.007)	0.001 (0.004)	0.002 (0.006)	0.002 (0.008)	0.914 (0.992)	0.184 (0.930)	0.294 (0.924)	0.301 (0.896)	0.606 (0.855)	0.277 (0.946)	0.046 (0.897)	0.220 (0.967)	0.110 (0.794)	0.153 (0.741)	1.000 (1.000)	0.909 (1.000)
Megasphaera	0.000 (0.000)	0.002 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.001 (0.004)	0.000 (0.001)	0.007 (0.058)	0.062 (0.707)	0.835 (1.000)	0.772 (1.000)	1.000 (1.000)	0.606 (0.855)	0.831 (1.000)	0.089 (0.897)	0.220 (0.967)	0.620 (0.942)	1.000 (1.000)	0.254 (1.000)	0.570 (0.973)

Merdimonas	0.005 (0.126)	0.002 (0.011)	0.002 (0.002)	0.005 (0.004)	0.005 (0.163)	0.032 (0.020)	0.003 (0.011)	0.004 (0.022)	0.356 (0.783)	0.937 (1.000)	0.017 (0.924)	0.340 (0.896)	0.760 (0.940)	0.741 (1.000)	0.906 (0.999)	0.787 (1.000)	0.309 (0.794)	1.000 (1.000)	0.941 (1.000)	0.161 (0.973)
Mesosutterella	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.005)	0.000 (0.000)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	0.257 (0.924)	0.551 (0.930)	0.302 (0.855)	0.499 (0.946)	0.190 (0.897)	0.239 (0.967)	0.453 (0.794)	0.571 (0.852)	0.440 (1.000)	0.307 (0.973)
Methylobacterium	0.005 (0.007)	0.009 (0.006)	0.006 (0.005)	0.004 (0.005)	0.004 (0.004)	0.005 (0.001)	0.006 (0.004)	0.006 (0.003)	0.758 (0.953)	0.440 (0.930)	0.315 (0.924)	0.797 (1.000)	0.571 (0.855)	0.876 (1.000)	0.667 (0.897)	0.548 (0.967)	0.886 (1.000)	0.412 (0.848)	0.945 (1.000)	0.476 (0.973)
Methylophilus	0.000 (0.000)	0.000 (0.000)	0.000 (0.004)	0.000 (0.001)	0.000 (0.000)	0.002 (0.001)	0.000 (0.001)	0.000 (0.000)	0.756 (0.953)	0.391 (0.930)	0.155 (0.924)	0.358 (0.896)	0.733 (0.938)	0.499 (0.946)	0.745 (0.926)	0.860 (1.000)	0.069 (0.794)	0.327 (0.741)	0.699 (1.000)	0.230 (0.973)
Microbacterium	0.009 (0.013)	0.009 (0.006)	0.011 (0.003)	0.011 (0.010)	0.002 (0.002)	0.008 (0.004)	0.009 (0.005)	0.012 (0.007)	0.252 (0.783)	0.050 (0.930)	0.850 (1.000)	1.000 (1.000)	1.000 (1.000)	0.268 (0.946)	0.517 (0.897)	0.714 (0.991)	0.245 (0.794)	0.073 (0.741)	0.295 (1.000)	0.352 (0.973)
Microvirga	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.001 (0.003)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.175 (0.930)	0.571 (0.924)	0.551 (0.930)	NA (0.924)	NA (0.930)	0.663 (0.897)	NA (0.991)	0.186 (0.794)	0.068 (0.741)	NA (1.000)	NA (0.973)
Mitsuokella	0.000 (0.000)	0.000 (0.000)	0.000 (0.007)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA (0.859)	NA (0.855)	NA (0.946)	0.063 (0.859)	0.329 (0.855)	0.523 (0.946)	NA (0.897)	NA (0.991)	NA (0.794)	0.571 (0.852)	0.440 (1.000)	NA (0.973)
Mixta	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.002 (0.007)	0.000 (0.003)	0.001 (0.002)	1.000 (1.000)	0.391 (0.930)	0.444 (0.924)	0.853 (1.000)	0.606 (0.855)	0.334 (0.946)	0.416 (0.897)	0.220 (0.967)	0.186 (0.794)	0.186 (0.741)	0.938 (1.000)	0.909 (1.000)
Mogibacterium	0.000 (0.002)	0.000 (0.001)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.019)	0.000 (0.002)	0.429 (0.783)	0.158 (0.930)	0.810 (1.000)	0.825 (1.000)	0.329 (0.855)	0.856 (1.000)	0.416 (0.897)	0.377 (0.967)	0.453 (0.794)	0.186 (0.741)	0.684 (1.000)	0.694 (0.973)
Monoglobus	0.015 (0.185)	0.000 (0.001)	0.002 (0.002)	0.002 (0.005)	0.029 (0.110)	0.001 (0.010)	0.000 (0.000)	0.000 (0.000)	0.243 (0.783)	0.481 (0.930)	0.743 (1.000)	0.405 (0.930)	0.541 (0.855)	0.246 (0.946)	0.369 (0.897)	0.125 (0.967)	0.110 (0.794)	0.018 (0.741)	1.000 (1.000)	0.238 (0.973)
Moraxella	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.001 (0.002)	0.000 (0.000)	0.000 (0.002)	0.000 (0.002)	0.224 (0.783)	0.685 (0.971)	0.571 (0.924)	0.062 (0.859)	NA (0.946)	0.136 (0.897)	0.663 (0.967)	0.377 (0.991)	0.186 (0.794)	1.000 (1.000)	0.935 (1.000)	0.287 (0.973)
Murdochella	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.641 (0.882)	0.391 (0.930)	0.674 (0.973)	0.325 (0.896)	NA (0.924)	NA (0.930)	0.663 (0.897)	NA (0.991)	0.453 (0.794)	NA (1.000)	NA (1.000)	0.307 (0.973)
Muribaculum	0.000 (0.004)	0.002 (0.002)	0.000 (0.002)	0.066 (0.039)	0.005 (0.002)	0.003 (0.051)	0.007 (0.011)	0.001 (0.004)	0.581 (0.868)	0.330 (0.930)	0.923 (1.000)	0.605 (0.953)	0.266 (0.855)	0.178 (0.946)	0.356 (0.897)	0.281 (0.967)	0.882 (1.000)	0.504 (0.852)	0.270 (1.000)	0.733 (0.973)
Muricoprocola	0.150 (0.146)	0.157 (0.272)	0.338 (0.240)	0.140 (0.155)	0.309 (0.188)	0.064 (0.061)	0.170 (0.091)	0.054 (0.039)	0.470 (0.799)	0.148 (0.930)	0.164 (0.924)	0.029 (0.859)	0.250 (0.855)	0.202 (0.946)	0.667 (0.897)	0.714 (0.991)	0.114 (0.794)	0.412 (0.848)	0.181 (1.000)	0.914 (1.000)
Mycobacterium	0.009 (0.018)	0.012 (0.015)	0.013 (0.005)	0.000 (0.003)	0.003 (0.004)	0.007 (0.008)	0.010 (0.009)	0.006 (0.013)	0.873 (0.985)	0.434 (0.930)	0.850 (1.000)	0.688 (0.970)	0.036 (0.855)	0.416 (0.946)	0.106 (0.897)	0.345 (0.967)	0.561 (0.895)	0.294 (0.741)	0.828 (1.000)	0.746 (0.973)
Natranaerovirga	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.001 (0.004)	1.000 (1.000)	0.617 (0.930)	0.571 (0.924)	0.662 (0.970)	0.606 (0.855)	0.310 (0.946)	0.663 (0.897)	0.220 (0.967)	NA (0.991)	NA (1.000)	0.053 (1.000)	0.149 (0.973)
Negativibacillus	0.005 (0.012)	0.000 (0.010)	0.002 (0.035)	0.000 (0.001)	0.001 (0.023)	0.002 (0.008)	0.000 (0.006)	0.000 (0.032)	0.385 (0.783)	0.584 (0.930)	1.000 (1.000)	0.893 (1.000)	0.525 (0.855)	0.386 (0.946)	0.608 (0.897)	0.878 (1.000)	1.000 (0.945)	0.757 (1.000)	0.935 (1.000)	1.000 (1.000)
Neglecta	0.130 (0.224)	0.054 (0.120)	0.083 (0.186)	0.032 (0.007)	0.108 (0.039)	0.035 (0.038)	0.045 (0.314)	0.079 (0.170)	0.351 (0.783)	0.503 (0.930)	0.527 (0.924)	1.000 (1.000)	0.250 (0.855)	0.639 (1.000)	0.833 (0.926)	0.381 (0.967)	0.200 (0.794)	1.000 (1.000)	1.000 (1.000)	0.476 (0.973)
Neisseria	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.006)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA (0.930)	0.182 (0.930)	NA (1.000)	NA (1.000)	NA (0.855)	NA (0.946)	NA (0.897)	NA (0.991)	0.453 (0.794)	0.257 (0.741)	NA (1.000)	NA (0.973)
Neobacillus	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.002 (0.002)	0.000 (0.001)	0.001 (0.003)	0.000 (0.001)	0.000 (0.002)	0.520 (0.825)	0.835 (1.000)	1.000 (1.000)	1.000 (1.000)	0.172 (0.855)	0.670 (1.000)	0.466 (0.897)	0.203 (0.967)	0.869 (1.000)	1.000 (1.000)	0.930 (1.000)	0.719 (0.973)
Nevskia	0.000 (0.000)	0.000 (0.002)	0.000 (0.002)	0.000 (0.002)	0.000 (0.001)	0.000 (0.001)	0.002 (0.002)	0.002 (0.003)	0.835 (0.969)	1.000 (1.000)	0.810 (1.000)	0.676 (0.970)	1.000 (1.000)	0.501 (0.946)	1.000 (1.000)	0.686 (0.991)	1.000 (1.000)	0.092 (0.741)	1.000 (1.000)	0.139 (0.973)
Nocardioides	0.007 (0.006)	0.005 (0.006)	0.004 (0.002)	0.002 (0.002)	0.001 (0.004)	0.002 (0.001)	0.005 (0.003)	0.005 (0.004)	0.138 (0.783)	0.105 (0.930)	0.507 (0.924)	0.438 (0.930)	0.294 (0.855)	0.755 (1.000)	0.356 (0.897)	0.358 (0.967)	0.885 (1.000)	0.394 (0.848)	0.520 (1.000)	0.593 (0.973)
Novosphingobium	0.007 (0.007)	0.005 (0.000)	0.004 (0.003)	0.005 (0.002)	0.003 (0.001)	0.006 (0.003)	0.006 (0.006)	0.006 (0.004)	0.210 (0.783)	0.089 (0.930)	0.412 (0.924)	0.125 (0.896)	1.000 (1.000)	0.624 (1.000)	0.833 (0.926)	0.714 (0.991)	0.029 (0.794)	0.391 (0.848)	1.000 (1.000)	0.352 (0.973)
Odoribacter	0.002 (0.013)	0.005 (0.008)	0.000 (0.000)	0.002 (0.005)	0.001 (0.003)	0.000 (0.001)	0.002 (0.007)	0.000 (0.002)	0.913 (0.992)	0.570 (0.930)	0.198 (0.924)	0.184 (0.896)	0.306 (0.855)	0.205 (0.946)	0.644 (0.897)	0.671 (0.991)	0.620 (0.942)	0.921 (1.000)	0.587 (1.000)	0.896 (1.000)
Oribacterium	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.835 (0.969)	0.391 (0.930)	1.000 (1.000)	0.802 (1.000)	0.733 (0.938)	0.105 (0.946)	1.000 (1.000)	0.239 (0.967)	0.453 (0.794)	NA (1.000)	NA (1.000)	0.307 (0.973)
Oscillibacter	0.019 (0.026)	0.000 (0.001)	0.000 (0.002)	0.000 (0.002)	0.001 (0.033)	0.000 (0.001)	0.000 (0.000)	0.001 (0.002)	0.015 (0.506)	0.389 (0.930)	1.000 (1.000)	0.041 (0.859)	1.000 (1.000)	0.523 (0.946)	0.779 (0.926)	1.000 (1.000)	0.620 (0.942)	0.186 (0.741)	0.257 (1.000)	0.631 (0.973)
Oxalobacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.002 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.073 (0.930)	0.571 (0.924)	0.354 (0.896)	0.329 (0.855)	0.394 (0.946)	0.663 (0.897)	0.637 (0.967)	0.069 (0.794)	0.101 (0.741)	1.000 (1.000)	0.540 (0.973)
Paludihabitans	0.007 (0.008)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.010 (0.017)	0.000 (0.000)	0.000 (0.002)	0.000 (0.002)	0.013 (0.506)	0.816 (1.000)	0.571 (0.924)	0.052 (0.859)	0.606 (0.855)	0.699 (1.000)	0.663 (0.897)	0.377 (0.967)	0.069 (0.794)	0.276 (0.741)	0.464 (1.000)	0.287 (0.973)
Panacagrimonas	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.001 (0.002)	NA (0.783)	NA (0.924)	NA (0.859)	0.233 (0.855)	0.606 (0.855)	1.000 (1.000)	NA (0.967)	0.220 (0.991)	NA (0.794)	0.571 (0.852)	0.337 (1.000)	0.149 (0.973)
Panacibacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	NA (0.924)	0.571 (0.859)	0.063 (0.855)	0.329 (0.855)	0.394 (0.946)	0.663 (0.897)	NA (0.991)	NA (0.794)	0.571 (0.852)	0.440 (1.000)	NA (0.973)
Parabacteroides	0.021 (0.018)	0.060 (0.043)	0.023 (0.051)	0.014 (0.021)	0.025 (0.019)	0.008 (0.044)	0.011 (0.012)	0.036 (0.039)	0.536 (0.825)	0.940 (1.000)	0.648 (0.971)	0.797 (1.000)	1.000 (1.000)	0.569 (0.991)	0.267 (0.897)	1.000 (1.000)	0.886 (1.000)	0.315 (0.741)	0.295 (1.000)	0.914 (1.000)
Parachlamydia	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	NA (0.924)	NA (0.930)	0.257 (0.924)	NA (0.896)	NA (0.855)	0.499 (0.946)	NA (0.897)	0.637 (0.967)	0.453 (0.794)	0.571 (0.852)	0.910 (1.000)	1.000 (1.000)

<i>Paracoccus</i>	0.002 (0.026)	0.002 (0.019)	0.007 (0.017)	0.002 (0.011)	0.000 (0.001)	0.004 (0.011)	0.008 (0.006)	0.002 (0.008)	0.957 (1.000)	0.144 (0.930)	0.391 (0.924)	0.840 (1.000)	0.653 (0.890)	0.807 (1.000)	0.906 (0.999)	1.000 (1.000)	0.027 (0.794)	0.033 (0.741)	0.350 (1.000)	0.454 (0.973)
<i>Paramuribaculum</i>	0.000 (0.000)	0.000 (0.003)	0.000 (0.002)	0.000 (0.001)	0.006 (0.019)	0.000 (0.000)	0.002 (0.003)	0.001 (0.002)	0.440 (0.783)	0.300 (0.930)	0.186 (0.924)	0.559 (0.930)	0.733 (0.938)	0.260 (0.946)	0.608 (0.897)	0.480 (0.967)	0.186 (0.794)	0.620 (0.882)	0.293 (1.000)	0.149 (0.973)
<i>Paraprevotella</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.064)	0.000 (0.003)	0.000 (0.000)	0.000 (0.005)	0.000 (0.002)	0.854 (0.971)	0.713 (0.971)	0.571 (0.924)	0.662 (0.970)	0.696 (0.926)	0.831 (1.000)	0.515 (0.897)	0.878 (1.000)	0.453 (0.794)	1.000 (1.000)	1.000 (1.000)	0.287 (0.973)
<i>Parasutterella</i>	0.000 (0.008)	0.000 (0.001)	0.002 (0.000)	0.000 (0.001)	0.001 (0.007)	0.001 (0.002)	0.000 (0.000)	0.000 (0.000)	0.301 (0.783)	0.933 (1.000)	0.913 (1.000)	1.000 (1.000)	0.359 (0.855)	0.046 (0.946)	0.779 (0.926)	0.596 (0.967)	0.645 (0.969)	0.186 (0.741)	1.000 (1.000)	0.512 (0.973)
<i>Paratractidigestivibacter</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.232 (0.783)	1.000 (1.000)	NA (1.000)	1.000 (0.855)	0.606 (1.000)	1.000 (0.855)	NA (0.967)	0.637 (0.967)	0.453 (0.794)	0.889 (1.000)	1.000 (1.000)	0.540 (0.973)
<i>Parvimonas</i>	0.002 (0.005)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.047 (0.707)	0.330 (0.930)	0.571 (0.924)	0.298 (0.896)	0.329 (0.855)	0.394 (0.946)	0.663 (0.897)	NA (0.794)	0.453 (0.882)	0.674 (1.000)	0.440 (1.000)	NA (0.973)
<i>Patulibacter</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	1.000 (1.000)	0.617 (0.930)	0.571 (0.924)	0.827 (1.000)	0.606 (0.855)	0.310 (0.946)	0.663 (0.897)	0.377 (0.967)	NA (1.000)	NA (1.000)	0.140 (1.000)	0.287 (0.973)
<i>Pedobacter</i>	0.002 (0.002)	0.000 (0.005)	0.002 (0.002)	0.002 (0.009)	0.000 (0.001)	0.000 (0.001)	0.000 (0.002)	0.002 (0.004)	0.912 (0.992)	0.516 (0.930)	0.444 (0.924)	0.785 (1.000)	1.000 (1.000)	0.298 (0.946)	0.627 (0.897)	1.000 (1.000)	1.000 (1.000)	0.913 (1.000)	0.153 (1.000)	0.306 (0.973)
<i>Pedomicrobium</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.617 (0.930)	0.571 (0.924)	0.551 (0.930)	0.302 (0.855)	0.499 (0.946)	0.745 (0.926)	0.239 (0.967)	NA (0.852)	0.571 (1.000)	0.440 (1.000)	NA (0.973)
<i>Peptococcus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.061)	0.000 (0.000)	0.000 (0.001)	0.000 (0.022)	0.000 (0.000)	0.000 (0.002)	0.450 (0.783)	0.540 (0.930)	0.257 (0.924)	0.194 (0.896)	0.329 (0.855)	0.394 (0.946)	NA (0.967)	0.377 (1.000)	1.000 (1.000)	0.889 (1.000)	0.499 (1.000)	0.896 (1.000)
<i>Peptostreptococcus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.001 (0.004)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.641 (0.882)	0.638 (0.942)	0.571 (0.924)	0.325 (0.896)	0.302 (0.855)	NA (0.926)	0.745 (1.000)	0.860 (0.794)	0.186 (0.741)	0.068 (1.000)	0.355 (1.000)	0.540 (0.973)
<i>Phascolarctobacterium</i>	0.068 (0.289)	0.017 (0.135)	0.027 (0.015)	0.004 (0.029)	0.004 (0.041)	0.002 (0.075)	0.005 (0.026)	0.041 (0.049)	0.252 (0.783)	0.189 (0.930)	0.394 (0.924)	0.438 (0.930)	0.571 (0.855)	0.143 (0.946)	0.383 (0.897)	0.548 (0.967)	1.000 (1.000)	0.923 (1.000)	0.172 (1.000)	0.331 (0.973)
<i>Phocaeicola</i>	0.230 (0.281)	0.156 (0.262)	0.073 (0.121)	0.039 (0.584)	0.055 (0.059)	0.082 (0.760)	0.021 (0.037)	0.082 (0.113)	0.536 (0.825)	0.106 (0.930)	0.788 (1.000)	0.112 (0.896)	0.786 (0.940)	0.432 (0.946)	1.000 (1.000)	0.905 (1.000)	1.000 (1.000)	0.315 (0.741)	0.234 (1.000)	1.000 (1.000)
<i>Phoea</i>	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.001 (0.003)	0.000 (0.000)	0.000 (0.002)	1.000 (1.000)	0.391 (0.930)	0.584 (0.924)	0.853 (1.000)	0.696 (0.926)	1.000 (1.000)	0.779 (0.926)	1.000 (1.000)	0.186 (0.794)	0.571 (0.852)	0.629 (1.000)	0.719 (0.973)
<i>Planococcus</i>	0.003 (0.007)	0.007 (0.007)	0.004 (0.002)	0.007 (0.006)	0.000 (0.000)	0.006 (0.006)	0.000 (0.004)	0.002 (0.000)	0.365 (0.783)	0.054 (0.930)	0.850 (1.000)	0.789 (1.000)	1.000 (1.000)	0.321 (0.946)	1.000 (1.000)	0.795 (1.000)	0.069 (0.794)	0.186 (0.741)	0.713 (1.000)	0.521 (0.973)
<i>Plesiomonas</i>	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.002)	0.120 (0.757)	0.777 (1.000)	0.257 (0.924)	0.616 (0.954)	0.606 (0.855)	0.310 (0.946)	NA (0.967)	0.377 (1.000)	1.000 (1.000)	0.257 (0.741)	0.140 (1.000)	0.896 (1.000)
<i>Pontibacter</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.713 (0.971)	0.571 (0.924)	0.354 (0.896)	0.329 (0.855)	0.394 (0.946)	0.663 (0.897)	0.637 (0.967)	0.453 (0.794)	0.674 (0.882)	0.910 (1.000)	0.540 (0.973)
<i>Prevotellamassilia</i>	0.000 (0.002)	0.000 (0.000)	0.000 (0.002)	0.004 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.005)	0.000 (0.000)	0.303 (0.783)	0.158 (0.930)	0.571 (0.924)	1.000 (1.000)	0.634 (0.886)	0.717 (1.000)	0.262 (0.897)	0.125 (0.967)	NA (0.741)	0.186 (1.000)	0.257 (1.000)	0.540 (0.973)
<i>Priestia</i>	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.004)	0.000 (0.003)	0.002 (0.007)	0.000 (0.000)	0.001 (0.002)	0.578 (0.868)	1.000 (1.000)	0.279 (0.924)	0.194 (0.896)	0.302 (0.855)	0.499 (0.946)	0.745 (0.926)	0.888 (1.000)	0.620 (0.942)	0.674 (0.882)	0.257 (1.000)	0.733 (0.973)
<i>Proteus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.001 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	0.037 (0.930)	0.571 (0.924)	NA (0.855)	0.302 (0.855)	NA (0.926)	0.745 (0.967)	0.239 (0.967)	0.186 (0.794)	0.068 (0.741)	NA (1.000)	NA (0.973)
<i>Pseudarthrobacter</i>	0.012 (0.004)	0.012 (0.007)	0.007 (0.007)	0.010 (0.009)	0.009 (0.006)	0.005 (0.007)	0.011 (0.006)	0.011 (0.005)	0.536 (0.825)	0.260 (0.930)	0.315 (0.924)	0.147 (0.896)	1.000 (1.000)	0.343 (0.946)	0.833 (0.926)	1.000 (1.000)	0.486 (0.794)	0.527 (0.852)	0.836 (1.000)	0.392 (0.973)
<i>Pseudoalteromonas</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.641 (0.882)	0.391 (0.930)	0.571 (0.924)	0.325 (0.896)	NA (0.855)	NA (0.946)	0.663 (0.897)	0.377 (0.967)	NA (1.000)	NA (0.741)	0.140 (1.000)	0.287 (0.973)
<i>Pseudokineococcus</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.232 (0.783)	0.391 (0.930)	NA (0.970)	0.676 (0.855)	0.329 (0.855)	0.499 (0.946)	NA (0.897)	NA (0.967)	NA (1.000)	0.327 (0.741)	0.212 (1.000)	NA (0.973)
<i>Pseudomonas</i>	0.021 (0.011)	0.012 (0.016)	0.026 (0.019)	0.049 (0.027)	0.009 (0.011)	0.010 (0.008)	0.021 (0.008)	0.013 (0.006)	0.174 (0.783)	0.076 (0.930)	0.927 (1.000)	1.000 (1.000)	0.393 (0.855)	0.530 (0.946)	0.183 (0.897)	0.381 (0.967)	0.686 (0.980)	0.073 (0.741)	0.101 (1.000)	0.476 (0.973)
<i>Pseudorhodoplanes</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.232 (0.783)	0.391 (0.930)	NA (0.896)	0.325 (0.855)	0.302 (0.855)	NA (0.946)	0.190 (0.897)	0.239 (0.967)	NA (1.000)	NA (0.741)	NA (1.000)	NA (0.973)
<i>Psychrobacter</i>	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.003)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.002)	0.352 (0.783)	0.540 (0.930)	0.810 (1.000)	0.551 (0.930)	0.302 (0.855)	0.261 (0.946)	0.779 (0.926)	0.878 (1.000)	1.000 (1.000)	1.000 (1.000)	0.930 (1.000)	0.694 (0.973)
<i>Ralstonia</i>	0.002 (0.003)	0.000 (0.002)	0.002 (0.002)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.002 (0.003)	0.000 (0.002)	0.258 (0.783)	0.208 (0.930)	0.186 (0.924)	1.000 (1.000)	0.525 (0.855)	0.866 (1.000)	1.000 (1.000)	1.000 (1.000)	0.453 (0.794)	0.216 (0.741)	0.587 (1.000)	0.287 (0.973)
<i>Raoultibacter</i>	0.005 (0.016)	0.007 (0.014)	0.009 (0.007)	0.021 (0.018)	0.009 (0.023)	0.011 (0.016)	0.005 (0.010)	0.001												

<i>Rhodoferax</i>	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.835 (0.969)	0.391 (0.930)	0.327 (0.924)	1.000 (1.000)	1.000 (1.000)	0.803 (1.000)	1.000 (1.000)	0.860 (1.000)	NA	0.571 (0.852)	0.910 (1.000)	0.540 (0.973)
<i>Romboutsia</i>	1.233 (4.623)	0.227 (0.697)	1.213 (3.647)	2.242 (1.479)	0.248 (0.765)	2.248 (2.238)	0.702 (1.459)	1.851 (1.531)	0.252 (0.783)	0.148 (0.930)	0.164 (0.924)	1.000 (1.000)	1.000 (1.000)	0.755 (1.000)	0.117 (0.897)	0.714 (0.991)	0.114 (0.794)	0.315 (0.741)	0.628 (1.000)	0.914 (1.000)
<i>Roseateles</i>	0.006 (0.011)	0.007 (0.002)	0.007 (0.000)	0.006 (0.005)	0.004 (0.009)	0.003 (0.009)	0.007 (0.010)	0.011 (0.007)	0.758 (0.953)	0.315 (0.930)	0.394 (0.924)	1.000 (1.000)	0.786 (0.940)	1.000 (1.000)	0.667 (0.897)	0.548 (0.967)	1.000 (1.000)	0.394 (0.848)	0.628 (1.000)	0.336 (0.973)
<i>Roseburia</i>	0.065 (0.116)	0.157 (0.168)	0.159 (0.090)	0.255 (0.314)	0.067 (0.028)	0.032 (0.064)	0.043 (0.432)	0.106 (0.105)	0.408 (0.783)	1.000 (1.000)	0.315 (0.924)	0.438 (0.930)	0.393 (0.855)	0.530 (0.946)	0.517 (0.897)	0.548 (0.967)	0.486 (0.794)	0.788 (0.945)	0.945 (1.000)	0.352 (0.973)
<i>Rothia</i>	0.003 (0.005)	0.007 (0.006)	0.002 (0.010)	0.012 (0.009)	0.006 (0.020)	0.000 (0.000)	0.002 (0.006)	0.000 (0.002)	0.458 (0.789)	0.587 (0.930)	0.009 (0.848)	0.893 (1.000)	1.000 (1.000)	0.679 (1.000)	0.833 (0.926)	0.203 (0.967)	0.069 (0.794)	0.439 (0.852)	0.313 (1.000)	0.287 (0.973)
<i>Rubellimicrobium</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.232 (0.783)	0.835 (1.000)	NA (0.896)	0.325 (0.896)	NA (0.946)	0.499 (0.946)	NA	NA	0.453 (0.794)	0.674 (0.882)	0.440 (1.000)	NA (0.973)
<i>Ructibacterium</i>	0.000 (0.005)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.001 (0.003)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.060 (0.707)	0.933 (1.000)	NA (0.896)	0.351 (0.896)	0.606 (0.855)	0.310 (0.946)	NA	NA	0.186 (0.794)	0.068 (0.741)	NA (1.000)	NA (0.973)
<i>Ruminococcoides</i>	0.003 (0.005)	0.000 (0.001)	0.000 (0.000)	0.004 (0.006)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.129 (0.783)	0.450 (0.930)	0.810 (1.000)	0.062 (0.859)	0.079 (0.855)	0.261 (0.946)	0.158 (0.897)	0.357 (0.967)	1.000 (1.000)	1.000 (1.000)	0.847 (1.000)	1.000 (1.000)
<i>Ruminococcus</i>	5.830 (3.344)	1.574 (3.960)	0.497 (1.749)	3.762 (3.801)	3.003 (2.493)	1.708 (3.835)	2.962 (3.060)	3.301 (4.954)	0.142 (0.783)	0.148 (0.930)	1.000 (1.000)	0.029 (0.859)	0.786 (0.940)	0.530 (0.946)	0.667 (0.897)	1.000 (1.000)	1.000 (1.000)	0.788 (0.945)	0.534 (1.000)	0.914 (1.000)
<i>Ruthenibacterium</i>	0.009 (0.063)	0.012 (0.009)	0.007 (0.015)	0.006 (0.004)	0.042 (0.061)	0.031 (0.054)	0.008 (0.007)	0.013 (0.029)	0.536 (0.825)	0.503 (0.930)	0.164 (0.924)	0.298 (0.896)	0.571 (0.855)	1.000 (1.000)	0.383 (0.897)	0.897 (1.000)	0.686 (0.980)	0.164 (0.741)	1.000 (1.000)	0.336 (0.973)
<i>Saccharibacteria</i>	0.002 (0.005)	0.002 (0.005)	0.002 (0.005)	0.000 (0.003)	0.006 (0.007)	0.002 (0.001)	0.002 (0.003)	0.001 (0.002)	0.957 (1.000)	0.313 (0.930)	0.847 (1.000)	1.000 (1.000)	0.751 (0.940)	0.613 (1.000)	0.808 (0.926)	0.888 (1.000)	0.343 (0.794)	0.153 (0.741)	1.000 (1.000)	0.660 (0.973)
<i>Sanguibacter</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.002)	0.641 (0.882)	0.391 (0.930)	0.889 (1.000)	0.452 (0.930)	0.329 (0.855)	0.629 (1.000)	0.663 (0.897)	0.377 (0.967)	0.453 (0.794)	0.327 (0.741)	0.794 (1.000)	0.694 (0.973)
<i>Schaalia</i>	0.051 (0.054)	0.023 (0.008)	0.034 (0.019)	0.047 (0.010)	0.025 (0.011)	0.034 (0.050)	0.039 (0.032)	0.025 (0.027)	0.012 (0.506)	0.076 (0.930)	0.927 (1.000)	0.298 (0.896)	0.571 (0.855)	0.876 (1.000)	0.067 (0.897)	0.381 (0.967)	0.886 (1.000)	0.412 (0.848)	0.366 (1.000)	0.762 (0.973)
<i>Segatella</i>	0.016 (0.012)	0.019 (0.025)	0.025 (0.097)	0.004 (0.006)	0.017 (0.016)	0.020 (0.038)	0.020 (1.448)	0.017 (0.016)	0.596 (0.875)	1.000 (1.000)	0.527 (0.924)	0.423 (0.930)	0.071 (0.855)	1.000 (1.000)	0.183 (0.897)	0.095 (0.967)	1.000 (1.000)	0.412 (0.848)	0.534 (1.000)	0.762 (0.973)
<i>Sellimonas</i>	0.049 (0.123)	0.002 (0.026)	0.007 (0.056)	0.000 (0.002)	0.042 (0.110)	0.000 (0.070)	0.002 (0.096)	0.006 (0.010)	0.320 (0.783)	0.696 (0.971)	0.680 (0.977)	0.677 (0.970)	0.341 (0.855)	0.869 (1.000)	0.466 (0.897)	0.345 (0.967)	0.301 (0.794)	0.394 (0.848)	0.612 (1.000)	0.570 (0.973)
<i>Senegalimassilia</i>	0.000 (0.127)	0.002 (0.177)	0.000 (0.000)	0.125 (0.214)	0.000 (0.001)	0.001 (0.061)	0.000 (0.103)	0.052 (0.124)	0.692 (0.927)	0.482 (0.930)	0.766 (1.000)	0.351 (0.896)	0.057 (0.855)	0.334 (0.946)	0.644 (0.897)	0.358 (0.967)	0.620 (0.942)	0.444 (0.852)	1.000 (1.000)	1.000 (1.000)
<i>Serratia</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.008)	0.007 (0.018)	0.000 (0.001)	0.000 (0.000)	0.001 (0.002)	0.877 (0.985)	0.396 (0.930)	0.889 (1.000)	0.325 (0.896)	0.302 (0.855)	NA (0.926)	0.745 (1.000)	0.888 (1.000)	0.408 (0.794)	0.068 (0.741)	0.053 (1.000)	0.337 (0.973)
<i>Skermanella</i>	0.000 (0.002)	0.000 (0.001)	0.002 (0.002)	0.002 (0.000)	0.000 (0.001)	0.000 (0.002)	0.002 (0.002)	0.001 (0.002)	0.797 (0.969)	0.777 (1.000)	1.000 (1.000)	0.154 (0.896)	0.393 (0.855)	0.160 (0.946)	0.224 (0.897)	0.694 (0.991)	1.000 (1.000)	0.410 (0.848)	0.881 (1.000)	0.810 (1.000)
<i>Slackia</i>	0.005 (0.104)	0.000 (0.093)	0.077 (0.049)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.012 (0.161)	0.002 (0.062)	0.560 (0.853)	0.095 (0.930)	0.810 (1.000)	0.275 (0.896)	0.127 (0.855)	0.804 (1.000)	1.000 (1.000)	0.500 (0.967)	0.453 (0.794)	0.101 (0.741)	0.713 (1.000)	0.139 (0.973)
<i>Solibaculum</i>	0.660 (0.963)	0.516 (0.363)	0.012 (0.686)	0.047 (0.244)	0.001 (0.347)	0.284 (0.286)	0.213 (1.483)	0.257 (0.549)	0.210 (0.783)	0.076 (0.930)	0.315 (0.924)	0.240 (0.896)	1.000 (1.000)	0.639 (1.000)	0.267 (0.897)	0.905 (1.000)	0.309 (0.794)	0.155 (0.741)	0.628 (1.000)	0.762 (0.973)
<i>Solimonas</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	NA (0.973)	0.674 (0.973)	0.233 (0.896)	0.606 (0.855)	0.310 (0.946)	0.663 (0.897)	NA (0.794)	0.453 (0.794)	NA (1.000)	NA (1.000)	0.307 (0.973)
<i>Solobacterium</i>	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.002)	0.060 (0.707)	0.598 (0.930)	0.257 (0.924)	0.113 (0.896)	0.302 (0.855)	NA (0.897)	0.190 (0.897)	0.878 (1.000)	1.000 (1.000)	0.257 (0.741)	0.140 (1.000)	0.896 (1.000)
<i>Sphingobacterium</i>	0.000 (0.000)	0.000 (0.003)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.002)	0.352 (0.783)	0.617 (0.930)	0.810 (1.000)	0.551 (0.930)	0.302 (0.855)	0.499 (0.946)	1.000 (1.000)	1.000 (1.000)	0.453 (0.794)	0.571 (0.852)	0.499 (1.000)	0.694 (0.973)
<i>Sphingobium</i>	0.000 (0.002)	0.000 (0.002)	0.002 (0.005)	0.002 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.001)	0.000 (0.002)	0.903 (0.992)	0.925 (1.000)	0.186 (0.924)	0.460 (0.930)	0.760 (0.940)	0.147 (0.946)	0.808 (0.926)	0.480 (0.967)	0.453 (0.794)	1.000 (1.000)	0.794 (1.000)	0.287 (0.973)
<i>Sphingomonas</i>	0.056 (0.014)	0.034 (0.025)	0.032 (0.010)	0.030 (0.008)	0.026 (0.012)	0.030 (0.026)	0.032 (0.018)	0.039 (0.017)	0.091 (0.735)	0.003 (0.831)	0.527 (0.924)	0.083 (0.896)	1.000 (1.000)	0.876 (1.000)	1.000 (1.000)	0.714 (0.991)	0.686 (0.980)	0.315 (0.741)	0.628 (1.000)	0.476 (0.973)
<i>Sporobacter</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.232 (0.783)	1.000 (1.000)	NA (0.896)	0.325 (0.896)	NA (0.946)	0.499 (0.946)	NA	NA	0.453 (0.794)	0.674 (0.882)	0.440 (1.000)	NA (0.973)
<i>Stagnimonas</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	NA (0.924)	NA (0.924)	0.257 (0.924)	NA (0.896)	NA (0.855)	NA (0.946)	NA (0.897)	0.637 (0.967)	0.453 (0.794)	NA (1.000)	0.355 (1.000)	1.000 (1.000)
<i>Staphylococcus</i>	0.000 (0.013)	0.000 (0.012)	0.006 (0.002)	0.002 (0.015)	0.008 (0.021)	0.000 (0.004)	0.000 (0.003)	0.006 (0.026)	0.761 (0.953)	0.933 (1.000)	0.810 (1.000)	0.275 (0.896)	0.571 (0.855)	0.098 (0.946)	0.521 (0.897)	1.000 (1.000)	0.408 (0.794)	0.606 (0.878)	0.587 (1.000)	0.471 (0.973)
<i>Stenotrophomonas</i>	0.000 (0.003)	0.002 (0.003)	0.000 (0.002)	0.000 (0.001)	0.000 (0.001)	0.000 (0.001)	0.000 (0.004)	0.000 (0.002)	0.955 (1.000)	0.598 (0.930)	0.216 (0.924)	0.941 (1.000)	0.733 (0.938)	0.856 (1.000)	0.627 (0.897)	1.000 (1.000)	1.000 (1.000)	0.584 (0.854)	0.684 (1.000)	0.694 (0.973)
<i>Streptococcus</i>	0.228 (0.212)	0.363 (0.221)	0.175 (0.539)	0.075 (0.146)	1.315 (3.957)	0.139 (0.273)	0.228 (0.394)	0.230 (0.513)	0.142 (0.783)	0.106 (0.930)	0.164 (0.924)	0.898 (1.000)	0.571 (0.855)	0.755 (1.000)	0.117 (0.897)	0.262 (0.967)	0.114 (0.794)	0.315 (0.741)	0.731 (1.000)	0.352 (0.973)
<i>Succinivibrio</i>	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.272)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.314 (0.783)	0.182 (0.930)	0.674 (0.973)	NA (0.896)	NA (0.855)	NA (0.946)	0.663 (0.897)	0.637 (0.967)	1.000 (1.000)	0.257 (0.741)	0.355 (1.000)	0.761 (0.973)
<i>Suilimivivens</i>	0.005 (0.008)	0.002 (0.001)	0.000 (0.000)	0.000 (0.004)	0.000 (0.001)	0.000 (0.002)	0.000 (0.001)	0.000 (0.000)	0.237 (0.783)	0.144 (0.930)	0.488 (0.924)	0.200 (0.896)	1.000 (1.000)	1.000 (1.000)	0.814 (0.926)	0.596 (0.967)	1.000 (1.000)	1.000 (1.000)	0.562 (1.000)	0.761 (0.973)

Sutterella	0.000 (0.000)	0.000 (0.003)	0.000 (0.004)	0.002 (0.004)	0.000 (0.001)	0.000 (0.003)	0.000 (0.002)	0.001 (0.004)	0.266 (0.783)	0.713 (0.971)	0.913 (1.000)	0.354 (0.896)	0.874 (1.000)	0.772 (1.000)	0.808 (0.926)	1.000 (1.000)	1.000 (1.000)	0.810 (0.952)	0.625 (1.000)	0.631 (0.973)
Thermus	0.000 (0.000)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	NA	NA	NA	0.063 (0.859)	0.329 (0.855)	0.394 (0.946)	NA	NA	NA	0.571 (0.852)	0.440 (1.000)	NA
Thomasclavelia	0.007 (0.091)	0.012 (0.022)	0.012 (0.015)	0.018 (0.008)	0.007 (0.002)	0.005 (0.013)	0.003 (0.025)	0.000 (0.005)	0.830 (0.969)	0.876 (1.000)	0.633 (0.968)	0.585 (0.949)	0.549 (0.855)	0.866 (1.000)	0.819 (0.926)	0.022 (0.967)	1.000 (1.000)	1.000 (1.000)	0.313 (1.000)	0.211 (0.973)
Tractidigestivibacter	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.438 (0.783)	0.617 (0.930)	0.327 (0.924)	0.551 (0.930)	0.302 (0.855)	0.499 (0.946)	1.000 (1.000)	0.860 (1.000)	NA	0.571 (0.852)	1.000 (1.000)	0.540 (0.973)
Traorella	0.002 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.005)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.013 (0.506)	0.516 (0.930)	NA	0.088 (0.896)	0.606 (0.855)	0.310 (0.946)	NA	0.637 (0.967)	0.453 (0.794)	0.257 (0.741)	0.355 (1.000)	0.540 (0.973)
Truepera	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.450 (0.783)	0.617 (0.930)	NA	0.551 (0.930)	0.302 (0.855)	0.261 (0.946)	0.190 (0.897)	0.239 (0.967)	NA	0.327 (0.741)	0.212 (1.000)	NA
Turicibacter	0.022 (0.332)	0.009 (0.017)	0.016 (0.122)	0.068 (0.032)	0.007 (0.005)	0.040 (0.165)	0.002 (0.069)	0.009 (0.019)	0.142 (0.783)	0.076 (0.930)	0.230 (0.924)	0.518 (0.930)	1.000 (1.000)	0.569 (0.991)	0.517 (0.897)	0.714 (0.991)	0.200 (0.794)	0.788 (0.945)	0.534 (1.000)	0.171 (0.973)
Unclassified	16.538 (6.697)	10.040 (1.053)	11.257 (5.731)	6.535 (2.750)	18.574 (9.492)	5.655 (4.261)	12.708 (7.384)	8.294 (2.433)	0.016 (0.506)	0.940 (1.000)	0.006 (0.848)	0.240 (0.896)	0.071 (0.855)	0.530 (0.946)	0.067 (0.897)	0.381 (0.967)	0.029 (0.794)	0.073 (0.741)	0.445 (1.000)	0.114 (0.973)
Variovorax	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	1.000 (1.000)	0.713 (0.971)	0.571 (0.924)	0.551 (0.930)	NA	0.499 (0.946)	0.663 (0.897)	NA	0.453 (0.794)	0.674 (0.882)	0.440 (1.000)	NA
Veillonella	0.009 (0.010)	0.009 (0.012)	0.009 (0.003)	0.017 (0.035)	0.009 (0.017)	0.002 (0.007)	0.008 (0.006)	0.008 (0.007)	0.596 (0.875)	0.587 (0.930)	0.155 (0.924)	1.000 (1.000)	0.036 (0.855)	1.000 (1.000)	0.383 (0.897)	0.262 (0.967)	0.460 (0.794)	1.000 (1.000)	0.836 (1.000)	0.240 (0.973)
Vescimonas	0.825 (1.465)	0.142 (0.174)	0.366 (0.297)	0.009 (0.071)	1.013 (1.599)	0.105 (0.049)	0.090 (0.234)	0.173 (0.698)	0.351 (0.783)	1.000 (1.000)	0.412 (0.924)	0.699 (0.970)	0.071 (0.855)	0.073 (0.946)	0.183 (0.897)	0.262 (0.967)	0.343 (0.794)	0.164 (0.741)	0.836 (1.000)	0.914 (1.000)
Xylanibacter	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.001 (0.002)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.877 (0.985)	0.638 (0.942)	0.889 (1.000)	1.000 (1.000)	0.606 (0.855)	1.000 (1.000)	0.663 (0.897)	0.637 (0.967)	0.620 (0.942)	0.743 (0.942)	0.847 (1.000)	1.000 (1.000)
Youxingia	0.000 (0.002)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.001)	0.000 (0.000)	0.000 (0.000)	0.060 (0.707)	0.598 (0.930)	0.257 (0.924)	0.534 (0.930)	0.606 (0.855)	0.803 (1.000)	NA	0.637 (0.967)	1.000 (1.000)	0.889 (1.000)	1.000 (1.000)	1.000 (1.000)
Zavarzinia	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.000)	0.000 (0.001)	0.000 (0.000)	NA	NA	NA	0.233 (0.896)	0.606 (0.855)	0.670 (1.000)	NA	NA	NA	0.327 (0.741)	0.212 (1.000)	NA

Supplementary Table 10: Differential abundance of bacterial genera from APOE3/3-TR (E3) and APOE4/4-TR (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing mice on the low-fat (LF) (10% kcal fat) diet.

Group Name	LF APOE4/4 $\bar{x}$ (SD)	LF APOE3/3 $\bar{x}$ (SD)	Fold-Change	P-value	FDR q
<i>Bacteroides</i>	4.34 (0.47)	7.62 (0.36)	1.76	2.20E-03	2.50E-02
<i>Dehalobacterium</i>	5.87 (0.09)	6.3 (0.17)	1.07	2.20E-03	2.50E-02
<i>Mucispirillum</i>	5.83 (0.65)	4.32 (0.48)	0.74	2.20E-03	2.50E-02
<i>Odoribacter</i>	5.93 (0.76)	1.96 (3.3)	0.33	5.00E-03	4.30E-02
<i>rc4-4</i>	-1.8 (1.22)	4.79 (2.4)	-2.66E+00	6.20E-03	4.30E-02
<i>SMB53</i>	-0.61 (2.64)	3.45 (0.44)	-5.67E+00	1.10E-02	0.066
<i>Dorea</i>	5.36 (0.37)	6.24 (0.48)	1.16	1.50E-02	0.076
<i>Anaerotruncus</i>	1.96 (0.87)	2.95 (0.74)	1.51	4.10E-02	0.152
<i>Desulfovibrio</i>	6.65 (0.36)	5.97 (0.6)	0.9	4.10E-02	0.152
<i>Butyricicoccus</i>	3.63 (0.73)	2.72 (0.33)	0.75	4.30E-02	0.152
<i>Parabacteroides</i>	3.18 (0.9)	4.53 (0.98)	1.42	0.065	0.189
<i>Ruminococcus</i>	6.63 (0.37)	6.33 (0.23)	0.95	0.065	0.189
<i>Faecalibacterium</i>	-0.21 (2.3)	1.86 (0.59)	-8.77E+00	0.073	0.198
<i>Blautia</i>	2.05 (1.37)	3.32 (0.5)	1.61	0.092	0.217
<i>Allobaculum</i>	6.52 (1.33)	5.51 (0.87)	0.85	0.093	0.217
<i>Proteus</i>	0.72 (2.02)	-1.3 (1.61)	-1.81E+00	0.112	0.245
<i>Lactobacillus</i>	4.77 (0.72)	4.37 (0.36)	0.92	0.24	0.495
<i>Others</i>	9.53 (0.11)	9.47 (0.15)	0.99	0.31	0.588
<i>Escherichia</i>	-0.21 (2.32)	-1.42 (1.39)	6.84	0.326	0.588
<i>Lactococcus</i>	4.37 (0.73)	4.26 (2.11)	0.98	0.336	0.588
<i>Turicibacter</i>	2.29 (3.91)	4.77 (1.48)	2.08	0.378	0.599
<i>AF12</i>	3.79 (3.28)	4.19 (0.61)	1.11	0.394	0.599
<i>[Ruminococcus]</i>	6.73 (0.3)	6.65 (0.17)	0.99	0.394	0.599
<i>Alistipes</i>	0.99 (1.68)	0.87 (0.77)	0.88	0.421	0.614
<i>Enterococcus</i>	-1.27 (1.68)	-0.65 (1.31)	0.51	0.485	0.679
<i>Deffluviitalea</i>	2.9 (0.49)	2.98 (0.58)	1.03	0.627	0.831
<i>Sporobacter</i>	-0.85 (1.63)	-0.15 (1.11)	0.18	0.676	0.831
<i>Staphylococcus</i>	-0.31 (1.61)	-0.54 (1.4)	1.76	0.676	0.831
<i>Adlercreutzia</i>	3.33 (0.62)	3.47 (0.57)	1.04	0.688	0.831
<i>Marvinbryantia</i>	-0.65 (1.31)	-1.04 (1.41)	1.59	0.729	0.851
<i>Roseburia</i>	-0.1 (3.43)	0.2 (3.88)	-2.09E+00	0.775	0.868
<i>Oscillospira</i>	8.09 (0.36)	8.19 (0.16)	1.01	0.818	0.868
<i>Streptococcus</i>	1.74 (2.13)	2.15 (0.88)	1.24	0.818	0.868
<i>Clostridium</i>	3.85 (0.66)	3.93 (0.35)	1.02	0.937	0.965
<i>Coproccoccus</i>	6.18 (0.26)	6.18 (0.42)	1	1	1

Supplementary Table 11: Differential abundance of the metabolome of APOE3/3-TR mice in the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing mice on the high-fat (HF) (45% kcal fat) diet to the High fat (45 kcal% from fat) supplemented with 6% flavan-3-ol rich cocoa (FLAV) diet.

Group Name	Flav APOE3/3 $\bar{x}$ (SD)	HF APOE3/3 $\bar{x}$ (SD)	Fold-Change	P-value	FDR q
Methanol	-1.95 (0.11)	-2.25 (0.05)	1.18	5.20E-04	3.20E-02
Xylose	-0.56 (0.27)	-0.05 (0.14)	0.08	4.70E-03	0.146
Lysine	1.17 (0.38)	1.81 (0.19)	1.69	8.40E-03	0.15
Hypoxanthine	0.14 (0.12)	0.33 (0.08)	2.1	1.20E-02	0.15
Valerate	-0.01 (0.27)	0.54 (0.25)	7.84	1.30E-02	0.15
2-methylbutyric acid	-1.54 (0.47)	-0.72 (0.37)	0.46	1.60E-02	0.15
Xanthine	0.48 (0.14)	0.78 (0.15)	1.57	1.70E-02	0.15
GTP	-1.9 (0.12)	-1.51 (0.21)	0.79	2.70E-02	0.183
1,3-Dihydroxyacetone	-3.78 (0.89)	-2.69 (0.19)	0.65	2.90E-02	0.183
3-Methyl-2-oxovalerate	-1.16 (0.28)	-0.62 (0.31)	0.6	3.00E-02	0.183
Malic	-0.48 (0.26)	-0.16 (0.14)	0.32	3.80E-02	0.201
Pyruvate	1.45 (0.27)	1.77 (0.14)	1.18	4.00E-02	0.201
2-Oxoisocaproate	-1.32 (0.23)	-0.85 (0.3)	0.72	4.20E-02	0.201
Methionine	0.69 (0.25)	0.98 (0.17)	1.64	0.057	0.235
Alpha-ketoisovaleric acid	-1.52 (0.27)	-1.06 (0.32)	0.76	0.057	0.235
Taurine	2.19 (0.21)	1.49 (0.5)	0.68	0.061	0.235
Tryptophan	-0.83 (0.39)	-0.48 (0.15)	0.49	0.084	0.308
Fructose	-0.32 (0.41)	0.06 (0.27)	-1.48E-01	0.117	0.387
Propionate	0.73 (0.51)	1.13 (0.15)	1.31	0.118	0.387
Acetate	2.75 (0.25)	3.04 (0.27)	1.09	0.128	0.396
AMP	-3.67 (0.21)	-3.29 (0.39)	0.92	0.148	0.436
Proline	0.31 (0.38)	0.6 (0.23)	2.52	0.165	0.466
Glucose	2.29 (0.12)	2.15 (0.16)	0.93	0.181	0.488
Succinate	-1.6 (1.57)	-0.65 (0.09)	0.83	0.197	0.488
Methylamine	-3.4 (0.12)	-3.18 (0.27)	0.91	0.202	0.488
Trimethylamine	-1.74 (0.38)	-1.27 (0.56)	0.67	0.205	0.488
Ornithine	-3.48 (2.7)	-1.95 (0.41)	1.05	0.225	0.518
Creatine	0.06 (0.15)	-0.21 (0.35)	-2.96E+00	0.235	0.521
Glycine	1.58 (0.32)	1.41 (0.13)	0.9	0.268	0.574
3-Phenylpropionate	-1.91 (0.34)	-3.58 (2.55)	1.74	0.281	0.582
Tyrosine	1.01 (0.33)	1.2 (0.23)	1.24	0.331	0.64
Uracil	0.59 (0.2)	0.69 (0.14)	1.08	0.345	0.64
Choline	-0.93 (1.18)	-0.37 (0.6)	0.29	0.347	0.64
Cytidine monophosphate	-1.46 (0.79)	-2.95 (2.68)	1.11	0.353	0.64
Phenylalanine	0.85 (0.37)	1.01 (0.12)	1.38	0.368	0.64
Formate	-1.65 (0.23)	-1.18 (0.91)	1.01	0.375	0.64
Galactose	0.4 (0.41)	0.21 (0.24)	0.85	0.382	0.64
Ethanol	0.9 (0.84)	0.57 (0.27)	0.97	0.405	0.644
Isoleucine	1.37 (0.29)	1.5 (0.16)	1.06	0.405	0.644
Ferulate	-2.8 (0.22)	-3.78 (2.12)	1.01	0.427	0.662
Fumarate	-2.88 (0.38)	-3.08 (0.36)	1.05	0.438	0.663
Leucine	1.81 (0.29)	1.93 (0.21)	1.1	0.457	0.675
Butyrate	1.17 (0.18)	1.33 (0.38)	1.14	0.475	0.679
Sn-Glycero-3-phosphocholine	-4.58 (2.6)	-3.41 (2.36)	0.48	0.484	0.679
Isobutyrate	-0.58 (0.37)	-0.42 (0.33)	0.73	0.493	0.679
Arabinose	-1.18 (0.27)	-1.06 (0.25)	0.86	0.509	0.686
Ribose	1.43 (0.1)	1.51 (0.2)	0.99	0.529	0.695
Urocanate	-2.48 (0.28)	-2.35 (0.35)	0.98	0.538	0.695
Threonine	1.32 (0.28)	1.23 (0.22)	0.95	0.566	0.717
Acetoin	-5.54 (2.19)	-4.6 (2.74)	0.69	0.586	0.727
Histidine	-0.34 (0.41)	-0.24 (0.23)	0.58	0.626	0.761
Glycerol	2.42 (0.99)	2.16 (0.77)	0.79	0.645	0.769
Valine	1.58 (0.29)	1.65 (0.18)	1.03	0.66	0.772
Dimethylamine	-3.71 (0.83)	-3.53 (0.6)	0.92	0.703	0.807
5-Aminopentanoic acid	-1.8 (0.54)	-1.69 (0.53)	0.84	0.764	0.861
Alanine	1.1 (0.54)	1.15 (0.16)	0.9	0.82	0.893
Lactaldehyde	-2.42 (0.44)	-2.35 (0.49)	0.93	0.821	0.893
Glutamate	2.2 (0.21)	2.18 (0.19)	1	0.863	0.922
Asparagine	0.23 (0.71)	0.28 (0.32)	0.84	0.902	0.933
Aspartate	-0.37 (0.32)	-0.35 (0.11)	0.78	0.903	0.933
Nicotinate	-1.09 (0.14)	-1.08 (0.09)	1.02	0.918	0.933
Glutamine	0.74 (0.37)	0.76 (0.22)	1.12	0.94	0.94

Supplementary Table 12: Differential abundance of the metabolome for APOE3/3-TR (E3) and APOE4/4-TR (E4) mice from the Cognitive Ageing Nutrition and Neurogenesis Animal (CANN-AN) Study. Comparing mice on the low-fat (LF) (10% kcal fat) diet.

Group Name	LF APOE4/4 x̄ (SD)	LF APOE3/3 x̄ (SD)	Fold-Change	P-value	FDR q
Xanthine	0.06 (0.19)	0.54 (0.16)	5.67	7.50E-04	4.70E-02
Aspartate	0.04 (0.32)	-0.65 (0.24)	-4.02E+01	2.10E-03	0.065
AMP	-3.13 (0.37)	-3.69 (0.2)	1.19	1.20E-02	0.176
Glucose	2.82 (0.46)	2.1 (0.33)	0.74	1.20E-02	0.176
Methanol	-2.31 (0.11)	-1.78 (0.38)	0.8	1.80E-02	0.176
Propionate	0.57 (0.27)	0.97 (0.23)	1.7	1.90E-02	0.176
Nicotinate	-0.81 (0.18)	-1.1 (0.18)	1.27	2.10E-02	0.176
Uracil	0.19 (0.25)	0.56 (0.23)	2.04	2.30E-02	0.176
Cytidine monophosphate	-0.83 (0.5)	-1.62 (0.59)	1.83	3.00E-02	0.181
Alanine	1.7 (0.17)	0.99 (0.61)	0.7	3.50E-02	0.181
Ferulate	-2.63 (0.15)	-2.96 (0.28)	1.18	3.70E-02	0.181
Ethanol	0.66 (0.27)	0.35 (0.06)	0.62	3.80E-02	0.181
Proline	0.43 (0.28)	0.04 (0.3)	0.23	4.10E-02	0.181
3-Phenylpropionate	-1.38 (0.31)	-1.86 (0.4)	1.43	4.30E-02	0.181
Asparagine	0.71 (0.08)	-0.34 (0.96)	-1.06E-01	4.40E-02	0.181
Xylose	-0.5 (0.27)	-0.16 (0.24)	0.36	4.80E-02	0.186
Urocanate	-1.91 (0.26)	-2.24 (0.26)	1.15	0.055	0.196
Lactaldehyde	-1.35 (0.46)	-1.82 (0.23)	1.36	0.057	0.196
Dimethylamine	-3.73 (0.41)	-4.19 (0.35)	1.13	0.063	0.206
Pyruvate	1.08 (0.34)	1.44 (0.24)	1.33	0.066	0.206
Trimethylamine	-0.9 (0.67)	-1.66 (0.66)	1.83	0.077	0.228
Butyrate	1.5 (0.44)	0.98 (0.58)	0.67	0.109	0.301
Methionine	0.95 (0.19)	0.7 (0.3)	0.8	0.116	0.301
Tyrosine	1.29 (0.23)	0.95 (0.42)	0.73	0.12	0.301
Glutamate	2.2 (0.19)	2.02 (0.19)	0.96	0.125	0.301
Creatine	0 (0.33)	-0.28 (0.25)	2.77	0.126	0.301
1,3-Dihydroxyacetone	-2.77 (0.37)	-3.37 (0.81)	1.15	0.139	0.307
Valine	1.73 (0.15)	1.47 (0.34)	0.92	0.139	0.307
Tryptophan	-0.42 (0.14)	-0.8 (0.56)	1.73	0.166	0.341
Hypoxanthine	0.18 (0.15)	0.3 (0.09)	1.43	0.166	0.341
Isoleucine	1.54 (0.28)	1.27 (0.34)	0.83	0.17	0.341
Arabinose	-1.1 (0.3)	-0.82 (0.36)	0.94	0.185	0.341
Glycine	1.52 (0.14)	1.32 (0.32)	0.98	0.189	0.341
Valerate	-0.1 (0.41)	-0.48 (0.51)	1.08	0.192	0.341
Lysine	1.76 (0.2)	1.49 (0.42)	0.96	0.193	0.341
Ornithine	-1.45 (0.31)	-2.72 (2.15)	1.34	0.21	0.349
Isobutyrate	-1.32 (0.4)	-0.95 (0.55)	0.82	0.215	0.349
Glutamine	0.96 (0.2)	0.71 (0.42)	0.83	0.219	0.349
Histidine	-0.03 (0.21)	-0.33 (0.5)	-7.39E+00	0.223	0.349
Leucine	1.96 (0.21)	1.71 (0.41)	0.9	0.228	0.349
Phenylalanine	1.06 (0.22)	0.81 (0.41)	0.85	0.231	0.349
Threonine	1.3 (0.23)	1.08 (0.38)	0.94	0.246	0.363
Sn-Glycero-3-phosphocholine	-6.17 (1.9)	-4.76 (2.4)	0.7	0.286	0.413
Fructose	-0.12 (0.57)	-0.43 (0.41)	-9.37E+00	0.309	0.436
Succinate	-0.88 (1.11)	-1.64 (1.47)	1.44	0.341	0.469
Malic	-0.17 (0.3)	-0.47 (0.72)	1.64	0.376	0.507
Taurine	2.23 (0.57)	2.02 (0.53)	0.95	0.532	0.695
Alpha-ketoisovaleric acid	-1.53 (0.41)	-1.41 (0.2)	1.02	0.542	0.695
2-Oxoisocaproate	-1.42 (0.49)	-1.28 (0.31)	1.04	0.566	0.695
3-Methyl-2-oxovalerate	-1.09 (0.28)	-1.25 (0.59)	0.98	0.567	0.695
5-Aminopentanoic acid	-1.69 (0.24)	-1.54 (0.58)	0.97	0.579	0.695
2-methylbutyric acid	-1.15 (0.71)	-1.78 (2.54)	0.72	0.583	0.695
Acetoin	-5.48 (2.28)	-4.78 (2.4)	0.73	0.615	0.72
Methylamine	-3.24 (0.76)	-3.09 (0.28)	0.9	0.664	0.762
Formate	-1.29 (0.29)	-1.23 (0.58)	1.03	0.817	0.922
Ribose	1.36 (0.39)	1.32 (0.2)	0.98	0.846	0.925
Fumarate	-3.08 (0.34)	-3.04 (0.22)	0.94	0.852	0.925
Galactose	0.05 (0.31)	0.01 (0.34)	-1.25E+00	0.865	0.925
Acetate	2.69 (0.3)	2.66 (0.38)	1.06	0.883	0.927
Glycerol	1.14 (0.62)	1.2 (1.26)	1.26	0.924	0.955
Choline	-0.36 (0.37)	-0.37 (0.33)	1.59	0.974	0.99
GTP	-1.86 (0.5)	-1.87 (0.49)	1	0.993	0.993

Supplementary Table 13: All urinary flavan-3-ol metabolites of the oral (poly)phenol challenge test (OPCT). Participants were split by age: younger (Y) 18-35 years old and older (O) 55+ years old. Sex and APOE4 Genotype (Gen) E4: APOE3/4 and APOE4/4 and Non E4: APOE2/2, APOE2/3, and APOE3/3. Compounds measured: Epicatechin-3-glucuronide (EC-3-GluAC), Epicatechin-3-sulfate (EC-3-S), Epicatechin-sulfate-glucuronide ion I (EC-S-GluAC I), Epicatechin-sulfate-glucuronide ion II (EC-S-GluAC II), Epicatechin-sulfate-glucuronide ion III (EC-S-GluAC III), Epicatechin-glucuronide (EC-GluAC), 5-(3',4',5'-Trihydroxyphenyl)-γ-valerolactone (3,4,5-TriHPVL), 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone (3,4-DiHPVL), 5-(3',5'-Dihydroxyphenyl)-γ-valerolactone (3,5-DiHPVL), 5-(3',5'-Dihydroxyphenyl)-γ-valerolactone-4'-glucuronide (3,5-DiHPVL-4-GluAC), 5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-glucuronide (3-HPVL-4-GluAC), 5-(3'-Hydroxyphenyl)-γ-valerolactone-4'-sulfate (3-HPVL-4-S), 5-(3'-methoxyphenyl)-γ-valerolactone-4'-glucuronide (3-MPVL-4-GluAC), 5-(4',5'-Dihydroxyphenyl)-γ-valerolactone-3'-glucuronide (4,5-DiHPVL-3-GluAC), 5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-glucuronide (5-HPVL-3-GluAC), 5-(5'-Hydroxyphenyl)-γ-valerolactone-3'-sulfate (5-HPVL-3-S), 5-(Dihydroxyphenyl)-γ-valerolactone-sulfate (DiHPVL-S), 5-(Hydroxy-methoxyphenyl)-γ-valerolactone-sulfate ion I (HMPVL-S I), 5-(Hydroxy-methoxyphenyl)-γ-valerolactone-sulfate ion II (HMPVL-S II), 5-(Methoxyphenyl)-γ-valerolactone-glucuronide (MPVL-GluAC), 5-(Phenyl)-γ-valerolactone-3'-glucuronide (PVL-3-GluAC), 5-(Phenyl)-γ-valerolactone-3'-sulfate (PVL-3-S), 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid-3'-sulfate (4-H-3-HPVA-3-S), 4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid-4'-glucuronide (4-H-3-HPVA-4-GluAC), 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-4HPVA-3-GluAC), 4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid-4'-sulfate (4-H-4-HPVA-4-S), 4-Hydroxy-5-(5'-hydroxyphenyl)valeric acid-3'-glucuronide (4-H-5-HPVA-3-GluAC), 4-Hydroxy-5-(dihydroxyphenyl)valeric acid-sulfate (4-H-DiHPVA-S), 4-Hydroxy-5-(hydroxy-methoxyphenyl)valeric acid-sulfate II (4-H-HMPVA-S II), 4-Hydroxy-5-(methoxyphenyl)valeric acid-glucuronide (3',4') (4-H-MPVA-GluAC (3,4), 4-Hydroxy-5-(phenyl)valeric acid-3'-glucuronide (4-H-PVA-3-GluAC), 4-Hydroxy-5-(phenyl)valeric acid-4'-glucuronide (4-H-PVA-4-GluAC), 4-Hydroxy-5-(phenyl)valeric acid-sulfate (4-H-PVA-S), 5-(Phenyl)valeric acid-4'-glucuronide (PVA-4-GluAC). Significant 3-way PERMUTATIONAL ANOVA (p < 0.05) values are highlighted in bold.

Sex Age Genotype	F				M				Gen	Sex	Age	Gen: Sex	Gen: Ag	Sex: Age	Gen: Sex: Age
	O	Y	O	Y	O	Y	O	Y							
Compound	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)	E4 ̄ μM/mol Creatine (SD)	Non E4 ̄ μM/mol Creatine (SD)							
EC-3-GluAC	0.182 (0.118)	0.241 (0.238)	0.133 (0.112)	0.147 (0.195)	0.086 (0.028)	0.188 (0.122)	0.097 (0.119)	0.060 (0.039)	0.345	0.077	0.089	0.955	0.212	0.857	0.505
EC-3-S	0.001 (0.002)	0.013 (0.065)	0.007 (0.007)	0.003 (0.007)	0.000 (0.000)	0.008 (0.011)	0.004 (0.006)	0.006 (0.009)	0.349	0.834	0.969	0.957	0.283	0.780	0.661
EC-S-GluAC I	0.040 (0.029)	0.049 (0.063)	0.047 (0.034)	0.039 (0.082)	0.042 (0.059)	0.067 (0.086)	0.025 (0.024)	0.021 (0.024)	0.695	0.715	0.210	0.669	0.369	0.250	0.803
EC-S-GluAC II	0.030 (0.021)	0.041 (0.063)	0.026 (0.025)	0.019 (0.028)	0.020 (0.029)	0.043 (0.038)	0.021 (0.034)	0.013 (0.012)	0.552	0.535	0.084	0.721	0.124	0.891	0.645
EC-S-GluAC III	0.021 (0.016)	0.026 (0.042)	0.023 (0.016)	0.011 (0.015)	0.027 (0.038)	0.024 (0.023)	0.010 (0.011)	0.009 (0.011)	0.608	0.595	0.035	0.862	0.437	0.366	0.346
EC-GluAC	0.015 (0.011)	0.021 (0.023)	0.011 (0.009)	0.011 (0.015)	0.000 (0.000)	0.013 (0.010)	0.006 (0.010)	0.004 (0.004)	0.184	0.025	0.178	0.706	0.102	0.391	0.508
3,4,5-TriHPVL	0.130 (0.261)	0.090 (0.121)	0.056 (0.102)	0.043 (0.069)	0.020 (0.016)	0.040 (0.062)	0.019 (0.047)	0.027 (0.042)	0.769	0.029	0.119	0.338	0.861	0.217	0.631
3,4-DiHPVL	0.021 (0.020)	0.020 (0.019)	0.014 (0.010)	0.026 (0.097)	0.004 (0.006)	0.012 (0.011)	0.004 (0.004)	0.007 (0.008)	0.579	0.168	0.911	0.979	0.834	0.893	0.604
3,5-DiHPVL	0.023 (0.014)	0.029 (0.030)	0.020 (0.017)	0.035 (0.063)	0.015 (0.002)	0.022 (0.022)	0.014 (0.019)	0.016 (0.023)	0.361	0.204	0.861	0.714	0.928	0.732	0.656
3,5-DiHPVL-4-GluAC	1.288 (1.525)	1.374 (1.680)	1.056 (0.885)	0.996 (1.380)	0.721 (0.934)	0.656 (0.959)	0.298 (0.550)	0.531 (0.800)	0.870	0.041	0.321	0.904	0.896	0.958	0.701
3-HPVL-4-GluAC	0.249 (0.134)	0.332 (0.244)	0.350 (0.213)	0.289 (0.259)	0.205 (0.216)	0.226 (0.160)	0.162 (0.163)	0.148 (0.146)	0.886	0.022	0.752	0.937	0.373	0.362	0.578
3-HPVL-4-S	24.728 (13.285)	25.173 (16.901)	27.628 (12.107)	22.267 (20.585)	24.082 (30.296)	23.737 (12.513)	12.462 (12.311)	12.977 (10.023)	0.743	0.079	0.131	0.717	0.732	0.132	0.650
3-MPVL-4-GluAC	0.224 (0.111)	0.240 (0.134)	0.219 (0.123)	0.168 (0.112)	0.191 (0.173)	0.181 (0.119)	0.112 (0.066)	0.113 (0.073)	0.677	0.018	0.036	0.815	0.594	0.504	0.446

4,5-DiHPVL-3-GluAC	0.354 (0.439)	0.396 (0.477)	0.241 (0.227)	0.282 (0.445)	0.175 (0.222)	0.178 (0.301)	0.091 (0.196)	0.153 (0.240)	0.670	0.068	0.328	0.961	0.865	0.732	0.861
5-HPVL-3-GluAC	1.284 (0.712)	1.423 (1.097)	1.216 (0.669)	1.168 (0.832)	0.753 (0.539)	1.175 (0.820)	0.748 (0.663)	0.720 (0.551)	0.533	0.036	0.323	0.698	0.425	0.861	0.742
5-HPVL-3-S	20.363 (17.545)	17.150 (14.669)	20.381 (14.437)	20.445 (20.039)	19.063 (23.486)	24.436 (18.379)	14.247 (21.024)	12.564 13.210	0.971	0.605	0.394	0.668	0.808	0.214	0.517
DiHPVL-S	4.794 (6.320)	5.713 (8.012)	4.665 (4.308)	3.868 (5.451)	3.981 (5.478)	3.363 (3.912)	1.227 (2.250)	2.339 (3.411)	0.904	0.109	0.247	0.942	0.998	0.711	0.482
5-MPVL-S 1	5.101 (6.822)	6.792 (9.521)	3.957 (3.483)	3.764 (4.892)	4.984 (6.625)	3.849 (4.239)	1.284 (2.614)	2.427 (3.548)	0.780	0.178	0.081	0.783	0.937	0.861	0.412
5-MPVL-S 1	0.975 (1.111)	1.417 (1.806)	0.933 (0.814)	0.761 (0.913)	1.291 (1.693)	0.901 (0.913)	0.305 (0.460)	0.648 (0.863)	0.835	0.363	0.070	0.758	0.907	0.599	0.199
5-MPVL-GluAC	0.020 (0.010)	0.018 (0.009)	0.014 (0.008)	0.013 (0.008)	0.010 (0.007)	0.013 (0.007)	0.007 (0.005)	0.007 (0.004)	0.877	0.001	0.007	0.420	0.813	0.905	0.676
PVL-3-GluAC	7.610 (8.770)	12.103 (17.033)	4.000 (6.231)	5.834 (7.137)	0.394 (0.135)	3.859 (5.422)	4.370 (4.696)	2.787 (3.198)	0.328	0.051	0.400	0.585	0.351	0.134	0.767
5-PVL-3-S	2.172 (2.448)	2.589 (3.184)	1.494 (1.122)	4.494 (9.667)	0.490 (0.613)	1.854 (4.638)	1.547 (1.813)	1.167 (1.438)	0.352	0.212	0.716	0.579	0.845	0.841	0.341
4-H-3HPVL-3-S	5.790 (2.962)	6.204 (4.632)	7.019 (3.943)	6.081 (8.773)	6.567 (8.636)	7.476 (5.183)	3.343 (4.437)	3.117 (2.766)	0.976	0.354	0.206	0.809	0.617	0.098	0.964
4-H-3HPVA-4-GluAC	0.043 (0.022)	0.054 (0.038)	0.048 (0.037)	0.038 (0.039)	0.024 (0.029)	0.038 (0.026)	0.019 (0.021)	0.023 (0.023)	0.539	0.013	0.314	0.589	0.335	0.794	0.720
4-H-4HPVA-3-GluAC	0.068 (0.037)	0.066 (0.038)	0.052 (0.031)	0.049 (0.045)	0.026 (0.025)	0.045 (0.027)	0.023 (0.024)	0.027 (0.023)	0.572	0.002	0.114	0.375	0.596	0.729	0.681
4-H-4HPVA-4-S	13.817 (12.278)	12.649 (13.403)	12.881 (8.556)	13.687 (14.428)	12.321 (14.952)	17.524 (16.101)	11.040 (19.323)	7.693 (9.391)	0.906	0.715	0.377	0.853	0.583	0.366	0.383
4-H-5HPVA-3-GluAC	0.057 (0.041)	0.061 (0.063)	0.049 (0.033)	0.049 (0.041)	0.026 (0.015)	0.049 (0.036)	0.028 (0.029)	0.029 (0.028)	0.493	0.043	0.339	0.611	0.535	0.949	0.676
4-H-DiHPVA-S	1.217 (1.367)	1.406 (2.004)	1.091 (1.051)	1.026 (1.692)	0.578 (0.818)	1.007 (1.175)	0.258 (0.479)	0.584 (0.861)	0.512	0.089	0.340	0.610	0.779	0.859	0.907
4-H-HMPVA-S	4.038 (4.962)	5.588 (8.966)	2.940 (2.595)	2.864 (4.269)	0.140 (0.199)	4.034 (4.693)	0.913 (2.170)	2.095 (3.261)	0.153	0.082	0.285	0.425	0.350	0.565	0.812
4-H-MPVA-GluAC (3',4)	0.122 (0.065)	0.125 (0.082)	0.106 (0.096)	0.088 (0.066)	0.099 (0.078)	0.105 (0.071)	0.049 (0.042)	0.056 (0.042)	0.973	0.042	0.021	0.678	0.746	0.478	0.734
4-H-PVA-3-GluAC	0.018 (0.023)	0.027 (0.037)	0.009 (0.009)	0.018 (0.028)	0.001 (0.001)	0.011 (0.014)	0.010 (0.010)	0.007 (0.009)	0.258	0.079	0.537	0.621	0.487	0.274	0.516
4-H-PVA-4-GluAC	0.034 (0.014)	0.035 (0.017)	0.034 (0.016)	0.029 (0.018)	0.020 (0.021)	0.027 (0.013)	0.017 (0.011)	0.019 (0.017)	0.789	0.003	0.277	0.379	0.522	0.768	0.897
4-H-PVA-S	8.399 (7.585)	6.388 (7.030)	4.337 (4.008)	7.743 (11.396)	3.278 (3.993)	6.318 (8.378)	6.801 (10.292)	4.037 (5.988)	0.836	0.401	0.845	0.881	0.962	0.603	0.148
PVA-4-GluAC	1.603 (1.562)	1.285 (1.161)	1.206 (0.925)	1.061 (1.261)	0.917 (0.854)	0.999 (0.810)	0.608 (0.619)	0.915 (1.123)	0.944	0.104	0.315	0.400	0.697	0.817	0.960