Effects of Defatted Rice Bran Fortified Bread on Gut Microbiota Composition of Healthy Adults with Low Dietary Fibre Intake: Protocol for a Crossover Randomised Controlled Trial

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[1]Department of Medicine, University of Otago, Christchurch Christchurch NZ
[2]High-Value Nutrition National Science Challenge Auckland NZ
[3]Riddet Institute, Massey University Palmerston North NZ
[4]AgResearch Grasslands Palmerston North NZ
[5]Edible Research Christchurch NZ
[6]Plant and Food Research, Ruakura Research Centre Hamilton NZ
[7]Biostatistics and Computational Biology Unit, University of Otago, Christchurch Christchurch NZ
[8]Department of Human Nutrition, University of Otago Dunedin NZ
[*]these authors contributed equally

Corresponding Author:
Nicole Clemence Roy PhD
Department of Human Nutrition, University of Otago
362 Leith Street
Dunedin North
Dunedin
NZ

Abstract

Background: Inadequate dietary fiber (DF) intake is associated with several human diseases. Bread is commonly consumed, and can increase its DF content by incorporating defatted rice bran (DRB).

Objective: This first human study on DRB fortified bread, primarily aims to assess the effect of DRB bread on the relative abundance of a composite of key microbial genera and species in stool samples. Secondary outcomes include clinical (cardiovascular risk profile), patient-reported (daily bread consumption and bowel movement, digestive comfort, general well-being, total DF intake), biological (stool microbiota gene abundances, stool and plasma metabolites). Exploratory outcomes include physiome (whole gut and regional transit time, and gas fermentation profiles) outcomes in healthy adults with low DF intake.

Methods: The BREAD (Bread Related Effects on Microbial Distribution) study is a two-armed, placebo-controlled, double-blind, randomized, crossover study. The study duration is fourteen weeks: two weeks lead-in, four weeks intervention per phase, two weeks washout, and two weeks follow-up. Sixty healthy adults with low DF intake (<18g/day (females), <22g/day (males)) were recruited in Christchurch, New Zealand between June and December 2022. Randomized participants consumed three (for females)/ four (for males) slices of fortified bread per day, then placebo white bread and vice versa. The DRB fortified bread provided 8 g (for females) and 10.6 g (for males) of total DF, while the placebo (a matched commercial white toast bread) provided 2.7 g for females and 3.6 g for males of total DF. Before and after each intervention phase, participants provided stool and blood samples to assess biological responses; completed a three-day food diary to assess usual intakes; online questionnaires to assess gut symptoms, general and mental well-being, daily bread intake and bowel movement via an app; underwent anthropometry and blood pressure measurements; drank blue food dye to assess whole gut transit time. In addition, 15 participants from the cohort ingested Atmo gas-sensing capsules to assess gut regional's fermentation gas profile.

Results: At the time of writing, data was still being analysed. Results will be published as separate manuscripts.

Conclusions: This study will offer insights into the prospect of consuming DRB fortified bread to effectively modulate health-

promoting gut microbes and their metabolism, and DF intake in healthy adults with low DF intake. Clinical Trial: ACTRN12622000884707

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[1] Department of Medicine, University of Otago, Christchurch, New Zealand
[2] High-Value Nutrition National Science Challenge, Auckland, New Zealand
[3] Riddet Institute, Massey University, Palmerston North, New Zealand
[4] AgResearch Grasslands, Palmerston North, New Zealand
[5] Edible Research, Christchurch, New Zealand
[6] Plant and Food Research, Ruakura Research Centre, Hamilton, New Zealand
[7] Biostatistics and Computational Biology Unit, University of Otago, Christchurch, New Zealand
[8] Department of Human Nutrition, University of Otago, Dunedin, New Zealand
[†] These authors contributed equally to this work and share first authorship

[*] Corresponding Author:
Nicole Clemence Roy, PhD
Department of Human Nutrition
University of Otago
362 Leith Street, Dunedin North
Dunedin 9016
New Zealand
Phone: +64 3 479 7945
Email: nicole.roy@otago.ac.nz

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[7] Biostatistics and Computational Biology Unit, University of Otago, Christchurch, New Zealand
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[*] Correspondence: nicole.roy@otago.ac.nz
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Abstract

Background: Inadequate dietary fiber (DF) intake is associated with several human diseases. Bread is commonly consumed, and can increase its DF content by incorporating defatted rice bran (DRB).
Objective: This first human study on DRB-fortified bread, primarily aims to assess the effect of DRB bread on the relative abundance of a composite of key microbial genera and species in faecal samples. Secondary outcomes include clinical (cardiovascular risk profile), patient-reported (daily bread consumption and bowel movement, digestive comfort, general well-being, total DF intake), biological (faecal microbiota gene abundances, faecal and plasma metabolites), physiome (whole gut and regional transit time, and gas fermentation profiles) in healthy adults with low DF intake.
Methods: The BREAD (Bread Related Effects on MicrobiAl Distribution) study is a two-armed, placebo-controlled, double-blind, randomised, crossover study. The study duration is fourteen weeks: two weeks lead-in, four weeks intervention per phase, two weeks washout, and two weeks follow-up. Sixty healthy adults with low DF intake (<18g/day (females), <22g/day (males)) were recruited in Christchurch, New Zealand between June and December 2022. Randomised participants consumed three (for females)/ four (for males) slices of DRB bread per day, then placebo white bread and vice versa. The DRB-fortified bread provided 8 g (for females) and 10.6 g (for males) of total DF, while the placebo (a matched commercial white toast bread) provided 2.7 g for females and 3.6 g for males of total DF. Before and after each intervention phase, participants provided faecal and blood samples to assess biological responses; completed a three-day food diary to assess usual intakes; online questionnaires to assess gut symptoms, general and mental well-being, daily bread intake and bowel movement via an app; underwent anthropometry and blood pressure measurements; drank blue food dye to assess whole gut transit time. In addition, 15 participants from the cohort ingested Atmo gas-sensing capsules to assess colonic gas fermentation profile, and whole and regional gut transit time.
Results: Recruitment of participants began in June 2022 and was completed in December 2022. Preliminary analysis included a total of 56 participants (n=33 females, 58.9%; n=23 males, 41.1%; New Zealand European n=41, 73.2%, Māori n=1, 1.8%). Due to the large dataset, data analysis for all outcomes was planned to be completed by the last quarter of 2024, with full results expected to be published in peer-reviewed journals by the end of 2024.
Conclusions: This first human study offers insights into the prospect of consuming DRB fortified

bread to effectively modulate health-promoting gut microbes and their metabolism, and DF intake in healthy adults with low DF intake.

Trial Registration: ACTRN12622000884707 https://www.anzctr.org.au/Trial/Registration/TrialReview.aspx?id=383814&isReview=true

Keywords: dietary fibre; defatted rice bran; bread; healthy adults; gut microbiota; metabolites; gut physiome; randomised controlled trial

Introduction

Diet strongly influences the gut microbiota composition [1-3]. Diets low in dietary fibres (DF) may contribute to gut dysbiosis, where specific bacterial taxa and microbial diversity are diminished [1, 2]. This phenomenon mostly occurs in industrialised populations consuming a low DF-high fat and protein diet [3]. However, alteration of the gut microbiota profile may be reversible by improving DF intake [2].

Dietary fibres are carbohydrates that are neither digestible nor absorbable in the small intestine. Some DF are fermented by gut microbes in the large intestine, which produce metabolites such as organic acids [4]. These metabolites, in particular the short chain fatty acids acetate, butyrate, and propionate, play major beneficial physiological and immunological roles for the human host [4]. They modulate the luminal pH and immune system, lower pathogenic bacteria load, act as a catalyst for calcium and magnesium absorption, and provide energy sources for colonic cells [4]. Therefore, increasing DF intake may provide the required substrates for microbial growth and energy, ultimately benefiting the human host's health [2].

Dietary fibres are found in a wide range of plant foods, yet inadequate DF intake is still a ubiquitous issue in the adult population worldwide [5-12]. Fortification of commonly consumed foods with synthesised or extracted DF may improve the DF intake of adult populations [2]. Bread is the main source of DF worldwide [6, 10, 12, 13] and is one of the oldest foods that offer health benefits to humans [13]. Bread is commonly consumed by all cultures and ethnicities, and is a staple food for many individuals. Further, bread is ideal for incorporating ingredients such as cereal brans to increase DF content [14, 15], which may modulate the gut microbiota.

Rice bran is a cheap cereal by-product of rice milling [14]. Rice bran has a purported hypo-allergenicity, and has a unique nutrient profile rich in DF, protein, antioxidants, and minerals as well as phytochemicals [13, 16-18]. Only a few human studies have been undertaken on rice bran [19-36]. These studies have focused on the use of modified arabinoxylan (a phytochemical) rice bran on subjective global assessments in participants with predominant-diarrhoea or mixed type irritable bowel syndrome [19], heat stabilised rice bran on modulation of the gut microbiota composition [20, 22] and metabolites (short chain fatty acids and bile acids) [20, 22] or rice bran fermented with *Lentinus edodes* on natural killer cell activity and cytokine production [30] in healthy adults, rice bran intervention in colorectal cancer survivors [21], individuals with a high risk of colorectal cancer [23] or cardiovascular diseases [24, 26-28, 31-36], or hydrolysed rice bran in patients with cervical cancer [29].

Among these published rice bran studies, only four human adult studies reported the impact of rice bran on the gut microbiota. When compared to baseline values, a daily consumption of 30 g of heat stabilised rice bran over two weeks increased the relative abundance of the genera *Methanobrevibacter, Paraprevotella, Ruminococcus, Dialister, Barnesiella, Anaerostipes*, and at four weeks, increased the genera *Bifidobacterium* and *Clostridium* [20], while 40 g over 24 weeks increased the taxa from the family *Veillonellaceae* in healthy adults [22]. Similarly, in a population at high risk of colorectal cancer, a 24-week intervention of 30 g rice bran increased the relative abundance of Firmicutes phylum, *Lactobacillus* genus, *Bifidobacterium* genus, and *Prevotella_9* species [23], compared to baseline levels. However, other measures of the microbial community based on the Firmicutes: Bacteroidetes ratio and alpha-diversity showed mixed effects. Heat stabilised rice bran reduced the Firmicutes: Bacteroidetes ratio at two weeks, with no difference at four weeks compared to baseline [21], yet an increased ratio following a 24-week rice bran intervention [23]. Whilst other studies did not report alpha diversity [19, 20, 22], one study found an increased diversity at four weeks, but not in two weeks [21] nor in 24 weeks compared to baseline values [23].

The interventions of these studies were either modified arabinoxylan rice bran or heat stabilised or

normal rice bran. They were administered to participants of varied health status and in various forms (powder, rice-bran enriched meals and snacks, oil) [19-32, 34-37]. However, to the best of our knowledge, no human studies have used defatted rice bran (DRB) in bread as an intervention. The process of defatting increases the insoluble fibre content, thereby increasing its proportion of DF [14]. Several studies have therefore, suggested using DRB as a value-added food ingredient [17, 38-41]. Taken together, bread, which is commonly eaten in adults, fortified with DRB, may help promote beneficial gut microbes and DF intake, ultimately may provide beneficial health effects to the adult population.

Hypotheses and Objectives

This study was based on the hypothesis that the consumption of three (for females)/ four (for males) slices of DRB-fortified bread per day for four weeks will increase the relative abundance of a composite of key genera and species in faecal samples (proxy of lower gut microbiota) known to be involved in plant glycan metabolism and/or known to be modulated by the DRB-fortified bread.
Secondary hypotheses were that consumption of DRB-fortified bread would lead to improvements in clinical, patient reported outcomes, biological measurements, whole gut and regional transit time, and gas fermentation profiles.
DF are complex compounds which require a network of microbes to degrade the complex structures of DF [42, 43]. Further, due to the inconsistent findings on the relative abundance of microbial taxa reported by existing rice bran studies, a microbiome composite index is a useful primary outcome for this study.
The primary aim of the study is to determine the influence of three (for females)/ four (for males) slices of DRB-fortified bread on the composition of selected genera and species of microbiota, which are known to be involved in plant glycan metabolism and/or known to be modulated by DRB-fortified bread intervention using shotgun metagenomics sequencing of faecal samples.
The secondary aims were to investigate the influence of three (for females)/four (for males) slices of DRB-fortified bread in comparison to placebo white bread on:
1. upper and lower gut comfort using validated gut-specific questionnaires
2. mental health and general well-being parameters using validated questionnaires
3. DF intake using three-day food diaries
4. relative abundance of individual taxa, predictive function (gene abundances) and diversity indices of the faecal gut microbiota using shotgun metagenomic sequencing, faecal and plasma metabolites using liquid chromatography mass spectrometry (LCMS)
5. cardiovascular risk profile based on anthropometry, blood pressure, and blood lipid concentrations
6. whole gut transit time using blue food dye in all participants
7. regional and whole gut transit time and gas fermentation profiles using Atmo gas capsules (Atmo Biosciences, Melbourne, Victoria, Australia) in a subset of participants.

Methods

Study Design

The protocol was put together in accordance with the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines for clinical trial protocols [44].

Study Overview

The Bread Related Effect on Microbial Distribution (BREAD) study was a double-blind, randomised, placebo-controlled, crossover trial of healthy adults with low DF intake. The design and management of the clinical study conformed with the CONSORT guidelines [45]. This design accounts for the recognised variability in individuals and enables each participant to be their own

control for their assigned interventions [46, 47]. The study duration was a nominal total of fourteen weeks, consisting of a two-week lead-in phase, two four-week intervention phases, separated by a two-week washout phase, and a final two-week follow-up phase (Table 1). During the first intervention period, one group of participants consumed three slices (for females) and four slices (for males) of DRB-fortified bread daily for four weeks, while the other group consumed placebo bread. Following a two-week washout period, participants who consumed the fortified bread in the first period crossed over to consume placebo bread in the second period and vice versa (**Error: Reference source not found**). Participants were instructed not to alter their diet during the two-week washout period. The washout period was selected based on gut microbiota studies reporting a return of microbiota abundances to baseline level within two weeks post-intervention [48].

Prior to study commencement, a mutually agreed schedule was discussed and set with each participant to ensure they understood all expected clinic/visit times and the study timeline. However, when a participant could not come to the clinic on the day a phase was ending, the participant's needs were accommodated by allowing earlier visits to the clinic for up to three days before the end of the intervention phase (minimum of 25 days of the 28-day intervention). This approach reduces the risk of participants exhausting intervention supplies before the visit and non-compliance or drop-out due to scheduling issues.

Table 1 Overview of the schedule of screening, enrolment, interventions, and assessments

			STUDY PERIOD						
	Screening	**Enrolment**	**Baseline 1**	**Allocation 1**	**Washout 1**	**Baseline 2**	**Allocation 2**	**Washout 2**	**Follow Up**
TIMEPOINT		D-14/ -Wk2	D 0/ Wk1	D 7-28/ Wk2-5	D 29-41/ Wk6-7	D 42/ Wk8	D 49-77/ Wk9-12	D 78-90/ Wk13-14	D 91/ Wk15
Screening									
Eligibility screen	X								
Informed consent	X								
Biochemical blood panel	X								X
Enrolment									
General health and living standard		X							
Allocations			X			X			
Interventions									
DRB, then placebo									
Placebo, then DRB									
Assessments									
Faecal samples [a]			X	X		X	X		X
Blood samples [a]			X	X		X	X		X
Anthropometry [a]	X		X	X		X	X		X
Blood pressure [a]	X		X	X		X	X		X
Atmo capsules [a]			X	X		X	X		
Blue dye [a]			X	X		X	X		
Digestive comfort			X	X		X	X		X
Anxiety and depression symptoms			X	X		X	X		X
Well-being and vitality			X	X		X	X		X
Fatigue symptoms			X	X		X	X		X
Three-day food diary [a]			X	X		X	X		X

Daily bowel movement and bread diaries	X	X	X	X	X	X	X	X

[a] Following enrolment, these were collected and/or conducted during clinic visit days 0 (week 1), 29 (week 5), 42 (week 8), 78 (week12) and 91 (week15).

Ethical Approval

The study was carried out in accordance with the International Conference of Harmonisation Guidelines, national and local requirements, and the Declaration of Helsinki. All participants gave written, informed consent prior to participating in the study. Ethical approval was sought from the University of Otago Human Ethics Committee for Health (H22/061), and consultation was done with the University of Otago Christchurch Māori Research Advisor. Prior to commencement, the study was registered at ANZCTR, registration number ACTRN12622000884707. Recruitment and interventions were conducted at the University of Otago, Christchurch, New Zealand.

Protocol Amendments

Any modifications to the protocol which may impact the conduct of the study, the potential benefit of the patient or may affect patient safety, including changes in study objectives, study design, patient population, sample sizes, study procedures, or significant administrative aspects, required a formal amendment to the protocol. Any amendments to the study protocol were required to report to the University of Otago Human Ethics Committee for Health and all other local approval committees. Changes pertaining to Māori (e.g., recruitment processes, analyses) were required to be reported to the Māori Research Advisor. However, the study had no amendments or changes to our protocol.

Recruitment

Recruitment of participants began in June 2022 and was completed in December 2022. Due to the large dataset, data analysis for all outcomes was planned to be completed by the last quarter of 2024, with full results expected to be published in peer-reviewed journals by the end of 2024.

Volunteers from the public were recruited through study posters, advertisements translated into Te Reo Māori, social media (online presence and Facebook), and word-of-mouth advertisements through Māori health nurses and general practitioners. Interested individuals contacted the research team via email or phone call. They were then given a patient information sheet, including a consent form via email, and individuals were provided with sufficient time to consider participating in the study. Once the individual agreed to participate, an online screening questionnaire was sent to ascertain their eligibility criteria, DF intake, approximate self-reported body mass index (BMI) and health conditions.

Eligibility Criteria

All interested volunteers were assessed for eligibility based on the inclusion and exclusion criteria.

Inclusion Criteria

Participants were deemed eligible according to the following criteria:

- Have good general health
- Aged between 18 and 65
- Have a BMI between 18 and 35

- Low DF intake, defined as <22 g/day for males and <18g/day for females estimated by a validated habitual DF intake short food frequency (DFI-FFQ) questionnaire. See below for further details.
- No history of bowel disease
- Non-smokers
- No fibre supplement consumption during the month prior to screening
- Willing to consume three slices (for females) and four slices (for males) of bread provided during the intervention periods

Exclusion Criteria

- Have indication of inability to comply with the study procedures
- Antibiotic use within the last month
- Allergies or intolerance to wheat, rice, or gluten
- Pregnant, breastfeeding or was planning a pregnancy in the three months post-screening or during the study period
- Alarm features associated with bowel habits such as recent changes in bowel habits (onset less than three months), rectal bleeding, sudden weight loss, occult blood in faecal, anemia, anal fissures, bleeding hemorrhoids and family history of gut cancer at an early age
- Known significant gut disorders and diseases such as chronic constipation, diarrhea, irritable bowel syndrome, inflammatory bowel disease, diverticulitis, celiac disease, or previous bowel resection
- Chronic diseases including cardiovascular disease, cancer, renal failure, previous upper or lower gut surgery other than cholecystectomy or appendectomy, neurological conditions such as multiple sclerosis, spinal cord injury, or stroke
- Known systemic conditions such as heart disease, kidney disease, diabetes, metabolic syndrome, and psychological disorder that could influence the gut directly or through medication use such as prokinetics, opioids, and opiate, or non-steroidal anti-inflammatory drug use
- Fasting blood glucose equal to or above 6.0 mmol/L
- Laxative, pre-, and probiotic supplement use, and indicated inability or unwilling-ness to stop using seven days prior to sample collections

Assessment of Low Dietary Fibre

Low DF intake was assessed during the preliminary screening process using a DFI-FFQ [49]. This questionnaire assesses the frequency of fruit, vegetable, bread and cereals, nuts and seeds, and legume consumption over the past year. The author of this questionnaire (Dr Genelle Healey) provided a DF calculation spreadsheet to the specified research team members. The raw food group frequency data were entered into the spreadsheet to calculate total DF intake (g/day).

Participants were grouped as either low, moderate, or high DF intake based on New Zealand gender-specific dietary fibre intake cut-offs. The cut-off points were low <18 g/d for females and <22 g/d for males; moderate 18-24.9 g/d for females and 22-29.9 g/d for males; and high >25 g/d for females and >30 g/d for males. Only participants meeting the low DF intake cut-offs were included in the study.

Non-Exclusion Criteria

Participants were included in the study if they were diagnosed and had stable health conditions (systemic conditions and diabetes) for over three months. Selective serotonin reuptake inhibitors, tricyclics, or non-steroidal anti-inflammatory drugs were permitted if the medication had been continually used and the condition was stable for over three months.

Screening

Individuals who fulfilled the inclusion and exclusion criteria were invited to an on-site full screening visit at research clinics in Christchurch, New Zealand. The screening visit involved a full explanation of the study, health history and dietary habit interviews, collection of written informed consent, standard blood tests (nine hours fast prior to visit) and blood pressure measurement. Height and weight were used to calculate BMI as body weight (kilograms (kg))/height (meter squared).
Participants specified unable to swallow capsules were not considered for the Atmo sub-study but were still eligible for the main study.

Enrolment

Questionnaires

Upon informing participants of their enrolment into the study, participants were emailed an individualised link to complete two online enrolment questionnaires. These questionnaires, which were administered via REDCap, took approximately 10 minutes to complete. The Modified Hunter New England Health Survey (ModHNES) includes the validated Short Form 12v2® health survey (SF-12v2®) and selected question domains from the New South Wales Population Health Survey for diabetes, smoking, alcohol consumption, and physical activity. The SF-12v2® assesses eight health domains (physical functioning, role-physical, bodily pain, general health, vitality, social functioning, role-emotional, and mental health). This questionnaire was used to assess the general well-being of each participant and raise any important issues. Only the SF-12 questionnaire was scored.
Participants also completed the Economic Living Standard Index short form ($ELSI_{SF}$), developed and validated by the New Zealand Ministry of Social Development, assessing the standard of living and socio-economic background.

Daily Bowel Movement and Daily Bread Diary App

Participants were instructed to download an app titled ‘BREAD STUDY’. The app consists of both the daily bowel movement and bread diary questions. The questions in the app were captured electronically via the REDCap data capture system, using the unique identifier allocated to the participants at the point of consent to the study. The app

was designed to be more user-friendly as compared to receiving daily texts or email links to questionnaires. These daily diaries aimed to obtain a comprehensive record of bowel habits, intervention compliance and other bread consumption during washout periods. More information pertaining to these questionnaires will be described later in the Measurements and Outcomes section.

Removal and Withdrawal Criteria

Participation in this study was voluntary, and participants could withdraw at any time without explanation, as stated on the participant information sheet.
In the case of the following occurrences, participants were withdrawn:

- The participant requested withdrawal from the study. Participants could decide to end their involvement in the study for any reason and at any time during the study.
- The investigator decided to withdraw a participant from the study when considered necessary. Examples include:
    1. non-respect of at least one of the selection criteria after inclusion
    2. non-compliance with the study protocol
    3. gastroenteritis, or antibiotic use
- The participant reported allergic reactions or adverse effects from either intervention.

Regardless of eligibility and/or continuing participation, an electronic case report form record (eCRF) was generated for each participant. The eCRF was designed specifically for the needs of this study and was the data collection instrument for the study. Therefore, all data requested on the eCRF was recorded, and all missing data was explained. Each eCRF had a unique five-digit identifier to link the participant who had completed the screening questionnaire to their own data in a de-identified manner. Collected de-identified data, such as laboratory information, demographic information, and obtained questionnaire data were stored in a password-protected customised database.

Termination Criteria for the Whole Study

In the case of serious safety concerns, the principal investigators could terminate or interrupt the study. If new information on the risk-to-benefit ratio of the intervention (including treatment and/or investigational processes) used in the study was obtained in the meantime, the principal investigators reserved the right to interrupt or terminate the project. Additionally, premature termination of the study was possible if the principal investigators noticed that participant recruitment was insufficient and could not be accelerated by appropriate measures.

Study Intervention

The main intervention was bread fortified with DRB. It is a commonly eaten grain and is safe for human consumption [14, 50-54]. Based on measured composition, the intervention had 18% replacement of the flour (cereal) weight used in the placebo bread and provided 8 g (for females) and 10.6 g (for males) of total DF. Of the 8 to 10.6 g of DF in three to four slices of fortified bread, 2.2 g (for females) and 2.9 g (for males) were

fibre from wheat and 5.8 g (for females) and 7.7 g (for males) were from the DRB. The placebo was a matched commercial white toast bread without DRB, providing 2.7 g DF for females (3 slices) and 3.6 g DF for males (4 slices). Toast slices were chosen for this study to ensure the production of DRB-fortified bread had a texture similar to the placebo white toast bread while providing the required DF content.

Blinding and Allocation Concealment

Participants were not informed of the bread ingredients until the completion of the study data collection to maintain blinding. Analysts and researchers were blinded to the order of treatment the participants received and to which group the participants were assigned during data collection and remained blinded until the statistical analysis of the primary outcome data was completed. The manufacturer of the bread was responsible for labelling both breads to maintain blinding of research team members. To further increase blinding, the manufacturer added coloured malt to the placebo bread to make both breads visibly indistinguishable. Specified research team members were responsible for the randomisation, enrolment and data collection of participants, the handout of the intervention and placebo bread, and the management of the stock. Unblinding was only permissible once statistical analysis of the primary outcome data was completed.

Randomisation

One week prior to the first baseline visit, participants were randomly assigned to a 1:1 allocation using randomised permuted blocks (block size 4) to either DRB or placebo bread. Randomisation was performed by drawing a folded note from a sealed opaque box.

Measurements and Outcomes

Timing of Assessments

Upon enrolment, there were five clinic visits in total. Study assessments were conducted at baseline 1 (day 0/week 1), post-intervention 1 (day 29/week 5), baseline 2 (day 42/week 8), post-intervention 2 (day 78/ week 12), and follow-up (day 91/ week 15). Fifteen participants who were part of the Atmo sub-study ingested capsules at four time points: baseline 1 (day 0/week 1), post-intervention 1 (day 29/ week 5), baseline 2 (day 42/ week 8), and post-intervention 2 (day 78/ week 12).

Biological Measurements

Faecal Samples

The primary outcome was the differences in the relative abundance of a composite of selected key genera and species of the gut microbiota known to be involved in plant glycan metabolism and/or known to be modulated by the DRB-fortified bread (see Sample Size section for more details) as compared to the placebo bread.

Once enrolled, participants received a posted cooler bag containing a faecal collection kit and ice pack. Participants collected a faecal sample into the provided faecal container 24 hours prior to their clinic visits at baseline, post-intervention of each phase, and follow-up. Participants were instructed to label the faecal container with the time and date collected and record again on the bowel movement diary app. Participants had to

refrigerate the collected faecal sample and transport it within 24 hours of sample collection in the provided cooler bag for each clinic visit. If participants could not provide a faecal sample 24 hours prior, they were encouraged to collect and bring the sample in the cooler bag as soon as possible following their clinic visits.
Five aliquots (1 g each) of a faecal sample were frozen for microbial composition, gene abundance and metabolite analyses. The samples were kept chilled until aliquoted in the laboratory. All aliquots were snap-frozen in liquid nitrogen before storage at -80 °C until analysis.

Microbial Composition and Gene Abundances

Taxonomic composition and gene abundance of the faecal microbiome were assessed by shotgun metagenomics. Libraries for metagenomics sequencing of the extracted deoxyribonucleic acid (DNA) were performed by Auckland Genomics at the University of Auckland, New Zealand. Microbial metagenomic libraries were then generated using the Seqwell PurePlex DNA library preparation approach. Briefly, genomic DNA was diluted to 5 ng/μL, then sheared and barcoded with unique dual indexes using transposases to produce a library with fragment sizes of 300-1,500 basepair (bp). Libraries were pooled in batches of 24 samples and subjected to a paramagnetic bead-based clean-up. The DNA samples underwent quality control checks for purity and concentration using a bioanalyser. The samples were shotgun sequenced using the Illumina Novaseq 6000 platform using 2 x 150 bp paired-end sequencing at Novogene in Singapore.
The quality of raw data was checked using FastQC (Version 0.11.9) [55]. The software Trimmomatic (Version 0.36) [56] was used for the removal of adapters, low quality (Phred scores < 30), and short (< 36 bp) sequencing reads. Read pairs were aligned to the human reference genome (RefSeq: GCF_000001405) using the “mem” algorithm of BWA (Version 0.7.17-r1188) [57], and fastq files were generated from the unmapped sequencing reads using the “fastq” function of samtools (Version 1.8) [58]. Read pairs were joined using PEAR (Version 0.9.6) [59] with default settings. Read pairs that did not join were pasted together with a string of N’s using the "fuse" function from the BBMAP package (Version 38.22-0) [60]. Joined and fused reads from different lanes from the same sample were compiled into a final “clean” read sample file. Metagenomics functions were obtained through the "blastx" function of Diamond (Version 0.9.22) [61], mapping the reads against the "non-redundant" National Centre for Biotechnology Information (NCBI) database [62]. Megan (Version 6 ultimate edition) [63] was used to assign putative functions to the alignment files produced by Diamond. This alignment was performed by Paul Maclean (Statistician, AgResearch).
Differential relative abundance was performed using DESeq2 in R (Version 1.40.2). This package analyses differential expressed taxa based on a negative binomial distribution. The effect of group was assessed by comparing between groups. The top 10 differentially abundant taxa were extracted based on adjusted p values for both baseline and post-intervention for each group. Taxa that changed within each group were identified by finding the set difference between the top taxa between baseline and post-intervention. Log2foldchange values were extracted for the identified changed taxa in each group [64]. Enrichment of functional gene attributes arranged hierarchically with Level 1 (broadest level of function, e.g., metabolism and cellular processes), Level 2 (specific functions, e.g., carbohydrate metabolism, amino acid metabolism) and Level 3 (detailed pathways,

e.g., glycolysis, glycan degradation), were analysed using the R package Microbiome Multivariable Associations with Linear Models (MaAsLin2), which uses general linear models that accommodates crossover designs [65]. Fixed effects included in the model were groups (DRB vs placebo bread).

Metabolome

The relative intensity of faecal metabolites was assessed in the polar, semi-polar, and non-polar fractions of faecal samples using untargeted metabolite profiling (metabolomics).

Faecal samples were freeze-dried under vacuum and extracted using a previously described method [66] with minor modifications. Briefly, 50 mg of lyophilised faecal samples were homogenised with a ceramic bead for 1 minute, and then 400 µL of 75% methanol/MilliQ water was added. The tubes were vortexed for 30 seconds, sonicated for 2 minutes and transferred into ice for 10 minutes. Afterwards, 1 mL of methyl tert-butyl ether was added, and the mixture was incubated for 1 hour at 450 revolutions per minute. Then, 550 µL of MilliQ water was added, and after 10 minutes of incubation, the mixture was centrifugated at 14,000 x g for 25 minutes to separate the aqueous (lower) and organic (upper) phases. The polar and lipid extracts were evaporated under a stream of nitrogen at room temperature. The dried samples were stored at -80 °C until LCMS analysis. The metabolite profiling analyses were carried out using high-resolution LCMS on a Shimadzu Q-Tof 9030 equipped with electrospray [67]. An aliquot of the polar extract was taken and analysed using hydrophilic interaction liquid chromatography [67], and semi-polar metabolites were resolved using Reverse Phase chromatography [68]. The organic phase was analysed using the lipidomic methodology [69].

Targeted Metabolites

Aliquots of 1 g of faecal samples were sent to Plant & Food Research, New Zealand, for organic acid (including short chain fatty acids) measurement using an LCMS method. Fourteen linear and branched organic acids (carbon 1 through to carbon 6) were derivatised with a probe using mass spectrometry (MS)-probe and stable isotope techniques as previously described [70] with modifications [71] and measured using targeted LCMS on a SCIEX LCMS/MS QTRAP 5500 instrument equipped with a Turbo V™ ion source and atmospheric pressure chemical ionisation probe (Sciex, Concord, ON, Canada) coupled to an Exion ultra high pressure liquid chromatography (UHPLC) (Shimadzu, Kyoto, Japan). Labelled internal standards for each organic acid were used to ensure accurate quantification [72]. See Table 2 the names and corresponding acronyms of the organic acids analysed.

Extraction methods for bile acid analysis followed those outlined elsewhere [73]. Briefly, 50 mg of wet faecal samples were extracted with 100 µL ice-cold methanol containing internal standards (10,000 nM of d5-CA and d5-CDCA). The mixture was homogenised for 30 seconds, incubated at -4 °C for 30 minutes and centrifuged at 18,000 x g for 20 minutes. Then, 20 µL of the supernatant was dissolved in 80 µL of 0.1% aqueous formic acid solution, vortexed and proceeded to HPLC for analysis. This analysis was carried out using high-resolution LCMS on a SCIEX LCMS/MS QTRAP 6500+ system coupled to an ExionLC (SCIEX, Victoria, Australia) [74]. See Error: Reference source not found the names and corresponding acronyms of the bile acids analysed.

Blood Samples

Two research team members trained in phlebotomy collected peripheral blood at each

clinic visit. At both screening and follow-up clinic visits, one aliquot (6 mL) of a blood sample was taken into a lithium-heparin vacutainer tube to measure fasting blood glucose, lipid panel (total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol and triglycerides), C-reactive protein and liver function/enzymes/general health markers, and one aliquot (4 mL) of blood into ethylenediaminetetraacetic acid (EDTA) tube (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) for a complete blood count. Prior to and following each intervention phase, one aliquot (6 mL) of a blood sample was taken into a lithium-heparin vacutainer tube (Becton, Dickinson and Company, Franklin Lakes, NJ, United States) to analyse the complete lipid profile. The collected blood samples were kept at room temperature and delivered to the Canterbury District Health Laboratory (Christchurch, New Zealand) for analysis within one hour of collection.

At all-time points except for the screening clinic visits, an additional 6 mL of blood was collected into 1 x 6 mL lithium-heparin vacutainer tubes to assess plasma metabolome and known metabolites (bile acids and organic acids). The 6 mL vacutainer was kept on ice and processed within one hour. The tubes were centrifuged at 4 °C for 5 minutes at 2000 x g, with high acceleration and slowest deceleration to separate plasma from cells. Plasma samples were distributed into 500 µL aliquots and stored at – 80 ° C until further analysis.

Metabolome

Polar metabolites were extracted using a single-phase extraction [68]. Briefly, 50 µL of plasma was extracted with 450 µL of pre-chilled acetonitrile:water (9:1 v/v). The mixture was shaken for 60 seconds, then centrifuged at 14,000 x g, 4 °C for 10 minutes, and then the extract was placed into an HPLC vial for analysis. Plasma semi-polar metabolite extraction was as described before [75]. Plasma semi-polar metabolite extraction was performed by adding 400 µL of ice chloroform:methanol (1:1 v/v) to 50 µL plasma samples, vortexed for 30 seconds, incubated for 1 hour at -20 °C. Then 200 µL of MilliQ water was added to the mixture, vortexed and centrifugated at 14,000 x g, 4 °C for 10 minutes. The supernatants were evaporated to dryness under a stream of nitrogen and stored at −80 °C until analysis. On the day of the analysis, the semi-polar extracts were thawed at room temperature (18 ± 2 °C) and re-dissolved in acetonitrile:water (1:9 v/v). The mixture was vortexed for 1 minute, then centrifuged (14000 x g, 4 °C, 10 minutes). Then, the extract was transferred into an HPLC vial for analysis. Lipid extraction was performed by placing 10 µL of plasma into an Eppendorf tube and adding 95 µL of butanol:methanol (1:1 v/v) and then spiked with 5 µL of internal standard SPLASH mix (Avanti Lipids, Merck KGaA, Darmstadt, Germany) [69]. The mixture was then vortexed for 1 minute, sonicated at room temperature for 60 minutes, and centrifuged at 14,000 x g for 10 minutes at 20 °C. Then, the extract was transferred into an HPLC vial and stored at -80 °C until LCMS analysis. The plasma polar, semi-polar and non-polar extracts were analysed using the LCMS methods for the plasma samples [69].

Targeted Metabolites

Table 2 shows the names and corresponding acronyms of organic acids analysed. Aliquots of 200 µL of heparin plasma were sent to Plant and Food Research, New Zealand, for analysis of organic acids via LCMS. Fourteen linear and branched organic acids (carbon 1 through to carbon 6) were derivatised with a probe using MS-probe and

stable isotope techniques as previously described [70] with modifications [71] and measured using targeted LCMS on a SCIEX LCMS/MS QTRAP 7500 instrument equipped with a Turbo V™ ion source and APCI probe (Sciex, Concord, ON, Canada) coupled to a Nexera UHPLC (Shimadzu, Kyoto, Japan). Labelled internal standards for each organic acid were used to ensure accurate quantitation [72].

Table 2 Plasma and faecal organic acids analysed using LCMS with the corresponding acronym.

Full Name	Acronym
Formic acid	FA
Lactic acid	LA
Acetic acid	AA
Propanoic acid	PA
Isobutyric acid (2-Methylpropanoic acid)	IBA
Butyric acid (Butanoic acid)	BA
Succinic acid	SucA
2-Methyl butyric acid (2-Methylbutanoic acid)	2MBA
Isovaleric acid (3-Methylbutanoic acid)	IVA
Valeric acid (Pentanoic acid)	VA
3-Methyl valeric acid	3MVA
4-Methyl valeric acid (Isocaproic acid)	4MVA
Caproic acid	CA
Hexanoic acid	HA

Table 3 Faecal bile acids analysed using LCMS with the corresponding acronym.

Full name	Acronym
β-Muricholic Acid	βMCA
Cholic acid	CA
Cheno deoxy cholic acid	CDCA
Deoxy-cholic acid	DCA
Glycocholic acid	GCA
Glycohyodeoxycholic acid	GHDCA
Glycoursodeoxycholic acid	GUDCA
Glyco-litho-cholic acid	GLCA
Hyocholic acid	HCA
Hyodeoxycholic acid	HDCA
Isolithocholic acid	ILA
Litho-cholic acid	LCA
Taurine	Taurine
Tauro-β-muricholic acid	TβMCA
Tauro-α-muricholic acid	TαMCA
Tauro-cholic acid	TCA
Tauro-cheno-deoxy cholic acid	TCDCA
Tauro deoxy cholic acid	TDCA
Tauro-urso-deoxy cholic acid	TUDCA
Urso-deoxy cholic acid	UDCA

Clinical Measurements

Cardiovascular Risk Profile

Anthropometry

Height, weight, BMI, and waist circumference were measured at each visit, i.e., screening, baseline, post-intervention, and follow-up according to the procedures established by the New Zealand Ministry of Health, New Zealand - Ministry of Health Protocols for Collecting Height, Weight and Waist Measurements [76].

Standing height was measured to the nearest 0.1 cm using a calibrated stadiometer. Body weight was measured to the nearest 0.1 kg using calibrated body weight scales. Both measurements were performed with participants removing heavy outer clothing and shoes. Waist circumference was taken over clothing, and hence, the level of the measurement was determined by the participant to identify their waist. This measurement was measured to the nearest 0.1 cm using a calibrated measuring tape. All measurements

were conducted twice, with a third reading when the two readings varied by more than 0.5 units. BMI was calculated by weight (in kilograms) divided by height (in metres squared).

Blood Pressure

Blood pressure was also measured at each morning clinic visit, i.e., during screening, at each phase of baseline, post-intervention, and follow-up, according to the recommendations established by the Australian Expert Consensus [77]. The measurement was taken using a validated, automated blood pressure monitor with a fitted-sized upper arm cuff. Participants were seated at rest for 5 minutes with feet flat on the floor, legs uncrossed, back and arm supported in a relaxed position, with the cuff at heart level. Measurements were taken on their non-dominant arm. The blood pressure readings were then recorded immediately and compared with those recorded in previous clinic visits. When a high blood pressure value reading was noted, participants were asked if any unusual incidents that may affect the readings. These were then recorded onto the eCRF of the participant.

Lipid Profile

As mentioned above, the full lipid profile was measured at each morning clinic visit, i.e., during screening, at each phase of baseline, post-intervention, and follow-up, according to the recommendations of the Canterbury District Health Laboratory (Christchurch, New Zealand). The lipid profile consisted of five components: total cholesterol (TC), LDL, HDL, triglycerides, and TC/HDL ratio. TC measures overall cholesterol level; LDL is a fat that circulates in the blood, moving cholesterol around the body where it is needed for cell repair and depositing it inside artery walls. HDL, also known as "good cholesterol," helps remove cholesterol from the bloodstream. TG represents the main lipid component of dietary fat. The TC/HDL ratio, a sensitive predictor of heart disease [78], was also calculated.

Patient Reported Outcomes

Daily Bowel Movement and Daily Bread Diary

Upon enrolment and throughout the study, participants were asked to complete a daily bowel movement and bread diary via an app. This app was developed by one of our research team members (Hilary Dewhurst from AgResearch, New Zealand), using a REDCap application programming interface token. The token is a unique code that is associated with a single user on a specific REDCap project, which allows the user to programmatically access data within the project. Upon installing the app by the participants, the app will download the diary questions from REDCap, linking the app (on that phone) to the corresponding participant’s allocated unique identifier at the point of consent to the study. All submissions were then recorded under the corresponding participants’ identifier. The submissions were stored in the app and submitted to REDCap with each entry. To ensure data integrity, the app “talks to” REDCap to retrieve the most recent record for the participant and increment accordingly, preventing any answers from being overwritten.

Each diary took approximately 2 minutes to complete each time. The bowel movement diary provided a comprehensive record of the bowel habits of the participants, which assessed the following variables:

1. frequency of bowel motions, which included questions on spontaneity and

completeness of bowel movement
2. ease of defecation/level of straining
3. faecal form based on the Bristol Stool Scale
4. menstruation (if applicable)
5. presence of blue dye (if applicable, see below for further details)

The daily bread diary assessed compliance with the consumption of intervention and general bread consumption preferences. Compliance of bread slices and days consumed for each participant was determined by the following equation:

Compliance for bread slices consumed = [(number of bread slices consumed/number of bread slices expected to be consumed) × 100%].

Compliance for days consumed = [(number of days consumed/number of days expected to be consumed) × 100%].

Compliance of intervention (bread slices and days consumed) were determined by calculating the mean percentage compliance for each bread and the proportion of participants meeting above 50% of compliance.

This bread diary also determined if:

1. the DRB-fortified and placebo breads were toasted
2. minutes of toasting (if applicable)
3. additional commercial sliced bread consumed (if applicable, during washout)
4. the type, brand, and amount of additional bread consumed (if applicable)

Online Questionnaires

One day prior to each phase of baseline, post-intervention and follow-up clinic visits, participants received an individualised email to six online questionnaires. Similarly, these online questionnaires, which were emailed to participants, were administered via the REDCap data capture system. All these questionnaires took approximately 15 minutes to complete each time.

Gastrointestinal Symptom Rating Scale (GSRS)

The GSRS, a validated instrument that assesses gut comfort, was assessed before and during the intake of intervention and placebo bread. The GSRS has a 1-week recall that assesses symptom severity using a 7-grade Likert scale, ranging from 1 (“no discomfort at all”) to 7 (“very severe discomfort”). The complete instrument consists of 15 primary items clustered into five domains: diarrhoea, constipation, reflux, abdominal pain, and indigestion [79-81].

Patient-Reported Outcome Measurement Information System (PROMIS): Anxiety, Depression

A validated system with multiple domains, where specific domains can be chosen to be integrated into diverse data collection tools. This chosen questionnaire evaluates anxiety and depression in the last seven days in detail, as well as mental symptoms rated by severity, from “not at all” to “very much” and from “never” to “always.” [82, 83].

World Health Organisation - Five Question Well-Being Index (WHO-5)

A short self-reported rating scale of current well-being. It consists of five statements concerning the past two weeks, adjusted to one week (All of the time = 5; Most of the time = 4; More than half of the time = 3; Less than half of the time = 2; Some of the time = 1; At no time = 0) [84].

Warwick-Edinburgh Mental Wellbeing Scales (WEMWBS)

A 14-item scale, adjusted to one week, was used to explore mental health and well-being over the past two weeks. The WEMWBS has five response categories, summed to provide a single score. The items are all worded positively and cover both feeling and functioning aspects of mental well-being. This questionnaire covers key aspects of psychological functioning: optimism, autonomy, agency, curiosity, clarity of thought and positive relationships; and positive affect (feelings): confidence, feeling relaxed, cheerful, having the energy to spare (ranging from none of the time; rarely; some of the time; often; all of the time) [85].

Multidimensional Fatigue Inventory Short Form (MFI-SF)

A 20-item self-report instrument with a 7-point scale indicates to what extent each statement applies to the participant, ranging from “yes, that is true” to “no, that is not true”, designed to measure fatigue. It covers the following dimensions: General Fatigue, Physical Fatigue, Mental Fatigue, Reduced Motivation and Reduced Activity [86].

Subjective Vitality Scale (SVS)

A 6-statement instrument assesses the state of feeling alive and alert, i.e., having energy available to oneself. The statements explore the current feeling on a scale of 1 to 7: 1= not at all true, 4= somewhat true, and 7= very true [87].

Dietary Intake

Paper copies of a non-consecutive three-day food diary were posted to all enrolled participants to complete prior to each of the five study visits, i.e., at baseline, post-intervention, and follow-up. All enrolled participants were provided verbal and written instructions by the study dietitian on completing a food diary. The written information included instructions on how to describe food using household measures. The food diary included written prompts for detailed information about when, where, who, the type of food or drink, brand details, preparation/cooking methods, quantity, and section for recipe details. Participants were also asked to record any reasons why their intake may have differed from their normal eating routines. Prior to each subsequent appointment, participants received reminder emails to start the food diary and the same written information on completing the food diary. Participants could contact the study dietitian if they required further guidance on completing the food diary. At each clinic visit, the study dietitian reviewed each food diary to ensure adequate detail was provided and if additional foods and beverages were consumed but not recorded. Two nutrition graduates trained in dietary assessment entered the food diaries into a dietary analysis software, FoodWorks Online Professional (Version 1.0, Xyris Software, Brisbane, Australia, 2021) [88]. The New Zealand Food Composition Data (FOODfiles 2018 – Version 1.0) was used to estimate energy and nutrient intakes. After the primary outcome was analysed and the study was unblinded to intervention, the macro- and micro-nutrients from the two types of study bread were manually added to the dataset based on the type of bread and actual reported bread consumption. This final dataset was then exported into Statistical Package for Social Sciences (SPSS) Version 29 (Armonk, New York, United States) and was treated as continuous data.

Physiome Measurements

Blue Food Dye

At each baseline and post-intervention clinic visit, participants ingested one teaspoon of Royal Blue Liqua-gel® (Chefmaster, United States) food colouring (12 drops/1.5 g) in water. The intake time and date were recorded. The dye was poorly absorbed or fermented, which allows for analysis of whole gut transit time upon passing by visual confirmation [89, 90]. Visual confirmation was recorded by the participant on the daily bowel movement diary app.

Atmo Gas-Sensing Capsule

A subset of participants (n=15) completed an assessment of colonic gas profiling and whole and regional gut transit time by the Atmo gas-sensing capsule. The capsule measures temperature, a range of gaseous fermentation by-products, including carbon dioxide and hydrogen, and an indication of oxygen level, capsule tumble and antenna reflectometry [91, 92]. The selected participants received a separate participant information sheet and completed a specific consent form before the transit assessment. The capsule was ingested at each phase of baseline and end of post-intervention clinic visits. These participants consumed a standardised cereal bar and an Atmo gas-sensing capsule (Atmo Biosciences, Melbourne, Victoria, Australia).

The selected participants were fitted with a sling bag containing a transponder. It consists of a receiver and smartphone with a custom app, which ‘listens’ to the capsule ingested. These participants wore or kept the Atmo transponder close to them (about one meter) until the exit of the capsule or for up to five days. They were also asked to enter additional daily bowel movement diary information into the Atmo transponder. The data was collected in real-time on the smartphone app and transmitted to an Atmo cloud during the passage to allow remote monitoring and troubleshooting. Once the participant confirmed the exit of the capsule, the participant returned the transponder to the research team. The diary data from the Atmo app were then synchronised to the Atmo cloud for review and analysis. Blinded and deidentified data synchronised onto the Atmo cloud were assessed using specialised Atmo software by trained Atmo team members. The analysis included colonic gas profiling (Hydrogen and Carbon Dioxide) and the determination of whole and regional gut transit times.

Adverse Events

Although the study is considered low risk, all participants were provided with information about managing possible side effects. If any, participants were encouraged to contact their healthcare provider, followed by our research team, in the event of side effects. Participants were encouraged to record any symptoms and may withdraw if they experienced any side effects. Any participants experiencing harm directly due to their involvement in the study were withdrawn from the study immediately.

Sample Size

Sample size calculations were based on the GutFeelingKB cohort [93]. The percentage relative abundance of a composite microbiome index (incorporating 15 operational taxonomic units as outlined below) was estimated to be 28.3% with a standard deviation (SD) of 14%. The sample size needed was 60 participants in this crossover study to

account for a 15% dropout rate. The crossover study had more than 80% power to detect a difference in the absolute increase in the relative abundance of approximately 6%, as statistically significant (2-tailed α=0.05). An increase of 6% compared to the baseline level of 28% equates to a relative increase of approximately 22%. The consumption of three (for females)/ four (for males) slices of DRB-fortified bread per day for 28 days is thought to affect individual and combined groups of the microbial taxa making up the composite.

The composite microbiome index included genera from five phyla found in the gut microbiota of healthy human adults. This index included *Prevotella* and *Barnesiella* genera and *Bacteroides ovatus* and *Bacteroides xylanisolvens* from the Bacteriodetes phylum; *Roseburia*, *Anaerostipes*, *Blautia*, *Eubacterium*, *Ruminococcus*, *Faecalibacterium*, *Lactobacillus* genera from the Firmicutes (Bacillota) phylum; *Bifidobacterium* and *Eggerthella* genera from the Actinobacteria (Actinomycetota) phylum; *Akkermansia* genera from the Verrucomicrobiota phylum; *Methanobrevibacter* genera from the Euryarcheaota phylum.

Statistical Analysis Plan

This study was conducted as a superiority trial. The Guidelines of the Committee for Proprietary Medicinal Products (now termed Committee for Medicinal Products for Human Use) require intention to treat analysis [94]. For a crossover design, the intention to treat analysis means that every enrolled participant who has consumed both the intervention and placebo bread is included despite dropout or non-compliance. All data from randomised participants who received at least one intervention dose and had at least one post-intervention measurement will be analysed.

The mean differences from the baseline of most outcomes will be compared between groups and referred as between-group comparison. For most outcomes, within-group analysis (post-intervention compared to baseline) will also be performed. For metabolome analyses, post-intervention values (not the mean differences) will be compared within groups and between groups.

All statistical analyses will be performed using IBM® SPSS® statistics version 29 (Armonk, New York, United States) or R version 4.3.1 (R Core Team 2021) by blinded researchers under the guidance of an independent biostatistician.

Baseline characteristics will be presented using descriptive statistics. The mean and SD, or median and range, will be used to describe continuous variables. Frequencies and/or percentages will be used to describe categorical variables. The group effects (i.e., the mean change from baseline between groups) will be assessed using (parametric) T-tests and (non-parametric) Mann-Whitney/Kruskal–Wallis tests for symmetrically and asymmetrically distributed data. Categorical variables will be assessed using Chi-squared tests (or Fisher's exact tests for small samples). A two-tailed $P<.05$ is determined as statistical significance. A $P\leq.10$ will be considered a trend for most variables except for the microbiome data.

For the primary outcome, to determine the microbiome composite, the selected genera, and species will be summed to give a composite relative abundance for each participant. Changes in the composite of the microbial taxa within and between groups will be assessed using the non-parametric Wilcoxon Signed Rank Test. The statistical

significance level is determined at $P<.05$, with a false discovery rate (FDR) of $P<.10$. A probable biological significance level (trend) is assumed at an unadjusted $P\leq.10$. For other faecal microbiome measures, univariate and multivariate statistical analyses will be used to assess alpha- and beta-diversity variations, individual relative abundance differences in taxa, and gene abundance differences between groups. Differences in alpha diversity between baseline and post-intervention, and between groups post-intervention will be assessed using the non-parametric Wilcoxon Signed Rank Test with Continuity Correction. The Benjamini-Hochberg method will be applied to control the FDR inherent in multiple-hypothesis testing for variables in microbiome and metabolome analyses. The resulting adjusted p values will be computed to address the inflated Type I error rates associated with numerous comparisons. $P<.05$ and FDR of $P<.10$ will be deemed significant, with a probable biological significant difference (trend) will be assumed at unadjusted $P\leq.10$.

Boxplots will be created using ggplot2 to compare groups for each Chao1 and Shannon Indices timepoint. The Permutational Multivariate Analysis of Variance (PERMANOVA) and the Analysis of Dissimilarity (ADONIS) functions will then be used to assess the statistical significance of microbial community composition between individuals. ADONIS uses permutation testing and provides statistical assessments and their significance using F tests, based on the sequential sums of squares from the permutations, to determine whether the observed differences in beta diversity are statistically meaningful. Microbial differential gene expression will be performed using quasi-likelihood F -tests. This test provides stricter error rate control by accounting for the uncertainty in dispersion estimation. Genes with an absolute log-fold change greater than 1.1 will be considered as differentially expressed.

As mentioned above, the comparisons will be between each group and baseline and between groups post-intervention for plasma or faecal metabolome measures. The partial least squares projection-discriminant analysis (PLS-DA) model will be performed to identify metabolites using *Soft Independent Modeling of Class Analogy*, Version 17 (Umetrics, Umea, Sweden). The PLS-DA models will be subject to 100-fold permutations to evaluate performance; the models will be validated using the predictive ability of the model (Q2) and the analysis of variance testing of cross-validated predictive residuals (CV-ANOVA *P*) [95]. The most discriminating features will be selected for building PLS-DA models. These models will be again subjected to prediction accuracy and overfitting assessment by the corresponding tests (CV-ANOVA *P*) and comparison of the Q2 afterwards. Features will be selected based on their variable importance in the projection score (VIP). Univariate statistical analysis and heatmaps will be performed using Metaboanalyst 5.0 [96]. The metabolite and lipid relative intensity differences between groups will be tested by T-test or Wilcoxon Rank-Sum Test. The multiple testing corrections will be controlled using FDR correction [97].

Data Quality and Assurance

The researchers collecting data were trained accordingly in data collection, maintaining security and health-related confidentiality.

Due to the large amount of data to be collected, there may be an inherent risk of errors during the data entry into the databank. All paper-based data were handled by blinded study personnel, and participants were strongly encouraged to enter survey data directly

into the database by email links to the surveys and by the app to minimise this risk.
The nutrition graduates who entered the raw food and beverage diary data maintained a data entry assumptions spreadsheet to ensure consistency of data entry. The study dietitian will independently audit 20% of the food diaries entered into the dietary analysis programme (FoodWorks Online, Version 1.0, Xyris Software, Brisbane, Australia, 2021) by the nutrition graduates. Diaries were checked for accuracy, consistency, and completeness. Extreme values or outliers for specific nutrients were identified and clarified by reviewing the assumptions spreadsheet and raw food and beverage diary data.
A formal Data Monitoring Committee was not required as the intervention and placebo breads in this study are considered a sufficiently low risk that no harm is anticipated to occur beyond the risks of standard care and everyday life.
Data Monitoring occurred through weekly meetings of the research team. The un-blinded research team-maintained concealment.

Confidentiality

The researchers collecting data were experienced in data collection, maintaining security and health-related confidentiality. No identifying or identifiable information about participants was reported from this study, including names, dates of birth, images, or aspects of their circumstances that could identify them. Identifying information of participants was gathered at enrolment, entered by researchers collecting data and accessed via REDCap only by these researchers.

Data Collection and Storage by Researchers

Data collection for questionnaires occurred electronically via the REDCap data capture system, using the unique identifier allocated to the participants at the point of consent to the study. Participants who preferred paper data collection were accommodated. Paper-based and telephone-collected data were entered directly into electronic data collection systems (using REDCap) as soon as they were received.
Biological and physiome data were analysed as per the aforementioned measures. The data files were stored in Excel for raw data and as data analysis files (IBM® SPSS® statistics version 29.0 (Armonk, New York, United States) or R statistical software version 4.3.1 (R Core Team 2021). This data was only identified by the unique code of each participant. Participants were identifiable to researchers only by their study code. Biological samples were stored in secure facilities with restricted access until publication of the results, but not longer than 10 years, after which they will be destroyed hygienically in accordance with NZS 4304:2002 Management of Healthcare Waste or with the appropriate karakia (for Māori participants).
Raw data collected in hard copy were stored after electronic data entry as part of the CRF in a locked filing cabinet. All electronic data files generated in the study were stored on a password-secured University of Otago server or Otago OneDrive cloud storage and were accessed (and downloaded, as needed) to the password-protected computers of named investigators, stored on locked premises. A master file containing participants' personal identifying information was only accessible to the researchers involved in data collection. The researchers (blind) had only access to de-identified raw data files and were responsible for the final data analysis.

Results

Recruitment of participants began in June 2022 and was completed in December 2022. Of the 66 individuals who completed the online preliminary screening questionnaire and attended an onsite screening, four individuals were excluded as they did not meet the inclusion criteria. Therefore, 62 participants were randomised. During the first intervention phase, six participants dropped out, resulting in a total of 56 participants (n=33 females, 58.9%; n=23 males, 41.1%; New Zealand European n=41, 73.2%; Māori n=1, 1.8%) included in the analysis.

The mean age for the entire cohort is 40.4 (SD: 13.4) years and a mean BMI of 21.9 (SD: 10.3) kg/m^2. Due to the large dataset, data analysis for all outcomes was planned to be completed by the last quarter of 2024, with full results expected to be published in peer-reviewed journals by the end of 2024.

Discussion

Anticipated findings

The primary aim of this study is to determine the influence of DRB-fortified bread on the relative abundances of a composite of selected key genera and species of the gut microbiota that are relevant in DF degradation and metabolism in healthy adults with low DF intake as compared to placebo white toast bread. Additionally, relative abundance/concentration of faecal and/or plasma biological markers of host and microbial interactions (microbial taxa, bile acids and organic acid concentrations) alongside cardiovascular health, patient-reported outcomes and physiome outcomes of the recruited healthy participants will be described. The findings from this study are anticipated to provide a better understanding of the habitual impact of DRB bread on the gut microbiome and all other assessed parameters in healthy individuals with low DF intake.

Preliminary analysis showed that during the first intervention phase, six participants dropped out, resulting in a total of 56 participants (73.2% New Zealand European, 1.8% Māori) included in the intention to treat analysis. Due to the large dataset, data analysis and publication of full results are expected to be completed by the end of 2024.

This is the first human study to explore DRB-fortified bread as a dietary intervention. Therefore, it was decided to recruit a generally healthy population of males and females with low DF intake prior to the commencement of the study. Additionally, participants were given a daily dose of three to four slices of DRB or white toast bread, which could easily be added to the diet. The bread slices were provided in separate doses tailored to the nutrient requirements for females (three slices) and males (four slices). Consequently, these strategies to improve DF intake among low DF consumers were practical and could be implemented in real life settings. Further, participants were specifically asked about their regular bread consumption. Therefore, only individuals who consumed bread were included in the study, which may reduce the generalisability of the study results. Nonetheless, these risk management measures aimed to minimise dropouts and enhance the cost-effectiveness of the study.

Faecal samples were collected to determine the gut microbiota composition, gene abundances, bile acid concentrations, organic acid concentrations and moisture content. Immediate analysis of fresh faecal samples is often unrealistic, raising concerns about

storage methods by participants, which may cause microbial DNA degradation, overgrowth and species death in faecal samples [98]. However, there are currently room temperature microbial stabilisation methods with high technical reproducibility and compositional stability [99], which should be considered for future studies. From a cultural perspective, these advanced methods offer an alternative for future studies where refrigerating faecal samples is not feasible, particularly in cases where it may be culturally insensitive, such as for Māori and Pasifika participants. Nonetheless, all participants were given clear written instructions, ice packs and a cooler bag for returning their samples during clinic visits, minimising potential confounding effects.

Strengths of the BREAD study include using shotgun metagenomics to analyse extracted faecal microbial DNA. This approach allowed the exploration of the composition and predictive functional aspects of the gut microbiota. These analyses provided insights into the community structure and diversity, as well as the identification of changes in taxa and genes associated with the study interventions [100]. Additionally, this study included other advanced -omic approaches (metabolomics), validated subjective questionnaires, and dietary records collected before and after each intervention. Integrating these data will allow a better understanding of the complex interactions between diet, gut microbiota, and other health outcomes of the healthy adults with low DF intake. Furthermore, this study is a blinded crossover study, allowing for participants to be their own control [47]. A crossover design was chosen due to the recognised inter- and intra-individual variabilities among populations, allowing a reduced sample size while achieving the required statistical power. Additionally, sequence effects were accounted for in the analysis [101].

Future studies may consider recruiting individuals with gut abnormalities, such as individuals with constipation, or to conduct further subgroup analysis on the same dataset to separate individuals with different BMI status, ethnicities, gut microbiota composition at baseline. These considerations may provide better understanding of the effects of DRB bread on all the assessed study outcomes.

Additionally, there is also a possibility that participants felt obligated to consume the interventions provided considering the perceived notion of being part of a research. Future studies should consider supplementing the study with qualitative interviews or surveys. Including consumer insights as part of the study would allow a better understanding of participants' perspectives and experiences. Subsequently, this may allow further generalisation to the general population should DRB bread be introduced to the market.

Conclusions

In this double-blinded, crossover randomised controlled trial, the collection of diet, clinical, biological (microbiota, metabolites, lipids) and physiome (transit time, gas profile) data will provide crucial information on the benefits of fortifying DRB in commonly consumed foods. This knowledge may help support the public health system and food industry in developing targeted interventions aimed at modulating health-promoting gut microbes, general and mental well-being, DF intake, whole and regional gut transit time, and fermentation gas profiles in healthy adults with low DF intake.

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Author Contributions

CW: Formal Analysis, Supervision, Writing – review & editing. CG: Formal Analysis, Investigation, Supervision, Writing – review & editing. CF: Formal Analysis, Supervision, Writing – review & editing. DC: Formal Analysis, Investigation, Methodology, Supervision, Writing – review & editing. HN: Data curation, Formal Analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. JMu: Formal Analysis, Investigation, Methodology, Supervision, Writing – review & editing. JC: Formal Analysis, Investigation, Methodology, Supervision, Writing – review & editing. JM: Data curation, Formal Analysis, Investigation, Project administration, Writing – original draft, Writing – review & editing. KF: Formal Analysis, Investigation, Methodology, Supervision, Writing – review & editing. MF: Formal Analysis, Supervision, Writing – review & editing. NR: Conceptualization, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. RG: Conceptualisation, Investigation, Methodology, Supervision, Writing – review & editing. SB: Investigation, Supervision, Validation, Writing – review & editing. TT: Investigation, Methodology, Writing – review & editing. WM: Formal Analysis, Investigation, Methodology, Supervision, Writing – review & editing. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. All authors work at independent research facilities or business and are not employees of the industry partner. The industry partner has had input into the study design; however, they were not involved in the data collection nor will be involved in the analysis or interpretation of results, manuscript writing or in the decision of results publication.

Abbreviations

%: Percent
ADONIS: Analysis of dissimilarity

ANOVA: Analysis of variance
ANZCTR: Australia New Zealand Clinical Trial Registration
BMI: Body mass index
BREAD: Bread Related Effects on microbiAl Distribution
Bp: Basepair
eCRF: Electronic case report form
CV-ANOVA P: Cross-validated predictive residuals
DA: Differential abundance
DF: Dietary fibre
DFI-FFQ: Dietary fibre intake-food frequency questionnaire
DNA: Deoxyribonucleic acid
DRB: Defatted rice bran
EDTA: Ethylenediaminetetraacetic acid
FDR: False discovery rate
HDL: High-density lipoprotein
HPLC: High pressure liquid chromatography
JMIR: Journal of Medical Internet Research
LCMS: Liquid chromatography mass spectrometry
LDL: Low-density lipoprotein
MBIE: Ministry of business, inovation and employment
MS: Mass Spectrometry
NCBI: National Centre for Biotechnology Information
PERMANOVA: Permutational multivariate analysis of variance
PLS-DA: Partial least squares projection-discriminant analysis
Q2: Predictive ability of the model
RCT: Randomised controlled trial
SPIRIT: Standard protocol items: Recommendations for interventional trials
SPSS: Statistical package for social sciences
VIP: Variable importance in the projection score

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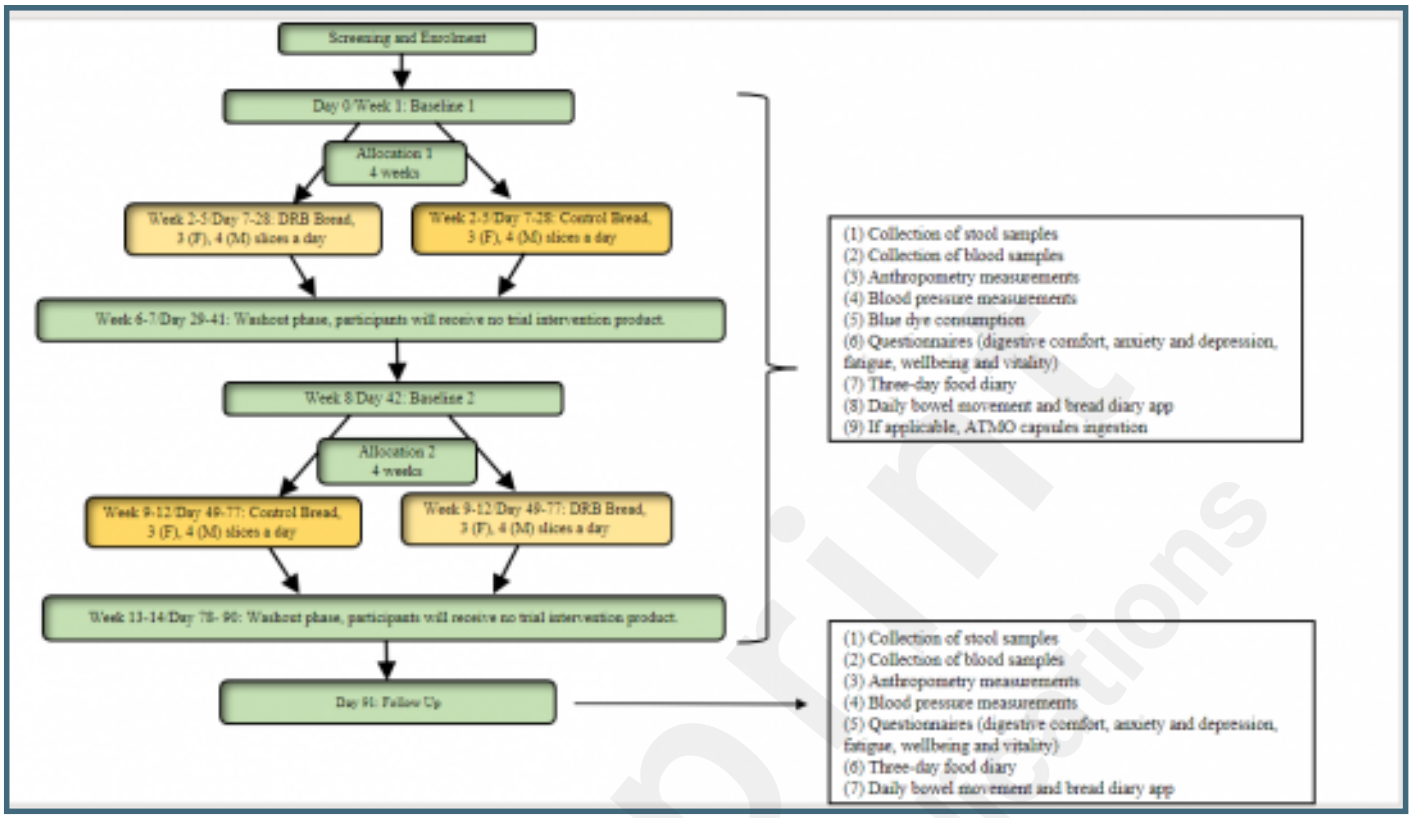
Supplementary Files

Figures

Study Flow Chart.



CONSORT (or other) checklists

SPIRIT Outcomes Checklist.
URL: http://asset.jmir.pub/assets/3f0ce14dd856cd7dc6b018aa4d0b525a.pdf