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# **The effect of pre- and probiotic supplementation on inflammatory markers in post-menopausal women.**

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A thesis presented in partial fulfilment of the requirements for  
the degree of Master of Science in Nutrition and Dietetics.

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## Abstract

**Background:** Chronic inflammation is linked with several deleterious diseases, including cardiovascular disease, diabetes mellitus, irritable bowel disease, and osteoporosis. Post-menopausal women are at a heightened risk of developing these diseases due to the remission of oestrogen, further amplifying a pro-inflammatory state.

**Objectives:** This study aimed to critically examine the combined effect of synbiotic supplementation and exercise in the form of  $\geq 7,000$  steps per day on inflammatory markers in sedentary post-menopausal women.

**Method:** Eighty-seven healthy post-menopausal women were allocated to receive either a synbiotic supplement or placebo for 12 weeks. Participants' demographics and physical activity levels were determined using questionnaires, and their diet was assessed using self-reported 3-day diet records. Body composition measures of height, weight and BMI were measured at baseline, while total body mass, lean body mass, total fat mass and total body fat percentage at baseline and week 12 using dual-energy X-ray absorptiometry. Fasted venous blood samples were collected to analyse inflammatory status before and after the intervention. Statistical analysis was performed using SPSS version 24. Outcome variables with multiple time points were analysed using repeated measures ANOVA with the model, including time, intervention group and their interaction as fixed effects.

**Results:** The results showed no significant differences between the intervention group's demographics, physical activity levels, and dietary intake. The 12-week study duration had a statistically significant effect on lowering hs-CRP, IL-8, IFN- $\gamma$ , TNF- $\alpha$  and increasing IL-6 and IL-10 in both groups. Additionally, the treatment type and study duration significantly impacted IL-10. However, the observed decrease in IL-1 $\beta$  over time was not significant. No significant interactions between time and group were observed across all inflammatory markers. Over time, there was a significant increase in total lean body mass and a decrease in total body fat percentage in both groups. However, the treatment type did not affect total lean body mass, total fat mass, total body mass and percentage body fat in both groups.

**Conclusion:** The current study showed no notable differences between groups, suggesting synbiotic supplementation is likely ineffective at reducing chronic inflammation in post-menopausal women. Future studies must confirm these findings and investigate the effects of exercise and synbiotic supplementation separately.

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## Abbreviations

<b>Abbreviation or Symbol</b>	<b>Definition</b>	<b>Abbreviation or Symbol</b>	<b>Definition</b>
CFU	Colony Forming Units	UC	Ulcerative Colitis
≥	Greater than or equal to	GI	Gastrointestinal
>	Greater than	ESR	Erythrocyte Sedimentation Rate
<	Less than	MPO	Myeloperoxidase
Hs-CRP	High sensitivity C-Reactive Protein	LPS	Lipopolysaccharide
IL-8	Interleukin- 8	LD	Low Dose
IFN-γ	Interferon Gamma	HD	High Dose
TNF-α	Tumour Necrosis Factor Alpha	FOS	Fructo-oligosaccharides
IL-6	Interleukin- 6	BMD	Bone Mineral Density
IL-10	Interleukin- 10	yr.	Year
IL-1β	Interleukin- 1 Beta	kg/m <sup>2</sup>	Kilogram per square meter
RCT	Randomised Control Trial	kJ/min	Kilo joules per minute
ROS	Reactive Oxygen Species	mg/L	Milligrams per litre
ROI	Reactive Oxygen Intermediates	nmol/L	Nanomoles per litre
CVD	Cardiovascular Disease	pg/mL	Picograms per millilitre
T2D	Type 2 Diabetes	μg	Micrograms
MetS	Metabolic Syndrome	DXA	Dual-energy X-ray absorptiometry
IBD	Irritable Bowel Disease	ANOVA	Analysis of Variance

# Chapter 1 Introduction

## 1.0 Background

Inflammatory-related diseases dominate as the leading causes of disability and mortality worldwide, with 50% of all-cause mortality able to be linked back to increased systemic inflammation (Furman et al., 2019; Nathan & Ding, 2010). Typically, inflammation occurs under normal physiological functioning of the inflammatory cascade (Schmid-Schönbein, 2006). This inflammatory cascade is critical for survival as it combines cellular and tissue-level reactions required for the removal and breakdown of damaged tissues and the passage of immune cells for infection control by stimulating changes in the microvascular system using cytokines (signalling molecules) (Schmid-Schönbein, 2006). Upon completion of tissue restoration or elimination of pathogens, the inflammatory cascade quickly resolves within a short duration (minutes- days) (Schmid-Schönbein, 2006). This form of inflammation is classified as acute. However, resolution is not always achieved in some individuals, leading to chronic inflammation, a warning sign for vulnerability to the development of organ and body system dysfunctions (Nathan & Ding, 2010; Schmid-Schönbein, 2006). These can cause a vast range of inflammatory-related diseases, such as cardiovascular disease, diabetes mellitus, irritable bowel disease, osteoporosis, rheumatoid arthritis, and auto-immune and neurodegenerative conditions (Furman et al., 2019; Nathan & Ding, 2010; Schmid-Schönbein, 2006; Sproston & Ashworth, 2018; Yang et al., 2017). For this reason, it is essential to identify individuals with an elevated inflammatory status to prevent the onset of disease and progression (Pepys & Hirschfield, 2003).

Screening plays a significant role in the prevention of chronic inflammatory diseases. One method for quantifying inflammation in the body is by measuring cytokine levels in the blood. Cytokines are a diverse group of proteins and peptides able to control intercellular communication. Therefore, they elicit beneficial or detrimental impacts on most biological processes in the body depending on their extracellular abundance and the activity of other cytokines present (Ilesanmi-Oyelere et al., 2019; Vulevic et al., 2008; Zhang & An, 2007). Cytokines can be subdivided into five main categories: interleukins (ILs),

chemokines, interferons (IFNs), colony-stimulating factors (CSF), and tumour necrosis factors (TNFs) and can then be further divided into pro-inflammatory or anti-inflammatory cytokines (Chen et al., 2018; Ilesanmi-Oyelere et al., 2019). From previous research, pro-inflammatory cytokines are involved in activating and proliferating immune cells and the release of cytokines necessary for fighting infection and disposal of tumour cells. However, pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-8, and IFN- $\gamma$  are also associated with the pathogenesis of chronic and auto-immune diseases, pain, discomfort, fatigue, low energy levels, and a general feeling of unwellness (Ilesanmi-Oyelere et al., 2019; Jang et al., 2021; Vulevic et al., 2008; Zhang & An, 2007). In comparison, anti-inflammatory cytokines inhibit and regulate the pro-inflammatory response from the acute phase. Major anti-inflammatory cytokines include IL-10, IL-33, and TGF- $\beta$ , where IL-10 is the most potent and can suppress the chronicity of TNF- $\alpha$  and IL-6 expression and promote the release of more anti-inflammatory cytokines to maintain balance (Ilesanmi-Oyelere et al., 2019; Kwok et al., 2022; Zhang & An, 2007). An alternative clinical indicator for detecting elevated inflammation is the acute phase protein, c-reactive protein (CRP). Being an acute phase protein means CRP is synthesised in the liver when the body initiates an inflammatory reaction to a stimulus (antigen) (Pepys & Hirschfield, 2003; Schmid-Schönbein, 2006). Therefore, CRP becomes concentrated in blood whenever systemic inflammation increases. Additionally, CRP is uninfluenced by diurnal and seasonal changes and very few medications, making it a sensitive marker for systemic inflammation (Pepys & Hirschfield, 2003; Schmid-Schönbein, 2006; Sproston & Ashworth, 2018). As vitamin D status is suggested to have an important role in the modulation of the immune system by downregulating and upregulating pro and anti-inflammatory cytokines, respectively (Agrawal & Yin, 2014; Mousa et al., 2016). 25-hydroxyvitamin D, the marker of Vitamin D status, may also be useful for identifying people at risk of inflammation-related health conditions (Agrawal & Yin, 2014; Mousa et al., 2016).

Prior research has shown that the inflammatory cascade fails to be resolved when the microbiome is in a state of dysbiosis (Kiousi et al., 2022; Patel et al., 2014; Valentini et al., 2015). Commensal bacteria, such as *Lactobacilli* and *Bifidobacteria*, are vital for maintaining a healthy microbiota as they generate a

protective barrier on the gut epithelium, preventing the establishment of opportunistic and pathogenic organisms and provide essential nutrients for the host. Therefore, when outcompeted, the gut becomes 'leaky,' allowing bacteria and toxins to enter the bloodstream, amplifying widespread inflammation and oxidative stress (Kiouisi et al., 2022; Patel et al., 2014; Valentini et al., 2015).

As a person ages, the microbiome undergoes a natural decrease in the number and variety of commensal bacteria (Patel et al., 2014; Vulevic et al., 2008). Therefore, a key population impacted by chronic systemic inflammation are post-menopausal women. However, in addition to chronic inflammation-associated ageing, termed "inflammaging," post-menopausal women are uniquely positioned due to hormonal and dietary changes associated with menopause, elevating inflammation in the body (Burch et al., 2014; Ginaldi et al., 2005; Mundy, 2007; Neyrinck et al., 2021). In particular, the recession of oestrogen (Hypoestrogenism) plays a prominent role in the upregulation of mitochondrial dysfunction leading to uncontrolled secretion of reactive oxygen species (ROS), consequently overwhelming the body's cellular antioxidant mechanism capacity, triggering unresolved cellular stress and a pro-inflammatory state (Au et al., 2016; Burch et al., 2014; Ginaldi et al., 2005; López-Armada et al., 2013; Mundy, 2007; Neyrinck et al., 2021; Sproston & Ashworth, 2018). Therefore, unrestrained secretion of pro-inflammatory cytokines and ROS causes chronic low-grade inflammation. Furthermore, oestrogen depletion is associated with increased central obesity, promoting the production of reactive oxygen intermediates (ROI) from adipocytes and induced mitochondrial damage through hypoxia. As a result, the body's anti-inflammatory mechanisms are disrupted, promoting the release of pro-inflammatory cytokines such as IL-6, TNF- $\alpha$  and IL- $\beta$  (Kozakowski et al., 2017; Nathan & Ding, 2010). Moreover, post-menopausal women, if sedentary, face an even greater risk of chronic inflammation and disease development than physically active women (Gleeson et al., 2011). This is because regular exercise has an anti-inflammatory effect on the body thought to occur through alterations in adipose tissue as part of weight loss or the release of the anti-inflammatory cytokine IL-10 into the bloodstream in response to elevated IL-6 post-exercise (Bianchi, 2018; Forsythe et al., 2008; Pedersen, 2006; Philips et al., 2010; Wärnberg et al., 2010).

One potential approach for mitigating chronic inflammation in post-menopausal women is restoring gut homeostasis through synbiotic supplementation (Ginaldi et al., 2005; Kazemi et al., 2020; Pandey et al., 2015). Synbiotics are a mixture comprising live microorganisms (probiotics) and substrate(s)(prebiotics), which are selectively utilised by host microorganisms to confer a health benefit on the host (Swanson et al., 2020). There are two classifications of synbiotics: complementary and synergistic. Complementary synbiotics combine probiotic(s) and prebiotic(s), which work independently to confer a health benefit(s) when administered at adequate doses. Synergistic synbiotics require a prebiotic substrate that is selectively utilised by the co-administered microorganism(s) in the probiotic component (Swanson et al., 2020).

The most widely utilised microbial species for probiotic supplementation are *Bifidobacterium* and *Lactobacillus* (Gibson & Delzenne, 2008; Plaza-Díaz et al., 2017; Yu et al., 2021). In healthy individuals, supplementation with these strains are associated with significant decreases in hs-CRP, TNF- $\alpha$ , IL-6 and IFN- $\gamma$  and increase in IL-4 and IL-10 levels (Groeger et al., 2013; Kekkonen et al., 2008; Schultz et al., 2003; Valentini et al., 2015). However, studies for specific inflammatory markers have a certain level of contradictions. In contrast, a meta-analysis of 42 publications reported that *Lactobacillus* and *Bifidobacteria* supplementation significantly decreases hs-CRP, IL-6, TNF- $\alpha$ , and IL-12; however, this is dependent on the dosage and health status of participants (Milajerdi et al., 2020). Lastly, in overweight and obese post-menopausal women, supplementation with a combination of these strains showed a significant decrease in TNF- $\alpha$  and IL-6 concentration in blood after 12 weeks (Majewska et al., 2020; Szulińska, Łoniewski, Skrypnik, et al., 2018).

When ingested, probiotics bind to epithelial cells, inhibiting opportunistic and pathogenic bacteria from colonising, thus helping maintain balance of the microbiome (Gibson & Delzenne, 2008; Plaza-Díaz et al., 2017; Yu et al., 2021). Furthermore, once bound, the probiotic bacteria activate a signalling pathway responsible for modulating the immune system and coordinating the release of beneficial antimicrobial chemicals (Manzoor et al., 2022). In contrast, prebiotics are stable dietary fibres naturally present in the human diet (Gibson & Delzenne, 2008; Manzoor et al., 2022; Rivièrè et al., 2016). Prebiotic fibres typically

utilised for supplementation include inulin, oligofructose, and fructo-oligosaccharides which, when consumed, remain undigested until they reach the large intestine, where they can influence the composition and activities of bacteria present directly or indirectly through their fermentation capacities (Gibson & Delzenne, 2008; Rivière et al., 2016). This fermentation positively influences the host's immune modulation and defence against pathogens, mineral absorption, bowel function, metabolic effects and appetite (Rivière et al., 2016; Sanders et al., 2019; Vulevic et al., 2008). Therefore, oral synbiotics could potentially mitigate or manage the development of inflammatory conditions and disorders in post-menopausal women when considering their role in immune modulation (Ginaldi et al., 2005; Kazemi et al., 2020; Pandey et al., 2015).

### **1.1 Purpose of the Study**

Synbiotics and their role in modulating inflammation have been a popular area of investigation in the last 15 years (McLoughlin et al., 2017). However, the effectiveness of synbiotics for treating chronic inflammation is still widely unknown, with many contradictions between study populations. Numerous studies show significant reductions in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines, while others demonstrate no significant impact on inflammatory markers (Asgharian et al., 2016; Brahe et al., 2015; Cicero et al., 2021; Farrokhian et al., 2019; Furrie et al., 2005; Kazemi et al., 2020; Lee et al., 2020; McLoughlin et al., 2017; Neto et al., 2013; Neyrinck et al., 2021; Yang et al., 2023). Heterogeneity between these studies regarding synbiotic effectiveness may result from various factors, including the specific prebiotic/ probiotic utilised, dose, duration, and intra and inter-individual differences in study participants. Therefore, when considering these factors, synbiotic supplements may be a plausible therapeutic strategy to help alleviate chronic inflammation in post-menopausal women.

In the same way, research involving exercises' impact on the inflammatory markers in post-menopausal women shows disparities across studies (Abd El-Kader & Al-Jiffri, 2019; Henke et al., 2018; Khalafi et al., 2021; Lopes et al., 2021; Masala et al., 2020; Monteiro et al., 2022; Philips et al., 2010; Tartibian et al., 2015). However, it is widely accepted that pro-inflammatory and anti-

inflammatory cytokines decrease and increase with exercise following an exercise intervention, regardless of exercise type, frequency and duration. Hence, exercise is crucial for promoting improvements in inflammatory cytokines in post-menopausal women.

Given the fact that minimal research has been conducted in the New Zealand context and in post-menopausal women as the sole focus, the research presented in this thesis aims to fill this knowledge gap. Our study investigated whether once-daily supplementation with  $6 \times 10^9$  CFU per 150 g of *Lactobacillus rhamnosus HN001*<sup>™</sup> and 10g Orafiti®Synergy 1 combined with exercise could actively decrease IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-8 and increase IL-10 levels in post-menopausal women. Thus, subsequently decreasing chronic inflammation, which is highly important, considering New Zealand has an ageing population, and age is associated with inflammatory conditions (Furman et al., 2019; López-Armada et al., 2013; Nathan & Ding, 2010). Therefore, investigating synbiotics' practicality as a cost-effective solution for promoting long-term health and independence for all New Zealanders is more urgent than ever, especially as our healthcare system is already under strain (Burch et al., 2014; Mundy, 2007).

## **1.2 Aims and Objectives**

### 1.2.1 Research Question

What are the effects of a dietary intervention with a synbiotic food supplement and weight-bearing exercise on inflammation status in post-menopausal women?

### 1.2.2 Aim

To investigate whether synbiotics can induce favourable changes in systemic inflammation of post-menopausal women.

### 1.2.3 Objectives

1. To determine if supplementation with synbiotics can decrease IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IL-8 pro-inflammatory cytokines and hs-CRP in post-menopausal women.

2. To determine if supplementation with synbiotics can increase the anti-inflammatory cytokine IL-10 in post-menopausal women.
3. To determine if supplementation with synbiotics is a viable therapy to reduce systemic inflammation.

#### 1.2.4 Hypothesis

1. I hypothesise that women allocated to the synbiotic group will have significantly reduced levels of the pro-inflammatory cytokines IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IL-8 and hs-CRP compared to women in the control group.
2. I hypothesise that women allocated to the synbiotic group will have significantly increased levels of the anti-inflammatory cytokine IL-10 compared to women in the control group.

### 1.3 Thesis Structure

This is a four-chapter thesis. **Chapter One** contains the study's background, intent, aim, objectives, hypotheses and researcher's contributions. **Chapter Two** is a literature review of the most current and relevant research concerning inflammation, probiotics, prebiotics and synbiotics with an emphasis on post-menopausal women. **Chapter Three** is the research study manuscript, which contains the abstract, introduction, methods, results, and discussion of findings. **Chapter Four** summarises the impact of this research in terms of the achievement of aims and objectives, strengths and limitations, recommendations and future directions for research. The **appendices** include the ethics approval letter, participant information sheet, consent form, questionnaires, and food diary.

## 1.4 Researcher Contributions

This study was part of a larger study, "COPES-4-Bones clinical study," executed by Dr. Ilesanmi-Oyelere as part of her post-doctoral research project.

**Table 1**

*Summary of researcher contributions*

Author	Contribution to Thesis
<b>Rebecca Branch</b> MSc Nutrition and Dietetic Candidate	The primary author of this thesis. Involved in completing the literature review, statistical analysis and interpretation of results for this study.
<b>Prof Marlena Kruger</b> Primary Academic Supervisor	Academic Supervisor advised on data analysis and assisted in dissemination. Revised and approved the thesis chapters and manuscript.
<b>Dr Lilian Ilesanmi-Oyelere</b> Co-Supervisor	Co-supervisor developed the study design and facilitated data collection. Assisted in the dissemination and approval of the thesis chapters and manuscript.
<b>Dr Barbara Kuhn-Sherlock</b> Statistician	Assisted with statistical data analysis.
<b>Dr Huan Zhao</b> Writing Consultant	Assisted with writing, editing, and proofreading of manuscripts.

# Chapter 2 Literature Review

## 2.0 Introduction

In this chapter, the current literature on the use of Prebiotics, Probiotics and Synbiotics to treat inflammatory conditions is discussed. Articles were selected using the search criteria shown below (Figure 1) in the databases PubMed, Google Scholar, Web of Science and Massey Discover. Reference lists from articles were used to find alternative relevant articles.

### Figure 1

*Literature review search strategy*

**Date searched:** November 2022 – October 2023

**Search Criteria:** Prebiotic\* OR Probiotic\* OR Synbiotic\* AND Postmenopas\* OR Post-menopas\* AND Inflamm\*

**Filters:** Past 15 years, nutrition and dietetics and peer reviewed.

**Electronic databases:** Massey Discover, Web of Science, PubMed, Google Scholar.

## 2.1. Inflammation and the Severity of Inflammatory-related Conditions

There is an accelerating increase in the number of people over 60 years old in New Zealand, calling for a heightened attention to their physiologic and health needs (Kiousi et al., 2022; Kwok et al., 2022). Ageing is associated with changes in the functioning of many body systems and increased nutritional requirements predisposing individuals to increased disease and infection rates (Kiousi et al., 2022; Kwok et al., 2022; Patel et al., 2014; Valentini et al., 2015). Of grave concern are changes in the immune system and the release of pro-inflammatory cytokines into the bloodstream as part of "inflammaging," which is associated with disability, frailty and mortality worldwide (Furman et al., 2019; Nathan & Ding, 2010; Neyrinck et al., 2021). Therefore, increased understanding and recognition

of these changes are required to improve the quality of life of New Zealanders and help alleviate the heavy burden on the healthcare system and its limited resources (Burch et al., 2014; Furman et al., 2019; Kwok et al., 2022; Livshits & Kalinkovich, 2022; Mundy, 2007; Nathan & Ding, 2010).

In healthy individuals, inflammation occurs under normal physiological functioning of the inflammatory cascade (Schmid-Schönbein, 2006). This inflammatory cascade is pivotal for survival as it combines cellular and tissue-level reactions required for tissue repair and infection control against endogenous and exogenous threats, e.g., pathogens, chemicals, trauma and environmental agents (López-Armada et al., 2013; Schmid-Schönbein, 2006). The inflammatory cascade works by inducing changes in the microvascular system and cellular reactions through inflammatory cytokines (signalling molecules), allowing the removal and breakdown of damaged tissues and the passage of immune cells (lymphocytes and macrophages) (López-Armada et al., 2013; Schmid-Schönbein, 2006). Upon completion of tissue restoration or elimination of pathogens, the inflammatory cascade quickly resolves within a short duration (minutes- days) (Schmid-Schönbein, 2006). However, resolution is not always achieved in some individuals, leading to an unregulated state of low-grade inflammation, otherwise known as chronic inflammation, a warning sign for vulnerability to developing organ and body system dysfunction (López-Armada et al., 2013; Nathan & Ding, 2010; Schmid-Schönbein, 2006). This can cause a vast range of chronic inflammatory-related conditions such as cardiovascular disease (CVD), diabetes mellitus, irritable bowel disease (IBD), osteoporosis, rheumatoid arthritis, and auto-immune and neurodegenerative conditions (Furman et al., 2019; Livshits & Kalinkovich, 2022; Nathan & Ding, 2010; Schmid-Schönbein, 2006; Sproston & Ashworth, 2018; Yang et al., 2017).

The mechanism behind the rise in systemic inflammation from the GI tract is triggered by a shift in commensal bacteria, known as dysbiosis of the microbiota (Al Bander et al., 2020). Commensal bacteria have a vital role in the maintenance of their host's health by generating a protective barrier against pathogenic organisms and providing essential nutrients. Therefore, it makes sense that changes in the microbiome composition impact health (Al Bander et al., 2020). As a person ages, their microbiome experiences a decrease in the

number and variety of beneficial bacteria, such as *Lactobacilli* and *Bifidobacteria*, while also subsequently becoming susceptible to the establishment of potentially pathogenic species such as facultative anaerobes and gram-negative bacteria (Patel et al., 2014; Vulevic et al., 2008). These bacteria can cause the gut to become 'leaky,' allowing bacteria and toxins the opportunity to enter the bloodstream, causing widespread inflammation. Furthermore, increased gut permeability amplifies low-grade local and systemic inflammation and oxidative stress, predisposing individuals to the onset of multimorbidity manifestations (Kiousi et al., 2022; Patel et al., 2014; Valentini et al., 2015).

A key population disproportionately impacted globally and in New Zealand by auto-immune diseases and inflammatory-related diseases is post-menopausal women, especially those in larger bodies (Al Bander et al., 2020; Szulińska, Łoniewski, Van Hemert, et al., 2018). These disproportionate levels are occurring due to the dietary and hormonal changes associated with menopause being positively correlated with elevated levels of several inflammatory markers (Burch et al., 2014; Ginaldi et al., 2005; Mundy, 2007). In particular, the remission of oestrogen (Hypoestrogenism) is a significant contributor, accelerating mitochondrial dysfunction and free radical accumulation in the body (Burch et al., 2014; Ginaldi et al., 2005; Kwok et al., 2022; Mundy, 2007; Szulińska, Łoniewski, Skrypnik, et al., 2018). Mitochondrial dysfunction adds another level of inflammation to an already disrupted immune system from inflammaging (López-Armada et al., 2013). Damaged or mutated mitochondria overproduce  $O_2^-$  ions that overwhelm the body's cellular antioxidant mechanism capacity, causing cellular stress, which cannot be effectively resolved. In cases where cellular stress is unresolved, it stimulates a pro-inflammatory response by activating either cell signalling, redox-sensitive inflammatory pathways or the inflammasome, allowing cleavage and subsequent activation of the inactive pro-inflammatory precursors IL-1 $\beta$  and IL-18 (López-Armada et al., 2013). Therefore, it is essential to identify individuals with an elevated pro-inflammatory status to prevent disease development and progression (Pepys & Hirschfield, 2003).

## 2.2 Inflammatory Markers

Cytokines are a diverse group of proteins and peptides used by researchers and health professionals to quantify the severity of inflammation in the body and monitor the effectiveness of treatments targeting immune responses (Ilesanmi-Oyelere et al., 2019; Vulevic et al., 2008; Zhang & An, 2007). Cytokines work as markers of inflammation due to their critical role in coordinating and proliferating immune responses in the form of signalling molecules. Because of their capacity to control intercellular communication, they elicit favourable or detrimental impacts on most biological processes in the body depending on the extracellular abundance and activity of cytokines present (Ilesanmi-Oyelere et al., 2019; Vulevic et al., 2008; Zhang & An, 2007). There are five main classes of cytokines: interleukins (ILs), chemokines, interferons (IFNs), colony-stimulating factors (CSF), and tumour necrosis factors (TNFs), which can be further divided into pro-inflammatory or anti-inflammatory cytokines (Ilesanmi-Oyelere et al., 2019). However, under the right circumstances, cytokines can have a dual role as part of a balancing act (Ilesanmi-Oyelere et al., 2019).

Pro-inflammatory cytokines such as IL-1 $\beta$ , IL-6, TNF-, IL-8, and IFN- $\gamma$  play a crucial role in the functioning of the immune system by combating infection and abnormal cell growth. However, they are shown to be notably high in post-menopausal women and individuals suffering from inflammation-related due to pro-inflammatory cytokine being associated with ageing, chronic inflammation and pain (Ilesanmi-Oyelere et al., 2019; Singh et al., 2022; Vulevic et al., 2008; Zhang & An, 2007).

In contrast, anti-inflammatory cytokines inhibit immune responses by regulating pro-inflammatory cytokine proliferation. There are many anti-inflammatory cytokines; however, the most potent is IL-10, as it directly suppresses the chronicity of TNF- $\alpha$  and IL-6 expression and stimulates the release of more anti-inflammatory cytokines to maintain homeostasis (Ilesanmi-Oyelere et al., 2019; Kwok et al., 2022; Zhang & An, 2007).

Along with specific cytokine analysis, C-reactive protein (CRP) is a practical and inexpensive clinical indicator for detecting elevated inflammation (Schmid-Schönbein, 2006). CRP is an acute-phase protein synthesised in the liver when

the body first initiates an inflammatory reaction to a stimulus (antigen) (Pepys & Hirschfield, 2003). Thus, it becomes concentrated in blood whenever systemic inflammation increases. Furthermore, as CRP is uninfluenced by diurnal and seasonal changes and very few medications, it makes it a sensitive marker for systemic inflammation (Pepys & Hirschfield, 2003; Schmid-Schönbein, 2006; Sproston & Ashworth, 2018).

### **2.3 Plausible Therapeutic Solution**

Even though a large proportion of our population is impacted daily, therapeutic solutions to treat inflammatory conditions are limited, leaving individuals to rely on multiple pharmacologic agents to alleviate disease symptoms (Kiousi et al., 2022; Sabico et al., 2019; Vaghef-Mehrabany et al., 2014). However, these agents can cause polypharmacy, drug interactions and many undesirable side effects (Kiousi et al., 2022; Sabico et al., 2019; Vaghef-Mehrabany et al., 2014). Therefore, an affordable and natural therapy that can modulate the root cause of chronic inflammation is needed more than ever—one that takes a nutraceutical approach to influence inflammatory status (Kiousi et al., 2022; Sabico et al., 2019).

It is well known that antibiotic therapy influences inflammation by inducing dysbiosis and that the microbiota is a modifiable cause of persistent low-grade inflammation when in dysbiosis regardless of ethnicity and study design (Livshits & Kalinkovich, 2022; Patel et al., 2014; Sanders et al., 2019; Vaghef-Mehrabany et al., 2014). Therefore, a therapeutic approach that potentially restores gut homeostasis, such as prebiotic, probiotic or synbiotic supplementation, may prevent the unresolved inflammatory cascade, efficiently controlling disease manifestation and progression (Patel et al., 2014; Sanders et al., 2019; Vaghef-Mehrabany et al., 2014; Valentini et al., 2015).

## **2.4 Probiotics for the management of inflammation**

### **2.4.1 Provision of Probiotics**

In accordance with the World Health Organisation, probiotics "are live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (World Health Organisation and Food and Agriculture Organisation of the United Nations, 2006). Probiotics are readily available and marketed to the public in a diverse range of foods, beverages, and supplements worldwide, with a vast majority being dairy products (Gibson & Delzenne, 2008; Yu et al., 2021). Lactic acid bacteria are an example of probiotic microorganisms (Plaza-Díaz et al., 2017). The most widely utilised strains for supplementation are *Bifidobacterium*, *Enterococcus*, and *Lactobacillus* species, which, when ingested, cause microbial transformation of the intestinal microbiota through various mechanisms, including immune modulation, production of organic acids and antimicrobial compounds, improving gut barrier integrity and enzyme formation (Gibson & Delzenne, 2008; Plaza-Díaz et al., 2017; Yu et al., 2021).

### **2.4.2 Effectiveness of probiotics in healthy individuals and different disease states.**

In recent years, a great deal of research has focused on the effects of probiotics on inflammatory markers and a range of inflammatory-related conditions. However, much of our general understanding and knowledge falls back on evidence from in-vitro, animal, cell culture or ex-vivo human model studies (Sanders et al., 2019). Therefore, conclusions from these studies cannot be carried over to humans easily.

#### *i. Healthy individuals*

In healthy individuals of the general population, oral consumption of probiotics was associated with a significant decrease in TNF- $\alpha$  and IL-6 secretion when 22 individuals were supplemented with  $1 \times 10^{10}$  CFU *Bifidobacterium infantis* 35,624 for 6-8 weeks (Groeger et al., 2013). However, no change was observed in CRP levels of the participants (Groeger et al., 2013). These findings are consistent with a second study that orally administered  $2 \times 10^9$  CFU *Lactobacillus rhamnosus ssp GG* for five weeks in ten healthy volunteers (Schultz et al., 2003). Supplementation significantly decreased TNF- $\alpha$ , IL-6 and IFN- $\gamma$  and

significantly increased IL-4 and IL-10 (Schultz et al., 2003). A third randomised, double-blind control study comparing the effects of three different bacteria on the inflammatory markers of 62 healthy volunteers did not agree with certain findings from the previous studies as only individuals supplemented with *L. rhamnosus GG* and *P. freudenreichii ssp. shermanii JS* had significantly lower hs-CRP levels compared to the *B. animalis ssp. lactis Bb12* and placebo groups (Kekkonen et al., 2008). Additionally, only individuals supplemented with *L. rhamnosus GG* had a significant decrease in TNF- $\alpha$  and *B. animalis ssp. lactis Bb-12*, a decrease in IL-2. No significant changes were found between the different bacteria groups and placebo for IL-6, IL-10, IL-12, IFN- $\gamma$ , IL-1 $\beta$  and IL-8 (Kekkonen et al., 2008). From these studies, there is some evidence to vouch for the utilisation of probiotics as an adjuvant therapy to mitigate inflammation in healthy adult subjects. Nevertheless, this tends to be strain specific.

## ii. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a debilitating auto-immune disease with an unknown aetiology characterised by inflammatory changes of the synovial tissue in joints, cartilage and bone (Hatakka et al., 2003; Vaghef-Mehrabany et al., 2014). When *Lacticaseibacillus rhamnosus* LC70536 was supplemented for 12 months in 21 RA patients 40-60 years, no significant changes in their hs-CRP, IL-6, TNF- $\alpha$ , serum MPO, IL-10, or IL-12 levels were detected compared to the placebo (Hatakka et al., 2003). However, the participants had slightly increased serum IL-1 $\beta$  and erythrocyte sedimentation rate (a non-specific biomarker for chronic inflammation) (Hatakka et al., 2003). Interestingly, patients who received the probiotic supplement reported a decline in the presence of swollen and tender joints post-intervention (Aldwin & Nath, 2019; Hatakka et al., 2003). However, in terms of inflammatory markers, probiotic supplementation could not reduce systemic inflammation levels in patients with RA. However, two studies disagree with this nil effect conclusion on inflammatory markers, as daily supplementation with  $1 \times 10^8$  CFU of *Lactobacillus casei 01* for eight weeks had a significant impact on reducing TNF- $\alpha$ , IL-6, IL-12, and hs-CRP while also increasing IL-10 in women 26-54 years with RA (Vaghef-Mehrabany et al., 2014). No significant changes were seen in IL-1 $\beta$  levels (Vaghef-Mehrabany et al., 2014). Additionally, multispecies probiotic supplementation for 60 days in

the third study caused a significant reduction in TNF- $\alpha$  and IL-6 compared to the control group. No significant impact was reported for hs-CRP, ESR, and IL-10 (Cannarella et al., 2021). Therefore, supplementation has the potential to regulate the immune system to reduce chronic inflammation in individuals with RA.

### iii. *Irritable bowel disease*

Irritable bowel disease (IBD) is a term used to describe four systemic gastrointestinal (GI) disorders: ulcerative colitis (UC), crohn's disease (CD), pouchitis, and microscopic colitis, in which shifts in the pathogenic to commensal bacteria ratios modify the GI's innate immunity (Groeger et al., 2013; Plaza-Díaz et al., 2017). In an RCT, *Bifidobacterium infantis* 35,624 was used to assess the impact of oral administration of 6-8 week supplementation on inflammatory markers and plasma cytokine levels in 22 UC patients (Groeger et al., 2013). Following supplementation, CRP and IL-6 levels were significantly reduced compared to the placebo. However, no effect was observed for TNF- $\alpha$  (Groeger et al., 2013). In comparison, another study conducted in 31 UC patients receiving  $1 \times 10^{10}$  CFU *reuteri* ATCC 55730 supplementation for eight weeks found a significant reduction in IL-1 $\beta$ , TNF- $\alpha$  and IL-8 levels and a significant increase in IL-10 levels compared to the placebo group (Oliva et al., 2012). A further study using a multispecies probiotics supplementation in 142 asymptomatic adults with IBD for four weeks led to no significant reductions in CRP and ESR, therefore, systemic inflammation (Bjarnason et al., 2019). For patients with UC specifically, there was a significant decrease in Calprotectin. Despite the disagreements in the outcomes of specific inflammatory markers, which may be due to differences in the strain of bacteria used in each probiotic, there is rising evidence that probiotic supplementation may help mitigate the increased inflammatory state of people with UC (Bjarnason et al., 2019). No papers looking at the inflammatory markers of the other types of IBD alone were found. Therefore, further research is needed to determine the effect of the probiotic in this area, especially in the other forms of IBD.

### iv. *Diabetes*

Diabetes is a chronic inflammatory disease in which the body's ability to produce or respond to the hormone insulin is impaired (Sabico et al., 2019). In an effort

to regulate the inflammation associated with this disease, probiotic supplementation was trialed in 61 individuals with diabetes aged 35 to 70. Unfortunately, CRP, TNF- $\alpha$ , IL-6, resistin and blood lipids remained unchanged after six months of supplementation with a  $5 \times 10^9$  CFU/g multispecies probiotic (Sabico et al., 2019). Similarly, multispecies probiotic supplementation at a dose of  $8 \times 10^9$  CFU/day for 12 weeks in men and women 45 to 85-year-old was ineffective at lowering TNF- $\alpha$  and TGF- $\beta$  levels compared to the placebo group (Mafi et al., 2018). However, gene expression of IL-1 and CRP was significantly reduced, as were glycaemic control markers and blood lipid levels in this study (Mafi et al., 2018). In a study of women with gestational diabetes, eight-week supplementation with a multispecies probiotic significantly decreased their hs-CRP, IL-6 and TNF- $\alpha$  levels (Jafarnejad et al., 2016). However, it was unable to induce changes in IL-10 and IFN- $\gamma$  (Jafarnejad et al., 2016). In conclusion, supplementation with probiotics may help modulate some inflammatory markers in this population, but more research is required to determine the dosage and strains most effective.

v. *Metabolic Syndrome*

Metabolic syndrome is an assemblage of physiological and biochemical risk factors linked to cardiovascular disease (CVD), Type 2 diabetes mellitus (T2D) and some cancers. These risk factors include dysglycemia, insulin resistance, dyslipidaemia, hypertension, and obesity (Alberti et al., 2009; Gentles et al., 2007; Kerekou-Hode et al., 2019). A Spanish study investigating the effects of probiotic supplementation on the inflammatory status of individuals with metabolic syndrome found that IL-6 was significantly reduced after 12 weeks of supplementation with *L. reuteri V3401* at a dose of  $5 \times 10^9$  CFU/ day (Tenorio-Jiménez et al., 2019). However, supplementation had no observed effects on CRP, TNF- $\alpha$ , IL-8, MCP-1 or resistin compared to the control group. Further research is required to confirm the accuracy of these findings.

vi. *Psoriasis*

Psoriasis is a chronic, inflammatory skin disease that causes itchy, scaly patches with no cure. However, the implications of probiotic supplementation in individuals with this disease are promising (Groeger et al., 2013; Zeng et al., 2021). An RCT conducted in 26 patients 18-60 years with mild to moderate

chronic plaque psoriasis reported significant reductions in CRP and TNF- $\alpha$  levels when  $1 \times 10^{10}$  CFU *Bifidobacterium infantis* 35,624 was consumed for 6-8 weeks (Groeger et al., 2013). Unfortunately, IL-6 levels remained unchanged compared to the control (Groeger et al., 2013). Another study of 46 patients reported considerable reductions in serum LPS, hs-CRP and IL-1 $\beta$  when taking  $1.6 \times 10^9$  CFU/g multispecies probiotics for two months (Moludi et al., 2022). Additionally, those in the supplement group had significantly improved Quality of Life (QoL) and Psoriasis Area and Severity Index (PASI) scores compared to the placebo group (Moludi et al., 2022). These studies demonstrate the potential use of probiotics to reduce systemic inflammation and associated symptoms with Psoriasis.

vii. *Alzheimer's disease*

Alzheimer's disease is a form of dementia, which is a progressive and fatal brain disease coupled with the reduction of cognitive ability and loss of memory (Tamtaji et al., 2019). To attenuate inflammation and oxidative stress associated with the disease, an RCT was conducted in 55–100-year-old individuals with Alzheimer's. The study found that co-supplementation with 200  $\mu$ g/day selenium and two capsules of  $2 \times 10^9$  CFU/day multispecies probiotic for 12 weeks resulted in a significant reduction in serum hs-CRP and TNF- $\alpha$  compared to the placebo group (Tamtaji et al., 2019). However, no significant effects were observed for IL-8 and TGF- $\beta$  levels (Tamtaji et al., 2019). Unfortunately, no sole probiotic arm was included in the study to contribute to the observed decrease in inflammation.

viii. *Meta-analysis*

From a meta-analysis of 42 randomised clinical trials, probiotic supplementation significantly reduced hs-CRP compared to the control group, but only if the strain of bacteria used was *Lactobacillus* and *Bifidobacteria* or at doses  $\geq 100$  CFU/day (Milajerdi et al., 2020). The health status of the participants also appeared to be an influencing factor of hs-CRP. Supplementation significantly reduced IL-6; however, this was dependent on the health status of the participants from the studies reported. TNF- $\alpha$  was significantly reduced if dosages were  $>100$  CFU/day and did not involve healthy individuals or those with allergies or auto-immune diseases as participants. Probiotic supplementation caused a significant

reduction in IL-12 if the studies' duration exceeded ten weeks. However, probiotics supplementation had no significant effect on the serum concentration of IL-1 $\beta$  unless performed solely in females or at doses  $< 1$  or  $\geq 1$  CFU/day. Supplementation caused a lower concentration of IL-4. However, no consensus decision can be made due to the lack of studies investigating changes in IL-4. Probiotic supplementation did not significantly affect IL-8, IL-10, IL-17 or IFN- $\gamma$ , regardless of strain, disease state or dosage (Milajerdi et al., 2020). Another meta-analysis solely looking at the effectiveness of probiotic yoghurt for reducing inflammation disagreed with the above findings. Oral consumption of 20-450 grams/d had no significant effect on IL-6 and TNF- $\alpha$  (Mousavi et al., 2020). However, CRP was significantly reduced, but only in individuals with a BMI  $>25\text{kg/m}^2$  or when 200g (at least one serving) of yoghurt was consumed for over  $>8$  weeks (Mousavi et al., 2020). Overall, from these analyses' there is limited evidence to support using probiotic supplementation to modify specific inflammatory markers.

### **2.4.3 Effectiveness of Probiotics in Post-menopausal Women**

#### *i. Healthy Individuals*

In individuals 65 to 85 years who had elevated hs-CRP concentrations  $> 3$  mg/L, consumption of two multispecies capsules per day containing  $1.12 \times 10^{11}$  CFU combined with personalised diets for eight weeks caused a significant reduction in ESR, fibrinogen and IL-6. No significant changes from baseline were recorded for TNF- $\alpha$  or IL-10. Unfortunately, no placebo arm was included to quantify the observed reduction of inflammation in this population (Valentini et al., 2015).

#### *ii. Osteoporosis*

Osteoporosis is a disease characterised by deteriorated bone mineral density, resulting in reduced bone strength and increased risk of fractures (Nilsson et al., 2018). As inflammatory cytokines stimulate the disease's progression by stimulating osteoclast activity and inhibiting osteoblast activity, probiotics have been trialled to attenuate low-grade chronic inflammation (Damani et al., 2023). In one study, administration of a multispecies probiotic (GeriLact) for six months in post-menopausal women 50-72 years with osteopenia showed significantly improved vitamin D status and reduced TNF- $\alpha$  levels. However, no change was

observed in IL-1 $\beta$  (Jafarnejad et al., 2017). In comparison, a second study conducted in women 75- 80 years with low BMD found that  $1 \times 10^{10}$  CFU/d *L. reuteri* 6475 for one year resulted in no significant improvements in their TNF- $\alpha$  and hs-CRP levels (Nilsson et al., 2018). Results from a systematic review and meta-analysis of 5 RCTs from across Denmark, Japan, Sweden and Iran looking at probiotic supplementation in post-menopausal women showed that daily supplementation between 5- 12 months was also ineffective at decreasing TNF- $\alpha$  compared to women receiving the placebo (Yu et al., 2021). Therefore, different studies in a similar population do not positively support the impact of probiotics on systemic inflammatory markers collectively.

### *iii. Obesity*

In obese post-menopausal women, multispecies probiotic supplementation for 12 weeks led to significant decreases in TNF- $\alpha$  and IL-6 (Szulińska, Łoniewski, Skrypnik, et al., 2018). These reductions in TNF- $\alpha$  and IL-6 appeared dose-dependent as women allocated to the Low Dose (LD) probiotic supplementation ( $2.5 \times 10^9$  CFU) saw a more significant decline in IL-6 compared to the High Dose Group (HD) supplemented with ( $1 \times 10^{10}$  CFU). In the case of TNF- $\alpha$ , the HD group saw a more significant decline than the LD group (Szulińska, Łoniewski, Skrypnik, et al., 2018). In contrast, 12-week supplementation of the  $2.5 \times 10^9$  CFU multispecies probiotic (Ecologic® BARIER) in a similar population effectively reduced TNF- $\alpha$  and homocysteine concentrations and improved the women's blood lipid profiles and total antioxidant potential (Majewska et al., 2020).

#### **2.4.4 Summary**

The results of probiotic use for attenuating chronic long-term systemic inflammation remain controversial as study outcomes in similar populations often have opposing conclusions. These differences may only partly be accounted for by differences in the duration of supplementation, dosage, the health status of participants, and the bacteria strain(s) utilised. Regardless, when pooled studies find evidence in favour of probiotic supplementation; therefore, probiotics cannot be dismissed as a simplistic and cost-effective way to reduce inflammation. Further research is necessary to determine the most

appropriate formulation and duration of treatment in terms of efficacy for specific chronic inflammatory diseases and how they contribute towards symptoms of the disease as related to inflammatory markers in post-menopausal women. In addition, they need to identify how each strain interacts with cell receptors and cell signalling cascades to influence inflammatory markers.

## **2.5 Prebiotics for the management of inflammation**

### **2.5.1 Provision of Prebiotics**

Prebiotics have been used for years to improve the health and well-being of people worldwide through a dietary-first approach that is affordable and accessible to the public (Sanders et al., 2019). Prebiotics are stable dietary substrates readily added to food, which, when consumed, remain undigested until they reach the intestines (Rivière et al., 2016). Once in the intestines, prebiotics influence the composition and activities of the bacteria present either directly or indirectly through their fermentation capacities (Rivière et al., 2016). This fermentation positively influences the host's immune modulation, defence against pathogens, mineral absorption, bowel function, metabolic effects and appetite (Rivière et al., 2016; Sanders et al., 2019; Vulevic et al., 2008). To date, the most well-known prebiotic fibres are carbohydrates found within plants such as chicory roots, wheat, onion and bananas (Gibson & Delzenne, 2008; Rivière et al., 2016). The most frequently studied prebiotic fibres include inulin, oligofructose, and fructo-oligosaccharides (FOS), which, when consumed, interact with the commensal bacteria such as *Lactobacilli* or *Bifidobacteria*, enhancing their activity and proliferation while inhibiting harmful bacteria such as *Enterobacteria* from becoming established (Gibson & Delzenne, 2008; Patel et al., 2014; Rivière et al., 2016; Vulevic et al., 2008).

### **2.5.2 Effectiveness of prebiotics in the general population and disease states.**

Analysis of literature from the past 15 years shows varying levels of contradiction between study outcomes.

i. *Healthy individuals*

A study of 19 healthy elderly from a nursing home saw positive outcomes when 8g of FOS prebiotic powder was supplemented daily for three weeks. (Guigoz et al., 2002) Supplementation significantly reduced IL-6 and promoted a 2-log increase of *bifidobacteria*, causing the women's microbiome to more closely resemble the expected microbiota composition of a young adult (Guigoz et al., 2002). Similar results were achieved from a 24-week cross-over intervention study in 44 older adults 64–79 years, where 55.5g/d prebiotic GOS mixture was able to significantly decrease the production of TNF- $\alpha$  and IL-6 by week five and IL-1 $\beta$  by week ten compared to the placebo groups (Vulevic et al., 2008). However, a significant reduction in IL-10 was observed at week five and another at week 10 (Vulevic et al., 2008). No significant effects were observed for IL-8. However, a clear positive association was witnessed between the number of *bifidobacteria* in the women's faecal samples and immune cell activity, showing that prebiotics worked indirectly by changing the microbiome's composition for better immunomodulation (Vulevic et al., 2008). This probable anti-inflammatory effect and increase in *bifidobacteria* are significant in the elderly as it is generally reported that the number of *bifidobacteria* in the microbiota diminishes with age, allowing the overgrowth of *clostridium perfringens*, *enterococci* and *enterobacteriaceae*, leading to gastritis, hypochlorhydria and alterations in the immune system's responsiveness (Vulevic et al., 2008). In comparison, another study involving 43 healthy free-living elderly  $\geq 70$  years showed no significant effect on IL-4, IFN- $\gamma$  and lymphocyte proliferation compared to the control group after 28 weeks of 6 g/d prebiotic mixture (Bunout et al., 2002). Thus, this study opposes the immunological finding of prebiotics observed in previous studies.

ii. *Overweight*

A mini-review including six studies with overweight and obese adults found that supplementation with either inulin or oligofructose caused a significant reduction in hs-CRP if the supplementation duration was greater than 30 days (da Silva Borges et al., 2019). In comparison, another study showed that oligofructose supplementation among overweight women for eight weeks significantly reduced both IL-6 and TNF- $\alpha$  (Dehghan et al., 2014). However, the observed increase in

IL-10 and decreases in hs-CRP and IFN- $\gamma$  were not significant, therefore opposing the mini-reviews findings.

*iii. Diabetes*

A meta-analysis of 11 publications on diabetics found that prebiotic supplementation significantly decreased CRP, IL-6, IL-12, LPS, TNF- $\alpha$ , endotoxin, and malondialdehyde (Colantonio et al., 2020). In addition, supplementation significantly increased IL-4 and the total antioxidant capacity of the participants involved in the studies, providing evidence that prebiotics may reduce inflammation in the diabetic population, making it an appropriate adjuvant therapy. However, as 70% of the participants were female, it is unclear whether the prebiotic's beneficial effect on inflammation carries over into the male diabetic population.

*iv. Meta-analysis*

A 29-study meta-analysis showed that administering prebiotics could significantly decrease serum CRP concentration, but upon subgroup analysis by prebiotic type, CRP only significantly decreased with oligosaccharide supplementation and not with polysaccharides (McLoughlin et al., 2017). Furthermore, prebiotic supplementation did not significantly affect TNF- $\alpha$  or IL-6, regardless of whether oligosaccharides or polysaccharides were used. Further investigation on the effects of other inflammatory markers from the meta-analysis was unattainable due to the lack of quality and quantity of studies solely looking at prebiotics.

### **2.5.3 Effectiveness of prebiotics in post-menopausal women.**

Unfortunately, no specific studies examining the effectiveness of prebiotics in post-menopausal women concerning inflammatory markers could be included in this literature review. Current research in post-menopausal women involving prebiotics tends to focus on managing or regulating menopause symptoms, lipids, insulin resistance and cancer risk (Shafie et al., 2022; Singh et al., 2022; Skiba et al., 2019; Wong et al., 2010). Therefore, further studies are needed to evaluate the impact of prebiotic supplementation, specifically on inflammatory markers in post-menopausal women.

## **2.5.4 Summary**

In summary, there is insufficient literature free from contradictions in humans available to fully determine whether prebiotics can positively impact systemic inflammation, particularly in post-menopausal women. This is because certain studies report no significant changes in pro and anti-inflammatory cytokines, while others reveal notable variations in inflammatory cytokine concentrations. However, what can be deciphered from the current literature is that not all bacterial strains in the microbiota interact with each prebiotic in a similar manner; each strain has a preferred prebiotic, and it takes different durations for specific changes in inflammatory cytokines to be observable in blood, highlighting the complexity of working with prebiotics for immunomodulation. Therefore, future research needs to prioritise the standardisation of study designs to provide clearer insights into the effectiveness of prebiotics as a therapeutic intervention for modulating systemic inflammation.

## **2.6 Synbiotics for the Management of Inflammation**

### **2.6.1 Provision of Synbiotics**

Synbiotics are also proposed as an effective therapeutic agent for managing inflammatory disorders and age-related changes (Plaza-Díaz et al., 2017). Synbiotics are mixtures comprising live microorganisms (probiotics) and substrate(s)(prebiotics), selectively utilised by host microorganisms that confer a health benefit on the host (Swanson et al., 2020). Furthermore, to be classified as a synbiotic, the probiotic(s) and prebiotic(s) components must have undergone a safety assessment for their intended use and show consistent performance. There are two categories of synbiotics: complementary and synergistic. Complementary synbiotics combine probiotic(s) and prebiotic(s), which work autonomously to confer a health benefit(s) when administered at adequate doses. In contrast, Synergistic synbiotics require a prebiotic that is selectively utilised by the co-administered probiotic bacteria. Currently, the majority of synbiotic formulations employed or available to the general public are complementary synbiotics (Swanson et al., 2020).

## 2.6.2 Effectiveness of synbiotics in healthy individuals and different disease states.

### *i. Healthy Individuals*

To improve inflammatory markers in middle-aged individuals, synbiotic supplementation with *Bifidobacterium animalis lactis* and FOS was utilised in a primarily female study sample. As a result, IL-6, IL-8, IL-17a and IFN- $\gamma$  were significantly lower after 30 days compared to the baseline values (Neyrinck et al., 2021). In contrast, an RCT in healthy men and women 18-65-year-olds found that  $1.5 \times 10^{10}$  CFU Ecologic® 825 and 10g FOS were unable to significantly modify TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-8 or MCP-1 in blood compared to the control group after a two week supplementation period (Wilms et al., 2016). These results were consistent in accordance with a double-blind pilot study where 3-month synbiotic supplementation with 6g FOS and multispecies probiotic did not significantly affect TNF- $\alpha$  and IL-6 levels in free-living 60-75-year-olds. Therefore, these synbiotics do not reduce inflammation in an older population (Neto et al., 2013). However, when 167 publications were analysed as part of systematic reviews and meta-analyses, oral probiotic/synbiotic supplementation affected a wide range of inflammatory markers in healthy individuals. A significant reduction in CRP levels was reported at supplemental doses greater than  $5 \times 10^{10}$  CFU and if participants had a BMI  $< 25 \text{ kg/m}^2$  (Kazemi et al., 2020). Overall, supplementation did not significantly affect IL-10 unless participants were  $> 49$  years old or given doses  $\geq 5 \times 10^{10}$  CFU. Supplementation caused a significant decrease in TNF- $\alpha$  and a marginal reduction in IL-1 $\beta$ . Furthermore, IL-6 was significantly reduced in participants  $< 49$  years. Additionally, synbiotic supplementation was more effective at reducing IL-6 than probiotics. However, IL-4, IL-8, IL-12 TGF- $\beta$ , IFN- $\gamma$  all remained uninfluenced by supplementation (Kazemi et al., 2020). Therefore, some evidence supports the use of synbiotic supplementation to mitigate inflammation in healthy individuals.

### *ii. Irritable Bowel Disease*

The effect of synbiotic supplementation for one month in 18 patients with UC significantly reduced TNF- $\alpha$ , IL-1 $\alpha$  and CRP levels (Furrie et al., 2005). However, no significant difference was seen in IL-10 compared to the placebo (Furrie et

al., 2005). Moreover, despite the reduction in overall systemic inflammation, no changes were detected in the antimicrobial peptide's human beta-defensins (indicators used to assess local inflammation of the gut epithelium and immunity in UC patients). However, the synbiotic, which contained  $2 \times 10^{11}$  CFU *Bifidobacterium longum* and inulin-oligofructose, was highly effective in inducing higher numbers of *Bifidobacteria* on the mucosal surface compared with placebo participants. Notably, the post-supplemental biopsies from the supplement group also showed visually less colitis at the macroscopic and microscopic levels compared to the placebo (Furrie et al., 2005). From the meta-analysis mentioned previously, supplementation significantly reduced CRP and TNF- $\alpha$  (Kazemi et al., 2020). However, supplementation did not significantly affect IL-4, IL-6, IL-8 and IL-10 (Kazemi et al., 2020). Therefore, opposing evidence exists regarding synbiotics' usefulness in decreasing systemic inflammation and disease state presentations.

### *iii. Metabolic Syndrome*

A synbiotic supplement containing four *Lactobacilli* species at a combined dose of  $6 \times 10^9$  CFU per capsule and the prebiotic fibres inulin and FOS was able to significantly reduce hs-CRP and TNF- $\alpha$  compared to the placebo and baseline measurements in elderly with MetS after 60 days (Cicero et al., 2021). Additionally, there were marked improvements in CVD risk factors, insulin resistance markers, fasting plasma insulin and lipid profiles (Cicero et al., 2021).

### *iv. Overweight*

A randomised, double-blinded control trial was conducted on overweight and/or diabetics 50–85 years (Farrokhian et al., 2019). Individuals in the intervention group received a multispecies probiotic at a concentration of  $6 \times 10^9$  CFU/g and 800 mg inulin daily. After 12 weeks of supplementation, participants saw a significant decrease in hs-CRP compared to baseline and the placebo group. However, no changes were observed in the oxidative stress of the participants (Farrokhian et al., 2019).

#### v. *Meta-Analysis*

A meta-analysis of 26 synbiotic studies primarily comprising of *Lactobacillus* or *Bifidobacterium* species combined with a single oligosaccharide prebiotic overall reported a significant reduction in CRP and TNF- $\alpha$  blood compared to the control group (McLoughlin et al., 2017). No significant difference in serum IL-6 was observed. Further investigation of synbiotic supplementation on inflammatory markers was not conducted due to the limited availability of studies (McLoughlin et al., 2017).

### **2.6.3 Synbiotics in Post-menopausal Women**

#### i. Healthy Individuals

Synbiotic supplementation in 37 otherwise healthy post-menopausal women caused a non-significant reduction in CRP after three weeks and a further decrease after a three-week washout period (Lee et al., 2020). The synbiotic drink containing multispecies bacteria, dietary fibre, FOS, xylo-oligosaccharides, and isomalto-oligosaccharides caused a significant increase in the commensal bacteria of the microbiota and an improvement in overall gut health. However, these both returned to their baseline measurements during the washout period (Lee et al., 2020). Further investigation into other inflammatory cytokine markers is required to assess synbiotics' ability to decrease systemic inflammation in this group.

#### i. Obesity

From a study involving 76 overweight or obese post-menopausal women with a history of hormone-receptor-positive breast cancer,  $1 \times 10^9$  CFU/day multispecies probiotic and 35g FOS significantly decreased TNF- $\alpha$ , hs-CRP and adiponectin after eight weeks (Raji Lahiji et al., 2021). In comparison, a Danish study in obese post-menopausal women ages 40–70 found that supplementation with  $9.4 \times 10^{10}$  CFU of *L. paracasei* F19 and 10g of flaxseed mucilage (30% dietary fibre) for six weeks had no significant effect on hs-CRP, TNF- $\alpha$  or IL-6 when compared to the placebo despite changes been seen in the microbiota (Brahe et al., 2015). Therefore, conflicting evidence supports synbiotic supplementation for reducing systemic inflammation.

#### **2.6.4 Summary**

In conclusion, the effectiveness of synbiotic supplementation for treating chronic inflammation varies across healthy individuals and those with health conditions. Current studies show significant reductions in pro-inflammatory cytokines and an increase in anti-inflammatory cytokines, while others demonstrate no significant impact on inflammatory markers. However, it is imperative to note that the heterogeneity around the effectiveness of synbiotic supplementation may depend on various factors, including the specific strain(s) and doses of probiotics used, prebiotic fibre, the duration of supplementation, intra- and inter-individual differences in the microbiome composition, genetics and nutritional intake related to differences in nationalities.

In terms of post-menopausal women, current evidence to support synbiotic supplementation is limited. Existing studies in post-menopausal women have shown significant and non-significant effects in modifying inflammation markers. Thus, further research which utilises standardised protocols between studies, larger sample sizes, and minimum intervention periods are needed to provide robust evidence of the potential benefits of synbiotics for modulating inflammatory markers in post-menopausal women.

#### **2.7 Safety Considerations**

Based on current evidence, oral supplementation with probiotics, prebiotics, or synbiotics has minimal risk in immunocompetent individuals (Barrea et al., 2023; World Health Organisation and Food and Agriculture Organisation of the United Nations, 2006). However, bacteraemia, sepsis, endocarditis, and cholangitis have been induced after supplementation; therefore, caution is advised for hospitalised or immunocompromised individuals due to a significantly increased risk of infection and morbidity (Barrea et al., 2023). In addition, concerns exist around regular supplementation being a potential vehicle for antibiotic resistance as drug resistance strains of *Lactobacilli* and *Bifidobacteria* may be in circulation due to no standardised international testing methods to decipher between drug-insensitive and sensitive strains (Gupta & Garg, 2009; Sanders et al., 2010; World Health Organisation and Food and Agriculture Organisation of the United Nations, 2006). Furthermore, it is unknown if

resistance is transmissible to other bacteria in the microbiota (Gupta & Garg, 2009; Sanders et al., 2010; World Health Organisation and Food and Agriculture Organisation of the United Nations, 2006). Moreover, as supplements are unregulated by Medsafe NZ, it falls on the manufacturer to ensure products are made to an acceptable quality and are safe for use (Gupta & Garg, 2009). Therefore, there is an element of risk associated with potentially unlabelled microorganisms and allergenic material being in the formulation, e.g., dairy proteins or *enterococci*, now shown to be associated with vancomycin resistance (Gupta & Garg, 2009; Sanders et al., 2010). Additionally, it is still unclear which supplementation type is the most effective and safest for decreasing inflammation, especially in post-menopausal women. Therefore, before commencing supplementation, a risk-to-benefit assessment is required to ensure the prevention, treatment, cure or mitigation of disease outweighs all associated risks in a target population.

## **2.8 Exercise**

Regular exercise is important for maintaining health and reducing the risk of non-communicable chronic diseases; therefore, it may be an alternative method for modulating inflammation in post-menopausal women (Khalafi et al., 2021; Monteiro-Junior et al., 2018). However, the exact mechanisms behind exercise's role in modulating inflammation is unascertained (Khalafi et al., 2021).

One potential hypothesis is that continual muscle contractions and subsequent muscle damage as part of exercise exponentially increases the production of IL-6, causing the release of anti-inflammatory cytokines IL-1 $\alpha$  and IL-10 into circulation, inhibiting the production of pro-inflammatory cytokines, TNF- $\alpha$ , IL-1 $\beta$  and IL-8 (Pedersen, 2006; Wärnberg et al., 2010; Woods et al., 2012). Additionally, contraction-induced IL-6 is thought to influence metabolism by stimulating glucose availability, leading to more efficient fuel utilisation during exercise, increasing mitochondrial density and enhanced fatty acid oxidation (Bianchi, 2018; Pedersen, 2006; Philips et al., 2010; Wärnberg et al., 2010).

A second hypothesis involves exercise reducing inflammatory markers over time by evoking weight loss (Bianchi, 2018; Forsythe et al., 2008). In particular, a reduction of adipose tissue in individuals who have a higher degree of adipose

tissue and adipocyte hypertrophy (increased size) causes a significant decline in CRP and pro-inflammatory cytokines like TNF- $\alpha$  allowing for stabilisation of the body's inflammatory profile and antioxidant defence systems (Bianchi, 2018; Forsythe et al., 2008; Wärnberg et al., 2010; Woods et al., 2012). Therefore, persistent acute bouts of exercise may reduce chronic inflammation over time.

### **2.8.1 Effects of exercise on inflammatory markers in post-menopausal women**

In healthy post-menopausal women, low-moderate intensity exercise for 16 weeks caused significant reductions in IL-6, IL-1 $\beta$  and TNF- $\alpha$  compared to baseline and the control group (Tartibian et al., 2015). In comparison, bi-weekly high-intensity interval training for one month in obese post-menopausal sedentary women also significantly decreased IL-6 (Henke et al., 2018). No significant change was observed for IL-1 $\beta$  and MCP-1. However, unfortunately, no control group was included to allow comparison to sedentary post-menopausal women (Henke et al., 2018).

Another study found that increased daily activities and at least one hour of strenuous physical activity per week for 24 months increased IL-1 $\alpha$  and IL-6 levels in healthy post-menopausal women. No significant effect was found for TNF- $\alpha$ , IL-1 $\beta$  and CRP levels (Masala et al., 2020). Similarly, a study on sedentary older women 60- 80 found that the sedentary group had significantly higher serum TNF- $\alpha$ , IL-1 $\beta$ , IFN- $\gamma$  and IL-17a and lower serum IL-10 than the exercise group (Lopes et al., 2021).

In sedentary, obese post-menopausal women, moderate- to high-intensity resistance training significantly increased IL-6 and decreased TNF- $\alpha$  after ten weeks (Philips et al., 2010). No changes in serum IL-1 $\beta$  were observed consequent to exercise time point or training (Philips et al., 2010). In contrast, resistance training for 12 weeks in obese post-menopausal women significantly reduced CRP and TNF- $\alpha$  while increasing IL-10 production compared to the control group. (Phillips et al., 2012). No significant effect occurred in adiponectin or IL-6 concentrations (Phillips et al., 2012). Furthermore, three months of either resistance or aerobic exercise in post-menopausal obese women significantly decreased TNF- $\alpha$ , IL-2, IL-4, IL-6, and CRP. However, the most prevalent

decreases occurred in the aerobic exercise group, meaning aerobic exercise is a more favourable exercise choice for modulating inflammatory markers in obese post-menopausal women (Abd El-Kader & Al-Jiffri, 2019). Alternatively, aerobic exercise alone in the form of bench steps for 12 weeks caused a significant reduction in IFN- $\gamma$  in Japanese post-menopausal women (Nishida et al., 2015).

When performing bodyweight training or strength and endurance training for 24 months, post-menopausal women in the bodyweight training group had significantly lower IL-6 levels, which may be explained as a result of changes in body composition in this group (Monteiro et al., 2022). However, at the end of the study, both groups had reduced TNF- $\alpha$  without differences in IL-10 (Monteiro et al., 2022).

Finally, a systematic review and meta-analysis of the effects of exercise training on inflammatory markers in post-menopausal women overall found that exercise lasting between 6-12 months was able to significantly reduce IL-6, TNF- $\alpha$  and CRP compared to the control group regardless of age (Khalafi et al., 2021). Further subgroup analysis demonstrated that aerobic, resistance and combined exercise training each caused different decreases in inflammatory markers, with aerobic exercise causing the most significant decrease in IL-6 compared to resistance or combined training (Khalafi et al., 2021).

## **2.9 Summary**

Despite a conflicting consensus among studies in the last 15 years, synbiotics and increased physical activity show potential for resolving chronic inflammation in individuals under varying health statuses through their ability to inhibit or enhance the production of various inflammatory cytokines (Asgharian et al., 2016; Cicero et al., 2021; Farrokhian et al., 2019; Furrie et al., 2005; Henke et al., 2018; Kazemi et al., 2020; Lopes et al., 2021; Masala et al., 2020; McLoughlin et al., 2017; Neto et al., 2013; Philips et al., 2010; Tartibian et al., 2015; Wilms et al., 2016; Yang et al., 2023). However, this literature review demonstrated a scarcity of relevant research around the effectiveness of synbiotics for modulating inflammatory cytokines in post-menopausal women and, to an extent, the effect of exercise on lesser-known inflammatory markers (Brahe et al., 2015; Lee et al., 2020; Lopes et al., 2021; Nishida et al., 2015;

Raji Lahiji et al., 2021). Therefore, there is a significant gap in our understanding regarding the efficacy and feasibility of synbiotics and exercise to slow the progression of chronic inflammation for disease prevention. For this reason, it is crucial to prioritise a comprehensive study to examine the relationship between synbiotics, increased physical activity and the modulation of inflammatory markers in post-menopausal women. Furthermore, as prior research involving prebiotic, probiotic and synbiotic supplements is primarily conducted in Western and Middle Eastern countries, a study conducted in New Zealand would provide invaluable data on the effectiveness of synbiotics and exercise for promoting long-term health and independence in this population group (Asgharian et al., 2016; Burch et al., 2014; Cicero et al., 2021; Farrokhian et al., 2019; Furrie et al., 2005; Henke et al., 2018; Kazemi et al., 2020; Lopes et al., 2021; Masala et al., 2020; McLoughlin et al., 2017; Mundy, 2007; Neto et al., 2013; Philips et al., 2010; Tartibian et al., 2015; Wilms et al., 2016; Yang et al., 2023)

# Chapter 3 Research Study Manuscript

## 3.0 Abstract

**Background:** Chronic inflammation is linked with several deleterious diseases, including cardiovascular disease, obesity, diabetes mellitus, irritable bowel disease, and osteoporosis (Furman et al., 2019; Nathan & Ding, 2010). Post-menopausal women are at a heightened risk of developing these diseases due to the remission of oestrogen, further amplifying a pro-inflammatory state (Burch et al., 2014; Neyrinck et al., 2021).

**Objectives:** This study aimed to critically examine the combined effect of pre- and probiotic supplementation (synbiotics) and exercise in the form of  $\geq 7,000$  steps per day on inflammatory markers hs-CRP, IL-1 $\beta$ , IL-6, IL-8, IL-10, INF- $\gamma$  and TNF- $\alpha$  in sedentary post-menopausal women.

**Method:** Eighty-seven healthy post-menopausal women were allocated to receive either a synbiotic supplement or placebo for 12 weeks. Participants' demographics and physical activity levels were determined using questionnaires, and their diet was assessed using self-reported 3-day diet records. Body composition measures of height, weight and BMI were measured at baseline, while total body mass, lean body mass, total fat mass and total body fat percentage at baseline and week 12 using dual-energy X-ray absorptiometry. Fasted venous blood samples were collected to analyse inflammatory status before and after the intervention. Statistical analysis was performed using SPSS version 24, where baseline continuous variables were analysed using one-way ANOVA or Mann-Whitney tests. The chi-squared test was used to investigate differences in categorical variables between the synbiotic and placebo groups. Outcome variables with multiple time points were analysed using repeated measures ANOVA with the model, including time (baseline vs 12 weeks), intervention group (placebo vs synbiotic), and their interaction as fixed effects.

**Results:** The results showed no significant differences between the intervention group's demographics, physical activity levels, and dietary intake ( $p > 0.05$ ). The 12-week study duration (time) was found to have had a statistically significant effect on lowering hs-CRP ( $p < 0.018$ ), IL-8 ( $p < 0.001$ ), INF- $\gamma$  ( $p$

<0.001), TNF- $\alpha$  ( $p < 0.001$ ) and increasing IL-6 ( $p < 0.001$ ) and IL-10 ( $p < 0.001$ ) in both groups. However, the observed decrease in IL-1 $\beta$  ( $p < 0.348$ ) over time was not significant. The intervention type (synbiotic or placebo) significantly impacted IL-10 ( $p < 0.003$ ). No significant interactions between time and group were observed across all inflammatory markers ( $p > 0.05$ ). An increase in total lean body mass ( $p < 0.015$ ) and decrease in total body fat percentage ( $p < 0.022$ ) overtime was observed in both the placebo and synbiotic groups. At the same time, the intervention type (synbiotic or placebo) had no effect on total lean body mass, total fat mass, total body mass and percentage body fat in both groups ( $p > 0.05$ ).

**Conclusion:** The current study showed no notable differences between the placebo and synbiotic groups, suggesting that synbiotic supplementation is likely ineffective at reducing chronic inflammation in overweight, sedentary post-menopausal women in New Zealand. However, future studies are needed to confirm these findings. Additionally, studies should investigate the effects of exercise and synbiotic supplementation separately in this population.

### 3.1 Introduction

Chronic inflammation has become a silent killer worldwide, with 50% of all-cause mortality able to be linked back to increased inflammation (Furman et al., 2019; Nathan & Ding, 2010). When elevated for an extended time, chronic inflammation inflicts substantial harm to the function and structural integrity of tissues and vital organs such as the brain, heart and kidneys (Chen et al., 2018). Due to this, a plethora of inflammatory-related diseases arise, including obesity, diabetes mellitus, irritable bowel disease, osteoporosis, rheumatoid arthritis, and auto-immune and mental health disorders (Furman et al., 2019; Nathan & Ding, 2010; Schmid-Schönbein, 2006; Sproston & Ashworth, 2018; Yang et al., 2017).

Nevertheless, inflammation is an inevitable part of being human, which occurs under normal physiological functioning of the inflammatory cascade (Schmid-Schönbein, 2006). This inflammatory cascade is pivotal for survival as it combines cellular and tissue-level reactions required for protection against endogenous and exogenous threats, e.g., pathogens, chemicals, trauma and

environmental agents (Chen et al., 2018; López-Armada et al., 2013; Schmid-Schönbein, 2006). Therefore, inflammation only becomes problematic when the inflammatory cascade and associated acute inflammation fail to resolve, creating a sustained pro-inflammatory state (Chen et al., 2018; Schmid-Schönbein, 2006).

A way to detect those most at risk is by monitoring the serum concentrations of specific cytokines released during the pro-inflammatory state. Cytokines such as IFN- $\gamma$ , IL-1 $\beta$ , IL-6, IL-8, IL-10 and TNF- $\alpha$  and the acute-phase proteins high sensitivity C-reactive protein (hs-CRP) are a few commonly used inflammatory markers (Colbert et al., 2004; Ilesanmi-Oyelere et al., 2019; Vulevic et al., 2008; Zhang & An, 2007).

Persuasive evidence from several papers has indicated that the inflammatory cascade fails to be resolved in the presence of a microbiome in dysbiosis, implying that the gut microbiota influences inflammatory responses (Kiousi et al., 2022; Patel et al., 2014; Valentini et al., 2015; Vulevic et al., 2008). This is particularly true for post-menopausal women. As they age, their microbiome undergoes a natural decrease in the number and variety of commensal bacteria (Patel et al., 2014; Vulevic et al., 2008). However, in addition to chronic inflammation-associated ageing termed “inflammaging,” post-menopausal women are uniquely positioned due to hormonal and dietary changes associated with menopause, elevating inflammation in the body (Burch et al., 2014; Ginaldi et al., 2005; Mundy, 2007; Neyrinck et al., 2021). In particular, the remission of oestrogen (Hypoestrogenism) plays a prominent role in accelerating mitochondrial dysfunction and free radical accumulation, overwhelming the body’s cellular antioxidant capacity, triggering a pro-inflammatory state (Au et al., 2016; Burch et al., 2014; Ginaldi et al., 2005; López-Armada et al., 2013; Mundy, 2007; Neyrinck et al., 2021; Sproston & Ashworth, 2018). Furthermore, sedentary post-menopausal women are at greater risk of chronic inflammation as they miss out on the anti-inflammatory properties exerted by exercise (Gleeson et al., 2011). The hypothesised mechanism behind exercise's ability to regulate inflammation is through changes in adipose tissue as part of weight loss or persistent bouts of exercise-induced IL-6 manipulating metabolism and the release of anti-inflammatory cytokines IL-1 $\alpha$  and IL-10 into circulation (Bianchi,

2018; Forsythe et al., 2008; Pedersen, 2006; Philips et al., 2010; Wörnberg et al., 2010).

A proposed solution to rectify chronic inflammation is restoring gut homeostasis through supplementation with synbiotics (Ginaldi et al., 2005; Kazemi et al., 2020; Pandey et al., 2015). Synbiotics combine the co-administration of one or more beneficial bacteria and prebiotic fibres which are selectively utilised by host microorganisms to confer a health benefit on the host (Swanson et al., 2020). Probiotics are live microorganisms, such as *Lactobacilli* and *Bifidobacteria*, that maintain balance by generating a protective barrier on the gut epithelium (Gibson & Delzenne, 2008; Plaza-Díaz et al., 2017; Yu et al., 2021). As a result, they prevent the establishment of opportunistic and pathogenic organisms responsible for making the gut 'leaky,' allowing bacteria and toxins to enter the bloodstream, amplifying widespread inflammation and oxidative stress (Kiousi et al., 2022; Patel et al., 2014; Valentini et al., 2015). Furthermore, probiotics have a direct role in activating signalling pathways responsible for modulating the immune system and providing essential nutrients for the host (Gibson & Delzenne, 2008; Manzoor et al., 2022; Plaza-Díaz et al., 2017; Yu et al., 2021). In contrast, prebiotics influence the composition and activity of bacteria in the gut directly or indirectly through fermentation (Gibson & Delzenne, 2008; Rivière et al., 2016). This fermentation positively influences immune modulation, mineral absorption, bowel function, metabolic effects and appetite (Rivière et al., 2016; Sanders et al., 2019; Vulevic et al., 2008).

Current research involving prebiotics, probiotics and synbiotics supplementation are still debating their effectiveness for treating chronic inflammation as numerous contradictions between studies in post-menopausal women exist (Brahe et al., 2015; Damani et al., 2023; Jafarnejad et al., 2017; Lee et al., 2020; Nilsson et al., 2018; Raji Lahiji et al., 2021; Szulińska, Łoniewski, Skrypnik, et al., 2018; Valentini et al., 2015; Yu et al., 2021). However, discrepancies between studies are likely the result of differences in supplementation type, doses and frequency utilised. Therefore, oral synbiotics should not be disregarded as a plausible method for alleviating chronic inflammation in post-menopausal women, especially as treating chronic

inflammation helps improve quality of life (Burch et al., 2014; Ginaldi et al., 2005; Kazemi et al., 2020; Mundy, 2007; Pandey et al., 2015).

Similarly, research involving exercise has varying levels of disagreement across studies in post-menopausal women (Abd El-Kader & Al-Jiffri, 2019; Henke et al., 2018; Khalafi et al., 2021; Lopes et al., 2021; Masala et al., 2020; Monteiro et al., 2022; Philips et al., 2010; Tartibian et al., 2015). However, the general consensus is that exercise decreases and increases pro-inflammatory and anti-inflammatory cytokines. Therefore, exercise is essential for endorsing improvements in inflammatory cytokines in post-menopausal women.

The present study aimed to report the effectiveness of the synbiotic *Lactocaseibacillus rhamnosus HN001*<sup>™</sup> and 10 g Orafti®Synergy 1 combined with  $\geq 7,000$  per day for improving the inflammatory status of post-menopausal women. Existing research is inconclusive in certain aspects and has not been widely conducted in New Zealand and post-menopausal women

## **3.2 Methodology**

### **3.2.1 Study Design**

This study was a 12-week randomised, single-blind, placebo-controlled trial investigating whether synbiotics can induce favourable changes in systemic inflammatory markers in post-menopausal women. The study was conducted in the Human Nutrition Research Unit at Massey University, Palmerston North, New Zealand. The flowchart (Figure 2) depicts the study protocol.

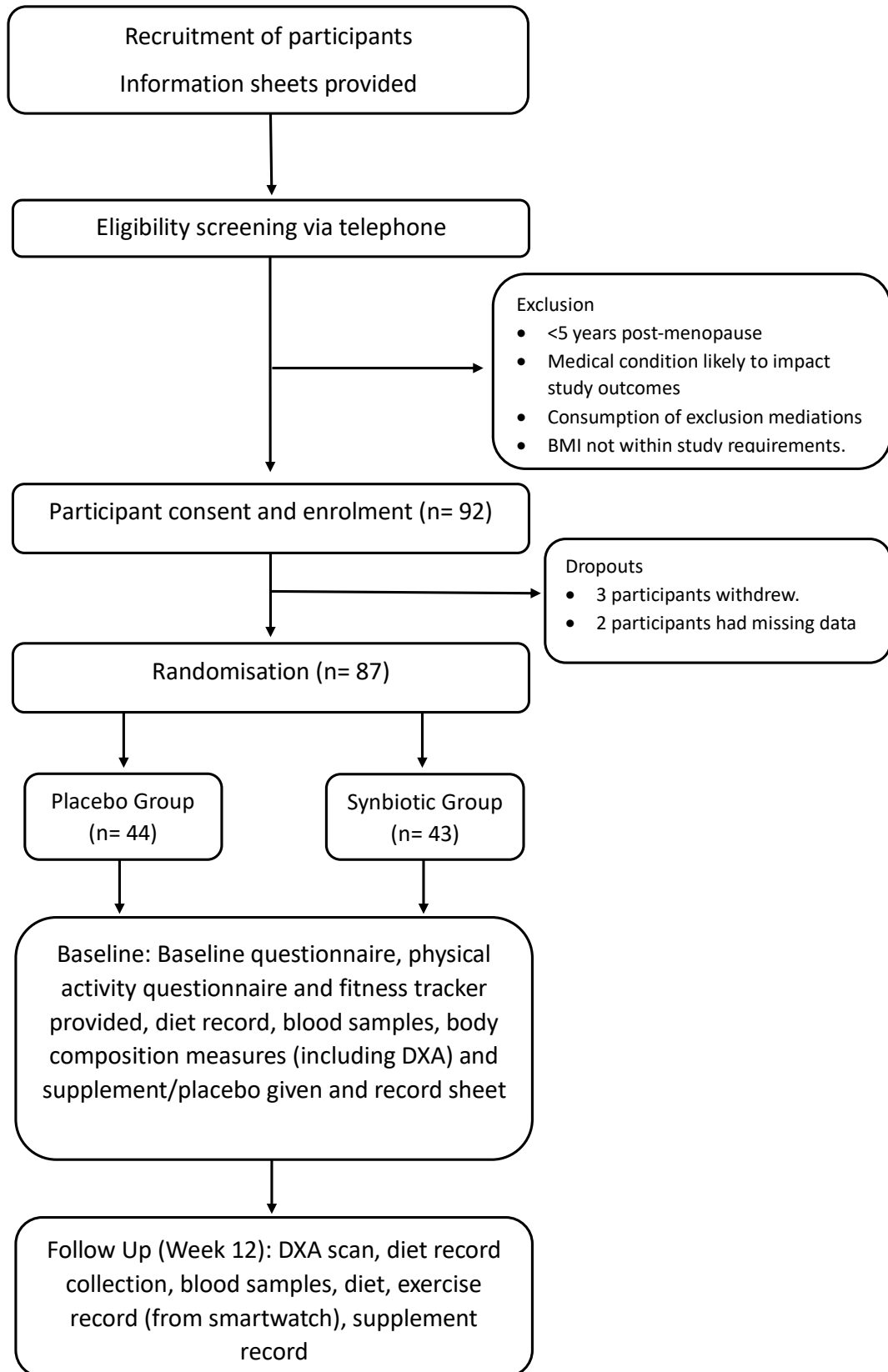
### **3.2.2 Ethics and Trial Registration**

The study was registered at the Australian New Zealand Clinical Trials Registry (ANZCTR), Trial registration number: ACTRN12620000998943 (Appendix 1).

The study was also approved by the Health and Disability Ethics Committee (HDEC) of New Zealand with the ethics reference number 21/NTB/47.

**Figure 2**

*Flow diagram summary of the study protocol*



### **3.2.3 Sample Size**

The sample size was calculated based on the post-doctoral COPES-4-Bones clinical study requirements, where the primary outcome variables were bone mineral density (BMD) and bone resorption (CTX-I). A minimum sample size of 100 participants was determined to detect a clinically significant difference at 80% power and 5% statistical significance, with an anticipated dropout rate of 20%. Hence, 50 subjects were required for each of the intervention and placebo groups. However, at completion, the COPES-4-Bones clinical study comprised only 43 women in the intervention group and 44 in the placebo due to dropouts.

### **3.2.4 Participants and Recruitment**

Recruitment of participants from the Manawatu-Whanganui region of New Zealand began in March 2022 by a recruitment agency named Trial Facts (<https://trialfacts.com/>). Ninety-two post-menopausal women 50-80 years, primarily of New Zealand European descent, enrolled in the study. However, only 87 women completed the study; three participants dropped out, and two had missing data. Data was collected from participants from the 11th of May 2022 to the 18th of November 2022.

### **3.2.5 Inclusion and Exclusion Criteria**

#### ***Inclusion Criteria***

The inclusion criteria were designed to recruit participants with an elevated inflammatory status, making changes in inflammatory markers easier to detect.

1. Assigned female at birth.
2.  $\geq 5$  years post-menopausal (based on the natural cessation of menstruation.)
3. 50-80 years
4. Body mass index (BMI) of 25-35 kg/m<sup>2</sup>
5. Sedentary
6. Presented with no major illnesses.

### **Exclusion criteria**

Individuals confirmed to have one or more of the following were excluded as participation could co-found the results or was deemed unsafe.

1. Allergic or intolerant to components of either probiotic or prebiotic supplements.
2. Use hormone replacement therapy (HRT).
3. Liver function test or creatinine above normal ranges, or suggested history of liver or kidney disease.
4. Have diabetes mellitus Type 1 or 2.
5. Antibiotics intake in the previous 6 months.
6. Smoking and intake of alcohol > 2 units per day

### **3.2.6 Intervention**

Of the 87 women who participated in this study, 43 were randomly allocated to receive synbiotic supplementation, while the remaining 44 were allocated the placebo. The synbiotic supplement consisted of 1 capsule of  $6 \times 10^9$  CFU per 150 mg of the probiotic *Lactocaseibacillus rhamnosus HN001*<sup>™</sup> and 10 g (1 scoop) of the prebiotic powder Orafti® Synergy 1, consisting of oligo- and polysaccharides composed of fructose units linked together by  $\beta$ -(2,1)-linkages. The probiotic was sourced from Fonterra Co-operative Group Limited, New Zealand and the prebiotic from BENEIO GmbH, Germany. Participants in the placebo group were provided with one capsule containing maltodextrin per day, provided by Fonterra.

Furthermore, as part of the intervention, both groups were required to complete a minimum of 7,000 steps per day. However, participants were welcome to complete more than 7,000 steps if desired. All participants were provided with smartwatches to ensure they completed the step requirement of the intervention. The average number of steps taken during the intervention period was documented at the study's conclusion. No additional analysis was conducted. The minimum number of steps required each day was chosen based on the assumption that most people typically complete 5,000 steps per day as part of their daily routines and that 10,000 steps per day may be too much for our study population as older adults.

### **3.2.7 Data Collection**

#### *i. Baseline Questionnaire and Consent form*

Participants' socio-demographics, health status, medication use and frequency, smoking status, and alcohol intake were captured using a short questionnaire (Appendix 2). All participants had to complete a written informed consent form before data collection (Appendix 3).

#### *ii. Physical Activity*

Participants' physical activity level was determined using the New Zealand Physical Activity Questionnaire- short form (Appendix 4).

#### *iii. Dietary Assessment*

To determine the participants' energy, protein, dairy, mineral, and vitamin intake, participants completed a 3-day diet record over non-consecutive days, including one weekend day. (Appendix 5) Participants used standard household measuring tools to estimate their food and beverage portion sizes. Data was analysed using FoodWorks version 10 Xyris software to produce daily nutrient intake (*FoodWorks 10*, 2019).

#### *iv. Body composition*

Height was measured without shoes to the nearest 0.1 cm using a stadiometer. Similarly, weight was measured wearing light clothing without shoes, jewellery or wallets to the nearest 0.1 kg using Detecto 437 eye-level weigh beam physician scales. Body mass index (BMI) was estimated using the equation  $\text{Body weight in kg} / \text{height in metre squared (kg/m}^2\text{)}$  and interpreted according to the Ministry of Health guidelines (Ministry of Health). Lean body mass, total body mass, total fat mass and body fat percentage were defined using dual-energy x-ray absorptiometry (DXA) scanned by a qualified operator at baseline and after 12 weeks (Horizon A bone densitometer, Hologic, Marlborough, Massachusetts,

US). Quality control was performed daily by standardisation of the densitometer to a standard phantom before the first participant scan.

#### *v. Blood Sampling*

Fasting blood samples were drawn at baseline and 12 weeks post-intervention. Systemic inflammation was measured using the concentration of inflammatory cytokines IFN- $\gamma$ , IL-10, IL-1 $\beta$ , IL-6, TNF- $\alpha$ , and IL-8. Samples were collected, centrifuged, and stored frozen at -80°C before completing Beckman Coulter's Gallio flow cytometer according to instructions from BioLegend LEGENDplex™ Multi-Analyte Flow Assay Kits. The resulting data was analysed by LEGENDplex™ data analysis software 8.0. Serum high sensitivity c- reactive protein (hs-CRP) was measured at Medlab Central, Palmerston North, using the electrochemiluminescence immunoassay "ELICA." Vitamin D status of participants was analysed at baseline by Canterbury Health Laboratories using HPLC Tandem mass spectrometry (LC-MS/MS).

### **3.2.8 Statistical Analysis**

Statistical analysis was performed using SPSS version 24 (IBM Corporation, n.d). Participants were described based on socio-demographic and dietary characteristics. Continuous variables were summarised using mean and standard deviation (SD) or median and interquartile range (IQR) for variables with normal distribution or skewed distribution, respectively. Kolmogorov-Smirnov and Shapiro-Wilk tests for normality were used to check the nature of the distribution of each variable. Categorical characteristics were presented as frequencies and percentages.

Further, inferential statistics were employed to investigate differences in characteristics between the synbiotics and the placebo groups. For variables assessed once, one-way ANOVA was used to investigate differences in continuous variables. Data was log<sub>10</sub> transformed if required to achieve homogeneity of variance. The chi-squared test was used to investigate differences in categorical variables between the synbiotic and placebo groups.

Outcome variables with multiple time points were analysed using repeated measures ANOVA with the model, including time (baseline vs 12 weeks),

intervention group (Placebo vs Synbiotic), and their interaction as fixed effects. Data was log<sub>10</sub> transformed if required to achieve homogeneity of variance of the residuals. Results are reported as least-squares means, 95% confidence limits, and p-values for the effect of the intervention group within and across time points.

### **3.2.9 Methodology Justification: Validity and Reliability**

When designing this study, careful consideration was given to ensure the validity and reliability of outcomes produced.

To ensure internal validity, a 12-week single-blinded RCT study design was deemed the most logical choice to detect a meaningful difference in inflammatory markers between the synbiotic and control groups. Random allocation of participants into the synbiotic and control group and the strict inclusion and exclusion ensured that participants recruited aligned with our ideal study population and minimised selection bias, ensuring both groups were similar at baseline and uninfluenced by confounding variables. Furthermore, the RCT study design allowed for the employment of statistical analysis to effectively determine the impact and significance of synbiotic supplementation on inflammatory markers of post-menopausal women, which is highly important for healthcare providers and policymakers to know when making informed choices for patients and deciding which treatments to prioritise and fund. The single-blind element of the study design further ensures the internal validity of our design by ensuring our results were uninfluenced by the participant's knowledge of intervention allocation, minimising bias and the placebo effect, therefore allowing observed changes in inflammatory markers to be attributed to the intervention easier. Furthermore, external validity was considered when registering with the Australian New Zealand Clinical Trials Registry (ANZCTR) and the Health and Disability Ethics Committee (HDEC) of New Zealand, demonstrating a commitment to ethical standards and transparency.

Standardised data collection methods and procedures previously utilised in relevant literature were employed to ensure our study's reliability, including questionnaires, physical activity assessments, dietary records, body composition measurements, and blood sampling. The utilisation of these methods increased

the reproducibility of our results by other researchers and prevented undisclosed bias or confounding variables. Additionally, measuring our outcome variables twice and using the average provided a more accurate representation of the real inflammatory marker or body composition values, reducing error. Moreover, completing a repeated measures ANOVA on our outcome variables enhanced the reliability and credibility of the study's findings concerning the impact of synbiotics on inflammatory markers in post-menopausal women. Finally, accounting for potential dropouts in the initial sample size calculation demonstrated a thoughtful approach to ensuring an appropriate sample size to detect significant differences between the synbiotic and control groups after attrition.

### **3.3 Results**

#### **3.3.1 Baseline Characteristics and Dietary Analysis**

Eighty-seven post-menopausal women (43 in the synbiotic group and 44 in the placebo group) completed the study. Baseline socio-demographic data for subjects who completed the study are presented in Table 2. No significant differences ( $p > 0.05$ ) were observed following the performance of independent t-tests or Mann-Whitney tests between placebo and synbiotic groups for age, weight, height, BMI, ethnicity, and alcohol intake (Table 2). Furthermore, there were no significant differences in the variables metabolic equivalent, activity expenditure and steps per day between intervention groups. Serum 25(OH) Vitamin D<sub>3</sub> was 74.0 nmol/L in both groups at baseline, as this value falls within the optimum range of 50 – 150 nmol/L; serum 25(OH) Vitamin D<sub>3</sub> levels were deemed adequate (Canterbury Health Laboratories, n.d).

Analysis of the 3-day food recalls showed no statistical differences between the medians and IQR for energy and macronutrient intake (Table 3), vitamin intake (Table 4) and mineral intake (Table 5) between the synbiotic and placebo groups ( $p > 0.05$ ).

**Table 2***Participant Socio-demographic Characteristics*

Characteristics	Synbiotics N=43	Placebo N=44	<i>p</i> -value <sup>d</sup>
Age (yr.) <sup>b</sup>	64.0 (61.0-68.0)	64.0 (61.0-69.0)	0.852
Weight (kg) <sup>b</sup>	70.4 (62.0-78.6)	67.5 (58.8-77.6)	0.380
Height (m) <sup>a</sup>	1.61 ± 0.06	1.64 ± 0.06	0.149
BMI (kg/m <sup>2</sup> ) <sup>a</sup>	27.14 ± 4.66	25.90 ± 5.17	0.240
Ethnicity <sup>c</sup>			0.269
Australian European	1 (2.3 %)	0 (0.00%)	
Irish	1 (2.3 %)	0 (0.00%)	
Māori/New Zealand European	2 (4.7%)	4 (9.1 %)	
NZ European	35 (81.4%)	38 (86.4 %)	
Other British	0 (0.00%)	2 (4.5%)	
Other Dutch	1 (2.3%)	0 (0.00%)	
Other French	1 (2.3%)	0 (0.00%)	
South African European	2 (4.7%)	0 (0.00%)	
Alcohol intake <sup>c</sup>	Yes= 36 (83.7 %) No= 7 (16.3 %)	Yes= 35 (79.5 %) No= 9 (20.5 %)	0.615
MET (min/day) <sup>b</sup>	184.6 (85.1-273.9)	241.1 (143.1-369.0)	0.259
AEE (kJ/min) <sup>b</sup>	472.2 (118.6-873.5)	446.7 (187.5-1,177.8)	0.444
Steps per day <sup>b</sup> (1,000 steps)	9,222.5 (8,051.1- 11,324.3)	9,189.9 (7,600.3- 10,656.5)	0.885
Serum 25(OH) Vitamin D <sub>3</sub> (nmol/L) <sup>b</sup>	74.0 (63.5-89.0)	74.0 (55.0-100.0)	0.992
MET = Metabolic equivalent of task, AEE=Activity energy expenditure			
<sup>a</sup> Reported as Mean ± SD			
<sup>b</sup> Reported as Median and 25-75 <sup>th</sup> percentiles			
<sup>c</sup> Obtained using Pearson Chi-Square test			
<sup>d</sup> Obtained through Independent t-test or Mann-Whitney test			

**Table 3***Estimated Energy and Macronutrient Intake by Group*

Characteristics	Synbiotics <sup>a</sup> n=43	Placebo <sup>a</sup> n= 44	Difference in median	p-value <sup>b</sup>
Energy (kJ)	8,680 (6,787-11,062)	8,875 (7,538- 11,177)	195.0	0.718
Protein (g)	89.9 (74.0-109.8)	88.0 (68.7-110.7)	-1.9	0.785
Total fat (g)	82.3 (55.8-114.1)	84.1 (66.4-120.8)	1.8	0.550
Saturated fat (g)	34.9 (19.1-43.2)	32.5 (22.8-45.9)	-2.4	0.550
Polyunsaturated fat (g)	10.3 (7.4-16.8)	12.0 (8.4-18.7)	1.7	0.444
Monounsaturated (g)	28.6 (19.5-45.7)	28.9 (23.6-42.5)	0.3	0.610
Cholesterol (mg)	262.3 (193.0-382.8)	222.9 (123.9- 373.2)	-39.4	0.043
Carbohydrate available (g)	214.3 (155.1-292.9)	211.4 (175.1- 305.1)	-2.9	0.712
Dietary fibre (g)	26.9 (22.1-33.4)	29.6 (23.0-38.9)	2.7	0.308
<sup>a</sup> Reported as median and 25-75 <sup>th</sup> percentiles.				
<sup>b</sup> Obtained by Mann-Whitney test.				

**Table 4***Estimated Vitamin Intake by Group*

Characteristics	Synbiotics <sup>a</sup> n=43	Placebo <sup>a</sup> n= 44	Difference in median	p-value <sup>b</sup>
Vitamin C (mg)	98.9 (47.4-142.7)	106.5 (60.7-139.7)	7.6	0.413
Vitamin D (µg)	4.1 (2.1-8.3)	6.0 (2.8-9.8)	1.9	0.316
Vitamin E (mg)	9.0 (5.8-13.1)	6.5 (6.9-15.5)	-2.5	0.604
Vitamin K (µg)	7.1 (1.6-27.1)	8.4 (1.1-23.0)	1.3	0.592
Total folate (µg)	393.0 (291.7-445.1)	372.0 (276.1- 477.1)	-21.0	0.860
Total vitamin A equivalents (µg)	850.5 (516.7- 1,170.7)	843.7 (643.9- 1,307.3)	-6.8	0.399
Retinol (µg)	278.2 (184.0-424.9)	285.9 (203.3- 392.2)	7.7	0.993
Beta carotene equivalents (µg)	3,317.0 (1,384.1- 4,337.9)	3,231.9 (1,824.5- 5,625.0)	-85.1	0.568
Beta carotene (µg)	1,756.3 (857.1-3,215.6)	1,581.7 (470.5- 3,911.6)	-174.6	0.487
Alpha carotene (µg)	318.0 (26.5-726.3)	66.0 (10.5-630.4)	-252.0	0.389
<sup>a</sup> Reported as median and 25-75 <sup>th</sup> percentiles.				
<sup>b</sup> Obtained by Mann-Whitney test.				

**Table 5***Estimated Mineral Intake by Group*

Characteristics	Synbiotics <sup>a</sup> n=43	Placebo <sup>a</sup> n= 44	Difference in median	p-value <sup>b</sup>
Potassium (mg)	3,601.4 (2,786.7- 4,319.5)	3,681.8 (3,079.9- 4,996.7)	80.4	0.315
Phosphorus (mg)	1,504.5 (1,197.1- 1,944.1)	1,490.5 (1,209.0- 2,099.6)	-14.0	0.758
Magnesium (mg)	394.0 (274.6-510.2)	365.3 (304.6- 588.5)	-28.7	0.498
Calcium (mg)	958.8 (621.9-1,287.0)	1,005.2 (718.5- 1,341.6)	46.4	0.399
Iron (mg)	13.0 (9.01-18.6)	11.8 (9.7-17.3)	-1.2	0.867
Zinc (mg)	10.9 (8.4-13.5)	9.8 (7.6-13.1)	-11.0	0.592
<sup>a</sup> Reported as median and 25-75 <sup>th</sup> percentiles.				
<sup>b</sup> Obtained by Mann-Whitney test.				

**3.3.2 Body Composition**

Table 6 shows the effect of time and intervention type on body composition variables and the interaction between time and intervention type participants received. The effects of time on total lean body mass ( $p < 0.015$ ) and total body fat percentage ( $p < 0.022$ ) showed that there was a statistically significant difference between the means at baseline and week 12 time points in both groups. In comparison, time did not significantly impact the variables total body mass ( $p < 0.882$ ) and total body fat mass ( $p < 0.323$ ). There was no significant effect of intervention type (synbiotic or placebo) on total lean body mass, total fat mass, total body mass and percentage body fat in both groups. In terms of the interaction between time and intervention group, there were no statistical

differences for total lean body mass, total fat mass, total body mass and percentage body fat in both groups.

### **3.3.3 Inflammatory Markers and hs-CRP**

The observed effects of time and intervention type on hs-CRP and the inflammatory markers are depicted in Table 7. When analysing the data, the 12-week study duration (time) was found to have had a statistically significant effect on lowering hs-CRP ( $p < 0.018$ ), IL-8 ( $p < 0.001$ ), IFN- $\gamma$  ( $p < 0.001$ ), TNF- $\alpha$  ( $p < 0.001$ ) and increasing IL-6 ( $p < 0.001$ ) and IL-10 ( $p < 0.001$ ). However, the observed decrease in IL-1 $\beta$  ( $p < 0.348$ ) over time was not significant.

Intervention type significantly affected IL-10 ( $p < 0.003$ ) but did not significantly affect hs-CRP, IL-8, IFN- $\gamma$ , TNF- $\alpha$ , IL-6, IL-10, and IL-1 $\beta$ . Regarding the interaction between time and intervention group, no statistical differences across all inflammatory markers and hs-CRP were found.

**Table 6***Participant body composition at baseline and week 12 by group*

	Time	Placebo			Treatment			Model P-value		
		EM Mean	LCL	UCL	EM Mean	LCL	UCL	Time	Supp	Time*Supp
Total body mass (kg)	T0	69.17	65.27	73.30	71.12	67.34	75.11	0.882	0.442	0.111
	T12	68.99	65.06	73.16	71.35	67.44	75.49			
Total lean body mass (kg)	T0	39.90	38.28	41.59	41.31	39.54	43.05	<b>0.015</b>	0.234	0.192
	T12	39.99	38.37	41.69	41.59	39.90	43.35			
Total body fat mass (kg)	T0	30.16	27.44	32.87	30.51	28.26	32.77	0.323	0.785	0.266
	T12	29.90	27.15	32.66	30.53	28.09	32.96			
Total % body fat	T0	42.09	40.56	43.61	41.85	40.63	43.08	<b>0.022</b>	0.819	0.958
	T12	41.79	40.23	43.35	41.57	40.25	42.90			

EM = Estimated marginal mean, LCL = Lower control limit, UCL = Upper control limit

**Table 7***Participant inflammatory markers at baseline and week 12 by group*

	Time	Placebo			Treatment			Model P-value		
		EM Mean	LCL	UCL	EM Mean	LCL	UCL	Time	Supp	Time*Supp
hs-CRP (mg/L)	T0	1.44	1.05	1.90	1.41	1.07	1.82	<b>0.018</b>	0.845	0.839
	T12	1.21	0.92	1.54	1.15	0.92	1.41			
IL-1 $\beta$ (pg/ml)	T0	73.52	54.32	99.42	63.73	48.92	82.93	0.348	0.438	0.971
	T12	68.31	51.57	90.41	58.85	42.87	80.64			
IL-6 (pg/ml)	T0	10.80	8.13	14.25	10.01	7.71	12.90	<b>&lt;0.001</b>	0.361	0.200
	T12	31.25	18.54	52.21	21.43	13.82	32.96			
IL-8 (pg/ml)	T0	162.64	143.01	184.95	155.46	144.01	167.81	<b>&lt;0.001</b>	0.601	0.809
	T12	73.17	57.01	93.84	68.21	57.24	81.26			
IL-10 (pg/ml)	T0	21.87	16.11	29.58	13.55	11.08	16.53	<b>&lt;0.001</b>	<b>0.003</b>	0.105
	T12	37.36	26.64	52.25	18.94	15.08	23.72			
IFN- $\gamma$ (pg/ml)	T0	17.05	11.65	24.75	12.47	9.08	16.99	<b>&lt;0.001</b>	0.195	0.973
	T12	20.95	14.42	30.26	15.32	10.96	21.28			
TNF- $\alpha$ (pg/ml)	T0	43.48	32.21	60.43	40.37	30.40	53.53	<b>&lt;0.001</b>	0.273	0.212
	T12	26.71	17.43	40.68	17.72	11.92	26.13			

EM = Estimated marginal mean, LCL = Lower control limit, UCL = Upper control limit

### **3.4 Discussion**

In this randomised control trial, we investigated the effects of daily synbiotic supplementation and exercise in the form of  $\geq 7,000$  steps per day on hs-CRP and six inflammatory markers among overweight and sedentary post-menopausal women. We found that 12-weeks administration of the synbiotic combined with exercise caused a significant decrease in hs-CRP and the inflammatory markers IL-8, IFN- $\gamma$ , and TNF- $\alpha$ . In contrast, the inflammatory markers IL-6 and IL-10 were increased, while the observed decrease in IL-1 $\beta$  was not significant. However, as the changes in hs-CRP and inflammatory markers also occurred in the placebo group, the intervention had no significant effect or interaction. However, we observed no differences between the group's demographics and dietary intake, and the women recruited for this study were overweight and sedentary prior to taking part. The increased number of steps per day completed in both our study groups likely influenced the activation of exercise's anti-inflammatory pathways in the body, leading to the observed decreases in IL-8, IFN- $\gamma$ , and TNF- $\alpha$  and increases in IL-6 and IL-10 at the end of the study in both groups. However, as there was no stand-alone exercise arm in our study, we cannot conclude that this is correct; although, it does align with previous research suggesting that exercise training is an effective strategy for reducing inflammation and central to preventing many chronic diseases (Khalafi et al., 2021). Therefore, if the supplementation of synbiotics in the present study was separated from exercise, a statistically significant correlation could be obtained in the exercise group and not the synbiotic.

#### **3.4.1 hs-CRP**

High-sensitivity C-reactive protein (hs-CRP) is an acute-phase protein secreted from hepatocytes in response to other cytokines, such as TNF- $\alpha$  and IL-6, during an immune response (Pepys & Hirschfield, 2003; Raji Lahiji et al., 2021). It is commonly used as a practical and inexpensive clinical indicator for detecting elevated inflammation as it is uninfluenced by diurnal and seasonal changes and very few medications (Pepys & Hirschfield, 2003; Schmid-Schönbein, 2006; Sproston & Ashworth, 2018).

In New Zealand, the clinical cut-off used in general medicine to define an elevated inflammatory state in adults using hs-CRP is  $<3$  mg/L (Canterbury

Health Laboratories, n.d.). However, hs-CRP Levels >2 mg/L are associated with an increased risk of cardiovascular disease development and stroke with poor prognosis, higher rates of complications, and death. Therefore, levels  $\leq$  1 mg/ L are optimal as they are associated with the least risk (Banait et al., 2022). At baseline of our study, hs-CRP levels in the placebo and synbiotic groups were 1.44 mg/L and 1.41 mg/L, respectively, dropping to 1.21 mg/L and 1.15 mg/ L. Although hs-CRP levels were already below the clinical cut-off at baseline, any decrease in hs-CRP is significant, as any improvement reduces the risk of developing chronic inflammatory diseases.

According to three RCT and two meta-analyses in healthy post-menopausal women and adults in general, serum hs-CRP significantly decreased following synbiotic supplementation regardless of supplement type and duration (Cicero et al., 2021; Farrokhian et al., 2019; Kazemi et al., 2020; McLoughlin et al., 2017; Raji Lahiji et al., 2021). However, this association between decreased hs-CRP and synbiotics is inconsistent with the result of our study, which found that the group participants were allocated in was redundant, meaning another factor was at play. Only one RCT could be found that supported our outcome in obese post-menopausal women. In this study, six weeks of synbiotic supplementation with  $9.4 \times 10^{10}$  CFU of *L. paracasei* F19 and 10g of flaxseed mucilage (30% dietary fibre) did not induce change in hs-CRP (Brahe et al., 2015).

When looking at the effects of exercise on hs-CRP in post-menopausal women, resistance, aerobic or combined exercise significantly decreased CRP regardless of age. (Abd El-Kader & Al-Jiffri, 2019; Khalafi et al., 2021) Likewise, two meta-analyses with older men and women reported that exercise could induce favourable changes in hs-CRP (Monteiro-Junior et al., 2018; Sardeli et al., 2018). However, a 24-month exercise intervention had no significant effect on CRP levels. (Masala et al., 2020). From our results, we saw a significant reduction in hs-CRP over time, which likely occurred through an overall increase in physical activity in the women's lives, which is consistent with the majority of previous research.

### **3.4.2 IL-6**

Interleukin- 6 (IL-6) is a pleiotropic cytokine, promptly and transiently released from macrophages and monocytes in response to infection and tissue damage, causing immune cell migration, phagocytosis and proliferation of cytokines and acute phase proteins (Monteiro-Junior et al., 2018; Tanaka et al., 2014).

Furthermore, IL-6 is also responsible for blood cell production and differentiation as part of haematopoiesis and mediating the release of immunoglobulins by B cells. However, the dysregulation of IL-6 is devastating as it is associated with the activation of signalling pathways related to ageing and increased susceptibility to chronic disease and cancer progression (Monteiro-Junior et al., 2018; Tanaka et al., 2014). Several studies have shown that elevated levels of IL-6 in post-menopausal women and the general population are significantly decreased following prebiotic, probiotic or synbiotic supplementation (Cannarella et al., 2021; Groeger et al., 2013; Kazemi et al., 2020; Milajerdi et al., 2020; Neyrinck et al., 2021; Schultz et al., 2003; Vaghef-Mehrabany et al., 2014; Valentini et al., 2015; Zaharuddin et al., 2019). However, an equal amount of studies report a similar conclusion to our results, that prebiotic, probiotic or synbiotic supplementation does not affect IL-6 concentrations in blood (Brahe et al., 2015; Dehghan et al., 2014; Hatakka et al., 2003; Kekkonen et al., 2008; McLoughlin et al., 2017; Mousavi et al., 2020; Neto et al., 2013; Wilms et al., 2016). Therefore, no consensus regarding synbiotic supplementation's ability to reduce IL-6 can be reached at this time.

Concerning IL-6 concentrations in healthy post-menopausal women, exercise had a prominent role in reducing circulating levels of IL-6 in four RCTs and one meta-analysis (Abd El-Kader & Al-Jiffri, 2019; Henke et al., 2018; Khalafi et al., 2021; Monteiro et al., 2022; Tartibian et al., 2015). In comparison, one RCT reported no significant change in IL-6 concentrations, while another two RCTs reported a significant increase in IL-6 following the exercise intervention (Masala et al., 2020; Philips et al., 2010; Phillips et al., 2012). This increase in IL-6 levels in the blood following exercise is the same increase we observed after our 12-week intervention period.

### **3.4.3 IL-1 $\beta$**

Similar to IL-6, IL-1 $\beta$  is a potent pro-inflammatory cytokine key for resistance to pathogens and tissue repair. However, it also exacerbates damage during chronic disease and acute tissue injury (Lopez-Castejon & Brough, 2011). According to four previous RCTs, prebiotic, probiotic or synbiotic supplementation caused a significant decrease in IL-1 $\beta$  in the general population with varying health statuses (Kazemi et al., 2020; Moludi et al., 2022; Oliva et al., 2012; Vulevic et al., 2008). However, our results showed no significance of synbiotic supplementation in reducing IL-1 $\beta$  thereby adding to previous research that found that IL-1 $\beta$  levels are uninfluenced by supplementation (Jafarnejad et al., 2017; Kekkonen et al., 2008; Wilms et al., 2016).

In post-menopausal women, the influence of introducing an exercise intervention is unclear as two RCTs showed a significant reduction in IL-1 $\beta$  following exercise training, contradictory to the outcome of three other RCTs, which observed no changes in IL- $\beta$  levels (Henke et al., 2018; Lopes et al., 2021; Masala et al., 2020; Philips et al., 2010; Tartibian et al., 2015). Based on the results obtained from our study, they support the hypothesis that exercise does not impact IL-1 $\beta$ .

### **3.4.4 TNF- $\alpha$**

Tumour necrosis factor (TNF- $\alpha$ ) is another pleiotropic cytokine that regulates cell survival, proliferation, differentiation, and death in response to tumours and almost all inflammatory responses (Jang et al., 2021; Monteiro-Junior et al., 2018). When in excess abundance, it has been strongly correlated with cancer and chronic disease development, specifically auto-immune diseases (Jang et al., 2021; Monteiro-Junior et al., 2018). In the present study, we found that TNF- $\alpha$  significantly decreased in both our placebo and intervention groups, suggesting that the synbiotic supplement was ineffective at reducing TNF- $\alpha$ . These findings are consistent with a study in obese post-menopausal women, which found that the synbiotic containing  $9.4 \times 10^{10}$  CFU of *L. paracasei* F19 and 10g of flaxseed mucilage (30% dietary fibre) for six weeks was unable to significantly reduce TNF- $\alpha$  levels in blood, after eight weeks (Brahe et al., 2015). Similarly, two RCT studies in healthy older adults were ineffective at reducing TNF- $\alpha$  following synbiotic supplementation (Neto et al., 2013; Wilms et al., 2016). However, a study by Raji Lahiji disagrees with this outcome, as synbiotic supplementation

with multispecies probiotic and 35g fructo-oligosaccharides significantly decrease TNF- $\alpha$  levels in blood after eight weeks (Raji Lahiji et al., 2021). Additionally, a large-scale meta-analysis of synbiotics' effect on the inflammatory markers of healthy adults showed that TNF- $\alpha$  significantly reduced post-supplementation (Kazemi et al., 2020). Therefore, no consensus regarding synbiotic supplementation's ability to reduce TNF- $\alpha$  production can be reached.

In contrast, exercise in healthy post-menopausal women, regardless of exercise type, duration, BMI and physical activity level, was shown to be highly effective in reducing TNF- $\alpha$  as significant reduction was found in seven RCT studies and one meta-analysis (Abd El-Kader & Al-Jiffri, 2019; Khalafi et al., 2021; Lopes et al., 2021; Monteiro et al., 2022; Philips et al., 2010; Phillips et al., 2012; Tartibian et al., 2015). Only one study opposed this conclusion, where the exercise intervention over 24 months had no observable effect on TNF- $\alpha$  levels in healthy post-menopausal women (Masala et al., 2020). However, as a whole, exercise supports the reduction of TNF- $\alpha$  in the body, aligning with the observed decrease in both groups in our study over the 12-week intervention.

### **3.4.5 IFN- $\gamma$**

Interferon-gamma (IFN- $\gamma$ ) is a pro-inflammatory cytokine secreted by Natural Killer and T cells. IFN- $\gamma$  is involved in orchestrating the activation and coordination of several immune responses, including cellular differentiation, proliferation, apoptosis and tumour elimination (Castro et al., 2018). Therefore, when unregulated, IFN- $\gamma$  induces profound tissue damage and necrosis, contributing to disease pathology and alterations in gut flora (Kak et al., 2018). In our study, we observed a significant decrease in IFN- $\gamma$  during the intervention period, but this decrease occurred in both our placebo and synbiotic group. This outcome is consistent with a meta-analysis in healthy men and women, which found that IFN- $\gamma$  remained uninfluenced by synbiotic supplementation even after subgroup analysis (Kazemi et al., 2020). Similarly, prebiotic supplementation in older adults and overweight women (Bunout et al., 2002; Dehghan et al., 2014) and probiotic supplementation in women with gestational diabetes or colon surgery (Jafarnejad et al., 2016; Zaharuddin et al., 2019) all reported no improvements in IFN- $\gamma$ . However, in middle-aged individuals, synbiotic supplementation with *bifidobacterium animalis lactis* and fructo-oligosaccharides

significantly reduced IFN- $\gamma$  after 30 days, as did  $2 \times 10^9$  CFU *Lactobacillus rhamnosus ssp GG* for five weeks (Neyrinck et al., 2021; Schultz et al., 2003).

In terms of exercise, there is limited evidence available to compare our results to, due to most research focusing only on the acute effects of exercise on inflammatory markers. In our study, the increased number of daily steps in both groups likely caused the significant reduction in IFN- $\gamma$  over time, especially as the women were sedentary before the study. This outcome is consistent with a Brazilian RCT in sedentary older women 60- 80 and a 12-week study in post-menopausal Japanese women, which found that the exercise significantly lowered serum IFN- $\gamma$  (Lopes et al., 2021; Nishida et al., 2015).

### **3.4.6 IL-8**

Interleukin-8 (IL-8) is a chemokine that local innate and epithelial cells release to sustain immune responses (Bickel, 1993). Unlike cytokines, as a chemokine, IL-8 is not pleiotropic and has a distinct role in recruiting and activating neutrophils to the inflammatory site, whereby recruitment increases oxidative stress and sustains localised inflammation responsible for chronic disease development (Bickel, 1993). Prior investigation into synbiotics for managing elevated IL-8 has proven supplementation ineffective at reducing IL-8 in healthy men and women and individuals with IBD, regardless of dosage and supplement duration (Kazemi et al., 2020; Wilms et al., 2016). Similarly, studies using solely prebiotics (Vulevic et al., 2008) and probiotics did not significantly affect IL-8 regardless of strain, fibre, disease state or dosage (Kekkonen et al., 2008; Milajerdi et al., 2020; Tamtaji et al., 2019; Tenorio-Jiménez et al., 2019). These findings are consistent with our results, as IL-8 levels decreased in both the synbiotic and placebo groups.

In terms of exercise, minimal research has been conducted on the long-term effects of exercise on IL-8. However, one RCT in older men and women reported exercise as an effective method for decreasing IL-8 following the 12-month intervention period (Beavers et al., 2010). However, this opposes the findings from our study, which showed that an increased number of daily steps decreased IL-8 over the intervention period.

### **3.4.7 IL-10**

Interleukin 10 (IL-10) is a potent anti-inflammatory cytokine that suppresses the chronicity and synthesis of pro-inflammatory cytokines TNF- $\alpha$ , IL-6 and IL-1 $\beta$  while also promoting the release of anti-inflammatory cytokines accordingly to maintain balance (Ilesanmi-Oyelere et al., 2019; Kwok et al., 2022; Zhang & An, 2007). In the present study, we observed a significant difference in IL-10 between our study groups after the 12-week intervention. However, previous literature on supplementation mostly disagrees with our findings, as five RCTs and two meta-analyses found no significant impact of supplementation on IL-10. (Dehghan et al., 2014; Furrie et al., 2005; Jafarnejad et al., 2016; Kazemi et al., 2020; Kekkonen et al., 2008; Milajerdi et al., 2020; Valentini et al., 2015) In contrast, only two RCTs supported the observed increase in IL-10 from our results (Schultz et al., 2003; Vulevic et al., 2008).

Of the studies available, exercise in post-menopausal women either increased the production of IL-10 significantly in blood (Lopes et al., 2021; Phillips et al., 2012) or remained unaffected after the intervention (Monteiro et al., 2022). Our evaluation of IL-10 indicated that increasing the amount of physical activity sedentary post-menopausal women complete daily is beneficial for increasing circulating IL-10 in blood.

However, despite IL-10 being higher at the end of the intervention period compared to baseline and IL-10 increasing more in the synbiotic group compared to the placebo group, there is no interaction between the intervention duration (time) and intervention type (placebo vs synbiotic). Therefore, the changes in IL-10 observed in blood are independent of whether participants received the synbiotic or placebo.

### **3.4.8 Body Composition**

Besides changes in inflammatory markers over time, we also observed a significant increase in the women's lean body mass and a decrease in total body fat percentage at the end of the study in both the placebo and synbiotic groups. As the women's dietary intake did not differ between the placebo and synbiotic groups, the relative increase in lean body mass (muscle mass) and decrease in body fat percentage is likely the result of increased exercise.

It is well known that regular exercise increases muscle mass and strength (Monteiro-Junior et al., 2018; Sardeli et al., 2018; Tuttle et al., 2020). However, three systematic reviews and meta-analyses have further shown that improvements in inflammatory markers following exercises are linked with increased muscle mass (Monteiro-Junior et al., 2018; Sardeli et al., 2018; Tuttle et al., 2020). Therefore, the decreases in hs-CRP, IL-8, IFN- $\gamma$ , TNF- $\alpha$  and increases in IL-6 and IL-10 induced over the intervention period in our study may have resulted from the increase in lean muscle mass in the women in both groups.

### **3.5 Limitations and Strengths**

A strength of the present study is that it was a randomised, single-blind trial with strict inclusion and exclusion criteria. Furthermore, it is one of the first to be conducted in New Zealand, specifically looking at the effects of synbiotic supplementation on inflammatory markers of post-menopausal women, a generally under-studied percentage of the population. Additionally, it included a diverse range of inflammatory markers. This is particularly important as previous research tends to focus on only reporting changes in hs-CRP, TNF- $\alpha$  and IL-6 when looking at the effects of synbiotics and increased exercise.

Limitations of this study include the absence of a placebo arm without exercise in this study's design due to funding restraints. Without this placebo arm, we cannot rule out whether the synbiotic supplementation did not affect the women's inflammatory markers in any way, even though there were no differences between the inflammatory markers in the control and placebo groups after the intervention. Secondly, the lack of ethnic diversity in our study to reflect the New Zealand population limited the generality of our results. Moreover, this lack of cultural diversity meant we could not observe the effects of the synbiotic supplementation across different ethnicities' microbiome compositions and those at greater risk of developing chronic inflammation. Another limitation of this study is that it does not explore whether the synbiotic chosen had an appropriate formulation and dosage to induce changes in the inflammatory markers of the women. Finally, due to financial and time constraints, our study did not analyse participant's microbial composition and the metabolites in their faeces; therefore, we have no proof of the colonisation

of *Lacticaseibacillus rhamnosus HN001*<sup>™</sup> in the microbiome and participant compliance with their assigned intervention.

### **3.6 Conclusion**

The current study showed notable differences in serum hs-CRP, IL-8, IFN- $\gamma$ , TNF- $\alpha$ , IL-6 and IL-10, total lean body mass, and percentage body fat in overweight, sedentary post-menopausal women over 12 weeks. However, as these changes occurred in both our placebo and synbiotic groups independent of participant demographics and diet, the synbiotic supplement (*Lacticaseibacillus rhamnosus HN001*<sup>™</sup> and 10 g Orafiti® Synergy 1) was deemed ineffective at reducing chronic inflammation. Instead, the increased number of daily steps in the placebo and control groups appeared to be the cause of the changes in inflammatory markers and body composition. Therefore, increased physical activity should be strongly encouraged to attenuate chronic low-level systemic inflammation and physical function. Future studies must aim to confirm these findings in overweight, sedentary post-menopausal women. Furthermore, they should investigate the effect of exercise and synbiotics separately in this population.

# Chapter 4 Conclusion & Recommendations

## 4.1 Overview

Chronic inflammation is undeniably linked to the development of diseases such as cardiovascular disease, obesity, diabetes mellitus, irritable bowel disease, and osteoporosis, which have the highest morbidity and mortality worldwide (Furman et al., 2019; Nathan & Ding, 2010). Post-menopausal women present as a particularly vulnerable percentage of the population to developing these diseases due to remission of oestrogen at menopause and inflammaging amplifying a pro-inflammatory state (Burch et al., 2014; Neyrinck et al., 2021). In order to slow the development or progression of chronic disease, it is, therefore, crucial to investigate practical and cost-effective solutions that can modulate the root cause of chronic inflammation, promoting long-term health and independence (Burch et al., 2014; Kiousi et al., 2022; Mundy, 2007; Sabico et al., 2019). To date, the feasibility of synbiotics to reduce inflammation is limited due to inconsistencies between studies or lack thereof. However, due to their role in modulating gut homeostasis, synbiotic supplementation may be a viable therapy to prevent the unresolved inflammatory cascade, efficiently controlling disease manifestation and progression (Patel et al., 2014; Sanders et al., 2019; Vaghef-Mehrabany et al., 2014; Valentini et al., 2015). Therefore, this study explored whether 12-week supplementation with *Lactocaseibacillus rhamnosus* HN001™ and Orafiti®Synergy 1 combined with  $\geq 7,000$  steps per day can influence the inflammatory status of post-menopausal women for disease prevention.

## 4.2. Main Findings

**Objective 1:** To determine if supplementation with synbiotics can decrease IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$  and IL-8 pro-inflammatory cytokines and hs-CRP in post-menopausal women.

**Findings:** At the end of our study, we found a significant decrease in IFN- $\gamma$ , TNF- $\alpha$ , IL-8 and hs-CRP. However, as these decreases were seen in both our synbiotic and placebo groups, our findings go against our initial hypothesis that only women allocated to the synbiotic group would experience a reduction in hs-CRP and the pro-inflammatory markers. Furthermore, our hypothesis that

women allocated to the synbiotic group would experience a significant decrease in IL-6 post-intervention was incorrect, as instead, there was a significant increase in IL-6 in both the synbiotic and placebo groups. In terms of IL-1 $\beta$ , there was no significant change in concentration in the women's blood following the intervention period, regardless of their assigned treatment group. Therefore, as hs-CRP, IFN- $\gamma$ , TNF- $\alpha$ , and IL-8 decreased, and IL-6 increased post-intervention regardless of group, the increased number of daily steps completed by the women as part of the intervention caused the relative increase or decrease in pro-inflammatory markers. However, caution should be had when interpreting these results as we cannot truly determine whether the synbiotic did not affect hs-CRP or the pro-inflammatory markers in these women without isolating the exercise and synbiotic intervention components.

**Objective 2:** To determine if supplementation with synbiotics can increase the anti-inflammatory cytokine IL-10 in post-menopausal women.

**Findings:** Our results showed that the 12-week synbiotic supplementation was unable to increase IL-10 concentrations in blood, as IL-10 significantly increased in both the synbiotic and placebo groups. Therefore, similar to our first objective, the increased number of daily steps completed by the women led to an observed increase in IL-10. These findings oppose our hypothesis, which predicted that only women allocated to the synbiotic group would have significantly increased levels of IL-10 at week 12. However, it is challenging to definitively ascertain whether the synbiotic did not impact IL-10 concentrations in these women in any form without separating the exercise and synbiotic interventions.

**Objective 3:** To determine if supplementation with synbiotics is a viable therapy to reduce systemic inflammation.

**Findings:** Our study reported an increase in IL-6 and IL-10, a decrease in hs-CRP, IL-8, IFN- $\gamma$  and TNF- $\alpha$ , and no change in IL-1 $\beta$  in both the synbiotic and placebo groups post-intervention. Based on these outcomes,  $6 \times 10^9$  CFU per 150 mg of *Lactocaseibacillus rhamnosus HN001*<sup>™</sup> and 10g Orafiti®Synergy 1 is an unviable therapy to reduce chronic inflammation in post-menopausal women. Instead, an increased daily step count serves as a promising approach for

decreasing chronic inflammation and disease development in post-menopausal women from New Zealand.

### **4.3 Research Impact**

Despite not achieving our desired therapeutic outcome of inflammation remission, our research has made a meaningful impact on expanding our knowledge and understanding of synbiotic supplementation's capacity to reduce inflammation in post-menopausal women. This is especially noteworthy given that minimal research has previously been conducted in New Zealand and among post-menopausal women, who are an understudied percentage of the population. Moreover, the inclusion of a diverse range of inflammatory markers, commonly excluded in research involving synbiotic supplementation and the long-term effects of increased exercise, sets our study apart from previous work in this field. By reporting the null effect observed with our chosen synbiotic *Lactocaseibacillus rhamnosus HN001*<sup>™</sup> and Orafiti®Synergy 1, we have helped minimise research redundancy, saving time and resources for future research. Furthermore, our shared insights into the complexities of working with synbiotic supplementation for inflammation reduction are invaluable and should be considered by researchers when designing future studies. Finally, our study provided an alternative approach for reducing inflammation through an increased daily step count. Based on the findings of our study, this is the most promising approach for reducing chronic inflammation and disease development in post-menopausal women from New Zealand.

Therefore, in its entirety, this study serves as a foundation for future research by encouraging researchers to consider using a more diverse range of inflammatory markers and a broader range of synbiotic alternatives to understand the benefits and limitations of synbiotic supplementation in post-menopausal women. Or alternatively, engage in further research on the effects of increased daily steps on inflammatory markers as a therapy to prevent chronic inflammatory disease development.

## **4.4 Strengths and Limitations**

Like all research, this study is not immune from limitations, but it also brings strengths

### **4.4.1 Strengths**

A core strength of this study was that its design was a randomised, single-blind trial with a prespecified analysis plan, helping to mitigate the chance of error and presence of confounding variables. Additionally, the study had a well-defined and categorised population, thanks to its inclusion and exclusion criteria, ensuring that the target population was represented within its participant pool.

To the best of our knowledge, this study is among the first to be conducted in New Zealand, specifically looking at the effects of synbiotic supplementation on inflammatory markers. Furthermore, as it included post-menopausal women as its primary focus, it provided invaluable data to build upon existing knowledge and literature in a generally under-studied percentage of the population.

Another strength of our study is the range of inflammatory markers included in its analysis. Existing studies that utilise prebiotics, probiotics and synbiotics supplementation tend to focus on only reporting changes in hs-CRP, TNF- $\alpha$  and IL-6. Therefore, other major cytokines that control the body's inflammatory response are overlooked, causing limited evidence on what happens to their concentrations in the body when supplementation occurs. Therefore, by including the inflammatory cytokines IFN- $\gamma$ , IL- $\beta$ , IL-8, and IL-10 in our study, we contributed to current knowledge of their relationship with synbiotics.

Furthermore, this study helped build upon existing knowledge of the long-term effects of increased exercise on inflammatory markers in the body, as most existing research papers only emphasise the acute effect of exercise in the general population and post-menopausal women. Furthermore, this study is one of few which reports on the effects of increased exercise on IFN- $\gamma$ , IL-8 and IL-10.

#### 4.4.2 Limitations

A major limitation in the present study, which resulted from limited funding, was the absence of a placebo arm without exercise. Without this arm of the study, we cannot rule out that the synbiotic supplementation did not affect the women's inflammatory markers in any way, even though there were no differences between the inflammatory markers in the control and placebo groups after the intervention. If a placebo arm without exercise was included, the results of our study could have been better interpreted to attribute the change in inflammatory markers solely to the exercise intervention or combined.

Additionally, the lack of ethnic diversity in our study to reflect the ethnic diversity of the New Zealand population limits the generality of our results as most participants were of NZ European descent. Furthermore, these women did not have an elevated inflammation level based on hs-CRP at the start of the study, which could prelude to a false negative result regarding the effectiveness of our chosen synbiotic supplement. It is well known that the prevalence of developing inflammatory diseases is much higher and steadily increasing in Māori and Pasifika populations than in non-Māori (Ministry of Health, 2001). Therefore, we may have missed observing the effects of the synbiotic supplementation in those predisposed to higher levels of inflammation at baseline. Moreover, as microbiome compositions have substantial divergences in the abundance of specific bacterium among healthy individuals from different ethnicities, our supplementation intervention may have likely responded differently in Māori and Pasifika individuals than non-Māori (Gupta et al., 2017).

Another limitation of this study is that it does not explore whether the synbiotic chosen had an appropriate formulation and dosage to induce changes in the inflammatory markers of the women. Prior meta-analysis shows that not all synbiotic supplements are created equal, as each bacterium in the microbiota has a preferred prebiotic fibre it uses to proliferate (Rivière et al., 2016). Therefore, if another prebiotic had been combined with *Lactocaseibacillus rhamnosus HN001*<sup>™</sup> or the synbiotic was given at a different dose (lower or higher), there may have been a significant change in our results. Furthermore, there was no exploration of the minimum intervention period required for the synbiotic to modulate inflammatory markers. Therefore, the effects of long-term

supplementation may have significantly affected the synbiotic group's inflammatory markers compared to the placebo groups.

A final limitation of this study is that due to financial constraints and time, no analysis of microbial composition and their metabolites in faeces was undertaken. Data received from faecal analysis would have confirmed whether or not colonisation of *Lactocaseibacillus rhamnosus HN001*<sup>™</sup> occurred in the intestinal tract, demonstrating the potential for metabolic activity. Moreover, faecal testing would have ascertained participant compliance with their designated intervention. However, the absence of gut microbiome data from our study does not necessarily mean an absence of colonisation and activity of the synbiotic.

## **4.5 Recommendations and Future Directions for Research**

Future research should build upon the findings of this study and existing gaps in our knowledge.

### **4.5.1 Future Study Design Recommendations**

Endeavour to prioritise the recruitment of ethnically diverse participants to represent the diversity of New Zealand, thereby increasing the generality of results.

Investigate the effect of the synbiotic supplementation in isolation from increased exercise to fully determine whether the positive change in inflammatory markers following the intervention occurred independently from the synbiotic treatment. Once confirmed, this will help inform the most appropriate routes for reducing chronic inflammation in post-menopausal women.

Explore and confirm the minimum number of steps needed per day to induce a positive change in the inflammatory markers of post-menopausal women to help inform public health recommendations. We know that completing 9,000 steps per day decreased IFN- $\gamma$ , TNF- $\alpha$ , IL-8, hs-CRP and increased IL-6 and IL-10 in both study groups. However, we do not know the exact number of steps at which

this effect begins or whether completing more than 9,000 steps per day positively impacts inflammatory markers further.

Include longitudinal studies that observe the long-term effects of synbiotic supplementation in post-menopausal women, helping to determine the mean duration length required to detect changes in inflammatory cytokines after commencing supplementation and any factors that influence this.

#### **4.5.2 Future Methodology Recommendations**

Investigate the characteristics of a healthy microbiome composition for each ethnic background found in New Zealand and then tailor supplementation to each ethnicity. Thus, enhancing the potential effectiveness of a synbiotic supplement, as different supplemental regimes and types will have varying results across each ethnicity.

Compare and contrast different synbiotic formulations and doses within the same RCT to determine the most effective dose, synbiotic composition and corresponding regime for promoting positive changes in inflammatory cytokines and chronic inflammation. Previous meta-analyses struggle to do this as the studies they include do not follow a single standardised study protocol or do not have enough high-quality evidence (Kazemi et al., 2020; McLoughlin et al., 2017; Milajerdi et al., 2020).

Investigate the effect of synbiotic supplementation, increased number of daily steps or both combined on other less studied inflammatory cytokines involved in regulating the immune response in the body. Potential anti-inflammatory cytokines to include are IL-4 and IL-13, as these are underrepresented in current literature and have been implicated in the development of various autoimmune diseases in recent studies (Iwaszko et al., 2021).

#### **4.5.3 Future Data Collection Recommendations**

Include analysis of microbial composition and metabolites in faeces to confirm participant compliance with prescribed intervention and colonisation of the intestinal tract by the probiotic component of the synbiotic supplement.

Identify how each synbiotic interacts with cell receptors and the cell signalling cascade to influence inflammatory markers.

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# Appendices

## Appendix 1: Ethics Approval Letter



**Health and Disability Ethics Committees**  
Ministry of Health  
133 Molesworth Street  
PO Box 5013  
Wellington  
6011  
0800 4 ETHICS hdecs@health.govt.nz

23 March 2021

Dr. Bolaji Lilian Ilesanmi-Oyelere  
Massey University  
Private Bag 11222  
Palmerston North 4442

Dear Dr. Ilesanmi-Oyelere,

Re:	<b>Ethics ref:</b>	<b>21/NTB/47</b>
	Study title:	Modulation of bone/joint biomarkers, gut microbiota and inflammation status by synbiotics (pre and probiotics) and weight-bearing exercise: A randomised controlled trial

I am pleased to advise that this application has been approved by the Northern B Health and Disability Ethics Committee. This decision was made through the HDEC-Expedited Review pathway.

### Conditions of HDEC approval

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

Standard conditions:

1. Before the study commences at *any* locality in New Zealand, all relevant regulatory approvals must be obtained.

2. Before the study commences at *any* locality in New Zealand, it must be registered in a clinical trials registry. This should be a WHO-approved registry (such as the Australia New Zealand Clinical Trials Registry, [www.anzctr.org.au](http://www.anzctr.org.au)) or <https://clinicaltrials.gov/>.
3. Before the study commences at *each given* locality in New Zealand, it must be authorised by that locality in Online Forms. Locality authorisation confirms that the locality is suitable for the safe and effective conduct of the study, and that local research governance issues have been addressed.

#### After HDEC review

Please refer to the *Standard Operating Procedures for Health and Disability Ethics Committees* (available on [www.ethics.health.govt.nz](http://www.ethics.health.govt.nz)) for HDEC requirements relating to amendments and other post-approval processes.

**Your next progress report is due by 23 March 2022**

#### Participant access to ACC

This clinical trial is to be conducted principally for the benefit of the manufacturer or distributor of the medicine or item being trialled. Section 32 of the Accident Compensation Act 2001 provides that participants injured as a result of treatment received as part of this trial will **not** be eligible for publicly-funded compensation through the Accident Compensation Corporation (ACC).

Please don't hesitate to contact the HDEC secretariat for further information. We wish you all the best for your study.

Yours sincerely,



Mrs Kate O'Connor

Chairperson

Northern B Health and Disability Ethics Committee

Encl: appendix A: documents submitted

appendix B: statement of compliance and list of members

## Appendix A

### Documents submitted

<i>Document</i>	<i>Version</i>	<i>Date</i>
CV for CI: CV for CI	Version 1	23 January 2021
CVs for other Investigators: CV for other investigators	Version 1	23 January 2021
CVs for other Investigators: CV for other investigators	Version 1	23 January 2021
Protocol: Protocol chart	Version 1	23 January 2021
Survey/questionnaire: The physical activity questionnaire	Version 1	23 January 2021
Survey/questionnaire: Baseline participants' questionnaire	Version 1	23 January 2021
Survey/questionnaire: Participants' screening questionnaire	Version 1	23 January 2021
Survey/questionnaire: 3-day diet diary	Version 1	23 January 2021
Covering Letter: Covering Letter for provisional approval	Version 2	12 March 2021
Evidence of scientific review: Evidence of scientific review	Version 1	26 January 2021
Evidence of scientific review: Evidence of scientific review	Version 1	26 January 2021
PIS/CF: Cleaned PIS/CF version	Version 3	12 March 2021
Protocol: Protocol	Version 1	27 January 2021
Evidence of scientific review: Evidence of scientific peer review	Version 2	15 February 2021
Protocol: Cleaned Version	Version 4	12 March 2021
Declined letter for previous application in respect of the same (or substantially similar) study: Previous invalid letter	Version 1	15 February 2021
Application		15 February 2021
Evidence of sponsor insurance	Version 1	12 March 2021
Evidence of CI indemnity	Version 1	12 March 2021
Protocol: COPES 4 Bones Research Proposal Clinical Study submitted for ethics_Mar 2021_TC.docx	version 4	01 March 2021
PIS/CF: COPES clinical study participant_information_sheetconsent-form_with update_Mar 2021_TC.doc	Version 3	01 March 2021
Response to Request for Further Information		

## Appendix B

### Statement of compliance and list of members

#### Statement of compliance

The Northern B Health and Disability Ethics Committee:

- is constituted in accordance with its Terms of Reference
- operates in accordance with the *Standard Operating Procedures for Health and Disability Ethics Committees*, and with the principles of international good clinical practice (GCP)
- is approved by the Health Research Council of New Zealand's Ethics Committee for the purposes of section 25(1)(c) of the Health Research Council Act 1990
- is registered (number 00008715) with the US Department of Health and Human Services' Office for Human Research Protection (OHRP).

#### List of members

<i>Name</i>	<i>Category</i>	<i>Appointed</i>	<i>Term Expires</i>
Mr John Hancock	Lay (the law)	14/12/2015	14/12/2018
Dr Nora Lynch	Non-lay (health/disability service provision)	19/03/2019	19/03/2026
Miss Tangihaere Macfarlane	Lay (consumer/community perspectives)	20/05/2017	20/05/2020
Mrs Kate O'Connor	Lay (ethical/moral reasoning)	14/12/2015	14/12/2018
Mrs Stephanie Pollard	Non-lay (intervention studies)	01/07/2015	01/07/2018
Mrs Leesa Russell	Non-lay (intervention studies), Nonlay (observational studies)	14/12/2015	14/12/2018
Ms Susan Sherrard	Lay (consumer/community perspectives)	19/03/2019	19/03/2022
Mrs Jane Wylie	Non-lay (intervention studies)	20/05/2017	20/05/2020

Unless members resign, vacate or are removed from their office, every member of HDEC shall continue in office until their successor comes into office (HDEC Terms of Reference)

<http://www.ethics.health.govt.nz>

## Appendix 2: Participant Baseline Questionnaire



MASSEY UNIVERSITY  
COLLEGE OF HEALTH  
TE KURA HAUORA TANGATA

ID:



**COPEs (combination of physical exercise and synbiotics) 4 Bones**

**Copes 4 BONES Study: Modulation of bone/joint biomarkers, gut microbiota and inflammation status by synbiotics (pre and probiotics) and weight-bearing exercise**

Thank you for expressing an interest in participating in our research project. To ensure you are eligible to participate in the research project we would appreciate it if you can answer the following questions.

If you have any comments or questions relating to the research project or the questionnaire, please feel free to contact **Lilian Ilesanmi-Oyelere** during working hours on **021 085 22308** or email **b.ilesanmi-oyelere@massey.ac.nz**

OR

**Professor Marlena Kruger** during working hours on  
**(06) 951 7571**

Date of birth: \_\_\_\_\_

Ethnicity (please tick all that apply):

NZ European  Maori  Samoan  Cook Island Maori   
Tongan Indian  Chinese  Other  please specify: \_\_\_\_\_

How were you delivered Caesarean  Normal  Don't know

Do you drink alcohol (please tick)? Yes  No

If yes, how many standard drinks do you consume per week? \_\_\_\_\_

*(1 standard drink is 1 can/bottle of standard beer (330ml), 100ml wine or 30ml of spirits)*

If yes, on how many occasions would you drink alcohol per week? \_\_\_\_\_

Were you breastfed as an infant? Yes  No  Don't know

Have you been diagnosed with or experienced any of the following (tick for yes)?

Osteoporosis	
Heart disease	
Stroke	
High cholesterol	
High blood pressure	
Cancer	
Inflammatory bowel disease	
Irritable bowel syndrome	
Food intolerance or allergies causing diarrhoea, bloating, cramping or constipation	
Long term diarrhoea or constipation	
Fractures	
If yes, how many?	
Other (please specify):	

**Are you taking any medications (traditional or homeopathic) or nutritional supplements?**

Type of medication/supplement	Taking? (please tick)	If you have answered <u>YES</u> for any of the medication or supplement options please provide the below information	
		<i>Medication/supplement name</i>	<i>Dose and frequency</i>
Antibiotics	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Blood pressure lowering	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Cholesterol lowering	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Vitamins	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Minerals	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Laxatives	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Metamucil or Benefibre	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Phloe or Kiwicrush	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Probiotics	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Prebiotics	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Antacids or anti-reflux	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Anti-inflammatory	Yes <input type="checkbox"/> No <input type="checkbox"/>		
Other	Yes <input type="checkbox"/> No <input type="checkbox"/>		

**Have you consumed prebiotic or probiotic yoghurt, or fermented drinks or foods within the past month** (i.e. Symbio probalance, Bio yoghurt, Yoplait Elivae, Activate, Bio farm organic, Yakult, Kefir, Sauerkraut, Kimchi, Kombucha) (please tick)?

Yes  No

If yes, please specify the product name and frequency of consumption: \_\_\_\_\_

---

**Have you taken antibiotics within the last 6 months** (please tick)?

Yes  No

If yes, please specify the name of the antibiotic and when you last took it:

---

**How often do you take antibiotics in a year** (please tick)?

Once  Twice  3 times  4 times or more

**Have you made any significant changes to your food intake over the past year** (i.e. become vegetarian/vegan, stopped consuming gluten, dairy or sugar, increased your fruit and vegetable intake or increased/decrease the amount of food you are eating) (please tick)?

Yes  No

If yes, what changes to your food intake have you made? \_\_\_\_\_

**Do you regularly experience any of the following** (please tick all that apply)?

Abdominal pain  Abdominal bloating   
 Flatulence/wind

**If you experience abdominal pain, bloating or flatulence/wind is it mild** (nagging/annoying), **moderate** (strong negative influence on your daily living) **or severe** (disabling) (please tick the boxes that apply)?

	<b>Absent</b>	<b>Mild</b>	<b>Moderate</b>	<b>Severe</b>
<b>Abdominal pain</b>				
<b>Abdominal bloating</b>				
<b>Flatulence/wind</b>				

***Thank you very much for taking the time to complete this questionnaire.***

## Appendix 3: Participant Information Sheet & Consent Form

### Participant Information Sheet



#### **COPES (Combination of Physical Exercise and Synbiotics) 4 Bones**

Formal Study title:

**Modulation of bone/joint biomarkers, gut microbiota and inflammation status by synbiotics (pre- and probiotics) and weight-bearing exercise; a randomised controlled trial**

Lead [Researcher / Study Doctor]: Dr. Bolaji Lilian Ilesanmi-Oyelere

Study Site: Human Nutrition Research Unit, Massey University, Palmerston North

Contact phone number: 021 0852 2308

Ethics committee ref.: 21/NTB/47

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You are invited to take part in a study on **modulation of bone/joint biomarkers, gut microbiota and inflammation status by synbiotics (pre- and probiotics) and weight-bearing exercise (The COPES-4-Bones clinical study)**. Whether or not you take part is your choice. If you don't want to take part, you don't have to give a reason, and it won't affect the care you receive. If you do want to take part now, but change your mind later, you can pull out of the study at any time.

This Participant Information Sheet will help you decide if you'd like to take part. It sets out why we are doing the study, what your participation would involve, what the benefits and risks to you might be, and what would happen after the study ends. We will go through this information with you and answer any questions you may have. You do not have to decide today whether or not you will participate in this study. Before you decide you may want to talk about the study with other people, such as family, whānau, friends, or healthcare providers. Feel free to do this.

If you agree to take part in this study, you will be asked to sign the Consent Form on the last page of this document. You will be given a copy of both the Participant Information Sheet and the Consent Form to keep.

This document is 11 pages long, including the Consent Form. Please make sure you have read and understood all the pages.

## VOLUNTARY PARTICIPATION AND WITHDRAWAL FROM THIS STUDY

Participation in this study is completely voluntary. You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- Decline to answer any particular question
- Withdraw from the study at any time
- Ask any questions about the study at any time during participation
- Provide information on the understanding that your name will not be used unless you give permission to the researcher
- Be given access to a summary of the project findings when it is concluded
- Withdrawing from the study, should you choose to, will not result in any disadvantage to you.

## What is the purpose of the study?

Osteoporosis is a health problem in the elderly with hip and spine fractures occurring commonly after the age of 70 while osteoarthritis is also a major health concern in older age. Low bone mineral density (BMD) at an early age is strong risk factor for osteoporosis, with early detection allowing precautionary measures. There are a lot of factors that affect BMD leading to osteoporosis including genetics, age, diet, physical activity, hormones, alcohol intake, smoking and body weight.

Synbiotics are known to have beneficial effect on the gut microbiota (GM) and inflammation status which in turn could affect the BMD especially at postmenopausal years. Synbiotics are a mix of friendly beneficial bacteria and fiber. The gut microbiota is the microbial make-up/communities of the intestinal tract. Studying the effect of synbiotics and weight-bearing exercise on the gut, inflammation status and bone health might help in the development of therapies for osteoporosis and osteoarthritis during post-menopause.

The aim of this project is to investigate the role of weight-bearing physical exercise, probiotics and prebiotics on bone markers, gut health and inflammation status in postmenopausal women.

## HOW IS THE STUDY DESIGNED?

The COPES clinical study will involve 120 eligible postmenopausal women 50 years and above. There will be 2 groups of 60 participants by stratified randomisation of exercise history. A method based on chance alone by which study participants are assigned to a treatment group. The chance of being in each group is 1/2. Participants will take part in online or telephone screening to check eligibility. If eligible they will visit the Human Nutrition Research Unit at Massey University twice at two time points; week 1 and week 12. Each visit will be for approximately 5 hrs for all the groups.

### Description of groups

Control group: Placebo and exercise (7,000 steps required per day) required

Synbiotic + Exercise group: Both exercise (7,000 steps per day) and synbiotic intake required

The intervention capsule will consist of the probiotic supplement (6 billion colony forming units (CFU) of *Lactobacillus rhamnosus*) and 10 grams of prebiotic fiber), meanwhile, the placebo will be a maltodextrin.

All participants will be required to have body composition measurements, bone density measurement and fill out questionnaires regarding health, demographics, lifestyle, physical activity and dietary intake. In addition, participants will be asked to provide fasted blood, urine and faecal samples.

## WHO CAN TAKE PART IN THE STUDY?

To be able to participate in this study you need to meet the following criteria:

- Aged 55 – 75 years
- 5 years past menopause
- Not taken antibiotics within the last 6 months
- Not taken laxatives, gastric motility medications, prebiotic or probiotic containing foods or supplements (i.e. Symbio probalance, Bio yoghurt, Yoplait Elivae, Activate, Bio farm organic, Yakult, Kefir, Psyllium, Sauerkraut, Kimchi, Kombucha) within the last month\*
- BMI between 17 and 40 kg/m<sup>2</sup>
- No significant weight loss or weight gain within the past year
- No food intolerances which cause gastrointestinal symptoms (i.e. lactose intolerance)
- Non-smoker
- Do not have a high intake of alcohol

\***Please note:** If you are taking prebiotic or probiotic containing foods or supplements and are willing to stop taking these foods or supplements for a month prior to starting the study and during the study then you are also eligible to take part in the study.

## What will my participation in the study involve?

**The following samples will be collected twice at two time points 12 weeks apart from all participants. And each appointment will be expected to take approximately 5 hours. Please kindly let Lilian or Marlena know if you will like the unused samples returned to you after the study.**

### **Fasting blood samples**

You will come into the lab around 9am having fasted since 9.00pm the night before. A light breakfast will be available. We will take a blood sample (25mL) by using a syringe for your fasting blood sample to analyse bone markers, C-Terminal telopeptide type I collagen (CTx), C-reactive protein (CRP), inflammation markers, 25-OH-D, Parathyroid hormone (PTH), COMP, a marker of cartilage degradation, osteocalcin etc. will be measured.

### **Faecal samples**

Faecal samples will be collected and processed by the extraction total genomic DNA and 16s ribosomal DNA amplification. The total genomic DNA is the genetic material of the gut microbiota and not for you as the participant.

### **Questionnaires**

You will be asked to complete questions about your dietary intake, demography and any physical activity or medications. You will be asked to fill in a screening questionnaire, 3-day food diary, Food Frequency Questionnaire (FFQ) and the New Zealand Physical Activity Questionnaire (NZPAQ).

### **Supplement**

You will need to take the synbiotic (probiotic + prebiotic) food supplement daily as instructed.

### **Exercise**

You will also need to make 7,000 steps daily.

Dual X-Ray Absorptiometry (DXA) measurement will be performed to measure body composition and bone density.

## WHAT WILL HAPPEN TO MY *BLOOD AND FAECAL* SAMPLES?

All samples will be labelled with the participant's unique identity code/number and not by the participant's name.

The blood samples will be stored in a -80 degree freezer for up to 12 months during which time the biochemical analyses will be conducted. While waiting for analysis for bone/joint biomarkers, samples will be kept in the freezer at the Nutrition laboratory at Massey University, Riddet Building, Palmerston North campus. Some serum/plasma samples will also be sent to the accredited laboratories, for example the University of Otago, Canterbury Health Laboratories, LabPlus to assess DNA sequencing, inflammatory markers and vitamin D status.

Participants may ask to withdraw their samples at any time during the study.

Māori participants will be fully informed and have time to make their decision to be a part of the study. They will be given full information regarding the disposal of samples and the opportunity to observe appropriate tikanga Māori practice while taking part in the study. For example, the research team will offer participants the opportunity to karakia while blood samples are being taken and the option for disposal of whole blood samples if they wish.

Bodily samples will be handled with integrity in the knowledge that the material is still considered living and therefore a treasure.

### What are the possible risks of this study?

Some people may have a fear of having a blood sample taken or experience discomfort when blood samples are taken. Occasionally a slight bruising will result. The bruising usually disappears within a day or two. Blood samples will be taken by a certified phlebotomist. There may be social or cultural discomfort from having a blood sample, body composition measurements or performance-based measurements taken, however, you will be treated with respect and privacy will be ensured. We will explain all measurements being taken and ask for your permission prior to undertaking these measurements. You may also be accompanied by a support person if required. Every effort will be made to ensure your comfort and respect your participation.

We will use the Hologic DXA machine to estimate body fat percentage and bone density. The DXA has X-ray beams at 2 different energies. This dose is very low and unlikely to cause harm. The total effective dose of radiation to which you will be exposed is 10 microsieverts ( $\mu\text{Sv}$ ), this is much lower than the range normally used in medical diagnostics. To put it in perspective, the amount of radiation you are exposed to during a flight to the United Kingdom return is 100  $\mu\text{Sv}$  and from a dental X-ray 50  $\mu\text{Sv}$ . The room is private, and you can enter the DXA machine in complete privacy. We will provide you with a gown to wear during this measurement.

### WHAT ARE THE POSSIBLE BENEFITS OF THIS STUDY?

A potential benefit of being involved in this study is that you will contribute to gaining a better understanding of the effect of synbiotics on the gut, inflammation status and bone health. If you are interested, you can also request to be sent information on your analyses and body composition measurements. You will also receive a summary of the main findings of the study which will either be posted or emailed to you.

### Will any costs be reimbursed?

Participant will not incur any costs as part of being involved in the study and will receive reimbursement for time and travel (\$75 in MTA vouchers).

### What if something goes wrong?

If you were injured in this study, you would be eligible **to apply** for compensation from ACC just as you would be if you were injured in an accident at work or at home. This does not mean that your claim will automatically be accepted. You will have to lodge a claim with ACC, which may take some time to assess. If your claim is accepted, you will receive funding to assist in your recovery.

If you have private health or life insurance, you may wish to check with your insurer that taking part in this study won't affect your cover.

## What will happen to my information?

During this study the researchers will record information about you and your study participation. This includes the results of any study assessments. You cannot take part in this study if you do not consent to the collection of this information.

### Identifiable Information

Identifiable information is any data that could identify you (e.g. your name, date of birth, or address). The following groups may have access to your identifiable information:

- Research staff (to complete study assessments)
- Government agencies, like HDEC, ACC and its representatives, if you make a compensation claim for study-related injury. Identifiable information is required in order to assess your claim.
- Your usual doctor, if a study test gives an unexpected result that could be important for your health. This allows appropriate follow-up to be arranged.

### De-identified (Coded) Information

To make sure your personal information is kept confidential, information that identifies you will not be included in any report generated by the researcher. Instead, you will be identified by a code. The researcher will keep a list linking your code with your name, so that you can be identified by your coded data if needed.

The results of the study may be published or presented, but not in a form that would reasonably be expected to identify you.

### Security and Storage of Your Information.

Your identifiable information is held at Massey University during the study. After the study it is transferred to a secure archiving site and stored for at least 10 years, then destroyed. Your coded information will be entered into electronic case report forms. Coded study information will be kept in secure, cloud-based storage indefinitely. All storage will comply with local and/or international data security guidelines.

The linked data in this study will be destroyed at the end of the study.

### Risks.

Although efforts will be made to protect your privacy, absolute confidentiality of your information cannot be guaranteed. Even with coded and anonymised information, there is no guarantee that you cannot be identified. The risk of people accessing and misusing your information (e.g. making it harder for you to get or keep a job or health insurance) is currently very small but may increase in the future as people find new ways of tracing information.

### Rights to Access Your Information.

You have the right to request access to your information held by the research team. You also have the right to request that any information you disagree with is corrected.

Please ask if you would like to access the results of your screening and safety tests during the study. You may access other study-specific information before the study is over, but this could result in you being withdrawn from the study to protect the study's scientific integrity.

If you have any questions about the collection and use of information about you, you should ask researcher.

#### Rights to Withdraw Your Information.

You may withdraw your consent for the collection and use of your information at any time, by informing the study researchers.

If you withdraw your consent, your study participation will end, and the study team will stop collecting information from you.

Information collected up until your withdrawal from the study will continue to be used and included in the study. This is to protect the quality of the study.

### **WHAT HAPPENS AFTER THE STUDY OR IF I CHANGE MY MIND?**

If you wish to withdraw from the study, please inform one of the research team. Information and data collected up until your withdrawal from the study will continue to be used and included in the study. This is to protect the quality of the study.

The data will be used for the purposes of this study, and fully anonymised, selected outcomes may be shared with other researchers on request for the purpose of accumulating data from individual studies. Only investigators and administrators of the study will have access to personal information, and this will be kept secure and strictly confidential. Participants will be identified only by a study identification number. Results of this project may be published or presented at conferences or seminars. No individuals will be able to be identified.

At the end of this study the list of participants and their study identification number will be disposed of. Any raw data on which the results of the project depend will be retained in secure storage for 10 years, after which it will be destroyed.

All participants will have access to a summary of the project findings and which treatment group they were in when the study is completed.

### **CAN I FIND OUT THE RESULTS OF THE STUDY?**

All participants will have access to a summary of the project findings when it is completed.

The study is registered with the Australian New Zealand Clinical Trials Registry and can be accessed at [www.ANZCTR.org.au](http://www.ANZCTR.org.au).

### **WHO IS FUNDING THE STUDY?**

This study is funded by Lottery Health Research, Fonterra and Massey University.

## WHO HAS APPROVED THE STUDY?

This study has been approved by an independent group of people called a Health and Disability Ethics Committee (HDEC), who check that studies meet established ethical standards. The Massey University Human Ethics Committee has approved this study.

## Who do I contact for more information or if I have concerns?

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

Dr. Bolaji Lilian Ilesanmi-Oyelere, Post-Doctoral Fellow

*Phone: 021 08522308*

*Email: [b.ilesanmi-oyelere@massey.ac.nz](mailto:b.ilesanmi-oyelere@massey.ac.nz)*

Prof. Marlena Kruger

Phone: + 6469517571

Email: [m.c.kruger@massey.ac.nz](mailto:m.c.kruger@massey.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](mailto:advocacy@advocacy.org.nz)

Website: <https://www.advocacy.org.nz/>

For Maori health support please contact:

*Dr. Sharon Henare, Senior Lecturer*

*Phone: +6469517289*

*Email: [s.j.henare@massey.ac.nz](mailto:s.j.henare@massey.ac.nz)*

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

Phone: 0800 4 ETHIC

Email: [hdecs@health.govt.nz](mailto:hdecs@health.govt.nz)

## **SCOPE**

The Covid-19 controls are over and above the existing Health and Safety policies within Massey University. They are intended to be read in addition to the University Operating Plan (appended) general guidelines for what the traffic light system means for Massey.

[https://www.massey.ac.nz/massey/about-massey/news/advice-on-coronavirus-outbreak/alert-levels/alert-levels\\_home.cfm](https://www.massey.ac.nz/massey/about-massey/news/advice-on-coronavirus-outbreak/alert-levels/alert-levels_home.cfm)

Where possible and appropriate, these guidelines can also be followed at other levels of the protection framework allowing a smooth transition if levels change.

## **Cleaning**

All cleaning of touch points will be undertaken with approved surface cleaning products, e.g. 70% ethanol wipes (e.g. Clinell hospital grade wipes) and sprays. (NB include any pens and clipboards used)

Extra cleaning will take place before and after the study visit including door handles, high use equipment and common areas. Note that cleaning is the responsibility of the research team.

After each participant visit (including visits to the toilet to provide a urine sample) researchers will clean the area and any equipment or surface they may have used or touched.

## **Basic Hygiene Protocols**

- Hands must be washed/sanitised upon entrance to the HNRU and at the beginning and end of each appointment.
- Cough or sneeze into your elbow or by covering your mouth and nose with tissues
- Place used tissues in the biohazard bin or a bag immediately and wash your hands
- Avoid touching your eyes, nose or mouth
- Items that have come into contact with bodily fluids and discarded PPE should be placed in a yellow biohazard waste bag located in the clinical lab and examination room; full bags should be disposed of via the normal medical waste disposal system.
- Wear a government-approved mask.

## **Risk Identification**

All participants will be asked to contact study staff if: they become aware they had contact with a confirmed or suspected case of Covid-19 within the 14 days prior to their visit to HNRU, or within two weeks following their study visit; they become unwell with symptoms consistent with Covid-19 (as per screening questions); if they are suspected of having Covid-19, or if they test positive for Covid-19.

The HNRU will stop operating if either a researcher or a participant is suspected or confirmed as having Covid-19 while an investigation is completed, and appropriate cleaning is carried out.

## **Social Distancing & Ventilation**

Physical distancing of at least 1 m (ideally 2 m) should be practised whenever possible. Obviously, there are some procedures (e.g. blood draws, anthropometry, DXA scans etc) where physical distancing guidelines cannot be adhered to; in this case, note the Ministry of Health guidelines that procedures should be carried out in as short a time-frame as possible (less than 15 minutes). Where physical distancing of at least 1 m is not possible, use of masks. Use of the air conditioning units in the HNRU aids ventilation.

Consider the vulnerability of your participants but remain aware that use of mask is important. Reassure them.

Note that the requirement for distancing may mean extra rooms will need to be booked.

## **Protection**

For non-contact activities, only social distancing and good hand hygiene is required.

When physical distancing of <1 m cannot be practised, an assessment must be made of the likelihood of contact with body fluids.

- Gloves must be worn for all contact procedures where contact with body fluids is possible) including blood-taking etc., and where possible for any contact with a participant e.g. positioning for a scan.
- Government-approved masks should be worn at all times by research staff.
- The number of participants in a single visit will be kept to a maximum of 2 unrelated participants. Participant arrival time will be staggered to reduce interaction between participants and contact between different groups

# Consent Form

## COPES (Combination of physical exercise and synbiotics) 4 Bones



Please tick to indicate you consent to the following

I have read, or have had read to me in my first language, and I understand the Participant Information Sheet.	<input type="checkbox"/>
I have been given sufficient time to consider whether or not to participate in this study.	<input type="checkbox"/>
I have had the opportunity to use a legal representative, whanau/ family support or a friend to help me ask questions and understand the study.	<input type="checkbox"/>
I am satisfied with the answers I have been given regarding the study and I have a copy of this consent form and information sheet.	<input type="checkbox"/>
I understand that taking part in this study is voluntary (my choice) and that I may withdraw from the study at any time without this affecting my medical care.	<input type="checkbox"/>
I consent to the research staff collecting and processing my information, including information about my health.	<input type="checkbox"/>
If I decide to withdraw from the study, I agree that the information collected about me up to the point when I withdraw may continue to be processed.	Yes <input type="checkbox"/> No <input type="checkbox"/>
I agree to an approved auditor appointed by the New Zealand Health and Disability Ethics Committees, or any relevant regulatory authority or their approved representative reviewing my relevant medical records for the sole purpose of checking the accuracy of the information recorded for the study.	<input type="checkbox"/>
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.	<input type="checkbox"/>
I understand the compensation provisions in case of injury during the study.	<input type="checkbox"/>

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I know who to contact if I have any questions about the study in general.

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I understand my responsibilities as a study participant.

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I wish to receive a summary of the results from the study.

Yes

No

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**Declaration by participant:**

I hereby consent to take part in this study.

Participant's name:

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Signature:

Date:

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**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:

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Signature:

Date:

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## Appendix 4: Physical Activity Questionnaire

# The New Zealand Physical Activity Questionnaires

Report on the validation and use of the NZPAQ-LF and  
NZPAQ-SF self-report physical activity survey instruments

JULY 2004

# New Zealand Physical Activity Questionnaire – Short Form (Version 1)

I am going to ask you about the time you spent being physically active in the last 7 days, from last xxx to yesterday. Do not include activity undertaken today.

By 'active' I mean doing anything using your muscles.

Think about activities at work, school or home, getting from place to place, and any activities you did for exercise, sport, recreation or leisure.

I will ask you separately about brisk walking, moderate activities, and vigorous activities.'

Start Time:

Ask questions 1–7 (8 is optional)

## Walking

1. During the last 7 days, on how many days did you **walk at a brisk pace** – a brisk pace is a pace at which you are breathing harder than normal? This includes walking at work or school, while getting from place to place, at home and at any activities that you did solely for recreation, sport, exercise or leisure.

Think *only* about brisk walking done for at least 10 minutes at a time.

\_\_\_\_\_ days per week (GO TO 2)

None (GO TO 3)

2. How much time did you typically spend walking at a brisk pace on each of those days?

\_\_\_\_\_ hours \_\_\_\_\_ minutes

## Moderate physical activity

3. During the last 7 days, on how many days did you do **moderate** physical activities? 'Moderate' activities make you breathe harder than normal, **but only a little** – like carrying light loads, bicycling at a regular pace, or other activities like those on this card (*Showcard 1 – Moderate Physical Activity*). Do not include walking of any kind.

Think *only* about those physical activities done for at least 10 minutes at a time.

\_\_\_\_\_ days per week (GO TO 4)

None (GO TO 5)

4. How much time did you typically spend on each of those days doing moderate physical activities?

\_\_\_\_\_ hours \_\_\_\_\_ minutes

### Vigorous physical activity

5. During the last 7 days, on how many days did you do **vigorous** physical activities? 'Vigorous' activities make you breathe a lot harder than normal ('huff and puff') – like heavy lifting, digging, aerobics, fast bicycling, or other activities like those shown on this card (*Showcard 2 – Vigorous Physical Activity*)?

Think only about those physical activities done for at least 10 minutes at a time.

\_\_\_\_\_ days per week (GO TO 6)

None (GO TO 7)

6. How much time did you typically spend on **each** of those days doing vigorous physical activities?

\_\_\_\_\_ hours \_\_\_\_\_ minutes

### Frequency of Activity

7. Thinking about all your activities over the last 7 days (including brisk walking), on how many days did you engage in:
- At least 30 minutes of moderate activity (including brisk walking) that made you breathe a little harder than normal, OR
  - At least 15 minutes of vigorous activity that made you breathe a lot harder than normal ('huff and puff')?

\_\_\_\_\_ days per week

None

### Stage of Change

*Note: This question is optional*

8. Describe your regular physical activity over the past six months. Regular physical activity means at least 15 minutes of vigorous activity (makes you 'huff and puff') or 30 minutes of moderate activity (makes you breathe slightly harder than normal) each day for 5 or more days each week. Include brisk walking.

I am not regularly physically active and do not intend to be so in the next 6 months

I am not regularly physically active but am thinking about starting in the next 6 months

I do some physical activity but not enough to meet the description of regular physical activity

I am regularly physically active but only began in the last 6 months

I am regularly physically active and have been so for longer than 6 months

Finish Time:

Notes:

# NZPAQ - Short Form Showcards

## Showcard 1: Moderate Physical Activity

Carrying light loads	
Electrical work	Badminton (social)
Farming	Ballroom dancing
Heavy gardening (digging, weeding, raking, planting, pruning, clearing section)	Bowls (indoor, outdoor/lawn)
Heavy cleaning (sweeping, cleaning windows, moving furniture)	Cricket (outdoors – batting and bowling)
House renovation	Cycling (recreational – less than 15 km/hr – not mountain biking)
Machine tooling (operating lathe, punch press, drilling, welding)	Deer hunting
Lawn mowing (manual mower)	Doubles tennis
Plastering	Exercising at home (not gym)
Plumbing	Golf
	Horse riding/equestrian
Kapa haka practice	Kayaking – slow
Waiata-a-ringa	Skate boarding
	Surfing/body boarding
	Yachting/sailing/dingy sailing

## Showcard 2: Vigorous Physical Activity

Carrying heavy loads	Boxing
Forestry	Aerobics
Heavy construction	Kayaking – fast
Digging ditches	Athletics (track and field)
Chopping or sawing wood	Aquarobics
	Skiing
Taiaha	Badminton (competitive)
Haka	Basketball
	Mountain biking
Soccer	Cricket – indoors (batting and bowling)
Rowing	Cycling – competitive
Rugby League	Cycling – recreational (not mountain biking) – more than 15 km/hr
Rugby Union	Rock climbing
Hockey	Exercise classes / going to the gym (other than for aerobics) / weight training
Race walking	Netball
Running/jogging/cross country	Judo, karate, other martial arts
Table tennis (competitive)	Softball (running and pitching only)
Singles tennis	Squash
Touch rugby	Surf life saving
Tramping	Swimming – competitive
Triathlon	Waterpolo
Volleyball	

## Interviewer Instructions: NZPAQ-SF (Version 1)

1. Administer the questionnaire only by face-to-face interview.
2. Do not use with children (<15 years).
3. Use translations when necessary (so far, Tongan and Samoan versions are available – please request these from Ministry of Health or SPARC if needed).
4. Note the start time for the interview in the box provided.
5. Begin with introductory statements, then proceed with the questionnaire, following the routing of questions as appropriate and showing the respondent the relevant showcard at the appropriate times.
6. Give the respondent the time they need to think about each question and formulate their response.
7. If the respondent requests clarification of a question, first read the question again. Then if clarification is still needed, try to use definitions or examples provided in the questionnaire and the showcards.
8. Once the interview has been completed, note the finishing time in the box provided (this is likely to be longer for older people, less educated people and those for whom English is a second language).
9. Also note (in the Notes box provided) if the information is possibly unreliable, together with reason for this, eg, lack of rapport, English is second language, cognitive defect, hearing/ speaking/communicating difficulty, lack of relevance (eg severe mobility disability).



## Appendix 5: Food Diary



MASSEY UNIVERSITY  
COLLEGE OF HEALTH  
TE KURA HAUORA TANGATA

ID:

# *Copes 4 Bones clinical Study*

## **3 Day Food Record**

***Thank you very much for taking part in the Copes 4 Bones clinical Study. We are extremely grateful for your time, effort and commitment!***

(2 week days and one weekend)

*If you have any questions, please contact Lilian Ilesanmi-Oyelere on 021-08522308 or email*

***b.ilesanmi-oyelere@massey.ac.nz***

*All information in this diary will be treated with the strictest confidence. No one outside the study will have access to this.*

*Please bring this diary with you for your second visit.*



## Reminders for your next appointments

- Bring this diary with you to your next appointment
- Wear comfortable, casual clothes including a top with either short-sleeves or no sleeves.
- Your appointment will last approximately 2 hours so bring something along to entertain yourself with – your laptop and personal DVDs, a book, magazines, iPod or study notes.

### Second Appointment

<b>Date</b>
<b>Time</b>

If for any reason you are going to be unable to come for this appointment at the scheduled time and/or day, please let us know as soon as possible.

Email: [b.ilesanmi-oyelere@massey.ac.nz](mailto:b.ilesanmi-oyelere@massey.ac.nz)

Phone: *Lilian Ilesanmi-Oyelere on 021-08522308*

## 3 day food diary - What to do?

- Record all that you eat and drink on the following dates (please include a weekend):

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- If possible record food at the time of eating or just after – try to avoid doing it from memory at the end of the day.
- Include all meals, snacks, and drinks, even tap water.
- Include anything you have added to foods such as sauces, gravies, spreads, dressings, etc.
- Write down any information that might indicate size or weight of the food to identify the portion size eaten.
- Use a new line for each food and drink. You can use more than one line for a food or drink. See the examples given.
- Use as many pages of the booklet as you need.

## Describing Food and Drink

- Provide as much detail as possible about the type of food eaten. For example **brand names and varieties / types** of food.

General description	Food record description
Breakfast example – cereal, milk, sugar	1 cup Sanitarium Natural Muesli 1 cup Pam's whole milk 1 tsp Chelsea white sugar
Coffee	1 tsp Gregg's instant coffee 1 x 200ml cup of water 2 Tbsp Meadow fresh light green milk
Pasta	1 cup San Remo whole grain pasta spirals (boiled)
Pie	Big Ben Classic Mince and Cheese Pie (170g)

- Give details of all the **cooking methods** used. For example, fried (sort of oil/fat used), grilled, baked, poached, boiled...

General description	Food record description
2 eggs	2 size 7 eggs fried in 2tsp canola oil 2 size 6 eggs (soft boiled)
Fish	100g salmon (no skin) poached in 1 cup of water for 10 minutes

- When using foods that are cooked (eg. pasta, rice, meat, vegetables, etc), please record the **cooked portion** of food.

General description	Food record description
Rice	1 cup cooked Jasmine rice (cooked on stove top)
Meat	90g lean T-bone steak (fat and bone removed)
Vegetables	½ cup cooked mixed vegetables (Wattie's peas, corn, carrots)

- Please specify the **actual amount of food eaten** (eg. for leftovers, foods where there is waste)

General description	Food record description
Apple	1 x 120g Granny Smith Apple (peeled, core not eaten – core equated to ¼ of the apple)
Fried chicken drumstick	100g chicken drumstick (100g includes skin and bone); fried in 3 Tbsp Fern leaf semi-soft butter

- Because we are especially interested in your calcium intake, please take care to list **all** the milk you consume, and record what type of milk it was.

General description	Food record description
hot chocolate	1 x cup hot chocolate made with Cadbury's powder and 150 mls Anchor Calcitrim milk, 100 ml hot water. No sugar

- **Record recipes** of home prepared dishes where possible: record how many the recipe fed and the proportion of the dish you ate. There are blank pages for you to add recipes or additional information.

## Recording the amounts of food you eat

It is important to also record the quantity of each food and drink consumed. This can be done in several ways.

- By using household measures – for example, cups, teaspoons and tablespoons. eg. 1 cup frozen peas, 1 heaped teaspoon of sugar.
- By weight marked on the packages – eg. a 425g tin of baked beans, a 32g cereal bar, 600ml Coke
- Weighing the food – this is an ideal way to get an accurate idea of the quantity of food eaten, in particular for foods such as meat, fruits, vegetables and cheese.
- For bread – describe the size of the slices of bread (eg. sandwich, medium, toast) – also include brand and variety.
- Using comparisons – eg. Meat equal to the size of a pack of cards, a scoop of ice cream equal to the size of a hen's egg.
- Use the food record instructions provided to help describe portion sizes.

General description	Food record description
Cheese (Edam)	1 heaped tablespoon of grated cheese 1 slice cheese (8.5 x 2.5 x 2mm) 1 cube cheese, match box size Grated cheese, size 10B

- If you go out for meals, describe the food eaten in as much detail as possible.
- ***Please eat as normally as possible - don't adjust what you would normally eat just because you are keeping a diet record and be honest! Your food record will be identified with a number rather than your name.***

Example day

<b>Time food was eaten</b>	<b>Complete description of food (food and beverage name, brand, variety, preparation method)</b>	<b>Amount consumed (units, measures, weight)</b>
<i>Example</i> 7:55am	Sanitarium weetbix	2 Weetabix
" "	Anchor Blue Top milk	150ml
" "	Chelsea white sugar	2 heaped teaspoons
" "	Orange juice (Citrus Tree with added calcium – nutrition label attached)	1 glass (275 ml)
10.00am	Raw Apple (gala)	Ate all of apple except the core, whole apple was 125g (core was ¼ of whole apple)
12.00pm	Home made pizza (recipe attached)	1 slice (similar size to 1 slice of sandwich bread, 2 Tbsp tomato paste, 4 olives, 2 rashers bacon (fat removed), 1 Tbsp chopped spring onion, 3 Tbsp mozzarella cheese)
1.00pm	Water	500ml plain tap water
3.00pm	Biscuits	6 x chocolate covered Girl Guide biscuits (standard size)
6.00pm	Lasagne	½ cup cooked mince, 1 cup cooked Budget lasagne shaped pasta, ½ cup Wattie's creamy mushroom and herb pasta sauce, ½ cup mixed vegetables (Pam's carrots, peas and corn), 4 Tbsp grated Edam cheese
6.30pm	Banana cake with chocolate icing (homemade, recipe attached)	1/8 of a cake (22cm diameter, 8 cm high), 2 Tbsp chocolate icing
" "	Tip Top Cookies and Cream ice cream	1 cup (250g)
7.30pm	Coffee	1 tsp Gregg's instant coffee 1 x 300ml cup of water 2 Tbsp Meadow fresh blue top milk 2 tsp sugar

















