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NEUROBIOLOGICAL IMPACTS OF KIWIFRUIT CONSUMPTION IN A PIG MODEL AND ITS EFFECTS ON SLEEP AND MOOD IN YOUNG ADULTS

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Alexander Putra Kanon

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To Tiffanie and Theodore

thank you for continuously reminding me

"That I can do all things 4:13".

Here I present my PhD thesis to you.

ABSTRACT

Kiwifruit (KF) positively impacts gut health, specifically in alleviating gastrointestinal symptoms and improving laxation. Emerging evidence also suggests that consuming KF influences sleep and mood, with most studies indicating improvements in subjective measures of these attributes. Previous research has explored the mechanisms behind these effects using *in vitro* and rodent models, which have considerable differences to human physiology. This study explores the impact of New Zealand KF on various brain physiological aspects in animal models and humans. It explores the antioxidant neuroprotective potential of KF, examines alterations in the gut microbiome composition and bioamine concentrations, analyses temporal bioamine concentration effects in plasma and brain regions, and assesses the acute effects on human sleep quality and mood.

Findings reveal that in one week, consumption of both green and gold KF reduced oxidative potential in plasma, increased concentrations of 5-Hydroxyindoleacetic Acid (5HIAA, a serotonin metabolite), and induced changes in the abundance of specific microbial genera along the colon of adult pigs, a more representative model of human physiology. Furthermore, green KF enhances antioxidant protective potential in plasma and various brain regions, while gold KF elevates plasma vitamin C levels and tends to reduce acetylcholinesterase activity across the entire brain. Temporal effects highlight distinct patterns in metabolite concentrations between green and gold KF, with γ-Aminobutyric Acid (GABA) and serotonin exhibiting notable interactions in different brain regions.

Good and poor sleepers consuming KF before sleep had improved sleep quality and mood. Fresh KF facilitates easier sleep onset for good sleepers, while freeze-dried KF leads to increased ease of awakening in the morning for poor sleepers. Notably, both forms of KF increase the urinary excretion of 5HIAA and reduce feelings of sleepiness while increasing alertness. The inclusion of the fruit skin appears to increase improvements in sleep quality, suggesting a more noticeable effect. These studies provide valuable insights into the neurobiological effects of KF and support its potential as a functional food to improve sleep in humans.

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LIST OF ABBREVIATIONS

5HIAA	5-Hydroxyindoleacetic Acid
5HT	Serotonin
5HTP	5-Hydroxytryptophan
AA	Amino Acids
ACE	Angiotensin-Converting Enzyme
Ach	Acetylcholine
AChE	Acetylcholinesterase
ACTH	Adrenocorticotrophic Hormone
ADMA	Asymmetric Dimetylarginine
AGEs	Advanced Glycation End Products
AgII	Angiotensin II
ANCOMBC	Analysis of the Composition of Microbiomes with Bias Correction
ANOVA	Analysis of Variance
ANS	Autonomic Nervous System
BAP	Biological Antioxidant Potential
BBB	Blood-Brain Barrier
BCAA	Branched-Chain Amino Acids
BCFA	Branched-Chain Fatty Acids
BChE	Butyrylcholinesterase
BDI	Beck Depression Inventory
BDNF	Brain-Derived Neurotrophic Factor
BMI	Body Mass Index
BW	Body Weight
CBF	Cerebral Blood Flow
CCK	Cholecystokinin
CNS	Central Nervous System
CRF	Corticotrophin-Releasing Factor
CRP	C-Reactive Protein
CTx	C-Telopeptide of Type I Collagen
CVD	Cardiovascular Disease
DPPH	N-E-(Hexanoyl)-Lysine And 2,2-Diphenyl-1-Picrylhydrazyl
d-ROMS	Diacron-Reactive Oxygen Metabolites
EAA	Essential Amino Acids
EDTA	Ethylenediaminetetraacetic Acid
EEG	Electroencephalogram
ENS	Enteric Nervous System
ESS	Epsworth Sleep Scale
FDR	False Discovery Rate
FRAP	Ferric Reducing Antioxidant Potential
GABA	γ-Aminobutyric Acid
GI	Glycaemic Index
GIT	Gastrointestinal Tract

GLUT2	Glucose Transporter 2
GPx	Glutathione Peroxidase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
HAM-D	Hamilton Rating Scale for Depression
HDL	High-Density Lipoprotein
HPA	Hypothalamic-Pituitary-Adrenal
HSS	High Strength Silica
IL-10	Interleukin 10
IL-6	Interleukin 6
ISI	Insomnia Severity Index
Keap1	Kelch-Like ECH-Associated Protein 1
KF	Kiwifruit
KISP	Kiwifruit Industry Strategy Project
LCMS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LDH	Lactate Dehydrogenase
LDL	Low-Density Lipoprotein
L-DOPA	3,4-Dihydroxyphenylalanine
LNAA	Large Neutral Amino Acids
LPS	Lipopolysaccharide
LSEQ	Leeds Sleep Evaluation Questionnaire
MAO	Monoamino Oxidase
MMSE	Mini-Mental Status Exam
MoCA	Montreal Cognitive Assessment
MRI	Magnetic Resonance Imaging
MRM	Multiple Reaction Monitoring
NEAA	Non-Essential Amino Acids
NF-κB	Nuclear Factor Kappa B
NO	Nitric Oxide
NREM	Non-Rapid Eye Movement
Nrf2	Nuclear Factor Erythroid 2-Related Factor
NZKMB	New Zealand Kiwifruit Marketing Board
OPA	Oxidative Potential Assay
ORAC	Oxygen Radical Absorbing Capacity
PANAS	Positive And Negative Affect Schedule
PCA	Principal Component Analysis
POMS	Profile Of Mood States
PSQI	Pittsburgh Sleep Quality Index
PVN	Paraventricular Nucleus
RAS	Renin–Angiotensin System
REM	Rapid Eye Movement
ROS	Reactive Oxidative Species
SCFA	Short-Chain Fatty Acids
SCN	Suprachiasmatic Nucleus

SDMA	Symmetric Dimethylarginine
SGLT1	Sodium-Glucose Co-Transporter 1
SOD	Superoxide Dismutase
SPE	Single Point Entry
sPLS-DA	Sparse Partial Least Squares Discrimination Analysis
SSS	Stanford Sleepiness Scale
TBARS	Thiobarbituric Acid
TNF-α	Tumor Necrosis Factor Alpha
ucOC	Uncarboxylated Osteocalcin
UPLC	Ultra Performance Liquid Chromatography

LIST OF PUBLICATIONS AND PRESENTATIONS

Published peer-reviewed papers

Kanon AP, Giezenaar C, Roy NC, McNabb WC, Henare SJ. Acute effects of fresh versus dried Hayward green kiwifruit on sleep quality, mood, and sleep-related urinary metabolites in healthy young men with good and poor sleep quality. Front Nutr. 2023;10:1079609. Published 2023 Mar 14. doi:10.3389/fnut.2023.1079609

Kanon AP, Giezenaar C, Roy NC, Jayawardana IA, Lomiwes D, Montoya CA, McNabb WC, Henare SJ. Effects of Green and Gold Kiwifruit Varieties on Antioxidant Neuroprotective Potential in Pigs as a Model for Human Adults. Nutrients. 2024; 16(8):1097. https://doi.org/10.3390/nu16081097

Publications in preparation

Kanon, A. P., Giezenaar, C., Jayawardana, I. Francis, M., A., Montoya, C. A., Fraser, K., Roy, N. C., McNabb, W. C., & Henare, S. J. (submitted to Nutritional Neuroscience) The temporal bioaminergic effects of actinidin in kiwifruit (*Actinidia deliciosa* cv. Hayward) on plasma and brain regions in growing pigs as an adult human model.

Kanon, A. P., Giezenaar, Roy, N. C., McNabb, W. C., & Henare, S. J. (in preparation for submission to Journal of Functional Foods) A narrative review of the neuroprotective potential of kiwifruit.

Kanon, A. P., Giezenaar, Roy, N. C., McNabb, W. C., & Henare, S. J. (in preparation to be submitted to Nutrients) A review of the clinical health benefits of kiwifruit.

Kanon, A. P., Roy, N. C., Mullaney, J., Giezenaar, C., Jayawardana, I. A., Francis, M., Montoya, C. A., Fraser, K., McNabb, W. C., & Henare, S. J. (in preparation for submission to Microorganisms) Effects of short-term green and gold kiwifruit supplementation on

the microbiota composition in the colon and metabolites in plasma and brain in a growing pig model.

Conference presentations

Kanon, A. P., McNabb, W. C., Balan, P., & Henare, S. J. Consumption of kiwifruit extract for improving health. Riddet Institute Annual Symposium, Wellington, New Zealand, 10 – 12 July 2018. (Poster presentation).

Kanon, A. P., The Kiwi Dream: investigating the sleep-promoting effects of NZ kiwifruit. Riddet Institute Visualise Your Thesis Competition 2019. Palmerston North, New Zealand, 22nd July 2019. (Video Presentation).

Kanon, A. P., Balan, P., McNabb, W. C., Roy N. C., & Henare, S. J. The Kiwi Dream: investigating the sleep-promoting effects of NZ kiwifruit. Riddet Institute Annual Symposium. Rotorua, New Zealand, 29 September 2019. (Oral presentation - awarded Best Student Presentation).

Kanon, A. P., Balan, P., McNabb, W. C., Roy N. C., & Henare, S. J. Kiwifruit: Sleep Superfood?. The 5th International Conference on Food Structures, Digestion and Health Conference. Rotorua, New Zealand, 30 September 3 October 2019. (Poster presentation).

Kanon, A. P., Balan, P., McNabb, W. C., Roy N. C., Chow, C. & Henare, S. J. Kiwifruit: Sleep Superfood? A study methodology. Sleep DownUnder 2019 Conference. Sydney, Australia, 16-19 October 2019. (Poster presentation).

Kanon, A. P., Giezenaar, C., Roy N. C., McNabb, W. C., & Henare, S. J. Can a single intake of kiwifruit improve sleep quality and mood? Riddet Institute Annual Symposium, Wellington, New Zealand, 7-9 April 2021. (Poster presentation – awarded best poster).

Kanon, A. P., Giezenaar, C., Roy N. C., McNabb, W. C., & Henare, S. J. Can eating a kiwifruit in the evening improve sleep quality and mood? The 6th International Conference on Food Structures, Digestion and Health Conference. Online, 16-19 November 2021. (ePoster presentation – awarded best poster).

Kanon, A. P., Giezenaar, C., Roy N. C., McNabb, W. C., & Henare, S. J. Acute evening consumption of green kiwifruit in young men enhances waking alertness, mood and increases 5-hydroxyindoleacetic acid in urine. Nutrition Society of New Zealand Annual Conference. Online, 2–3 December 2021. (Online oral presentation).

Kanon, A. P., Giezenaar, C., Roy N. C., McNabb, W. C., & Henare, S. J. Can a single intake of kiwifruit improve sleep quality and mood? High Value Nutrition Foodomics Conference, Auckland, New Zealand, 8-9 September 2022. (Poster presentation).

Kanon, A. P., Giezenaar, C., Roy N. C., McNabb, W. C., & Henare, S. J. Acute effects of green kiwifruit on sleep quality and mood in healthy males. High Value Nutrition Foodomics Conference, Wellington, New Zealand, 19-21 March 2024. (Oral presentation).

CHAPTER 1. Introduction and project overview

1.1 Background

Kiwifruit (KF) is New Zealand's leading horticultural export, pivotal in the country's flourishing economy. In 2022, the industry witnessed remarkable growth, with exports surpassing $2.9 billion, marking a 15% increase from 2020. This success highlights the sector's economic resilience and capacity to meet the surging global demand for this nutritious fruit, evidenced by a 20% rise in sales volumes. The industry's accomplishment bolsters New Zealand's economic standing and showcases the nation's adaptability and competitiveness in the global fruit trade. As one of the world's foremost KF exporters, New Zealand is primarily recognised for the ‘Hayward’, ‘SunGold’ and ‘RubyRed’ varieties marketed under the Zespri brand. KF is marketed for being nutritious, tasty, and has a unique appearance. It has become more popular over the years and has prompted numerous research programmes to unravel the health benefits of KF (1). An overview of health benefits is depicted in Figure 1.1.

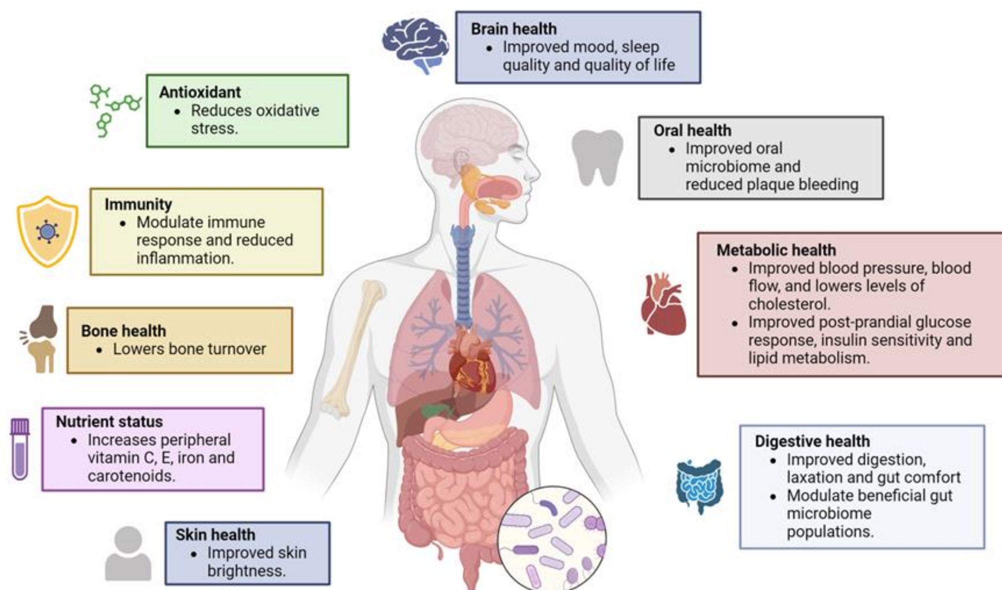


Figure 1.1 Overview of KF health benefits. Figure Created with BioRender.com.

An area of growing interest is the exploration of neurobiological or brain health effects, specifically the neurochemistry and connectivity within the brain structure that contribute to the overall well-being and optimal functioning of the brain. The brain is crucial for cognitive functions such as learning, memory, decision-making, and emotional and sleep regulation which are also influenced by genetics, lifestyle choices, and environmental elements. Adequate nutrition, regular physical exercise, and sufficient sleep also support neurobiological health. Conversely, factors like chronic stress, poor dietary habits, lack of exercise, and substance abuse can adversely impact neurobiological health, potentially leading to cognitive decline, mood disorders, poor sleep, and other neurological conditions. The elaborate interplay of age, genetics, neurotransmitters, gut microbiome, hypothalamic-pituitary-adrenal (HPA) axis activity, inflammation, oxidative stress, cardiovascular function, blood flow, and glycaemic control collectively influence brain health (2). Diet and certain foods may contribute to or influence aspects that collectively shape the brain's overall health.

KF has been shown to benefit mood (3, 4) and sleep quality (5-7). Additionally, as highlighted in Figure 1.1, KF may contribute to various factors that influence the underlying mechanisms of brain function. For instance, KF has demonstrated the ability to modulate neurotransmitter concentrations (8), positively affecting glycaemic control (9), and may influence the HPA axis (10). Clinical studies further indicate improvements in gut health (11) and the growth of beneficial gut microbes (12) in response to KF. This positive influence extends to mitigation of oxidative stress (13) and inflammation (14, 15). The evidence shows that KF complexly impacts different factors contributing to optimal brain function. Studies using KF have focused on humans or animals with health

challenges or external stressors (10, 16). Limited information is available regarding the effects of KF in healthy subjects or animals.

This thesis aims to explore the effects of consuming KF on specific factors influencing brain function, focusing on antioxidants, the gut microbiome, and neurotransmitters. Additionally, the study will investigate the acute impact of consuming KF on sleep and mood.

1.2 Research objectives

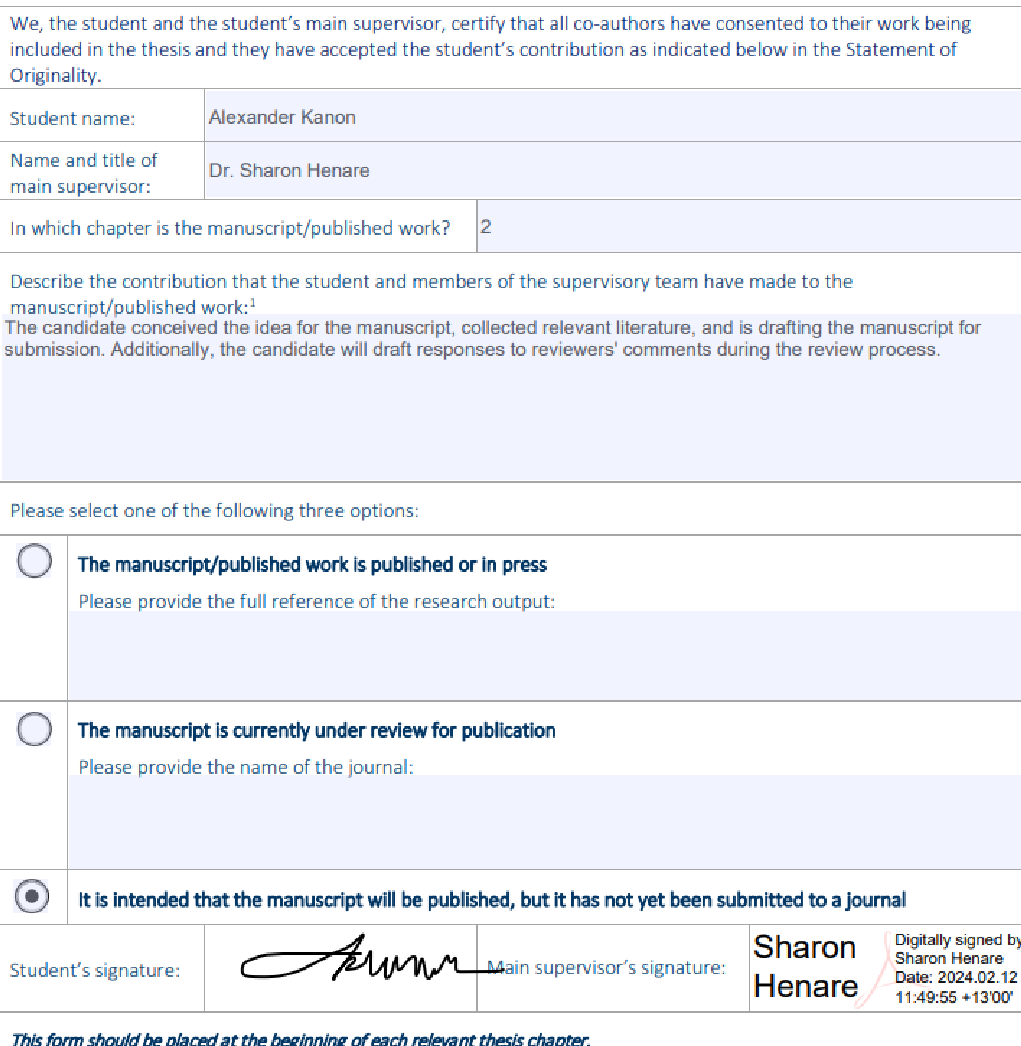
This research thesis project is a component of an industry initiative undertaken by Alpha Group, a nutraceutical company based in New Zealand, specialising in producing natural products tailored to the Chinese market. Zespri International, a cooperative consortium of KF growers in New Zealand, also provided in-kind support. The project underwent a significant realignment in response to disruptions caused by the COVID-19 pandemic. The primary focus transitioned towards a study of the influence of KF on sleep quality.

Complications arising from the pandemic-induced lockdowns rendered the initially planned clinical trials unfeasible. In response, the research strategy utilised samples from an alternative research project, which aimed to understand the potential of KF in mitigating the production of immunogenic peptides from gluten. Consequently, this doctoral project underwent a further adjustment with the primary objective of comprehending the neurobiological effects of KF consumption.

This research hypothesised that *Actinidia Deliciosa*, a green KF-containing the actinidin enzyme, would provide additional brain health benefits compared to a gold KF variety that was devoid of actinidin. An additional hypothesis was that green KF skin could have added benefits to sleep and mood due to the added polyphenols. The specific research objectives are outlined as follows:

1. To examine the differences between green and gold KF consumption on markers of oxidative stress and antioxidant systems in peripheral plasma and four brain regions in a growing pig model (Chapter 3).
2. To examine the differences between green and gold KF consumption on the colonic bacterial population and metabolites in plasma and four brain regions in a growing pig model (Chapter 4).
3. To characterise the temporal postprandial bioaminergic response to green kiwifruit that contains actinidin compared to gold KF that does not contain actinidin on peripheral plasma and four brain regions in a growing pig model (Chapter 5).
4. To determine the acute effect of an evening supplementation with fresh green KF or whole freeze-dried green KF on sleep quality, mood, and urinary metabolites in healthy males with good and poor sleep quality (Chapter 6).

Statement of contribution



STATEMENT OF CONTRIBUTION
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1

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May 2023

CHAPTER 2. Literature review

2.1 KF: cultivars, nutrient composition, and health benefits

Kiwifruit (KF), belonging to the genus *Actinidia*, is a woody, dioecious plant. *A. deliciosa* is one of the most prevalent species among the cultivated KFs. The first KF seeds were introduced to New Zealand in 1904, originating from Yichang, China, courtesy of a teacher named Isabel Fraser (17). In 1927, a New Zealander, Hayward Wright, bred the cultivar known today as the 'Hayward,' which has since become the standard KF globally, constituting 90% of the total production. Originally sold as the Chinese gooseberry or Yang Tao, the fruit acquired its current name, KF, in 1959 (18).

From the 1980s onwards, KF production in New Zealand experienced significant growth. By 1987, the quantity of exported goods surged from 22,000 tonnes in 1981 to 203,000 tonnes. This surge, however, resulted in a decline in grower returns, dropping from $7.81 per tray in 1981 to $3.00 in 1987. Faced with an impending equity crisis, the industry contemplated a Single Point Entry (SPE) system to regulate product management and sales. A referendum ensued, establishing the New Zealand Kiwifruit Marketing Board (NZKMB) by late 1989. The board replaced the previous multi-export regimes and remains the current regulatory structure in New Zealand. In 1993, NZKMB underwent division into three entities, focusing on growers, operations, and marketing (branded as Zespri). In 2014, the Kiwifruit Industry Strategy Project (KISP) emerged to formulate strategies for achieving long-term market, strategic, and financial goals.

Currently, KF enjoys global popularity and is available in the market in various forms, including fresh, frozen, and processed. In 2011, the global area dedicated to KF cultivation was nearly 175,000 hectares, which has grown by 111,000 hectares over the past decade (19). The three leading KF producers are China, New Zealand, and Italy. In

2021, New Zealand ranked the second-largest producer, cultivating over 628,000 tonnes and contributing 14% of the global volume (19).

2.1.1 Cultivars and structure of KF

There are approximately seventy-five distinct varieties of KF, with the majority remaining understudied and only a few having undergone cultivation from the wild to establish new generations. Among the cultivated varieties, the most prominent are *A. deliciosa* 'Hayward' and *A. chinensis* 'Gold3' (marketed as Zespri 'SunGold' KF). They were initially believed to be the same species and are now recognised as distinct (20). Another commercially significant minor species is *A. argute*, marketed as kiwi berry, along with the introduced red KF, *A. chinensis*, in March 2019 (Figure 2.1).

All KF species manifest as perennial climbing vines, each exhibiting varying degrees of vigour. Properly stringing these vines, especially during peak production, is fundamental (18). Flowering commences once the vines reach 3-4 years of age, with growth in spring and bud break occurring diversely among taxa. Flowering takes place approximately two months post bud break, and depending on the species, the flowers display pentamerous or tetramerous configurations adorned with petals in shades of red, pink, white, yellow, or green (21).

Figure 2.1 Left: cross-section from different *Actinidia* species showing different colours and sizes. Right: the three dominant species currently marketed (L to R- Red, Gold, Green). Source (22)

KF fruits are classified as berries, featuring numerous tiny seeds embedded in the flesh in 3-5 clusters. KF characteristics exhibit considerable variation, including size, shape, skin and flesh colour, composition, flavour, skin texture, and hairiness (23). Despite these differences, the maturity, harvest, and storage timing remain relatively consistent. The structural composition of the KF comprises a core enveloped by a pericarp and skin, further divided into inner and outer pericarps, with the inner pericarp housing locules containing seeds (24).

2.1.2 Nutrient composition of KF

KF is recognised as a good source of essential nutrients, including dietary fibre, vitamin C, and folate. The nutrient adequacy score, calculated by averaging the percentages of recommended daily intake values for the nutrients outlined in the model (17 nutrients: protein, fibre, vitamin A, C, E, B1, B2, B3, B5, B6, B9, B12, calcium, iron, magnesium, potassium and zinc) (25), highlights KF's superiority compared to other fruits. Notably, the green and gold KF varieties scores are 12.5 and 13.7, respectively. In contrast, fruits like strawberries (source of vitamin C) and bananas (rich in fibre) are lower with scores of 8.0 and 4.9, respectively (26). Notably, the nutrient composition analysis primarily focuses on commercial varieties, with a specific focus on ‘Hayward’ (green KF) and Zespri’s Gold3 (‘SunGold’ KF). A detailed breakdown of the nutrient composition for the three main KF varieties available in New Zealand is presented in Table 2.1 (27).

Table 2.1 Proximate compositional analysis of green, gold and red KF (28).

	Nutrients	Unit/100 g	Zespri Green[1]	Zespri SunGold[1]	Zespri Red[1]
	Energy	kJ	202	215	232
	Water	g	82.2	81.5	80.4
	Protein	g	0.8	0.9	0.9
	Actinidin	mg	80-430	N/A	N/A
	Fat	g	0.6	0.3	0.4
	Carbohydrate	g	9.8	11.1	11.9
	Sugars, total	g	9.7	11	11.4
	Starch, total	g	0.1	0.1	0.5
	Dietary fibre	g	2.3	1.1	1.8
	Fibre, water-insoluble	g	1.8	0.8	1.3
	Fibre, water-soluble	g	0.6	0.3	0.5
Fat Soluble Vitamins	Vitamin A	µg	4	3	8
	Vitamin E (tocopherols)	mg	1.2	1.1	1.6
	Alpha-tocopherol	mg	1.1	1.1	1.6
	Beta-tocopherol	mg	0.08	0.03	0.05
	Gamma-tocopherol	mg	0.11	0.04	0.06
	Vitamin K	µg	15	6.5	6.8
Water Soluble Vitamins	Folate	µg	73	82	69
	Niacin	mg	0.41	0.47	0.3
	Riboflavin	mg	0.06	0.04	0.03
	Thiamine	mg	0.01	0.02	0.02
	Vitamin B_{12}	µg	0	0	0
	Vitamin B_6	mg	0.22	0.18	0.1
	Vitamin C	mg	87.8	152	189
Minerals	Calcium	mg	27	14	16
	Copper	mg	0.13	0.12	0.19
	Iron	mg	0.2	0.19	0.2
	Magnesium	mg	14	12	12
	Potassium	mg	300	298	338
	Selenium	µg	0.3	0.6	0.7
	Sodium	mg	1	1	1
	Zinc	mg	0.11	0.06	0.07
Carotenoids	Alpha-carotene	µg	1	1	3
	Beta-carotene	µg	26	20	49

[1] Source: New Zealand food composition database, 2021 N/A: Data not available

The nutrient composition of fruit exhibits variations based on species and variety, with additional factors such as environmental conditions, storage, ripening, and the analytical methods employed contributing to these differences (26). The comprehensive nutrient profile of KF, showcases its diverse range of nutrients that support various potential

health claims. Overall, KF is a rich folate and vitamin C source, providing potassium, vitamin B6, and vitamin E. Each KF variant boasts unique nutritional attributes, with green KF serving as a source of dietary fibre, gold KF renowned for its vitamin C content, and red KF distinguished for its vitamin C and anthocyanin levels.

Delving into the proteins and amino acids (AA) in KF (Table 2.2), the primary protein in green KF is actinidin, a cysteine protease enzyme (29). Other minor proteins include kiwellin and its peptides, kissper (30) and KiTH (29), along with a thaumatin-like protein (31). Actinidin has a broad pH activity range of 3-8 (17, 32) and exhibits resistance to pepsin degradation, remaining active until reaching the colon, suggesting the potential to aid digestion (33). Kiwellin, a cysteine-rich protein, is cleaved by actinidin to form KiTH and kissper (30). The biological functions of KiTH and kissper remain poorly understood. However, kissper might form ion-channel-like pores in the phospholipid membrane and may possess anti-inflammatory properties (34).

Green KF stands out for its richness in glutathione (GSH), arginine, and γ-aminobutyric acid (GABA), and moderate levels of serotonin (5HT), tryptophan, and tryptamine (Table 2.2) (35-38). GSH, a tripeptide, synergistically functions with vitamins C and E as a potent antioxidant, although its survival through digestion may be limited (39). Asparagine, arginine, and glutamine are conditional amino acids necessary for GABA synthesis, an inhibitory neurotransmitter. 5HT, derived from the essential amino acid (EAA) tryptophan, is also an inhibitory neurotransmitter, while tryptamine is a 5HT receptor agonist.

Table 2.2 Green KF minor protein components.

Nutrient (mg)	Amount per 100 g of green KF edible flesh
Asparagine[1]	0.17
Arginine and GABA[1]	7.7
Glutamine[1]	1.3
Glutathione[2]	22.5
Serotonin[3]	0.6 - 1.0
Tryptamine[3]	0.6 - 0.9
Tryptophan[3]	0.3 - 0.7

[1]Source: (35)
[2]Source: (36)
[3]Source: (37)(38)

In addition to being a rich source of essential nutrients, KF contains many non-nutrient phytochemicals, with polyphenols being particularly abundant, especially in the skin, as opposed to the flesh and seeds (Table 2.3) (40). This rich nutritional content in KF has prompted numerous studies exploring the potential health benefits associated with its consumption.

Table 2.3 Polyphenolic content of the different regions of 'Hayward' green and gold 'Jinlong' KF.

	Green KF			Gold KF		
Polyphenols[1] (mg/g)	Peel	Flesh	Seed	Peel	Flesh	Seed
Gallic acid	8.48	6.80		3.77	2.60	3.29
Protocatechuic acid	65.95	23.80	24.85	14.50	16.88	20.20
Catechin		61.16	45.34	169.41	17.45	33.70
P-hydroxybenzoic acid		16.50		46.42	4.75	70.69
Chlorogenic acid	108.32	1.89	23.25	223.39	29.79	48.43
Vanillic acid			14.53	103.36	37.42	
Caffeic acid	97.31		49.10			27.14
L-epicatechin	445.62	67.58			36.08	
Syringic acid	65.99	9.27	8.45	149.80	4.14	14.58
Vanillin			17.37			14.30
P-coumaric acid	142.69	0.71	53.38	24.20	4.86	6.00
Ferulic acid	37.29	0.53	6.36			
Rutin	24.33		22.10	76.77	11.27	32.3
Phlorizin	52.19		24.11			
Quercetin	54.04	33.16		168.29	100.85	106.49
Kaempferol	53.85	27.80	40.09	49.94	73.09	25.23
Total	1156.06	249.20	328.93	1029.85	339.18	402.35

[1]Source: (40)

2.1.3 Health benefits of KF

Extensive research has been dedicated to exploring the potential health benefits of consuming KF (Figure 1.1). This research spans various methodologies, including *in vitro*, *ex vivo*, and *in vivo* studies. Table 2.4 comprehensively lists all published human studies involving fresh KF and KF products, categorised based on the clinical endpoints under investigation. Additional detailed information on these studies can be found in APPENDIX A.

The range of effects studied is broad, encompassing digestive health (19 studies), impact on gut microbiota (4 studies), markers of oxidative stress (8 studies), markers of inflammation (5 studies), risk factors for metabolic diseases (19 studies), bone health (1 study), nutrient status (14 studies), oral health (5 studies), and neurobiological health (6 studies). Notably, 32 studies utilised *A. deliciosa*, 20 focused on *A. chinensis*, 7 incorporated both species and three did not specify the KF species used. This wealth of research highlights the multifaceted potential health benefits of KF consumption, shedding light on its impact on various physiological aspects. From digestive and metabolic considerations to psychological well-being and beyond, the diverse effects studied contribute to understanding the positive implications of integrating KF into dietary habits.

Table 2.4 List of KF studies investigating biological or clinical markers for pre-disease and disease conditions in human clinical studies. ↓= reduced ↑ = increased

Health condition	Effect of fresh KF or KF products supplementation on measures and markers for listed conditions
Digestive health	↓Bloating (41) ↓ Flatulence (42) ↓ Days of laxative use (43) ↓ Indigestion (11, 44) ↓ Constipation (11, 14, 41, 44-46)

	↑ Bowel movements (11, 41-43, 45, 47-50) ↓ Abdominal pain (11, 14, 42, 44, 48) ↑ Stool consistency (11, 41-43, 47, 49, 51, 52) ↓ CH_4 and H_2 breath production (53) ↓ Transit time (43, 51) ↑ Relaxation time of ascending colon (52)
Gut microbiota	↑*Faecalibacterium prausnitzii, Clostridiales, Dorea spp* (12) ↑*Lactobacilli and Bifidobacteria* (54) ↑ *Coriobacteriaceae* (55) No changes in microbial diversity (56)
Oxidative stress	↑ BAP ↓d-ROMs, ↑BAP/d-ROMs ratio (57) ↓ Lipid oxidation (13) ↓ Oxidative stress(10, 58) ↑ Antioxidant capacity (59, 60) No change in antioxidant measures (13, 15, 61) SOD enzyme (13) and lipid oxidation (15)
Inflammation	↓TNF-α (14, 15) ↓ Plasma hs-CRP (62) No change on IL-6, (14, 15, 61, 63) IL-10, (14) and CRP (13, 14, 63)
Risk factors for diabetes and cardiovascular disease (metabolic)	↑ Blood flow (48) Improved anthropometric measures (15, 55) Improved lipid markers(15, 60, 62, 64-66) ↓Blood pressure (15, 55, 67-69) ↓ Platelet aggregation (60, 66, 68, 70) ↓ ACE activity (68) and angiotensin II (AgII) (15) ↓Postprandial glycaemic responses (71-75) ↓ Hunger (72, 74)
Bone and skin health	↓ Bone turnover (56) ↑ Skin brightness (48)
Nutrient status	↑ Plasma vitamin C (4, 13, 55, 60, 61, 64, 76-79) ↑Neutrophil vitamin C (76) (80) ↑Urinary vitamin C (77, 80) ↑Skeletal muscle ascorbate (80) ↑Mononuclear cell ascorbate (80) ↑Vitamin E (3, 13, 64) ↑Serum D3 (3) ↑Serum ferritin (81)

	↑ Zeaxanthin (13, 81) ↑Lutein (13, 67, 81) ↑Urinary 5HIAA (8) Earlier peak ↑ of EAA, BCAA, leucine (82)
Oral Health	↓Bleeding, plaque, and attachment loss in teeth (79) ↓ Volatile sulphur compound concentration (83-85) ↓Tongue coating (84, 85) ↓Harmful oral bacteria (85, 86)
Neurobiological health	↑Quality of life (51) ↓ General and sports stress (7) ↓ Total mood disturbance and depression (3, 4) ↑ Sleep quality and daytime function(5-7)

2.1.3.1 Digestive health

KF has long been used in China for medicinal purposes, especially for digestion and reducing irritability. Research supports that green KF improves digestive health and promotes abdominal comfort (43, 49, 50). The putative mechanism of KF on normal gastrointestinal function has been extensively reviewed by others (87). KF has a unique combination of soluble and insoluble fibres, polyphenols and actinidin, improving laxation and reducing abdominal discomfort in healthy individuals with constipation and those with irritable bowel syndrome (IBS) (87). This has allowed Zespri to successfully obtain an European Food Safety Authority (EFSA) claim that 'consumption of KF contributes to the maintenance of normal defecation' (88).

Studies using pigs and human clinical studies have provided information on how KF increases water retention and faecal bulking (43, 46, 49, 89, 90). The fibre in KF enables better lubrication by increasing hydration, which assists in the propulsion of digesta through the colon (91). The insoluble fibres add bulk to faeces and alleviate constipation (92). Animal studies suggest that the pectic components of the KF are highly fermented in the hindgut (93, 94). This intestinal fermentation depends on the amount of KF

consumed (95), suggesting that an optimal amount may be required to elicit the beneficial effects of fermentation in the gut.

Actinidin enhances protein digestion when added to various protein-based meals *in vitro* (96, 97). In a pig model study, in which beef proteins were fed in the presence or absence of actinidin, the rate of AA digestibility in the small intestine was highly dependent on the rate of the digested proteins entering the small intestine (95). Furthermore, a clinical study assessing AA uptake in peripheral blood from beef showed an earlier peak increase of EAA, branched-chain amino acids (BCAA) and leucine after green KF when compared to gold ('Hort16A' – variant with no actinidin) (82).

2.1.3.2 Gut microbiome

The gastrointestinal tract (GIT) hosts a diverse array of bacterial species key for maintaining metabolic homeostasis within the host. Some studies (12, 50, 54, 98, 99) have demonstrated that the consumption of KF induces notable changes in the human gut microbiome. Short-term feeding of freeze-dried green KF triggers an increase in beneficial *Lactobacillus* and *Bifidobacteria* species, accompanied by a significant reduction in harmful *Clostridia* and *Bacteroides* species in humans. However, it is worth noting that these effects were temporary (54).

In a four-week study, Blatchford, Stoklosinski (12) observed an increase in the *Faecalibacterium prausnitzii* species among individuals consuming gold KF powder. This species is considered health-promoting due to its butyrate production and anti-inflammatory effects (100). Consistent findings in *in vitro* fermentation models of KF include increased *Bifidobacteria* (101), while pig models fed with KF fibre experienced a decrease in *Enterobacteria* (102). In a rat model fed with KF, there was an observed increase in the *Lachnospiraceae* family (103). A recent human study involving the

consumption of two fresh 'SunGold' KF daily for twelve weeks demonstrated significant increases in the *Coriobacteriaceae* family (55). The role of this family is less understood and requires further investigation regarding its potential contributions to human health. However, other research has stipulated that polyphenols enhance the growth of this family (104).

The alterations in microbiota induced by KF consumption may play a role in suppressing harmful microbes and promoting the growth of beneficial microbes, although the specific families affected can vary. The precise mechanisms driving these shifts have yet to be precisely known, as results differ in which microbes are changed. This variability could be attributed to differences in methods and cohorts tested. Nevertheless, a prevalent theme among studies suggests that the fibre or polyphenol in KF could contribute to these observed changes. In summary, while the exact mechanisms require further investigation and may be influenced by study-specific factors, KF consumption enhances beneficial gut microbes, potentially improving overall gut health.

2.1.3.3 Markers of oxidative stress and inflammation

The antioxidant and anti-inflammatory properties of KF are attributed to the presence of vitamins C and E, polyphenols, 5HT, and carotenoids. These bioactive compounds play a critical role in enhancing the body's defence against oxidative stress, and may influence various markers of oxidative stress, including plasma/serum oxygen radical absorbing capacity (ORAC), ferric reducing antioxidant potential (FRAP), SOD, glutathione peroxidase (GPx), lipid peroxidation, thiobarbituric acid (TBARS), diacron-reactive oxygen metabolites (d-ROMs), biological antioxidant potential (BAP), salivary cortisol, uric acid, and urinary 8-hydroxy-2-deoxyguanosine and N-e-(hexanoyl)-lysine

and 2,2-diphenyl-1-picrylhydrazyl (DPPH). However, the value of *in vitro* measures of oxidative stress (ORAC, FRAP, total antioxidant status [TAS]) remains debatable.

Studies involving KF have focused on measuring inflammatory markers such as plasma/serum C-reactive protein (CRP), high-sensitivity CRP (hs-CRP), and pro-inflammatory cytokines (interleukin 10 [IL-10], interleukin 6 [IL-6], tumour necrosis factor-alpha [TNF-α]), which collectively indicate inflammatory status. Notably, TNF-α and IL-6 are pro-inflammatory cytokines, hs-CRP is a general marker of inflammation, and IL-10 is anti-inflammatory.

KF may modulate inflammatory cytokines. *In vitro* tissue culture and mouse models of inflammatory bowel disease have been utilised to assess the anti-inflammatory effects of green and gold KF extracts (105). These extracts demonstrated a reduction in nitric acid and cytokine (IL-6, IL-10, TNF-α) production in lipopolysaccharide (LPS)-activated macrophages and intestinal epithelial cells (105). Additionally, green KF peel extract inhibited the production of inflammatory cytokines (IL-6, IL-1β, TNF-α) in LPS-stimulated human THP-1 monocytes (106).

Studies in humans have shown that KF consumption decreases plasma TNF-α (14, 15), plasma IL-6 and hs-CRP (62). However, conflicting results exist regarding IL-6 (14, 15, 63), IL-10 (14) and CRP measures (13, 14, 63), which may be attributed to variations in cohorts and intervention periods. The proposed mechanisms underlying these anti-inflammatory improvements suggest that KF may modulate fundamental pathways, including Nuclear Factor kappa B (NF-κB) (a key regulator of the inflammatory response in cells), as observed *in vitro* (107). However, as of now, no human studies have explored these pathways.

Both acute and long-term benefits on oxidative stress markers have been observed with KF consumption. Acute consumption of 300 g of green KF increased plasma ORAC (59) while consuming green or gold KF for one week decreased urinary concentrations of oxidative stress markers (58). Long-term studies revealed increased FRAP with four weeks of green/gold KF consumption (60) and reduced plasma lipid peroxidation with two weeks of gold KF consumption (13). These longer-term studies were accompanied by increases in plasma vitamin C, which may explain the improvements in oxidative markers. However, some studies reported no differences in FRAP, ORAC, SOD, and lipid oxidation when consuming green or gold KF (13, 15, 61).

To evaluate the oxidative stress-reducing capability of KF, studies have induced stress through exercise (108). One study found that both 'SunGold' KF and a matched vitamin C drink reduced exercise-induced uric acid, suggesting potential benefits for alleviating oxidative stress in women (10). Another study with male medium and long-distance runners consuming 'SunGold' KF daily for two months observed decreased d-ROMs (oxidative stress) and increased BAP/s-ROMs ratio, indicating potential benefits for athletes routinely exposed to oxidative stress (57).

Moreover, KF has been shown to activate the nuclear factor erythroid 2 (Nrf2) signalling pathway, which adds another layer of understanding to its observed antioxidant effects (109, 110). Nrf2 is known for its role in inducing the transcription of antioxidant enzymes, thereby enhancing the cellular defence mechanisms against oxidative stress. This dual action of KF—simultaneously boosting antioxidant defences through Nrf2 and mitigating inflammation by reducing NF-κB—provides a mechanistic insight into its potential to counteract oxidative stress and inflammation. The modulation of these

critical pathways may well explain some of the varied antioxidant outcomes observed in studies, shedding light on the multifaceted nature of KF's impact on cellular health.

2.1.3.4 Metabolic health

Metabolic health is a critical determinant of overall well-being, with imbalances potentially leading to physiological disruptions. Such irregularities encompass hypertension, abnormal glucose metabolism, vascular inflammation, dyslipidaemia (characterised by elevated blood total cholesterol, low-density lipoprotein cholesterol, triglycerides, and reduced high-density lipoprotein cholesterol), and hematologic disorders. These factors play pivotal roles, contributing to morbidities like diabetes, cardiovascular disease (CVD), stroke, and dementia.

Recent studies shed light on the positive impact of regular KF consumption on key physiological markers associated with diabetes, cardiovascular disease, and stroke (111). The low carbohydrate content and fibre swelling capacity of KF contribute to delayed glucose absorption (9, 73, 93). Mishra, Willis (71) show a significant reduced glycaemic response was observed after ingesting 200 g of green KF, attributed to fruit sugars and non-digested fibre components. Additional components in KF, such as the cell wall and remnants of polyphenols, further increase its potential to improve glycaemic response (72). Polyphenols, for example, have the potential to inhibit sodium-glucose co-transporter 1 (SGLT1) and glucose transporter 2 (GLUT2), thus impeding the uptake of glucose by enterocytes in the small intestines and their subsequent entry into circulation.

KF shows promise in normalising blood lipid profiles, influencing the balance between low-density lipoprotein (LDL) and high-density lipoprotein (HDL) cholesterol. HDL is key in removing excess cholesterol, while LDL, the primary cholesterol carrier, operates in

the opposite direction. The daily consumption of two green KFs for eight weeks lead to a significant increase in serum HDL concentrations and a decrease in TC:HDL ratios in a hypercholesterolemic cohort (64). Similarly, positive outcomes in HDL were observed in hypercholesterolemic men following a four-week study involving the consumption of green KFs (65). The mechanisms by which KF may alter HDL levels are believed to involve the upregulation of the expression and production of apolipoprotein A1, a key component of HDL cholesterol. However, an increase in apolipoprotein A1 has not been observed in response to KF treatment (62).

Conversely, a study involving consumption of two 'SunGold' KFs for six weeks in overweight and obese participants significantly decreased serum HDL levels; however, it is noteworthy that the HDL levels remained within the normal range (15). In contrast, studies on healthy cohorts consuming green KF for three weeks showed no significant differences in total cholesterol, LDL, glucose, or blood pressure (112, 113). These diverse responses emphasise the potential impact of individual characteristics on the effectiveness of KF in influencing lipid profiles.

The consumption of KF holds promise for reducing blood pressure and enhancing blood flow, as indicated by various studies. Notably, Svendsen, Tonstad (67) observed a significant decrease in 24-hour systolic and diastolic blood pressure after three weeks of daily green KF consumption compared to daily apple intake. The suggested mechanisms driving this improvement point to the elevated intake of antioxidants and potassium through the KF intervention (67). Similar positive effects on blood pressure were noted in a prediabetic cohort consuming 'SunGold' KF for 12 weeks (55) and obese cohorts for six weeks (15). Furthermore, a study involving women showed significant improved finger blood flow after four weeks of green KF consumption (48). Additionally,

male smokers experienced beneficial effects on blood pressure when consuming three green KFs daily for eight weeks compared to an antioxidant-rich diet (68). However, it is important to note the lack of consistent results, exemplified by Gammon, Kruger (114), who found no blood pressure differences in men with hypercholesterolemia after four weeks of green KF consumption compared to a healthy diet alone.

Despite the positive outcomes, the exact antihypertensive mechanisms of KF remain unclear. One such mechanism is the vasodilatory property of KF through its ability to upregulate nitric oxide (NO), a potent vasodilator, by activating or increasing the expression of NO synthase. However, assessment of biomarkers of NO metabolism (asymmetric dimethylarginine [ADMA], symmetric dimethylarginine [SDMA], L-arginine, L-arginine/ADMA ratio) showed no effect of KF on these biomarkers (67). Studies exploring platelet aggregation and the renin–angiotensin system (RAS) in blood samples from hypercholesterolemic (68) and obese cohorts (15) showed reduction in these and hint at the potential pathways. Decreased platelet aggregation and angiotensin-converting enzyme (ACE) activity, leading to reduced Angiotensin II (AgII) levels, suggest that certain constituents in KF may counteract processes contributing to metabolic diseases. Further research is needed to elucidate these mechanisms and establish KF's potential role in managing metabolic health.

2.1.3.5 Nutrient status

KF is a nutrient-dense fruit packed with essential nutrients, with studies primarily focusing on its impact on vitamin C, E, and folate levels. Vitamin C, crucial for iron absorption, is abundantly present in green and gold KF, enhancing mineral absorption. *In vitro* studies using human epithelial cell line Caco-2 cell models and pig models have demonstrated the ability of green and gold KF extracts to increase calcium and

phosphorus retention, like the effects observed with ascorbic acid supplementation (115).

In human clinical studies, adding two gold KF to the diet has significantly enhanced iron absorption from iron-fortified breakfast cereals in women with mild iron deficiency (81). Furthermore, KF consumption has been linked to increased plasma vitamin C levels, neutrophil vitamin C, urinary vitamin C, skeletal muscle ascorbate, and mononuclear cell ascorbate (4, 13, 55, 60, 64, 76-79). Notably, as little as half a gold KF has impacted plasma vitamin C levels, and a four-week intervention with two gold KF approached saturation levels (78). The increased plasma vitamin C levels resulting from KF consumption have been associated with reducing the severity and duration of upper respiratory infection symptoms (13) and mood disturbances (3). These increase in plasma vitamin C are consistent across diverse cohorts, including the young, elderly, and prediabetic individuals (55). Moreover, KF's bioavailability is comparable to synthetic vitamin C supplements (77).

Studies of green and gold KF consumption have shown increased plasma vitamin E concentrations (3, 13, 64), possibly due to the higher bioavailability of vitamin E from KF (116). Vitamin E, a potent antioxidant, protects cells from damage and supports skin, eye, and immune health, while folate is essential for DNA synthesis and foetal development. While the direct impact of KF consumption on folate status is not well-established, some evidence suggests plasma folate is increased with 'SunGold' KF consumption over 12 weeks (55).

Additionally, KF has been shown to significantly improve carotenoid status in plasma, with gold KF consumption increasing plasma zeaxanthin (13, 81) and lutein (13, 67, 81) concentrations. Lutein and zeaxanthin support eye health by forming the macular

pigment, filtering harmful light, and acting as antioxidants, potentially reducing the risk of age-related macular degeneration and cataracts. Nevertheless, limited studies have explored whether KF could improve these specific nutrient statuses and how they would relate to DNA synthesis and antioxidant protection.

2.1.3.6 Bone health

The impact of KF supplementation on bone health is not established. Early evidence from a study of postmenopausal women showed a reduction of uncarboxylated osteocalcin (ucOC; a marker of bone turnover and vitamin K status) and no change in C-telopeptide of type I collagen (CTx; a marker of bone resorption) when consuming two green KF with 50 mg of isoflavone (daidzein and genistein) for six weeks (56). On the contrary, there was an increase in ucOC when consuming the isoflavone supplement alone. The reduction in ucOC, which is generally considered beneficial for bone health, suggests a potential positive effect of the KF supplementation. The study did not assess the impact of KF alone, so it is unclear whether the KF alone was responsible for this decrease in ucOC. However, the authors hypothesise that the improvement was due to additional vitamin K, from the KF.

2.1.3.7 Oral health

Emerging evidence suggests that KF consumption or KF-based products may benefit oral health. Consumption of two KF had a beneficial effect on periodontal parameters and systemic health before oral hygienist treatment (79). These parameters included bleeding, plaque build-up and attachment loss. Use of KF-containing oral care tablets reduced volatile sulphur compound concentration, Winkel tongue-coating index, the number of total bacteria and *Fusobacterium nucleatum* (periodontal pathogen

implicated in oral malodour) (83-85). These studies suggest the potential benefits of KF and KF products on oral health, especially around the oral microbiome.

2.1.3.8 Brain health

The effects of KF on brain health has garnered attention, particularly regarding cognitive performance, mood, and sleep quality. To our knowledge only one study has explored the acute effects of KF on cognitive performance (117). Participants received two servings of green KF alongside a carbohydrate-based breakfast, with cognitive testing conducted at intervals pre- and post-meals. No significant effects were observed; however, trends suggest improved performance on reaction time and serial sevens were noted, particularly over a three-hour period with the KF treatments. Additional studies with more comprehensive methodologies are needed to validate these findings (117).

Research on green KF supplementation and sleep quality is expanding. Consumption of two green KF one hour before bed has shown promise in significantly improving sleep quality for individuals experiencing poor sleep quality (5), insomnia (6) and for athletes (7). Reported benefits include reduced sleep onset latency, increased sleep efficiency, longer sleep duration and reduced general and sports stress. While biomarker support for these improvements is lacking, one study hinted at a potential explanation by noting an increase in the 5HT metabolite 5HIAA in urine following KF consumption (8). Furthermore, both green and gold KF peel extracts have demonstrated the ability to enhance pentobarbital-induced sleep onset (118) and positively impact sleep architecture in mice (119).

Regarding mood and well-being, studies indicated a positive impact of KF consumption. For instance, elderly individuals with constipation reported a significant increased quality of life after incorporating KF into their diet (51). Similarly, a study involving

students using the Profile of Mood States (POMS) revealed decreased total mood disturbance scores following six weeks of consuming two gold KF servings, among those with higher baseline mood scores. The rise in plasma ascorbic acid levels suggests a potential role of vitamin C in enhancing mood (3). Additionally, a follow-up study found significantly improved mood and well-being among participants consuming KF compared to those receiving a placebo or vitamin C tablets (4) suggesting an added benefit of consuming KF as opposed to vitamin C alone.

With the COVID-19 pandemic highlighting issues like "brain fog", addressing brain health has become even more urgent (120). Furthermore, given the increased prevalence of mood disorders, sleep disturbances, and cognitive impairments and the promising but still emerging findings regarding the potential benefits of KF on aspects of brain health, further research in this area is warranted.

2.2 Neurobiological health

Neurobiological health encompasses the brain's structural integrity, functional efficiency, and chemical balance. Its influence permeates various aspects of daily life, affecting bodily functions, cognitive sharpness, decision-making, emotional stability, social interactions, and sleep quality. Other symptoms of compromised neurobiological health may include difficulties falling asleep, morning grogginess, poor concentration, mental fatigue, and reduced alertness. In 2021, 28% of the population in New Zealand reported poor mental health (121). Additionally, approximately 27% of people in New Zealand report experiencing sleep problems (122), underscoring the importance of maintaining optimal neurobiological function.

Individuals often use various health methods to address nervous system-related issues, including meditation, yoga, massage therapy, natural remedies, and supplements (123).

The underlying neurobiological brain mechanisms are complex, involving various factors such as age, genetic predispositions, cardiovascular health (124), blood circulation, oxidative stress, inflammation, glycaemic control, gut microbiome composition and neurotransmitter balance (2). Each factor plays a vital role and will be explained in more detail in section 0.

2.2.1 Foundations of neurobiology

2.2.1.1 Brain structure

The brain serves as the core of neurological health, orchestrating the intricate relationship of cognitive, emotional, and physiological functions essential for human well-being. The brain is a complex organ housed within the skull and serves as the command centre for the human body. The brain is made up of billions of neurons, the building blocks of the nervous system. The brain comprises interconnected regions that contribute to a variety of functions, including cognition, emotions, and motor skills. While certain areas may be more active during specific tasks, these functions often overlap and involve networks that integrate multiple regions working together (125). Neurons communicate through synapses, forming sophisticated networks that facilitate the transmission of electrical signals. This structure enables the brain to process information, regulate bodily functions, and adapt to an ever-changing environment.

The human brain is complex, with its functions divided among distinct regions known as lobes. The frontal lobe, situated at the front of the brain, plays a crucial role in decision-making, problem-solving, and voluntary movement, as well as regulating emotions and social behaviour (125). Behind the frontal lobe lies the parietal lobe, which is responsible for processing sensory information such as touch, temperature, and pain while aiding in

spatial awareness and navigation. The temporal lobe, found on the sides of the brain, is primarily involved in processing auditory stimuli, language comprehension, and memory formation. Finally, the occipital lobe, located at the back of the brain, is dedicated to visual processing, enabling us to interpret and make sense of the world around us. These lobes work together, allowing us to perceive, understand, and interact with our environment (125) (Figure 2.2).

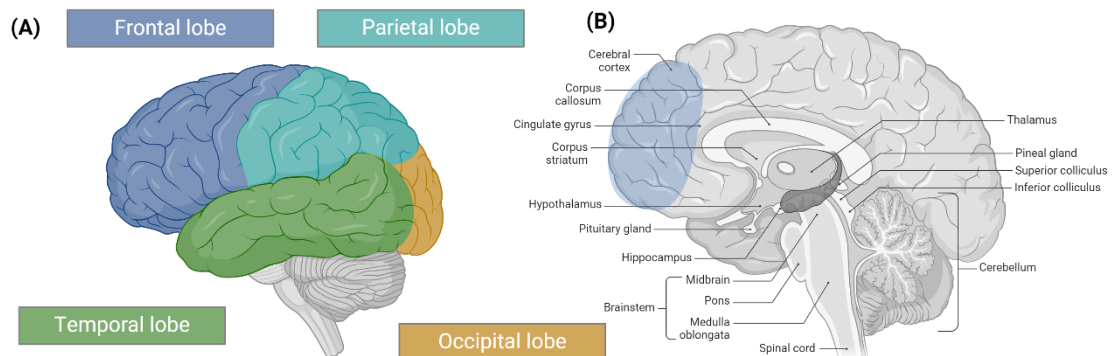


Figure 2.2 Lobes of the brain (A) and structure of the brain (B). Figure Created with BioRender.com.

The prefrontal cortex, situated in the frontal lobe just behind the forehead, serves as the brain's executive centre, overseeing higher cognitive functions such as decision-making, planning, and impulse control. It also plays a role in personality expression and social behaviour regulation (125). The hippocampus, nestled deep within the temporal lobe, is integral to memory formation and spatial navigation, necessary for learning and remembering past events and environments (125). The hypothalamus, located beneath the thalamus at the base of the brain, serves as a control centre for various bodily functions, including regulating hunger, thirst, body temperature, and the sleep-wake cycle, as well as influencing hormone secretion from the pituitary gland (125). Moving to the brain's centre, the corpus striatum, found within the basal ganglia in the forebrain, aids in motor control and procedural learning, helping to coordinate voluntary

movements and habits (125). The cerebellum, at the brain's base, fine-tunes motor control, balance, and timing of movements while aiding motor learning and cognitive functions like attention and language (125). Lastly, the brainstem, located at the base of the brain, connects the cerebrum with the spinal cord and controls essential functions such as breathing, heart rate, and arousal. It also serves as a conduit for sensory and motor pathways, facilitating communication between the brain and the rest of the body (125). These structures play vital roles in regulating cognitive and physiological processes essential for human functioning.

2.2.1.2 Signalling in the brain

The fundamental building blocks of the brain are neurons, which play a fundamental role in signalling processes. These specialised cells communicate with each other to orchestrate brain functions and enable the transmission of information between different regions. This communication occurs through neurotransmitters, which are chemical messengers released by neurons to transmit signals across synapses, the tiny gaps between neurons. Neurotransmitters like 5HT, dopamine, and glutamate are significant in this process (126). They play pivotal roles in regulating various neurobiological health outcomes, including sleep, mood, cognition, behaviour, and physiological processes. Table 2.5 provides a breakdown of neurotransmitters, elucidating their precursor AA, essential nutrients, pivotal enzymes in synthesis, breakdown enzymes, and relevant receptors. As highlighted, essential macro and micronutrients are vital factors in producing these signalling molecules.

Neurotransmitters do not operate in isolation. Another important component of brain communication is the network of neuroglial cells, particularly astrocytes, the most abundant glial cells in the brain, which maintain the chemical environment essential for

neurotransmission. They regulate neurotransmitter concentration in the synaptic cleft, ensuring efficient signal transmission while preventing excessive excitation or inhibition. Additionally, astrocytes release gliotransmitters, signalling molecules that modulate neuronal activity and synaptic strength, actively participating in synaptic communication and stabilising and regulating the blood-brain barrier (BBB) (127).

Another factor of relevance are the neurotrophic factors. Neurotrophins are a group of proteins essential for the growth, survival, and maintenance of neurons. One key neurotrophins is Brain-Derived Neurotrophic Factor (BDNF), which is crucial for neurotransmission, promoting neuronal survival, growth, and synaptic plasticity (137). BDNF modulates synaptic strength and plasticity, essential for learning, memory formation, and adaptive responses. By binding to its receptors, BDNF initiates signalling cascades that regulate gene expression, synaptic structure, and neurotransmitter release, fostering synapse formation and improving synaptic transmission (137). Other neurotrophins, such as Nerve Growth Factor, support sensory and sympathetic neurons (138), while Neurotrophin-3 is vital for the development and survival of neurons in the central and peripheral nervous systems. Neurotrophin-4 contributes to synaptic plasticity and neuron differentiation (139). These neurotrophins work through Trk and p75 receptors (140), playing a key role in neuroplasticity, cognitive function, and recovery from neural injuries. Dysfunctional neurotrophic factor signalling is implicated in neurological and psychiatric disorders, highlighting its therapeutic potential (141).

Table 2.5 Overview of chemical messengers: precursors, enzymes, receptors, and roles in brain signalling.

Chemical messengers	Precursor amino acid	Other co-factors required	Enzymes involved in synthesis	Breakdown enzymes	Receptors	Role in brain signalling	References
Acetylcholine	Choline	Acetyl-CoA	Choline acetyltransferase	Acetylcholinesterase	Nicotinic, muscarinic	Excitatory neurotransmitter	(128)
Dopamine	Tyrosine	Tetrahydrobiopterin, Vitamin B6, Iron	Tyrosine hydroxylase, Aromatic, L-amino acid decarboxylase	Monoamine oxidase, Catechol-O-methyltransferase	D_1, D_2, D_3, D_4, D_5	Both excitatory and inhibitory functions	(129)
Serotonin	Tryptophan	Vitamin B6, Iron	Tryptophan hydroxylase	Monoamine oxidase	$5HT_1$, $5HT_2$, $5HT_3$	Inhibitory neurotransmitter	(130)
Norepinephrine	Tyrosine	Tetrahydrobiopterin, Vitamin C, Iron	Tyrosine hydroxylase, Dopamine beta-hydroxylase, Phenylethanolamine N-methyltransferase	Monoamine oxidase	α_1, α_2, β_1, β_2, β_3	Both excitatory and inhibitory functions	(131)
GABA	Glutamate	Vitamin B6, Magnesium	Glutamate decarboxylase	GABA transaminase	$GABA_A$, $GABA_B$	Inhibitory neurotransmitter	(132)
Glutamate	Glutamine	None	Glutaminase	Glutamate dehydrogenase	NMDA, AMPA, kainate, metabotropic	Primary excitatory neurotransmitter,	(133)
Histamine	Histidine	Vitamin C, Copper	Histidine decarboxylase	Diamine oxidase	H_1, H_2, H_3, H_4	Excitatory neurotransmitter	(134)
Melatonin	Tryptophan	None	Serotonin N-acetyltransferase (AANAT), Acetylserotonin O-methyltransferase (ASMT)	N-acetylserotonin deacetylase	MT_1, MT_2	Sleep onset	(135)
Adenosine	Adenosine Triphosphate (ATP)	None	Adenosine kinase, Adenosine deaminase	Adenosine deaminase	A1, A2A, A2B, A3	Inhibitory neurotransmitter	(136)

Furthermore, neurotransmitter signalling in the brain is linked to the gut-brain axis, a bidirectional communication system between the gut and the brain (142). Several neurotransmitters in brain function also play crucial roles in modulating gut physiology and vice versa. For instance, 5HT, primarily known for its mood-regulating role (126), is abundant in the gastrointestinal tract, regulating intestinal motility, secretion, and sensation. Dopamine, associated with reward and motivation (126), influences intestinal motility and secretion. Moreover, through their production of neurotransmitters and metabolites, the gut microbiota can influence neurotransmitter signalling in the brain, impacting mood, cognition, and behaviour (142). Understanding the communication between neurotransmitter signalling in the brain and the gut-brain axis is central for unravelling the complex mechanisms underlying gut-brain communication and its implications for health and disease.

2.2.1.3 Nutrient Communication: The Gut-Brain Axis

Nutrients play a vital role in the gut-brain communication pathway. The absorption of nutrients in the intestine influences various physiological processes, including neurotransmitter synthesis, hormone regulation, and immune function, which in turn affect brain health and function. As mentioned above, AA, and certain nutrients are required to produce neurotransmitters. Additionally, there is growing evidence for underlying microbiota-gut-brain communication and the development of novel therapeutic strategies targeting the gut microbiome to improve brain health (143).

The communication between the brain and the gut occurs through a bidirectional pathway known as the gut-brain axis. This sophisticated network involves various components, including the central nervous system (CNS), enteric nervous system (ENS), and the gut microbiota. There are three bidirectional communication pathways

between the gut and brain: the neuroendocrine pathway, the immunoregulatory pathway, and the pathway via the autonomic nervous system (ANS) (Figure 2.3).

The ANS facilitates efferent and afferent signals between the brain and gut, mediated through the vagus nerve. Nutrients and the gut microbiome have been shown to influence these bidirectional pathways (144). Animal studies have demonstrated that vagal nerve stimulation can modulate various aspects of intestinal function, such as motility and secretion (145), and affect brain function and behaviour (146). Additionally, clinical studies in humans have explored the therapeutic potential of vagal nerve stimulation for conditions such as depression, epilepsy, and inflammatory bowel disease (147), highlighting the importance of this neural pathway in gut-brain communication.

The neuroendocrine pathway affects both the CNS and the HPA axis by regulating the secretion of neurotransmitters and metabolites (143). The HPA axis's activation primarily aims to prepare the body for the "fight or flight" response (148). When homeostasis is disrupted, corticotrophin-releasing factor (CRF) is released from the paraventricular nucleus (PVN) of the hypothalamus, prompting the secretion of adrenocorticotrophic hormone (ACTH) from the anterior pituitary. ACTH is then released into the systemic circulation and acts on the adrenal cortex, eventually releasing glucocorticoids, including cortisol (149). Cortisol is important in various physiological processes, such as metabolism, immune response, and stress regulation, influencing mood, appetite, and stress responses (150). Besides the HPA axis communication, the enteroendocrine cells release neuroendocrine compounds (151), including ghrelin, somatostatin, cholecystokinin (CCK), peptide YY, and 5HT, which may have a direct effect through systemic circulation or interaction with the vagus nerve (149).

The third pathway, the immunoregulatory pathway, represents another communication channel between the gut and the brain. This involves the complex relationship between immune cells, gut microbiota, and metabolites (143). The intestinal tract houses a significant portion of the body's immune system, ensuring and maintaining tissue integrity (152). Gut-derived inflammatory signals may influence neural messages and communicate with the brain to inform health status and potentially regulate brain function and behaviour. Furthermore, there is potential for these messages to activate immune cells, also known as microglia, in the brain, potentially leading to neuroinflammation implicated in various neurological disorders, including depression, anxiety, and neurodegenerative diseases (153).

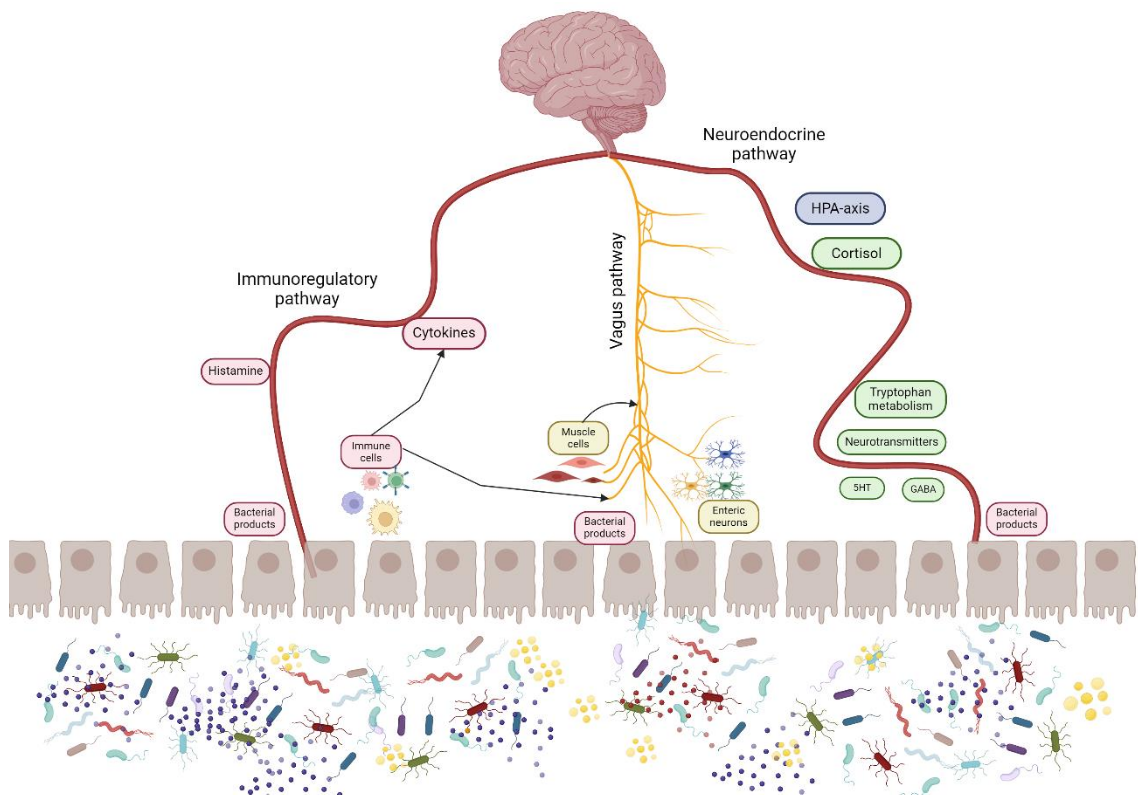


Figure 2.3 Bidirectional communication between the gut environment (microbiota and metabolites) and the brain. Figure Created with BioRender.com.

The intermediatory between peripheral communication of the gut and the brain is the BBB. The BBB is a highly selective semipermeable membrane that separates circulating

blood from the brain's extracellular fluid. This barrier regulates the passage of substances such as neurotransmitters, hormones, and immune cells, ensuring proper brain function and homeostasis (154). For example, intestinal-derived 5HT does not cross the BBB (155), while large neutral amino acids (LNAA) and branched-chain amino acids (BCAA) do (156). The BBB protects the brain from potentially harmful substances, allowing essential nutrients and molecules to pass through (154). Dysfunction of the BBB has been implicated in various neurological disorders, highlighting its significance in maintaining the delicate balance of communication between the gut and the brain (157).

2.2.1.4 Interplay of brain with cognition, mood, and sleep

As mentioned, the brain is vital for overall life, significantly impacting neurological outcomes that influence everyday experiences. This section provides background information to understand how the brain influences these aspects of life.

Cognition, the process of acquiring knowledge and understanding through thought, experience, and the senses, relies on sophisticated neural networks within the brain. Several brain regions are involved in various aspects of cognition, including perception, attention, memory, language, and problem-solving (158). As discussed earlier in section 2.2.1.1, the lobes and specific areas of the brain each play distinct roles in various aspects of cognition. Dysfunction or damage to these brain regions can lead to cognitive impairments, affecting attention, memory, language, and other cognitive abilities (159).

Mood often describes the subjective emotional state, which influences how individuals perceive and experience the world around them. It encompasses a range of feelings and emotions, including happiness, sadness, anger, anxiety, and contentment, which can fluctuate in response to various internal and external factors (160).

Mood regulation involves a complex interaction of biological, psychological, and environmental factors, including brain chemistry, neurotransmitter activity, hormonal balance, cognitive processes, and social interactions (161). Mood regulation involves the coordinated activity of several key brain regions responsible for emotional processing and regulation. The amygdala, located deep within the brain's temporal lobe, plays a central role in processing emotions, particularly fear and pleasure responses (162). The prefrontal cortex modulates emotional responses by exerting inhibitory control over the amygdala and other limbic regions (163). Additionally, dysfunction or dysregulation in the hippocampus and the hypothalamus may disrupt mood regulation mechanisms, contributing to mood disorders such as depression, anxiety, and bipolar disorder (164). Sleep is a vital physiological process essential for overall health and well-being (165), cellular repair of the body and is associated with learning (166), memory consolidation (167), emotional regulation (168) and recovery (169). During sleep, the brain undergoes a series of complex processes, transitioning through different stages that involve changes in brain wave activity and physiological functions (170). Various brain structures regulate sleep, each playing a distinct role in orchestrating the sleep-wake cycle. The suprachiasmatic nucleus (SCN) of the hypothalamus (171), often called the "master clock" of the brain, controls the circadian rhythm, which dictates the timing of sleep and wakefulness (172-174).

Furthermore, the brainstem, particularly the reticular formation, regulates arousal and wakefulness, while the thalamus serves as a relay station for sensory information, filtering out external stimuli during sleep. The basal forebrain releases neurotransmitters such as GABA and adenosine, promoting sleep by inhibiting

wakefulness-promoting regions (175). These brain structures work in concert to coordinate the transitions between different sleep stages.

Non-Rapid Eye Movement (NREM) sleep, characterised by slower brain waves, facilitates physical restoration and replenishment. Rapid Eye Movement (REM) sleep, marked by increased brain activity and vivid dreaming (170), is vital for cognitive functions such as memory consolidation and emotional processing. Adequate sleep duration and quality are essential for optimal cognitive function, mood regulation, and overall mental health. Sleep quality refers to how restful and refreshing sleep feels and includes sleep duration and ease of falling and staying asleep. Disturbances, like difficulty falling asleep or frequent awakenings, can lower sleep quality. Chronic sleep deprivation or poor sleep quality can increase health risks of inflammation (156), hypertension (157, 158), diabetes (159), obesity (160), and CVD (176).

Understanding human cognition, mood, and sleep requires a comprehensive assessment combining subjective and objective measures. Subjective measures, such as self-reports and questionnaires, rely on individuals' perceptions and are easy to administer and cost-effective, but they can be biased by memory recall and social desirability. Objective measures, like physiological data and brain imaging, provide precise, unbiased data but may overlook individual nuances and can be costly or invasive. A combined approach, using both subjective and objective measures, often provides a more comprehensive understanding by integrating personal insights with quantifiable data. Table 2.6 summarises the critical validated subjective and objective measures used to assess cognition, mood, and sleep, highlighting the diverse approaches employed in understanding these psychological domains.

Table 2.6 Summary of subjective and objective measures for assessing cognition, mood, and sleep.

Aspect	Subjective	Objective
Cognition	- Completion of standardised cognitive tests (e.g., MMSE, MoCa)	- Brain imaging techniques (e.g., fMRI, EEG) - Engagement in computerised cognitive tasks
Mood	- Completion of mood questionnaires (e.g., BDI, HAM-D, POMS, PANAS)	- Physiological markers (e.g., heart rate variability, cortisol levels, facial expressions)
Sleep	- Recording sleep patterns in sleep diaries - Completion of validated sleep questionnaires (e.g., PSQI, ISI, LSEQ, ESS, SSS)	- Actigraphy monitors (wearable devices tracking movement patterns and sleep-wake cycles) - Polysomnography (PSG; monitoring various physiological parameters during sleep) to allow for sleep architecture quantification

Abbreviations: MMSE = Mini-Mental State Examination, MoCA = Montreal Cognitive Assessment, BDI = Beck Depression Inventory, HAM-D = Hamilton Depression Rating Scale, POMS = Profile of Mood States, PANAS = Positive and Negative Affect Schedule, PSQI = Pittsburgh Sleep Quality Index, ISI = Insomnia Severity Index, LSEQ = Leeds Sleep Evaluation Questionnaire, ESS = Epworth Sleepiness Scale, SSS = Stanford Sleepiness Scale, fMRI = Functional Magnetic Resonance Imaging, EEG = Electroencephalogram

As highlighted, neurotransmitters are fundamental in regulating various aspects of cognition, mood, and sleep. These chemical messengers facilitate communication between neurons in the brain and are essential for maintaining proper brain function. Dysregulation of neurotransmitter systems can lead to disruptions in cognitive processes, mood disorders, and sleep disturbances. Table 2.7 outlines neurotransmitters and their functions in these domains.

Table 2.7 Overview of the functions of neurotransmitters on cognition, mood, and sleep.

Neurotransmitter	Function in Cognition	Function in Mood	Function in Sleep	Reference
Acetylcholine	Enhances attention, learning and memory	Influences mood regulation	Promotes REM sleep	(177, 178)
Dopamine	Modulates attention, motivation, and reward processing	Plays a role in mood regulation and pleasure	Inhibits REM sleep	(179)
Serotonin	Regulates mood, emotion, and cognition	Regulates mood and promotes feelings of well-being	Contributes to sleep onset and maintenance	(180, 181)
Norepinephrine	Enhances alertness, attention, and arousal	Influences mood and stress response	Promotes wakefulness and suppresses REM sleep	(182, 183)

GABA	Inhibits excitatory neurotransmission, regulates neuronal activity	Has anxiolytic and calming effects	Promotes deep sleep and relaxation	(184, 185)
Glutamate	Excitatory neurotransmitters involved in synaptic plasticity and learning	May influence mood	Involved in the regulation of REM sleep and overall sleep-wake cycle	(186, 187)
Histamine	Modulates attention, and cognitive function	May impact mood and arousal	Promotes wakefulness and alertness	(188, 189)
Melatonin	-	May influence mood regulation	Regulates sleep onset and maintenance, helps synchronise circadian rhythms	(190, 191)
Adenosine	Inhibits excitatory neurotransmission	May affect mood and cognition	Accumulates during wakefulness, promotes sleepiness and sleep onset	(192, 193)

2.2.2 Consequences of poor neurobiological health

Poor neurobiological health can lead to numerous adverse outcomes across various critical domains. It is important to note that these consequences are well-characterized and can disrupt all aspects of life (Table 2.8). Furthermore, the reciprocal relationship between neurobiological health and overall health outcomes emphasises the bidirectional nature of this interaction. For instance, impaired sleep, mood, and cognitive abilities can further exacerbate neurobiological imbalances, perpetuating a cycle of dysregulation and compromising overall mental well-being.

Table 2.8 Consequences of poor neurobiological health on aspects of cognition, mood, and sleep (194-196).

Cognitive ability	**Mood**	**Sleep quality**
- Reduced attention and concentration - Memory problems - Slowed reaction times. - Decreased cognitive flexibility. - Impaired learning - Decreased academic and work performance. - Impaired problem-solving skills	- Worsening of depression and anxiety - Increased irritability - Mood swings - Emotional Instability - Difficulty regulating emotions. - Heightened stress response - social withdrawal, - strained interpersonal relationships, - diminished resilience to psychosocial stressors	- Reduced sleep quality - Difficulty falling asleep. - Difficulty staying asleep. - Frequent awakenings during the night - Less alert - Reduced daytime function. - Difficulty getting up in the morning. - Altered sleep architecture.

2.2.3 Factors that contribute to neurobiological health

A plethora of information about the impacts of different factors on neurobiological health exists. One of the all-encompassing factors is lifestyle, which includes habits of exercise, diet, sleep, stress management, and cognitive stimulation, all interconnected in some form. Underlying these lifestyle factors are mechanistic contributors to neurobiological health. These include age and genetics, cardiovascular function, cerebral blood flow (CBF), glycaemic control, the HPA axis, oxidative stress and inflammation, the microbiome, and nutritional factors (2) (Figure 2.4). These will be covered in the following section.

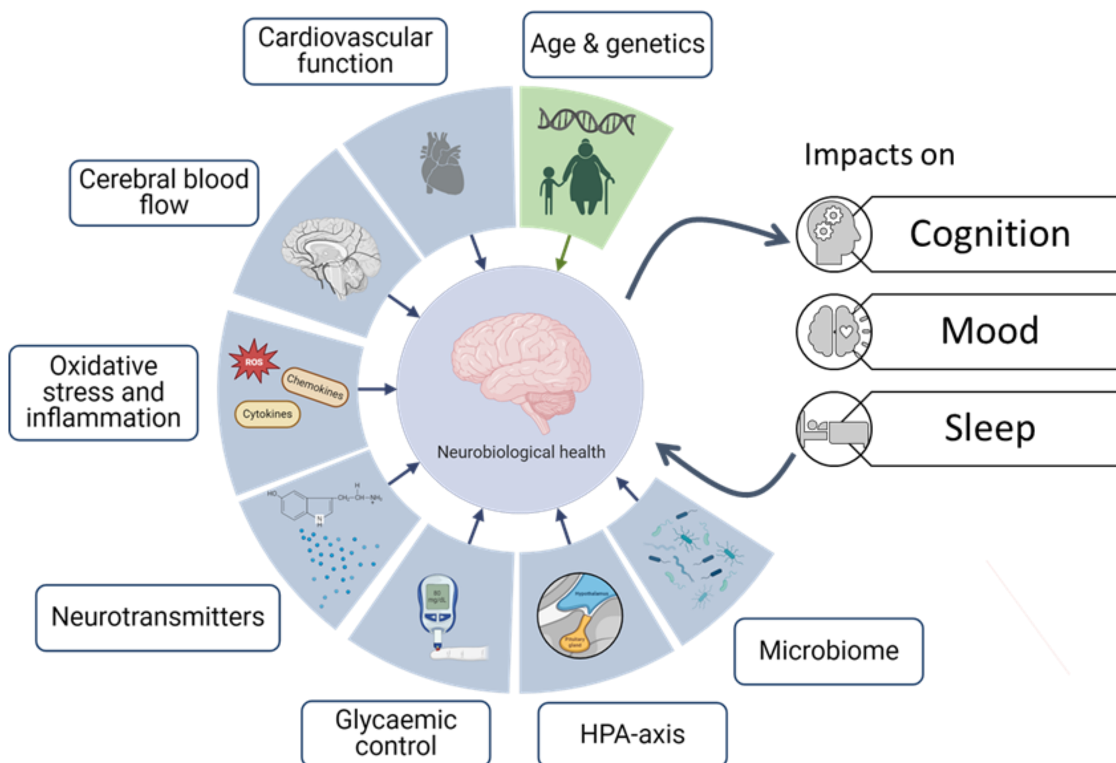


Figure 2.4 Influences on neurobiological health. Processes in the green box are non-modifiable factors, whereas blue are areas where diet can impact. HPA, hypothalamic-pituitary-adrenal. Figure Created with BioRender.com. Adapted from (2).

2.2.3.1 Age and genetics

Age and genetics represent two fundamental and non-modifiable factors that influence neurobiological health throughout an individual's life. Ageing is a natural and inevitable process that brings about changes at the neurobiological level. This may manifest in changes in brain size, morphology and vasculature (197). Over time, the neurons experience wear and tear, and the brain's ability to repair and regenerate declines (198). This also includes an increased accumulation of oxidative damaged products, mitochondrial dysfunction, impaired adaptive stress response signalling, compromised DNA repair, aberrant neuronal network activity, and dysregulated neuronal Ca^{2+} handling (reviewed in (198, 199)). The relationship between these elements dictates the structure and function of the nervous system, impacting cognitive abilities, memory, and overall brain health.

Genetics, on the other hand, contributes to an individual's baseline neurobiological framework. The genetic code inherited from parents, shapes the development and organisation of the nervous system from the very beginning (200, 201). Certain genetic variations can influence synaptic connections, neurotransmitter levels, and overall brain structure, laying the foundation for an individual's cognitive profile (202). While environmental factors and lifestyle choices can modulate the impact of genetics to some extent, the core genetic blueprint remains immutable.

2.2.3.2 Cardiovascular function

Cardiovascular function plays a pivotal role in supporting and influencing neurobiological health. The cardiovascular system is responsible for delivering nutrients to the brain. Its principal function is transport, which includes the constant supply of oxygen and glucose to neurons, essential for their metabolic processes and overall

function. Additionally, blood transports other vital nutrients, such as AA and fatty acids, to the brain, supporting energy production, neurotransmitter synthesis, and neuronal maintenance. Furthermore, cardiovascular function supports neuroprotective mechanisms by delivering growth factors and antioxidants, helping to defend against oxidative stress and inflammation. The cardiovascular system also aids in removing waste products, toxins, and carbon dioxide from the brain, maintaining a conducive environment for optimal neural function. The interaction between cardiovascular health and neurobiological function is evident in studies linking cardiovascular risk factors, such as hypertension or atherosclerosis, to an increased risk of cognitive decline and neurodegenerative disorders (203-206).

2.2.3.3 Cerebral blood flow (CBF)

CBF specifically pertains to the blood supply to the brain through transport across the BBB, delivering oxygen and nutrients while removing waste products. Healthy cardiovascular function is associated with better CBF (207). However, CBF operates independently of cardiovascular health and has elaborate mechanisms for neurobiological health. Firstly, CBF facilitates neurometabolic coupling, ensuring that active brain regions receive sufficient oxygen and nutrients in response to increased neuronal activity. This tight regulation maintains optimal brain function and prevents neurobiological dysfunction (208). Secondly, neurovascular coupling enables bidirectional communication between neurons and blood vessels, allowing for precise adjustment of CBF per local neuronal demands (209). Dysfunction in this coupling mechanism can disrupt CBF regulation, compromising brain function and contributing to neurobiological abnormalities (210). Lastly, CBF plays an important role in neuroprotection by supporting brain resilience and safeguarding against ischemic injury

and neurodegenerative diseases (208). Chronic reductions in CBF, as seen in various cardiovascular conditions, can heighten the risk of stroke, cognitive decline, and neurodegeneration, underscoring the vital importance of preserving adequate CBF for sustaining optimal neurobiological health (211).

2.2.3.4 Glycaemic control

As much as the brain needs a healthy transport system of nutrients, regulating blood sugar levels has a vital role in neurobiological health. The brain relies heavily on a steady supply of glucose for energy, and fluctuations in blood sugar levels can affect cognitive function and overall brain health (212). For example, prolonged exposure to high blood sugar, as seen in conditions like diabetes or insulin resistance, can lead to chronic inflammation and oxidative stress—factors associated with accelerated brain ageing (213, 214). Long-standing hyperglycaemic conditions can contribute to developing advanced glycation end products (AGEs), compounds formed when sugars react with proteins (215). AGEs can accumulate in the brain and impair the function of neurons and supporting cells, potentially contributing to cognitive decline and the progression of neurodegenerative diseases (216).

Moreover, episodes of hypoglycaemia (low blood sugar) can have immediate and detrimental effects on brain cellular health (217), leading to oxidative stressors, which may damage vulnerable brain regions, ultimately leading to altered cognitive ability. The brain is particularly vulnerable to fluctuations in glucose levels and maintaining stable blood sugar is critical for sustaining optimal cognitive performance.

2.2.3.5 HPA axis

As mentioned above, the HPA axis is vital for managing stress responses in the body. Prolonged activation of the HPA axis, which leads to elevated cortisol levels, has been

associated with adverse effects on the brain (218). Chronic exposure to high cortisol can negatively impact the structure and function of neurons, particularly in brain regions responsible for memory and emotional regulation (219). It may also impair neuroplasticity, the brain's ability to adapt and form new connections, which is essential for learning and cognitive function (220).

Moreover, cortisol plays a dual role in neuroprotection, depending on the context of its release. In response to acute stress, cortisol can enhance cognitive function and memory formation, facilitating adaptive responses to challenging situations (221-224). However, chronic stress-induced dysregulation of the HPA or excessive cortisol exposure, characteristic of prolonged stress, can have detrimental effects on the brain (225, 226), which could lead to impaired neuroplasticity and potentially increasing susceptibility to neurodegenerative diseases over time. Furthermore, elevated cortisol levels can initially suppress inflammation and regulate the immune system. Chronic cortisol elevation can impair immune function by inducing resistance, leading to heightened inflammation and compromised immunity (227). Determining the delicate equilibrium remains unclear, necessitating additional research to investigate methods of harnessing this system for neurobiological benefit.

2.2.3.6 Oxidative stress and inflammation

Oxidative stress and inflammation impact neurobiological health. The brain is highly susceptible to oxidative damage due to its elevated metabolic activity. Oxidative stress arises from an imbalance between reactive oxygen species (ROS) production and the body's antioxidant capacity. ROS exhibit diverse effects depending on their concentration and cellular context. ROS include superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), peroxynitrite ($ONOO^-$), and hydrogen peroxide (H_2O_2) (228).

Elevated ROS levels induce oxidative damage to lipids, proteins, and DNA, leading to cellular dysfunction, inflammation, and cell death. ROS-induced damage is implicated in various pathological conditions such as ageing, neurodegenerative diseases, and cardiovascular diseases (229).

The cells have a robust antioxidant defence system comprising chemical antioxidants and antioxidant enzymes to counteract ROS. Chemical antioxidants like vitamins C and E, GSH, carotenoids and flavonoids directly scavenge ROS, preventing oxidative damage (230). Meanwhile, antioxidant enzymes such as SOD, GPx, catalase, and peroxiredoxins play fundamental roles in neutralising ROS by catalysing their breakdown (230). The Nrf2 pathway coordinates cellular antioxidant defence mechanisms. Under oxidative stress, Nrf2 is released from its inhibitor Keap1 (Kelch-like ECH-associated protein 1) and translocates into the nucleus, activating genes encoding antioxidant and detoxification enzymes (231, 232).

Alternatively, inflammation is the natural response to injury or infection. The NF-κB pathway regulates inflammation (232). Upon activation by various stimuli such as pro-inflammatory cytokines, microbial products, or oxidative stress. The NF-κB pathway induces the expression of pro-inflammatory mediators such as cytokines (e.g., IL-6, IL-8), chemokines, adhesion molecules (233) and enzymes. These molecules orchestrate the recruitment and activation of immune cells, amplify inflammatory responses, and contribute to tissue damage and dysfunction (233). Dysregulated NF-κB signalling is implicated in the pathogenesis of various inflammatory diseases. Chronic low-grade inflammation contributes to mood disorders like depression and anxiety, highlighting its broader impact on mental health (234).

The crosstalk between Nrf2 and NF-κB pathways enables cells to effectively integrate oxidative stress and inflammation signals, maintaining cellular homeostasis (235). Understanding this relationship holds promise for developing therapeutic strategies to target oxidative stress and inflammation-related diseases, thereby mitigating neurobiological dysfunction and the progression of neurological disorders. The relationship between oxidative stress and inflammation is bidirectional; oxidative stress can trigger inflammatory pathways, while inflammation can promote ROS generation, exacerbating oxidative damage.

2.2.3.7 Gut microbiome

The gut microbiota refers to thousands of microorganisms colonising the gut. These species live together, metabolising and interacting to produce metabolites that can impact host metabolism, immune system, and neurobiological function. The present species can influence the physiology and development of the individual and the maintenance of the host's health. The dominant bacterial phyla in the human GIT are *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. While there have been proposals to rename these phyla to *Bacillota*, *Bacteroidota*, *Actinomycetota*, *Pseudomonadota* and *Verrucomicrobiota* respectively, these new names have not been universally implemented. Therefore, the older names will continue to be used. *Firmicutes* and *Bacteroidetes* phyla comprise the vast majority, ranging up to 90% of the total bacteria (236).

The gut microbiome modulates the gut-brain axis. Disruptions in the composition and balance of the gut microbiota, known as dysbiosis, have been linked to various neurological conditions and mental health disorders (143). As highlighted in the previous section, the gut microbiome influences neurobiological health via the

interaction with the gut-brain-axis; this occurs through several mechanisms, including the metabolism of nutrients, production of neurotransmitters (142, 237) (Table 2.9), as well as the generation of bioactive compounds, such as SCFA (238) modulation of immune responses (143, 239). Mounting evidence suggests a connection between the gut microbiome and conditions like anxiety, depression, and even neurodegenerative disorders such as Alzheimer's and Parkinson's diseases. Lifestyle factors such as diet, antibiotic use, and stress can influence the microbiome's composition, impacting its ability to support neurobiological health (143).

Table 2.9 Summary of the neurochemical production shown by specific genera (142).

Genus	**Neurochemical**
Lactobacillus and *Bifidobacterium*	GABA
Lactobacillus and *Bacillus*	Acetylcholine
Escherichia, *Streptococcus*, *Enterococcus, Lactobacillus and Lactococcus*	Serotonin
Escherichia and *Bacillus*	Norepinephrine
Escherichia, *Bacillus, Lactobacillus, Lactococcus and Streptococcus*	Dopamine
Lactobacillus, Lactococcus, Streptococcus and *Enterococcus*	Histamine

2.2.3.8 Neurotransmitters

As highlighted in the section 2.2.1.2, neurotransmitters are the central chemical messengers facilitating communication between neurons in the brain and play a fundamental role in neurobiological processes. Imbalances in neurotransmitter systems are associated with a spectrum of neurological and psychiatric disorders. For instance, deficiencies in 5HT are linked to mood disorders such as depression (240), while disruptions in the dopaminergic system are implicated in conditions like Parkinson's disease and schizophrenia (241). The GABAergic system, responsible for inhibiting neural activity, is associated with anxiety disorders when dysregulated (242).

Dysfunctions in neurotransmitter systems may contribute to attention disorders, sleep disturbances, and neurodegenerative diseases.

2.3 Kiwifruit effects on neurobiological health

2.3.1 Evidence from *in vitro* and animal studies

The impacts of KF on neurobiological health have been investigated using *in vitro*, animal models, and human studies. In *in vitro* studies, brain cells have been used to evaluate the potential protective effects of KF and KF extracts against induced oxidative damage (Table 2.10). For example, extracts of the KF varieties 'Biden' and 'Qinmei' increased the viability of PC-12 brain cells subjected to damage induced by $A\beta_{1-42}$ (16) and lead (243). The protective effects of *Actinidia callosa* and *arguta* KF were also observed in other cell lines, such as Neuro-2A and human neuroblast cells MC-IXC, where KF extracts demonstrated protective effects against oxidative damage induced by glucose (244) and H_2O_2 (245). Additionally, all these experiments showed decreased intracellular ROS generation compared to cells treated with stressors alone. Moreover, studies have investigated the mechanisms behind these effects, revealing decreased lactate dehydrogenase (LDH, a marker of cellular damage) (244) levels and reduced activation of caspase-3/7 and caspase-9 (two critical enzymes involved in the process of apoptosis) (109, 244) in brain cell lines with KF treatments (Table 2.10), indicating the potential involvement of cell senescence. However, it remains unclear which specific compounds in KF may be responsible for these effects, although researchers have suggested that polyphenols could be the active constituents (109, 244).

Table 2.10 *in vitro* studies assessing the effects of KF and KF products on brain health protection.

Consumption type	Main findings	Reference
KF extract *Actinidia eriantha* cv. Bidan	Increased cell viability decreased intracellular ROS generation and decreased Caspase 3/7 activation. (PC-12 cells treated with Aβ_{1-42})	(16)
KF extract *Actinidia arguta* cv. Autumn Sense	Decreased intracellular ROS, increased cell viability, decreased LDH. (Human neuroblasts MC-IXC treated with glucose)	(244)
KF extracts Hayward, Xuxiang, Qinmei, Jinkui, and Wancui	Increased cell viability decreased intracellular ROS. (PC-12 cells treated with lead)	(243)
KF extract *Actinidia arguta* cv. Mansu, cv. Haeyeon, cv. Chiak	Decreased intracellular ROS, increased cell viability, (PC-12 and SH-SY5Y treated with H_2O_2) inhibition of AChE and BCHE – *in vitro*	(245)
KF extracts *Actinidia callosa* peel (kiwi fruit)	Increased cell viability in pre-treatment, decreased intracellular ROS, attenuated caspase-3 and -9 activities. (Neuro-2A cell treated with Methylglyoxal)	(109)
Green and gold KF 'Hayword' and 'Zesy002'	Modulation of bioamines Caco-2	(246)

Bioassays have provided evidence for the neurotransmitter modulatory potential of KF. For instance, KF juice was shown to reduce monoamine oxidase (MAO) enzyme activity (247), the enzymes involved in the breakdown of bioamines. Additionally, KF was found to reduce the activity of acetylcholinesterase (AChE) and butyrylcholinesterase (BCHE) enzyme activities (245). *In vitro* studies have also explored the modulatory potential of KF on neurotransmitter concentrations, including GABA, 5HT, and 3,4-Dihydroxyphenylalanine (L-DOPA) (246). Moreover, research has indicated a potential modulatory role of KF on the expression of period and clock genes in cell lines, suggesting a possible influence on sleep/wake control (246). These findings suggest the potential role of KF in neurotransmitter modulation.

Similarly, in pre-clinical animal models investigating the impact of KF on the brain, researchers have employed a comparable approach to examine the underlying mechanisms of improvement. These animal studies have assessed behavioural and

measurable neurobiological outcomes, focusing on cognitive ability and sleep impacts (Table 2.11).

In a study involving mice treated with Aβ1-42 to induce neurological damage, treatment with Bidan KF led to improved spatial cognitive ability and short-term memory compared to untreated mice (16). These improvements were accompanied by increased activity of antioxidant enzymes such as SOD and catalase. Similarly, in a study of rats exposed to lead and treated with or without 'Qinmei' extract, increased SOD and GPx activity and expression were observed, along with reductions in inflammatory markers TNF-α and IL-1β. This was associated with learning and memory development improvements, which were impaired by lead exposure (243). Lastly, rats fed a high-fat diet exhibited impaired cognitive processing capabilities, as evidenced by performance in the Morris water maze and Y-maze tests, which were alleviated by administering KF extract from arguta. These improvements were concurrent with enhanced SOD and GSH levels in the brain, as well as reduced levels of lipid peroxidation products (244).

Regarding sleep, KF peels improved sleep onset latency and duration in male mice treated with pentobarbital (118). Similarly, green KF peel also improved aspects of sleep architecture, increasing the amount of deep sleep and decreasing sleep onset latency (119). The authors of these studies suggest the potential role of KF in eliciting an effect on the GABAergic pathways, which are responsible for calming, which may explain the observed effects.

Table 2.11 *In vivo* animal studies of KF on brain measures.

Consumption type	Neurobiological outcomes	Biomarkers	Reference
KF extract - *Actinidia eriantha* cv. Bidan /ICR mice treated with $A\beta_{1-42}$	Improved spatial cognitive ability, no changes in motor ability, improved short-term memory, cognitive impairment	Increased SOD, catalase compared to both control and AB and GSH/GSSG ratio - same to control/higher than AB	(16)
KF extract – Qinmei/ Sprague-Dawley (SD) rats treated with Pb	Morris water maze and significantly repaired Pb-induced learning and memory deficit	Increased dendritic spine density in the hippocampus, increased SOD and GPx activity and expression compared to Pb treatment only, reduced Iba1 marker, TNF-α and IL-1β	(243)
KF extract - *Actinidia arguta*/ C57BL/6 mouse obese induced with high fat	Y-maze Increased alternation behaviour. Improved step-through latency MWM, Long-term memory and spatial navigation	Reduced fasting glucose, improved glucose tolerance, lowered AUC. Increased ACh and decreased AChE activity. Increased SOD and GSH and lowered MDA in the brain compared to high fat only. Rescued mitochondrial ROS, membrane potential increase to normal, increased ATP	(244)
Green and gold protein extracts/Caenorhabditis elegans	Rescued the degeneration and death of motor neurons and locomotion impairment	-	(248)
KF peel extracts - Actinidia *deliciosa* and *Actinidia chinensis*/ Male ICR mice treated with pentobarbital	Acute administration decreases sleep latency and an increases sleep duration	-	(118)
KF peel extracts - Actinidia deliciosa/ C57BL/6N mice	Acute and sub-chronic administration increased in NREM and decreased sleep latency	-	(119)

2.3.2 Evidence from human studies

As mentioned in section 2.1.3, few studies have shown the positive benefit of KF on neurobiological outcomes, including mood and sleep quality as summarised in Table 2.12**.** The impact of KF consumption on vitality, well-being, and mood is becoming increasingly evident. In a randomised parallel human study, 35 young males received either half or two gold KF (*A. chinensis* var. Hort16A) daily for six weeks. POMS, recognised as the gold standard for mood research (249), was measured before and

after the intervention period. The group supplemented with two gold KF experienced a decrease in total mood disturbance scores, with a subgroup analysis indicating a reduction in individuals with higher baseline mood scores (3). These findings suggest that KF supplementation may enhance the mood of individuals with moderate mood disturbance. Moreover, increased plasma and urinary ascorbic acid levels suggest improved mood could be attributed to elevated vitamin C levels.

In a subsequent three-arm parallel study involving young males and females, participants were assigned to receive either a placebo, 250 mg vitamin C tablet, or two gold KF (*A. chinensis* var. chinensis 'Zesy002'/’SunGold’) for four weeks. POMS and other vitality measures were assessed. Plasma vitamin C levels reached saturation within two weeks for both the KF and vitamin C groups. The participants consuming KF showed reduced mood disturbance, decreased fatigue, and improved well-being as measured by POMS, though these were not sustained in the washout period. Vitamin C tablet consumption alone improved well-being after two weeks, enhanced subjective mood, and reduced subjective fatigue in participants with consistently low vitamin C levels during the lead-in to the study. Eating KF improved vitality in adults with low vitamin C levels. Vitality improved in participants taking vitamin C tablets, albeit to a lesser extent than with KF, indicating that other nutrients in KF may also boost vitality (4).

Accumulating evidence in sleep quality suggests that consuming two green KF one hour before bedtime for four weeks may improve various aspects of sleep. Three studies have investigated this. The first study was a pre- and post-study conducted in 24 males and females with poor sleep quality. They measured sleep via actigraphy and found that consumption of two green KF was associated with increased sleep time and efficiency, as well as a decrease in the time taken to fall asleep, waking time after sleep onset, and

the Pittsburgh Sleep Quality Index (PSQI) score (5). In a randomised, parallel study, 74 males and females with insomnia were given either 130 g of green KF or pear. The improvement from baseline to post-treatment showed greater efficacy in the KF condition compared to the pear condition regarding sleep quality and daytime function. However, the objective measures using actigraphy failed to see any impact (6). Similarly, in a cohort of 15 athletes given the same protocol (two green KF, 1 hr before bed for four weeks), athletes felt that their sleep quality had improved and that they were better able to cope with stress, including sports-related stress compared to their pre-intervention period. One notable aspect of these human sleep studies is the lack of biomarker analysis, making it difficult to understand the mechanisms of action. However, the authors suggest that the benefits may be attributed to added vitamins, polyphenols acting as antioxidants, and the potential 5HT provided by KF.

Overall, the human studies investigating the effects of KF on neurobiological health have established a robust foundation for future research. They provide valuable insights into potential beneficial outcomes, highlighting specific areas warranting further exploration. For instance, based on current research, gold KF may be particularly beneficial for mood enhancement, while green KF could potentially improve sleep quality. It is worth mentioning that all these human studies have used fresh KF, which may limit the generalisability of the results to powdered or juice formats, which are also available. Understanding these options is beneficial as these formats may have longer shelf lives and offer greater convenience. Future studies should also aim to confirm these findings by incorporating additional biomarkers to assess the underlying mechanisms of action, particularly those directly influencing neurobiological health.

Table 2.12 Clinical studies of the effects of KF consumption on neurobiological measures.

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size	Outcome measures	Biological samples measured	Main effects	Reference
Pre and post	Two green KF Flesh for 4 weeks 1 h before bed	None	Mixed cohort 34.4 ± 12.9 y Bad sleep	24	Sleep quality	None	Sleep diary - ↓WASO, SOL, CPSQI, ↑TST, SE $P<0.05$ Actigraphy = ↑TST, SE $P<0.05$	(5)
Parallel RT	130 g green KF Flesh 4 weeks 1 h before bed	Pear	Mixed cohort 24.5 ± 3.6 y Insomnia	37	Sleep quality	None	↑sleep quality and daytime functioning	(6)
Parallel	0.5 or 2 flesh of gold KF. 6 weeks	KF doses	Males 21 ± 3 SD y	36	Mood	Plasma Urine	↓ total mood, depression ↑Vit E and serum D3, plasma vitamin C, urine vitamin c	(3)
Parallel	4 weeks 2 flesh of gold KF	Vitamin C or Placebo	Mixed cohort 20 ± 2 y	57	Mood Sleep quality Well-being	Plasma	↑ plasma vitamin C, mood, and well-being	(4)
Quasi-experimental	Two green KF Flesh for 4 weeks 1 h before bed	None	Mixed cohort 23.2 ± 3.9 y Athletes	15	Sleep quality Stress	None	↑sleep quality ↓ stress	(7)

2.3.3 Potential mechanism of KF on neurobiological health

Previous research has explored the numerous health benefits of consuming fresh KF or KF products. These benefits highlight the promising potential of KF in enhancing various aspects of health, all of which may ultimately impact neurobiological health. Studies indicate the beneficial effects on cardiovascular function, including improvements in blood pressure (15, 55, 67, 68), lipid profiles (64, 65), and anthropometric measures (15, 55), potentially mediated through the ACE pathway (68). While direct research on CBF is lacking, preliminary findings suggest green KF may improve peripheral blood flow (48). Moreover, KF exhibits promising implications for glycaemic control by attenuating insulin and glucose responses (71-75), indicative of its potential in metabolic health. Furthermore, KF demonstrates effects on oxidative stress (13), inflammation (14, 15, 62) and antioxidant protection (59, 60), suggesting a holistic impact on systemic health.

The effect of KF extends to the gut microbiome, where it induces discernible changes (12, 54, 55). Evidence also suggests potential neurotransmitter modulation by KF, as evident from *in vitro* (246) and biobased assays (247), as well as human studies which potentiate alterations in peripheral amino acid profiles (82). However, further *in vivo* exploration is necessary to elucidate these mechanisms fully. Overall, KF has the potential to impact neurobiological health through its multifaceted effects on physiological pathways. At its core, the nutrient density of KF could be what is allowing for all the associated neurobiological health benefits (Table 2.13).

The impact of KF nutrients on neurobiology is demonstrated through three interconnected mechanisms. Firstly, the array of nutrients in KF, including vitamins, minerals, amino acids, neurotransmitters, and polyphenols, may provide

neuroprotective effects and serves as precursors for neuroactive compounds. This dual role emphasises their significance in supporting neurobiological health and function.

Table 2.13 Overview of the potential influence of KF nutrients on neurobiological health.

Nutrients	Potential mode of action in the brain	KF components and attributes	Potential
Carbohydrates Fibre	Insulin response Postprandial absorption of amino acids Growth of specific GM that produce NCs	Low GI Low sugar content Source of dietary fibre	Moderate Moderate
Fats	Reduction of inflammation	Source of Omega-3	Low
Protein	Supply of amino acids for metabolism to sleep/wake NCs. Impact on gut hormones leptin, Ghrelin, CCK etc	Rich and moderate amounts of amino acids Kiwellin, KiTH, kissper	Low
Vitamin C	Tryptophan Metabolism Antioxidant - reduction of stress/inflammation	Good source	Moderate
Vitamin D	Antioxidant - reduction of stress/inflammation	Not known to contain	None
Vitamin E	Antioxidant - reduction of stress/inflammation	Source	Moderate
Vitamin B_{12}	Amino acid metabolism into NCs		Low
Vitamin B_9	Amino acid metabolism into NCs	Good source	Moderate
Vitamin B_6	Amino acid metabolism into NCs	Source	Low
Vitamin B_3	Tryptophan Metabolism		Low
Potassium	Amino acid metabolism into NCs	Source	Low
Magnesium	Amino acid metabolism into NCs		Low
Zinc	Amino acid metabolism into NCs		Low
Enzymes	Breakdown of protein into AA Reduction of inflammation	Actinidin	High
Polyphenols	Growth of specific GM that produce NCs Antioxidant - reduction of stress/inflammation Blood flow Enzyme inhibition	Catechin, L-epicatechin, Quercetin	High
Neurochemicals	Supply of NCs	Serotonin	High

Secondly, the presence of the digestive enzyme actinidin in green KF ‘Hayward’ variety and to lesser extent in ‘SunGold’ indirectly contributes to the neurobiological effects of the first-mentioned mechanism by facilitating protein utilisation. This accelerated protein digestion and absorption of dietary protein ensures the availability of EAA for neurotransmitter synthesis and supports overall metabolic processes crucial for brain function.

Lastly, emerging research suggests that KF may modulate the composition of gut microbiota, potentially leading to alterations in the production of neuroactive

substances. This interaction between KF nutrients, digestive enzymes, and gut microbiota highlights the multifaceted nature of KF's impact on neurobiology (Figure 2.5).

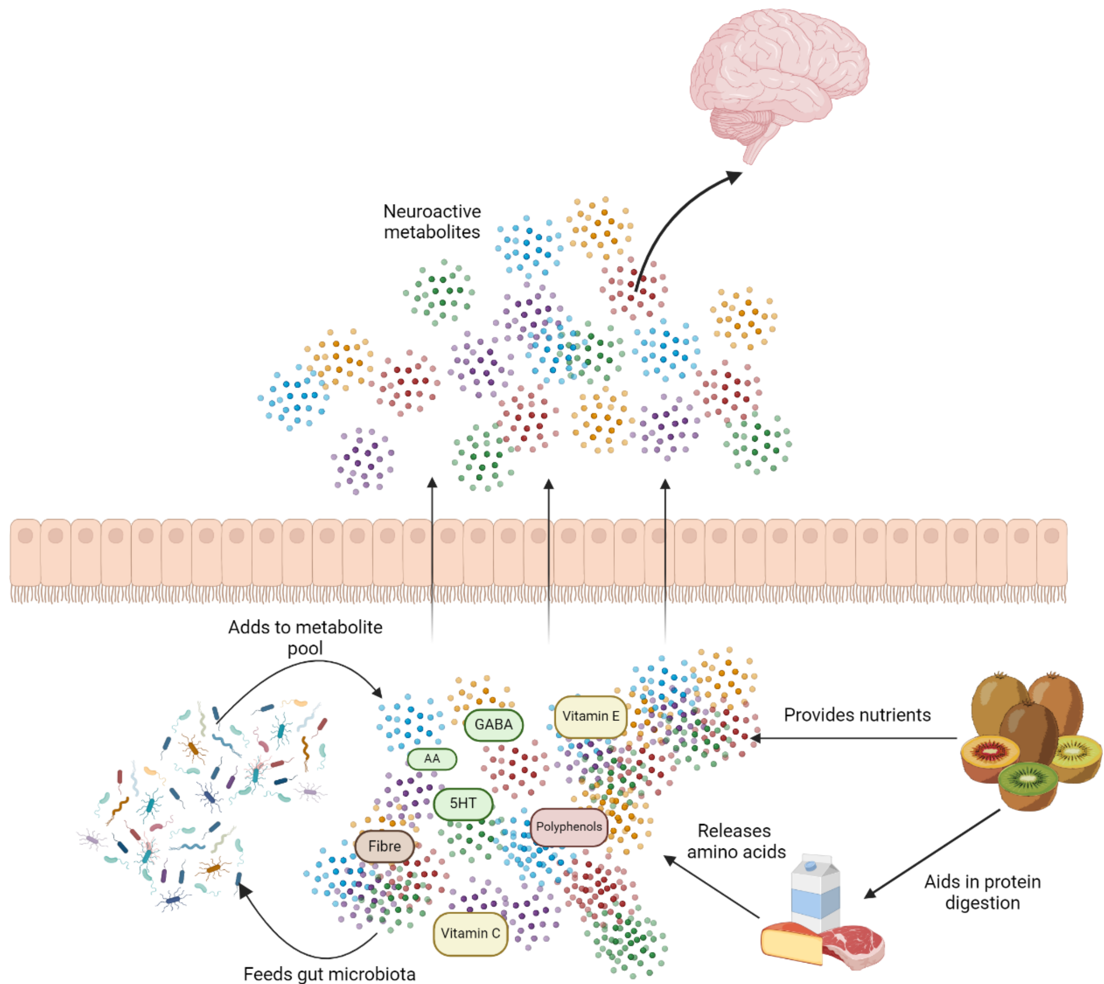


Figure 2.5 Potential mechanism of KF improving neurobiological outcomes. Figure Created with BioRender.com.

2.3.3.1 Effects of nutrients from KF on neurobiological health

The macro and micronutrient bundle provided by KF may have varying effects on neurobiological outcomes. The carbohydrates and fibre content of KF may be a critical component in health. Observational studies have found a negative association between reduced fibre intake and sleep quality (250). Increasing fibre intake through KF may

improve sleep quality. Furthermore, KF is known to be a low-sugar and glycaemic index (GI) fruit, as indicated by previous studies (9, 72). Low-GI breakfast foods have been shown to improve cognitive function (251), in contrast clinical studies on KF and carbohydrate breakfasts did not observe similar effects on cognition (117).

KF is notable for its richness in compounds such as GSH, arginine, and GABA, alongside moderate 5HT levels, tryptophan, and tryptamine (Table 2.2) (35-38). Studies have shown increased excretion of the major metabolite of 5HT, 5-Hydroxyindoleacetic acid (5HIAA), after consumption of green KF (8). 5HT plays central roles in various bodily functions, particularly in the intestine, for processes such as peristalsis and other critical digestive functions (252-257), with around 90% of it being produced in the gut (258). However, it is important to note that 5HT does not cross the BBB when transported in the bloodstream (259). Additionally, tryptophan must cross this barrier to undergo conversion along this pathway. Therefore, increased 5HT levels due to KF consumption may not necessarily translate into effects within the brain.

Furthermore, studies have shown that oral administration of GABA can elevate systemic plasma GABA levels after 30 minutes (260), its impact on brain GABA levels remains uncertain. Understanding GABA's ability to cross the BBB is complex. Traditionally, it was believed that GABA could not pass this barrier; however, some research suggests otherwise. Nevertheless, no human studies have conclusively demonstrated GABA's permeability across the BBB (184).

Besides the macronutrient content, KF is also highly recognised for its high levels of vitamins C and E, which may contribute to neurological improvement. Vitamin C deficiency has been linked to sleep disturbances (261), although few detailed studies have directly examined the effects of vitamin C and E supplementation on sleep.

Compared to a purified vitamin C supplement, the vitamin C in KF seems to have a more sustained effect on mood improvement (4). The vitamin E in KF may also have higher bioavailability due to its unique structure (116). Both of these vitamins are potent antioxidants, capable of protecting neural cells from the damaging effects of ROS (230) and they also serve as cofactors in neurotransmitter metabolism (262). These two vitamins, from KF, are likely to play a significant role in improving neurological outcomes, especially when supplemented in cohorts with deficiencies, as demonstrated in a study of male students (3).

KF is a rich source of folate and potassium, both of which have demonstrated potential benefits for sleep improvement in various sleep studies (263, 264), cognitive studies (265, 266) and mood enhancement (267). Folate is crucial for brain health, supporting the production of neurotransmitters and DNA synthesis. Similarly, potassium is essential for proper brain function, as it helps maintain the electrochemical balance across neurons.

KF contains a natural combination of B vitamins, and numerous studies on B vitamin supplementation have demonstrated improvements in sleep, cognition, and mood (264, 267-275). These B vitamins play essential roles individually, contributing to converting amino acids into neurochemicals. Specifically, they are integral C1 carbon metabolism and the production of S-adenosylmethionine, a key methyl donor for neurotransmitter production (275).

Finally, KF also contains a considerable concentration of polyphenols, with much of it concentrated in the skin (refer to section 2.1.2 Nutrient composition of KF). There is a wealth of research on the benefits of polyphenols for neurobiological health, encompassing various impacts such as cardiovascular health improvement (276), CBF

regulation (277), gut microbiome modulation (278) and neurotransmitter synthesis. Much of this literature focuses on polyphenols found in chocolate (279, 280), tea (281, 282) and berries (283, 284), suggesting that similar benefits may extend to KF consumption. Examples include the modulatory effects on enzymes involved in neurotransmitter metabolism, such as MAO (247), AChE (245) and GABA metabolism enzymes (285). However, detailed research is needed to confirm whether these mechanisms are at play in the context of KF consumption.

2.3.3.2 Actinidin's role in protein digestion and neurotransmitter synthesis

Beyond the AAs, as mentioned, the presence of actinidin may also contribute to greater neurochemical production. Research has shown that beef protein, in the presence of actinidin from fresh green KF, is digested faster, leading to an earlier release of AAs (82). This accelerated digestion could increase AAs availability for uptake into the brain. However, it is important to note that while adding KF may expedite the release of AAs, it does not guarantee faster absorption into the brain. The absorption of tryptophan by the brain is heavily influenced by the ratio of other LNAA (286). Studies have demonstrated that KF induces a slower glycaemic response, which could slow the absorption of other LNAA (9, 73, 93), and therefore could be counterproductive.

Therefore, when exploring the direct impacts of KF neurochemicals, it is important to consider timing and whether KF is co-ingested with other meals, as this could affect absorption. For instance, in sleep studies involving KF consumption, participants were instructed to consume KF one hour before bedtime to maximise its protective antioxidant effects (287). However, no information regarding the timing and composition of any meal consumed prior to eating the KF makes it challenging to draw definitive conclusions. It is possible that the stomach still contained food, and KF might

have assisted in its digestion of the proteins in this meal. Therefore, it's likely that antioxidants are not the only mechanisms that could have an effect. Further research is needed to understand the mechanisms and implications of KF consumption on neurochemical production.

Additionally, KF contains Kiwellin, which, when cleaved by actinidin, forms KiTH and kissper (30). Kissper is thought to create ion-channel-like pores and may have anti-inflammatory properties (34). The potential impact of these compounds on neurobiological health remains unexplored.

2.3.3.3 KF's influence on gut microbiota and neuroactive substances

Beyond fibre having a direct effect on gut health, research indicates that KF consumption may induce a reversible shift in gut microbiota, primarily attributed to its fibre content (12, 50, 98, 99, 288). Certain microorganisms within the intestine produce neurochemicals and vitamins that play roles in brain signalling (142, 289-292). For instance, consumption of freeze-dried green KF has been linked to changes in *Lactobacillus* and *Bifidobacterium* genera, both known for synthesising GABA. This suggests that KF might affect brain function by altering gut microbiota composition, affecting neurochemical production. However, whether such alterations directly contribute to health outcomes remains to be confirmed.

2.4 Concluding remarks

KF may play a significant role in neurobiological impact through various mechanisms. *In vitro* studies suggest that KF exhibits strong antioxidant properties. Similarly, animal studies support this notion, with an additional consideration for potential neurotransmitter control. Human clinical studies contribute to the growing body of

evidence, indicating measured psychological benefits associated with KF, particularly in stress, mood, and sleep quality improvements.

2.5 Organisation of the thesis

The research objectives in Section 1.2 were completed in a single *in vivo* study using pigs as a model for the human adult and a clinical study. The thesis contains four experimental chapters: Chapters 3 to 5 report on data from a single *in vivo* study (growing pig model), and Chapter 6 reports on data from a human clinical study. Chapters 3 to 5 present the results derived from analyses of samples collected during the animal study conducted by Dr Isuri Jayawardana as part of her doctoral research. Her study specifically focused on how actinidin containing KF influenced gluten digestion and immunogenic peptides in a growing pig model.

The animal study had three treatments: a control group receiving only bread and two treatments involving KF varieties (one with green KF 'Hayward' and the other with gold KF 'Hort16a'). The research in this thesis used samples from this animal study to explore three distinct areas contributing to brain health: antioxidant mechanisms, gut microbiota and metabolites, and temporal responses of neurotransmitters.

Previous studies have emphasised the antioxidant protective capabilities of KF in stress-induced rodent models and clinical examinations of borderline disease conditions (16) (243). However, no analysis has elucidated the impact of KF in humans or animals in healthy states or in an animal model more closely aligned with human physiology. Chapter 3, therefore, investigated the three treatments on antioxidant systems within circulatory blood plasma and four brain regions (brain stem, corpus striatum, hippocampus, and prefrontal cortex). Various bioassays were employed, encompassing the quantification of antioxidant protective potential, oxidative stress-inducing

potential, and the activities and expression of antioxidant enzymes (catalase and superoxide dismutase [SOD]) in brain tissues.

In vitro and clinical evidence suggests that KF may influence gut microbiota composition, potentially affecting downstream metabolism of bioamines. Bioamines are crucial determinants of brain function, contributing to neural membrane structure, receptor membrane functions, cell signalling, synaptic plasticity, sleep/wake regulation, mood, and memory formation. However, there is a lack of *in vivo* data on the impact of KF on bioamine profiles in brain regions associated with sleep or mood. Chapter 4 explored the effects of the abovementioned KF treatments on the gut microbiome composition of the proximal and distal colon and also explored the impact on peripheral blood plasma and brain regional (brain stem, corpus striatum, hippocampus, and prefrontal cortex) bioamine concentrations using a Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) approach.

Clinical evidence elucidated the temporal effects of actinidin-containing KF on amino acid metabolism (82). Nevertheless, a notable gap existed in the available data concerning the temporal effects of KF on bioamines within peripheral plasma and various brain regions. Consequently, Chapter 5 investigated the temporal effects of green-fleshed actinidin-containing KF 'Hayward' compared to gold-fleshed KF 'Hort16A' which contains no actinidin on bioamines. This examination focused on postprandial effects (within five hours) of consuming actinidin-containing KF compared to gold-fleshed KF without actinidin in peripheral blood plasma and bioamine concentrations in specific brain regions (brain stem, corpus striatum, hippocampus, and prefrontal cortex) utilising a LC-MS/MS approach.

Lastly, emerging evidence has provided the basis for the beneficial impacts of KF on sleep quality and mood (3, 5). Previous studies have focused on long-term intervention periods (four weeks or more), with no biochemical markers besides vitamin C being quantified. Furthermore, most clinical studies exploring these benefits have used fresh fruit and have yet to incorporate the use of a dried powder product. Therefore, Chapter 6 explored the effect of acute consumption of two forms of green KF, a dried and fresh KF, on sleep quality, mood, and urinary biomarkers in adult males with good or poor sleep quality.

The thesis concludes in Chapter 7, wherein a comprehensive discussion amalgamates the primary outcomes delineated in Chapters 3 through 6. This discourse elucidates the significance and applicability of these findings, the strengths, and limitations inherent in the research, and contemplates potential directions for future research endeavours. Figure 2.6 provides a summary of the experimental approach used in this thesis and how this relates to the research objectives.

Overall objective:

To understand the neurobiological impacts of kiwifruit

Kiwifruit selected for *in vivo* study

- *Actinidia deliciosa* cv. Hayward
- *Actinidia chinensis* cv. Hort16A

Kiwifruit selected for clinical study

- *Actinidia deliciosa* cv. Hayward
- Consumed in two forms:
  1. Fresh flesh only
  2. Freeze dried powder skin included

Experimental approach

***In vivo* study**

Research Objective 1

Determine the differential effect of kiwifruit varieties on regional brain antioxidant systems

Chapter 3

Brain regional and plasma measures of:

- Oxidative stress
- Antioxidant markers
- Vitamin C distribution
- Enzyme activity

Research Objective 2

Investigate the gut-brain axis relationship in response to kiwifruits

Chapter 4

Effect of kiwifruit on:

- Gut microbial populations Proximal and distal colon
- Plasma and brain concentrations of bioamines

Research Objective 3

Characterise the temporal bioamine responses in brain regions and plasma

20 60 120 300 min

Chapter 5

Postprandial response to two different kiwifruit varieties on plasma and brain region concentrations of:

- Amino acids
- Tyrosine metabolites
- Tryptophan metabolites
- Glutamatergic metabolites
- Polyamines

Clinical study

Research Objective 4

Determine clinical effects of two different forms kiwifruit on sleep and mood in human participants

Chapter 6

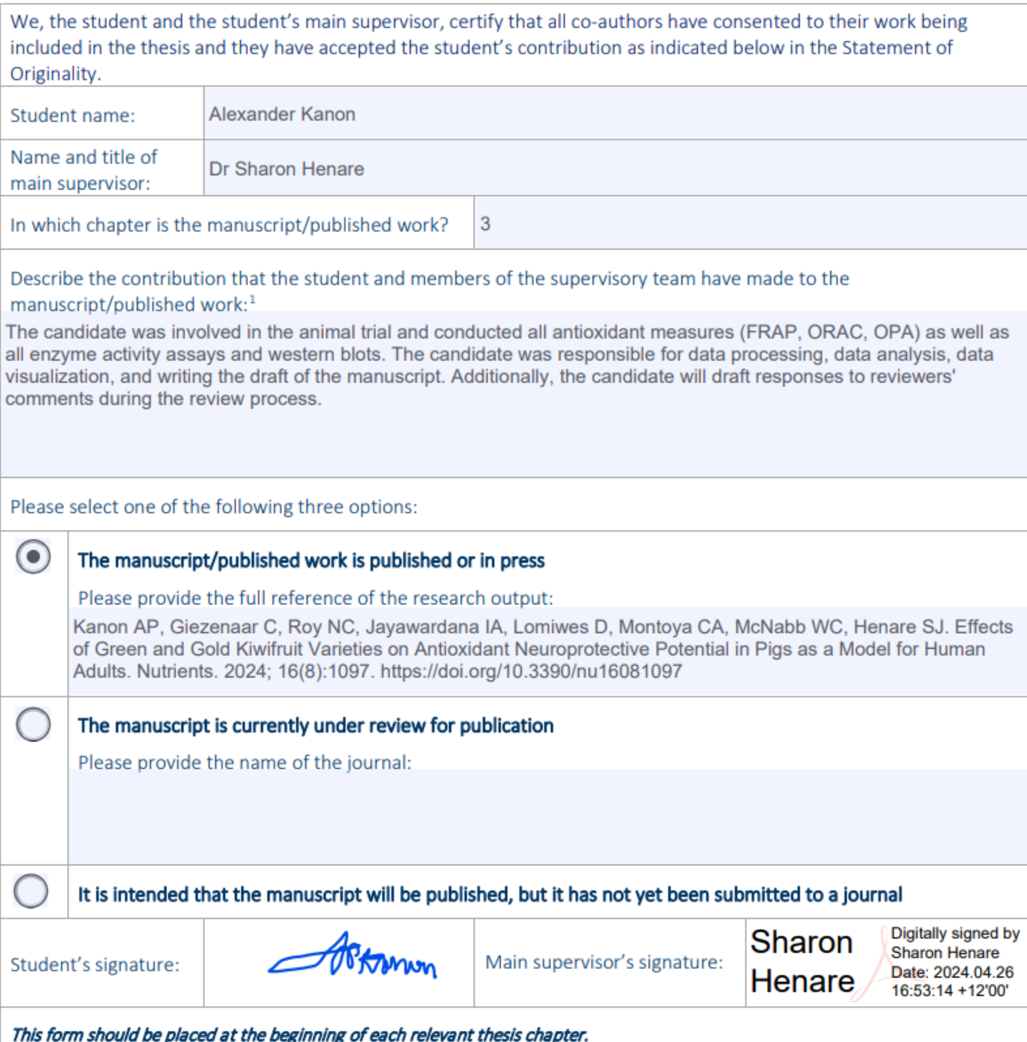
In healthy males, the acute effect of fresh vs powder kiwifruit with skin on:

- Objective and subjective sleep quality
- Subjective mood
- Urinary metabolites

Figure 2.6 Summary of the experimental measures used in this thesis and how they relate to the research objectives.

Statement of contribution

STATEMENT OF CONTRIBUTION DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS



We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Alexander Kanon
Name and title of main supervisor:	Dr Sharon Henare
In which chapter is the manuscript/published work?	3

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

The candidate was involved in the animal trial and conducted all antioxidant measures (FRAP, ORAC, OPA) as well as all enzyme activity assays and western blots. The candidate was responsible for data processing, data analysis, data visualization, and writing the draft of the manuscript. Additionally, the candidate will draft responses to reviewers' comments during the review process.

Please select one of the following three options:

(●) **The manuscript/published work is published or in press**

Please provide the full reference of the research output:

Kanon AP, Giezenaar C, Roy NC, Jayawardana IA, Lomiwes D, Montoya CA, McNabb WC, Henare SJ. Effects of Green and Gold Kiwifruit Varieties on Antioxidant Neuroprotective Potential in Pigs as a Model for Human Adults. Nutrients. 2024; 16(8):1097. https://doi.org/10.3390/nu16081097

( ) **The manuscript is currently under review for publication**

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Doctoral Research Committee
May 2023

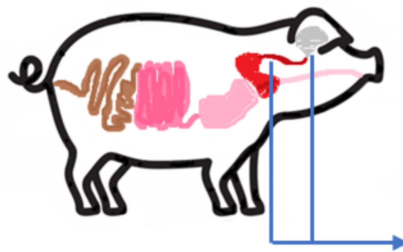
CHAPTER 3. Effects of green and gold kiwifruit varieties on antioxidant neuroprotective potential in pigs as a model for human adults[1]

Prologue

Previous studies have demonstrated the antioxidant protective potential of kiwifruit (KF) both in vitro *and in rodent trials. These investigations primarily relied on induced damage models, employing treatments such as high-fat diets, lead supplementation, and antibody injections. While these findings underscore KF's ability to mitigate neurological stress, they lack exploration into its impact on antioxidant markers under normal physiological conditions. Moreover, no study has examined KF's antioxidant protective potential on the brain using a pig model, which offers the advantage of whole fruit consumption, mirroring human dietary habits.*

The present study seeks to fill this gap by evaluating the effects of green and gold KF varieties on antioxidant systems. This encompasses analysis of chemical antioxidants in both plasma and brain tissue, alongside investigation into the activity and expression of key enzymes like superoxide dismutase (SOD) and catalase. Additionally, we assess changes in vitamin C levels and quantify acetylcholinesterase (AChE) activity in brain regions, providing insight into KF's potential role in preserving brain health.

[1]Part of the contents of this chapter has been published as a peer-reviewed paper:
Kanon AP, Giezenaar C, Roy NC, Jayawardana IA, Lomiwes D, Montoya CA, McNabb WC, and Henare SJ (2024) Effects of Green and Gold Kiwifruit Varieties on Antioxidant Neuroprotective Potential in Pigs as a Model for Human Adults. Nutrients; 16(8):1097. https://doi.org/10.3390/nu16081097



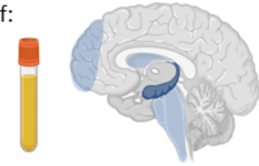
Research Objective 1

Determine the differential effect of kiwifruit varieties on regional brain antioxidant systems

Chapter 3

Brain regional and plasma measures of:

- Oxidative stress
- Antioxidant markers
- Vitamin C distribution
- Enzyme activity
- Brain health



Highlights

- *There was no difference in plasma oxidative stress markers after treatments, but there was increased antioxidant potential after green KF and decreased oxidative generation potential after both KF varieties compared to bread only.*
- *Gold KF consumption increased plasma ascorbate. Ascorbate content did not differ between regions of the brain following KF consumption.*
- *Antioxidant potential increased in all brain regions following consumption of green KF compared to bread and gold KF.*
- *Differential acetylcholinesterase activity was observed across whole brain regions with a reduction occurring following gold KF.*
- *Catalase activity was greatest in the brain stem regardless of treatments.*
- *SOD activity differences across regions Hippocampus > Corpus striatum > brain stem.*

The chapter is presented in a manuscript format and published in "Nutrients".

3.1 Abstract

KF has shown neuroprotective potential in cell-based and rodent models by augmenting the capacity of endogenous antioxidant systems. This study aimed to determine whether KF consumption modulates the antioxidant capacity of plasma and brain tissue in growing pigs. Eighteen male pigs were divided equally into three groups: (1) bread, (2) bread + *Actinidia deliciosa* cv. 'Hayward' (green-fleshed), and (3) bread + *A. chinensis* cv. 'Hort16A' (yellow-fleshed). Following consumption of the diets for eight days, plasma and brain tissue (brain stem, corpus striatum, hippocampus, and prefrontal cortex) were collected and measured for biomarkers of antioxidant capacity, enzyme activity, and protein expression assessments. Green KF significantly increased ferric-reducing antioxidant potential (FRAP) in plasma and all brain regions compared with the bread-only diet. Gold KF increased plasma ascorbate concentration and trended towards reducing acetylcholinesterase activity in the brain compared with the bread-only diet. Pearson correlation analysis revealed a significant positive correlation between FRAP in the brain stem, prefrontal cortex, and hippocampus with the total polyphenol concentration of dietary interventions. These findings provide exploratory evidence for the benefits of KF constituents in augmenting the brain's antioxidant capacity that may support neurological homeostasis during oxidative stress.

Keywords: kiwifruit, antioxidant, neuroprotection, actinidin, brain regions, acetylcholinesterase

3.2 Introduction

Numerous physiological and pathological processes increase the body's reactive oxygen species (ROS) concentration, including physiological or emotional stress and sleep disturbances. Stressful conditions can result in the excessive production of ROS, overwhelming the body's ability to neutralise them and leading to oxidative stress and damage. This process depletes endogenous antioxidants and disrupts the homeostatic mechanisms that maintain cellular health. The imbalance between pro-oxidants and antioxidants in the presence of ROS is a key factor in neuronal damage and the development of neurodegenerative diseases. Maintaining a delicate balance between these factors is essential for preserving cellular health and preventing oxidative damage in the brain (293). The brain's sensitivity to oxidative stress is attributed to its high energy demands, the abundance of lipids and weak antioxidant capacity. This susceptibility to oxidative damage can cause neuronal impairment and functional decline through ROS-mediated brain tissue damage, ultimately increasing the risk of neurodegenerative diseases (294). Specifically, ROS play a crucial role in this process by increasing the brain's susceptibility to damage. Diet and nutrients have been shown to reduce these risks by enhancing the antioxidant system activities thereby protecting from the deleterious effects of oxidative stress and ultimately leading to improved brain health (230, 295, 296).

SOD is essential in reducing oxidative stress by converting ROS into hydrogen peroxide (H_2O_2), which is subsequently converted into inert molecules by other antioxidant enzymes. Malondialdehyde (MDA) is a marker of lipid oxidation and excessive MDA induced by oxidative stress is associated with a major depressive disorder (297). AChE,

the enzyme responsible for breaking down acetylcholine (ACh) and decreased levels of ACh are associated with neurological disorders. Likewise, increasing AChE activity, is associated with neurological pathogenesis (298). This has led to the development of therapeutics that target the treatment of these disorders by inhibiting or reducing brain AChE activity.

KF has been shown to improve subjective mood (3, 4) and sleep quality (5) in human clinical studies. Furthermore, studies have also shown that consumption of fresh KF may regulate antioxidant and inflammatory states (13, 59, 60). KF is a rich source of vitamin C, vitamin E and polyphenols, which serve as antioxidants and it is hypothesised that this may be a mechanism by which KF improves mood (3) and sleep quality (5). The majority of the clinical trials incorporating KF supplementation have used a standardised serving size of the flesh from two fresh KF, adhering to established norms. Several *in vitro* studies have demonstrated that KF is neuroprotective through its efficacy in attenuating cellular ROS while increasing cell viability (16, 243-245). Rodent models have demonstrated the benefit of KF on brain health. A study of mice fed a high-fat diet (to induce stress) reported reduced oxidative stress in brain regions of mice supplemented with an extract of the KF *Actinidia Arguta* (known for its high actinidin activity and content). The reduction in ROS reported in this study was concurrent with increased ACh, improved SOD activity and reduced MDA and AChE activity in brain tissue (244). In another study, Sprague-Dawley rats with lead-induced cognitive dysfunction and neurodegeneration that were fed 'Qinmei' KF had higher hippocampal SOD and glutathione peroxidase (GPx) activities compared with those fed the placebo intervention (243).

While rodent models serve a valuable purpose, translating findings from these models to humans is constrained by significant physiological and metabolic differences. For instance, rodents exhibit a higher mass-specific metabolic rate, and their digestive physiology is less complex than that of humans. Additionally, it is important to comprehend the potential advantages of KF in healthy states, as rodent studies assessing the impact of KF have introduced an external stressor through diet or supplementation. Compared with rodents, the physiological function and brain anatomy of pigs share a closer affinity to humans (299). Moreover, pigs can consume KF in a manner like humans, whereas in rodent studies, the KF needs to be freeze-dried and ground, potentially resulting in a loss of some of its properties. Thus, employing pig models to investigate the effects of KF interventions on brain health outcomes may offer a more accurate reflection of these outcomes in the human brain compared to rodent models.

This study primarily explored the antioxidant effects of KF, and the samples were collected as part of a larger study aiming to map the digestion of gluten and gluten-derived immunogenic peptides in the gastrointestinal tract (GIT) of a pig model that was fed a meal containing gluten. The aim of this study was to evaluate the effect of consumption of green and gold KF interventions over one week, compared to a control diet without KF, on plasma and brain antioxidant levels in a healthy pig model. The distinguishing features between the two KF interventions is the micronutrient composition and the presence of actinidin, a protease enzyme, in the green KF variety, while the tested gold variety had none. Actinidin is an enzyme needed for the cleavage of kiwellin (a minor protein in KF) into KiTH and kissper (29). Kissper is suggested to form ion-channel-like pores by integrating itself into the phospholipid membrane (30) and

may have anti-inflammatory properties (34). This, in turn, could influence ROS. Whether these proteins are responsible for the beneficial effects in the brain has yet to be determined *in vivo.* We hypothesised that both KF interventions would improve both plasma and brain antioxidant markers, and the green KF variety may be more effective due to the presence of actinidin and extra micronutrients.

3.3 Materials and methods

3.3.1 Animals and treatments

Briefly, a total of 18 entire male pigs (n = 6 per diet; body weight (BW): 19.9 ± 0.5 kg, mean ± SE) of nine weeks of age were used in this study. Pigs were housed individually in metabolism crates (1.5 m × 1.5 m) in a room with a 12 h:12 h light/dark cycle and maintained at 22 ± 2 °C (Animal Production Unit, Massey University). Water was provided *ad libitum*. The Massey University Animal Ethics Committee, Palmerston North, New Zealand, approved all animal-related procedures (MUAEC protocol 20/46) (300). All experiment stages, from group allocation to data analysis, were known to the researchers and technicians involved.

On arrival, pigs were fed the same commercial grower mix provided on the farm. From the following day, experimental diets replaced this commercial diet gradually per day by 33%-increments. Each pig received 90 g dry matter (DM) of feed per kilogram of metabolic BW per day (metabolic BW was calculated $BW^{0.75}$) (301). The pigs were provided with a solid meal portion size of 2:1 at 0900 and 1600 h, respectively. The pigs were adapted to their experimental diet for eight days and on the final day were fasted and euthanised. Pigs were assigned to the following experimental diets:

1. Soda bread only
2. Soda bread + gold KF (Actinidia chinensis cv. 'Hort16A')
3. Soda bread + green KF (Actinidia deliciosa cv. 'Hayward')

To limit the digestive system's exposure to proteins other than those derived from wheat, soda bread was employed in the study, as the focus was to evaluate peptides originating from gluten. For the adaptation diets, pure gluten was introduced to reach the daily protein requirements. Before feeding the pigs, soda bread slices were cut into small pieces and KF pulp was crushed manually before mixing it thoroughly with the bread. The fresh KF included in the diets comprised 19% of the daily DM intake, equivalent to two fresh KF eaten with a meal (301). The actinidin activities were 27.08 ± 1.20 U/g of DM and 0.22 ± 0.15 U/g of DM for green KF and gold KF respectively (300). Average daily nutrient intake was calculated from when pigs were on experimental diets (Table 3.1). Commercially available vitamin and mineral premixes were added to the adaptation diets according to the National Research Council (302) recommendations for the pigs' daily nutritional requirements.

Table 3.1 Average determined nutrient (g/kg of body weight (BW) per pig) intake of diets with bread only, bread + gold KF and bread + green KF over the supplementation period.

Composition (unit/kg BW per pig)	Bread only	Bread + gold KF	Bread + green KF
Dry matter (g)	40.81	40.61	41.68
Protein (g)	2.80	2.91	3.00
Starch (g)	14.34	14.82	15.23
Fibre (g)	2.09	2.15	2.24
Gross energy (kJ)	493.91	510.51	525.12
Vitamin C (mg)	0.27	13.27	4.69
Vitamin E (mg)	3.38	2.87	3.30
Vitamin K (µg)	0.18	0.15	0.17
Vitamin B3 (mg)	1.64	1.48	1.56
Vitamin B6 (mg)	0.30	0.31	0.28
Vitamin B9 (µg)	23.06	19.16	19.44
Total polyphenol content (mg GAE)	0.22	0.63	1.17

GAE: gallic acid equivalents

3.3.2 Euthanasia and tissue collection

On the final day of the experimental diet, pigs were euthanised 300 min after their final meal. This final meal was smaller than their adaptation meals to mimic a standard meal size of bread intake by humans, consisting of four slices of bread (178.2 g DM) and two KF of their allocated KF intervention (25.6 g DM of green KF and 27.2 g DM of gold KF). The pigs were anaesthetised through intramuscular injection in the neck at a dose rate of 120 µL/kg of an anaesthetic cocktail (Zoletil 100 (50 mg/mL), Ketamine (50 mg/mL) and Xylazine (50 mg/mL)). Pigs were transferred from the cage to the surgery room, where blood samples (~4 mL) from the anterior jugular vein (which carries blood from the brain to the heart) were collected. Blood samples were collected into ethylenediaminetetraacetic acid (EDTA)-containing tubes (BD Vacutainer®, Becton, Franklin Lakes, NJ, USA) and kept on ice for 10 min before centrifugation for separation of plasma at 3,000 g for 5 min. The plasma samples were frozen on dry ice immediately and stored at -80 °C until analysis. Once blood samples were collected, the pigs were euthanised by intracardial injection of sodium pentobarbitone (0.3 mL/kg BW of Pentobarb 300; Provet NZ Pty Ltd, Christchurch, New Zealand) (301).

Once pigs were euthanised, a registered veterinarian removed their head from the body and placed it into clamps. The skull plate was cut carefully using a bone saw, ensuring the brain remained undamaged. Once the skull plate was cut, the plate was levered up to give access to the brain. The brain was carefully scooped out, and the two lobes were gently pulled apart. The left hemisphere was used for consistency with other studies (303) and the brain stem (regulation of vital life functions, e.g., breathing, blood pressure), corpus striatum (reward and reinforcement circuit), hippocampus (role in

learning and memory) and prefrontal cortex (central role in cognitive control functions) were carefully dissected out. All the samples were snap-frozen in liquid nitrogen, kept on dry ice, and stored at -80 °C for analysis. All brain samples were collected within 15 min of euthanasia.

3.3.3 Biochemical measures

Prior to biochemical analyses, the brain tissue regions were lysed in Neuronal Protein Extraction Reagent (N-PER™, Thermo Scientific, Waltham, MA, USA) with a cocktail of Protease and Phosphatase inhibitors included (Pierce Protease and Phosphatase Inhibitor Mini Tablets, Thermo Scientific, Waltham, MA, USA) at a ratio of 10 ml per 1 g of tissue. This mix was then placed on ice for 10 min and centrifuged at 10,000× g for 10 min at 4 °C. The Bradford Assay (Bio-Rad Laboratories, Hercules, CA, USA) measured the supernatant protein concentrations with bovine serum albumin as the standard. As described in the following sections, the supernatant was aliquoted and stored at -80 °C for downstream analyses.

3.3.3.1 Plasma oxidative stress measures

Plasma MDA levels (lipid peroxidation biomarker) and plasma protein carbonyls (protein peroxidation biomarker) were used to evaluate the oxidative stress state of animals. MDA was assessed by High-Performance Liquid Chromatography (HPLC) using a modified method described by Karatepe (304) against MDA standards. MDA levels were calculated against MDA standards and presented as µmol/L. Protein carbonyls were measured using the modified version of a colourimetric end-point assay described by Levine, Williams (305). Carbonyl levels were calculated as nmol/L.

3.3.3.2 Plasma and brain tissue antioxidant measures

3.3.3.2.1 Oxidative potential assay (OPA)

ROS generating potential capacity was assessed using a modified car-boxy-dihydro-2′,7′-dichlorohydrofluorescein diacetate (carboxy-H2DCFDA) kinetic assay described by Wang and Joseph (306). The assay consisted of hydrolysing 10 µM carboxy-H2DCFDA (Thermo Scientific, Waltham, MA, USA) into the product dichlorofluorescein (DCF), which is fluorescent when oxidised. Car-boxy-H2DCFDA was hydrolysed with an equal volume of methanol and 1 M potassium hydroxide for one h at room temperature. Five µL of diluted plasma (1:5) and brain lysates (1:100) were added to black 96-well plates and then 1 µM H_2O_2 was added. Immediately after, 5 µL of DCF was added to each well. The changes in fluorescence intensity were measured over 5 min at 37 °C using a fluorescence plate reader with excitation and emission wavelengths of 485 and 528 nm, respectively. All plasma and brain extracts were assayed in triplicate, with the CV of replicate measures being < 10%. Data were presented as the percentage change in relative fluorescence intensity after 5 min ($\%\Delta FI_{5min}$) and for brain tissue, standardised to a milligram protein content.

3.3.3.2.2 Ferric-reducing antioxidant power (FRAP)

The FRAP assay was measured using the standard method described by Benzie and Strain (307). Briefly, plasma and brain lysates diluted in acetate buffer (1:8) were added to an equal amount of FRAP reagent (containing TPTZ [2,4,6-tripyridyl-s-triazine] and ferric chloride in a hydrochloric acid solution). After 15 min of incubation at room temperature, the absorbance was measured in a plate reader set at a wavelength of 593 nm. All plasma and brain extracts were assayed in triplicate, with the coefficient of

variation (CV) of replicate measures being < 10%. Plasma and brain lysates' antioxidant capacity was measured against a standard curve of Trolox (Merck, Auckland, NZ), calculated as µM Trolox equivalents and standardised to a milligram protein content of plasma and brain tissue.

3.3.3.2.3 Oxygen Radical Absorbance Capacity (ORAC)

The ORAC assay was measured using the standard method described by Prior, Hoang (308). Briefly, plasma and brain lysates were diluted in PBS (plasma 1:40 and brain 1:40), and fluorescein was added and incubated at 37 °C for 30 min. 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was added and the fluorescence intensity was measured on a plate reader with excitation and emission wavelengths of 485 and 520 nm, respectively for 90-120 min at 2 min intervals. All plasma and brain extracts were assayed in triplicate, with the CV of replicate measures being < 10%. The ORAC was measured against the standard curve of Trolox (Merck, Auckland, NZ), calculated as µM Trolox.

3.3.3.2.4 Vitamin C (ascorbate) concentration

The ascorbate content of plasma and brain tissue was measured on reverse-phase HPLC with a Synergi 4 micron Hydro-RP 80-A column and an ESA coulochem II electrochemical detector (78). Ascorbate concentrations in plasma were expressed in µM, and those in brain tissue were expressed in nmol/mg of wet-weight tissue.

3.3.3.2.5 Catalase activity

The Catalase activity was determined in the brain regions using the Catalase Colorimetric Activity Kit (Invitrogen™, Waltham, MA, USA). A bovine catalase standard was used to generate a standard curve for the assay and all tissue lysates read off the standard curve. Brain lysates were diluted at 1:20 in the provided assay buffer and added to the wells of a half-area transparent plate. H_2O_2 was added to each well and the plate was incubated at room temperature for 30 min. Following this incubation, the supplied colourimetric detection reagent was added, followed by diluted horseradish peroxidase, and incubation at room temperature for 15 min. The coloured product was read at 560 nm. All brain extracts were assayed in triplicate, with the CV of replicate measures being $< 10\%$. The activity of catalase was standardized to protein and expressed as U/mg protein.

3.3.3.2.6 Superoxide Dismutase Activity

The SOD activity was determined in the brain regions using a SOD Activity Assay Kit (Sigma-Aldrich, St. Louis, MO, USA), based on the procedure described by Peskin and Winterbourn (309). A bovine SOD standard was used to generate a standard curve for the assay and all tissue lysates read off the standard curve. Briefly, samples were diluted at 1:1000 with the provided assay buffer and added to the wells. The activity of SOD was determined by measuring the decrease in superoxide anions (generated by the enzyme xanthine oxidase [XO]). The superoxide anions react with water-soluble tetrazolium (WST) dye, producing a colour read at a 450 nm wavelength. The decrease in signal is proportional to SOD inhibition activity. All brain extracts were assayed in triplicate with

the CV of replicate measures being < 10%. The activity of SOD was standardised to protein and expressed as U/mg protein.

3.3.3.2.7 Western blot of antioxidant enzymes

A western blot analysis was conducted to examine the protein levels of antioxidants in the brain regions. The biochemical analysis section described the brain lysates used for this analysis. Aliquots containing 25 µg of total protein were boiled in a loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. Catalase was resolved onto wells of 12% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA). SOD enzymes were resolved onto wells of 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis of all gels was conducted in Bio-Rad Criterion cell systems at room temperature under normal running buffer (Tris/Glycine/SDS buffer).

Proteins from the gels were blotted onto Immun-Blot® PVDF Membrane (Bio-Rad Laboratories, Hercules, CA, USA) and then blocked with 5% non-fat dry milk powder in PBS-Tween (0.08 M Na2PO4, 0.02 M NaH2PO4, 0.1 M NaCl, 0.1% Tween 20). After washing with PBS-Tween three times for 10 min each, the membranes were cut at approximately 50kD and 30kD and incubated with primary polyclonal antibodies for 1 h at room temperature. Polyclonal antibodies used included rabbit anti-catalase (Thermo Scientific, Waltham, MA,USA, BS-2302R), rabbit anti-SOD2 (Thermo Scientific, Waltham, MA,USA, PA1-31072), rabbit anti-SOD1 antibodies (Abcam, Cambridge, UK, ab13498) and rabbit anti-GAPDH (Thermo Scientific, Waltham, MA,USA, PA5-85074) diluted to 1:1,000, 1:5,000, 1:5,000 and 1:10,000, respectively, in PBS-Tween. After washing with PBS-Tween, membranes were incubated with a secondary antibody, mouse anti-rabbit

IgG HRP conjugate (BioLegend, San Diego, CA, USA Cat. 410404), diluted to 1:2,000 in PBS-Tween for 1 h at room temperature. The secondary antibody bound to the membrane was detected with an ECL Western blot substrate kit (Bio-Rad Laboratories, Hercules, CA, USA). The resulting luminescent bands were captured with a G:Box Chemi HR16 (Syngene, Cambridge, United Kingdom). Relative quantitation was calculated by normalisation to housekeeping gene GAPDH.

3.3.3.2.8 Acetylcholinesterase (AChE) activity

The AChE activity was determined using an Amplex™ Acetylcholine/Acetylcholinesterase Assay Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, to tissue lysates and H_2O_2 standards (0.01-2 mM) and phosphate buffer control, Amplex Red substrate was added. The change in fluorescence was measured at 37 °C over 10 min (530–560 and 590 nm excitation and emission wavelengths, respectively) in a fluorescence plate reader. Brain regional AChE activity was calculated against H_2O_2 standards and expressed as nM H_2O_2 produced /µg protein/min. All brain extracts were assayed for AChE activity in triplicate with the CV of replicate measures being < 10%.

3.3.4 Data analysis

Statistical analyses were performed using SPSS software (version 28; IBM, Armonk, NY, USA). Effects of treatment on plasma biomarkers were determined using a one-way analysis of variance (ANOVA). Outliers were assessed by examination of studentised residuals for values greater than ±3. Normality was assessed by Shapiro-Wilk's test ($p > 0.05$). The effects of treatment and brain region and their interaction were determined

using a repeated measures mixed-effects model. Residual plots were inspected to confirm that the normality and constant variance model assumptions were met. An unstructured covariance structure was used to account for the repeated regions. Post hoc comparisons, adjusted for multiple comparisons using Bonferroni's correction, were performed when there were significant main or interaction effects. For some antioxidant measures, data were log-transformed before analysis to stabilise the variance. Statistical significance was accepted at a probability inferior to 0.05 ($p < 0.05$). A statistical trend was observed with a p-value between 0.05 and 0.10. All data are presented as means ± standard error of means (SEM). Pearson correlation was also performed between all regional brain biomarkers and nutrient intakes. Furthermore, where interaction effects were present in brain regions, Pearson correlation was also performed with corresponding plasma measures. Correlations with $p < 0.05$ and R-value > ± 0.5 were considered significant (310).

3.4 Results

3.4.1 Plasma oxidative and antioxidant markers

Treatment effects were found for plasma FRAP [$F(2, 14) = 4.272$, $p = 0.0036$], OPA [$F(2, 14) = 33.214$, $p < .0005$] and plasma ascorbate concentrations [$F(2, 15) = 7.402$, $p = 0.006$]. FRAP was higher in pigs fed bread + green KF (25.9 ± 1.4 µM) compared to pigs fed bread only (14.0 ± 3.1 µM; post hoc $p = 0.030$) (Figure 3.1A). OPA was higher after bread only (4.4 ± 0.11 ΔFI_{5min}) compared to both bread + gold KF (3.5 ± 0.09 ΔFI_{5min}; post hoc $p < 0.005$) or bread + green KF (3.6 ± 0.03 ΔFI_{5min}; post hoc $p < 0.005$) (Figure 3.1B). Ascorbate concentrations were higher in pigs fed bread + gold KF (5.8 ± 0.9 µM) compared to pigs fed with bread + green KF (2.7 ± 0.4 µM; post hoc $p = 0.006$) and bread

only (3.4 ± 0.5 µM; post hoc p = 0.030) (Figure 3.1C). There were no effects of treatment on plasma oxidative stress markers (MDA and protein carbonyls) (APPENDIX B).

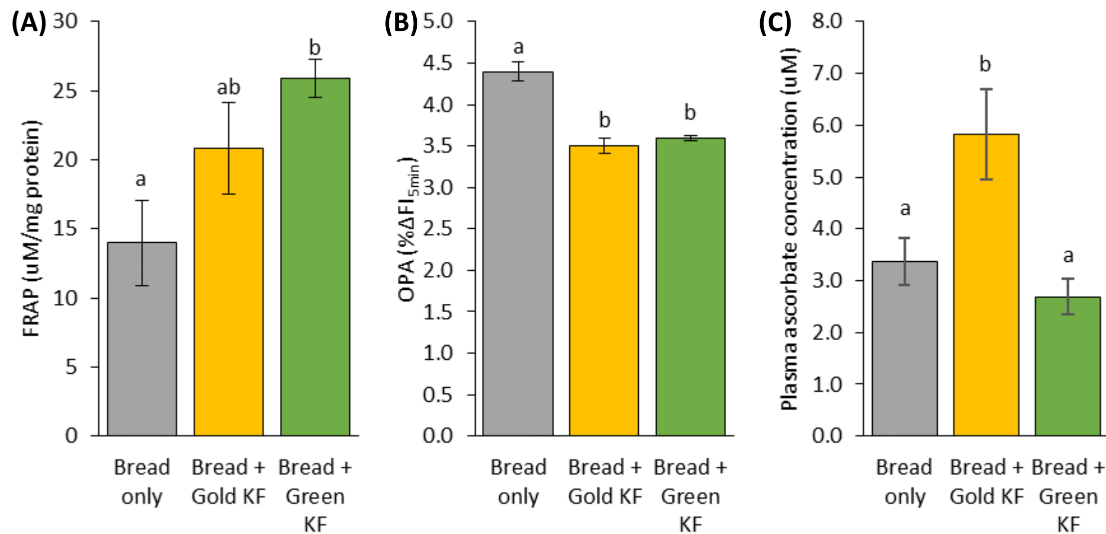


Figure 3.1 Estimated marginal means and standard error of means (SEM) for plasma ferric reducing antioxidant potential (FRAP) values (A), Oxidative potential assay (OPA) (B) and ascorbate concentrations (C) in growing pig model in response to the bread, bread with gold KF and bread with green KF treatments. Bars in each graph without a common letter are significantly different (*p* < 0.05).

3.4.2 Antioxidant enzyme activity and capacity in different brain regions

A summary of antioxidant and enzyme activity measures is presented in Table 3.2. The main effects of brain region were identified for ORAC [F(3, 15) = 27.03, p < 0.001], OPA [F(3, 15) = 41.07, p < 0.001], catalase activity [F(3, 15) = 30.54, p < 0.001], SOD activity [F(3, 15) = 211.87, p < 0.001], SOD2 expression [F(3, 15) = 6.24, p = 0.006], ascorbate concentration [F(3, 15) = 379.62, p < 0.001] and AChE activity [F(3, 15) = 3045.85, p < 0.001].

Irrespective of treatment, ORAC was significantly higher in the brain stem (mean ± SEM across brain regions, 39.13 ± 1.70 µM Trolox/mg protein), compared to corpus striatum (23.70 ± 1.26 µM Trolox/mg protein, post hoc p < 0.005), hippocampus (27.86 ± 1.70 µM Trolox/mg protein; post hoc p < 0.005) and prefrontal cortex (26.39 ± 1.49 µM Trolox/mg

protein; post hoc $p < 0.005$). OPA values were higher in the prefrontal cortex (mean ± SEM across brain regions, 29.49 ± 5.18 ΔFI_{5min}) than in the hippocampus (8.97 ± 1.81 ΔFI_{5min}), corpus striatum (4.93 ± 2.15 ΔFI_{5min}) and brain stem (-1.42 ± 1.30 ΔFI_{5min}).

Table 3.2 Measures of antioxidant and enzyme activities across brain regions in growing pig model in response to the intake of bread, bread with gold KF and bread with green KF treatments.

Measure	Treatment	*Brain stem*	*Corpus striatum*	*Hippocampus*	*Prefrontal cortex*	*Factors*	*F*	*p*
FRAP (μM/mg protein)	Bread only	0.52 ± 0.04[bcd]	0.50 ± 0.02[cde]	0.47 ± 0.02[cde]	0.37 ± 0.02[ef]	Treatment	52.52	<0.001
	Bread + Gold KF	0.52 ± 0.04[bcd]	0.44 ± 0.02[de]	0.37 ± 0.02[ef]	0.28 ± 0.02[f]	Brain region	49.48	<0.001
	Bread + Green KF	0.78 ± 0.04[a]	0.58 ± 0.02[bc]	0.65 ± 0.02[ab]	0.50 ± 0.02[cde]	Treatment x regions	4.33	0.010
ORAC (μM/mg protein)	Bread only	34.01 ± 2.94	23.40 ± 2.18	29.86 ± 2.94	26.01 ± 2.57	Treatment	0.27	0.764
	Bread + Gold KF	43.64 ± 2.94	22.21 ± 2.18	27.37 ± 2.94	25.20 ± 2.57	Brain region	27.03	<0.001
	Bread + Green KF	39.73 ± 2.94	25.50 ± 2.18	26.34 ± 2.94	27.98 ± 2.57	Treatment x regions	1.72	0.184
OPA (%ΔFI_{5min}/mg protein)	Bread only	-0.47 ± 2.26	8.76 ± 3.72	10.19 ± 3.13	24.49 ± 8.97	Treatment	0.01	0.995
	Bread + Gold KF	-2.65 ± 2.26	6.76 ± 3.72	8.13 ± 3.13	29.72 ± 8.97	Brain region	41.07	<0.001
	Bread + Green KF	-1.14 ± 2.26	-0.72 ± 3.72	8.59 ± 3.13	34.26 ± 8.97	Treatment x regions	0.73	0.631
Ascorbate (nmol/mg tissue)	Bread only	0.64 ± 0.02	0.80 ± 0.04	1.25 ± 0.03	1.00 ± 0.07	Treatment	1.00	0.392
	Bread + Gold KF	0.65 ± 0.02	0.77 ± 0.04	1.31 ± 0.03	1.12 ± 0.07	Brain region	379.62	<0.001
	Bread + Green KF	0.69 ± 0.02	0.79 ± 0.04	1.30 ± 0.03	0.97 ± 0.07	Treatment x regions	1.25	0.338
Catalase activity (U/mg protein)	Bread only	15.53 ± 0.73	13.08 ± 0.37	13.92 ± 0.75	13.07 ± 0.78	Treatment	1.11	0.354
	Bread + Gold KF	17.04 ± 0.73	12.10 ± 0.37	11.98 ± 0.75	12.62 ± 0.78	Brain region	30.54	<0.001
	Bread + Green KF	15.72 ± 0.73	11.30 ± 0.37	12.10 ± 0.75	13.04 ± 0.78	Treatment x regions	2.10	0.114
Catalase (relative expression)	Bread only	1.02 ± 0.07	0.98 ± 0.11	1.11 ± 0.06	1.01 ± 0.09	Treatment	2.53	0.113
	Bread + Gold KF	1.11 ± 0.07	1.17 ± 0.11	0.97 ± 0.06	1.02 ± 0.09	Brain region	0.78	0.525
	Bread + Green KF	1.01 ± 0.07	0.95 ± 0.11	0.79 ± 0.06	0.93 ± 0.09	Treatment x regions	1.26	0.331
SOD activity (U/mg protein)	Bread only	101.5 ± 18.3	327.1 ± 37.9	664.2 ± 41.6	458.8 ± 89.1	Treatment	0.30	0.746
	Bread + Gold KF	115.4 ± 18.3	334.6 ± 37.9	623.6 ± 41.6	578.9 ± 89.1	Brain region	211.87	<0.001
	Bread + Green KF	88.3 ± 18.3	392.7 ± 37.9	707.3 ± 41.6	394.5 ± 89.1	Treatment x regions	0.92	0.510
SOD1 (relative expression)	Bread only	1.10 ± 0.30	0.96 ± 0.10	1.21 ± 0.07	1.04 ± 0.21	Treatment	0.41	0.668
	Bread + Gold KF	0.80 ± 0.30	1.01 ± 0.10	0.97 ± 0.07	1.15 ± 0.21	Brain region	0.53	0.669
	Bread + Green KF	1.34 ± 0.30	0.88 ± 0.10	0.88 ± 0.07	0.82 ± 0.21	Treatment x regions	1.73	0.181
SOD2 (relative expression)	Bread only	0.96 ± 0.11	1.51 ± 0.15	1.00 ± 0.10	0.92 ± 0.28	Treatment	0.31	0.736
	Bread + Gold KF	1.09 ± 0.11	1.12 ± 0.15	0.87 ± 0.10	0.99 ± 0.28	Brain region	6.24	0.006
	Bread + Green KF	1.11 ± 0.11	1.10 ± 0.15	0.92 ± 0.10	1.33 ± 0.28	Treatment x regions	0.91	0.516
AChE activity (nM H_2O_2/μg protein/min)	Bread only	45.97 ± 1.02	93.19 ± 1.01	39.86 ± 1.02	28.48 ± 1.04	Treatment	2.90	0.086
	Bread + Gold KF	45.45 ± 1.02	93.20 ± 1.01	37.43 ± 1.02	24.96 ± 1.04	Brain region	3045.85	<0.001
	Bread + Green KF	46.63 ± 1.02	93.55 ± 1.01	38.02 ± 1.02	26.02 ± 1.04	Treatment x regions	1.77	0.172

FRAP: Ferric reducing antioxidant potential (values that share the same letters in each region are not significantly different ($p < 0.05$)), ORAC: Oxygen Radical Absorbance Capacity, OPA: Oxidative potential assay, CAT: catalase, SOD: superoxide dismutase, AChE: Acetylcholinesterase. Representative Western blot figures of catalase, SOD1, and SOD2 are supplied in Figure B.1-3.

Catalase activity was significantly higher in the brain stem (mean ± SEM across brain regions, 16.01 ± 0.42 U/mg protein) compared with all other regions (corpus striatum, 12.16 ± 0.22 U/mg protein; hippocampus, 12.67 ± 0.43 U/mg protein; prefrontal cortex, 12.91 ± 0.45 U/mg protein). SOD activity was significantly lower in the brain stem (101.72 ± 10.57 U/mg protein) compared with the corpus striatum (351.45 ± 21.85 U/mg protein), hippocampus (665.03 ± 23.99 U/mg protein) and prefrontal cortex (477.39 ± 51.41 U/mg protein). SOD2 protein expression was higher in the corpus striatum (1.26 ± 0.09 relative expression) than in the hippocampus (0.92 ± 0.06 relative expression). Ascorbate concentration was significantly higher in the hippocampus (1.28 ± 0.02 nmol/mg tissue), compared with the prefrontal cortex (1.02 ± 0.04 nmol/mg tissue), corpus striatum (0.79 ± 0.03 nmol/mg tissue) and brain stem (0.65 ± 0.01 nmol/mg tissue). AChE activity was significantly higher in the corpus striatum (93.30 ± 0.51 nM H_2O_2/µg protein/min), compared with the prefrontal cortex (27.10 ± 0.64 nM H_2O_2/µg protein/min), hippocampus (38.84 ± 0.46 nM H_2O_2/µg protein/min) and brain stem (46.09 ± 0.65 nM H_2O_2/µg protein/min).

3.4.3 Antioxidant enzyme activity and capacity after KF intake

An interaction effect of treatment by brain region were found for FRAP [$F(6, 15) = 4.33$, $p = 0.010$]. Within the brain stem and prefrontal cortex, FRAP was significantly higher after bread + green KF, compared to both bread + gold KF and bread only ($p < 0.05$; Figure 3.2A). Furthermore, the greatest FRAP was recorded in the brain stem, when bread + green KF was consumed, and the lowest FRAP was in the prefrontal cortex when bread + gold KF was consumed.

The analysis also determined a trend towards a main effect of treatment for AChE activity [$F(2, 15) = 2.90$ $p = 0.086$; Figure 3.2B]. Gold KF (44.60 ± 1.02 nM H_2O_2/μg protein/min) trended to be lower in AChE activity compared to bread only (46.96 ± 1.02 nM H_2O_2/μg protein/min, $p = 0.090$). No additional interaction or treatment effects were observed on any other measures of antioxidants in the brain regions.

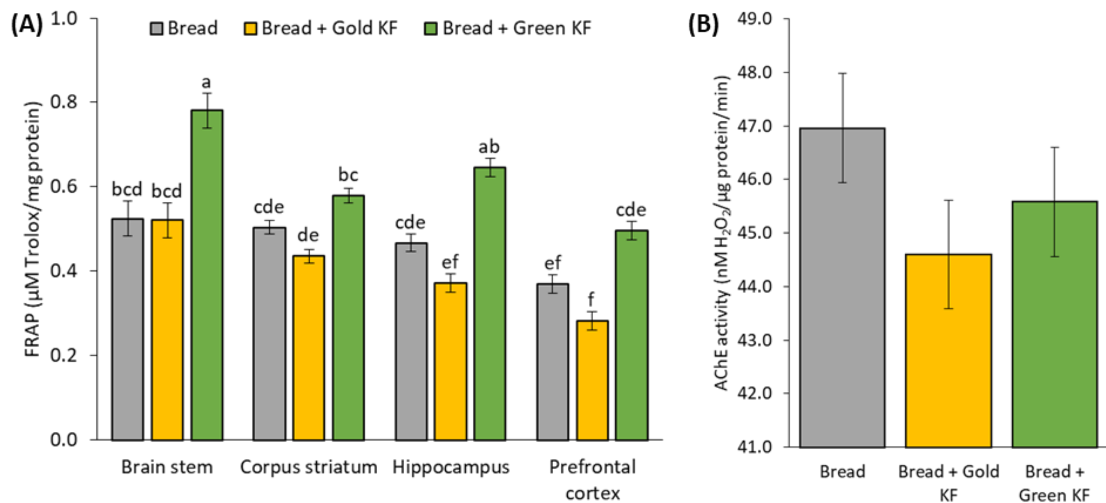


Figure 3.2 Estimated marginal means and standard error of means (SEM) for brain regional ferric reducing antioxidant potential (FRAP) values (A) and Acetylcholinesterase (AChE) activity across all brain regions (B) of in growing pig model in response to the bread, bread with gold KF and bread with green KF treatments. Values in the graphs with the same letters in each brain region are not significantly different ($p < 0.05$).

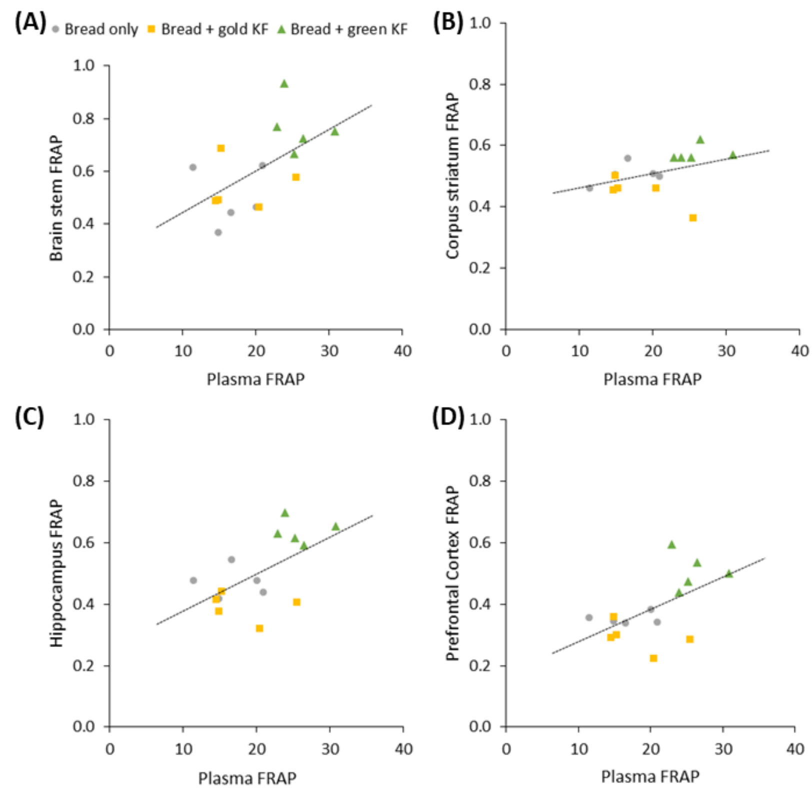


Figure 3.3 Linear correlations between plasma ferric-reducing antioxidant potential (FRAP) and brain regional FRAP in the brain stem (A) (r = 0.57, *p* = 0.027), corpus striatum (B) (r = 0.41, *p* = 0.135), hippocampus (C) (r = 0.59, *p* = 0.022), and prefrontal cortex (D) (r = 0.57, *p* = 0.028) in a growing pig model (n = 15) fed a bread-based diet alone or supplemented with gold or green kiwifruit (KF). Single outliers per treatment group were detected with scatter plots, leaving each treatment group with an n = 5.

3.4.4 Correlations between plasma and brain regional FRAP

With treatments and interaction having a significant effect on FRAP measurements, a correlation analysis between plasma FRAP and brain regional FRAP were conducted. Single outliers per treatment group were detected with scatter plots, and hence were removed. There were statistically significant positive correlations between plasma FRAP and brain stem FRAP (r = 0.57, *p* = 0.027; Figure 3.3A), hippocampus FRAP (r = 0.59, *p* = 0.022; Figure 3.3C) and prefrontal cortex FRAP (r = 0.57, *p* = 0.028; Figure 3.3D). However, there was no statistically significant relation between plasma FRAP and corpus striatum FRAP (r = 0.41, *p* = 0.135; Figure 3.3B).

3.4.5 Correlation between nutrient intakes and antioxidant capacity in the brain regions

Pearson correlation analysis revealed significant correlations between FRAP and various macro- and micro-nutrients in all brain regions (Figure 3.4). FRAP in the corpus striatum, hippocampus and prefrontal cortex was significantly positively correlated with vitamin E intake (r = 0.54, 0.58 and 0.57, respectively). Similarly, FRAP in the brain stem, hippocampus and prefrontal cortex significantly correlated with total polyphenol content (TPC) intake (r = 0.71, 0.57 and 0.57, respectively). FRAP was also significantly correlated with protein and fibre intake in the brain stem (r = 0.51 and 0.55, respectively). Conversely, FRAP in all the brain regions was negatively correlated with vitamin B6 intake; brain stem (r = -0.60), corpus striatum (r = -0.83), hippocampus (r = -0.75) and prefrontal cortex (r = -0.75).

Protein, starch, fibre, energy and TPC intakes were found to be significantly negatively correlated with catalase activity in the corpus striatum (r = -0.74, -0.73, -0.74, -0.73 and -0.67, respectively) and catalase protein expression in the hippocampus (r = -0.59, -0.58, -0.60, -0.58 and -0.70, respectively). SOD1 protein expression in the hippocampus also significantly negatively correlated with protein, starch, fibre, energy and TPC intakes in the hippocampus (r = -0.56, -0.55, -0.55, -0.55 and -0.60, respectively). Conversely, catalase activity and SOD1 protein expression in the hippocampus were significantly correlated with vitamin B9 intake (r = 0.52 and 0.53, respectively).

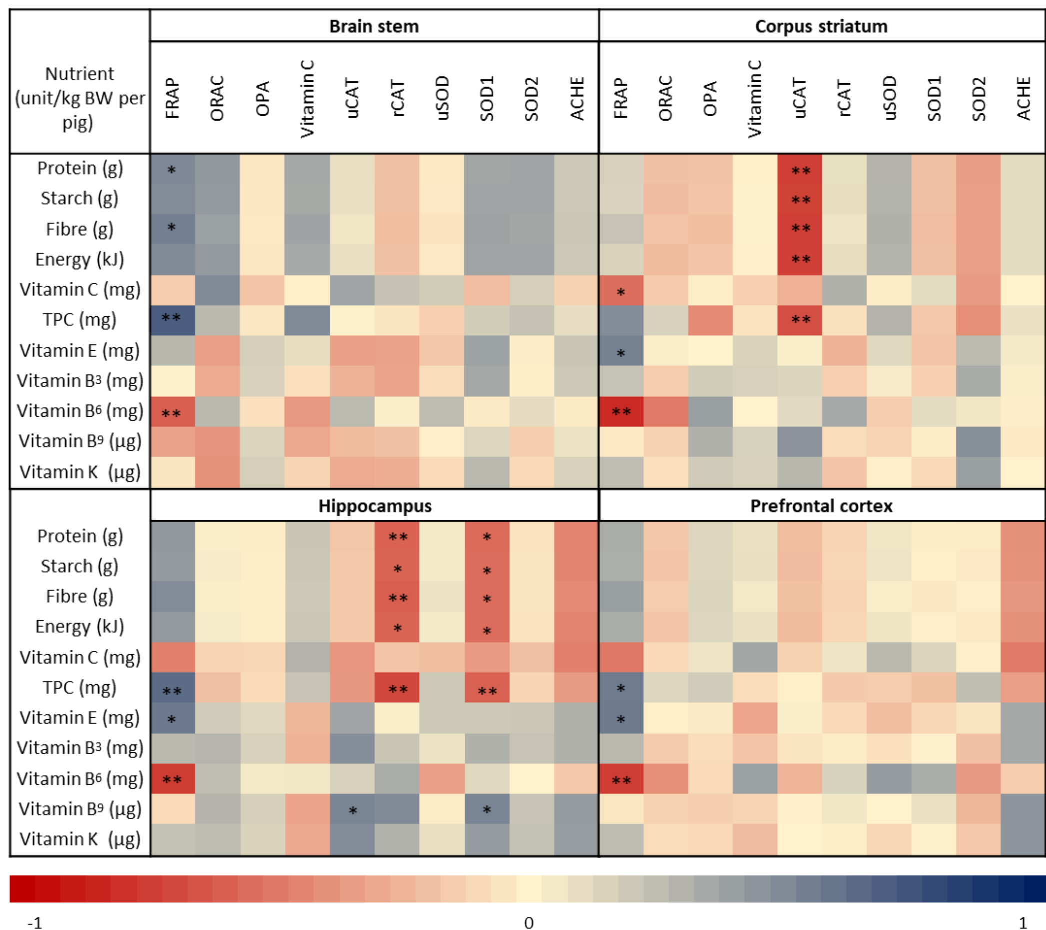


Figure 3.4 Pearson correlation plot between nutrient intake and biochemical markers in different brain regions (brain stem, corpus striatum, hippocampus, and prefrontal cortex) obtained from pigs fed bread only, bread with gold KF, and bread with green KF. The plot is colour-coded to indicate the strength and direction of the correlation, with blue representing a positive correlation and red representing a negative correlation. Asterisk (*) symbols indicate different levels of statistical significance. *$p < 0.05$; **$p < 0.01$. TPC: Total polyphenol content, FRAP: Ferric reducing antioxidant potential, ORAC: Oxygen Radical Absorbance Capacity, OPA: Oxidative potential assay, uCAT: catalase activity, rCAT: catalase expression, uSOD: superoxide dismutase activity, AChE: Acetylcholinesterase activity.

3.5 Discussion

This study sought to investigate how the addition of green or gold KF to a bread-based diet affected the antioxidant capacity of different brain regions in pigs. The current study shows that KF supplemented to pigs consuming a bread-based diet resulted in changes in antioxidant measures in plasma and brain regions. Green KF increased antioxidant capacity and decreased oxidative generating potential in plasma, while gold KF increased plasma ascorbate concentrations and decreased oxidative generating

potential in plasma. Additionally, green KF increased chemical antioxidant capacity in all brain regions, and gold KF showed a trend towards reducing AChE activity. Findings from this study also highlight the differences in antioxidant capacity, antioxidant enzyme activity and protein expression between brain regions. This finding is supported by higher catalase in the prefrontal cortex and SOD activities in the brain stem, compared with other brain regions. Conversely, oxidative generating potential and AChE activity were lower in the brain stem and prefrontal cortex, respectively compared with other brain regions. Interestingly, FRAP was found to be positively correlated with total polyphenol intake in all brain regions. Altogether, these findings suggest the effect of green KF consumption in improving brain antioxidant capacity, may have benefits for neuroprotection from oxidative stress.

One of the promoted health benefits of KF is its potential as a dietary antioxidant. This benefit may be attributed to the concentrations of antioxidant micronutrients in KF (i.e., vitamin C, vitamin E, carotenoids, and polyphenolics) that are chemically powerful antioxidants. KF has also been reported to activate the nuclear factor erythroid 2-related factor (Nrf2) signalling pathway (110) responsible for inducing the transcription of antioxidant enzymes and reducing transcription factor nuclear factor kappa B (NF- κB) (107), a key transcription factor that regulates the inflammatory response in cells.

3.5.1 Effects of KF on peripheral antioxidant capacity

The increased plasma antioxidant potential after the KF treatments reported from the current study are consistent with other KF interventions in humans, which showed an increase in plasma FRAP (60) and ORAC (59). The increase in FRAP observed in this study has also been observed in pigs fed a wheat diet with polyphenol-rich fruit blackcurrants

(311). The interpretation of these results depends on the method of antioxidant measure. Both methods measure a different 'antioxidant capacity', which may explain the divergent results. A FRAP measurement indicates the capacity to reduce (electron transfer), while an ORAC measurement indicates the capacity to scavenge radicals (hydrogen atom transfer) (312). The results suggest that KF might impact electron transfer, which could lead to a decrease in the generation of ROS. This observation is consistent with the finding that KF treatments reduced the ROS generating potential capacity and may prime the body to respond to periods of oxidative stress and protect from the detrimental effects of excessive ROS. This interpretation is supported by a recent study reporting reduced levels of oxidative stress in women who consumed a gold KF drink before engaging in an acute high-intensity exercise (10).

3.5.2 Effects of KF on brain antioxidant capacity

Regulating oxidative stress in the brain is crucial for maintaining cognitive health, as excessive oxidative damage has been implicated in neurodegenerative disorders. Research has shown that dietary interventions, such as increased intake of fruits and vegetables, can enhance antioxidant capacity, as evidenced by improvements in FRAP (313), potentially mitigating neurodegenerative risks. The hippocampus and prefrontal cortex are reported to be the most susceptible brain regions to undergo functional decline following exposure to oxidative stress (314). The susceptibility of these brain regions to oxidative stress has been linked to behavioural and cognitive deficits in rodents exposed to stress (315).

While detrimental effects of oxidative stress on these brain regions, and their cognitive consequences have been characterised, it is evident that these regions may also be most

reactive to oxidative stress. OPA results showed that both the prefrontal cortex and the hippocampus showed the highest ROS generating capacity of the four regions tested. Like previous studies in rats (316), SOD activity was highest in the hippocampus of pigs compared with other brain regions, indicating that the higher endogenous antioxidant activity in the hippocampus provides some resilience from oxidative stress.

KF consumption has been demonstrated to have neuroprotective effects in *in vitro* models and in rodents, attenuating cellular ROS and protecting cells from the detrimental effects of oxidative stress (16, 243-245). Reduction in oxidative stress and increase in antioxidant markers from KF consumption corresponded with reduced decrements in and cognitive behavioural assessments in stressed rodents (243, 244). In this study, green KF supplementation induced greater antioxidant protective potential in all brain regions than gold KF and bread only. Besides having differing vitamin C, E and polyphenols contents, as mentioned previously, actinidin is required for the cleavage of kiwellin, a minor protein of KF, into KiTH and kissper (29). The latter has demonstrated anti-inflammatory properties (34), potentially leading to the downregulation of systemic ROS. Whether these proteins are responsible for the beneficial effects in the brain has yet to be determined and could be explored in future research.

Like findings from human dietary intervention studies (4, 13, 55, 60, 64, 76-79), the current findings showed that consuming gold KF increased plasma concentrations of ascorbate in pigs. However, this result was not observed in brain tissue where KF consumption had no effect on regional brain ascorbate concentrations and could be because pigs, in contrast to humans, can synthesise ascorbate and so is not considered essential for the species (317). Additionally, the brain retains higher concentrations of

ascorbate than does plasma, which is maintained throughout states of deficiency (317); thus any additional dietary intake of ascorbate would result in no observable changes in the brain.

Mapping ascorbate distribution of the human brain (318, 319), like the results observed in this study, showed the highest concentrations of ascorbate in the hippocampus, followed by the prefrontal cortex. The transportation of ascorbate can explain the differential concentrations of ascorbate across the brain. Ascorbate is transported by the sodium-dependent vitamin C Transporter type 2 (SVCT2) (320), which is more abundant in brain regions that contain many neurons, such as the cortex and hippocampus (321).

Compared to rodents (322), the present findings showed that AChE activity was the highest in the corpus striatum region in pigs. This is because, in this region, cholinergic interneurons spontaneously release ACh and AChE (323). Rodent studies reveal that curcumin (324) and green tea (325) can inhibit AChE activity, hinting at their cognitive health benefits. KF extracts inhibits AChE *in vitro* (326). A trend in reduced AChE activity across the whole brain after gold KF treatments was observed in the current results, corresponding with the findings in the brains of high-fat diet-induced obese mice supplemented with Hardy KF extracts (244). This finding could be due to the polyphenols that gold KF provides (quercetin, caffeic, gallic acid, catechin) which could be present in lower concentrations in green KF. These polyphenols have been shown to have inhibitory effects on AChE (327, 328) and is worth exploring further.

3.5.3 Correlations between peripheral and brain antioxidant capacity

The strong correlations between FRAP in plasma and in the different brain regions suggest that plasma FRAP measurement may serve as a potential biomarker of brain antioxidant capacity. Whether increased peripheral FRAP directly influences the antioxidant capacity of brain regions is unclear and further research is needed to elucidate the clinical relevance of this correlation.

3.5.4 Associations between KF composition and total antioxidant capacity

The analysis revealed that the increase in antioxidant potential in the brain regions were strongly correlated with higher concentrations of total polyphenols and vitamin E. Given that both vitamin E and polyphenols are known to influence FRAP (329) and that both these constituents are present at higher concentrations in green KF than in gold KF (Table 1), it is likely that greater concentrations of these constituents consumed by pigs explains the greater FRAP in brain tissue. This is observed in pigs who consumed green KF compared with those who consumed gold KF and bread only diets.

3.5.5 Strengths, limitations, and future studies

This study was the first to assess the effect of KF with a bread-based diet on the endogenous antioxidant systems of plasma and brain regions in a pig model. Although the results do not show many effects on the antioxidant systems, they provide some evidence, which could direct further research. A key strength of this study is the use of pigs; besides having digestive physiology like humans (330), the pig brain is also more anatomically similar to humans than mice or rat models. Like humans, pigs have gyrencephalic brains, and their white-to-grey matter ratio is 60:40 (331). Moreover, pig

and human brains have homologous resting-state networks (332). Additionally, a notable strength lies in the fact that the dosage of fresh KF used mirrors what is commonly considered a standard serving in humans, enhancing the translational relevance of the results compared to *in vitro* experiments or studies involving rodent models using extracts.

Nevertheless, this study has several limitations. Firstly, caution is advised when interpreting the results, as the research was conducted on healthy pigs, and it is unclear whether KF provides any additional benefits in diseased or unhealthy individuals. Secondly, while previous studies in rodents showed enhanced antioxidant systems, the models used in these studies had increased oxidative stress due to external stressors like a high-fat diet (244), lead exposure (243) and causative agent Aβ injection (16) which may have amplified the protective effects of KF on antioxidant systems. Thirdly, the study's sample size was constrained due to its amalgamation with another study.

Future research should incorporate a larger animal cohort to enhance result reliability. Our findings suggest the efficacy of green KF in elevating plasma antioxidant protection. However, to validate these results, a sample size of 40 (20 per treatment, Power = 80%, α = 0.05) is required based on plasma FRAP mean and standard deviation comparisons between green KF with bread and bread alone. Additionally, the diet used in this study was limited to bread only, which is not representative of a normal diet for humans.

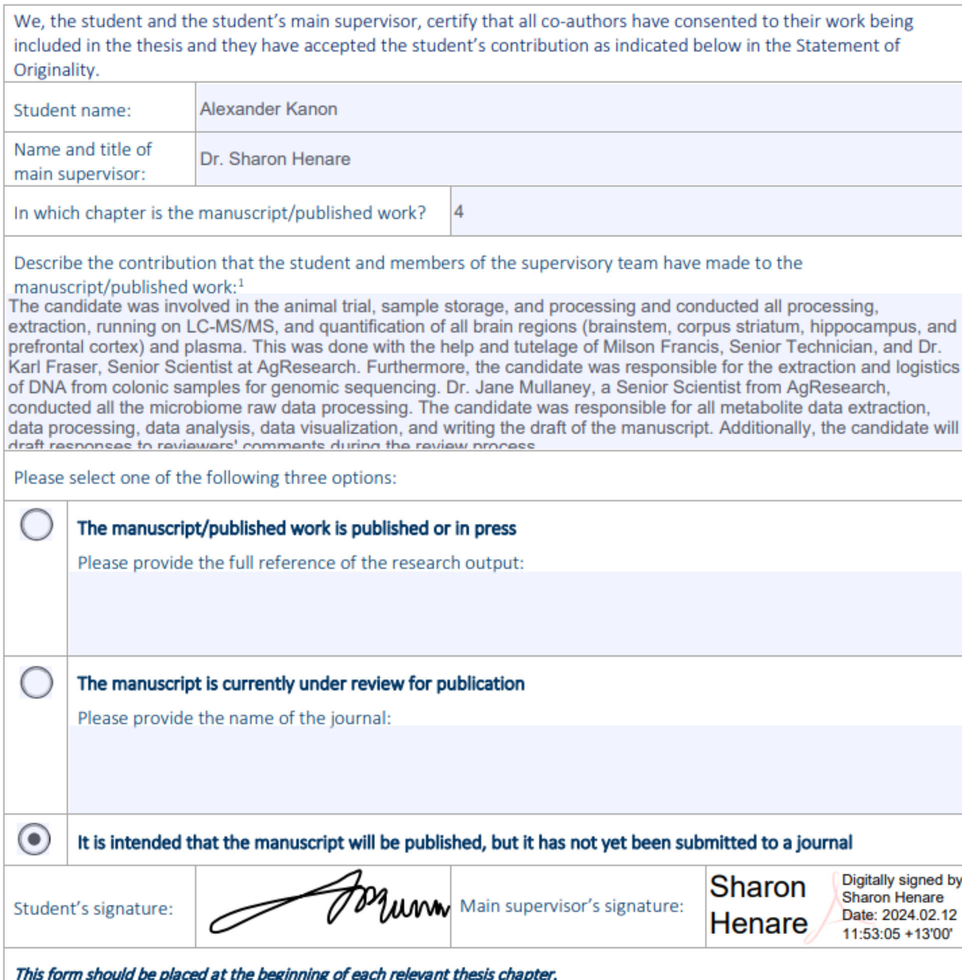
As previously highlighted, future studies on the neuroprotective effects of KF in pig models should consider manipulating stress through dietary interventions (Western or high fat) (333, 334) or chronic stressors. Cognitive measures (335) and the investigation of specific components of KF, such as pure vitamins or KF skin, similar to other studies

(40), could provide valuable insights on which KF constituents contribute to the antioxidant modulatory properties of KF in the brain. Comparisons with other polyphenol-rich fruits or purified polyphenol compounds could also be informative. Assessing additional biomarkers like BDNF, which plays a crucial role in neuronal growth and survival, would further enhance our understanding (137). Human clinical studies, particularly with populations experiencing high levels of stress, could provide relevant information. Administering KF or a placebo prior to cognitive tests and assessing antioxidant status through blood samples would be a valuable approach, consistent with similar studies (336).

3.6 Conclusions

Oxidative stress caused by the inability of endogenous antioxidant systems to counteract ROS formation in the brain leads to neurodegeneration, consequently resulting in cognitive and behavioural decline. Overall, this study is the first to demonstrate the efficacy of green and gold KF consumption in differentially augmenting the antioxidant capacity of plasma and brain regions in growing pigs. Specifically, consuming green KF increased FRAP and gold KF tended to reduce AChE activity across all brain regions. These findings would suggest the potential neuroprotective effect of green or gold KF during oxidative stress by augmenting the brain's antioxidant capacity. Further studies would need to be conducted to elucidate the neuroprotective potential of KF during stress.

Statement of contribution



STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Alexander Kanon
Name and title of main supervisor:	Dr. Sharon Henare
In which chapter is the manuscript/published work?	4

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

The candidate was involved in the animal trial, sample storage, and processing and conducted all processing, extraction, running on LC-MS/MS, and quantification of all brain regions (brainstem, corpus striatum, hippocampus, and prefrontal cortex) and plasma. This was done with the help and tutelage of Milson Francis, Senior Technician, and Dr. Karl Fraser, Senior Scientist at AgResearch. Furthermore, the candidate was responsible for the extraction and logistics of DNA from colonic samples for genomic sequencing. Dr. Jane Mullaney, a Senior Scientist from AgResearch, conducted all the microbiome raw data processing. The candidate was responsible for all metabolite data extraction, data processing, data analysis, data visualization, and writing the draft of the manuscript. Additionally, the candidate will draft responses to reviewers' comments during the review process

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1

Doctoral Research Committee
May 2023

CHAPTER 4. Effects of short-term green and gold kiwifruit supplementation on the microbiota composition in the colon digesta and metabolites in plasma and brain in a growing pig model[2]

Prologue

Consumption of kiwifruit (KF) can alter the gut microbiome, as evidenced by in vitro *fermentation and human studies. For instance, green KF reduced Enterobacteria and Escherichia coli in pigs, while regular intake increased* Faecalibacterium prausnitzii *in humans. These effects are attributed to KF nutrients, which influence systemic nutrient concentrations and protein metabolism. KF fermentation also promotes beneficial bacteria like Bifidobacterium spp.,* Veillonellaceae*, and* Lachnospira *spp., correlating with increased production of 3,4-Dihydroxyphenylalanine (L-DOPA) and γ-Aminobutyric Acid (GABA). Based on this evidence, this study aims to evaluate how the consumption of green and gold KF alters colonic microbiota composition and alters bioaminergic metabolite concentrations in plasma and brain tissues. Samples collected for this study were obtained from the study described in the previous chapter.*

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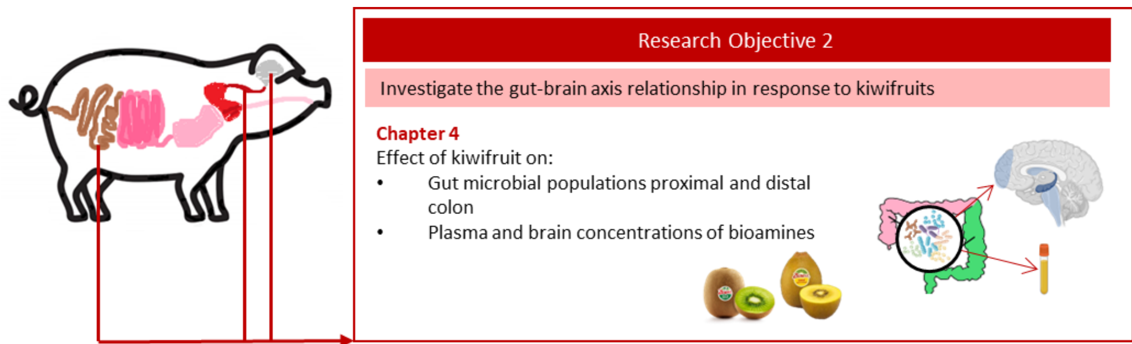
[2]Parts of this chapter is in preparation to be submitted to Microorganisms:
Kanon, A. P., Roy, N. C., Mullaney, J., Giezenaar, C., Jayawardana, I. A., Francis, M., Montoya, C. A., Fraser, K., McNabb, W. C., & Henare, S. J. (in preparation to be submitted to Microorganisms) Effects of short-term green and gold kiwifruit supplementation on the microbiota composition in the colon digesta and metabolites in plasma and brain in a growing pig model.



Highlights

- *The study demonstrates the effects of short-term green or gold KF supplementation on proximal and distal colonic microbiota composition in a growing pig model.*
- *Green or gold KF supplementation compared to a control increased the relative abundance of butyrate-producing and flavonoid-degrading microbial genera.*
- *Green or gold KF supplementation compared to a control decreased the relative abundance of potential pathogenic microbial genera.*
- *Green or gold KF supplementation compared to a control increased the relative abundance of* Lachnospira *and* Intestinimonas *genera and decreased the relative abundance of* Enterococcus *and* Streptococcus *genera.*
- *Green or gold KF supplementation compared to a control increased plasma* 5HIAA *concentrations.*

The chapter is presented in a manuscript format but has not been published.

4.1 Abstract

In vitro research has provided initial evidence of the potential effects of KF on microbial composition, organic acid production, and bioamine metabolites in the colon. This study aimed to investigate how KF consumption modulates the colonic microbiome and assesses its impact on plasma and brain tissue bioamine concentrations in pigs. Eighteen pigs were fed a control diet of bread only or bread with either gold-fleshed KF (*Actinidia chinensis* 'Hort16A') or green-fleshed KF (*A. deliciosa* 'Hayward') for eight days. Proximal and distal colon digesta samples and plasma and brain tissue samples were collected 300 minutes postprandial. Shotgun metagenomics sequencing was conducted on DNA extracted from digesta samples, and bioamine concentration was quantified in plasma and brain tissue samples. A significant increase in alpha diversity of the proximal colon microbiota was seen with green KF compared to control bread only. Significant reductions in the relative abundance of genera *Prevotella*, *Streptococcus*, *Galactobacillus*, *Megasphaera*, *Lactimicrobium*, and *Enterococcus* were observed in both the proximal and distal colon in pigs receiving either KF treatments compared to those on the control diet. Conversely, significant increases were observed in the relative abundance of genera *Faecalibacterium*, *Pseudoflavonifractor*, Flavonifractor, *Intestinimonas*, *Faecalicoccus*, *Butyricicoccus*, *Eggerthella*, and *Slackia* with either KF treatment (compared to the control diet) in both the proximal and distal colon. Plasma concentration of 5-hydroxyindoleacetic acid was significantly increased with both KF treatments, compared to the control. However, no differences were observed in other plasma and brain metabolite concentrations. In conclusion, the addition of KF to a control diet altered colonic microbiota composition in both proximal and distal regions,

reducing some potential opportunistic pathogens. KF supplementation affected plasma 5-hydroxyindoleacetic acid, an important metabolite, in the tryptophan pathway.

Keywords: green kiwifruit, gold kiwifruit, microbiome, *Faecalibacterium*, 5-Hydroxyindoleacetic acid, proximal and distal colon, pig, plasma, brain

4.2 Introduction

The gut microbiota is home to thousands of species and is involved in important processes, including digestion, immunity, and protection against pathogens (337, 338). Changes to the gut microbiota have been associated with various pathologies, including obesity, type 2 diabetes mellitus and inflammatory bowel diseases (339, 340). Microbial compositional abundance changes along the length of the colon as the environment changes. The colon, spanning 1.5 meters, undergoes environmental changes from anaerobic and slightly acidic conditions in the proximal colon to a more neutral pH in the distal colon (341). The proximal colon, rich in carbohydrates and water, undergoes rapid fermentation, reducing carbohydrate concentration in the distal colon, primarily composed of insoluble carbohydrates. Consequently, the proximal colon exhibits higher fermentative capability, elevated hydrogen levels, and a pH of around 5.6, which rises to about 6.6 in the distal colon (342). As the colon lengthens, water concentration diminishes due to reabsorption (343). Taxa from the Firmicutes phylum are more abundant in the acid-tolerant proximal colon, while the distal colon typically features a higher abundance of *Bacteroides* genus taxa (344-346). Similarly, the production of short-chain fatty acids (SCFA) decreases along the colon (347), while concurrently, the levels of branched-chain fatty acids (BCFA) increase along the colon (348).

Recent findings emphasise the crucial role of the gut microbiota in shaping brain activity and cognition through the microbiota-gut-brain axis (349). However, the precise nature of this interaction and its impact on brain function remains a subject of ongoing research. The gut microbiota utilises host dietary components as substrates and supplies energy and metabolites to the host. These microbial-produced metabolites could include but are not limited to B vitamins (350) and other neuroactive metabolites,

including neurotransmitters. For example, species of *Streptococcus*, *Escherichia* and *Enterococcus* genera produce 5HT; *Bacillus* produce dopamine; *Bacillus* and *Saccharomyces* produce norepinephrine; *Lactobacillus* produce acetylcholine and *Lactobacillus* and *Bifidobacterium* produce, gamma-amino butyric acid (GABA) (142, 289, 351). The brain relies on these excitatory (e.g., acetylcholine, dopamine) and inhibitory (e.g., GABA, glycine, 5HT) neurotransmitters for various functions like movement, emotion, learning, and memory (352). Dysregulation in the concentration of these neurotransmitters can result in diminished cognitive performance and poor sleep quality, and if left untreated, can result in neurological and psychological conditions such as Alzheimer's Disease, Parkinson's Disease, anxiety, and depression (353, 354). Additionally, intestinal dysbiosis is believed to potentially contribute to systemic inflammation, which, in turn, may increase neuroinflammation and cause damage to neurons (355).

Consumption of KF may alter the gut microbiome. *In vitro* fermentation studies found an increase in the relative abundance of *Bacteroides* spp. with both green and gold KF flesh, while the relative abundance of *Bifidobacterium* spp. was increased only with green KF (99). Green KF has also modulated the colonic microbiota in growing pigs, with increased relative abundances of the *Bacteroides* genus and lowered relative abundances of *Enterobacteria* genera and *Escherichia coli* (102).

Clinical studies have explored the effects of KF on the gut microbiota composition. Regular consumption of freeze-dried green KF increased the relative abundance of faecal *Lactobacillus* and *Bifidobacterium* genera in healthy individuals after four days (54). Additionally, consuming gold KF-containing capsules in functionally constipated

individuals increased the relative abundance of faecal *Faecalibacterium prausnitzii* after four weeks (12). Another study found that individuals with prediabetes supplemented with fresh gold KF increased the relative abundance of the *Coriobacteriaceae* family after twelve weeks (55). Beyond the prebiotic potential, clinical evidence suggests that consuming either green or gold KF cultivars can improve subjective measures of mood (3) and sleep quality (5).

The enhanced health outcomes have been ascribed to the nutrients in KF, which contribute to increased systemic nutrient concentrations that alter protein and amino acid (AA) metabolism and metabolite production. For example, consuming gold KF (rich in vitamin C) for four weeks significantly increased plasma vitamin C concentration in young adults (3, 55). Consumption of green KF with beef, compared to consumption of gold KF with beef, caused an earlier and faster-increased concentration in EAAs and BCAAs in an elderly cohort (82). Furthermore, *in vitro* fermentation studies of KF have correlated increases in the relative abundances of *Bifidobacterium* spp. and *Veillonellaceae* family with KF, leading to elevated fermenta concentration of L-DOPA. Additionally, the KF fermentation increased *Lachnospira* spp., which was correlated with an elevation in the fermentation concentration of GABA (246). As a result, it is plausible that KF may directly increase plasma metabolite concentrations or influence the gut microbiota, thereby impacting the supply and production of these essential neurochemicals.

Based on this evidence, it was hypothesised that adding green or gold KF to a meal will alter the compositional profile of the colonic microbiota and increase the concentration of bioaminergic metabolites in plasma and brain samples. Therefore, this study's

primary aim was to assess the impact of consuming two green or gold KF on microbial communities (shotgun metagenomics) in the proximal and distal colon and neurotransmitter concentrations in plasma and brain tissues (liquid chromatography-tandem mass spectrometry (LCMS/MS)). Using the pig model, which closely mirrors human digestive and brain physiology, allows the sampling of various GIT regions and the collection of the brain, which human studies cannot explore. Data obtained using a pig model would enable further understanding of the impact of KF in altering the colon microbiome and how this may relate to the modulation of neurochemicals peripherally and in the brain. Shotgun metagenomics yields microbial taxonomic data and gene abundance data; the latter was not included within the scope of this chapter and, therefore, is not reported.

4.3 Materials and methods

The pigs used in this study were the same as those described in Chapter 3. Below are details of additional analyses relevant to Chapter 4.

4.3.1 Animal, housing, and treatment

Animal housing and treatment were described in Chapter 3 (Section 3.3.1).

4.3.2 Sampling day collection

The sample collection is described in Chapter 3 (Section 3.3.2), with the additional steps taken to collect the colonic digesta samples. Following euthanasia, the abdomen of each animal was opened, and the entire stomach, jejunum, duodenum, terminal ileum, caecum, proximal colon, and distal colon were removed. Any blood was washed away using sterile deionised water. Each section was carefully divided and dried using absorbent paper towels. Digesta samples from the proximal and distal colon were

collected and stored in microcentrifuge tubes and temporarily kept on dry ice until the completion of the collection process (approximately five hours). Digesta samples were then stored in a −80°C freezer until analysed. The plasma and brain regions were collected as described in 0.

4.3.3 Gut microbiome analysis

The proximal and distal colonic digesta samples were thawed on ice, and approximately 200 mg was subsampled for genomic DNA extraction. DNA extraction of the digesta samples was conducted using the Machery-Nagel NucleoSpin® Soil kit (Machery-Nagel, Düren, Germany) according to the manufacturer's instructions. The extracted DNA was then stored at -80°C until further use. DNA purity and concentration were assessed via agarose gel electrophoresis and absorbance measurements at 260 nm and 280 nm using the Nanodrop 1000 (Thermo Fisher Scientific, Waltham, MA, USA). The optimal DNA purity ratio is 1.80, with the elution buffer from the DNA kit serving as the blank. These tests were conducted on the extraction day, each using a 2 µL sample.

OSS Technology Hong Kong conducted shotgun metagenomics sequencing on the extracted DNA samples, ensuring quality control checks for purity and concentration. The sequencing utilised the Illumina HiSeq platform with 2x150bp paired-end sequences. Following sequencing, the raw data underwent quality control using FastQC (v.0.11.9) (356). Subsequently, Trimmomatic (v.0.36) was employed for the removal of adapters, elimination of low-quality reads (Phred scores < 30) and discarding short reads (< 36 bp) (357). Host read pairs were aligned to the *Sus scrofa* reference genome Sscrofa11.1 (RefSeq: GCF_000003025.6) using the "mem" algorithm of bwa (v.0.7.17-r1188) (358), and unmapped sequencing reads were transformed into fastq files using

the "fastq" function of samtools (v.1.8) (359). PEAR (v.0.9.6) with default settings facilitated the joining of read pairs (360), while unjoined pairs were concatenated with a string of N's through the "fuse" function from the BBMAP package (v.38.22-0) (361). Subsequently, merged, and fused reads from distinct lanes within the same sample were consolidated into a final "clean" read sample file.

4.3.4 Bioamine analysis

Plasma proteins were precipitated by taking 200 µL of plasma and adding 800 µL of ice-cold acetonitrile. The samples were vortexed briefly, then centrifuged at 12,100 rpm for 10 min at 4 °C. From this, 200 µL of the supernatant was derivatised by the sequential addition of 100 µL of 100 mM sodium carbonate, 100 µL of BzCl (2% (v/v) in acetonitrile), and 100 µL of an internal standard mixture. 500 µL of water was added to reduce the samples' organic content for the reverse-phase LCMS/MS analysis.

The brain tissues were homogenised for three 30 sec intervals (QIAGEN TissueLyser II, QIAGEN, Hilden, Germany), and 20 mg of brain tissue was added to 80 µL of ice-cold acetonitrile. The samples were vortexed briefly and then centrifuged at 12,100 rpm for 10 min at 4°C. From this, 20 µL of the supernatant was derivatised by the sequential addition of 10 µL of 100 mM sodium carbonate, 10 µL of BzCl (2% (v/v) in acetonitrile), and 10 µL of the internal standard mixture. Fifty µL of water was added to reduce the organic content of the samples for the LCMS/MS analysis.

The analysis of the derivatised plasma and brain tissue was based on the method developed by Wong, Malec (362). Derivatised plasma and brain tissue samples were analysed using a SCIEX LCMS/MS QTRAP 6500+ system coupled to an ExionLC (SCIEX, Victoria, Australia). Five µL of the sample was injected into a Waters Aquity C18 High

Strength Silica (HSS) Ultra Performance Liquid Chromatography (UPLC) column (Massachusetts, USA). The flow rate was set at 100 µL/min, and the autosampler temperature was kept ambient while the column temperature was maintained at 27 °C. Positive electrospray ionisation at 4 kV was used. The mobile phase A was 10 mM ammonium formate with 0.15% formic acid, and the mobile phase B was acetonitrile. Gradient elution was as follows; initial, 0% B; 0.01 min, 15% B; 0.5 min, 17% B; 14 min, 55% B; 14.5 min, 70% B; 18 min, 100% B; 19 min, 100% B; 19.1 min, 0% B; and 20 min, 0% B. An additional 10 min of column equilibration was required at 0% B to achieve reproducible chromatography. The gas temperature was 350 °C, the gas flow was 11 L/min, and the nebuliser was 15 psi. Data was captured using Analyst (V1.6) software and processed on SCIEX software MultiQuant (V3.0.2).

Forty-four metabolites were analysed in both plasma and brain tissue samples. The metabolites included were tyrosine metabolites (3-methoxytyramine, dopamine, L-DOPA, normetanephrine, octopamine, phenylalanine, phenethylamine, synephrine, tyrosine and tyramine), tryptophan metabolites (5HIAA, 5HT, 5-hydroxytryptophan [5HTP], kynurenine, 3-Hydroxykynurenine, N-acetylserotonin, tryptophan and tryptamine), glutamate metabolites (GABA, glutamine, glutamate and glycine), AAs (asparagine, histidine, isoleucine, leucine, lysine, methionine, proline, serine and valine), non-proteinogenic AAs (citrulline, homocysteine and ornithine), polyamine (agmatine, N-acetylputrescine, putrescine, spermidine, spermine) and other metabolites involved in the gut-brain-axis (adenosine, anserine, ethanolamine, GSH, kyotorphin).

Calibration curves were made using standards at 10, 50, 100, 500, 1000, 2500, 5000 nM for 3-hydroxykynurenine, asparagine, GSH, glycine, and serine; 1, 5, 10, 50, 100, 250,

500 nM for 5HIAA, 5HTP, adenosine, agmatine, anserine, citrulline, ethanolamine, glutamate, glutamine, histidine, homocysteine, kynurenine, methionine, N-acetylputrescine, ornithine, phenylalanine, proline, tryptophan, and valine; 0.1, 0.5, 1, 5, 10, 25, 50 nM for 3-methoxytyramine, GABA, dopamine, kyotorphin, L-DOPA, isoleucine, leucine, lysine, N-acetylserotonin, normetanephrine, octopamine, phenethylamine, putrescine, 5HT, spermidine, spermine, synephrine, tryptamine, tyramine, and tyrosine. An internal standard stock was prepared with 500 µM 3-Hydroxykynurenine, asparagine, GSH, glycine, and serine; 50 µM 5HIAA, 5HTP, adenosine, agmatine, anserine, citrulline, ethanolamine, glutamate, glutamine, histidine, homocysteine, kynurenine, methionine, N-acetylputrescine, ornithine, phenylalanine, proline, tryptophan, and valine; 5 µM 3-methoxytyramine, GABA, dopamine, kyotorphin, L-DOPA, leucine, lysine, N-acetylserotonin, octopamine, phenethylamine, putrescine, 5HT, spermidine, spermine, synephrine, tryptamine, tyramine, and tyrosine. The internal standard was derivatised with ^{13}C BzCl solution. Calibration standards were prepared in Millipore water. Calibration standard and internal standard stocks were frozen at −80 °C in aliquots to prevent multiple freeze/thaw cycles, and a single internal standard stock aliquot was thawed the day of use, diluted 100-fold in 20% (v/v) acetonitrile containing 1% (v/v) sulphuric acid.

4.3.4.1 Optimisation of each metabolite detection

Each target compound was run before sample analysis to optimise multiple reaction monitoring (MRM) conditions and separation of compounds. Mass spectral detection was performed in negative electrospray ionisation mode using MRM for 44 compounds and the 45 stable isotope BzCl derivative labelled internal standards using electrospray ionisation. Data was captured using Analyst (V1.6) software and processed on SCIEX

software MultiQuant (V3.0.2). Metabolite concentrations were generated from standard curves of standard injections for all 45 compounds. The transitions monitored (Q1 and Q3) and their optimised DP, CE and CXP parameters are listed in Table 4.1.

4.3.4.2 Validation of the method

Pooled sample matrixes (plasma and brain tissue) were spiked with none, low (50nM) and high (500nM) concentrations of GABA, 5HT, tryptophan, ornithine, phenylethylamine, tyrosine, L-DOPA to validate the method and test recovery. Two hundred µL of spiked plasma and 20 mg of brain tissues were precipitated with 800 µL and 80 µL, respectively, of ice-cold acetonitrile, followed by centrifugation for 10 min at 4 °C 12,100×g. 200 µL pooled plasma and 20 µL pooled brain of supernatant were derivatised by sequential addition of 10 µL of 100 mM sodium carbonate, 10 µL of BzCl (2% (v/v) in acetonitrile), and 10 µL of the internal standard mixture. Fifty µL of water was added. Three spiked samples of each matrix (plasma and brain) were extracted and derivatised in parallel for triplicate analysis. This process was repeated on three occasions to account for the day-to-day variation. Recoveries were calculated and ranged between 60-100%, concurrent with Wong, Malec (362) findings.

Table 4.1 Multiple reaction monitoring transitions are used for metabolites.

Q1 Mass (Da)	Q3 Mass (Da)	Retention time (min)	Abbreviation	Name	DP	CE	CXP
260.1	213.9	5.4	His	Histidine	61	19	24
345.2	170.0	5.5	Ans	Anserine	76	29	18
237.1	220.0	5.6	Asn	Asparagine	26	13	24
210.1	105.0	5.7	Ser	Serine	91	17	12
251.1	105.1	5.7	Gln	Glutamine	41	17	12
165.9	105.0	5.8	Eta	Ethanolamine	26	19	12
280.1	262.9	5.8	Cit	Citrulline	36	11	30
180.2	105.0	5.9	Gly	Glycine	36	13	12
252.1	104.9	6.0	Glu	Glutamate	36	17	12
234.9	105.0	6.5	Agm	Agmatine	66	35	10
234.9	105.0	6.5	NAP	N-Acetylputrescine	51	27	12
207.9	105.0	6.6	GABA	γ-aminobutyric acid	26	17	10
372.2	237.0	6.9	Ado	Adenosine	96	17	26
220.2	104.9	7.0	Pro	Proline	41	19	10
253.9	105.0	8.5	Met	Methionine	31	25	12
222.2	176.0	8.5	Val	Valine	36	13	20
341.2	174.0	8.9	Orn	Ornithine	56	19	20
516.0	440.8	9.3	GSH	Glutathione	66	17	22
355.3	188.0	9.5	Lys	Lysine	56	21	20
236.2	190.0	9.9	Ile	Isoleucine	36	13	22
296.9	105.0	9.9	Put	Putrescine	56	27	12
270.1	224.0	10.2	Phe	Phenylalanine	36	13	26
309.2	263.0	10.4	Trp	Tryptophan	46	17	30
236.2	104.9	10.4	Leu	Leucine	36	21	48
545.9	344.0	11.3	Kyo	Kyotorphin	151	41	34
458.2	336.0	12.4	Spd	Spermidine	66	25	38
323.2	263.9	12.6	Nas	N-Acetylserotonin	46	23	28
226.2	104.9	12.7	Phet	Phenethylamine	91	19	12
264.9	144.0	12.8	TrpA	Tryptamine	81	19	16
313.3	295.9	12.9	5HIAA	5-Hydroxyindoleacetic acid	31	11	32
344.2	221.9	13.2	Hcy	Homocysteine	41	13	26
417.2	277.9	13.5	Kyn	Kynurenine	51	21	30
618.9	497.2	13.7	Spm	Spermine	96	37	54
429.1	382.9	14.0	5HTP	5-Hydroxytryptophan	61	21	42
373.8	105.0	14.2	Nm	Normetanephrine	86	23	12
390.2	240.0	14.4	Tyr	Tyrosine	56	21	26
343.8	105.0	14.5	Oa	Octopamine	91	21	12
537.1	414.8	14.7	3HK	3-Hydroxykynurenine	41	13	22
357.8	105.0	14.9	Syn	Synephrine	121	25	12
384.9	264.1	16.2	5HT	Serotonin	1	27	26
509.8	382.0	16.5	L-DOPA	3,4-Dihydroxyphenylalanine	1	17	18
376.2	104.9	16.6	3MT	3-Methoxytyramine	41	23	48
346.3	104.9	16.7	Tyra	Tyramine	96	27	12
466.1	104.9	17.5	DA	Dopamine	76	21	50

4.3.5 Data analysis

4.3.5.1 Calculations

The sum of the AAs was grouped into BCAA (leucine, isoleucine, valine), EAA (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, valine), non-essential amino acids (NEAA; asparagine, serine, glutamine, glutamate, glycine, proline, tyrosine) and large neutral amino acids (LNAA; phenylalanine, tyrosine, tryptophan, leucine, isoleucine, valine, histidine, methionine).

4.3.5.2 Statistical analysis

All microbiome statistical analyses were performed using the R packages vegan (V2.5-7) and analysis of the composition of microbiomes with bias correction (ANCOMBC) (V1.0.5) (363, 364). Firstly, all reads were filtered to retain those classified as not microbial, and then the data were standardised to the mean (rarefaction). Taxa absent more than three times in at least 20% of the samples were removed to prevent any very low abundance taxa from skewing the results. Multiple measures of alpha-diversity are reported in the literature. The measures selected were the Chao1 and Shannon indexes, calculated after filtering for microbes but before standardising and filtering the very low abundance data. Chao1 estimates species richness (total abundance), whereas the Shannon index quantifies species diversity (variety of taxa). A one-way non-parametric Kruskal-Wallis and multiple comparison correction with Tukey testing were conducted using Graphpad Prism V9.3.1.

Multiple cluster analyses were conducted to explore relationships among treatment groups. Initially, a principal component analysis (PCA) was conducted to evaluate the similarities between treatment groups using Euclidean distances, facilitating data visualisation, interpretation, and analysis. A supervised analysis using sparse partial least squares discriminant analysis (sPLS-DA) was also conducted to assess the model's generalisation capabilities and reduce dimensionality while considering class labels. Following this, plot loadings derived from sPLS-DA to pinpoint the specific taxa responsible for the observed differences. Total or relative abundance was visualised on stacked barplots using phyloseq (365) and significantly different genera were graphed using Graphpad Prism V9.3.1.

The beta-diversity comparing microbiotas across diets (Bread only, bread + gold KF and bread + green KF) was then examined using a non-parametric permutation ANOVA using ANCOMBC (364, 366), where data were agglomerated to the genus level. ANCOMBC corrects for bias due to sample differences and estimates sampling fractions. It tests the null hypothesis of equal sample dispersion, which is more accurate with larger sample sizes ($n > 5$). The global 'p' value is significant when $p < 0.05$. The 'q' value, adjusted for false discovery rate (FDR), indicates the probability of a false positive (e.g., $q < 0.01$ means a 1% chance). The 'w' value represents the number of rejected null hypotheses (statistical significance). This study considered only q values <0.001 and w values >0.

Metabolite statistical analysis was done using MetaboAnalyst v5.0 (367). There were no missing data points. The data was normalised; data underwent log10 transformation and mean-centred before analysis. ANOVA and sPLS-DA of the plasma and each brain tissue region samples were performed. Heatmap visualisation and Ward's Method clustering algorithm were used for hierarchical clustering analysis. An integrative analysis of the microbiome and metabolites was done using MicrobiomeAnalyst v2.0 (368).

4.4 Results

4.4.1 Microbial DNA purity and concentrations

The purity and concentration of extracted DNA from the proximal and distal colon were assessed using Nanodrop and agarose gel electrophoresis before sequencing. All samples had an optimal DNA purity ratio ≥ 1.80. These quality checks were also independently performed by OSS Technology Hong Kong. Unfortunately, four samples

failed to meet quality standards and were not sequenced. These included one sample from the proximal colon with gold KF, two from the distal colon with bread only, and one from the distal colon with bread and gold KF. All the other samples passed the quality checks and were sequenced. After quality trimming, there were a total of 228 M reads. The median number of paired-end reads per sample was 7.1 M, ranging from a minimum of 0.46 M to a maximum of 9.7 M.

4.4.2 Microbial taxonomical composition

The alpha diversity was measured using CHAO1 and Shannon indexes. As shown in Figure 4.1A and B, there was a significant difference in the CHAO1 index for the distal colon between pigs fed bread only vs bread with green KF ($P < 0.05$). No other significant treatment effects were found for the CHAO1 or Shannon Index.

A PCA plot was drawn to examine the similarities between treatment groups and colonic regions (Figure 4.1C). Samples belonging to pigs fed bread only form a distinct cluster, whereas an overlap was observed for clusters of samples belonging to pigs fed gold or green KF. Furthermore, it shows that the treatment, not the colonic region, was responsible for the observed cluster formation. The high explained variance percentage (78%) by the first two principal components provides a strong confidence level in the reliability of these observations.

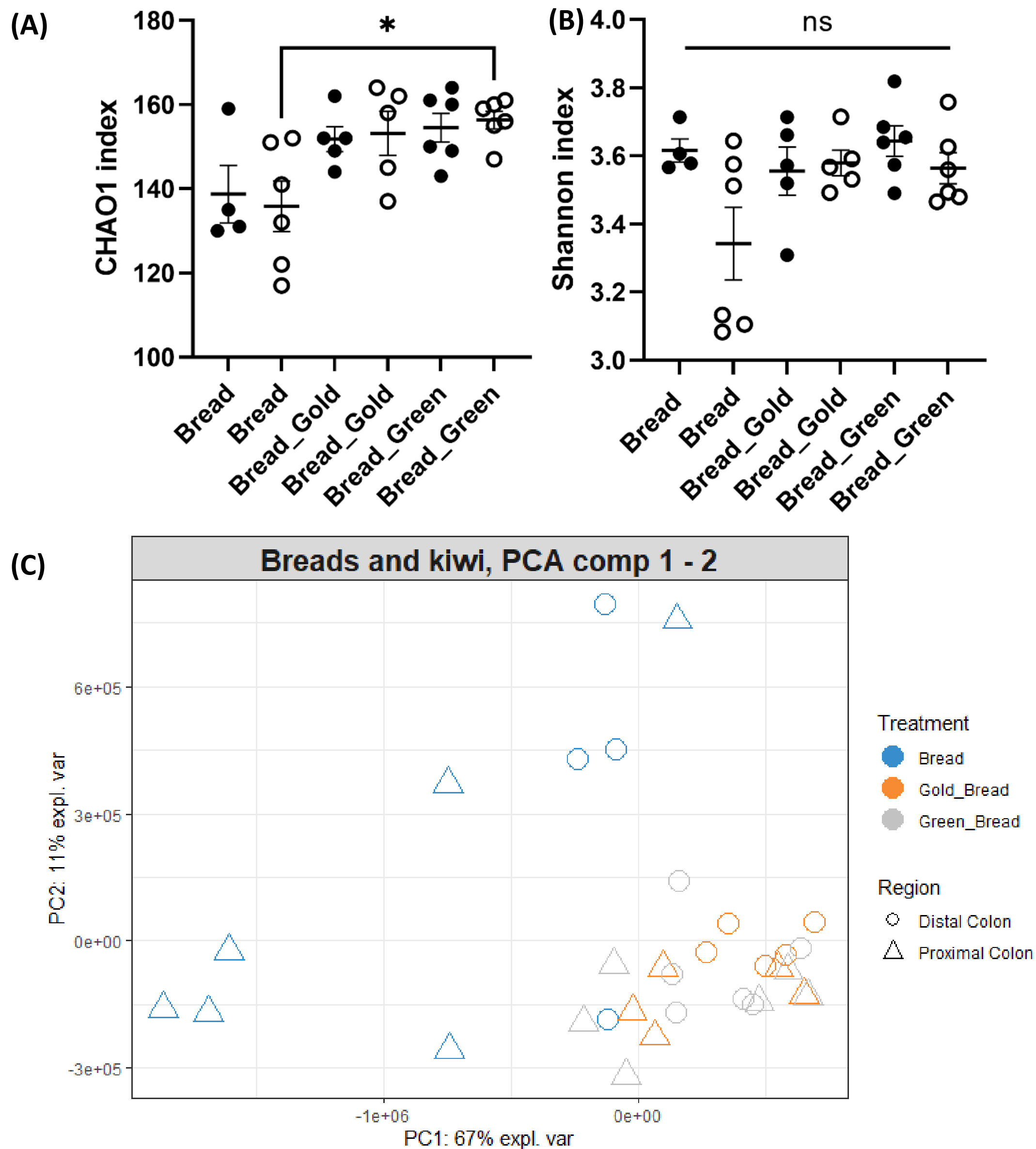


Figure 4.1 CHAO1 (A) and Shannon (B) Diversity Indexes of the proximal (white circles) and distal (black circles) colonic microbiota of digesta samples of pigs fed bread only or bread only and green KF or bread only and gold KF * P < 0.05. Principal component analysis plot (C) of both the proximal and distal colonic microbiota samples with all treatments included.

4.4.2.1 Proximal colon microbiota

The results from the sPLS-DA show that samples from the bread-only group form a distinct cluster, with both KF treatments overlapping (Figure 4.2A). The cluster for bread with green KF treatment was more compact, whereas the clusters for bread only and bread with gold KF treatments were spread out.

In addition, the sPLS-DA of the plot (Figure 4.2A) captured a third of the variation: sPLS-DA 1 captured 26% and sPLS-DA 2 captured 10%. In the plot loadings of the first component (Figure 4.2B), bacteria from the *Lachnospiraceae* family found in pigs fed green KF exhibited the most significant influence on this variable, followed by the order Clostridiales found in pigs fed gold KF. Conversely, in the second component, bacteria from the *Muribaculaceae* family in pigs fed green KF had the greatest (inverse) impact on this variable (Figure 4.2C).

The total abundance of each sample at the phylum, family and genus levels was visualised in a stacked bar-plot (Figure 4.3A-C). ANCOMBC analyses of proximal colon reads at genus level identified 22 differentially abundant taxa between different KF treatments and the control diet (Table 4.2). The largest proportion of bacteria in pigs fed gold or green KF treatments belonged to the Firmicutes phylum. In contrast, the bread-only group appears predominantly dominated by the phylum Bacteroidetes in most pigs (Figure 4.3A). Pigs fed bread with gold or green KF had the largest proportion of bacteria that was unclassified (unlabelled pink top group); the next belonged equally to the families *Prevotellaceae* and *Ruminococcaceae* (Figure 4.3B).

In contrast, pigs fed only bread had a higher total abundance of the *Prevotellaceae* family, though not consistently across all samples. The *Streptococcaceae* family was also more abundant in most pigs fed bread only (Figure 4.3B). None of these observed differences between treatment groups were statistically significant. Similarly at the genus level, the largest proportion of bacteria was unclassified (unlabelled pink top group), which was consistent for all treatment groups (Figure 4.3C).

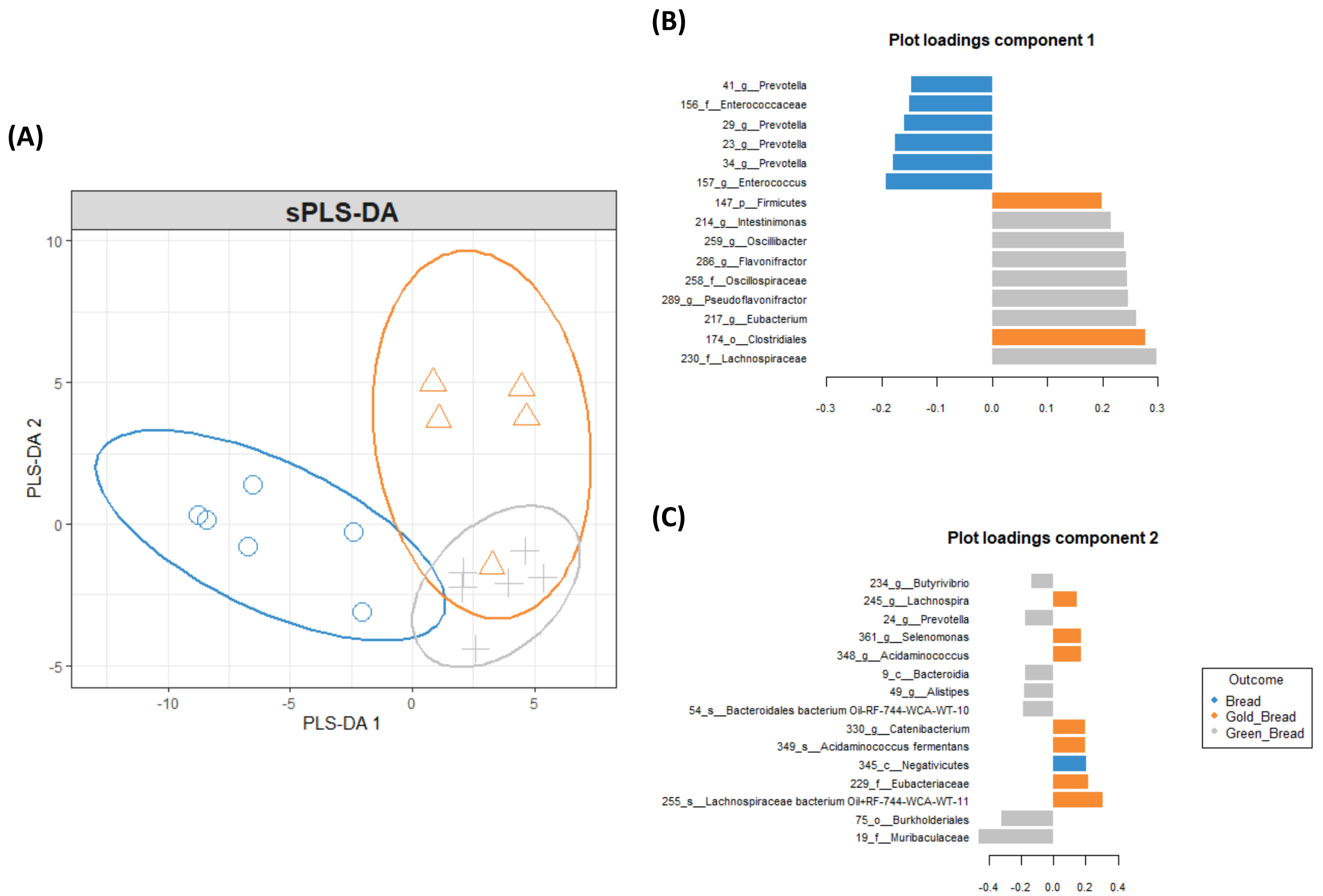


Figure 4.2 sPLS-DA plot of proximal colon microbiota of pigs fed bread (blue circles), bread with gold KF (orange triangles) or bread with green KF (grey plus signs) (A). Plot loading of component 1 (B) and component 2 (C) of pigs consuming bread only (n=6), bread with gold KF (n=5) and bread with green KF (n=6).

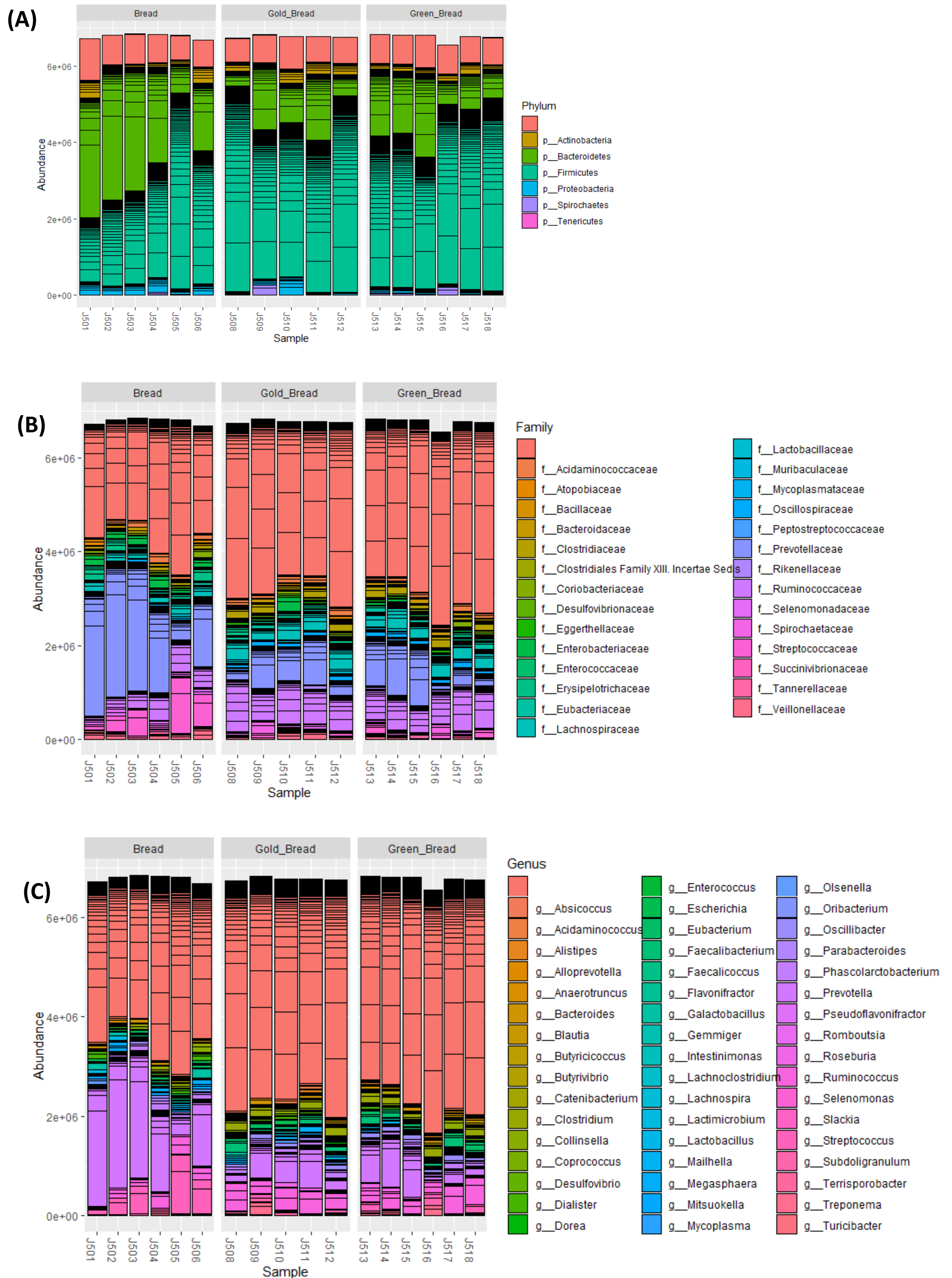


Figure 4.3 Stacked bar-plot after standardisation of the total abundance of proximal colonic microbiota at phylum (A) Family (B) and Genus (C) levels of pigs consuming bread only (n=6), bread with gold KF (n=5) and bread with green (n=6).

Both KF treatment groups compared to the bread-only control had significantly ($P<0.01$) decreased relative abundance of bacteria belonging to genera *Prevotella, Streptococcus, Galactobacillus, Megasphaera, Lactimicrobium, Escherichia* and *Enterococcus* (Table 4.2). Correspondingly, in both KF treatments compared to the bread-only control, there were greater relative abundances of *Clostridium, Faecalibacterium, Treponema, Alistipes, Anaerotruncus, Flavonifractor, Intestinimonas, Pseudoflavonifractor, Faecalicoccus, Butyricicoccus, Eggerthella, Lachnospira, Alloprevotella* and *Slackia* genera (Table 4.2). The *Mailhella* genus was not present in pigs fed bread with gold KF (p=0.0000, q=0.0000, w=25.2), and the *Enterococcu*s genus was not present in pigs fed bread with green KF (p=0.0000, q=0.0000, w=998.9). In pigs fed bread only the genera *Butyricicoccus* (p=0.0000, q=0.0000, w=722.0), *Eggerthella* (p=0.0000, q=0.0000, w=35.6), *Lachnospira* (p=0.0000, q=0.0000, w=35.5) and *Slackia* (p=0.0000, q=0.0000, w=20.4) were not present (Table 4.2).

Table 4.2 Relative abundance of statistically significant taxonomic genera of proximal colonic microbiota of pigs consuming bread only (n=6), bread with gold KF (n=5) and bread with green KF (n=6). Global p-value, significant when p<0.05. q value is the p-value adjusted for the false discovery rate. w value reports the number of times the null hypothesis was rejected. Data is presented as mean ± SEM.

Phylum	Family	*Genus*	Bread only	Bread + gold KF	Bread + green KF	global p	global q	global w
Genus that was decreased in with KF								
Bacteroidetes	Prevotellaceae	*Prevotella*	40.665 ± 7.015	16.155 ± 3.228	21.855 ± 3.896	0.0000	0.0001	32.9
Firmicutes	Streptococcaceae	*Streptococcus*	15.486 ± 4.340	3.331 ± 0.982	3.777 ± 1.372	0.0001	0.0003	28.5
Firmicutes	Erysipelotrichaceae	*Galactobacillus*	3.627 ± 1.127	0.888 ± 0.252	0.300 ± 0.125	0.0000	0.0000	46.0
Firmicutes	Veillonellaceae	*Megasphaera*	2.030 ± 0.356	2.184 ± 0.584	1.282 ± 0.227	0.0001	0.0004	27.2
Firmicutes	Erysipelotrichaceae	*Lactimicrobium*	1.867 ± 0.866	0.113 ± 0.077	0.021 ± 0.021	0.0000	0.0000	17.9
Proteobacteria	Enterobacteriaceae	*Escherichia*	1.718 ± 0.220	1.554 ± 0.698	0.447 ± 0.124	0.0000	0.0000	34.3
Firmicutes	Enterococcaceae	*Enterococcus*	0.225 ± 0.040	0.091 ± 0.048	0.000 ± 0.000	0.0000	0.0000	998.9
Genus that was increased in with KF								
Firmicutes	Clostridiaceae	*Clostridium*	1.653 ± 0.440	9.846 ± 3.546	9.337 ± 1.588	0.0003	0.0008	25.0
Firmicutes	Ruminococcaceae	*Faecalibacterium*	0.645 ± 0.173	4.154 ± 0.831	4.295 ± 0.747	0.0000	0.0000	34.9
Spirochaetes	Spirochaetaceae	*Treponema*	0.382 ± 0.219	1.676 ± 1.080	2.351 ± 0.944	0.0002	0.0006	26.1
Bacteroidetes	Rikenellaceae	*Alistipes*	0.147 ± 0.085	1.318 ± 0.572	2.043 ± 0.485	0.0024	0.0060	20.1
Firmicutes	Ruminococcaceae	*Anaerotruncus*	0.064 ± 0.036	0.183 ± 0.042	0.481 ± 0.138	0.0007	0.0021	22.8
Firmicutes	Ruminococcaceae	*Flavonifractor*	0.060 ± 0.031	0.256 ± 0.035	0.312 ± 0.032	0.0001	0.0004	27.1
Firmicutes		*Intestinimonas*	0.060 ± 0.032	0.258 ± 0.015	0.307 ± 0.032	0.0002	0.0006	26.3
Firmicutes	Ruminococcaceae	*Pseudoflavonifractor*	0.058 ± 0.030	0.277 ± 0.043	0.326 ± 0.038	0.0001	0.0004	27.5
Firmicutes	Erysipelotrichaceae	*Faecalicoccus*	0.059 ± 0.030	0.195 ± 0.047	0.276 ± 0.018	0.0000	0.0001	30.6
Firmicutes	Clostridiaceae	*Butyricicoccus*	0.000 ± 0.000	0.191 ± 0.047	0.221 ± 0.024	0.0000	0.0000	722.0
Actinobacteria	Eggerthellaceae	*Eggerthella*	0.000 ± 0.000	0.158 ± 0.045	0.124 ± 0.051	0.0000	0.0000	35.6
Firmicutes	Lachnospiraceae	*Lachnospira*	0.000 ± 0.000	0.155 ± 0.042	0.048 ± 0.029	0.0000	0.0000	35.5
Bacteroidetes	Prevotellaceae	*Alloprevotella*	0.018 ± 0.018	0.137 ± 0.089	0.096 ± 0.045	0.0000	0.0000	21.4
Actinobacteria	Eggerthellaceae	*Slackia*	0.000 ± 0.000	0.143 ± 0.050	0.111 ± 0.042	0.0000	0.0000	20.4
Proteobacteria	Desulfovibrionaceae	*Mailhella*	0.092 ± 0.033	0.000 ± 0.000	0.059 ± 0.043	0.0000	0.0000	25.2

4.4.2.2 Distal colon microbiota

Like the proximal colon results, the sPLS-DA results show that both KF clusters overlap, while the bread-only group forms a separate and distinct cluster (Figure 4.4A). The cluster for the green KF treatment group was more compact, whereas the clusters for the bread-only control group and gold KF treatment group were more spread out. In addition, the sPLS-DA of the plot captured a third of the variation: sPLS-DA 1 captured 24% and sPLS-DA 2 captured 10%. In the plot loadings of component 1, the genus *Butyricicoccus* found in greatest abundance in pigs fed green KF and *Enterococcus* found

in greatest abundance in pigs fed bread only exert the most significant influence on this variable (Figure 4.4B). In contrast, in component 2, the class Betaproteobacteria found in greatest abundance in pigs fed bread only, and species of *Dorea* found in pigs fed gold KF exhibits the greatest impact on this variable (Figure 4.4C).

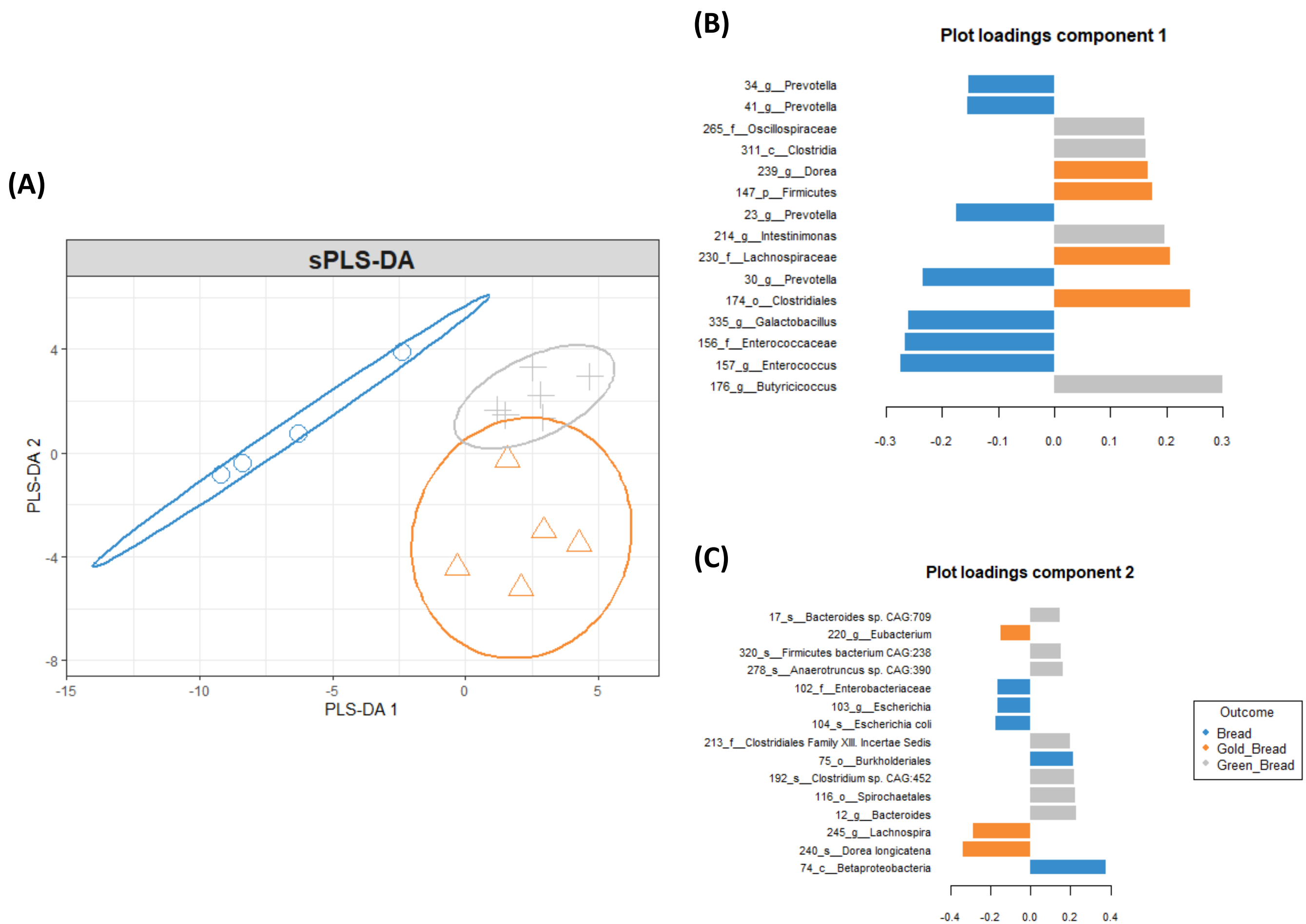


Figure 4.4 sPLS-DA plot of distal colon microbiota of pigs fed bread (blue circles), bread with gold KF (orange triangles) or bread with green KF (grey plus signs) (A). Plot loading of component 1 (B) and component 2 (C) of pigs consuming bread only (n=4), bread with gold KF (n=5) and bread with green KF (n = 6).

A stacked bar plot was used to visualise the overall total abundance of the taxa in each sample at the phylum, family, and genus levels (Figure 4.5). ANCOMBC analyses of distal colon reads at the genus level identified 16 differentially abundant taxa between different KF treatments and the control diet (Table 4.3). Like the proximal colon, pigs fed bread with either gold or green KF had the largest proportion of bacteria belonging to the phylum Firmicutes in the distal colon (Figure 4.5A). Unlike in the proximal colon, pigs fed bread only had the largest proportion of the Firmicutes phylum (Figure 4.5A), however, this was not consistent between all samples, with the Bacteroidetes phylum being higher in some samples. At the family level, pigs fed bread with gold or green KF had the largest proportion of bacteria that was unclassified (unlabelled pink top group). The next belonged to the family *Ruminococcaceae* (Figure 4.5B).

In contrast, in pigs fed bread-only, the largest proportion of bacteria belonged to the family *Prevotellaceae*, however, this was not consistent between all samples. There is also a greater proportion of bacteria from the *Streptococcaceae* family in pigs fed bread only (Figure 4.5B). None of these observed differences between treatment groups were statistically significant. Similarly, at the genus level, the largest proportion of bacteria was unclassified (unlabelled pink top group), which was consistent for all treatment groups (Figure 4.5C).

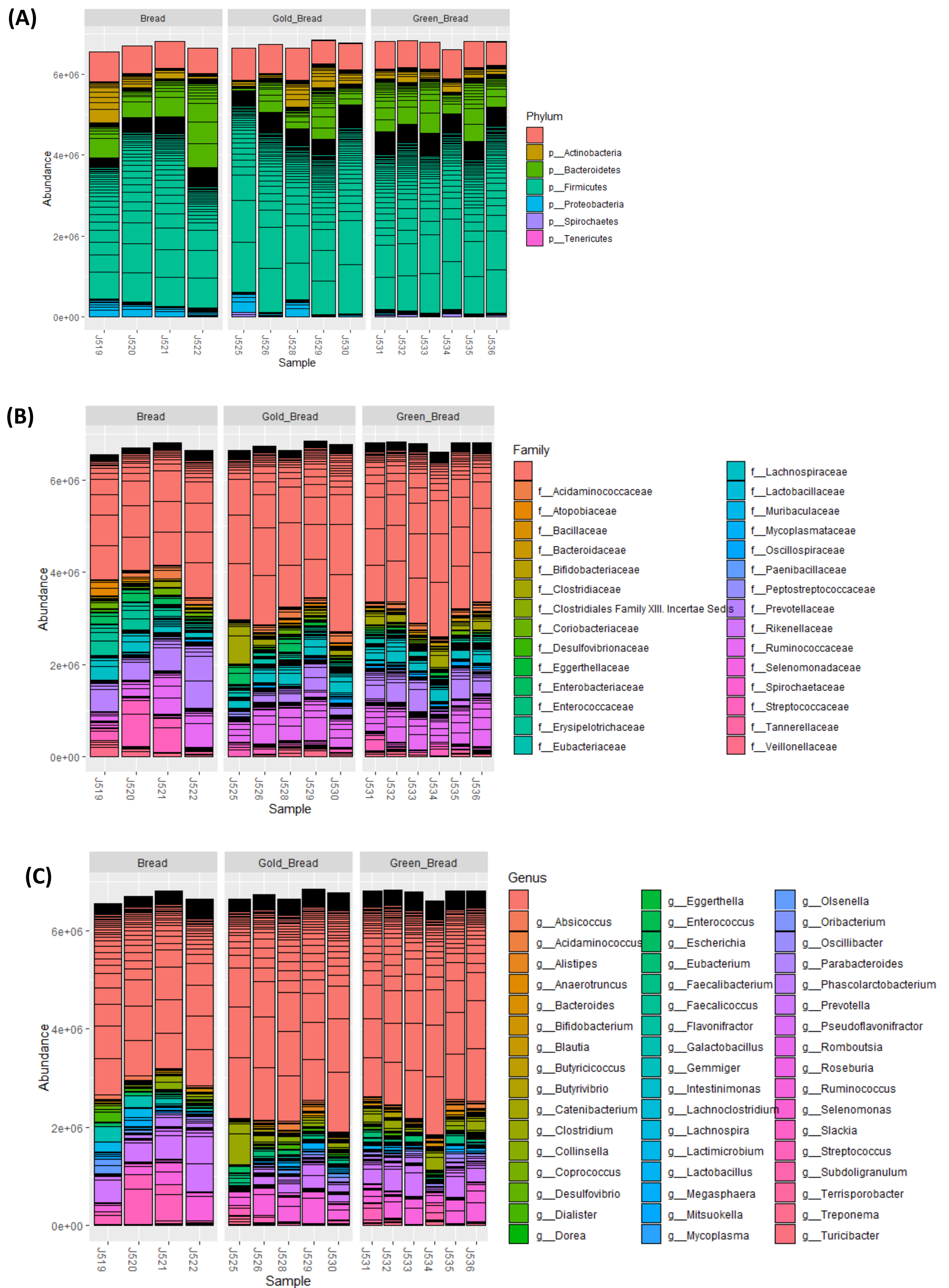


Figure 4.5 Stacked bar-plot after standardisation of the total abundance of distal colonic microbiota at phylum (A) Family (B) and Genus (C) of pigs consuming bread only (n=4), bread with gold KF (n=5) and bread with green KF (n=6).

The relative abundance of the *Prevotella* genus was significantly lower in KF treatments, compared to the bread only (*p*=0.0000, *q*=0.0000, *w*=31.1) (Table 4.3). In addition, both KF treatments, compared to bread-only control, also had significantly (global $p<0.01$) lowered relative abundance of the bacteria belonging to genera *Galactobacillus, Lactimicrobium, Collinsella, Escherichia* and *Enterococcus* in the distal colon (Table 4.3) In contrast, both KF treatments, compared to bread-only control, had significantly ($P<0.01$) increased relative abundance of *Faecalibacterium*, *Roseburia*, *Pseudoflavonifractor*, *Flavonifractor*, *Intestinimonas* and *Faecalicoccus* genera (Table 4.3). *Lactimicrobium* and *Enterococcus* genera were absent in pigs fed the green KF treatment (*p*=0.0000, *q*=0.0000, *w*=11.5 and 678.0, respectively). *Lachnospira, Slackia, Eggerthella* and *Butyricicoccus* genera were absent in pigs fed bread only (*p*=0.0000, *q*=0.0000, *w*=6.7, 9.1, 29.8 and 578.0 respectively).

Table 4.3 Relative abundance of statistically significant taxonomic genera of distal colonic microbiota of pigs consuming bread only (n=4), bread with gold KF (n=5) and bread with green KF (n = 6). Global p-value, significant when p<0.05. q value is the p-value adjusted for the false discovery rate. w value reports the number of times the null hypothesis was rejected. Data is presented as mean ± SEM.

Phylum	Family	Genus	Bread only	Bread + gold KF	Bread + green KF	global p	global q	global w
Genus that was decreased in with KF								
Bacteroidetes	Prevotellaceae	*Prevotella*	26.226 ± 6.860	11.177 ± 3.380	19.989 ± 4.107	0.0000	0.0000	31.1
Firmicutes	Erysipelotrichaceae	*Galactobacillus*	6.104 ± 2.298	0.714 ± 0.313	0.476 ± 0.223	0.0004	0.0031	17.2
Firmicutes	Erysipelotrichaceae	*Lactimicrobium*	3.438 ± 1.962	0.140 ± 0.140	0.000 ± 0.000	0.0000	0.0000	11.5
Actinobacteria	Coriobacteriaceae	*Collinsella*	2.413 ± 0.727	2.342 ± 0.793	1.961 ± 0.371	0.0060	0.0243	11.6
Proteobacteria	Enterobacteriaceae	*Escherichia*	1.993 ± 0.477	2.130 ± 1.215	0.208 ± 0.145	0.0000	0.0000	26.1
Firmicutes	Enterococcaceae	*Enterococcus*	0.345 ± 0.044	0.137 ± 0.085	0.000 ± 0.000	0.0000	0.0000	678.0
Genus that was increased in with KF								
Firmicutes	Ruminococcaceae	*Faecalibacterium*	0.599 ± 0.216	3.079 ± 0.706	3.582 ± 0.925	0.0045	0.0205	12.2
Firmicutes	Lachnospiraceae	*Roseburia*	0.672 ± 0.261	1.382 ± 0.403	1.331 ± 0.505	0.0006	0.0043	0.0
Firmicutes	Ruminococcaceae	*Pseudoflavonifractor*	0.061 ± 0.061	0.264 ± 0.079	0.330 ± 0.069	0.0039	0.0205	12.5
Firmicutes	Ruminococcaceae	*Flavonifractor*	0.062 ± 0.062	0.240 ± 0.066	0.317 ± 0.061	0.0041	0.0205	12.4
Firmicutes		*Intestinimonas*	0.066 ± 0.066	0.253 ± 0.026	0.313 ± 0.042	0.0068	0.0243	11.4
Firmicutes	Erysipelotrichaceae	*Faecalicoccus*	0.055 ± 0.055	0.217 ± 0.062	0.266 ± 0.025	0.0034	0.0205	12.8
Firmicutes	Clostridiaceae	*Butyricicoccus*	0.000 ± 0.000	0.209 ± 0.056	0.236 ± 0.010	0.0000	0.0000	578.0
Actinobacteria	Eggerthellaceae	*Eggerthella*	0.000 ± 0.000	0.222 ± 0.057	0.219 ± 0.086	0.0000	0.0000	29.8
Actinobacteria	Eggerthellaceae	*Slackia*	0.000 ± 0.000	0.131 ± 0.080	0.150 ± 0.070	0.0000	0.0000	9.1
Firmicutes	Lachnospiraceae	*Lachnospira*	0.000 ± 0.000	0.127 ± 0.062	0.012 ± 0.012	0.0000	0.0000	6.7

4.4.3 Plasma and brain bioamine concentrations

4.4.3.1 Plasma metabolite concentrations

In the sPLS-DA analysis of plasma, component 1 captures 29.7% of the variance in plasma; it does not seem to be the primary driver of group separation, as evidenced by overlapping clusters in the scores plot (Figure 4.6A). Instead, it represents the overall variability in the dataset. In contrast, component 2, explains 11.5% and emerges as the more intriguing set of loadings. The separation between groups becomes evident in the direction of component 2, as reflected in the scores plot. As shown in the plot loading of component 2, 5HIAA from pigs fed bread with gold KF exerts the most significant influence on the component. This observation suggests that component 2 is pivotal for discriminating between groups. The sPLS-DA analysis resulted in classification error rates less <20% (APPENDIX C).

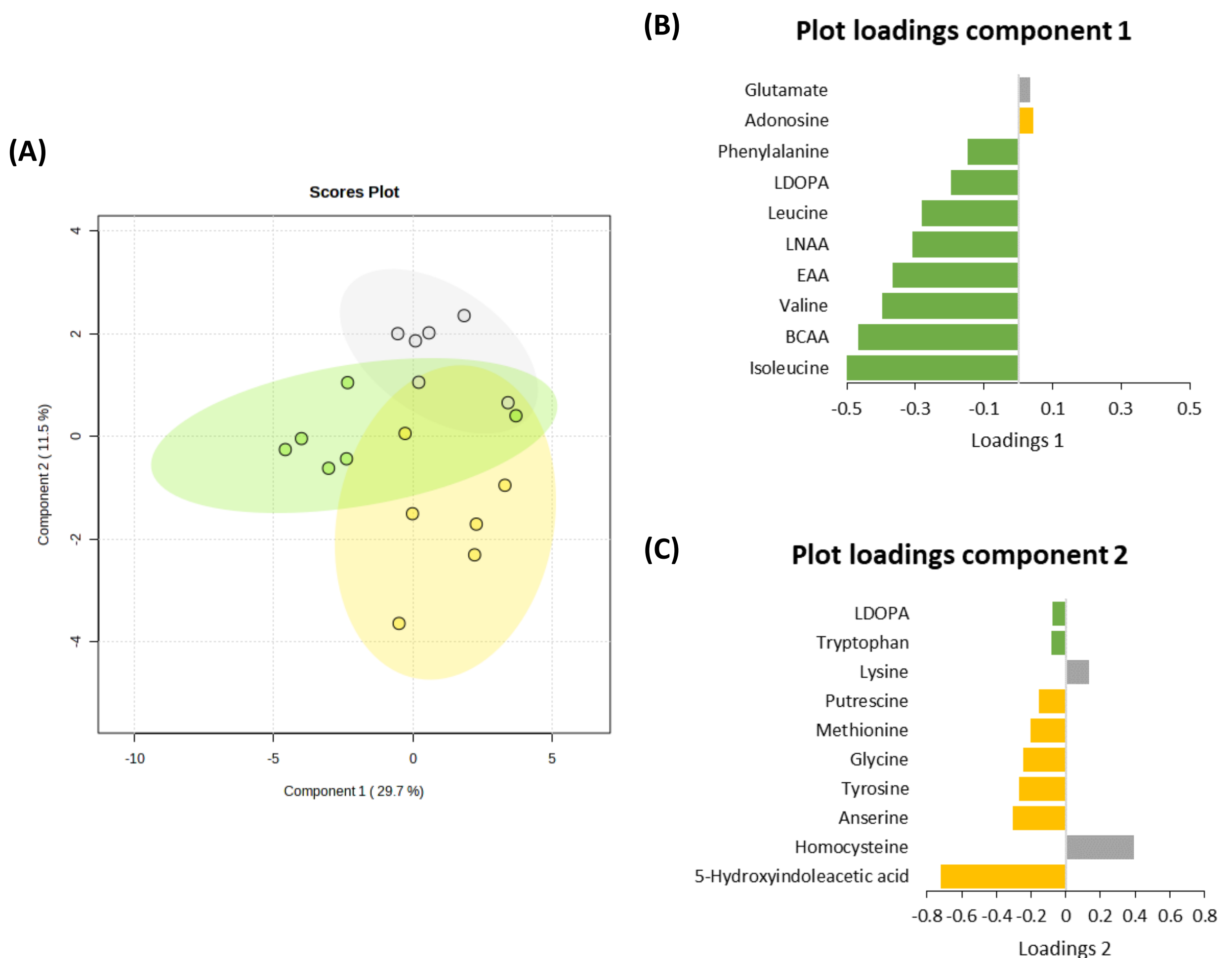


Figure 4.6 sPLS-DA score plot of plasma (A) the score plot were created using MetaboAnalyst (ver. 5.0, https://www.metaboanalyst.ca), plot loading of component 1 (B) and component 2 (C) of pigs consuming bread only, bread with gold KF and bread with green KF (n = 6 animals per group).

Hierarchical clustering analysis showed that bread-only and bread with gold KF groups clustered in plasma samples, while bread with green KF group formed a separate cluster (Figure 4.7A). Furthermore, the clustering analysis indicates that the green KF treatment exhibited the highest levels among 16 out of 31 metabolites in plasma. The gold KF treatment showed the highest levels in 11 out of 31 metabolites. Conversely, only four out of 31 metabolites demonstrated the highest levels with bread alone. The univariate analysis showed that the plasma concentration of one of the metabolites, 5HIAA, was significantly different (Fisher's LSD test, FDR adjusted p-value <0.05) across the three

treatments (Figure 4.7B). Both gold and green KF had a greater plasma concentration of 5HIAA compared to the control.

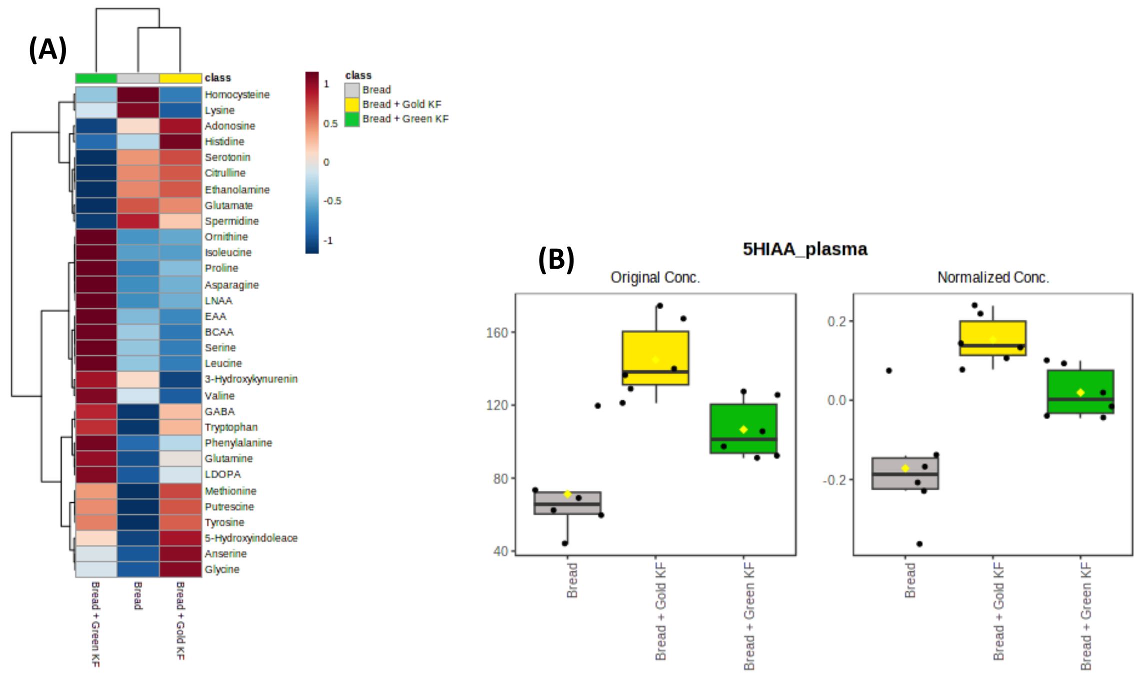


Figure 4.7 Heatmap of mean metabolites in plasma samples, red = high concentration and blue = low concentration levels (A) and unnormalised concentration (left) and normalised log10 transformed and mean-centred concentration (right) boxplots of 5HIAA in plasma (n = 6 animals per group) in pigs fed bread only, bread with gold KF or bread with green KF. The images were created using MetaboAnalyst (ver. 5.0, https://www.metaboanalyst.ca).

4.4.3.2 Brain region bioamine concentrations

In the sPLS-DA analysis of the four brain regions, component 1 explained varying percentages of variance: 83.1% for the brain stem, 44.1% for the corpus striatum, 28.4% for the hippocampus, and 28.6% for the prefrontal cortex. Meanwhile, component 2 accounted for 5.8%, 14.8%, 38.6%, and 30% of the variance in the same regions, respectively (Figure 4.8A-D).

In the brain stem, both KF treatments clustered closely, with some overlap, while the bread-only group exhibited a broader distribution but also encompassed the KF treatment clusters (Figure 4.8A). The corpus striatum displayed overlapping clusters (Figure 4.8B). No distinct clustering was observed among KF treatments in the hippocampus, leading to a more scattered pattern (Figure 4.8C). In the prefrontal cortex, the samples formed overlapping clusters (Figure 4.8D).

The absence of sample separation suggests a potentially small treatment effect. Upon examining the classification metrics, the sPLS-DA analysis for each brain region consistently produced classification error rates exceeding 50%, indicating a potential overfitting issue and a lack of robustness in the analysis. The hierarchical clustering analysis of the measured metabolites unveiled shared and distinct patterns in various brain regions. In the brain stem and prefrontal cortex, clustering was similar between gold KF and green KF treatment groups, while the bread-only control group formed a separate cluster (Figure 4.9A and D). In the corpus striatum (Figure 4.9B) and hippocampus (Figure 4.9C), pigs fed gold KF and green KF formed separate clusters, respectively. Pigs fed the KF treatments generally displayed higher concentrations of metabolites in all brain regions, while pigs fed the bread-only diet consistently had reduced levels in all regions (Figure 4.9). However, the univariate analysis did not reveal

any statistically significant variations in the measured metabolite concentrations across any regions.

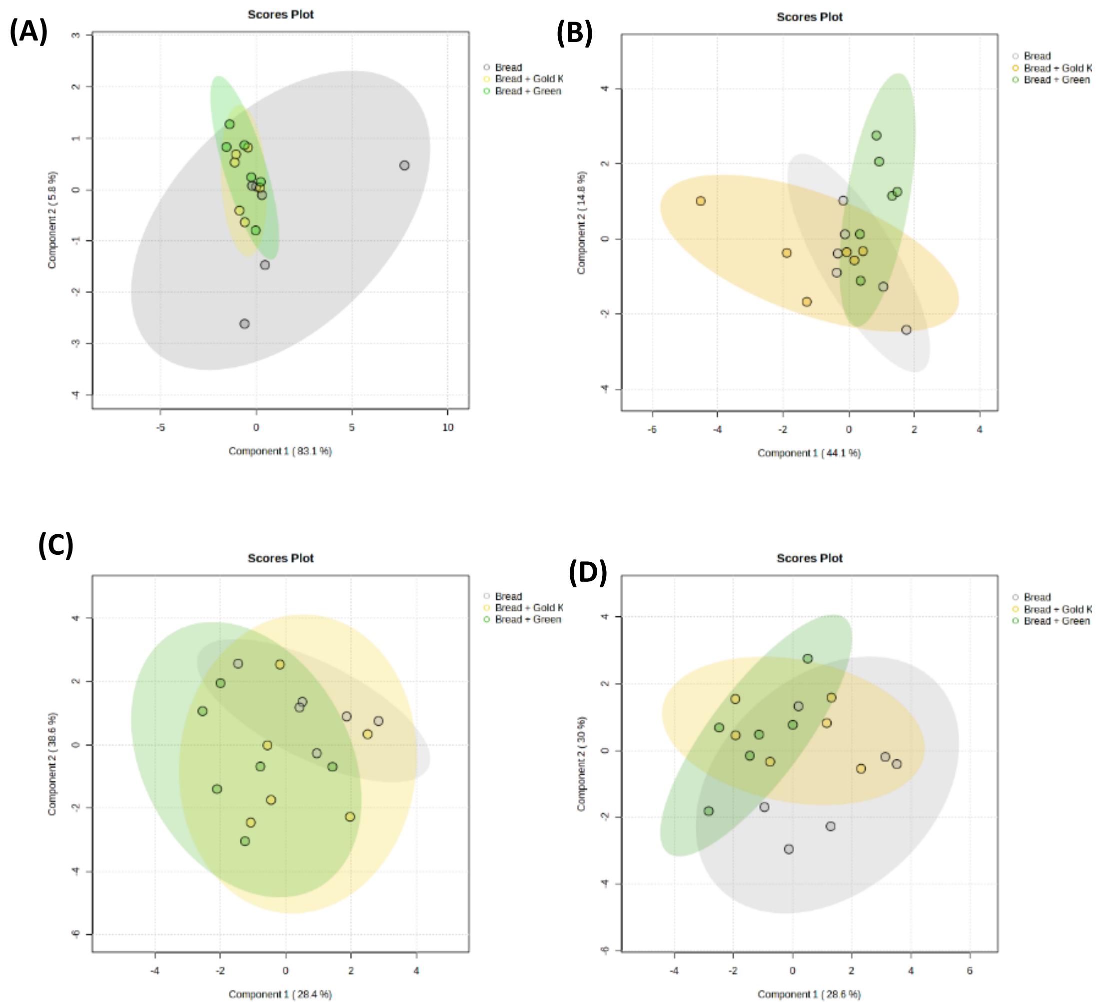


Figure 4.8 sPLS-DA score plot of the brain stem (A), corpus striatum (B), hippocampus (C) and prefrontal cortex (D) of pigs fed bread only (n = 6 animals), bread with gold KF (n = 6 animals) or bread with green KF (n = 6 animals). Data was normalised log10 transformed and mean-centred. The images were created using MetaboAnalyst (ver. 5.0, https://www.metaboanalyst.ca).

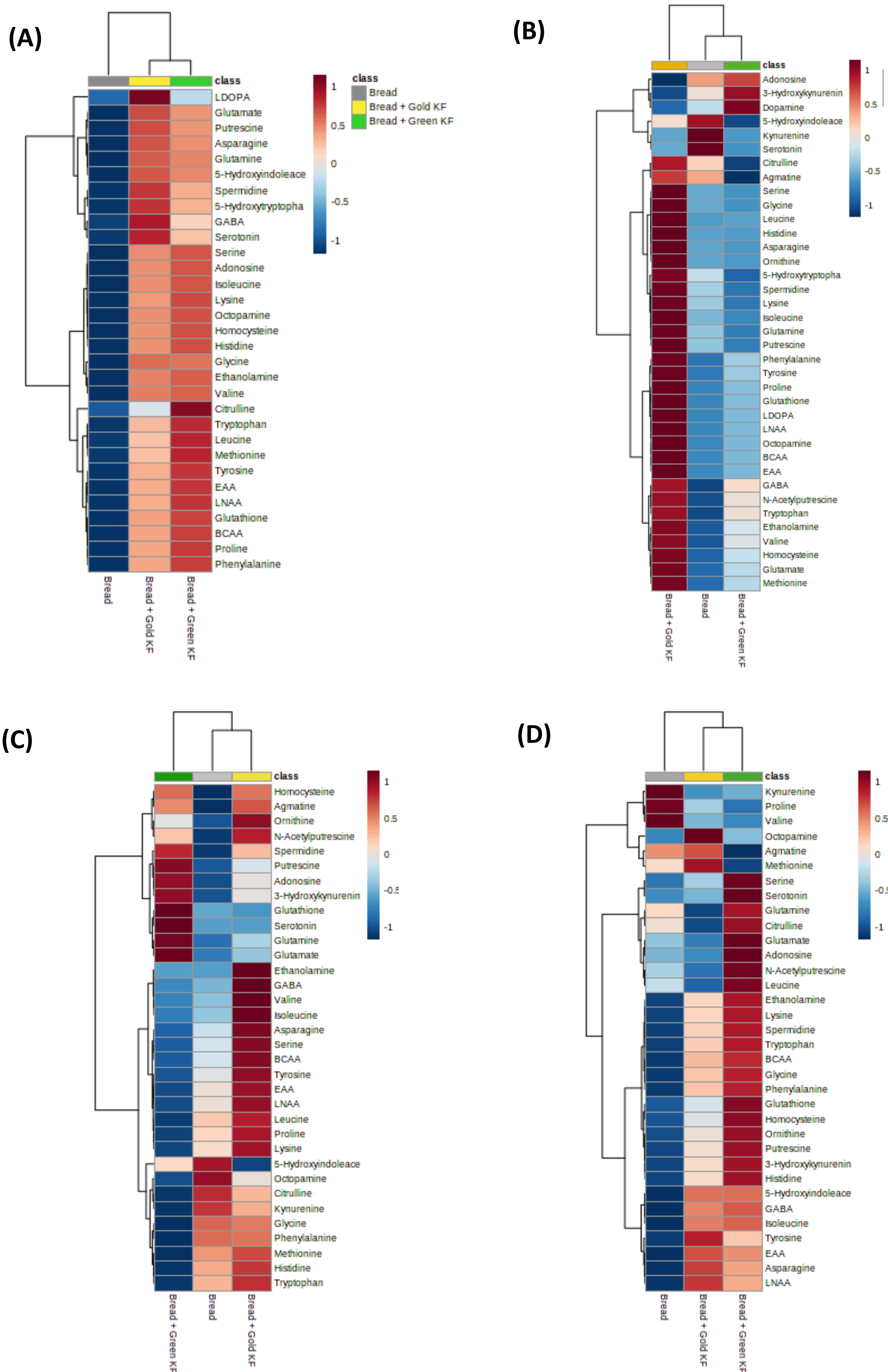


Figure 4.9 Heatmap of mean metabolites in brain stem (A), corpus striatum (B), hippocampus (C) and prefrontal cortex (D) (n = 6 animals) in pigs fed bread only, bread with gold KF or bread with green KF. Data was normalised log10 transformed and mean-centred. The images were created using MetaboAnalyst (ver. 5.0, https://www.metaboanalyst.ca).

4.4.4 Correlations between bacteria and metabolites

A Data Integration Analysis for Biomarker discovery using Latent variable approaches for omics studies (DIABLO) analysis (369) was initially considered for this dataset. The evidence shows that adding KF to the diet may impact the proximal and distal colonic microbiome composition. The only discernible difference was in a single metabolite within the plasma, with no observable alterations detected in the brain. Multiplicity in the data is often required for successful integration. Given that there was a single different metabolite, an integrated analysis was not conducted as it is unlikely to yield any overt changes or meaningful insights based on the current data.

4.5 Discussion

Supplementation of a bread diet with green KF increased microbial diversity (CHAO1) in the proximal colon compared to a bread-only diet in pigs. No other differences in diversity indexes reported here were observed in response to treatment in any colonic region. Reductions in the relative abundance of the genera *Prevotella*, *Streptococcus*, *Galactobacillus*, *Megasphaera*, *Lactimicrobium* and *Enterococcus* were observed in both the proximal and distal colons for both KF treatments. Conversely, increased relative abundance of the genera *Faecalibacterium*, *Pseudoflavonifractor*, *Flavonifractor*, *Intestinimonas*, *Faecalicoccus*, *Butyricicoccus*, *Eggerthella*, and *Slackia* were observed with either KF treatment in both the proximal and distal colon. The concentration of 5HIAA in plasma increased with both KF treatments compared to the control; no other plasma or brain metabolites showed concentration differences between treatments.

4.5.1 Colonic microbiota

Both gold or green KF supplementation decreased the relative abundance of potential pathogenic genera, *Prevotella*, *Enterococcus*, *Escherichia*, *Galactobacillus* and *Lactimicrobium*, in both the proximal and distal colon. The current study confirms results from *in vitro* fermentation studies showing that fresh gold KF (25 g) reduces the relative abundance of the *Prevotella* genus (370). Additionally, an oral care tablet containing green KF powder reduced the relative abundance of the *Prevotella* genus in the oral cavity (86). The genus *Prevotella* plays a role in fermenting dietary fibre and breaking down polysaccharides, and its abundance is associated with plant-rich diets high in carbohydrates and fibre (371). *Prevotella* is recognised as a beneficial genus due to its considerable prevalence in the healthy human body and its infrequent association with infections. However, it is proposed that specific strains of *Prevotella* may have the potential to function as pathobionts within the intestinal environment (372).

The lower relative abundance of *Enterococcus* and *Escherichia* genera with the green KF treatment reported here agrees with a study with healthy growing pigs, where the *Escherichia* genus was also reduced in response to green KF (102). Similarly, *Enterococcus* and *Escherichia* genera are common bacteria found in the GIT, with most strains being non-pathogenic commensals (373). However, both genera also have strains that may be opportunistic pathogens that may cause various infections. The genus *Enterococcus* (most notably *E. faecalis* and *E. faecium*) has been the most common cause of infection in hospitalised patients, resulting in intra-abdominal infections and endocarditis (374). *E. coli* has a broad diversity of disease-causing genotypes (375).

This is the first instance where KF is shown to influence both *Galactobacillus* and *Lactimicrobium* genera. It is particularly noteworthy given that *Galactobacillus* and *Lactimicrobium* are newly discovered genera, first isolated in the breastmilk of healthy mothers (376). To date, only two other research papers have mentioned these genera (377, 378), and there is no data about the effect of KF supplementation. A *Galactobacillus* species displayed an inverse correlation with serum immunoglobulin E levels in children with allergies (asthma and rhinitis) (377), while the *Lactimicrobium* genus was found in healthy cats but absent in obese cats (378). The functional implications of the involvement of both *Galactobacillus* and *Lactimicrobium* genera in human health remain unexplored. Therefore, caution is warranted in drawing conclusions or formulating hypotheses regarding the potential translational relevance of the observed phenomena due to KF in human physiological contexts.

The decrease in the relative abundance of potential pathogenic genera (*Prevotella*, *Enterococcus*, *Escherichia*, *Galactobacillus* and *Lactimicrobium*) in response to KF treatments may be due to the proliferation of alternative microbial species. Microbial metabolic by-products, such as SCFA, can hinder the growth of pathogens (379). Moreover, symbiotic microbes produce enzymes that transform primary bile acids into secondary bile acids, which have antimicrobial properties against specific pathogens (380). Therefore, the supplementation with green or gold KF, known for its rich fibre content, vitamins, and polyphenols, could have promoted the growth of microbes capable of producing metabolites that restrict the proliferation of harmful pathogens. However, the concentrations of SCFA or bile acids were not measured here.

The green or gold KF supplementation enhances the relative abundance of known butyrate-producing gut bacteria from the genera *Faecalibacterium*, *Flavonifractor*, *Intestinimonas*, *Pseudoflavonifractor*, *Faecalicoccus*, *Butyricicoccus*, *Eggerthella* and *Slackia*. Prior studies found that gold KF-based supplements increased the relative abundance of the *Faecalibacterium* genus in subjects with functional constipation (12). The *Faecalibacterium* genus is one of the key components of the gut microbiota and the primary butyrate-producing bacteria in the human colon; hence, a plethora of research is targeted at methods of increasing its abundance (100). Butyrate provides numerous benefits to the host, including anti-inflammatory, immunoregulatory, anti-diabetes, cardiovascular protective, and neuroprotective effects (381). This genus has been negatively correlated with depressive symptoms (382). Additionally, the *Faecalicoccus* genus has shown positive associations with neuropsychological performance and sleep efficiency (383). This finding suggests that green or gold KF supplementation may facilitate neurological effects by altering the gut microbiome, but further research from human studies is needed to confirm this finding.

Both green and gold KF increase the abundance of the genera, *Flavonifractor*, *Pseudoflavonifractor*, *Eggerthella*, and *Slackia*, which are recognised as flavonoid-degrading bacteria (384-386). This finding is significant as the breakdown of flavonoids enhances their bioactivity compared to their precursors (387), potentially amplifying the downstream beneficial effects of added polyphenols to the diet. In a study involving prediabetic adults, supplementation with gold KF led to an increase in the relative abundance of the family *Coriobacteriaceae* and the *Actinobacteria* phylum, including the genera *Eggerthella* and *Slackia* (55). Additionally, research on rats fed a high-fat diet showed that gold KF seed oil extract increased the relative abundance of *Flavonifractor*

and *Pseudoflavonifractor*, while concurrently reducing the concentration of circulating inflammatory cytokines(388). Moreover, a higher relative abundance of the *Pseudoflavonifractor* genus has been linked to greater success in weight loss (389). These collective findings suggest that KF could positively affect metabolic conditions, possibly mediated through alterations in the gut microbiome. This finding underscores the necessity for more in-depth exploration and investigation in future research to elucidate the mechanisms and potential therapeutic implications of KF in metabolic health.

Ultimately, the findings from this study indicate that the variety of KF supplemented may have differential region-specific and genus-specific alterations in the colon microbiome. Significant alterations in the relative abundance of bacterial genera were observed in response to green or gold KF supplementation compared to a control group. Specifically, the proximal colon exhibited changes in the genera *Streptococcus*, *Clostridium*, *Treponema*, *Alistipes*, *Anaerotruncus*, *Alloprevotella* and *Mailhella*, while the distal colon showed variations in *Collinsella* and *Roseburia*. Green KF supplementation decreased *Escherichia* in both the proximal and distal colon, while gold KF did not, exhibiting a profile like that of the control group.

The differential impact of KF on these bacterial populations suggests that the composition of nutrients in different KF varieties may be a contributing factor. Green KF, containing the protease enzyme actinidin, potentially influences the proximal colon by reducing intact protein levels. This is crucial, as excessive undigested proteins in the colon can promote the growth of pathogens and protein-fermenting bacteria (390). Moreover, the green KF treatment exhibits higher polyphenol and vitamin E contents,

both associated with promoting beneficial bacteria and metabolites (391, 392). Conversely, the gold KF variety lacks actinidin ('Hort16A') or contains a lower level ('SunGold') but has elevated levels of vitamin C, linked to shifts in gut bacterial populations (393).

In vitro evidence supports the notion that green-fleshed KF selectively increases the relative abundance of the *Lachnospira* genus, while gold-fleshed KF increases that of the *Akkermansia* genus (370). Additionally, rat studies revealed that the type of fibre from KF may also play a role. Rats fed a high-fat diet supplemented with insoluble KF fibre extract exhibited an increased relative abundance of the *Alistipes* genus, while those supplemented with soluble KF fibre showed an increase in the *Roseburia* genus (394). The varying nutrient profiles of green and gold KF varieties might contribute to these differences, highlighting the potential of KF to selectively influence the growth of specific bacterial genera along the colon.

4.5.2 Bioamines

There were limited effects on the concentration of bioamines, with no observable changes in the brain. However, a single metabolite change was identified in the plasma. As seen in the present study, both KF treatments increased the plasma concentration of 5HIAA (a metabolite of 5HT) compared to the control. Elevated levels of 5HIAA are significant as they indicate increased serotonin production and turnover, which plays a crucial role in regulating mood and sleep-wake cycles. Previous *in vitro* digestion studies have shown gold KF increases the 5HT precursor 5HTP in the gastric digesta and green KF increases both in the gastric and intestinal digesta, suggesting that during gastrointestinal transit, more 5HTP are generated, which may be available for entry into

the circulatory system (246). Furthermore, consumption of green KF increased urinary excretion of 5HIAA, the primary metabolite of 5HT (8). These occurrences may be attributed to the nutrient richness of both KF. Firstly, KF is a fruit known to be rich in tryptophan and 5HT compared to other fruits (27, 37); thus, the additional tryptophan and 5HT provided by KF may be responsible for the observed increase in plasma 5HIAA levels. Secondly, KF is considered a good source of vitamin C (with gold KF having higher levels), which functions as a co-factor in the tryptophan metabolism pathway. Taken together, it is possible that both KF varieties affect 5HIAA levels through the supply of precursor metabolites and essential co-factors, therefore potentially leading to systemic concentration changes.

The hierarchical clustering results unveil intriguing patterns in how pigs respond to KF. In plasma, gold KF and bread were clustered together, while green KF clustered separately, implying the potential distinction due to the actinidin enzyme present in green KF, which may lead to different metabolic effects. Furthermore, clustering patterns varied across brain regions, indicating region-specific treatment effects. The clustering of KF treatments in the brain stem and prefrontal cortex suggests similar effects in these regions, potentially influenced by the connections between the prefrontal cortex and monoamine cell bodies in the brainstem (163). In contrast, the corpus striatum and hippocampus exhibited distinct gold and green KF clusters, respectively. These diverse clusters suggest variations in uptake, metabolism, or functional specificity, prompting further investigation into the detailed molecular and cellular mechanisms.

4.5.3 Strengths, limitations, and future studies

Strengths of the study included using shotgun metagenomics sequencing technology to improve coverage and multiple quality checks before sequencing to ensure adequate sample quantity and quality for analysis. Furthermore, the ANCOMBC analyses, specifically designed to analyse microbiome studies, offered insights into statistical significance without making distributional assumptions and included bias correction. Additionally, the pig model, closely mirroring human digestive and brain physiology, enabled the collection of specific gastrointestinal and brain regions that would otherwise remain unattainable.

Some limitations are noteworthy. It is essential to recognise that this investigation was part of a larger study conducted over seven days to assess the impact of KF on gluten digestion. This short duration may not induce measurable changes in brain chemistry, especially without stressors that could amplify the observed differences. Previous studies of the impact of KF on the gut microbiota have been up to eight weeks longer, which enhances the likelihood of efficiently monitoring their long-term effects on the microbiota. The colon digesta microbiota was analysed approximately eight days after KF was introduced. Concurrently, a single timepoint does not reflect the dynamic nature of colonic bacteria composition, which may change even more as time progresses. Therefore, adding the collection of faecal samples over the intervention period (for example, every two days) to the study would be advantageous since they can be collected repeatedly from the same animals during the feeding regimen.

In vitro evidence (246) suggests that the most significant metabolite increases due to KF are likely to occur in the digesta. In future studies, assessing the composition of

bioamines in the colon digesta would provide *in vivo* evidence of how changes in colonic digesta could translate to peripheral circulation and tissue. Furthermore, exploring the gene abundance of microbes in the colonic digesta and the expression of genes in brain tissue would provide valuable insights into the interaction dynamics.

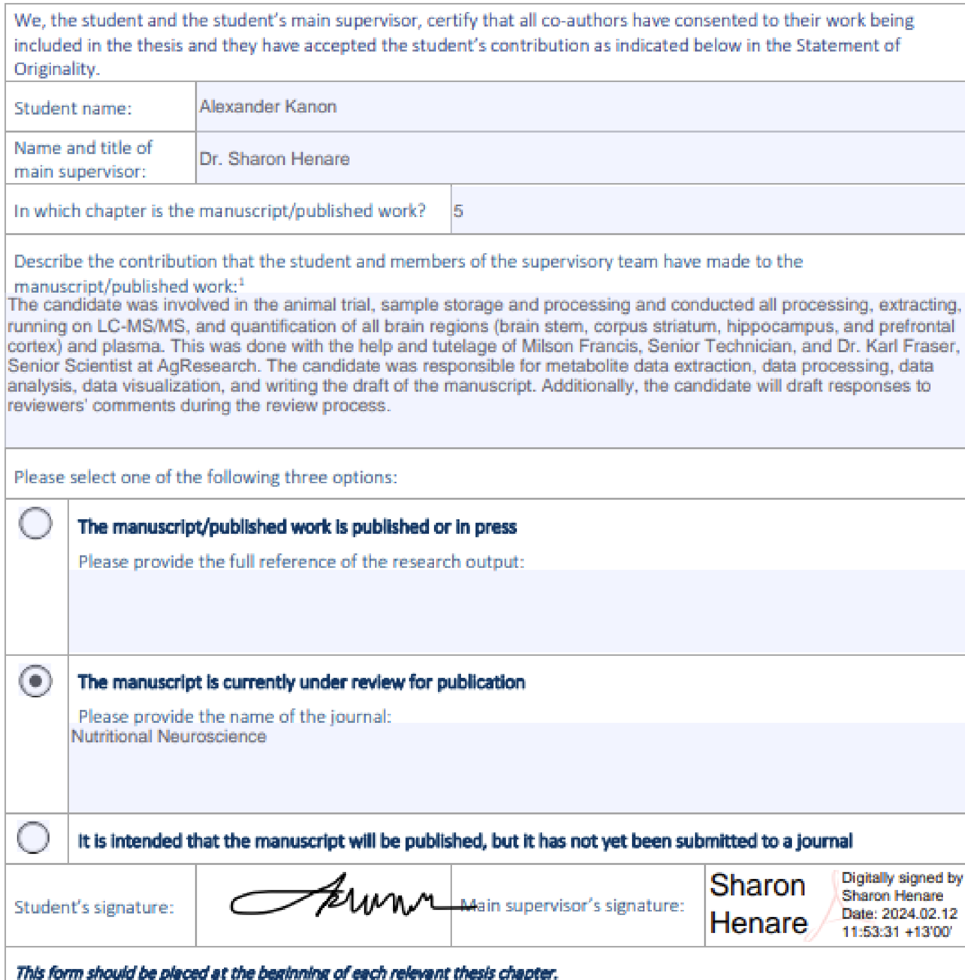
Additionally, this study involved healthy animals, and no external stressors or interventions were applied to induce a diseased state, which could have provided valuable insights into the potential of green or gold KF in mitigating intestinal-related issues. Gut dysbiosis is associated with increased neuroinflammation and neuronal loss (395, 396). To gain a more comprehensive understanding of the prebiotic and nootropic potential of KF, future research should consider incorporating models that induce gut dysbiosis to elucidate this better. Additionally, as highlighted, several studies have explored the prebiotic potential of KF in humans; however, none have explored the potential links with brain neurochemistry. Future research could utilise functional Magnetic Resonance Imaging methods in human studies to quantify brain metabolites.

4.6 Conclusions

In summary, the supplementation with green or gold KF may have inhibited potentially harmful pathogenic bacteria, as shown by their reduced relative abundance in colon digesta with KF treatments. This observation could be attributed to the increased relative abundance of butyrate-producing and flavonoid-degrading microbial genera. The results also suggest that KF has the potential to modulate the plasma concentration of 5HIAA, a metabolite derived from the tryptophan degradation pathway.

Statement of contribution

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS



We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Alexander Kanon
Name and title of main supervisor:	Dr. Sharon Henare
In which chapter is the manuscript/published work?	5

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

The candidate was involved in the animal trial, sample storage and processing and conducted all processing, extracting, running on LC-MS/MS, and quantification of all brain regions (brain stem, corpus striatum, hippocampus, and prefrontal cortex) and plasma. This was done with the help and tutelage of Milson Francis, Senior Technician, and Dr. Karl Fraser, Senior Scientist at AgResearch. The candidate was responsible for metabolite data extraction, data processing, data analysis, data visualization, and writing the draft of the manuscript. Additionally, the candidate will draft responses to reviewers' comments during the review process.

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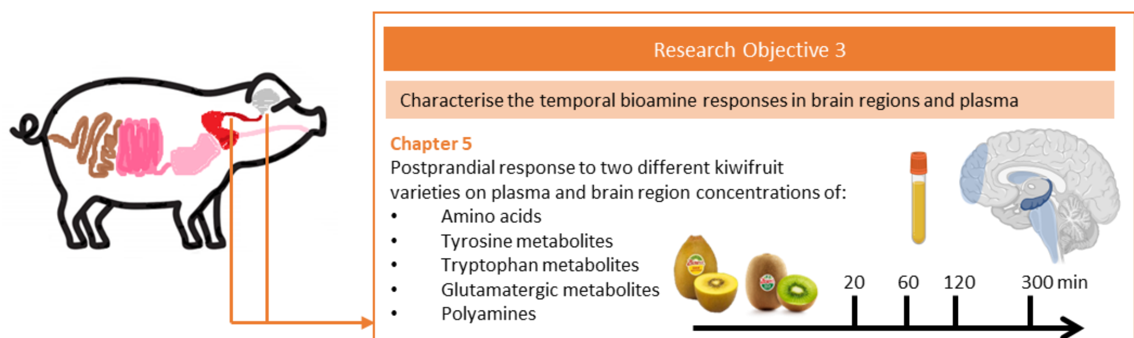
1 Doctoral Research Committee May 2023

CHAPTER 5. Temporal effects of high compared to low actinidin-containing kiwifruit (*Actinidia*) on postprandial biogenic amine concentrations in peripheral plasma and brain regions of a pig model[3]

Prologue

Clinical evidence suggests that consuming green KF positively affects mood and sleep quality. It also influences bioaminergic responses, increasing dopamine and 5HT *precursor concentrations in gastrointestinal digests. Fermentation of these digests further elevates bioamine levels like L-DOPA and GABA, crucial for brain and body signalling. Despite these findings,* in vivo *studies examining KF's effects on neurochemical modulation are lacking. Samples collected for this study were obtained from the study described in chapter 3. The pig model allows sampling of various brain regions (brain stem, corpus striatum, hippocampus, and prefrontal cortex) to quantify bioamines, which human studies cannot achieve. The aim of this study was to assess the impact of actinidin-containing KF on postprandial peripheral plasma and brain neurotransmitter concentrations using a pig model.*



[3] This chapter has been submitted to Nutritional Neuroscience:
Kanon, A. P., Giezenaar, C., Roy, N. C., Jayawardana, I. A., Montoya, C. A., Fraser, K., McNabb, W. C., & Henare, S. J. (Nutritional Neuroscience) The temporal bioaminergic effects of actinidin in kiwifruit (*Actinidia deliciosa* cv. Hayward) on plasma and brain regions in growing pigs as an adult human model.

Highlights

- *The study demonstrates the differential effects of green and gold KF on bioaminergic metabolite concentrations in plasma and specific brain regions of a growing pig model.*
- *Green KF supplementation compared to gold KF led to an earlier increase in plasma histidine concentrations.*
- *Brain stem shows the least interaction or treatment effects, suggesting it is less susceptible to modulation by the KF supplementation.*
- *Corpus striatum exhibits the most interaction and treatment effects, indicating a higher degree of metabolite manipulation in response to KF supplementation.*
- *Both the hippocampus and prefrontal cortex show similar capacity for modulation by KF-based diets.*
- *Gamma-amino butyric acid emerges as a metabolite with interaction effects in all brain regions, increasing with green KF and decreasing with gold KF within the first two hours*.

The chapter is presented in a manuscript format but has not been published.

5.1 Abstract

This study investigated whether the bioamine modulation observed in in vitro digestion and fermentation studies with green KF translates to changes in the brain. This study investigated the temporal and differential effects on bioamine concentrations in plasma and brain regions of growing pigs fed KF containing the enzyme actinidin, a protease found in green KF (*Actinidia deliciosa* cv. 'Hayward') and a gold KF without this enzyme (*A. chinensis* cv. 'Hort16A'). Entire male pigs (n=48, starting bodyweight 21.2 ± 2.1 kg) were fed whole wheat bread either with green KF (+ actinidin) or gold KF (- actinidin). The pigs were euthanised at 20 min, 1, 2, and 5 hours postprandially (n=6 for each time point). Jugular plasma and brain tissues (brain stem, corpus striatum, hippocampus, and prefrontal cortex) were collected to determine bioamine concentration using a liquid-chromatography mass-spectrometry. After green KF intake, peripheral plasma histidine rose greater than with gold KF. Most metabolite concentrations in all regions increased over time ($P<0.05$), peaking two hours after green KF intake, while adding gold KF decreased metabolite concentrations during the same period before increasing to similar levels of green. Gamma-amino butyric acid (GABA) concentrations increased in all regions in response to green KF over time but decreased with gold KF. Green KF led to sustained serotonin concentrations in the hippocampus over time, while gold KF resulted in an initial increase followed by a decrease after two hours. This study is the first to report that brain bioamine concentrations increase more after green KF than gold KF supplementation, providing the first evidence that actinidin in green KF has bioaminergic modulation potential *in vivo*.

Keywords: kiwifruit, actinidin, neurochemicals, biogenic amines, gamma-amino butyric acid, serotonin, brain regions, pig

5.2 Introduction

Clinical evidence showed that consuming green KF may have a positive neurobiological impact. Consumption of gold KF was reported to increase mood (3, 4), enhance sleep (5) and improve cognition (117). Potential mechanisms for this mediated response could occur by supplying nutrients from the KF. Macronutrients such as fibre from KF have been purported to aid in digestive health (87) and the growth of beneficial gut bacteria (102). The vitamin C and 5HT contents are postulated to be the reasons for improved mood and sleep (3, 5). Green KF (*Actinidia deliciosa* cv. 'Hayward') contains actinidin, a protease enzyme specific to the *Actinidia* species, which is also reported to affect digestive health. In *in vitro* (97) and *in vivo* studies (96), actinidin containing KF enhanced protein digestion, which causes an earlier release of amino acids (AAs) in the gut contents of rats (90), pigs (95) and into the peripheral bloodstream in humans (82) compared to non-actinidin containing KF.

Green KF has also been shown to modulate bioaminergic responses in *in vitro* systems, causing increased concentration at the gastric and intestinal stages of the dopamine precursor L-DOPA and the 5HT precursor 5HTP in the gastrointestinal digests (246). Furthermore, upon fermentation of digested green KF with human faecal bacteria for 18 h, there were increases in the concentration of L-DOPA and GABA compared to gold KF treatment and controls (inulin or water) (246). These bioamines are essential for signalling in the brain and the rest of the body, and imbalances are linked to various neurological disorders (126). However, *in vivo* studies exploring the effects of KF on the modulation of neurochemicals peripherally and in the brain have yet to be conducted.

The pig has been used as a model for digestion in humans (330). Using the pig model allows sampling of various brain regions to quantify bioamines, which human studies cannot explore. Here, the effects of actinidin-containing KF on the temporal postprandial response of metabolites in plasma and the prefrontal cortex, hippocampus, corpus striatum and brain stem were determined by analyses of brain samples of growing pigs fed bread supplemented with two KFs, either actinidin-containing green KF 'Hayward' or gold 'Hort16A' KF which does not contain actinidin. It was hypothesised that green and gold KF, with differential actinidin activity, would lead to differential bioaminergic responses in plasma and brain regions. Therefore, the primary aim of this study was to assess actinidin impact on postprandial peripheral plasma and brain neurotransmitter concentrations using a pig model. The current study is a secondary analysis of a study focusing on the digestion and release of immunogenic gluten peptides (300).

5.3 Materials and methods

The pigs used in this study were the same as those described in Chapter 3. Below are details of additional analyses relevant to Chapter 5.

5.3.1 Animals, housing, and treatments

Animal housing and treatment were described in Chapter 3 (Section 3.3.1). A total of 48 entire male pigs (21.2 ± 2.1 kg, average ± SE) of nine weeks of age were used in this study. On the morning of the ninth day, pigs were fed a single meal and euthanised at either 20 min, 1 h, 2 h, or 5 h postprandial to mimic the postprandial meal response. There were six animals in each postprandial time and treatment.

5.3.2 Experimental diets

In the study, two experimental diets were used: 1) green KF (*Actinidia deliciosa* cv. 'Hayward') + soda bread (treatment) and 2) gold KF (*Actinidia chinensis* cv. "Hort16A") + soda bread (control). The "Hort16A" variety of gold KF was used as the control as the variety lacks actinidin. Still, it contains other compounds in green KF, particularly dietary fibre, which may influence gastrointestinal transit time and digestion. The last meal consisted of four slices of soda bread with two KF (either green or gold) (Table 5.1). For the main study, titanium dioxide was added to the diets to track the flow of the last meal through the GIT.

Table 5.1 Nutrient and chemical composition of final meal pigs given.

	Final meal (g dry matter)	
	Bread + Gold KF	Bread + Green KF
Meal composition		
Gold KF	27.20	-
Green KF	-	25.60
Bread	178.10	178.10
Titanium dioxide	1.02	1.01
Nutrient composition		
Energy (kJ)	3677	3677
Carbohydrates		
– Dietary Fibre	14.60	18.13
– Starch	105.09	104.01
Protein and amino acids		
– Protein, total	22.20	22.76
– Threonine	0.66	0.69
– Valine	1.12	1.14
– Methionine	0.52	0.48
– Isoleucine	0.77	0.79
– Leucine	1.51	1.53
– Tyrosine	0.88	0.85
– Phenylalanine	1.26	1.27
– Histidine	0.63	0.67
– Lysine	0.71	0.71
Fat		
– Fats, total	11.09	11.28
Micronutrients		
– Vitamin C	4.57	1.54
– Vitamin E	0.14	0.43
– Vitamin B_3	0.21	0.21
– Vitamin B_6	0.09	0.08
– Vitamin B_9	0.21	0.21
– Total polyphenols*	0.28	0.43

*Gallic acid equivalence

5.3.3 Sampling day collection

The plasma and brain tissue collection were conducted as described in Chapter 3 (Section 3.3.2). Samples were snap-frozen in liquid nitrogen and stored at -80°C for analysis. All brain tissue was collected within 15 minutes of euthanasia. An overview of the study allocation is presented in Figure 5.1

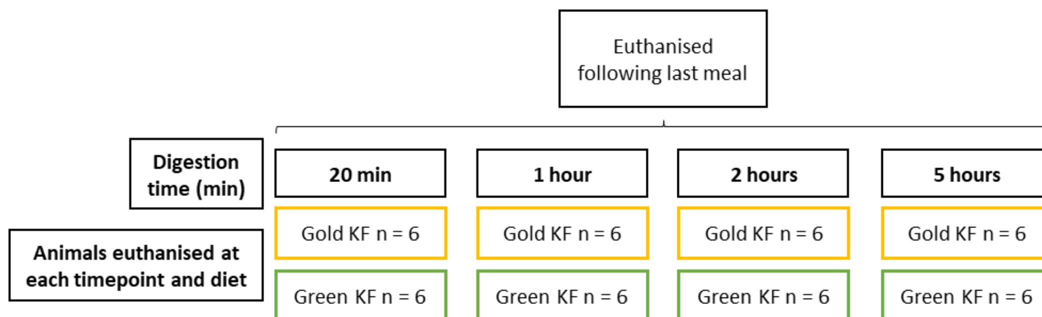


Figure 5.1 Overview of study group allocation.

5.3.3.1 Sample extraction and preparation

Sample extraction and preparation was conducted as described in Chapter 4 (Section 4.3.4).

5.3.4 Bioamine analysis

Bioamine analysis was determined using LCMS/MS as described in Chapter 4 (Section 4.3.4).

5.3.5 Data and statistical analysis

5.3.5.1 Calculations

The sum of AAs was calculated as described in Chapter 4 (Section 4.3.5.1)

5.3.5.2 Data analysis

The data were analysed using the statistical software SAS (SAS/STAT version 9.4). A polynomial regression analysis (up to the cubic order) was first conducted on each factor

(i.e., treatment and time) and their interaction. Only significant ($P < 0.05$) terms were kept in the final polynomial model, which was selected by comparing full models with reduced models (i.e., removing predictors that did not affect the response variable) by using the log-likelihood ratio test. Using the log-likelihood ratio test, the selected polynomial model was then compared with a selected model with time as a categorical variable. Time as a numerical variable for all response variables gave the best model fit. The model diagnostics (e.g., normal distribution) for each response variable were tested using the ODS Graphics procedure and the repeated statement of SAS.

5.4 Results

5.4.1 Plasma metabolites

Of the 44 metabolites, 16 were below the limit of detection. Therefore, 28 metabolites in the plasma were quantifiable (APPENDIX D - Table D.1). Six of these metabolites showed a significant interaction between time and type of KF. These metabolites included the AAs, histidine, valine, lysine and isoleucine, neuroactive AA glycine, dipeptide antioxidant anserine and the calculated sum of BCAA (Figure 5.2). The interaction responses of different metabolites varied, indicating diverse trends. Histidine exhibited the most distinct trend (Figure 5.2A); within the first two hours, the increase was 105.6 nM/min KF for pigs fed the green KF and only 37.6 nM/min for those fed the gold KF. However, after five hours, both KF treatments resulted in similar concentrations of histidine. Both valine (Figure 5.2B) and glycine (Figure 5.2E) concentrations showed significant opposite linear trends, with valine increasing and glycine decreasing concentrations over time with green KF supplementation. Isoleucine (Figure 5.2D) and BCAA (Figure 5.2G) concentrations were similar between KF

treatments during the first two hours but were higher at five hours with the green KF diet compared to the gold KF diet. Lysine concentration decreased over time (Figure 5.2C), while anserine concentrations varied with each KF treatment (Figure 5.2F). The concentration of asparagine and 5HIAA exhibited significant main effects of KF treatment. Asparagine concentrations decreased over time for both KF treatments, with the pigs fed the green KF showing higher concentrations than those fed the gold KF. In contrast, 5HIAA levels increased during the first two hours, peaked, and then decreased, with higher concentrations observed after gold KF compared to green KF throughout the 5-hour postprandial period.

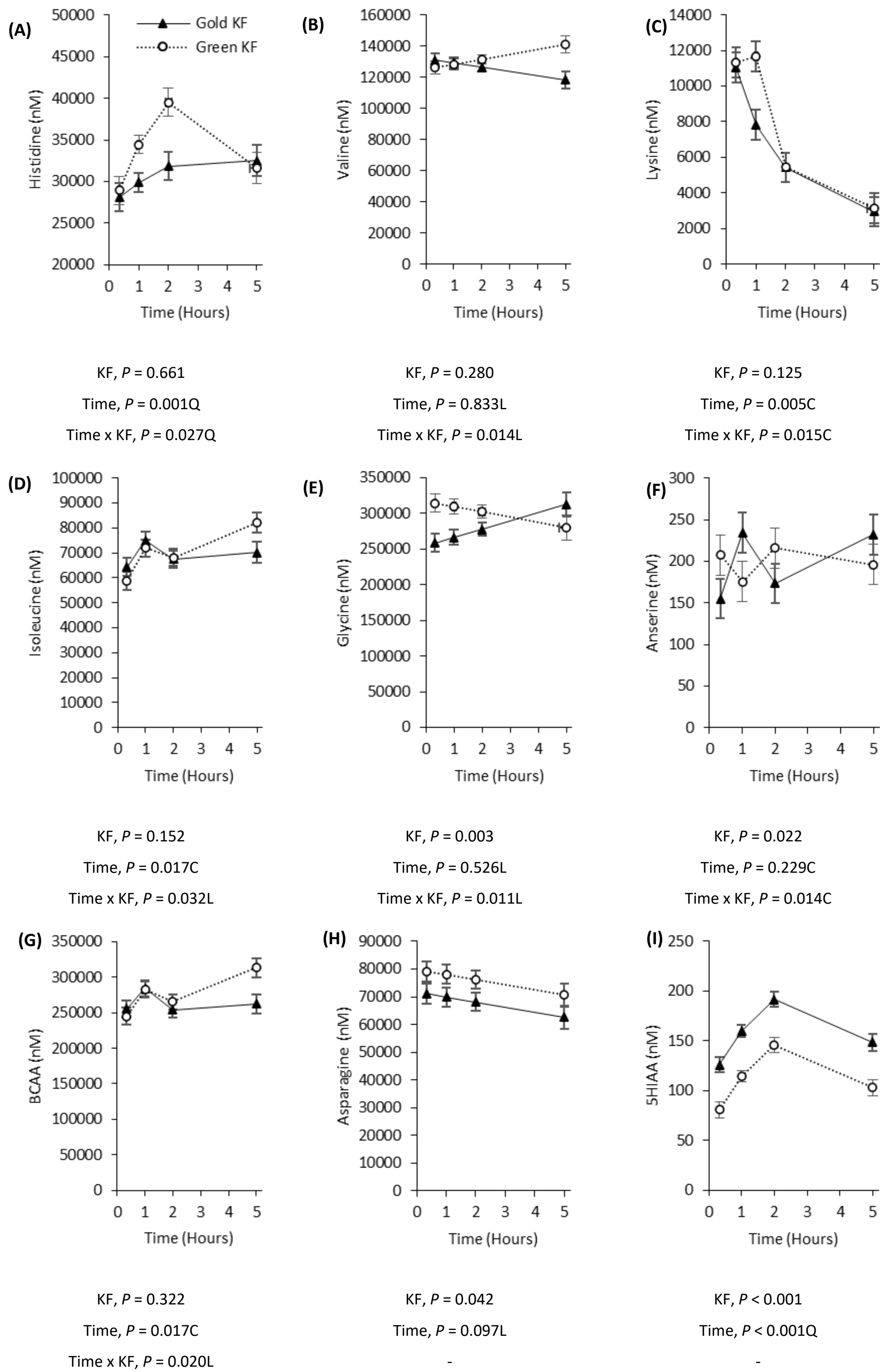


Figure 5.2 Postprandial plasma concentrations of histidine (A), valine (B), lysine (C), isoleucine (D), glycine (E), anserine (F), Branched Chain Amino Acids (BCAA) (G), asparagine (H) and 5HIAA (I) in response to gold or green KF in growing pigs. Values are means ± SEMs. [1]L, Q or C, linear, quadratic, or cubic effect for the time factor, respectively.

5.4.2 Brain regions

Generally, regardless of treatment, metabolite concentrations increased over sampling time (20 min - 5 hours) in all brain regions examined, including the brain stem, corpus striatum, hippocampus, and prefrontal cortex. Multiple metabolites showed significant interactions (P<0.05) between KF type and sampling time, as discussed below per brain region (APPENDIX D). Only metabolites with a treatment or treatment time interaction are discussed for each brain region studied.

In general, it was observed that green KF supplementation led to an increase in brain metabolite concentrations within the first two hours, while gold KF supplementation caused a decrease during the same period. Both treatments reached similar metabolite levels at the five-hour mark. Adding green KF to the diets consistently resulted in higher metabolite concentrations at the two-hour mark for most metabolites measured compared to gold KF-supplemented diets. Figure 5.3 presents the proportional changes in GABA concentrations in measured brain regions in response to the two KF treatments. The intensity of the colour corresponds to the relative concentration, with brighter reds showing higher concentrations and darker greens indicating lower concentrations. For example, GABA concentrations in the brain stem, show the levels in the green KF group increase steadily over time, transitioning from darker green to lighter green and finally to red. In contrast, the gold KF group starts at a much higher concentration but then decreases (from dark red to lighter red and dark green) before finally returning to darker red.

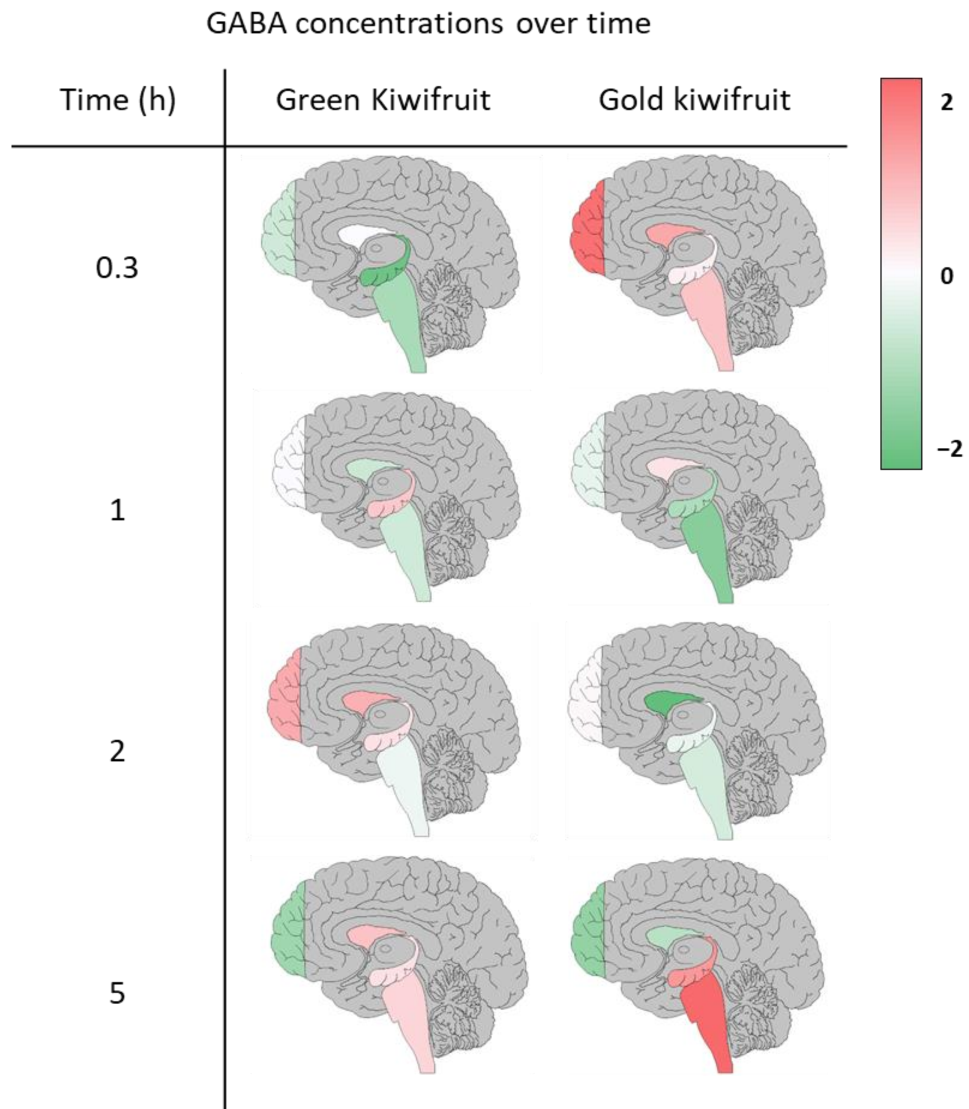


Figure 5.3 Proportional changes in γ-Aminobutyric Acid (GABA) across time in brain regions in response to gold or green KF in growing pigs. Red and green hues indicate higher and lower concentrations, respectively (scaled by region).

5.4.2.1 Brain stem

Of the 44 targeted metabolites, 17 metabolites in the brain stem were below the detection limit. Twenty-eight of the 45 metabolites in the brain stem were quantifiable (APPENDIX D - Table D.2). Among these, three metabolites, 5HIAA, GABA, and L-DOPA, showed a significant interaction between time and KF type. Generally, after gold KF, 5HIAA and GABA concentrations (Figure 5.4A and B) decreased within the first two hours. However, at five hours postprandially, the concentration of 5HIAA was similar

between gold KF and green KF supplementations, while the concentration of GABA was higher after consuming gold KF compared to green KF-supplemented diets.

In contrast, the green KF-supplemented diet steadily increased 5HIAA, and GABA concentrations in the brain stem over the five-hour postprandial period. Compared to the response observed for 5HIAA and GABA, the concentration of L-DOPA exhibited an opposite trend (Figure 5.4C). After consuming gold KF, the concentration of L-DOPA increased within the first two hours, while for green KF, the concentration decreased during the same period. However, at five hours postprandially, the concentrations of L-DOPA were similar between gold KF and green KF-supplemented diets.

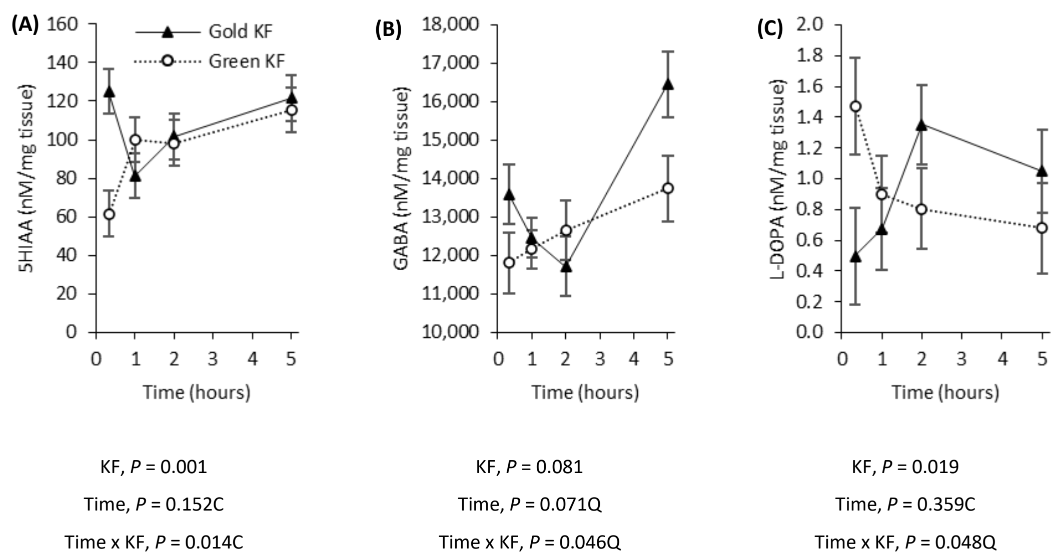


Figure 5.4 Postprandial brain stem concentrations of 5HIAA (A), γ-aminobutyric acid (GABA) (B) and L-dihydroxyphenylalanine (L-DOPA) (C) in response to gold or green KF in growing pigs. Values are means ± SEMs. [1]Q or C, quadratic or cubic effect for the time factor, respectively.

5.4.2.2 Corpus striatum

Of the 44 targeted metabolites, 11 metabolites in the corpus striatum were below the detection limit. Thirty-four of the 45 metabolites were quantifiable (APPENDIX D - Table D.3). Of these, 19 metabolites displayed significant ($P<0.05$) interaction effects between time and type of KF. These included three tyrosine metabolites (octopamine, tyrosine and phenylalanine), three tryptophan metabolites (5HTP, 5HT and tryptophan), one GABA metabolite (glycine), three polyamines (spermine, putrescine and agmatine), ten AAs (histidine, valine, lysine, proline, methionine, tyrosine, phenylalanine, tryptophan, and glycine), citrulline, ethanolamine and the calculated sums of EAA and LNAA. Two metabolites, L-DOPA, and GABA trended ($P<0.055$) towards significant interaction effects between time and type of KF.

The following metabolites displayed similar patterns in the corpus striatum: histamine, octopamine, tyrosine, phenylalanine, tryptophan, valine, proline, methionine, ethanolamine, EAA, and LNAA. Within two hours, there was an increased concentration ($P<0.05$,Figure 5.5) of these metabolites. In contrast, the pigs fed the gold KF diet had a decreasing concentration, reaching its lowest concentration within two hours. However, the trend shifted after two hours, with the pigs fed the green KF diet appearing to slow the rate of increase based on visual inspection of the slope, while gold KF showed a greater increase. Similar concentrations were obtained after five hours. The histidine response to gold KF supplementation was consistent with other AAs. Citrulline concentration increased over the five hours in response to green KF treatment, while it decreased over the same duration for gold KF treatment (Figure 5.5D).

Unlike other tyrosine pathway metabolites (tyrosine and phenylalanine), L-DOPA showed a unique concentration response in the corpus striatum (Figure 5.6A). Both green KF and gold KF interventions did not affect concentration over time. However, their responses during the first hour differed. L-DOPA concentration decreased initially in response to green KF before subsequently rising. Conversely, L-DOPA increased in the first hour after gold KF, followed by a subsequent decrease and rise.

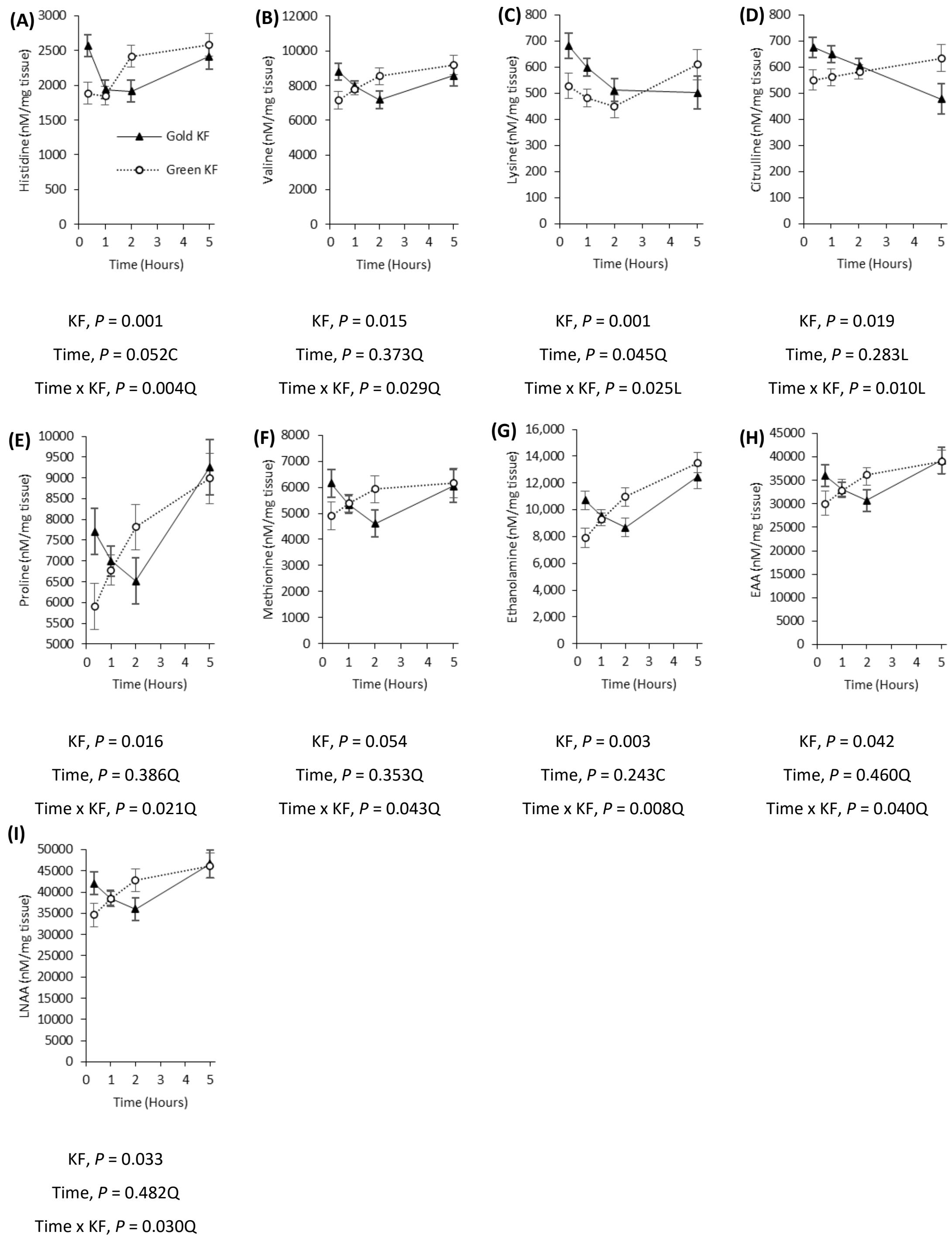


Figure 5.5 Postprandial corpus striatum concentrations of histidine (A), valine (B), lysine (C), citrulline (D), proline (E), methionine (F), ethanolamine (G), Essential Amino Acids (EAA) (H) and Large Neutral Amino Acids (LNAA) (I) in response to gold or green KF in growing pigs. Values are means ± SEMs. [1]Q or C, quadratic or cubic effect for the time factor, respectively.

The responses of 5HT (Figure 5.6F) and 5HTP (Figure 5.6G) differed from their precursor AA, tryptophan (Figure 5.6E), in the corpus striatum. The addition of green KF in the diet decreased 5HT concentration over time, while concentrations of 5HT increased after gold KF, peaking at two hours. However, the 5HT concentration rapidly decreased in the following hours, reaching levels similar to the green KF response after five hours. 5HTP concentrations remained consistent over the five hours following green KF ingestion, whereas the concentrations consistently increased after gold KF consumption. Concentrations of both GABA (Figure 5.6H) and glycine (Figure 5.6I) increased over time in response to green KF supplementation. The addition of gold KF to the diet exhibited contrasting effects on these metabolites, initially reducing GABA concentration in the first two hours before it began to rise while glycine concentration remained constant over the five hours.

Three polyamines, namely agmatine (Figure 5.6J), putrescine (Figure 5.6K), and spermidine (Figure 5.6L), displayed distinct concentration responses in the corpus striatum. Green KF consumption gradually decreased agmatine concentration over time, reaching its lowest point after five hours. In contrast, putrescine concentration showed an upward trend ($P=0.048$) following ingestion of green KF. It is noteworthy that gold KF consumption had contrasting effects on these two polyamines compared to green KF consumption. Spermidine exhibited a delayed increasing concentration trend, like putrescine, where the concentration decreased initially for one hour before rising. On the other hand, gold KF consumption showed an initial increase in concentration within the first hour, followed by a subsequent decrease.

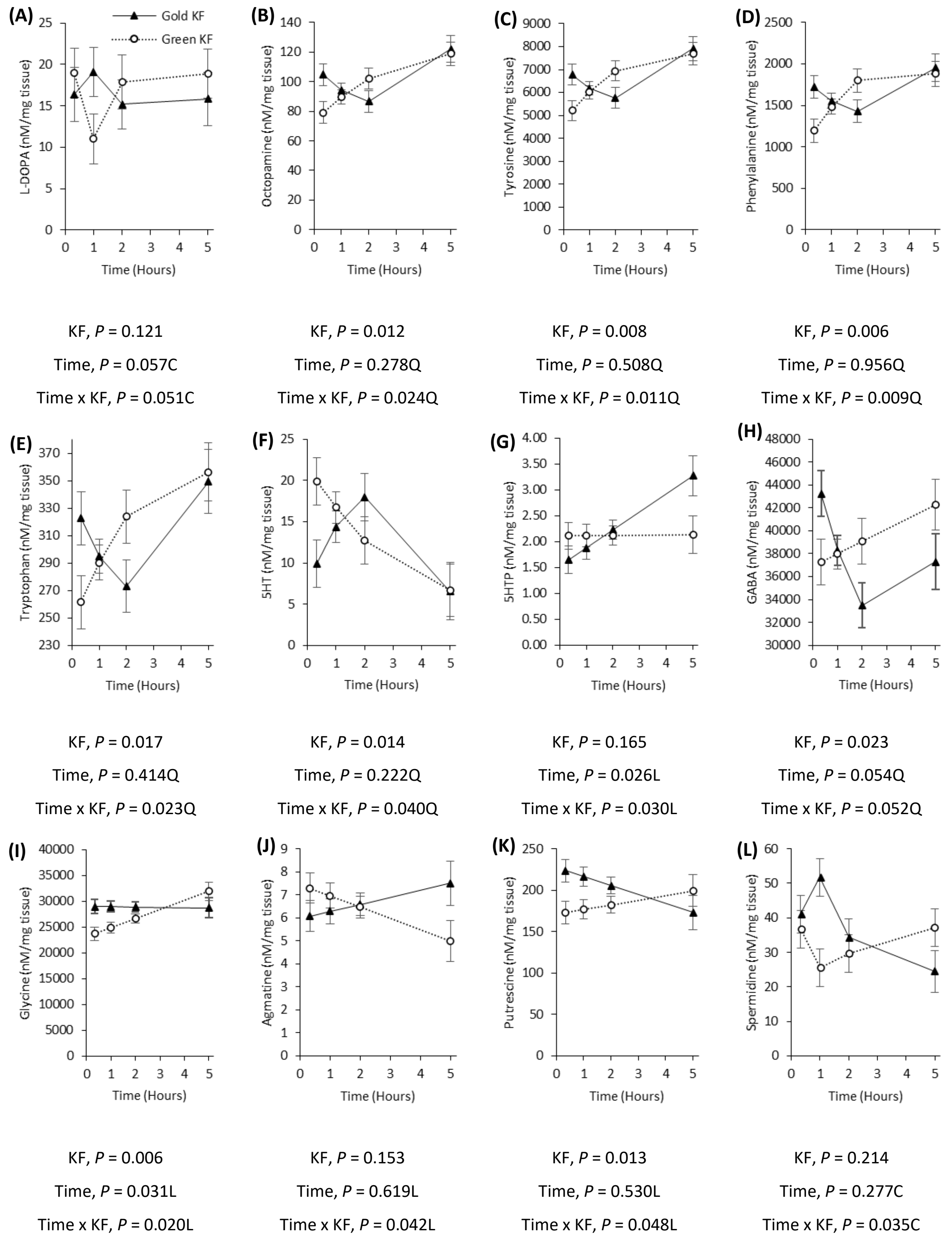


Figure 5.6 Postprandial corpus striatum concentrations of L-DOPA (A), octopamine (B), tyrosine (C), phenylalanine (D) tryptophan (E), Serotonin (5HT) (F), 5-Hydroxytryptophan (5HTP) (G), γ-aminobutyric acid (GABA) (H), glycine (I), agmatine (J), putrescine (K) and spermidine (L) in response to gold or green KF in growing pigs. Values are means ± SEMs. [1]L, Q or C, linear, quadratic, or cubic effect for the time factor, respectively.

5.4.2.3 Hippocampus

Of the 44 targeted metabolites, 14 metabolites in the hippocampus were below the detection limit. Therefore, 31 of the 45 metabolites in the hippocampus were quantifiable (APPENDIX D - Table D.4). Five metabolites had significant interactions ($P<0.05$) between time and type of KF, and two metabolites trended toward significant interactions ($P<0.1$) (Figure 5.7). These metabolites included two tryptophan metabolites (tryptophan and 5HT; Figure 5.7A and B), GABA (Figure 5.7C), phenylalanine (Figure 5.7D), and three AA; proline, leucine and methionine (Figure 5.7E, F and G). Overall, green KF showed a rapid and increasing trend until concentrations in the hippocampus peaked at two hours. Contrastingly, after gold KF supplementation, concentrations decreased slowly and reached their lowest levels after two hours. The concentration for most metabolites was lower five hours after green KF consumption than gold KF consumption. The calculated values for BCAA, EAA and LNAA (Figure 5.7H, I and J) also showed significant interaction effects of time by type of KF. They followed similar trends to those described above. In contrast to other metabolites, the concentration of 5HT increased and peaked at two hours in response to adding gold KF to the diet, then decreased for the remaining period. At the same time, green KF supplementation resulted in a sustained release over the five hours.

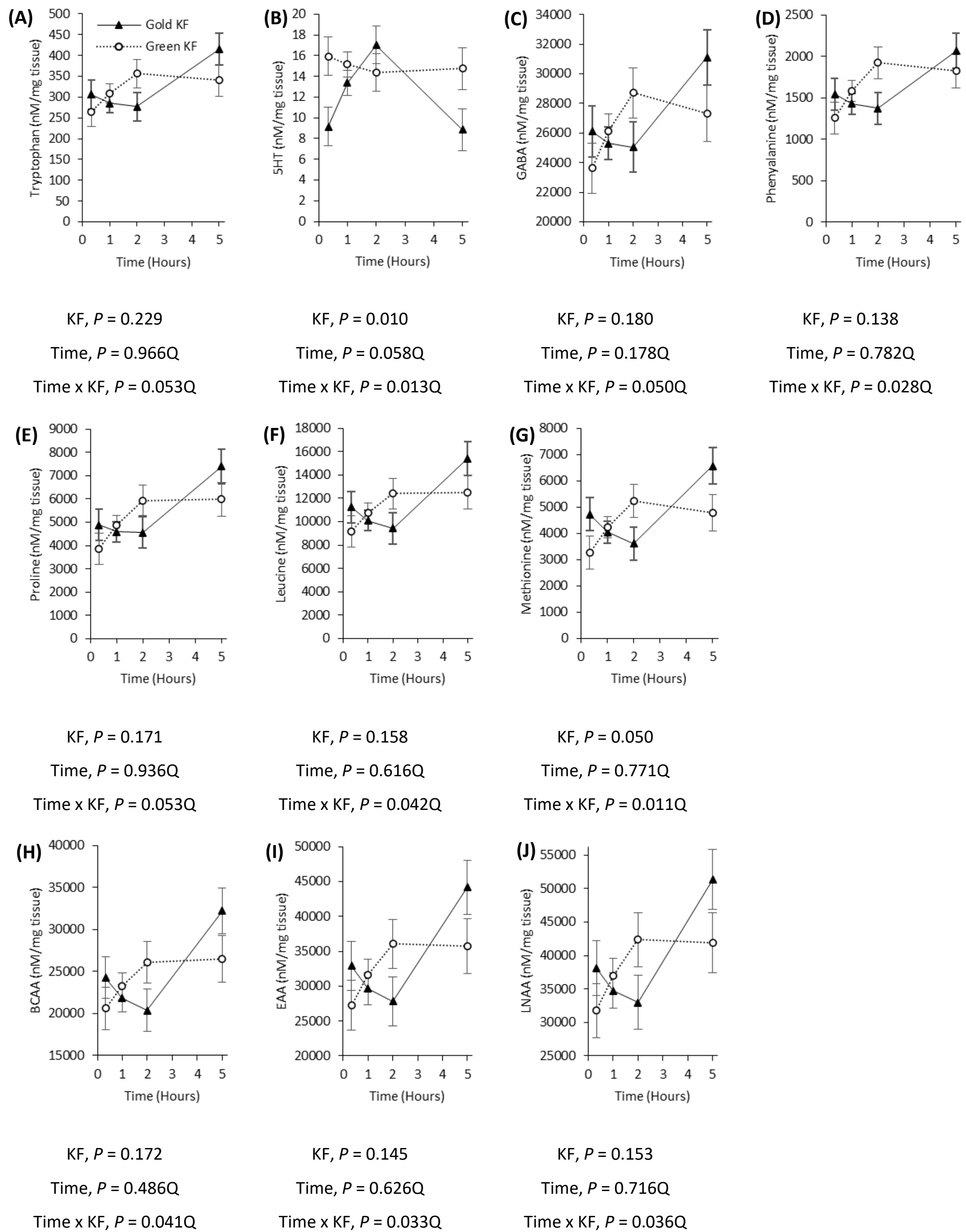


Figure 5.7 Postprandial hippocampus concentrations of tryptophan (A), Serotonin (5HT) (B), γ-aminobutyric acid (GABA) (C), phenylalanine (D, proline (E), leucine (F), methionine (G), BCAA (H), EAA (I) and LNAA (J) in response to gold or green KF in growing pigs. Values are means ± SEMs. [1]Q or C, quadratic or cubic effect for the time factor, respectively.

5.4.2.4 Prefrontal Cortex

Of the 44 targeted metabolites, 14 metabolites in the prefrontal cortex were below the detection limit. Therefore, 31 of the 45 targeted metabolites in the prefrontal cortex were quantifiable (APPENDIX D - Table D.5). Eight had significant interactions between time and KF type (Figure 5.8). These metabolites included 5HIAA (Figure 5.8A), GABA (Figure 5.8B), tyrosine metabolites (phenylalanine; Figure 5.8C)), two polyamines (N-acetylputrescine and agmatine; Figure 5.8D and H respectively) and three AAs; valine (Figure 5.8E), leucine (Figure 5.8F) and proline (Figure 5.8G). Like the hippocampal region, a rapid upward trend for metabolite concentrations in the prefrontal cortex was observed after green KF consumption during the initial two hours. In contrast, the concentration levels of metabolites in pigs fed the gold KF-supplemented diet decreased gradually and reached a nadir at two hours. After five hours, the pigs fed the green KF-supplemented diet had decreased levels and comparable levels to those fed the gold KF-supplemented diet. The calculated values for BCAA, EAA, and LNAA concentrations (Figure 5.8I, J and K) also showed significant interaction effects of time by KF type and followed similar patterns.

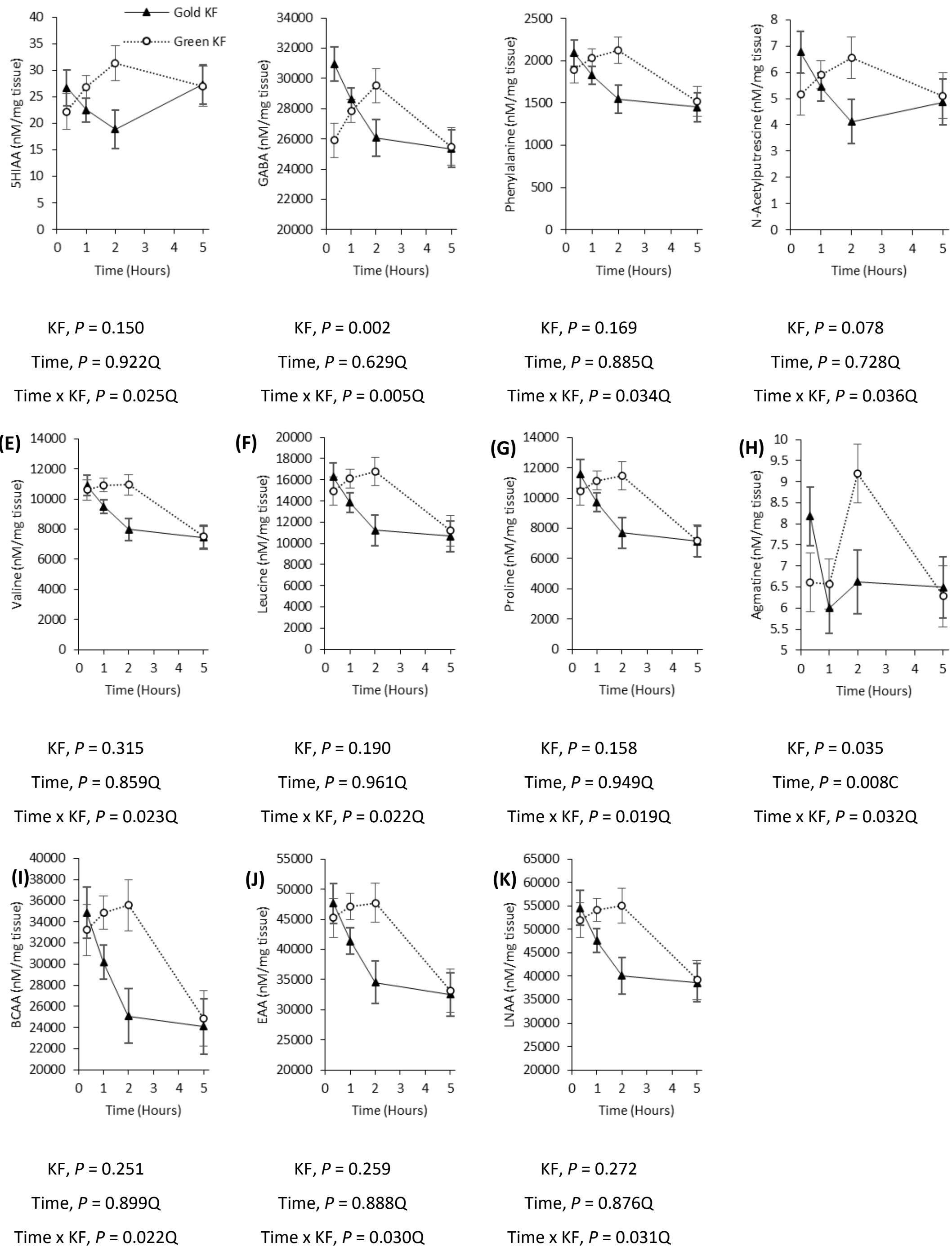


Figure 5.8 Postprandial prefrontal cortex concentrations of 5HIAA (A), γ-aminobutyric acid (GABA) (B), Phenylalanine (C), N-acetylputrescine (D), valine (E), leucine (F), proline (G), agmatine (H), Branched Chain Amino Acids (BCAA) (I), Essential Amino Acids (EAA) (J) and Large Neutral Amino Acids (LNAA) (K) in response to gold or green KF in growing pigs. Values are means ± SEMs. [1]Q or C, quadratic or cubic effect for the time factor, respectively.

5.5 Discussion

The study showed the differential effects of green and gold KF-supplemented diets on modulating bioaminergic metabolite concentrations in plasma and specific brain regions of a growing pig model. Notably, green KF supplementation led to increased plasma metabolite concentrations. Furthermore, the results clearly distinguished brain region responses between green and gold KF treatments. The most consistent trend observed was that gold KF resulted in a declining concentration of metabolites before eventually rising, while green KF induced a rapid increase in metabolite concentrations.

The brain stem had the least interaction or treatment effects among the different brain regions examined, suggesting it was the least susceptible brain region to modulation by the type of KF. At the same time, the corpus striatum had the most interaction and treatment effects of KF, which suggested it has a higher degree of metabolite modulation in response to KF supplementation. Based on treatment and interactions, the hippocampus and prefrontal cortex exhibit a similar capacity for modulation by KF type.

Additionally, GABA emerges as a metabolite with interaction effects in all brain regions, showing an increase in concentration with the green KF-supplemented diet and a decrease in gold KF within the first two hours. Furthermore, 5HT concentrations were sustained over time in the hippocampus after the green KF-supplemented diet but not with the gold KF-supplemented diet.

5.5.1 Plasma metabolites

Supplementation with green KF resulted in heightened plasma metabolite concentrations. This differential response may be attributed to actinidin in green KF,

which potentially enhances the digestive process, leading to a faster availability of nutrients peripherally and to the brain. The driving force for AA absorption is the concentration gradient established by the digestion of dietary proteins and active transport. As shown from previous studies of pigs, the administration of green KF (which contains actinidin) increased rates of digestion and release of AA in the digesta of the small intestine (90, 301), potentially contributing to an increase in the concentration gradient and absorption, and ultimately leading to higher concentrations in plasma (95).

In a study of elderly adults consuming beef mince with either green KF or gold KF ('Hort16A'), green KF resulted in earlier and greater increases in peripheral plasma AA (82). Higher plasma concentrations of leucine, BCAA, and EAA were observed at 40 and 60 minutes after ingesting beef mince with green KF compared to beef mince with gold KF ('Hort16A'). Additionally, with green KF, the rate of appearance of phenylalanine was greater at 60- and 120-min post-consumption compared to gold KF. However, there was no difference between the green KF and gold KF treatments on the total appearance of AA (82).

The plasma BCAA results in this study did not reflect what has been observed in elderly adults, where an early increase is seen. This discrepancy could be attributed to actinidin's differential effects on the different protein sources (i.e., beef in an elderly study and gluten in this study), which has been highlighted in a rat study (90). Furthermore, other factors such as the amount of protein consumed, the AA profile of each protein source, the type of food and other nutrients in the experimental meal could all potentially contribute to what is observed.

5.5.2 Brain region metabolites

Despite a similar trend in AAs in plasma between the two KF treatments, several AAs and neurotransmitters exhibit vastly different metabolite responses in the brain depending on the actinidin content of KF consumed. As discussed previously, the actinidin in green KF may explain this beyond its role in digesting proteins in the gastrointestinal tract. It is plausible that actinidin is transported in the bloodstream. While this study did not confirm it, other research has shown that fruit-derived cysteine proteases like bromelain (found in pineapples) can appear in the blood plasma within an hour of ingestion (397) and does retain its proteolytic activity (398), which could further aid AA metabolism or processing in peripheral tissues. The higher levels of polyphenols and vitamin E in green KF may contribute to the observed differences between the two treatments. These compounds help improve blood-brain barrier integrity and reduce inflammation (399), thus protecting the brain. For example, in the same study, we observed that actinidin-containing green KF enhances antioxidant protective potential in brain regions (400). Future studies may aim to characterise the effects of KF on blood-brain barrier models to clarify these mechanisms.

The corpus striatum exhibits the most significant metabolite concentration shifts compared to other brain regions in response to the KF treatments. These findings likely stem from the striatum's densely populated neuronal projections (401) and complex neurotransmitter networks (402), which contributes to its heightened sensitivity and metabolic plasticity (403). This may explain the pronounced metabolite concentration shifts. Figure 5.9 highlights directional changes in metabolite concentrations in the corpus striatum within the first two hours following KF treatment, suggesting differential and opposing effects of KF on catecholamine and GABAergic signalling

pathways. Specifically, green KF stimulates synthesis pathways, while gold KF appears to inhibit them, affecting neurotransmitter availability over time. As highlighted, GABA levels diverge, increasing with green KF and decreasing with gold KF. Tryptophan and 5HTP levels rise with green KF but either decrease or remain unchanged with gold KF. Similarly, metabolites involved in catecholamine synthesis (phenylalanine and tyrosine) increase with green KF and decrease with gold KF.

Green KF consumption increased GABA concentrations in all brain regions over time, while gold KF reduced them in the brain stem and hippocampus, except at five hours postprandial (Figure 5.3). GABA, a key inhibitory neurotransmitter, regulates neuronal activity and promotes relaxation (404). Therapeutics targeting GABAergic inhibition aim to balance excitatory and inhibitory neurotransmission (405). How KF nutrients induce this acute effect in the brain remains to be fully understood. KF contains higher concentrations of GABA compared to other fruits (406), suggesting a possible direct influence of dietary GABA on brain concentrations (35). *In vitro* fermentation of green ('Hayward') and gold ('Sungold', contains less actinidin) KF increases GABA concentrations compared to controls (water and inulin) (246), potentially involving the gut microbiome. Moreover, it is hypothesised that KF interacts with GABA receptor subunits in the enteric nervous system, influencing GABA levels, which may explain how green and gold KF peel extracts improve sleep onset and architecture in mice (118, 119) and green KF flesh in humans (5, 407). This study supports the hypothesis, as no difference in plasma GABA concentrations was observed. This finding suggests that KF does not affect peripheral GABA concentrations, though further research is needed.

KF consumption affects metabolites in the tryptophan and tyrosine pathways across all brain regions, including precursor AAs (tryptophan, phenylalanine, tyrosine) and downstream metabolites like 5HIAA, 5HT, 5HTP, octopamine, and L-DOPA (Figure 5.9). Gold KF increased 5HT in the hippocampus and striatum, followed by a decline, while green KF triggered sustained 5HT release in the hippocampus and decreased corpus striatum. This may be attributed to the different plasma BCAA responses between green and gold KF, as plasma BCAA can affect tryptophan and tyrosine uptake into the brain (286), affecting their metabolism into 5HT and dopamine. *In vitro* fermentation and human studies show green KF increases L-DOPA production (246) and 5HIAA excretion (407), suggesting modulation of these metabolites. Furthermore, KF, rich in 5HT (406), may influence 5HT levels by interacting with enteric 5HT receptors, potentially like its interaction with GABA.

Additionally, the flavonoid composition of KF may have influenced the tyrosine, tryptophan and glutamate metabolites. Key enzymes in these metabolic pathways, include GABA transaminase, glutamic acid decarboxylase, hydroxylases, aromatic L-amino acid decarboxylase, and monoamine oxidases (MAO) A and B, may have been modulated by nutrients found in KF (Figure 5.9). *In vitro* bioassays using rat brain homogenates indicated that flavonoid extracts from various plants could inhibit GABA transaminase and stimulate glutamic acid decarboxylase, both of which were crucial for regulating GABA levels (285). Green KF may have inhibited the activity of both MAO isomers, as suggested by *in vitro* assays (247). Specific polyphenols in KF, such as quinic acid, caffeic acid and catechin, were associated with MAO inhibition. However, neither animal nor human studies have explored the modulatory potential of KF and its components in these enzymes

This study is the first to explore the postprandial effects of KF consumption on polyamines in any system. Spermidine and putrescine in the corpus striatum and N-acetylputrescine in the prefrontal cortex are affected differently in response to the KF varieties. Polyamines may be involved in the pathophysiological process impacting blood flow in the brain (408). Also, polyamines regulate microglial cell proliferation, maturation, and functional activation (409). Polyamines can influence microglial phenotype and function (410), modulating their response to various stimuli and contributing to immune and inflammatory processes in the brain (411). Diet has been implicated in regulating macrophage function, and whether different KF varieties may be impacting microglial differentiation and blood flow would be compelling to explore further.

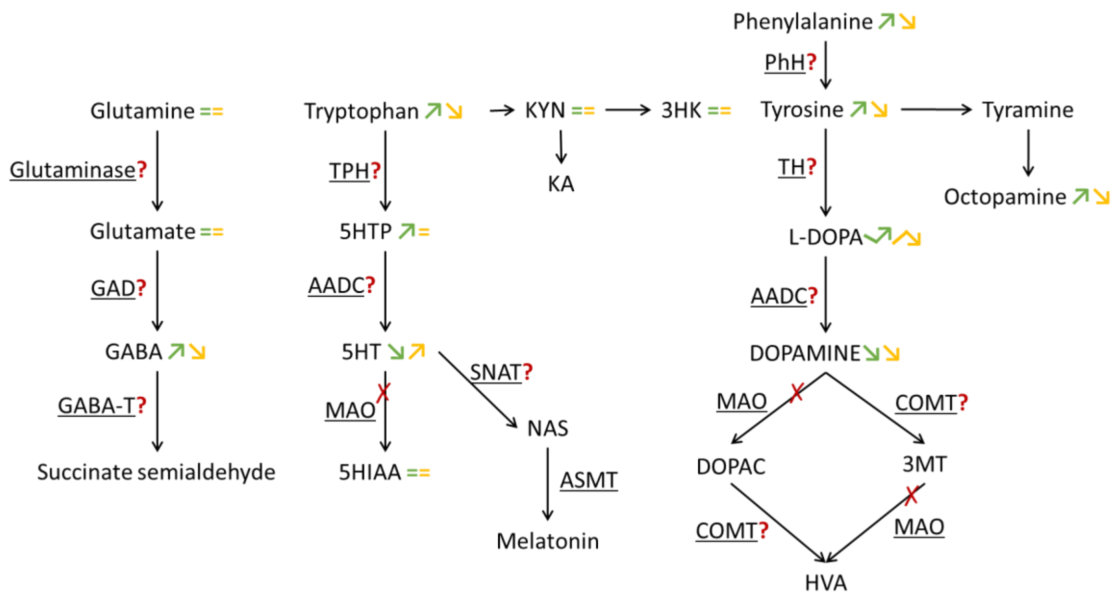


Figure 5.9 Overview of the biosynthesis of GABA, 5HT and dopamine. Abbreviations: GAD, glutamic acid decarboxylase; GABA-T, GABA transaminase; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; PhH, phenylalanine hydroxylase; AADC, aromatic L-amino acid decarboxylase; MAO, monoamine oxidase; COMT, catechol-O-methyl-transferase; HVA, homovanillic acid; 5HIAA,5-hydroxy indole acetic acid; NAS, N-Acetyk-5-Hydroxytryptamine; SNAT, serotonin N-acetyltransferase; ASMT: N-acetyltraptamine methyltransferase. ✗ depicts a potential inhibition of the enzyme with KF (247). ↘ **or** ↗ depicts the direction of metabolite concentration in the first two hours in the corpus striatum; metabolites with no arrows were not measured, and the colour of the arrow specifies KF (green arrow for green KF and yellow for gold KF).

5.5.3 Strength, limitation, future studies

Strengths of this study include the first report of differences in temporal bioamine responses in plasma and various brain regions in response to the consumption of diets supplemented with green and gold KF varieties. The results provide evidence for the modulatory potential of the actinidin-containing KF variety in the brain, which should be used to inform further research. A main advantage of using pigs as a model organism is their closer anatomical resemblance to humans, compared to smaller animals like mice or rats, making pigs a more relevant model for studies on the complex interactions between the gut and brain.

Nevertheless, this study has limitations. Firstly, what the response would be without any KF added to the diet needs to be clarified due to the lack of a no-KF treatment arm being

included. Furthermore, although 'Hort16A' is a valuable comparator as it lacks the actinidin enzyme, it differs in the flesh colour, driven by the lack of chlorophyll (21), which may have an effect. Future studies should have another group where purified actinidin is added to another gold KF group to function as a positive control or a green KF group where the enzyme is deactivated. Secondly, there was no baseline data, so how KF may shift from their baseline response could not be explored. Thirdly, the background diet used in this study was bread, which makes it difficult to compare with *in vitro*, animal, and human data, which used beef as their background meals (82, 90, 97). Fourthly, different pigs were killed at each time point, meaning for each group, an inter-individual variability bias may exist. Lastly, the methods used do not ascertain all AA nor bioamines, which may have been impacted but cannot be quantified.

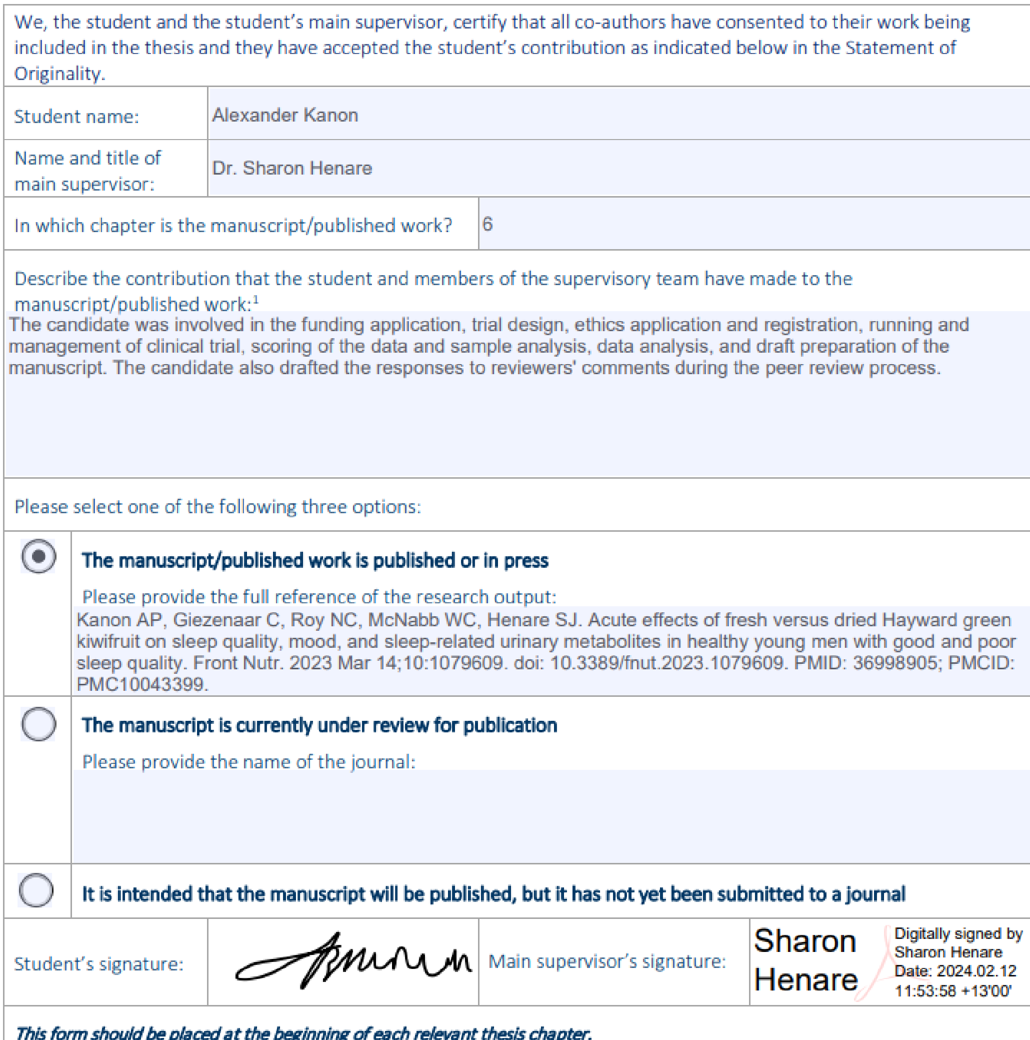
Future studies assessing the bioaminergic concentration responses to different KF consumption should consider assessing the expression and activities of glutamic acid decarboxylase, GABA transaminase, hydroxylases, aromatic l-amino acid decarboxylase and monoamine oxidase in the brain regions to be able to explain the observed trends. Furthermore, vitamins C and B could also be quantified in each brain region as they are required for AA metabolism. These vitamins could explain the prevalence of varietal differences between the different cultivars. Additionally, to evaluate the specific impacts of actinidin in green KF or added vitamin C from the gold KF, a comparator group in which actinidin is deactivated or vitamin C is added would be advantageous. Finally, considering that the observed effects may be attributed to the added polyphenols present in green KF, it would be beneficial to include KF skin as another group or combined with the flesh, as the skin is known to be the most abundant source of polyphenols compared to the entire fruit (40).

To better understand KF's modulatory effects on bioaminergic pathways, a human clinical study could assess platelet monoamine oxidase inhibition, peripheral bioamine concentrations, and the modulation of GABA and 5HT levels in the brain using a cognitive stress protocol (336, 412). Exploring the timing and co-consumption of KF with other foods, conducting longer-term interventions, and utilising advanced techniques like an electroencephalogram (EEG) (413) to assess electrical signalling, near-infrared spectroscopy to measure CBF (414) and positron emission tomography to map neurotransmitter responses (415) could further enhance our understanding of these effects. Overall, a direct supply of metabolites, including polyphenols, vitamins, GABA, 5HT, fibre, and actinidin from the KF can impact nutrient availability, digestion, metabolism, enzyme activity, and the gut microbiota (416).

5.6 Conclusions

Overall, this study represents the first evidence of the effects of green and gold KF consumption on postprandial bioamine metabolite concentrations in both plasma and distinct brain regions: brain stem, corpus striatum, hippocampus, and prefrontal cortex. The data indicate that GABA exhibits a heightened susceptibility to modulation by a green KF-supplemented diet across all examined brain regions. Furthermore, metabolites derived from tyrosine, tryptophan, and polyamines show region-specific modulation following green KF consumption. These findings underscore the significance of considering the influence of dietary interventions with KF on metabolites within specific brain regions. Nonetheless, additional investigations are warranted to elucidate the mechanisms through which green KF exerts these effects when added to the diet.

Statement of contribution



STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the student and the student's main supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the student's contribution as indicated below in the Statement of Originality.

Student name:	Alexander Kanon
Name and title of main supervisor:	Dr. Sharon Henare
In which chapter is the manuscript/published work?	6

Describe the contribution that the student and members of the supervisory team have made to the manuscript/published work:[1]

The candidate was involved in the funding application, trial design, ethics application and registration, running and management of clinical trial, scoring of the data and sample analysis, data analysis, and draft preparation of the manuscript. The candidate also drafted the responses to reviewers' comments during the peer review process.

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Kanon AP, Giezenaar C, Roy NC, McNabb WC, Henare SJ. Acute effects of fresh versus dried Hayward green kiwifruit on sleep quality, mood, and sleep-related urinary metabolites in healthy young men with good and poor sleep quality. Front Nutr. 2023 Mar 14;10:1079609. doi: 10.3389/fnut.2023.1079609. PMID: 36998905; PMCID: PMC10043399.

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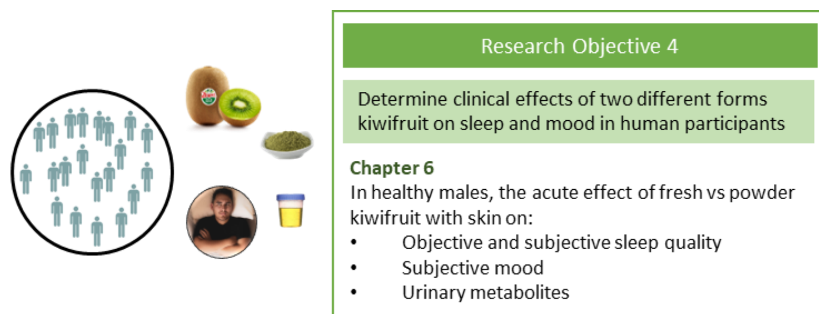
1 Doctoral Research Committee May 2023

CHAPTER 6. Acute effects of fresh and dried green kiwifruit in males with good and bad quality sleep[4]

Prologue

*Consuming kiwifruit (KF) for four weeks or more has shown associations with improved sleep quality and mood. KF contains serotonin (*5HT*), vitamin C, and B-vitamins, known for their roles in sleep regulation and mood enhancement. However, the acute effects of KF consumption are not well understood. This investigated the immediate impact of consuming two forms of KF on sleep quality and mood in a randomised controlled trial.*



Highlights

- *Irrespective of sleeper type, dried KF has more beneficial impacts.*
- *Regardless of sleep quality group, morning sleepiness, alertness upon awakening, and vigour were improved after dried KF consumption compared to control.*
- *Both fresh and dried KF treatments compared to control tended toward improved esteem and total mood disturbance.*
- *No effects of KF were found with objective sleep measures.*
- *Both KF treatments compared to control increased urinary concentration of the* 5HT *metabolite 5-hydroxyindoleacetic acid (*5HIAA*).*
- *Poor sleepers excreted less urinary B-vitamins compared to good sleepers.*

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[4] Part of the contents of this chapter has been published as a peer-reviewed paper: Kanon AP, Giezenaar C, Roy NC, McNabb WC, and Henare SJ (2023) Acute effects of fresh versus dried Hayward green kiwifruit on sleep quality, mood, and sleep-related urinary metabolites in healthy young men with good and poor sleep quality. Front. Nutr. 10:1079609. doi: 10.3389/fnut.2023.1079609

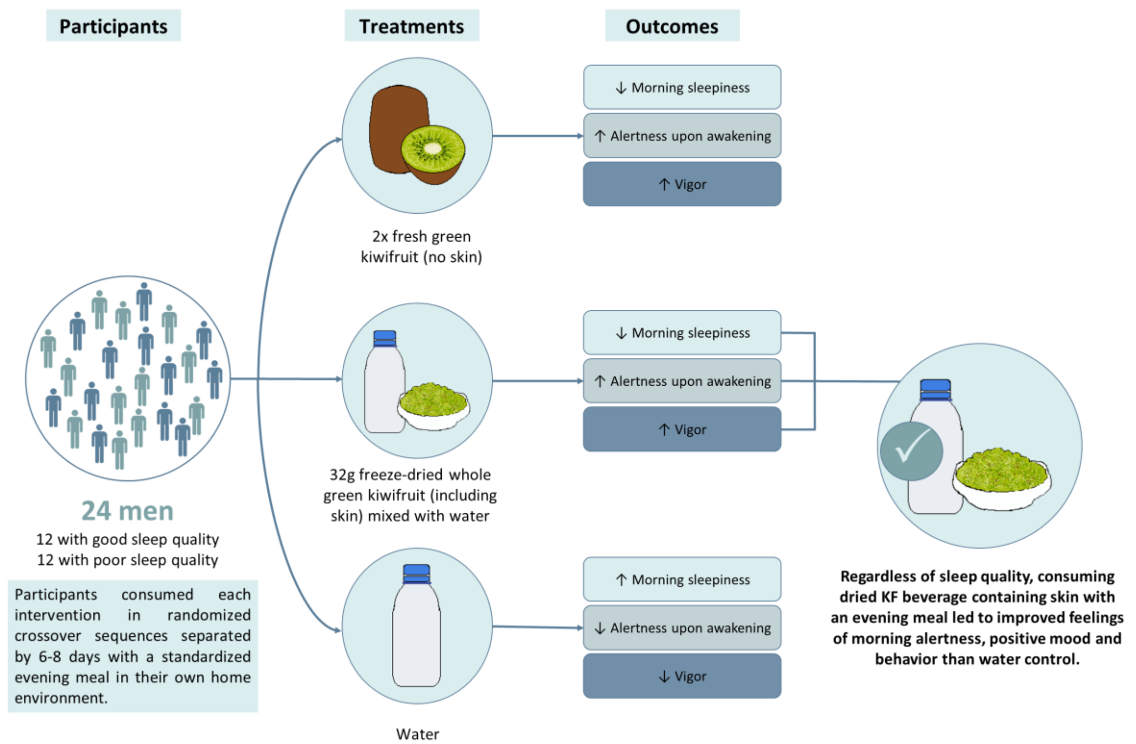
The chapter is presented in manuscript form and has been published in "Frontiers Nutrition".

6.1 Abstract

Daily KF consumption has been associated with improved sleep quality, but underlying physiological mechanisms are unknown. This study examined the acute effects of fresh and dried green KF, compared with a water control, on sleep quality, mood, and urinary 5HT and melatonin metabolite concentrations. 24 men (age: 29 ± 1 yrs, body mass index: 24 ± 1 kg/m2) with poor (n = 12) or good (n = 12) sleep quality participated in a randomised, single-blind crossover study. One of three treatments was consumed with a standardized evening meal; 1) the flesh of two fresh green KF; 2) dried green KF powder (including skin; equivalent to dry matter of two fresh KF) mixed with water; or 3) a water control, in their own home. Subjective and objective sleep quality, mood, waking urinary 5HIAA, 6-sulfatoxymelatonin (aMT6s), vitamin C and B-vitamin concentrations were determined. Regardless of sleep quality group, compared to control, morning sleepiness, alertness upon awakening, and vigour were improved ($p < 0.05$) after dried KF consumption. Compared to control, both fresh and dried KF treatments tended ($p < 0.1$) toward improved esteem and total mood disturbance. Both KF treatments increased (fresh +1.56 ± 0.4 ng/g, p=0.001; dried: +1.30 ± 0.4 ng/g, p=0.004) urinary concentration of the 5HT metabolite 5HIAA compared to the control (4.32 ± 0.4 ng/g). In poor sleepers, ease of awakening improved by 24% after dried KF consumption ($p = 0.005$) and tended to improve by 13% after fresh KF intake ($p = 0.052$) compared to the control. Good sleepers tended towards 9% improved ratings of getting to sleep with fresh KF ($p = 0.053$) compared to the control. Poor sleepers had lower amounts of some B-vitamins compared to good sleepers ($p < 0.05$). Consumption of dried or fresh KF with a standard evening meal, was associated with improved aspects of sleep quality and mood, possibly mediated through changes in 5HT metabolism. This trial was registered at www.anzctr.org.au as ACTRN12621000046808.

Key words: sleep, mood, kiwifruit, melatonin (6-sulfatoxymelatnonin), serotonin, alertness

Graphical abstract



6.2 Introduction

Sleep is essential for the cellular repair of the body. Inadequate sleep is associated with increased health risks such as inflammation, diabetes, hypertension, and obesity. (417-421). Insufficient sleep can also lower cognitive performance and cause mood changes (422). One night of sleep disturbance can affect a person's ability to concentrate; increasing technical errors and their overall mood the following day (423-425). Sleep quality is an assessment of a person's contentment with their sleep period. This includes measures of one's sleep duration, latency, efficiency, and waking after sleep. Good sleep quality has positive effects including improved daily function and feeling rested. Poor sleep quality outcomes include increased irritability and fatigue (426).

The circadian clock in the hypothalamus, which is affected by the light and dark cycles, controls sleep-wake cycles in humans. The neurohormone melatonin is secreted from pinealocytes in the pineal gland, and its precursor 5HT plays a vital role in regulating this. The urinary melatonin metabolite 6-sulfatoxymelatonin (aMT6s) is related to subjective and objective sleep quality measures (427) and the urinary concentration of the 5HT metabolite 5HIAA is related to mood (428). Moreover, first-morning urine concentration of aMT6s accurately reflected peak and total nocturnal plasma melatonin secretion (429).

The consumption of fruit and vegetables is known to affect plasma and urinary melatonin and 5HT metabolite concentrations. Consumption of vegetables (sweet corn, bitter gourd, Japanese radish sprout, shumeji and shiitake mushrooms) (430), tomatoes (431), and cherries (432), which are rich in melatonin, increased morning urinary aMT6s concentration (430-432) and improved sleep quality (431-433). Consumption of serotonin-rich fruits, such as KF (406) and a Jerte Valley cherry-based product (434)

increased urinary levels of 5HIAA (8, 434) and improved mood (434). Vitamin C and B-vitamins are essential co-factors in catecholamine biosynthesis, supporting neurotransmitter production that regulates mood, stress, and sleep. Vitamin C also helps reduce oxidative stress, and studies show increased urinary levels with vitamin C-rich foods. Furthermore, daily consumption of two green KF one hour before bed for four weeks was associated with improved sleep quality (5). KF is high in 5HT, vitamin C and B-vitamins (27). However, the relationships between biochemical measures and sleep quality with KF consumption are unknown.

The aim of this study was to determine the effect of consuming the flesh of two fresh green KF (without the skins), whole freeze-dried green KF (with the skins included equivalent to two green KF), or a water control with a standardized evening meal on urinary concentrations of metabolites of melatonin, 5HT, vitamin C and B-vitamins as well as objective (actigraphy) and subjective sleep quality and mood measures in healthy young men. We hypothesised that sleep quality and mood would improve, and urinary metabolite concentrations would increase after the KF interventions compared to the control intervention.

6.3 Materials and methods

6.3.1 Participant recruitment and screening

Twenty-four healthy men (eligibility: age 18-35 years, Body Mass Index (BMI) 18.5-30kg/m2) were recruited by advertisement from the Massey University campus and community in Palmerston North, Manawatu, New Zealand, between January 2021 and May 2021 amid the COVID-19 pandemic. The PSQI was used to determine subjective sleep quality. Participants were classified as 'good sleeper' when their global PSQI score was ≤5, and as 'poor sleeper' when their global PSQI score was >5 (435). Exclusion

criteria included smoking, excessive alcohol intake of >21 standard drinks per week, use of prescribed or non-prescribed medications and antibiotics, physically active for more than two hours a day, food intolerances and allergies, consuming a vegan/vegetarian diet, gastrointestinal disorders; chronic conditions, such as cardiorespiratory, diabetes mellitus, high cholesterol/blood pressure; psychiatric conditions; diagnosed sleep conditions and working night shift or irregular work hours. In addition, participants who experienced significant weight loss (>5%) three months prior to the start of the study or consumed strict diets were excluded.

The study protocol was approved by the Massey University Human Ethics Committee (Massey University HEC: Southern A application-20/52), and the study was conducted according to the guidelines in the Declaration of Helsinki. The study was registered as a clinical trial with the Australian New Zealand Clinical Trial Registry (www.anzctr.org.au; ACTRN12621000046808). All participants provided written informed consent before the clinical trial and could withdraw at any time for any reason.

6.3.2 Study design, intervention, and protocol

This intervention was a randomised, single-blind, cross-over study. The study involved two green KF interventions of different forms and control. The two KF interventions were: 1) two fresh green KFs (Actinidia deliciosa cv. Hayward) (flesh only; approximate 200 g), 2) freeze-dried whole (flesh and skin), green KF (32 g, equivalent to the dry matter of two fresh green KFs) with 200 ml of water or 3) control of 200 ml of water. The nutritional composition of each treatment is shown in Table 6.1. The researcher administering and analysing the data (AK) was blinded to treatment allocation until the completion of analyses, and a separate researcher (CG) was unblinded and responsible

for preparing the interventions. The researcher preparing the interventions was not involved in analysing the data.

Randomisation was conducted using a 6 × 3 Williams design balanced for the order of presentation and carryover effects. The trial CONSORT flow diagram is shown in Figure 6.1. The study was conducted in the participant's home environment. Before the start of study evenings, a familiarization evening was conducted. The familiarization occurred one to three days before the first evening. The same procedure described below was followed without the provision of a standardized evening meal and intervention.

Participants were studied on three separate occasions, separated by six to eight days. Each participant consumed an intervention with a standardized evening meal [Pub Size Spaghetti and Meatballs (McCain Foods), ∼720 kcal, P 26.5 g, C 75.0 g, F 33.5 g] Enrolled participants were asked to refrain from eating KF from enrolment until the completion of the study period. On the day of the study, participants were asked to collect a data collection pack (Figure 6.2). The pack contained the evening meal, an intervention they were randomised to receive, an actigraphy watch, a urine sample collection container, and a survey booklet. It was confirmed that the participant refrained from consuming any restricted foods during the two days prior to their study day. The restricted foods included oranges, pineapples, bananas, mangos, papayas, plums, grape, cherries, strawberries, tomatoes, capsicum, pistachios, plantains, mushrooms, chocolate, teas, coffee, and caffeinated beverages as they are known to contain and alter levels of 5HT and melatonin in urine (8, 436, 437).

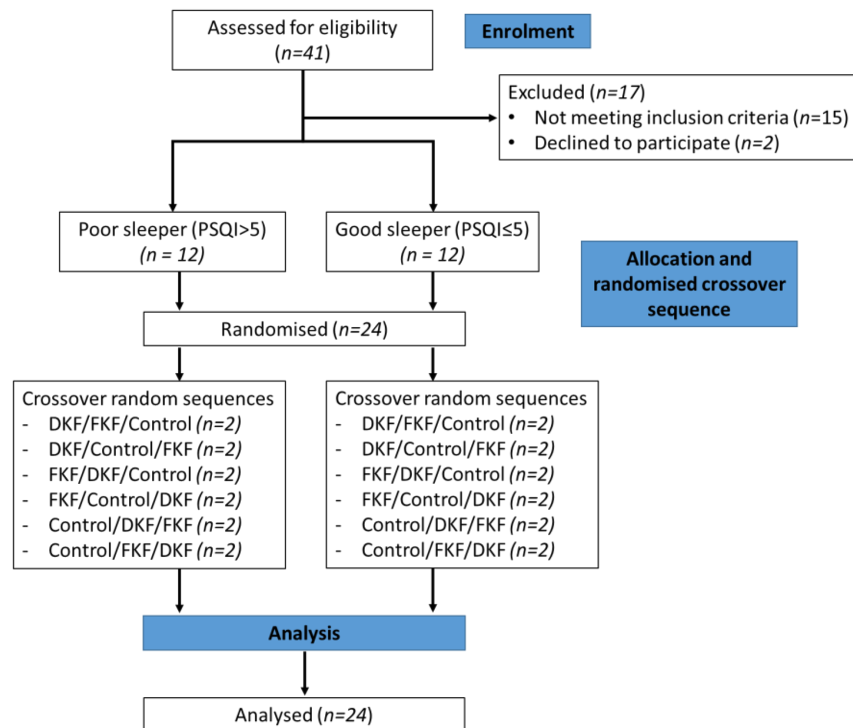


Figure 6.1 Consolidated Standards of Reporting Trials (CONSORT) flow diagram of the recruitment, enrolment, and random assignment process. DKF (Dried KF—32 g freeze-dried green KF powder), FKF (fresh KF—approx. 200 g fresh green KF).

The participant consumed the standardized evening meal four hours before their usual sleep time, followed immediately by the allocated intervention taking note of the time. Participants were asked not to eat any food or drinks, except water, until the following morning. Immediately before going to bed, participants were asked to rate their sleepiness level using the Stanford Sleepiness Scale (SSS). The participant was asked to activate the phase marker on the actigraphy watch when they were in bed and ready to sleep. Upon waking the following day, participants were asked to activate the phase marker, collect the whole first-morning urine sample noting the time, and complete a set of surveys including another SSS, Leeds Sleep Evaluation Questionnaire (LSEQ), and the abbreviated POMS. The urine sample and surveys were delivered to the laboratory within two hours of waking (Figure 6.2). Arrangements were made if the participant could not deliver samples within this time.

Table 6.1 Composition of total daily treatments for the Fresh KF, Dried KF and control.

	QUANTITY PER SERVE		
Nutrient	**≈ 200 g fresh KF[a]**	**32 g freeze-dried whole green KF powder with 200 ml water[b]**	**200 ml water**
Energy (kcal)	95.00	95.00	0
Carbohydrates			
Carbohydrate, total (g)	18.20	21.30	0
Sugars			
Sugar, total (g)	17.60	20.77	0
Fructose (g)	9.40	10.30	0
Galactose (g)	0.00	0.03	0
Glucose (g)	8.20	8.93	0
Lactose Anhydrous (g)	0.00	0.03	0
Lactose monohydrate (g)	0.00	0.03	0
Maltose (g)	0.00	0.03	0
Sucrose (g)	0.00	1.41	0
Unavailable			
Dietary fibre, total (g)	6.00	4.16	0
Fats			
Fat, total (g)	1.40	0.61	0
Minerals			
Calcium (mg)	54.00	41.92	0
Iron (mg)	0.44	0.26	0
Potassium (mg)	602.00	550.40	0
Magnesium (mg)	28.00	18.24	0
Sodium (mg)	4.00	6.05	0
Zinc (mg)	0.20	0.16	0
Protein and amino acid			
Protein (g)	2.40	1.31	0
Tryptophan (mg)	100.00	43.10	0
Vitamins Water Soluble			
Vitamin B_{12}	0.00	0.02	0
Vitamin B_3 (mg)	1.66	0.40	0
Vitamin B_6 (mg)	0.14	0.36	0
Vitamin B_9 (mg)	76.00	48.00	0
Vitamin C (mg)	170.20	98.56	0
Vitamins Fat Soluble			
Vitamin E (mg)	1.72	2.69	0
Other			
Ash (g)	1.40	1.18	0
Water (g)	167.00	200.00	200.00
Total Phenolic Content (mg GAE)[b]	168.30**	217.60**	0

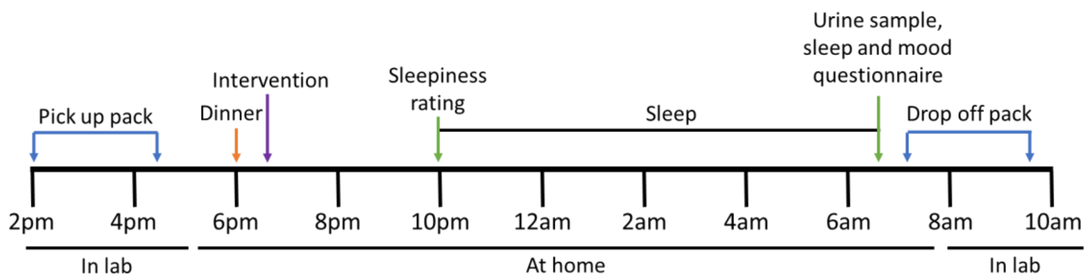


Figure 6.2 Study protocol for each intervention. At approximately 6 pm, the participant consumed a standardized evening dinner followed by one of three interventions. Before bedtime, at approximately 10-11 pm, the participant was asked to rate their sleepiness.

6.3.3 Subjective measures for sleep quality and mood

Two questionnaires to measure subjective sleep quality were used. The SSS measures sleepiness using a one-item scale; the participants select one of seven statements that best represent their current sleepiness. For example, a score of one indicates feeling active, vital, and alert, while a score of seven indicates no longer fighting sleep, sleep onset soon (438). The LSEQ is ten Visual Analogue Scales (VAS) as a subjective self-measure to assess sleep quality changes throughout psychopharmacological treatment interventions (439). The scale evaluates four domains of sleep: i) ease of getting to sleep (mean of questions 1, 2 and 3), ii) quality of sleep (mean of questions 4 and 5), iii) ease of awakening from sleep (mean of questions 6 and 7), and iv) alertness upon awakening (mean of questions 8, 9, and 10). A 100 mm VAS scale was used for scoring, and scores were averaged per domain to determine the domain score. Higher scores indicate a better sleep quality domain.

Mood was assessed using the abbreviated POMS questionnaire (440). This form contains 40 mood-related adjectives rated on a 5-point Likert-type scale, ranging from 0 (not at all) to 4 (extremely). The data is then categorized into seven mood scales (maximum scores indicated): i) tension, ii) depression, iii) anger, iv) fatigue, v) confusion,

vi) vigour and vii) esteem. Finally, a Total Mood Score (TMD) was calculated by adding tension, depression, anger, fatigue, and confusion scores, then subtracting the vigour and esteem scores.

6.3.4 Objective sleep measures

Participants were provided with an Actiwatch Spectrum Plus (Philips Respironics, Murrysville, Pennsylvania, USA) to wear on the wrist of their non-dominant arm during the study evenings. The medium threshold setting was used due to its use in studies using actigraphy to assess sleep (441), and 30 seconds was selected as the epoch length. The data was downloaded and subject to a mathematical algorithm in Actiware 6.0.9 (Philips Respironics, Murrysville, Pennsylvania, USA) to quantify sleep onset latency (SOL), sleep efficiency (SE), total sleep time (TST), wake after sleep onset (WASO), number of awakenings and the average length of awakenings.

6.3.5 Biochemical measures

Urine samples were collected in 500 ml containers with 1.0 g of EDTA. Participants were provided with two containers. Upon arriving at the laboratory, urine samples were weighed, and their volume was recorded. The urine was aliquoted into 1.5 ml Eppendorf tubes and stored at -80°C until analysis of aMT6s and vitamin C. Ten millilitres of urine were acidified with 6 M HCL and aliquoted and stored at -80°C until analysis of 5HIAA. Urinary aMT6s and 5HIAA analyses were analysed according to the manufacturer's instructions using an enzyme-linked immunosorbent assay (ELISA) kit (IBL International, Hamburg, Germany). Assays were performed in duplicate and averaged. The average intra-assay coefficient of variance for the aMT6s assay was 5.2%, and the inter-assay was 2.9%. The average intra-assay coefficient of variance for the 5HIAA assay was 7.5%,

and the inter-assay was 3.5%. The ascorbate content of the samples was determined by reverse-phase high-performance liquid chromatography (HPLC) with electrochemical detection Pullar, Bayer (442).

B-vitamins and vitamers were measured using high-performance liquid chromatography coupled with mass spectrometry (UHPLC-MS/MS) technique (443). The B vitamins measured included pantothenic acid, 4-pyridoxic acid, nicotinic acid, nicotinamide, nicotinuric acid, pyridoxal, biotin, riboflavin, folic acid, pyridoxamine and thiamine. The concentrations of measured metabolites and vitamins were corrected to creatinine to adjust for variation in urine dilution. Creatinine was measured using the colorimetric Jaffe method (444) (Nutrition Laboratory, Massey University).

6.3.6 Data and statistical analysis

A power analysis was performed using ease of getting to sleep VAS from LSEQ as the dependent variable to estimate the required sample size. Estimates of variance components were conducted based on data from a study examining caffeine ingestion's effect on VAS getting to sleep in healthy subjects (445). When there are three treatments and a sample size of two in each of the six sequences, a 6 x 3 Williams Crossover inequality test of paired differences will have 94.2% power to detect a minimum difference of 20 mm or greater, assuming that the standard deviation of the paired differences is 13 mm (445) at the 5% significance level. The Bonferroni adjustment was used to keep the family-wise error at the specified error level. Thus, with the total number of pairwise comparisons equal to 3, each pairwise test was at the two-sided 1.67% significance level.

Statistical analyses were performed using SPSS software (version 25; IBM, Armonk, NY, USA). No outliers for any outcome measures were noted by examination of studentized

residuals for values greater than ±3. A paired t-test was used to compare nutritional compositional and demographic data. Effects of sleeper type and treatment and their interaction effect were determined using a repeated measures mixed-effects model. An unstructured covariance structure was used to account for the repeated treatments by the subject. Post hoc comparisons, adjusted for multiple comparisons using Bonferroni's correction, were performed when there were significant main or interaction effects. Residual plots were inspected to confirm that the normality and constant variance model assumptions were met. Statistical significance of the mixed effects models was accepted at a probability inferior to 0.05 ($p < 0.05$). A trend was noted at a probability lower than 0.10 but higher than 0.05. To assess the within-subject correlations between urinary metabolites and sleep quality and mood measures, a univariate model with sleep and mood measures as the dependent variable, urinary metabolites as the covariate, and subject as the fixed factor were performed (446). Correlations with $p < 0.05$ and R-value > ± 0.4 were considered significant (310). All data are presented as means ± standard errors of the mean (SEM).

6.4 Results

6.4.1 Participants

Twenty-four young men (18-35 years) completed the study: 12 with good sleep quality (age: mean ± SEM: 29 ± 0.9 years; BW: 79.3 ± 2.8 kg; BMI: 24.4 ± 0.6 kg/m2) and 12 with poor sleep quality (29 ± 1.2 years; 76.4 ± 2.2 kg; 24.3 ± 0.7 kg/m2) (Table 6.2). The demographics between the groups were similar except for PSQI, which was significantly lower in good sleepers (3 ± 1) compared to poor sleepers (8 ± 2, $p = 0.005$) (Table 6.2). Main effects of sleeper type were identified for evening sleepiness (SSS) [$F(2, 22) = 5.71$ $p = 0.026$], getting to sleep (LSEQ) [$F(2, 22) = 4.57$ $p = 0.044$] and quality of sleep (LSEQ)

[F (2, 22) = 7.91 p = 0.010]. Regardless of treatment, good sleepers rated themselves sleepier in the evening (mean ± SEM across treatments; 4.4 ± 0.3) compared to poor sleepers (3.4 ± 0.3; post hoc p = 0.026). Good sleepers rated themselves as harder getting to sleep and had worse sleep quality (mean ± SEM across treatments; 47.8 ± 2.6, 46.6 ± 2.7 respectively) compared to poor sleepers (55.6 ± 2.6; post hoc p = 0.044, 57.2 ± 2.7; post hoc p = 0.010). All subjects completed and tolerated the study protocol. None of the participants had been diagnosed with COVID-19.

Table 6.2 Characteristics of participants, data presented as mean ± SEM, range, median (IQR).

Characteristic of participants	Whole group (n =24)	Poor sleeper (n = 12)	Good sleeper (n = 12)
Age, y	29 ± 4, 19-34, 28.5 (2)	29 ± 3, 19-34, 28 (5.3)	29 ± 3, 24-34, 28.5 (2)
BW, kg	77.8 ± 8.7, 77.2 (12.2)	76.4 ± 7.7, 76.6 (6.3)	79.3 ± 9.8, 79.8 (14.8)
BMI, kg/m^2	24.3 ± 2.3, 24.1 (2.9)	24.3 ± 2.5, 24.2 (2.3)	24.4 ± 2.2, 24.1 (2.9)
PSQI Score	5 ± 3, 1-12, 3 (1.3)	8 ± 2, 6-12, 7 (2.5)***	3 ± 1, 1-5, 3 (1.3)
Daily screen time, hours	8.0 ± 3.6, 2-14, 6 (5.1)	9.1 ± 3.9, 2.5-14, 10 (5.75)	8.0 ± 3.6, 3-12.5, 6 (5.13)
Daily caffeine intake, cups	1.7 ± 1.5, 0-5, 1.0 (1.75)	2.0 ± 1.6, 0-5, 1.5 (2.13)	1.4 ± 1.3, 0-4, 1.0 (1.63)
Ethnicity (n)			
– **European**	15/24	8/12	7/12
– **Asian**	3/12	0/12	3/12
– **Indian**	3/24	1/12	2/12
– **African**	1/24	1/12	0/12
– **Latin American**	2/24	2/12	0/12

BMI = Body Mass Index, PSQI = Pittsburgh Sleep Quality Index. *** = significant difference between poor and good sleepers, p<0.005. SEM: standard error of mean.

6.4.2 Subjective measures of sleep and mood

6.4.2.1 Sleep measures

Table 6.3 shows a summary of subjective sleep measures. Analyses determined interaction effects of treatment by sleeper type for ease of getting to sleep (LSEQ) [F (2, 22) = 4.81, p = 0.018] and ease of awakening following sleep (LSEQ) [F (2, 22) = 3.85, p = 0.037]. Within good sleepers, ratings of ease of getting to sleep tended to increase after the fresh KF treatment (54.1 ± 4.1) compared to the control (45.1 ± 3.4; post hoc p =

0.053; Figure 6.3a). Within poor sleepers, ratings of ease of waking were higher after the freeze-dried KF (62.8 ± 5.1; post hoc p = 0.005) and tended to be higher after the fresh KF treatment (51.9 ± 5.0; post hoc p = 0.052), compared to the control (38.6 ± 5.1, Figure 6.3b).

Main effects of treatment were identified for morning sleepiness (SSS) [F (2, 22) = 5.47, p = 0.012] and alertness upon awakening (LSEQ) [F (2, 22) = 5.11, p = 0.015]. Irrespective of sleeper type, morning sleepiness ratings were higher after the control (3.00 ± 0.26; post hoc p = 0.013) and fresh KF (2.6 ± 0.2; post hoc p = 0.034) interventions compared to the freeze-dried KF treatment (2.1 ± 0.2; Figure 6.3c). Ratings of alertness upon awakening were higher after the freeze-dried KF (57.1 ± 2.4; post hoc p = 0.018) and tended to be higher following the fresh KF treatment (53.1 ± 3.4; post hoc p = 0.082) compared to the control (44.2 ± 2.8, Figure 6.3d).

Table 6.3 Subjective sleep outcomes for the fresh KF, dried KF and water control treatments in poor and good sleepers. Post hoc estimated marginal means and standard error of means (SEM) are presented with F and p values of the main effects and interaction from the linear mixed models.

Variable	Treatment	Poor sleeper (n = 12)	Good sleeper (n = 12)	Main effects		
					F	*p*
Evening Sleepiness (1-7)	Fresh KF	3.5 ± 0.3	4.8 ± 0.3	Treatment	0.51	0.608
	Dried KF	3.3 ± 0.4	4.3 ± 0.4	Sleeper type	5.70	0.026
	Control	3.5 ± 0.4	4.2 ± 0.4	Treatment x Sleeper type	0.36	0.699
Morning Sleepiness (1-7)	Fresh KF	2.6 ± 0.2	2.6 ± 0.2	Treatment	5.47	0.012
	Dried KF	1.8 ± 0.2	2.4 ± 0.2	Sleeper type	0.15	0.706
	Control	3.2 ± 0.4	2.8 ± 0.4	Treatment x Sleeper type	1.94	0.167
Getting to Sleep (0-100)	Fresh KF	52.0 ± 4.1	54.1 ± 4.1	Treatment	1.00	0.383
	Dried KF	60.8 ± 3.6	44.0 ± 3.6	Sleeper type	4.57	0.044
	Control	54.1 ± 3.4	45.1 ± 3.4	Treatment x Sleeper type	4.81	0.018
Quality of Sleep (0-100)	Fresh KF	60.0 ± 5.3	53.1 ± 5.3	Treatment	1.48	0.249
	Dried KF	58.0 ± 3.9	41.9 ± 3.9	Sleeper type	7.91	0.010
	Control	53.6 ± 4.4	44.7 ± 4.4	Treatment x Sleeper type	0.54	0.589
Awake Following Sleep (0-100)	Fresh KF	51.9 ± 5.0	54.9 ± 5.0	Treatment	3.23	0.059
	Dried KF	62.8 ± 5.1	50.3 ± 5.1	Sleeper type	0.07	0.787
	Control	38.6 ± 5.1	52.5 ± 5.1	Treatment x Sleeper type	3.85	0.037
Alertness upon awakening (0-100)	Fresh KF	55.7 ± 4.8	50.5 ± 4.8	Treatment	5.11	0.015
	Dried KF	63.9 ± 3.4	50.2 ± 3.4	Sleeper type	2.28	0.145
	Control	42.8 ± 4.0	45.7 ± 4.0	Treatment x Sleeper type	1.96	0.165

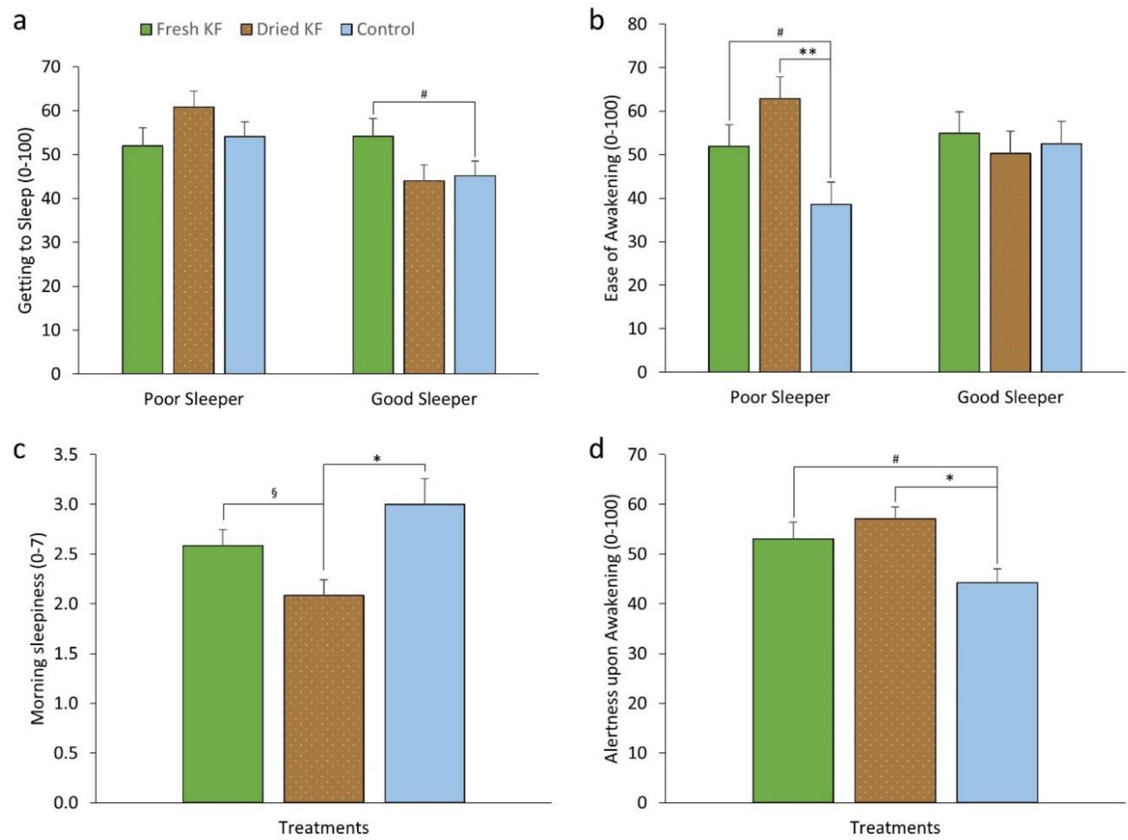


Figure 6.3 Estimated marginal means and standard error of means (SEM) for post-treatment subjective ratings of morning sleepiness (SSS) (a), getting to sleep (LSEQ) (b), ease of waking (LSEQ) (c) and behaviour following wake (LSEQ) (d). $^*p < 0.05$ represents statistical difference from control values. $^{\S}p < 0.05$ represents the statistical difference from Dried KF values. $^{**}p < 0.01$ represents statistical difference from control values. $^{\#}p < 0.1$ represents statistical difference from control values.

6.4.2.3 Mood measures

A summary of subjective mood measures is presented in Table 6.4. Main significant effects of treatment for esteem-related affect were identified [F (2, 22) = 4.56, p = 0.022], vigour [F (2, 22) = 4.34, p = 0.026], and total mood disturbance [F (2, 22) = 3.82, p = 0.038]. Furthermore, main trend effects of treatment for fatigue [F (2, 22) = 3.17, p = 0.062] and confusion [F (2, 22) = 3.37, p = 0.053] were identified. Irrespective of sleeper type, ratings for esteem-related affect tended to be higher following freeze-dried KF (12.6 ± 0.8; p = 0.060) and fresh KF (12.3 ± 0.6; post hoc p = 0.054) compared to the control (10.8 ± 0.5; Figure 6.4a). Ratings for vigour were higher following freeze-dried KF (6.58 ± 0.91; post hoc p = 0.030), but not fresh KF (4.9 ± 0.7; post hoc p = 0.487) treatments compared to the control (3.9 ± 0.6; Figure 6.4a). Ratings for total mood disturbance tended to be lower following the freeze-dried KF (86.9 ± 2.5; post hoc p = 0.054) and fresh KF (90.3 ± 1.8; post hoc p = 0.063) treatments compared to the control (96.4 ± 2.1, Figure 6.4b). No main effects for sleeper type or interactions were identified for any mood measures.

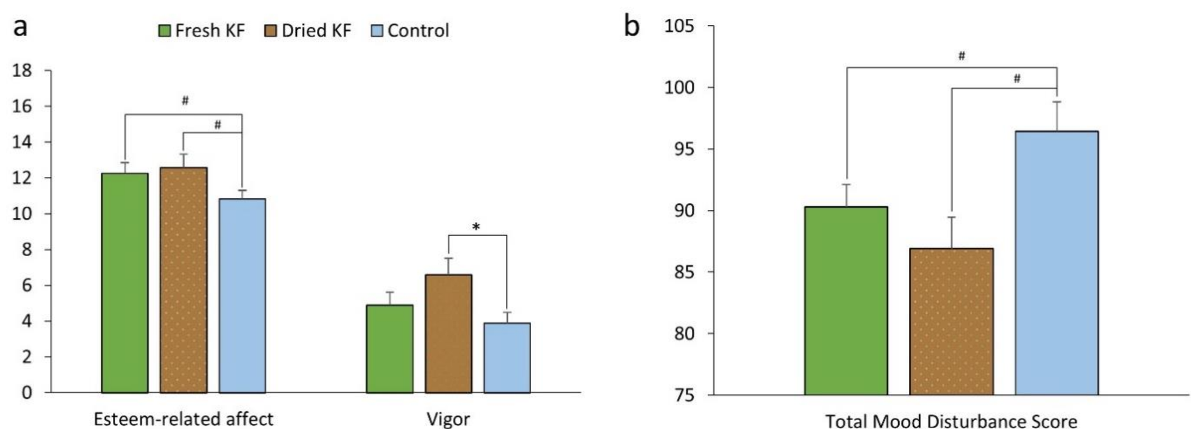


Figure 6.4 Estimated marginal means and standard error of means (SEM) for post-treatment subjective mood ratings (abbreviated Profile of Mood States) of esteem and vigour (a) and total mood disturbance (b). *$p < 0.05$ represents statistical difference from control values. #$p < 0.1$ represents statistical difference from control values.

Table 6.4 Subjective mood outcomes for the fresh KF, dried KF and water control treatments in poor and good sleepers. Post hoc estimated marginal means and standard error of means (SEM) are presented with F and p values of the main effects and interaction from the linear mixed models.

Variable	Treatment	Poor sleeper (n = 12)	Good sleeper (n = 12)	Main effects	*F*	*p*
Tension (0-24)	Fresh KF	2.4 ± 0.7	1.8 ± 0.7	Treatment	1.09	0.352
	Dried KF	2.3 ± 0.8	2.3 ± 0.8	Sleeper type	0.51	0.482
	Control	2.9 ± 0.6	1.8 ± 0.6	Treatment x Sleeper type	1.10	0.350
Anger (0-24)	Fresh KF	0.8 ± 0.4	0.8 ± 0.4	Treatment	1.85	0.180
	Dried KF	0.5 ± 0.3	0.5 ± 0.3	Sleeper type	0.04	0.850
	Control	1.7 ± 0.8	1.4 ± 0.8	Treatment x Sleeper type	0.02	0.976
Fatigue (0-20)	Fresh KF	2.5 ± 0.7	1.9 ± 0.7	Treatment	3.17	0.062
	Dried KF	2.1 ± 0.5	2.1 ± 0.5	Sleeper type	1.58	0.222
	Control	4.6 ± 0.9	2.8 ± 0.9	Treatment x Sleeper type	0.70	0.508
Depression (0-28)	Fresh KF	0.7 ± 0.6	0.9 ± 0.5	Treatment	1.77	0.193
	Dried KF	0.6 ± 0.6	0.8 ± 0.6	Sleeper type	0.01	0.909
	Control	2.0 ± 0.9	1.2 ± 0.8	Treatment x Sleeper type	0.36	0.699
Esteem-related Affect (0-24)	Fresh KF	11.9 ± 0.8	12.6 ± 0.8	Treatment	4.56	0.022
	Dried KF	12.6 ± 1.1	12.6 ± 1.1	Sleeper type	0.41	0.530
	Control	10.3 ± 0.7	11.4 ± 0.7	Treatment x Sleeper type	0.36	0.703
Vigour (0-20)	Fresh KF	4.9 ± 1.0	4.8 ± 1.0	Treatment	4.34	0.026
	Dried KF	6.6 ± 1.3	6.6 ± 1.3	Sleeper type	0.01	0.921
	Control	3.7 ± 0.9	4.1 ± 0.9	Treatment x Sleeper type	0.08	0.928
Confusion (0-20)	Fresh KF	1.8 ± 0.7	2.0 ± 0.7	Treatment	3.37	0.053
	Dried KF	0.8 ± 0.3	0.8 ± 0.3	Sleeper type	0.03	0.869
	Control	2.5 ± 0.8	1.9 ± 0.8	Treatment x Sleeper type	0.60	0.560
Total Mood disturbance	Fresh KF	91.0 ± 2.6	89.6 ± 2.6	Treatment	3.82	0.038
	Dried KF	86.9 ± 3.6	86.9 ± 3.6	Sleeper type	0.68	0.417
	Control	99.3 ± 3.4	93.5 ± 3.4	Treatment x Sleeper type	0.43	0.654

6.4.3 Objective measures of sleep quality

A summary of objective sleep measures is presented in Table 6.5. There was an interaction effect between treatment and sleeper type for the number of awakenings [F (2, 22) = 4.06, p = 0.032]. Within poor sleepers, the number of awakenings tended to be lower after the fresh KF treatment (35.9 ± 2.76; p = 0.099) than the control (43.4 ± 3.5, Figure 6.5b). Conversely, among good sleepers, the number of awakenings tended to increase after the freeze-dried KF treatment (42.8 ± 3.81; post hoc p = 0.080) compared to the control (33.3 ± 3.54, Figure 6.5b).

There were main effects of treatment for wake after sleep onset [F (2, 22) = 3.74, p = 0.040]. Irrespective of sleeper type, the number of awakenings was higher after the freeze-dried KF treatment (41.4 ± 3.2; p = 0.045) but not the fresh KF treatment (34.4 ± 2.7; p = 0.888) compared to the control (37.0 ± 4.3) (Figure 6.5a). No main effects of treatment for sleeper type were identified for any objective sleep measures.

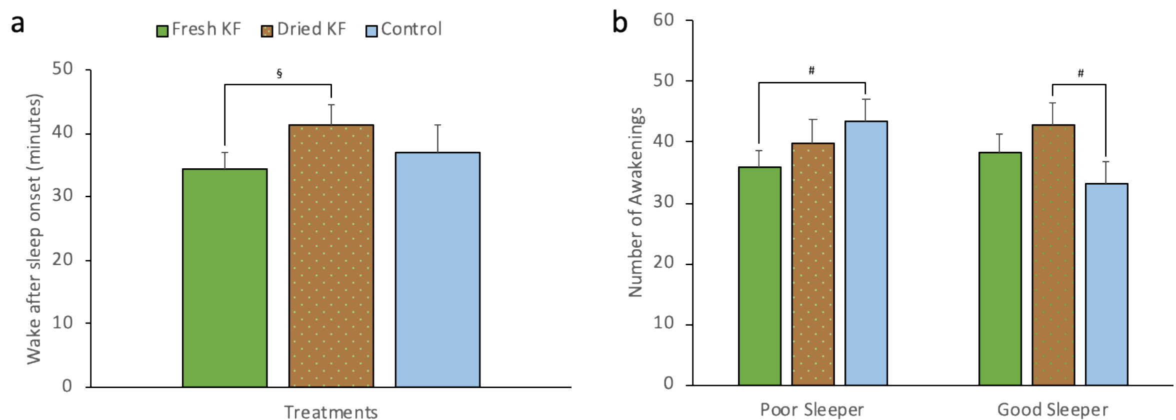


Figure 6.5 Estimated marginal means and standard error of means (SEM) for post-treatment objective sleep (actigraphy) measures of wake after sleep onset (a) and the number of awakening (b). §p < 0.05 represents the statistical difference from Dried KF values. #p < 0.1 represents statistical difference from control values.

Table 6.5 Objective sleep outcomes for the Fresh KF, dried KF and water control treatments in poor and good sleepers. Post hoc estimated marginal means and standard error of means (SEM) are presented with F and p values of the main effects and interaction from the linear mixed models.

Variable	Treatment	Poor sleeper (n = 12)	Good sleeper (n = 12)	Main effects		
					F	*p*
Latency (minutes)	Fresh KF	17.3 ± 4.3	8.8 ± 4.3	Treatment	1.45	0.256
	Dried KF	10.6 ± 3.4	12.1 ± 3.4	Sleeper type	0.49	0.491
	Control	20.8 ± 6.5	17.0 ± 6.5	Treatment x Sleeper type	1.23	0.312
Efficiency (%)	Fresh KF	85.4 ± 1.6	87.3 ± 1.6	Treatment	0.31	0.740
	Dried KF	86.4 ± 1.2	85.7 ± 1.2	Sleeper type	0.15	0.698
	Control	84.9 ± 1.9	85.6 ± 1.9	Treatment x Sleeper type	0.46	0.638
Total Sleep Time (minutes)	Fresh KF	389.3 ± 15.3	400.1 ± 15.3	Treatment	0.67	0.520
	Dried KF	409.5 ± 13.3	403.2 ± 13.3	Sleeper type	0.01	0.938
	Control	406.8 ± 20.9	398.1 ± 20.9	Treatment x Sleeper type	0.32	0.731
Wake after sleep onset (minutes)	Fresh KF	35.4 ± 3.8	33.4 ± 3.8	Treatment	3.74	0.040
	Dried KF	41.1 ± 4.5	41.7 ± 4.5	Sleeper type	0.64	0.433
	Control	43.1 ± 6.1	31.0 ± 6.1	Treatment x Sleeper type	1.37	0.274
Number of Awakenings	Fresh KF	35.9 ± 2.8	38.4 ± 2.8	Treatment	1.52	0.240
	Dried KF	39.9 ± 3.8	42.8 ± 3.8	Sleeper type	0.17	0.684
	Control	43.4 ± 3.5	33.3 ± 3.5	Treatment x Sleeper type	4.06	0.032
Average awakening length (minutes)	Fresh KF	1.0 ± 0.1	0.9 ± 0.1	Treatment	1.43	0.260
	Dried KF	1.0 ± 0.1	1.0 ± 0.1	Sleeper type	0.36	0.553
	Control	1.0 ± 0.1	0.9 ± 0.1	Treatment x Sleeper type	0.17	0.845

6.4.4 Urinary metabolites

A summary of urinary measures is in Table 6.6. A significant main effect of treatment for urinary 5HIAA concentration was identified [F (2, 22) = 19.15, p < 0.001 with post hoc comparisons identifying freeze-dried KF (6.2 ± 0.4 mg/g creatinine; p < 0.001) and fresh KF (6.6 ± 0.4 mg/g creatinine; p = 0.001) treatments as showing significantly higher 5HIAA concentration, as compared to the control (4.9 ± 0.4 mg/g creatinine) (Figure 6.6a). A significant main effect of sleeper type for urinary B-vitamin concentration was identified for nicotinamide [F (2, 22) = 6.11, p = 0.022], biotin [F (2, 22) = 5.77, p = 0.025], riboflavin [F (2, 22) = 6.85, p = 0.016], pyridoxamine [F (2, 22) = 7.71, p = 0.011], thiamine [F (2, 22) = 7.58, p = 0.012] and was near significant for pyridoxal [F (2, 22) = 4.28, p = 0.051]. The post hoc comparison revealed that good sleepers had significantly higher concentrations of all measured B-vitamins than poor sleepers (Figure 6.6b). No interaction effects were identified for any urinary metabolite measures. Additionally, the urinary concentration of 5HIAA and vitamin C in good sleepers was negatively related to total mood disturbance (5HIAA; r = -0.41, p = 0.04) and latency (vitamin C; r = -0.72, p < 0.005). A full within correlation table is presented in APPENDIX E.

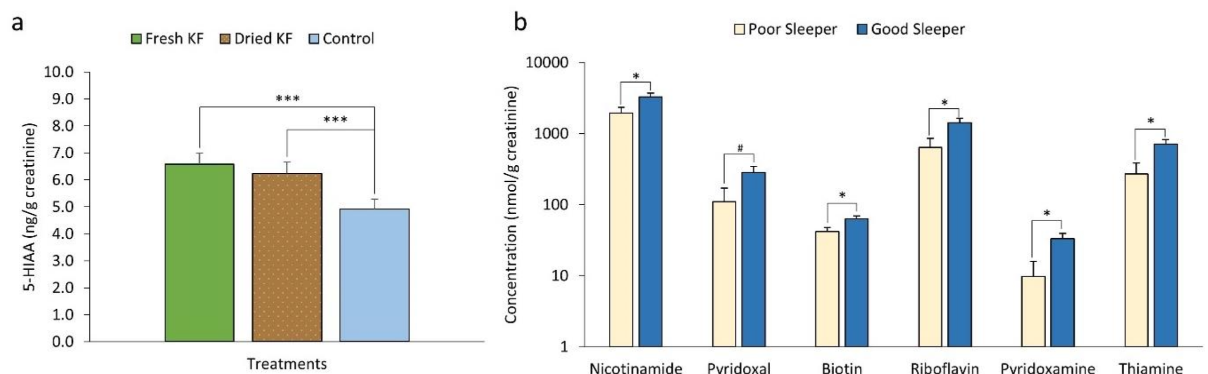


Figure 6.6 Estimated marginal means and standard error of means (SEM) for post-treatment urinary 5HIAA (a) and urinary B-vitamins. #p < 0.1 represents statistical difference between sleeper type. *p < 0.05 represents the statistical difference between sleeper types. ***p < 0.005 represents statistical difference from control concentrations.

Table 6.6 Urinary 5HIAA, aMT6s, vitamin C and B-vitamins for the Fresh KF, dried KF and water control treatments in poor and good sleepers. Post hoc estimated marginal means and standard error of means (SEM) are presented with F and p values of the main effects and interaction from the linear mixed models.

Variable	Treatment	Poor sleeper (n=12)	Good sleeper (n=12)	Main effects		
					F	*p*
5HIAA (ng/g creatinine)	Fresh KF	6.6 ± 0.6	6.5 ± 0.6	Treatment	19.15	0.001
	Dried KF	5.7 ± 0.6	6.7 ± 0.6	Sleeper type	0.30	0.591
	Control	4.8 ± 0.5	5.0 ± 0.5	Treatment x Sleeper type	1.72	0.202
aMT6s (ng/g creatinine)	Fresh KF	49,570 ± 5,771	39,644 ± 5,771	Treatment	0.27	0.769
	Dried KF	45,420 ± 4,523	40,819 ± 4,523	Sleeper type	1.02	0.323
	Control	46,983 ± 5,036	40,857 ± 5,036	Treatment x Sleeper type	0.79	0.466
Vitamin C (µmol/mmol creatinine)	Fresh KF	26.5 ± 15.0	27.9 ± 15.0	Treatment	1.98	0.161
	Dried KF	24.0 ± 17.6	27.3 ± 17.6	Sleeper type	0.00	0.983
	Control	13.9 ± 7.2	8.1 ± 7.2	Treatment x Sleeper type	0.06	0.938
Pantothenic Acid (nmol/g creatinine)	Fresh KF	5,488 ± 1,174	7,108 ± 1,174	Treatment	0.03	0.975
	Dried KF	5,753 ± 1,186	6,450 ± 1,186	Sleeper type	0.92	0.349
	Control	5,511 ± 1,173	6,597 ± 1,173	Treatment x Sleeper type	0.12	0.887
4-Pyridoxic Acid (nmol/g creatinine)	Fresh KF	4,241 ± 1,075	5,559 ± 1,075	Treatment	1.73	0.201
	Dried KF	3,975 ± 478	3,455 ± 478	Sleeper type	0.28	0.604
	Control	4,130 ± 1,016	4,754 ± 1,016	Treatment x Sleeper type	1.05	0.367
Nicotinamide (nmol/g creatinine)	Fresh KF	1,889 ± 880	3,960 ± 880	Treatment	0.55	0.587
	Dried KF	1,963 ± 455	2,871 ± 455	Sleeper type	6.11	0.022
	Control	1,973 ± 409	3,056 ± 409	Treatment x Sleeper type	0.68	0.515
Pyridoxal (nmol/g creatinine)	Fresh KF	121.9 ± 47.9	273.9 ± 47.9	Treatment	0.62	0.549
	Dried KF	109.7 ± 58.0	260.0 ± 58.0	Sleeper type	4.28	0.051
	Control	99.4 ± 79.5	316.4 ± 79.5	Treatment x Sleeper type	1.06	0.363
Biotin (nmol/g creatinine)	Fresh KF	42.5 ± 10.4	67.0 ± 10.4	Treatment	0.37	0.694
	Dried KF	41.7 ± 9.3	57.3 ± 9.3	Sleeper type	5.77	0.025
	Control	40.8 ± 9.9	66.1 ± 9.9	Treatment x Sleeper type	0.38	0.688
Riboflavin (nmol/g creatinine)	Fresh KF	505.2 ± 466.2	2,112.3 ± 466.2	Treatment	1.43	0.261
	Dried KF	600.4 ± 230.4	938.7 ± 230.4	Sleeper type	6.85	0.016
	Control	820.9 ± 328.3	1,226.8 ± 328.3	Treatment x Sleeper type	1.72	0.202
Pyridoxamine (nmol/g creatinine)	Fresh KF	9.9 ± 7.0	35.9 ± 7.0	Treatment	0.71	0.504
	Dried KF	9.4 ± 7.9	34.8 ± 7.9	Sleeper type	7.71	0.011
	Control	10.2 ± 4.7	28.9 ± 4.7	Treatment x Sleeper type	0.54	0.593
Thiamine (nmol/g creatinine)	Fresh KF	265.6 ± 181.4	923.1 ± 181.4	Treatment	0.92	0.414
	Dried KF	298.6 ± 155.4	547.5 ± 155.4	Sleeper type	7.58	0.012
	Control	242.0 ± 222.6	672.1 ± 222.6	Treatment x Sleeper type	1.48	0.250

6.5 Discussion

The current study indicates that regardless of participants' sleep quality, consuming a single intake of freeze-dried KF led to feelings of increased alertness and decreased sleepiness. Both fresh and freeze-dried KF increased urinary excretion of 5HIAA. Furthermore, poor sleepers felt it was easier to wake up in the morning after consuming freeze-dried KF in the evening and this was also moderately felt when consuming the fresh KF. Good sleepers rated themselves as getting to sleep easier when consuming the fresh KF. Interestingly, poor sleepers tended to wake more often in the night after consuming freeze-dried KF with the evening meal, while good sleepers woke less often during the night after consuming fresh KF with the evening meal.

The beneficial impacts observed when consuming KF on aspects of sleep quality are consistent with other KF sleep intervention studies that showed improvement in subjective daytime function ratings (6) and sleep quality measures (5). In addition, in a mouse model fed KF skin extracts sleep onset latency was reduced (118), thus providing further evidence supporting the beneficial impact of KF on sleep. Sleep is a complex process controlled by internal and external factors. There are potentially at least three explanations that could explain the observed outcomes. Firstly, it is known that KF is rich in phenylalanine, tyrosine, tryptophan, glutamic acid (27) and 5HT (37) that are further metabolized to produce dopaminergic, serotoninergic, and GABAergic neurochemicals. These neurotransmitters are vital in the regulation of sleep/wake cycles. Secondly, green KF contains the enzyme actinidin, which has been shown to cause an earlier peak increase in plasma circulating amino acids in adults when compared to KF without actinidin (82). Therefore, potentially increasing the concentration of bioavailable amino acids to be further metabolized into neurochemicals. Lastly, KF may act on improving

sleep via potentiating the bioaminergic responses. This has been demonstrated in *in vitro* studies (246). Given this, KF may impact sleep through the supply and modulation of peripheral neurochemical responses, which may impact systemic concentration modifications throughout the rest of the body. Further studies are needed to determine which postprandial biomarkers are increased in sleep and upon waking the following morning to elucidate how KF may impact sleep and subsequent mood.

The current study also shows that irrespective of sleep quality participant experience, acutely supplemented freeze-dried KF resulted in improved vigour compared to the control. Likewise, fresh, and freeze-dried KF treatments tended to improve esteem-related affects, and freeze-dried KF tended to decrease total mood disturbance score compared to the control. The impacts of consuming KF on aspects of waking mood in this study are consistent with earlier KF mood studies that showed improvement in mood (3, 4).

Similarly, the various underlying regulatory mechanisms of mood are complex. Firstly, vitamin C is a potent water-soluble antioxidant with distinct roles in the body, including reducing systemic inflammation and as co-factors in production of neurotransmitters. Consumption of KF reduces oxidative stress and inflammatory markers (111). Additionally, higher vitamin C status has been correlated with decreased total mood disturbances (447), and regular consumption of KF increases vitamin C in the body (76). Secondly, neurochemicals, such as 5HT, provided by KF, may also enhance mood (181). Lastly, KF is rich in other vitamins and polyphenols, which may have facilitated these mood improvements by improving oxidative stress and metabolism. Given this, KF may have impacted mood via the supply of vitamin C and other nutrients, which altered the metabolism of neurochemicals and may also have affected oxidative stress. One cannot

rule out that improved sleep might have led to improved mood outcomes and vice versa (448).

No differences between KF treatments were observed for urinary aMT6s and vitamin C. The correlation analysis of urinary metabolites with sleep and mood measures indicated that urinary aMT6s and vitamin C morning excretion were negatively related to sleep latency. Waking urinary aMT6s can be used to predict nocturnal plasma melatonin (429) and lower urinary aMT6s excretion has been associated with lowered alertness (427). The results of aMT6s can be interpreted in two ways; first, the lack of treatment effects on excreted aMT6s may be due to insufficient samples collected or only one sample collected upon waking. Studies have shown that a 24–48-hour collection period may better reflect these changes in urinary aMT6s (449). Dim light melatonin onset, a circadian phase marker measured over a five to seven-hour window in plasma or saliva before bed, could be measured to assess whether KF may alter the circadian phase. Second, the finding may suggest that another mechanism not related to melatonin may facilitate the acute improvements in sleep quality and mood by KF. However, regardless of sleep quality, consumption of fresh and dried KF treatments increased urinary excretion of 5HIAA compared to the control, which is consistent with data on the acute effects of KF consumption on urinary 5HIAA (8). Comparably, in a different study, participants consuming tart cherries increased morning urinary 5HIAA excretion and improved mood (434). 5HIAA is the primary metabolite of 5HT and is involved with mood regulation (181). The results here suggested that consumption of fresh or freeze-dried KF may have increased circulating and brain levels of 5HT, which impacted mood. Concentrations of excreted B-vitamins were greater for good sleepers than for poor sleepers, regardless of the treatment received. Excretion of B-Vitamins can be used as

an indicator of baseline B-vitamin status, with higher excretion suggesting saturation and lowered amounts indicating deficiency (450). The lower amount of B-vitamins may be explained by a lower background dietary intake of B-vitamins, however this is speculation and should be interpreted with caution as no dietary records were collected. It may be suggested that poor sleepers utilize more B-vitamins for cellular metabolic processes. For example, nicotinamide (a form of B3), pyridoxine and pyridoxamine (forms of B6) are required as co-factors in metabolizing neurotransmitters (451). Additionally, low levels of B-vitamins have also been associated with low-grade inflammation (452) and poor sleep caused increased circulating inflammatory markers (453). However, this study did not measure any inflammatory markers, suggesting that inflammatory status is worth exploring in future studies. Other factors that were not assessed that may also impact B-vitamins include exercise status, which is known to increase the requirements for B-vitamins (454).

Irrespective of sleep quality participant experience, acutely supplemented freeze-dried KF improved morning sleepiness compared to both fresh KF treatment and control. This finding could be explained by the structural and compositional differences between KF interventions; a KF drink that contained the skin (liquid) or KF flesh eaten fresh without the skin (solid). Including skin in the freeze-dried powder altered the polyphenolic content, and as mentioned previously, KF skin extracts potentiate sleep induction in a pentobarbital-induced sleep mouse model (118). Thus, the freeze-dried powder may be improving sleep onset, thus causing better morning alertness. The results on sleep onset suggested that this may occur in poor sleepers. Furthermore, the added polyphenols may be impacting the body by affecting the expression of clock genes (455), improving

CBF (thus, improving mood (456)), altering the permeability of the BBB and/or altering neurotransmissions (457).

Nevertheless, it is worth noting that the food structure may influence stomach emptying rate and satiety and cause differential interactions with other food matrices, consequently impacting gut comfort. For example, consumption of a carbohydrate-rich meal with KF lowered postprandial hunger compared to consumption of a carbohydrate meal only (72). Also, consuming fresh green actinidin-containing KF has improved gastric comfort compared to KF without actinidin (44). These findings suggest that improvements in sleep quality may be due to reduced feelings of bloating and hunger; however, these parameters were not measured in this study.

Contrary to the previous KF and sleep quality studies, this study was the first to assess the acute and separate effects of a fresh KF or dried (containing skin) KF treatment on sleep quality and mood in a healthy male cohort with good or poor-quality sleep. The urinary 5HIAA and aMT6s concentrations suggest a novel potential mechanism underpinning the relationship between KF and sleep. Additionally, the results provide evidence for the repurposing of KF skin and lower-quality fruit, which would otherwise be wasted, into products that can easily be stored with extended shelf-life.

This study is not without limitations. One of the limitations was the low subject numbers, the study was powered using a different intervention to this (445), as this was the first study to assess acute impact of KF on LSEQ. Secondly, due to the nature of the intervention, it was impossible to blind participants to their treatment. This is evident in the ratings for getting to sleep and quality of sleep in good sleepers. Fresh KF was on average higher than both the control and freeze-dried KF, suggesting that some participants may have preconceived notions about one treatment over another. Thirdly,

because the study was conducted only on men, it is difficult to interpret the results for women. Fourthly, the control used was not iso-calorically matched, suggesting participants may have rated themselves poorly on the control intervention due to other factors such as appetite and hunger, which were not measured. Furthermore, urine samples were limited to morning samples, with no other timepoints collected. Collecting samples other than urine at different time points may have allowed a better understanding of the postprandial changes that have occurred. Collection of plasma and saliva pre and post intervention and up to five hours before bedtime may have provided a better understanding of melatonin circulation, which may have influenced sleep (449). In addition, saliva could have been collected upon waking and used to quantify cortisol awakening response (CAR), which could provide a simple measure of the reactive capacity of the HPA axis.

Although actigraphy was used here and in other acute settings (458), results should be interpreted with caution. For instance, a person lying still while awake may be recorded as asleep due to immobility. Actigraphy does not account for these events. Using polysomnography was not feasible in this study due to the in-home setting, but would have provided a better understanding of the actual acute impacts of KF and sleep quality and architecture (459). Future studies could incorporate a larger cohort with participants of mixed cohort of males and females consuming differing doses of the freeze-dried KF. Furthermore, future studies could use an energy matched placebo with the same amounts of vitamin C and B-vitamins. Additionally, other cohorts worth exploring include older adults due to age-related sleep disturbances (460), University students under high stress (461), or inpatients in planned care services (462).

6.6 Conclusions

Overall, this study is the first to demonstrate that a single evening meal with KF, whether fresh or freeze-dried, improved sleep quality and mood in males with good or poor-quality sleep. This effect may have been mediated through increased 5HT metabolism. Further studies should be conducted to elucidate the impact of freeze-dried KF on those who experience poor sleep and the potential mechanisms by measuring other biomarkers in urine, plasma, and saliva. Nonetheless, as beneficial effects of sleep were identified following supplementation with KF in young men, these data help provide additional evidence for the role of KF in facilitating healthy sleep regulation.

CHAPTER 7. General discussion

The initial aim of this thesis was to investigate the effects of KF on sleep quality and explore the potential mechanisms by measuring a selection of biomarkers in a range of biospecimens. However, due to the global pandemic, this was not feasible. Therefore, a shift in approach was needed, focusing on understanding the neurobiological impacts of gold and green KF.

This research hypothesised that *Actinidia deliciosa* (green KF-containing actinidin) would have more benefits compared to a gold KF variety (without actinidin). Additionally, it was hypothesised that green KF skin could enhance sleep and mood due to its polyphenol content. This research used a multifaceted approach to explore three different mechanisms which could all contribute to neurobiological impacts (Figure 7.1).

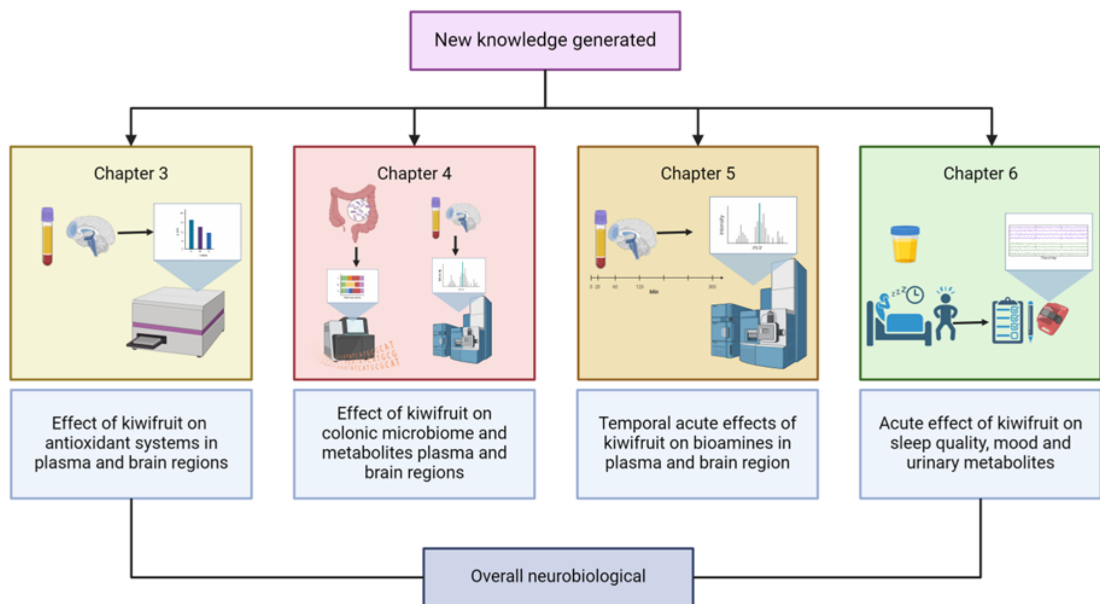


Figure 7.1 Novel insights contributed by this PhD research findings. Figure Created with BioRender.com.

In this doctorate thesis, a series of experiments were conducted to explore the potential effects of KF on neurobiological aspects. This investigation explored how KF may modulate plasma and brain regional antioxidant systems and bioamine concentrations and the proximal and distal colonic gut microbiome in a pig model of human metabolism. The beneficial effects of KF using samples generated from the same pig study are highlighted in Chapters 3, 4 and 5. Finally in Chapter 6, a human study was conducted to explore the potential benefits of the acute consumption of KF on sleep quality and mood while trying to uncover how this may be modulated by measuring urinary metabolites. The research findings from both studies contribute to understanding the potential mechanism by which KF may positively impact the brain and highlight mechanisms that may potentiate benefits for both sleep and mood.

7.1 Main findings

In summary, both green and gold KF demonstrated significantly decreased oxidative generating potential in plasma (Chapter 3). Additionally, green KF significantly increased the antioxidant protective potential in plasma and each measured brain region. Gold KF significantly elevated plasma vitamin C levels and exhibited a trend towards reducing AChE activity across the entire brain. Significant increases were observed in the relative abundance of several genera, including *Faecalibacterium*, *Pseudoflavonifractor*, *Flavonifractor*, *Intestinimonas*, *Faecalicoccus*, *Butyricicoccus*, *Eggerthella*, and *Slackia* in Chapter 4. Conversely, a significant reduction in relative abundance was noted in the genera *Prevotella*, *Streptococcus*, *Galactobacillus*, *Megasphaera*, *Lactimicrobium*, and *Enterococcus* in both the proximal and distal colon in response to both green and gold KF. Lastly, both green and gold KF demonstrated significant increased peripheral plasma concentrations of 5HIAA over an eight-day intervention period.

The benefits of actinidin-containing green KF in plasma and brain samples collected within five hours after consumption on the ninth day of the intervention are highlighted in chapter 5, using the same pigs discussed in Chapters 3 and 4 of the pig study. The results reveal that green KF consumption increased plasma histidine compared to gold KF. A clear pattern appeared in the measurement of metabolites in the brain in response to the two treatments. Gold KF showed a decline in metabolites before increasing, while green KF led to a rapid increase. However, at five hours postprandial, the metabolite levels appeared to equalise to similar concentrations. These observations show that green KF causes an increased concentration of metabolites for the first two hours when consumed with a meal, although the dynamics between 2 to 5 hours remain unclear. GABA emerged as a metabolite with interaction effects in all brain regions, showing an increase in concentration with the green KF-supplemented diet and a decrease with gold KF within the first two hours.

Furthermore, green KF supplementation maintained 5HT levels in the hippocampus over time but did not with gold KF. The brain stem showed minimal response to KF variety. Conversely, the corpus striatum had significant time and treatment interaction effects, while the hippocampus and prefrontal cortex responded similarly to both types, suggesting a similar capacity for modulation by KF type.

When compared to a control, KF demonstrated a positive impact on sleep quality and mood aspects in individuals with good and poor sleep quality in Chapter 6. Good sleepers reported significantly easier sleep onset and woke less often during the night after consuming fresh KF with the evening meal compared to the control. Interestingly, poor sleepers tended to experience more night-time awakenings after consuming

freeze-dried KF with the evening meal compared to the control. Both fresh and freeze-dried KF compared to the control were found to significantly increase the urinary excretion of 5HIAA. Regardless of the participants' sleep quality, the acute supplementation of freeze-dried KF significantly led to reduced feelings of sleepiness and increased alertness compared to the control. Additionally, including the fruit skin appeared to significantly enhance improvements in sleep quality, potentially exerting a more pronounced and potent effect.

Overall, KF contributes to oxidative protective potential in the body and brain, enhances growth of beneficial colonic microbiome, modulates the concentration of some neurotransmitter metabolites, and provides acute benefits for sleep and mood.

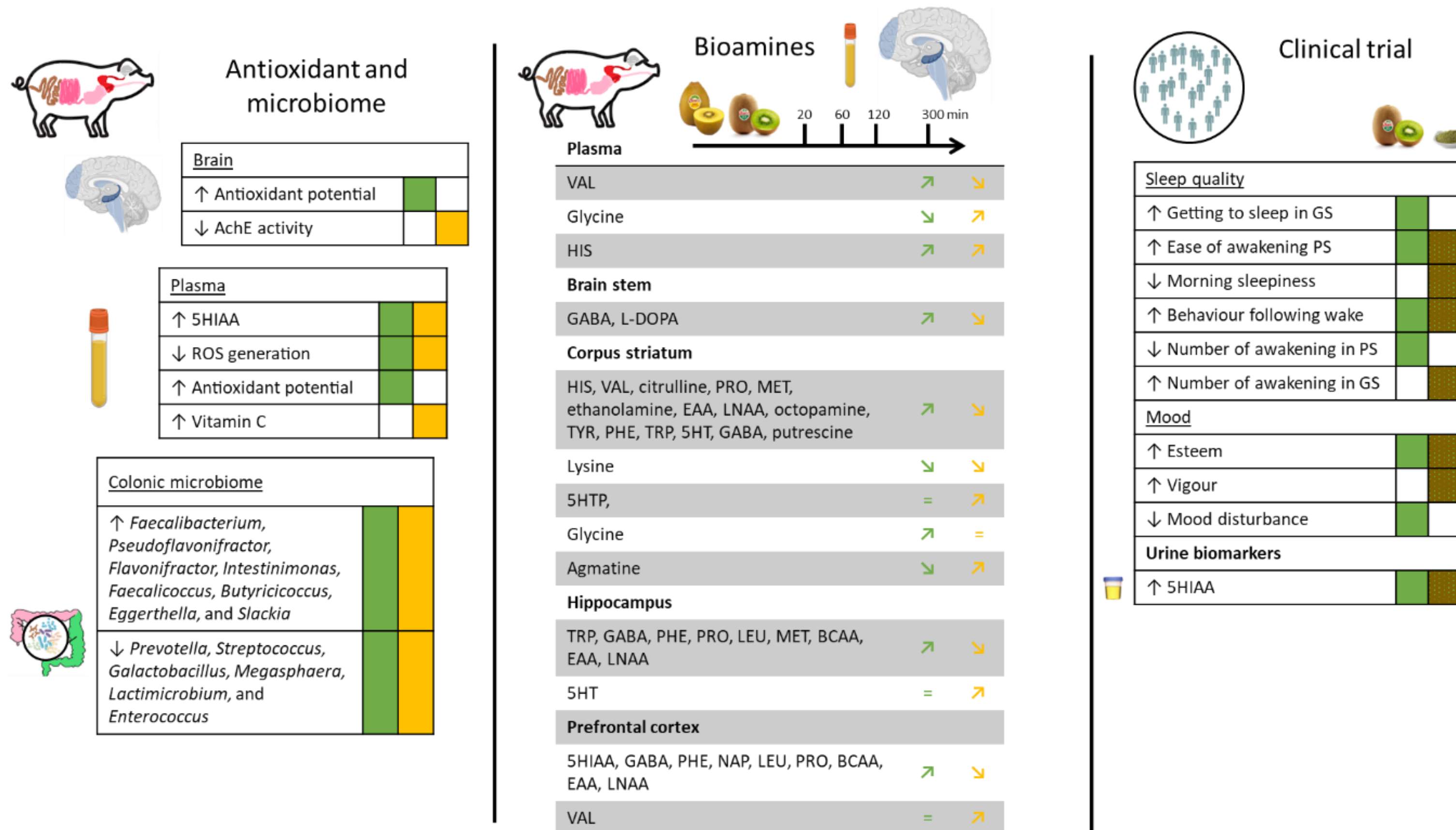


Figure 7.2 Overview of the effects of KF supplementation from this thesis on antioxidant and colonic microbiome, bioamine concentrations in plasma and brain regions, and clinical trial. In the antioxidants and microbiome, the coloured boxes indicate which KF variety had an impact and directional effect on measured outcomes. In the bioamines, the arrows indicate the trend direction in the first two hours and the arrow's colour indicates the type of KF. Lastly, in the clinical trial, the coloured boxes indicate which KF treatment (fresh without skin vs powder with skin) had a specific directional impact on the measured outcome. AChE, Acetylcholinesterase; 5HIAA, 5-Hydroxyindoleacetic acid; ROS, Reactive oxidative species; VAL, valine; HIS, histidine; GABA, Gamma-aminobutyric acid; L-DOPA, 3,4-dihydroxyphenylalanine; PRO, proline; MET, methionine; EAA, essential amino acids; LNAA, large neutral amino acids; TYR, tyrosine; PHE, phenylalanine; TRP, tryptophan; 5HT, serotonin; 5HTP, 5-Hydroxytryptophan; LEU, leucine; BCAA, branched-chain amino acids; NAP, n-acetylputrescine; GS, good sleeper; PS, poor sleeper.

7.1.1 Kiwifruits contribute to brain antioxidant protection.

In this study, the two KF varieties demonstrated a reduction in the generation of plasma ROS, which might be attributed to the presence of micronutrients that play a crucial role in scavenging free radicals, contribute to preventing cellular damage and maintaining overall cellular health. This observation aligns with other research showing the protective potential of KF against oxidative stress *in vitro* (106), rodent models (243, 244) and particularly in women who consumed gold KF before a bout of acute high-intensity exercise (10).

Furthermore, research has shown KF consumption's potential impact on the gut microbiome (12). This study observed increases in known microbial SCFA producers, such as the genus *Faecalibacterium*, in the proximal and distal colon. This genus is associated with anti-inflammatory effects in the intestine, producing SCFAs, especially butyrate, which is linked to reducing inflammation in the intestinal lining (463). While it is not conclusively known whether this increase in the genus *Faecalibacterium* relates to a reduction in systemic oxidative stress, nor is it measured here, evidence indicates the potential to at least control oxidative stress within the intestine. Further evidence would be required to substantiate this hypothesis.

7.1.2 Kiwifruit may facilitate neurochemicals metabolism

The intricate interplay between KF consumption and its potential influence on various physiological processes, including amino acid modulation, gut microbiome composition, and subsequent effects on neurotransmitter synthesis and the brain, represents an intriguing area of research. It is understood from previous research that KF may influence tryptophan metabolism. KF consumption increased gastric and intestinal

digesta of 5HTP (246) and urinary 5HIAA (8). This PhD research contributes to the current understanding by demonstrating that KF led to elevated plasma (Chapter 4) and urinary 5HIAA (Chapter 6) levels compared to the control group. Furthermore, it provides evidence for the temporal differential response of the green and gold KF treatments (Chapter 5). Both KF varieties exhibited the same consistent rise in plasma 5HT levels over the 5-hour period, while 5HIAA, peaked within the first two hours before declining. However, their effects on tryptophan metabolites in the brain differed between the two KF varieties. Green KF showed an increase in tryptophan levels in the corpus and hippocampus during the initial two hours, along with a simultaneous rise of 5HTP in the corpus and 5HIAA in the prefrontal cortex and brain stem. In contrast, gold KF displayed a decrease in concentrations of these before mentioned metabolites during the first two hours in the brain. Notably, the concentrations of these metabolites converged to similar levels at the 5-hour mark after both treatments. This suggests that green KF influences tryptophan metabolism in the brain, indicating its potential therapeutic role in modulating neurological pathways. However, additional studies are needed to confirm these findings.

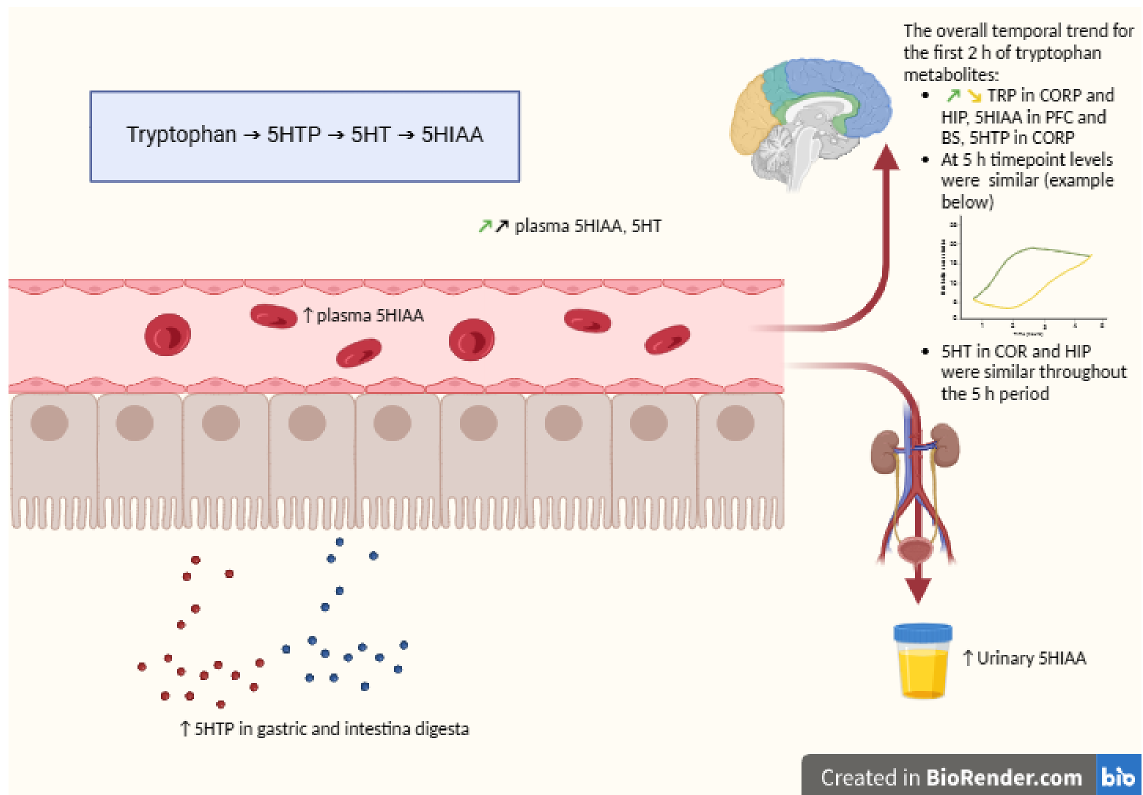


Figure 7.3 Simplified diagram of the potential impact of KF in gut, peripheral, brain, and excretion on tryptophan metabolite concentrations. 5HIAA, 5-Hydroxyindoleacetic acid; 5HT, serotonin; 5HTP, 5-Hydroxytryptophan; CORP, corpus striatum; PFR, prefrontal cortex; Hip, hippocampus; BS, brain stem. Figure Created with BioRender.com.

The neurological impact of green KF on GABA concentrations starkly contrasts from that of gold KF (Chapter 5). Green KF increased GABA concentration in all brain regions during the initial two hours, whereas gold KF showed a decreasing trend. These results support existing hypotheses regarding KF's interaction with GABA receptors and have the potential to explain observations from animal studies, where the peel extract of green KF has demonstrated greater effectiveness in promoting sleep compared to its gold counterpart (118).

Several factors may contribute to these observed effects, including actinidin, recognised for enhancing the release of amino acids during small intestinal digestion (95) and appearance in plasma (82). While the pig study controlled for protein intakes, the

persistence of differences in KF responses underscores the influence of the KF cultivar. It is plausible that actinidin plays a crucial role in producing peptides with potential barrier-altering properties (30).

Additionally, despite both KF varieties being similar in macronutrients, specific micronutrient profiles differ. The two types of KF exhibit variations in vitamin C, vitamin E, and polyphenol content. Vitamin C, crucial for neurotransmitter metabolism, is in higher quantities in the gold KF. Consequently, one would expect the gold KF to offer additional benefits on neurotransmitter concentrations, which does not align with the observed results. A potential explanation lies in the U-shaped relationship of nutrients (464) where moderate levels are advantageous, but both deficiency and excess can cause problems. Further research is necessary to validate this.

The observed effects (including trends) on metabolite concentrations may also be attributed to the microbiota community. The gut microbiome plays a pivotal role in digestion, nutrient absorption, and overall health. KF is considered a prebiotic, and its fibre and polyphenol contents may influence the composition and activity of the gut microbiota. This, in turn, has implications for overall health and might be linked to neurotransmitter synthesis. For example, species of the *Streptococcus*, *Escherichia*, and *Enterococcus* genera produce 5HT, while *Lactobacillus* and *Bifidobacterium* genera produce GABA (142). In our pig study, both KF varieties reduced the abundance of known microbial 5HT producers while not affecting known microbial GABA producers. These observations suggest that the differences in peripheral and brain neurotransmitters concentrations might be influenced by other unknown bacteria, or that the effects of gut microbiota on neurotransmitter concentrations could be

apparent in the intestinal contents, which were not measured. More research is required to confirm this.

7.1.3 Kiwifruit provides acute benefits on sleep and mood

From the results of the pig study, it was decided that green KF would be selected to progress into the human study. The skin in the powder form was also used to confirm the beneficial impacts found in rodent studies (118, 119). Study participants reported improved sleep onset, awakening, and behaviour after consuming green KF once. Furthermore, subjective mood assessments demonstrated positive changes, with individuals reporting reduced mood disturbances and enhanced overall vigour.

Sleep and mood have long been associated with changes in tryptophan metabolism. Our results confirm this observation, demonstrating that KF affects these metabolites. Both the pig study and clinical trials revealed increases in metabolites along the tryptophan metabolic pathway, suggesting the potential role of KF in this process. This could be attributed to KF serving as a natural source of tryptophan, a precursor for 5HT and melatonin, as well as being a source of 5HT itself. The clinical study results indicate that KF improves sleep quality and increases the excretion of 5HIAA. Additionally, findings from the pig study support the potential impact of KF on sleep by providing evidence of its influence on tryptophan metabolites, as mentioned previously.

KF is considered rich in GABA, adding another dimension to its impact on sleep and mood. GABA is the primary inhibitory neurotransmitter in the brain, contributing to relaxation and calmness. As observed in the temporal study, the consumption of green KF steadily increases GABA concentration in various brain regions over 5 hours.

Understanding this and considering how it explains the observed impacts on sleep in the clinical study, it is plausible that GABA plays a role in improving sleep onset, as reported by participants.

Beyond neurotransmitters, KF also exerts an influence on the gut microbiota. The prebiotic properties of KF support the growth of beneficial gut microbes (12, 54, 55), potentially alleviating mood disorders and sleep disturbances linked to gut dysbiosis. Notably, KF was found to increase the relative abundance of family members of *Lachnospiraceae*, a family positively correlated with sleep efficiency and total sleep time (465). Additionally, KF was observed to enhance the relative abundance of *Faecalibacterium*, which has been positively correlated with sleep quality scores (466). This effect is hypothesised to be linked to butyrate production by this genus, with other studies suggesting the potential of butyrate as a bacterial-derived sleep-promoting signal (467). While these are intriguing hypotheses, it is essential to note that the current study did not measure faecal or plasma fatty acid concentrations. Notably, the human clinical study was limited to a single serving, with no stool samples collected to quantify the microbiome population, which presents a limitation in exploring this potential link.

Furthermore, the antioxidant content of KF, including vitamins C and E and phytochemicals, offers protection against oxidative stress. This finding becomes especially relevant during sleep when the brain actively removes excess free radicals generated throughout the day (468). It is plausible that adding KF enhances this process, as indicated by the increased antioxidant protection capacity five hours post-consumption.

7.2 Research significance

7.2.1 Scientific knowledge

This research aimed to investigate the brain effects of KF in a pig model, which better aligns with human physiology, addressing the current lack of information in this area. Notably, this research represents the first scientific exploration of the effects of KF on antioxidants and neurochemicals in a pig model, leading to the following accomplishments:

1. Antioxidant neuroprotective potential of KF within specific brain regions observed during a short intervention period.
2. Distinct effects on the colonic microbiome of two types of KF in a pig model, along with the impact on bioamine concentrations in both plasma and the brain.
3. Temporal response of bioamine concentrations to KF in a growing pig model's plasma and various brain regions, providing insights into the dynamic nature of these effects.
4. The potential acute benefits of fresh and freeze-dried green KF formats on sleep quality and mood in participants with self-reported good and poor sleep quality.

This research contributes essential data to understanding the potential impact of KF consumption on antioxidant concentrations, neurochemical concentrations, sleep quality, and mood. These findings advance our understanding of KF and provide valuable implications for its potential applications in human health.

7.2.2 Applications

This research reveals insights applicable to human health. Recognising GABA's role in sleep onset, our data suggests that consuming green KF five hours before bedtime may enhance sleep quality. While concerns about daytime consumption causing sleepiness may exist, sleep signals are mainly influenced by light exposure and circadian rhythms

(469). Thus, daytime green KF intake may promote calmness without inducing sleep. Regulating light exposure before bedtime is crucial for maximising sleep benefits.

The implications of green KF extend beyond sleep to impact cognitive function. The highest concentration of bioamines in specific brain regions, induced by green KF, peaks within two hours post-ingestion. This suggests that to optimise the benefits of consuming KF, they should be ingested two hours before stressful or cognitively demanding activities.

Additionally, to promote SCFA-producing gut bacteria growth, the study recommends consuming two doses of either gold or green KF daily. This regimen may enhance gastrointestinal health, as evidenced in humans (12). Similarly, for brain antioxidant benefits, consumption of the two green KF varieties is advised. This intake of green and gold KF may prime the body to produce fewer ROS, potentially mitigating free radical effects, reducing oxidative stress and inflammation. This protective effect bolsters overall health, fortifies the immune system, and may aid in preventing chronic diseases.

However, caution is advised when applying this research, as a weekly intake was used in the pig study and may not yield the same benefits in humans and needs further testing. Furthermore, we cannot directly conclude whether the effects would improve with longer-term consumption or if the benefits would be sustained if interventions were stopped.

7.3 Limitations

The research provides valuable insights into the potential benefits of KF consumption, but it is vital to acknowledge and explore its limitations. Firstly, one limitation is using

healthy pigs in the animal study. While this choice is common in research, it raises concerns about the generalisability of the findings to populations with underlying health conditions. Individuals with pre-existing health issues may respond differently to KF interventions, hindering the broader applicability of the research. Secondly, the animal study was not designed to investigate changes in antioxidants, the gut microbiome, or metabolites. This limitation is crucial as it restricts the comprehensive understanding of the mechanisms underlying the observed effects of the intervention. Thirdly, another notable limitation is the need for more behaviour measures in the animal study. While the study measured physiological changes of antioxidants and metabolites, the absence of behavioural assessments makes it challenging to establish whether the observed changes in physiological parameters translate into positive behavioural outcomes.

Fourthly, the absence of a 'control with no KF' arm in the temporal study further diminishes the robustness of the findings. Including a ‘no KF’ control arm in studies is crucial for comparing the effects of KF interventions, ensuring observed changes are attributed solely to KF and enhancing result reliability compared to comparing KF against other cultivars. Fifthly, an inherent limitation of the study is the use of bread as the sole background diet, simplifying the experimental design to investigate gluten but not accurately representing general population dietary habits. Future research with a more diverse diet would show how KF interacts with a broader range of food constituents in real-world scenarios. However, these samples were collected as part of another study, so this limitation was beyond the scope of the intended PhD research. Moreover, a limitation of both pig and human studies is the exclusive assessment of males. This gender bias restricts the generalisability of the findings to the broader population, as

potential gender-specific effects or responses to the intervention may not be adequately represented.

The study's limitations also apply to the scope of metabolite measurements used, which focused on bioamines and their metabolites, and may not capture all KF-induced biochemical changes. Employing untargeted metabolomics could reveal new biomarkers or pathways associated with KF consumption, despite its limitations. Lastly, the study's acute nature prompts investigation into the longevity of the observed benefits of consuming KF. Additionally, the suitability of the chosen controls in the study needs to be examined. Future research with extended intervention periods and carefully selected controls will provide a clearer understanding of the temporal dynamics and sustained effects of KF consumption.

In conclusion, while the thesis offers valuable insights, the outlined limitations underscore the need for caution in extrapolating the findings to broader populations and contexts. Future studies addressing these limitations could provide a more robust foundation for understanding the potential benefits of fresh KF consumption on neurobiological outcomes.

7.4 Future recommendations

For future research, the core recommendation for any subsequent research is the selection of the appropriate KF cultivar. Green and gold KF varieties, as shown in this thesis, notably affect measured outcomes, suggesting each cultivar may target specific brain aspects differently. Previous studies have also explored other KF cultivars, such as ‘hardy’ and ‘Qinmei’, demonstrating positive health benefits. Additionally, further

studies could investigate the format of KF (fresh, powder or juice) and explore different extraction techniques as well as isolating specific components (skin, flesh, or seeds). After selecting a specific KF ingredient, the following suggestions are presented systematically, utilising tissue culture, pig, and human studies. They seek to improve our comprehension of KF's impact on the brain.

7.4.1 Tissue culture experiments

Defining the study's primary aim is essential in considering tissue culture experiments. Different cell lines facilitate testing various hypotheses. The following outlines potential future cell culture experiments:

- Exploring the PC12 cell line from a rat adrenal medulla pheochromocytoma could offer new insights into KF's impact on the GABA signalling pathway, complementing past antioxidant pathway studies. Neuro-2a and SH-SY5Y cells provide additional options for neurobiological investigations.
- Utilising the cerebromicrovascular endothelial cell line hCMEC/D3 as a model for the human BBB could provide initial insights into the effects of KF. Applying whole KF or isolates like kwellin/kissper could be attempted.
- Additionally, exploring the impact of KF on monocyte differentiation is of interest. PMA-induced differentiation of THP-1 coculture with polyamines has shown changes in macrophage differentiation into M1 or M2 nature (470). A similar approach could be applied to KF to assess potential differential phenotypes of macrophages.

- Subsequently, a comparable method could be applied to microglial tissue culture experiments using a differentiation protocol of peripheral blood mononuclear cells (471).
- Exploring co-culture, tri-culture, and organoid methods could effectively mimic cell type relationships in native tissue, overcoming inefficiencies typically seen with single cell lines alone.

Utilising this diverse set of cell lines allows for a more holistic examination of the potential beneficial effects of KF on various tissues and systems within the body.

7.4.2 Animal studies

- To comprehensively study stress impact on animals, external stressors like diet manipulation or controlled environmental stressors could be introduced.
- Quantify metabolites in the digestive system, blood, and brain tissue to understand metabolic aspects and the gut microbiome's role in health.
- Incorporate behavioural measurements, advanced imaging like magnetic resonance imaging (MRI), for a longitudinal brain structure and function study.
- An approach combining external stressors, metabolic profiling, behavioural assessments, and advanced imaging techniques will provide a more comprehensive understanding of the intricate relationship between stress and its physiology.

7.4.3 Human clinical studies

For comprehensive future studies on KF's impact on sleep and cognition, consider refining the methodology across various aspects:

- Incorporating iso-caloric meals as a control group is critical to isolate KF effects from calorie-related variables, ensuring accurate outcome interpretation.
- Future sleep research should explore sleep architecture, circadian rhythms, dim light melatonin onset, and cortisol awakening responses for deeper insights into KF's impact on sleep mechanisms.
- Adding waking cognitive performance assessments and extending them throughout the day would provide a more thorough understanding of KF's cognitive impact.
- Conducting KF dose-response studies is vital to establish dosage recommendations.
- Investigating the optimal timing of KF consumption (evening, one hour before bedtime, morning) could reveal important efficacy nuances.
- Considering age and sex variations explicitly in participant selection enhances understanding of KF's effects on sleep and cognition.
- Longitudinal studies are crucial to assess KF's long-term impact and consider the influence of lifestyle factors.
- Including subjective measures like self-reported sleep quality and cognitive function, such as self-reported assessments and participant feedback, adds qualitative depth to quantitative data, offering a holistic view of participant experiences and study outcomes.

As mentioned previously, the pandemic interrupted this PhD research. Some considerations to improve the clinical study have been integrated into a subsequent trial

design which has ethical approval. The study protocol is available for review in the APPENDIX F.

7.5 Concluding remarks

In summary, this doctoral thesis represents a novel examination of the neurobiological effects of New Zealand KF. The research explores the impact of KF on antioxidants, gut microbiome, neurochemicals, and clinical outcomes through animal and clinical studies. Notably, this study is the first comprehensive report on the potential neuroprotective properties of KF in a pig model, which is a model that has close relevance to human subjects. The findings reveal decreased oxidative potential and increased plasma concentrations of 5HIAA, accompanied by changes in the relative abundance of specific beneficial genera within the colonic microbiota in response to green and gold KF. Green KF demonstrates increased antioxidant potential and alterations in brain region bioamines. The temporal aspects highlight distinct metabolite patterns between green and gold KF, especially in the corpus striatum. KF's influence on GABA and 5HT concentrations in the brain varies over time, revealing the complexity of its neurochemical effects. Acute clinical benefits are evident, with positive impacts on sleep quality and mood for individuals with varying sleep quality. This thesis enhances our understanding of the multifaceted effects of KF, paving the way for future research and potential applications in nutritional interventions.

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APPENDIX A

SUPPLEMENTARY MATERIALS TO CHAPTER 2

Table A.1 Effects of kiwifruit and products made from kiwifruit on biological and clinical markers of human health. Main findings reported were all that were statistically significant (P<0.05).

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Pre and post	2 flesh of green KF for 4 weeks 1h before bed	N/A	Mixed cohort 34.4 ± 12.9 y Bad sleep	24	Sleep quality	None	Sleep diary ↓WASO, SOL, CPSQI, ↑TST, SE P<0.05 Actigraphy = ↑TST, SE P<0.05	(5)
Parallel	130 g flesh of green KF for 4 weeks 1hr before bed	Pear	Mixed cohort 24.5 ± 3.6 y Insomnia	37	Sleep quality	None	↑sleep quality and daytime functioning	(6)
Parallel	0.5 or 2 flesh of gold KF (Hort16A) for 6 weeks	KF doses	Males 21 ± 3 y	36	Mood	Blood Urine	↓ total mood, depression ↑Vit E and serum D3, plasma vit C, urine vit C	(3)
Parallel	2 flesh of SunGold KF for 4 weeks	Vitamin C or placebo	Mixed cohort 20 ± 2 y	57	Mood	Blood	↑ plasma vitamin C, mood, and well-being	(4)
Pre and post	2 flesh of green KF for 4 weeks 1h before bed	N/A	Mixed cohort 23.2 ± 3.9 y Athletes	15	Sleep quality Stress	None	↑sleep quality ↓ stress	(7)
Parallel	2 flesh of green KF for 4 weeks	No KF	Female 31.5 ± 1.3 y	11	Digestive health Blood flow Skin health	None	↑ bowel movement, blood flow on fingers, and skin brightness ↓ bowel discomfort	(48)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Crossover	2 flesh of green KF + isoflavone supp. (50 mg/day aglycone daidzein and genistein) for 6 weeks	Isoflavone supp. only	Post-menopausal females 56.1 ± 0.8 y	33	Bone health Gut microbiome	Stool Blood	↓ucOC (lower bone turnover) No changes to microflora or other bone markers	(56)
Crossover	2 flesh of SunGold KF consumed 90 min before exercise-induced stress	300 mg Vit C + exercise Placebo + exercise Placebo + rest	Females 30.9 ± 7.3 y	10	Stress	Saliva	↓salivary uric acid (oxidative stress)	(10)
Parallel	2 flesh of SunGold KF for 4 weeks	No KF	Male mid-long runners 20.5 ± 0.8 y	15	Stress	Blood	↑BAP/d-ROMs ratio ↓d-ROMs	(57)
Pre and post	2 flesh of SunGold KF for 8 weeks	N/A	Male long-distance runner 20.4 ± 1.0 y	20	Stress	Blood	↑BAP and BAP/d-ROMs ratio	(57)
Crossover	4 flesh of SunGold KF for 4 weeks (2 consumed fresh and 2 consumed as 2 freeze-dried)	2 freeze-dried bananas	Older mixed cohort 56.1 ± 0.8 y	32	Flu symptoms Antioxidants Stress Immune function	Blood	↓severity and duration of head congestion, duration of sore throat, lipid peroxidation ↑ vitamin C, a-tocopherol, and lutein/zeaxanthin conc, erythrocyte folate No difference ORAC, FRAP, SOD, Glutathione peroxidase, Glutathione, Homocysteine	(13)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Pre and post	2 flesh of SunGold KF for 4 weeks	N/A	Males 23 ± 4 y	14	Immune function	Blood	↑ plasma vitamin C, neutrophil vitamin C, neutrophil chemotaxis, and oxidant generation No effect on neutrophil extracellular trap formation or spontaneous apoptosis	(76)
Crossover	3 flesh of green or gold (Hort16A) KF for 1 week, 1 at breakfast, lunch, and dinner	KF varieties	Healthy cohort No other info provided	3	Stress	Urine	↓ 8-OHdG (oxidative stress) and HEL (lipid oxidation) ↑ polyphenol excretion on day 7	(58)
Crossover	2 or 3 flesh of green KF for 4 weeks	KF doses	Mixed cohort 20 - 50 y	30	Metabolic markers Nutrient status Antioxidant	Blood	↑plasma FRAP and Vit. C ↓ platelet aggregation and triglycerides	(60)
Crossover	1 or 2 flesh of gold KF (Hort16A) for 4 weeks	KF doses	Mixed cohort 20 - 57 y	24	Metabolic markers Nutrient status Antioxidant	Blood	↓triglyceride concentrations ↓ H_2O_2-induced DNA damage ↓FPG-sensitive site in lymphocyte DNA decrease ↓endonuclease III-sensitive site in lymphocyte DNA ↓Whole blood platelet aggregation No effect on FRAP, lipid peroxidation, carotenoids, and vitamin C	(66)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Parallel	3 flesh of green KF for 8 weeks	Control diet Antioxidant rich diet	Male smokers 45-73 y	34	DNA damage	Blood	↓DNA strand breaks, nucleotide excision repair	(472)
Crossover	1, 2 or 3 flesh of green KF for 3 weeks	KF doses	26-54 y	14	Antioxidant DNA damage	Blood	↓endogenous oxidation of pyrimidines and purines ↑antioxidant status, DNA repair and Vit C	(473)
Parallel	1 flesh of green KF every 30kg body weight for 3 weeks	No KF	Mixed cohort 40 (SD 7) y	6	Metabolic markers	Blood	↑ability of leukocytes to repair DNA breakage by free radicals No changes to metabolic markers	(112)
Parallel	3 flesh of green KF for 8 weeks	Control diet Antioxidant rich diet	Male smokers 45-73 y	10	Antioxidant DNA damage Nutrient status	Blood	↑ genes of regulation of cellular stress defence, such as DNA repair, apoptosis and hypoxia, regulatory motifs for aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (AhR/ARNT), antioxidant, polyphenols/carotenoids	(474)
Parallel	0.5 or 2 flesh of gold KF (Hort16A) for 6 weeks	KF doses	Males <average plasma Vit, C conc. 22 ± 4 y	36	Nutrient Status	Urine Blood Muscle biopsy	↑skeletal muscle ascorbate, Mononuclear cell, and neutrophil ascorbate concentrations	(80)
Parallel	2 flesh of gold KF (Hort16A) with Fe-fortified cereal for 16 weeks	Banana with Fe-fortified cereal	Females with low Fe stores 31 IQR 15.5 y	33	Nutrient Status	Blood	↓ soluble transferrin receptor ↑Serum ferritin	(81)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Sequential	Sequential consumption of gold KF (Hort16A) 0.5 KF for 4 weeks 1 KF for 6 weeks 2 KF for 6 weeks 3 KF for 4 weeks	KF doses	Males <average plasma Vit, C conc. 21 ± 2.2 y	15	Nutrient Status	Urine Blood	↑plasma Vit. C with as little as 0.5 KF and urinary Vit. C with 2 KF	(78)
Parallel	0.5 flesh of gold KF (Hort16A) for 6 weeks	50 mg Vit. C tablet	Males 22 ± 4 y	18	Nutrient Status	Urine Semen Blood Muscle biopsy	= bio availability of Vit. C from KF compared to synthetic in all samples collected	(77)
Pre and post	2 flesh of SunGold KF for 12 weeks	N/A	Prediabetic	26	Nutrient Status Metabolic markers Gut microbiome	Stool Blood	↓waist circumference, diastolic and systolic blood pressure and HbA1c ↑plasma Vit. C and *Coriobacteriaceae* family	(55)
Crossover	3% actinidin in a 2 g oral care tablet for 1 week 3 times a day	Placebo	Males 23-54 y	14	Oral health	Breath Tongue coating	↓volatile sulphur compound concentration	(83)
Parallel	2 flesh of KF (variety not specified) for 5 months	No KF	Mixed cohort 52.4 ± 9.2 y	25	Oral health Metabolic markers Nutrient status	Blood	↓Diastolic blood pressure, bleeding, plaque, and attachment loss in teeth ↑ plasma Vit. C	(79)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Crossover	Oral care tablet containing KF powder	Tongue brushing	Mixed cohort 21.5 ± 2.1 y	32	Oral health	Breath Tongue coating	↓volatile sulphur compound (VSC) conc, Winkel tongue-coating index (WTCI), total bacteria, Fusobacterium nucleatum in tongue coating	(85)
Crossover	Oral care tablet containing KF powder	Tongue brushing	Mixed cohort 21.5 ± 2.1 y	32	Oral health	Tongue coating	↓relative abundance of *Prevotella* and *Porphyromonas* ↑*Firmicutes/Bacteroidetes* ratio	(86)
Pre and post	32 g of green KF powder for 4 days at breakfast	N/A	Females 18-25 y	6	Gut microbiome	Stool	↑*Lactobacilli* and *Bifidobacteria*	(54)
Crossover	KF supplements consumed for 4 weeks: Low dose Actazin (600 mg/day) High dose Actazin (2400 mg/day) Livaux (2400 mg/day) Actizin prod. from green KF and Livaux from gold KF	Placebo (isomalt)	Mixed cohort 25-56 y	20 healthy 9 functional constipated	Gut microbiome	Stool	↑*Faecalibacterium prausnitzii* (Livaux), *Clostridiales* (Livaux), *Dorea spp* (Actazin H) No significant differences in organic acid production in treatments	(12)
Pre and post	6x capsules of freeze-dried KF for 4 weeks	N/A	Mixed cohort 67 ± 6 y	42	Digestive health	None	↑evacuation time and stool quality, Quality of life (increased external appearance, decreased - lower back pain, headache)	(51)
Crossover	100 g flesh of green KF every 30 kg	No KF	Mixed cohort 71 + 3 y	38	Digestive health	None	↑laxation, bulkier and softer stool, frequency	(49)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Pre and post	2 flesh of green KF for 4 weeks	N/A	Mixed cohort Healthy 50.8 ± 14 y Constipated 49.9 ± 12 y	22 Healthy 33 constipated	Digestive health	None	↑SBM, satisfaction of bowel habit, rectal sensation ↓bothersomeness of constipation, days of laxative used, transit time, rectal sensation	(46)
Parallel	2 flesh of green KF for 4 weeks in healthy and constipated cohort	Placebo - Glucose capsule	Mixed cohort Healthy KF 30.7 ± 13.3 y Constipated control 22.8 ± 3.4 y Constipated KF 28.6 ± 8.3 y	16 healthy KF 13 constipated control 41 constipated KF	Digestive health	None	↓ transit time ↑weekly defecation frequency	(43)
Parallel	5.5 g Kivia powder as capsule for 4 weeks Kivia prod. from green KF contain Zyactinase®	Placebo - lemon powder, vital spirulina, citric acid, fructose, sucralose, and tropical flavour	Mixed cohort 38 ± 14 y	43	Digestive health	None	↓ flatulence, urgent bowel movement, Abdominal discomfort, or pain ↑spontaneous bowel movements, smoother and softer bowel movement	(42)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Crossover	KF supplements consumed for 4 weeks: Low dose Actizin (600 mg/day) High dose Actizin (2400 mg/day) Livaux (2400 mg/day) Actizin prod. from green KF and Livaux from gold KF	Placebo (isomalt)	Mixed cohort 25-56 y	20 healthy 9 functional constipated	Digestive health	None	↑ number of daily bowel movement (Actazin H and Livaux) in healthy cohort No effects seen of any treatment on constipated cohort	(50)
Crossover	Green KF supplement =1 g/day for 4 weeks	Placebo (magnesium stearate)	Mixed cohort with < 3 bowel movement a week 41 ± 12.5 y	32	Digestive health	None	No Change	(475)
Crossover	2 flesh of green or gold (Hort16A) KF with 400 g lean beef steak	KF varieties	Males 34 ± 10 y	10	Digestion Satiety	None	↓bloating after green No changes in VAS satiety or energy consumption at ad libitum meal	(44)
Crossover	2 flesh of green KF before 3 h measure of CH_4 and H_2 breath production	1 Royal gala apple 15 g lactulose 35 g fructose 50 g lactose	Females 38 ± 14 y	10 healthy 10 IBS	Digestion Satiety	Breath	↓CH4 and H2 breath production	(53)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Parallel	2.16 g/day Zyactinase® pill for 1 week Zyactinase® produced from green KF	Placebo (isomalt)	Mixed cohort with < 3 bowel movement a week 23-65 y	28	Digestive health	None	↓ abdominal discomfort ↑defecation frequency and faecal score	(476)
Crossover	3 flesh of SunGold KF for 4 weeks	14.75 g Metamucil	Females 23-65 y	9 functional constipated 23 IBS-C	Digestive health	None	↓abdominal pain, constipation, and indigestion ↑CSBM per week, softer stool and less straining	(11)
Crossover	2 flesh of green KF for 4 days	Isocaloric Maltodextrin	Mixed cohort 26 ± 4 y	14	Digestive health using MRI	None	↑T1 relaxation time of ascending colon, small bowel water content (SBWC), colon volume, stool frequency, stool form (Bristol)	(52)
Crossover	2 flesh of green KF for 2 weeks	No KF	Mixed cohort 29-23 y	11	Digestive health	None	↑ bowel movement, looser stools (Bristol)	(47)
Crossover	2 flesh of green or gold (Hort16A) KF for 2 weeks before with 100 g beef mince on trial day	KF varieties	Mixed cohort 72.5 ± 1.9 y	12	Digestion	Blood Muscle biopsy	peak ↑ of EAA, BCAA, leucine earlier with green KF = EAA absorbed, whole body protein kinetics	(82)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Parallel	2 flesh of green KF for 4 weeks	100 g Prunes or 12 g Psyllium	Mixed cohort with < 3 bowel movement a week mean age = 42.7 y	29	Digestive health	None	↑CSBM, stool consistency, straining, ↓bloating least AE reported	(41)
Crossover	2 flesh of SunGold KF for 2 weeks	7.5 g Psyllium	Mixed cohort 18-65 y	32 healthy 11 functional constipated 13 IBS-C	Digestive health	None	↓constipation score, straining ↑CSBM	(45)
Crossover	3 flesh of SunGold KF or 3 whole SunGold KF (include. skin) for 4 weeks	KF varieties	Mixed cohort 44 (22-65) y	19 healthy 19 with IBS-C	Digestive health Inflammation	Blood	↓TNF-α, constipation and abdominal pain ↑IL-10	(14)
Acute crossover	300 g flesh of green KF	280 g red grapes 300 g strawberries	Females 18-40 y	7	Antioxidant	Blood	↑antioxidant capacity	(59)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Pre and post	2 flesh of green KF for 8 weeks	N/A	Mixed cohort with hyperlipidaemia 44.6 ± 13.3 y men and 43.0 ± 11.2 y females	43	Metabolic health Antioxidant Nutrient status	Blood	↑HDL-C, Vitamin C, vitamin E ↓ LDL cholesterol/HDL-C ratio, total cholesterol/HDL-C	(64)
Crossover	2 flesh of green KF with healthy diet for 4 weeks	Healthy diet alone	Hypercholesterolaemia males 45 y (27-73)	85	Metabolic health Inflammation	Blood	↑HDL-C ↓(TC): HDL-C APOE4 allele carrier, ↓TAG	(65)
Parallel	3 flesh of green KF for 8 weeks	Control diet Antioxidant rich diet	Male smokers 45-73 y	34	Metabolic health	Blood	↓systolic BP, diastolic BP, platelet aggregation, angiotensin-converting enzyme activity	(68)
Acute crossover	500 mg sugar-free KF extract (equiv. to 7.5 KF) with 10 g of margarine	10 g margarine	Mixed cohort	9	Metabolic health	Blood	↓platelet aggregation	(70)
Crossover	2 flesh of green KF with healthy diet for 4 weeks	Healthy diet alone	Hypercholesterolaemia males 45 y (27-73)	85	Metabolic health	Blood	No changes in finger pulse pressure, stroke volume (SV), cardiac output (CO), and total peripheral resistance (TPR)	(114)
Parallel	3 flesh of green KF for 8 weeks	170 g royal gala apple	High BP mixed cohort 55 ± 9 y	58	Metabolic health Nutrient status	Blood	↓systolic and diastolic BP ↑lutein	(67)
Acute crossover	200 g flesh of green or SunGold KF	Glucose	Mixed cohort 36.7 ± 8.1 y	20	Metabolic health	Blood	↓glycaemic response, insulin response	(73)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Acute crossover	2 flesh of green KF + 30 g of wheat biscuit and 100 mg sodium [1-13C] acetate	30 g of wheat biscuit and 100 mg sodium [1-13C] acetate with: Sugar (control) Sugar + organic acids KF juice Neutralised KF	Mixed cohort 18-40 y	9	Metabolic health	Blood Breath	↓glycaemic response and iAUC due to KF Similar trend for observed for 13C release in breath CO2	(75)
Parallel	2 flesh of SunGold KF + carbonated water for 7 weeks	Carbonated water	Mixed Asian cohort in NZ 21.9 ± 3.5 y	20	Metabolic health	Blood	↓Systolic blood pressure	(69)
Crossover	2 flesh of SunGold KF for 6 weeks	No KF	Males 25-60 y	24	Metabolic health	Urine Blood	No significant results in biomarkers nor metabolic health markers	(63)
Parallel	2 flesh of SunGold KF for 12 weeks	Bottled water	Mixed cohort 55.3 ± 8.3 y	16	Metabolic health Nutrient status	Blood	↑ vitamin C	(61)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
Crossover	2 flesh of green KF with healthy diet for 4 weeks	Healthy diet alone	Hypercholesterolaemia males 45 y (27-73)	70	Metabolic health Inflammation	Blood	↓HDL-C, TC/HDL-C ratio, plasma hs-CRP, IL-6 in people who were medium inflammation group	(62)
Acute crossover	200 g flesh of green or SunGold KF with wheat biscuit	Glucose Wheat biscuit	Mixed cohort 36.7 ± 8.1 y	20	Metabolic health Satiety	Blood	↓postprandial glycaemic response amplitude compared to control with both KF, ↓ incremental area under the blood glucose response curve	(71)
Acute crossover	2 flesh of green KF + 47.3 g of wheat biscuit	47.3 g of wheat biscuit with: Sugar (control) Sugar + guar gum Guar gum + KF	Mixed cohort 36 y	20	Metabolic health Satiety	Blood	↓postprandial blood glucose response amplitude lower in KF than control, hunger at 180 min (longer satiating)	(72)
Pre and post	2 flesh of SunGold KF 6 weeks	N/A	Overweight and obese cohort 21.9 ± 0.3 y	22	Metabolic health Inflammation Stress Antioxidant	Blood	↓HDL-C, reduction of angiotensin II (AgII) concentration and systolic blood pressure (who had higher SBP), body fat %, hip circumference, (TNF)-a No change for, HBA1c, TG, TC,	(15)

Design	Dosage and length of supplementation	Comparator	Cohort	Sample size in KF group	Outcome measures	Biomarkers measured	Main findings (P<0.05)	Reference
							LDL-C, AST, ALT, Adiponectin, leptin, MDA, FRAP, IL-6	

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APPENDIX B

SUPPLEMENTARY MATERIALS TO CHAPTER 3

B.1 Supplementary Tables

Table B.1 Plasma oxidative stress and antioxidant biomarkers. All values are presented as mean ± SE. Significantly different values within the same row are represented by different letters (ab; $p < 0.05$).

Plasma biomarkers	Bread only (n = 6)	Bread + Gold KF (n = 6)	Bread + Green KF (n = 6)	*F*	*p*
Oxidative stress markers					
MDA (µM)	32.6 ± 1.1	35.0 ± 2.5	25.6 ± 5.8	1.76	0.21
Protein carbonyl (µM)	21.5 ± 3.1	13.9 ± 2.7	17.0 ± 3.7	1.44	0.27
Antioxidant markers					
FRAP (μM_{Trolox}/mg protein)	14.0 ± 3.1^{a}	20.8 ± 3.3^{ab}	25.9 ± 1.4$^{b\wedge}$	4.27	0.04
ORAC (μM_{Trolox}/mg protein)	21.5 ± 1.0	18.3 ± 1.5	19.8 ± 1.2	1.52	0.25
OPA (%ΔFI_{5min})	4.4 ± 0.1^{a}	3.5 ± 0.1^{b}	3.6 ± 0.0$^{b\wedge}$	33.21	0.00

MDA: Malondialdehyde, FRAP: Ferric reducing antioxidant potential, ORAC: Oxygen Radical Absorbance Capacity, OPA: Oxidative potential assay. ^an outlier was detected with studentised residuals ≥ ±3, leaving these groups with an *n* = 5.

B.2 Supplementary Figures

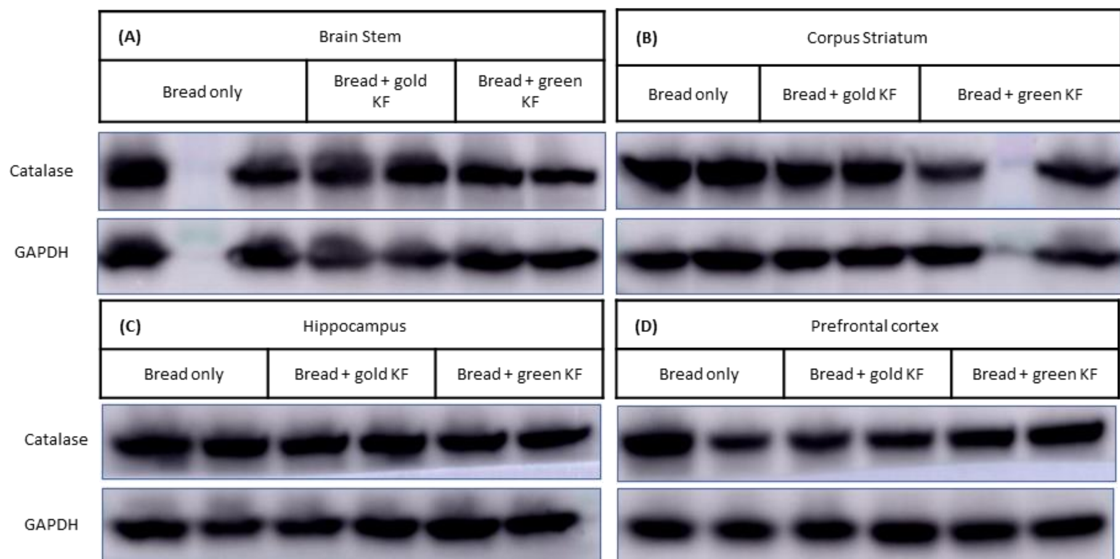

Figure B.1 Representative western blot analysis of catalase and GAPDH expression in brain stem (A), corpus striatum (B), hippocampus (C) and prefrontal cortex (D) in a growing pig model in response to the bread, bread with gold kiwifruit (KF) and bread with green KF treatments.

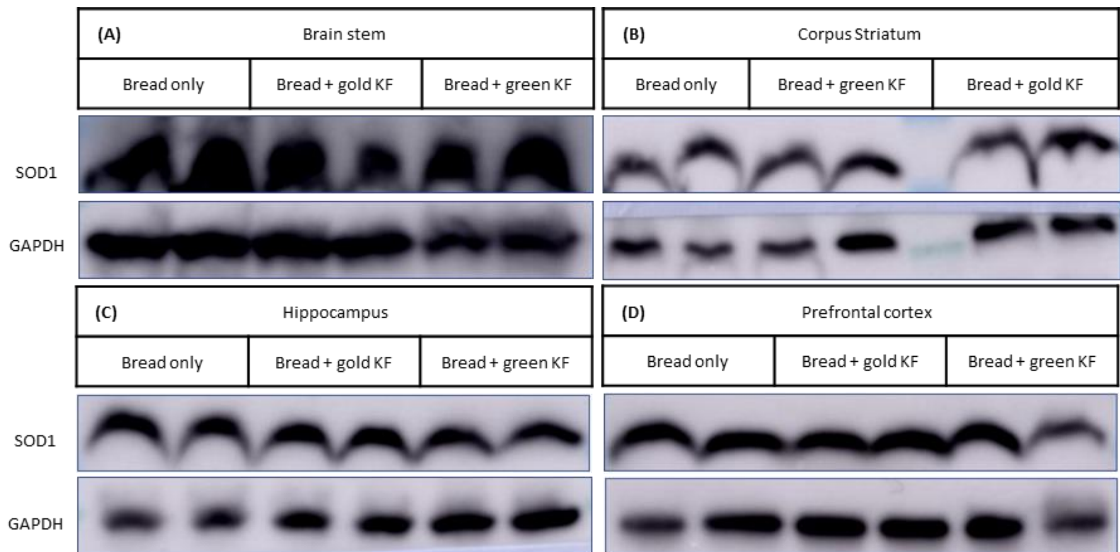

Figure B.2 Representative western blot analysis of SOD1 and GAPDH expression in brain stem (A), corpus striatum (B), hippocampus (C) and prefrontal cortex (D) in a growing pig model in response to the bread, bread with gold kiwifruit (KF) and bread with green KF treatments.

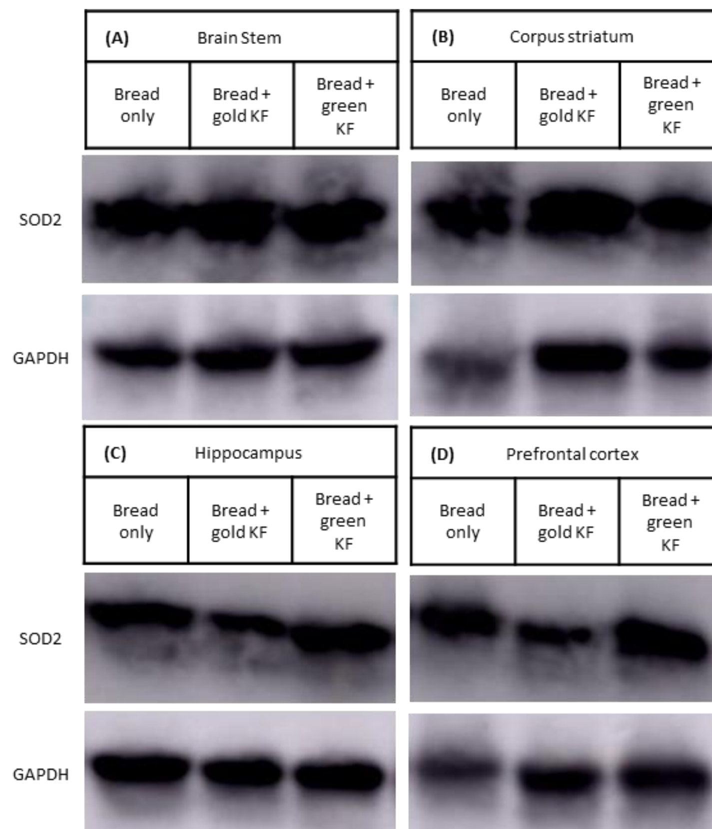

Figure B.3 Representative western blot analysis of SOD2 and GAPDH expression in brain stem (A), corpus striatum (B), hippocampus (C) and prefrontal cortex (D) in a growing pig model in response to the bread, bread with gold kiwifruit (KF) and bread with green KF treatments.

APPENDIX C

SUPPLEMENTARY MATERIALS TO CHAPTER 4

C.1 Supplementary Tables

Table C.2 Plasma metabolite concentrations of growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. All timepoints had n = 6 pigs per treatment group.

Plasma Metabolite (nM)	Bread	Bread + Gold KF	Bread + Green KF
Anserine	170.1 ± 9.8	232.2 ± 27.4	196.4 ± 21.1
Asparagine	63,250.7 ± 4,446.0	63,919.9 ± 5,495.5	68,307.3 ± 6,099.7
Serine	43,277.8 ± 3,746.5	42,273.5 ± 2,754.3	46,430.8 ± 5,231.0
Glutamine	139,107.4 ± 11,282.8	143,557.5 ± 4,727.9	153,322.1 ± 14,127.7
Citrulline	30,314.1 ± 2,591.5	30,675.1 ± 2,857.6	28,702.7 ± 3,486.3
Glycine	254,319.0 ± 17,994.6	317,101.8 ± 32,734.9	279,608.3 ± 24,299.5
Glutamate	9,212.0 ± 751.0	9,359.5 ± 1,390.4	7,574.0 ± 521.0
GABA	62.9 ± 4.1	66.5 ± 4.3	67.9 ± 3.3
Proline	168,920.4 ± 10,579.6	171,861.9 ± 11,860.9	187,021.0 ± 15,246.9
Adenosine	190.9 ± 5.9	255.8 ± 68.7	172.6 ± 30.0
Ornithine	4,889.0 ± 385.7	4,926.3 ± 392.5	5,583.6 ± 467.0
Spermidine	230.9 ± 6.1	229.3 ± 2.7	226.8 ± 3.7
5HIAA	71.3 ± 10.5	144.8 ± 8.7	106.6 ± 6.6
Homocysteine	648.2 ± 103.9	379.6 ± 74.8	386.1 ± 38.5
3-Hydroxykynurenine	1,240.0 ± 126.7	1,240.8 ± 286.4	1,473.9 ± 333.7
Putrescine	903.2 ± 63.9	1,012.6 ± 49.5	1,004.0 ± 67.6
Ethanolamine	3,758.1 ± 158.8	3,838.8 ± 328.4	3,511.8 ± 262.0
Serotonin	176.1 ± 78.0	683.4 ± 611.3	157.9 ± 71.1
LDOPA	362.6 ± 1.0	364.3 ± 1.3	366.6 ± 1.6
Histidine	31,925.1 ± 2,400.0	32,445.6 ± 1,550.3	31,596.3 ± 2,473.6
Valine	126,530.3 ± 5,185.3	117,738.9 ± 4,369.3	141,098.5 ± 7,959.4
Lysine	3,523.5 ± 331.0	2,956.6 ± 359.7	3,142.9 ± 286.3
Isoleucine	69,491.4 ± 2,501.2	69,379.4 ± 2,011.0	82,838.9 ± 5,740.0
Leucine	75,930.1 ± 3,928.6	72,987.2 ± 4,608.3	92,267.4 ± 8,882.3
Phenylalanine	12,605.5 ± 515.3	13,292.1 ± 427.0	15,253.4 ± 1,625.1
Tryptophan	7,462.4 ± 405.4	8,056.6 ± 460.8	8,317.7 ± 580.4
Tyrosine	73,090.7 ± 4,502.7	84,775.1 ± 4,633.4	84,178.1 ± 5,055.9
Methionine	18,151.5 ± 1,453.7	21,771.3 ± 1,890.0	21,330.2 ± 2,252.1
BCAA	271,951.8 ± 10,942.1	260,105.4 ± 9,805.3	316,204.7 ± 19,769.0
EAA	345,619.9 ± 11,042.8	338,627.6 ± 11,770.6	395,845.3 ± 25,069.6
LNAA	415,187.1 ± 12,208.2	420,446.2 ± 12,593.1	476,880.5 ± 29,814.8

Table C.3 Brain stem metabolite concentrations of growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. All timepoints had n = 6 pigs per treatment group

Brain Metabolite (nM/mg tissue)	Bread	Bread + Gold KF	Bread + Green KF
Asparagine	4,353 ± 917	5,348 ± 442	5,084 ± 433
Serine	5,594 ± 1,165	6,872 ± 663	7,574 ± 1,143
Glutamine	25,420 ± 4,807	28,783 ± 1,954	27,946 ± 1,952
Citrulline	579 ± 169	536 ± 82	711 ± 105
Glycine	27,527 ± 5,482	35,113 ± 2,542	34,781 ± 2,257
Glutamate	16,679 ± 4,610	17,232 ± 1,922	16,111 ± 1,058
GABA	12,070 ± 2,157	16,416 ± 570	13,762 ± 1,102
Proline	3,182 ± 605	3,984 ± 374	4,556 ± 507
Adenosine	585 ± 237	710 ± 93	964 ± 396
Glutathione	1,880 ± 683	2,159 ± 498	2,219 ± 377
Spermidine	54 ± 26	56 ± 16	57 ± 20
5-Hydroxyindoleacetic acid	85 ± 17	122 ± 13	115 ± 13
Homocysteine	415 ± 211	265 ± 22	302 ± 63
5-Hydroxytryptophan	2.4 ± 0.5	3.7 ± 0.5	3.1 ± 0.3
Octopamine	40.3 ± 8.1	50.7 ± 4.1	56.3 ± 7.9
Putrescine	124 ± 37	150 ± 22	143 ± 22
Ethanolamine	6,914 ± 1,467	9,779 ± 727	10,406 ± 1,100
Serotonin	46.1 ± 8.6	57.0 ± 2.2	51.2 ± 4.2
LDOPA	2.6 ± 0.6	2.6 ± 0.2	2.2 ± 0.2
Histidine	942 ± 211	1,138 ± 117	1,273 ± 199
Valine	3,788 ± 740	4,790 ± 303	4,947 ± 356
Lysine	254 ± 70	296 ± 50	324 ± 56
Isoleucine	2,084 ± 408	2,590 ± 237	2,781 ± 294
Leucine	6,115 ± 1,166	7,237 ± 823	8,661 ± 883
Phenylalanine	904 ± 185	1,076 ± 102	1,216 ± 118
Tryptophan	164 ± 32	190 ± 23	220 ± 25
Tyrosine	3,860 ± 757	4,807 ± 490	5,580 ± 623
Methionine	2,963 ± 637	3,729 ± 407	5,093 ± 1,014
BCAA	11,986 ± 2,284	14,618 ± 1,235	16,388 ± 1,463
EAA	17,213 ± 3,363	21,047 ± 1,869	24,514 ± 2,811
LNAA	20,169 ± 3,917	24,778 ± 2,263	28,878 ± 3,305
NEAA	86,616 ± 17,308	102,139 ± 7,391	101,631 ± 6,800

Table C.3 Corpus striatum metabolite concentrations of growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. All timepoints had n = 6 pigs per treatment group

Brain Metabolite (nM/mg tissue)	Bread	Bread + Gold KF	Bread + Green KF
Asparagine	9,774 ± 940	16,465 ± 7,535	9,689 ± 943
Serine	14,279 ± 1,584	26,271 ± 14,175	13,922 ± 1,053
Glutamine	72,476 ± 8,597	99,933 ± 31,875	68,363 ± 5,783
Citrulline	712 ± 50	1,389 ± 881	641 ± 32
Glycine	32,454 ± 2,201	49,891 ± 20,132	31,950 ± 1,866
Glutamate	36,373 ± 4,499	65,488 ± 35,262	38,330 ± 4,318
GABA	37,988 ± 2,346	50,698 ± 13,394	42,241 ± 2,611
Proline	8,489 ± 580	11,812 ± 2,650	8,956 ± 924
Adenosine	5,982 ± 883	5,716 ± 1,005	6,041 ± 832
Ornithine	95 ± 19	650 ± 587	89 ± 9
Glutathione	3,952 ± 963	7,406 ± 4,802	4,097 ± 1,142
Spermidine	48 ± 13	326 ± 302	37 ± 4
5-Hydroxyindoleacetic acid	52.4 ± 4.1	48.0 ± 3.6	43.8 ± 4.4
Homocysteine	158 ± 13	352 ± 185	184 ± 23
Kynurenine	5.7 ± 0.8	3.7 ± 0.5	3.8 ± 0.6
5-Hydroxytryptophan	7.9 ± 0.3	9.5 ± 0.7	7.1 ± 0.3
3-Hydroxykynurenine	202 ± 18	183 ± 21	230 ± 28
Dopamine	122.5 ± 12.6	115.5 ± 9.6	139.8 ± 24.5
Octopamine	115 ± 12	159 ± 38	118 ± 10
Putrescine	235 ± 52	735 ± 564	203 ± 17
Agmatine	7.3 ± 1.3	8.0 ± 1.5	5.2 ± 0.8
Ethanolamine	12,443 ± 852	16,536 ± 4,143	13,545 ± 1,047
N-Acetylputrescine	7.8 ± 0.7	8.3 ± 0.6	8.1 ± 0.8
Serotonin	17 ± 4	12 ± 1	12 ± 1
LDOPA	28 ± 6	38 ± 12	28 ± 5
Histidine	2,655 ± 343	4,807 ± 2,395	2,574 ± 152
Valine	8,514 ± 615	10,961 ± 2,435	9,193 ± 620
Lysine	675 ± 120	2,180 ± 1,662	591 ± 68
Isoleucine	5,943 ± 498	7,386 ± 1,022	5,773 ± 478
Leucine	12,481 ± 938	15,133 ± 2,342	12,511 ± 961
Phenylalanine	1,815 ± 185	2,252 ± 331	1,878 ± 139
Tryptophan	320 ± 22	395 ± 48	355 ± 28
Tyrosine	7,388 ± 688	8,922 ± 1,084	7,656 ± 584
Methionine	5,663 ± 431	8,162 ± 2,171	6,138 ± 450
BCAA	26,937 ± 1,928	33,479 ± 5,768	27,477 ± 1,646
EAA	38,066 ± 2,752	51,276 ± 12,286	39,014 ± 2,418
LNAA	43,638 ± 3,198	57,946 ± 13,025	44,791 ± 2,843
NEAA	181,233 ± 16,550	278,782 ± 112,326	178,865 ± 10,191

Table C.4 Hippocampus metabolite concentrations of growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. All timepoints had n = 6 pigs per treatment group.

Brain Metabolite (nM/mg tissue)	Bread	Bread + Gold KF	Bread + Green KF
Asparagine	9,452 ± 896	11,536 ± 1,893	9,108 ± 1,328
Serine	14,898 ± 629	16,260 ± 1,820	14,698 ± 1,414
Glutamine	48,905 ± 2,646	49,889 ± 3,723	50,428 ± 2,034
Citrulline	814 ± 101	837 ± 181	653 ± 66
Glycine	26,510 ± 1,272	27,903 ± 3,455	26,166 ± 3,440
Glutamate	39,623 ± 2,364	43,377 ± 6,381	45,701 ± 3,061
GABA	27,494 ± 1,148	31,050 ± 2,144	27,441 ± 2,358
Proline	6,521 ± 851	7,387 ± 1,320	6,026 ± 1,046
Adenosine	2,652 ± 344	3,034 ± 347	3,595 ± 502
Ornithine	39.2 ± 3.5	52.4 ± 7.8	48.2 ± 9.3
Glutathione	2,795 ± 706	2,433 ± 199	3,616 ± 531
Spermidine	48 ± 7	68 ± 12	72 ± 8
5-Hydroxyindoleacetic acid	38 ± 5	33 ± 2	37 ± 6
Homocysteine	132 ± 7	169 ± 21	177 ± 28
Kynurenine	5.8 ± 0.8	5.7 ± 0.8	5.2 ± 0.5
3-Hydroxykynurenine	242 ± 25	251 ± 26	256 ± 22
Octopamine	121 ± 14	118 ± 19	105 ± 17
Putrescine	307 ± 22	365 ± 65	385 ± 41
Agmatine	6.8 ± 0.7	7.6 ± 0.8	7.5 ± 0.7
Ethanolamine	5,598 ± 365	6,525 ± 941	6,020 ± 994
N-Acetylputrescine	4.6 ± 0.5	6.1 ± 0.7	5.5 ± 0.6
Serotonin	14.1 ± 1.8	14.7 ± 2.5	20.7 ± 1.8
Histidine	2,020 ± 239	2,235 ± 335	1,746 ± 238
Valine	8,084 ± 753	8,922 ± 1,243	8,162 ± 1,054
Lysine	559 ± 65	664 ± 114	549 ± 107
Isoleucine	6,108 ± 792	7,851 ± 1,516	5,921 ± 880
Leucine	13,781 ± 1,680	15,421 ± 2,573	12,629 ± 2,034
Phenylalanine	2,025 ± 327	2,066 ± 337	1,843 ± 290
Tryptophan	388.7 ± 52.4	414.4 ± 55.2	342.3 ± 54.0
Tyrosine	6,967 ± 717	7,826 ± 1,137	6,724 ± 861
Methionine	5,598 ± 676	6,563 ± 1,392	4,813 ± 779
BCAA	27,973 ± 3,193	32,194 ± 4,693	26,712 ± 3,881
EAA	38,563 ± 4,403	44,136 ± 6,817	36,005 ± 5,317
LNAA	43,505 ± 4,650	49,897 ± 7,582	40,886 ± 5,864
NEAA	152,875 ± 5,034	164,178 ± 17,502	158,849 ± 11,762

Table C.5 Prefrontal cortex metabolite concentrations of growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. All timepoints had n = 6 pigs per treatment group

Brain Metabolite (nM/mg tissue)	Bread	Bread + Gold KF	Bread + Green KF
Asparagine	7,697 ± 744	8,296 ± 641	8,369 ± 1,045
Serine	11,782 ± 1,175	11,862 ± 928	12,679 ± 1,463
Glutamine	41,514 ± 4,180	38,735 ± 1,602	43,090 ± 4,589
Citrulline	918 ± 83	798 ± 48	1,021 ± 74
Glycine	25,170 ± 1,289	27,560 ± 1,839	29,173 ± 3,022
Glutamate	28,916 ± 2,951	29,299 ± 4,531	33,053 ± 3,011
GABA	24,036 ± 868	25,259 ± 1,241	25,463 ± 1,636
Proline	7,426 ± 504	7,069 ± 281	7,169 ± 803
Adenosine	2,941 ± 738	2,691 ± 500	3,452 ± 240
Ornithine	37.6 ± 5.7	45.5 ± 6.1	55.0 ± 7.5
Glutathione	1,735 ± 227	2,114 ± 279	2,607 ± 235
Spermidine	21.6 ± 3.6	26.6 ± 4.8	28.2 ± 2.4
5-Hydroxyindoleacetic acid	35.1 ± 4.4	36.5 ± 3.8	36.1 ± 3.2
Homocysteine	196 ± 50	215 ± 34	251 ± 23
Kynurenine	12.0 ± 1.6	10.1 ± 0.4	10.6 ± 1.4
3-Hydroxykynurenine	200 ± 19	220 ± 22	251 ± 48
Octopamine	87.5 ± 9.2	93.4 ± 5.9	89.6 ± 11.5
Putrescine	162 ± 23	184 ± 29	199 ± 20
Agmatine	6.4 ± 0.5	6.5 ± 0.6	6.3 ± 0.6
Ethanolamine	11,659 ± 761	12,544 ± 337	13,518 ± 1,294
N-Acetylputrescine	4.8 ± 0.5	4.8 ± 0.5	5.1 ± 0.6
Serotonin	11.9 ± 2.3	11.5 ± 0.8	14.1 ± 1.9
Histidine	1,935 ± 199	1,960 ± 90	2,073 ± 220
Valine	7,632 ± 590	7,397 ± 374	7,510 ± 802
Lysine	359 ± 48	386 ± 38	420 ± 50
Isoleucine	5,324 ± 407	5,980 ± 347	6,142 ± 572
Leucine	10,820 ± 870	10,644 ± 562	11,186 ± 1,426
Phenylalanine	1,430 ± 131	1,446 ± 58	1,524 ± 184
Tryptophan	267 ± 20	276 ± 15	293 ± 39
Tyrosine	6,053 ± 613	6,425 ± 398	6,423 ± 775
Methionine	4,276 ± 440	4,371 ± 266	4,068 ± 486
BCAA	23,777 ± 1,760	24,021 ± 1,114	24,839 ± 2,694
EAA	32,044 ± 2,506	32,460 ± 1,434	33,216 ± 3,547
LNAA	36,667 ± 2,957	37,438 ± 1,648	38,115 ± 4,075
NEAA	128,559 ± 10,353	129,245 ± 9,218	139,956 ± 14,187

C.2 Supplementary Figures

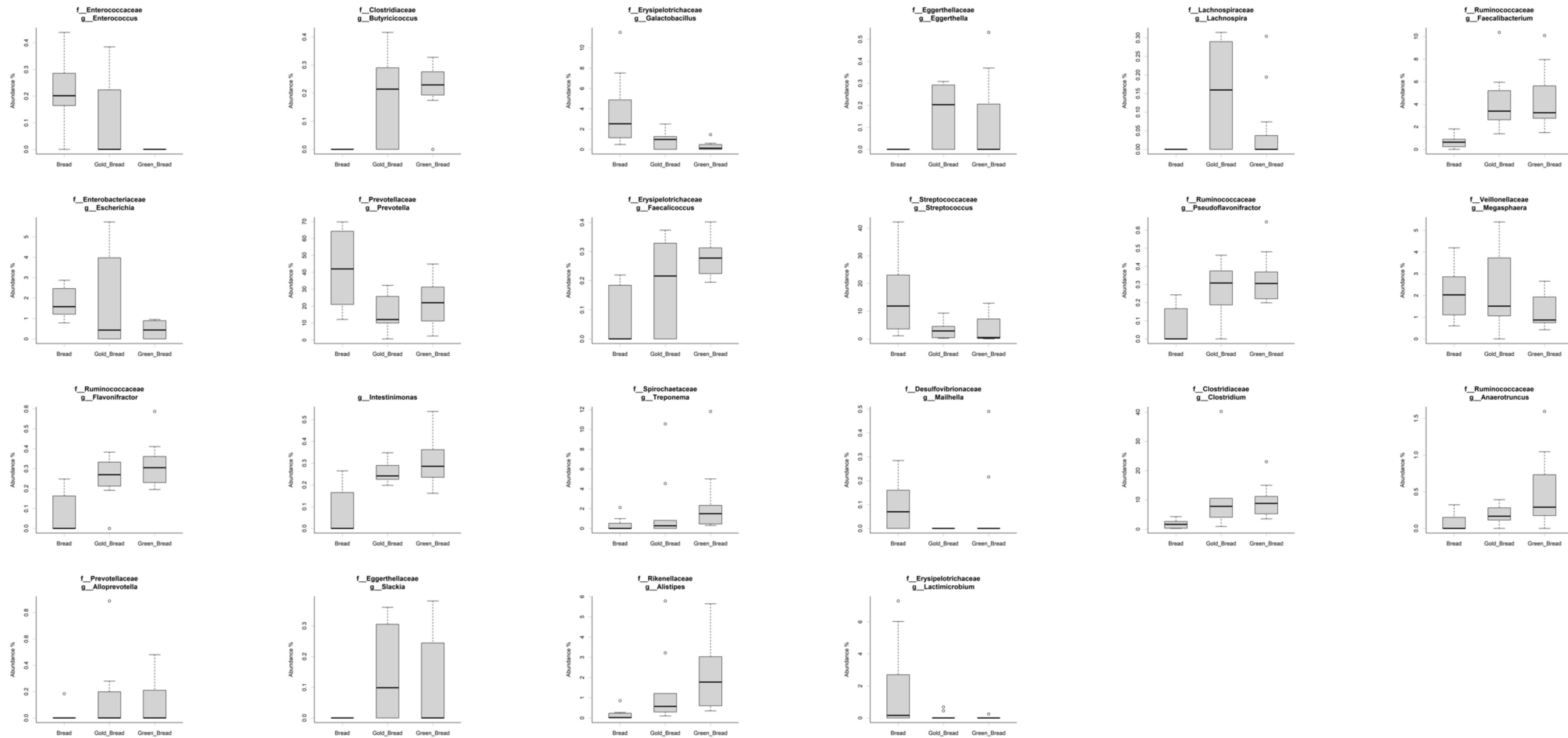

Figure C.4 Box plots of the relative abundance of statistically significant taxonomic genera of proximal colon microbiota of digesta samples collected from pig fed bread, bread + gold KF or bread + green KF.

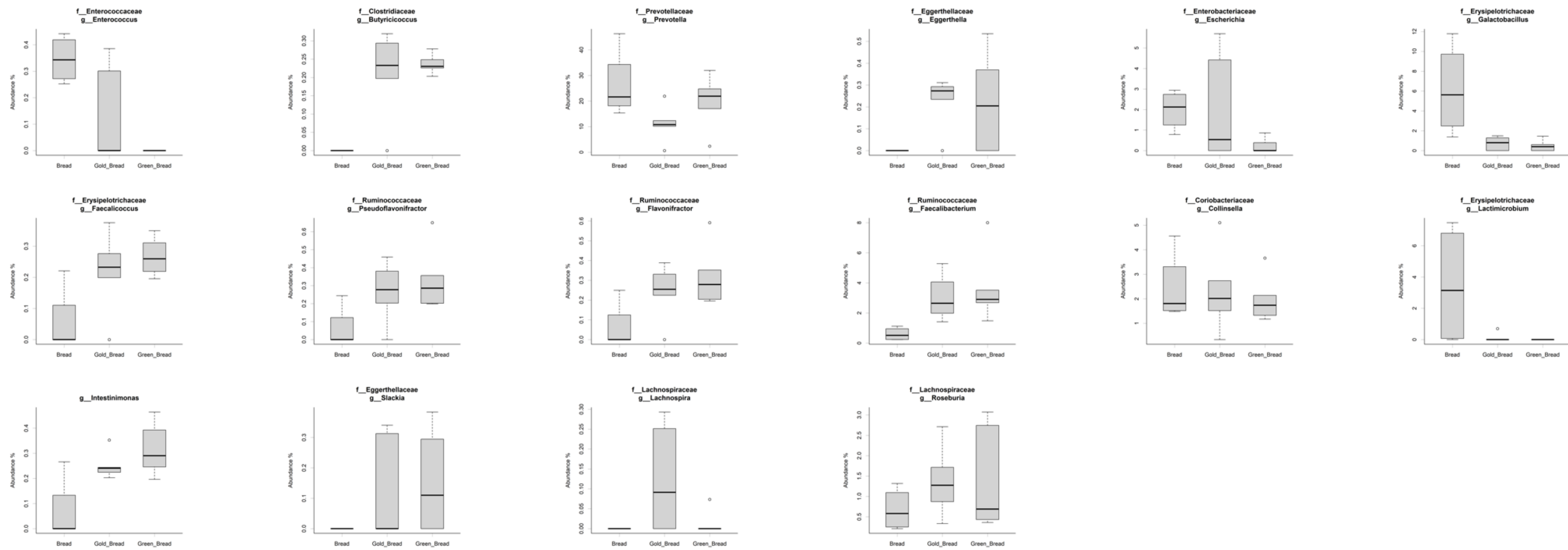

Figure C.5 Box plots of the relative abundance of statistically significant taxonomic genera of distal colon microbiota of digesta samples collected from pig fed bread, bread + gold KF or bread + green KF.

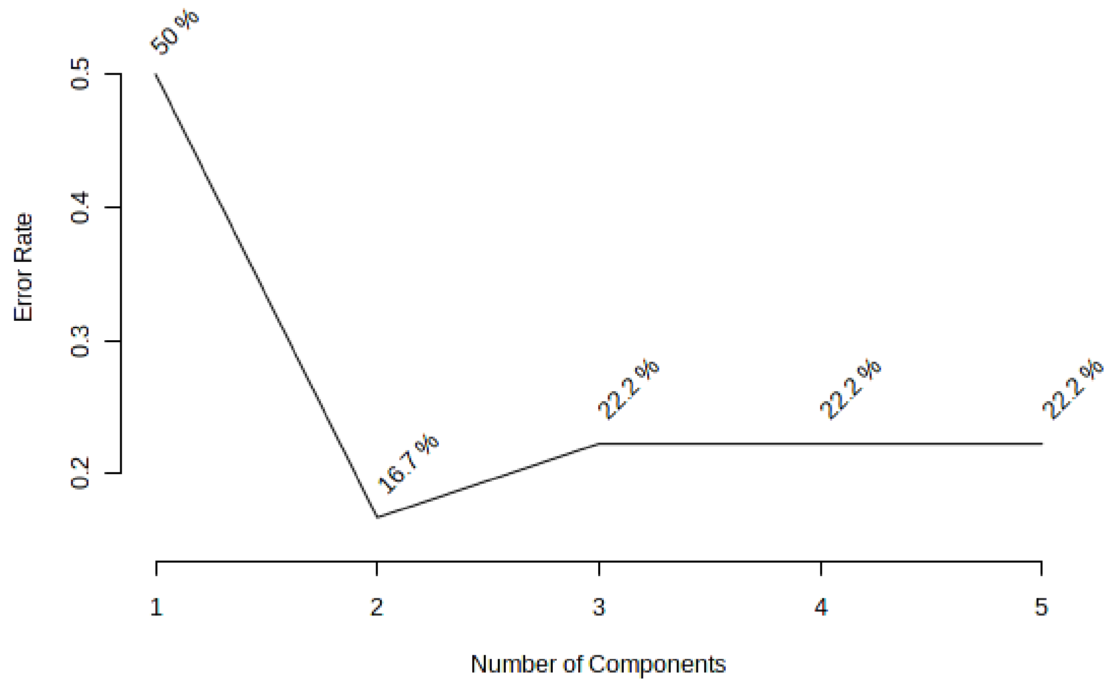

Figure C.6 sPLS-DA classification error rates for plasma metabolites.

APPENDIX D

SUPPLEMENTARY MATERIALS TO CHAPTER 5

D.1 Supplementary Tables

Table D.1 Plasma metabolite concentrations over time for growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. [1]L, Q or C, linear, quadratic, or cubic effect for the time factor, respectively. All timepoints had n = 6 pigs per time and the treatment group.

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Amino acids								
Isoleucine	Gold	64,209 ± 3,640	74,903 ± 3,455	67,427 ± 3,335	70,177 ± 4,129	0.152	0.017C	0.032L
	Green	58,757 ± 3,640	71,925 ± 3,455	68,159 ± 3,335	82,042 ± 4,129			
Leucine	Gold	61,244 ± 4,614	72,519 ± 4,614	61,541 ± 4,614	79,650 ± 4,614	0.156	0.024C	-
	Green	67,198 ± 4,614	78,473 ± 4,614	67,495 ± 4,614	85,604 ± 4,614			
Valine	Gold	131,032 ± 4,125	129,199 ± 3,446	126,449 ± 2,949	118,199 ± 5,642	0.280	0.833L	0.014L
	Green	126,032 ± 4,125	128,196 ± 3,446	131,443 ± 2,949	141,182 ± 5,642			
Proline	Gold	103,522 ± 9,116	169,712 ± 6,826	233,979 ± 9,061	174,648 ± 9,803	0.301	<0.001Q	-
	Green	112,700 ± 9,116	178,891 ± 6,826	243,158 ± 9,061	183,826 ± 9,803			
Asparagine	Gold	71,155 ± 3,326	69,933 ± 2,975	68,099 ± 2,735	62,597 ± 4,177	0.042	0.097L	-
	Green	79,241 ± 3,326	78,018 ± 2,975	76,184 ± 2,735	70,682 ± 4,177			
Serine	Gold	40,130 ± 1,903	40,578 ± 1,702	41,250 ± 1,565	43,266 ± 2,389	0.06	0.284L	-
	Green	44,399 ± 1,903	44,847 ± 1,702	45,519 ± 1,565	47,535 ± 2,389			
Histidine	Gold	28,083 ± 1,706	29,879 ± 1,117	31,844 ± 1,693	32,496 ± 1,872	0.661	0.001Q	0.027Q
	Green	28,948 ± 1,706	34,457 ± 1,117	39,511 ± 1,693	31,575 ± 1,872			
Lysine	Gold	11,042 ± 827	7,827 ± 827	5,429 ± 827	2,957 ± 827	0.125	0.005C	0.015C
	Green	11,323 ± 827	11,667 ± 827	5,430 ± 827	3,143 ± 827			
Methionine	Gold	22,549 ± 1,129	22,208 ± 1,010	21,697 ± 928	20,163 ± 1,418	0.187	0.171L	-
	Green	24,309 ± 1,129	23,969 ± 1,010	23,457 ± 928	21,923 ± 1,418			

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Non-proteinogenic amino acids								
Homocysteine	Gold	394 ± 40	391 ± 36	387 ± 33	376 ± 51	0.387	0.768L	-
	Green	435 ± 40	432 ± 36	428 ± 33	417 ± 51			
Ornithine	Gold	3,269 ± 294	4,209 ± 220	5,197 ± 292	5,124 ± 316	0.325	0.001Q	-
	Green	3,551 ± 294	4,491 ± 220	5,479 ± 292	5,406 ± 316			
Citrulline	Gold	25,807 ± 1,599	26,586 ± 1,430	27,753 ± 1,315	31,257 ± 2,008	0.281	0.030L	-
	Green	23,778 ± 1,599	24,557 ± 1,430	25,724 ± 1,315	29,228 ± 2,008			
Glutamate metabolites								
Glutamine	Gold	107,435 ± 7,636	144,048 ± 7,636	145,914 ± 7,636	145,103 ± 7,636	0.334	0.041C	-
	Green	114,108 ± 7,636	150,721 ± 7,636	152,587 ± 7,636	151,776 ± 7,636			
Glutamate	Gold	9,666 ± 832	9,523 ± 744	9,307 ± 684	8,660 ± 1,044	0.789	0.430L	-
	Green	9,406 ± 832	9,262 ± 744	9,046 ± 684	8,400 ± 1,044			
Glycine	Gold	258,558 ± 12,603	266,298 ± 10,528	277,908 ± 9,010	312,736 ± 17,240	0.003	0.562L	0.011L
	Green	314,644 ± 12,603	309,684 ± 10,528	302,243 ± 9,010	279,921 ± 17,240			
GABA	Gold	57 ± 3.5	54 ± 3.5	66 ± 3.5	66 ± 3.5	0.418	0.055C	-
	Green	60 ± 3.5	56 ± 3.5	68 ± 3.5	69 ± 3.5			
Tryptophan metabolites								
Tryptophan	Gold	7,293 ± 329	7,413 ± 294	7,592 ± 270	8,131 ± 413	0.503	0.101L	-
	Green	7,551 ± 329	7,671 ± 294	7,850 ± 270	8,389 ± 413			
3-Hydroxykynurenine	Gold	1,092 ± 121	1,125 ± 108	1,174 ± 99	1,320 ± 151	0.326	0.219L	-
	Green	1,231 ± 121	1,264 ± 108	1,313 ± 99	1,459 ± 151			
Serotonin	Gold	137 ± 156	189 ± 139	267 ± 127	500 ± 198	0.931	0.144L	-
	Green	121 ± 159	173 ± 143	251 ± 132	484 ± 204			
5-Hydroxyindoleacetic acid	Gold	126 ± 7.7	160 ± 5.7	191 ± 7.6	148 ± 8.2	<0.001	<0.001Q	-
	Green	81 ± 7.7	114 ± 5.7	146 ± 7.6	103 ± 8.2			
Tyrosine metabolites								
Phenylalanine	Gold	9,078 ± 577	13,450 ± 577	13,051 ± 577	13,814 ± 577	0.0821	0.0004	-
	Green	9,996 ± 577	14,368 ± 577	13,969 ± 577	14,732 ± 577			
Tyrosine	Gold	57,660 ± 2,667	65,878 ± 1,997	75,441 ± 2,651	84,234 ± 2,868	0.929	0.012Q	-
	Green	57,890 ± 2,667	66,107 ± 1,997	75,670 ± 2,651	84,464 ± 2,868			

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
L-DOPA	Gold	362 ± 1.0	364 ± 0.8	366 ± 1.0	365 ± 1.1	0.175	0.008Q	-
	Green	364 ± 1.0	366 ± 0.8	368 ± 1.0	366 ± 1.1			
Polyamines								
Putrescine	Gold	1,039 ± 40	1,029 ± 36	1,016 ± 33	974 ± 50	0.197	0.291L	-
	Green	1,100 ± 40	1,090 ± 36	1,077 ± 33	1,035 ± 50			
Spermidine	Gold	231 ± 2.4	230 ± 2.1	229 ± 2.0	227 ± 3.0	0.531	0.259L	-
	Green	233 ± 2.4	232 ± 2.1	231 ± 2.0	229 ± 3.0			
Other metabolites part of the GBA								
Adenosine	Gold	202 ± 16	205 ± 15	208 ± 13	220 ± 21	0.449	0.469L	-
	Green	187 ± 16	190 ± 15	194 ± 13	206 ± 21			
Anserine	Gold	155 ± 24	234 ± 24	174 ± 24	232 ± 24	0.022	0.229C	0.014C
	Green	207 ± 24	176 ± 24	216 ± 24	196 ± 24			
Ethanolamine	Gold	4,684 ± 156	4,565 ± 140	4,385 ± 128	3,845 ± 196	0.197	0.001L	-
	Green	4,447 ± 156	4,327 ± 140	4,147 ± 128	3,608 ± 196			
Calculations								
BCAA	Gold	255,448 ± 11,857	284,478 ± 11,252	253,931 ± 10,861	262,692 ± 13,449	0.322	0.017C	0.020L
	Green	244,874 ± 11,857	282,690 ± 11,252	265,322 ± 10,861	313,619 ± 13,449			
EAA	Gold	320,454 ± 13,911	361,556 ± 13,911	333,074 ± 13,911	357,462 ± 13,911	0.124	0.025C	-
	Green	340,003 ± 13,911	381,105 ± 13,911	352,623 ± 13,911	377,011 ± 13,911			
NEAA	Gold	671,963 ± 30,945	764,513 ± 23,173	857,240 ± 30,760	803,520 ± 33,280	0.094	<0.001Q	-
	Green	722,887 ± 30,945	815,437 ± 23,173	908,164 ± 30,760	854,444 ± 33,280			
LNAA	Gold	366,092 ± 15,419	420,906 ± 15,419	402,194 ± 15,419	439,313 ± 15,419	0.182	0.028C	-
	Green	384,793 ± 15,419	439,608 ± 15,419	420,895 ± 15,419	420,895 ± 15,419			

Table D.2 Brain stem metabolite concentrations over time for growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. [1]L, Q or C, linear, quadratic, or cubic effect for the time factor, respectively. All timepoints had n = 6 pigs per time and treatment group.

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Amino acids								
Isoleucine	Gold	1,666 ± 176	1,824 ± 157	2,060 ± 145	2,769 ± 221	0.447	<0.001L	-
	Green	1,510 ± 176	1,667 ± 157	1,903 ± 145	2,612 ± 221			
Leucine	Gold	4,167 ± 484	3,923 ± 362	4,030 ± 481	7,744 ± 520	0.373	0.017Q	-
	Green	4,586 ± 484	4,342 ± 362	4,449 ± 481	8,163 ± 520			
Valine	Gold	3,194 ± 171	3,415 ± 153	3,747 ± 140	4,743 ± 214	0.839	<0.001L	-
	Green	3,234 ± 171	3,456 ± 153	3,788 ± 140	4,783 ± 214			
Proline	Gold	2,200 ± 223	2,471 ± 199	2,877 ± 183	4,095 ± 280	0.638	<0.001L	-
	Green	2,323 ± 223	2,593 ± 199	2,999 ± 183	4,218 ± 280			
Asparagine	Gold	3,990 ± 226	4,165 ± 202	4,427 ± 186	5,213 ± 284	0.532	<0.001L	-
	Green	3,825 ± 226	4,000 ± 202	4,262 ± 186	5,048 ± 284			
Serine	Gold	5,492 ± 399	5,697 ± 357	6,005 ± 328	6,928 ± 501	0.706	0.022L	-
	Green	5,669 ± 399	5,874 ± 357	6,181 ± 328	7,104 ± 501			
Histidine	Gold	704 ± 68	768 ± 61	865 ± 56	1,155 ± 85	0.421	<0.001L	-
	Green	768 ± 68	833 ± 61	929 ± 56	1,219 ± 85			
Lysine	Gold	335 ± 29	287 ± 22	242 ± 29	298 ± 31	0.353	0.026Q	-
	Green	361 ± 29	313 ± 22	268 ± 29	324 ± 31			
Methionine	Gold	1,571 ± 316	1,932 ± 282	2,474 ± 260	4,100 ± 397	0.494	0.001L	-
	Green	1,824 ± 316	2,186 ± 282	2,728 ± 260	4,354 ± 397			
Non-proteinogenic amino acids								
Homocysteine	Gold	230 ± 63	381 ± 63	193 ± 63	247 ± 63	0.204	0.019C	-
	Green	303 ± 63	454 ± 63	266 ± 63	320 ± 63			
Citrulline	Gold	350 ± 42	319 ± 31	314 ± 41	599 ± 45	0.210	0.015Q	-
	Green	401 ± 42	370 ± 31	365 ± 41	650 ± 45			
Glutamate metabolites								
Glutamine	Gold	27,828 ± 1,373	27,883 ± 1,228	27,964 ± 1,129	28,208 ± 1,724	0.528	0.857L	-
	Green	26,813 ± 1,373	26,867 ± 1,228	26,949 ± 1,129	27,193 ± 1,724			

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Glutamate	Gold	16,214 ± 997	16,225 ± 891	16,243 ± 820	16,294 ± 1,252	0.934	0.958L	-
	Green	16,311 ± 997	16,322 ± 891	16,339 ± 820	16,391 ± 1,252			
Glycine	Gold	28,714 ± 1,438	29,563 ± 1,286	30,835 ± 1,182	34,653 ± 1,805	0.828	0.009L	-
	Green	28,349 ± 1,438	29,198 ± 1,286	30,470 ± 1,182	34,288 ± 1,805			
GABA	Gold	13,604 ± 779	12,463 ± 510	11,720 ± 773	16,462 ± 855	0.081	0.071Q	0.046Q
	Green	11,804 ± 779	12,165 ± 510	12,654 ± 773	13,751 ± 855			
Tryptophan metabolites								
Tryptophan	Gold	117 ± 10	128 ± 9	146 ± 8	197 ± 12	0.599	<0.001L	-
	Green	123 ± 10	134 ± 9	152 ± 8	203 ± 12			
5-Hydroxytryptophan	Gold	2.38 ± 0.21	2.54 ± 0.18	2.78 ± 0.17	3.49 ± 0.26	0.669	<0.001L	-
	Green	2.28 ± 0.21	2.44 ± 0.18	2.68 ± 0.17	3.39 ± 0.26			
Serotonin	Gold	76.4 ± 5.4	83.6 ± 4.0	87.7 ± 5.3	50.7 ± 5.8	0.879	0.002Q	-
	Green	75.6 ± 5.4	82.8 ± 4.0	86.9 ± 5.3	49.9 ± 5.8			
5-Hydroxyindoleacetic acid	Gold	125 ± 12	81 ± 12	102 ± 12	122 ± 12	0.001	0.152C	0.014C
	Green	62 ± 12	100 ± 12	98 ± 12	115 ± 12			
Tyrosine metabolites								
Phenylalanine	Gold	551 ± 551	630 ± 630	750 ± 750	1,109 ± 1,109	0.793	<0.001L	-
	Green	568 ± 568	647 ± 647	767 ± 767	1,125 ± 1,125			
Tyrosine	Gold	2,463 ± 259	2,831 ± 232	3,383 ± 213	5,038 ± 325	0.691	<0.001L	-
	Green	2,583 ± 259	2,951 ± 232	3,503 ± 213	5,158 ± 325			
Octopamine	Gold	26.9 ± 3.0	30.4 ± 2.7	35.8 ± 2.4	51.7 ± 3.7	0.877	<0.001L	-
	Green	27.4 ± 3.0	31.0 ± 2.7	36.3 ± 2.4	52.3 ± 3.7			
L-DOPA	Gold	0.50 ± 0.31	0.67 ± 0.26	1.35 ± 0.26	1.05 ± 0.27	0.019	0.359C	0.048Q
	Green	1.47 ± 0.31	0.90 ± 0.25	0.80 ± 0.26	0.68 ± 0.30			
Polyamines								
Putrescine	Gold	161 ± 14	157 ± 13	151 ± 12	133 ± 18	0.425	0.192L	-
	Green	174 ± 14	170 ± 13	164 ± 12	146 ± 18			
Spermidine	Gold	53.4 ± 8.1	52.9 ± 7.3	52.3 ± 6.7	50.3 ± 10.2	0.344	0.806L	-
	Green	62.4 ± 8.1	61.9 ± 7.3	61.3 ± 6.7	59.3 ± 10.2			

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Other metabolites part of the GBA								
Adenosine	Gold	612 ± 99	638 ± 89	676 ± 82	792 ± 125	0.920	0.240L	-
	Green	624 ± 99	649 ± 89	688 ± 82	804 ± 125			
Glutathione	Gold	3,609 ± 427	3,397 ± 382	3,078 ± 351	2,121 ± 536	0.501	0.026L	-
	Green	3,946 ± 427	3,734 ± 382	3,415 ± 351	2,458 ± 536			
Ethanolamine	Gold	6,040 ± 523	6,494 ± 468	7,175 ± 430	9,218 ± 657	0.076	<0.001L	-
	Green	7,144 ± 523	7,598 ± 468	8,279 ± 430	10,322 ± 657			
Calculations								
BCAA	Gold	8,356 ± 698	9,295 ± 625	10,703 ± 574	14,928 ± 877	0.711	<0.001L	-
	Green	8,659 ± 698	9,598 ± 625	11,006 ± 574	15,231 ± 877			
EAA	Gold	11,596 ± 1,123	13,049 ± 1,005	15,228 ± 924	21,768 ± 1,411	0.611	<0.001L	-
	Green	12,265 ± 1,123	13,718 ± 1,005	15,898 ± 924	22,437 ± 1,411			
NEAA	Gold	85,873 ± 3,730	87,805 ± 3,336	90,704 ± 3,068	99,399 ± 4,684	0.813	0.021L	-
	Green	86,902 ± 3,730	88,835 ± 3,336	91,733 ± 3,068	100,429 ± 4,684			
LNAA	Gold	13,762 ± 1,356	15,585 ± 1,213	18,321 ± 1,115	26,527 ± 1,703	0.617	<0.001L	-
	Green	14,525 ± 1,356	16,349 ± 1,213	19,084 ± 1,115	27,290 ± 1,703			

Table D.3 Corpus striatum metabolite concentrations over time for growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. [1]L, Q or C, linear, quadratic, or cubic effect for the time factor, respectively. All timepoints had n = 6 pigs per time and treatment group.

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Amino acids								
Isoleucine	Gold	4,926 ± 300	5,134 ± 269	5,446 ± 252	6,382 ± 396	0.120	0.003Q	-
	Green	4,367 ± 303	4,575 ± 269	4,888 ± 246	5,824 ± 383			
Leucine	Gold	9,872 ± 600	10,228 ± 539	10,761 ± 503	12,363 ± 792	0.552	0.011L	-
	Green	10,295 ± 605	10,651 ± 539	11,185 ± 493	12,786 ± 766			
Valine	Gold	8,806 ± 505	7,959 ± 331	7,196 ± 501	8,579 ± 607	0.015	0.373Q	0.029Q
	Green	7,155 ± 505	7,797 ± 331	8,540 ± 501	9,193 ± 554			
Proline	Gold	7,707 ± 554	7,001 ± 362	6,520 ± 549	9,251 ± 665	0.016	0.386Q	0.021Q
	Green	5,907 ± 554	6,778 ± 362	7,815 ± 549	8,986 ± 607			
Asparagine	Gold	7,930 ± 434	8,127 ± 390	8,421 ± 364	9,305 ± 573	0.652	0.049L	-
	Green	7,699 ± 438	7,895 ± 390	8,189 ± 357	9,073 ± 554			
Serine	Gold	13,131 ± 572	13,093 ± 514	13,035 ± 480	12,861 ± 756	0.859	0.747L	-
	Green	13,011 ± 578	12,972 ± 514	12,914 ± 470	12,741 ± 731			
Histidine	Gold	2,567 ± 156	1,940 ± 135	1,917 ± 156	2,413 ± 179	0.001	0.052C	0.004Q
	Green	1,883 ± 156	1,848 ± 135	2,418 ± 156	2,578 ± 163			
Lysine	Gold	682 ± 48	599 ± 35	511 ± 44	502 ± 63	0.010	0.045Q	0.025L
	Green	527 ± 48	482 ± 35	450 ± 43	610 ± 58			
Methionine	Gold	6,148 ± 519	5,331 ± 340	4,608 ± 515	6,049 ± 624	0.054	0.353Q	0.043Q
	Green	4,902 ± 519	5,386 ± 340	5,920 ± 515	6,154 ± 570			
Non-proteinogenic amino acids								
Homocysteine	Gold	102 ± 15	109 ± 14	120 ± 13	153 ± 20	0.133	0.040L	-
	Green	130 ± 15	137 ± 14	148 ± 12	180 ± 19			
Ornithine	Gold	7,930 ± 434	8,127 ± 390	8,421 ± 364	9,305 ± 573	0.352	0.049L	-
	Green	7,699 ± 438	7,895 ± 390	8,189 ± 357	9,073 ± 554			
Citrulline	Gold	676 ± 39	648 ± 32	605 ± 28	478 ± 57	0.019	0.283L	0.010L
	Green	550 ± 39	562 ± 32	580 ± 28	634 ± 53			
Glutamate metabolites								
Glutamine	Gold	71,333 ± 2,537	70,883 ± 2,279	70,209 ± 2,129	68,186 ± 3,351	0.446	0.432L	-

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
	Green	69,038 ± 2,561	68,588 ± 2,279	67,914 ± 2,085	65,891 ± 3,240			
Glutamate	Gold	32,464 ± 2,296	32,608 ± 2,063	32,824 ± 1,927	33,471 ± 3,033	0.838	0.781L	0
	Green	33,018 ± 2,318	33,162 ± 2,062	33,377 ± 1,887	34,024 ± 2,932			
Glycine	Gold	28,993 ± 1,302	28,949 ± 1,083	28,884 ± 946	28,687 ± 1,927	0.006	0.031L	0.020L
	Green	23,695 ± 1,297	24,867 ± 1,083	26,625 ± 927	31,900 ± 1,774			
GABA	Gold	43,243 ± 1,998	38,320 ± 1,308	33,483 ± 1,982	37,306 ± 2,401	0.023	0.054Q	0.052Q
	Green	37,241 ± 1,998	37,978 ± 1,308	39,073 ± 1,982	42,284 ± 2,192			
Tryptophan metabolites								
Tryptophan	Gold	323 ± 19	295 ± 13	273 ± 19	349 ± 23	0.017	0.414Q	0.023Q
	Green	262 ± 19	290 ± 13	324 ± 19	356 ± 21			
Kynurenine	Gold	3.53 ± 0.28	3.55 ± 0.26	3.59 ± 0.24	3.71 ± 0.38	0.311	0.675L	-
	Green	3.18 ± 0.29	3.21 ± 0.26	3.25 ± 0.23	3.37 ± 0.36			
3-Hydroxykynurenine	Gold	176 ± 13	179 ± 12	183 ± 11	195 ± 17	0.970	0.355L	-
	Green	176 ± 13	179 ± 12	183 ± 11	196 ± 17			
5-Hydroxytryptophan	Gold	1.65 ± 0.26	1.88 ± 0.22	2.23 ± 0.19	3.28 ± 0.39	0.165	0.026L	0.030L
	Green	2.11 ± 0.26	2.12 ± 0.22	2.12 ± 0.19	2.13 ± 0.36			
Serotonin	Gold	9.9 ± 2.9	14.4 ± 1.9	18.0 ± 2.9	6.6 ± 3.5	0.014	0.222Q	0.040Q
	Green	19.9 ± 2.9	16.7 ± 1.9	12.8 ± 2.9	6.7 ± 3.2			
5-Hydroxyindoleacetic acid	Gold	30.3 ± 2.3	30.9 ± 2.0	31.8 ± 1.9	34.6 ± 3.0	0.954	0.264L	-
	Green	30.4 ± 2.3	31.1 ± 2.0	32.0 ± 1.9	34.7 ± 2.9			
Tyrosine metabolites								
Phenylalanine	Gold	1,721 ± 139	1,554 ± 91	1,429 ± 138	1,951 ± 167	0.006	0.956Q	0.009Q
	Green	1,193 ± 139	1,483 ± 91	1,798 ± 138	1,882 ± 153			
Tyrosine	Gold	6,779 ± 443	6,182 ± 290	5,762 ± 440	7,915 ± 533	0.008	0.508Q	0.011Q
	Green	5,204 ± 443	6,013 ± 290	6,941 ± 440	7,677 ± 486			
Octopamine	Gold	105 ± 7	94 ± 5	87 ± 7	122 ± 9	0.012	0.278Q	0.024Q
	Green	79 ± 7	90 ± 5	102 ± 7	119 ± 8			
L-DOPA	Gold	16.38 ± 3.26	19.12 ± 2.98	15.18 ± 2.98	15.82 ± 3.26	0.121	0.057C	0.051C
	Green	18.99 ± 2.98	10.99 ± 2.98	17.90 ± 3.26	18.85 ± 2.98			
Dopamine	Gold	345 ± 46	310 ± 41	257 ± 39	98 ± 61	0.309	0.001L	-

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
	Green	401 ± 47	365 ± 41	312 ± 38	154 ± 59			
Polyamines								
Agmatine	Gold	6.08 ± 0.66	6.28 ± 0.54	6.59 ± 0.48	7.50 ± 0.97	0.153	0.619L	0.042L
	Green	7.29 ± 0.65	6.96 ± 0.54	6.47 ± 0.47	4.98 ± 0.89			
N-Acetylputrescine	Gold	6.94 ± 0.51	6.33 ± 0.38	5.91 ± 0.51	8.09 ± 0.58	0.745	0.022Q	-
	Green	6.78 ± 0.51	6.17 ± 0.38	5.74 ± 0.51	7.92 ± 0.56			
Putrescine	Gold	224 ± 14	216 ± 12	206 ± 10	173 ± 21	0.013	0.530L	0.048L
	Green	173 ± 14	177 ± 12	182 ± 10	199 ± 19			
Spermidine	Gold	41.1 ± 5.4	51.7 ± 5.4	34.3 ± 5.4	24.5 ± 6.0	0.214	0.277C	0.035C
	Green	36.6 ± 5.4	25.5 ± 5.4	29.8 ± 5.4	37.2 ± 5.4			
Other metabolites part of the GBA								
Adenosine	Gold	5,782 ± 784	5,759 ± 704	5,724 ± 658	5,618 ± 1,036	0.739	0.895L	-
	Green	6,091 ± 791	6,068 ± 704	6,033 ± 644	5,928 ± 1,001			
Glutathione	Gold	4,912 ± 711	4,631 ± 639	4,209 ± 597	2,944 ± 940	0.541	0.084L	-
	Green	5,427 ± 718	5,146 ± 639	4,725 ± 585	3,460 ± 908			
Ethanolamine	Gold	10,704 ± 699	9,534 ± 458	8,665 ± 694	12,432 ± 841	0.003	0.243Q	0.008Q
	Green	7,897 ± 699	9,270 ± 458	10,972 ± 694	13,512 ± 767			
Calculations								
BCAA	Gold	22,555 ± 1,088	23,266 ± 977	24,333 ± 913	27,532 ± 1,437	0.933	0.005L	-
	Green	22,448 ± 1,098	23,159 ± 977	24,225 ± 894	27,424 ± 1,389			
EAA	Gold	35,947 ± 2,321	33,011 ± 1,519	30,735 ± 2,303	39,228 ± 2,789	0.042	0.460Q	0.040Q
	Green	29,883 ± 2,321	32,831 ± 1,519	36,230 ± 2,303	39,059 ± 2,546			
NEAA	Gold	171,871 ± 6,211	164,725 ± 4,654	158,852 ± 6,174	176,138 ± 7,035	0.419	0.054Q	-
	Green	166,954 ± 6,207	159,807 ± 4,665	153,934 ± 6,170	171,220 ± 6,797			
LNAA	Gold	42,011 ± 2,694	38,601 ± 1,763	36,029 ± 2,673	46,621 ± 3,237	0.033	0.482Q	0.030Q
	Green	34,593 ± 2,694	38,356 ± 1,763	42,680 ± 2,673	46,141 ± 2,955			

Table D.4 Hippocampus metabolite concentrations over time for growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. [1]L, Q or C, linear, quadratic, or cubic effect for the time factor, respectively. All timepoints had n = 6 pigs per time and treatment group.

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Amino acids								
Isoleucine	Gold	4,949 ± 471	5,211 ± 422	5,604 ± 388	6,784 ± 592	0.715	0.014L	-
	Green	4,748 ± 471	5,010 ± 422	5,403 ± 388	6,582 ± 592			
Leucine	Gold	11,243 ± 1,334	10,074 ± 873	9,426 ± 1,323	15,431 ± 1,463	0.158	0.616Q	0.042Q
	Green	9,173 ± 1,334	10,734 ± 873	12,400 ± 1,323	12,533 ± 1,463			
Valine	Gold	6,901 ± 484	7,124 ± 433	7,459 ± 398	8,462 ± 608	0.954	0.039L	-
	Green	6,869 ± 484	7,092 ± 433	7,426 ± 398	8,430 ± 608			
Proline	Gold	4,883 ± 670	4,592 ± 439	4,562 ± 665	7,402 ± 736	0.171	0.936Q	0.053Q
	Green	3,857 ± 670	4,867 ± 439	5,941 ± 665	5,986 ± 736			
Asparagine	Gold	7,722 ± 642	8,138 ± 574	8,762 ± 528	10,633 ± 806	0.476	0.005L	-
	Green	7,186 ± 642	7,602 ± 574	8,226 ± 528	10,097 ± 806			
Serine	Gold	13,519 ± 724	13,854 ± 647	14,355 ± 595	15,860 ± 909	0.621	0.039L	-
	Green	13,100 ± 724	13,435 ± 647	13,936 ± 595	15,441 ± 909			
Histidine	Gold	1,570 ± 133	1,639 ± 119	1,744 ± 109	2,057 ± 167	0.620	0.020L	-
	Green	1,493 ± 133	1,562 ± 119	1,667 ± 109	1,980 ± 167			
Lysine	Gold	512 ± 49	526 ± 44	547 ± 41	609 ± 62	0.894	0.200L	-
	Green	504 ± 49	518 ± 44	539 ± 41	602 ± 62			
Methionine	Gold	4,732 ± 634	4,046 ± 415	3,610 ± 629	6,570 ± 695	0.050	0.771Q	0.011Q
	Green	3,256 ± 634	4,233 ± 415	5,225 ± 629	4,784 ± 695			
Non-proteinogenic amino acids								
Homocysteine	Gold	82 ± 17.8	118 ± 17.8	77 ± 17.8	155 ± 17.8	0.030	0.036C	-
	Green	117 ± 17.8	154 ± 17.8	113 ± 17.8	191 ± 17.8			
Ornithine	Gold	21.1 ± 3.8	25.8 ± 3.4	32.8 ± 3.1	53.8 ± 4.7	0.421	<0.0001L	-
	Green	17.5 ± 3.8	22.2 ± 3.4	29.2 ± 3.1	50.2 ± 4.7			
Citrulline	Gold	595 ± 52	617 ± 47	650 ± 43	750 ± 66	0.921	0.057L	-
	Green	589 ± 52	611 ± 47	644 ± 43	744 ± 66			
Glutamate metabolites								

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Glutamine	Gold	50,107 ± 1,807	50,087 ± 1,616	50,057 ± 1,486	49,967 ± 2,269	0.989	0.960L	-
	Green	50,077 ± 1,807	50,058 ± 1,616	50,028 ± 1,486	49,938 ± 2,269			
Glutamate	Gold	39,146 ± 2,328	40,011 ± 2,082	41,310 ± 1,915	45,206 ± 2,924	0.855	0.094L	-
	Green	38,648 ± 2,328	39,514 ± 2,082	40,813 ± 1,915	44,709 ± 2,924			
Glycine	Gold	21,878 ± 1,475	22,796 ± 1,319	24,173 ± 1,213	28,304 ± 1,852	0.324	0.006L	-
	Green	20,170 ± 1,475	21,087 ± 1,319	22,464 ± 1,213	26,595 ± 1,852			
GABA	Gold	26,119 ± 1,715	25,310 ± 1,123	25,048 ± 1,702	31,105 ± 1,882	0.180	0.1781Q	0.050Q
	Green	23,618 ± 1,715	26,157 ± 1,123	28,710 ± 1,702	27,322 ± 1,882			
Tryptophan metabolites								
Tryptophan	Gold	306 ± 35	285 ± 23	277 ± 35	415 ± 38	0.229	0.966Q	0.053Q
	Green	264 ± 35	309 ± 23	356 ± 35	340 ± 38			
Kynurenine	Gold	4.88 ± 0.39	4.90 ± 0.35	4.92 ± 0.32	4.99 ± 0.49	0.586	0.866L	-
	Green	4.63 ± 0.39	4.65 ± 0.35	4.67 ± 0.32	4.74 ± 0.49			
3-Hydroxykynurenine	Gold	198 ± 14	202 ± 12	207 ± 11	221 ± 17	0.208	0.270L	-
	Green	218 ± 14	222 ± 12	227 ± 11	242 ± 17			
Serotonin	Gold	9.2 ± 1.8	13.4 ± 1.2	17.0 ± 1.8	8.9 ± 2.0	0.010	0.058Q	0.013Q
	Green	15.9 ± 1.8	15.2 ± 1.2	14.4 ± 1.8	14.7 ± 2.0			
5-Hydroxyindoleacetic acid	Gold	19.2 ± 2.1	20.2 ± 1.9	21.7 ± 1.7	26.4 ± 2.7	0.821	0.031L	-
	Green	19.7 ± 2.1	20.8 ± 1.9	22.3 ± 1.7	26.9 ± 2.7			
Tyrosine metabolites								
Phenylalanine	Gold	1,545 ± 189	1,427 ± 124	1,371 ± 188	2,066 ± 208	0.138	0.782Q	0.028Q
	Green	1,256 ± 189	1,583 ± 124	1,921 ± 188	1,827 ± 208			
Tyrosine	Gold	5,504 ± 433	5,770 ± 387	6,169 ± 356	7,366 ± 543	0.856	0.007L	-
	Green	5,412 ± 433	5,678 ± 387	6,077 ± 356	7,275 ± 543			
Octopamine	Gold	77.7 ± 7.3	82.4 ± 6.6	89.4 ± 6.0	110.5 ± 9.2	0.910	0.005L	-
	Green	78.7 ± 7.3	83.4 ± 6.6	90.4 ± 6.0	111.5 ± 9.2			
Polyamines								
Agmatine	Gold	5.74 ± 0.30	6.02 ± 0.27	6.44 ± 0.25	7.70 ± 0.38	0.428	<0.001L	-
	Green	5.46 ± 0.30	5.75 ± 0.27	6.17 ± 0.25	7.43 ± 0.38			
N-Acetylputrescine	Gold	3.81 ± 0.28	4.12 ± 0.25	4.58 ± 0.23	5.97 ± 0.36	0.129	<0.001L	-

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
	Green	3.30 ± 0.28	3.60 ± 0.25	4.07 ± 0.23	5.46 ± 0.36			
Putrescine	Gold	316 ± 23	322 ± 20	333 ± 19	363 ± 29	0.448	0.183L	-
	Green	336 ± 23	343 ± 20	353 ± 19	383 ± 29			
Spermidine	Gold	49.5 ± 6.4	51.9 ± 5.8	55.4 ± 5.3	65.9 ± 8.1	0.914	0.103L	-
	Green	50.4 ± 6.4	52.7 ± 5.8	56.2 ± 5.3	66.7 ± 8.1			
Other metabolites part of the GBA								
Adenosine	Gold	3,897 ± 391	3,105 ± 292	2,343 ± 388	3,118 ± 420	0.312	0.008Q	-
	Green	4,282 ± 391	3,490 ± 292	2,728 ± 388	3,503 ± 420			
Glutathione	Gold	2,774 ± 340	2,757 ± 304	2,732 ± 279	2,656 ± 426	0.178	0.821L	-
	Green	3,313 ± 340	3,297 ± 304	3,272 ± 279	3,196 ± 426			
Ethanolamine	Gold	4,099 ± 375	4,411 ± 335	4,879 ± 308	6,283 ± 471	0.944	<0.001L	-
	Green	4,130 ± 375	4,442 ± 335	4,910 ± 308	6,314 ± 471			
Calculations								
BCAA	Gold	24,270 ± 2,512	21,813 ± 1,644	20,373 ± 2,492	32,212 ± 2,756	0.172	0.486Q	0.041Q
	Green	20,599 ± 2,512	23,236 ± 1,644	26,070 ± 2,492	26,494 ± 2,756			
EAA	Gold	32,904 ± 3,551	29,649 ± 2,324	27,806 ± 3,524	44,162 ± 3,897	0.145	0.626Q	0.033Q
	Green	27,261 ± 3,551	31,558 ± 2,324	36,073 ± 3,524	35,724 ± 3,897			
NEAA	Gold	142,369 ± 6,654	145,479 ± 5,951	150,144 ± 5,472	164,141 ± 8,356	0.655	0.037L	-
	Green	138,890 ± 6,654	142,000 ± 5,951	146,665 ± 5,472	160,662 ± 8,356			
LNAA	Gold	38,092 ± 4,082	34,730 ± 2,671	32,972 ± 4,050	51,342 ± 4,478	0.153	0.716Q	0.036Q
	Green	31,747 ± 4,082	36,925 ± 2,671	42,359 ± 4,050	41,865 ± 4,478			

Table D.5 Prefrontal cortex metabolite concentrations over time for growing pigs fed a bread diet containing fresh gold KF or fresh green KF pulp. Values are means ± SEMs. [1]L, Q or C, linear, quadratic, or cubic effect for the time factor, respectively. All timepoints had n = 6 pigs per time and treatment group.

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Amino acids								
Homocysteine	Gold	220 ± 20	218 ± 18	215 ± 17	206 ± 25	0.053	0.649L	-
	Green	266 ± 20	264 ± 18	261 ± 16	252 ± 25			
Ornithine	Gold	51.72 ± 5.41	51.75 ± 4.86	51.81 ± 4.48	51.98 ± 6.75	0.938	0.9746	-
	Green	52.21 ± 5.33	52.25 ± 4.77	52.30 ± 4.39	52.47 ± 6.70			
Citrulline	Gold	1,204 ± 76	1,160 ± 68	1,093 ± 63	894 ± 95	0.6479	0.009L	-
	Green	1,245 ± 75	1,200 ± 67	1,134 ± 62	934 ± 94			
Isoleucine	Gold	7,217 ± 390	6,994 ± 350	6,659 ± 323	5,655 ± 486	0.1136	0.0104	-
	Green	7,946 ± 384	7,722 ± 343	7,388 ± 316	6,383 ± 482			
Leucine	Gold	16,276 ± 1,333	13,849 ± 897	11,225 ± 1,418	10,665 ± 1,457	0.19	0.961Q	0.022Q
	Green	14,961 ± 1,328	16,116 ± 869	16,790 ± 1,318	11,191 ± 1,457			
Valine	Gold	10,893 ± 678	9,503 ± 456	7,978 ± 721	7,432 ± 741	0.315	0.859Q	0.023Q
	Green	10,595 ± 675	10,924 ± 442	10,938 ± 670	7,518 ± 741			
Proline	Gold	11,575 ± 940	9,718 ± 632	7,695 ± 1,000	7,126 ± 1,028	0.1581	0.949Q	0.019Q
	Green	10,468 ± 937	11,157 ± 613	11,465 ± 929	7,173 ± 1,028			
Asparagine	Gold	9,806 ± 532	9,587 ± 478	9,259 ± 441	8,274 ± 664	0.6458	0.061L	-
	Green	10,091 ± 524	9,872 ± 469	9,544 ± 431	8,559 ± 659			
Serine	Gold	13,823 ± 684	13,629 ± 614	13,340 ± 566	12,471 ± 853	0.9989	0.194L	-
	Green	13,824 ± 674	13,631 ± 603	13,341 ± 554	12,472 ± 846			
Histidine	Gold	2,457 ± 143	2,388 ± 128	2,285 ± 118	1,976 ± 178	0.5024	0.030L	-
	Green	2,568 ± 141	2,500 ± 126	2,397 ± 116	2,088 ± 177			
Lysine	Gold	664 ± 46	626 ± 41	570 ± 38	399 ± 57	0.609	<0.001L	-
	Green	692 ± 45	654 ± 40	597 ± 37	426 ± 56			
Methionine	Gold	6,605 ± 403	6,232 ± 362	5,673 ± 333	3,996 ± 503	0.4559	<0.001L	-
	Green	6,956 ± 397	6,583 ± 355	6,024 ± 326	4,347 ± 498			
Non-proteinogenic amino acids								
Homocysteine	Gold	220 ± 20	218 ± 18	215 ± 17	206 ± 25	0.053	0.649L	-
	Green	266 ± 20	264 ± 18	261 ± 16	252 ± 25			

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
Ornithine	Gold	51.72 ± 5.41	51.75 ± 4.86	51.81 ± 4.48	51.98 ± 6.75	0.938	0.975L	-
	Green	52.21 ± 5.33	52.25 ± 4.77	52.30 ± 4.39	52.47 ± 6.70			
Citrulline	Gold	1,204 ± 76	1,160 ± 68	1,093 ± 63	894 ± 95	0.648	0.009L	-
	Green	1,245 ± 75	1,200 ± 67	1,134 ± 62	934 ± 94			
Glutamate metabolites								
Glutamine	Gold	42,947 ± 1,745	42,804 ± 1,566	42,590 ± 1,444	41,946 ± 2,177	0.707	0.703L	-
	Green	42,183 ± 1,719	42,040 ± 1,538	41,825 ± 1,414	41,182 ± 2,159			
Glutamate	Gold	26,756 ± 2,097	27,622 ± 1,882	28,921 ± 1,735	32,820 ± 2,616	0.5332	0.060L	-
	Green	25,232 ± 2,065	26,098 ± 1,847	27,397 ± 1,698	31,296 ± 2,594			
Glycine	Gold	28,813 ± 1,191	28,704 ± 1,069	28,541 ± 986	28,051 ± 1,486	0.6804	0.671L	-
	Green	29,385 ± 1,173	29,276 ± 1,049	29,113 ± 965	28,623 ± 1,473			
GABA	Gold	30,957 ± 1,146	28,625 ± 771	26,084 ± 1,219	25,349 ± 1,253	0.002	0.692Q	0.005Q
	Green	25,904 ± 1,142	27,852 ± 748	29,521 ± 1,133	25,499 ± 1,253			
Tryptophan metabolites								
Tryptophan	Gold	360.6 ± 18.9	347.4 ± 17.0	327.6 ± 15.6	268.2 ± 23.6	0.1895	0.002L	-
	Green	389.8 ± 18.6	376.6 ± 16.6	356.8 ± 15.3	297.4 ± 23.4			
Kynurenine	Gold	11.91 ± 0.67	11.58 ± 0.60	11.09 ± 0.56	9.62 ± 0.84	0.2654	0.028L	-
	Green	12.79 ± 0.66	12.46 ± 0.59	11.97 ± 0.54	10.49 ± 0.83			
3-Hydroxykynurenine	Gold	222 ± 22	217 ± 19	210 ± 18	189 ± 27	0.1344	0.314Q	-
	Green	259 ± 21	255 ± 19	248 ± 18	227 ± 27			
Serotonin	Gold	3.91 ± 1.14	4.29 ± 1.03	4.86 ± 0.95	6.57 ± 1.43	0.558	0.128L	-
	Green	4.69 ± 1.13	5.07 ± 1.01	5.64 ± 0.93	7.35 ± 1.41			
5-Hydroxyindoleacetic acid	Gold	26.7 ± 3.4	22.5 ± 2.3	18.9 ± 3.6	27.3 ± 3.7	0.15	0.922Q	0.025Q
	Green	22.2 ± 3.4	26.8 ± 2.2	31.3 ± 3.4	27.0 ± 3.7			
Tyrosine metabolites								
Phenylalanine	Gold	2,092 ± 159	1,830 ± 107	1,545 ± 169	1,451 ± 174	0.1692	0.885Q	0.034Q
	Green	1,897 ± 158	2,037 ± 104	2,125 ± 157	1,520 ± 174			
Tyrosine	Gold	7,161 ± 374	7,013 ± 336	6,791 ± 310	6,124 ± 467	0.1757	0.071L	-
	Green	7,757 ± 369	7,609 ± 330	7,387 ± 303	6,721 ± 463			
Octopamine	Gold	112 ± 6	108 ± 6	103 ± 5	89 ± 8	0.449	0.0196	-

Metabolites	KF Treatment	20 min	1 hour	2 hours	5 hours	KF	Time[1]	KF x Time[1]
	Green	117 ± 6	114 ± 6	109 ± 5	94 ± 8			
Polyamines								
Agmatine	Gold	8.18 ± 0.69	6.00 ± 0.60	6.62 ± 0.75	6.50 ± 0.73	0.0345	0.008C	0.032Q
	Green	6.62 ± 0.69	6.57 ± 0.60	9.19 ± 0.69	6.29 ± 0.73			
N-Acetylputrescine	Gold	6.76 ± 0.80	5.45 ± 0.54	4.13 ± 0.85	4.86 ± 0.87	0.078	0.728Q	0.036Q
	Green	5.16 ± 0.80	5.90 ± 0.52	6.55 ± 0.79	5.10 ± 0.87			
Putrescine	Gold	160 ± 17	190 ± 13	219 ± 18	194 ± 19	0.899	0.031Q	-
	Green	157 ± 18	187 ± 13	217 ± 18	192 ± 19			
Spermidine	Gold	25.60 ± 3.65	25.50 ± 3.27	25.35 ± 3.02	24.91 ± 4.55	0.778	0.900L	-
	Green	26.80 ± 3.59	26.70 ± 3.21	26.55 ± 2.96	26.11 ± 4.51			
Other metabolites part of the GBA								
Adenosine	Gold	1,770 ± 335	1,917 ± 301	2,137 ± 277	2,796 ± 418	0.562	0.047L	-
	Green	1,997 ± 330	2,143 ± 295	2,363 ± 271	3,023 ± 414			
Glutathione	Gold	1,496.6 ± 231.7	1,639.9 ± 207.9	1,854.8 ± 191.7	2,499.6 ± 289.1	0.790	0.006L	-
	Green	1,424.9 ± 228.2	1,568.2 ± 204.1	1,783.1 ± 187.7	2,428.0 ± 286.6			
Ethanolamine	Gold	12,539 ± 421	12,522 ± 378	12,496 ± 349	12,419 ± 526	0.036	0.850L	-
	Green	13,593 ± 415	13,576 ± 371	13,550 ± 341	13,473 ± 521			
Calculations								
BCAA	Gold	34,870 ± 2,417	30,168 ± 1,626	25,091 ± 2,571	24,089 ± 2,642	0.251	0.899Q	0.022Q
	Green	33,218 ± 2,408	34,862 ± 1,576	35,552 ± 2,389	24,840 ± 2,642			
EAA	Gold	47,663 ± 3,305	41,384 ± 2,223	34,542 ± 3,515	32,563 ± 3,613	0.259	0.888Q	0.030Q
	Green	45,234 ± 3,293	47,145 ± 2,155	47,742 ± 3,267	33,181 ± 3,613			
NEAA	Gold	139,843 ± 5,678	139,346 ± 5,095	138,600 ± 4,698	136,362 ± 7,083	0.983	0.684L	-
	Green	139,986 ± 5,592	139,488 ± 5,002	138,742 ± 4,599	136,504 ± 7,023			
LNAA	Gold	54,564 ± 3,747	47,605 ± 2,521	40,091 ± 3,986	38,609 ± 4,097	0.272	0.876Q	0.031Q
	Green	51,978 ± 3,734	54,216 ± 2,444	55,031 ± 3,705	39,183 ± 4,096			

APPENDIX E

SUPPLEMENTARY MATERIALS TO CHAPTER 6

E.1 Supplementary Tables

Table E.1 Within-subject correlations between urinary 5HIAA, aMT6s and Vitamin C and subjective and objective measures of sleep quality and subjective mood in poor and good sleepers.

	Within-subjects correlations					
	Combined		Poor sleeper (n= 12)		Good sleeper (n= 12)	
	r	*p*	*r*	*p*	*r*	*p*
5HIAA						
Subjective Sleep Quality						
Evening Sleepiness	-0.20	0.17	-0.34	0.09	-0.10	0.64
Morning Sleepiness	-0.12	0.12	-0.14	0.50	-0.09	0.66
Getting to Sleep	-0.06	0.70	-0.02	0.92	-0.10	0.63
Quality of Sleep	0.03	0.85	0.09	0.68	-0.03	0.87
Awake Following Sleep	-0.06	0.70	-0.02	0.92	-0.12	0.56
Behaviour Following Wakening	0.14	0.35	0.24	0.25	0.00	0.99
Objective Sleep Quality						
Latency	-0.24	0.09	-0.09	0.66	-0.35	0.08
Efficiency	0.11	0.47	-0.04	0.85	0.22	0.30
Total Sleep Time	-0.07	0.66	-0.17	0.41	0.08	0.70
Wake after sleep onset	-0.07	0.64	-0.36	0.08	0.29	0.15
Number of Awakenings	0.03	0.81	-0.29	0.15	0.38	0.06
Average Awakening Length	-0.10	0.51	-0.25	0.24	0.04	0.86
Subjective Mood						
Tension	-0.05	0.73	-0.10	0.63	0.02	0.92
Anger	-0.10	0.51	0.05	0.80	-0.38	0.06
Fatigue	-0.23	0.11	-0.19	0.35	-0.35	0.09
Depression	-0.13	0.37	-0.04	0.86	-0.30	0.14
Esteem-related Affect	0.21	0.15	0.24	0.25	0.19	0.37
Vigour	-0.18	0.21	0.22	0.29	0.21	0.30
Confusion	-0.08	0.58	0.03	0.89	-0.20	0.33
Total Mood Disturbance	-0.21	0.14	-0.14	0.49	-0.41	0.04
6aMTs						
Subjective Sleep Quality						
Evening Sleepiness	0.19	0.19	0.00	0.99	0.35	0.09
Morning Sleepiness	0.24	0.10	0.26	0.21	0.22	0.29
Getting to Sleep	0.14	0.33	-0.34	0.10	0.14	0.51
Quality of Sleep	0.02	0.88	0.05	0.82	-0.01	0.97
Awake Following Sleep	0.14	0.33	0.17	0.40	0.09	0.66
Behaviour Following Wakening	0.16	0.27	0.19	0.36	0.12	0.56

	Within-subjects correlations					
	Combined		Poor sleeper (n= 12)		Good sleeper (n= 12)	
	r	***p***	***r***	***p***	***r***	***p***
Objective Sleep Quality						
Latency	-0.29	0.05	0.22	0.29	-0.35	0.09
Efficiency	-0.12	0.41	-0.05	0.83	0.19	0.36
Total Sleep Time	-0.27	0.06	-0.33	0.11	-0.19	0.37
Wake after sleep onset	-0.05	0.75	0.09	0.66	0.02	0.93
Number of Awakenings	0.11	0.45	0.11	0.60	0.11	0.60
Average Awakening Length	0.02	0.91	-0.05	0.81	0.09	0.68
Subjective Mood						
Tension	0.09	0.54	0.33	0.10	-0.33	0.11
Anger	0.07	0.64	0.17	0.42	-0.13	0.55
Fatigue	0.08	0.57	0.15	0.47	-0.05	0.80
Depression	-0.13	0.36	-0.02	0.93	-0.37	0.07
Esteem-related Affect	0.00	0.98	-0.08	0.72	0.08	0.69
Vigour	-0.18	0.21	0.36	0.08	0.04	0.84
Confusion	0.07	0.61	0.09	0.65	0.05	0.82
Total Mood Disturbance	0.09	0.56	0.21	0.30	0.20	0.35
Vitamin C						
Subjective Sleep Quality						
Evening Sleepiness	0.03	0.82	0.05	0.82	0.02	0.91
Morning Sleepiness	-0.04	0.80	-0.16	0.45	0.23	0.26
Getting to Sleep	-0.09	0.55	-0.24	0.25	0.15	0.48
Quality of Sleep	-0.13	0.38	-0.26	0.21	0.04	0.83
Awake Following Sleep	-0.08	0.60	0.07	0.75	-0.37	0.07
Behaviour Following Wakening	-0.18	0.21	-0.07	0.73	-0.38	0.06
Objective Sleep Quality						
Latency	-0.43	0.00	-0.12	0.55	-0.72	0.00
Efficiency	0.13	0.37	-0.03	0.88	0.29	0.16
Total Sleep Time	0.05	0.71	-0.01	0.95	0.17	0.41
Wake after sleep onset	0.17	0.24	0.13	0.55	0.25	0.23
Number of Awakenings	0.11	0.45	-0.02	0.93	0.28	0.18
Average Awakening Length	0.06	0.67	0.07	0.74	0.05	0.80
Subjective Mood						
Tension	-0.11	0.46	-0.20	0.33	0.06	0.77
Anger	0.03	0.85	0.08	0.72	-0.08	0.71
Fatigue	-0.15	0.30	-0.11	0.61	-0.27	0.19
Depression	0.05	0.75	0.09	0.68	-0.04	0.87
Esteem-related Affect	0.22	0.13	0.27	0.19	0.16	0.43
Vigour	0.18	0.21	0.12	0.56	0.27	0.20
Confusion	-0.05	0.73	-0.23	0.28	0.19	0.37
Total Mood Disturbance	-0.15	0.31	-0.13	0.53	-0.20	0.33

E.2 Supplementary Documents

Document E.1 Massey University ethics committee letter of approval.

Date: 11 December 2020

Dear Alex Kanon

Re: Ethics Notification - **SOA 20/52 - Acute effect of kiwifruit on sleep quality, mood and metabolites associated with sleep in healthy young men**

Thank you for the above application that was considered by the Massey University Human Ethics Committee: **Human Ethics Southern A Committee** at their meeting held on **Friday, 11 December,**

Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Professor Craig Johnson
Chair, Human Ethics Chairs' Committee and Director (Research Ethics)

Research Ethics Office, Research and Enterprise
Massey University, Private Bag 11 222, Palmerston North, 4442, New Zealand **T** 06 350 5573; 06 350 5575 **F** 06 355 7973
E humanethics@massey.ac.nz **W** http://humanethics.massey.ac.nz

Document E.2 Recruitment poster

HEALTHY MALES WANTED

WE ARE EXPLORING THE ACUTE EFFECTS OF KIWIFRUIT ON METABOLITES, SLEEP & MOOD

In this study you will:

- Eat a prepared meal with or without kiwifruit on three separate occasions
- Give donations of urine to determine how kiwifruit may be impacting your sleep quality and mood

We are interested in talking to men in good general health who:

- Are 18-35 years of age
- Have a BMI of 18.5-30kg/m^2
- Are not allergic to kiwifruit
- Are not vegetarian or vegan
- Have no medical health problems

Participants selected to take part will be compensated for their time.

For more information email Alex at a.kanon@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 20/52. If you have any concerns about the conduct of this research, please contact Dr Negar Partow, Chair, Massey University Human Ethics Committee: Southern A, telephone 04 801 5799 x 63363, email humanethicsoutha@massey.ac.nz.

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Kiwifruit, sleep, metabolites and mood in men – an acute study

INFORMATION SHEET

Who are we?
This is a PhD research project being conducted by Alex Kanon (PhD Student), Dr Sharon Henare, Dr Caroline Giezenaar, Dr Prabhu Balan, Prof Nicole Roy, and Prof Warren McNabb of the Riddet Institute and Massey University. We are investigating if eating kiwifruit improves your sleep and mood.

Why are we doing this trial?
Sleep is an essential aspect of our lives. It is vital for the repair of the body, as well as being critical for learning and memory formation. Poor sleep can harm the body and has been linked to health concerns, such as diabetes, cardiovascular disease and hypertension. Studies have assessed the effects of nutrients on sleep and have been shown to have positive impacts on sleep. Evidence from other researchers has shown that consuming two green kiwifruits before bed may improve sleep. However, previous kiwifruit sleep studies have not explained how and what may be causing this. In this study, we would like to compare the effect of eating fresh or dried kiwifruit before going to bed on:

1. Changes in biomarkers related to sleep (serotonin, melatonin metabolites and other vitamins) as measured in urine collected the next morning
2. Sleep quality
3. Changes in your mood

Would you like to take part?
We want to invite 24 male participants aged 18 to 35 to join our study who are either good or bad sleepers. There are some reasons why you may not be able to take part in our research. These are:

- your Body Mass Index (BMI) is less than 18.5 or more than 30 kg/m^2 (To calculate your BMI, divide your weight in kilograms by your height in metres, squared)
- you suffer from a sleep disorder: periodic leg movements, sleep apnoea, narcolepsy, REM sleep behaviour disorder
- you have had a history of gastrointestinal surgery or gastrointestinal disorders including inflammatory bowel disease (IBD), ulcerative colitis, coeliac disease, or Crohn's disease. Those with medical conditions (e.g., cardiovascular or respiratory diseases, diabetes mellitus, bleeding disorders, autoimmune disorders) or psychiatric conditions (e.g., major depressive disorder, schizophrenia)
- Do you have any other medical conditions/illnesses (e.g. asthma, diabetes, epilepsy, claustrophobia, or gallbladder, pancreatic, cardiovascular or respiratory diseases)?
- you smoke cigarettes

- you are a shift worker who works during the night
- you undertake more than 2 hours of strenuous exercise per day
- you have had antibiotics in the last month
- you consume more than two glasses of wine or 600 ml of beer a day
- you take particular prescribed medication or use recreational drugs
- you have an allergy or intolerance to foods such as dairy, eggs, cereal, soy and kiwifruit
- you are currently taking supplements, and would not be willing to stop using these during the study
- you are on a controlled diet or dietary weight loss regimen within the last six months
- you are vegetarian or vegan
- you are currently experiencing any flu-like symptoms (fever, headache, dehydration, weight loss, lethargy) or have been tested and diagnosed with COVID-19

If you have any queries about these, please contact us by phone.

What is involved?

If you would like to participate, we will ask you to attend the Human Nutrition Research Unit (HNRU) at Massey University to complete a consent form and a screening questionnaire. In this screening questionnaire, we will ask you some questions about your general health, your current sleep quality and measure your height and weight. We will also explain what you will need to do in this study at this session. It will also be an excellent time for you to ask questions about any aspect of the study.

If the results of the screening questionnaires show you are eligible to take part, we will enroll you in the study. From this point on (screening session) we ask that you refrain from eating kiwifruit until the end of the study. We will then arrange a day and time for you to return to the unit for your first trial day.

Specific details of the study and what is expected of you

Trial day

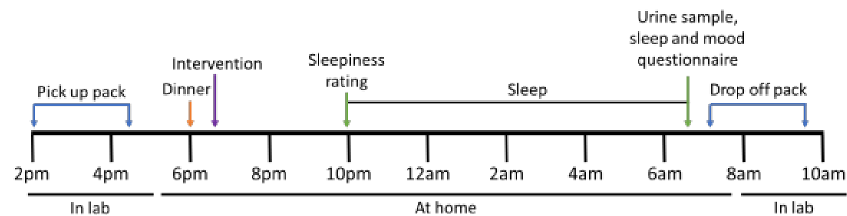
We will ask you to come to the HNRU between 2-4 pm on four different days, with a gap of at least one day between the first and second visit, and one week between visits after that. The first visit will serve as a baseline (to understand your regular habits) and the remaining three visits will be your intervention visits. Two days before each intervention visit, we will confirm your participation in the study. We will remind you not to eat certain foods that may affect the results of the study. The foods are on the restricted food list (page 6). We will also ask that you do not do any strenuous exercise (no more than 1 hour of exercise) on the three intervention days that you visit us.

When you arrive at the unit, we will confirm that you have not eaten any of the foods on the list and have not done any strenuous exercise. If you have, the study visit will be postponed. If you have not eaten the foods and not done any strenuous exercise, you will be provided with a data collection pack to take home. The pack will contain your standardised evening meal [Man Size Spaghetti and Meatballs (McCain Foods), 720 kcal], one of the three interventions, an actiwatch, a urine sample collection kit (container, cleansing wipe, gloves, Ziplock bag, label, icebox and ice bricks) and a survey booklet. The meal and intervention will not be provided on the first visit, eat as you usually would on this day. The actiwatch we lend you will allow us to measure your activity and sleep.

The interventions you will receive will be given to you in random order and include water, two green kiwifruit or dried green kiwifruit to be mixed with water. We ask that you consume your evening provided meal four hours before your usual bedtime and after finishing the entire meal, consume the

intervention you have been supplied with. We would then ask that you do not consume any more food after this. You may then continue to do what you usually would do until bedtime.

Just before you go to bed, we would like you to answer some questions in the survey booklet. After, once you are in bed, please press the marker button on actiwatch to indicate you will start sleeping. Then go to sleep as you usually would. When you wake up in the morning, press the marker button once again to indicate you have woken. We would then like you to collect a urine sample. All the things you will need to collect your urine will be given to you with instructions in the data collection pack. After this, please complete the survey booklet. Once this is done, we would ask that you return the sample and survey booklet to the HNRU at Massey or we will arrange to collect them from your home once completed. Details of what we would like you to do are summarised below. Please note that the timings are approximate and will vary between people.



This study will take approximately 4 days. The estimated total time commitment will be no more than 56 hours over 3 weeks (about 32 hours of this time you will be sleeping). This will be composed of a:

- Screening visit: 1 hour
- Study period (collection of intervention, consumption of standardised dinner and intervention, collection of urine and completion of surveys): 56 hours (about 32 hours of this sleeping).
- Travel time: 2 hours (based on 15 min journey each way)

As part of this study, we will use your urine for several different tests. When we separate your urine into smaller volumes for testing, we will not be able to return any part of these samples to you. However, any unused urine samples not used for testing may be returned to you at the end of the study if requested.

What are we going to measure?

Medical assessment before you start the study: We will ask you questions about your health and current medications. The use of the questionnaire in the screening session will allow us to understand whether you are a suitable participant.

Body measurements: We will measure your height and weight to estimate your body mass index. These measurements will be conducted in private. Bodyweight will be measured using ordinary weighing scales (you will be asked to remove your shoes and outer clothes), and standing height will be measured using a wall measure.

Urine: A morning urine sample will be collected. These samples will be used to assess changes in biomarkers related to sleep. Some of the biomarkers we are interested in are serotonin, melatonin metabolites and vitamins.

<u>Sleep quality and mood:</u> Sleep is vital for daily function. Moos is shown to be enhanced due to improved sleep. We want to gain a better understanding of how mood and sleep change with the kiwifruit intervention, to determine the possible mechanisms of these changes. Sleep will be measured using an Actiwatch 2 (activity watch) – this involves wearing a wristwatch which measures how active you are. They will tell us your activity, as well as inform us of your sleep at home. From this, we will be able to calculate how long you sleep each night, how active you are during the night, and how fast you fall asleep. Please remember to press the marker button when you are in bed and ready to sleep, and when you wake up in the morning. Surveys will also be used to measure your sleep quality and mood.

Figure 1 - Actiwatch 2 activity watch

Will I get financial compensation?
We recognise that this is a considerable amount of time and commitment and will reimburse you for that reason. Participants will receive voucher compensation of $20 after each submission of urine, actiwatch and questionnaire data.

Are any of the procedures harmful of painful?
None of the procedures are harmful or painful.

Who will see the information about me?
When you join the trial, you will be given a number and after that, all your information will be filed with a code number and stored in a locked filing cabinet accessed by the research team only. When data from all the participants has been pooled and made anonymous, it will be used in presentations to academic societies, scientific publications and a thesis as partial fulfilment of the requirements for a PhD in Nutritional Science. Only Alex Kanon will have access to names or personal details. All personal data will be destroyed at the end of the trial. You have a right to receive feedback about the overall results of this study. You can tell us if you wish to receive feedback by circling the relevant box on the consent form. This feedback will be in the form of a one-page summary. You will receive this feedback after the study has finished.

Who is funding this research?
This research is funded by the Riddet Institute and the Alpha-Massey Natural Nutraceutical Research Centre.

Compensation for injury
If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic, and your request will be assessed by ACC per the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted, you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

What are your rights?
You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question (although if you do not want to answer questions about your health before the study begins, we will not be able to recruit you)
- Withdraw from the study at anytime
- Ask any questions about the study at any time during participation
- Provide information on the understanding that your name will not be used unless you give permission to the researcher
- Be given access to a summary of the project findings when it is concluded

Will your employment be affected by choosing or not choosing to participate?
If you are employed by Massey University, your employment status or relationships with other staff members will not be affected by either your agreement or refusal to participate in this study. The same applies should you agree to participate in the study and then withdraw later. In other words, your employment with Massey University is entirely independent of your participation in the study.

Will your grades be affected by choosing or not choosing to participate?
If you are a student at Massey University, then you should understand that under no circumstances will your grades nor academic relationships with Massey University staff members be affected by either agreement or refusal to participate in this study.

Who can you contact for further information?
If you are interested in participating in this study or would like further information, please contact:

Alex Kanon: a.kanon@massey.ac.nz (022 650 7798) Dr Sharon Henare: S.J.Henare@massey.ac.nz

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 20/52. If you have any concerns about the conduct of this research, please contact Dr Negar Partow, Chair, Massey University Human Ethics Committee: Southern A, telephone 04 801 5799 x 63363, email humanethicsoutha@massey.ac.nz.

List of foods to avoid 48 hours before the overnight main study day

Here is a list of common foods that are high in serotonin and melatonin. The trial co-ordinator will ask you to avoid eating these foods 48 hours before and during the main trial days of the study.

Fruit and fruit juices:

Avocado	Bananas	Blackcurrants
Plums	Kiwifruit	Strawberry
Cranberries	Grapes (black & red)	Blackberries
Blueberries	Cherries	Raspberry
Tomatoes	Pineapple	Mango
Papayas	Passionfruit	Citrus (oranges, grapefruit, lemons)

Vegetables and vegetable juice:

Spinach	Beans (red, kidney)	Beetroot
Capsicums/Bell peppers	Mushrooms	Plantains (Cooking bananas)
Chinese cabbage	Lettuce	Potato/sweet potato (red & purple)

Miscellaneous:

Wine (red)	Chocolate	Pistachios
Walnuts		

List of drinks to avoid 24 hours before the overnight main study day

Tea (black & green)	Coffee	Caffeinated beverages

If you have any queries about other foods that you may eat as part of your regular diet, please ask the trial co-ordinator

Document E.4 Consent form.

Riddet Institute (PN 445)
Massey University
Private Bag 11 222
Palmerston North 4442
Telephone: +64 6 951 7295
www.riddet.ac.nz

Kiwifruit, metabolites, sleep and mood in men – an acute study

PARTICIPANT CONSENT FORM - INDIVIDUAL

I have read and I understand the Information Sheet. I have had the details of the study explained to me, any questions I had have been answered to my satisfaction, and I understand that I may ask further questions at any time. I have been given sufficient time to consider whether to participate in this study and I understand participation is voluntary and that I may withdraw from the study at any time.

1. I agree to participate in this study under the conditions set out in the Information Sheet.

Declaration by Participant:

I ____[print full name] hereby consent to take part in this study.

Signature: _______________________________ Date: _____________________________________

I would like any unused urine sample to be returned to me	YES	NO
I would like to receive feedback about the overall results of this study	YES	NO

Email: ____

Participant Consent Form v2 (2020) Page 1 of 1

Document E.5 Data collection booklet.

Study Code: | | | | | | |

Kiwifruit, sleep, metabolites and mood in men – an acute study

Data collection book

If you have any comments or questions relating to the research project or the questionnaire please feel free to contact Alex Kanon during working hours on 022 650 7798 or email a.kanon@massey.ac.nz

Instructions:

In this questionnaire, we will ask you to report on your sleep behaviour and mood. Please follow the instructions on the next page

Participant code________________ Date_________________

Study visit_________________

Study Code: | | | | | |

Hi, thank you for taking part of our study. You are provided with:

1. Actigraphy watch – to be worn on non-dominant arm
2. Data collection book (this booklet)
3. Urine collection containers, label, gloves, ziplock bag and instructions
4. Cleansing wipe, ice box and ice brick – **Note**: place provided ice brick in the freezer to freeze overnight
5. Meal (spaghetti) – only during intervention days
6. Intervention - only during intervention days

Please consume the meal and intervention four hours before your usual bedtime. Directions are below on how to heat provided meal:

1. Remove frozen meal from carton. Place meal into a microwave oven.
2. Cook on high for 6 minutes.
3. Remove meal from microwave, peel back film lid and stir meal.
4. Place film back over the meal and return to microwave. Cook on HIGH for a further 2 – 3 minutes.
5. Remove meal from microwave and stand for two minutes.
6. Eat the entire meal and then consume your intervention.
7. Do not consume any more food until tomorrow morning.
8. Please **ONLY** consume water and **DO NOT** drink any water two hours before bed.

Urine collection procedure – refer to laminated card on collection procedure (All pictures shown are for illustration purpose only. Actual items may vary)

Study Code: | | | | | | |

Please answer the following questions if you are ready for bed.

1. What time is it right now? ____
2. What time did you have dinner? _____________________________________
3. What time did you have the intervention? _________________________________
4. How much water did you drink between your intervention and now? ____________ml
5. Have you gone to the bathroom within the last two hours? YES/NO
6. According to the below scale, how would you rate your sleepiness right now? (circle/highlight)

Stanford Sleepiness Scale (SSS)

Degree of Sleepiness	**Scale Rating**
Feeling active, vital, alert, or wide awake	1
Functioning at high levels, but not at peak; able to concentrate	2
Awake, but relaxed; responsive but not fully alert	3
Somewhat foggy, let down	4
Foggy; losing interest in remaining awake; slowed down	5
Sleepy, woozy, fighting sleep; prefer to lie down	6
No longer fighting sleep, sleep onset soon; having dream-like thoughts	7

7. Once you are in bed, press the marker button on actigraphy watch to indicate you are ready to sleep. Press this again once you wake up tomorrow morning.

PLEASE be sure you have answered every item

PLEASE continue to the next page tomorrow morning when you wake up

Study Code: | | | | | | |

Good morning,

Please collect your whole urine sample in the containers provided – follow laminated instruction card. You have been provided with two containers should you need both. Do not forget to press the marker button on actigraphy watch. Please then answer the following questions once you have collected this.

1. What time is it right now? ____
2. What time did you wake up? ____
3. What time did you go to bed? ____
4. Did you wake up in the middle of the night? YES/NO
    - If yes, how many times and what for? ________________________________
5. What time did you collect your urine sample? ________________________________
6. Have you put the urine sample in the ice box with the ice brick? YES/NO
7. According to the below scale, how would you rate your sleepiness right now? (circle/highlight)

Stanford Sleepiness Scale (SSS)

Degree of Sleepiness	**Scale Rating**
Feeling active, vital, alert, or wide awake	1
Functioning at high levels, but not at peak; able to concentrate	2
Awake, but relaxed; responsive but not fully alert	3
Somewhat foggy, let down	4
Foggy; losing interest in remaining awake; slowed down	5
Sleepy, woozy, fighting sleep; prefer to lie down	6
No longer fighting sleep, sleep onset soon; having dream-like thoughts	7

PLEASE be sure you have answered every item

PLEASE continue to the next page

Study Code: | | | | | | |

Abbreviated POMS (Revised Version)

Below is a list of words that describe feelings people have. Please circle the number that best describes **HOW YOU FEEL RIGHT NOW**.

	Not at All	A Little	Moderately	Quite a lot	Extremely
Tense	0	1	2	3	4
Angry	0	1	2	3	4
Worn Out	0	1	2	3	4
Unhappy	0	1	2	3	4
Proud	0	1	2	3	4
Lively	0	1	2	3	4
Confused	0	1	2	3	4
Sad	0	1	2	3	4
Active	0	1	2	3	4
On-edge	0	1	2	3	4
Grouchy	0	1	2	3	4
Ashamed	0	1	2	3	4
Energetic	0	1	2	3	4
Hopeless	0	1	2	3	4
Uneasy	0	1	2	3	4
Restless	0	1	2	3	4
Unable to concentrate	0	1	2	3	4
Fatigued	0	1	2	3	4
Competent	0	1	2	3	4
Annoyed	0	1	2	3	4
Discouraged	0	1	2	3	4
Resentful	0	1	2	3	4
Nervous	0	1	2	3	4
Miserable	0	1	2	3	4
Confident	0	1	2	3	4
Bitter	0	1	2	3	4
Exhausted	0	1	2	3	4
Anxious	0	1	2	3	4

Study Code: | | | | | | |

	Not at All	A Little	Moderately	Quite a lot	Extremely
Helpless	0	1	2	3	4
Weary	0	1	2	3	4
Satisfied	0	1	2	3	4
Bewildered	0	1	2	3	4
Furious	0	1	2	3	4
Full of Pep	0	1	2	3	4
Worthless	0	1	2	3	4
Forgetful	0	1	2	3	4
Vigorous	0	1	2	3	4
Uncertain about things	0	1	2	3	4
Bushed	0	1	2	3	4
Embarrassed	0	1	2	3	4

PLEASE be sure you have answered every item

PLEASE continue to the next page

Study Code: | | | | | | |

Leeds Sleep Evaluation Questionnaire

Place a vertical mark on the line to indicate your self-evaluation

How would you describe the way you currently fall asleep in comparison to usual?

1. More difficult than usual	____________________	Easier than usual
2. Slower than usual	____________________	More quickly than usual
3. I feel less sleepy than usual	____________________	More sleepy than usual

How would you describe the quality of your sleep compared to normal sleep?

4. More restless than usual	____________________	Calmer than usual
5. With more wakeful periods than usual	____________________	With less wakeful periods than usual

How would you describe your awakening in comparison to usual?

6. More difficult than usual	____________________	Easier than usual
7. Requires a period of time longer than usual	____________________	Shorter than usual

How do you feel when you wake up?

8. Tired	____________________	Alert

How do you feel now?

9. Tired	____________________	Alert

How would you describe your balance and co-ordination upon awakening?

10. More disrupted than usual	____________________	Less disrupted than usual

Thank you for your cooperation. PLEASE be sure you have answered every item

Stop here and deliver this booklet and sample to researchers within two hours. If you have arranged to have this picked up, call/text researcher.

APPENDIX F

SUPPLEMENTARY MATERIALS TO CHAPTER 7

F.1 Supplementary Documents

Document F.6 Human and disability ethics committee letter of approval.

Health and Disability Ethics Committees
Ministry of Health
133 Molesworth Street
PO Box 5013
Wellington
6011
hdecs@health.govt.nz

Ethics reference: 2021 EXP 11089

24 November 2021

Mr Alexander Kanon

Massey University
Private Bag 11 222
Palmerston North
4442
New Zealand

Tēnā koe Mr Kanon

APPROVAL OF APPLICATION

Study title: Acute effect of dried kiwifruit on sleep onset, metabolites associated with sleep, mood and morning cognitive performance – a randomised controlled trial in healthy males with poor sleep

I am pleased to advise that your application was **approved** by the Central Health and Disability Ethics Committee (the Committee). This decision was made through the expedited review pathway.

Conditions of HDEC approval

HDEC approval for this study is subject to the following conditions being met prior to the commencement of the study in New Zealand. It is your responsibility, and that of the study's sponsor, to ensure that these conditions are met. No further review by the Central Health and Disability Ethics Committee is required.

Standard conditions:

- Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.
- Before the study commences at *any* locality in New Zealand, it must be registered in a clinical trials registry. This should be a registry approved by the World Health Organization (such as the Australia New Zealand Clinical Trials Registry, www.anzctr.org.au or https://clinicaltrials.gov/).
- Before the study commences at *each given* locality in New Zealand, it must be authorised by that locality in Ethics RM. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

After HDEC review

Please refer to the *SOPs* for HDEC requirements relating to amendments and other post-approval processes.

Your next progress report is due by 20 November 2022.

Participant access to compensation

The Central Health and Disability Ethics Committee is satisfied that your study is not a clinical trial that is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Participants injured as a result of treatment received as part of your study may therefore be eligible for publicly-funded compensation through the Accident Compensation Corporation.

Further information and assistance

Please contact the HDECs Secretariat at hdecs@health.govt.nz or visit our website at www.ethics.health.govt.nz for more information.

Nāku noa, nā

Mrs Helen Walker

Chair

Page 1 of 3

Central Health and Disability Ethics Committee

Encl: Appendix A: documents submitted

Appendix A: Documents submitted

Document Type	File Name	Date	Version
Evidence of CI Indemnity	Massey 2020 PI		
CV for Coordinating Investigator	Alex Kanon CV		
Surveys/questionnaires	20210923 Kiwi Sleep Acute - Surveys and Questionaires_V1	23/09/2021	1
Surveys/questionnaires	20210923 Kiwi Sleep Acute - Mood surveys and questionaires_V1	23/09/2021	1
Protocol	Protocol v1.1 240921 Acute Kiwi Sleep	24/09/2021	1.1
PIS/CF	PIS CF Acute Kiwi Sleep 250921	25/09/2021	1.1
Advertisement	Acute Kiwi Sleep - Recruitment Poster V1 20211001	01/10/2021	1
Data and Tissue Management Plan	20211001 Kiwi Sleep Acute - DMP and Tissue Managment Plan_v1	01/10/2021	1
Scientific Peer Review	hdec-peer-review_ ProfMarlenaKruger for A Kanon	04/10/2021	
Evidence of Consultation	Alex Kanon (HDEC) - Letter of Support	20/10/2021	1

Confidential

Acute effect of dried kiwifruit on sleep onset, metabolites associated with sleep, mood and morning cognitive performance – a randomised controlled trial in healthy males with poor sleep

Project Numbers: RM18873
Trial Registration Number: In process
Universal Trial Number: U1111-1268-2664
HDEC Application Number: In process

Primary Researcher:
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Supervisory Team:

Dr Sharon Henare	Dr Caroline Giezenaar	Prof Warren McNabb	Prof Nicole Roy
Chief supervisor	Co-supervisor	Co-supervisor	Co-supervisor
School of Health Sciences	School of Food and Advanced Technology	Riddet Institute	Department of Human Nutrition
Massy University	Massy University	Massy University	University of Otago
s.j.henare@massey.ac.nz	C.Giezenaar@massey.ac.nz	W.McNabb@massey.ac.nz	Nicole.roy@otago.ac.nz

Date: 24/09/2021
Version: 1.1

This study will be conducted in compliance with the protocol, Good Clinical Practice and all other applicable regulatory requirements, including archiving of essential documents.

Confidential information: No use or disclosure of this protocol is permitted without prior written authorisation from the investigators.

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Study Schedule

	Screening	Familiarisation (Day -7)		Treatment 1 (Day 0)		Treatment 2 (Day 7)		Treatment 3 (Day 14)	
Visit time		PM	AM	PM	AM	PM	AM	PM	AM
Visit number	1	2		3		4		5	
Research unit visit	x	x	x	x	x	x	x	x	x
Informed consent	x								
Eligibility screen	x								
Chronotype questionnaire	x								
Mental stress assessment	x								
Anthropometric measures	x		x		x		x		x
Subjective sleep quality assessment	x			x	x	x	x	x	x
Dietary assessment				x		x		x	
Sleep diary		x	x	x	x	x	x	x	x
Objective sleep quality assessment - Actigraphy		x	x	x	x	x	x	x	x
Mood assessment				x	x	x	x	x	x
Physical activity				x		x		x	
Weekly stress				x		x		x	
Blood samples				x		x		x	
Urine sample				x	x	x	x	x	x
Saliva sample					x		x		x
Cognitive assessment			x		x		x		x

1 Introduction and study rationale

1.1 Background

Sleep is essential for the body's cellular repair and is associated with learning (1) and memory consolidation (2). In New Zealand, insomnia approximately affects a quarter of adults (3). Insomnia symptoms include difficulties falling asleep and maintenance of sleep. Current treatment options for insomnia include Cognitive behavioural therapy (CBT). Other popular remedies used to treat sleep difficulties include prescribed sedatives, herbal extracts and complementary medicines, regular physical activity, and avoidance of stimulants such as caffeine before sleeping. These difficulties can have short and long-term health consequences. Short-term difficulties are associated with lower cognitive performance and concentration ability, increasing technical errors, and overall mood the following day (4). Long term consequences of sleep difficulties include increased health risks associated with inflammation, diabetes, hypertension, and obesity. (5-9).

Sleep studies have shown that changes in sleep and wake-promoting neurochemicals until bedtime impact sleep onset and maintenance. The primary neurotransmitters involved in sleep onset include melatonin (Mel), serotonin, gamma-aminobutyric acid (GABA) and adenosine (10). The neurotransmitters activate neurons in the brain's ventrolateral preoptic nucleus (VLPO) region, inhibiting the ascending wake/arousal system, thus causing sleepiness (11). Diet has been shown to impact the levels of these neurotransmitters. Consumption of a tart cherry (melatonin-rich) drink has improved subjective insomnia symptoms (12), nocturnal rest (13), sleep duration and onset (14). Daily consumption of two green kiwifruits (serotonin-rich) for four weeks improved subjective quality of sleep in people with sleep problems (15) and chronic insomnia (16). The subjective daytime function was also improved in the chronic insomnia cohort (16). The high levels of vitamins, minerals and serotonin are thought to be responsible for this effect. Furthermore, green kiwifruit skin extracts potentiate pentobarbital-induced sleep in mice via a GABAergic mechanism (17). This would suggest that supplementation of foods can impact neurochemicals after sleep onset.

Based on our recent findings, a single evening consumption of dried green kiwifruit (including skin) mixed with water significantly improved subjective sleep quality measures and increased urinary concentrations of hydroxyindoleacetic acid (5-HIAA, metabolite of serotonin) when compared to water alone. Specifically, subjective perception of getting to sleep improved, and falling asleep time decreased after consuming dried green kiwifruit powder. Subjective perceptions of ease of awakening, behaviour following awakening, morning alertness, and mood were significantly improved after consuming dried green kiwifruit powder compared to water alone. This provides evidence that consuming kiwifruit before bedtime positively impacts subsequent sleep and mood the following day. The results also suggest that kiwifruit is acting via the metabolites associated with sleep.

Therefore, the primary aim of this study is to confirm whether dried kiwifruit significantly improves getting to sleep compared to control and whether this corresponds with changes in metabolites associated with sleep onset. As a secondary aim for this study, we also aim to determine whether kiwifruit consumption influences mood and cognition and whether this is correlated with changes in metabolites related to sleep quality.

1.2 Hypothesis

- That a single evening consumption of dried green kiwifruit (including skin) will improve subjective measures of getting to sleep by the Leeds Sleep Evaluation Questionnaire (LSEQ) in males with poor sleep.
- That the dried green kiwifruit (including skin) will improve all other aspects of sleep quality, reduce sleep onset, and improve mood measures of fatigue, depression, vigour, esteem, and mood disturbances.
- Dried green kiwifruit (including skin) will change levels of sleep-wake metabolites and immune profiles. These differences can be used to understand better the mechanisms by which dried green kiwifruit influences sleep quality.
- Dried green kiwifruit (including skin) will improve morning alertness, cognitive ability and mood measures.

2 Study aims and objectives

The primary aim is:

a) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit) on falling asleep, determined using the LSEQ in healthy, young male adults who have difficulty falling asleep.

Secondary aims include:

b) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit) on other subjective (LSEQ) and objective (actigraphy) sleep parameters in healthy, young male adults who have difficulty falling asleep.

c) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit) on other mood in healthy, young male adults who have difficulty falling asleep.

d) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit) on plasma melatonin circulating levels before bed in healthy, young male adults who have difficulty falling asleep.

e) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit) on urinary metabolites of melatonin and serotonin in healthy, young male adults, who have difficulty falling asleep.

f) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit) on neuroactive metabolites, amino acids, enzyme activity, inflammation, and oxidative stress markers before bed in healthy young male adults, who have difficulty falling asleep.

g) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit) on waking salivary cortisol in healthy, young male adults who have difficulty falling asleep.

h) To determine the effect of an evening consumption of whole freeze-dried green kiwifruit (equivalent to two green kiwifruit), on a range of morning cognitive tasks measuring aspects of working memory, information processing speed, attention and global cognition, subjective mood, alertness and mental fatigue in healthy, young male adults, who have difficulty falling asleep.

3 Study design

3.1 Study overview

This is a double-blind, placebo-controlled, parallel cross-over design intervention study that will allow us to evaluate whether acute supplementation with green kiwifruit powder (which includes the skin) on; objective and subjective sleep quality and determines the potential mechanisms of action by measuring plasma, urinary and salivary concentrations of sleep/wake neurotransmitters. Additionally, we seek to determine impacts on other metabolites, mood and cognition.

The study includes five visits, spread over approximately four weeks. The first will be a screening visit, followed by one familiarisation visit (evening and morning) and the intervention visits. Each intervention visit will be separated by at least seven days before the next visit will occur. The study schedule and Figure 1 shows the general study design. We will set mutually agreed times with all participants before the study. This is to ensure participants understand all expected research unit visit times and the timeline of the study. From enrolment, participants will be asked to refrain from eating kiwifruit until the end of the study and maintain a sleep schedule.

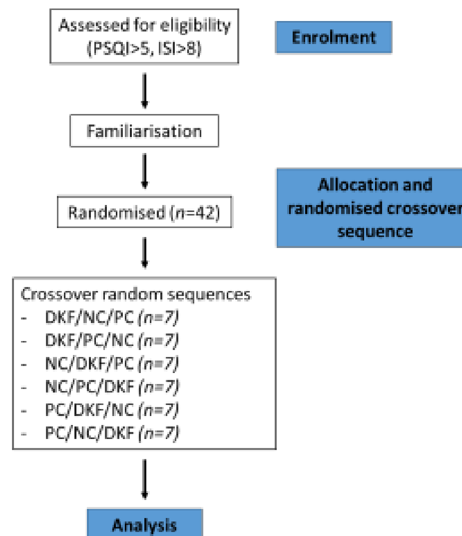

Figure 1 - CONSORT flow diagram. DKF = Dried green kiwifruit, PC = Positive control (matched sugar, flavour, and colour), NC = Negative control (Flavour and colour)

The dietary interventions will be:

1. Control (Flavour and colour) mixed with 200ml water
2. Control sugar (matched sugar, flavour, and colour) dissolved in 180ml water.
3. Freeze-dried whole (flesh and skin) green kiwifruit (32g which is equivalent to two green kiwifruits) dissolved in 170 ml of water

The intervention will be consumed within 10 minutes after a standardised evening meal. Sleep is a complex process, and it is not precisely known what dietary factors may impact sleep. Hence a flavoured water-only reference will be included as this gives certainty that no dietary factors or bioactives that may impact sleep will be given to the participants. A sugar control will also be included in the study. Studies have shown that a high carbohydrate meal before bed may decrease sleep onset (18). Tryptophan (Trp) is an amino acid that serves as a precursor for brain serotonin (a precursor of Mel) and is influenced by carbohydrates. The promotion of the entry of Trp into the brain is its plasma concentration relative to that of the other large neutral amino acids (LNAAs: tyrosine, phenylalanine,

leucine, isoleucine, valine, and methionine). It is known that high glycaemic index carbohydrates can increase the circulating ratio of Trp to LNAAs via a direct action of insulin, which promotes a selective muscle uptake of LNAAs. Kiwifruit sugars may be impacting sleep through this pathway. Still, it is also possible that other nutrients outside of sugar are responsible for the benefit seen. Hence a sugar control is being included in this study. The whole kiwifruit powder will include skin, which has higher levels of phytochemicals and actinidin (19, 20) than kiwifruit flesh. All interventions will be prepared in a food-safe facility by a member of the research team.

Randomisation will be conducted using a 3 x 6 Williams design. Randomisation will be performed using the method of randomly permuted blocks (https://www.randomizer.org/). Participants and researchers administering the intervention will be blinded to the treatment groups. The researchers involved in the analyses of the study results will be blinded to what intervention participants are receiving. Specified members of the research team will be responsible for the randomisation, the blinding of the other team members, the handout of the interventions, and the management of the interventions (Figure 1).

3.2 Study plan

Healthy males with sleep complaints will be eligible for this study (see section 4 Study population). Prospective participants will be provided with the study information sheet and invited to the Human Nutrition Research Unit (HNRU) at Massey University. They will meet with the study's primary researcher to sign the consent form and undergo screening. This session will be used to explain and clarify any aspect of the study participants may not understand.

After written informed consent has been collected, the participant will receive a unique identifier to link them to all data in an anonymous manner. The schedule for the participant will then be devised to ensure optimised timing for data and biological sample collection.

Collected questionnaire data will be stored and managed in a de-identified manner (also see section 8.3, Format of data). This includes a customised database, which is password protected and will consist of laboratory information, demographic information, and obtained questionnaire data. Diet data will be entered into Foodworks software (version 10; Xyris Software Pty Ltd).

Best practice will be the basis for the collection, processing, and storage of all biological samples. All samples will be stored at -80° C. Blood samples will be spun down and the plasma will be collected into eight aliquots, two for analysis of untargeted and targeted metabolites, two for neurotransmitters, two for immune and stress markers and two for vitamins and amino acids, a platelet-rich pellet will also be aliquoted for enzyme activity assay before being frozen and stored at -80° C. For more details on the collection, processing, and analyses to be performed, please see section 5 Measurement and samples methods

3.3 Study endpoint

The study's endpoint is when all questionnaires and biological datasets are collected from all enrolled participants, approximately the end of May 2022. The study aims to enrol a minimum of 42 participants.

3.4 Study outcomes

3.4.1 Primary outcomes

The primary outcome of this study is improvement in sleep quality as indicated by a statistically significant reduction in the time to fall asleep as measured by the LSEQ.

3.4.2 Secondary outcomes

Changes in objective and subjective sleep quality, subjective mood, plasma, urinary and salivary metabolites, cognitive function in response to an evening dinner supplemented with water mixed with flavour matched control, whole freeze-dried green kiwifruit, or matched sugar control.

4 Study population

4.1 The participants

The participants of this study are males with issues related to falling asleep which will be determined using the insomnia severity index (ISI) (minimum of 42 participant).

Participants will be recruited through advertisements placed on Massey University noticeboards and emailed to staff and students at Massey University, and partner organisations including the Riddet Institute, AgResearch and Plant & Food Research. The poster will also be shared on social media to increase exposure to potential participants. Should recruitment be challenging, permission will be requested from the ethics committee to engage with trial recruitment agency, Trial facts.

If potential participants are interested in the study, they will be provided with an information sheet. Potential participants will therefore self-identify. A screening questionnaire will be used to determine the suitability of the participants during the screening process.

4.2 Inclusion criteria

- Healthy males
- Aged between 18-45 years of age
- Body mass index (BMI) (18.5-30kg/m^2)
- Strenuous exercise no more than 2 hours per day
- Poor sleepers, defined as having an ISI (Insomnia Severity Index) score of >8 (21) and the Pittsburgh Sleep Quality Index (PSQI) score >5 (22)
    i. Regular bedtimes of approx. 11-12 to 7-8am

4.3 Exclusion criteria

- Inability to give informed consent
- Taken antibiotics within one month prior to the start of the study
- Medical history of gastrointestinal surgery or disorders (inflammatory bowel disease, ulcerative colitis, coeliac disease, Crohn's disease), cardiorespiratory problems, diabetes mellitus, bleeding disorders, sleep disorders, psychiatric conditions (major depressive disorder, schizophrenia)
- Significant weight loss (>5% of total body weight) during the past six months
- Significant dietary changes within one month prior to the start of the study (i.e. being on a controlled diet or dietary weight loss regimen)
- Night shiftworker
- Vegetarian/vegan
- Food intolerances (i.e., dairy, eggs, cereal, soy, kiwifruit)
- Smokers
- Aversion to blood sampling
- Excessive alcohol intake >20g of pure alcohol (2 drinks)/d on average. (>21 standard drinks a week)
- Donated blood in the past 12 weeks
- Use of certain prescribed medication (see table 2) or recreational drugs use

Table 1 - Exclusions by medications and supplements

Diuretics	Antacids
Oral or inhaled steroids	Cholesterol-lowering medications
Cholinergic antispasmodics	Proton pump inhibitors (acid reflux treatments)
Lactulose	Vitamin/mineral supplements
Metamucil	Heparin

Antibiotics	Antidepressants
Sedative/hypnotic medication	Laxatives

4.4 Restrictions during study

- Participants are asked to abstain from alcohol, caffeinated beverages, vigorous exercise twenty-four hours before their averaged bedtimes on trial visit days.
- Forty-eight hours before the in-lab visit, participants are asked to refrain from eating melatonin and serotonin rich foods.
- Participants are asked to abstain from eating kiwifruit for the entire duration of the study.

4.5 Number of participants

A power analysis was performed to estimate variance components using data from our study where dried kiwifruits were given and sleep was measured. Calculations were done on the primary endpoint subjective measures of getting to sleep (0.44) and sleep onset latency (0.51). The model-based upon an estimated error variance was taken from a recently completed study and calculated. Using the power of 80% suggests that the number of subjects required is estimated to fall between 33-36 participants. Given the potential for participants to withdraw from the study and ensure the design is balanced for order and carryover effects, recruiting 42 people will allow 80% power even after potential withdraws.

4.6 Participation and withdrawal from study

Participation in this study is voluntary. Participants may choose to withdraw at any time without explanation, as stated on the participant information sheet.
In case of the following occurrences, participants will be withdrawn:

- The participant requests withdrawal from the study. Participants can decide to end their involvement in the study for any reason during the study.
- The investigator can decide to withdraw a participant from the study if they consider it required, for example non-respect of at least one of the selection criteria after inclusion or non-compliance with the study protocol, gastroenteritis, or antibiotic use.

4.7 Reimbursement for participation

The participants will receive compensation for taking part in this study. Participants will receive compensation of $20 after the familiarisation night, $60 after the first intervention trial arm, $100 after the second intervention arm and $120 upon completion of the final intervention arm. The total value per participant is equal to $300.

5 Measurement and samples methods

5.1 Screening measures

Potential participants will complete the following screening measures to determine their eligibility in the study:

- Age
- Anthropometry – height and weight
- No hypnotic or sedating medications
- List of all medication taken in the last two months
- List of weekly physical activity and exercise
- Sleep difficulties – assessed by DSM-V diagnostic criteria - insomnia severity index (ISI) and Pittsburgh Sleep Quality Index (PSQI)
- Chronotype test – Morningness and Eveningness Questionnaire (MEQ)

5.2 Participant measures

Upon enrolment, participants will be invited to HNRU at the beginning of the study period for a familiarisation visit. They will then come in for their intervention visits. This is illustrated in the Study Schedule.

5.3 Outcome measures

5.3.1 Overview

Overview of all study outcome measurements

Clinical outcomes and measures	
PRIMARY OUTCOME	• Getting to sleep based on LSEQ
SECONDARY OUTCOMES	
Subjective	• Other measures from LSEQ • Sleepiness • Mood
Objective	• Actigraphy • Cognitive performance • Nutrient intakes
Biochemical measurements	
Blood	Plasma • Melatonin and cortisol • Neurotransmitters • Composite measure of inflammatory biomarkers and composite oxidative stress parameters • Composite measure of Hypothalamic–pituitary–adrenal (HPA) axis hormones • Macronutrients and micronutrients • Metabolome Platelets • Monoamine Oxidase (MAO) activity
Urine	• 6-sulfatoxymelatonin • 5-hydroxyindoleacetic
Saliva	• Cortisol – used for calculating cortisol awakening response (CAR)

5.3.2 Primary measurements

Sleep quality will be measured using LSEQ. The LSEQ is 10-item subjective self-measure to assess changes in sleep quality throughout psychopharmacological treatment intervention (23, 24). The scale

evaluates four domains of sleep, ease of initiating sleep (GTS), quality of sleep (QOS), ease of waking and behaviour following wakefulness. Scoring is on a 100mm scale which is then averaged to give a score of each domain. The LSEQ will be completed the morning after each intervention.

5.3.3 Secondary measurements

5.3.3.1 Subjective measures

- The Stanford sleepiness scale (SSS) (25) is a subjective measure of sleepiness that consists of a one-item question, with a scale which requires respondents to select one of seven statements that best represent their current sleepiness. During blood sampling at each trial visit, participants will be asked to rate their perceived sleepiness based on the Stanford Sleepiness Scale. Ratings will be asked at baseline upon arrival at the laboratory and 30, 60, 120, 180 and 240 min after complete ingestion of the standardised meal and intervention.
- The mood will be assessed using the POMS questionnaire (26, 27). This form contains 40 mood-related adjectives rated on a 5-point Likert-type scale, ranging from 0 (not at all) to 4 (extremely). The data is then categorised into six mood scales, tension-anxiety, depression-dejection, anger-hostility, vigour-activity, fatigue-inertia, and confusion-bewilderment. A Total Mood Score (TMD) will be calculated by adding depression, fatigue, tension, anger, and confusion scores, then subtracting the vigour score from this. Three mood surveys will be completed during each trial visit. That is upon arrival, before leaving the research unit, and at home upon waking.
- Visual analogue scale (VAS) of participants awakening state will also be measured. Participants will be asked to rate their current subjective state by marking on a 100mm line anchored with "not at all" (left-hand end) and "very much" so (right hand). Questions using these scales include, "how rested do you feel?", "how energetic do you feel?", "how relaxed do you feel?", "how irritable do you feel?", "how ready do you feel to perform?" and "have you had a good night's sleep?". The VAS will be completed the morning after each intervention.

The following measures will be used as covariates in analysis

- International Physical Activity Questionnaire (IPAQ) short form consists of 7 questions and measures total physical activity of week.
- Weekly Stress Inventory Short Form (WSI-SF) consists of 25 stress-related situations, which is rated on a 0-point liker scale, ranging from 0 (did not occur) to 7 (extremely stressful). Two scores are obtained, the event score (WSI-SFE) and the impact score (WSI-SFI).
- General sleep questions.
- Actigraphy data from five-seven for use in the respective analysis.
- 3-day food dairy.

5.3.3.2 Objective measures

- Actigraphy - this is a non-invasive method for monitoring cycles of rest and activity (28). Participants will be asked to wear an actigraphy wristwatch that uses a motion logger to log activity over time. Data will be downloaded to a computer programme for analysis. This allows you to measure sleep onset latency (SOL), sleep efficiency (SE), total sleep time (TST), wake after sleep onset (WASO) and number of awakenings. The actigraphy watch will be used for the entire duration of the study.
- Cognitive – Computerised Mental Performance Assessment System (COMPASS) is a digital cognitive battery that has been used to measure cognitive function in response to a nutritional intervention (29). Assessments will focus on attention and vigilance, working memory and executive function. The tests that will be used include digit vigilance, Rapid Visual Information

Processing (RVIP) and Stroop. The test will be repeated five times in succession. Before starting the tests and each repetition, a Bond-Lader VAS will be administered and participants will be asked to rate how mentally fatigued and alert they feel. Cognitive assessment will be completed at each morning trial visit after a standardised breakfast.

5.3.3.3 Biochemical measures

Briefly, at each visit, six blood samples will be collected. Samples collected will include baseline upon arrival at the laboratory and 30, 60, 120, 180 and 240 min after complete ingestion of the standardised meal and intervention. Upon the participant's arrival, a qualified phlebotomist will insert a catheter for blood sampling (Figure 2). Blood will be drawn in the following order at every time point: 4.0ml BD Vacutainer® Heparin and 6.0ml BD Vacutainer® K2EDTA. The vacutainers will be gently inverted and encased in aluminium foil. Blood will be placed on wet ice in the dark and centrifuged (Eppendorf Centrifuge 5702 R) for 10 minutes at 1,000 g at 4 °C within 10 minutes of collection. The resulting plasma will be removed and aliquoted into Eppendorf tubes for further centrifugation. A second centrifugation step will be done to achieve platelet free plasma and platelet-rich pellet; this will be centrifuged (Eppendorf Centrifuge 5415 R) for 10 minutes at 10,000 g at 4 °C (30). Both the pellet and plasma samples from the second centrifugation step will be stored at -80 °C until analysis. The plasma will be aliquoted into eight Eppendorf tubes. The area under the curve (AUC) will be calculated using the trapezoidal rule for all biochemical measures.

- Melatonin and cortisol will be quantified from plasma using a commercial assay kit. The results of the melatonin will be used to calculate the Dim Light Melatonin Onset (DLMO) timing (31).
- A targeted metabolomics approach of 75 different neurochemicals and metabolites (including serotonin, GABA and adenosine) will be quantified using established methods of AgResearch.
- Composite inflammatory biomarkers (TNFα, IL-6, IL-1β, IL-8, IL-12p70) and oxidative stress parameters (reactive oxygen species (ROS)/reactive nitrogen species, superoxide dismutase activity, catalase activity and lipid peroxidation concentrations) will be assayed using a bead-based multiplex panel and measured by flow cytometry. In addition, oxidative stress parameters will be measured using a commercial assay kit.
- Composite measures of HPA axis hormones (adrenocorticotropic, prolactin and growth hormone) in plasma will be measured using a commercial assay kit.
- Plasma vitamin C, amino acids and lipids will be measured by High-Performance Liquid Chromatography with electrochemical detection (HPLC-ECD) using established methods.
- Untargeted multiomic analysis of plasma samples using validated LC-MS techniques.
- Monoamine Oxidase (MAO) activity will be quantified from platelets using a commercial assay kit. MAO is responsible for the metabolism of neurotransmitters.

Three urine samples will be collected during each trial visit. That is upon arrival, before leaving the research unit, and at home upon waking. Urine will be collected into 500ml containers with 1g of chelating agent EDTA inside. Participants will be provided with multiple containers.

- Urine will be analysed for aMT6-s and 5-HIAA. The concentration will be corrected to creatinine to adjust for variation in dilution of urine. aMT6-s and 5-HIAA analyses will be conducted using a commercial assay kit.

Four waking salivary samples will be collected the morning after each trial visit using Salivette® containers. That is upon waking, and 30, 45, 60 min post wake.

- Saliva will be analysed for cortisol using a commercial assay kit.

5.4 Statistical analysis

Demographic and anthropometric characteristics will be summarised using descriptive statistics. All outcome variables will be analysed using a mixed-effects linear model ANOVA to account for the repeated measurements that yield period, sequence, and carryover effects and to model the various intra-patient and inter-patient variability sources. Where the repeated measures linear mixed model ANOVA is significant, Bonferroni post hoc analysis will be used to compare conditions. Relations of measures and metabolites will be tested using Pearson's Correlation Coefficient. Statistical significance will be accepted at $P < 0.05$. All data will be presented as means ± SEs. Heat maps will be generated to enable better data visualisation. Where appropriate, the original data will be transformed to achieve normality and constant variance in the residuals. Statistical significance for all parameters will be set at $P < 0.05$ with a confidence level of 95%.

6 Study visits

6.1 Screening visit

Prospective participants will be provided with the study information sheet and invited to a Human Nutrition Research Unit (HNRU) screening session to sign the consent form and have height and weight measured, complete health questionnaires, and sleep quality questionnaires (PSQI, and ISI). Eligible participants will be enrolled. From this point on, participants will be asked to refrain from eating kiwifruit until the end of the study and maintain a sleep schedule.

A case report form record (CRF) will be generated for all participants who have signed the consent form for the study, regardless of eligibility and/or continuing participation.

6.2 Familiarisation

Seven days before the first trial visits, participants will be invited for a familiarisation session. Participants will be asked to arrive at the HNRU between 6-8pm. The HNRU will be dimly lit to collect metabolites of interest. Once arrived, participants will be talked through exactly what would happen on a trial day. They will be provided with an actigraphy watch to be used until the completion of the study and a survey booklet to be completed before the next trial visit. The survey booklet will contain a three-day food diary and daily sleep diary. Participants will be told to activate the phase marker on the actigraphy watches when they are about to sleep and upon waking.

Once everything is complete, the participant will be escorted to on-site accommodation and asked to return the following morning fasted at an agreed time. Upon arriving in the morning, participants will be provided a standardised breakfast and asked to complete a cognitive test; this is used to ensure practice and learning effects are mitigated for subsequent trial visits.

6.3 Intervention visits

Two days before the scheduled trial visit, participants will be asked to refrain from consuming oranges, pineapples, bananas, mangos, papayas, plums, grapes, cherries, strawberries, tomatoes, capsicum, pistachios, plantains, mushrooms, chocolate, teas and coffee as these are known foods to contain and impact levels of serotonin and melatonin in urine (32-34).

On the day of the trial, participants will attend HNRU between 5-6:30 pm. Participants will be asked to have their last meal five hours before the lab visit and only take water until the visit. On arrival, the participant will be asked whether they have consumed any of the restricted foods two days before or undergone exercise on this day. If the participant has consumed restricted items or exercised, the study visit will be rescheduled. If the participant has not, they will continue with the trial visit and follow the schedule below.

Participants will be asked to complete a set of questionnaires (including abbreviated Profile of Mood States (POMS), International Physical Activity Questionnaire (IPAQ), Weekly Stress Inventory (WSI)) and provide a urine sample. A person trained in cannulation and venepunctures will insert a venous cannula in the arm vein of the participant to collect blood samples across multiple times points. At each blood sample, the participant's level of sleepiness will be rated according to the validated Stanford Sleepiness Scale (SSS). Approximately twenty minutes after the first blood sample, participants will be provided with a standardised evening meal [Man Size Spaghetti and Meatballs (McCain Foods), ~720 kcal], and the intervention to consume. The intervention will be in an opaque white bottle to mask what intervention the participant may be consuming. The dietary interventions will be given in a random order and will include (1) control (Flavour and colour) mixed with 200ml water, (2) control sugar (matched sugar, flavour, and colour) dissolved in 180ml water or (3) freeze-dried whole (flesh and skin) green kiwifruit (32g which is equivalent to two green kiwifruits) dissolved in 170 ml of water. Once the meal is finished, participants will be asked to sit and do light activities

such as read a book. They will also be asked not to use mobile phones or computers during their time in the lab. Six blood samples will be collected (~100mL each) beginning on arrival and 30, 60, 120, 180, 240 min post evening meal.

The last blood sample will be collected at approximately 10-11 pm (differs for each participant) and the cannula will be removed after this. The participant will be asked to complete the POMS survey and provide a urine sample. They will then be provided with a urine sample collection container, saliva collection containers and survey booklet to be completed in their room the following morning. Participants will be escorted to on-site accommodation. Once settled, when the participant is ready for bed, they are asked to activate the phase marker on the actigraphy watch.

Upon waking the following day, participants will be asked to activate the phase marker, collect the whole first-morning urine sample, collect four saliva samples (0, 30, 45, 60 min after waking) and complete the set of surveys. The validated surveys that will be completed in the morning are the SSS, Leeds Sleep Evaluation Questionnaire (LSEQ) and POMS. Participants will be asked to come into HNRU at the allocated time, fasted and with their urine, saliva samples and completed booklet.

Upon arriving at the HNRU, participants will be provided with a standardised breakfast. The participant will be asked to complete a cognitive test as they did at the familiarisation session. Once they have completed this, the participant will be free to go. Participants will repeat the in-lab visit two more times separated by seven days until each intervention has been consumed.

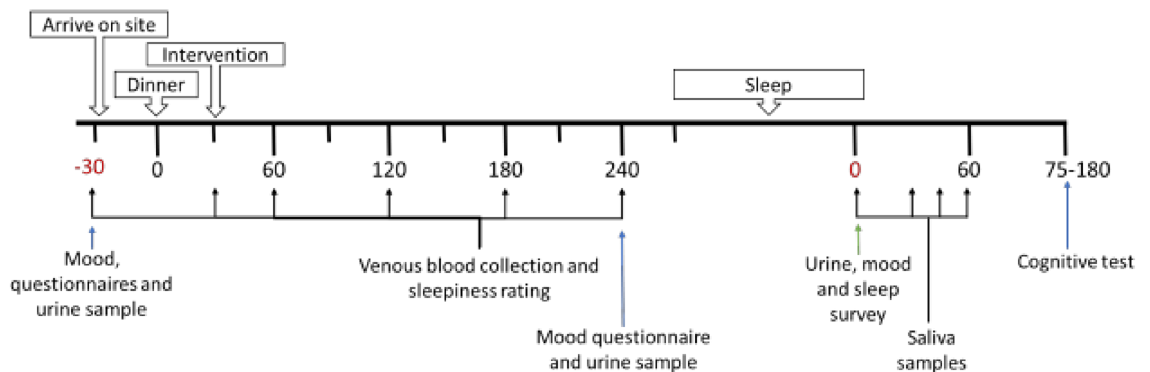

Figure 2 - Study protocol for each trial visit. At 1830h participant arrives at HNRU and completes a set of surveys, provides a urine sample, a cannula is inserted and the participant will consume a standardised dinner 1900h. Following this the intervention will be consumed. Blood samples will be collected on arrival and 30, 60, 120, 180, 240 min post evening meal. After the final blood sample, a mood survey will be completed, and urine sample collected. The participant will be escorted to a room and allowed to sleep. Upon waking the following day, urine and saliva samples (0, 30,45, 60 min) will be collected and a survey booklet completed. The sample is to be bought to HNRU and a cognitive test completed.

7 Risk assessment

7.1 Possible effect of kiwifruit

- Kiwifruit is shown to be well tolerated without any adverse side effects in human intervention studies. However, KF affects gastrointestinal function and causes laxation. For this reason, participants will be asked to report on experiences of mild bloating, nausea, vomiting, diarrhoea or abdominal cramps. If symptoms persist past 24 hours, they will be asked if they would like to continue or withdraw from the study.
- Kiwifruit is known to cause allergic reactions in a small proportion of the general population. For this reason, only participants who are not allergic to kiwifruit can take part in the study.

7.2 Possible effect of controls

- Sugar, flavours and colour are commonly used food additives. The health risk associated with this include:
    - High blood sugar: It has a high glycaemic index and can cause spikes in blood sugar levels; The main symptoms of increased blood sugar include sudden headaches, increased thirst, trouble concentrating, blurred vision, and fatigue. People with diabetes will be excluded from the study.
    - Allergies or intolerance: Many food additives can cause allergies or intolerance.
        - Symptoms of allergic reactions may include rash, skin irritation, swelling, and difficulty breathing
- The sugar amount is matched to the amount found in the dried kiwifruit powder.
- The amount of flavour and colour added to our control beverage is below the acceptable amount as stated in Australia New Zealand Food Standards Code – Standard 1.3.1 – Food Additives.

7.3 Possible risk of cannulation

- Slight pain may be experienced during the blood draw when the needle enters the arm, usually a prick or sting. Afterwards, there may be throbbing, excessive bleeding, bruising, fainting or feeling light-headed, and in rare cases, infection.
- To minimise risks, the researcher collecting the samples is trained in phlebotomy.
- In case of negative experiences during venepuncture, the research team needs to be informed.

7.4 Possible risk of fasting for venepuncture

- Since the participants will be required to fast after lunch (avoid all food and beverages except water for >5 hours) for the blood collection appointments, they may experience feelings of queasiness and shaking due to low blood sugar. We will provide their standardised evening meal and beverages after sample collection to remedy this.

7.5 Possible risk of data entry errors

- The quality and accuracy of the data collection will be checked by the researcher completing the data spreadsheet after each visit. All study team members are qualified and experienced scientists in human nutrition intervention studies. The data collection methods are previously established measures. Supervisors will also undertake monitoring visits at the unit before, during and after the trial. The supervisory team will audit data, consent forms and accountability logs after blocks within the study.

8 Data management

8.1 Purpose of data collection

We will only be collecting data explicitly relevant to answering the research questions. There are no plans to link with any other data sets. No harm is anticipated to occur with collecting this data beyond the risks of standard care and everyday life.

8.2 Data description

This study will generate quantitative data including questionnaires, laboratory analysis, physiological measurement, and clinically relevant information regarding symptoms.

8.3 Format of data

The data will be in the form of electronic files - excel spreadsheets, word documents, and data analysis files (R, SAS, Stata, or SPSS) and paper questionnaires. All participants will have a unique identifying code that will be recorded and used instead of any identifying information in all data files, including labels on the samples and data collected during the study period. The identifying data is kept separate to all surveys, including the screening survey.

8.4 Data collection, storage and access

8.4.1 Data collection strategy and storage by researchers

- Training requirements of data collectors: The researchers collecting data are experienced in data collection, data security and health-related confidentiality.
- Questionnaires: Most of the data collection will occur on paper. Paper-based collected data will be entered directly into an electronic data file as soon as it is received. The data will be inputted and be stored as excel for raw data and as data analysis files (R, SAS, Stata, or SPSS). The unique code will only identify this data for the participant.
- Software-based data: will be exported and data files will be stored as excel for raw data and as data analysis files (R, SAS, Stata, or SPSS). The unique code will only identify this data for the participant.
- Biochemical data will be analysed as per the measures mentioned above. The data files will be stored as excel for raw data and analysis files (R, SAS, Stata, or SPSS). The unique code will only identify this data for the participant. Participants are identifiable to researchers only by their study code.
- All electronic data files generated in the study will be stored on a password-secure Massey University server or Massey University OneDrive cloud storage and will be accessed (and downloaded, as the need arises) to the password-protected computers of named investigators, stored on locked premises.
- Raw data collected on hard copies will be stored as part of the CRF in a locked filing cabinet after electronic data entry.

8.4.2 Data access and sharing

- As indicated above, a master file containing participants' personally identifying information will only be accessible to the researchers undertaking data collection.
- The researchers (blind) will have only access to de-identified raw data files and will be responsible for final data analysis.

8.5 Data preservation strategy

All raw data collected in hard copy will be held for 10 years in locked cabinets on locked premises of the Massey University. Only the research team will have access to the data. No personal information will be revealed, and participants' confidentiality and data privacy will be protected in the

dissemination of research results. After 10 years hard copy documents will be destroyed and digital data deleted.

8.6 Data security and confidentiality of potentially disclosing information

The confidentiality of each participant will always be respected, and data will not be shared with third parties. The principles of the New Zealand Privacy Act 2020 will be followed. All data will be stored on password-protected files, backed up to Massey University servers and stored on computers on locked premises. The data is stored in Massey university data servers. Server backups are taken daily and are stored within the data centre. The server is secured with TLS Certificates, encrypting end-to-end communications. Security updates are applied to the server on a regular basis. Only the research team will have access to the data. No personal information will be revealed, and the confidentiality of participants and privacy of their data will be protected in the dissemination of research results.

8.7 Data monitoring

A formal Data Monitoring Committee will not be required as this study's intervention and control treatments are considered sufficiently low risk. No harm is anticipated to occur beyond the risks of standard care and everyday life. Data Monitoring will occur through the regular meeting of the research team. Procedures for recruitment and safety monitoring and adverse events are detailed in this full study protocol. The unblinded research team will maintain concealment. Ethical approval is sought from the Health and Disability Ethics Committee and locality authorisation for the study site before study commencement.

8.8 Case report forms

The CRF was explicitly designed for the needs of this study and contained all the data collected for the entire study. All data requested on the CRF must be recorded, and missing data must be explained. The CRF will only be identified by the unique code for the participant.

9 Dissemination of results

Results of the study will be provided to participants in the form of a one-page lay summary. The overall results of this research project will be used as part of a PhD thesis. The research results will also be published in scientific journals and/or book chapters and presented in conferences and/or in media.

10 Ethical and general considerations

10.1 Ethical conduct of study

The study will be carried out following this protocol, International Conference of Harmonisation (ICH) guidelines, national and local requirements, and the ethical principles originating in the Declaration of Helsinki.

10.2 Ethics approval and registration

Ethical approval will be sought from the Health and Disability Ethics Committee (HDEC) before the start of the study (https://ethics.health.govt.nz/).
The study will be prospectively registered on the Australian New Zealand Clinical Trials Registry (www.anzctr.org).

10.3 Protocol amendments

Any amendments to the study protocol will be reported to HDEC and all other local approval committees.

10.4 Consent

The researcher will invite eligible participants to consent to the study, emphasising that participation is voluntary and that the decision to participate will not influence the quality of care they receive. Participants will be advised that they are welcome to take time to consider consenting to the study and to discuss participation with their friends, whanau or other support persons. Adequate English language to undertake a discussion of consent is an inclusion criterion for the study; thus, translation of consent materials will not be offered. If at any time the participant's circumstances change in ways that could affect ongoing consent to participate in the study, they will be advised to raise that with the researchers or to notify they wish to withdraw consent to participate.

10.5 Confidentiality

No identifying or identifiable information about participants will be reported in any way from this study, including names, images or aspects of their circumstance that could identify them. For details on maintaining the confidentiality of health data (e.g., secure data storage) see 8.4

10.6 Funding source

Funding for this study has come from Riddet Institute and Massey University and in-kind from Zespri International Ltd.

10.7 Ethical considerations

Participants being coerced or perceiving to be coerced to take part

- All participants will be advised verbally and in written form that participation is voluntary and will not impact on them in any way, now or in the future.
- The Patient Information Sheet (PIS) and Consent Form (CF) state that participants are welcome to withdraw at any point without explanation by notifying the research team.

Participants may be concerned about the potential for breach of their privacy in the information shared

- Samples and data will be coded. The identity will only be known to the investigators and not transferred to third parties.
- All data will be inputted digitally, where the only identifier is the participant code.
- All devices on which study data are accessible are password protected and stored in locked premises.

- A code sheet linking participant identity to the code will be kept as a single hard copy and locked in a cabinet.
- Only the primary researcher shall have access. This coding sheet and the original hard documents of data shall be shredded upon completing the PhD project.
- No identifying data/ information will be shared with other researchers or collaborators.

Participants may experience side effects of the intervention/control or measures

- Participants will be provided with information about the management of likely side effects.
- Participants will be encouraged to contact their health care provider followed by the researcher in the event of side effects.
- Participants may withdraw if they experience any side effects; participants will be encouraged to record these symptoms as they are clinically relevant.
- Collection of venous blood samples can be slightly painful and cause discomfort, to reduce this a trained phlebotomist will do this. Participants will also be asked before enrolment if they have an aversion to blood sampling.
- Urine and saliva collections may be a cause of embarrassment for participants. To minimise this, the collection of these samples will be done solely by participants in their private bathrooms and in home.
- There will always be two researchers in the unit when conducting the study. The primary researcher is trained in first aid. The after-hours access protocol of Massey University will be followed in each instance.

Time commitment

- Participation in this study will require multiple visits and multiple hours of commitment to complete outcome measures; the researcher will include this information in the participant information sheet and provide a schedule of visits and measures at the time of enrolment.

Return of bodily material samples to participants

- Participants will be informed that they are given a choice for the return of remaining unused bodily material samples (such as blood, urine and saliva) after sample processing or that the remaining samples will be hygienically disposed of with appropriate karakia. Participants will be advised that bodily material samples sent for analysis to study collaborators cannot be returned.

Risks to researcher

- Blood, urine and saliva may pose a risk to researchers. It will be handled according to the Massey University Guideline for Handling Human Tissue in Research (2008). The primary researcher who will be exposed to the biological risk has been immunised against Hepatitis B.

11 References

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Document F.3 Recruitment Poster

Riddet Institute
ADVANCING FRONTIERS IN FOOD SCIENCE
MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

TROUBLE GOING TO SLEEP?

WE ARE EXPLORING THE ACUTE EFFECTS OF KIWIFRUIT ON SLEEP, METABOLITES, MOOD & COGNITION

In this study you will:

- See whether drinking kiwifruit helps you sleep
- Visit the lab four separate times
- Give donations of blood, urine and saliva to determine how kiwifruit may be helping you to sleep
- Do cognitive tests to determine if kiwifruit can help you

We are interested in talking to healthy males who:

- Are 18-45 years of age
- Have a BMI of 18.5-30kg/m^2
- Have trouble going to sleep
- Have no medical health problems
- Are not vegetarian or vegan
- Can attend study visits once a week over approximately 4 weeks

Participants selected to take part will be compensated for their time.

For more information email Alex at

a.kanon@massey.ac.nz

This project has been reviewed and approved by the Central Health and Disability Ethics Committee (reference: 11089)

Participant Information Sheet & Consent Form

Study title: The acute effects of dried kiwifruit on sleep onset, metabolites and cognition in men

Locality:	Massey University Manawatu (Turitea) Tennent Drive Palmerston North 4474 New Zealand	Ethics committee ref.:	TBC
Lead investigator:	Alex Kanon (PhD Student)	Contact phone number:	06 951 7292
Co-Investigator:	Dr Sharon Henare		
Co-Investigator:	Dr Caroline Giezenaar		
Co-Investigator:	Prof Nicole Roy		
Co-Investigator:	Prof Warren McNabb		

You are invited to take part in a study on whether drinking kiwifruit with your evening meal can help you fall asleep. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is ***11*** pages long, including the Consent Form. Please make sure you have read and understood all the pages.

Voluntary participation and withdrawal from this study

Participation in this study is voluntary and you are free to decline to participate at any time without having to give an explanation. In addition, you may withdraw your participation from this study at any practicable time without experiencing any disadvantage.

What is the purpose of the study?

Sleep is an essential aspect of our lives. It is vital for the repair of the body and is important for learning and memory formation. Poor sleep can negatively impact the body and has been linked to health concerns, such as diabetes, cardiovascular disease, and hypertension. Evidence from researchers has shown that consuming two green kiwifruits before bed may improve sleep onset. However, previous kiwifruit sleep studies have not shown how and what may be causing this. In this study, we aim to compare the effect of three different kiwifruit drinks on:

1. Sleep onset and quality
2. Biomarkers related to sleep (GABA, adenosine, serotonin, melatonin and tryptophan metabolites) in the blood, urine and saliva
3. Mood and mental ability

Riddet Institute — Advancing Frontiers in Food Science
Massey University — Te Kunenga ki Pūrehuroa — University of New Zealand | College of Health — Te Kura Hauora Tangata

This research is funded by the Riddet Institute and hosted at Massey University.

This study has been approved by an independent group of people called a Health and Disability Ethics Committee (HDEC), who check that studies meet established ethical standards. The **[insert Committee name]** has approved this study.

Who can take part in the study?

We want to invite 42 male participants aged 18 to 45 who have difficulty sleeping to join our study. There are some reasons why you may not be able to take part in our study. These are:

- your Body Mass Index (BMI) is less than 18.5 or more than 30 kg/m^2 (To calculate your BMI, divide your weight in kilograms by your height in meters, squared)
- you suffer from sleep disorders: periodic leg movements, sleep apnea, narcolepsy, REM sleep behaviour disorder
- you have had a history of gastrointestinal surgery or gastrointestinal disorders (e.g., inflammatory bowel disease (IBD), ulcerative colitis, coeliac disease, or Crohn's disease), chronic medical conditions (e.g., cardiovascular or respiratory diseases, diabetes mellitus, bleeding disorders, autoimmune disorders, asthma, epilepsy, claustrophobia, or gallbladder, pancreatic) or psychiatric conditions (e.g., major depressive disorder, schizophrenia)
- you smoke cigarettes
- you are a shift worker who works during the night
- you consume more than two standard drinks of alcohol a day
- you take prescribed medication or use recreational drugs
- you have an allergy or intolerance to kiwifruit, dairy, eggs, cereals and soy
- you undertake more than 2 hours of strenuous exercise per day
- you are currently taking supplements and would not be willing to stop using these during the study, as well as for two weeks before the study begins
- you have had antibiotics in the last month
- you have donated blood in the previous three months
- you are on a controlled diet or dietary weight loss regimen within the last six months
- you are vegetarian or vegan
- you have a fear of blood sampling or veins that are difficult to cannulate
- you are currently experiencing any flu-like symptoms

If you have any queries about these, please contact us by phone – Alex 022 650 7798.

What will my participation in the study involve?

If you would like to participate, we will ask you to attend the Human Nutrition Research Unit (HNRU) at Massey University on nine visits.

Specific details of the study and what is expected of you

Screening visit ~30min

The first visit is a screening session, during which we will explain what you will need to do if you are enrolled in this study. You will complete a consent form and a screening questionnaire. During this session, we will ask you some questions about your general health and current sleep habits. Your weight and height will be measured. This session is also an excellent time for you to ask questions about any aspect of the study.

If the results of the screening questionnaires show you are eligible to take part, we will enrol you in the study. From this point on (screening session), we will ask that you refrain from eating kiwifruit

until the end of the study and maintain a sleep schedule. We will then arrange a day and time for you to return to the unit for your familiarisation and trial visits.

Familiarisation visit~ 2 hours

Seven days before your first trial visit, you will be invited for a familiarisation session. When you come in for this visit, you will need to bring your sleeping clothes because you will be staying the night. Linen and towels will be provided, and you will have access to a shared shower and toilet facility. You will be asked to arrive at HNRU between 6-8 pm. The substances that we want to measure in the blood are affected by bright light, so the research unit will be dimly lit when you arrive. We will not be collecting any blood at this session. We will talk through exactly what will happen on a trial day. You will be provided with an actigraphy watch to use for the entire study and a survey booklet to be completed before the next trial visit. The survey booklet will contain a three-day food diary and daily sleep diary. We will show you how to activate the phase marker on the actigraphy watch so you can record when you go to sleep and when you wake up. Once everything is complete, you will be escorted to campus accommodation where you will spend the night to get you used to the environment. The next morning you will need to return to the HNRU in a fasted state at a time agreed with the researcher. That means you will not have eaten any food before you arrive. You can however drink water before coming into the unit.

At the unit you will be provided with breakfast and asked to complete a cognitive test. This is a test that tells us about the processes of your brain that are involved in almost every aspect of your life. This includes thinking, memory, judgment, and the ability to learn new things.

We will then arrange a day and time for you to return to the unit for your trial visits. You will be asked to come into the HNRU for **three trial visits**, each visit lasting 5 hours in the evening and 1.5 hour the following morning. Two days before each visit, we will confirm your upcoming visit to the unit. We will remind you not to eat certain foods that may affect the results of the study. These foods are on the restricted food list (page 9). We will also ask that you do not do any exercise on the three days you visit us. Once completed, you will be free to leave.

Trial visits ~ 7 hours

For these visits, we need you to arrive between 5-6:30 pm and be in a fasted state. That means you will not be able to eat anything for five hours before you arrive. You can however drink water during those five hours. When you arrive, we will confirm you have not consumed any of the restricted foods or done any strenuous exercise. If you have, the study visit will need to be rescheduled. If you have not, we will continue with the in-trial visit and follow this schedule.

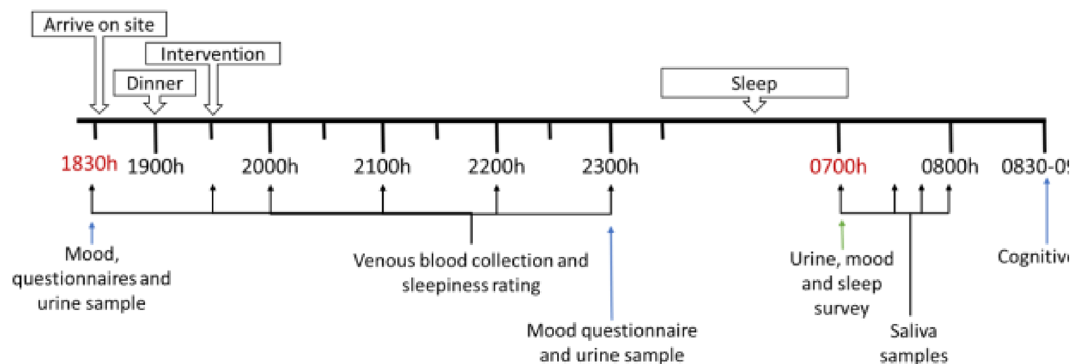
We will ask you some questions about how you feel, your sleep and physical activity. We will ask you to provide a urine sample and insert a cannula into your forearm to collect blood samples. A cannula is a small plastic tube and valve device taped to your arm that allows repetitive withdrawal of blood to be collected without having the need for insertion of a needle into the vein every time we need a blood sample. After your first blood sample, we will provide you with a standardised evening meal [Man Size Spaghetti and Meatballs (McCain Foods)] and one of the three different kiwifruit drinks. During your time at the unit, you will not be allowed to eat any other food/drinks other than what is provided.

Throughout the evening, we will collect six small blood samples (~100 ml in total, less than half a cup) using the cannula. You will not be able to use your mobile phone or computers during your time in the lab because the light from electronic devices influences the metabolites, we are interested in. So please bring some reading material for yourself. Once we have collected your last blood sample between 10-11 pm the cannula will be removed, you will complete a final survey and be asked to provide another urine sample. In the first session at the unit, you will be guided through what will happen. Once everything is complete, you will be escorted to campus accommodation. A data

collection pack will be given to you to take to the accommodation. The pack contains a urine and saliva sample collection kit (instructions for collecting a sample, urine container, saliva tubes, cleansing wipe, gloves, Ziplock bag, label, icebox and ice bricks) and a survey booklet to complete in the morning before coming into the lab.

When you wake up in the morning, you will need to collect a urine sample, saliva samples and complete the survey booklet. Once this is done, we would like you to return the urine and saliva samples and survey booklet to the HNRU. At the HNRU, we will give you breakfast, and you will then complete a cognitive test which will take one hour to complete. When you have finished the test, you will be free to leave.

Details of what we would like you to do during a trial visit are summarised below. Please note that the timings are a guideline to give you an idea about when things will happen and will vary depending on your schedule.



This study will take approximately four weeks. The estimated total time commitment will be around 63 hours over the four weeks (about 32 hours of this time, you will be asleep). This will be composed of:

- Screening and familiarisation process (including an overnight familiarisation sleep): 10.5 hours (about 8 hours of this time, you will be sleeping at onsite accommodation)
- Three trial visits (completion of surveys, consumption of intervention, collection of blood, urine and saliva samples, overnight sleep study): 45 hours (about 24 hours of this time, you will be sleeping at onsite accommodation)
- In-home food and sleep diary: 5 hours (one minute daily for sleep diary, and thirty minutes daily for food diary, three times a week, three times)
- Travel time: 2.5 hours (based on 15 min journey each way)

Reassurances

On first reading, this study may appear complicated and too much to remember. To reassure you, the trial investigator will be present throughout the whole trial and commit to the following:

- Provide you with the necessary information for you to decide whether you would like to take part in this study.
- Keep you informed about what is expected from you at each step of the study.
- Monitor your wellbeing at each step of the study.
- Provide drink and food and an area to rest and relax when required.

What are we going to measure?

Screening assessment before you start the study: we will ask you questions about your health and any medications you are currently taking. The use of the questionnaire in the screening session will allow

us to understand whether you are able to take part in the study and give us an understanding of your general health that may affect sleep, mood, and cognition (which includes your attention, memory, decision making).

Body measurements: we will measure your height and weight to calculate your BMI. These measurements will be done in private. Bodyweight will be measured using ordinary weighing scales (you will be asked to remove your shoes and outer clothes) and standing height will be measured using a wall measure.

Sleep measures: Actiwatch 2 (activity watch) involves wearing a wristwatch that measures how active you are. We will ask you to press the marker button on the device every time you are in bed and about to sleep and when you get up in the morning. From this, we will calculate how long you sleep each night, how active you are during the night, and how fast you fall asleep. We will also use a sleep questionnaire to see whether the kiwifruit drinks change how you perceive your sleep quality.

Biomarkers in blood, urine & saliva: Each evening you are in the unit we will collect some of your blood. We will collect approx. 100mL (less than half a cup) of blood as six samples per visit. Three urine samples will be collected per visit, two at the unit and one at home. Four saliva samples will be collected at home. These samples will be used to assess changes in biomarkers related to sleep. Some of the biomarkers we are interested in are serotonin, melatonin and tryptophan metabolites. We will also look at other biomarkers including neuroactive metabolites, amino acids, vitamins, enzyme activity, inflammation and oxidative stress markers.

Mood and cognition: It is well established that cognitive function (which includes your attention, memory, decision making) is improved when a person has a good sleep. Mood is also shown to be improved when we have a better night's sleep. We want to understand how kiwifruit improves mood, cognition, and sleep. We will use surveys and a computer based cognitive test to assess this.

What will happen to my blood, urine and saliva samples?

As part of this study, we will determine concentrations of several components from your blood, urine and saliva. Specific components are unable to be returned after separation. However, any unused plasma and urine samples may be requested and returned to you at the end of the study. All samples collected from you will be labelled with a unique code in a way that will not identify you as the donor. Your samples will be stored in a secure lab with restricted access to members of the research team. Your samples will ONLY be used for the purpose of this study. Your blood or its products will not be stored for future research and will not be used for research purposes outside this project. Blood samples collected from you in this study will not be passed on to anybody else outside of the project team. After all analysis has been completed, if you require it, we will return the rest of your samples to you after analysis. Otherwise, it will be disposed of hygienically (in accordance with NZS 4304:2002 "Healthcare Waste Management") or with the appropriate karakia if you wish

You may hold beliefs about a sacred and shared value of all or any tissue samples removed. The cultural issues associated with storing tissue samples collected from you should be discussed with your family/whānau as appropriate. There is a range of views held by Māori around these issues; some iwi disagree with storage of samples citing whakapapa and advise their people to consult before participating in research where this occurs. However, it is acknowledged that individuals have the right to choose.

If you decide to withdraw from this study at any point, any samples collected from you will be chemically destroyed or returned to you if requested.

What are the possible benefits of this study?

You may or may not benefit from taking part in this study. There is no guarantee that you will experience any changes in sleep quality or satisfaction from taking any of the study products. This study will provide insight on the impact of dried green kiwifruit on sleep quality, mood and cognitive ability. It will also provide new knowledge on the changes in metabolites that may change be seen in blood is linked to observed changes in sleep quality. Findings from this study will be used in presentations to academic societies, scientific publications, and a thesis as partial fulfilment of the requirements for a PhD in Nutritional Science. No names or personal details will be given to anyone outside of the research team.

What are the possible risks of this study?

Food restrictions and kiwifruit: You may feel unsure about what foods, drinks and supplements you are able to eat before doing this study. Moreover, you may feel uneasy about stopping taking dietary supplements and/or kiwifruit. There is a small chance that you may experience a tingling and itching sensation in your mouth or a skin rash after consuming kiwifruit.

You will be reassured by the trial investigators that will closely monitor and advise you on what foods will be fine to eat and drink during the trial period. You will be given a list of foods to refrain from consuming during the study. If you are uncomfortable with refraining from these foods, you may withdraw from the study without having to give an explanation.

The amount of green kiwifruit you will be asked to consume as a drink is equivalent to consuming two green kiwifruits, which is the size of a standard serve. It should have no adverse effect on your health. However, if you do experience adverse side effects from consuming the kiwifruit drink, you are asked to let the trial investigators know and consult with your health care provider.

Blood sampling: You may feel uneasy about giving blood. There may be a minor physical discomfort and you may experience some stinging while donating blood. You may also feel light-headed and dizzy after donating blood. This is not caused by blood loss from the amount of blood that you will be asked to donate in this study, but due to the possibility that you may feel queasy from the appearance of blood. You may also have concerns about donating blood.

To ease any concerns, you may have about giving blood, only a fully trained phlebotomist, who holds a certificate in venepuncture, will collect venous blood donations from you. A First Aider will be present during the blood sampling. The amount of blood we are asking you to donate is well below what is deemed safe to donate in one day. Nevertheless, if you feel light-headed after having blood taken, you will be asked to lie down with your feet slightly elevated, which will minimise the feeling of any light headiness/dizziness after giving blood. This only happens in a small percentage of the population. If you have cultural concerns about giving blood, you may want to talk to your family or whānau before taking part in this study. We can assure you that the blood donated in this study will not be given to anyone else and any unused plasma may be requested and returned to you, or we will destroy it after the trial.

Will any costs be reimbursed?

We recognise that this is a considerable amount of time and commitment, and we will reimburse you for that reason. Therefore, participants will receive voucher compensation of $20 after the familiarisation visit, $60 upon completion of the first intervention trial arm, $100 upon completion of the second intervention arm and $120 upon completion of the final intervention trial arm. Free parking is provided in front of the HNRU.

What if something goes wrong?

If you were injured in this study, you would be eligible to apply for compensation from ACC just as you would be if you were injured in an accident at work or at home. This does not mean that your claim will automatically be accepted. You will have to lodge a claim with ACC, which may take some time to assess. If your claim is accepted, you will receive funding to assist in your recovery.

If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won't affect your cover.

What will happen to my information?

During this study the lead researcher will record information about you and your study participation. This includes the results of any study assessments.

Identifiable Information: Identifiable information is any data that could identify you (e.g., your name, date of birth, or address). Only researchers will have access to your identifiable information:

- The project research team who will complete the study assessments.
- Massey University and its representatives if you make a compensation claim for study-related injury. Identifiable information is required to assess your claim.
- The sponsor, ethics committees, or government agencies from New Zealand or overseas, if the study or site is audited. Audits are done to make sure that participants are protected, the study is run properly, and the data collected is correct.

De-identified (Coded) Information: To make sure your personal information is kept confidential, information that identifies you will not be included in any report generated by the research team. Instead, you will be identified by a code. The lead investigator will keep a list linking your code with your name, so that you can be identified by your coded data if needed. The results of the study may be published or presented, but not in a form that would reasonably be expected to identify you.

Future Research Using Your Information: Your coded information collected in this study will only be used for this study and will not be used for other medical and/or scientific research that is unrelated to the current study.

Security and Storage of Your Information: Your identifiable information is held at Massey University during the study. After the study it is transferred to a secure archiving site and stored for at least 10 years, then destroyed. Your coded information will be entered into electronic files and stored on secure server at Massey University. Coded study information will be kept by the sponsor in secure, cloud-based storage indefinitely. All storage will comply with local and/or international data security guidelines.

Risks: Although efforts will be made to protect your privacy, absolute confidentiality of your information cannot be guaranteed. Even with coded and anonymised information, there is no guarantee that you cannot be identified. The risk of people accessing and misusing your information (e.g., making it harder for you to get or keep a job or health insurance) is currently very small, but may increase in the future as people find new ways of tracing information.

Rights to Access Your Information: You have the right to request access to your information held by the research team. You also have the right to request that any information you disagree with is corrected. Please ask if you would like to access the results of your screening during the study. If you have any questions about the collection and use of information about you, please talk with the lead investigator.

Rights to Withdraw Your Information: Your participation in this study is voluntary and you are free to decline to participate. If you agree to participate, you may withdraw from the study at any stage without giving a reason.

You may also withdraw your consent for the collection and use of your information at any time by informing the Lead Investigator. If you withdraw your consent, your participation will end, and the study team will stop collecting information from you. If you agree, information collected up until your withdrawal from the study will continue to be used and included in the study. You may ask for it to be deleted when you withdraw unless you withdraw after the study analyses have been undertaken.

Ownership Rights: Information from this study may lead to discoveries and inventions or the development of a commercial product. The rights to these will belong to Massey University. You and your family will not receive any financial benefits or compensation, nor have any rights in any developments, inventions, or other discoveries that might come from this information.

What happens after the study or if I change my mind?

The Lead Investigator will give you an overview of the main findings of the study and your personal results and answer any questions you have about this research upon request. A significant delay may occur between data collection and the publication of any results, which is normal. Data will be stored for a maximum of 10 years and will be the responsibility of the Lead Investigator, after which it will be destroyed. All data collected in this study will not be used for future related studies or unrelated research.

If you decide to withdraw from the study, your health questionnaire and survey can be returned to you upon request, otherwise it will be destroyed. Any blood, urine and saliva sample collected from you will be destroyed or returned to you if requested.

Will your employment be affected by choosing or not choosing to participate?

If you are employed by Massey University, your employment status or relationships with other staff members will not be affected by either your agreement or refusal to participate in this study. The same applies should you agree to participate in the study and then withdraw later. In other words, your employment with Massey University is entirely independent of your participation in the study.

Will your grades be affected by choosing or not choosing to participate?

If you are a student at Massey University, then you should understand that under no circumstances will your grades nor academic relationships with Massey University staff members be affected by either agreement or refusal to participate in this study.

What do I do if I have concerns about this research?

If you want to talk to someone who isn't involved with the study, you can contact an independent health and disability advocate on:

- Phone: 0800 555 050
- Fax: 0800 2 SUPPORT (0800 2787 7678)
- Email: advocacy@advocacy.org.nz
- Website: https://www.advocacy.org.nz/

For Māori health support please contact:

- Dr Sharon Henare, Senior Lecturer, School of Health Sciences, Massey University, Palmerston North
- Telephone number: +64 (06) 356 9099 ext. 84259
- Email: s.j.henare@massey.ac.nz

You can also contact the health and disability ethics committee (HDEC) that approved this study on:

- Phone: 0800 4 ETHICS
- Email: hdecs@health.govt.nz

Who do I contact for more information?

Alex Kanon (PhD Student and Lead Investigator)
School of Health Science & Riddet Institute
Massey University
Palmerston North 4474
Phone: 022 650 7798
Email: a.kanon@massey.ac.nz

List of foods to avoid 48 hours prior to the overnight trial days

Here is a list of common foods that are high in serotonin and melatonin. The trial co-ordinator will ask you to avoid eating these foods 48 hours before and during the main trial days of the study.

Fruit and fruit juices:

Avocado	Bananas	Blackcurrants
Plums	Kiwifruit	Strawberry
Cranberries	Grapes (black & red)	Blackberries
Blueberries	Cherries	Raspberry
Tomatoes	Pineapple	Mango
Papayas	Passionfruit	Citrus (oranges, grapefruit, lemons)

Vegetables and vegetable juice:

Spinach	Beans (red, kidney)	Beetroot
Capsicums/Bell peppers	Mushrooms	Plantains (Cooking bananas)
Chinese cabbage	Lettuce	Potato/sweet potato (red & purple)

Miscellaneous:

Wine (red)	Chocolate	Pistachios
Walnuts		

List of drinks to avoid 24 hours before the overnight main study day

Tea (black & green)	Coffee	Caffeinated beverages

If you have any queries about other foods that you may eat as part of your regular diet, please ask the trial co-ordinator

Riddet Institute
ADVANCING FRONTIERS IN FOOD SCIENCE

MASSEY UNIVERSITY
TE KUNENGA KI PŪREHUROA
UNIVERSITY OF NEW ZEALAND

COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

Consent Form

Study title: The acute effects of dried kiwifruit on sleep onset, metabolites and cognition in men

Please tick to indicate you consent to the following		
I have read or have had read to me in my first language, and I understand the Participant Information Sheet.		
I have been given sufficient time to consider whether or not to participate in this study.		
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.		
I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.		
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.		
I consent to the research staff collecting and processing my information, including information about my health.		
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes ☐	No ☐
I agree to an approved auditor appointed by the New Zealand Health and Disability Ethics Committees, or any relevant regulatory authority or their approved representative reviewing my relevant medical records for the sole purpose of checking the accuracy of the information recorded for the study.		
I understand that my participation in this study is confidential and that no material, which could identify me personally, will be used in any reports on this study.		
I understand the compensation provisions in case of injury during the study.		
I know who to contact if I have any questions about the study in general.		
I understand my responsibilities as a study participant.		
I wish to receive a summary of the results from the study.	Yes ☐	No ☐

I wish to receive all unused biological samples collected at the end of the study.	Yes ☐	No ☐

Declaration by participant:

I hereby consent to take part in this study.

Participant's name:	
Signature:	Date:

Declaration by member of research team:

I have given a verbal explanation of the research project to the participant and have answered the participant's questions about it.

I believe that the participant understands the study and has given informed consent to participate.

Researcher's name:	
Signature:	Date:

The acute effects of dried kiwifruit on sleep onset, metabolites and cognition in men

Health Screening Questionnaire

Thank you for expressing an interest in participating in our research project. To ensure you are eligible to participate in the research project, we would appreciate it if you would answer the following questions.

Basic Information

Name:

Age:

Current Weight (kg):

Height (m):

Ethnicity (please highlight all that apply)

European	Maori	Pasifika
Asian	Middle Eastern/Latin American/African	Other___________

Email address:

Telephone number:

Postal address:

Primary Emergency Contact Name:

Relationship:

Phone Home:

Health Information (Please delete as appropriate)

1. Do you smoke cigarettes? YES / NO
2. Do you drink alcohol? YES / NO
   - If **yes**, how many standard drinks do you consume per week? (1 standard drink is 1 can/bottle of standard beer (330ml), 100ml wine or 30ml of spirits)____________________
   - If **yes**, how many occasions would you drink alcohol per week?________________________

3. At each visit are you happy to have blood samples taken via cannulation? YES / NO

4. Have you given blood in the last **three months**? YES / NO

5. Are you a night sift worker? YES / NO

6. Are you currently experiencing flu-like symptoms (fever, headache, dehydration, weight loss, lethargy) or have you been tested and diagnosed with COVID-19? YES / NO

7. In the last **month**, have you taken antibiotics? YES / NO

8. Are you vegetarian or vegan? YES / NO

9. Are you allergic to dairy, eggs, cereal, soy or kiwifruit? YES / NO

   - If **yes**, which ones? ____

10. Are you currently on or in the last **month been** on a controlled diet or weight loss regimen? YES / NO

    - If **yes**, what type of diet or weight loss regimen? ______________________

11. In the last **six months** have you experienced significant weight loss? (>5 kg) YES / NO

12. How many caffeinated drinks do you have in a day? ______________________

13. How many hours of screen time do you have in a day? ______________________

14. What time do you have dinner? ____________________________________

15. Do you live with children? YES / NO

    - If **yes**, do they wake you in the middle of the night? ______________________

Have you been diagnosed with or experienced any of the following ('x' for yes)?	Yes
Heart disease	
Stroke	
High cholesterol	
High blood pressure	
Asthma	
Kidney disease	
Diabetes (type 1 or 2, or prediabetes)	
Bleeding disorders	
Inflammatory bowel disease	
Irritable bowel syndrome	
Bowel or gastrointestinal surgery	
Food intolerance or allergies causing diarrhoea, bloating, cramping or constipation	
Long term diarrhoea or constipation	
Autoimmune disease (e.g. Coeliac disease, Rheumatoid arthritis, Multiple sclerosis)	
Liver disease	
Psychiatric conditions (e.g. Major depressive disorder, Schizophrenia)	
Sleep disorders (e.g. periodic leg movements, sleep apnoea, narcolepsy)	
Other (please specify):	

Are you taking any medications (traditional or homoeopathic) or nutritional supplements?

Type of medication/supplement	Taking (delete as appropriate)	If you have answered YES for any of the medication or supplement options, please provide the information below	
		Medication/supplement name	Dose and frequency
Oral or inhaled steroids	YES / NO		
Cholinergic antispasmodics	YES / NO		
Lactulose	YES / NO		
Metamucil	YES / NO		
Antibiotics	YES / NO		
Sedative/hypnotic medication	YES / NO		
Laxatives	YES / NO		
Diuretics	YES / NO		
Antacids	YES / NO		
Cholesterol-lowering medications	YES / NO		
Proton pump inhibitors (acid reflux treatments)	YES / NO		
Vitamin/mineral supplements	YES / NO		
Heparin	YES / NO		
Antidepressants	YES / NO		
Recreational drugs	YES / NO		

16. Are you willing to stop taking your nutritional supplement for the duration of the study? YES / NO

17. Would you like to take part in the sleep architecture study? YES/NO

What statement would best describe your day to day activity level (please mark with an 'x')?

Chair-bound or bed-bound	□
Seated work with no option of moving around and little or no strenuous leisure activity	□
Seated work with no option of moving around but strenuous leisure activity	□
Seated work with some moving around but with little or no strenuous leisure activity	□
Seated work with some moving around and strenuous leisure activity	□
Standing work with little or no strenuous leisure activity	□
Standing work with strenuous leisure activity	□
Strenuous work or very active leisure	□
Strenuous work and very active leisure	□

Please note: strenuous leisure activity is defined as 30-60 minutes of activity (i.e. walking) or sport, 4-5 times a week. Very active leisure would exceed strenuous activity requirements.

PLEASE be sure you have answered every item

PLEASE continue to the next page

Sleep quality assessments

The Insomnia Severity Index (ISI) has seven questions.

For each question, please highlight the number that best describes your answer. Please rate the **CURRENT (LAST 2 WEEKS)** SEVERITY of your insomnia problem(s).

Insomnia Problem	None	Mild	Moderate	Severe	Very Severe
1. Difficulty falling asleep	0	1	2	3	4
2. Difficulty staying asleep	0	1	2	3	4
3. Problems waking up too early	0	1	2	3	4

4. How SATISFIED/DISSATISFIED are you with your CURRENT sleep pattern?

Very Satisfied	Satisfied	Moderately Satisfied	Dissatisfied	Very Dissatisfied
0	1	2	3	4

5. How NOTICEABLE to others do you think your sleep problem is in terms of impairing the quality of your life?

Not at all Noticeable	A Little	Somewhat	Much	Very Much Noticeable
0	1	2	3	4

6. How WORRIED/DISTRESSED are you about your current sleep problem?

Not at all Worried	A Little	Somewhat	Much	Very Much Worried
0	1	2	3	4

7. To what extent do you consider your sleep problem to INTERFERE with your daily functioning (e.g. daytime fatigue, mood, ability to function at work/daily chores, concentration, memory, mood, etc.) CURRENTLY?

Not at all Interfering	A Little	Somewhat	Much	Very Much Interfering
0	1	2	3	4

PLEASE be sure you have answered every item

PLEASE continue to the next page

The Pittsburgh Sleep Quality Index (PSQI)

The following questions relate to your usual sleep habits **DURING THE PAST MONTH** only. Your answers should indicate the most accurate reply for most days and nights in the past month. Please answer all questions. (for the table please mark with an 'x'/highlight)?

During the past month,

1. When have you usually gone to bed? ____

2. How long (in minutes) did it take you to fall asleep each night? ___________________________

3. What time did you usually get up in the morning? _________________________________

4. A. How many hours of actual sleep did you get at night? _________________________________

B. How many hours were you in bed? ____

5. During the past month, how often have you had trouble sleeping because you	Not during the past month (0)	Less than once a week (1)	Once or twice a week (2)	Three or more times a week (3)
A. Cannot get to sleep within 30 minutes				
B. Wake up in the middle of the night or early morning				
C. Have to get up to use the bathroom				
D. Cannot breathe comfortably				
E. Cough or snore loudly				
F. Feel too cold				
G. Feel too hot				
H. Have bad dreams				
I. Have pain				
J. Other reason (s), please describe, including how often you have had trouble sleeping because of this reason (s):				
6. During the past month, how often have you taken medicine (prescribed or "over the counter") to help you sleep?				
7. During the past month, how often have you had trouble staying awake while driving, eating meals, or engaging in social activity?				
8. During the past month, how much of a problem has it been for you to keep up enthusiasm to get things done?				
9. During the past month, how would you rate your sleep quality overall?	Very good (0)	Fairly good (1)	Fairly bad (2)	Very bad (3)

PLEASE be sure you have answered every item

PLEASE continue to the next page

MORNINGNESS-EVENINGNESS QUESTIONNAIRE

Self-Assessment Version (MEQ-SA)

For each question, please select the answer that best describes you by circling the point value that best indicates how you have felt in recent weeks.

1. *Approximately* what time would you get up if you were entirely free to plan your day?
   [5] 5:00 AM–6:30 AM *(05:00–06:30 h)*
   [4] 6:30 AM–7:45 AM *(06:30–07:45 h)*
   [3] 7:45 AM–9:45 AM *(07:45–09:45 h)*
   [2] 9:45 AM–11:00 AM *(09:45–11:00 h)*
   [1] 11:00 AM–12 noon *(11:00–12:00 h)*

2. *Approximately* what time would you go to bed if you were entirely free to plan your evening?
   [5] 8:00 PM–9:00 PM *(20:00–21:00 h)*
   [4] 9:00 PM–10:15 PM *(21:00–22:15 h)*
   [3] 10:15 PM–12:30 AM *(22:15–00:30 h)*
   [2] 12:30 AM–1:45 AM *(00:30–01:45 h)*
   [1] 1:45 AM–3:00 AM *(01:45–03:00 h)*

3. If you usually have to get up at a specific time in the morning, how much do you depend on an alarm clock?
   [4] Not at all
   [3] Slightly
   [2] Somewhat
   [1] Very much

4. How easy do you find it to get up in the morning (when you are not awakened unexpectedly)?
   [1] Very difficult
   [2] Somewhat difficult
   [3] Fairly easy
   [4] Very easy

5. How alert do you feel during the first half hour after you wake up in the morning?
   [1] Not at all alert
   [2] Slightly alert
   [3] Fairly alert
   [4] Very alert

6. How hungry do you feel during the first half hour after you wake up?
   [1] Not at all hungry
   [2] Slightly hungry
   [3] Fairly hungry
   [4] Very hungry

7. During the first half hour after you wake up in the morning, how do you feel?
   [1] Very tired
   [2] Fairly tired
   [3] Fairly refreshed
   [4] Very refreshed

8. If you had no commitments the next day, what time would you go to bed compared to your usual bedtime?
   [4] Seldom or never later
   [3] Less that 1 hour later
   [2] 1-2 hours later
   [1] More than 2 hours later

9. You have decided to do physical exercise. A friend suggests that you do this for one hour twice a week, and the best time for him is between 7-8 AM *(07-08 h).* Bearing in mind nothing but your own internal "clock," how do you think you would perform?
   [4] Would be in good form
   [3] Would be in reasonable form
   [2] Would find it difficult
   [1] Would find it very difficult

10. At *approximately* what time in the evening do you feel tired, and, as a result, in need of sleep?
   [5] 8:00 PM–9:00 PM *(20:00–21:00 h)*
   [4] 9:00 PM–10:15 PM *(21:00–22:15 h)*
   [3] 10:15 PM–12:45 AM *(22:15–00:45 h)*
   [2] 12:45 AM–2:00 AM *(00:45–02:00 h)*
   [1] 2:00 AM–3:00 AM *(02:00–03:00 h)*

11. You want to be at your peak performance for a test that you know is going to be mentally exhausting and will last two hours. You are entirely free to plan your day. Considering only your "internal clock," which one of the four testing times would you choose?
   [6] 8 AM–10 AM *(08–10 h)*
   [4] 11 AM–1 PM *(11–13 h)*
   [2] 3 PM–5 PM *(15–17 h)*
   [0] 7 PM–9 PM *(19–21 h)*

12. If you got into bed at 11 PM *(23 h)*, how tired would you be?
   [0] Not at all tired
   [2] A little tired
   [3] Fairly tired
   [5] Very tired

13. For some reason you have gone to bed several hours later than usual, but there is no need to get up at any particular time the next morning. Which one of the following are you most likely to do?
   [4] Will wake up at usual time, but will not fall back asleep
   [3] Will wake up at usual time and will doze thereafter
   [2] Will wake up at usual time, but will fall asleep again
   [1] Will not wake up until later than usual

14. One night you have to remain awake between 4-6 AM *(04-06 h)* in order to carry out a night watch. You have no time commitments the next day. Which one of the alternatives would suit you best?
   [1] Would not go to bed until the watch is over
   [2] Would take a nap before and sleep after
   [3] Would take a good sleep before and nap after
   [4] Would sleep only before the watch

15. You have two hours of hard physical work. You are entirely free to plan your day. Considering only your internal "clock," which of the following times would you choose?
    [4] 8 AM–10 AM *(08–10 h)*
    [3] 11 AM–1 PM *(11–13 h)*
    [2] 3 PM–5 PM *(15–17 h)*
    [1] 7 PM–9 PM *(19–21 h)*

16. You have decided to do physical exercise. A friend suggests that you do this for one hour twice a week. The best time for her is between 10-11 PM *(22-23 h).* Bearing in mind only your internal "clock," how well do you think you would perform?
    [1] Would be in good form
    [2] Would be in reasonable form
    [3] Would find it difficult
    [4] Would find it very difficult

17. Suppose you can choose your own work hours. Assume that you work a five-hour day (including breaks), your job is interesting, and you are paid based on your performance. At *approximately* what time would you choose to begin?
    [5] 5 hours starting between 4–8 AM *(05–08 h)*
    [4] 5 hours starting between 8–9 AM *(08–09 h)*
    [3] 5 hours starting between 9 AM–2 PM *(09–14 h)*
    [2] 5 hours starting between 2–5 PM *(14–17 h)*
    [1] 5 hours starting between 5 PM–4 AM *(17–04 h)*

18. At *approximately* what time of day do you usually feel your best?
    [5] 5–8 AM *(05–08 h)*
    [4] 8–10 AM *(08–10 h)*
    [3] 10 AM–5 PM *(10–17 h)*
    [2] 5–10 PM *(17–22 h)*
    [1] 10 PM–5 AM *(22–05 h)*

19. One hears about "morning types" and "evening types." Which one of these types do you consider yourself to be?
    [6] Definitely a morning type
    [4] Rather more a morning type than an evening type
    [2] Rather more an evening type than a morning type
    [1] Definitely an evening type

PLEASE be sure you have answered every item

PLEASE continue to the next page

Kessler psychological distress scale (K10)

These questions concern how you have been **FEELING OVER THE PAST 30 DAYS**. Mark the choice that best applies to you and your situation with an "X" in the box.

In the past four weeks:	**None of the time (1)**	**A little of the time (2)**	**Some of the time (3)**	**Most of the time (4)**	**All of the time (5)**
1. about how often did you feel tired out for no good reason?					
2. about how often did you feel nervous?					
3. about how often did you feel so nervous that nothing could calm you down?					
4. about how often did you feel hopeless?					
5. about how often did you feel restless or fidgety?					
6. about how often did you feel so restless you could not sit still?					
7. about how often did you feel depressed?					
8. about how often did you feel that everything was an effort?					
9. about how often did you feel so sad that nothing could cheer you up?					
10. about how often did you feel worthless?					

Thank you for your cooperation

PLEASE be sure you have answered every item

Subject Code_______________ Date:____________________ Day:____________________

The acute effects of dried kiwifruit on sleep onset, metabolites and cognition in men

Data collection book

If you have any comments or questions relating to the research project or the questionnaire please feel free to contact Alex Kanon during working hours on 022 650 7798 or email a.kanon@massey.ac.nz

Bedtime/waking

Please complete the following table with the approximate time you went to bed/awoke and how long it took you to fall asleep the following morning for each of the seven days. The date for each day should also be completed.

Day of the week	Date	Approximate Bedtime	Approximate waking time the following morning	Approximate time to fall asleep (min)

Subject Code_______________ Date:____________________ Day:____________________

3-Day diet record

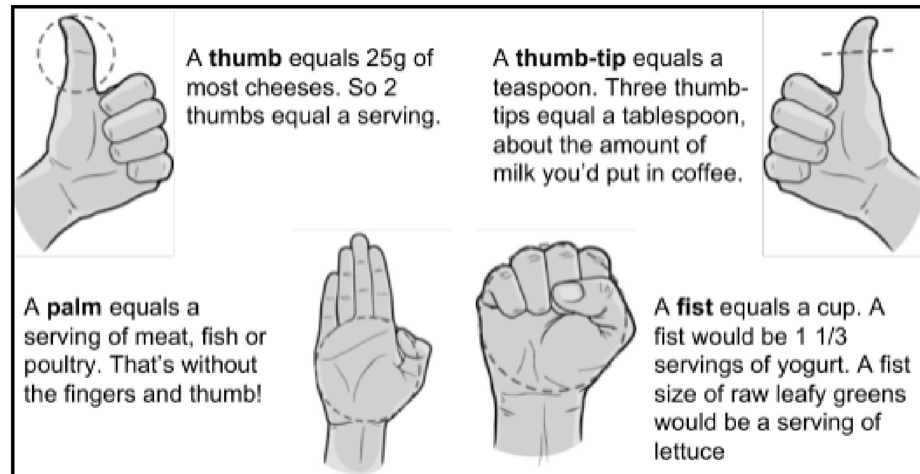
Instructions

Please fill in this food intake record on any 2 days of the week and 1 day during the weekend.

- The days in which food intake is recorded do not need to be consecutive but need to be within the same 1-week period.
- You should fill in details immediately after you make and eat each meal or snack. Please do not wait till the end of the day to complete.
- Directions for how to fill in the food record are at the top of each day. An example of one day is on the following page.

Instructions for completing the food record chart

- Write down everything that you eat and drink over one day from waking to going to sleep. It is important that you do this straight away after you eat or drink, rather than waiting till the end of the day. This also includes any snacks, water, vitamin and mineral supplements, and thickening powders you may add to drinks.
- Use a new line for each food, drink or supplement.
- Record the type of eating occasion in the appropriate column. For example, breakfast, lunch, dinner, morning tea, afternoon tea or snack.
- Record each food individually. For example, while you may state 'tuna sandwich' in the first row for lunch, you should then list each of the individual foods contained in the sandwich in the rows below, together with their amounts.
- Include the amounts in quantities, household measures or natural portion sizes. For example, 100grams, 2 slices of bread, ½ cup rice, ¼ cup peas.
- If you wish to use abbreviations for spoon measures please use the following:
  - 1 teaspoon = 1 tsp   1 tablespoon = 1 TBSP
- Count the number of food items if practical: E.g. 20 green grapes, 15 baby carrots, 8 medium-sized shrimp.
- Record amounts of fluid in millilitres (mL) or in cups. Remember 1 cup = 250mL
- Use your hand to estimate portion size quickly:
  - Whole Thumb = 1 tablespoon
  - Tip of your thumb = 1 teaspoon
  - Palm of your hand = 85g of meat
  - Fist = 1 cup (250mL)



- Record the cooking method used, if applicable. For example, grilled, BBQ, deep fried, pan fried, baked, roasted, boiled, steamed, minced, pureed etc.
- Give a detailed description of the food or drink and include brand names where possible. For an example Arnott's Milk Arrowroot® biscuit; Yalla Humus; Cobram Estate Extra Virgin Olive Oil; Flavour Creations Thickened Fluid.
- Don't forget to include any sauces, mayonnaise, dressings or gravies that are used. We are interested to find out about your usual eating patterns, so please keep your food intake as usual.
- If you record a day that is not typical, please indicate in the box at the end of each food

When in doubt... include more details!

Subject Code_______________ Date:____________________ Day:____________________

EXAMPLE - FOOD INTAKE RECORD (WEEKDAY)

Write down everything that you eat and drink over one day from waking to going to sleep. It is important that you **do this straight away after you eat or drink**, rather than waiting till the end of the day. This also includes any snacks, water, vitamin and mineral supplements, and thickening powders you may add to drinks.

EXAMPLE

Date:			Day:		
Location	Time	Meal/Eating occasion	Food/Drink/Water/Supplement	Cooking method (where applicable)	Amount/Size EATEN
Home	7am	Breakfast	Full cream milk (Countdown)	-	250ml
			Weet-bix (Sanitarium)	-	2 Weetbix
			Fresh yoghurt Plain (yoplit)	-	1 small tub
			Blueberries	-	8 fruit
			Coffee powder (nescafe)		1tsp
			Hot water		200ml
			Full cream milk (Countdown)		50ml
Uni	10:30am	Snack	Fresh Apple		1 med fruit
			Orange Juice - Countdown		200ml
Uni	1pm	Lunch	Sandwich		
			Whole wheat bread – Tip top		2 slice
			Chicken breast	roast	40g
			Lettuce		30g
			Tomato slices		20g
			Mayo		20g
			Water		1 cup
Uni	3pm	Snack	Protein bar (Tasti) salted caramel		1 bar
			Coffee – Cappuccino, skim milk		250ml
Club	7pm	Dinner	Fish	Grilled	1 filet 80g
			Potato, peeled	Boiled	50g
			Carrots, peeled	Roast	¼ cup
			Broccoli, peeled	Roast	50g

Subject Code_______________ Date:____________________ Day:____________________

Date:			Day:		
Location	Time	Meal/Eating occasion	Food/Drink/Water/Supplement	Cooking method (where applicable)	Amount/Size EATEN
			Vanilla ice cream		½ cup
			Chocolate brownie	Baked	80g
			Water		250ml
			Coke Zero		250ml
Home	9pm	Supper	Water		250ml

EXAMPLE

Was your intake unusual in any way? No □ ✓ Yes

If yes, in what way? _________Had dinner at club once a month______________________

Subject Code_______________ Date:____________________ Day:____________________

DAY 1: FOOD INTAKE RECORD (WEEKDAY)

Write down everything that you eat and drink over one day from waking to going to sleep. It is important that you **do this straight away after you eat or drink**, rather than waiting till the end of the day. This also includes any snacks, water, vitamin and mineral supplements, and thickening powders you may add to drinks.

Date:			Day:		
Location	Time	Meal/Eating occasion	Foods/Drinks/Water/Supplements	Cooking method (where applicable)	Amount/Size EATEN

Subject Code_______________ Date:____________________ Day:____________________

Date:			Day:		
Location	Time	Meal/Eating occasion	Foods/Drinks/Water/Supplements	Cooking method (where applicable)	Amount/Size EATEN

Was your intake unusual in any way? No □ Yes □

If yes, in what way? ____

Subject Code_______________ Date:____________________ Day:____________________

DAY 2: FOOD INTAKE RECORD (WEEKDAY)

Write down everything that you eat and drink over one day from waking to going to sleep. It is important that you **do this straight away after you eat or drink**, rather than waiting till the end of the day. This also includes any snacks, water, vitamin and mineral supplements, and thickening powders you may add to drinks.

Date:			Day:		
Location	Time	Meal/Eating occasion	Foods/Drinks/Water/Supplements	Cooking method (where applicable)	Amount/Size EATEN

Subject Code______________ Date:___________________ Day:___________________

Date:			Day:		
Location	Time	Meal/Eating occasion	Foods/Drinks/Water/Supplements	Cooking method (where applicable)	Amount/Size EATEN

Was your intake unusual in any way? No □ Yes □

If yes, in what way? ____

Subject Code_______________ Date:____________________ Day:____________________

DAY 3: FOOD INTAKE RECORD (WEEKEND)

Write down everything that you eat and drink over one day from waking to going to sleep. It is important that you **do this straight away after you eat or drink**, rather than waiting till the end of the day. This also includes any snacks, water, vitamin and mineral supplements, and thickening powders you may add to drinks.

Date:			Day:		
Location	Time	Meal/Eating occasion	Foods/Drinks/Water/Supplements	Cooking method (where applicable)	Amount/Size EATEN

Subject Code_______________ Date:____________________ Day:____________________

Date:			Day:		
Location	Time	Meal/Eating occasion	Foods/Drinks/Water/Supplements	Cooking method (where applicable)	Amount/Size EATEN

Was your intake unusual in any way? No □ Yes □

If yes, in what way? ____

Participant code______________ Date______________ Study visit__________________

The acute effects of dried kiwifruit on sleep onset, metabolites and cognition in men

Data collection book – in unit

Instructions: In this questionnaire, we will ask you to report on your typical sleep behaviour, mood, stress you may experience. Please follow the instructions on the next page

Weight:________________

Stanford Sleepiness Scale (SSS)

During each blood sample you will be asked to rate how you feel according to the below scale.

Degree of Sleepiness	Scale Rating
Feeling active, vital, alert, or wide awake	1
Functioning at high levels, but not at peak; able to concentrate	2
Awake, but relaxed; responsive but not fully alert	3
Somewhat foggy, let down	4
Foggy; losing interest in remaining awake; slowed down	5
Sleepy, woozy, fighting sleep; prefer to lie down	6
No longer fighting sleep, sleep onset soon; having dream-like thoughts	7

Blood sample	Rating scale from above
1	
2	
3	
4	
5	
6	

Abbreviated POMS (Revised Version)

Below is a list of words that describe feelings people have. Please circle the number that best describes **HOW YOU FEEL RIGHT NOW**.

	Not at All	A Little	Moderately	Quite a lot	Extremely
Tense	0	1	2	3	4
Angry	0	1	2	3	4
Worn Out	0	1	2	3	4
Unhappy	0	1	2	3	4
Proud	0	1	2	3	4
Lively	0	1	2	3	4
Confused	0	1	2	3	4
Sad	0	1	2	3	4
Active	0	1	2	3	4
On-edge	0	1	2	3	4
Grouchy	0	1	2	3	4
Ashamed	0	1	2	3	4
Energetic	0	1	2	3	4
Hopeless	0	1	2	3	4
Uneasy	0	1	2	3	4
Restless	0	1	2	3	4
Unable to concentrate	0	1	2	3	4
Fatigued	0	1	2	3	4
Competent	0	1	2	3	4
Annoyed	0	1	2	3	4
Discouraged	0	1	2	3	4
Resentful	0	1	2	3	4
Nervous	0	1	2	3	4
Miserable	0	1	2	3	4
Confident	0	1	2	3	4
Bitter	0	1	2	3	4
Exhausted	0	1	2	3	4
Anxious	0	1	2	3	4
Helpless	0	1	2	3	4
Weary	0	1	2	3	4
Satisfied	0	1	2	3	4
Bewildered	0	1	2	3	4
Furious	0	1	2	3	4
Full of Pep	0	1	2	3	4
Worthless	0	1	2	3	4
Forgetful	0	1	2	3	4
Vigorous	0	1	2	3	4
Uncertain about things	0	1	2	3	4
Bushed	0	1	2	3	4
Embarrassed	0	1	2	3	4

Physical Activity

International Physical Activity Questionnaire (IPAQ)

We are interested in finding out about the kinds of physical activities that people do as part of their everyday lives. The questions will ask you about the time you spent being physically active in the **last 7 days**. Please answer each question even if you do not consider yourself to be an active person. Please think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

Think about all the **vigorous** activities that you did in the **last 7 days**. **Vigorous** physical activities refer to activities that take hard physical effort and make you breathe much harder than normal. Think *only* about those physical activities that you did for at least 10 minutes at a time.

1. During the **last 7 days**, on how many days did you do **vigorous** physical activities like heavy lifting, digging, aerobics, or fast bicycling?

   _____ **days per week**

   ☐ No vigorous physical activities → ***Skip to question 3***

2. How much time did you usually spend doing **vigorous** physical activities on one of those days?

   _____ **hours per day**
   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about all the **moderate** activities that you did in the **last 7 days**. **Moderate** activities refer to activities that take moderate physical effort and make you breathe somewhat harder than normal. Think only about those physical activities that you did for at least 10 minutes at a time.

3. During the **last 7 days**, on how many days did you do **moderate** physical activities like carrying light loads, bicycling at a regular pace, or doubles tennis? Do not include walking.

   _____ **days per week**

   ☐ No moderate physical activities → ***Skip to question 5***

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

   _____ **hours per day**
   _____ **minutes per day**

   ☐ Don't know/Not sure

Think about the time you spent **walking** in the **last 7 days**. This includes at work and at home, walking to travel from place to place, and any other walking that you have done solely for recreation, sport, exercise, or leisure.

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?

_____ **days per week**

☐ No walking → ***Skip to question 7***

6. How much time did you usually spend **walking** on one of those days?

_____ **hours per day**
_____ **minutes per day**

☐ Don't know/Not sure

The last question is about the time you spent **sitting** on weekdays during the **last 7 days**. Include time spent at work, at home, while doing course work and during leisure time. This may include time spent sitting at a desk, visiting friends, reading, or sitting or lying down to watch television.

7. During the **last 7 days**, how much time did you spend **sitting** on a **week day**?

_____ **hours per day**
_____ **minutes per day**

☐ Don't know/Not sure

PLEASE be sure you have answered every item

PLEASE continue to the next page

Weekly Stress Inventory (WSI) Short Form

Below are listed a variety of events that may be viewed as stressful or unpleasant. Read each item carefully and decide whether or not that event happened to you during this past week. If the event did not happen this week, fill in the circle labelled X to the right of the item. If the event did happen, show the amount of stress that it caused you by filling in the circle numbered 1 to 7 to the right of that item (see scale below).

X	1	2	3	4	5	6	7
Did not happen	Happened not stressful	Slightly stressful	Mildly stressful	Moderately stressful	Stressful	Very stressful	Extremely stressful

	X	1	2	3	4	5	6	7
Had a pet peeve violation (someone fails to knock, etc.)		1	2	3	4	5	6	7
Was excluded or left out		1	2	3	4	5	6	7
Was without privacy		1	2	3	4	5	6	7
Was ignored by others		1	2	3	4	5	6	7
Was stared at		1	2	3	4	5	6	7
Was lied to, fooled, or tricked		1	2	3	4	5	6	7
Competed with someone		1	2	3	4	5	6	7
Had a minor injury (stubbed toe, sprained ankle, etc)		1	2	3	4	5	6	7
Had too many responsibilities		1	2	3	4	5	6	7
Was forced to socialise		1	2	3	4	5	6	7
Did something you were not good at		1	2	3	4	5	6	7
Dealt with rude waiter, waitress, or salesperson		1	2	3	4	5	6	7
Was interrupted while talking		1	2	3	4	5	6	7
Was clumsy (spilled or knocked something over)		1	2	3	4	5	6	7
Not enough time for fun (movie, eating out) or recreation		1	2	3	4	5	6	7
Had someone disagree with you		1	2	3	4	5	6	7
Did poorly because of others		1	2	3	4	5	6	7
Argued with a friend		1	2	3	4	5	6	7
Not enough time to socialize		1	2	3	4	5	6	7
Forgot something		1	2	3	4	5	6	7
Was told what to do		1	2	3	4	5	6	7
Lost or misplaced something (wallet, keys)		1	2	3	4	5	6	7
Spoke or performed in public		1	2	3	4	5	6	7
Did not hear from someone you expected to		1	2	3	4	5	6	7
Had someone cut in front of you in line		1	2	3	4	5	6	7

Once finished, stop here and advise researcher you are finished.

Abbreviated POMS (Revised Version)

Below is a list of words that describe feelings people have. Please circle the number that best describes **HOW YOU FEEL RIGHT NOW**. **Once finished, stop here and advise researcher you are finished.**

	Not at All	A Little	Moderately	Quite a lot	Extremely
Tense	0	1	2	3	4
Angry	0	1	2	3	4
Worn Out	0	1	2	3	4
Unhappy	0	1	2	3	4
Proud	0	1	2	3	4
Lively	0	1	2	3	4
Confused	0	1	2	3	4
Sad	0	1	2	3	4
Active	0	1	2	3	4
On-edge	0	1	2	3	4
Grouchy	0	1	2	3	4
Ashamed	0	1	2	3	4
Energetic	0	1	2	3	4
Hopeless	0	1	2	3	4
Uneasy	0	1	2	3	4
Restless	0	1	2	3	4
Unable to concentrate	0	1	2	3	4
Fatigued	0	1	2	3	4
Competent	0	1	2	3	4
Annoyed	0	1	2	3	4
Discouraged	0	1	2	3	4
Resentful	0	1	2	3	4
Nervous	0	1	2	3	4
Miserable	0	1	2	3	4
Confident	0	1	2	3	4
Bitter	0	1	2	3	4
Exhausted	0	1	2	3	4
Anxious	0	1	2	3	4
Helpless	0	1	2	3	4
Weary	0	1	2	3	4
Satisfied	0	1	2	3	4
Bewildered	0	1	2	3	4
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Forgetful	0	1	2	3	4
Vigorous	0	1	2	3	4
Uncertain about things	0	1	2	3	4
Bushed	0	1	2	3	4
Embarrassed	0	1	2	3	4

The acute effects of dried kiwifruit on sleep onset, metabolites and cognition in men

Data collection book – at accommodation

Participant code________________Date_________________Study visit_________________

If you have any comments or questions relating to the research project or the questionnaire please feel free to contact Alex Kanon during working hours on 022 650 7798 or email a.kanon@massey.ac.nz

Instructions:

In this questionnaire, we will ask you to report on your sleep behaviour and mood. Please follow the instructions on the next page

Hi, thank you for taking part of our study.

1. Once you are in bed, press the marker button on actigraphy watch to indicate you are ready to sleep. Press this again once you wake up tomorrow morning.

PLEASE continue to the next page tomorrow morning when you wake up

Good morning,

Please collect your whole urine sample and saliva samples in the containers provided – follow the laminated instruction card. There are two containers for urine samples should you need both. You have been provided with four Salivette®containers for saliva samples. Please collect this within 15 minutes of waking up, then collect every 15minutes. Do not forget to press the marker button on the actigraphy watch.

Please then answer the following questions once you have collected this.

1. What time is it right now? ____
2. What time did you wake up? ____
3. What time did you go to bed? ____
4. How long did it take you to sleep? _____________________________________min
5. Did you wake up in the middle of the night? YES/NO
    - If yes, how many times and what for? ______________________________
6. What time did you collect your urine sample? ______________________________
7. What time did you collect your saliva samples?______________________________
8. Have you put the urine sample in the ice box with the ice brick? YES/NO
9. According to the below scale, how would you rate your sleepiness right now? (circle/highlight)

Stanford Sleepiness Scale (SSS)

Degree of Sleepiness	**Scale Rating**
Feeling active, vital, alert, or wide awake	1
Functioning at high levels, but not at peak; able to concentrate	2
Awake, but relaxed; responsive but not fully alert	3
Somewhat foggy, let down	4
Foggy; losing interest in remaining awake; slowed down	5
Sleepy, woozy, fighting sleep; prefer to lie down	6
No longer fighting sleep, sleep onset soon; having dream-like thoughts	7

PLEASE be sure you have answered every item

PLEASE continue to the next page

Abbreviated POMS (Revised Version)

Below is a list of words that describe feelings people have. Please circle the number that best describes **HOW YOU FEEL RIGHT NOW**. **Once finished, stop here and advise researcher you are finished.**

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Ashamed	0	1	2	3	4
Energetic	0	1	2	3	4
Hopeless	0	1	2	3	4
Uneasy	0	1	2	3	4
Restless	0	1	2	3	4
Unable to concentrate	0	1	2	3	4
Fatigued	0	1	2	3	4
Competent	0	1	2	3	4
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Anxious	0	1	2	3	4
Helpless	0	1	2	3	4
Weary	0	1	2	3	4
Satisfied	0	1	2	3	4
Bewildered	0	1	2	3	4
Furious	0	1	2	3	4
Full of Pep	0	1	2	3	4
Worthless	0	1	2	3	4
Forgetful	0	1	2	3	4
Vigorous	0	1	2	3	4
Uncertain about things	0	1	2	3	4
Bushed	0	1	2	3	4
Embarrassed	0	1	2	3	4

Leeds Sleep Evaluation Questionnaire

Place a vertical mark on the line to indicate your self-evaluation

How would you describe the way you currently fall asleep in comparison to usual?

1. More difficult than usual	______________________	Easier than usual
2. Slower than usual	______________________	More quickly than usual
3. I feel less sleepy than usual	______________________	More sleepy than usual

How would you describe the quality of your sleep compared to normal sleep?

4. More restless than usual	______________________	Calmer than usual
5. With more wakeful periods than usual	______________________	With less wakeful periods than usual

How would you describe your awakening in comparison to usual?

6. More difficult than usual	______________________	Easier than usual
7. Requires a period of time longer than usual	______________________	Shorter than usual

How do you feel when you wake up?

8. Tired	______________________	Alert

How do you feel now?

9. Tired	______________________	Alert

How would you describe your balance and co-ordination upon awakening?

10. More disrupted than usual	______________________	Less disrupted than usual

Thank you for your cooperation. PLEASE be sure you have answered every item

Stop here and deliver this booklet and sample to researchers within one hours.

Morning awakening state

Place a vertical mark on the line to indicate your self-evaluation

1. **How rested do you feel?**

   Not at all ____ Very much so

2. **How energetic do you feel?**

   Not at all ____ Very much so

3. **How relaxed do you feel?**

   Not at all ____ Very much so

4. **How irritable do you feel?**

   Not at all ____ Very much so

5. **How ready do you feel to perform?**

   Not at all ____ Very much so

6. **Have you had a good night's sleep?**

   Not at all ____ Very much so

Thank you for your cooperation. PLEASE be sure you have answered every item

Stop here and deliver this booklet and sample to researchers at your allocated time slot.