Exploring the Influence of a Flavonoid-Rich Blackcurrant Beverage on the Gut Microbiomes of Healthy Women

A Secondary Analysis of the LINK Study

Ella May Risbrook

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Health Sciences in Nutrition and Dietetics, the University of Auckland, 2024.
Abstract

**Background:** The gut microbiome plays a crucial role in human health, driving interest in potential therapeutic dietary interventions. Blackcurrants, rich in the flavonoid class of polyphenols, show promise in modulating the gut microbiome, although human trials are lacking.

**Objective:** To measure changes in the composition and functional potential of the gut microbiome in healthy adult women following four weeks of supplementation with a flavonoid-rich blackcurrant beverage. Secondly, to explore how baseline diet quality influences the gut microbiome response to intervention.

**Method:** Forty healthy females (18-45 years) participated in this double-blinded, placebo-controlled, cross-over randomized controlled trial (RCT). The study sample was balanced during recruitment for equal representation of ‘optimal’ and ‘suboptimal’ baseline diet quality, as assessed by the Dietary Screening Tool (DST). The active intervention was a commercially available flavonoid-rich blackcurrant beverage (Ārepa performance beverage, 300mL), containing 150 mg anthocyanins, 200 mg L-theanine, and 150 mg Pine Bark Extract, while the placebo was a taste- and colour-matched beverage. Participants were randomly allocated to consume either the active or placebo beverage daily for four weeks, followed by a four week wash-out period, before consuming the other beverage in the second four-week intervention arm. Faecal samples were collected at the beginning and end of each arm for shotgun metagenomic sequencing.

**Results:** The active beverage did not significantly alter participants’ gut microbiome. However, the placebo reduced the abundance of the probiotic *Bifidobacterium* genus (p = 0.001) and related family (*Bifidobacteriaceae, p = 0.001*), phylum (Actinobacteria, p = 0.03) and metabolic pathway (*Bifidobacterium* P124-PWY, p < 0.0001) in participant gut microbiomes. Baseline diet quality had a small but significant impact on the active intervention response, with a *Clostridium* species AM22-11AC (p = 0.001) increasing in abundance in the suboptimal diet group. Additionally, clustering in overall gut bacterial community composition based on diet group was observed, accounting for 4% of the total variance at baseline (p = 0.004) and 3% at follow-up (p = 0.001).

**Conclusion:** While the flavonoid-rich blackcurrant beverage did not produce significant alterations in the gut microbiome, further research is warranted to elucidate the complex relationship between blackcurrants and the gut microbiome. Notably, the placebo beverage, which shared similar constituents with the active beverage except for the bioactive ingredients, exhibited significant potentially negative effects, suggesting potential masking of the active intervention’s effects.
Dedication

To my exceptional supervisor, Dr Nicola Gillies, your kindness, support and guidance have been invaluable in bringing this thesis to fruition. Thank you for providing me with structure, belief in my abilities and prompt feedback on my many drafts. I couldn’t have asked for anything more from a supervisor, and feel immensely fortunate to have had your guidance and mentorship.

I’d also like to extend my gratitude to my co-supervisor, Dr Brooke Wilson, for your guidance and support, particularly regarding the methods and data analysis in R.

I extend my sincere appreciation to the women who participated in the LINK study. Your generous contributions of time, involvement, and even faecal samples made this research possible and are very much appreciated.

I would like to thank the past and present dietetics course coordinators for your continual support and encouragement, and for making the programme truly enriching and enjoyable.

A special thank you to Donna, Tristan and friends at The Workshop co-working space, where the majority of this thesis took shape. The support, enthusiasm about this research, and, of course, endless coffee were invaluable in keeping me focused and motivated.

Thank you to my beloved family and friends for going above and beyond to support me. Like the prebiotics to myself as the gut microbiome, you have nourished me with laughter, welcome distractions, and endless kindness. I am truly fortunate to have had your wraparound support, especially during the final stretches of writing.

Lastly, I dedicate this work to my beautiful friend and dietetics classmate, Georgia, who passed away during the writing of this thesis. Your personal strength and pursuit for excellence has inspired me to persevere through the ups and downs since your passing, and will continue to do so. All my aroha to you.
## Statement of contribution

<table>
<thead>
<tr>
<th>Researcher</th>
<th>Contribution to thesis</th>
</tr>
</thead>
</table>
| **Ella Risbrook (MHSc Student)**    | • Primary thesis author  
|                                     | • Assisted with extraction of DNA from faecal samples  
|                                     | • Adapted exemplar R scripts and conducted data analysis in R  
|                                     | • Interpreted and integrated findings with previously published research               |
| **Dr Nicola Gillies (Academic Supervisor)** | • Application for ethical approval  
|                                     | • Developed study design, and conducted randomised controlled trial  
|                                     | • Reviewed thesis chapters                                                               |
| **Dr Brooke Wilson (Co-Supervisor)** | • Extracted DNA from faecal samples  
|                                     | • Bioinformatic processing of sequencing reads  
|                                     | • Developed exemplar R scripts  
|                                     | • Supported data analysis in R  
|                                     | • Reviewed thesis chapters                                                               |
# Table of contents

Abstract ................................................................................................................................. 2  
Dedication ............................................................................................................................. 3  
Statement of contribution ..................................................................................................... 4  
Table of contents .................................................................................................................. 5  
List of tables .......................................................................................................................... 8  
List of figures ........................................................................................................................ 9  
Glossary ................................................................................................................................. 11  

## Chapter 1. Literature review ............................................................................................. 12  
1.1 Introduction ..................................................................................................................... 12  
1.2 The role of the gut microbiome in the human host ......................................................... 12  
1.3 Studying the human gut microbiome .............................................................................. 13  
    1.3.1 Taxonomical classification .................................................................................... 13  
    1.3.2 Measurement tools ............................................................................................. 14  
    1.3.3 Metrics to describe the microbiome .................................................................... 15  
1.4 What constitutes a “healthy” gut microbiome? ............................................................... 16  
1.5 Diet and the gut microbiome ......................................................................................... 17  
1.6 Polyphenols and the gut microbiome ............................................................................ 18  
    1.6.1 Two-way interplay between polyphenols and the gut microbiome .................... 20  
1.7 Blackcurrants and modulation of the gut microbiome .................................................. 20  
    1.7.1 *In vitro* studies .................................................................................................. 20  
    1.7.2 Animal studies .................................................................................................... 22  
    1.7.3 Human studies .................................................................................................... 26  
    1.7.4 Summary of the evidence base .......................................................................... 26  
1.8 Rationale ........................................................................................................................ 27  

## Chapter 2. Methods ............................................................................................................ 28  
2.1 Study design .................................................................................................................. 28  
    2.1.1 The LINK study: polyphenol-rich drink for gut and brain health ....................... 28  
    2.1.2 The current study ............................................................................................... 28  
    2.1.3 Thesis objectives and hypotheses ...................................................................... 28  
2.2 Participants .................................................................................................................... 29  
    2.2.1 Recruitment ....................................................................................................... 29  
    2.2.2 Inclusion and exclusion criteria ......................................................................... 29  
    2.2.3 Dietary screening tool ....................................................................................... 30
2.3 Intervention ..............................................................................................................31
2.4 Randomisation and blinding..................................................................................31
2.5 Procedures ..............................................................................................................32
  2.5.1 Participant timeline .......................................................................................32
2.6 Data collection .......................................................................................................34
  2.6.1 Intervention adherence ..................................................................................34
  2.6.2 Sample collection .........................................................................................34
  2.6.3 DNA extraction of stool samples ....................................................................34
  2.6.4 Metagenomic sequencing and processing .......................................................34
2.7 Sample size calculation .........................................................................................36
2.8 Statistical analysis.................................................................................................36
2.9 Ethical considerations ...........................................................................................37

Chapter 3. Results ......................................................................................................38
3.1 Participant characteristics .....................................................................................38
3.2 Intervention effects ...............................................................................................41
  3.2.1 Taxonomic profiling ......................................................................................41
  3.2.2 Firmicutes:Bacteroidetes ratio ......................................................................42
  3.2.3 Richness and diversity metrics ......................................................................43
  3.2.4 Functional potential ......................................................................................45
3.3 Exploratory analyses: effect of baseline diet quality on gut microbiota response to active (BB) intervention.................................................................46
  3.3.1 Taxonomic profiling ......................................................................................46
  3.3.2 Firmicutes:Bacteroidetes ratio ......................................................................47
  3.3.3 Richness and diversity metrics ......................................................................48
  3.3.4 Functional potential ......................................................................................50

Chapter 4. Discussion .................................................................................................52
4.1 Overview .................................................................................................................52
4.2 Key findings ............................................................................................................52
4.3 Interpretation of findings in the context of existing literature.................................53
4.4 Strengths and limitations .......................................................................................57
  4.4.1 Limitations .....................................................................................................57
  4.4.2 Strengths .........................................................................................................59
4.5 Social implications .................................................................................................60
4.6 Future directions ...................................................................................................62
4.7 Conclusion ..............................................................................................................64

References..................................................................................................................65
Appendices.................................................................................................................................................. 79
Appendix A: Participant Information Sheet (PIS) .................................................................................. 79
Appendix B: Participant consent form ..................................................................................................... 88
Appendix C: Dietary Screening Tool (DST) ............................................................................................. 90
Appendix D: Intervention beverage ingredients ....................................................................................... 92
Appendix E: Comparison of study standard replicates and reference values ...................................... 93
Appendix F: Sequencing read counts from quality control steps ......................................................... 94
List of tables

Table 1.1. Key findings from animal studies (with control groups) examining the impact of blackcurrant supplementation on the gut microbiome .................................................................23
Table 2.1. LINK study inclusion and exclusion criteria.................................................................30
Table 2.2. Nutritional composition of active (BB) and placebo beverages ...........................31
Table 3.1. Characteristics of LINK trial participants at enrolment..........................39
Table 3.2. Significant changes in the abundance of specific genera, families and phyla in participant gut microbiomes from baseline to post-placebo intervention, as identified from generalised linear modelling (MaAsLin2 analysis) .................................................................42
List of figures

Figure 1.1. Illustrative examples of taxonomic gut microbiota classification .........................14
Figure 1.2. Classification of phenolic compounds with examples of their rich dietary sources ...............................................................................................................................19
Figure 2.1. Schematic overview of LINK study enrolment, intervention, and assessment ......33
Figure 3.1. CONSORT diagram illustrating the flow of participants through the current study .40
Figure 3.2. Relative abundance of bacterial phyla in participant gut microbiomes at baseline and post-placebo or active (BB) intervention .........................................................41
Figure 3.3. Firmicutes:Bacteroidetes ratio of participant gut microbiomes at baseline and post-placebo or active (BB) intervention .................................................................42
Figure 3.4. Species richness counts of participant gut microbiomes at baseline and post-placebo or active (BB) intervention ..............................................................................43
Figure 3.5. Species-level alpha diversity, as measured by Shannon diversity index, of participant gut microbiomes at baseline and post-placebo or active (BB) intervention........43
Figure 3.6. Non-metric multi-dimensional scaling plots based on species-level Bray Curtis dissimilarities, illustrating variations in participant gut microbiomes at baseline and post-placebo or active (BB) intervention ..........................................................................................45
Figure 3.7. Shifts in gut microbiome composition of participants from baseline to post-placebo or active (BB) intervention, based on species-level Bray Curtis dissimilarities ..........45
Figure 3.8. Gene and pathway richness counts of participant gut microbiomes at baseline and post-placebo or active (BB) intervention ........................................................................46
Figure 3.9. Relative abundance of Clostridium species AM22-11AC in participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups ....................................................................................................................47
Figure 3.10. Firmicutes:Bacteroides ratio of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups ........................................47
Figure 3.11. Species richness counts (normalised by sequencing depth) of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups ....................................................................................................................48
Figure 3.12. Species-level alpha diversity (Shannon diversity index) of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups ....................................................................................................................48
Figure 3.13. Non-metric multi-dimensional scaling plots based on species-level Bray Curtis dissimilarities illustrating variations in participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups ....................................................................................................................50
Figure 3.14. Shifts in gut microbiome composition of participants from baseline to post-active (BB) intervention, based on species-level Bray Curtis dissimilarities and split by optimal and suboptimal diet groups ....................................................................................................................50
Figure 3.15. Gene and pathway richness counts (normalised by sequencing depth) of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups.
## Glossary

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Årepa/BB</td>
<td>Commercially available flavonoid-rich blackcurrant beverage (BB) used as the active intervention beverage for this study. Contains 308 mg polyphenols, of which 151 mg anthocyanins, 200 mg L-theanine, and 150 mg Enzogenol®.</td>
</tr>
<tr>
<td>Enzogenol®</td>
<td>Commercial New Zealand-grown pine-bark extract product</td>
</tr>
<tr>
<td>L-theanine</td>
<td>Unique non-proteineic amino acid commonly used as a cognitive supplement</td>
</tr>
<tr>
<td>LINK study</td>
<td>Polyphenol-rich drink for gut and brain health, the RCT that this thesis is a sub-study of</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomised Controlled Trial</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>DST</td>
<td>Dietary Screening Tool</td>
</tr>
<tr>
<td>R</td>
<td>R software used for data analysis</td>
</tr>
<tr>
<td>F:B ratio</td>
<td>Ratio of Firmicutes phyla to Bacteroidetes phyla</td>
</tr>
</tbody>
</table>
Chapter 1. Literature review

1.1 Introduction

Over the past decade, the gut microbiome has been linked to many complex disease states, including metabolic syndrome, cancer, mental health conditions and functional gut disorders (1,2). At the same time, the significance of the gut microbiome extends beyond disease prevention, as it plays a crucial role in the normal physiological functioning of both the gut and its human host (3,4). This paradigm shift has revolutionised our understanding of health and disease, revealing the gut microbiome as the potential missing factor in mediating numerous host-disease relationships that have long puzzled the scientific community (2,5). Consequently, targeting the gut microbiome has emerged as a promising strategy for addressing disease states and promoting host health (2).

The community of microbes (‘microbiota’), the genes they possess (‘metagenome’), as well as their theatre of activity, including microbial structures, metabolites, mobile genetic elements and relic DNA, are collectively known as the gut microbiome (6). The human gut microbiota is highly diverse (7,8) and dynamic (9). It encompasses tens of trillions of microbial cells from different kingdoms, including bacteria, fungi, viruses, archaea and protozoa, of which the bacterial population is the most abundant and well-characterised (10–12). The diversity of the gut microbiota gives rise to an incredible metabolic potential, with approximately 150 times more genes than the human genome (13).

Given the pivotal role of the gut microbiome in host health, coupled with the modifiable nature of the gut microbiome, there is a burgeoning interest in the potential therapeutic implications of dietary interventions. Among these, blackcurrants have emerged as a promising intervention due to their rich phytochemical profile, with a particularly high concentration of the anthocyanin class of flavonoids. This chapter provides an overview of the human gut microbiome, its physiological significance, methods for its assessment, and the characteristics of a ‘healthy’ gut microbiome. Next, it explores the potential of blackcurrant polyphenols as dietary modulators of the gut microbiome, summarising existing literature on their mechanisms and effects. Lastly, this review identifies gaps in knowledge regarding flavonoids and gut microbiota, providing a rationale for the objectives of this thesis.

1.2 The role of the gut microbiome in the human host

The gut microbiota and their human host exist in a symbiotic mutualistic relationship – the gut provides an environment conducive to microbial development (pH, temperature, moisture, nutrients), while the microbiota, when in a state of proper functioning (known as eubiosis), fulfils crucial metabolic, immunological, and structural defence roles (14–16).
The gut microbiota’s vast metabolic capacity enables it to synthesise a myriad of specialised enzymes essential for breaking down complex substrates, such as dietary fibre, which the human host alone cannot metabolise efficiently (17). This results in the production of a catalogue of metabolites with a wide spectrum of activities for the human host, such as modulation of energy metabolism, nutrition absorption, intestinal barrier and gut motility (17). These microbial metabolites are thought to be the critical link between the gut microbiome and host health (1,18). Gut microbiota metabolites originate in three ways: 1) produced by gut microbiota from dietary components, such as short chain fatty acids (SCFA’s) and indole derivates; 2) generated by the host but modified by gut microbiota, such as secondary bile acids, and 3) produced de novo, such as polysaccharide A (17). Other microbially-derived metabolites within these categories include neurotransmitters (e.g. GABA, serotonin, dopamine, acetylcholine) and gasotransmitters (e.g. H₂S, NO), shown to have important roles in the regulation of host metabolism and intestinal homeostasis (13,17).

SCFA’s are one of the most extensively researched microbial metabolites and are produced by bacterial fermentation of dietary fibre in the large intestine, with acetate, propionate, and butyrate produced in the greatest abundance (19). SCFAs accumulate in the body and are responsible for various biological activities, including modulation of host metabolism, appetite, immunity and inflammation, and intestinal barrier defence (20,21). For example, butyrate is utilised as an energy source by colon cells, contributing to maintaining the integrity of the colonic epithelium (22).

Although less relevant to the current thesis, the gut microbiota also has well-established roles in nutrient synthesis (23), immunity and pathogen resistance (24), and communication with other organ systems such as through the gut-brain axis (12).

1.3 Studying the human gut microbiome

1.3.1 Taxonomical classification

Bacteria are taxonomically grouped into phyla, classes, orders, families, genera, species, and strains, as illustrated in Figure 1.1. Currently, 25 different bacterial phyla have been identified to inhabit the gut microbiome (25), with the six most prevalent being Firmicutes, Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria and Fusobacteria (2). Of these, Bacteroidetes and Firmicutes phyla alone comprise approximately 90% of the bacterial load (15). While different strains exist within individual species, most remain uncharacterised (26).
1.3.2 Measurement tools

The study of gut microbial ecology has advanced significantly since Antonie van Leeuwenhoek’s initial observation of “very prettily moving animalcules” while examining his excrement under a microscope in 1681 (28). Traditional methods relied on culture staining and microscopy techniques to characterise microbes. However, these methods were culture-dependent, necessitating laboratory cultivation of organisms. Due to the difficulty in cultivating anaerobic bacteria, which are abundant in the gut, this resulted in a limited view of the gut microbiome (29,30).

The emergence of culture-independent techniques, which analyse DNA extracted directly from samples, allowed the evaluation of taxonomic diversity within microbial communities (30,31). While early DNA-based methods, including Quantitative Polymerase Chain Reaction (qPCR) and Fluorescent In Situ Hybridisation (FISH), allowed the broad evaluation of taxonomic diversity, they were not efficient in investigating microbial communities at scale (30,31).

The advent of high-throughput sequencing in 2005 and subsequent technological advancements such as next-generation sequencing and bioinformatics empowered the profiling of entire microbial communities. Nowadays, most microbiome studies employ high-throughput sequencing (31,32).

Amplicon-based sequencing, whereby a specific sequence present in the organism of interest is targeted, is one of the most widely used and affordable high-throughput methods. Due to its bacterial specificity, the 16S ribosomal RNA (rRNA) gene is the most commonly used target.
(30,31), a method known as **16S rRNA gene sequencing**. However, this method excludes other types of microorganisms, including archaea, viruses and eukaryotes, and only provides taxonomic resolution at the genera level or above, with no information available at the species level. Further, it does not discern between living and dead bacteria or provide predictive functional insights (10,31).

**Shotgun metagenomic sequencing**, albeit more costly and computationally intensive than 16S rRNA gene sequencing, is a high-throughput method providing a deeper level of taxonomical classification (30,31). Unlike amplicon-based sequencing, shotgun metagenomics does not target specific genomic loci but rather fragments all DNA within a sample into millions of tiny strands that are independently sequenced and then restructured computationally. Shotgun metagenomic sequencing provides species or even strain-level resolution, and encompasses archaea, viruses and eukaryotes in addition to bacteria. It provides information on the presence of microbes, as well as genes (e.g. antibiotic resistance) and metabolic pathways (e.g. carbohydrate metabolism), i.e. functional potential (30,31). However, functional potential doesn’t necessarily imply functional activity, as this sequencing method alone cannot assess which microbes are active and which genes and metabolic pathways are being expressed. To obtain a more comprehensive picture of microbiome function, shotgun metagenomics can be complemented with other omic technologies, such as metatranscriptomics, metaproteomics, and metabolomics, which assess active microbial genes, proteins and metabolites, respectively (31,33,34).

Current sampling methods for extraction of DNA before gut microbiome sequencing include faecal specimens, mucosal biopsy and intestinal aspiration, though faecal samples are most commonly used due to their non-invasive nature (29). However, it is acknowledged that a faecal sample is not necessarily representative of the entire gastrointestinal tract, as the composition and function of the gut microbiome varies considerably along its length (29,35,36).

### 1.3.3 Metrics to describe the microbiome

The highly complex nature of the gut microbiome poses a challenge in transforming a large amount of high-dimensional data into meaningful metrics. There are many different metrics to analyse and present microbiome data, with those used most frequently in the literature described below (12).

**Comparative relative abundance**

The most common and straightforward method of comparing two samples is comparing the presence of specific microbes. Due to the nature of the output of current sequencing tools, including 16S and shotgun metagenomic sequencing, comparisons between samples most
commonly rely on relative abundance rather than absolute counts of microbes (12,31). However, it is important to be aware of this distinction, as relative abundance can fluctuate with changes in the abundance of other microbial taxa within the sample, even when absolute levels of the taxon of interest remain constant. Similarly, changes in total microbial biomass can affect absolute counts of individual taxa without necessarily affecting their relative abundances (12).

**Population diversity**

Expanding beyond assessing comparative relative abundance, the concept of microbiome diversity offers a method to quantify the degree of heterogeneity within a sample or the difference between two samples. Various formulas exist for measuring diversity, broadly categorised into two main measures: alpha and beta diversity (30,37). Alpha diversity estimates the diversity within a sample, considering factors such as species richness (number of different species present), species evenness (distribution of abundances of these species), and how phylogenetically distant they are from one another (12). On the other hand, beta diversity assesses the heterogeneity of microbial composition between samples, providing valuable insights into shifts in population structure over time or between different individuals or groups. Different formulas for measuring alpha and beta diversity assign varying weights to factors like species richness or evenness. It’s worth noting that although species-level diversity has been discussed here, diversity can be calculated at various taxonomic levels, such as phyla or family (12).

**Functional metagenomics**

Beyond taxonomic profiling, functional metagenomics allows us to explore the potential metabolic capabilities and ecological functions of microbial communities (12). Metagenomic sequencing provides information on the relative abundances of microbial genes, identified using databases such as UniRef90 (38). The abundance of metabolic pathways can then be predicted by mapping these genes against pathway databases, such as MetaCyc (38).

**1.4 What constitutes a “healthy” gut microbiome?**

Gut microbiome composition is highly individualised, with no two microbiomes identical (39). Even within a single individual, the gut microbiome undergoes fluctuations throughout each day (40–42) and gradually shifts in composition over time (43,44). Thus, defining a “healthy” gut microbiome remains challenging due to this immense interindividual and intraindividual diversity (39). Nevertheless, there is widespread agreement that microbial diversity and stability are key indicators of gut microbiome health (39). A species-rich microbiome exhibits resilience against environmental influences, owing to the functional redundancy of microbes, which allows for compensation if one microbe fails to fulfil its metabolic function (3,39,45).
Certain bacterial genera have been identified as potentially beneficial or pathogenic, with the ratio thought to influence disease susceptibility (2). Genera such as *Bifidobacterium*, *Lactobacillus* and *Faecalibacterium* are commonly regarded as beneficial due to their health-promoting effects, such as the production of beneficial metabolites, immune modulation, and prevention of pathogenic colonisation (24,27). Conversely, potentially pathogenic bacteria, such as species of *Clostridium*, *Escherichia coli*, and *Enterococcus*, have been associated with disruptions in gut homeostasis and the onset of disease states through mechanisms such as toxin production, induction of inflammation, and compromise of gut barrier function (27,46–48). An example of this shift in balance is the ratio between the two dominant phyla in the gut microbiome, Firmicutes and Bacteroidetes (F:B ratio), which has been linked with several disease states, including obesity (49), inflammatory bowel disease (49,50) and non-alcoholic fatty liver disease (50), albeit with inconsistent findings (51).

Some researchers propose defining a “healthy” gut microbiome based on functional capacity rather than taxonomical composition (52). Despite compositional diversity, two seminal projects, the Human Microbiome Project (HMP) and the Metagenomics of the Human Intestinal Tract (MetaHIT) project, found that individuals possess functionally similar microbiomes (31,52). Thus, it has been suggested that identifying key microbial genes and metabolic pathways consistently present in the gut microbiota of healthy individuals, described as the “functional core”, may be a useful way to define a “healthy” gut microbiome (45,53,54).

Conversely, dysbiosis is an imbalance or disruption in the microbiota associated with adverse functional outcomes on gut physiology, including disturbed metabolic processing or localised inflammation (3,55). Dysbiosis is associated with, and may contribute to the development of various disease states, including diabetes, obesity and functional gut disorders (3,55), although the causal relationship remains uncertain (56).

In summary, while the scientific community continues to seek measurable gut microbiome characteristics associated with human health benefits, a consensus has yet to be reached (2,52). Nevertheless, regardless of exact definitions, targeting the gut microbiome through dietary interventions offers a promising avenue for the potential prevention and treatment of disease states (20).

### 1.5 Diet and the gut microbiome

While the core gut microbiota tends to remain relatively stable in adulthood, environmental factors have been shown to shape its composition (2). These include diet, exercise, stress, and medications (57–61). Diet has been identified to be one of the most critical factors modulating the composition of the gut microbiome and, importantly, is a modifiable risk factor (12,56,62). Acute dietary changes have been shown to rapidly (albeit transiently) modify gut microbiome composition and functionality in as brief a timeframe as 24–48 hours (63,64),
whilst long-term dietary pattern changes may lead to more enduring shifts in composition (65–70). Interestingly, this ability of the gut microbiome to rapidly shift has been linked to our volatile hunter-gatherer past, where the ability of the microbial community to shift in their functional repertoire to respond to rapid dietary changes (e.g. from herbivorous to carnivorous) was an advantage (64). By modulating microbial composition in the gut, dietary changes have also been observed to influence microbial enzymatic capacity and metabolite production, such as SCFA's (62,71,72). However, responses to gut microbiota-targeted dietary intervention vary widely among individuals (73,74), and emerging evidence suggests that diet-microbe interactions are highly personalised and dependent on an individual’s baseline microbiota (62,75).

Prebiotics are a particularly promising dietary intervention to shape gut microbiome composition and output (76). Prebiotics are defined by the International Scientific Association for Probiotics and Prebiotics as “a substrate that is selectively used by host microbiota conferring a health benefit” (77). They stimulate the growth and/or activity of potentially beneficial bacteria in the host’s gut microbiota by serving as substrates for said bacteria (77). Commonly used prebiotics are fermentable fibres such as fructooligosaccharides, galactooligosaccharides and inulin (78). While the concept of prebiotics was previously limited to specific non-digestible carbohydrates, this definition has been revisited with recent evidence indicative of polyphenols having a prebiotic effect (79).

**1.6 Polyphenols and the gut microbiome**

Although primarily known for their antioxidant and anti-inflammatory properties, there is growing evidence to show that dietary polyphenols may favourably impact the gut microbiome (71,80). Polyphenols are a diverse group of chemical compounds characterised by hydroxylated phenyl moieties (13). Population statistics estimate we consume 500-1000 mg/day of these compounds daily, which occur naturally in various plant foods and beverages, such as fruits, vegetables, coffee, tea, cocoa, legumes and cereals (57,81,82). Polyphenols are classified into two main groups: flavonoids and nonflavonoids (e.g. phenolic acids, stilbenes and lignans). Flavonoids are the most abundant polyphenolic group and include flavones, flavonols, flavanones, flavan-3-ols, isoflavones and anthocyanins (Figure 1.2) (83).
Polyphenols have been shown to have a prebiotic effect on gut microbiota. This is achieved by promoting specific bacteria containing the enzymes required to catalyse the cleavage and breakdown reactions of polyphenols, such as *Bifidobacterium* and *Lactobacillus* (24,85). Furthermore, polyphenols show antimicrobial activity against human pathogens (13,57). A systematic review and meta-analysis concluded that polyphenol supplementation stimulates the abundance of health-promoting species *Lactobacillus* and *Bifidobacterium* whilst inhibiting potentially pathogenic *Clostridium* species (80).

Numerous pre-clinical and clinical studies have demonstrated that polyphenol-rich foods or extracts induce favourable shifts in the gut microbiome, including alterations in composition (86–91), increased SCFA production (92–94), modulation of bacterial enzyme activity (95), and enhancement of intestinal barrier function (96,97). Notably, the most robust body of evidence supporting these favourable changes pertains to the flavonoid class of polyphenols (98). Not only are flavonoids the most extensively researched subclass, but they also represent the polyphenol class most commonly consumed (99).

For example, one clinical study investigating the consumption of an anthocyanin-rich wild blueberry drink daily for six weeks revealed an increased abundance of *Bifidobacterium*, particularly *Bifidobacterium longum infantis*, which has been shown to have immunomodulatory capabilities and promote the regression of several gut disorders (100,101). Another trial involving three weeks of daily supplementation with red wine and de-alcoholised red wine, both rich in anthocyanins, observed increased *Bifidobacterium* and *Lactobacillus*, along with decreased abundance of potentially pathogenic bacteria *Escherichia coli* and *Enterobacter cloacae* (102).
1.6.1 Two-way interplay between polyphenols and the gut microbiome

As discussed above, polyphenols may exert a prebiotic effect on gut microbiota composition. However, it is worth noting here that the gut microbiota also has a critical reciprocal role in polyphenol metabolism. Owing to the complicated chemical structures and high molecular weights of many polyphenols, they have very low bioavailability, and so the activity of polyphenols for the human host is mainly mediated through interaction with gut microbes (103). Approximately 90-95% of polyphenols are not absorbed in the small intestine, reaching the colon where they are metabolised by the enzymatic activity of resident bacteria into bio-accessible phenolic metabolites, including phenolic acids and flavonoids (104). These can then be absorbed via the intestinal mucosa with a perhaps greater physiological significance for the host (1,104–106). Different microbial species possess varying abilities to metabolise polyphenols, influencing the final metabolites produced (105,107,108). Consequently, an individual’s unique microbiota structure, with differing capacities to metabolise polyphenols, is thought to influence the impact of polyphenols on their health (105).

Thus, it is evident that a two-way interaction between polyphenols and the gut microbiota exists (13,109). Polyphenols nourish health-promoting bacteria in our gut, while gut microbes aid in breaking down polyphenols. This enhances their bio-accessibility and biological activity, ultimately influencing their health benefits (13,109). Understanding this dynamic is crucial to leverage the potential health benefits of polyphenol intake and supplementation, although this relationship remains incompletely understood (13,110).

1.7 Blackcurrants and modulation of the gut microbiome

This section presents the research exploring the potential of blackcurrants to modulate the gut microbiome, from mechanistic in vitro evidence to animal studies and human clinical trials. As previously discussed, the modulatory activity of polyphenols against various gut microbes is well documented. Blackcurrants (Ribes nigrum L.) are berry fruits with a rich nutritional profile, containing multiple bioactive compounds, including flavonoids and vitamin C (111). They have a rich concentration of polyphenols relative to other berries (112,113), particularly anthocyanins, which are within the flavonoid class of polyphenols. The predominant phenolic constituents are 3-O-glucosides and 3-O rutinosides of anthocyanins delphinidin and cyanidin (114). They also contain smaller quantities of other phenolic structures, such as phenolic acids (115).

1.7.1 In vitro studies

In vitro studies generally show a dual-action effect of blackcurrant on gut microbial strains, with both the proliferation of beneficial bacteria and inhibition of potentially pathogenic bacteria. This has been observed with various blackcurrant formats, including freeze-dried...
powder (116), extracts (117,118), concentrates (119), cordial (120), and phenolic extracts (116,119,121). For example, berry extracts, including those from blackcurrants, have been shown to inhibit potentially harmful microbes *Salmonella enterica* and *Escherichia coli* while promoting the growth of health-promoting bacteria *Lactobacillus rhamnosus* and *Lactobacillus paracasei in vitro* (118).

However, the literature is inconsistent. There is variation in the specific microbes affected, and some studies observe only proliferative effects on beneficial microbes, while others report solely antimicrobial effects. For example, phenolic extracts of blackcurrants inhibited the growth of potentially pathogenic microbes *Helicobacter pylori, Bacillus cereus, Staphylococcus aureus, Clostridium perfringens, Escherichia coli,* and *Salmonella enterica serovar Typhimurium* (121). In contrast, a recent study that employed a human faecal culture model (wherein a culture medium was inoculated with faeces from six healthy human volunteers) found that blackcurrant extract powder increased the abundance of beneficial bacteria *Bifidobacteriaceae* and did not exert any antimicrobial effects (111). The human faecal culture model offers a more realistic representation of gut bacterial strains and their behaviour in vivo, which may account for the observed discrepancy in findings compared to other in vitro studies utilising a model (111). Discrepancies may also exist in the research due to variations in blackcurrant concentrations (ranging from 0.5 mg/ml - 16 mg/ml), strains of microorganisms, formats (pure polyphenol solution vs plant extract vs whole food), preparation (e.g. acetone or methanol extraction), and incubation time (ranging from 3 hours to 6 days) (104,122). Regarding intervention format, the plant food matrix has been shown to affect the bioavailability and activity of blackcurrant polyphenols, thus influencing the interactions with the gut microbiota (79).

Some studies have attempted to tease out the effect of blackcurrants from that of their phenolic components alone. For instance, one study investigated the impact of blackcurrant concentrates versus purified blackcurrant anthocyanins. The purified blackcurrant anthocyanin mix did not influence the growth of microorganisms, whereas the concentrate inhibited potentially pathogenic microbes *Staphylococcus aureus, Enterococcus faecium,* and *Escherichia coli* (119). This aligns with the findings of another study, which observed that freeze-dried blackcurrant supplementation inhibited potentially harmful microbes *Salmonella enterica serovar Typhimurium* and *Staphylococcus aureus,* while phenolic extracts isolated from blackcurrants only inhibited *Staphylococcus aureus* (116). These observations suggest that the antimicrobial activity of blackcurrants may stem from the synergistic effects of various bioactive compounds within the blackcurrant extracts rather than solely the phenolic components (116).

Regarding the mechanism underlying blackcurrants' effect on gut microbiota composition, their prebiotic effect is thought to be due to polyphenols acting as a nutritional substrate for the health-promoting microbes, which possess the enzymes to release their stored energy.
These enzymes release glucose from the polyphenol structure, providing fuel to the microbes which possess them (24). However, regarding their antimicrobial effect, more research is required to elucidate the exact mechanism, though it has been suggested that multiple mechanisms may be at play (122). Anthocyanins appear to adhere to bacterial cell membranes, potentially immobilising harmful bacteria and inhibiting their growth (121). Additionally, blackcurrant has been observed to inhibit the adhesion of pathogens to gut epithelial cells in vitro, thereby preventing pathogenic bacteria colonisation (123). Other suggested mechanisms include the permeabilisation of bacterial plasma membranes, inhibition of extracellular microbial enzymes, interference with microbial metabolism, and deprivation of substrates needed for microbial growth (13,104,121,122).

In summary, in vitro studies have demonstrated that blackcurrants, in a variety of formats, may have a proliferative effect on ‘good’ bacteria, in parallel with an antimicrobial effect. However, in vivo efficacy of dietary interventions is influenced by interactions with other members of an individual’s gut microbiota and their metabolic capacity, human digestive enzymes, host health or interactions with other components of the food matrix. Thus, in vitro studies only indicate possible benefits that may occur from in vivo blackcurrant intake, with in vivo experimentation required to confirm these findings (104).

### 1.7.2 Animal studies

A summary of pre-clinical in vivo animal research on blackcurrant interventions is presented in Table 1.1. The pre-clinical research corroborates the prebiotic effect of blackcurrant observed in in vitro studies while also contributing insight into richness counts, diversity metrics and predictive function, which cannot be evaluated in vitro. This is discussed further in the following two sections.
Table 1.1. Key findings from animal studies (with control groups) examining the impact of blackcurrant supplementation on the gut microbiome.

<table>
<thead>
<tr>
<th>Reference</th>
<th>BC format</th>
<th>Dose &amp; duration</th>
<th>Model (Sample Sze, Animal Age)</th>
<th>Study Design</th>
<th>Method of Microbiota Analysis/ Sample Type</th>
<th>Key Findings¹</th>
<th>Richness and Diversity²</th>
<th>Taxa Relative Abundance</th>
<th>Functional Outputs³</th>
</tr>
</thead>
<tbody>
<tr>
<td>(124)</td>
<td>BC extract</td>
<td>2 mL 3 times weekly for 4 weeks</td>
<td>Male Sprague-Dawley rats (n = 40, 8 weeks)</td>
<td>Parallel RCT</td>
<td>FISH/Caecal samples</td>
<td>NE</td>
<td>↑ Bifidobacterium</td>
<td>↑ Lactobacillus</td>
<td>⁰β-glucuronidase activity ³Caecal pH (SCFA production NE)</td>
</tr>
<tr>
<td>(125)</td>
<td>BC pomace extract</td>
<td>1.5% w/w diet for 4 weeks</td>
<td>Male white rabbits (n = 10, 1 month)</td>
<td>Parallel RCT</td>
<td>Caecal and colonic samples</td>
<td>NE</td>
<td>NE</td>
<td>NE</td>
<td>⁰Caecal β-glucuronidase activity ³Caecal and colonic putrefactive SCFA’s ⁴Caecal butyric acid</td>
</tr>
<tr>
<td>(126)</td>
<td>BC pomace</td>
<td>7.7% w/w diet for 4 weeks</td>
<td>Male Wistar rats (n = 48, 4 weeks)</td>
<td>Parallel RCT</td>
<td>Caecal samples</td>
<td>NE</td>
<td>NE</td>
<td>↑ α-galactosidase &amp; β-glucuronidase activity (SCFA production NE)</td>
<td></td>
</tr>
<tr>
<td>(127)</td>
<td>BC powder</td>
<td>1.14 mg/day anthocyanins for 12 weeks</td>
<td>Obese male C57BL/6J mice fed high fat diet (n = 16, 6 weeks)</td>
<td>Parallel RCT</td>
<td>qPCR/Faecal samples</td>
<td>NE</td>
<td>↑ Bacteroidetes</td>
<td>↑ Actinobacteria</td>
<td>NE</td>
</tr>
<tr>
<td>(79)</td>
<td>BC extract</td>
<td>700 mg/day for 6 weeks</td>
<td>Male Sprague-Dawley rats (n = 64, 6 weeks)</td>
<td>Parallel RCT</td>
<td>qPCR/Caecal samples</td>
<td>NE</td>
<td>↑ Lactobacillus</td>
<td>↑ Bifidobacterium</td>
<td>↑ Clostridium perfringens</td>
</tr>
<tr>
<td>(128)</td>
<td>BC extract</td>
<td>1% w/w diet for 4 months</td>
<td>Female C57BL/6J mice fed high or low-fat diet (n = 12, 3 or 18 months)</td>
<td>Parallel RCT</td>
<td>qPCR/Faecal samples</td>
<td>NC</td>
<td>↑ Incertae sedis Otu0340</td>
<td>↑ Roseburia Otu0142</td>
<td>↑ Marvinbryantia Otu0254</td>
</tr>
<tr>
<td>Reference</td>
<td>BC format</td>
<td>Dose &amp; duration</td>
<td>Model (Sample Sze, Animal Age)</td>
<td>Study Design</td>
<td>Method of Microbiota Analysis/ Sample Type</td>
<td>Key Findings¹</td>
<td>Taxa Relative Abundance</td>
<td>Functional Outputs³</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-----------</td>
<td>-----------------</td>
<td>--------------------------------</td>
<td>-------------</td>
<td>-------------------------------------------</td>
<td>---------------</td>
<td>------------------------</td>
<td>---------------------</td>
<td></td>
</tr>
</tbody>
</table>
| (129)     | BC powder | 100-900 mg/kg BW/day for 8 weeks | Non-obese T2DM male partially pancreatectomised Sprague-Dawley rats fed high-fat diet (n = 40, 8 weeks) | Parallel RCT | Metagenome sequencing/Faecal samples | ↑ α-diversity | Altered β-diversity | ↑ Lachnospiraceae  
↑ Ruminococcaceae  
↑ Bacteroides  
↑ Ruminococcus  
↑ Akkermansia  
↑ Akkermansia muciniphila  
↑ Clostridaceae  
↑ Lactobacillaceae  
↑ Coriobacteriaceae  
↑ Lactobacillus  
↑ Bacteroides  
↑ Oxidative phosphorylation genes  
↑ Glycine, serine, threonine metabolism genes  
↑ Phosphatidylinositol signalling genes  
↓ Carbohydrate digestion and absorption genes (SCFA production and enzyme activity NE) |
| (130)     | BC anthocyanin powder | 150 mg/kg BW/day for 12 weeks | Obese male C57BL/6J mice fed high fat diet (n = 24, 4 weeks) | Parallel RCT | 16S/Faecal samples | Altered β-diversity | ↑ Akkermansia muciniphila  
↑ Parabacteroides distasonis  
↑ Clostridiales bacterium CIEAF 020 |
| (131)     | BC powder | 6% w/w diet for 4 months | C57BL/6J mice fed high-fat diet (n = 30, 22 weeks) | Parallel RCT | 16S/Caecal samples | ↑ α-diversity  
↑ OTU richness | ↑ Firmicutes:Bacteroidetes  
↑ Clostridiales  
↑ Lachnospiraceae  
↑ Unclassified genera from S24-7  
↑ Proteobacteria  
↓ Desulfovibrionaceae  
↑ Unclassified genera from Ruminococcaceae  
↓ Prevotella2 |
| (132)     | BC powder | 150 mg/day for 4 weeks | C57BL/6J mice with DSS-Induced Colitis (n = 24, 5 weeks) | Parallel RCT | 16S/Faecal samples | ↑ α-diversity  
↑ OTU richness | ↑ Lactobacillus  
↓ Bacteroides |

¹Significant findings presented only (p < 0.05). Diversity metrics limited to α- and β-diversity. ³Functional capacity metrics limited to microbial genes, metabolic pathways, SCFA production, stool pH and enzyme activity. Abbreviations: RCT, Randomised Controlled Trial; NE, Not Explored; NC, No Change; BC, Blackcurrant; FISH, Fluorescence in situ hybridisation; qPCR, Quantitative real-time polymerase chain reaction; DSS-Induced Colitis, Dextran Sulfate Sodium-Induced Colitis, T2D, Type 2 Diabetes Mellitus; OTU, Operational Taxonomic Unit, a cluster of closely related sequences, grouped together based on a predefined sequence similarity threshold. OTU’s serve as a proxy for species-level identification where species are not yet identified (31).
Gut microbiome composition

In general, pre-clinical in vivo research shows that blackcurrant supplementation proliferates beneficial bacterial species. This was observed in pre-clinical in vivo studies spanning different time frames (4 weeks – 4 months), formats (blackcurrant powders, extracts and pomace, constituent anthocyanin extracts) and doses. Doses were reported differently between studies (e.g. anthocyanin content vs percentage of blackcurrant format in diet vs weight of blackcurrant format per day), making comparison difficult. For example, rats gavaged with a commercial blackcurrant extract powder three times weekly for four weeks exhibited a significant proliferation of health-promoting Bifidobacterium and Lactobacillus genera and inhibition of potentially pathogenic Bacteroides genus and Clostridia perfringens and Clostridia histolyticum species compared to the control group (124). A similar prebiotic-like effect was observed in a later rodent study regarding the number of Lactobacillus and Clostridium, although not with Bifidobacterium (79). While specific bacterial species seen to alter in abundance differs, a consistent trend of increasing health-promoting species was observed across the research. However, animal studies generally demonstrate lower antimicrobial efficacy compared to mechanistic in vitro studies, with fewer animal studies showing a reduction in potentially pathogenic species (79,133). Among the studies that investigated richness and diversity, nearly all observed changes, including increased operational taxonomic unit richness (OTU, a proxy for unidentified species) (131), and enhanced alpha diversity (129,131,132) with blackcurrant supplementation. Clustering of gut microbiome composition according to intervention group (altered beta diversity) was also seen (129,130,132). Additionally, opposing effects on Firmicutes:Bacteroidetes ratio have been observed (128,131).

Functional outputs

Animal studies have shown various impacts of blackcurrant supplementation on functional outputs, including modulation of SCFA production, stool pH, microbial enzymes and microbial genes. Results regarding SCFA production are mixed. For example, one study documented a decrease in caecal acetic and butyric acid production alongside increased propionic acid production (79), while another observed reduced caecal butyric acid production and decreased colonic putrefactive SCFA’s (125,134). As a likely corollary to SCFA production, stool pH decreased in rats supplemented with blackcurrant extract (124). Studies on enzyme activity also yielded diverse effects. Blackcurrant pomace extract decreased caecal β-glucuronidase activity in one study (125), whilst a subsequent study reported a decrease in caecal β-glucosidase activity alongside increased caecal α-galactosidase (126). Findings related to microbial gene abundance are currently limited to a single study, which demonstrated an increased abundance of microbial genes involved in: 1) oxidative phosphorylation, 2) glycine, serine, and threonine metabolism and 3) phosphatidylinositol signalling whilst predicting the downregulation of genes related to 1) carbohydrate digestion and absorption, and 2) type 2 diabetes mellitus risk. This prediction of function was made possible through the use of shotgun metagenomic sequencing (129).
1.7.3 Human studies

Evidence in humans is currently limited to a single clinical trial by Molan et al. (2014), which found that two weeks of blackcurrant extract supplementation (672 mg/day; 200 mg anthocyanins/day) in 30 healthy adults (aged 20-60 years) enhanced the growth of beneficial bacteria *Lactobacillus* and *Bifidobacterium*, and inhibited the growth of *Bacteroides* and potentially pathogenic *Clostridium* (133). These changes resolved after a two-week washout period, with the exception of *Clostridium* inhibition, which persisted. These findings suggest that blackcurrant extract may have a transient prebiotic and antimicrobial effect on gut microbiota in healthy humans. Additionally, decreased faecal pH, as well as alterations in enzymatic activity, were noted in the faecal samples of subjects post-supplementation, including a decrease in β-glucuronidase activity by approximately 25% and an increase in β-glucosidase activity by around 30%. Notably, the study lacked a control and did not utilise a cross-over design, introducing the possibility of confounding factors influencing the observed outcomes. Therefore, placebo-controlled, cross-over RCTs are needed to confirm these preliminary findings (133).

1.7.4 Summary of the evidence base

The existing evidence regarding the impact of blackcurrant supplementation on the gut microbiome consists primarily of *in vitro* and animal studies. These studies generally show a proliferation of health-promoting bacteria, increased alpha diversity, and altered overall community composition (beta diversity) with blackcurrant supplementation. The suppression of potentially pathogenic species demonstrated in *in vitro* studies and the one human trial, however, was inconsistent in the animal studies. Changes to functional outputs such as intestinal pH, metabolite production and microbial gene abundance were also observed, though the directionality of these changes is less clear.

Findings do vary widely between studies, and compounding this, it is a challenge to summarise the literature due to variations in study methodology. For example, differences in blackcurrant format used, animal type and health status, length and dose of supplementation, location of microbiota sample (e.g. faecal vs caecal), and different gut microbiome metrics measured. For example, although most studies looked at taxonomic composition, some did not specifically investigate Firmicutes:Bacteroidetes ratio, even when a specific change in one of these phylum was reported (127).

Further, the translatability of these findings to human subjects remains uncertain, with human trials in this domain very limited.
1.8 Rationale

This literature review highlights the pivotal role of the gut microbiome in human health and disease, as well as the potential of flavonoid-rich blackcurrants to positively influence gut microbiome composition and metabolic function.

While pre-clinical studies have shown promising outcomes, heterogeneity in the findings exists across studies. Variations in aspects of study methodology, such as dosage, intervention duration and animal models, likely contribute to these inconsistencies. Translating these preclinical findings to human populations necessitates further investigation, given the scarcity of well-designed trials.

Consequently, there is a need for robust, double-blinded, placebo-controlled, randomised clinical trials in diverse human population groups to validate the potential use of blackcurrant for improving markers of gut microbiome health in humans. Addressing these research gaps will advance our understanding of the intricate interplay between blackcurrants and the gut microbiome, thereby facilitating the development of nutritional interventions aimed at optimising gut health and mitigating disease risks across diverse demographics.

This thesis aims to investigate the impact of a flavonoid-rich blackcurrant beverage on the human gut microbiome. Leveraging data from the previously conducted LINK study – a double-blinded, randomised placebo-controlled, cross-over clinical trial – this thesis presents an opportunity to address some of the research gaps regarding the effect of blackcurrants on the human gut microbiome.
Chapter 2. Methods

2.1 Study design

2.1.1 The LINK study: polyphenol-rich drink for gut and brain health

The LINK study was a randomised, cross-over, double-blind, placebo-controlled clinical trial conducted in Auckland, New Zealand, investigating the effects of Ārepa, a commercial flavonoid-rich blackcurrant beverage on several markers of the gut-brain axis, including neurocognitive function, biochemical markers, and gut microbiota composition. Additionally, it investigated the potential moderating effect of baseline diet quality and gut microbiota on any observed changes. The study was conducted between July 2022 and January 2023 at the University of Auckland Clinical Research Centre (Faculty of Medical and Health Sciences) in Grafton, Auckland. The trial was funded by High-Value Nutrition (National Science Challenges, grant #1968), with intervention and control beverages supplied in-kind by Arepa IP Ltd. Funders had no contribution to the design or analysis of this investigator-initiated study.

2.1.2 The current study

The current study focuses on the effect of Ārepa, hereafter referred to as BB (blackcurrant beverage), on gut microbiome outcomes.

2.1.3 Thesis objectives and hypotheses

Primary objective
To measure changes in gut microbiota composition of healthy female adults following four weeks of BB supplementation, compared to placebo.

Secondary objectives
- To measure changes in gut microbiota functional potential following BB supplementation compared to placebo.
- To examine whether there are any differences in changes to gut microbiota composition and functional potential according to baseline diet quality following BB supplementation.
Hypotheses

- BB supplementation will have a beneficial effect on the gut microbiota, resulting in the proliferation of health-promoting microbes, increased alpha diversity and altered overall community composition (beta diversity), compared to placebo.
- Changes to microbial genes and pathways (i.e., functional potential of gut microbiota shifts) will be observed following BB supplementation, as compared to placebo.
- Changes in gut microbiome composition following BB supplementation will differ according to baseline diet quality grouping.

2.2 Participants

2.2.1 Recruitment

LINK study recruitment took place between July and September 2022. Women were recruited from the Auckland region via various online and print strategies, including social media platforms (Facebook), internal University of Auckland staff emails, and printed flyers across University of Auckland campuses and facilities (e.g. gyms and libraries). Study information was also listed on the University of Auckland Faculty of Medical and Health Sciences Clinic Trials website. After expressing interest in the trial through either an online form or directly contacting the research team, individuals were emailed the participant information sheet and consent form to read (Appendix A and B) and asked to complete an online screening questionnaire and the Dietary Screening Tool (DST). Eligible and willing women were invited to attend an enrolment visit at the University of Auckland Clinical Research Unit. As compensation for participant’s time, a $50 petrol or shopping voucher was given as a koha/gift after both clinic visits two and four. Participants were offered access to free parking for each study visit at the Clinical Research Unit and the option to receive summary information, including information on their gut microbiome and response to intervention after the trial.

2.2.2 Inclusion and exclusion criteria

In brief, the LINK study population was healthy female adults with no history of conditions known to affect the gut-brain axis. Specific inclusion/exclusion criteria are summarised in Table 2.1.
Table 2.1. LINK study inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Females aged between 18 - 45 years.</td>
<td>• Diagnosis of gastrointestinal disorders (e.g. Coeliac disease, inflammatory bowel disease) or past major gastrointestinal surgery likely to affect study outcomes (e.g. ileostomy)</td>
</tr>
<tr>
<td>• Residing in Auckland</td>
<td>• Treated for anxiety, depression or psychiatric disorders within the past two years</td>
</tr>
<tr>
<td>• BMI of 18 - 30 kg/m²</td>
<td>• History of neurological disorders (e.g. Parkinson's Disease, epilepsy, stroke, serious head trauma), cognitive impairment, or metabolic disease (e.g., diabetes, cardiovascular disease)</td>
</tr>
<tr>
<td>• Not pregnant or intending to become pregnant during the trial</td>
<td>• Use of medications expected to interfere with study outcomes (e.g. proton pump inhibitors, laxatives)</td>
</tr>
<tr>
<td>• Non-smoker</td>
<td>• Antibiotic use within four weeks prior to the study</td>
</tr>
<tr>
<td>• Access to the internet and a computer/smartphone/tablet</td>
<td>• Unwillingness to cease intake of pre- or probiotics, herbal extracts, or supplementations expected to interfere with digestion, cognition or mood for the four weeks prior to (and during) the intervention</td>
</tr>
<tr>
<td>• Not participating in another interventional clinical research trial four weeks prior to and during the study period</td>
<td>• Self-reported alcohol intake exceeding moderate level (&gt; 15 standard drinks/week)</td>
</tr>
<tr>
<td>• Comprehends and is willing and able to comply with all study procedures</td>
<td>• Regular use of recreational or illicit drugs</td>
</tr>
<tr>
<td>• Willing and able to provide written informed consent</td>
<td>• Allergy or intolerance to the investigational product or any of its ingredients</td>
</tr>
</tbody>
</table>

2.2.3 Dietary screening tool

To determine baseline diet quality, participants were screened with the DST during recruitment. This allowed the sorting of potential participants into ‘optimal’ and ‘suboptimal’ diet groups to balance (50%) equally for these in the study population. Initially developed for older adults (135), the DST has been modified and validated for Australian middle-aged adults (136,137). Notably, it showed discrimination in nutrient intakes (vitamin E, magnesium, zinc, and fibre), biochemical status (serum vitamin B6, folate, saturated fatty acids), and cognitive function (Stroop processing scores) between ‘optimal’ and ‘suboptimal’ diet groups (136). The tool was contextualised to food products and brands in Aotearoa, with ‘Hungry Jacks’ replaced with ‘Burger King’ in one item. The 20-item questionnaire had a maximum score of 104, with higher scores indicating better diet quality and lower nutritional risk. For example, higher scores indicate greater intakes of fruits, vegetables, legumes, nuts, olive oil and less processed foods, including confectionery, baking, sugar-sweetened beverages, and processed meats. Eighteen questions were related to the frequency of consumption of particular foods and food groups, and two were related to the number of servings consumed (Appendix C). In the LINK study, participants who scored ≥ 60 were categorised in the ‘optimal’ diet group, and those who scored ≤ 59 were placed in the ‘suboptimal’ diet group, aligning with the validated criteria established (136).
2.3 Intervention

Participants were administered the following interventions orally daily for four weeks each, with each intervention period separated by a four-week washout period:

1. Active intervention (BB) – Ŵëpap performance drink (300 mL), containing blackcurrant juice and extracts, along with 150 mg Pine Bark Extract Enzogenol® and 200 mg L-theanine. Total polyphenol content is 308 mg, with 151 mg specifically attributed to anthocyanins.

2. Control intervention – Placebo beverage (300 mL) with negligible polyphenol content (22 mg)

Table 2.2 displays the nutritional content of the intervention beverages; see appendix D for the ingredient breakdown. The control beverage was purpose-designed to match the taste, colour, macronutrient composition and energy density of the BB intervention and had been used successfully in a prior randomised controlled trial (138). The BB and control beverages were manufactured and packaged by Ŵëpap IP Ltd. The packing for both beverages was identical, except for the printed batch codes and expiry dates, which were concealed as per the blinding procedures outlined below. The intervention and control beverages were produced in separate batches and stored in a dark, temperature-controlled room prior to distribution to study participants.

| Table 2.2. Nutritional composition of active (BB) and placebo beverages. |
|---------------------------------|---------------------------------|
|                                | Blackcurrant beverage, BB (300mL) | Placebo beverage (300 mL) |
| Total Polyphenols (mg)         | 308                              | 22                         |
| Anthocyanins (mg)              | 151                              | 7                          |
| L-theanine (mg)                | 200                              | 0                          |
| Enzogenol® (mg)                | 150                              | 0                          |
| Vitamin C (mg)                 | 90                               | 90                         |
| Energy (kJ)                    | 155                              | 155                        |
| Protein (g)                    | 0.4                              | 0.4                        |
| Fat, total (g)                 | 0                                | 0                          |
| Fat, saturated (g)             | 0                                | 0                          |
| Carbohydrate (g)               | 23                               | 23                         |
| Sugars (g)                     | 14.8                             | 14.8                       |
| Sodium (g)                     | 8                                | 8                          |

2.4 Randomisation and blinding

Using an equal (1:1) allocation ratio, participants were randomly assigned to an intervention sequence (BB then placebo, or placebo then BB). Intervention sequences were balanced for diet group (50% suboptimal and optimal). The randomisation sequence was computer-
generated by an independent researcher (139), and randomisation was managed centrally using REDCap during the trial (140,141). Both participants and investigators were blinded to the identity of treatments for the duration of the trial and data analysis.

2.5 Procedures

2.5.1 Participant timeline

Study timeline and procedures, including participant recruitment, study visits and intervention arms, are outlined in Figure 2.1. Note that this section describes the procedures used in the LINK trial rather than the current thesis alone to contextualise the overall participant burden. The LINK trial involved five study visits at the Clinical Research Centre: enrolment, weeks 1 and 4 (arm 1), and weeks 8 and 12 (arm 2), with participants also completing weekly and fortnightly online questionnaires.

**Enrolment visit**

At the enrolment visit, eligibility was confirmed, details of the study discussed, and the opportunity was given for any questions to be clarified. If eligible, participants were asked to provide written informed consent (Appendix B). Baseline data questionnaires were conducted to collect self-reported information on demographics, medical and lifestyle history, medication and supplement use, and physical activity (using IPAQ-SF; International Physical Activity Questionnaire – Short Form). Weight and the average of two height measurements using a stadiometer were taken in clinic using standardised protocol, and BMI was calculated. Participants were then familiarised with the purple multitasking framework (MTF, a computerised cognitive stressor) to minimise the impact of learning effects when used in subsequent study visits. Participants were also familiarised with three online mood/well-being questionnaires (POMS, Profile of Mood States; WHO-5 well-being index; PSQI; Pittsburg Sleep Quality Index). Participants were provided with a stool sample collection kit and instructions for at-home collection, to be done 24 hours before study visits 1 - 4. After the enrolment visit, participants were randomly allocated to an intervention sequence.

**Study visits 1 -4**

Two weeks after the enrolment visit, participants had study visit 1, marking the beginning of the first intervention arm. For each participant, all four of their study visits were scheduled at the same time of day. Participants were instructed to fast overnight (avoid eating or drinking anything except water from 10 pm), as well as abstain from alcohol (for 24 hours) and caffeine (for 12 hours) before each study visit. At study visits 1-4, stool samples were collected from participants and stored in freezers immediately. Participants then completed mood questionnaires to assess stress reactivity immediately before and after completing a 20-minute cognitive stressor (purple-MTF). A fasted blood sample was then collected.
Participants were asked to complete the three mood and sleep questionnaires within 24 hours of their study visit. Participants were also required to complete a weekly online intervention adherence questionnaire that assessed compliance to intervention beverages, whether any significant dietary changes to diet/lifestyle were made, and whether any adverse events occurred. Participants received intervention beverages at study visits 1 and 3 (either BB or placebo), as prepared by a researcher unblinded to allocation. Participants were instructed to consume one beverage per day while (to the best of their ability) maintaining their usual diet and lifestyle. Between intervention arms 1 and 2, participants had a four-week washout period where they did not consume any study beverages. Participants were instructed to maintain their usual diet and lifestyle.

Figure 2.1. Schematic overview of LINK study enrolment, intervention, and assessment.
2.6 Data collection

2.6.1 Intervention adherence
During intervention periods, adherence was tracked through a weekly online questionnaire. Participants recorded the number of study beverages they had consumed, reasons for any missed beverages, and any changes to their usual dietary and lifestyle habits in that week. A minimum adherence threshold of 80% of beverages consumed per intervention arm (28 beverages per arm) was set. Research staff monitored adherence weekly.

2.6.2 Sample collection
Faecal samples were collected at home in the 24 hours before study visits 1-4, using faecal collection kits including instructions, gloves, a pottle with a scoop, freezer packs for transport to the clinic, and a labelled bag with participant ID and space to fill in the time and date of stool sample collection. Participants were instructed to freeze their samples immediately after collection before bringing them to the study visit, where they were stored in the fridge during clinic visits, allowing them to defrost sufficiently before aliquoting and mixing 100 mg stool with 900 μL sample preservation solution (DNA/RNA Shield™, Zymo Research, California, USA) for storage at -80°C until DNA extraction.

2.6.3 DNA extraction of stool samples
DNA extractions were performed in a disinfected U.V. sterilised class II hood at room temperature, using the ZymoBIOMICS™ 96 MagBead DNA kit from Zymo Research, California, USA (#D4308), which employs magnetic bead-based extraction. Mechanical bead beating was performed using a 1600 MiniG (SPEX SamplePrep), shaking at full speed (1500 rpm) for 5 minutes. Up to 800 μL of stool solution was used as input, and the procedure for each sample followed the manufacturer’s instructions, with DNA/RNA Shield™ from Zymo Research used (added in a prior step) in place of lysis solution to improve DNA integrity. DNA extract concentration and purity was initially screened by spectrophotometry, and then concentration was assessed using the Qubit dsDNA Broad Range Assay Kit (Invitrogen, USA, #Q33266). This DNA extraction protocol was employed for DNA extraction in: 156 LINK study samples, a blank extraction control (800μL DNA/RNA shield), and three replicates of a 21-strain ZymoBIOMICS™ Gut Microbiome Standard (#D6331), providing a known microbial composition for validating our experimental procedures (see Appendix E for comparison of study standards to reference values).

2.6.4 Metagenomic sequencing and processing
DNA extracts were sent to a commercial provider (Novogene, Beijing, China) for shotgun metagenomic library preparation and sequencing. DNA libraries were prepared using the NEBNext®Ultra™ DNA Library Prep Kit for Illumina® (NEB, Massachusetts, USA, #E7370), and sequencing was performed on an Illumina®NovaSeq6000 platform, generating an average
sequencing depth of 21.8 ± 1.4 million paired-end reads/sample (range 17.4 – 29.4 million). Raw sequencing files underwent processing using a custom-built bioinformatic script, executed on the cloud-native platform Terra (142). The script was derived from one available on GitHub (https://github.com/brookewilson/gutbugs_microbiome). Co-supervisor Dr. Brooke Wilson ran this initial bioinformatics pipeline. In the first step of the script, raw sequencing reads underwent quality control, which included the removal of adapter sequences, low-quality reads and human sequences using KneadData v0.11.0 (143). Subsequent steps in the pipeline involved taxonomic and functional profiling.

**Taxonomic profiling**
Quality-filtered reads were mapped against a database of marker genes from many different bacteria, archaea, viruses and eukaryotes, using MetaPhlAn3 v3.1 (38) with default parameters. This generated relative abundance profiles of identified taxa within each metagenome sample. Resulting read counts were normalised by the length of the clade-specific marker genes in each taxonomic group and converted into relevant abundances that sum to 100.

**Functional potential**
Following taxonomic profiling, characterisation of the species-stratified gene content and metabolic potential was performed by HUMAnN3 v3.6 utilising UniRef90 and MetaCyc databases (38). For each sample, default abundances of UniRef90 gene families and MetaCyc Pathways, expressed as reads per kilobase (RPK), were normalised by the total number of read counts and multiplied by a million to give copies-per-million (CPM).

**Richness and diversity metrics**
Downstream processing and analysis of microbiome data was conducted in R (v4.2.2). For each sample, species, gene and pathway richness counts were calculated using the relative abundance tables generated from MetaPhlAn3 and HUMAn3 analysis, and counts were normalised by sequencing depth. Based on species-level relative abundance profiles, Shannon diversity index was used to estimate alpha diversity, and Bray-Curtis dissimilarity index was used to estimate beta diversity. Alpha diversity was calculated using the ‘diversity()’ function and Bray-Curtis dissimilarity index using the ‘vegdist()’ function, both from the vegan package (v2.6.4). To visualise variations in microbiome composition between intervention groups at each time point, ordinations on the Bray-Curtis dissimilarity index were performed. This ordination was performed using non-metric multi-dimensional scaling using the ‘metaMDS()’ function in the vegan package.
2.7 Sample size calculation

Power calculations to determine adequate sample size for the LINK study were based on measures of stress as the primary endpoint. Based on published data of stress reactivity measures after an intervention containing similar doses of anthocyanins compared to a control (144), a sample size of 36 subjects was estimated to be adequate for a between-group difference of 20% to identify significant differences at a level of 5% (α = 0.05 and β = 0.8). A sample size of 40 allowed for a drop-out rate of less than 15%. It is difficult to base power calculations on shifts in gut microbiota composition due to the multifactorial nature of the data. However, we expected to be able to see shifts with this sample size based on prior studies investigating anthocyanin supplementation in healthy adults. For example, differences in the relative abundance of *Bifidobacteria* in 15 healthy adults were reported following supplementation with anthocyanin-rich blueberry powder in a cross-over study (100).

2.8 Statistical analysis

Statistical analysis and visualisation of microbiome data was performed in R (v 4.2.2) whilst blinded to group allocation. Statistical significance was defined as p < 0.05 unless otherwise specified. The analysis adhered to an intention-to-treat approach, including available data from all 40 participants, including the 2 participants with data exclusively from the first intervention arm.

To test the effect of intervention from baseline to follow-up on the relative abundance of microbiome features (such as specific taxa and metabolic pathways), generalised linear modelling was performed using MaAsLin2 (v1.15.1) (145). Time point was used as a fixed effect, with participant ID added as a random effect variable, including each intervention group in turn (i.e. BB and placebo profiles run separately). Taxa profiles were tested at the species, genus, family, and phylum levels. Relative abundances were log-transformed and rare taxa and metabolic pathways present in <10% of samples were excluded. Nominal p-values were adjusted for multiple testing using false discovery rate correction (FDR) using the Benjamini-Hochberg procedure, with q < 0.25 considered statistically significant. To assess differences in beta diversity between intervention groups, permutational multivariate analysis of variance (PERMANOVA) tests were performed cross-sectionally at each time point, with 999 permutations, using the ‘adonis2()’ function from the vegan package (v2.6.4) (146). Non-parametric paired Wilcoxon signed rank tests were performed to assess changes in Firmicutes:Bacteroidetes ratio, Shannon diversity index, and species, gene and pathway richness counts from baseline to follow-up in both intervention groups. The same test also assessed the Bray-Curtis dissimilarity to baseline between intervention groups.
For the exploratory analyses investigating the influence of diet group on intervention effects, differences in response to the intervention according to diet group were assessed using the same statistical tests described previously for each metric, but with analyses stratified by diet group.

2.9 Ethical considerations

The LINK trial was not initiated until approval was gained by the Institutional Review Board/Ethics Committee (IRB/EC). The Health and Disability Ethics Committee approved the trial on 27/05/2022 (reference number: 2022 EXP 12513). Individuals interested in the study were provided comprehensive information on the trial and participant requirements. In accordance with the International Conference on Harmonisation (ICH) guidelines, properly executed written informed consent was obtained from each participant. Official enrolment into the trial was finalised after this consent was given. The researchers ensured that the trial was conducted in accordance with the Declaration of Helsinki, including relevant institutional regulations. The trial was registered with the Australian New Zealand Clinical Trials Registry (ACTRN12622000850774).
Chapter 3. Results

3.1 Participant characteristics

Of 110 participants who registered their interest in the LINK study, 40 healthy females were enrolled in the study. As shown in Figure 3.1, 27 potential participants were excluded for not meeting the inclusion criteria, 17 declined to participate, and 26 were not appropriate as their dietary subgroup was already full. The study employed a stratified random allocation strategy of participants. 20 participants were randomly allocated to receive either the placebo beverage before the BB (placebo > BB) and 20 to receive the BB before the placebo (BB > placebo). Within each sequence group, participants were further stratified based on their diet, resulting in equal representation from suboptimal and optimal diet groups (i.e. 10 suboptimal, 10 optimal within each sequence group). 38 participants completed both intervention arms of this cross-over trial (95% retention rate), with 2 participants on the placebo > BB sequence withdrawing before commencing their second intervention arm due to COVID-related health reasons. All participants met the minimum 80% adherence to the intervention requirement. All available data from the 40 participants was included in the intention-to-treat analysis.

Participant characteristics are outlined in Table 3.1, including both the total LINK study population, as well as according to optimal and suboptimal diet groups. In brief, this is a population of healthy females with a mean age of 29.8 years (± 6.9), with the majority rating their health as either excellent (35%) or very good (37.5%). Most participants are European/Pākehā (62.5%), 25% are Asian, 5% Māori, and 7.5% Middle Eastern/Latin American/African. The majority of participants regularly engaged in moderate (37.5%) or high-intensity exercise (52.5%), and had undergraduate (45%) or postgraduate (47.5%) university degrees. Baseline participant characteristics appear reasonably well-matched between optimal and suboptimal diet groups, except for a greater proportion of participants identifying as NZ European in the optimal diet group.
<table>
<thead>
<tr>
<th>Table 3.1. Characteristics of LINK trial participants at enrolment.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total sample</strong></td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
</tr>
<tr>
<td>Alcohol Intake (drinks/week)</td>
</tr>
<tr>
<td>Ethnicity¹</td>
</tr>
<tr>
<td>European/Pākehā</td>
</tr>
<tr>
<td>Māori</td>
</tr>
<tr>
<td>Asian</td>
</tr>
<tr>
<td>MELAA²</td>
</tr>
<tr>
<td>Education</td>
</tr>
<tr>
<td>NCEA L3 or equivalent</td>
</tr>
<tr>
<td>University - undergraduate</td>
</tr>
<tr>
<td>University - postgraduate</td>
</tr>
<tr>
<td>Exercise level³</td>
</tr>
<tr>
<td>High</td>
</tr>
<tr>
<td>Moderate</td>
</tr>
<tr>
<td>Low</td>
</tr>
<tr>
<td>Self-rated health</td>
</tr>
<tr>
<td>Excellent</td>
</tr>
<tr>
<td>Very good</td>
</tr>
<tr>
<td>Good</td>
</tr>
<tr>
<td>Fair/poor</td>
</tr>
<tr>
<td>Supplement use⁴</td>
</tr>
<tr>
<td>Vitamins and/or minerals</td>
</tr>
<tr>
<td>Herbals and homeopathics</td>
</tr>
<tr>
<td>Omega-3</td>
</tr>
<tr>
<td>Creatine</td>
</tr>
<tr>
<td>Probiotics</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Medication use⁵</td>
</tr>
<tr>
<td>Yes</td>
</tr>
<tr>
<td>No</td>
</tr>
</tbody>
</table>

Data presented as mean ± SD for continuous variables or n (%) for categorical variables; ¹Ethnicity was classified according to Te Whatu Ora Level 1 ethnic codes (147); ²MELAA denotes Middle Eastern/Latin American/African ethnicity; ³Exercise level was self-reported via the International Physical Activity Questionnaire (IPAQ) – Short Form, with one participant from the suboptimal diet group not completing this questionnaire; ⁴Supplements refers to usual supplement intake, although participants abstained from supplement intake four weeks prior to and for the duration of the trial. As some participants were taking more than one type of supplement, percentages may not sum to 100. Supplement categories were based on the FSANZ Classification of Foods and Dietary Supplements (148); ⁵Medications taken during the trial included antihistamines, contraception, blood clotting medication, and asthma inhalers; Abbreviations: BMI, Body Mass Index; NCEA, National Certificate of Educational Achievement.
Figure 3.1. CONSORT diagram illustrating the flow of participants through the current study.

Within each sequence group, participants were stratified based on their diet group to ensure equal representation from both suboptimal and optimal diet groups, resulting in 10 participants from each diet group within each sequence group. Abbreviations: BB, Blackcurrant Beverage.
3.2 Intervention effects

3.2.1 Taxonomic profiling

Of the 40 participants in the study, 38 (19 optimal diet group, 19 suboptimal diet group) provided stool samples at all four collection time points. Therefore, microbiome analysis was performed on a subset of the original study population and included a total of 156 stool samples (placebo > BB, n = 80 samples; BB > placebo, n = 76 samples). These samples were analysed by shotgun metagenomic sequencing, at a mean sequencing depth of $21 \pm 1.25$ million reads per sample (see Appendix F for breakdown of read counts after quality control steps).

Taxonomic profiling was performed, and the relative abundance of bacterial species, genera, families, and phyla in participants’ stool samples compared before and after the BB or placebo intervention. Using phyla as an illustrative example (Figure 3.2), it was observed that participant gut microbiomes were dominated by Bacteroidetes and Firmicutes phyla (average abundance 53% and 40% respectively), with lower relative abundances of Actinobacteria (2.4%), Proteobacteria (2.2%), and Verrucomicrobia (1.5%). An estimated 45% of the total microbiome sample had unknown taxonomy (SD: 8%, range 25-67%).

![Figure 3.2](image-url)

**Figure 3.2.** Relative abundance of bacterial phyla in participant gut microbiomes at baseline and post-placebo or active (BB) intervention. Each column represents an individual participant.
Using generalised linear modelling implemented in MaAsLin2, no species, genera, families, or phyla were found to be significantly differentially abundant between baseline and following BB intervention ($p > 0.05$). However, the Actinobacteria phylum and related genus and family were significantly reduced in abundance from baseline to post-placebo intervention ($q < 0.25$), see Table 3.2.

Table 3.2. Significant changes in the abundance of specific genera, families and phyla in participant gut microbiomes from baseline to post-placebo intervention, as identified from generalised linear modelling (MaAsLin2 analysis).

<table>
<thead>
<tr>
<th>Level</th>
<th>Intervention Group</th>
<th>Coefficient</th>
<th>p-value</th>
<th>q-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium</td>
<td>Genus</td>
<td>-0.752</td>
<td>0.001</td>
<td>0.074</td>
</tr>
<tr>
<td>Bifidobacteriaceae</td>
<td>Family</td>
<td>-0.752</td>
<td>0.001</td>
<td>0.038</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>Phylum</td>
<td>-0.421</td>
<td>0.027</td>
<td>0.239</td>
</tr>
</tbody>
</table>

$^1$Microbiome features showed significance at $p < 0.05$ and remained significant after correcting for False Discovery Rate ($q < 0.25$).

3.2.2 Firmicutes:Bacteroidetes ratio

At baseline, participant microbiomes’ on average displayed a larger proportion of Bacteroidetes phyla than Firmicutes (mean F:B ratio 0.26 ± 0.33 in placebo group; 0.24 ± 0.36 in BB group). The F:B ratio of participants’ gut microbiomes did not significantly change after either the BB or placebo interventions (Wilcoxon signed-rank test; placebo, $p = 0.48$; BB, $p = 0.33$ (Figure 3.3).

![Figure 3.3. Firmicutes:Bacteroidetes ratio of participant gut microbiomes at baseline and post-placebo or active (BB) intervention. Each box represents the median and interquartile range (IQR), and the whiskers the data range (expanding up to 1.5 x IQR).](image)
3.2.3 Richness and diversity metrics

On average, 100 species ± 21 (range 40 – 148) were detected per faecal sample. Neither BB nor placebo interventions altered the richness counts (normalised by sequencing depth) of species from baseline levels, as shown by Wilcoxon signed-rank tests (placebo, p = 0.40, BB, p = 0.38) (Figure 3.4).

Figure 3.4. Species richness counts of participant gut microbiomes at baseline and post-placebo or active (BB) intervention, normalised by sequencing depth.

Similarly, there was no significant difference in alpha diversity, as measured by Shannon diversity index, from baseline to follow-up in either intervention group (Wilcoxon signed-rank test, placebo; p = 0.38, BB; p = 0.23) (Figure 3.5).

Figure 3.5. Species-level alpha diversity, as measured by Shannon diversity index, of participant gut microbiomes at baseline and post-placebo or active (BB) intervention.
Differences in the overall microbial composition profile between intervention groups at baseline and post intervention (species-level Bray Curtis dissimilarities) were visualised by non-metric multi-dimensional scaling (Figure 3.6) and tested by PERMANOVA. PERMANOVA tests found no significant difference in microbial composition between intervention groups both at baseline and following the intervention (pre, p = 1.00; post, p = 0.99).

Participant microbiomes' displayed similar levels of species-level microbiome drift from baseline to follow-up with BB or placebo intervention, as measured by Bray-Curtis dissimilarity to baseline (Figure 3.7, Wilcoxon rank-sum test, p = 0.21). The median species-level microbiome drift from baseline was 0.30 ± 0.09 with placebo intervention, and 0.26 ± 0.14 with BB intervention, with a higher value indicating a larger degree of shift from baseline. Of interest, the gut microbiomes of eight participants appeared to have shifted more on the BB intervention, (i.e. were at the higher end of the Bray-Curtis dissimilarity to baseline index (as can be seen in Figure 3.7). However, due to the low sample size, no further sub-analysis was appropriate.
Figure 3.6. Non-metric multi-dimensional scaling plots based on species-level Bray Curtis dissimilarities, illustrating variations in participant gut microbiomes at baseline and post-placebo or active (BB) intervention. Each point represents a microbiome sample, with points clustering together indicating similarity in community structure.

Figure 3.7. Shifts in gut microbiome composition of participants from baseline to post-placebo or active (BB) intervention, based on species-level Bray Curtis dissimilarities.

3.2.4 Functional potential

The relative abundance of microbial pathways in participant’s stool samples were compared before and after BB or placebo intervention. Generalised linear modelling (MaAsLin2) identified no pathways that were differentially abundant between baseline and post-active
(BB) intervention (p > 0.05). However, 1 pathway (P124-PYW: *Bifidobacterium* shunt) was significantly reduced in abundance from baseline to post-placebo intervention (coefficient = -0.72, p < 0.0001, q = 0.10).

On average, 233,000 ± 42,661 genes (range 103 k - 325 k), and 283 ± 35 pathways (range 219 - 377) were detected per faecal sample. Neither BB nor placebo interventions altered the richness counts (normalised by sequencing depth) of genes or pathways from baseline levels, as shown by Wilcoxon signed-rank tests of genes (placebo, p = 0.93, BB, p = 0.69), and pathway (placebo, p = 0.89, BB, p = 0.08) richness (Figure 3.8).

![Figure 3.8. Gene and pathway richness counts of participant gut microbiomes at baseline and post-placebo or active (BB) intervention, normalised by sequencing depth.](image)

### 3.3 Exploratory analyses: effect of baseline diet quality on gut microbiota response to active (BB) intervention

This section describes results from analyses further split based on diet group (suboptimal or optimal), with the objective to explore whether any differences in intervention effects exist according to baseline diet quality. Given the small sample size and exploratory nature, this analysis is restricted to the BB intervention only.

#### 3.3.1 Taxonomic profiling

Generalised linear modelling (MaAsLin2) identified one species, but no genera, families or phyla that were differentially abundant between diet groups at baseline and post BB intervention (p > 0.05). In the suboptimal diet group, a *Clostridium* species (*Clostridium*...
species AM22-11AC) was significantly increased in abundance from baseline following the BB intervention (coefficient = 1.43, p = 0.001, q = 0.13) (Figure 3.9).

**Figure 3.9.** Relative abundance of *Clostridium* species AM22-11AC in participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups.

### 3.3.2 Firmicutes:Bacteroidetes ratio

The F:B ratio of participants’ gut microbiomes from baseline to follow up with the BB intervention beverage were not significantly impacted by diet group, with no change seen in either diet group (Wilcoxon signed-rank test; suboptimal, p = 0.13, optimal, p = 0.83) (Figure 3.10).
3.3.3 Richness and diversity metrics

Neither optimal nor suboptimal diet groups showed altered species richness counts (normalised by sequencing depth) from baseline levels with the BB intervention beverage, as shown by Wilcoxon signed-rank tests (suboptimal, $p = 0.29$ optimal, $p = 0.24$) (Figure 3.11).

Figure 3.10. Firmicutes:Bacteroides ratio of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups.

Figure 3.11. Species richness counts (normalised by sequencing depth) of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups.
Similarly, there was no significant difference in alpha diversity from baseline to follow-up with BB intervention in either diet group, as measured by Shannon diversity index from baseline to post-BB intervention (Wilcoxon signed-rank test, suboptimal, p = 0.62, optimal, p = 0.17) (Figure 3.12).

![Figure 3.12. Species-level alpha diversity (Shannon diversity index) of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups.](image)

Differences in microbial composition between diet groups at baseline and post-BB intervention (species-level Bray Curtis dissimilarities) were visualised by non-metric multi-dimensional scaling (Figure 3.13) and tested by PERMANOVA. Whilst diet group-related difference in beta diversity was not immediately obvious with no clear clustering of microbiome samples, PERMANOVA testing showed that diet group was a significant factor on microbial composition at baseline and post-BB intervention, contributing to approximately 4% of the total variance seen in the baseline data (p = 0.004), and 3% at follow-up (p = 0.001).

The mean species-level microbiome drift from baseline with BB intervention, assessed by Bray-Curtis dissimilarity to baseline, was $0.25 \pm 0.11$ for participants in the optimal diet group, and $0.30 \pm 0.17$ for those in the suboptimal diet group. Comparison between the two diet groups revealed no significant difference in species-level microbiome drift from baseline (Figure 3.14, Wilcoxon rank-sum test, p = 0.11).
Figure 3.13. Non-metric multi-dimensional scaling plots based on species-level Bray Curtis dissimilarities illustrating variations in participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups. Each point represents a microbiome sample, and points that are more similar to each other in community structure cluster together.

Figure 3.14. Shifts in gut microbiome composition of participants from baseline to post-active (BB) intervention, based on species-level Bray Curtis dissimilarities and split by optimal and suboptimal diet groups.

3.3.4 Functional potential

The relative abundance of microbial pathways in participant’s stool samples were compared before and after BB or placebo intervention. Generalised linear modelling (MaAsLin2) identified no pathways that were differentially abundant between diet groups at baseline and post-BB intervention (p > 0.05).
Neither optimal nor suboptimal diet groups showed altered gene or pathway richness counts (normalised by sequencing depth) from baseline levels with the BB intervention, as shown by Wilcoxon signed-rank tests of gene (suboptimal, p = 0.80, optimal, p = 0.72) and pathway (suboptimal, p = 0.06, optimal, p = 0.36) richness (Figure 3.15).

Figure 3.15. Gene and pathway richness counts (normalised by sequencing depth) of participant gut microbiomes at baseline and post-active (BB) intervention, split by optimal and suboptimal diet groups.
Chapter 4. Discussion

4.1 Overview

This chapter summarises findings from a double-blinded, placebo-controlled, cross-over RCT investigating the impact of four weeks of daily intake of a flavonoid-rich blackcurrant beverage, providing a similar quantity of polyphenols to that found in around 80 blackcurrants (149,150) on the gut microbiomes of healthy women as part of the LINK study. This chapter interprets the study findings in the context of existing literature, outlines the study’s strengths and limitations, examines theoretical and clinical implications, and suggests future research directions.

4.2 Key findings

Taxonomic profiling revealed a predominance of Bacteroidetes and Firmicutes phyla in participants’ gut microbiomes, with lower abundances of Actinobacteria, Proteobacteria and Verrucomicrobia present, mirroring the characteristic phyla distribution observed in healthy individuals in the literature (2).

No significant changes in taxonomic composition, richness, diversity or functional potential were observed following the active (BB) intervention. However, alterations were noted with the placebo intervention. Significant reductions were observed in a related group of bacterial taxa, specifically the probiotic Bifidobacterium genus (151) and its related Bifidobacteriaceae family and Actinobacteria phylum. Bifidobacterium are a common health-promoting taxa, well-known for their role in the metabolism of complex carbohydrates (151,152). Additionally, a significant reduction in the metabolic pathway Bifidobacterium P124-PWY, responsible for carbohydrate metabolism (153), was observed following the placebo intervention. The reduction in this pathway likely corresponds with the observed decrease in Bifidobacterium taxa abundance.

In an exploratory analysis, participants were stratified by baseline diet quality into ‘optimal’ and ‘suboptimal’ diet groups. PERMANOVA testing revealed a significant association between diet group and overall bacterial community composition (beta diversity), contributing to 4% of the total variance at baseline and 3% at follow-up. A significant increase in Clostridium species AM22-11AC from baseline to follow-up was observed in the suboptimal diet group following the BB intervention, though no significant differences in changes to richness counts, alpha diversity or functional potential between suboptimal and optimal diet groups were seen.
4.3 Interpretation of findings in the context of existing literature

This study marks the second investigation into the influence of blackcurrant supplementation on the human gut microbiome, yet is the first to utilise a cross-over and placebo control. A prior clinical trial conducted by Molan et al., (2014) explored the effects of two weeks of blackcurrant extract supplementation (200 mg anthocyanins/day) in 30 healthy adults, observing favourable modulation of gut microbiome composition and altered functional outputs (133). Despite its exploratory nature, due to its lack of a control group or cross-over design, this 2014 study aligned with evidence from several pre-clinical in vitro and in vivo animal studies showing a similar modulatory effect of blackcurrant supplementation on the gut microbiome. As outlined in Chapter 1, this pre-clinical evidence shows a general consensus of a proliferation of health-promoting bacteria, increased alpha diversity, and altered overall community composition (beta diversity), as well as changes to functional outputs such as intestinal pH, metabolite production and microbial gene abundance. However, findings vary widely between studies. The current study contributes to this variability, as, contrary to the current literature consensus, consumption of the flavonoid-rich blackcurrant intervention beverage was not significantly associated with changes in the gut microbiome, including taxa abundance, richness counts, diversity metrics, microbial gene and pathway abundance.

Next, possible explanations for the lack of significant findings will be discussed within the context of existing literature, including intervention format, dose and duration, and study sample characteristics, such as age and health.

**Intervention format**

As presented in Table 1.1 in Chapter 1 of this thesis, most studies investigating blackcurrants and the gut microbiome utilise extract-based blackcurrant interventions. In contrast, the intervention in this study contained a blackcurrant juice component, alongside blackcurrant extract. Perhaps a greater effect of the BB intervention would have been observed with an extract-based intervention, considering that potential counteractive or synergistic interactions may have occurred between other components within the blackcurrant juice and influenced the study findings, such as blackcurrant fruit sugars or vitamin C (154,155). Furthermore, the study found a significant potentially negative influence of the placebo beverage on the gut microbiome, suggesting that the placebo was not inert (further discussed below). Thus, as the placebo beverage shared similar constituents with the active beverage, except for the bioactive ingredients, using the placebo as a control may have potentially masked effects of the active intervention. For example, fruit sugars and sweeteners Erythritol and Stevia are in both beverages, and may have exerted counteractive or synergistic interactions influencing the effect of the active intervention. A review of pre-clinical evidence concluded that Stevia consumption may have a potential benefit by increasing alpha diversity, though findings from
subsequent human trials vary, with one observing significant gut microbiome modulation (156), and others observing no significant effect (157, 158). Additionally, human consumption of date fruit sugars (glucose, fructose, and sucrose) increased the abundance of *Bifidobacteria* and decreased the abundance of *Bacteroides* (154). While the intervention format may have contributed to the absence of an intervention effect, it could also be considered a strength of the study and a valuable contribution to the literature. Utilising a beverage as the supplementation vehicle aligns more closely with real-life consumption patterns, as polyphenols are typically consumed within a food matrix, such as in fruits, juices, and whole foods (82). Additionally, this beverage-based intervention adds diversity to an evidence base dominated by extract or powder-based interventions (Table 1.1).

**Intervention dose**

The BB intervention dose of 308 mg polyphenols (150 mg anthocyanins) may have been insufficient to have a significant impact on the gut microbiome. There is currently no consensus on the optimal dosage for eliciting the effects of blackcurrant supplementation on the gut microbiome. Scoping the research for a dose effect is difficult due to large variability in how doses were reported (e.g. flavonoid content vs percentage of blackcurrant format in diet vs weight of blackcurrant format per day), making it difficult to compare between studies. The only other clinical trial of blackcurrant supplementation utilised a blackcurrant extract containing 200 mg anthocyanins and observed significant modulation of gut microbiota with daily intake. However, this study was limited by its lack of a control group (133). Looking more broadly at polyphenols, a systematic review and meta-analysis found that polyphenol supplementation had a dose-dependent effect on microbiota abundance, and 396 mg/day was recommended to stimulate the abundance of *Lactobacillus* and *Bifidobacterium* health-promoting species and decrease the abundance of potentially pathogenic *Clostridium* species present in human gut microbiome (80). The BB intervention dose being less than this recommendation may have contributed to the insignificant findings.

**Age and health of study cohort**

As a reminder, the study cohort consisted of healthy female adults (aged 18-45 years) with no history of conditions known to affect the gut-brain axis. Perhaps a more pronounced response to the BB intervention may be seen in health conditions characterised by gut dysbiosis, such as with ageing, and in certain clinical conditions, such as obesity (3, 55, 159). For example, 30-days of supplementation with red wine polyphenols increased the relative abundance of health-promoting *Bifidobacterium* and *Lactobacillus* species in patients with metabolic syndrome, but not their healthy controls (102). Many of the animal studies forming the evidence base for blackcurrants influence on the gut microbiome have investigated models of disease or obesity, though these findings do appear consistent with those in ‘healthy’ animals. (Table 1.1). For example, blackcurrant anthocyanin supplementation of obese mice enriched
Akkermansia muciniphila, a species positively linked with intestinal health and leanness, as well as beta diversity (130). Currently, there is a lack of studies comparing ‘healthy’ and diseased animals with the same intervention, which would be helpful in understanding whether intervention effects are more pronounced in conditions typically characterised by dysbiosis.

Another relevant effect to consider here is age, where the blackcurrant literature suggests there may be an age-related intervention effect. Animals investigated ranged in age from 4 weeks (125,126,130) to 18 months (128). Cao et al. (2020) observed an age-related effect of blackcurrant supplementation, with the downregulation of Firmicutes in young mice and downregulation of Verrucomicrobia in old mice (128). This may indicate the role of age in mediating the effects of blackcurrant supplementation on the gut microbiome. However, due to the small sample size (n = 3 per intervention group) of this study by Cao et al., the findings are considered exploratory (128). Nevertheless, this study finding aligns with broader insights from clinical research on berry fruits, which showed an age-related effect of supplementation on gut microbiota composition in women of different ages (160). Therefore, perhaps only including young to middle-aged participants may have contributed to the lack of significant findings (164).

**Intervention duration**

This study used a 4-week intervention period to assess the short-term effects of blackcurrant supplementation on the gut microbiome. As discussed in Chapter 1, there is large variation in the intervention duration of blackcurrant supplementation studies, ranging from 4 weeks (124–126,132) to 4 months (128,131) in animal studies alone. However, intervention effects seem to be similar for both shorter and longer time frames. Looking more widely at the evidence for duration of dietary intervention studies, there is currently no consensus in the literature on the optimal duration to elicit gut microbiome modulatory effects, warranting further exploration (63). Therefore, it is difficult to say whether clearer differences might have emerged with an extended intervention period, though it is an important consideration alongside other factors discussed (161).

**Summary**

The lack of significant intervention effect observed in this study could be attributed to intervention format, dose, duration, and characteristics of the study cohort. However, it is important to acknowledge that the absence of significant findings may also be because there is no relationship between blackcurrant intake and the gut microbiome. Most of the current research on blackcurrants and the gut microbiome has been conducted in animals, with only a single prior clinical trial. Further research is therefore required to understand the complex relationship between these variables, as discussed in the future directions section.
‘Suboptimal’ vs ‘Optimal’ diet quality

This study aimed to explore the influence of baseline diet quality, as assessed by the DST, on the gut microbiome response to blackcurrant intervention, an aspect not yet investigated in the current literature on blackcurrants or polyphenols. Given the intricate relationship between polyphenols and gut microbiota, where the effectiveness of polyphenols depends on microbial utilisation (13,109), alongside the observed influence of diet on the gut microbiome (12,56,62), this was an important consideration for this study (63). Moreover, broader insights from studies on various dietary interventions suggest that baseline diet quality may impact the responsiveness of the gut microbiome to dietary changes. For example, a study involving a date fruit intervention in humans observed that individuals with higher baseline dietary fibre intake were less responsive compared to those with lower intake (162). In another study, lower baseline dietary fibre intake resulted in a more significant increase in SCFA production following prebiotic intervention (163).

The current study detected an impact of baseline diet quality on the effect of the BB intervention, noting an increase in the relative abundance of a single Clostridium species (AM22-11AC) within the suboptimal diet group. However, it is difficult to draw further conclusions given the exploratory nature of this analysis (which solely examines the active group without comparison to placebo control), and the lack of association of this particular species with any health outcomes (164). Additionally, PERMANOVA testing showed a significant association between diet group and overall microbial community composition at baseline and follow-up.

Overall, baseline diet quality appeared to have a limited influence on the response of participant gut microbiomes to intervention, with only a single species differing. It is worth considering that the discriminatory power of the DST may have impacted changes seen according to baseline diet quality, though it may be a true finding, given the lack of response to the BB intervention seen in the main analysis. The DST was chosen for use in the LINK study due to its ability to discriminate cognitive function, more relevant to the primary outcome of the wider LINK study. However, future gut microbiome studies should consider the use of dietary indices more closely associated with gut microbiome composition, such as the Healthy Eating Index, to better capture gut microbiota variance attributable to habitual diet in comparable populations (165).

Nevertheless, this study contributes to the growing body of evidence demonstrating the importance of considering baseline participant characteristics, such as diet quality, in understanding individual responses to interventions targeting the gut microbiome.
4.4 Strengths and limitations

4.4.1 Limitations

This study has several limitations that must be acknowledged.

Firstly, the characteristics of the study cohort may restrict the generalisability of the results. The findings, derived from a cohort of ‘healthy’ individuals, may not apply to those with underlying health conditions due to inherent physiological differences. Secondly, the distribution of ethnicities in the study cohort is not representative of the general population in Aotearoa, particularly underrepresenting Māori (16.5% in Aotearoa vs 5% in this study sample) (166). This may have some impact on the study generalisability, though possibly what is more pertinent is the social implications, as will be discussed in Section 4.5 below. Additionally, sex-specific differences in gut microbiome exist (167–170). Thus, these findings from an exclusively female sample may not apply to men. Furthermore, whilst the specific focus on young to middle-aged females aimed to mitigate age-related variations in gut microbiota composition (171), age may impact the effect of blackcurrant intervention, as discussed above. Overall, while this selection of healthy women aged 18-45 years was deliberate and enhances internal validity, it restricts the extrapolation of results to other demographic groups.

A second important limitation of this study pertains to the multicomponent nature of the intervention beverage. The intervention, Ārepa, is a pre-existing commercial beverage containing multiple bioactive ingredients, including blackcurrant juice and extract, L-theanine, and Enzogenol® (Pine Bark Extract). This complexity makes it challenging to isolate the effects observed to a specific ingredient, limiting the generalisability of the findings. New Zealand pine bark extract (Enzogenol®) contains a variety of flavonoid compounds, with a particularly high concentration (85%) of proanthocyanidins (172). As previously discussed in Chapter 1, flavonoids have been shown to have gut microbiome modulatory effects (173). L-theanine is a plant-derived amino acid (174,175) commonly used for cognitive enhancement to promote calmness, attention and reduce anxiety (175). While some pre-clinical studies suggest a potential gut modulatory effect of L-theanine, the current evidence is limited and inconsistent (176–180). However, synergistic or counteractive interventions between the various bioactive components in the beverage could have influenced our study outcomes.

Although a taste-, colour-, sodium-, sugar-, vitamin C-, and macronutrient-matched placebo beverage was employed to maintain blinding, used previously in an RCT (138), the study findings suggest it may not have been inert. This is illustrated in the observed reduced abundance of the probiotic *Bifidobacterium* genus and related family, phylum and metabolic pathway in participant gut microbiomes following the placebo intervention. The placebo contains New Zealand Apple Juice and Elderberry Juice concentrate, though the polyphenol
content from these ingredients was very low. It also contained fruit sugars, and two sweeteners Erythritol and Stevia, of which their possible gut microbiome modulation effects have been discussed above. Perhaps ingredients in the placebo beverage itself may have exerted synergistic or counteractive effects on the gut microbiome, influencing the study outcomes.

Thirdly, the broader LINK study was powered in sample size to detect differences in stress reactivity, not microbiome differences. Consequently, the lack of significant intervention effect findings could perhaps be a type II error, attributed to study underpowering. Furthermore, the gut microbiome is highly variable between individuals which makes it difficult to find consistent changes associated with nutritional interventions, such as prebiotics, particularly with a small sample size (39,181). Metabolic redundancy amongst microbes (i.e. they utilise the same substrates for growth) can contribute to a variable effect of prebiotics seen across individuals. For example, species X might increase in response to BB in one individual, whereas species Y might increase in another person (39,181). Thus, adequately powering gut microbiome studies investigating efficacy of possible prebiotic interventions is essential (63).

Lastly, while shotgun metagenomic sequencing utilised in this study provides valuable insight into the functional potential of the gut microbiome, it does not directly measure functional outcomes. Certain metabolic pathways may be predicted to be present based on gene abundance, but this doesn’t guarantee their activation or effectiveness in vivo. Factors such as microbial interactions, host-microbe interactions, and environmental factors can all influence whether predicted metabolic pathways are expressed and contribute to physiological health (31). Furthermore, bacterial genome databases are currently incomplete, with the majority of genes in the human gut microbiome unable to be functionally assigned, an issue exacerbated by a current lack of knowledge of both the dynamic and biological effect of the vast numbers of polymorphisms and other structural variations of the microbiome (182). To address limitations in predicting function, future research efforts should consider integrating complementary approaches, such as metatranscriptomics to assess gene expression levels, metabolomics analysis to evaluate metabolite levels, or targeted functional assays like SCFAs measurement or enzyme activity assays. Metabolites produced by bacteria are the primary agents which mediate host-microbe and microbe-microbe interactions (183). Thus, studying metabolites as well as bacterial genes is important to provide a comprehensive and accurate assessment of collective community function and its implications for physiological health (17).
4.4.2 Strengths

Despite the acknowledged limitations, this study has several notable strengths.

The main strength of this study lies in its robust study design: a randomised, double-blind, placebo-controlled, cross-over human intervention trial, with a washout period between intervention arms. This design limits biases and confounding variables, thereby increasing the internal validity.

While the microbiome is a relatively objective measurement, employing a placebo-controlled trial is important to ensure that participant perception of the intervention arm doesn’t influence compliance or other lifestyle and dietary habits. Rigorous adherence to double-blinding until data analysis completion minimises both participant performance bias and expectation effects, as well as researcher bias, further strengthening the internal validity. The only other clinical trial investigating blackcurrant supplementation on the gut microbiome, by Molan et al (2014), lacked a placebo control group, so this study adds a novel aspect to the evidence base. However, the use of a more straightforward intervention format, such as powder or capsules, might have more easily facilitated the use of an inert placebo while maintaining blinding, avoiding the need for other ingredients which may have synergistic or counteractive effects (as discussed above).

The cross-over design, wherein participants act as their own control, reduces the confounding effects of inter-individual variation. Participants can have vastly different baseline microbiomes and responses to dietary intervention, and cross-over reduces the sample size needed to detect differences by at least half (63). Cross-over intervention studies with a washout period are the most optimal study design for dietary intervention studies (63) and have been successfully implemented in diet-microbiome studies (184–186). However, most studies assessing the effect of blackcurrant supplementation on the gut microbiome utilised a parallel study design, so this study adds a novel aspect to the research space. Despite a cross-over design increasing the complexity of data analysis, study duration and participant burden, the high compliance to treatment suggests that any intervention ‘fatigue’ in participants may have been minimal and not impactful on study outcomes. An imperative aspect of this cross-over study design is an adequate washout period to prevent carryover effects from the first intervention arm (187). The washout period of this study was four weeks, based on prior evidence. Previous studies have shown that gut microbiota changes following a prebiotic fibre intervention recovered to baseline after a 28-day washout period (188). Furthermore, gut microbiota changes resolved after a three-week washout period following a two-week probiotic yoghurt intervention (189).

The use of shotgun sequencing is a notable strength. Unlike the predominant use of 16S sequencing in prior studies on blackcurrants and gut microbiome (Table 1.1), which offers
limited species-level taxa resolution and predictive functional insights, shotgun metagenomic sequencing provides a deeper level of taxonomic classification. It also encompasses microbes from various kingdoms of life, and enables the detection of functional genes and metabolic pathways within the microbial community. Although no significant effect of the active intervention on composition or functional potential was seen in this study, the use of shotgun sequencing augments the evidence base.

Another strength is the use of a novel approach for recruitment, aiming to reduce the pervasive issue of self-selection sampling bias inherent in dietary intervention studies (190). Typically, such studies are susceptible to the overrepresentation of ‘health-conscious’ individuals, potentially skewing findings towards a population more likely to have optimal nutrient status and healthier habits prior to intervention. This scenario neglects baseline diet and nutrient status, disregarding its potential impact on treatment effects, thus increasing the risk of type II error in nutrition research (190,191). To limit this bias, individuals interested in the LINK study were screened with the Dietary Screening Tool (DST) to assess their baseline diet quality. This allowed us to balance the study population, ensuring equal representation of ‘optimal’ and ‘sub-optimal’ diet groups. While this approach did require additional time and increased participant burden during recruitment, it reduced this self-selection bias, increasing the integrity and generalisability of the findings. Interestingly, this recruitment phenomenon was demonstrated during recruitment, where 26 potential participants were turned away because the ‘optimal’ diet group was already at capacity (Figure 3.1).

Lastly, the study benefited from good study conduct, as evidenced by high intervention adherence and low dropout rates. All participants met the 80% adherence to intervention requirement, as measured through weekly online adherence questionnaires. Furthermore, only two participants dropped out of the study (due to COVID-19 related health reasons). This enhances the internal validity and reliability of the findings and indicates the feasibility and tolerability of the intervention within the target population, strengthening the generalisability of the findings. The robust study conduct speaks to the effectiveness of strategies employed to promote engagement, including regular study visits, ongoing support, and the appeal of receiving personal health information post-study.

4.5 Social implications

While this research contributes to our scientific understanding of blackcurrants and the gut microbiome, it would be remiss not to consider the implications of this research within the social landscape of Aotearoa.

As previously noted, the study sample notably underrepresents Māori. This raises concerns about the applicability of the findings to Māori. Ethnicity has been found to account for some of the intraindividual variation in gut microbiome composition (192,193). Thus, the
underrepresentation of Māori in the study sample is concerning given their overrepresentation in poor health outcomes, including non-communicable diseases (NCDs) such as cancers, diabetes, metabolic illness, and heart disease (194,195). Recent research has shown that many NCDs are associated with, and influenced by the gut microbiome (196). For example, gut dysbiosis was present in stroke and transient ischaemic attack patients (197), and reduced diversity of the gut microbiome has been associated with obesity and insulin resistance (198). Thus, due to the presence of these health inequities associated with outcomes of this research, the LINK study should have prioritised Māori in recruitment to ensure equal explanatory power in the study sample, vital to rebalance the demographic dominance of non-Māori (199). When Māori are not appropriately represented in health research, non-Māori realities stay in the foreground and inform policy and practice, thus further perpetuating health inequities, a direct conflict with Te Tiriti commitments (199,200).

This would require a different approach in participant recruitment and study design and, importantly, the involvement of Māori researchers from inception to implementation (199,200). Ethical and cultural challenges specific to research involving human tissue and genetics, such as in this study, require Māori perspectives and leadership in study design (200,201). For example, storing faecal samples in the freezer may have been a point of sensitivity for some Māori (200). This could be mitigated through the use of different stool collection protocols, with samples placed directly into tubes containing DNA stabiliser, preserving the microbes at the point of collection and circumventing the need to be stored in a freezer (202). Though more expensive, this could be one way to ensure the study design is more appropriate for Māori (199). Further, the extracted DNA from participant faecal samples was sent overseas, moving beyond the jurisdiction of New Zealand law, which is another point of sensitivity to some Māori (200). In some cases, this can be somewhat mitigated by ensuring that the overseas laboratory is quality-assured according to internationally agreed standards (203). Such issues must be considered during study design and clarified during the consultation and consenting process (199). With gut microbiome research being a relatively new space, researchers have the critical opportunity and responsibility to improve from the past, and ensure equitable research in this space to ensure equitable health outcomes, services and systems.

Another point to consider here is that fad foods and health products have made the gut microbiome popular among the more affluent and health-conscious demographics (201,204). In contrast, the benefits of the microbiome and its role as a determinant of health are likely less understood (and utilised) among communities facing greater socioeconomic barriers, of which Māori are overrepresented (195,201). This situation inadvertently sidelines those who stand to gain the most from advances in understanding the connection between gut microbiome and health (201). The use of a relatively costly commercial intervention beverage in this study, currently sold for $6.99 at major supermarkets (205), may inadvertently perpetuate the existing disconnect of socioeconomically disadvantaged populations with
microbiome research. Despite the broader aim of this research to be around flavonoids, which are readily available through dietary sources like fruits and vegetables, the use of this commercial product may imply to readers that consuming this beverage is necessary to yield an effect.

To conclude, future research in Aotearoa should ensure equal explanatory power of Māori in study cohorts where health inequities related to the research are present, and involve Māori in study design consultation early and consistently (199,200). Furthermore, researchers should prioritise the accessibility of interventions to improve the relevance of the research to all consumers. These considerations are important in combatting health inequities in Aotearoa.

4.6 Future directions

The lack of significant intervention effect seen in this study adds more uncertainty to the literature on blackcurrant supplementation, underscoring the complex relationship between polyphenols and the gut microbiome. While the absence of significant findings may be perceived as a limitation, it also identifies critical avenues for future research.

1. High-quality RCTs are needed

Primarily, more high-quality RCTs investigating blackcurrant supplementation on composition and metabolic function of the gut microbiome are needed. Such studies would provide valuable insights into the efficacy of blackcurrants in favourably modulating the gut microbiome, potentially leading to increased consumption and integration of blackcurrants into consumers’ diets.

These RCTs should be rigorously controlled, adequately powered, employ cross-over designs, involve larger sample sizes, encompass diverse populations and assess functional outputs, alongside functional potential. For example, through the use of complementary omics technologies, as well as functional assays.

2. Head-to-head comparisons within studies are needed

Given the considerable variation in study methodologies, there is a need for these robust RCTs to compare a single hypothesised factor influencing efficacy, such as intervention duration, dose, format, participant age and health condition, within the study. These comparisons are essential for deciphering the heterogeneity in the literature and understanding how these factors impact intervention efficacy. Particularly, investigating health conditions characterised by dysbiosis, and where a beneficial effect of polyphenols on the gut microbiome has been observed, is of particular research interest as perhaps a greater effect may be seen here. For example, in depression (206).
3. **Research into personalised responses to polyphenol supplementation is needed**

It is becoming increasingly evident that there is considerable inter-individual variability in gut microbiota response to dietary interventions (207). While emerging evidence of the impact of habitual diet has been discussed above, baseline gut microbiota may also influence intervention response (208). However, the evidence base for polyphenol interventions is still limited.

Looking more broadly at dietary intervention studies, a short-term dietary fibre intake intervention in 19 healthy adults led to differing gut microbiome responses among participants. Participants with a higher baseline microbial richness, a characteristic associated with microbiota stability and resilience to change, were less responsive to the dietary fibre intervention (209,210). Further, several studies have observed an association between lower baseline *Bifidobacterium* concentrations and more significant increases in *Bifidobacterium* concentration in response to intervention (211–215). Furthermore, responses to fibre interventions based on measurement of SCFA production have revealed responder and non-responder baseline microbial phenotypes (216,217). Perhaps one day it may be possible in research to stratify participants according to baseline gut microbiome, though current sequencing timeframes and costs are significant barriers currently preventing the incorporation of this type of baseline assessment. Participants could be selected or stratified with the aim of including a range of microbiome compositions at baseline to capture the breadth of possible responses to intervention. Alternatively, only those with a certain baseline microbiome type could be selected, for example by the dominance of particular bacterial groups (e.g. *Prevotella*-dominant) to increase statistical power and investigate how interventions depend on the presence of particular microbes or microbiome characteristics (63). Lastly, individuals with a specific bacterial group expected to be involved in the intervention’s mechanism of action could be targeted (63,171). For example, when studying a particular prebiotic, researchers might want to select a cohort of individuals that harbour the specific microbe expected to be enriched by that prebiotic.

To summarise, it is becoming apparent how challenging it is to predict how the gut microbiome will respond to a dietary intervention. Thus, gaining insight into the factors that influence gut microbiota responsiveness to intervention will be essential to determine the true efficacy of dietary interventions, so these can be controlled for, i.e. ensuring that gut microbiota responds consistently to prescribed dietary interventions, irrespective of factors such as baseline gut microbiome or habitual diet. This may help to improve the success of nutritional interventions and consequently improve human health outcomes (218).
4.7 Conclusion

In this study, supplementation with a flavonoid-rich blackcurrant beverage did not significantly impact gut microbiome composition or functional potential among a cohort of healthy women. However, interestingly, the placebo beverage elicited a significant reduction in the abundance of the probiotic *Bifidobacterium* genus and related family (*Bifidobacteriaceae*), phylum (*Actinobacteria*) and metabolic pathway (*Bifidobacterium* P124-PWY) in participant gut microbiomes. This suggests that, using the placebo beverage as a control, which shared similar constituents with the active beverage except for the bioactive ingredients, may have potentially masked effects of the active intervention. Furthermore, the study’s exploration of the effect of baseline diet quality on gut microbiome response to the BB intervention revealed a small, but significant effect, with a single *Clostridium* species AM22-11AC increasing in relative abundance in the suboptimal diet group. The absence of significant findings with the active intervention contrasts to the existing evidence base, which is mostly in animals, but suggests a favourable shift in gut microbiome composition with blackcurrant supplementation. Thus, these study outcomes add uncertainty to the evidence base, underscoring the complexities of blackcurrant supplementation and its effect on the gut microbiome. Further research is needed to elucidate the potential effects of blackcurrants on gut microbiome health. In particular, high-quality RCTs are required in diverse human populations, involving within-study comparisons of potential efficacy factors such as dose, duration, participant age and health condition. Additionally, they should integrate high-throughput sequencing with multi-omics technologies and functional assays to comprehensively assess functional outputs.
References


142. Terra [Internet]. [cited 2024 Apr 10]. Available from: https://app.terra.bio

143. bioBakery KneadData: Quality control tool on metagenomic and metatranscriptomic sequencing data, especially data from microbiome experiments [Internet]. [cited 2024 Feb 26]. Available from: https://github.com/biobakery/kneaddata


149. Rosenblat M, Volkova N, Attias J, Mahamid R, Aviram M. Consumption of polyphenolic-rich beverages (mostly pomegranate and black currant juices) by healthy subjects for a


Appendices

Appendix A: Participant Information Sheet (PIS)

PARTICIPANT INFORMATION SHEET

Polyphenol-rich drink for gut and brain health (LINK Study)

Invitation

You are invited to take part in this study which aims to understand the effects of a blackcurrant-based beverage on markers of the gut-brain axis. As a volunteer, it is important for you to understand why we are doing this research, and to understand what will be involved if you decide to participate.

This participant information sheet will help you decide if you would like to take part. It describes 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. Please take time to read this information sheet carefully, and contact us if anything is not clear or you would like further information. Please also talk to other people like family/whānau, friends, or healthcare providers to help you decide whether you would like to take part in the study. You will have the opportunity discuss the information presented here with the study team, who can answer any questions you might have.

If you agree to take part in this study, you will be asked to find the Consent Form attached at the end of this document. You will be given a copy of this information sheet and the signed consent forms.

What Is the LINK study?

The gut microbiome (gut bacteria) plays an important role in many aspects of our health, particularly our mood and mental wellbeing. We are carrying out the LINK study to find out whether a blackcurrant-based beverage (Ārepa) can provide benefits to markers of the gut-brain axis, including the gut microbiome, neurocognitive responses (cognition, mood, sleep), and related markers in the blood. We will also explore whether baseline diet or the gut microbiome mediates the effects of the blackcurrant drink on study outcomes.

The LINK study will recruit 40 healthy female adults to participate in a 3-month long intervention. The study period includes four weeks on one arm of the intervention (Ārepa performance beverage or placebo beverage), four weeks of a ‘wash-out’ period, and then four weeks on the second arm of the intervention. The order of intervention will be randomised, and there will be 5 study visits in total. The first will be a screening visit and take approximately 1-hour, the following four will be study visits conducted in the morning which take approximately 45-minutes. We will be collecting blood and stool (poop) samples, and you will also complete questionnaires and cognitive tasks during the study.

What is the purpose of the LINK study?

There is an increasing number of people with functional gastrointestinal disorders, like irritable bowel syndrome. These conditions are thought to be driven by altered communication along the gut-brain axis. Strategies which improve microbiota-gut-brain interactions are thought to be important in optimising gastrointestinal health and preventing the onset of functional gastrointestinal disorders. Translational to practical solutions are still needed though.
Several dietary components might have positive or negative impacts on aspects of microbiota-gut-brain interactions. The New Zealand blackcurrant cultivar Black Adder is rich in anthocyanins, a class of plant-based compounds called polyphenols. Previous data in animals and humans has shown a prebiotic effect of anthocyanins, leading to the growth of health-promoting bacterial species in the gut. Supplementation with the Black Adder cultivar has also been shown to favourably impact brain physiology and behaviour. Integrative studies which simultaneously measure the microbiota, neurocognitive responses, and related blood biomarkers are needed to better understand the potential of polyphenols.

The Ārepa beverage is rich in anthocyanins and other plant-based ingredients known to provide neurocognitive benefits (L-theanine, pine-bark extract). We hypothesize that this unique formula will enhance microbiota-gut-brain interactions through dual effects on the gut microbiota and neurocognitive responses.

**Who are the researchers?**

This study involves a team of researchers from the University of Auckland (Dr Nicola Gillies, Dr Tommi Vatanen, Dr Andrea Braakhuis), the University of Otago (Prof Nicole Roy) and Swinburne University of Technology (Prof Andrew Scholey). These are the researchers who have designed this study. Dr Nicola Gillies is the Principal Investigator and will be managing the study. The research team includes registered dietitians, neuroscientists, and specialists in research on the gut microbiota.

**Who is funding the study?**

The LINK study is funded by grants from the Ministry of Business and Innovation (MBIE) and AlphaGen Ltd through the National Science Challenges – High Value Nutrition [www.highvaluenutrition.co.nz](http://www.highvaluenutrition.co.nz).

**Who can participate in the LINK study?**

This study may be suitable for you if you are a healthy female, and aged between 18-45 years at the time of enrolment.

We cannot include people who have taken antibiotics in the four weeks prior to the study starting, or who are diagnosed with gastrointestinal disorders (e.g., Coeliac Disease, Inflammatory Bowel Disease), who have been treated for anxiety, depression or psychiatric disorders within the past two years, or who have a history of neurological disorders (e.g., Epilepsy, serious head trauma) or cognitive impairment. This is because these conditions might impact our study outcomes.

If you are taking prebiotic, probiotic or other supplements which might impact our study outcomes we ask that you stop taking these four weeks prior to the study starting and for the duration of the study period. You can ask us if you’re sure about which supplements might be relevant here.

No one has to take part in the LINK study, and it is completely up to you to decide whether or not to take part in the study. If you do decide to enrol in the study, you can withdraw at any time without giving a reason.
What will my participation in the LINK study involve?

Online screening: If you are interested in participating, you will complete an online screening questionnaire to make sure that you meet the criteria for taking part. If you are eligible according to this questionnaire, you will be invited to attend a screening visit at the Grafton Clinical Research Centre (University of Auckland).

Screening visit: If you agree to attend this study visit, researchers will review this participant information sheet with you in person, explaining the study in detail and answering any questions you may ask about participation. If you are satisfied and agree to take part, we will ask you to sign the consent form which can be found at the end of this document. At this point you will be officially enrolled into the LINK study.

After enrolment, you will be familiarised with the computerised multi-tasking programme we use in the study to trigger cognitive stress. You will also complete some questionnaires at this visit, which are repeated online during the study. These questionnaires will give us information on your mood, sleep quality, dietary intake, and physical activity. At the end, you will be given instructions and materials for stool (poo) sample collection which allows us to measure your gut bacteria. This screening visit will take around 1 hour in total.

Study period: Approximately two weeks after your first study visit you will start the study, which takes three months in total. This involves four weeks on the first study beverage ("arm 1") where you will consume one 300ml beverage daily, four weeks in a ‘wash-out’ period where you do not consume any study beverage, and then four weeks on the second study beverage ("arm 2") where you will consume the other 300ml beverage daily. You will be supplied with all the beverages needed for your participation in this study at no cost.

This trial is ‘blinded’, which means that neither you nor the researchers will know whether you are having the study treatment (Arepas) or the taste- and colour-matched placebo beverage. This information is only known to someone unconnected with the trial. The order in which you consume the study beverages will be randomly assigned after you are enrolled in the study. This study design helps to make sure that the researchers interpret the results in a fair and appropriate way, and avoids researchers or participants jumping to conclusions.

Both beverages are manufactured in a registered facility that complies with Food Standards Australia and New Zealand guidelines, with respect to manufacturing standards and compliance with food safety requirements.

Data collection: There are 4 visits during the study period, where data will be collected in person. These happen at the start and end of each intervention arm (i.e., every four weeks), and are expected to take around 45 minutes each. Each study visit will have similar testing procedures. All data will be collected at the Clinical Research Centre in Grafton, Auckland. You will be asked to avoid alcohol the day before your study visit, caffeine in the 12h prior to your appointment, and to not eat after 10pm. Except for water, you will fast overnight and attend your scheduled study visit in the morning.
You will provide researchers with a stool (poo) sample which was collected at home, in the 24h before your study visit using the kit and instructions provided at previous study visits.

Height and weight will be taken, and blood samples will then be collected. You will be asked to rest on a bed or chair, and a small needle will be placed into your arm vein. This can be slightly painful, and can cause discomfort. The researcher will then take approximately 15mL of blood which will be used to measure inflammatory markers (circulating cytokines), neurocognitive markers (monoamine oxidase B, brain derived neurotropic factor), and amino acids and their metabolites (serum tryptophan, kynurenine).

You will then complete questionnaires which evaluate your mood in the present moment. After this, you will complete the cognitive stressor which takes approximately 20 minutes. Finally, you will repeat the questionnaires on your mood. This allows us to measure your mood under conditions of stress or no stress.

You will complete online questionnaires at fortnightly intervals during the study which provide information on your diet, mood, sleep, and physical activity. These will take approximately 15 minutes of your time or less. At weekly intervals you will need to confirm that you have consumed the study beverage through a quick online questionnaire.
What will happen to my blood and stool samples?

**Blood samples:** Your blood will be used to analyse inflammatory markers, neurocognitive markers, and amino acids and their metabolites. These markers are related to the gut and/or brain and provide insights into whether there are differences in the response to the intervention or placebo beverage, and how the intervention might be achieving these outcomes.

Blood samples will be collected, prepared, and stored at the Nutrition Department Laboratory (Faculty of Medical and Health Sciences, University of Auckland). Analysis of blood samples will take place at the Liggins Institute Laboratory (University of Auckland) or Plant and Food Research (Palmerston North, New Zealand). After these analyses have been performed, it will not be possible to return any unused samples to you. You can request the return of your blood prior to any analysis; this would mean we would not use your information in the study.

**Stool samples:** We will measure changes in gut microbiota (through identifying and classifying the types of gut bacteria present), microbiota diversity, and the predictive function of your gut bacteria (through measuring the genetic material of the gut bacteria) from your stool samples. These samples will be processed at the University of Auckland. A small sample will be sent to an overseas laboratory (Beijing, China), frozen on dry ice, for expert analysis of gut bacteria that cannot take place in New Zealand.

**Transport and storage:** All blood and stool samples will be transported locally, nationally, and internationally according to international guidelines for the transport of human tissue. All samples will be labelled with your LINK study ID number and not your name, to maintain confidentiality.

Iwi, hapu, and whānau might disagree with transport of tissue samples due to issues with the loss of rights to your whakapapa. It is acknowledged that individuals have the right to choose, and these concerns might also apply to non-Māori. We encourage you to consult with your whānau before agreeing to participate. You can also have a support person contact us or attend your screening visit if you have any questions.

All samples will be labelled with your LINK study ID and will be stored in secure freezers in an access-restricted area at the University of Auckland until analysis. Any unused samples collected in this study will be kept for a total of 10 years. At the end of this time, a medical waste contractor will dispose of your tissue. If you would like a karakia said at this time, please indicate so in the consent portion of this form. Any samples for disposal by karakia will be clearly marked. It is possible that the entire sample will be used for analysis, in that case there will be no need for disposal and a karakia will not be possible. Karakia ceremonies take place through the Auckland District Health Board. *Stored tissue will not be used for any future unspecified research purposes.*

**Detection of abnormalities:** We will advise you of any abnormal test results found as part of the study which might have implications for your future health, including results from questionnaires, blood samples, and stool samples. These findings will be provided to you, along with a letter for your doctor. We will contact your doctor or an appropriate specialist if you agree.

If you do not wish to be informed of any results indicating a possible medical concern, you cannot participate in this study.
What are the benefits or risks of taking part in the LINK study?

**Risks:** There are no major risks associated with taking part in this study. You do need to be aware that this study will involve collection of a blood sample. This will be performed by an experienced researcher. A blood sample may hurt a little, and some people get a small bruise where the needle goes in. Occasionally, this can become infected but this is very rare and most people have no problems. If you ever faint with blood samples or when you see blood, please let the researchers know beforehand. That way, we can be prepared for this and take the sample while you are lying down.

There is minimal risk in other procedures associated with the trial, but acknowledge that the multi-tasking activity and questionnaires can bring about disturbances in mood which people might find uncomfortable. Psychological assessments will be checked weekly by the research team. If any questionnaires raise concerns, these will be reviewed by a neuroscientist in the research team, who may suggest referral to counselling services if needed with your permission.

Researchers will check in on your safety regularly through the trial. Any adverse events (e.g., reaction to the beverages such as gastrointestinal upset) or serious adverse events that emerge or worsen relative to your usual state will be recorded and reported to the Health and Disability Ethics Committee.

**Benefits:** There are many benefits to being involved in this research. You will have several assessments which are not usually available through standard care, receive information about how you respond to an experimental stressor, and receive information about your gut microbiota. You will be acknowledged in publications (anonymously), and be provided with the study findings. Your involvement in this study is of great value to the researchers and will help to advance understanding on how dietary components affect the gut-brain axis, thank you for considering taking part.

How will my confidentiality be protected?

Your confidentiality and protection of personal information will be treated very seriously, and individual results from this study will be kept strictly confidential. On entering the study, you will be given a unique study identification number/ID, which will be used on all forms, questionnaires, measurements, and blood/stool samples. Researchers will remove any personal information provided by you, and there is no risk that you will be able to be identified if samples are sent nationally or overseas. It is important to note that privacy protections in other countries may be different to those offered in New Zealand, but using your study ID will protect your identity. Researchers will analyse the whole study group's data and report on averages in any scientific publications and presentations and no person will be identifiable.

Any hard-copy documents, including paper copies of questionnaires, data collection forms or measurements will be stored in a locked filing cabinet in a secure swipe-access area at the University of Auckland, where only the research team has access. Any information on electronic files will only be accessible by the research team. The LINK study researchers responsible for data collection and management have been trained by an independent committee at the Greenlane Coordinating Centre (GLCC), who ensure the study is carried out according to guidelines for Good Clinical Practice.
What happens if I suffer harm, injury, or complications because of the study?

It is unlikely that you will suffer any harm or complications because of this study. If you were injured as a result of treatment given as part of this study, you won't be eligible for compensation from ACC. However, Dr Nicola Gillies has satisfied the XXX Health and Disability Ethics Committee that approved this study that it has up-to-date insurance for providing participants with compensation if they are injured as a result of taking part in the study.

New Zealand ethical standards require compensation for injury to be at least ACC equivalent. Compensation should be appropriate to the nature, severity and persistence of your injury and should be no less than would be awarded for similar injuries by New Zealand's ACC scheme. Some sponsors voluntarily commit to providing compensation in accordance with guidelines that they have agreed between themselves, called the Medicines New Zealand Guidelines (Industry Guidelines). These are often referred to for information on compensation for commercial clinical trials. There are some important points to know about the Industry Guidelines:

- On their own they are not legally enforceable and may not provide ACC equivalent compensation.
- There are limitations on when compensation is available, for example compensation may be available for more serious, enduring injuries, and not for temporary pain or discomfort or less serious or curable complaints.
- Unlike ACC, the guidelines do not provide compensation on a no-fault basis. The Sponsor may not accept the compensation claim if:
  - Your injury was caused by the investigators, or;
  - There was a deviation from the proposed research plan, or;
  - Your injury was caused solely by you.

An initial decision whether to compensate you would be made by the sponsor and/or its insurers. If they decide not to compensate you, you may be able to take action through the Courts but it could be expensive and lengthy, and you might require legal representation. You would need to be able to show that your injury was caused by participation in the trial. You are strongly advised to read the Industry Guidelines and ask questions if you are unsure about what they mean for you.

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.

You may have your friend, family, or whānau support help you understand the risks and/or benefits of this study or any other explanation you require. You are also welcome to have a friend or whānau support with you during every session.
What will happen to my information?

All data will be stored electronically in password-protected files on a secure network drive managed by the University of Auckland. With your consent, your study records will be stored securely for 10 years after the study is completed and then destroyed. Your blood and stool samples will also be stored for 10 years after the study ends, which will be stored securely in the Nutrition Department freezers at the University of Auckland. If you decide to withdraw from the study, you may request that your samples are disposed of.

What happens with the results of the study?

If you give us your permission by signing the Consent Form, findings from the study will be used in internal reports, scientific/professional conference presentations, and scientific journal. The findings may also be featured in the media. You will not be identified in any presentations or publications.

At the end of the study, we will provide you with a summary of results from the study. Please note that there may be a delay between your study visit and publication of the results.

What happens if I change my mind?

You have the right to withdraw from the study at any time. Your contribution is entirely voluntary. If you decide to withdraw from the study, data that has already been obtained may be kept and used to contribute to the overall results. However, you can request that any data or information relating to you can be destroyed and we will ensure that this happens.

Will taking part in the LINK study cost me anything, and will I be reimbursed?

You will not incur any costs for taking part in this study except for your time, for which we thank you. All study treatments and data collection will be paid for by the study funders.

We appreciate that taking part in this study involves 5 visits to our research centre, and approximately 4-5h of your time. All participants will receive a kōhā (gift) in the form of vouchers as an expression of thanks for dedicating time to this research. Vouchers will be provided at completion of each arm of the intervention (study visits 3 and 5). When visiting the Grafton Campus for research visits reserved parking will be arranged for you by the study team. Please let the study team know if there is a problem getting to your appointment, as arrangements can be made.
Who do I contact for more information or if I have concerns?

Once you have read this information, a member of the study team will discuss it with you and answer any questions you may have.

If you have any questions, concerns, or complaints about the study at any stage you can contact:

Dr Nicola Gillies, Principal Investigator  
Discipline of Nutrition,  
Faculty of Medical and Health Sciences  
The University of Auckland, New Zealand  
Email: n.gillies@auckland.ac.nz

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 cultural support, talk to your whānau in the first instance. Alternatively, you may contact the administrator for He Kameka Weiora (Māori Health Team) by telephoning 09 486 8324 ext 2324

You may also contact the health and disability ethics committee (HDEC) that has approved this study:

Phone: 0800 4 ETHICS  
Email: hdec@moh.govt.nz

APPROVED BY THE HEALTHY AND DISABILITY ETHICS COMMITTEE ON 27/05/2022, Reference Number (2022 EXP 12513)
Appendix B: Participant consent form

CONSENT FORM

THIS FORM WILL BE HELD FOR A PERIOD OF 10 YEARS

Polyphenol-rich drink for gut and brain (LINK Study)

Principal Investigator: Dr Nicola Gillies
Co-Investigators: Dr Tommi Vatanen, Prof Nicole Roy, Prof Andrew Scholey, Dr Andrea Braakhuis

The investigators conducting this research abide by the principles governing the ethical conduct of research as set out by the World Medical Association, Declaration of Helsinki (2008) and the National Health and Medical Research Council’s National Statement on Ethical Conduct in Human Research (2007) and at all times vow to protect the interests, comfort, and safety of all subjects. This form and the accompanying participant information sheet have been given to you for your own protection.

As a participant, please tick to indicate consent to the following:

<table>
<thead>
<tr>
<th>Statement</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>I have read the Participant Information Sheet, have understood the nature of this research and why I have been selected. I have had the opportunity to ask questions and have them answered to my satisfaction.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I agree to take part in this study, and have been given sufficient time to consider whether or not to participate.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I have had the opportunity to use support from whānau/family or a friend to help me ask questions and understand the study.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>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 (please circle)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>I understand that blood and stool samples will be collected and used for research.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I wish for a karakia to be said at the time of my tissue disposal (please circle)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>I understand that any test results found to be outside the normal healthy range will be conveyed to me, and that if I do not wish to be informed I cannot participate in this study.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I consent to my GP or current provider being informed about any significant abnormal results obtained during the study, with my permission (please circle)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>I agree to my stool samples being sent to an overseas laboratory (Beijing, China) and I am aware that these samples will be disposed of using established guidelines for discarding biohazard waste.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>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.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
I understand my responsibilities as a study participant, and know who to contact if I have any questions about the study in general.

I wish to receive a summary of the results from the study *(please circle)*

| Yes | No |

I understand that the results from this study will be used for scientific publication and presentations.

I agree not to restrict the use of any data or results that arise from this research, provided that such a use is only for scientific purposes.

**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:**

**APPROVED BY THE HEALTH AND DISABILITY ETHICS COMMITTEE ON 27/05/2022, Reference Number 2022 EXP 12513**
Appendix C: Dietary Screening Tool (DST)

<table>
<thead>
<tr>
<th>Dietary Screening Tool</th>
<th>Options</th>
</tr>
</thead>
<tbody>
<tr>
<td>How often do you usually eat wholegrain breads or crackers?</td>
<td>Never OR less than once a week, 1 or 2 times a week, 3 or more times a week</td>
</tr>
<tr>
<td>How often do you usually eat wholegrain cereals? (e.g. Weetbix, porridge, muesli)</td>
<td>Never OR less than once a week, 1 or 2 times a week, 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat beef or lamb (including mince)?</td>
<td>Never or once a week or less, 2-3 times a week, 4-5 times a week, More than 5 times per week</td>
</tr>
<tr>
<td>How often do you eat fish or seafood that IS NOT fried (including tinned)?</td>
<td>Never, Less than once a week, Once a week, More than once per week</td>
</tr>
<tr>
<td>How many servings of LOW FAT milk, cheese, or yoghurt do you usually have each DAY?</td>
<td>None, Once, Two or more</td>
</tr>
<tr>
<td>How many different vegetable servings do you usually have at your main meal of the day?</td>
<td>None, One, Two or more</td>
</tr>
<tr>
<td>How often do you eat carrots, sweet potatoes, or pumpkin?</td>
<td>Never, Less than once a week, 1 or 2 times a week, 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat rocket, spinach, or silverbeet?</td>
<td>Never, Less than once a week, 1 or 2 times a week, 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat broccoll or cauliflower?</td>
<td>Never, Less than once a week, 1 or 2 times a week, 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat fruit (not including juice)? Please include fresh, canned, or frozen fruit</td>
<td>Never or less than once a week, 1 or 2 times a week, 3-5 times a week, Every day or almost every day</td>
</tr>
<tr>
<td>Question</td>
<td>Options</td>
</tr>
<tr>
<td>-------------------------------------------------------------------------</td>
<td>----------------------------------------------</td>
</tr>
<tr>
<td>How often do you consume olive oil?</td>
<td>- Never</td>
</tr>
<tr>
<td></td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 to 5 times a week</td>
</tr>
<tr>
<td></td>
<td>- Every day or almost every day</td>
</tr>
<tr>
<td>How often do you consume legumes, such as lentils or chickpeas?</td>
<td>- Never</td>
</tr>
<tr>
<td></td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 to 5 times a week</td>
</tr>
<tr>
<td></td>
<td>- Every day or almost every day</td>
</tr>
<tr>
<td>How often do you consume fresh nuts, such as almonds, cashews, walnuts,</td>
<td>- Never</td>
</tr>
<tr>
<td>or brazil nuts?</td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 to 5 times a week</td>
</tr>
<tr>
<td></td>
<td>- Every day or almost every day</td>
</tr>
<tr>
<td>How often do you drink (non-diet) soft drinks or cordials?</td>
<td>- Never</td>
</tr>
<tr>
<td></td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 or more times a week</td>
</tr>
<tr>
<td>How often do you usually eat sweets or chocolate?</td>
<td>- Never</td>
</tr>
<tr>
<td></td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 or more times a week</td>
</tr>
<tr>
<td>How often do you usually eat chips, twisties, or something similar?</td>
<td>- Never</td>
</tr>
<tr>
<td></td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat pies, sausage rolls, or hot chips?</td>
<td>- Never</td>
</tr>
<tr>
<td></td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat cakes, biscuits, ice creams, or doughnuts?</td>
<td>- Never</td>
</tr>
<tr>
<td></td>
<td>- Less than once a week</td>
</tr>
<tr>
<td></td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat lunchmeats or deli meats (e.g. ham, bacon, or</td>
<td>- Never or less than once a week</td>
</tr>
<tr>
<td>sausage)?</td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 or more times a week</td>
</tr>
<tr>
<td>How often do you eat takeaway meals, such as McDonalds, KFC, Pizza</td>
<td>- Never or less than once a week</td>
</tr>
<tr>
<td>Hut, or Burger King?</td>
<td>- 1 or 2 times a week</td>
</tr>
<tr>
<td></td>
<td>- 3 or more times a week</td>
</tr>
</tbody>
</table>
## Appendix D: Intervention beverage ingredients

### Table D1. Ingredients in active (BB) and control beverages.

<table>
<thead>
<tr>
<th>Blackcurrant beverage, BB</th>
<th>Control beverage</th>
</tr>
</thead>
<tbody>
<tr>
<td>New Zealand Neuroberry®, Blackcurrant Juice, Ultra-filtered Water, New Zealand Apple Juice, Erythritol (Natural Sweetener), Natural Flavours, New Zealand Blackcurrant Extract, L-theanine Suntheanine®, New Zealand Pine Bark Extract Enzogenol®, Natural Acidity Regulator (Citric), Stevia (Natural Sweetener)</td>
<td>New Zealand Apple Juice, Ultra-Purified Water, Erythritol (Natural Sweetener), Natural Flavours, Elderberry Juice Concentrate, Natural Colours, Artificial Flavours, Natural Acidity Regulator (Citric), Ascorbic Acid, Stevia (Natural Sweetener)</td>
</tr>
</tbody>
</table>
Appendix E: Comparison of study standard replicates and reference values

Figure E1. Mean microbial species composition of three replicates of the 21-strain ZymoBIOMICS® Gut Microbiome Standard (#D6331) utilised in this study, as compared to the theoretical reference values (219). In this figure, the category ‘Escherichia coli’ encompasses five strains: JM109, B-3008, B-2207, B-766, B-1109.
Appendix F: Sequencing read counts from quality control steps

Figure F1. Total number of reads before (‘raw’), during (‘trimmed’ and ‘human removal’), and after (‘final’) quality control filtering with KneadData.