The impact of cranberry polyphenols on the brain and gut microbiome in healthy ageing

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ABSTRACT

Ageing is associated with increasing risk of cognitive decline, and modifiable lifestyle factors including diet have been shown to significantly affect the progression of age-related neurodegeneration. Specific dietary components, particularly polyphenol-rich berries such as cranberries, have been increasingly recognised for their effects on the mechanisms underlying age-related neurodegeneration, including the alteration of the gut microbiome and its functions. However, the impact of cranberries on cognition, brain function and the gut microbiome in healthy older adults remains little explored. A 12-week randomised placebo-controlled trial of freeze-dried cranberry powder was conducted in 60 healthy older adults aged between 50 and 80 years. Cognitive assessment, including memory and executive function, neuroimaging, and blood, stool and urine sample collection were conducted before and after the intervention to assess the impact of daily cranberry consumption on cognition, brain function, and the structure and function of the gut microbiome. Cranberry supplementation significantly improved visual episodic memory, with mechanisms of action underpinned by increased regional perfusion in the right entorhinal cortex, the nucleus accumbens area and the caudate. A beneficial shift in microbial abundances in bacterial families relating to polyphenol degradation was also detected in the cranberry group, which correlated with increased levels of circulating polyphenol metabolites in plasma. Gut bacteria-derived metabolites such as TMAO and hippuric acid were also significantly related to improved episodic memory in the cranberry group, although common polyphenol metabolites did not relate to cognitive performance or brain perfusion. These results indicate that daily cranberry supplementation over a 12-week period improved episodic memory performance and neural functioning, corresponding with a beneficial shift in the composition and function of the gut microbiome. These findings provide a basis for future investigations

to determine efficacy of intake of high-polyphenol cranberry in the context slowing the onset of neurodegenerative diseases, such as Alzheimer's disease.

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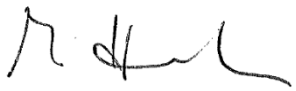
SUPERVISOR SIGNATURE

I, Michael Hornberger (primary supervisor), confirm that any required taught courses have been satisfactorily completed:



AUTHOR'S DECLARATION

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Parts of this work have been presented at conferences and submitted to academic journals.

ETHICAL APPROVAL

The study was conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the University of East Anglia's Faculty of Medicine and Health Sciences ethical review committee (Reference: 201819 – 039) and the Health Research Authority (IRAS number: 237251; HRA number: 18/HRA/1339; ClinicalTrial.gov: NCT03679533). All study participants provided written informed consent before participating in the study. Participants took part in the study on a voluntary basis and as such were able to withdraw from the study at any time. Withdrawal from the study would take place if participants lost capacity to consent during the study, in the event of a serious adverse event, non-compliance to taking the study powder, or if continuation of the study would be detrimental to the participant.

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ORAL PRESENTATIONS RESULTING FROM THIS PHD

The impact of Cranberries On the Microbiome, Brain and Ageing sTudy (COMBAT). UEA Dementia Open Forum, 25th February 2021 13:30 – 14:30, Norwich Research Park, UK.

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ABBREVIATIONS

ACE-III: Addenbrooke's Cognitive Examination III

AD: Alzheimer's disease

ADNI: Alzheimer's Disease Neuroimaging Initiative

APOE: Apolipoprotein E

ASL: Arterial Spin Labelling

BBB: Blood Brain Barrier

BDNF: Brain-Derived Neurotrophic Factor

BMI: Body Mass Index

CBF: Cerebral Blood Flow

CBI-R: Cambridge Behavioural Index Revised

CCI: Cognitive Change Index

COVID-19: Coronavirus disease 2019

CPT: Cell Preparation Tube

DNA: Deoxyribonucleic acid

DS: Digit Span

EDTA: Ethylenediaminetetraacetic acid

ELISA: Enzyme Linked ImmunoSorbent Assay

FINGER: Finnish Geriatric Intervention Study to Prevent Cognitive Impairment and Disability

FLAIR: Fluid Attenuated Inversion Recovery

GAD-7: Generalised Anxiety Disorder 7

GGT: Gamma Glutamyl Transferase

HELIAD: The Hellenic Longitudinal Investigation of Aging and Diet

HPA: Hypothalamic-Pituitary-Adrenal

IPAQ: International Physical Activity Questionnaire

IR-FSPGR: Inversion-Recovery Fast Spoiled Gradient Recalled Echo

MCI: Mild Cognitive Impairment

MCV: Mean Corpuscular Volume

MedEx-UK: The Mediterranean Diet, Exercise and Dementia Risk Reduction Programme

MTL: Medial Temporal Lobe

MRI: Magnetic resonance imaging

NDNS: National Diet and Nutrition Survey

NMNA: N-methyl-nicotinic acid

NMR: Nuclear Magnetic Resonance

NNUH: Norfolk and Norwich University Hospital

OPLS: Orthogonal Projection to Latent Structures

PAG: Phenylacetylglutamine

PHQ-9: Patient Health Questionnaire 9

PSQI: Pittsburgh Sleep Quality Index

PREDIMED: Prevención con Dieta Mediterránea study

RCT: Randomised Controlled Trials

RCF: Rey Complex Figure

RNI: Recommended Nutrient Intake

ROI: Region of Interest

SCFA: Short-Chain Fatty Acids

SCG-FFQ: Scottish Collaborative Group Food Frequency Questionnaire

SST: Serum Separation Tube

TMA: Trimethylamine

TMAO: Trimethylamine N-oxide

TMT: Trail Making Test

WHO: World Health Organisation

WMH: White Matter Hyperintensities

CHAPTER 1: GENERAL INTRODUCTION

As a result of astounding advances in medical science and socio-economic conditions over the past century people are now living considerably longer than ever before. Despite this triumph in extending the length and quality of our lives, these gains in life expectancies carry with them the increased prevalence of age-related chronic disease such as cardiovascular disease, diabetes, and dementia. Ageing is the greatest single risk factor for the development of neurodegenerative diseases that impact daily functioning and cause dementia such as Alzheimer's disease (AD), presenting a devastating personal and societal burden. By recent estimates, the global prevalence of dementia is expected to double every 20 years, with 152 million people projected to be affected by 2050 (*Alzheimer's Disease International. World Alzheimer Report 2019: Attitudes to dementia*, 2019). Although some degree of cognitive decline is to be expected with normal ageing as is well documented in studies involving both animals and humans, it is the significant loss of function and self that is associated with age-related neurodegenerative disease which poses a highly detrimental cost to individuals and their families.

To date there are currently no effective pharmacological treatments that directly curtail the progression of the mechanisms underlying these conditions, instead often targeting only single aspects of disease pathology such as amyloid deposition or treating clinical symptoms. This is despite significant effort and resources having been invested over several decades to identify a pharmacological cure for dementia. This failure so far to produce a cure for age-related neurodegeneration is partly owing to the exact mechanisms underlying these diseases still being elucidated. The factors causing these conditions cannot be explained by genetics alone, for example with identifiable genetic mutations accounting for only 1-2% of AD cases (Bettens, Sleegers, & Van Broeckhoven, 2010), leaving the vast majority of cases being 'sporadic' and having no clear family history or presence of a known genetic mutation

associated with disease onset. The pathophysiological processes leading to neurodegeneration, like many other diseases of the body, are proposed to involve the dysfunction of multiple systems. Neurodegeneration is hypothesized to be characterised primarily by the deposition of aggregated proteins within the brain, modulated by progressive changes in several interlinked cellular and molecular mechanisms which include chronic neuroinflammation, oxidative stress and metabolic imbalances, and loss of vascular integrity and function. Such mechanisms underlie not only pathological but also normal brain ageing, resulting in loss of neural plasticity and neuronal death (Foster, 2006).

Furthermore, the disease processes that lead to dementia are now understood to commence long before symptom onset, with slow atrophy of the brain occurring for decades prior to significant symptoms and clinical diagnosis. This trajectory is proposed to be determined by a complex interplay of genetic, endogenous and environmental factors (Livingston et al., 2017). This means that it is currently very difficult to determine when these processes begin and who is at greater risk, although great efforts are being made to this end. However, this also means that there is a significant window of opportunity to curtail the disease processes before they significantly impact cognition and function to a clinical degree. As a result, attention has turned from identifying a cure towards alternative methods of preventing neurodegeneration before disease onset which ideally are low cost, safe, and easy to implement, and that target multiple factors contributing to the disease process.

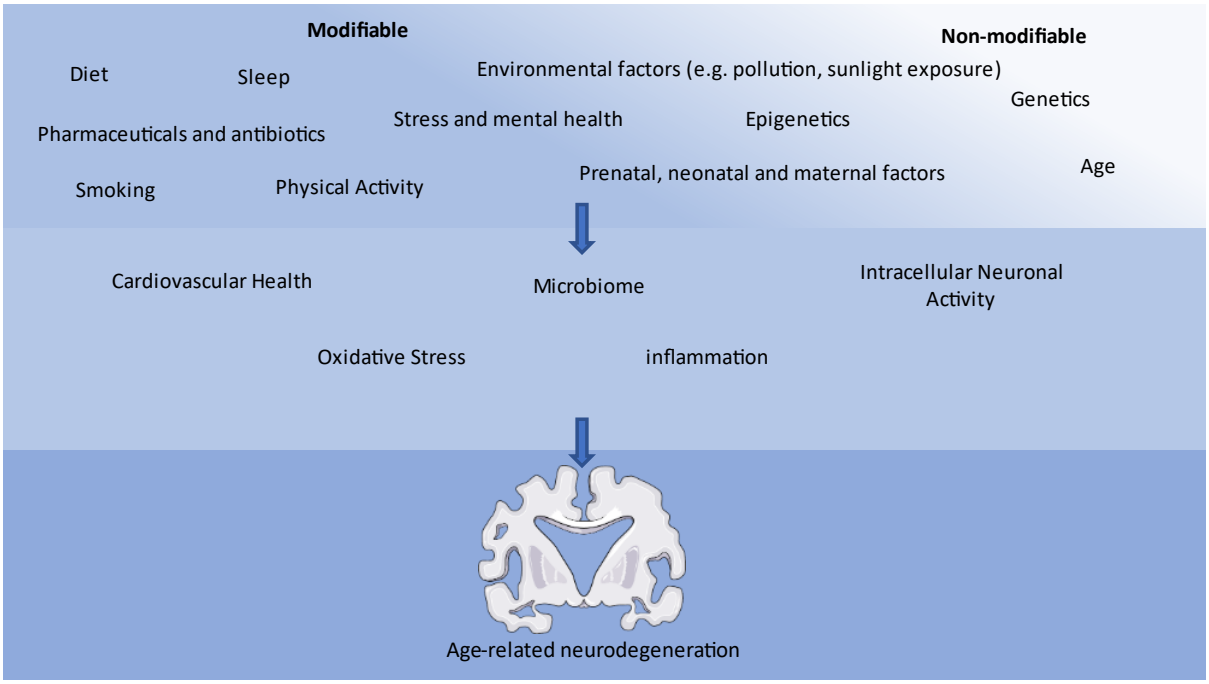


Figure 1.1. Influences on neurocognitive ageing including therapeutic and lifestyle targets for preventing age-related neurodegeneration.

Indeed, in 2019 the World Health Organisation (WHO) published its first guidelines for reducing the risk of cognitive decline and dementia (World Health Organization, 2019). These guidelines were based on a growing body of clinical trial evidence supporting the efficacy of lifestyle interventions to target modifiable risk factors and disease mechanisms for age-related cognitive decline (see Figure 1.1.). Evidence from large-scale epidemiological studies such as the Three City Study (Amadieu et al., 2017) and the HELIAD study (Dardiotis, Kosmidis, Yannakoulia, Hadjigeorgiou, & Scarmeas, 2014) has indicated that diet could be a key factor in delaying the progression of age-related neurodegeneration. This has initiated a surge in large-scale clinical trials involving humans investigating the impact of diet on outcomes relating to cognitive ageing, often in conjunction with other modifiable lifestyle factors such as physical activity. For example, the FINGER (Finnish Geriatric Intervention

Study to Prevent Cognitive Impairment and Disability) study emerged to investigate the impact of a 24-month multi-domain approach including diet, exercise, cognitive training and vascular risk factor management to preventing cognitive decline in older at-risk individuals aged > 60 years (Ngandu et al., 2015). Several more randomised controlled trials (RCTs) which include changes to dietary patterns, often a Mediterranean-style diet, have been conducted with promising results. The Prevención con Dieta Mediterránea (PREDIMED) RCT produced beneficial effects of a Mediterranean diet supplemented with nuts or olive oil on cognitive function (Martinez-Lapiscina et al., 2013; Martínez-Lapiscina et al., 2014; Valls-Pedret et al., 2015). Currently, the Mediterranean Diet, Exercise and Dementia Risk Reduction Programme (MedEx-UK) study (Shannon et al., 2021) is a multi-centre trial investigating whether adherence to a Mediterranean dietary pattern, in addition to physical activity, impacts cognition and neural function in older adults identified as at risk of developing dementia.

The mechanisms by which beneficial dietary patterns exert their effects on health also remain to be fully understood, particularly when it comes to which specific nutrients or foods within these patterns are driving health benefits in humans. The majority of existing evidence for the health potential of specific nutrients come from epidemiological and preclinical studies, although RCT's investigating the impact of certain nutrients on health in humans are becoming more prevalent. Indeed, certain nutrients have been identified to exhibit protective effects against some of the mechanisms causing this decline such as improving cardiovascular health, neuronal signalling and function, and the diversity and health and function of the gut microbiome, as well as improving outcomes of cognitive performance and reducing risk of neurodegenerative disease onset. Likely candidates for these specific nutrients (and foods that contain them) include B vitamins (Lefevre-Arbogast et al., 2016)), vitamin D (Koduah, Paul, & Dorr, 2017), long-chain omega-3 fatty acids, (Samieri et al.,

2008; Samieri et al., 2012; Yu Zhang et al., 2015) and plant-derived polyphenols. Polyphenols and their neuroprotective potential and the possible mechanisms by which they support brain health and cognition across the lifespan will be explored further here.

POLYPHENOLS

Attention has been increasingly drawn to the health-promoting benefits of polyphenols. Polyphenols are a complex family of non-nutritive plant-derived compounds found abundantly in fruit, vegetables, cocoa, and certain beverages such as coffee and tea. Polyphenols can be further divided into flavonoids and non-flavonoids, which is determined by the number of phenol rings they contain and how these rings interact. Dietary flavonoids are the most prevalent polyphenolic compounds in the human diet, and include approximately 6000 different types, and the relative consumption of the different types of flavonoids can vary greatly between individuals depending on diet (Manach, Scalbert, Morand, Remesy, & Jimenez, 2004; Manach, Williamson, Morand, Scalbert, & Remesy, 2005). Flavonoids are comprised of two aromatic rings bound by three carbon atoms and can be further divided into flavonols, flavones, flavanones, isoflavones, anthocyanidins and flavanols (G. R. Beecher, 2003). These phytochemicals have received attention due to their implicated role in directly impacting the development of non-communicable diseases, including cancer, type-II diabetes mellitus and cardiovascular disease, as well as the disease mechanisms which have been identified as underlying the progression of neurodegeneration.

POLYPHENOLS AND COGNITIVE DECLINE

Polyphenol-rich whole foods and extracts have been shown to protect against the development or slow the progression of neurological conditions, including dementia and age-related cognitive decline (Dai, Borenstein, Wu, Jackson, & Larson, 2006; Devore, Kang, Breteler, & Grodstein, 2012). Indeed, nutritional epidemiological studies have reported that higher dietary intake of polyphenol-containing foods is associated with slower rates of cognitive decline

(Devore et al., 2012; Letenneur, Proust-Lima, Le Gouge, Dartigues, & Barberger-Gateau, 2007; Shishtar, Rogers, Blumberg, Au, & Jacques, 2020) and dementia (Lefèvre-Arbogast et al., 2018). For example, in the Three City Study, higher reported intakes of fruit and vegetables were found to be associated with a lower risk of cognitive decline, which was attributed to the higher concentrations of specific polyphenols contained in these foods (Barberger-Gateau et al., 2007). Furthermore, higher intake of polyphenol-rich foods was associated with a significantly lower risk of dementia in a large cohort of older adults aged 65 years and over (Commenges et al., 2000), and higher polyphenol consumption in middle age corresponded with better cognitive function in later life (Kesse-Guyot et al., 2012).

Polyphenols are also in a higher concentration among certain diets that have been found to have protective health benefits, such as the Mediterranean-style diets (Wu & Sun, 2017), attributed largely to the higher proportion of fruit and vegetable intake in these dietary patterns. By contrast, results from the UK National Diet and Nutrition Survey (NDNS) indicate that at least 70% of the UK population are consuming below the recommended intake (Bates et al., 2014). Adults aged over 65 years in the UK are estimated to achieve approximately 1035.1 ± 544.3 mg/day of polyphenols (Ziauddeen et al., 2019), although estimates can vary between studies and can depend on the methods of dietary data collection and cohort selection (Vogiatzoglou et al., 2015; Zamora-Ros et al., 2016; Zamora-Ros et al., 2013). The main dietary contributors to polyphenol intake in the UK are non-alcoholic beverages (coffee and tea) followed by chocolate, fruit juice, and then fruit in general (Zamora-Ros et al., 2016).

Several specific polyphenols have been identified as producing the most salient protective effects for health, which has sparked investigation into how these compounds could be producing these results in human subjects at concentrations that are feasibly attainable through dietary intake or supplementation. Berries in particular have a polyphenolic profile

which is high in concentrations of several of these beneficial polyphenols, and the growing amount of evidence supporting the potential benefits of berry intake on cognitive ageing and neurodegeneration will be detailed further in the next section.

Berry Polyphenols

Many epidemiological studies which have found that higher intake of polyphenol-rich foods supports health do not specify which particular polyphenols may be carrying these benefits. Of the foods containing higher concentrations of polyphenols that have also been shown to exert effects on the disease mechanisms considered to underpin neurodegeneration, berries have gathered particular attention due to their specific profile of health-promoting polyphenols. Berries that have received the most focus for their health promoting benefits include blueberry, strawberry, acai, grapes, bilberry, raspberry and cranberry. The chemical composition of berries can vary greatly depending on their genetics as well as the conditions of their growth, storage, and processing. In general, however berries are high in concentrations of flavonoids including anthocyanidins (delphinidin, cyanidin, petunidin, peonidin and malvidin), flavanols (catechin, epicatechin, proanthocyanidins B type) and flavonols (quercetin and myricetin) (Rothwell et al., 2013). Other phenolic compounds such as ellagitannins and derivatives of hydroxybenzoic and hydroxycinnamic acids are also present. Anthocyanins and proanthocyanidins are found in particularly high concentrations in berries, which has caused an increased interested in the potential health benefits of increasing dietary intake of berries. Cranberries (*Vaccinium macrocarpon*) are especially rich in anthocyanins (delphinidin, cyanidin, petunidin, peonidin and malvidin), flavanols or flavan-3-ols (catechin, epicatechin, proanthocyanidins type), and flavonols such as quercitrin and myricitrin (Vvedenskaya et al., 2004).

Of the polyphenols found in higher concentrations in berries, anthocyanins are of particular interest because they are amongst the most commonly consumed polyphenols in a normal

diet and as such are generally regarded as safe in higher concentrations (Scalbert & Williamson, 2000). Anthocyanins are largely responsible for the pigmentation present in berry fruits, and the most common anthocyanins are composed of one of six anthocyanidin bases which differ in structure, particularly with regards to their B ring, with an extra sugar moiety added to the C-ring. Anthocyanins are also found in other foods but to a lower extent, as they lack the sugars that stabilise the charge (Wrolstad, Skrede, Lea, & Enersen, 1990). Proanthocyanidins, also referred to as condensed tannins, are oligomers or polymers of flavan-3-ols and can involve either A- or B-type linkages between constituents. Specifically, the B-type dimers are linked in either the C4-C6 or C4-C8 position while A-type are linked in the C4-C8 position with an additional C2-O-7 linkage (see Figure 1.2). These polyphenols are also found particularly abundantly in berry fruits, and also have a suggested role in protecting against neurodegenerative disorders such as dementia (Zhao, Zhang, Yang, Li, & Rong, 2019). Proanthocyanidins with B-type linkages are the most common, however the more unusual A-type linkages can be found in greater abundance in certain berries such as cranberries (Foo, Lu, Howell, & Vorsa, 2000). Proanthocyanidins with A-type linkages have been particularly implicated in underlying health benefits associated with these polyphenols, particularly for their anti-adhesion activity for uropathogenic *Escherichia coli* (Howell et al., 2005). There are also other specific polyphenols found in berries to which neuroprotective benefits have been attributed, although to a lesser extent, such as the flavonol quercetin (quercitrin without the deoxy sugar rhamnose) due to its potential cholinesterase inhibitory potential (Orhan, 2021). Indeed, quercetin has been demonstrated *in vitro* to inhibit the aggregation of certain proteins associated with AD, such as amyloid beta plaques, alpha synuclein and tau, by directly interacting with misfolding proteins resulting in the stabilisation of oligomers and the inhibition of fibril growth (Dhouafli et al., 2018), and to

protect against cognitive deficits in a transgenic mouse model of AD, while also reducing Alzheimer's pathology (Sabogal-Guaqueta et al., 2015).

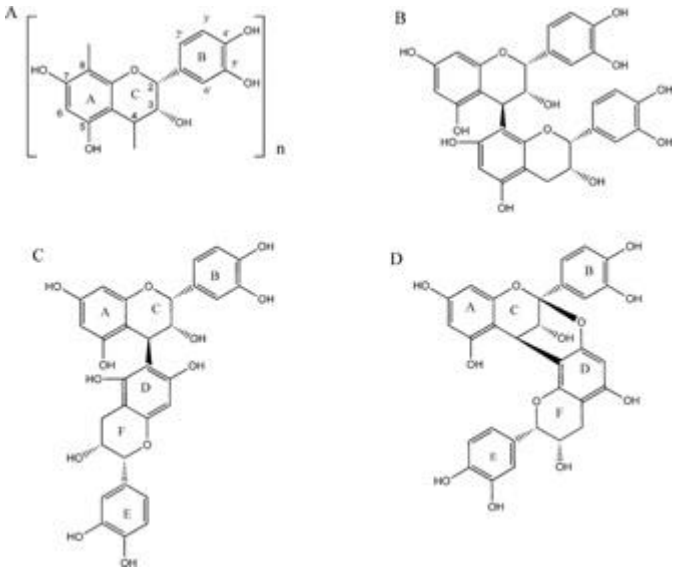


Figure 1.2. Representative linkages within proanthocyanidin molecules. (A) Monomeric representation with carbon 4 and 8 shown as potential linkages. Structure of (−)-epicatechin shown as example. Letters within rings identify individual phenolic or heterocyclic ring. n may equal 2 (dimer) to ~ 50. (B) Example of B type (4→8) linkage. Specific compound is procyanidin B2 (dimer), epicatechin-(4β→8)-epicatechin. (C) Example of B type (4→6) linkage. Specific compound is procyanidin B5 (dimer), epicatechin-(4β→6)-epicatechin. (D) Example of A-type (4→8, 2→7) linkage. Specific compound is procyanidin A2 (dimer), epicatechin-(2β→7, 4β→8)-epicatechin. From Beecher, G. R. (2004). Proanthocyanidins: Biological activities associated with human health. Pharmaceutical Biology, 42(sup1), 2-20 (Gary R. Beecher, 2009).

Berry Polyphenols and Cognitive Decline

Supplementation of berries have been found to improve cognition in both animal models and humans. Animal models have allowed for higher levels of direct manipulation and control over polyphenol doses and conditions that are not always possible in studies involving humans. In rodents, berry supplementation has been shown to improve spatial memory (Casadesus et al., 2004; Shukitt-Hale et al., 2015; C. M. Williams et al., 2008), object recognition memory (Goyarzu et al., 2004) and inhibitory fear conditioning learning (Barros et al., 2006). Blueberry appears to have a pronounced effect on short-term memory (Ramirez et al., 2005), and has also been shown to improve long-term reference memory following 8 weeks of supplementation in aged rats (Rendeiro et al., 2012; C. M. Williams et al., 2008). Supplementation with a mix of blueberry and grape in ageing beagle dogs was able to protect against cognitive impairments (Fragua et al., 2017).

Regarding intervention studies investigating how increased intake of dietary polyphenols impact cognitive performance in humans, while there have been findings of improved global cognitive performance (Kean et al., 2015), it has emerged that specific cognitive domains may particularly be affected. Furthermore, the cognitive domains that appear to be most impacted by increased intake of dietary polyphenols, particularly those from berries and other fruits high in anthocyanins, are domains that are also impacted by age-related cognitive decline. These include episodic memory, working memory and executive function (for summary of human intervention studies involving the impact of berries or high-anthocyanin fruits on cognitive performance, see Table 1.1.). However, the evidence from these studies is not conclusive, as there still exists significant inconsistencies between studies regarding which cognitive domains appear to be impacted by increased dietary polyphenol intake. Indeed, the inconsistency in findings of positive effects, even between studies using the same cognitive tests, have led to several authors to be tentative in concluding that berry polyphenols positively impact cognitive performance (Kent, Charlton, Netzel, & Fanning, 2017; Travica et al., 2020), including in older

age (Solfrizzi et al., 2018) and within the context of dementia (Colizzi, 2019). The reasons for this inconsistency appear to be due to significant disparities in methodologies between studies, including sample and test selection, as well as the duration of interventions.

First of all, the cognitive domains that are affected by these interventions appear to depend on the characteristics of the sample. Many studies investigating the impact of berry polyphenols on cognition in humans involve samples with younger age groups (Barfoot et al., 2019; Watson et al., 2019; Whyte, Cheng, Butler, Lamport, & Williams, 2019), where verbal episodic memory and executive function performance appear to be most commonly affected where they are measured, even following acute doses (Barfoot et al., 2019; Whyte, Schafer, & Williams, 2016; Whyte & Williams, 2015). However, these improvements have not been consistently demonstrated even when using the same cognitive tests (Barfoot et al., 2021; Whyte, Lamport, Schafer, & Williams, 2020). In older age groups, the impact of berry polyphenols on cognition in middle-aged and older adults has also been demonstrated to improve episodic memory performance, however the evidence for episodic memory improvement from studies involving older adults is scarcer and less robust (Bowtell, Aboo-Bakkar, Conway, Adlam, & Fulford, 2017; Cook, Sandu, & Joyce Ph, 2020; Crews et al., 2005). Furthermore, these interventions appear to have the most impact where measurable memory deficits are already present, where participants have either MCI significant subjective memory complaints, or a clinical diagnosis of dementia (Kent, Charlton, Roodenrys, et al., 2017; Krikorian et al., 2012; Krikorian et al., 2020; Krikorian, Nash, Shidler, Shukitt-Hale, & Joseph, 2010; Krikorian, Shidler, et al., 2010; Whyte, Cheng, Fromentin, & Williams, 2018), although significant impacts on other cognitive domains such as working memory have not been as robustly demonstrated in these groups (Boespflug et al., 2018). Furthermore, although improvements on delayed recall trials have been detected, recognition trials rather than retention appears to be commonly improved in middle-aged to older age groups (McNamara et al., 2018; Whyte et al., 2018; Whyte et al.,

2021), which suggests at least a partial role of executive function to inhibit interference items rather than memory performance alone. Indeed, findings of improvements on tests of executive function or scores on memory tests that are understood to be influenced by executive function (such as inhibition of repetition errors) have also been detected in these older age groups with or without existing cognitive deficits (Krikorian et al., 2020; Miller, Hamilton, Joseph, & Shukitt-Hale, 2018). As such, differences in test selection may impact the consistency of findings of improved cognitive performance between studies. While tests of verbal episodic memory are the most commonly used for measuring memory performance, tests of visuospatial episodic memory appear to be more commonly significantly impacted in studies where tests of both verbal and visual episodic memory performance are used (Krikorian et al., 2020; Lamport, Lawton, et al., 2016). Previous findings have also suggested that effects on cognitive performance are more pronounced on more cognitively demanding tests (Whyte et al., 2021). This is especially relevant for studies involving healthy older adults without existing cognitive deficits, as it is unlikely that benefits of a dietary intervention on cognitive performance is likely to be detected if participants are already performing at ceiling on these cognitive tests. Therefore, it could be expected in healthy older adults that performance on measures of episodic memory, particularly visuospatial memory, and executive function would be expected to be impacted by increased intake of berry polyphenols, particularly if tasks are sufficiently cognitively demanding.

It also appears that a number of trials that did not find significant effects or found trends that only approached significance were not adequately powered in order to detect group differences in cognitive performance due to small sample sizes (Boespflug et al., 2018; Bowtell et al., 2017; Cook et al., 2020; Schrager, Hilton, Gould, & Kelly, 2015) compared with trials with larger sample sizes (Lamport, Lawton, et al., 2016; Miller et al., 2018; Whyte et al., 2021). The exceptions to this appears to be cases where measurable cognitive impairment is already

present (Krikorian et al., 2012; Krikorian et al., 2020; Krikorian, Nash, et al., 2010; Krikorian, Shidler, et al., 2010). As such, when investigating the impact of polyphenols in healthy older adults without pre-existing cognitive deficits, a larger sample size appears to be necessary to observe effects on cognitive performance.

However, relatively larger sample sizes have not guaranteed positive results for cognitive performance in healthy older adults (Crews et al., 2005). The length of trial may also be critical for detecting measurable impacts on cognitive performance in this age group. Although acute improvements on cognitive performance have been demonstrated in younger age groups (Barfoot et al., 2019; Whyte & Williams, 2015) and that acute doses may assist in resistance to cognitive fatigue in adult age groups (Watson et al., 2015; Whyte et al., 2021), acute interventions have not similarly produced significant improvements in cognitive performance in adults (Hendrickson & Mattes, 2008). Instead, improvements in cognitive performance in adults, particularly older age groups, are measured after chronic intake over a period of several weeks. Regarding the length of trials required to detect improvements in cognitive performance in these age groups, the length of the intervention also appears to be critical. Interventions of up to 6 weeks have not produced significant improvements in cognitive performance (Cook et al., 2020; Crews et al., 2005), whereas significant differences have been detected following longer durations of 12 weeks (Kent, Charlton, Roodenrys, et al., 2017; Krikorian et al., 2012; Krikorian, Nash, et al., 2010; Krikorian, Shidler, et al., 2010; Lamport, Lawton, et al., 2016; Whyte et al., 2018). Furthermore, the study by Lamport, Lawton, et al. (2016) found effects of Concord grape juice on verbal memory and executive function appeared to endure for some time after consumption, replicating similar findings of an enduring effects of an 8-week citrus juice trial on memory and executive function in a group of healthy older adults (Kean et al., 2015). As such, it appears based on previous findings that longer-term chronic intake, specifically over 12 weeks, is required to observe measurable impacts of polyphenol-rich

berries on cognitive performance in healthy older adults, that may persist possibly due to longer term modulation of the underlying mechanisms that contribute to cognitive function. Such mechanisms contributing to cognitive decline in older age and the possible impact of will be explored in the next section.

Table 1.1. Human intervention studies investigating the impact of berry or high-anthocyanin fruit polyphenols on cognitive performance.

Reference	Study design	Study Duration	Sample population	Age (years)	Sample size	Polyphenol source	Cognitive Domains	Neuropsychological Measures	Key Findings
Ahles et al. (2020)	Double-blind, randomised, placebo-controlled parallel	24 weeks	Healthy middle-aged adults	40-60	34 90mg 35 150mg 32 placebo	90mg Chokeberry extract 150mg Chokeberry extract	Executive function Attention and psychomotor speed	Stroop test Grooved peg-board test Number cross-out test	Psychomotor speed improved compared to placebo. Attention and cognitive flexibility (executive function) not significantly improved.
Barfoot et al. (2019)	Single-blind, randomised, placebo-controlled, parallel	Acute (2 hours)	Healthy children	7-10	29 wild blueberry 25 placebo	Wild blueberry drink	Verbal episodic memory Executive function Reading efficiency	RAVLT Modified Attention Network Task MANT	Significantly faster reaction time on MANT (executive function) for wild blueberry group. Better performance on verbal episodic memory recall and learning.
Barfoot et al. (2021)	Single-blind, randomised, placebo-controlled, parallel	4 weeks	Healthy children	7-10	8 Wild blueberry 7 placebo	Wild blueberry drink (freeze-dried and mixed with water and orange squash)	Verbal Episodic memory Executive function	RAVLT MANT	No significant impact on memory performance, but impact on more difficult components of executive function task, with higher accuracy on incongruent trials in the wild blueberry group (MANT).
Boespflug et al. (2018)	double-blind, randomised, placebo-	16 weeks	Older adults with MCI	67 and older	8 blueberry 8 placebo	Freeze-dried blueberry	Working memory	*N*-back task	No improvement on working memory performance.

	controlled, parallel								Increased regional BOLD activation during working memory task-based fMRI (see Table 1.2).
Bowtell et al. (2017)	Double-blind, randomised, placebo-controlled parallel	12 weeks	Healthy older adults	65 and older	12 blueberry 14 placebo	Blueberry extract	Verbal and spatial episodic memory Psychomotor function Executive Function Working memory	CogState Ltd battery: Detection task Groton maze learning test Identification task Shopping list task *N*-back tasks Stroop test	Trends towards improved working memory performance in blueberry group. Regional increases in perfusion as measured on ASL for blueberry group, and activation during task-based (Stroop) fMRI (see Table 1.2).
Cook et al. (2020)	Double-blind, randomised, placebo-controlled, crossover	1 week	Healthy older adults	M=69±4	14	600mg/day New Zealand blackcurrant extract	Verbal episodic memory Working memory Executive Function Attention and psychomotor speed Social and emotional cognition	CANTAB	No effect of blackcurrant extract on cognitive performance.
Crews et al. (2005)	Double-blind, randomised, placebo-controlled, parallel	6 weeks	Healthy older adults	60 and older	25 cranberry 25 placebo	27% cranberry juice	Verbal and visual episodic memory Executive function	Selective reminding test Stroop Test WMS-III Faces I and Faces II TMT WAIS-III Digit Symbol Coding	No significant effect of cranberry on cognitive performance.

Hendrickson and Mattes (2008)	Double-blind, randomised, placebo-controlled, crossover	Acute (immediate)	Adult smokers	M=26	35	Concord grape juice	Implicit memory	Word Fragmentation Test	No significant effect on cognition.
Kent, Charlton, Roodenrys, et al. (2017)	Double-blind, randomised, placebo-controlled, parallel	6, 12 weeks	Older adults with mild to moderate dementia	70 and older	24 cherry 25 control	Anthocyanin-rich cherry juice (vs. apple juice)	Verbal episodic memory Working memory Semantic memory Executive function	RAVLT TMT DS (backwards) Self-ordered pointing task Boston naming test Verbal fluency	Improved verbal fluency (which can be impacted by deficits in executive function), immediate, and delayed verbal episodic memory performance at 12 weeks.
Krikorian, Nash, et al. (2010)	Double-blind, randomised, placebo-controlled, parallel	12 weeks	Older adults with MCI	M=78.2 (±5.0)	5 concord grape juice 7 placebo	Concord grape juice	Verbal and visual episodic memory	CVLT Spatial Paired Associate Learning Test	Improved performance on verbal learning. Non-significant trend towards improved verbal and spatial recall.
Krikorian, Shidler, et al. (2010)	Double-blind, randomised, placebo-controlled parallel	12 weeks	Older adults with MCI	M= 76.2 (±5.2)	9 blueberry 7 placebo	Blueberry juice	Verbal episodic memory	V-PAL CVLT	Improved memory performance in the blueberry group, particularly learning and recall.
Krikorian et al. (2012)	Double-blind, randomised, placebo-controlled parallel	16 weeks	Older adults with MCI	68-90	10 concord grape juice 11 placebo	Concord grape juice	Verbal episodic memory	CVLT-II	Concord grape juice produced significantly reduced interference during recognition memory. No effects on learning or retention were observed.

Krikorian et al. (2020)	Double-blind, randomised, placebo-controlled parallel	16 weeks	Older adults with MCI	77.3 (±6.3) 77.0 (±5.4)	16 blueberry 21 placebo	Freeze-dried blueberry	Verbal and visual episodic memory Executive Function Attention and Psychomotor Speed	HVLT SPAL COWAT TMT	Improved visual episodic memory and semantic fluency in the blueberry group. Trend for improved performance on verbal episodic memory, and executive function (as measured by the TMT).
Lamport, Lawton, et al. (2016)	Double-blind, randomised, placebo-controlled, crossover	12 weeks	Healthy middle-aged adults	40-50	25	Concord grape juice	Verbal and visual episodic memory Executive function Attention	VVLT (visual analogue to RAVLT) VSLT RVIP Grooved pegboard Tower of Hanoi	Improvements in immediate spatial memory compared to placebo, which endured for those who crossed over into the placebo arm.
McNamara et al. (2018)	Double-blind, randomised, placebo-controlled, parallel	24 weeks	Older adults with subjective cognitive impairment	69 (±5.2) fish oil 68 (±3.9) blueberry 68 (±4.7) blueberry and fish oil 67 (±4.9) placebo	17 fish oil 19 blueberry 20 blueberry and fish oil 20 placebo	Freeze-dried blueberry (and combined with fish oil)	Verbal episodic memory Executive function Attention and Psychomotor Speed	HVLT TMT COWAT	Blueberry group showed improved recognition discrimination (but not when combined with fish oil).
Miller et al. (2018)	Double-blind, randomised, placebo-controlled, parallel	90 days	Healthy older adults	67.8 (±4.6) blueberry 67.3 (±4.8) placebo	18 blueberry 19 placebo	Freeze-dried blueberry	Verbal and spatial episodic memory Working memory Executive Function Attention and Psychomotor Speed	CVLT-II vMWM DS TST TMT ANT	Improved executive function (fewer repetition errors on CVLT and reduced switching cost on TST) in blueberry group.

Schrager et al. (2015)	Double-blind, randomised, placebo-controlled, parallel	6 weeks	Healthy older adults	60 years and older	13 blueberry 7 placebo	Flash-frozen whole blueberries (vs. carrot juice drink control)	Executive function	TMT Reciting days of the week backwards during adaptive gait task	No impact on cognitive performance
Traupe et al. (2018)	Randomised, parallel	2 weeks	Middle aged adults (20 day pre- and 3 hours, 24 hours post-general anaesthetic)	66.0 (±4.1) blueberry 67.0 (±3.0) control	13 blueberry 13 control (no placebo)	Blueberry juice	Verbal episodic memory Executive function Attention and psychomotor speed	Prose Memory test TMT Attentional Matrices	The blueberry group did not show the same decreases in performance on verbal episodic memory, attention, or executive function following general anaesthetic, compared to the control group).
Watson et al. (2015)	Double-blind, randomised, placebo-controlled, crossover	Acute (2.5 hours)	Healthy young adults	18-35	36	Blackcurrant extract or cold-pressed blackcurrant juice	Attention and psychomotor speed Executive function Working Memory	Digit vigilance Stroop test RVIP Logical reasoning	The blackcurrant extract group showed better performance and possible attenuation of repetition fatigue on higher demand attention and working memory tasks.
Watson et al. (2019)	Double-blind randomised, placebo controlled, crossover	Acute (2 hours)	Healthy young adults	M= 23	9	Blackcurrant juice (500mg total polyphenols)	Attention and Psychomotor Speed	CogTrack™ System: Simple reaction time Digit vigilance Choice reaction time	Significant treatment effect for reaction time during choice reaction time task. No other significant impacts on cognitive performance were detected as a result of the blackcurrant juice.
Whyte and Williams (2015)	Randomised, placebo-controlled, crossover	Acute (2 hours)	Healthy children	8-10	14	143 mg anthocyanins blueberry drink	Verbal episodic memory Working memory	RAVLT Go No-Go Stroop test Visual *n*-back test	Delayed verbal recall was improved in the blueberry drink group, although no

							Executive Function	Object location test	treatment effects were detected for executive function or spatial memory tests.
Whyte et al. (2016)	Double-blind, randomised, placebo-controlled crossover	Acute (1.15, 3, 6 hours)	Healthy children	7-10	21	15g and 30g Freeze-dried wild blueberry	Verbal episodic memory Executive function Processing speed	RAVLT Modified Flanker Test Go No-go Picture Matching Task	Wild blueberry produced significant improvements in acquisition and recognition, and resistance to interference effects.
Whyte, Schafer, and Williams (2017)	Double-blind, randomised, placebo-controlled crossover	Acute (3 hours)	Healthy children	7-10	21	30g freeze-dried wild blueberry powder	Executive function Attention	MANT	The wild blueberry group showed better performance compared to controls on cognitively demanding components of tasks.
Whyte et al. (2018)	Double-blind, randomised, placebo-controlled, parallel	0, 12, 24 weeks	Older adults with subjective memory complaints	65-80 M= 70.8 (±3.88)	30 500mg 31 1000 31 100mg extract 30 placebo	500mg whole wild blueberry freeze-dried powder 1000mg whole wild blueberry freeze-dried powder 100mg purified extract	Verbal episodic memory Working memory Executive function Attention	RAVLT Object recognition Corsi block Test Serial subtractions MANT Stroop test	Improved delayed word recognition on the RAVLT at 12 weeks. Non-significant trend for better performance on Corsi bloc test at 3 months.
Whyte et al. (2019)	Single-blind, randomised, placebo controlled parallel	Acute (2, 4, 6 hours)	Healthy young adults	20-30 M= 22.8 (±2.46) berry M= 22.8 (±2.8) placebo	20 mixed berry 20 placebo	Mixed berry smoothie (blueberry, strawberry, raspberry, blackberry)	Executive function Attention	MANT TST	The mixed berry group showed maintained accuracy on both tasks over the course of the 6 hours compared to placebo.
Whyte et al. (2020), a	Double-blind, randomised, placebo-	Acute (75 minutes)	Healthy children	7-10 M= 8.8 (±0.67)	17	Wild blueberry drink (freeze-dried mixed	Verbal and visual episodic memory	RAVLT Brown-Peterson task Picture recognition test	No impact of wild blueberry on verbal or visual episodic or working memory

	controlled, crossover					with water and squash)	Working memory	VSGT	performance, however there was evidence of faster visual processing on VGST.
Whyte et al. (2020), b	Double-blind, randomised, placebo-controlled, crossover	Acute (3 hours)	Healthy children	7-10 M= 8.4 (±0.4)	18	Wild blueberry drink (freeze-dried mixed with water and squash)	Executive function	Stop-go test TST ANT	There were benefits for the wild blueberry group for the selective attention (ANT) but not other executive function aspects of measures.
Whyte et al. (2021)	Double-blind, randomised, placebo-controlled, crossover	Acute (2, 4, 6, 8 hours)	Middle-aged adults	40-65 M= 51 (± 8)	35	Wild blueberry freeze-dried powder	Verbal episodic memory Executive function Attention	RAVLT Go No-go MANT	There was significant treatment effects for verbal recognition, with maintained performance over time compared to placebo. Benefits were also observed for the more cognitively demanding aspects of executive tasks, and faster response times over time compared to placebo.

ANT= Attention Network Task; CANTAB= Cambridge Neuropsychological Test Automated Battery; COWAT= Controlled Oral Word Association Test; CVLT= California Verbal Learning Test; DS= Digit Span; fMRI = functional Magnetic Resonance Imaging; HVLT= Hopkins Verbal Learning Test; MANT = Modified Attention Network Test; RAVLT=Rey's Auditory Verbal Learning Test; RVIP= Rapid Visual Information Processing; SPAL= Spatial Paired Associate Learning Test; TMT= Trail Making Test; TST= Task Switching Test; vMWM= virtual Morris Water Maze; V-PAL= Verbal Paired Associate Learning Test; VSGT= Visuospatial grid task; VVLT= Visual Verbal Learning Test.

MECHANISMS OF ACTION OF POLYPHENOLS ON BRAIN AGEING

Classically, the effects of certain polyphenols on promoting health have previously been attributed to their ability to reduce cell damage by directly scavenging free radical species (Young & Woodside, 2001), according to evidence from *in vitro* studies as well as their antioxidant actions in plants. However, this may not be a complete explanation, as the concentrations of polyphenols that seem to be required to exert sufficient antioxidant activity to produce this effect is significantly higher than would be achieved through diet in humans, at least to have an impact on oxidative stress beyond the gastrointestinal tract. Many polyphenols furthermore have very limited bioavailability as they are efficiently metabolised before being able to exert their antioxidant effects (R. J. Williams, Spencer, & Rice-Evans, 2004). Following ingestion some dietary polyphenols are quickly absorbed into the blood stream, the initial site of absorption likely the stomach (Passamonti, Vrhovsek, Vanzo, & Mattivi, 2003). Other polyphenols, such as anthocyanins, are poorly absorbed in the stomach, and are metabolised lower down the digestive tract, after which they are able to be transported via the bloodstream to target multiple tissues. This includes the central and peripheral nervous systems, as polyphenols and their metabolites have been shown to be able to pass the BBB (Andres-Lacueva et al., 2005; Youdim et al., 2003), with some polyphenols including anthocyanins proposed to also be able to interact with P-glycoprotein transporters in order to enter the brain, although this is largely based on evidence from *in vitro* studies (Youdim, Shukitt-Hale, & Joseph, 2004). Evidence from studies involving rodents suggests that intravenously administered anthocyanins are able to be rapidly taken up into the brain within seconds, with constituent compounds also being detected after minutes (Fornasaro et al., 2016), and that anthocyanins are then able to accumulate and persist in tissues for some time, including the brain (Passamonti, Vrhovsek, Vanzo, & Mattivi, 2005) and digestive tract (Talavera et al., 2005). However, although anthocyanins have been found to accumulate in

key neural regions, it is important to note that they have comparatively low bioavailability compared to their metabolites (Kay, Kroon, & Cassidy, 2009). Furthermore, evidence from intravenous administration bypasses the primary metabolism of these polyphenols within the digestive tract, and there is growing understanding of a critical role of gut microbes in degrading polyphenols to active metabolites which will be discussed further in a later section.

Other mechanisms by which polyphenols exert their health benefits are still being understood. The majority of available research into the mechanisms by which dietary polyphenols exert their effects has come from studies involving extracts of foods high in concentrations of these compounds, rather than the effects of the pure polyphenols directly. Despite this, results from studies involving polyphenol-rich foods and extracts have suggested that these compounds have effects on multiple disease-causing mechanisms such as by reducing inflammation, supporting vascular integrity and function, influencing neuronal signalling and survival and interacting with the gut microbiome (for review, see Rodriguez-Mateos et al. (2014)). As such, polyphenols appear to exert effects on multiple mechanisms believed to contribute to age-related neurodegeneration making them an attractive therapeutic option. Furthermore, such intervention is in line with current research efforts to develop multi-target-directed ligands to combat the disease processes underlying dementia subtypes such as AD (Bajda, Guzior, Ignasik, & Malawska, 2011), either acting alone or providing a launchpad to develop new treatments of this nature. Indeed, investigations are already underway in the area of identifying novel therapies for AD using specific polyphenols and their metabolites as a base, such as flavones and isoflavones (for review, see Jalili-Baleh et al. (2018)). Still, an understanding of how polyphenols support brain health across the lifespan is still being actively explored.

Neural Functioning

Age-related cognitive decline is associated with reduced functioning in key neural regions responsible for memory, learning, and executive functioning, as well as sometimes other cognitive domains such as spatial navigation. The hippocampus and supporting medial temporal regions are associated with memory and learning functioning, whereas activity in the prefrontal cortex is related more to executive functions including set-shifting and inhibition. A study by (Andres-Lacueva et al., 2005) in rats found that following 8-10 weeks feeding with 2% blueberry diet resulted in better spatial learning and memory compared to controls, and that this improvement corresponded with localisations of anthocyanins in hippocampal and striatal regions, key regions for learning and memory, which were not detected in the control rats. Furthermore, they found that there was a direct association between the amount of anthocyanins present in these brain regions and improved performance on cognitive tests. Subsequent studies have confirmed the benefit of blueberry supplementation for cognition, particularly spatial memory, in rat models (Rendeiro et al., 2012; C. M. Williams et al., 2008). In another study, rats fed either 2% strawberry or blueberry supplemented diets showed improved cognitive and motor performance, including working memory, and that this corresponded with increased hippocampal neurogenesis and insulin-like growth factor 1 expression (Shukitt-Hale et al., 2015).

The precise sites of the interactions between polyphenols and neuronal signalling pathways remain to be determined, based on existing evidence suggesting that polyphenols may exert their effects through 1) modulating signalling cascades that control neuronal apoptosis; 2) modulating the expression of specific genes and 3) impacting mitochondrial activity (Vauzour, 2012)). Polyphenols have the potential to activate neuronal pathways that regulate cell transcription, translation, proliferation, growth and survival, and also regulate pathways involved in synaptic plasticity. As such, they have a suggested modifier role in learning and

memory processes. Polyphenols could also support neuronal cell health as they have been shown to stimulate key neurotrophic factors such as brain-derived neurotrophic factor (BNDF).BDNF is a central and peripheral central nervous system protein that supports neuronal survival, differentiation, and synapse formation, and has an established involvement in learning and memory by supporting hippocampal neuronal connectivity and synaptic efficacy (Lu & Chow, 1999). A reduction in BDNF has been linked to the development of neurodegenerative disease (Bercik et al., 2011; Bistoletti et al., 2019), with increased hippocampal BDNF associated with slower cognitive decline in AD (Laske et al., 2011). Blueberry consumption has been suggested to support to neuroplasticity in hippocampal and frontal structures via the stimulation of BDNF production, which corresponds with improved spatial memory performance in rats (Rendeiro et al., 2013; C. M. Williams et al., 2008). However, in human clinical trials, using a large sample size (n=101) Ahles and colleagues (Ahles et al., 2020) found a positive impact of a 24-week chokeberry intervention on psychomotor performance but not for other cognitive domains such as attention and cognitive flexibility or BDNF levels in overweight (body mass index (BMI) of 25-35) middle-aged adults aged 40-60 years. As such, the effects of berry supplementation on BDNF in humans is less clear when it is measured peripherally in humans as opposed to directly observing concentrations in neural regions in animal models, although there are other studies investigating the impact of high-flavonoid intake more generally that have found positive results for increased BDNF measured peripherally in humans (Neshatdoust et al., 2016).

There also exist only a few studies directly investigating the impact of the consumption of polyphenol rich foods on neural functioning in humans by using functional imaging techniques such as arterial spin labelling (ASL) to measure cerebral blood flow (CBF) (for further details of these studies, see Table 1.2). Novel neuroimaging techniques such as ASL have enabled more objective investigation into the impact of nutritional interventions on brain structure and

function, and as such have advanced understanding of the impact of specific nutrients on brain health and activity. Investigations involving these neuroimaging techniques however remain limited, particularly regarding research into the impact of polyphenols on brain function. There is evidence of consumption of other polyphenols, such as flavanones in particular hesperidin found in citrus fruits and flavanols in cocoa, relating to changes in CBF in human subjects, with effects measurable often quite soon after consumption. For example, a study looking at the impact of a flavanone-rich juice in 24 young adults found improved CBF in the inferior and middle frontal regions in conjunction with improved cognitive function 2 hours post-consumption, compared to a placebo matched for both energy and vitamin-C content (Lamport, Pal, et al., 2016). The same group found improved regional cerebral blood flow following consumption of cocoa flavanols in middle aged adults (50-65 years), particular in the anterior cingulate and parietal regions (Lamport et al., 2015). Another study by (Kennedy et al., 2010) found a dose-dependent increase in CBF during executive test performance 45 minutes following resveratrol administration. A recent study investigating the impact of three active beverages containing either apple, blueberry and coffee berry extracts in combination with beetroot, sage and ginseng found an increase in oxygen saturation during executive cognitive tests for all three beverages, however due to the inclusion of the additional extracts it is difficult to draw conclusions regarding the independent impact of berry polyphenols on cerebral blood flow (Jackson et al., 2020). One mechanism by which polyphenols are expected to improve neural functioning and CBF is suggested to be due at least in part to their vasodilatory properties (Furuuchi et al., 2018). However, there is also an increasing understanding of the role of polyphenols in modifying the structure and function of the gut microbiome, and how this in turn can improve the bioavailability of these polyphenols through the production of active metabolites. This will be explored in more detail in the next section.

Table 1.2. Human intervention studies investigating the impact of dietary polyphenols on neural function.

Reference	Study Design	Study Duration	Sample population	Age (years)	Study size	Polyphenol source	Imaging Modality	Findings	Other relevant findings
Bowtell et al. (2017)	Double-blind, randomised, placebo-controlled parallel	12 weeks	Healthy older adults	65 and older	12 blueberry 14 placebo	Blueberry extract	ASL fMRI Task-based fMRI (BOLD)	Increased regional CBF in parietal and occipital lobe gray matter after blueberry supplementation. Increased activation in Brodman areas 4, 6, 10, 21, 40, 44, 45, precuneus, anterior cingulate, insula and thalamus during the Stroop test.	Non-significant trend for improved working memory. No increases in serum BDNF concentration following blueberry supplementation.
Brickman et al. (2014)	Double-blind, randomised, placebo-controlled parallel	3 months	Healthy older adults	50-69	10 high flavanol + active exercise 11 high flavanol 10 low flavanol + active exercise 10 low flavanol	900mg daily high-flavanol cocoa supplement (vs 45mg in the low flavanol groups)	Task-based fMRI (BOLD)	High-flavanol increased dentate gyrus BOLD activation.	
Decroix et al. (2016)	Double-blind, placebo-controlled, randomised, crossover	Acute (95 minutes)	Healthy young adults (male)	M= 30 (±3)	12	900mg high-flavanol cocoa milk (vs 15mg low-flavonol chocolate milk)	Task-based NIRS	Increased cerebral oxygenation in the prefrontal cortex at rest (vs post-exercise) during Stroop test.	No improvement on cognitive performance

Francis, Head, Morris, and Macdonald (2006)	Double-blind, randomised, placebo-controlled, crossover	5 days	Healthy young adults (female)	18-30	16	172mg high-flavanol cocoa drink (vs. 13mg low flavanol cocoa drink)	Task-based fMRI (BOLD)	Greater BOLD signal change between activation and baseline components on cognitive test, in regions that showed task-related activity (right medial and lateral PFC, cerebellum, parietal cortex and ACC), and significantly increase the CBF to grey matter.	No impact of higher flavanol dose on cognition.
Jackson et al. (2020)	Double-blind, randomised, placebo-controlled, crossover	Acute (1, 3, 6 hours)	Healthy adults	18-49	32	Base beverage containing beetroot, sage and ginseng extract with either: -234mg flavanols apple extract -300mg anthocyanins blueberry extract -440mg Chlorogenic acid coffee berry extract	Task-based NIRS	Increased total hemoglobin and oxygen saturation in right hemisphere following apple and coffee berry extract beverages, and increased total hemoglobin following apple extract and increase oxygen saturation for the blueberry group for each post-dose measurements compared to placebo.	No differences between placebo and extracts found for cognitive performance, although more errors on delayed word list recall were detected for the coffee berry compared to placebo.

Kennedy et al. (2010)	Double-blind, randomised, placebo-controlled, crossover	Acute	Healthy young adults	18-25	24	250mg and 500mg resveratrol	Task-based NIRS	Increased CBF (dose-dependent) in the prefrontal cortex during task performance.	No improvement in cognitive performance
Kobe et al. (2017)	Double-blind, randomised, placebo-controlled, parallel	26 weeks	Older adults with MCI	50-80	18 resveratrol 20 placebo	200mg resveratrol/day (vs 1015mg olive oil/day placebo)	Resting state functional connectivity fMRI	Higher functional connectivity between right anterior hippocampus and right angular cortex compared to placebo.	No different in memory performance between groups.
Krikorian et al. (2012)	Double-blind, randomised, placebo-controlled, parallel	16 weeks	Older adults with MCI	68 and older	10 Concord grape juice 11 placebo	Concord grape juice	Task-based fMRI (BOLD)	Out of 6 pre-determined ROI selected for their supposed role in working memory, there was greater activation in the right middle PFC and superior parietal regions in the Concord grape juice group during an *n*-back task.	Participants in the grape juice group showed less semantic interference on a test of verbal episodic memory.
Lamport et al. (2015)	Double-blind, randomised, placebo-controlled, crossover	Acute (2 hours)	Healthy older adults	55-65	18	High (494mg flavanols) cocoa drink (vs. low (23mg) flavanol cocoa drink)	ASL fMRI	Increased reginal perfusion in the anterior cingulate cortex and central opercular cortex in the high flavanol cocoa condition.	
Lamport, Pal, et al. (2016)	Single-blind, randomised,	Acute (2 hours)	Healthy young adults	18-30	16	Flavanone-rich citrus juice (70.5-mg flavonoids)	ASL fMRI	Significantly increased regional	

	placebo-controlled, crossover							perfusion in inferior and middle right frontal gyrus at 2 hours relative to baseline and placebo.	
Lee, Torosyan, and Silverman (2017)	Double-blind, randomised, placebo-controlled, parallel	6 months	Older adults with MCI	65 and older	5 grape 5 placebo	Freeze-dried Californian grapes	FDG-PET	The active grape group did not show the same metabolic decline over 6 months compared to the placebo group, specifically in the right PCC, and left superior posterolateral temporal cortex (sVOI), and left PFC and cingulate (SPM). Metabolism in right superior parietal cortex and left inferior anterior temporal cortex was correlated with improvements in working memory performance.	No differences between groups in cognitive performance.
Wightman, Haskell, Forster, Veasey, and Kennedy (2012)	Double-blind, randomised, placebo-controlled, crossover	Acute (45 minutes)	Healthy young adults	18-30	27	Green tea 135 mg and 270 mg EGCG (vs. inert placebo)	Task-based NIRS	Reduced CBF in the prefrontal cortex.	No improvement on cognitive performance

Wightman et al. (2014)	Double-blind, randomised, placebo-controlled crossover	Acute (40 minutes)	Healthy young adults	19-34	23	250mg trans-resveratrol and trans-resveratrol + 20mg piperine	Task-based NIRS	Piperine and trans-resveratrol improved CBF in the frontal cortex during task performance.	No differences in cognitive performance.
Wightman et al. (2015)	Double-blind, randomised, placebo-controlled, parallel	Acute (45 minutes) and 4 weeks	Healthy young adults	18-30	30 resveratrol 30 placebo	500mg/day resveratrol	Task-based NIRS	Improved prefrontal CBF parameters during task performance at acute measurement (45 minutes).	Limited differences in cognitive performance on serial subtraction task at acute measurement (45 minutes).
Witte, Kerti, Margulies, and Floel (2014)	Double-blind, randomised, placebo-controlled, parallel	26 weeks	Healthy overweight older adults	50-75	23 resveratrol 23 placebo	200mg/day resveratrol	Resting state functional connectivity fMRI (hippocampus)	Resveratrol group had significant increases in hippocampal functional connectivity.	Improved retention on verbal episodic memory task in resveratrol group.

ACC= Anterior Cingulate Cortex; ASL= Arterial Spin Labelling; BDNF= Brain-Derived Neurotrophic Factor; BOLD= Blood Oxygen Level Dependent; CBF= Cerebral Blood Flow; EGCG= Epigallocatechin gallate; FDG-PET= FluoroDeoxyGlucose Positron Emission Tomography ; fMRI= functional Magnetic Resonance Imaging; NIRS= Near-Infrared Spectroscopy; PCC= posterior cingulate cortex; PFC= Prefrontal Cortex; ROI= Region of Interest; SPM= Statistical Parametric Mapping; sVOI= standardized Volumes Of Interest.

The Gut Microbiome

Research focusing on characterising the structure and function of microbial communities in the human body has increased in the past century, as a result of the emergence of the holobiont conception of how hosts interact with their associated microbiota alongside the development of techniques in the study of metagenomics for identifying specific microorganisms directly without requiring *in vitro* cultivation. This has led to a recent explosion in research interest in the "gut-brain axis" and the complex bidirectional relationship between the functioning of gut microbiota and brain health and function, particularly its role in the development of neurodegenerative disease (for review see Castillo-Alvarez and Marzo-Sola (2019)). The gut microbiota have a relatively newly established role in supporting human health and homeostasis, operating between the enteric and central nervous systems via various pathways including along the vagus nerve, the hypothalamic-pituitary-adrenal (HPA) axis, or indirectly via peripheral signalling including by modulating tryptophan metabolism and neurotransmitters and the immune system. Manipulation of the structure and function of the gut microbiota could provide a target for the prevention and treatment of neurodegenerative disease. There is evidence that this can be modulated with interventions such as use of probiotics, prebiotics and postbiotics, faecal microbiota transplants (Khoruts & Sadowsky, 2016), and dietary changes.

The gastrointestinal tract is populated by a highly diverse range of microorganisms including bacteria, fungi, viruses, and protozoa. It was initially been believed that the microbiota were mostly comprised of bacteria which did not have a particularly strong role in either supporting or being detrimental to health, with the exception of significant intestinal infections resulting from invasion of pathogenic species into a compromised gut ecosystem (Servin & Coconnier, 2003). However, it is now understood that the functioning of the gut and its integrity relies on a fragile homeostatic balance where certain bacteria populations can

produce both significantly positive or negative impacts on health (Rakoff-Nahoum, Paglino, Eslami-Varzaneh, Edberg, & Medzhitov, 2004). Depending on the location within the gastrointestinal tract, the abundance of these live bacteria can range from an estimated 10^2 bacteria/ml at the stomach level to the highest density at approximately 10^{14} bacteria/ml in the colon (Savage, 1977), the latter being where the largest concentration of commensal bacteria is expected to reside within the body. This includes an estimated over 35,000 bacterial species (Frank et al., 2007), which largely consist of *Actinobacteria, Proteobacteria, Firmicutes,* and *Bacteroidetes*. Higher abundance of certain species of bacteria is associated with health benefits, and include *Bifidobacterium* spp., *Lactobacillus* spp., *Faecalibacterium prausnitzii, Bacteroides* spp., *Prevotella* spp, and *Akkermansia* spp., whereas increased prevalence of other species of bacteria including *Escherichia coli* are associated with negative health effects. The commensal species *Akkermansia muciniphila* is of particular interest due to its implicated role in modulating inflammation and intestinal adaptive immune responses (Ansaldo et al., 2019). Bacterial diversity is decreased in microbial dysbiosis (Kim, Kim, & Park, 2016; Zhernakova et al., 2016), or a change or imbalance in the composition of gut microbial communities often characterised by lower diversity and altered metabolic functioning of microbiota, which could be a contributing factor in the development of a variety of chronic diseases, including diseases beyond those affecting the gastrointestinal tract such as neurological conditions including cognitive decline and AD (Akbari et al., 2016; Brandscheid et al., 2017; Frohlich et al., 2016).

Although having its origins in the placenta prior to birth, the gut microbiota can be modulated across the lifespan via interactions between both the host's genetics and environment, such as infections, antibiotic use, and changes in lifestyle factors including diet (Marchesi et al., 2016; Messer, Liechty, Vogel, & Chang, 2017; Sonnenburg & Backhed, 2016). Although the composition of gut microbiota and relative phylum abundances can vary greatly between

individuals, an understanding is gradually being developed of what constitutes a healthy human gut microbiota (Marchesi et al., 2016). The composition of the microbial communities in the gastrointestinal tract changes across the lifespan, with the prevalence of certain types of bacteria populations in older adults aged >65 years into older age particularly observed to undergo changes (Claesson et al., 2011; Claesson et al., 2012). These changes with older age are attributed to factors such as immuno-senescence and increased systemic inflammation with older age, as well as changes in dietary habits and increased incidence of comorbid illnesses and associated medications and hospitalisations. These changes are generally characterised by a decrease in abundance of *Firmicutes,* and *Clostridium* (Ribera, 2016) and relative increases in *Bacilli Bifidobacterium* and *Bacteroidetes* populations compared to proportions found in younger adults, with further emergence of *Proteobacteria* into elderly years which in particular can lead to pathologies caused by dysbiosis (Nagpal et al., 2018). These morphological and functional changes of the microbiome due to ageing in turn could be impacted by changes in lifestyle as people move into older age, such as decreases in physical activity, changes in diet and medication and digestive tract changes, including changes in digestion and absorption of nutrients (Camilleri, Lee, Viramontes, Bharucha, & Tangalos, 2000). As such, although the composition of gut microbiota changes with age, there is the potential for these changes to be influenced by environmental factors such as changes to diet.

POLYPHENOLS AND THE GUT MICROBIOME

Polyphenols can directly alter the composition and function of the gut microbiota, particularly the diversity of bacteria and abundance of certain strains of beneficial bacteria, while inhibiting the adhesion of harmful bacteria. Diet may reversibly alter the human faecal microbiome at the species and genera level, although the microbiome is stable at the phylum level in long-term studies (Martinez, Muller, & Walter, 2013). For example, in rats the

administration of two polyphenols (curcumin and resveratrol) resulted in changes in the bacteria groups *Bacteroidetes* and *Clostridium* which in turn related to metabolic improvements including glycaemic control (Sreng et al., 2019), and similar increases in abundances of these bacterial populations were also found in rats with diets supplemented with lowbush wild blueberry (Lacombe, Li, et al., 2013). The modulation of gut microbiota, including increased abundance of *Akkermansia muciniphila* and restoration of their colonic epithelial mucus layer in diet-induced obese mice fed with wild blueberry, was attributed to the high concentration of proanthocyanidins over anthocyanins and phenolic acids present after the different polyphenol fractions present were analysed (Rodriguez-Daza, Daoust, et al., 2020). Supplementation of black raspberries similarly showed an increased abundance of *Akkermansia muciniphila*, along with improved intestinal functions associated with the gut microbiome including amino acid and carbohydrate metabolism, and vitamin biosynthesis in mice (P. Tu et al., 2018). In humans, consumption of wild blueberry drink derived from freeze-dried fruit in 20 healthy adult men daily for 6 weeks resulted in increased *Bifidobacteria* populations (Guglielmetti et al., 2013). As such, there is an increasing body of evidence that supplementation with berries could produce beneficial shifts in the structure of the gut microbiome.

Polyphenols present in berries have also shown inhibitory actions against *Bacillus cereus, Campylobacter jejuni, Clostridium perfingens, Helicobacter pylori, Staphylococcus aureus, Staphylococcus epidermidis,* and *Candida albicans* (Nohynek et al., 2006). An analysis of the fractions of berry which have the most beneficial effects on increasing microbial diversity in the gut found that anthocyanin and proanthocyanidins had the most impact, particularly in older participants (Ntemiri et al., 2020). Proanthocyanidins are particularly effective at inhibiting bacterial adhesion, and furthermore have been found to improve gut barrier function in mouse models (Pierre et al., 2014). Dietary proanthocyanidins have also been

shown to improve mucin production in the gut in mice (Pierre et al., 2013), and as *Akkermansia* are mucin degrading bacteria this increase in mucin could have in turn supported increase in *Akkermansia* populations. In vivo, proanthocyanidins in cranberry, in particular due to their A-type linkages, have been implicated as important inhibitors of primarily P-fimbriated *Escherichia coli* adhesion to uroepithelial cells (Howell et al., 2005). *In vitro* studies have shown dose-response effects of cranberry polyphenols on the inhibition of microbial invasion of gut and bladder epithelial cells (de Llano et al., 2015; Feliciano, Meudt, Shanmuganayagam, Krueger, & Reed, 2014). In animal models, whole cranberry increased the abundances of purportedly beneficial species of bacteria including *Lactobacillus* and *Bifidobacterium* while decreasing the prevalence of potentially harmful bacteria such as *Sutterella* and *Bilophila* in the gastrointestinal tracts of mice (Cai et al., 2019). Cranberries have been shown to stimulate the probiotics *Lactobacillus rhamnosus* (Lacombe, McGivney, Tadepalli, Sun, & Wu, 2013) and *Bacillus coagulans* (Majeed et al., 2018), and mice supplemented with cranberry have shown increased abundance of *Akkermansia* spp. which corresponded with protection from intestinal inflammation, as well as insulin-resistance and obesity (Anhe et al., 2015).

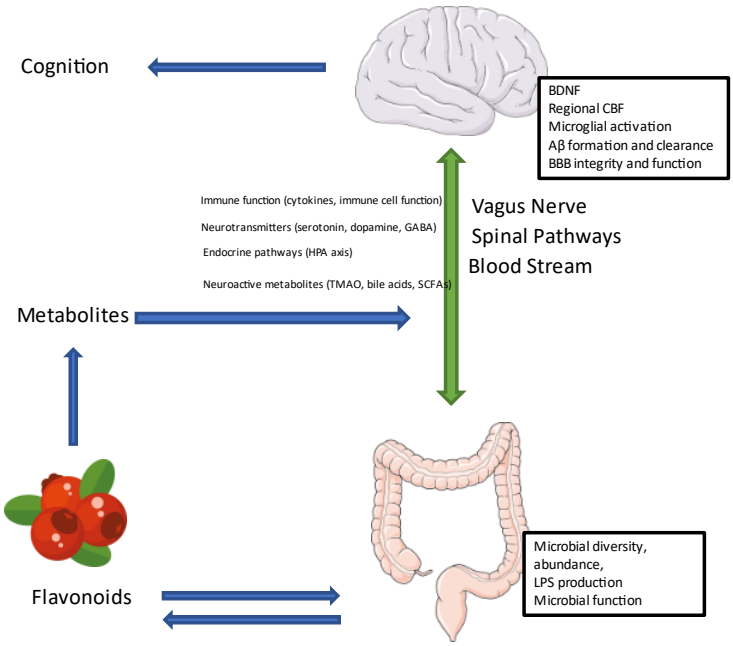


Figure 1.3. Illustration of the bidirectional relationship between brain and gut microbiota function, and the potential modifying role of dietary polyphenols on the gut-brain axis in the context of age-related cognitive decline.

In humans, cranberries have long been used as part of treatment for urinary tract infections (Maki et al., 2016). When it comes to clinical trials, an intervention of daily consumption of freeze-dried whole cranberry for 5 days produced a decreased abundance of *Firmicutes* and increase in *Bacteroidetes* (Rodriguez-Morato, Matthan, Liu, de la Torre, & Chen, 2018). There have been placebo-controlled trials of cranberry juice in subjects positive for *Helicobacter pylori* with positive results (Gao et al., 2021), as well as findings of the attenuation of the impact of an animal-based diet in a small placebo-controlled crossover trial in 11 human subjects for 5 days (Rodriguez-Morato et al., 2018), and limited effects on the microbiome of women with urinary tract infections who took cranberry daily for 24 weeks compared to placebo (Straub et al., 2021). The addition of sweetened dried cranberry to the diet of 10 human subjects daily for 2 weeks influenced the composition of the faecal microbiome (Bekiares, Krueger, Meudt, Shanmuganayagam, & Reed, 2018), with 7 subjects

showing increased microbial diversity. As such, the specific types of polyphenols in cranberries, particularly A-type proanthocyanidins, could directly alter the composition and function of the gut microbiome, particularly the diversity of bacteria and abundance of certain strains of beneficial bacteria, while inhibiting the adhesion of harmful bacteria. However, the impact of cranberry on the microbiome in the context of healthy aging remains virtually unexplored.

POLYPHENOLS AND MICROBIAL-DERIVED METABOLITES

On the other hand, microbiota in the gut are able to enhance the health benefits of polyphenols, largely through metabolising them down into their constituent components. The microbiota can metabolize a large variety of nutrients, including fibre, which are then able to exert additional beneficial health effects beyond their parent compounds (Sanchez et al., 2017). This is partly owing to the ability for intestinal microbes to utilise nutrients that have not been absorbed during digestion. Polyphenols are able to have their structure and function modified by the microbiota in the intestinal tract which are able to exert different actions than the parent molecules. Cranberries contain a diversity of microbial substrates including polyphenols, as well as polysaccharides (fibres), which are unable to be directly digested in the upper intestinal tract and often reach the colon intact (Yan, Murphy, Hammond, Vinson, & Neto, 2002). In fact, it is estimated that between 90-95% of ingested polyphenols reach the colon undigested, which is where they are expected to undergo the metabolising and fermentation by the gut microbiota (Clifford, 2004). For example, the intestinal microbiota are necessary to break down anthocyanins into metabolites such as protocatechuic acid (Gu et al., 2019). As such, the functions of the microbiota in the gut are necessary to depolymerize these compounds so that they are then able to be utilised by the body (El Kaoutari, Armougom, Gordon, Raoult, & Henrissat, 2013; van Duynhoven et al., 2011). Indeed, although in the case of polyphenols such as anthocyanins there is a lot of evidence to suggest

their direct beneficial impacts on health, the bioavailability of these compounds *in vivo* could be actually much less than their more stable metabolites (Kay et al., 2009; Keppler & Humpf, 2005). Some of the more notable of these metabolites of anthocyanins are phenolic acids, to which many of the beneficial effects of a higher concentration anthocyanin diet due the observation of their tendency to accumulate at higher levels throughout the body (Sandhu et al., 2018). Furthermore, the interaction between intestinal microbiota and proanthocyanidins from grape seeds results in the phenolic acids, 3-hydroxybenzoic acid and 3-(3-hydroxyphenyl) propionic acid, which have in turn been found to inhibit AD pathogenesis in a mouse model of AD (Q. Sun, Jia, Li, Yang, & Chen, 2019).

In addition to polyphenols, microbiota also break down other dietary compounds into metabolites that impact gut function, including secondary bile acids, short chain fatty acids (SCFA) and trimethylamine, and this metabolism can be influenced in turn by polyphenols. Polysaccharides from plant cell walls in berries can also exert prebiotic effects, such that they are non-digestible dietary components that can promote the growth of certain intestinal bacteria (Gibson et al., 2017). Specifically, cranberry cell walls are composed of cellulose and hemicellulose, as well as pectin rich in arabinans and arabino-xyloglucans (White, Howard, & Prior, 2010), the latter of which has been linked to inhibition of *Escherichia coli* adhesion in epithelial cells *in vitro* (Hotchkiss et al., 2015). In addition, polyphenols can stimulate the breakdown of these prebiotic fibres into SCFAs through certain gut bacteria (Parkar, Trower, & Stevenson, 2013). SCFAs are produced from the bacterial fermentation of predominantly dietary fibres in the colon as well as amino acids, and include butyrate, formate, valerate, isobutyrate, isovalerate, acetate and propionate. These SCFAs have a role in maintaining the homeostasis of the gut and brain via supporting the intestinal barrier and also the BBB (Hoyles, Snelling, et al., 2018), as well as regulating energy supply, and activation of trophic and inflammatory factors. SCFAs have also been implicated in

mechanisms that support brain health such as activating glial cell activity to influence neuronal connectivity and plasticity (Sadler et al., 2020), as well as supporting metabolic functions via gut-brain pathways (De Vadder et al., 2014). Polysaccharides are also able to interact with specific polyphenols present through diet, including proanthocyanidins (Bindon, Smith, & Kennedy, 2010; Le Bourvellec, Bouchet, & Renard, 2005). In humans, freeze-dried cranberry consumption has been shown to attenuate both the reduction of SCFAs and the increase of detrimental secondary bile acids that as induced by a high animal-based diet (Rodriguez-Morato et al., 2018). The cranberry also induced an increase in *Bacteroidetes* and decrease in *Firmicutes* in contrast to the control group. As such, there is emerging evidence that cranberry could support the production of beneficial microbial-derived product (such as SCFA's) and decrease detrimental compounds by changing the structure and function of the gut microbiota. Taken together, berry supplementation has been found to promote shifts in the structure of the gut microbiota, attributed to their unique polyphenolic profiles. These shifts can produce changes in the metabolising of key active compounds which can support brain health and function.

THE CURRENT THESIS

To date there are no clinical trials investigating the impact of cranberries on cognition, brain function, and gut microbiome diversity and function in cognitively healthy older adults, with many existing studies involving other berry types with similar nutrient profiles such as blueberry. However, cranberries are more abundant in A-type proanthocyanidins which have been linked to inhibition of adherence of detrimental bacterial populations. A recent systematic review identified a total of 18 studies that had been published focusing on cognitive performance following supplementation with berry anthocyanins (Ahles, Joris, & Plat, 2021). Of these, the majority focused on blueberry supplementation, and the review did not include any studies focusing on the impact of cranberry intake. There has been one study

to focus on the short term (6 week) intervention in older adults (aged ~60 years) in a placebo-controlled trial of cranberry juice (n=25 in each the cranberry and placebo group) and its impact on a range of cognitive outcomes, with no positive results found. When it comes to investigating the impact of cranberry on the gut microbiome, there are no clinical trials in cognitively healthy older adults, and much of the evidence to date regarding the impact of cranberry on the microbiome comes from animal studies particularly involving rodents (Chettaoui, Mayot, De Almeida, & Di Martino, 2021; Liu et al., 2021; Renaud et al., 2021), as well as *in vitro* studies (D. Zhang et al., 2021) and simulator models (O'Connor et al., 2019). Furthermore, to date there are no studies in older adults investigating the impact of long-term intake of cranberry on both cognition and neural function and its relationship to corresponding shifts in the gut microbiota and their actions on producing key metabolites.

As a result, the aims of the current thesis are:

1. To investigate the impact of a 12-week randomised placebo-controlled intervention of a high-polyphenol containing cranberry powder on cognition in healthy older adults.
2. To investigate the impact of the intervention on gut microbiome composition..
3. To investigate potential underlying mechanisms, including neural blood perfusion and the production gut microbially-derived metabolites, and how these relate to observed changes in cognition and gut microbiome composition.

Therefore, the primary outcomes for this thesis are:

1. To measure differences between cranberry and placebo groups in mean cognitive performance on measures on memory and executive function between baseline and follow-up.
2. To measure differences between cranberry and placebo groups in microbial taxonomic abundances between groups between baseline and follow-up.

Secondary outcomes include:

1. To measure differences between cranberry and placebo groups for mean neural regional blood perfusion between baseline and follow-up visits, and its relationships with changes in cognitive performance.
2. To measure differences between cranberry and placebo groups in gut-derived metabolites, including levels of common polyphenol metabolites, between baseline and follow-up, and their relationship to changes in cognitive outcomes, regional neural perfusion, and microbiota abundances.

Tertiary outcomes include:

3. To measure differences between cranberry and placebo groups for other cognitive domains understood to be impacted by age-related neurodegeneration such as spatial navigation.
4. To measure differences between cranberry and placebo groups in plasma biomarkers of neuronal signalling (BDNF) between baseline and follow-up, and its relationship to changes in cognition and regional neural perfusion.
3. To measure differences between cranberry and placebo groups in structural grey and white matter integrity between baseline and follow-up.
4. To analyse intra- and inter-individual changes in microbial diversity and richness, as measured by alpha and beta diversity analyses.

Based on existing evidence, it could be hypothesized that:

1. Long-term daily cranberry intake will relate to improvements in cognition, particularly episodic memory and executive function, and these improvements will relate to increased blood perfusion in key neural regions that are sensitive to age-related changes such as the medial temporal lobe and prefrontal cortex.

2. Cranberry intake will produce beneficial shifts in gut microbiome composition, which will relate to increases in gut bacteria derived metabolites, including secondary polyphenol metabolites.
3. Shifts in gut microbiome structure and function as measured by metabolites will relate to changes in cognition and neural function.

CHAPTER 2: INTERVENTION METHODOLOGY

PARTICIPANT RECRUITMENT

Participants were identified using online recruitment databases (Join Dementia Research https://www.joindementiaresearch.nihr.ac.uk/), existing research databases within the Norwich Medical School, University of East Anglia where participants had previously consented to be contacted about research studies, and community-based advertising (e.g. recruitment posters, leaflets, talks).

This study aimed to include 60 (i.e. n=30 each in the control and treatment groups) healthy older adults. Volunteers aged 50-80 years old with no subjective or objective memory complaints were recruited. Married couples who live together were particularly targeted to reduce the variability in background diet patterns, however participants were able to take part in the study on their own.

Participants were first screened over the telephone for eligibility for the study using a screening questionnaire. Telephone screening took between 15 and 30 minutes.

Participants were included in the study if they fell within the following criteria:

- Aged between 50 and 80 years old.
- Male and female.
- Generally fit and healthy.
- Willing and able to provide written informed consent.
- Fluent in written and spoken English.
- Normal or corrected to normal vision and hearing.

- Understood and were willing and able to comply with all study procedures.

Participants were excluded from the study if they met any of the following criteria:

- Diagnosis of any form of dementia or significant neurological condition.
- Significant subjective memory complaints.
- History or MRI evidence of brain damage, including significant trauma, stroke, learning difficulties or serious neurological disorder, or a previous loss of consciousness for more than 24 hours.
- Currently smoking or ceased smoking less than 6 months ago.
- Chronic fatigue syndrome, liver disease, diabetes mellitus, or gall bladder abnormalities including gall bladder removal.
- History of alcohol or drug dependency.
- Clinically diagnosed psychiatric disorder, or currently on antidepressant or antipsychotic medication.
- Existing diagnosed gastrointestinal disorder, or currently on any medication which alters the function of the gastrointestinal tract.
- Major cardiovascular event, such as myocardial infarction, within the last 12 months.
- A current diagnosis of cancer, or treatment for cancer that had concluded less than 12 months prior.
- Known or suspected allergy to the intervention supplement or placebo ingredients.
- Any other significant medical condition likely to affect participation.
- Currently a participant or have been a participant in any other study involving an investigational product within the last 4 weeks.

Participants were eligible for the study if they were on blood pressure lowering medication only if the dose had been stable for at least 2 months prior to starting the intervention.

Participants were not eligible for the study if they were prescribed anticoagulant medicine such as warfarin, due to the potential for interaction with the active cranberry powder.

Regarding supplements and diet, participants were not eligible if they take and were unwilling or otherwise unable to stop taking the following:

- Flavonoid-containing supplements.
- High estimated daily flavonoid intake defined as > 15 portions of flavonoid-rich foods per day.
- Any other supplements that could have a significant impact on the outcome measures.

Participants were not eligible to undergo the neuroimaging component of the study if they had a cardiac pacemaker, any metal surgical implants that would not be safe within the MRI, or experienced claustrophobia in small spaces. If participants were unable to undergo the neuroimaging component of the study, they were still able to take part in the other components of the study.

Participants took part in the study on a voluntary basis and as such were able to withdraw from the study at any time. Withdrawal from the study would take place if participants lost capacity to consent during the study, in the event of a serious adverse event, non-compliance to taking the study powder, or if continuation of the study would be detrimental to the participant.

STUDY DESIGN

The study design was a single-centre, 12-week, double-blind, placebo-controlled parallel intervention. Participants attended 3 visits in total: a screening visit, a baseline visit at the beginning of the 12-week intervention, and a follow-up visit at the end of the intervention. Figure 2.1 details all contact points and visits made with participants and the research

activities completed at each. Participants were reimbursed £25 for completion of the study, with attendance at all visits necessary to receive the full amount.

The intervention was provided in the form of sachets of freeze-dried cranberry powder designed to be incorporated into food and beverages twice daily. Apart from the addition of the study powder, participants were asked to not modify their dietary intake in any additional way, including any deliberate changes to their caloric intake. However, participants were asked to limit their intake of certain vitamin supplements and foods with a higher concentration of polyphenols (see Supplementary Table 2 on food intake guidance). Participants were also asked not to modify their medication intake and to inform the study team of any changes to their medication. Participants were also encouraged not to commence taking new over-the-counter supplements while taking part in the trial, unless instructed to by their GP. Participant compliance, safety, and medication changes were monitored with frequent email and phone contact during the intervention, including within the first week of starting the intervention. Participants were also asked to return all remaining cranberry sachets at the end of the 12-week treatment period, with the number of leftover sachets taken as one measure of compliance. Adherence to treatment was also determined by measuring total plasma (poly)phenol metabolite concentrations which were quantified at baseline and following the 12-week intervention, as described previously (Favari et al., 2020).

To minimise bias participants were randomised into either the active cranberry or placebo group using a randomisation algorithm. Both participants and the study team were blinded to treatment group for the duration of the study and analysis. The sachets of study powder were identified only by an alphanumeric serial code which did not indicate to the study team or participants whether the powder was active or placebo, ensuring that the study team and participants remained blinded to the contents of the sachets.

On the day before the screening, baseline and follow-up visits participants were required to fast from 10pm the day before for the purposes of the blood sample collection. Participants were provided with the study powder during the baseline visit and asked to take the powder the evening immediately following the baseline visit.

Due to safety concerns surrounding the outbreak of COVID-19 in the UK in 2020, some follow-up visits were conducted entirely remotely to ensure the safety of participants. As a result, some research components could not be conducted at follow-up for 14 of the participants, which included blood sample collection, neuroimaging, physical measurements and some cognitive tests which required face-to-face administration. Spatial navigation tests such as the Supermarket Test were only conducted remotely during COVID-19 restrictions if the participant had access to their own iPad to download and complete the test during the remote testing session, otherwise these tests were not completed.

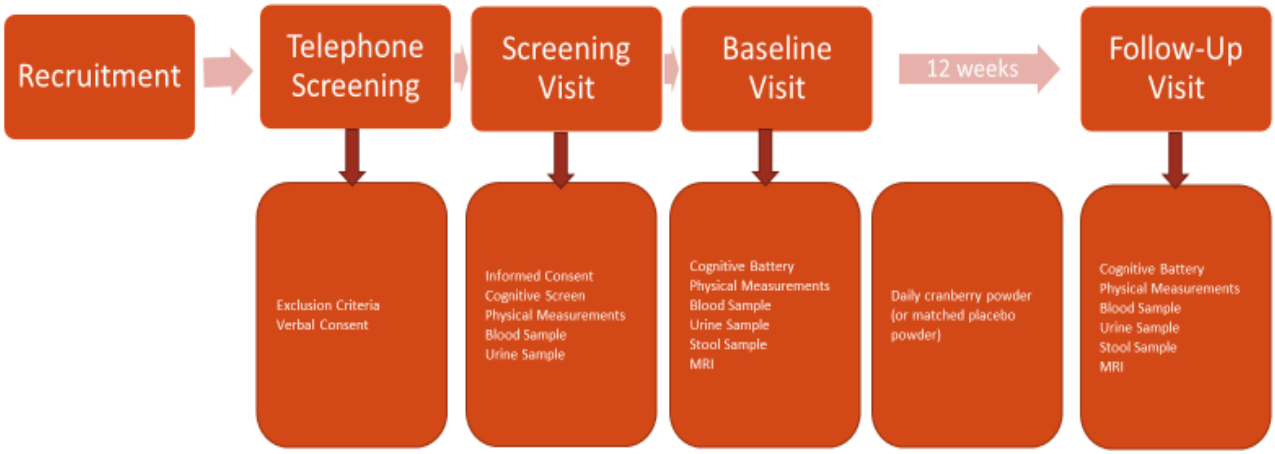


Figure 2.1. Participant flow through study and research activities conducted at each contact or visit.

STUDY COMPOUND

The freeze-dried cranberry powder was sourced from The Cranberry Institute, Massachusetts USA. The dosage was calculated to provide 281 mg proanthocyanidins over two doses taken daily, consisting of 20 mg flavonols and 58mg anthocyanins per day. Participants were instructed to take two sachets per day (4.5 grams each), one in the morning and one in the evening to maximise the physiological impact based on current understanding of bioavailability (Rodriguez-Mateos et al., 2014). The daily dosage of cranberry powder was selected as it is closely equivalent to consuming 1 cup of cranberry, or a typical serving of fresh fruit, to be at a tolerable level to prevent compliance and retention issues due to potential gastrointestinal upset and astringency. This dosage of the freeze-dried cranberry powder would provide the same amount of proanthocyanidins, anthocyanins and flavonols as previous studies that have explored dose responsiveness to improve cognition and serum BDNF with high-polyphenol intake (Neshatdoust et al., 2016) and the dosage of flavonoids of short-term consumption of a 76% concentrated cranberry juice required to produce positive effects on vascular function (Rodriguez-Mateos et al., 2016).

The placebo powder was designed to match the active cranberry powder for taste, colour, calories and macronutrient content. The placebo powder contained a blend of water, maltodextrin (CPC Maltrin M-180), citric acid, artificial cranberry flavour (Lorann oils), fructose, red colour (Lorann oils), and grape shade (Esco Foods) that had been freeze-dried. Calcium Silicate was also added as a flow agent.

DATA COLLECTION METHODS

Table 2.1. Overview of research activities at each time point.

Research Activity	First Contact	Screening	Baseline	Follow-up
Telephone Screening	X			
Participant Information Sheet	X			
Informed Consent		X		
QRisk3		X		X
Questionnaires			X	X*
ACE-III		X		X
Cognitive Assessment			X	X
Blood Pressure		X	X	X
Physical Measurements		X	X	X
Blood Sample		X	X	X
Urine Sample		X	X	X
Stool Sample			X	X
Neuroimaging			X	X

*****Some questionnaires only required completion at baseline.

PHYSICAL MEASUREMENTS

Height and weight were taken at each study visit. Participants were fully clothed with shoes removed for these measurements. Body mass index was calculated from the measurements taken.

Blood pressure was measured twice: once with the participant seated and following a five-minute rest period. Measurements were calculated using a sphygmomanometer and an appropriately sized cuff.

COGNITIVE ASSESSMENT

Participants completed all of the following cognitive tests at the baseline and follow-up visits (see Table 2.1 for full details of the data collection measures completed at each contact or visit). The only exception to this was the Addenbrooke's Cognitive Examination III (ACE-

III), which was conducted at the screening visit in the first instance to assess eligibility for the study (i.e. total score >88) and then repeated at the follow-up visit.

Addenbrooke's Cognitive Examination III (ACE-III)

The ACE-III (Hsieh, Schubert, Hoon, Mioshi, & Hodges, 2013) is a measure of global cognitive functioning which is considered a gold standard measure typically used in clinical settings to detect the presence of age-related cognitive decline specific to the onset of dementia. It covers domains including attention and orientation, memory, fluency, language, and visuospatial function. This test takes approximately fifteen minutes to complete. A score of 88 has previously been determined to be the most sensitive to detecting the presence of cognitive decline (Hsieh et al., 2013).

The Trail Making Test (TMT)

The TMT (Reitan, 1992) is a well-established short test of processing speed, attention, and set-shifting. It consists of two parts in which the subject is instructed to connect a set of 25 dots as quickly as possible while still maintaining accuracy. It is often used in clinical settings to detect the presence of executive dysfunction, particularly using the second part of the test (Trail B) which requires the shifting between sequential numeric and alphabetical sets. This test takes less than five minutes to complete, and both the time taken to complete and the number of errors made are recorded.

Digit Span (DS)

Digit span is a subtest from the Weschler Adult Intelligence Scale – third edition (WAIS III) (Kaufman & Lichtenberger, 1999) that assesses attention and working memory. Digit span is composed of two tasks administered independently of each other: digits forwards and digits backwards. For each digits forwards item, participants are presented with a series of digits in increasing length, and must immediately repeat them to the examiner in the same order as

presented. For digits backwards, the participant is required to repeat the number sequence in the reverse order. Maximum spans for both the forwards and backwards components of the tests are then determined. This test takes approximately five minutes to administer.

Rey Complex Figure (RCF) Test

The RCF test (Meyers & Meyers, 1995) is a well-established and commonly used measure of visual memory and visuospatial constructional ability, which has application to detect memory and visuospatial processing deficits in a range of brain diseases and psychiatric disorders, but particularly for the assessment of dementia. Participants are asked to copy a complex figure onto a blank piece of paper, before the original stimulus is taken away. Following a delay (often three minutes), the participant is then asked to draw the picture from memory. The accuracy and placement of elements of the figure are then scored. This study included the copy and three-minute recall trials of the test and takes approximately ten minutes in total to complete.

The Supermarket Test

The Supermarket Test (S. Tu et al., 2015) is a computer- and tablet-based assessment of spatial orientation which involves movement through a simulation of a supermarket environment. As spatial disorientation is a feature of early AD, appearing alongside or even before the emergence of episodic memory deficits (Morris, 1999), this test was included to detect the earliest cognitive symptoms typical of some forms of dementia. Participants viewed a series of videos from a first-person perspective travelling through a virtual supermarket and were required to maintain orientation to the starting location. When the video ends they are then asked to 1) indicate the direction they would need to turn to face the starting position, 2) indicate their final position on a top-down map of the virtual supermarket, and 3) indicate on a map which direction they were facing at the end of the

video. There are allocentric (using landmarks) and egocentric (using the self as reference) components to this test. This test takes approximately fifteen minutes to complete.

QUESTIONNAIRES

Background Questionnaire

A background questionnaire produced for this study was provided to participants at the screening visit to collect demographic and general health information about the participant, including medical history and a full list of medications. This background questionnaire took approximately fifteen minutes to complete. A shorter review questionnaire was provided to participants at the follow-up visit to collect information relating to any GP or hospital visits during the study and medication changes.

Cognitive Change Index (CCI)

The CCI (Rattanabannakit et al., 2016) is a brief self-rated measure of the participant's perceived cognitive status and decline compared to five years ago. Participants rate the severity of a range of cognitive changes compared to five years ago from 1 to 5 (1=no changes, 2=minimal change, 3=some change, 4=clearly noticeable change, 5=much worse), with a possible total score range between 20 – 100. The test also provides a sub score for subjective memory performance based on the first 12 items (total scores 12-60), a score for executive function from items 13-17 (total scores 5-25) and a score for language function based on items 18 – 20 (total scores 3-15). This questionnaire was provided to participants at the screening visit only as a measure of subjective cognition. The CCI takes approximately five minutes to complete.

Cambridge Behavioural Index Revised (CBI-R)

The CBI-R (Wear et al., 2008) is a brief self-rated measure of changes in the participant's behaviour. The behavioural domains measured include memory and orientation, everyday

skills, self-care, abnormal behaviour, mood, beliefs, eating habits, sleep, stereotypic and motor behaviours, and motivation. The CBI measures the frequency of these behaviours over the past month on a five-point scale, from 'never' to 'constantly'. Participants completed this questionnaire at screening and again at follow-up to determine whether there had been any perceived changes in behaviour during the study. The CBI-R takes approximately five minutes to complete.

Patient Health Questionnaire 9 (PHQ-9)

The PHQ-9 (Lowe, Unutzer, Callahan, Perkins, & Kroenke, 2004) is a 9-item self-report measure of the severity of depressive symptoms in the participant. Participants completed this questionnaire at screening and follow-up to measure levels of low mood. Participants rate the presence of depressive symptoms over the past two weeks between 0 ('not at all') and 3 ('nearly every day') giving a total score of 27, with scores between 0-4 indicating no symptoms of depression, 5-9 indicating mild symptoms, 10-14 indicating moderate symptoms, 15-19 indicating moderately severe symptoms and 20-27 indicating severe symptoms of depression. The PHQ-9 takes less than five minutes to complete.

Generalized Anxiety Disorder Questionnaire 7 (GAD-7)

The GAD-7 (Spitzer, Kroenke, Williams, & Lowe, 2006) is a 7-item self–report questionnaire measuring for screening symptoms of generalized anxiety disorder in the participant. The GAD-7 takes less than five minutes to complete. Participants are asked to self-rate symptoms of generalised anxiety over the past two weeks between 0 and 3 for a total score of 21, with scores of 0-4 indication no symptoms, 5-9 indicating mild symptoms, 10-14 indicating moderate symptoms, and 15-21 severe symptoms of generalised anxiety. The GAD-7 was completed by the participant both at screening and follow-up.

The QRISK3 Cardiovascular Screen (QRISK3)

The QRISK3 (Hippisley-Cox, Coupland, & Brindle, 2017) is a well-established screening tool for identifying individuals at high risk of developing cardiovascular disease. This questionnaire score was calculated based on the participant's medical history as well as their blood pressure measurement and cholesterol ratio (total:HDL) results from the screening visit.

Scottish Collaborative Group Food Frequency Questionnaire (SCG-FFQ)

Participants were asked to fill in the SCG-FFQ (version 6.6) (Hollis et al., 2017), a validated, semi-quantitative dietary assessment instrument that has been developed to estimate and rank the dietary intake of a wide range of nutrients in large-scale UK epidemiological studies (Hollis et al., 2017; Masson et al., 2003). The SCG-FFQ covers 169 food items grouped into 21 categories (e.g. breads and breakfast cereals). Possible responses for frequency of consumption range from 'rarely', to once in a month, to seven days a week, and the number of serves (from 1 to 5+ per day) for the amount usually consumed. Standard household measures (e.g. one tablespoon and one teaspoon) or items (e.g. one small cake and one medium slice) are listed as portion size responses (Crawley, 1988). The SCG-FFQ was used to describe each participant's habitual diet over the previous two to three months. Participants completed the paper-based questionnaire at baseline and were asked to return it within 1 week. Responses were then entered using a purpose-built, web-based, data-entry system. SCG-FFQ data were analysed using the UK food composition tables (McCance & Widdowson, 2014).

International Physical Activity Questionnaire (IPAQ)

The long version of the IPAQ is a reliable and well validated measure (Booth, 2000; Craig et al., 2003) which assesses physical activity across 5 domains including leisure time physical activities, domestic and gardening activities, work-related physical activity, and transport-

related physical activity. Participants are asked to report the number of minutes spent performing activities in each of the five domains over the past 7 days, including time engaged in walking, moderate, and vigorous activity. Levels of activity were then classed as 'inactive', 'minimally active' and 'health-promoting physical activity (HEPA) active' according to guidelines provided by the developers of this questionnaire (Committee, 2005).

Pittsburgh Sleep Quality Index (PSQI)

The PSQI (Buysse, Reynolds, Monk, Berman, & Kupfer, 1989) is a self-report questionnaire asking participants about their sleep habits and quality over the past month. The PSQI contains 19 self-rated questions and 5 questions rated by the bed partner or roommate (if one is available). Only self-rated questions are included in the scoring. The 19 self-rated items are combined to form seven "component" scores, each of which has a range of 0-3 points. In all cases, a score of "0" indicates no difficulty, while a score of "3" indicates severe difficulty. The seven component scores are then added to yield one "global" score, with a range of 0-21 points, with "0" indicating no difficulty and "21" indicating severe difficulties in all areas.

MAGNETIC RESONANCE IMAGING

DATA ACQUISITION

MRI scans were conducted in all eligible and willing participants at baseline and the end of the intervention and took approximately 30 minutes. In order to monitor structural brain information across the study, a T_1-weighted 3D gradient-echo MR sequence was conducted at each testing visit. A T_2-weighted fluid attenuated inversion recovery (FLAIR) scan and Arterial spin labelling (ASL) was also conducted during the study visits. ASL has previously been used to monitor changes in cerebral blood flow (CBF) in AD and MCI patients (Sierra-Marcos, 2017; N. Zhang, Gordon, & Goldberg, 2017).

All data were acquired on a 3 tesla Discovery 750w widebore MR system (GE Healthcare, Milwaukee, WI, USA) with a 12-channel phased-array head coil for signal reception. After localisers, T_1-weighted structural data were acquired using a 3D inversion-recovery fast spoiled gradient recalled echo (IR-FSPGR) sequence with repetition time (TR) = 7.7 ms; echo time (TE) = 3.1 ms; inversion time = 400 ms; field-of-view = 256 × 256 mm; acquired matrix = 256 × 256; 200 sagittal sections of 1 mm thickness; flip angle = 11°; and ASSET acceleration factor = 2 in the phase-encoding direction. Furthermore, a 3D T_2-weighted fluid attenuated inversion recovery (T_2w FLAIR) sequence was prescribed as follows: TR = 4800 ms; TE = 129 ms; inversion time = 1462 ms; field-of-view = 256 × 256 mm; acquired matrix = 256 × 256; 182 sagittal sections of 1 mm thickness; flip angle = 90°; an ARC acceleration factor of 2 in the phase-encoding direction; and a "HyperSense" compressed sensing subsampling factor of 2. The ASL scan consisted of a 3D spiral pseudo-continuous ASL (pCASL) acquisition with the following parameters: TE = 10.7 ms, TR = 4854 ms, 8 spiral interleaves with 512 sample points, field-of-view = 240 × 240 × 128 mm with a reconstructed resolution of 1.9 × 1.9 × 4 mm; post-label delay = 1500 ms, number of excitations = 3. Before analyses, all participant scans were visually inspected for significant head movements and artefacts.

BIOLOGICAL SAMPLES

COLLECTION OF BIOLOGICAL SAMPLES

BLOOD SAMPLES

A fasted blood sample was taken at each assessment visit, using a standard gauge needle and vacutainer system. Of these samples, 7mL (5mL in serum separation tube (SST), 2ml Fluoride) were sent to accredited pathology laboratories at the Norfolk and Norwich University Hospital (NNUH) for determination of markers of general health (See Table 2.2. for total samples collected at each study visit). An additional 4mL Ethylenediaminetetraacetic

acid (EDTA) tube was sent at screening to the same pathology laboratories for full blood count.

Additional samples (2 x 5ml SST, 2 x 4ml EDTA) were collected at baseline and follow-up, and a 8ml sodium heparin cell preparation tube (CPT) sample was also collected at baseline only for genetic analysis. These samples were separated by centrifugation (at a speed / force, and temperature, consistent for the future assessment of each analyte) and then aliquoted into sub-fractions into polypropylene tubes (to provide sufficient material for duplicate analysis). Similarly for serum separation, blood samples were left to coagulate (in clot activating gel tubes) following manufacturers guidelines (i.e. ≥ 30mins) to be followed by centrifugation and removal of the resultant serum into sub-fractions. Samples collected were labelled only with volunteer identification code, age, gender, visit number and time and date of collection. Aliquoted plasma, buffy coat, mononuclear cell and serum samples were stored at −80°C at the Norwich Biorepository for further analysis (as described in subsequent sections).

Table 2.2. Summary of biological samples collected at each study visit.

	Visit		
Test Type	Screening	Baseline	Follow-Up
Biochemistry screening (NNUH)	1 x 5ml SST (yellow) 1 x 4ml EDTA (purple) 1 x 2ml Fluoride (grey) 1 x urine (Dipstick urinalysis only)	1 x 5ml SST (yellow) 1 x 2ml Fluoride (grey)	1 x 5ml SST (yellow) 1 x2 ml Fluoride (grey)
Gene Variants		1x8ml sodium heparin cell preparation tube	
Biomarkers		1 x urine 2 x 5ml SST (yellow) 2 x 4ml EDTA (purple) 2x stool samples	1 x urine 2 x 5ml SST (yellow) 2 x 4ml EDTA (purple) 2x stool samples
Total blood collected:	11ml	33ml	25ml
Total blood tubes:	3	7	6

URINE SAMPLES

At least 100ml urine was collected at screening, baseline and follow-up visits. A dipstick urinalysis was conducted at each visit (see further details below). At baseline and follow-up visits the urine sample was aliquoted into 4 x 2ml storage cryotubes and stored at the Norwich Biorepository before further analyses were conducted.

STOOL SAMPLES

In total, two faecal samples were collected (within 48 hours of the baseline and follow-up visits) by participants at home, using the collection kits, gloves and vessels provided by the research team (NHS approved EasySampler collection kit supplied by Co-vertec Limited). Briefly, these collection kits included a flushable collection sheet that fits over the toilet seat, allowing for samples to be collected and stored into 10mL specimen tubes. The storage container was then placed in a cool dry location prior to returning to the research facility at the earliest opportunity (i.e. at the study visit). In cases where the sample was unable to be provided to the study team at a visit, the samples were posted to the Norwich Biorepository using Royal Mail Safeboxes, where they were placed immediately in -80C freezers on arrival. Participants were asked to indicate the time and date of stool sample collection on a separate study document, and in cases where samples were posted they were advised to post as soon as possible and to not freeze the sample before posting.

ANALYSIS OF BIOLOGICAL SAMPLES

SCREENING OF BLOOD BIOCHEMISTRY

The following were measured by the NNUH pathology laboratories, following standardised procedures:

- Full blood count analysis (screening visit only) (white cell count (lymphocytes, neutrophils, monocytes, eosinophils, basophils), red cell count, haemoglobin, haematocrit, mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), platelet count).
- Urea and electrolytes (sodium, potassium, bicarbonate, urea and creatinine).

- Liver function (total bilirubin, total protein, albumin, globulin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), gamma glutamyl transferase (GGT)).
- Full lipid panel (Including total cholesterol, lipoprotein, high density cholesterol, cholesterol (HDL cholesterol), triglycerides)

URINALYSIS

The midstream spot urine sample collected at the clinical screen was tested to assess pH, the presence of protein, glucose, ketones and blood, as indicators of general health (using a Siemens Multistix 10SG urinalysis dipstick method or equivalent). This procedure was also repeated at baseline and follow-up visits.

BIOMARKERS OF NEURONAL ACTIVITY (BDNF)

Commercially available enzyme linked immunosorbent assay (ELISA) kits (R&D Systems, UK) were used to analyse plasma samples at baseline and follow-up for biomarkers of neuronal activity including BDNF.

APOE GENOTYPING

DNA extraction and APOE genotyping were conducted on buffy coat samples from EDTA samples by LGC Genomics Ltd., Hoddeson, United Kingdom.

GUT MICROBIOME COMPOSITION AND FUNCTION

SHOTGUN METAGENOMICS, DATA PROCESSING AND ANALYSES

DNA extraction from faecal water, quality assessment and library preparation were conducted at the Quadram Institute. Genomic DNA was extracted from all faecal samples using a commercially available kit (Maxwell® RSC PureFood GMO and Authentication Kit, Cat. #AS1600). Around 50 mg of faecal pellet was used, following manufacturer's instructions, with an additional bead beating step using the FastPrep (MP Biomedicals, USA), protocol previously described by Kellingray et al. (2017). DNA concentrations of each

sample were evaluated using Qubit® dsDNA High Sensitivity Assay Kit (Cat. Q32851) with Qubit® 2.0 Fluorometer, following manufacturer's instructions. All concentrations ranged between 2.4 - 26.8 ng/µL dsDNA samples. Remaining faecal pellets were also stored at –80°C for faecal water extraction for subsequent metabolomic analysis. The sequencing library was prepared using the Nextera XT DNA Library Preparation Kit (Illumina), according to the manufacturer's instructions. The pre-made library was sent to Novogene Europe for sequencing.

Libraries were prepared using a novel modified Illumina DNA prep tagmentation approach (formerly called Nextera DNA Flex Illumina Library Prep). A 20-fold reduced tagmentation reaction followed by PCR barcoding, quantification and pooling. The resultant pool was outsourced to run on an Illumina Novaseq S4 flowcell with paired end 151bp reads with dual 8bp barcodes. Briefly, genomic DNA was normalised to 5ng/µl with EB (10mM Tris-HCl). 0.5 µl of TB1 Tagment DNA Buffer was mixed with 0.5 µl BLT, Tagment DNA Enzyme (Illumina Catalogue No. 20018704) and 4 µl PCR grade water in a master mix and 5ul added to a chilled 96 well plate. 2 µl of normalised DNA (10ng total) was pipette mixed with the 5 µl of the tagmentation mix and heated to 55 ºC for 15 minutes in a PCR block. A PCR master mix was made up using 4 ul kapa2G buffer, 0.4 µl dNTP's, 0.08 µl Polymerase and 4.52 µl PCR grade water, contained in the Kap2G Robust PCR kit (Sigma Catalogue No. KK5005) per sample and 9 µl added to each well need to be used in a 96-well plate. 2 µl of each P7 and P5 of Nextera XT Index Kit v2 index primers (Illumina Catalogue No. FC-131-2001 to 2004) were added to each well. Finally, the 7 µl of Tagmentation mix was added and mixed. The PCR was run with 72ºC for 3 minutes, 95ºC for 1 minute, 14 cycles of 95ºC for 10s, 55ºC for 20s and 72ºC for 3 minutes. Following the PCR reaction, the libraries were quantified using the Promega QuantiFluor® dsDNA System (Catalogue No. E2670) and run on a GloMax® Discover Microplate Reader. Libraries were pooled following quantification in equal

quantities. The final pool was double-SPRI size selected between 0.5 and 0.7X bead volumes using KAPA Pure Beads (Roche Catalogue No. 07983298001). The final pool was quantified on a Qubit 3.0 instrument and run on a D5000 ScreenTape (Agilent Catalogue No. 5067-5579) using the Agilent Tapestation 4200 to calculate the final library pool molarity.

Sequence data (n=120 samples) were generated on an Illumina Novaseq 4000 (paired-end; 150 bp). Sequencing data were checked using fastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), with an average of 5.29 Gb (± 1.19 Gb) generated for each sample. Data processing and analysis of shotgun sequencing data was conducted by Lesley Hoyles, Nottingham Trent University with computing resources UK MEDical BIOinformatics partnership (UK Med-Bio, supported by Medical Research Council grant number MR/L01632X/1). Nextera transposase contamination was removed from samples using Trimmomatic v0.39 (Bolger, Lohse, & Usadel, 2014). Human DNA within samples was detected by mapping reads against the human genome (GRCh38/hg38; https://hgdownload.soe.ucsc.edu/downloads.html) using bwa mem v0.7.17-r1188 (Li, 2013). Non-human DNA was extracted from read files using samtools v1.10 (http://www.htslib.org/). These human-filtered files were uploaded to the Sequence Read Archive and are available under BioProject PRJNA681337 (embargoed until 31/12/2023) and used for all subsequent analyses. They had an average of 3.93 Gb (± 1.03 Gb) sequence data per sample. SPAdes v3.13.1 (meta) (Bankevich et al., 2012; Nurk, Meleshko, Korobeynikov, & Pevzner, 2017) was used to assemble sequence data for each of the 120 datasets, with contigs <500 nt in length removed from analyses using reformat.sh in BBMap. Genes (nucleotide, amino acids) in assemblies were predicted using Prodigal v2.6.3 (Hyatt et al., 2010). A total of 33,190,228 genes was predicted across all samples. Protein sequences of ≥30 amino acids (n=32,622,164) were clustered using UCLUST (Edgar, 2010) with a 90 % cut-off identity. Centroid sequences from each cluster (n=4,161,937) were used to generate a

non-redundant gene catalogue for determination of gene abundances and functional predictions. Normalised gene abundances in each sample were determined as described previously (Hoyles, Fernandez-Real, et al., 2018). The eggNOG-mapper v2 (eggNOG 5.0) was used to generate functional predictions for the dataset (Huerta-Cepas et al., 2017; Huerta-Cepas et al., 2019).

MICROBIAL DIVERSITY

Measures of alpha and beta diversity were determined using Phyloseq v1.30.0 (McMurdie & Holmes, 2012), with species-level data (where counts had ≥1 % cumulative relative abundance across all metagenomes) rarefied to 3,389,582 reads prior to analysis. Non-rarefied count data were subject to analyses (paired Wilcoxon rank sum tests for comparisons within control and treated groups) using ALDex2 v.1.14.1 (Fernandes et al., 2014). To characterize the phylogenetic composition of bacterial communities in our samples, we firstly compared the alpha-diversity of microbiota of the separate groups. For maintaining the blinding during the analysis we kept the following codes: A_BL (Cranberry group at baseline); A_FU (Cranberry group at follow up); B_BL (Placebo group at baseline) and B_FU (Placebo group at follow-up).

Alpha diversity refers to the diversity in a specific area or ecosystem in terms of species richness. According to species richness in the list of OTUs in the faecal sample, diversity, richness, coverage, and evenness estimations were calculated for all data sets. The observed species metric is an estimator of phylotype richness, whilst the Shannon or Inverse Simpson indices of diversity reflects both the richness and community evenness.

We then explored the variation of phylogenetic diversity among all tested samples using the beta diversity analysis, which represents the extent of similarity between different microbial communities. Principal coordinate analysis (PCoA) of the species abundance data set was

used to test whether we could separate samples that belonged to diverse groups, when phylogenetic information about detected sequences was taken into account.

RELATIVE SPECIES ABUNDANCES

The distribution of the relative abundances of different bacterial classes among samples and groups was investigated using taxonomic abundance and read count data for archaea and bacteria which were generated using Kraken2 2.0.8-beta (Wood, Lu, & Langmead, 2019) and the pre-compiled Kraken2 GTBD_r89_54k index (downloaded on 3 May 2020) available from https://bridges.monash.edu/ndownloader/files/16378439 (Méric, Wick, Watts, Holt, & Inouye, 2019).

MICROBIAL GENE RICHNESS AND FUNCTIONALITY

As an additional measure to assess diversity, gene richness was examined (i.e. the number of unique genes) in the shotgun metagenomic sequencing data. This measure is used to divide samples into those of low versus high gene richness, with those of high gene richness generally considered to have higher diversity (Ilett et al., 2019). Microbial gene richness was determined as described previously (Hoyles, Fernandez-Real, et al., 2018). Data were downsized to adjust for sequencing depth and technical variability by randomly selecting 8 million reads mapped to the merged gene catalogue (of 4,161,937 genes for each sample and then computing the mean number of genes over 30 random drawings).

METABOLOMICS

URINARY METABOLISM PHENOTYPES

Metabolic phenotypes were derived from plasma and urine samples at baseline and follow-up and analysed by Professor Jonathan Swann and colleagues at the University of Southampton. Metabolic phenotypes were measured using ^{1}H nuclear magnetic resonance (NMR) spectroscopy. For urine samples, 300 µL of urine was combined with 300 µL of sodium phosphate buffer (pH 7.4, 100% D_2O) containing 1 mM of the internal standard, 3-(trimethylsilyl)-[2,2,3,3,-$^{2}H_4$]-propionic acid (TSP). Samples were vortexed to mix,

centrifuged at 13,000 *g* for 10 minutes, and the supernatant was transferred to 5 mm NMR tubes. For plasma samples, 300 µL was combined with 300 µL saline (100% D_2O), vortexed to mix, centrifuged at 13,000 *g* for 10 minutes, and 550 µL was transferred to a 5 mm NMR tube.

One dimensional NMR experiments were performed using a Bruker 700 MHz NMR spectrometer equipped with a cryoprobe (Bruker Biospin GmbH, Rheinstetten, Germany). A standard one-dimensional solvent suppression pulse sequence (relaxation delay, 90° pulse, 4 µs delay, 90° pulse, mixing time, 90° pulse, acquire FID) was used (Beckonert et al., 2007). For each sample, 64 transients (8 dummy scans) were collected in 64K frequency domain points with a spectral window set to 20 ppm. A relaxation delay of 4 s, a mixing time of 10 ms, an acquisition time of 2.73 s and 0.3 Hz line broadening was used. An additional Carr-Purcell-Meiboom-Gill (CPMG) NMR experiment (64 scans; 8 dummy scans; 64K data points) was performed for the plasma samples to allow the study of low molecular weight metabolites. For the urine spectra, profiles were referenced to the TSP resonance at δ 0.0 and for the plasma spectra, the doublet arising from glucose (δ 5.223) was used. Spectral phasing and baseline correction were automatically performed using Topspin 4.0 (Bruker Biospin GmbH, Rheinstetten, Germany). Raw NMR spectra were digitized using in-house scripts in MATLAB (Version 2018a, Mathworks Inc). After digitization of the spectra, redundant peaks (TSP, H_2O and urea) were removed followed by manual alignment of the spectra to reference peaks using a recursive segment-wise peak alignment approach (Veselkov et al., 2009). Aligned spectra were normalized by probabilistic quotient normalization (Dieterle, Ross, Schlotterbeck, & Senn, 2006).

POLYPHENOL METABOLISM

Polyphenol metabolites in baseline and follow-up plasma were processed and analysed by Professor Daniele del Rio and colleagues at the University of Parma.

Plasma extraction of polyphenol metabolites was performed using microelution solid phase extraction (µSPE) according to validated protocols, with some modifications (Feliciano et al., 2016; Feliciano et al., 2017). Briefly, plasma samples (350 µl) were diluted (1:1) with phosphoric acid 4% to reduce phenolic-protein interactions. Each sample (600 µl) was loaded on a 96 well µSPE plate, washed with water (200 µl) and 0.2% acetic acid (200 µl) and finally eluted with methanol (60 µl). The 96 well collection plates were directly put in the UHPLC autosampler for immediate analysis. Plasma samples were analyzed through UHPLC DIONEX Ultimate 3000 fitted with a TSQ Vantage Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with a heated-electrospray ionization source (H-ESI-II; Thermo Fisher Scientific Inc.). Separations were performed with a Kinetex EVO C18 (100 × 2.1 mm), 2.6 µm particle size (Phenomenex). For UHPLC, mobile phase A was water containing 0.01% formic acid and mobile phase B was acetonitrile containing 0.01% formic acid. The gradient started with 5% B, keeping isocratic conditions for 0.5 min, reaching 95% B at 7 min, followed by 1 min at 95% B and then 4 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 thermostatted at 40°C. The MS worked in negative ionization mode with capillary temperature at 270°C, while the source was at 300°C. The sheath gas flow was 60 units, while 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). Compounds were monitored in selective reaction monitoring (SRM) mode, and characteristic MS conditions (S-lens RF amplitude voltage and collision energy) were optimized for each compound. Chromatograms, mass spectral data and data processing were performed using Xcalibur software 2.1 (Thermo Fisher Scientific Inc.). Quantification was performed with calibration curves of standards, when available; when not available, metabolites were quantified with the most structurally similar compound. Due to failure in

collecting follow-up plasma due to COVID-19 restrictions, 14 volunteers were not considered for the calculations of plasma metabolite content.

COVID-19 TESTING

Faecal samples for the 14 participants who had follow-up visits that occurred from March 2020 onwards were tested for the presence of COVID-19 in collaboration with the 'Inactivation of SARS-CoV-2 in human stool samples and saliva samples' study (IRAS ID 284252, HRA REC REF 20-NI-0076). None of the participant samples tested were found to be positive for presence of the COVID-19 virus.

STATISTICAL ANALYSIS PLAN

The sample size was estimated based on the false discovery rate (FDR) control (Pawitan, Michiels, Koscielny, Gusnanto, & Ploner, 2005). For the analysis of the microbiome in particular, the following assumptions were used: 50% of microbiome profiles are not associated with the case-control status and at least 80% power. The calculation indicates that with a sample size of 30 in each experimental group, it would be expected to control FDR between 0.5% and 4%. FDR of 4% is considered low and hence it is proposed that a sample size of 30 in each group is adequate to control FDR at reasonable levels. This number is consistent with other studies investigating changes in the gut microbiome associated with diabetes (a risk factor for cognitive decline) (He, Shan, & Song, 2015). This sample size is also consistent with the only other study to date investigating the impact of cranberry on cognition, despite only non-significant trends being detected as a result of a shorter intervention period (6 weeks) (Crews et al., 2005).

Unless stated otherwise, demographic, behavioural, neuroimaging and biological data were analysed using the Statistical Package for the Social Sciences (SPSS; v28.0), applying

standard statistical thresholds ($p<0.05$). Data were also tested for normality using the Shapiro-Wilk test.

To assess the baseline characteristics and identify any inequalities between cranberry and placebo groups at the outset of the study, as most variables were not normally distributed these group differences were determined using non-parametric Mann-Whitney U Independent Samples tests. Differences in group proportions were measured using chi-squared tests.

For cognitive and neuroimaging outcomes, one-way ANCOVA's were first used to detect any baseline differences in cognition, regional neural perfusion, WMH between groups controlling for age, education and gender – which are variables that are expected to potentially impact cognitive outcomes. To address the primary outcome of changes in cognitive performance due to the intervention, linear mixed models were used to detect group x time interactions between groups over the course of the 12 week intervention, allowing for covariate adjustment for age given the large age range of the target sample, as well as education and gender. Similar linear mixed modelling was performed on neural regional mean perfusion with age entered as a covariate to determine whether there were any changes that might relate to changes in cognitive performance. To determine relationships between changes in cognition, regional neural perfusion and BDNF as a result of the intervention, Spearman correlations between significant cognition, regional perfusion and BDNF at follow-up were also conducted.

For the analysis of changes to the genetic structure of the gut microbiome as a result of the cranberry intervention, Wilcoxon rank sums analysis was conducted within groups to detect differences between baseline and follow-up in relative microbial taxonomic abundances at the class, order, family, genus and species levels. Differences in intra- and inter-individual microbial diversity was then analysed, with alpha and beta diversity determined using

Phyloseq v1.30.0 (McMurdie & Holmes, 2012), and compared between groups using Wilcoxon rank sums (paired). To characterize the phylogenetic composition of bacterial communities in our samples, the alpha-diversity of microbiota of the separate groups was compared using Wilcoxon rank sums (paired).

To determine metabolic profiles, urinary metabolites were analysed using orthogonal projection to latent structures discriminant analysis (OPLS-DA) models by Prof Jonathan Swann and colleagues. OPLS-DA models were built in R using the MetaboMate package. To determine the relationship between metabolic profiles and cognitive and changes in microbiota species abundances detected as a result of the cranberry intervention, significant cognitive and microbiome outcomes were used as response vectors. Common polyphenol metabolites from plasma were also quantified, and linear mixed modelling used to determine differences between placebo and cranberry groups over the course of the intervention. Finally, changes in levels of polyphenol metabolites were related to changes in cognition and regional neural perfusion.

To further analyse the number of responders vs. non-responders between cranberry and placebo groups, participants were grouped according to whether their significant cognitive performance, regional neural blood perfusion, or polyphenol metabolite biomarkers increased between baseline and follow-up (responders) or decreased or remained the same (non-responders). A chi-squared test was then performed to determine whether groups significantly differed in their proportions of responders and non-responders, including further grouping based on the number of outcomes for which individual participants were classed as responders.

With the exception of the microbiome analysis which involved a large number of pairwise comparisons and as such were corrected with FDR adjustments to *p*-values, corrections for

multiple comparisons were not applied to the analyses of baseline characteristics, cognitive outcomes, neuroimaging results or metabolite analyses, as the study presented in this thesis was conducted as a feasibility trial in part to determine effect sizes for larger future trials. As such, the results presented here and their significance as they are presented in this thesis should be interpreted provisionally.

CHAPTER 3: BASELINE CHARACTERISTICS OF THE STUDY SAMPLE

INTRODUCTION

To date there are very few other clinical trials investigating the impact of cranberries on brain function and the gut microbiome in cognitively healthy older adults. A recent systematic review identified a total of 18 studies in total that had been published focusing on cognitive performance following supplementation with berry anthocyanins (Ahles et al., 2021). Of these, the majority focused on blueberry supplementation, and the review did not include any studies focusing on the impact of cranberry intake. There has been one placebo-controlled trial of chronic cranberry juice intake (6 week) in a sample of healthy older adults (aged >60 years) and its impact on a range of cognitive outcomes, with no positive results found (Crews et al., 2005). Comparison of participant baseline characteristics in the current study with similar studies involving older adults would need to focus on interventions supplementing other types of berries with similar polyphenolic profiles high in flavonoids such as anthocyanins and proanthocyanidins, which most often involve supplementation of either common or wild blueberry strains (Boespflug et al., 2018; Bowtell et al., 2017; Krikorian, Shidler, et al., 2010; Miller et al., 2018).

Among the blueberry studies that are most comparable to the current study, a 12-week placebo-controlled intervention of daily blueberry concentrate supplementation in healthy older adults (ages over 65 years) found increases in resting brain perfusion, but results for cognitive performance did not quite reach significance in a smaller sample of 26 participants (Bowtell et al., 2017). When it comes to investigating the impact of cranberry on the gut microbiome, there are no clinical trials in cognitively healthy older adults and much of the evidence to date regarding the impact of cranberry on the microbiome comes from animal studies particularly involving rodents (Chettaoui et al., 2021; Liu et al., 2021; Renaud et al., 2021), as well as *in vitro* studies (D. Zhang et al., 2021) and simulator models (O'Connor et

al., 2019). The most similar studies in humans include a placebo-controlled trial of cranberry juice in subjects positive for *Helicobacter pylori* with positive results (Gao et al., 2021), attenuation of the impact of an animal-based diet in a small placebo-controlled crossover trial in 11 human subjects for 5 days , and a trial that produced limited effects on the microbiome in women with urinary tract infections who took cranberry daily for 24 weeks (Straub et al., 2021). Therefore, there are currently no studies investigating the impact of cranberry on the gut microbiome in healthy older adults available to compare baseline sample characteristics required to detect positive results.

Here the baseline characteristics and group differences at the outset of the study between the placebo and cranberry groups are described, in the context of the few existing clinical trials investigating the impact of berry supplementation on cognition, brain function and the gut microbiome in older adults.

METHODS

DEMOGRAPHICS

Participant demographics were collected using a brief study-specific questionnaire at the screening visit and completed by participants themselves. Information collected included date of birth, highest level of education, details of any tertiary educational degrees or diplomas, brief work history, medical history and current diagnoses, and smoking status and any history of smoking.

PHYSICAL MEASUREMENTS AND BLOOD RESULTS

Height, weight, and lying blood pressure were collected at screening, baseline and follow-up visits. Blood samples were also collected at each visit and sent to the pathology laboratory at the Norfolk and Norwich University Hospital for full blood biochemistry, lipid profile, and haematology. A urine sample was also collected at each study visit for dipstick urinalysis.

All blood biochemistry results at each visit (and haematology at screening) were reviewed by a clinician and who indicated whether follow-up on abnormal results and notification of the participant's GP would be required. Instances of results that indicated chronic conditions that would exclude the participant from taking part (eg. Type 2 diabetes) were also indicated and these participants were informed of the results and that they would not be able to continue with the study. Similarly, urinalysis results were reviewed during each visit by a research nurse, who advised whether results were abnormal and required further action.

A QRISK3 (Hippisley-Cox et al., 2017) risk prediction algorithm was used to calculate the 10 year risk of cardiovascular disease based on the baseline results from blood pressure, height and weight readings, total:HDL cholesterol ratio, demographic details and current medications reported.

Medications

Information regarding medications being taken by participants was collected at the screening visit and also requested again at follow-up to determine whether there had been any changes in medications during the trial.

Background Diet

Participants were asked to fill in the SCG-FFQ version 6.6 (Hollis et al., 2017) to allow for calculation of detailed nutrient intake over the previous two to three months based on self-reported frequency of habitual food consumption. The SCG-FFQ was completed either during the baseline visit or given to participants to complete at home after the baseline visit to be returned within 1 week. Further details of the SCG-FFQ can be found in Chapter 2.

Sleep and Physical Activity

Sleep and physical activity questionnaires were collected at the baseline visit and were self-rated by the participant. The sleep and physical activity questionnaires were also collected

again at the follow-up visit. Full details of these questionnaires are available in Chapter 2. The PSQI (Buysse et al., 1989) was used to assess participants' sleep quality, duration and habits. The long version of the IPAQ (Booth, 2000) was used to assess participant's self-reported level of physical activity.

Mood, Subjective Memory and Behaviour

All mood, cognition and behaviour questionnaires were self-rated by the participant. Full details of the questionnaires assessing behaviour, cognition, and mood are available in Chapter 2. Participants completed the CBI-R (Wear et al., 2008) to indicate behavioural changes associated with the onset of dementia. Subjective memory changes were self-reported using the CCI (Rattanabannakit et al., 2016), which measures the participant's perceived cognitive decline and changes over the past 5 years. The PHQ-9 (Lowe et al., 2004) was used to assess symptoms of depression over the previous two weeks. Similarly, the GAD-7 (Spitzer et al., 2006) questionnaire was used to measure self-reported symptoms of generalised anxiety over the previous two weeks. The CBI-R, PHQ-9 and GAD-7 were completed by participants at the screening visit and then repeated at follow-up, and the CCI was completed at baseline only.

APOE Genetic Status

DNA extraction and *APOE* genotyping was conducted on buffy coat samples by LGC Genomics Ltd., Hoddeson, United Kingdom (Chapter 2).

Statistical Analyses

As most variables were not normally distributed, group differences were determined using non-parametric Mann-Whitney U Independent Samples tests. Differences in group proportions of gender, working status, subjective sleep quality, frequency of sleep medication, and APOE genetic status were measured using chi-squared tests.

RESULTS

DEMOGRAPHICS

In total, 60 healthy male and female older adults aged 50-80 years old (M=65.68, SD= 5.17) with mean years of education of 14.50 (SD=2.80) and with no subjective memory complaints were recruited in this study. There were 29 participants randomised into the cranberry group and 31 participants into the placebo group. All participants randomised into the intervention completed the intervention to the 12-week follow-up visit. Of the participants who began the intervention, there were 11 cohabiting married dyads.

Of the participants consented to the study, 10 participants did not make it past the screening visit. Of these, 3 participants declined further participation, and 7 did not meet screening criteria. Five participants were excluded due to severe uncontrolled hypertension, one participant scored below the cut-off on the ACE-III total score (i.e. <88/100), and one participant had screening blood and urinalysis results indicative of a possible undiagnosed diabetic condition which was consequently diagnosed with the participant's GP, and so this participant was not enrolled into the intervention. There were no participants who were ineligible for an MRI scan due to contraindications, however 9 participants declined to undergo an MRI scan (see Figure 3.1 for full details).

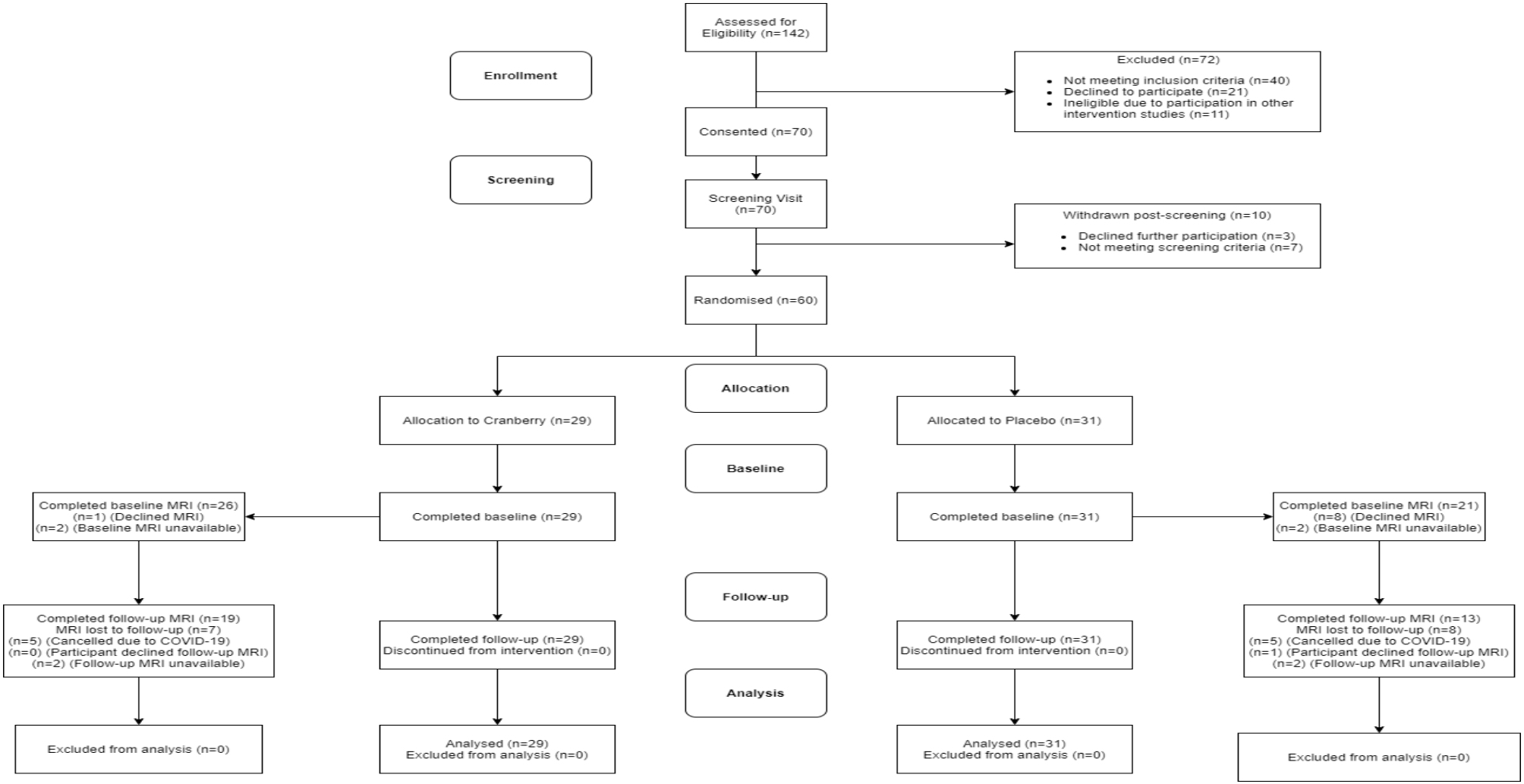


Figure 3.1. Consolidated Standards of Reporting Trials (CONSORT) flowchart diagram. MRI: magnetic resonance imaging.

There were no participants withdrawn from the study for adverse reactions to the study product, loss of capacity, or non-compliance to taking the study powder. No serious adverse events were reported during the study. Compliance to taking the study product based on returned sachets was good, with an average of 6.9 (SD=7.92) sachets returned, or 96% compliance. The minimum number of sachets returned was 0 by 14 participants, and the maximum number was 38 sachets (indicating 77% compliance) returned by a participant in the placebo group. Plasma measurements of total polyphenol metabolites also indicated excellent adherence, with the concentration of polyphenol metabolites increasing by 1.82 ± 0.57 µM in the cranberry group.

There were no significant differences at baseline between groups for age, gender, years of education, or global cognitive performance on the ACE-III total score, *p*'s > .05 (see Table 3.1.). In total, 39 of the 60 participants entered into the study were retired, 2 indicated that they were not currently working but not retired, 13 participants were working part-time and 6 participants were working full time. There were no differences between cranberry and placebo groups for working status, $\chi 2(3)=3.89$. p=.274.

Table 3.1. Demographic characteristics of the subjects at randomisation into the intervention (n=60). Significance of group comparisons determined using Mann-Whitney U Independent Samples and Pearson Chi-Squared Tests.

Characteristics	**Cranberry**		**Placebo**		***p* value**
N	29		31		
Gender (Male/Female)	12/17		13/18		.965
	Mean	SD	Mean	SD	
Age (years)	65.86	5.51	65.32	4.91	.929
Education (years)	14.38	2.60	14.61	3.01	.610
ACE-III Total (/100)	96.58	2.39	96.10	2.80	.644

ACE-III: Addenbrooke's Cognitive Examination III.

PHYSICAL MEASURES AND BLOOD BIOCHEMISTRY

Table 3.2 includes group data for physical measurements, QRISK3 calculations and blood biochemistry results. The BMI of the total sample ranged from 18.11 to 38.78. Systolic blood pressure ranged between 104 mmHg and 173mmHg (M=133.37, SD=16.09), and diastolic blood pressure between 49 mmHg and 107mmHg (M=82.08, SD=10.67). Participants who were found to have significant hypertension and were not undergoing treatment for it were not entered into the study.

The screening haematology results for all participants who were entered into the study were within normal range. The screening biochemistry results for one participant had liver enzyme results above the normal range, which were forwarded to their GP. This participant was able to enter the study when they were re-screened with normal liver enzyme results.

Table 3.2. Physical measurements, QRISK3 10-year risk of cardiovascular disease and blood biochemistry results for participants in the cranberry and placebo groups at baseline. significance of group comparisons determined using Mann-Whitney U Independent Samples tests.

	Cranberry		**Placebo**		
	M	**SD**	**M**	**SD**	***p* value**
BMI	24.86	3.99	25.72	4.17	.348
Systolic Blood Pressure (mmHg)	137.03	15.91	135.21	14.24	.646
Diastolic Blood Pressure (mmHg)	79.38	8.69	81.77	10.63	.318
QRISK3 (%)	14.49	7.89	12.88	5.22	.594
Blood Biochemistry					
Fasted Glucose (mmol/L)	4.71	.45	4.80	.48	.660

Total Protein (g/L)	71.21	3.67	71.92	3.60	.349
Creatinine (umol/L)	72.64	12.52	73.00	14.72	.750
Total Bilirubin (umol/L)	11.00	4.04	14.58	5.03	.027
Albumin (g/L)	40.54	2.33	40.12	2.40	.516
Globulin (g/L)	30.68	3.61	31.81	3.21	.154
Urea (mmol/L)	5.09	1.04	4.98	1.00	.314
Calcium (mmol/L)	2.34	.10	2.38	.08	.419
Adjusted Calcium (mmol/L)	2.36	.09	2.40	.07	.239
Phosphate (mmol/L)	1.00	.67	1.02	.19	.594
Bicarbonate (mmol/L)	27.04	3.28	25.92	2.19	.260
Sodium (mmol/L)	139.50	2.33	133.54	24.49	.122
Potassium (mmol/L)	4.48	.32	4.50	.34	.551
ALP (U/L)	70.14	17.43	73.27	23.42	.544
AST (U/L)	22.57	4.59	22.00	3.68	.476
ALT (U/L)	17.82	5.34	18.04	8.81	.196
Lipids					
Total Cholesterol (mmol/L)	5.58	1.16	5.51	1.02	.728
LDL (mmol/L)	3.44	1.05	3.35	.93	.813
HDL (mmol/L)	1.67	.36	1.61	.411	.451
Total:HDL ratio	3.54	1.19	3.56	1.28	.801
Triglycerides (mmol/L)	1.027	.36	1.19	.64	.278

ALP: Alkaline phosphatase; ALT: Alanine transaminase; AST: Aspartate transaminase; BMI: body mass index; HDL: High-density lipoprotein; LDL: Low-density lipoprotein.

MEDICATIONS

No participants reported changes to prescribed medication between baseline and follow-up visits. Eleven participants (9 male, 2 female) were using blood pressure medication for at

least 3 months before entering the study (one on Perindopril and Atenolol, one on Lisinopril and Amlodipine, one on Verapamil and Ramipril, one on Candesartan and Tamulosin, one on Bendroflumethiazide, Felodopine and Lisinopril, one on Lisinopril only, one on Propranolol, one on Amlodipine and Ramipril, one on Lercanidipine and Ramipril, one on Perindopril, one on Bendroflumethiazide, Viazem and Peridopril). Additionally, 9 participants (not mutually exclusive with participants taking blood pressure medication) were using statin medication for at least 3 months before entering the study (three on Avorstatin, one on Pravastatin, four on Simvastatin, one not specified). One participant using medication for arrhythmia (Lignocaine).

Three participants were using hormone replacement therapy (one Kliofem, one Evorel Conti, one Premarin), and one participant was using a dihydrotestesterone inhibitor (Dutasteride). Four participants were on prostate medication (one on Finasteride, one Alfuzosin, two Tamsulosin). Four participants were using thyroid medication (three using Levothyroxine, one Thyroxin). One participant (female) was taking anti-epileptic medication (Gabapentin). One participant (female) on medication to assist with urinary incontinence (Neditol). Three participants were using asthma medication (one Beclamethadone, one Seretide, one Beclomethasone and Salbutamol).

Five participants were regularly taking non-essential vitamin supplements that could impact the outcome measures and were asked to refrain from taking these during the intervention. Three participants were regularly taking non-prescribed supplements containing omega-3, one participant was taking a daily vitamin C supplement and one participant was taking a multivitamin daily.

BACKGROUND DIET

There was no difference between the placebo and cranberry groups on the macronutrient content of their background diets, *p*'s > .05 (Table 3.3). The mean background total flavonoid intake at baseline was 222.69mg/day (SD=142.92).

Table 3.3. Total energy and nutrient intake as calculated from the SCG-FFQ for participants in the cranberry and placebo groups at baseline. Significance of group comparisons determined using Mann-Whitney U Independent Samples tests.

	Cranberry		**Placebo**		
	M	**SD**	**M**	**SD**	***p* value**
Energy					
kCal/d	2126.44	758.55	2006.68	645.27	.629
kJ/d	8963.25	3184.40	8466.62	2710.37	.646
Fat (g/d)	85.87	33.48	78.60	28.81	.385
Saturated (g/d)	32.11	13.44	29.26	11.64	.403
Monounsaturated (g/d)	27.68	9.30	25.66	9.34	.658
Polyunsaturated (g/d)	13.53	4.58	13.44	4.90	.844
Trans (g/d)	2.25	1.16	2.08	.88	.974
Cholesterol (mg/d)	255.84	103.68	256.99	100.87	1.000
Proteins (g/d)	84.66	30.05	78.08	25.39	.512
Carbohydrates (g/d)	256.50	107.92	244.59	87.82	.987
Total sugars (g/d)	125.93	57.01	126.69	60.10	.781
Glucose (g/d)	22.20	9.15	25.64	20.01	.896
Fructose (g/d)	24.58	9.84	28.41	21.07	.909
Sucrose (g/d)	46.91	21.95	45.48	17.37	.883

Fibres (g/d)	21.38	9.12	21.93	11.19	.935
AOAC Fibres (g/d)	5.65	3.64	6.86	4.43	.471
Starch (g/d)	125.11	60.96	114.34	39.84	.806
Vitamins					
Vitamin C (mg/d)	116.37	50.21	131.73	66.94	.441
Vitamin D (ug/d)	5.01	2.97	3.49	1.75	.039
Vitamin E (mg/d)	12.38	5.87	10.97	4.24	.461
Thiamin (mg/d)	1.80	.64	1.71	.46	.974
Riboflavin (mg/d)	2.53	1.13	2.24	.72	.376
Vitamin B6 (mg/d)	2.35	.89	2.14	.64	.670
Niacin (mg/d)	19.93	7.26	18.91	5.74	.793
Folic acid (ug/d)	352.97	153.23	359.84	127.16	.600
Vitamin B12 (ug/d)	7.40	3.49	5.84	2.55	.105
Vitamin K (mg/d)	56.62	29.06	116.49	122.40	.085
Minerals					
Sodium (mg/d)	2525.99	894.55	2777.36	1039.89	.279
Potassium (mg/d)	4130.41	1457.59	3855.29	1248.62	.555
Calcium (mg/d)	1296.32	637.48	1167.11	411.59	.623
Magnesium (mg/d)	396.44	134.81	359.61	120.54	.310
Phosphorus (mg/d)	1732.47	696.73	1556.14	453.89	.544
Iron (mg/d)	13.40	4.84	13.16	4.91	.883
Copper (mg/d)	1.71	.89	1.49	.77	.359
Zinc (mg/d)	11.07	4.06	9.79	2.82	.350
Chloride (mg/d)	3964.27	1413.41	4301.59	1593.84	.294
Manganese (mg/d)	4.98	1.84	5.05	1.89	.915
Selenium (ug/d)	60.26	27.38	49.85	17.79	.105
Iodine (ug/d)	270.07	157.55	232.73	88.35	.857
Flavonoids (mg/d)	216.81	134.97	228.38	152.30	.952
Flavonols (mg/d)	35.70	18.84	42.14	35.23	.891
Flavan-3-ols (mg/d)	121.27	89.98	123.90	84.36	.903

Flavones (mg/d)	2.24	3.00	4.20	13.10	.544
Proanthocyanidins (mg/d)	35.19	24.67	39.35	22.90	.705
Flavanones (mg/d)	27.29	20.52	33.39	30.10	.677
Other					
Caffeine (mg/d)	240.96	130.01	189.68	91.14	.164
Alcohol (g/d)	7.86	8.64	9.94	9.41	.279
Water (mL/d)	2454.49	907.76	2328.17	771.85	.566
Diet Quality Index (%)	66.60	16.33	68.32	18.74	.688

AOAC=Association of Analytical Chemists.

LIFESTYLE QUESTIONNAIRES

Details of sleep as measured by the PSQI and physical activity as measured by the IPAQ are detailed in Table 3.4.

Regarding subjective sleep quality, in the cranberry group 22 participants indicated that their sleep quality was ‘Very Good’, 5 indicated ‘Fairly Good’, 2 ‘Fairly Bad’, and no participants indicated their sleep quality was ‘Very Bad’. In the placebo group, 24 participants indicated their sleep was ‘Very Good’, 5 indicated it was ‘Fairly Good’, 1 indicated it was ‘Fairly Bad’ and 1 indicated it was ‘Very Bad’. There was no difference between groups in proportion of participants indicating their sleep quality according to these ratings, $\chi 2(3) = 1.355$, p=.716. For use of sleep medication, in the cranberry group 25 participants indicated they had not taken any sleep medication during the last month, 1 had indicated that they took these less than once a month, 2 indicated that they took these once or twice a week, and 1 indicated that they took these three or more times a week. In the placebo group, 30 indicated that they had not taken these medications during the past month, and 1 indicated that they took these 3 or more times a week. There were no differences in proportions of participants in their frequency of sleep medication intakes, $\chi 2(3)=3.392$, p=.335.

When categorising participants into activity levels (inactive, minimally active, and HEPA active), in the cranberry group 17 participants were HEPA active and 12 were minimally active, whereas in the placebo group 21 participants were HEPA active and 10 were minimally active. No participants in either group were classified as inactive. The proportions of participants belonging to each of activity levels did not differ between the two treatment groups, $\chi 2(1)=.537$, $p=.464$.

Table 3.4. Summary of questionnaire assessing sleep and physical activity for participants in the cranberry and placebo groups. Significance of group comparisons determined using Mann-Whitney U Independent Samples tests.

	Cranberry		**Placebo**		
	M	**SD**	**M**	**SD**	***p* value**
PSQI					
Subjective Sleep Quality (/3)	.86	.74	.84	.454	.831
Sleep Latency (minutes)	19.03	22.03	19.42	15.00	.770
Sleep Duration (hours)	7.103	1.160	7.00	.796	.836
Habitual Sleep Efficiency (%)	83.11	14.86	82.17	13.04	.950
Sleep Disturbances (/3)	1.34	.48	1.35	.49	.936
Daytime Dysfunction (/3)	.55	.63	.55	.51	.834
Global PSQI Score (/21)	5.41	3.28	5.29	7.75	.964
IPAQ					

Total overall MET-minutes/week	4812.86	3008.68	5305.68	3449.98	.739

PSQI=Pittsburgh Sleep Quality Index; IPAQ=International Physical Activity Questionnaire; MET=multiples of resting metabolic rate.

MOOD AND BEHAVIOUR

For the PHQ-9, 53 participants had scores that indicated no symptoms of depression (0-4), 5 participants had scores indicating mild depression symptoms (5-9), and 2 participants had scores indicating moderate depression symptoms (10-14). The GAD-7 scores showed similar proportions of symptom severity, with 51 participants indicating no symptoms of generalized anxiety (0-4), 6 participants indicating mild symptoms (5-9), and 3 participants had scores indicating moderate symptoms (10-14).

Table 3.5. Summary of totals and subtotals from self-reported measures of subjective cognitive decline, behaviour, and mood for participants in cranberry and placebo groups at baseline, and comparisons of group differences. Significant of group differences determined using Mann-Whitney U Independent Samples tests.

	Cranberry		**Placebo**		
	M	**SD**	**M**	**SD**	***p* value**
CCI (/100)	27.38	7.61	27.00	8.80	.732
Episodic Memory Subscore (/60)	17.99	5.32	17.41	6.16	.577
Executive Subscore (/25)	5.83	1.67	6.06	2.07	.553
Language Subscore (/15)	3.62	1.24	3.52	1.09	.550

CBI-R					
Memory and Orientation (%)	10.69	14.06	7.74	8.18	.757
Everyday Skills (%)	.34	1.29	.32	1.80	.544
Self-Care (%)	0.00	0.00	0.00	0.00	1.0
Abnormal Behaviour (%)	2.59	5.53	4.00	7.58	.713
Mood (%)	8.52	12.15	7.29	9.59	.800
Beliefs (%)	0.00	0.00	.26	1.43	.333
Eating (%)	4.97	10.60	2.81	8.25	.417
Sleep (%)	17.41	18.73	17.58	16.38	.818
Stereotypic and Motor Behaviours (%)	4.97	10.27	6.03	12.52	.993
Motivation (%)	3.10	7.12	4.03	9.70	.696
PHQ-9 (/27)	1.78	3.13	1.97	2.39	.443
GAD-7 (/21)	1.70	2.91	1.90	2.66	.291

CCI=Cognitive Change Index; CBI-R=Cambridge Behavioural Inventory Revise; PHQ-9=Patient Health Questionnaire 9; GAD-7=Generalized Anxiety Disorder 7.

APOE GENETIC TESTING

Buffy coat samples were available for all 60 participants who entered the intervention. No participants were found to carry 2 copies of the *APOE*-4 mutation. Furthermore, there were no differences between the cranberry and placebo groups for distribution of *APOE* genetic types, $\chi 2$ (3) = 2.60, p=.457 (see Table 3.6).

Table 3.6. *APOE* Genetic status of participants in the cranberry and placebo groups, and overall totals.

APOE Genotype	Cranberry	Placebo	Total
E2/E3	3	4	7
E2/E4	0	2	2
E3/E3	21	22	43
E3/E4	5	3	8

APOE= Apolipoprotein E.

DISCUSSION

The cranberry and placebo groups were well-matched for the main demographics of age, gender, and education. The cranberry and placebo groups were also well-matched for almost all baseline characteristics and measures, with very minor differences detected for estimated daily vitamin D intake and liver enzymes; however the results for these were not generally above the normal ranges for either group. How the baseline characteristics of this sample relates to other similar trials will be discussed further here.

The mean age of this sample (65.68 ± 5.17) was younger than the similar 12-week study involving blueberry juice in older adults with early memory changes by Krikorian, Shidler, et al. (2010), that found positive results regarding episodic memory performance. The current sample had very similar demographic characteristics to the 12-week blueberry studies by Bowtell et al. (2017) and Miller et al. (2018) which found limited results for improvements in cognition, however the current study involved a sample twice the size. The sample in the current thesis was also of a very similar size and age as participants in the study by Crews and colleagues (Crews et al., 2005), which did not find positive results regarding the impact

of 6-week cranberry intake on cognition, however this disparity in results could be due to the shorter length of the trial in the previous study. The proportions of male and female subjects also mirror comparable berry trials, as does the education level of the total sample, which is particularly critical for cognitive outcomes which have been suggested to be possibly influenced by these factors (Herlitz & Rehnman, 2008; Le Carret, Lafont, Mayo, & Fabrigoule, 2003) with years of education even suggested to have a protective role against age-related cognitive decline (Colsher & Wallace, 1991; Evans et al., 1993).

There were no differences in blood biochemistry between cranberry and placebo groups except for a significant baseline difference in total bilirubin, with the placebo group showing slightly higher mean readings compared to the cranberry group. However, the normal range for total bilirubin is <20, which the results of the majority of participants in both groups fell within. Four participants had results just above this normal range, which were reviewed by the study clinician and flagged to their GP's but was determined not to indicate that they could not continue with the study when taken into account with their other results.

The two groups were generally well-matched for background diet, including for the flavonoid intakes measured, with the main exception being a significant difference between groups in baseline vitamin D intake favouring the cranberry group. Although the dietary intake of vitamin D significantly differs between groups in this sample, both groups are on average (3.49-5.01µg/day) intaking less than the UK's Reference Nutrient Intake (RNI) of daily recommended vitamin D of 10 µg per day for older adults or the Institute of Medicine's 2011 proposed RNI of 15 µg per day for individuals aged up to 70 years, and 20 µg per day for individuals older based on conditions of minimal sun exposure (Ross et al., 2011).

The estimated average total daily intake at baseline of total flavonoids, flavonols, flavan-3-ols were significantly lower than the average levels detected in a similar age group in the UK

NDNS, although the average intake of flavanones was comparable (Ziauddeen et al., 2019). However, the estimated intake reported here was more in line with UK estimates based on international Food Balance Sheets of 182mg/day per capita (Beking & Vieira, 2011). Flavan-3-ols also contributed the most to this total estimated flavonoid intake, which is in line with findings from population surveys in the UK (Ziauddeen et al., 2019), with these flavonoids predicted to derive predominantly from tea, chocolate, wine, fruit, and vegetables in adults (Ziauddeen et al., 2019). The daily total flavonoid intakes in this sample were also significantly lower than the UK-EPIC general population estimates of 1020mg/day (Zamora-Ros et al., 2016). Although the predicted intakes of the polyphenols measures in this sample are lower than what would be expected, it could also be taken to indicate that participants entered into the study were successfully screened for high baseline intake of high-polyphenol concentration foods and therefore would potentially be more likely to benefit from an increased intake of polyphenols as a result of the intervention, especially since participants were instructed to maintain their normal diets throughout the duration of the intervention.

There were no differences between groups at baseline for estimates of physical activity. There is growing understanding of a complex interaction of lifestyle factors which includes both incidental and structured physical activity and its impact on ageing well, particularly when it comes to cognitive ageing (Blondell, Hammersley-Mather, & Veerman, 2014; Laurin, Verreault, Lindsay, MacPherson, & Rockwood, 2001; Raffin et al., 2021). Indeed, several large-scale intervention trials aimed at preventing age-related cognitive decline have emerged that incorporate physical activity programs alongside changes to dietary patterns (Ngandu et al., 2015; Shannon et al., 2021). Furthermore, our results indicate that there were no differences between groups for self-reported sleep quality, and the subjective ratings given by participants indicated that there were very few participants who rated their own sleep quality as poor. Emerging evidence suggests that disturbed sleep poses a potentially increased

risk for developing dementia (Shi et al., 2018), and older adults have been found to have more disturbed sleep compared to younger adults (Ohayon, Carskadon, Guilleminault, & Vitiello, 2004). The results of the mood questionnaires also indicated that there were no differences between groups for anxiety or depression, and the scores for these tests indicate that the vast majority of participants reported mild or no symptoms of anxiety or depression. Although it is unclear whether significant depression is a risk factor for age-related cognitive decline, or if rather it coincides with dementia rather than precedes it (Richard et al., 2013), there is evidence suggesting that raised levels of depression and anxiety can impact on cognitive performance (Maloney, Sattizahn, & Beilock, 2014; McDermott & Ebmeier, 2009).

None of the participants enrolled into the intervention were carriers of two copies of the *APOE*-4 genetic mutation, which is the allele of the three available mutations (*APOE*-2, *APOE*-3, and *APOE*-4) that has been found to confer the highest risk of developing AD, particularly at an earlier onset (Corder et al., 1993), with this risk decreasing with a decreasing number of *APOE*-4 alleles. Approximately 13% of participants in the current sample carried one copy of the *APOE*-4 gene, with the majority of participants carrying two copies of the lower-risk *APOE*-3 mutation, and approximately 97% of the sample carrying at least one allele. The proportions of the frequencies of these alleles are in line with UK prevalence estimates previously reported (P. P. Singh, Singh, & Mastana, 2006), although the prevalence of the *APOE*-3 allele in this sample is slightly higher than most of the UK estimates listed in the previous report. Therefore, the genetic risk of AD or pre-clinical cognitive decline is not higher in the current sample than would be expected in the general UK population.

In conclusion, the cranberry and placebo groups were well-matched for almost all baseline characteristics and measures, with very minor differences detected for estimated daily vitamin D intake and liver enzymes, however for these results were not generally above the

normal ranges for either group. Therefore, there are very few characteristics at baseline that would be predicted to influence differences detected between groups as a result of the intervention or confound any of the primary outcomes of interest in this study.

CHAPTER 4: COGNITION AND NEURAL FUNCTION

INTRODUCTION

Epidemiological studies have reported that higher dietary intake of flavonoids is associated with slower rates of cognitive decline (Devore et al., 2012; Letenneur et al., 2007; Shishtar et al., 2020) and dementia (Lefèvre-Arbogast et al., 2018), and foods rich in anthocyanins and proanthocyanidins such as berries have also been shown to improve cognition , supported by a growing body of preclinical data , as well as emerging clinical evidence (Boespflug et al., 2018; Krikorian, Nash, et al., 2010; Krikorian, Shidler, et al., 2010; Lamport, Lawton, et al., 2016; Lee et al., 2017; Travica et al., 2020; Whyte et al., 2021).

When it comes to clinical trials involving humans, consumption of blueberry has produced short-term (2-6 hours) improvement in executive function and memory in school-aged children (Whyte et al., 2017) and similarly using mixed berries for young healthy adults (Whyte et al., 2019). In older age-groups, a single dose of wild blueberry produced improvements in executive function on the Go/No-Go task compared to placebo in middle-aged adults aged 40-65 years (Whyte et al., 2021). Over the longer term, in older adults with early memory changes wild blueberry juice supplementation for 12 weeks improved episodic memory performance (Krikorian, Shidler, et al., 2010), and a similar 12-week randomised control trial involving Concord grape juice resulted in a significantly better cognitive performance compared to placebo in elderly adults with memory changes (Krikorian, Nash, et al., 2010). A 6-month trial found improvements in cognition and brain metabolism in older adults with mild cognitive impairment (McNamara et al., 2018), in line with other findings from similar trials involving grape juice supplementation improving cognition across the lifespan in younger and spatial memory in middle-aged adults (Lamport, Lawton, et al., 2016). In the latter study by Lamport, Lawton, et al. (2016), effects of the Concord grape juice on verbal memory and executive function appeared to endure for some time after consumption, replicating similar findings of

an enduring effect of an 8-week citrus juice consumption trial on memory and executive function .

In addition to improvements in cognitive function, the intake of a high-flavonoid citrus drink produced increases in blood perfusion in inferior and medial frontal brain regions (Lamport, Pal, et al., 2016). Similarly, another study by the same authors detected regional increases in brain blood perfusion in the anterior cingulate cortex and left parietal regions in a sample of older adults (50-65 years) following the consumption of a cocoa study drink high in flavonoids compared with a matched drink with lower concentration of flavonoids (Lamport et al., 2015). However, these results come from studies involving doses of high concentration flavonoid intake over a short period of time. A longer trial of cocoa consumption for 3 months in middle-aged to older adults (ages 50-69 years) found changes in performance on a test of object recognition along with changes in the dentate gyrus as measured by fMRI using a region-of-interest approach focusing on the hippocampal circuit (Brickman et al., 2014), although these findings were not replicated in a more recent 12-week trial of cocoa by the same group (Sloan et al., 2021). Regarding berries, a 12-week blueberry supplementation in healthy older adults (mean age 67.5) was related with increases in task-related (Stroop test) regional perfusion in parietal and occipital regions in addition to an improvement in working memory performance between pre- and post-supplementation compared to placebo (Bowtell et al., 2017).

Increased levels of BDNF are considered to support learning and memory function via its role in increasing neural survival and synaptic growth, with the hippocampus being a major site for its expression (Neeper, Gomez-Pinilla, Choi, & Cotman, 1996), based largely on evidence from animal studies (Cirulli, Berry, Chiarotti, & Alleva, 2004). Indeed, findings from animal models have suggested a beneficial impact of berry intake on BDNF expression in the hippocampus in conjunction with improved spatial memory performance (Rendeiro et al., 2012; C. M. Williams et al., 2008). Findings from clinical trials in humans using peripherally measured levels of

BDNF, for example from circulating blood samples, are less clear however. Although high-flavonoid intake from fruit and vegetables has previously been found to increase serum BDNF levels (Neshatdoust et al., 2016), a study by Ahles et al. (2020) with a large sample size of 101 middle-aged adults found no impact of 12-week supplementation of chokeberry on serum BDNF. A recently published study involving 60 older adults aged 50-75 found no impact of a 12-week supplementation with red berry mixture, cocoa or a combination of both on BDNF (García-Cordero et al., 2022), however there was a significant association found between BDNF levels and a test of executive functioning.

Here is described the impact of the 12-week cranberry intervention on cognitive performance in addition to neural function detected for regional CBF as measured by ASL, and plasma BDNF, in this sample of cognitive healthy older adults.

METHODS

COGNITIVE ASSESSMENT

Further details of cognitive tests are available in Chapter 2. Global cognition was assessed using the ACE-III (Hsieh et al., 2013). Executive function and working memory were measured by using the TMT (Reitan, 1992) and the Digit Span (DS) test. Visual episodic memory was evaluated using the RCF test (Meyers & Meyers, 1995). The Supermarket Test was used as an assessment of spatial orientation.

A composite executive function score was also calculated out of the ACE-III Category Fluency score (/7), the Digit Span Backwards Raw Score (/14), and the Scaled Trails B from the Trail Making Test based on previously published normative data (Tombaugh, 2004).

DATA ACQUISITION

MRI scans were conducted in all eligible and willing participants at baseline and end of intervention and took approximately 30 minutes. In order to monitor structural brain information across the study, a T_1-weighted 3D gradient-echo MR sequence was conducted at each testing visit. A T_2-weighted fluid attenuated inversion recovery (FLAIR) scan was also conducted during the study visits. Arterial spin labelling (ASL) was used to detect changes in cerebral blood flow (CBF) based on regional blood perfusion. Further details of imaging data acquisition and the sequences can be found in Chapter 2.

IMAGING ANALYSIS

Voxel-Based Morphometry (VBM) was used on whole-brain T_1-weighted scans using the VBM package in FSL (FMRIB Software Library, Oxford, UK) to confirm that there were no grey matter structural differences between the cranberry and placebo groups at baseline, or whether there were any changes over the course of the intervention (Good et al., 2001).

White matter hyperintensities (WMH) were rated using Multi-image Analysis GUI (Mango version 4.1, Research Imaging Institute, UTHSCSA, San Antonio, TX, USA) by one rater (EF). A well-established rating scale developed by Fazekas et al. (Fazekas, Chawluk, Alavi, Hurtig, & Zimmerman, 1987) was used to qualitatively rate WMH in periventricular (PWMH) and deep (DWMH) regions using FLAIR images. WMH in the periventricular areas was rated as 0 = absent, 1 = "caps" or pencil-thin lining, 2 = smooth "halo", or 3 = irregular, whereas DWMH were rated as 0 = absent, 1 = punctate foci, 2 = beginning confluence of foci, or 3 = large confluent areas.

For regional perfusion (ASL), equilibrium magnetisation (M_0) and perfusion-weighted images were calculated in-line on the scanner workstation. All further analyses were performed using

a processing pipeline written in bash and Python (v3.6, Python Software Foundation, www.python.org), which was run on the ADA high-performance computing cluster at the University of East Anglia. The pipeline closely resembled that used for the Alzheimer's Disease Neuroimaging Initiative (ADNI, adni.loni.usc.edu) ASL sub-study, substituting FastSurfer for brain segmentation instead of FreeSurfer (Henschel et al., 2020). In brief, M_0 and perfusion-weighted images were scaled and used to calculate CBF maps in physical units of arterial water density (mL/min/100g). T_1-weighted data were then segmented using FSL's FAST algorithm and the derived grey matter probability maps were used to register the ASL perfusion-weighted images to T_1 space—via FSL's FLIRT algorithm. ROIs from the FastSurfer segmentation were then used to determine ROI-wise CBF statistics: minimum maximum, mean, median, and standard deviation.

Statistical analyses

One-way ANCOVA's were used to detect baseline differences in cognition between groups controlling for age, education and gender. The impact of treatment on the cognitive outcomes of interest was established using mixed linear model with time and treatment as independent variables and with age, education and gender entered as covariates. Whole-brain differences in grey matter intensities were analysed between cranberry and placebo groups using VBM at baseline and follow-up with age added as a covariate. Periventricular and deep white matter hyperintensisties were compared between cranberry and placebo groups at baseline and follow-up using ANCOVAs with age added as a covariate. Mean regional perfusion derived from ASL scans were analysed using mixed linear modelling with age entered as a covariate to determine and group x time interactions. Spearman correlations between significant cognition and regional perfusion at follow-up were also conducted. To further analyse the number of responders vs. non-responders between cranberry and placebo groups, participants were grouped according to whether their significant cognitive performance or regional

perfusion increased between baseline and follow-up (responders) or if their performance or regional perfusion remained the same or decreased (non-responders). Where there were more than one test or neural region that was shown to have significant group x time interaction effects from the linear mixed modelling, participants were further grouped based on how many tests or regions they were classed as responders for to detect whether there were patterns of participants consistently responding across all significant outcomes. A chi-squared test was then performed to determine whether groups significantly differed in their proportions of responders. Finally, differences at baseline between groups for plasma concentrations of BDNF was measured using non-parametric Mann-Whitney U tests, and linear mixed modelling used to determine group x time interactions as a result of the intervention. BDNF was then related with performance on cognitive tests and regions of CBF which showed significant changes as a result of the intervention using Spearman correlations.

RESULTS

COGNITIVE PERFORMANCE

There were no differences between cranberry and placebo groups at baseline on the ACE-III total score or on the sub-scores (Attention and Orientation, Memory, Fluency, Language, Address Delayed Recall and Category Fluency) (Table 4.1). No difference at baseline was also observed for any RCF scores, DS backwards, TMT A-B Scaled Score, or for the composite executive function score but there was a significant difference at baseline on overall visuospatial performance in the ACE-III ($p = 0.008$).

Table 4.1. Cognitive performance at baseline and follow-up. Baseline differences determined using Mann-Whitney U Independent-Samples Test, and group x time interactions on linear mixed modelling.

	Baseline	**Follow-up**	**Group x Time**

Measures		**Treatment**	**M**	**SD**	***p***	**M**	**SD**	***p***
ACE-III	Attention	Cranberry	17.65	.72	.219	17.66	.55	.467
		Placebo	17.35	1.11		17.60	.62	
	Memory	Cranberry	24.97	1.18	.726	25.10	1.05	.498
		Placebo	24.84	1.70		25.67	1.95	
	Fluency	Cranberry	12.45	1.53	.512	13.24	.91	.164
		Placebo	12.26	1.44		12.43	1.48	
	Language	Cranberry	25.79	.49	.305	25.76	.51	.401
		Placebo	25.58	.54		25.83	.46	
	Visuospatial	Cranberry	15.62	.73	**.008**	15.76	.51	.120
		Placebo	15.97	.18		15.83	.38	
	Address	Cranberry	6.10	1.05	.466	6.34	.81	.332
	Delayed	Placebo	6.19	1.28		6.07	1.46	
	Recall							
	Category	Cranberry	6.34	1.17	.759	6.66	.61	.431
	Fluency	Placebo	6.32	.91		6.43	.68	
RCF	Copy Score	Cranberry	34.52	2.61	.994	35.34	1.05	.092
		Placebo	35.00	1.29		34.97	1.28	
	Delayed	Cranberry	18.59	7.67	.416	23.41	5.96	**.028**
	Recall Score	Placebo	20.53	5.92		22.25	6.06	
DS	Forwards	Cranberry	11.28	2.28	.437	11.41	2.01	.309
	Raw Score	Placebo	10.84	2.28		11.39	2.50	
	Backwards	Cranberry	7.76	2.18	.781	7.86	2.5	.165
	Raw Score	Placebo	7.58	2.03		8.30	2.55	
TMT	A-B	Cranberry	37.41	21.84	.416	35.93	16.22	.639
		Placebo	33.29	16.89		34.14	13.73	
	B Scaled	Cranberry	14.83	1.79	.756	15.03	1.66	.127
		Placebo	15.13	1.73		15.37	1.97	
Executive Composite		Cranberry	28.93	3.50	.964	29.34	3.73	.430
Score		Placebo	29.03	3.41		29.93	3.45	

Supermarket	Egocentric	Cranberry	3.32	1.84	.383	3.76	1.96	.906
Test	Score 1	Placebo	3.67	1.76		3.92	1.91	
	Egocentric	Cranberry	5.28	1.95	.917	5.68	1.65	.936
	Score 2	Placebo	5.04	2.01		5.58	1.79	
	Egocentric	Cranberry	8.60	3.29	.656	9.44	3.29	.885
	Total	Placebo	8.71	3.36		9.50	3.27	
	Allocentric	Cranberry	11.72	4.35	.324	12.54	8.31	.267
	Error 1	Placebo	13.00	4.94		11.96	5.81	
	Allocentric	Cranberry	14.28	3.00	.164	15.47	10.54	.481
	Error 2	Placebo	14.11	4.55		13.27	4.76	
	Allocentric	Cranberry	13.00	3.14	.964	13.98	8.88	.325
	Error Total	Placebo	13.55	4.47		12.70	4.70	
	Allocentric	Cranberry	5.64	1.50	.562	5.92	1.80	.417
	Heading 1	Placebo	5.33	1.66		6.00	1.25	
	Allocentric	Cranberry	5.60	1.50	.489	5.84	1.34	.697
	Heading 2	Placebo	5.38	1.41		5.79	1.29	
	Allocentric	Cranberry	11.24	2.67	.386	11.76	8.44	.413
	Heading	Placebo	10.71	2.46		11.79	2.23	
	Total							

ACE III: Addenbrooke's cognitive examination III; RCF: Rey complex figure test; TMT: Trail making test.

At follow-up, a significant group × time interaction ($F(1, 55) = 5.060$; $p = 0.028$) was observed in performance of the RCF test delayed recall such that the cranberry group showed a significant improvement in performance between baseline and follow-up compared to the placebo group (Figure 4.1). Post-hoc analysis revealed a significant effect of time in the cranberry group, $F(1,28)=30.44$, $p<.001$, but not the placebo group, $F(1, 29.64)=2.893$, $p=.099$. Linear mixed modelling to detect group × time interactions between the groups between baseline and follow-up did not reveal any differential impact of the intervention on groups pre- to post-treatment for the copy score or on ACE-III, TMT or DS scores ($p > 0.05$) (Table 4.1.).

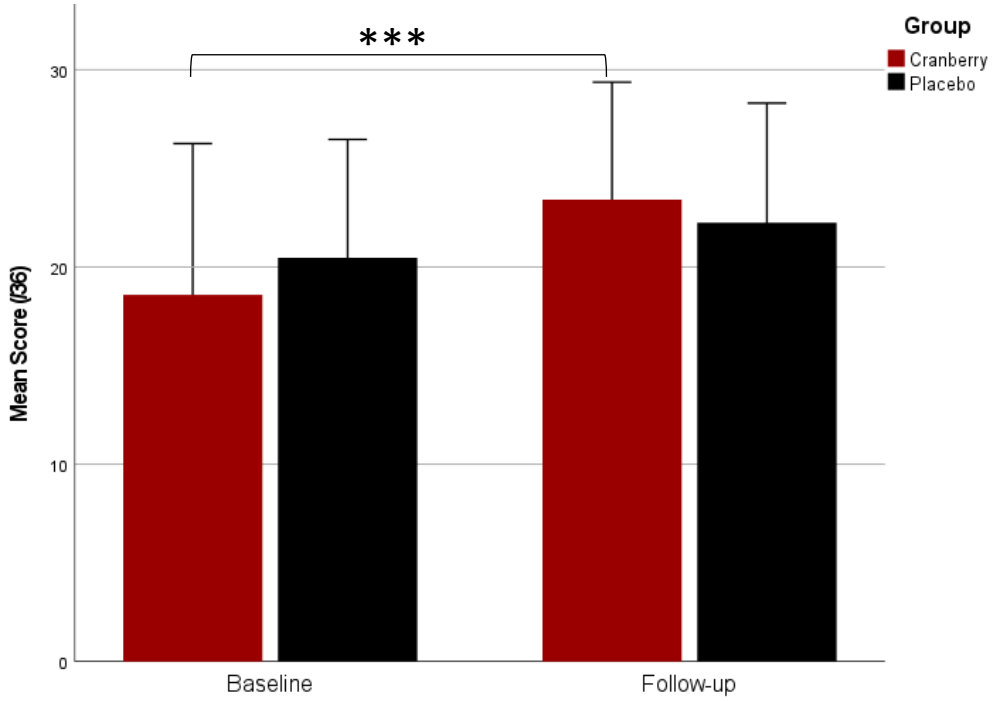


Figure 4.1. RCF Delay performance between groups baseline to follow-up. Error bars represent 1 SD. *** p<.001. RCF = Rey Complex Figure.

No significant differences were detected between groups on the egocentric, allocentric error or allocentric heading subtotals and totals of the Supermarket Test (Table 3). When the linear mixed modelling was run on these subtotals and totals, no significant group × time interactions were found ($p > 0.05$ in all cases).

Magnetic Resonance Imaging

Among our study population, 47 participants were eligible and underwent the neuroimaging component of the study (26 in the cranberry group and 21 in the placebo group). Due to COVID-19 restrictions and reduced capacity of hospital facilities, 10 follow-up scans could not be conducted. An additional 5 follow-up scans could not be scheduled due to participants (n = 1) or scanning facilities (n = 4) being unavailable during the critical follow-up time window. Additionally, ASL data for 2 baseline scans and 1 follow-up scan were not able to be

included in the analysis due to significant motion artefacts in the images. As such, 17 cranberry and 11 placebo participants had complete baseline and follow-up scans.

Structural brain changes

There were no differences in whole brain grey matter intensity between cranberry and placebo groups found at either baseline or follow-up scans. There similarly weren't any statistically significant differences observed between groups in periventricular white matter hyperintensities between the cranberry and placebo groups at baseline (p=.688) or follow-up (p=.833), or for deep white matter hyperintensities at baseline (p=.693) or follow-up (p=.723) using ANCOVA's at each time point with age added as a covariate. Two participants were rated as '3' for periventricular white matter hyperintensities (i.e. 'irregular'), with the remaining participants being rated between 0-2.

Blood perfusion brain changes

Supplementary Table 3 details mean regional blood perfusion from arterial spin labelling for the cranberry and placebo groups at baseline and follow-up. No significant differences in regional brain blood perfusion between cranberry and placebo groups were detected at baseline (p's > 0.05). Mixed linear modelling controlling for age and education detected significant group × time interactions for the right caudate ($F(1, 29.275) = 4.207$, $p = 0.049$), right nucleus accumbens ($F(1, 31.744) = 4.916$, $p = 0.034$), and right entorhinal cortex ($F(1, 30.558) = 5.202$, $p = 0.030$) (Figure 4.2). All models showed an increase in brain blood perfusion between baseline and follow-up in the cranberry group compared to a relative decrease in brain blood perfusion over time in the placebo group.

As a significant group × time interaction was found for the delayed recall of the RCF, a correlation analysis was performed between the follow-up RCF delay scores and follow-up regional perfusion data, however no significant correlation was found for either group (p's > 0.05).

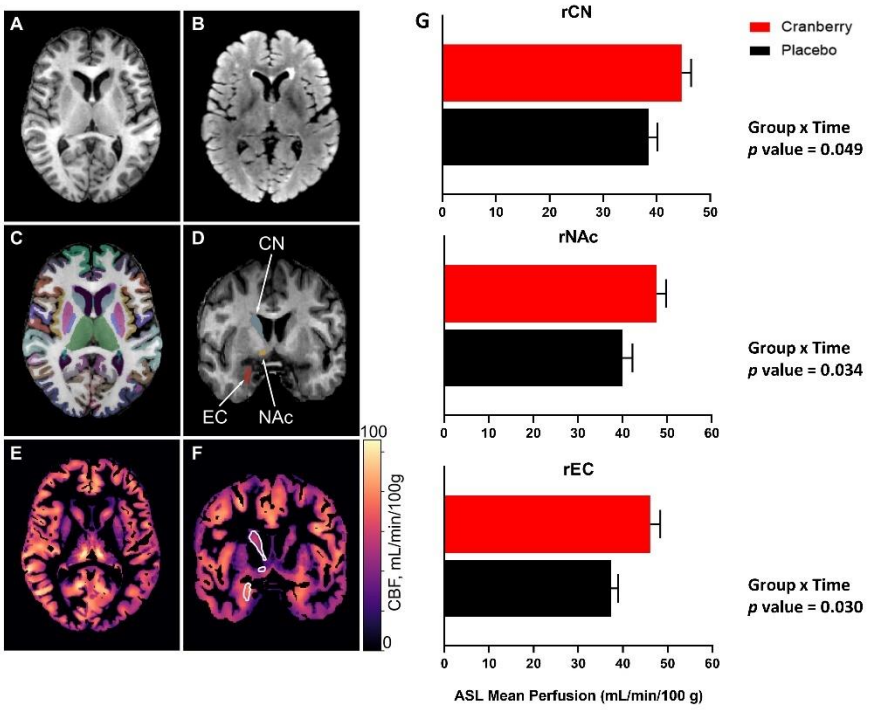


Figure 4.2. Representative magnetic resonance imaging data from this study, obtained in a 61-year-old male/female subject (COMBAT_041 baseline). A) axial T1-weighted inversion recovery fast spoiled gradient echo (IR-FSPGR) image; B) axial T2-weighted fluid attenuated inversion recovery (FLAIR) image; C) an axial view of FastSurfer cortical and subcortical segmentations superimposed on a T1-weighted image; D) a coronal view of the T1-weighted image indicating regions that showed significantly increased perfusion after 12 weeks consumption of cranberry extract—namely, from superior to inferior, the right caudate nucleus, accumbens area, and entorhinal cortex; E) an axial cerebral blood flow (CBF) map, in the T1 space, derived from arterial spin labeling data; F) a coronal CBF map highlighting the regions indicated in D, and G) differences in mean blood perfusion for the right caudate nucleus (rCN), right nucleus accumbens (rNAc) and right entorhinal cortex (rEC), with p values represented

for group x time interaction effects between cranberry and placebo groups from baseline to follow-up. MRI images generated by Dr Donnie Cameron.

RESPONDERS AND NON-RESPONDERS

Regarding responders vs. non-responders, 24/29 (82.8%) participants in the cranberry group showed improved RCF performance at follow-up compared to baseline, whereas 16/30 (53.3%) of participants in the placebo group showed improved performance, and these proportions of responders vs. non-responders differed significantly between the groups ($\chi 2(1)=5.85$, $p=.016$). For individual slopes of RCF performance between baseline and follow-up, see Figure 4.3.

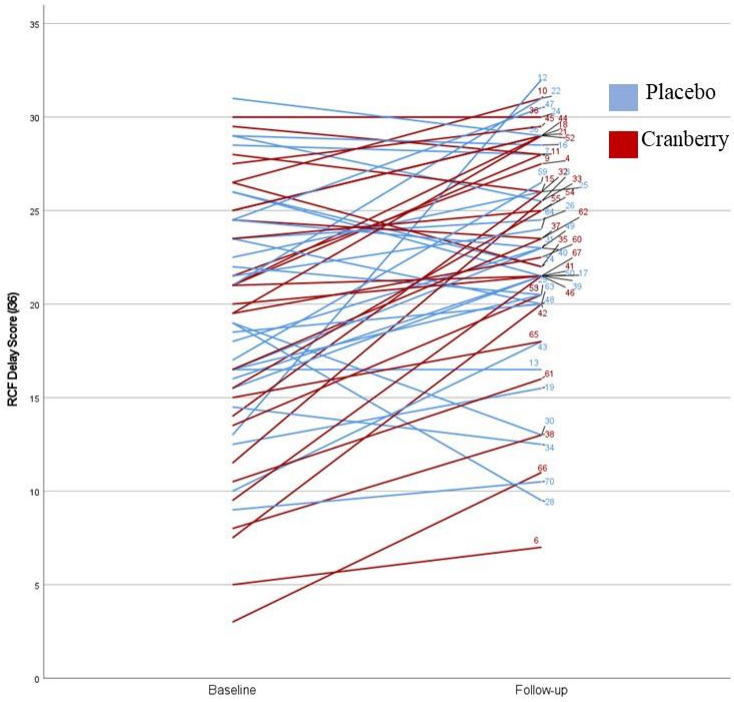


Figure 4.3. Individual participant slopes for Rey Complex Figure (RCF) performance at baseline and follow-up. Labels represent participants' study identification numbers.

Regarding the regions which found significant group x time interactions for mean blood perfusion (see Figure 4.4 for individual slopes), the number of responders in the cranberry group for the right nucleus accumbens were 10/17 (58.8%), compared to 5/11 in the placebo

group (45.5%), however this difference in proportions between groups was not significant ($\chi 2(1)=.480$, $p=.488$). For the right caudate nucleus, 10/17 (58.8%) cranberry participants were responders compared to 4/11 (36.4%) in the placebo group, however again these proportions did not differ between groups ($\chi 2(1)=1.348$, $p=.246$). Finally, regarding the right entorhinal cortex, 10/17 (58.8%) cranberry and 4/11 (36.4%) placebo participants were classed as responders, with again these proportions not found to be significantly different between groups ($\chi 2(1)=1.348$, $p=.246$).

Furthermore, among participants who were classed as responders to any regions, in the cranberry group 8/13 (61.5%) were responders to all 3 regions, 3/13 (23.1%) were responders to 2 regions and 2/13 (15.4%) were classed as responders to only 1 region. In the placebo group, 3/6 (50%) participants classed as responders for any regions were responders for all 3 regions, 1/6 (16.7%) were responders for 2 regions and 2/6 (33.3%) were responders for 1 region. However, the difference in proportions between groups for types of responders (that is, whether they were responders to 1, 2, or 3 regions) were not significant ($\chi 2(2)= .682$, $p=.711$).

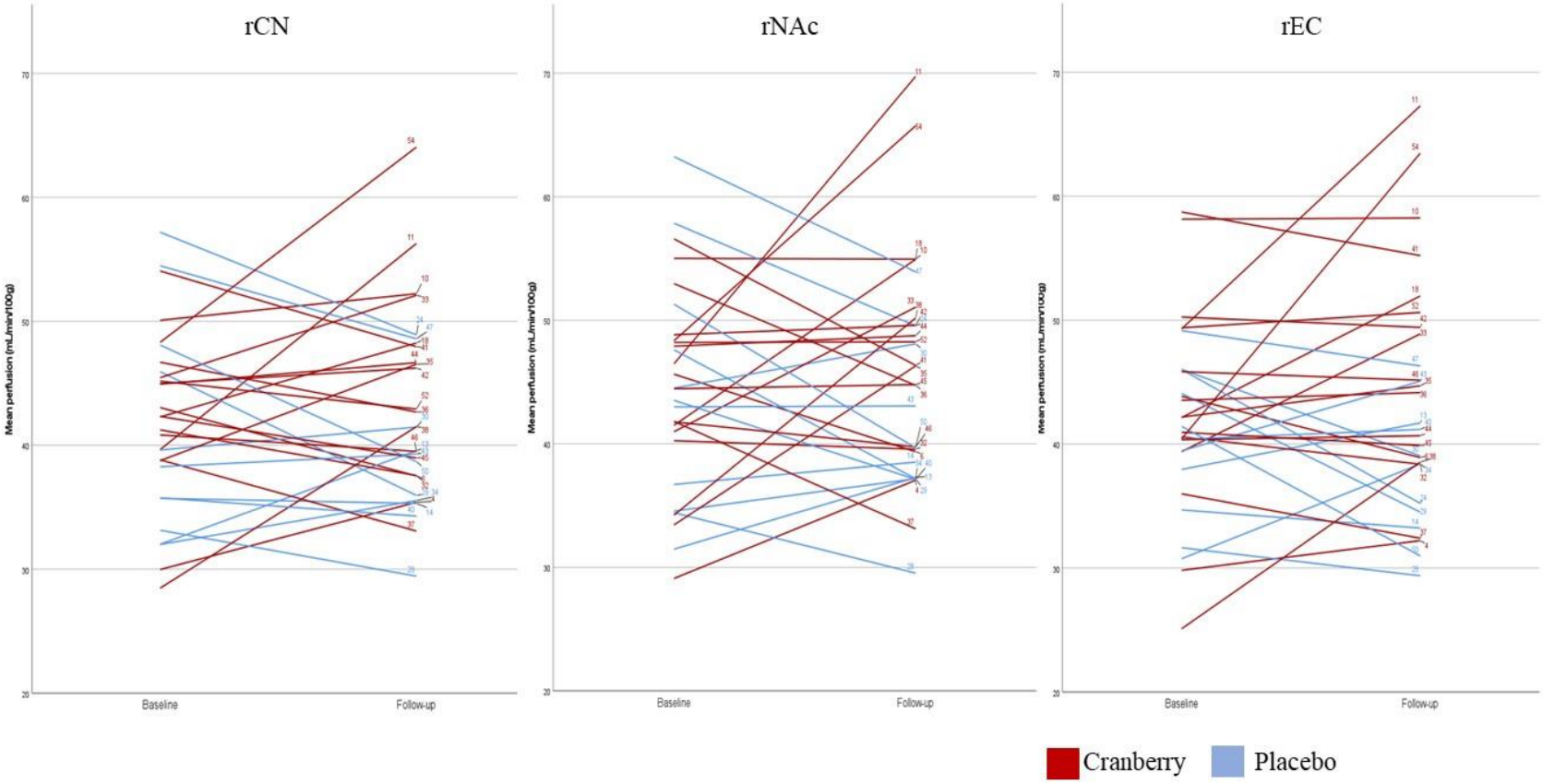


Figure 4.4. Individual participant slopes for regional perfusion as measured by ASL at baseline and follow-up. Labels represent participants' study identification numbers.

rCN = right caudate nucleus; rNAc = right nucleus accumbens; rEC: right entorhinal cortex.

MARKERS OF NEURONAL SIGNALLING

The placebo and cranberry groups did not differ in plasma levels of BDNF significantly at baseline, Mann-Whitney $U(1, 39) = 1.510$, $p = .219$. There was no significant group x time interaction for BDNF concentration between cranberry and placebo over the course of the intervention ($F(1, 39.76)=.300$, $p = 0.587$). Although concentrations appeared higher in the cranberry group than in the placebo group at follow up (Figure 4.5.), this difference was not significant, Mann-Whitney $U(1, 39)=.575$, p=.488. BDNF at the follow-up visit did not correlate significantly with follow-up RCF delayed score or regional CBF in either treatment group (p's >.05).

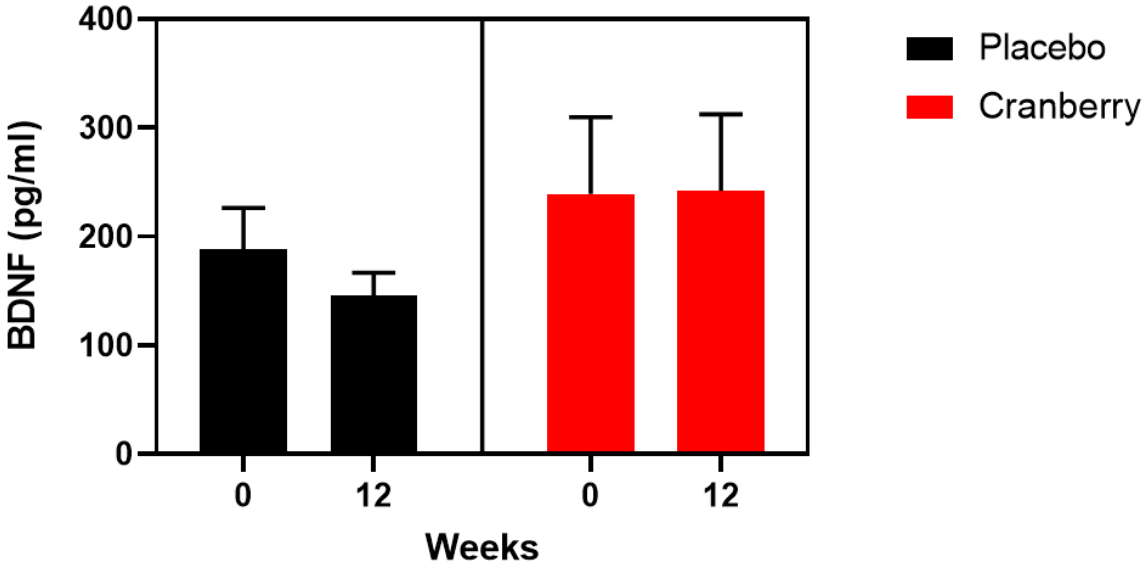


Figure 4.5. Impact of cranberries and placebo on circulating concentrations of the brain derived neurotrophic factor (BDNF) over a 12-week period and as measured by ELISA in plasma.

DISCUSSION

The results reported here show that daily cranberry supplementation over 12 weeks produced promising results for cognitive performance and blood perfusion compared to the placebo

group. Daily supplementation with the freeze-dried cranberry led to significant improvements in visual episodic memory performance, which coincided with increased blood perfusion of key neural areas which support cognition. Interestingly, our results are in direct contrast to a previously conducted clinical investigation in which no significant change in memory performance was established following cranberry intake (Crews et al., 2005). The discordance between these results likely relates to experimental inconsistencies such as duration of the intake (6 weeks versus 12 weeks) or product formulation (cranberry juice versus freeze-dried whole cranberry powder). The improvements in episodic memory performance is in line however with longer interventions (~12 weeks) in older adults with early memory changes involving blueberries (Krikorian, Shidler, et al., 2010) and high-anthocyanin Concord grape (Krikorian, Nash, et al., 2010).

Surprisingly, our cranberry intervention had no further impact upon additional neurocognitive domains. Working memory and executive functioning (including the executive functioning composite score), remained unaltered despite the contrary being reported by others investigating flavonoid-rich juices (Lamport, Lawton, et al., 2016). This could in part relate to the distinct polyphenolic composition of each distinct intervention. Conversely, this may be a product of cognitive test choice (de Jager et al., 2014) in contrast to other studies which used more laboratory-based measures such as the Stroop test (Stroop, 1935). It could also relate to task difficulty, since as this was a group of cognitively healthy adults it is possible that the task difficulty of the DS and TMT tasks was not sufficient to detect differences over time between these groups. Indeed, previous studies have detected more pronounced effects of berry supplementation on cognition for more cognitively-demanding tests (Whyte et al., 2021). In agreement with our results, a longer-term 12-week intervention with wild blueberry juice (Krikorian, Shidler, et al., 2010) led to similar improvements upon episodic memory performance in older adults. When taken together, these findings suggest that longer duration

of supplementation may be required to establish episodic memory enhancement associated with high anthocyanin berries.

Although other interventions investigating the impact of polyphenol intake on spatial navigation are not known, there have been observational findings of lower ratios of omega-6 to omega-3 fatty acid relating to better performance on virtual navigation tasks, suggesting that the cognitive processes underlying spatial navigation may represent a target for nutritional intervention. However, this cranberry intervention did not produce significant improvements in spatial navigation. Although deficits in spatial navigation has been proposed specifically as an early marker for the development of Alzheimer's disease, even before detectable episodic memory changes (Fu et al., 2017), evidence suggests that similar spatial disorientation may not be exhibited by cognitively healthy older adults (Lithfous, Dufour, & Despres, 2013; Serino, Morganti, Di Stefano, & Riva, 2015) while episodic memory function can still be impacted in normal ageing (Park et al., 2002). Furthermore, the specificity of spatial navigation deficits appears to be to the early stages of AD compared to other forms of neurodegenerative conditions (S. Tu, Spiers, Hodges, Piguet, & Hornberger, 2017; Yew, Alladi, Shailaja, Hodges, & Hornberger, 2013). Therefore, it is possible that this sample of healthy older adults did not exhibit detectable deficits in spatial navigation more typical of early AD, and as such no improvements in performance as a result of the cranberry intervention were detected.

As anticipated, the cranberry intervention had no impact upon differences in structural grey matter between groups, nor did it influence differences in white matter hyperintensities over the 12-week period of investigation. However, a relative increase in brain blood perfusion was detected in the cranberry group between baseline and follow-up compared to the placebo group the entorhinal cortex, the caudate and nucleus accumbens. The entorhinal cortex forms part of the medial temporal lobe (MTL), a collection of structures which are implicated in supporting memory function (Scoville & Milner, 1957; Sperling et al., 2003). The caudate and nucleus

accumbens form part of the striatum, and although their relationship to cognitive aging and neurodegeneration is less specific, they do form part of frontal-striatal circuits which underpin cognitive, affective and motor processes (Alexander & Crutcher, 1990; Lehéricy et al., 2004). Among the studies that are most comparable to the current study, a 12-week placebo-controlled intervention of daily blueberry concentrate supplementation in healthy older adults (ages over 65 years) found increases in brain blood perfusion in parietal and occipital lobes, in conjunction with results approaching significance for improvements in working memory which corresponded to task-associated activation in the anterior cingulate, precuneus, insula and thalamus (Bowtell et al., 2017). In another study, Boespflug et al. (2018) found a 16-week blueberry intervention in older adults with MCI produced increased BOLD activation in frontal and parietal regions. Regarding interventions involving high concentration polyphenol foods more generally, Lamport, Pal, et al. (2016) found increased perfusion in the middle and inferior frontal gyrus in younger adults following consumption of a high-flavonone citrus drink, in addition to improved executive performance, and the same group found increased perfusion in anterior cingulate and parietal regions following cocoa intake in older adults (Lamport et al., 2015). As such, our findings for regional blood perfusion in the brain do not overlap with those of previous polyphenol interventions, which in turn have been largely inconsistent in their findings. However, the finding of increased entorhinal cortex perfusion in particular as a result of this cranberry intervention is in line with our findings of improved episodic memory performance.

When the results were further analysed to identify responders compared to non-responders to the intervention, when categorised based on whether visual episodic memory performance as measured by the RCF increased between baseline and follow-up at the individual level, there were significantly more participants classed as responders in the cranberry group compared to the placebo group. Specifically, a large majority (82.8%) of cranberry participants showed

increased performance at follow-up, compared to a significantly lower percentage (53.3%) in the placebo group, the latter of which is a rate of responders vs. non-responders closer to what would be expected by chance. It is important to note that classifying responders based on increased performance between baseline and follow-up is vulnerable to influence from other factors such as practice effects, which is of particular relevance to the current study as alternate versions of the RCF were not used at baseline and follow-up. However, the fact that the proportion of responders was significantly greater in the cranberry group indicates that practice effects alone would not account for the improved performances between baseline and follow-up due to this difference between groups in the proportion of participants who improved. Regarding regional neural perfusion, there was a larger proportion of participants in the cranberry group who were classed as responders due to increased regional perfusion over the duration of the study for all three regions that were found to be differentially impacted by the intervention, however in these cases the proportions of responders vs. non-responders did not differ between groups. As only approximately half the full sample had complete baseline and follow-up ASL scans available, the analysis of group differences in proportions may not have been adequately powered to detect significant differences. By contrast, the linear mixed modelling method which detected significant group x time interactions in mean perfusion for these regions was chosen in part because it is more robust the impact of missing data points. Furthermore, when analysing further which individuals classed as responders were showing improvements in perfusion consistently across multiple regions, the majority of responders in the cranberry group (8/13) were responders across all three regions, followed by only 3 who were responders for two regions and only 2 participants were responders for just one region. The number of total responders in the placebo group was smaller with only 6 participants responders to at least one region, of which half were responders to all three regions and the remainder responders to one or two regions. Again, a larger sample of participants with

available scans may have revealed a more significant disparity in number of responders in each group, however the proportions of responders and non-responders in each group and furthermore the number of participants who were impacted consistently across all significant regions are in proportions that would be expected if the cranberry intervention were having an effect on neural function.

Furthermore, when observing individual slopes for both RCF delay performance and regional perfusion, it is clear that the individual trajectories of participants between baseline and follow-up are not uniform, and that some participants show considerably steeper increases and decreases over time. This may not be completely attributed to effects of the cranberry intervention, as both cranberry and placebo participants show steep trajectories in either direction (that is, increases and decreases over time). Furthermore, particularly regarding individual slopes of neural regional perfusion, it is apparent that some participants show consistently steep increases (such as cranberry participants 11 and 54) and decreases (such as placebo participant 28) across all regions. However, observation of individual slopes shows generally more increases over time in the cranberry group (represented by red slopes) compared to the placebo group (represented by blue slopes), which is supported by results of the other analyses.

Finally, although it was anticipated that BDNF may increase in the cranberry group in line with improved memory performance, the results from this study did not indicate this. Previous studies focusing on the impact of high flavonoid intake from fruit and vegetables have found a positive increase in serum BDNF in older adults (Neshatdoust et al., 2016), and the dosage of proanthocyanidins, anthocyanins and flavonols in the cranberry powder used in the current intervention was selected to approximate the levels used by that study. One likely reason for this might be that a high concentration of BDNF was measured at baseline which masked the overall impact of cranberries at follow-up. Such increased concentration may be related to

participants' higher consumption of caffeine in this group at baseline, although this difference did not reach significance. Additionally, since a number of follow-up plasma samples were unable to be collected due to COVID-19 restrictions it is possible that the sample was not large enough to detect differences between groups. However, a similar study by Ahles and colleagues (Ahles et al., 2020) investigating the impact of 24-weeks chokeberry on cognitive and vascular outcomes did not detect a change in serum BDNF with a sample of 101 participants. This is in contrast with an earlier study which found higher polyphenol intake generally from fruit and vegetables produced increased levels of BDNF (Neshatdoust et al., 2016).

These findings are however certainly encouraging that sustained intake of cranberry over a 12-week period produced significant improvements in memory and neural function in older adults who were cognitively healthy, however underlying mechanisms relating to improvements in neuronal signalling as reflected by BDNF levels cannot be suggested based on these results.

CHAPTER 5: GUT MICROBIOME COMPOSITION AND FUNCTION

INTRODUCTION

Polyphenols in berries, particularly proanthocyanins, have been found to directly alter the relative abundance of bacterial populations in the body. High-polyphenol berry supplementation has been shown to stimulate changes in gut bacterial abundances in animal models, particularly for increasing beneficial species such as *Akkermansia* (Anhe et al., 2015; Rodriguez-Daza, Daoust, et al., 2020; P. Tu et al., 2018). Regarding cranberry, whole cranberry has been found to increase the abundance of beneficial species of bacteria including *Lactobacillus* and *Bifidobacterium* while decreasing the prevalence of potentially harmful bacteria such as *Sutterella* and *Bilophila* in the gastrointestinal tracts of mice (Cai et al., 2019). Polyphenols present in berries have also shown inhibitory actions against bacterial populations that are considered detrimental to health (Nohynek et al., 2006).

In humans there have been very scarce investigations of gut bacterial changes resulting from berry supplementation. One study found that the consumption of a wild blueberry drink derived from freeze-dried fruit in 20 healthy adult men daily for 6 weeks resulted in increased abundance of beneficial microbial species such as *bifidobacterial* populations (Guglielmetti et al., 2013). The addition of sweetened dried cranberry to the diet of 10 human subjects daily for 2 weeks influenced the composition of the faecal microbiome (Bekiares et al., 2018), with 7 subjects showing increased microbial diversity. Another short-term intervention found that daily consumption of freeze-dried whole cranberry for 5 days produced a decreased abundance of *Firmicutes* and increase in *Bacteroidetes and* attenuated the impact of an animal-based diet on the gut microbiome (Rodriguez-Morato et al., 2018). The impact of longer-term cranberry consumption has been little explored, particularly in healthy older adults, and furthermore no studies investigating the relationship between these changes in gut microbiome composition and function and cognitive ageing.

The microbiota is also able to enhance the health benefits of polyphenols, largely through metabolising them down into their constituent components (El Kaoutari et al., 2013; van Duynhoven et al., 2011). Berry polyphenols are able to be converted by bacteria into several key bioactive secondary metabolites, such as benzaldehydes and hippuric, benzoic, cinnamic, phenylacetic and (phenyl)propanoic acids (Chandra et al., 2019). Indeed, microbial-derived metabolites of anthocyanins make up the majority of phytochemical compounds absorbed from berries (Kay, Pereira-Caro, Ludwig, Clifford, & Crozier, 2017; Williamson, Kay, & Crozier, 2018), and increase the impact of dietary polyphenols on a number of systems including the brain via circulation in the blood stream.

The previous chapter has shown that the cranberry intervention had a significant effect on cognitive performance and brain blood perfusion. In this chapter we explore how the cranberry intervention changed the gut microbiota and how this might have related to the results for cognition and brain health. The aim was to measure abundance and functionality changes in the microbiome as a result of the cranberry versus placebo interventions, as measured by shotgun metagenomics from both baseline and follow-up faecal samples. Additionally, metabolites detected in urine and circulating plasma samples were measured and related to cognitive performance and shifts in microbial abundances. Finally, changes in key active phenolic compounds metabolised from polyphenols were measured to detect changes due to the intervention and their relationship to changes in cognition and regional neural blood perfusion detected previously.

METHODS

MICROBIOME ANALYSIS

DNA EXTRACTION, QUALITY ASSESSMENT AND LIBRARY PREPARATION

DNA extraction from faecal water, quality assessment and library preparation was conducted at the Quadram Institute. Genomic DNA was extracted from all faecal samples collected at baseline and follow-up, and DNA concentrations of each sample were evaluated and pre-made library prepared according to methods described in Chapter 2, before the pre-made library was sent to Novogene Europe for sequencing. Data processing and analysis of shotgun metagenomic sequencing data was conducted by Professor Lesley Hoyles, Nottingham Trent University.

METABOLOMICS

URINARY METABOLOMICS

Metabolic phenotypes were derived from plasma and urine samples at baseline and follow-up and analysed by Professor Jonathan Swann and colleagues at the University of Southampton. Metabolic phenotypes were measured using NMR spectroscopy, with methods described in Chapter 2.

STATISTICAL ANALYSIS

For maintaining the blinding during the analysis the following codes were kept for the metagenomics and metabolomics analyses: A_BL (Cranberry group at baseline); A_FU (Cranberry group at follow up); B_BL (Placebo group at baseline) and B_FU (Placebo group at follow-up). Measures of alpha and beta diversity were determined using Phyloseq v1.30.0 (McMurdie & Holmes, 2012), with species-level data (where counts had ≥1 % cumulative relative abundance across all metagenomes) rarefied to 3,389,582 reads prior to analysis.

Non-rarefied count data were subject to analyses (Kruskal-Wallis for differences between groups, and paired Wilcoxon rank sum tests for comparisons within control and cranberry groups) using ALDex2 v.1.14.1 (Fernandes et al., 2014). To characterize the phylogenetic composition of bacterial communities in our samples, the alpha-diversity of microbiota of the separate groups was compared using Wilcoxon rank sums (paired). Wilcoxon rank sums analysis was also conducted within groups to detect differences between baseline and follow-up in relative microbial abudances at the class, order, family, genus and species levels.

Urinary metabolites were analysed using orthogonal projection to latent structures discriminant analysis (OPLS-DA) models by Prof Jonathan Swann and colleagues. OPLS-DA models were built in R using the MetaboMate package. Urinary metabolic profiles served as the descriptor (X) matrix and various measures were used as the response vector (*e.g.* RCF delay score, faecal *Eggerthellaceae* counts). The predictive ability (Q^2Y) of the model was calculated using a seven-fold cross validation approach. Permutation testing was performed to establish the validity of the model.

Plasma polyphenol metabolites were compared between groups at baseline using non-parametric Mann-Whitney U analysis. Mixed linear modelling was used to detect within group differences between baseline and follow-up, as well as group x time interactions of polyphenol levels. Responders and non-responders were grouped according to whether their significant polyphenol metabolite levels increased (responders) or did not increase (non-responders) between baseline and follow-up. A chi-squared test was then performed to determine whether placebo and cranberry groups significantly differed in their proportions of responders. Spearman correlations were conducted between polyphenol levels and follow-up RCF delayed score and regional blood perfusion from MRI ASL in regions found to be impacted by the cranberry intervention in the previous chapter.

RESULTS

MICROBIOME ANALYSIS

Unpaired and/or duplicate metagenomes and those metagenomes with 10-fold coverage lower sequence coverage than the average across samples were removed from analyses, leaving 28 and 27 paired metagenomes, respectively, for the cranberry and placebo groups. One participant had 10-fold lower sequencing coverage than other samples so this participant's corresponding baseline sample were removed from analyses. In total, 28 samples from the cranberry group and 27 samples from the placebo group were included in the analysis.

MICROBIAL DIVERSITY

ALPHA DIVERSITY

Kruskall-Wallis comparisons for each group (cranberry, placebo) between baseline and follow-up determined no significant differences were observed on species richness (observed species), indicating no major shift in bacterial community richness and diversity following 12 weeks supplementation with either cranberries or a placebo (Figure 5.1). However, Wilcoxon rank sums (paired) showed significant difference in the Shannon diversity index ($p = 0.02294$) for B_BL vs B_FU, indicating changes in both abundance and evenness of the placebo samples between baseline and follow-up.

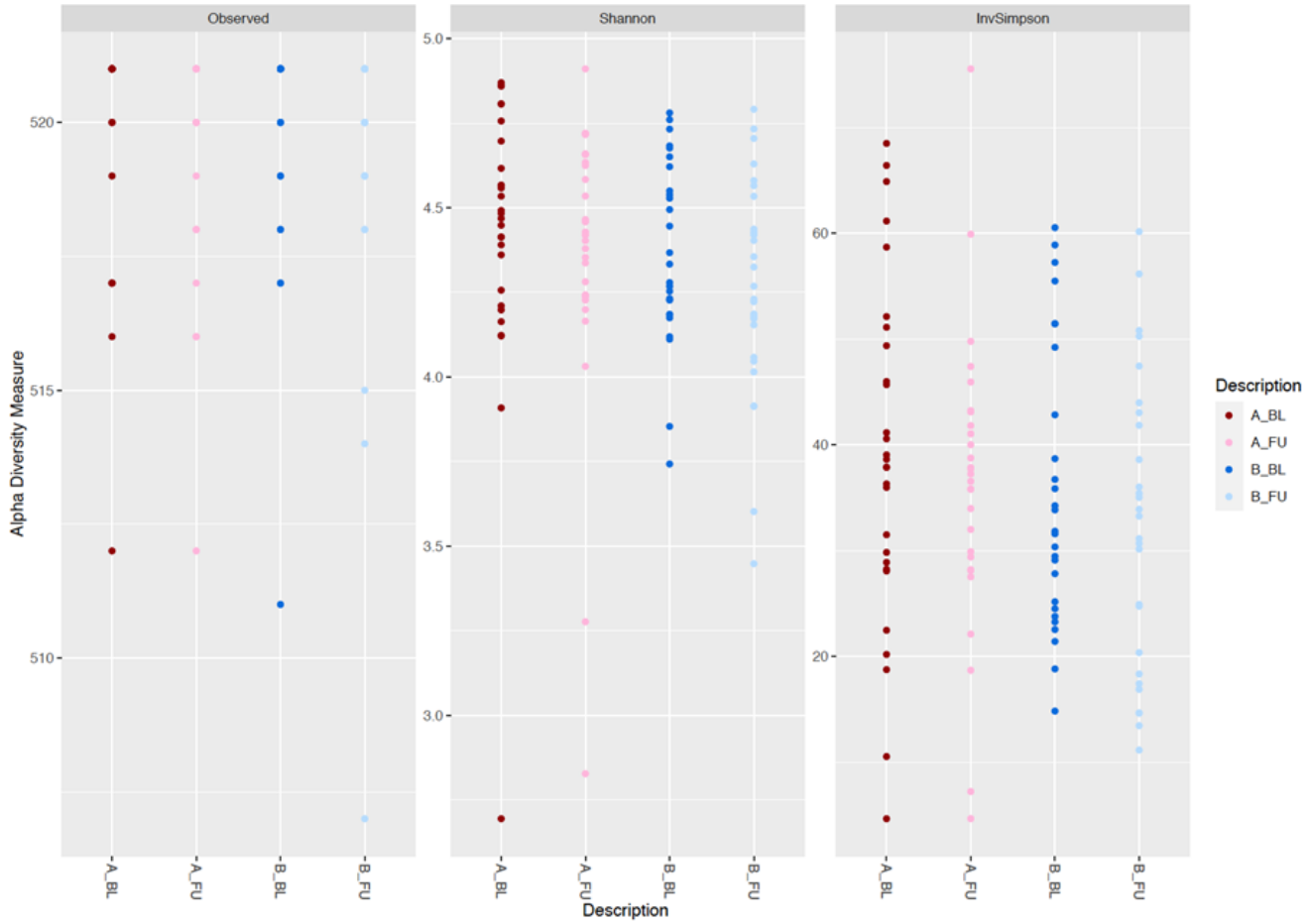


Figure 5.1. Estimation of the alpha diversity within faecal samples from the four groups. Kruskal-Wallis statistics showed no significant differences among groups (FDR < 0.2). Wilcoxon rank sums (paired) showed significant difference in the Shannon index (P = 0.02294) for B_BL vs B_FU. Cranberry group at baseline: A_BL; Cranberry group at follow up: A_FU; Placebo group at baseline: B_BL and Placebo group at follow-up: B_FU. Figure created by Professor Lesley Hoyles.

BETA DIVERSITY

As observed in Figure 5.2, Bray-Curtis dissimilarity detrended correspondence analysis (DCA) comparison of microbial communities in the samples did not produce a clear separation of the samples in the PCoA plots. Further analysis separating by treatment at

baseline and follow-up indicated that participants tended to cluster with themselves in the beta diversity metrics (Figure 5.2-B and C).

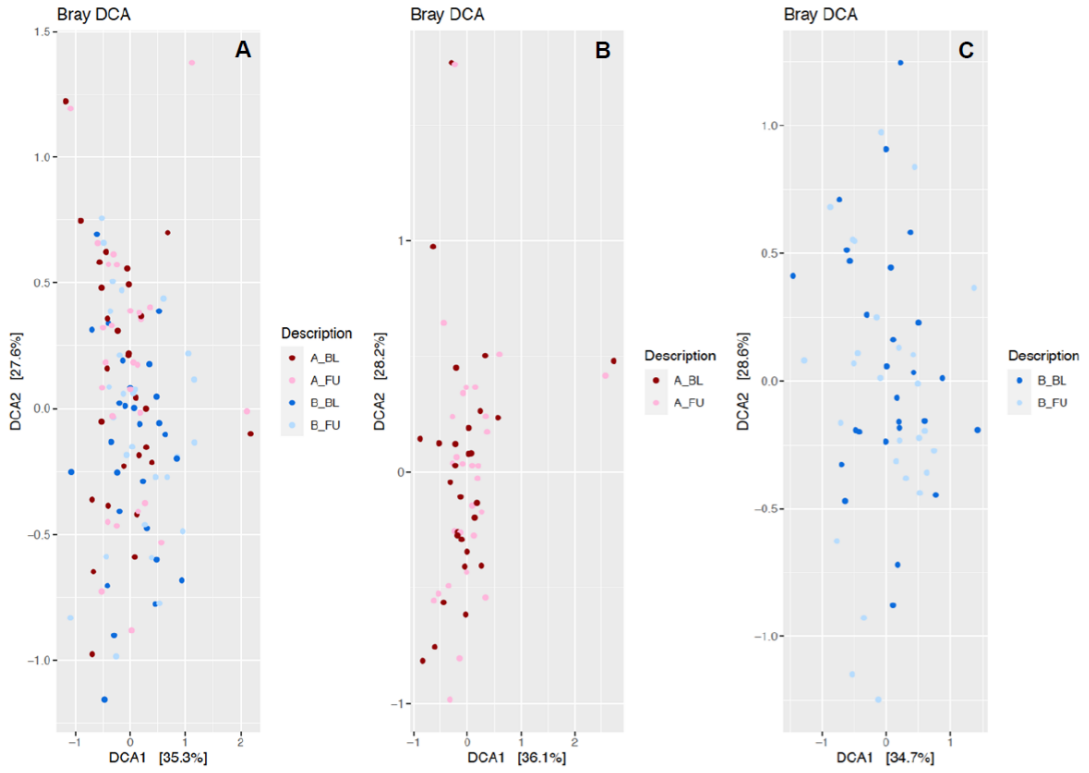


Figure 5.2. Beta-diversity analyses performed on shotgun metagenomic data. Bray-Curtis dissimilarity detrended correspondence analysis (DCA) analysis did not show any clear separation of the samples. Cranberry group at baseline: A_BL; Cranberry group at follow up: A_FU; Placebo group at baseline: B_BL and Placebo group at follow-up: B_FU. Figure created by Professor Lesley Hoyles.

RELATIVE SPECIES ABUNDANCES

There were no significant differences between groups at the Phylum level, with differences emerging between groups from the order level downwards.

Clostridia were by far the most abundant class in all 120 samples profiled, with an average of 60% and 55% of the total sample abundance in the placebo group and cranberry groups respectively. *Bacteroidia* (22.8% and 24.6%), *Actinobacteria* (4.9% and 5.2%),

Gammaproteobacteria (3.5% and 4.7%), *Coriobacteriia* (2.6% and 2.6%), *Verrucomicorbiae* (1.9% and 2.6%) and *Bacilli* (1.3% and 1.4%) were also well represented (see Supplementary Table 4 for more details).

Wilcoxon rank sums (unpaired) analysis highlighted significant increase in *Actinobacteria* abundance in the placebo group, whilst *Coriobacteria* were increased in the cranberry group between baseline and follow-up (Figure 5.3).

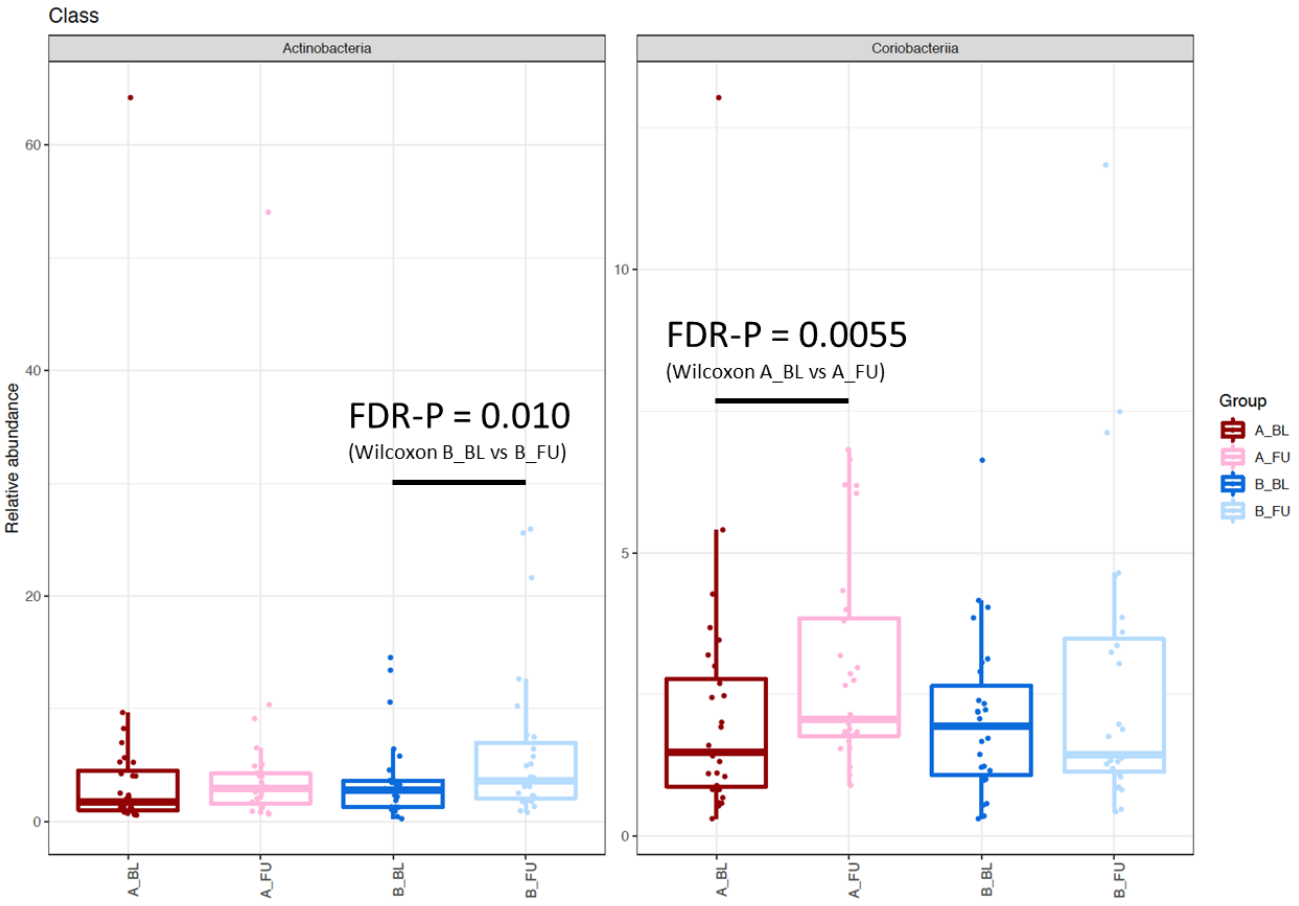


Figure 5.3. Microbial abundance at the class level. Only significant taxa are shown (Wilcoxon rank sums (unpaired), FDR <0.05). Cranberry group at baseline: A_BL; Cranberry group at follow up: A_FU; Placebo group at baseline: B_BL and Placebo group at follow-up: B_FU. Figure created by Professor Lesley Hoyles.

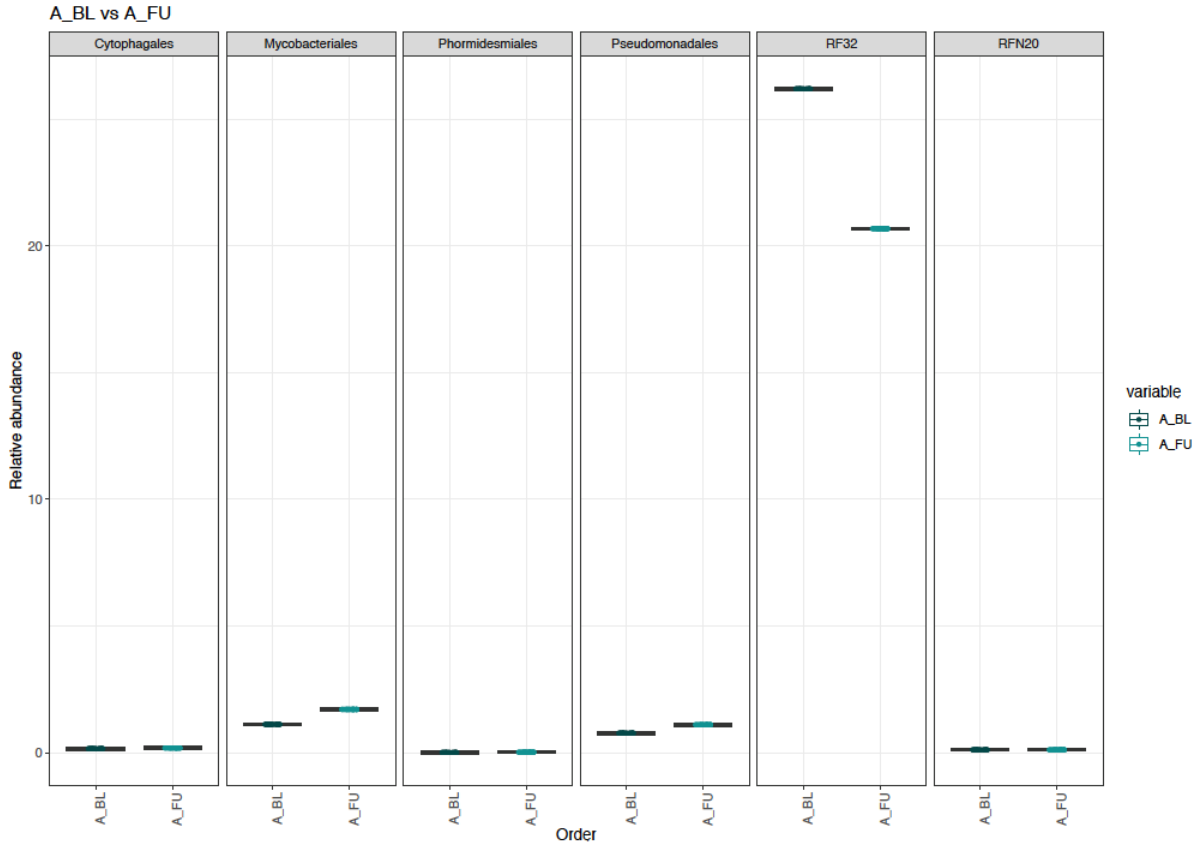


Figure 5.4. Microbial abundance at the order level. Only significant taxa are shown (Wilcoxon rank sums (unpaired), FDR <0.02). Cranberry group at baseline: A_BL; Cranberry group at follow up: A_FU; Placebo group at baseline: B_BL and Placebo group at follow-up: B_FU. Figure created by Professor Lesley Hoyles.

The distribution of relative abundances among different bacterial order, family and genera is shown in Figures 5.4, 5.5 and 5.6. Similar to the spread of class abundances, the faecal microbiota was dominated by relatively few genera. Statistical analysis (Kruskal-Wallis) of the genera levels revealed significant differences among groups (FDR < 0.05). Wilcoxon rank sums (unpaired) analysis highlighted significant differences within groups as shown in Figure 5.5 and 5.6 (CAG-1427 belongs to the *Eggerthellaceae* family and *Collinsella, Senegalimassilia* belong to the *Coriobacteriaceae* family).

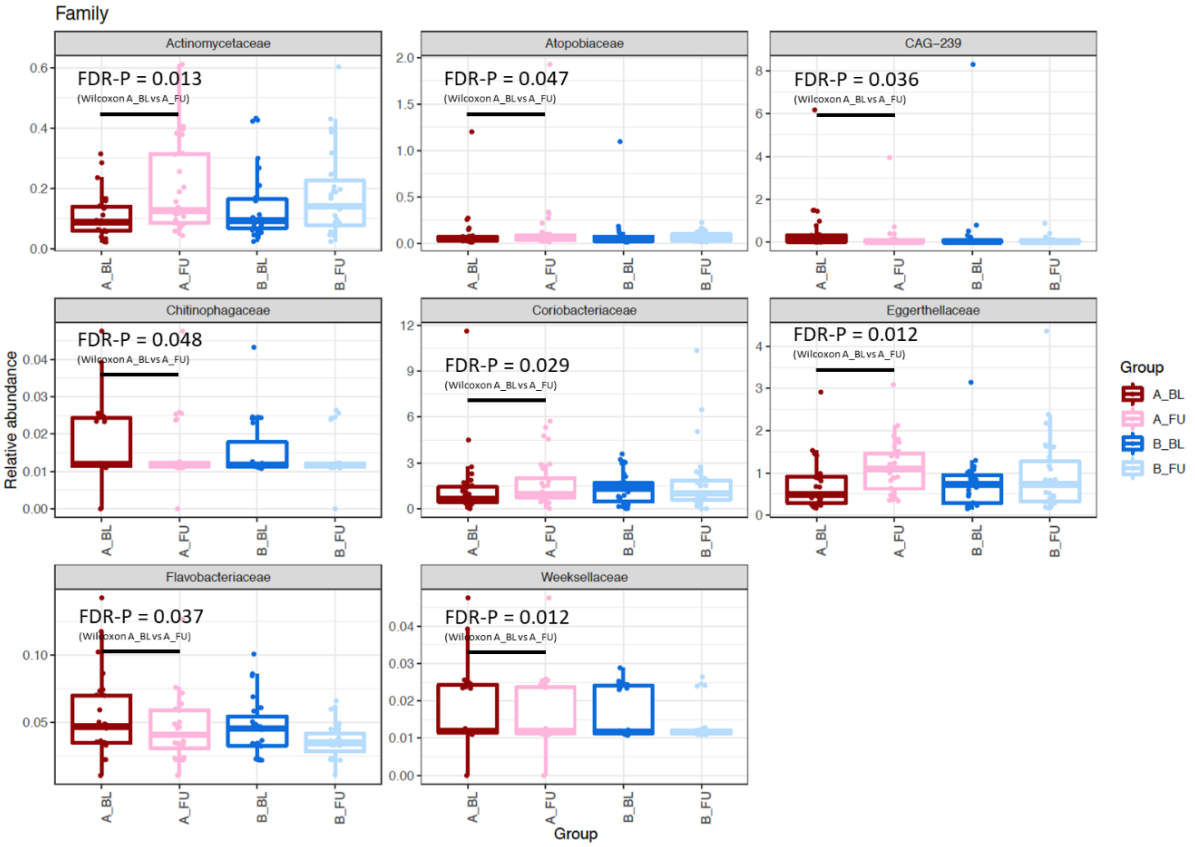


Figure 5.5. Microbial abundance at the family level. Only significant taxa are shown (Wilcoxon rank sums (unpaired), FDR <0.05). Cranberry group at baseline: A_BL; Cranberry group at follow up: A_FU; Placebo group at baseline: B_BL and Placebo group at follow-up: B_FU. Figure created by Professor Lesley Hoyles.

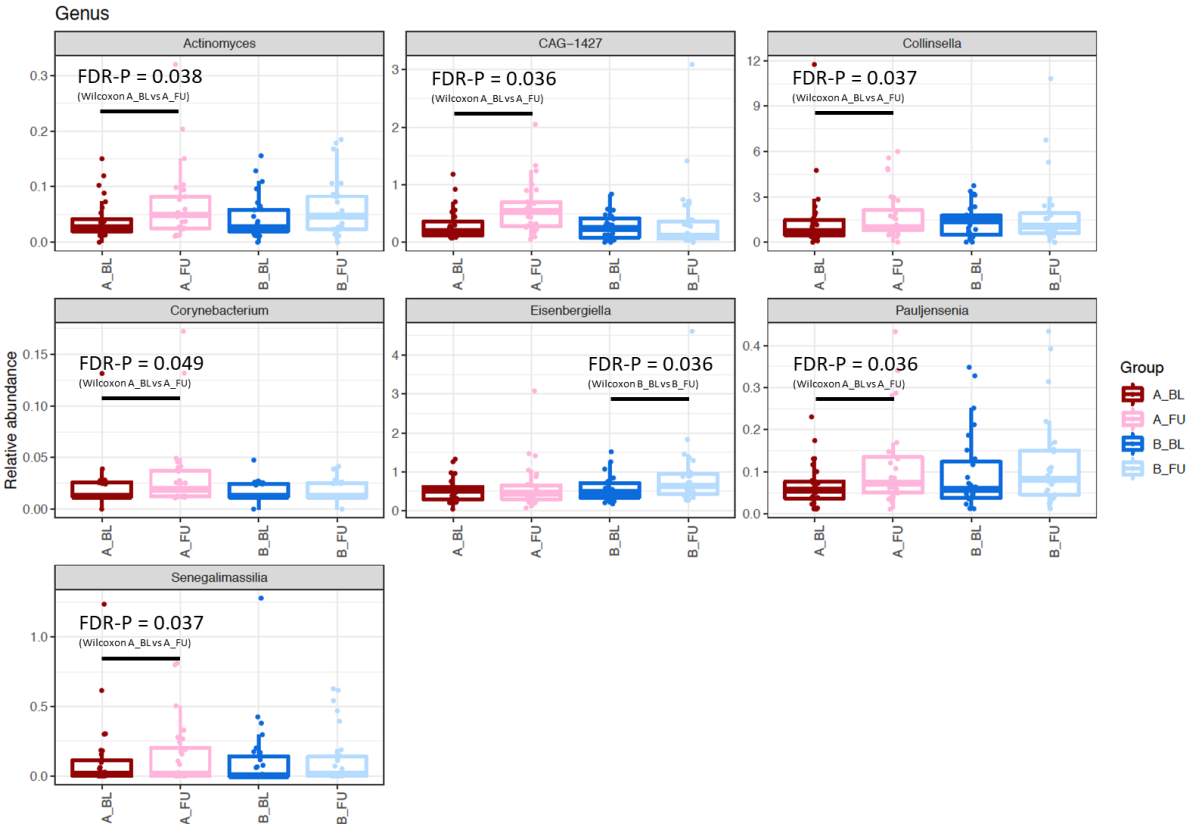


Figure 5.6. Microbial abundance at the genus level. Only significant taxa are shown (Wilcoxon rank sums (unpaired), FDR <0.05). Cranberry group at baseline: A_BL; Cranberry group at follow up: A_FU; Placebo group at baseline: B_BL and Placebo group at follow-up: B_FU. Figure created by Professor Lesley Hoyles.

MICROBIAL GENE RICHNESS

A total of 33,190,228 genes was predicted across all samples. Only those ≥30 aa in length were used in subsequent analyses: n= 32,622,164 and clustered at 90 % identity to create a non-redundant database of 4,161,937 proteins used to determine microbial gene richness and functional predictions. Kruskal-Wallis test across all groups showed no significant differences between the groups (FDR-P < 0.20). However, Wilcoxon rank sum test (paired) showed a significant difference between placebo at baseline and placebo at follow up (p = 0.03621) (Figure 5.7).

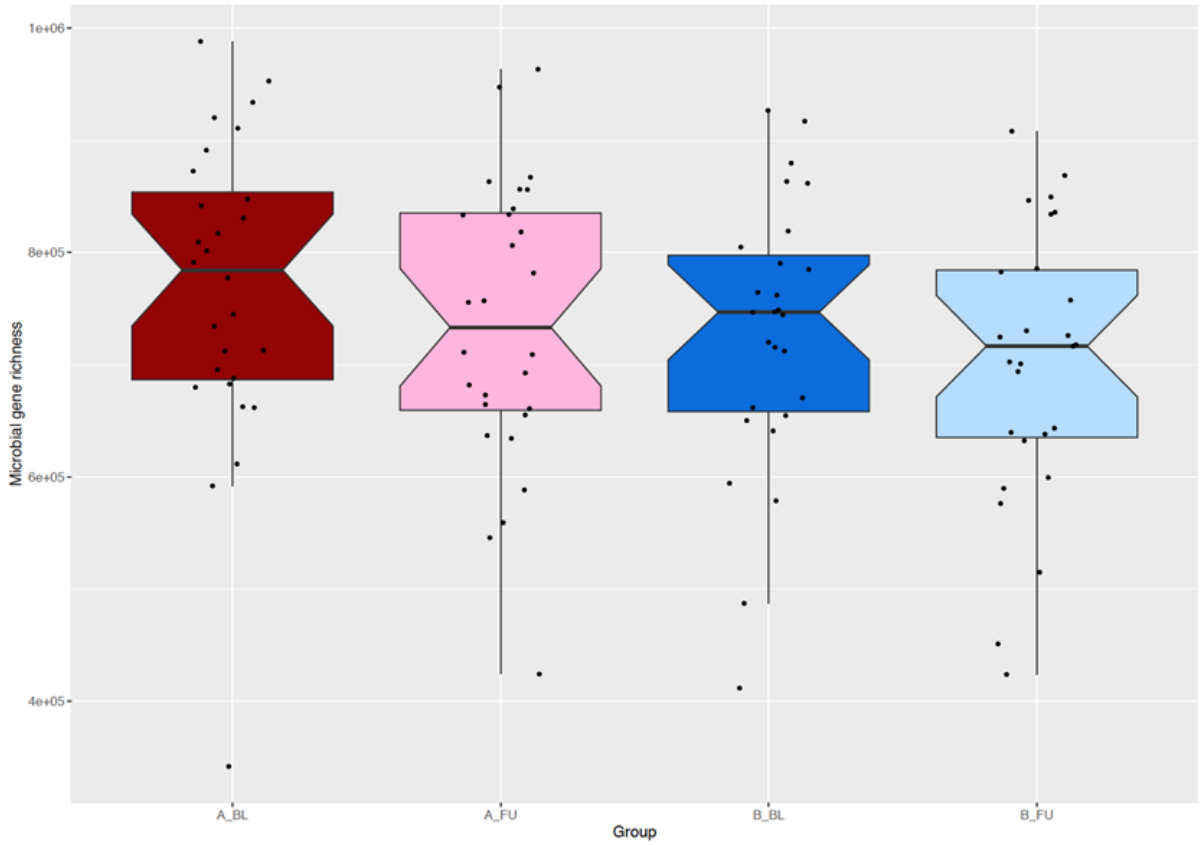


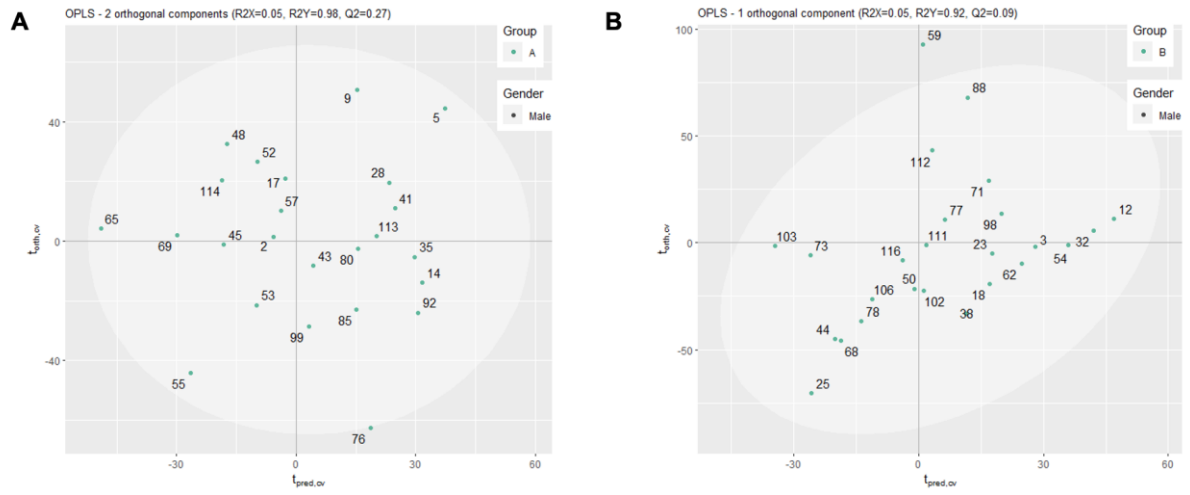
Figure 5.7. Microbial gene richness. Cranberry group at baseline: A_BL; Cranberry group at follow up: A_FU; Placebo group at baseline: B_BL and Placebo group at follow-up: B_FU Figure created by Professor Lesley Hoyles.

METABOLOMICS

URINARY METABOLOMICS

Orthogonal Projections to Latent Structures (OPLS) models were constructed on the urinary metabolic profiles of all participants at baseline. No significant models were obtained comparing the study groups (placebo vs intervention) indicating that all participants were biochemically comparable at the start of the study. No differences were seen between the placebo and treatment groups at baseline when stratifying by sex.

OPLS models were constructed on the urinary metabolic profiles at both sampling points to identify biochemical variation associated with RCF delay (measured at follow up) (Figure 5.8. A and B). Significant OPLS models were obtained for the males in the cranberry group and the males in the placebo group. Interestingly, the predictive ability of the model constructed on the metabolic profiles from the cranberry group was stronger ($Q^2Y = 0.27$) than that constructed from the placebo group (Q^2Y) = 0.09). Inspection of the coefficients plot from the cranberry model (Figure 5.8C) identified that creatinine, trimethylamine-*N*-oxide (TMAO, *tentative assignment*), formate, *cis*-aconitate, hippurate, *N*-methyl-nicotinamide (NMND) and ribose (*tentative assignment*) excretion was positively associated with the RCF delay score. Conversely, trimethylamine (TMA), trigonelline (*N*-methyl-nicotinic acid, NMNA), 4-aminohippurate (*tentative assignment*), *p*-cresol-sulfate, and phenylacetylglutamine (PAG) was negatively associated with RCF delay score.



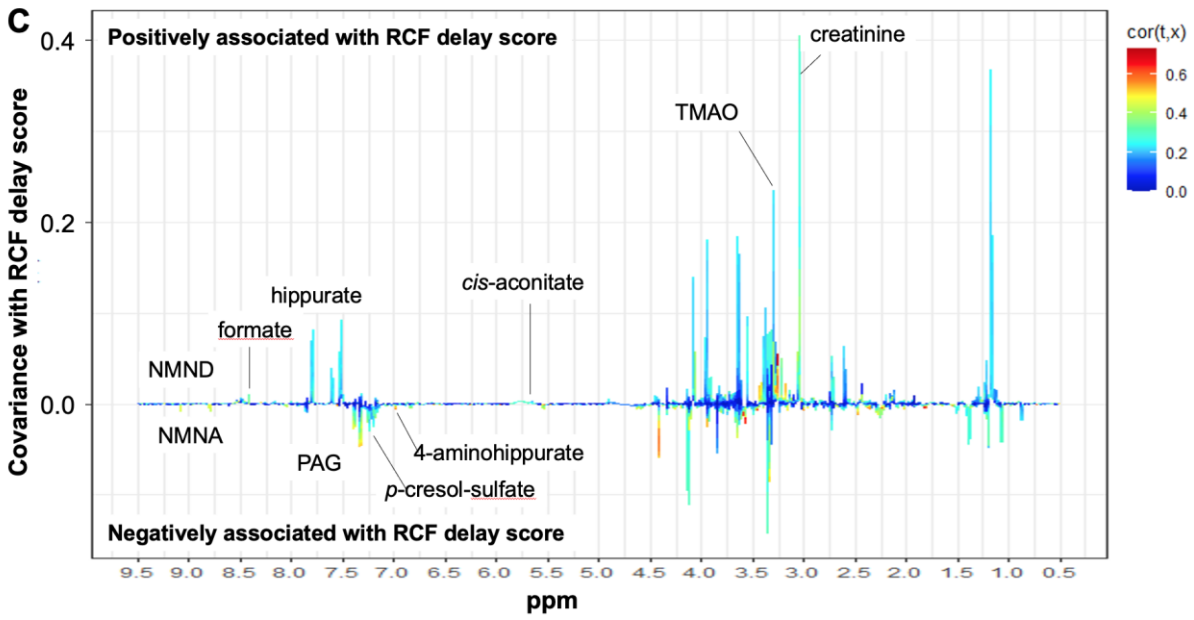


Figure 5.8. Orthogonal projection to latent structures (OPLS) models identifying urinary metabolic variation in the male participants associated with RCF delay scores. Scores plots built on the urinary metabolic profiles from individuals receiving A) cranberries (Q2Y = 0.27) and the B) placebo (Q2Y = 0.09) at all sampling points. C) Coefficients plot from the cranberry model. Figure created by Professor Jonathan Swann.

URINARY METABOLIC PHENOTYPES AND BACTERIAL GROUPS

Several OPLS models were constructed on the urinary metabolic phenotypes to illuminate biomolecular signatures associated with different bacterial groups. A significant OPLS model was obtained investigating urinary metabolites related to *Eggerthellaceae* in the cranberry group at follow-up (Figure 5.9; Q2Y = 0.15). This model identified that *Eggerthellaceae* was positively correlated with *cis*-aconitate excretion and negatively associated with NMND excretion.

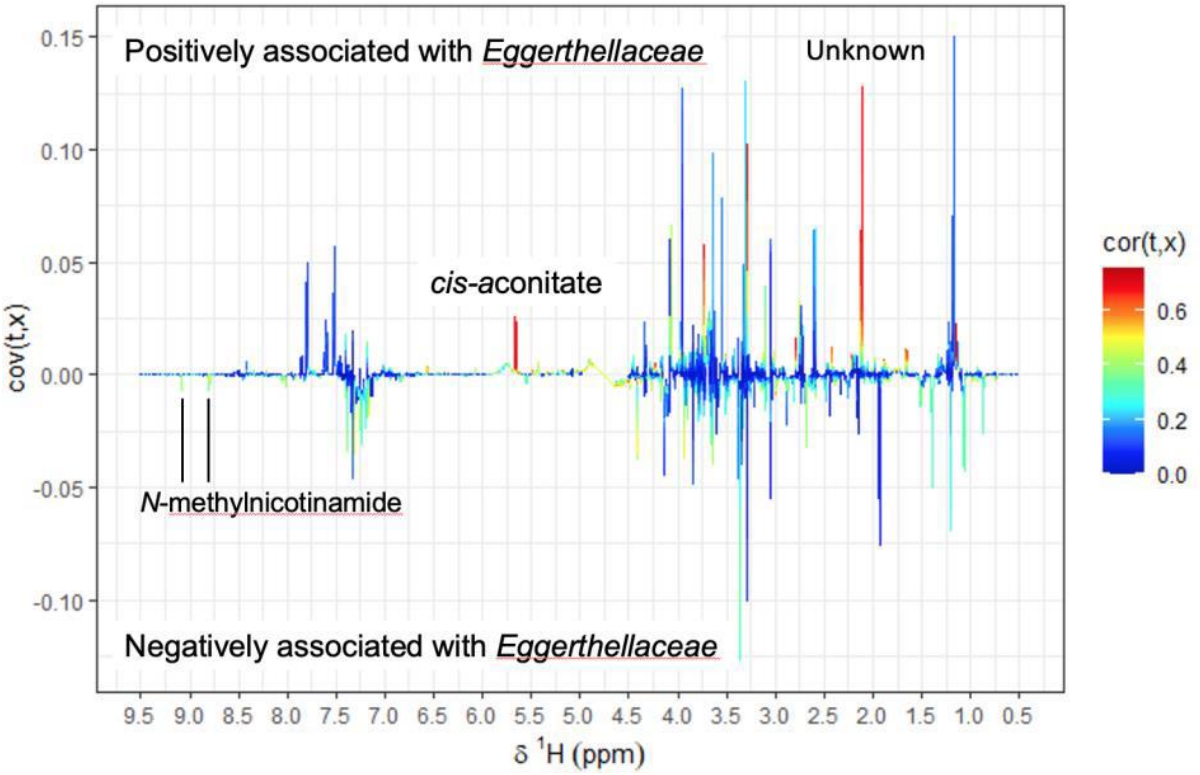


Figure 5.9. Significant OPLS model identifying urinary metabolites associated with faecal *Eggerthellaceae* in the cranberry group at follow-up (Q2Y = 0.15). Figure created by Professor Jonathan Swann.

Another significant model was obtained identifying urinary metabolic variation associated with *Flavobacteriaceae* in the cranberry group at follow up (Q2Y = 0.14). Here, this bacterial group was positively associated with acetate, trimethylamine, PAG, NMNA, and *p*-cresol sulfate excretion (Figure 5.10).

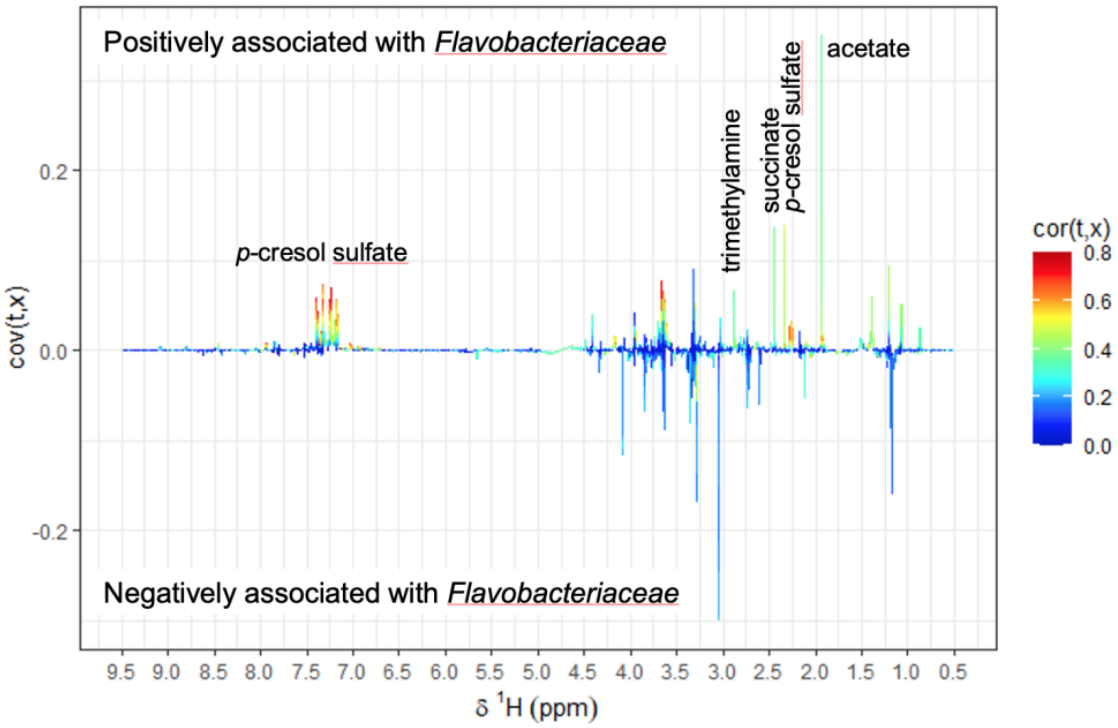


Figure 5.10. Significant OPLS model identifying urinary metabolites associated with faecal *Flavobacteriaceae* in the cranberry group at follow-up (Q2Y = 0.14). Figure created by Professor Jonathan Swann.

In the placebo profiles at follow up, a significant OPLS model was obtained comparing faecal 'CAG-1427' counts with urine biochemical signatures (Figure 5.11**,** Q2Y = 0.22). This bacterial genus was positively correlated with urinary hippurate.

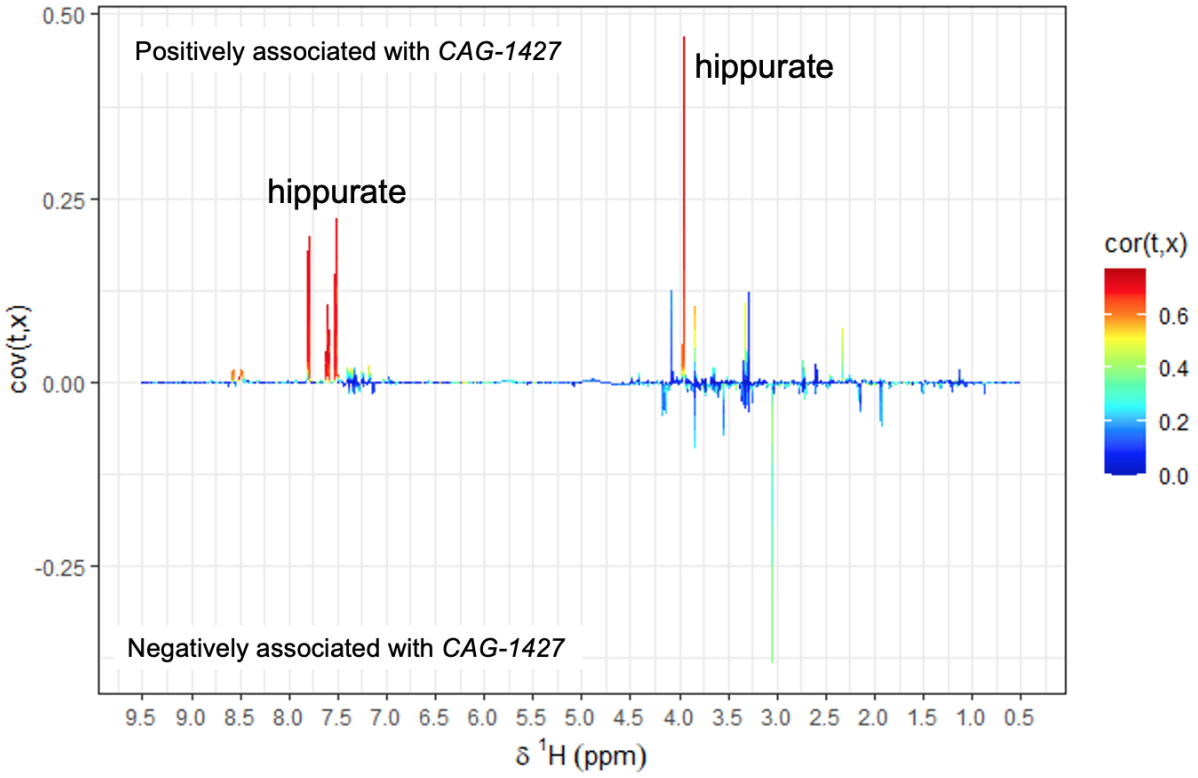


Figure 5.11. Significant OPLS model identifying urinary metabolites associated with faecal 'CAG-1427' in the placebo group at follow-up (Q2Y = 0.22). Figure created by Professor Jonathan Swann.

POLYPHENOL METABOLISM

PLASMA POLYPHENOL METABOLITES

The only significant group difference at baseline was for Kaempferol-3-glucuronide from flavonols (Mann Whitney $U(1, 43)=351.00$, $p=.026$), with placebo group showing higher total circulating levels at baseline compared to the cranberry group. There were no significant differences between cranberry and placebo groups at baseline for circulating plasma levels of metabolites measures (p's >.05) (Table 5.1.).

Table 5.1. Plasma (poly)phenol metabolites at baseline and follow-up visits in umol/L. Significance of baseline group differences determined by non-parametric Mann-Whitney U Independent Samples Tests, and main effects of time within group between baseline and follow-up and interactions between group and time determined using Linear Mixed Modelling.

Metabolite	**Group**	**Baseline (umol/L)**		**Baseline Difference**	**Follow-up (umol/L)**		**Group x Time**
		M	**SD**	***p***	**M**	**SD**	***p***
Kaempferol-3-glucuronide (Flavonols)	Cranberry	.017	.018	.026	.019	.020	.274
	Placebo	.033	.034		.028	.022	
4-Methylcatechol-sulfate isomer 1 (Catechols)	Cranberry	.033	.016	.617	.042	.015	.003
	Placebo	.032	.012		.024	.010	

4-Hydroxybenzaldehyde (Benzaldehydes)	Cranberry	.013	.006	.609	.019	.013	.307
	Placebo	.013	.006		.018	.009	
Hippuric acid	Cranberry	2.82	1.58	.892	4.29	1.94	.002
	Placebo	2.76	1.12		2.53	1.78	
4-Hydroxyhippuric acid	Cranberry	.039	.020	.540	.043	.025	.508
	Placebo	.043	.023		.041	.025	
4-Hydroxybenzoic acid	Cranberry	.024	.022	.056	.027	.012	.358
	Placebo	.032	.079		.038	.054	
Benzoic acid-4-sulfate	Cranberry	.023	.023	.107	.028	.024	.192
	Placebo	.015	.009		.014	.009	
Benzoic acid-3-sulfate	Cranberry	.026	.038	.609	.020	.021	.588

	Placebo	.015	.014		.012	.011	
Caffeic acid (3',4'-Dihydroxycinnamic acid)	Cranberry	.021	.012	.856	.032	.014	.005
	Placebo	.020	.008		.019	.013	
Ferulic acid-4-glucuronide	Cranberry	.018	.020	.927	.025	.015	.061
	Placebo	.016	.015		.012	.010	
3-Hydroxyphenylacetic acid (Phenylacetic acids)	Cranberry	.078	.041	.496	.087	.042	.811
	Placebo	.068	.033		.074	.028	
3-(3'-Hydroxyphenyl)propanoic acid (Phenylpropanoic acids)	Cranberry	.051	.043	.115	.039	.036	.166

	Placebo	.077	.054		.041	.049	
5-(Phenyl)-γ-valerolactone-methoxy-glucuronide (3',4') isomer 1 (Phenyl-γ-valerolactones)	Cranberry	.018	.011	.751	.017	.010	.190
	Placebo	.017	.009		.013	.008	
Total Metabolites	Cranberry	3.18	1.68	.820	4.69	2.01	.002
	Placebo	3.14	1.20		2.87	.321	

In the cranberry group, there was a significant increases in 4-Methylcatechol-sulfate isomer 1 (catechols) (F (1, 21)=4.553, p=.045), hippuric acids (F(1, 21)=14.784, p<.002, 4-Hydroxybenzaldehyde (benzaldehyde) (F(1,21)=11.783, p=.002, 3',4'-Dihydroxycinnamic acid (caffeic acid), F(1, 21)=12.460, p=.002, and total polyphenol metabolites (F(1,21)=14.035, p=.001. Between baseline and follow-up there was a significant decreases for the placebo group in 4-Methylcatechol-sulfate isomer 1 (F(1,22)=5.488, p=.029), 3-(3'-Hydroxyphenyl)propanoic acid (Phenylpropanoic acids), F(1, 22)=6.515, p=.018, and phenyl-y-valerolactones (F(1,22)=4.708, p=.041), and an increase in 4-Hydroxybenzaldehyde (F(1,22)=6.785, p=.016).

Regarding group differences in changes between baseline and follow-up, there were significant group x time interactions for catechols, F(1, 43)=9.999, p=.003, Caffeic acid (3',4'-Dihydroxycinnamic acid), F(1, 43)=8.867, p=.005, hippuric acid, F(1,43)=10.942, p=.002, and total metabolites F(1,43)=11.338, p=.002, with the cranberry group showing a relative increase in circulating levels of these metabolites between baseline and follow-up compared to the placebo group (Figure 5.12).

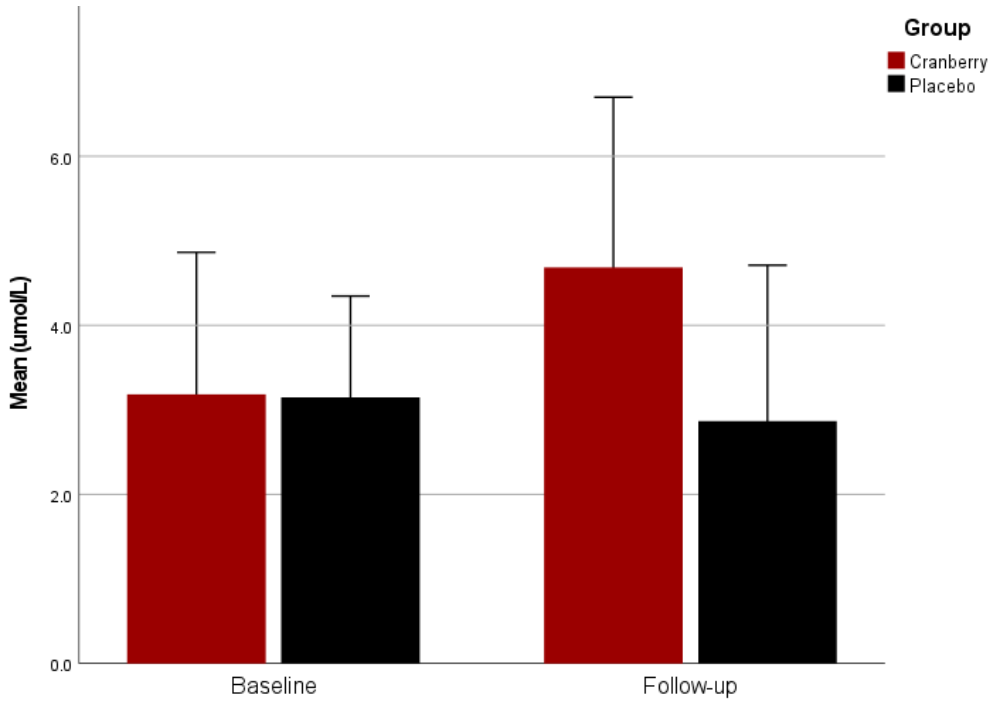


Figure 5.12. Total plasma polyphenol metabolites at baseline and follow-up for cranberry and baseline groups (umol/L). Error bars represent 1 SD.

RESPONDERS AND NON-RESPONDERS

Responders vs. non-responders based on whether individual participants showed an increase between baseline and follow-up were then identified for the plasma polyphenol metabolites which had significant group x time interactions. For catechols, 15/22 (68.2%) of cranberry and 8/23 (34.8%) of placebo participants showed increases between baseline and follow-up, and the difference between groups for these distributions was significant, $\chi^2(1) = 5.020$, $p = .025$. For caffeic acids, 18/22 (81.8%) of cranberry participants showed an increase from baseline to follow-up, compared to 10/23 (43.5%) in the placebo group, with these proportions again differing significantly between groups, $\chi^2(1) = 7.032$, $p = .008$. Regarding hippuric acid, 18/22 (81.8%) of cranberry and 9/23 (39.1%) of placebo participants showed increases since baseline, which again differed between the groups, $\chi^2(1) = 8.538$, $p = .003$. Finally, for total metabolites, 18/22 (81.8%) of participants showed an increase between

baseline and follow-up in the cranberry group compared to 14/23 (39.1%) in the placebo group, with these distributions significantly different, $\chi^2(1) = 8.538$, $p = .003$.

Furthermore, among the participants classed as responders, 20/22 (90.9%) cranberry and 12/23 (52.2%) placebo participants showed increases in at least one of the significant polyphenol metabolites (not including total metabolites). Of these, 13/20 (65%) cranberry and 5/12 (41.7%) placebo showed increases in all 3 metabolites, 5/20 (25%) cranberry and 5/12 (41.7%) placebo showed increases in 2 metabolites, and 2/20 cranberry (10%) and 2/12 (16.7%) placebo showed increases in only 1 of the metabolites. The differences in these distributions did not significantly differ between groups however, $\chi^2(2) = 1.659$, $p = .436$.

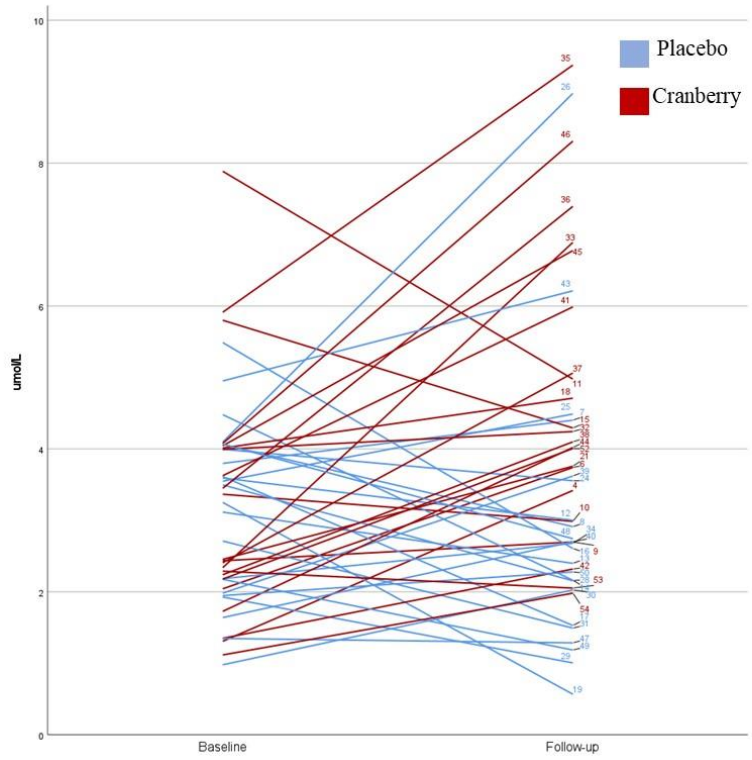


Figure 5.13. Individual participant slopes for total polyphenol metabolites at baseline and follow-up. Labels represent participants' study identification numbers.

When correlated with performance on cognitive tests which showed significant improvement in the cranberry group over the placebo group (RCF delay score), there were no significant correlations between metabolites which were significantly increased as a result of the intervention (4-Methylcatechol-sulfate isomer 1, hippuric acid, caffeic acid, total metabolites) and RCF delay score at follow-up, *p*'s > .05. The follow-up RCF delay score also did not significantly correlate with any metabolites at follow-up in the placebo group, *p*'s > .05.

For correlations between changed (poly)phenol metabolites and neural regions that showed significant group x time interactions for regional blood perfusion (right caudate nucleus, right nucleus accumbens, right entorhinal cortex), there were not significant correlations found in the cranberry group, *p*'s >.05. In the placebo group, there was a positive correlation between phenyl-y-valerolactones and the right caudate nucleus and phenyl-y-valerolactones, $r = .680$, $p = .015$, and the right nucleus accumbens, $r = .724$, $p = .008$.

DISCUSSION

The results of this chapter suggest that there was a change in microbial abundance due to the intervention. Many of the changes in microbial abundances in the cranberry group due to the intervention were detected from the order level down. Where dietary interventions have been found to reversibly impact the human gut microbiome as measured by faeces it is often at the species and genera level, although less often the phylum level at which the microbiome tends to be more stable over the longer term (Martinez et al., 2013). Furthermore, the results from the beta diversity analysis that there was not a clear clustering of samples based on treatment, which might indicate that the differences between our samples are caused by bacterial species and genera spread out among different phylogenetic groups rather than all clustered within one family, order, or class.

Many of the bacterial populations that significantly increased in relative abundances at follow-up in the cranberry group are associated with microbial use of flavonoids, including *Slackia, Eggerthella,* and *Coriobacteriia* classes, within which *Coriobacteriaceae*, *Atopobiaceae*, and *Eggerthellaceae*, and *Arabia*, *Eggerthella*, *Enorma*, *Enteroscipio*, *Libanicoccus*, *Olsenella*, *Raoultibacter*, *Rubneribacter*, *Senegalimassilia*, and *Slackia* genera were found to be changed by the intervention. In particular, there were increases in microbial abundances in the *Eggerthellaceae* and *Coriobacteriaceae* families, which are both implicated in the degradation of polyphenols, and furthermore have been shown to produce bioactive secondary polyphenol metabolites (González-Sarrías, Espín, & Tomás-Barberán, 2017). These bacterial populations have previously been found to change in abundance following a cranberry fruit powder intervention in an animal model (Rodriguez-Daza, Roquim, et al., 2020). Diet enrichment with wild blueberries in rodents (Lacombe, Li, et al., 2013; Rodriguez-Daza, Daoust, et al., 2020) has been found to produce increases in abundance of bacteria belonging to the *Coriobacteriaceae* family, often in conjunction with increases in symbiotic *Bifidobacteriaceae* microbes, although similar increases in the latter microbial family was not detected in the cranberry group in this study.

There was also an increase in *Actinobacteria* at the class level in the placebo group as a result of the intervention. This class of bacteria is one of the largest families of bacteria which are diverse in biological function, and as there were no differences as a result of the intervention detected from this class at more specific levels it is difficult to determine which specific microbial populations are driving this increase. There were also increases detected at the genus level of *Eisenbergiella,* which belongs to the *Lachnospiraceae* family within the *Clostridia* class, although differences were not detected due to the intervention for these higher levels in the placebo group. The reason for this increase in *Eisenbergiella* in the placebo group is unclear, and there is currently limited functional information for this genus,

but increased levels of species within this genus of bacteria been previously tenuously linked to obesity (Togo et al., 2016) and a high saturated fat diet combined with low fibre intake (Bailen et al., 2020). The placebo group did have a higher mean BMI in the overweight range, although the difference from the BMI in the cranberry group was not significant. Furthermore, as background diet was not measured during and after the intervention it is not clear whether this could provide an explanation for the increase detected here in the group that weren't supplemented with cranberry, especially since the placebo powder is largely composed of maltodextrin and hence poor in fibres.

One main difference between the microbial diversity results produced here and previous works involving cranberry and berry supplementation generally is that there was no change detected in *Akkermansia* abundances as a result of the cranberry intervention. Much of the evidence that exists from previous studies investigating the impact of berry polyphenols on microbes belonging to the *Akkermansia* family have been conducted in rodents (Anhe et al., 2015; Rodriguez-Daza, Daoust, et al., 2020; P. Tu et al., 2018), with the existing human trials involving subjects of younger ages taking sweetened dried cranberry (Bekiares et al., 2018). The relevance of the sweetened as opposed to unsweetened cranberry as provided in this study is that *Akkermansia* has a suggested role in glycaemic control (Gerard & Vidal, 2019). This clearly needs to be further investigated in future studies involving humans.

The analysis of metabolic phenotypes results indicated that there were several metabolites produced in the cranberry group that could impact brain function and cognition. Key metabolites were found to relate to RCF delayed memory performance in the cranberry group, including hippurate and TMAO. Hippurate has long been associated with the metabolism of polyphenols (Blatherwick, 1922; Cathcart-Rake et al., 1975). TMAO is metabolised from choline by microbes (Zhu, Wang, Tang, & Hazen, 2017), and may protect the brain via supporting the integrity of the BBB and improve cognition (Hoyles et al., 2021),

although other findings from an Alzheimer's mouse model suggests that it is associated with decreased synaptic plasticity as it increases with age (Govindarajulu et al., 2020). Formate and creatinine were also positively associated with RCF performance. Formate is a SCFA which has been determined to be of microbial origin due to the absence of its formation detected in germ-free mice (Hughes et al., 2017), and decreased plasma levels of formate have been associated with dementia (Smith & Refsum, 2016) and MCI (V. Singh, Mishra, Prajapati, Ampapathi, & Thakur, 2020), as was creatinine in the latter study. Metabolites that were negatively associated with RCF performance at follow-up include trimethylamine (TMA), trigonelline (N-methyl-nicotinic acid, NMNA), 4-aminohippurate, p-cresol sulfate, and phenylacetylglutamine (PAG). These metabolites are gut microbiota-derived (Evenepoel, Meijers, Bammens, & Verbeke, 2009; Zeisel, Wishnok, & Blusztajn, 1983) and have been previously either described as toxins or associated with age-related cognitive decline and poor brain health (Sankowski et al., 2020; C. Y. Sun et al., 2020; Vogt et al., 2018; Yu et al., 2021). As such, there were several gut bacteria-derived metabolites previously associated with the onset of age-related cognitive decline which showed significant associations with RCF performance at follow-up. The relationship between the shift in bacterial abundance and microbially produced metabolites was further supported by findings in the cranberry group, where there were significant models detected for urinary metabolic phenotypes relating to the bacterial groups *Eggerthellaceae* and *Flavobacteriaceae* families. This included a strong positive association between hippurate and 'CAG-1427' genus, which is a member of the polyphenol-degrading *Eggerthellaceae* family.

The circulating plasma polyphenol metabolites indicated that both groups were well matched at baseline, apart from flavanols which were higher in the placebo group before the intervention. The results of the background diet questionnaires also indicated that the placebo group had a higher average intake of flavonols, although this difference between groups was

not significant. There were increases in total metabolites in the cranberry group as a result of the intervention, which appeared to be driven largely by significant increases in catechols, hippuric acids, and caffeic acids. Interestingly, there was also a significant decrease in catechols and y-valerolactones in the placebo group as a result of the intervention. The reason for these decreases is unclear, however it could be due to the dietary restrictions on high-polyphenol foods including berries that all participants were asked to adhere to throughout the intervention.

Furthermore, as would be expected there were significantly more participants who were classed as responders to the intervention in the cranberry group for all common polyphenol metabolites that were found to increase compared to placebo over the duration of the intervention. The cranberry group showed more consistency in responders across all three metabolite types such that more participants were classed as responders to all metabolite types compared to placebo. However, these proportions did not differ significantly between groups in this subset of participants who had been classed as responders for at least one type of metabolite. Similar to analyses of individual slopes in the previous chapters for cognitive performance and neural perfusion, an observation of individual slopes shows a general increase over time in total metabolites for cranberry participants compared to placebo, with exceptions such as participants 11, 26 and 15, further supported by the analysis of responders and non-responders.

Further contrary to expectations, plasma polyphenol metabolite concentrations did not relate to either RCF delay score or regional blood perfusion within the regions found to be differentially changed between groups as a result of the intervention. It is possible therefore that the mechanisms underpinning the changes in cognition and regional blood perfusion in the brain were not the direct interaction between these metabolites and neural functioning, but

rather more related to the beneficial shift in specific bacteria (eg. *Eggerthella*) that are responsible for producing other beneficial metabolites described above that did relate to markers of brain function.

In summary, these results indicate that the cranberry intervention produced positive shifts in microbial abundance, particularly for species relating to polyphenol metabolism. Indeed, the intervention also produced increases in metabolites and metabolic phenotypes which in turn related to improvements in memory performance previously detected. However, metabolites specific to polyphenol degradation did not significantly relate to improvements in cognition and regional CBF observed in the previous chapter.

CHAPTER 6: GENERAL DISCUSSION

This thesis aimed to explore the impact of dietary cranberry on brain function and the gut microbiome, and explore possible relationships between these outcomes. As was hypothesized, daily intake of cranberry over a 12-week intervention produced positive effects on episodic memory performance and regional brain perfusion, although surprisingly not an improvement in executive function performance. In addition, beneficial shifts in the structure and function of the gut microbiome were also suggested from these findings, with several microbial-derived metabolites related to the improvements in memory performance and microbial families found to be increased in abundance as a result of the intervention, although no similar associations were found for metabolites commonly produced from polyphenols. These results, their implications for understanding of the impact of cranberry on the brain and gut microbiome with future directions, and limitations are discussed further here.

SUMMARY OF FINDINGS

The placebo and cranberry groups were very well-matched at baseline for the main demographics of age, gender, and education. The cranberry and placebo groups were well-matched for almost all characteristics and measures, with very minor differences detected for estimated daily vitamin D intake and liver enzymes, however the results for both of these were within the normal ranges for each group. The sample used in the current intervention had comparable demographics to previous similar clinical trials which found positive results regarding the impact of berry intake on similar outcomes of interest with comparable intervention lengths (~12 weeks). Therefore, the impact of confounding factors that could have influenced the main outcomes of interest in this study were minimised.

Daily supplementation with freeze dried whole cranberry (equivalent to one small cup of cranberries) led to significant improvements in episodic memory performance, which coincided with increased perfusion of key neural areas which support memory in older adults

such as the entorhinal cortex in the MTL, as well as striatal regions including the caudate nucleus and nucleus accumbens. However, contrary to predictions, there was no beneficial impact of the intervention on executive function and working memory, possibly owing to insufficient task difficulty in this sample of cognitively healthy adults, or on BDNF levels measured in plasma.

The results of this intervention study also suggest that there was a shift in microbial abundances as a result of the cranberry intervention. There were changes in abundances from the order level down, without a clear clustering of species within particular phylogenetics group suggesting that group differences are spread out among different families, orders and classes. There were increases in abundance in the cranberry group in bacterial families associated with polyphenol degradation (*Eggerthellaceae* and *Coriobacteriaceae* families). Furthermore, there were significant increases in circulating plasma polyphenol metabolites detected in the cranberry group as a result of the intervention, such as from catechols and hippuric acids, although not from phenyl-y-valerolactones which are a class of metabolites from flavan-3-ols including proanthocyanidins. The results also indicate that there were several metabolites produced in the cranberry group that could impact brain function and cognition. Key metabolites were found to relate to RCF delayed memory performance in the cranberry group, including hippurate and TMAO. However, contrary to expectation in the cranberry group there was no positive relationships between circulating plasma metabolites derived from polyphenols and cognition or blood perfusion in regions found to change as a result of the intervention.

IMPLICATIONS OF FINDINGS

Our results of improved episodic memory in the group supplemented with daily freeze-dried cranberry are in direct contrast to a previously conducted 6-week clinical investigation of cranberry in healthy older adults by Crews et al. (2005). The findings are more in line with

other longer-term (~12-week) studies involving older adults with MCI and supplementing either blueberry (Krikorian, Shidler, et al., 2010) or high-anthocyanin Concord grape (Krikorian, Nash, et al., 2010). This is despite the current study involving cognitively healthy adults and using a visual rather than verbal (often word lists) measure of episodic memory, although the sample size was substantially larger in this study. This possibly indicates that this longer duration of supplementation is required to establish episodic memory enhancement associated with high anthocyanin and proanthocyanin containing berries. In line with results suggesting that the cranberry intervention led to improved episodic memory performance, differences in brain blood perfusion in response to the intervention were detected between cranberry and placebo groups, including in the entorhinal cortex, a key medial temporal lobe area supporting memory consolidation and retrieval (Scoville & Milner, 1957; Spencer, 2009; Sperling et al., 2003). Episodic memory performance declines with age (Park et al., 2002), coinciding with changes in MTL structures even in the context of normal ageing (Hackert et al., 2002; Head, Rodrigue, Kennedy, & Raz, 2008; O'Brien, Desmond, Ames, Schweitzer, & Tress, 1997). Indeed, accelerated changes to these regions are detected in pathological as compared to normal ageing (Driscoll et al., 2009; Jobst et al., 1994). With regards to potential actions of berry polyphenols, anthocyanins have previously been detected in key neural regions that support cognition including the MTL in rats (Andres-Lacueva et al., 2005) and has related to enhanced performance on behavioural measures of spatial working memory in Listar rats (C. M. Williams et al., 2008), although it also related to increased hippocampal levels of BDNF which was not replicated here by measuring plasma levels of BDNF in our human subjects. Anthocyanins have also been suggested to be able to pass the BBB (Youdim et al., 2004), although the mechanisms by which this occurs is still yet to be fully understood. Furthermore, anthocyanins have been able to be detected in animal brain tissue even when not detected in circulating plasma levels (Kalt et al., 2008). Taken together with improvements in episodic

memory detected as a result of the cranberry intervention, it could be that this improvement in memory performance were at least partially mediated by increased blood perfusion in MTL regions, with these regions a potential target for the high anthocyanin concentration provided by the cranberry. However, this proposed role of cranberry polyphenols in episodic memory improvement is based on evidence from previous animal studies, rather than being confirmed using peripheral measurements of these polyphenols and their metabolites in this human sample.

Surprisingly, in contrast with other similar studies and expectations, the cranberry intervention had no further impact upon additional neurocognitive domains such as working memory and executive functioning (including the executive functioning composite score), which remained unaltered despite the findings of improved executive function being reported by others investigating berries and polyphenol-rich juices (Lamport, Lawton, et al., 2016; Lamport, Pal, et al., 2016; Whyte et al., 2021). Interestingly, a 12-week intervention in a group of healthy older adults (60-75 years) who were provided with the daily equivalent of one cup of blueberries produced significant improvements in cognitive performance on some tests including executive function and episodic memory using a smaller sized sample (n=37) (Miller et al., 2018). This could in part relate to the distinct polyphenolic composition of each intervention. The tests measuring executive function and working memory selected for the current intervention were chosen as they are gold standard measures which are typically used in a clinical setting to detect changes in these domains due to neurodegenerative conditions such as dementia. In addition, a composite executive score was calculated based on letter fluency from the ACE-III, a scaled score for TMT Trail B and backwards Digit Span to increase the variability of these scores, however group differences as a result of the intervention were still not detected. Other tests used in previous studies such as the Go/No Go (Drewe, 1975) and Stroop tests (Stroop, 1935) are more commonly used in laboratory settings. It is possible that

in this sample of healthy older adults that there were ceiling effects in performance for the tests used in this intervention, therefore limiting differences over time being detected. A previous set of recommendations and criteria for selecting cognitive tests for detecting the impact of nutritional interventions (de Jager et al., 2014) argued that tests such as the TMT may be less sensitive for detecting changes in a non-clinical population, although this was based on the two previous studies published at the time investigating the impact of high-polyphenol foods on performance on this test including berries and cocoa (Crews et al., 2005; Crews, Harrison, & Wright, 2008). Furthermore, one of the studies referenced, the previous 6-week cranberry intervention study by Crews et al. (2005) found no impact of cranberry on either the TMT or Stroop test performances. As it stands, in the context of berry supplementation further investigation is required to determine the impact on executive function, and which tests are most sensitive to detecting these changes.

Further to this, the cranberry group also did not show significantly differentially improved perfusion in prefrontal structures over the course of the trial, although a group by time interaction was approaching significance in this direction for the right medial orbitofrontal cortex ($p=.065$) as well as the right lateral orbitofrontal cortex ($p=.089$). The patterns in perfusion changes in this region was in line with other regions for which significant results were found, with a relative increase in the cranberry group over the course of the intervention compared to the placebo group. The prefrontal cortex has long been established as particularly vulnerable to both normal ageing processes (Jolles, 1986; Stuss & Knight, 2013; Tisserand & Jolles, 2003), underpinning changes in executive function and working memory (Chadick, Zanto, & Gazzaley, 2014), as well as memory retrieval (Kramer et al., 2005). Since complete baseline and follow-up MRI data were only available for a subset of the sample, it is possible that greater availability of these data for more participants may have produced more robust results for these regions.

Changes in spatial navigation performance as a result of the intervention were also not detected. Although deficits in spatial navigation has been proposed specifically as an early marker for the development of Alzheimer's disease, even before detectable episodic memory changes (Fu et al., 2017), evidence suggests that similar spatial disorientation is not experienced by healthy older adults (Lithfous et al., 2013; Serino et al., 2015) while episodic memory is impacted in normal ageing (Park et al., 2002). Furthermore, the specificity of spatial navigation deficits to the early stages of AD compared to other forms of neurodegenerative conditions (S. Tu et al., 2017; Yew et al., 2013). Therefore, it is possible that this sample of healthy older adults did not exhibit detectable deficits in spatial navigation more typical of early AD in order to detect improvements as a result of the cranberry intervention. Otherwise, as to current knowledge there have not been other studies to date investigating the impact of nutritional interventions on spatial navigation performance it is not certain whether spatial navigation performance represents a target for these interventions, although MTL structures found to be impacted by the current intervention have been implicated in the formation of internal representations required for navigation through space (Howard et al., 2014).

The involvement of increased perfusion in the nucleus accumbens and caudate as a result of the cranberry intervention are less clear. These structures are anatomically proximal to each other, the nucleus accumbens forming part of the ventral striatum and the caudate part of the dorsal striatum. The striatum is a critical node in the brain which receives input from multiple cortical regions and is a key component in motor, affective, and cognitive circuits (Alexander & Crutcher, 1990; Lehéricy et al., 2004). Furthermore, evidence from neuroimaging has suggested that the roles of distinct anatomical regions within the striatum in supporting cognition are less important than the complex connectivity between these regions and cortical regions, particularly frontal regions (Bertoux, O'Callaghan, Flanagan, Hodges, & Hornberger, 2015). Although the striatum, particularly the caudate, is not generally considered a vulnerable

region in AD when taken in relation to whole-brain atrophy (Looi et al., 2009), other findings have suggested that changes are observable in these regions even in early preclinical cognitive impairment (Hilal et al., 2015). This area is within its circuit with the ventromedial prefrontal cortex is particularly implicated in reward-related cognition (Schultz, Tremblay, & Hollerman, 2000), which was not specifically reflected in the cognitive tests used in this thesis, so the contribution of these striatal regions to the observed improvements in cognition would be more clear with the use of tests which tap into these specific cognitive processes.

The results reported in this thesis suggest that there was a change in microbial abundance as a result of the intervention, with many of the changes in the cranberry group between baseline and follow-up were detected from the order level down. Where dietary interventions have been found to reversibly impact the human gut microbiome as measured by faeces it is often at the species and genera level, although less often the phylum level at which the microbiome tends to be more stable over the longer term (Martinez et al., 2013). Many of the increases in relative microbial abundance between baseline and follow-up observed in the cranberry group were in species associated with microbial metabolism of polyphenols. Specifically, there were increases in microbial abundances in the *Eggerthellaceae* family and the *Coriobacteriaceae* family, which are both implicated in the degradation of polyphenols, and furthermore have been shown to produce bioactive secondary polyphenol metabolites (González-Sarrías et al., 2017). Indeed, these bacterial populations have previously been found to change in abundance following cranberry fruit powder intervention in an animal model (Rodriguez-Daza, Roquim, et al., 2020).

Coriobacteriaceae is a family of bacteria found to be involved in the degradation of polyphenols, in doing so having the potential to activate bioactive phenolic metabolites associated with producing health beneficial effects (González-Sarrías et al., 2017). For example, microbes in the *Coriobacteriaceae* are suggested to be able to metabolize γ-

valerolactones and their catabolites, phenolic acids, from catechins (Braune & Blaut, 2016). Diet enrichment with wild blueberries in rodents (Lacombe, Li, et al., 2013; Rodriguez-Daza, Daoust, et al., 2020) has been found to produce increases in abundance of bacteria belonging to this family, often in conjunction with increases in symbiotic *Bifidobacteriaceae* microbes, although similar increases in the latter microbial family was not detected in the cranberry group in this study.

One main difference between the microbial diversity results produced here and previous works involving cranberry and berry supplementation generally is that there was no change detected in *Akkermansia* abundances as a result of the cranberry intervention. Much of the evidence that exists from previous studies investigating the impact of berry polyphenols on microbes belonging to the *Akkermansia* family have been conducted in rodents (Anhe et al., 2015; Rodriguez-Daza, Daoust, et al., 2020; P. Tu et al., 2018), with the existing human trials involving subjects of younger ages taking sweetened dried cranberry (Bekiares et al., 2018). The relevance of the sweetened as opposed to unsweetened cranberry as provided in this study is that *Akkermansia* has a suggested role in glycaemic control (Gerard & Vidal, 2019). This clearly needs to be further investigated in future studies involving humans.

The analysis of metabolic phenotypes results indicated that there were several metabolites produced in the cranberry group that could impact brain function and cognition. Key metabolites were found to relate to RCF delayed memory performance in the cranberry group, including hippurate and TMAO. Hippurate has long been associated with the metabolism of polyphenols (Blatherwick, 1922; Cathcart-Rake et al., 1975). TMAO is metabolised from choline by microbes (Zhu et al., 2017), and may protect the brain via supporting the integrity of the BBB and improve cognition (Hoyles et al., 2021), although other findings from an Alzheimer's mouse model suggests that it is associated with decreased synaptic plasticity as it increases with age (Govindarajulu et al., 2020). The SCFA formate

also related to RCF performance, and is one of the major products of the fermentation of non-digestible carbohydrates which are not absorbed in the small intestine. The role of this metabolite in the gut remains little understood, decreased plasma levels of formate have been associated with dementia (Smith & Refsum, 2016) and MCI (V. Singh et al., 2020). Formate is a SCFA which has been determined to be of microbial origin due to the absence of its formation detected in germ-free mice (Hughes et al., 2017), and decreased plasma levels of formate have been associated with dementia (Smith & Refsum, 2016) and MCI (V. Singh et al., 2020), as was creatinine in the latter study. In the cranberry group in particular, there were significant models detected for urinary metabolite phenotypes and the bacterial groups *Eggerthellaceae* and *Flavobacteriaceae* families, the latter of which is one of the largest families of the *Bacteroidetes* phylum which has been implicated in carbohydrate metabolism and particularly the production of SCFAs (Louis & Flint, 2017), although primarily acetate and proprionate, and are also become more abundant in older adults relative to a decrease in *Firmicutes* populations (Claesson et al., 2011).

Metabolites that were negatively associated with RCF performance at follow-up included TMA, NMNA, 4-aminohippurate, p-cresol sulfate, and PAG. The metabolite p-cresol sulfate is considered a uremic toxin and is produced through metabolism of tyrosine largely by *Coriobacteria* in the large intestine (Evenepoel et al., 2009; Saito, Sato, Nomoto, & Tsuji, 2018), and has been closely linked with diseases of the central nervous system (Sankowski et al., 2020) including those that cause dementia (C. Y. Sun et al., 2020). TMA is metabolised by gut bacteria largely from choline (Zeisel et al., 1983), and has been associated with the onset of Alzheimer's disease (Vogt et al., 2018). PAG is also metabolised from phenylalanine by gut bacteria (Moldave & Meister, 1957) and has been associated with increased WMH burden and risk of ischaemic stroke (Yu et al., 2021). As such, there were

several gut bacteria-derived metabolites previously associated with the onset of age-related cognitive decline which showed significant associations with RCF performance at follow-up.

When measuring compounds typically metabolised from polyphenols, there were increases in total metabolites in the cranberry group as a result of the intervention, which appeared to be driven largely by significant increases in catechols and hippuric acid which was not also detected in the placebo group. The metabolism of berry polyphenols by bacteria has previously been demonstrated (Vuong, Martineau, Ramassamy, Matar, & Haddad, 2007). Interestingly, there was also a significant decrease in catechols in the placebo group as a result of the intervention. One proposed role for catechols metabolised from higher order polyphenols is the regulation of carbohydrate metabolism (Nachar et al., 2017), particularly recently in the context of reducing insulin sensitisation in type 2 diabetes in humans (Zamora-Ros et al., 2014). Contrary to expectations, despite changes in plasma polyphenol metabolite concentrations as a result of the intervention, these did not relate to either RCF delay score or regional blood perfusion within the regions found to be differentially changed between groups as a result of the intervention. It is possible therefore that the mechanisms underpinning the changes in cognition and regional blood perfusion in the brain were not the direct interaction between these metabolites and neural functioning, but rather more related to the beneficial shift in specific bacteria (eg. *Eggerthella*) that are responsible for producing other beneficial metabolites described above that did relate to markers of brain function.

Overall, the findings presented in this thesis do contribute to an understanding of how berry polyphenols, particularly those in high concentrations in cranberry, may influence cognition and brain function and modify the structure and function of the gut microbiome. However, there were limitations to the study design which limits the generalisability and conclusions that are possible from the findings, which are discussed in the next section.

LIMITATIONS

SAMPLE SIZE AND IMPACT OF COVID-19

As this study was part of a feasibility intervention, the relatively small sample size may have been a limiting factor particularly with regards to having sufficient power to detect significant differences between groups resulting from the intervention. However, the current sample was larger than several previous studies reporting a positive impact of berries on brain function (Bowtell et al., 2017; Miller et al., 2018). Nonetheless, regarding the neuroimaging data, several regions showed non-significant trends for perfusion indicating relatively increased blood perfusion in the cranberry group as a result of the intervention, such as PFC regions (see Supplementary Table 2),, which may have reached significance if more participants' scans were available. Only a subsection of the study sample was able to have complete baseline and follow-up MRI scans, due to practical constraints and the impact of COVID-19 lockdowns on hospital imaging facilities during the critical follow-up window for several (n = 14) patients, ultimately reducing the power to detect the impact of the intervention on several variables. This also led to a more pronounced imbalance in sample size between groups for these measures collected at follow-up. Specifically, 19 participants in the cranberry group had an MRI scan at follow-up and 13 in the placebo group. Also due to safety concerns during the first COVID-19 lockdown in 2020, follow-up blood samples also couldn't be collected for these participants, so the results from plasma analyses such as measurement of circulating metabolites were not available at follow-up for these participants. Stool and urine samples however were able to be collected remotely during COVID-19 restrictions, so results derived from these samples were closer to complete for the whole sample.

SUBJECTIVE MEMORY COMPLAINTS

The current study also used the same criteria used by Bowtell and colleagues (Bowtell et al., 2017) of >88/100 on the total score of the Addenbrooke's Cognitive Examination III (ACE-III) at screening to exclude participants with cognitive deficits, as this cut-off has been found

to be the most sensitive (1.00) to detecting cognitive decline while still having high specificity (.96) (Hsieh et al., 2013). Participants were also asked at the telephone screening whether they have experienced any significant memory changes over the past 2-3 years that concern them, to exclude participants with significant subjective memory complaints. It however should be noted that the baseline CCI scores, particularly the CCI memory sub score, could indicate that a number of participants who were included into the study experienced subjective memory complaints. No cut-offs based on the CCI were used for exclusion from the study as currently no established cut-offs exist. Furthermore, there were no differences between cranberry and placebo groups at baseline on either the overall CCI score or the memory, executive function or language sub scores. Nonetheless, despite all participants performing normally on cognitive screening and reporting cognitive complaints at the telephone screen, it is possible based on the results of the CCI that a portion of the sample experienced subjective memory complaints. There have been unpublished cut-offs between 20 and 28 (ranging from more sensitive to more specific) suggested for the memory sub-score by relating CCI scores to a combination of MRI results and cognitive performance in cognitively normal older adults and participants with significant memory concerns in the ADNI cohort (Risacher et al., 2017), but no established cut-offs have been published for this questionnaire. Other self-report questionnaires used in this study such as the CBI-R also indicate quite low degrees of changes in memory performance, with <11% change endorsed, although this is over the course of the previous month compared to the past five years as measured by the CCI, even though this was the highest scoring domain on the CBI-R. Overall, despite higher scores on the CCI indicating possible subjective memory complaints, when considered in conjunction with other cognitive and behavioural screening measures it does not appear that the participants included in this sample have significant memory decline that could have impacted the primary outcome measures of this study.

Impact of other non-polyphenol nutrients

It is important to note that food sources containing high concentrations of polyphenols such as berries also contain other health-promoting compounds and nutrients, including fibre and other micronutrients, making it sometimes difficult to determine whether it is in fact these specific polyphenols producing observed health effects. For example, nutrients such as fermentable fibres can influence gut microbial metabolism of polyphenols (Mansoorian, Combet, Alkhaldy, Garcia, & Edwards, 2019). Regarding the food composition of the cranberry itself, the degree of processing of these foods can greatly impact the actions of these compounds, such that more processing produces less potent benefits. Direct comparison between different studies therefore comes with a degree of difficulty, as a number of factors can influence the efficacy of the dietary intervention or supplementation. As such, fruits and fruit extracts that have been minimally processed would be predicted to be the most ideal method of dietary delivery to produce the most benefits. Therefore, the freeze-dried whole cranberry powder used in this study would be expected to deliver the most health impacts due to its minimal processing compared to cranberry juice, which has been used in the few other trials investigating the impact of cranberry on cognition (Crews et al., 2005).

Medications

In total, there were eleven participants taking blood pressure medication for at least 2 months prior to entering the study, which did not exclude these participants from the study based on the inclusion criteria however is worth noting. Furthermore, although also not listed as an exclusion criterion, nine participants were taking cholesterol-lowering medication. Although participants were explicitly allowed to enter the study if they were taking blood pressure medication at a stable dose for at least 2 months and these medications did not change during the study, it is not clear whether these medications may have impacted cardiovascular responses to the study intervention or in turn study outcomes that could be influenced by cardiovascular functioning.

Baseline Differences in Vitamin D

The two groups were generally well-matched for background diet, including for flavonoid intakes measured, with the main exception being a significant difference in baseline vitamin D intake favouring the cranberry group. A difference in this nutrient is of particular note, since there has been a growing body of evidence suggesting that vitamin D could be protective against age-related neurodegenerative conditions (Koduah et al., 2017). Dietary intake of vitamin D is particularly of concern in areas of the world where the UVB-synthesis in skin from sunlight exposure is insufficient to meet physiological needs, which includes the UK (Rhodes et al., 2010; Zgaga et al., 2011), where vitamin D deficiency is especially prevalent. Indeed, although the dietary intake of vitamin D significantly differed between groups in this sample at baseline, both groups were on average intaking less (3.49-5.01ug/day) than the UK RNI of daily recommended vitamin D of 10mcg per day for older adults or the Institute of Medicine's 2011 proposed RDA of 15ug per day for individuals aged up to 70 years, and 20 ug per day for individuals older based on conditions of minimal sun exposure (Ross et al., 2011), so it is unlikely that the higher baseline levels of dietary vitamin D intake in the cranberry group conferred a neuroprotective advantage compared to the placebo group.

Impact of Omega-3 fatty acids

Omega-3 fatty acids have received considerable attention in the context of nutrition and ageing regarding their possible role in reducing the risk of age-related cognitive decline, and are proposed to be one of the key nutrients underpinning the benefits of a Mediterranean style dietary pattern (Barberger-Gateau et al., 2007). Indeed, observational studies such as the Three City Study showed that higher blood levels of the long-chain omega-3 fatty acid eicosapentaenoic acid (EPA) were associated with lower dementia risk (Samieri et al., 2008), and there have also been findings of higher blood levels of DHA to be associated with lower dementia risk (Y. Zhang et al., 2016). Clinical trials of omega-3 fatty acids involving healthy

older adults however have produced less clear benefits to cognition (van de Rest et al., 2008), with improvements to cognitive performance possibly limited to specific domains in the absence of dementia (Mazereeuw, Lanctot, Chau, Swardfager, & Herrmann, 2012). Despite this, one potential limitation of this study is that omega-3 fatty acids were not specifically measured using the background diet or screening questionnaires, and physiological levels were also not measured via serum samples. Therefore, it is unknown whether participant groups were balanced from the outset in terms of habitual intake, or furthermore whether these differences might have modulated the impact of the cranberry intervention on our outcomes of interest.

Background diet measurement

Participant's background diet was measured at baseline using a food frequency questionnaire where participants estimated their dietary intake of a range of food types over the past 2-3 months, to measure whether there were any differences in habitual dietary intakes at baseline that could modulate the impact of the cranberry supplementation. Although these food frequency questionnaires were comprehensive in measuring nutrient intake based on 169 common food types, there was no direct measurement of participants' actual dietary intake during the intervention which could have been achieved with the use of data collections methods such as food diaries. Furthermore, participants were recruited throughout the year, meaning that there could also have been an impact of seasonality on dietary intake. Participants would estimate their intake at baseline over the past 2-3 months, and since the intervention then took place over the next 3 months it is possible that significant shifts in diet took place during the study due to changes in food habits and availability throughout that period. Nonetheless, these self-report instruments rely on participants' subjective estimates of food intake, which have been suggested to be limited in their validity to reflect actual intake (Archer, Hand, & Blair, 2013) particularly when compared to biomarkers from blood samples

(Yuan et al., 2018). Furthermore, actual nutrient composition of food can be highly variable (Kuhnle, 2018), for example, even for fruit harvested from the same tree (Wilkinson & Perring, 1961) such that food composition data provide only an approximate marker of dietary patterns rather than a measurement of actual intake. One way of overcoming this limitation would be to supplement these self-report measures of nutrient intake with the measurement of nutrient biomarkers in blood samples, particularly for nutrients which could influence the outcomes of interest of this thesis (for example, vitamin D or omega-3 fatty acids as mentioned above).

Other potential mechanisms of action and causality

Other mechanisms such as chronic neuroinflammation, mitochondrial function and compromised vascular integrity and function are increasingly becoming understood to be key mechanisms which also contribute to age-related cognitive decline and neurodegenerative conditions and provide targets for interventions to curtail the disease processes contributing to age-related neurodegeneration (for review, see Flanagan, Muller, Hornberger, and Vauzour (2018)). Indeed, these mechanisms are also among the targets of nutritional interventions including those involving high-polyphenol foods, particularly in light of their suggested bidirectional relationships with the function of gut microbiota. The focus of the intervention discussed in this thesis was the impact of a long-term cranberry intervention on brain and gut microbial function, and as such the investigation of the impact on these additional mechanisms fell outside the scope of this project. However, these mechanisms are important to note as they form part of a complex interplay of processes alongside gut and neural health that are hypothesized to underpin age-related neurodegeneration.

Further to this, it is important to note that results from a singular study is not sufficient to fully explain the causal mechanisms which led to the observed changes presented here, or to eliminate all possible bias or confounds that may not have been accounted for by the study

design. Elements of the study design such as randomisation, concealment at recruitment of future treatment arm allocation and maintaining blindness to randomization for both participants and investigators throughout the intervention are key to reducing bias and to balance sample characteristics and outside influences on results. Although RCTs are currently the gold standard approach to allowing for results to be interpreted in a way that possible mechanisms of action can be discussed, there will always be limits to the amount of bias that can be accounted for by the study design, and other factors such as missing data and loss to follow-up can reduce the validity of the final analysis and the generalisability of results.

Furthermore, our understanding of the mechanisms discussed in this thesis, particularly the actions of the gut-brain axis and its potential impact on age-related neurodegeneration, are only just beginning to be understood and are based largely on correlational group analyses of changes in proportions of specific bacteria or microbial abundances in relation to disease onset or differences between groups for certain health outcomes. It is becoming clearer that differences in the structure and function gut microbiota can vary significantly within individuals dependent on a variety of intrinsic and extrinsic factors that are not fully understood. Advances in our understanding of neuropsychology and the specific domains that are sensitive to age-related cognitive decline and reflect patterns of the underlying neurodegeneration, as well as the development of higher granularity in functional neuroimaging and genomic sequencing and analysis techniques that can assist in approaching a better understanding of the complex relationships between the function of the gut and brain and how they might be modified by lifestyle interventions. As such, the results presented here are intended to be interpreted amongst a larger body of evidence to improve current understanding of the role that dietary polyphenols may have in supporting brain and gut microbiome function across the lifespan.

These findings are however certainly encouraging that sustained intake of cranberry over a 12-week period produced significant improvements in memory and neural function in conjunction to beneficial shifts in the structure and functioning of the gut microbiome in older adults who were cognitively healthy. The results presented in this thesis provide a promising basis for the investigation of the dosage and duration of dietary cranberry intake required to produce benefits to neural function and cognition in larger clinical trials, particularly to determine whether these changes then persist over the longer term. In particular, replication of these methods in a larger sample size might also produce more robust results, particularly with regards to neuroimaging and biomarker findings. Future studies investigating whether these changes translate to a clinical population of cognitively impaired adults in the context of neurodegenerative conditions such as mild cognitive impairment or AD is warranted based on these results, as well as determining whether these changes are sustained following the cessation of intake, for how long and to what degree.

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APPENDICES

Supplementary Methods 1. Identification and quantification of phenolic compounds in the cranberry powder by uHPLC–MSn analysis

Quantification of phenolic compounds in the cranberry powder provided to participants for this study was conducted by Professor Daniele del Rio and team at the University of Parma, Italy. Two packages of cranberry powder were extracted in triplicate as reported previously (Mena et al., 2016), with some modifications. Briefly, 50 mg of powder were added with 1 mL of 50% aqueous methanol acidified with formic acid (0.1%). The solution was vortexed for 1 minute, sonicated for 25 min, vortexed for 1 minute, and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was collected. The pellet was re-extracted twice using 0.5 mL of the same solvent, following the same procedure, and the three supernatants were pooled. Finally, extracts of the powders were diluted with acidified water (0.1 formic acid) (1:20, 1:10, 1:5 and 1:2) before uHPLC-MSn analysis.

The identification and quantification of the (poly)phenols present in cranberry powder was performed using an untargeted, full-scan, MS2/3 analysis. Powder extracts were analysed by ultra-high performance liquid chromatography (uHPLC) coupled with mass spectrometry (MS), using an Accela uHPLC 1250 apparatus equipped with a linear ion trap MS (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San José, CA, USA), fitted with a heated-ESI (H-ESI-II) probe (Thermo Fisher Scientific Inc.). Separation was carried out by means of Kinetex EVO C18 column (100 x 2.1 mm; 2.6 µm particle size; Phenomenex, CA, USA) installed with a precolumn cartridge (Phenomenex). Phenolic compounds were analysed in negative ionization mode, with the exception of anthocyanins, which were detected in positive ionization mode. For both methods, mobile phase, pumped at a flow-rate of 0.4 mL/min, consisted of a mixture of acidified acetonitrile (0.1% formic acid) (solvent A) and 0.1% aqueous formic acid (solvent B). Following 0.5 min of 5% solvent A in B, the

proportion of A was increased linearly to 51% over a period of 8.5 min. Solvent A was increased to 80% in 0.5 min, maintained for 2 min and then the start conditions were re-established in 0.5 min and maintained for 5 min to re-equilibrate the column (total run: 17 min). For negative mode, 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 4 kV, the capillary voltage was -42 V and tube lens voltage was -118 V. For anthocyanin analysis, the H-ESI-II interface was set to a capillary temperature of 275 °C and the source heater temperature was 300 °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 4.5 kV, and the capillary voltage and tube lens voltage were +20 and +95 V, respectively. Both in positive and in negative ionization mode, a collision induced dissociation (CID) equal to 35 (arbitrary units) was used to obtain MS2 and MS3 fragmentation. Quantification was performed using calibration curves built with pure standards, when available, or using the curves built with the most structurally similar compound.

Supplementary Table 1. Quantification of (poly)phenolic compounds in the cranberry powder. Individual (poly)phenols were quantified using uHPLC–MS^n analysis.

(9 grams, 2 sachets of 4.5 grams)	**Quantity (mg)**
Sum of Polyphenols	**522.6**
Total Proanthocyanidins (PACs) and catechins	**374.6**
Soluble PACs (c-PAC, DMAC)	280.8
Insoluble PACs (BuOH-HCl)	93.4
Epicatechin (LC-MS)	0.387
Catechin (LC-MS)	0.072
Total Flavonols	**19.7**
Quercetin-3-rhamnoside (LC-MS)	16.1
Quercetin (LC-MS)	2.3
Kaempferol (LC-MS)	0.045
Isorhamnetin (LC-MS)	0.34
Myricetin (LC-MS)	0.64
Laricitrin (LC-MS)	0.08
Syringetin (LC-MS)	0.23
Total Anthocyanins	**58.5**
Cyanidin-3-*O*-galactoside (LC-MS)	6.8
Cyanidin-3-*O*-arabinoside (LC-MS)	3.8
Peonidin-3-*O*-galactoside (LC-MS)	35.0
Peonidin-3-*O*-arabinoside (LC-MS)	12.9
Total Phenolic acids	**69.8**
Benzoic acid (LC-MS)	2.3
3,4-Dihydroxybenzoic acid (Protocatechuic acid) (LC-MS)	6.9
Cinnamic acid (LC-MS)	2.0
p-Coumaric acid (LC-MS)	1.9
Caffeic acid (LC-MS)	0.8
5-Caffeoylquinic acid (LC-MS)	4.7
Coumaroyl-glucose (LC-MS)	24.3

Feruloyl-glucose (LC-MS)	8.9
Feruoylquinic acid (LC-MS)	0.4
Sinapoyl-glucose (LC-MS)	3.6
Caffeoyl-glucose I (LC-MS)	2.4
Caffeoyl-glucose II (LC-MS)	4.8
Glucosyl-caffeoyl-glucose (LC-MS)	6.8

BuOH-HCl: butanol – hydrochloric acid; c-PAC: cranberry proanthocyanidins; DMAC: 4-(Dimethylamino)cinnamaldehyde; LC-MS: Liquid chromatography–mass spectrometry; PAC: proanthocyanidins.

Supplementary Table 2. Food intake guidance provided to all participants at baseline.

"*The following is a list of foods and beverages, which should be consumed less often during the study. Please try and check whether the foods to avoid are part of ready-made and packet meals. Other foods can be consumed without limitations."*

Food	Guidance for Intake	Alternative Foods
Berries, red/black/purple grapes or foods containing them	Please try to avoid during the study	banana, orange, mango, melon, , peach, pear, nectarine
Dark chocolate (e.g. >70% cocoa solids)	Please try to avoid during the study	milk chocolate, white chocolate
Cocoa/dark chocolate drink	Please try to avoid during the study	malted drink such as Horlicks
Tea (including herbal), *medium mug*	Try to limit to 4 or fewer mugs per day	malted drink such as Horlicks, coffee, sugar-free soft drinks, water

Supplementary Table 3. Mean regional perfusion at baseline and follow-up for cranberry and placebo groups, and significance of baseline and within group differences between baseline and follow-up for each region, and group x time interaction.

			Baseline perfusion (mL/min/100g)		**Baseline differences**	**Follow-up perfusion (mL/min/100g)**		**Group x time**
Region		**Group**	**M**	**SD**	***p***	**M**	**SD**	***p***
Thalamus	Left	Cranberry	51.578	9.598	.259	53.910	11.123	.129
		Placebo	44.431	8.918		42.314	8.823	
	Right	Cranberry	51.429	8.949	.260	52.374	11.111	.317
		Placebo	45.563	8.695		43.529	7.828	
Caudate	Left	Cranberry	41.625	5.822	.925	44.318	6.918	.132
		Placebo	40.566	7.649		39.641	5.954	
	Right	Cranberry	42.509	6.252	.960	44.972	7.868	**.049**
		Placebo	41.113	8.981		38.824	5.871	
Putamen	Left	Cranberry	46.107	7.083	.896	47.883	6.765	.181
		Placebo	44.737	7.647		43.018	4.808	
	Right	Cranberry	44.812	6.426	.674	46.849	8.228	.149
		Placebo	43.138	5.914		40.787	4.672	
Pallidum	Left	Cranberry	35.305	5.301	.356	36.777	5.391	.393
		Placebo	33.046	4.472		33.025	3.248	
	Right	Cranberry	37.185	5.668	.261	38.213	6.663	.096
		Placebo	34.163	5.073		31.734	4.044	
Brain Stem		Cranberry	40.585	8.405	.306	41.429	8.753	.292
		Placebo	36.219	5.600		34.643	6.047	
Hippocampus	Left	Cranberry	50.911	8.701	.234	53.381	10.996	.313
		Placebo	44.128	6.016		43.989	7.106	

	Right	Cranberry	51.699	9.006	.354	52.977	10.891	.249
		Placebo	45.607	6.451		43.878	5.710	
Amygdala	Left	Cranberry	47.780	7.803	.506	49.688	9.087	.237
		Placebo	43.417	5.377		41.844	6.023	
	Right	Cranberry	46.878	8.542	.638	49.685	10.671	.088
		Placebo	43.494	6.802		41.429	5.831	
Nucleus	Left	Cranberry	44.759	7.669	.637	46.807	7.658	.133
Accumbens		Placebo	42.644	8.098		40.543	7.401	
	Right	Cranberry	44.308	7.319	.929	48.015	9.337	**.034**
		Placebo	44.383	10.095		41.014	7.017	
Ventral	Left	Cranberry	42.059	6.513	.192	43.763	8.304	.057
Diancephalon		Placebo	37.505	5.119		35.154	5.521	
	Right	Cranberry	42.796	6.213	.259	43.460	8.202	.171
		Placebo	38.509	6.531		35.994	5.202	
Choroid Plexus	Left	Cranberry	50.926	14.603	.510	52.926	18.117	.321
		Placebo	41.436	11.535		40.234	7.995	
	Right	Cranberry	50.742	11.528	.374	53.262	15.572	.207
		Placebo	42.066	8.667		40.938	7.198	
Caudal Anterior	Left	Cranberry	53.247	7.600	.990	56.506	9.544	.207
Cingulate		Placebo	50.731	10.893		50.751	8.957	
	Right	Cranberry	53.930	7.917	.940	56.538	11.119	.224
		Placebo	51.802	11.037		51.337	9.003	
Caudal middle	Left	Cranberry	50.833	8.958	.747	52.708	10.544	.406
frontal		Placebo	48.799	11.437		48.882	10.233	
	Right	Cranberry	52.407	9.518	.715	55.648	13.623	.213
		Placebo	47.331	11.583		46.658	10.792	
Cuneus	Left	Cranberry	52.986	12.434	.390	55.851	14.406	.331
		Placebo	44.465	11.071		44.304	13.331	
	Right	Cranberry	55.969	13.078	.424	59.485	16.229	.332
		Placebo	49.640	10.730		49.275	9.835	

Entorhinal cortex	Left	Cranberry	43.825	7.062	.425	46.333	8.547	.162
		Placebo	38.689	4.819		38.240	5.911	
	Right	Cranberry	43.115	8.401	.461	46.675	9.917	**.030**
		Placebo	40.149	6.024		37.734	5.564	
Fusiform gyrus	Left	Cranberry	42.003	7.871	.494	44.464	9.811	.122
		Placebo	37.679	7.384		36.268	9.624	
	Right	Cranberry	42.817	8.650	.466	44.928	10.072	.271
		Placebo	39.117	6.503		38.386	5.024	
Inferior parietal	Left	Cranberry	47.184	7.824	.701	49.956	11.035	.265
gyrus		Placebo	43.726	8.267		43.177	11.028	
	Right	Cranberry	59.820	10.177	.962	63.779	15.628	.154
		Placebo	55.973	11.981		53.647	11.060	
Inferior temporal	Left	Cranberry	38.540	6.192	.616	40.294	7.726	.222
gyrus		Placebo	35.520	7.550		34.697	7.985	
	Right	Cranberry	41.638	7.774	.844	44.406	9.814	.115
		Placebo	39.635	7.608		38.100	6.961	
Isthmus cingulate	Left	Cranberry	66.546	13.345	.695	69.520	15.069	.302
		Placebo	61.114	10.806		59.208	12.476	
	Right	Cranberry	68.620	13.761	.845	71.803	16.386	.253
		Placebo	64.506	12.110		62.240	10.983	
Lateral occipital	Left	Cranberry	39.202	9.371	.879	42.773	11.749	.129
		Placebo	35.625	8.270		34.744	9.939	
	Right	Cranberry	45.882	11.182	.921	50.562	15.859	.123
		Placebo	41.984	9.160		40.652	8.124	
Lateral	Left	Cranberry	43.114	7.661	.837	44.847	7.216	.188
orbitofrontal		Placebo	40.917	5.183		39.854	5.459	
cortex	Right	Cranberry	42.739	7.765	.890	45.286	8.576	.089
		Placebo	41.205	5.714		39.276	6.397	
Lingual gyrus	Left	Cranberry	49.388	10.867	.318	51.645	12.398	.266
		Placebo	41.421	7.520		40.694	10.058	

	Right	Cranberry	50.806	10.420	.576	53.480	12.907	.225
		Placebo	45.920	9.364		44.870	7.006	
Medial	Left	Cranberry	41.078	7.943	.654	43.394	7.981	.115
orbitofrontal		Placebo	38.210	4.260		37.010	4.826	
cortex	Right	Cranberry	40.704	7.305	.799	42.796	7.850	.065
		Placebo	38.726	5.236		36.426	4.148	
Middle temporal	Left	Cranberry	46.394	6.380	.950	48.196	8.513	.266
gyrus		Placebo	44.526	8.315		43.453	8.535	
	Right	Cranberry	53.777	8.655	.838	56.461	11.884	.103
		Placebo	52.072	8.859		48.940	8.483	
Parahippocampal	Left	Cranberry	44.595	7.659	.245	46.262	9.611	.170
gyrus		Placebo	38.904	5.212		37.329	7.696	
	Right	Cranberry	44.581	7.982	.411	45.350	8.678	.310
		Placebo	39.701	5.334		38.526	4.421	
Paracentral gyrus	Left	Cranberry	61.648	10.858	.313	64.278	12.569	.378
		Placebo	54.794	9.812		55.410	7.592	
	Right	Cranberry	63.752	11.427	.383	67.322	14.730	.264
		Placebo	56.897	10.851		56.665	8.306	
Pars opercularis	Left	Cranberry	55.308	8.351	.684	56.906	9.674	.229
		Placebo	54.346	9.215		52.959	8.009	
	Right	Cranberry	57.554	8.166	.956	59.771	10.623	.168
		Placebo	55.450	9.317		53.019	7.739	
Pars orbitalis	Left	Cranberry	48.249	7.945	.368	49.454	8.426	.147
		Placebo	48.166	5.689		45.967	7.621	
	Right	Cranberry	50.413	7.010	.913	52.484	9.601	.134
		Placebo	49.480	6.763		46.806	7.974	
Pars triangularis	Left	Cranberry	51.841	8.423	.665	53.179	8.389	.294
		Placebo	50.467	7.694		49.272	7.846	
	Right	Cranberry	52.363	52.363	.703	54.465	9.283	.225
		Placebo	52.437	8.628		50.395	7.429	

Pericalcarine	Left	Cranberry	54.206	13.153	.311	55.924	15.300	.422
gyrus		Placebo	44.076	11.313		43.734	12.717	
	Right	Cranberry	56.682	13.398	.390	59.092	17.183	.511
		Placebo	49.551	11.586		50.065	10.309	
Postcentral gyrus	Left	Cranberry	47.319	7.669	.835	49.260	9.147	.404
		Placebo	45.345	7.656		45.503	8.657	
	Right	Cranberry	53.635	10.032	.846	56.484	13.291	.239
		Placebo	50.000	9.127		49.122	8.050	
Posterior cingulate	Left	Cranberry	69.584	10.615	.471	73.005	13.098	.305
		Placebo	62.915	13.411		62.978	11.728	
	Right	Cranberry	71.630	10.091	.557	75.673	13.394	.179
		Placebo	65.401	13.447		64.275	11.540	
Precentral gyrus	Left	Cranberry	50.074	8.062	.841	52.159	9.343	.322
		Placebo	47.759	8.797		47.856	8.982	
	Right	Cranberry	54.395	9.558	.642	57.293	12.748	.190
		Placebo	49.980	9.244		48.946	8.589	
Precuneus	Left	Cranberry	65.048	12.161	.394	68.248	15.603	.303
		Placebo	57.527	11.394		56.561	12.953	
	Right	Cranberry	66.277	13.042	.574	69.769	17.425	.359
		Placebo	59.796	12.338		59.729	12.065	
Rostral anterior	Left	Cranberry	52.766	10.183	.910	55.630	10.427	.192
cingulate		Placebo	50.362	9.350		49.547	8.104	
	Right	Cranberry	51.239	9.402	.677	53.613	10.447	.154
		Placebo	50.171	9.005		48.307	8.241	
Rostral middle	Left	Cranberry	51.581	9.785	.491	53.173	10.436	.306
frontal		Placebo	51.271	10.971		49.935	10.924	
	Right	Cranberry	51.296	9.418	.865	53.413	13.013	.234
		Placebo	49.402	10.022		47.830	10.093	
Superior frontal	Left	Cranberry	49.854	9.516	.860	51.644	10.106	.328
gyrus		Placebo	47.229	9.531		46.770	8.719	

	Right	Cranberry	50.401	9.438	.964	52.421	11.388	.194
		Placebo	47.418	9.950		46.018	8.638	
Superior parietal	Left	Cranberry	43.022	8.966	.703	46.786	12.981	.224
gyrus		Placebo	38.798	8.122		39.028	10.275	
	Right	Cranberry	49.990	11.498	.612	54.170	16.816	.226
		Placebo	45.014	11.217		44.597	11.239	
Superior temporal	Left	Cranberry	46.966	6.273	.744	49.081	7.259	.238
gyrus		Placebo	45.938	6.156		45.183	7.381	
	Right	Cranberry	52.328	7.999	.719	55.103	10.033	.082
		Placebo	51.418	7.240		48.596	7.194	
Supramarginal	Left	Cranberry	49.229	6.560	.760	51.203	8.007	.325
gyrus		Placebo	48.050	8.427		47.551	9.250	
	Right	Cranberry	59.367	9.102	.881	62.928	12.679	.104
		Placebo	56.949	11.830		54.386	9.487	
Transverse	Left	Cranberry	61.465	8.057	.729	65.014	9.404	.231
temporal		Placebo	60.435	8.226		60.360	9.284	
	Right	Cranberry	65.395	10.405	.940	67.556	11.845	.119
		Placebo	62.050	9.454		58.439	8.475	
Insula	Left	Cranberry	52.786	7.791	.779	56.297	9.309	.163
		Placebo	52.393	8.502		52.064	8.125	
	Right	Cranberry	55.707	8.726	.945	58.964	11.772	.056
		Placebo	53.947	7.570		50.853	7.000	

Supplementary Table 4. Classes present (>0.5%) at different relative abundances in human samples. Data presented are showing mean and standard deviation of the classes of bacteria in the different samples. Percentage of total representation is also provided for each group.

	Placebo baseline			**Placebo follow-up**			**Cranberry baseline**			**Cranberry follow-up**		
	Mean	**SD**	**%**	**Mean**	**SD**	**%**	**Mean**	**SD**	**%**	**Mean**	**SD**	**%**
Clostridia	54.6	10.5	60.9	53.0	10.9	59.1	49.7	10.0	56.4	47.3	11.9	53.5
Bacteroidia	20.4	9.3	22.8	20.4	10.5	22.7	22.3	10.1	25.4	21.0	8.5	23.8
Actinobacteria	3.5	3.4	3.9	5.4	6.2	6.0	4.5	11.2	5.1	4.6	9.3	5.2
Gammaproteobacteria	3.6	7.8	4.0	2.7	6.1	3.0	2.9	2.8	3.3	5.4	6.4	6.1
Coriobacteriia	2.2	1.8	2.4	2.4	2.3	2.7	2.0	2.3	2.3	2.7	1.7	3.0
Verrucomicrobiae	1.3	2.0	1.4	2.1	3.3	2.3	1.9	2.7	2.1	2.7	5.0	3.0
Bacilli	1.3	0.5	1.4	1.2	0.6	1.3	1.4	0.9	1.6	1.2	0.8	1.3
Negativicutes	1.0	0.7	1.1	1.0	1.1	1.1	0.9	0.8	1.0	1.3	1.6	1.5
Alphaproteobacteria	0.7	1.3	0.7	0.5	0.3	0.5	1.0	1.1	1.1	0.7	0.7	0.8

Supplementary Table 5. Differences in relative family abundances, , cranberry group baseline vs. follow-up (FDR-P < 0.2).

Family	***p***	**FDR-P**
Eggerthellaceae	0.000103961	0.02760876
Actinomycetaceae	0.000164636	0.027728672
Micrococcaceae	0.00036554	0.037371016
Atopobiaceae	0.000585173	0.04768734
RUG033	0.002562919	0.079297045
GCA-900066905	0.002122734	0.083875328
Pseudomonadaceae	0.00259163	0.089631963
Geodermatophilaceae	0.004925621	0.104657126
Mycobacteriaceae	0.004232282	0.107985819
CAG-239	0.004317854	0.109848711
Rhodocyclaceae	0.006746513	0.11486362
Phormidesmiaceae	0.007807377	0.116161529
Cyclobacteriaceae	0.00672045	0.117061146
CAG-288	0.006536444	0.121604959
Cellulomonadaceae	0.007871591	0.130915047
Weeksellaceae	0.008049648	0.130980764
QAMH01	0.008078719	0.132112677
Arcobacteraceae	0.011377503	0.146676778
GWC2-71-9	0.016690861	0.152786942
CAG-449	0.014656907	0.154197319
Microbacteriaceae	0.012873124	0.158107711
Solirubrobacteraceae	0.022220296	0.17333366
UBA2241	0.029470417	0.17371783
CAG-272	0.016732574	0.174802624
SM23-33	0.03282935	0.174925186
Chitinophagaceae	0.022497306	0.17712066
Nakamurellaceae	0.032657153	0.177303336
GWC2-55-46	0.040712536	0.177368092
UBA11471	0.019685437	0.178066879

CAG-313	0.019828508	0.178140498
CAG-314	0.020456955	0.181439178
Nostocaceae	0.023218121	0.182264444
Alcanivoracaceae	0.028595349	0.184210109
UBA1212	0.036220459	0.184246296
Flavobacteriaceae	0.023179566	0.189389779
Neisseriaceae	0.035229296	0.193253791
Lutisporaceae	0.034190811	0.195644027
Frankiaceae	0.030336959	0.196753392
Chloroflexaceae	0.046088144	0.197392254

Supplementary Table 6. Differences in relative genus abundances, cranberry group baseline vs. follow-up (FDR-P < 0.2).

Genus	***p***	**FDR-P**
RUG013	2.46E-05	0.017504184
Slackia	0.000117968	0.029765813
UBA9715	0.000184212	0.030073426
Pauljensenia	0.000175153	0.032648811
CAG-1427	0.000236228	0.033841526
Raoultibacter	0.000297125	0.035406477
Libanicoccus	0.000533035	0.040991633
Actinomyces	0.000460261	0.041037096
F0332	0.000671099	0.04411781
DTU033	0.00079124	0.045016697
UBA7748	0.000754209	0.045547478
Senegalimassilia	0.000657918	0.046186802
Olsenella_E	0.00076505	0.048329674
Corynebacterium	0.000941288	0.051849583
Chryseobacterium	0.001142138	0.055744878
Olsenella	0.00118864	0.059320164
Enteroscipio	0.001329647	0.062400433
An7	0.001658032	0.064151285
Arabia	0.001468763	0.064378394
UBA7477	0.002699767	0.078292329
UBA1409	0.003089041	0.080937504
Eggerthella	0.002406335	0.083655425
Rubneribacter	0.002796891	0.088087757
Algoriphagus	0.003756363	0.088977609
DNF00809	0.003270903	0.090438797

RUG033	0.00416485	0.095257625
CAG-724	0.00357761	0.095782234
CAG-841	0.003631569	0.097206786
GCA-900066905	0.003654828	0.097870474
Slackia_A	0.003879496	0.10035556
CAG-495	0.004367805	0.108307219
Prochlorococcus_A	0.005647738	0.11131594
Serratia	0.006675809	0.114303185
CAG-45	0.005204589	0.118297695
Atopobium	0.007108892	0.120243042
Olsenella_B	0.006190131	0.120778137
UBA4675	0.009354758	0.121824252
Pedobacter	0.007026339	0.122020661
CAG-603	0.006599866	0.129713881
Olsenella_C	0.007945979	0.136400952
CAG-314	0.009266163	0.139018426
CAG-313	0.008303162	0.13977364
RUG721	0.01190752	0.142930439
Bacillus_AA	0.012366511	0.143168384
CAG-632	0.009736546	0.144751482
Adlercreutzia	0.008398856	0.146004694
UBA4716	0.010997488	0.14838309
Olegusella	0.009411402	0.148432774

Phoenicibacter	0.010419169	0.148701792
QAMH01	0.009487597	0.149493286
Lancefieldella	0.009651401	0.151317639
Desnuesiella	0.016220115	0.152383354
Olsenella_D	0.011345942	0.152673926
Frigoribacterium	0.015376457	0.154657311
UBA737	0.010144259	0.157440852
Atlantibacter	0.012749945	0.157617398
CAG-521	0.015097867	0.159607728
UBA4636	0.012866308	0.162105032
UBA1212	0.018982057	0.165211762
Kosakonia	0.013716228	0.166045762
UBA1367	0.015555693	0.167373402
Clostridium_B	0.017705412	0.171995441
Hungatella	0.013032802	0.17475508
UBA3766	0.018747121	0.180802239
UBA10281	0.01644087	0.181243424
UBA11471	0.016407667	0.190014382
Blastococcus	0.021612448	0.19088119
UBA10677	0.018534704	0.195051394
Zag1	0.018753063	0.195253398
UBA1394	0.018548471	0.198309361

Supplementary table 7. Differences in relative species abundances, cranberry group baseline vs. follow-up (FDR-P < 0.2).

Species	***p***	**FDR-P**
RUG013 sp001486445	5.19E-05	0.04442455
Actinomyces naeslundii	0.00012475	0.0524133
Corynebacterium durum	0.00032207	0.05628574
Pauljensenia bouchesdurhonensis	0.00031587	0.05739078
F0332 sp000466165	0.00050439	0.05786175
Collinsella phocaeensis	0.00044988	0.05880601
Slackia heliotrinireducens	0.00046723	0.05892854
Actinomyces sp000195595	0.00057887	0.05898997
Collinsella stercoris	0.00040096	0.05909592
Senegalimassilia sp002431805	0.00051919	0.05923402
Olsenella sp001189515	0.00072905	0.06201393
Collinsella intestinalis	0.00075981	0.0625971
Pauljensenia keddieii	0.00087562	0.06341972
Collinsella tanakaei	0.00085427	0.06358713
Senegalimassilia anaerobia	0.00087106	0.06445149
Olsenella_E sp002159495	0.00146351	0.06459542
Pauljensenia sp000278725	0.0010402	0.06551392
Libanicoccus massiliensis	0.00108463	0.0655869

Pauljensenia sp000466265	0.00109888	0.06614641
Collinsella aerofaciens_F	0.0011025	0.06619913
Actinomyces oris_E	0.00119394	0.06674383
Actinomyces massiliensis	0.00121008	0.06688281
Collinsella vaginalis	0.00131264	0.06767226
Collinsella aerofaciens	0.00133317	0.06798568
Raoultibacter timonensis	0.0013568	0.06831339
Pauljensenia sp000185285	0.00145362	0.06929262
Collinsella sp002437815	0.00147635	0.06943785
Actinomyces graevenitzii	0.00162293	0.0703976
Collinsella sp002305035	0.00162032	0.07041808
Collinsella sp000763055	0.00172324	0.07158136
Lachnospira sp000436475	0.00173723	0.07180078
Actinomyces dentalis	0.00178659	0.07234606
CAG-313 sp000433035	0.00205826	0.07381599
RC9 sp000431015	0.0020541	0.07446347
Slackia exigua	0.00210739	0.07523212
Actinomyces gerencseriae	0.00211079	0.07553858
CAG-495 sp000436375	0.00227233	0.0768865
Actinomyces viscosus	0.00228413	0.07717678
Olsenella_E provencensis	0.0023476	0.07720281
UBA9715 sp002371495	0.00254577	0.07852086

Actinomyces sp000220835	0.00245862	0.07860074
Rubneribacter sp002159915	0.00272127	0.08107129
F0332 sp001652275	0.00320235	0.08141976
Enteroscipio rubneri	0.00278409	0.08145591
Collinsella bouchesdurhonensis	0.00280062	0.08193993
Collinsella ihuae	0.00285374	0.08238641
GCA-900066905 sp900066905	0.00292488	0.0829508
Actinomyces oris_C	0.00297697	0.08304476
CAG-495 sp001917125	0.00294284	0.08337322
Collinsella sp002232035	0.00303379	0.08392679
Actinomyces johnsonii	0.00314182	0.08474332
Pauljensenia hongkongensis	0.0031977	0.08521639
Enteroscipio sp000270285	0.00338544	0.08700345
Collinsella aerofaciens_E	0.00335396	0.08721922
Eggerthella timonensis	0.00351171	0.08846757
Raoultibacter massiliensis	0.00355883	0.08867432
Collinsella sp000434535	0.00351193	0.08871331
Olsenella_E mediterranea	0.00400408	0.08883379
Arabia massiliensis	0.00362387	0.08939606
UBA7748 sp900314535	0.00385874	0.08985944
Pauljensenia odontolyticus_A	0.00371252	0.09010997
CAG-724 sp003524145	0.00379001	0.09072992

Eggerthella lenta	0.00401698	0.09324034
CAG-448 sp000433415	0.00419842	0.09438192
Pauljensenia cellulosilytica	0.00427471	0.09527857
CAG-1427 sp000436075	0.00424362	0.09541592
Pauljensenia meyeri	0.00456186	0.09548738
Olsenella uli	0.00433756	0.09549054
Sutterella wadsworthensis_A	0.00444029	0.09571273
Collinsella aerofaciens_A	0.00434638	0.0962643
Collinsella sp003487125	0.00437851	0.09658774
CAG-521 sp000437635	0.00532402	0.09894788
Eubacterium_F sp000434115	0.00473466	0.09931147
Collinsella provencensis	0.00491637	0.10063634
Olsenella sp001457795	0.00560919	0.10235485
Pauljensenia sp001838165	0.00505418	0.10250623
UBA7597 sp003448195	0.00521654	0.10328979
Collinsella sp002391315	0.00510708	0.103343
DNF00809 sp000814825	0.0057944	0.10839127
Pauljensenia sp000411415	0.0056882	0.10850904
RUG033 sp900314665	0.0065298	0.11201718
CAG-314 sp000437915	0.00673256	0.11253224
CAG-45 sp900315735	0.00693965	0.11726942
Atlantibacter hermannii	0.00757632	0.11787533

Eubacterium_G ventriosum	0.0066832	0.11814988
Pauljensenia odontolyticus	0.00730752	0.11821795
Slackia_A piriformis	0.00681591	0.11843626
CAG-1427 sp000434775	0.00740253	0.11940512
DTU033 sp003519645	0.00814924	0.12099512
CAG-841 sp000437375	0.0073115	0.12102272
Coprococcus sp900066115	0.00730035	0.12357759
Adlercreutzia equolifaciens	0.00738139	0.12429838
Adlercreutzia mucosicola	0.00779369	0.12733625
Olsenella_E sp002160255	0.00929001	0.12864079
Enorma phocaeensis	0.00955244	0.12930261
UBA2882 sp900317505	0.008702	0.12943879
Eubacterium_F sp002431395	0.00829088	0.13072116
UBA4675 sp002405165	0.01040792	0.13122769
Butyrivibrio fibrisolvens_C	0.00958991	0.13202406
CAG-180 sp002490525	0.009614	0.13292539
UBA7477 sp001940995	0.00947002	0.13405004
Rubneribacter badeniensis	0.00887658	0.13626257
Pauljensenia odontolyticus_B	0.00941748	0.13952287
Gordonibacter massiliensis	0.00991157	0.14479281
Phoenicibacter massiliensis	0.01102397	0.14645696
An7 sp002159335	0.01192018	0.14687274

Actinomyces oris_A	0.01066092	0.14699944
CAG-1427 sp000435675	0.01042076	0.14812068
Lachnospira sp003537285	0.01072069	0.14873454
UBA4636 sp002405915	0.01204831	0.15069837
Eubacterium_R sp000434995	0.01078069	0.15147625
Ruminococcus flavefaciens_O	0.01481745	0.15582452
Olsenella_E sp003150175	0.01260505	0.15685628
CAG-1435 sp003537755	0.01220268	0.15781272
UBA1367 sp002449555	0.01597477	0.1596082
Olsenella_E sp002407785	0.01522093	0.16127089
UBA11471 sp000434215	0.0129545	0.16456706
Olsenella_D sp002331575	0.01397773	0.16644267
Eubacterium_R sp002494125	0.01709703	0.16888203
Desnuesiella massiliensis	0.01827086	0.17023627
Olsenella_B sp000752675	0.01410056	0.17069414
RUG721 sp900321745	0.01676022	0.17380291
QAMH01 sp003149935	0.01423267	0.17497596
Pauljensenia turicensis	0.015564	0.17566173
Duodenibacillus massiliensis	0.01698401	0.17629942
Olsenella_B sp900111695	0.01854129	0.17915059
CAG-603 sp900314525	0.01726201	0.18168153
UBA10677 sp003533505	0.01616664	0.18218889

14-2 sp001940225	0.01696054	0.18238637
CAG-841 sp002479075	0.01896025	0.18272495
UBA7748 sp900313905	0.01859065	0.18287568
Serratia proteamaculans_B	0.02186612	0.18396226
Actinomyces sp001278845	0.01703743	0.1845861
CAG-45 sp900066395	0.01547964	0.18513081
CAG-791 sp900315055	0.0191128	0.18583952
Corynebacterium pyruviciproducens	0.02038614	0.18632846
Lachnospira eligens	0.01623976	0.18795127
UBA10281 sp000437515	0.01870016	0.19191429
Citrobacter_B koseri	0.01821825	0.19212218
Olsenella_C umbonata	0.0175175	0.1928425
TF01-11 sp003524945	0.01755199	0.19717117
Bifidobacterium animalis	0.01719299	0.19718024
Pauljensenia odontolyticus_C	0.01856873	0.1989477
UBA4716 sp002438865	0.02033518	0.19980449