Effect of New Zealand Greenshell™ Mussel on Osteoarthritis Biomarkers and Inflammation in Healthy Postmenopausal Women

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For a six-week period from March 26 to April 27 2020, New Zealand was placed under very strict lockdown conditions (Level 4 – Lockdown), with students and staff unable to physically access University facilities, unless they were involved in essential research related to Covid-19. All field work ceased and data collection with humans was restricted to online methods, if appropriate. The restrictions were partially lifted on April 27, but students and staff were not generally allowed back into University facilities until May 13.

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This Alert Level system continues to be utilised throughout 2021, and in particular from 17 August 2021 when the whole of New Zealand again moved to Level 4 lockdown for an extended period. The Auckland region remained in alert level 3 or 4 for a number of months. Please see the NZ Government website for more information on lockdown dates.

These changing Alert Levels have meant that some research students had experimental, clinical, laboratory, field work, and/or data collection or analysis interrupted, and consequently may have had to adjust their research plans. For some students, the impacts of Covid-19 have been substantial as they may have had to significantly revise their research plans.

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Due to multiple lockdowns and changes in alert levels and advice from Massey university on physical distancing during this period, the data collection was halted for 6 months. No members from the community were allowed on campus. The study involved the collection of blood samples as well as body composition measurements which requires close contact between people. The university Covid response required us to stop using the Human Nutrition Research Unit, and we were not allowed to continue till restrictions went to level 2. Even with these, significant measures had to be taken to minimise contact and risk to participants as well as staff. We were however able to continue recruitment and phone screening during this period.

The study originally proposed to measure the levels of C-terminal propeptide type II collagen (CPII), a commonly measured collagen type II synthesis biomarker. The only vendor is in Canada, which then required shipment through the US. Essentially the FedEx office were short of staff and the assay kits were sitting in the US for a period of time before they were shipped to NZ. So delivery of the assay kits was severely delayed due to COVID-19 impacts on global transport systems, and the kits when received proved to be unusable as they were not held in dry ice anymore and have reached room temperature. As these delays still continue, and the kits are expensive, we did not attempt to order the kits again.

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Abstract

New Zealand Greenshell™ Mussel (GSM) showed chondroprotective effects in a pre-clinical study using a rat model of metabolic osteoarthritis, warranting further assessment in a human study. This PhD project aimed to assess the effect of GSM supplementation on cartilage degradation biomarkers in humans, and to develop novel biomarkers through a metabolomic approach. A double-blind, placebo-controlled, longitudinal clinical trial was carried out in overweight postmenopausal women who were given 3 g/day whole meat GSM powder or placebo (sunflower seed protein) for 12 weeks. Plasma samples from the pre-clinical rat trial were assessed through an untargeted metabolomic approach, followed by metabolomic analysis of plasma samples from the clinical trial.

In participants with active knee pain, the cartilage turnover biomarker C-terminal telopeptide of type II collagen was significantly lower in GSM participants compared to placebo at weeks 6 and 12. GSM significantly reduced joint pain and improved knee-related symptoms. GSM but not placebo altered the faecal microbiota population and reduced the rate at which body fat accumulation increased. However, no changes in inflammatory cytokines were found.

The metabolomic analysis of rat plasma samples revealed that GSM supplementation regulated the alteration in plasma triglyceride and other lipids caused by a high-fat diet. In the plasma of human participants, GSM supplementation increased long-chain polyunsaturated fatty acids (PUFA), ceramide, and some other lipids. In both rats and humans, GSM suppressed the sphingomyelin synthesis pathway. Polar metabolites including threonine, histidine and pipecolic acid were significantly impacted in both rat and human and are potential metabolic biomarkers for the impact of GSM powder supplementation in metabolic osteoarthritis.

In conclusion, consumption of GMS powder may provide cartilage protection and reduce joint pain, particularly in women with symptomatic knees. However, no significant impact was observed on circulating inflammatory cytokines, suggesting that GSM may exert anti-inflammatory effects at the microenvironmental rather than systemic level. The bioactive compounds present in GSM powder such as omega-3 PUFA and chondroprotective glycosaminoglycans may be responsible for the beneficial effect through inhibiting the breakdown of type II collagen in cartilage, regulating gut microbe

abundance, improving body composition, and the metabolite profile which needs to be investigated in future research.

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Visual Analogue Scale (VAS)

Western Ontario and McMaster Universities Arthritis Index (WOMAC)

World Health Organization (WHO)

Chapter 1 Introduction

Osteoarthritis (OA) is the most common type of degenerative joint disease, contributing to progressive pain and functional loss. It is estimated to affect 33.6% of the elderly over 65 years old (Lawrence et al., 2008; Woolf & Pfleger, 2003). OA is viewed as a whole joint disease involving cartilage degeneration, synovial inflammation, and subchondral bone thickening. The OA symptoms include joint pain, bone deformity and stiffness which develop over time (Loeser et al., 2012). The public health burden of OA is rising in parallel with aging and the obesity epidemic. With a significant economic burden and quality life years loss, early prevention of OA has a considerable impact on public health and the economy.

OA is more prevalent in women compared to men, and the incidence tends to consistently increase following menopause (Srikanth et al., 2005). Oestrogen depletion is an important risk factor that exacerbates OA development, possibly due to presence of oestrogen receptors (ERs) in joint tissues. Furthermore, menopausal transition is accompanied by weight gain which contributes to several health risks including musculoskeletal diseases (Watt, 2018). Increased body mass index (BMI) is strongly related to increased risk of knee and hip OA (Holliday et al., 2011). Excess body weight or fat tissue not only increases the OA risk at weight-bearing joints due to mechanical loading; it has also been associated with increased OA risk in non-weight-bearing joints such as hands (Oliveria et al., 1999). Increased adipose tissue secretes adipokines and pro-inflammatory cytokines contributing to low-grade systemic inflammation (Hauner, 2005). Tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) play a crucial role in cartilage degradation and bone resorption in the pathogenesis of OA. These cytokines induce the production of cartilage degrading enzymes and suppress the synthesis of collagen type II and other cartilage matrix components (Wang & He, 2018). Moreover, sarcopenia or low lean mass is another complication of obesity that has been linked with joint instability and malalignment (Roubenoff, 2004).

So far, no therapeutic strategy has been proven and conventional treatments, particularly non-steroidal anti-inflammatory drugs (NSAIDs) have been used to reduce the inflammation and symptoms, however, prolonged use of these medications have been associated with adverse side effects (Lanas et al., 2003). Hence, identification of safe

interventions for disease prevention and the treatment of early OA have become increasingly important.

Green-lipped mussel (*Perna canaliculus*) or Greenshell™ Mussel (GSM) is a known commercial marine species native to New Zealand. GSM contains high amounts of long-chain omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (20:5 n-3 EPA) and docosahexaenoic acid (22:6 n-3 DHA) (Miller et al., 2011). With potent anti-inflammatory properties, GSM extracts have shown a beneficial effect in chronic inflammatory diseases such as OA and rheumatoid arthritis (RA) (Coulson et al., 2012; Gruenwald et al., 2004). Chondroprotective effects of GSM have been reported in a recent animal study using a pre-clinical rat model of metabolic syndrome-associated OA (MetOA) where a biomarker for cartilage degradation (C-telopeptide collagen type II (CTX-II)) decreased in rats fed the GSM diet. In a longer trial, half the study rats were ovariectomized (OVX) to produce a postmenopausal model and histopathological assessment of knee cartilage (Mankin score) was used to measure the severity of cartilage damage. Histological examination of the rats fed with GSM demonstrated a lower Mankin score (a histological measure of cartilage damage) for knee joint health. In addition, body composition data from this study revealed that GSM inclusion in a high-fat/high (HFHS) sugar diet resulted in increased lean mass compared to HFHS-fed rats (Siriarchavatana et al., 2019). Results from this promising pre-clinical study supported the potential for an intervention study supplementing human subjects with whole meat GSM to attenuate the development of OA.

Collagen breakdown, predominately type II collagen, and proteoglycan depletion lead to OA. Type II collagen is synthesized as a pre-propeptide with C- and N-terminal domains. During the aggregation and formation of type II collagen fibrils, these domains are cleaved. The procollagen II C-propeptide (PIICP) also referred as (CPII) is utilized as a marker of collagen synthesis (Shinmei et al., 1993). The proteolysis of collagen triple helix results in generation of 3/4 and 1/4 lengths. C-terminal telopeptides of type II collagen (CTX-II) is a linear six amino acid sequence cleaved from ¼ length. The urinary concentration of CTXII has been extensively studied and utilized as a biomarker of cartilage degradation and OA (Jayabalan & Sowa, 2014). Cartilage oligomeric matrix protein (COMP) is a non-collagenous cartilage matrix protein which binds to type I, II, and IX collagen fibres, fibronectin, and aggrecan (Tseng et al., 2009). Serum COMP is

used as a biomarker for OA diagnosis, prognosis, and monitoring an intervention (Clark et al., 1999).

Obesity predisposes individuals to subclinical inflammation and concurrently reduces iron absorption and systemic iron availability from cellular iron stores (Weiss & Goodnough, 2005). Obese people are at risk of developing iron deficiency, leading to an escalating disease burden (Zhao et al., 2015). GSM contains high concentrations of both haem and non-haem iron as well as iron absorption enhancers including myofibrillar proteins, low-molecular-weight aminoglycans and omega-3 PUFA (Murphy et al., 2003). An *in vitro* study showed that GSM digestate enhanced the non-haem iron uptake in human intestinal epithelial (Caco-2) cells to a similar extent to that of beef (Stewart et al., 2012). For this reason, we hypothesised that GSM will reduce the chronic low-grade inflammation and may potentially improve iron status.

The gut microbiome is linked to the pathogenesis of many disorders. It was recently recognized that obesity-associated OA is an inflammatory disorder, potentially or in part driven by gut microbial dysbiosis (Schott et al., 2018). Therefore, probiotics, prebiotics, and nutritional supplements which aid gut microbiota restoration can represent a therapeutic approach for this disease. A recent clinical study on knee OA ascertained that a whole GSM extract may lead to prebiotic activity in the intestinal microbiota and in turn may exhibit beneficial effects on reducing OA symptoms (Coulson et al., 2013). Further research is warranted to elucidate the role of GSM on gut microbiota homeostasis.

Metabolomics has proven to be a useful technique to study the global changes of metabolites from biological specimens to reveal disease status. The application of a metabolomic approach is increasingly used in OA studies to understand the metabolism of disease and to identify new biomarkers or potential therapeutic targets (Jiang et al., 2013). Serum and synovial fluid lipidomic profiles are predictive biomarkers of the radiographic stage of obesity associated OA (Wu et al., 2017).

The existing studies on GSM extracts have predominantly focused on OA clinical symptoms and less attention has been paid to OA biomarkers as outcome measure to assess treatment efficacy. To the best of our knowledge, this is the first randomised controlled trial (RCT) that will determine the effect of whole meat GSM powder on cartilage metabolism, bone resorption, and inflammation biomarkers in healthy overweight and obese postmenopausal women who are at high risk of developing OA.

The current study builds on a promising pre-clinical trial (Siriarchavatana et al., 2019) which confirmed the chondroprotective effect of whole meat GSM powder in an experimental model of metabolic OA. GSM may improve joint health, although it remains less clear what broader impact it has on metabolic regulation. Therefore, this study will explore how GSM intervention may alter metabolic function through a metabolomics approach with the aim of revealing the mechanism of action and targets of GSM in prevention or treating OA. Further, bioactive compounds from GSM may positively interact with gut microbiota and represent a novel therapeutic approach for inflammatory diseases such as OA. Therefore, this study aimed to determine the effect of whole meat GSM powder on the gut microbe profile.

1.1 Initial research question

Through the recently completed high-value nutrition (HVN) contestable funded programme "Musseling Up", new evidence of novel health benefits of Greenshell™ Mussels (GSM) foods were shown. This newly demonstrated bioactivity of whole GSM in protecting joint health was a lead that we aimed to further investigate in a clinical intervention study. This PhD project aimed to address the following research objectives:

1.2 Specific objectives

1) To evaluate the effect of GSM on cartilage metabolism in healthy overweight/obese postmenopausal women by monitoring the change in cartilage degradation biomarkers (urinary CTX-II, and serum COMP).
2) To determine the effect of GSM on inflammatory markers including C-reactive protein (CRP) and cytokines along with joint pain and knee-related symptoms.
3) To determine the effect of GSM on improving gut microbe abundance, body composition (fat mass, lean mass and fat percentage) and iron status markers.
4) To develop the novel biomarkers/fingerprint through untargeted metabolomics to assess metabolites associated with GSM supplementation in model of MetOA

1.3 Importance of study

The results of this study have implications as the importance of biomarkers for OA continues to expand. Importantly, the clinical data developed by this study could benefit healthy or at-risk individuals for protection against OA and other joint degenerative diseases that impair the quality of life. Further, the findings of this study will provide new evidence for health claims and marketing across the GSM industry to raise value and export revenue.

1.4 Thesis structure

The following chapters include a literature review which is comprised of four sections. The first section provides an overview of OA (including synovial joint structure, pathophysiology and classification of OA, epidemiology, and burden of disease). The second section discusses biomarkers of OA, and the third section provides evidence on the role of obesity, inflammation, and gut microbiota in OA. In the final section, potential health benefits of New Zealand GSM and its role in OA treatment (evidence from *in vitro*, animal, and human studies) are discussed.

This chapter is followed by chapter 3, a systematic review of clinical trials on the effect of GSM extract supplementation on OA clinical symptoms. This chapter was published in "Inflammopharmacology" and is presented in the thesis in the format of a manuscript. Following this are chapters 4, 5, 6 and 7 that include the methodology and results sections, each including different outcome findings.

Chapter 4 reports the results of untargeted metabolomic and lipidomic analysis revealing the effect of HFHS diet and GSM feeding on plasma lipids and metabolites of OVX rats.

Chapter 5 examines the effect of GSM supplementation on biomarkers of cartilage metabolism, inflammation, and joint pain in postmenopausal women.

Chapter 6 discusses the effect of GSM supplementation on faecal microbiota abundance, body composition and iron status in postmenopausal women.

Finally, chapter 7 reports the impact of GSM supplementation on the plasma lipids and metabolites in postmenopausal women through untargeted metabolomic approach. All these chapters are presented in manuscript format.

Chapter 8 includes an overview of findings and discussion, assessment of the strengths and limitations, conclusion, followed by recommendations and suggestions for future research.

Appendices consist of each chapter's supplementary files, and an information sheet and consent forms, and questionnaires.

Chapter 2 Literature review

2.1 Synovial joint structure and function

A joint is a structure where two or more bones connect, and which enables movement and mechanical support. Synovial joints are the most common joint present in the human body. Synovial joints consist of articular cartilage, synovial membranes, ligaments, and a fibrous capsule. These components are different in shape, architectural organization, and biomechanical function according to the movements and loading requirement of the joint (Buckwalter et al., 1999). The synovial joint is encased in a synovial capsule, which consist of a fibrous membrane outside and synovial membrane inside that encompass the articulating surfaces of bones (Figure 2. 1).

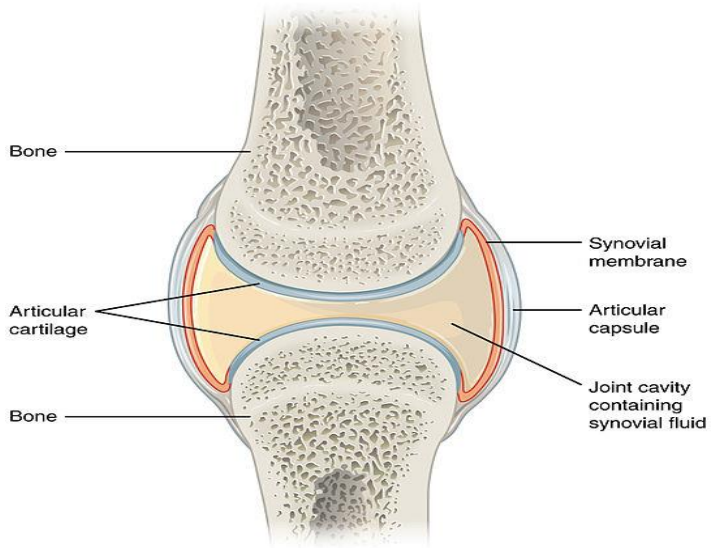

Figure 2.1 Schematic of the simple synovial joint.

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2.2 Cartilage components

Cartilage is a highly specialized connective tissue that covers and protects the ends of bones at the joint. The principal function of cartilage is to provide a smooth, wear-resistant structure to enable bones to slide over one another, with low friction and absorb the rigorous forces. (Wachsmuth et al., 2006). Articular cartilage is the most abundant type of cartilage found in synovial joints and is composed of two main components:

extracellular matrix (ECM) and chondrocytes. Chondrocytes are the only cellular component of healthy cartilage, originated from the mesodermal stem cell and sparsely distributed throughout the cartilage tissue (Sophia Fox et al., 2009). Chondrocytes, collagen and other ECM components are distributed in a highly organized order from the articular surface to subchondral bone subdivided into four distinct zones including a superficial zone (tangential zone/zone I), middle zone (intermediate/transitional zone/zone II), deep zone (radial zone/zone III) and the calcified zone (zone IV) (Clark, 1990). Synovial fluid is a viscous and lubricating ultrafiltrate of blood that provides nourishment and waste product removal during the normal physiological functioning of the joint. The main function of synovial fluid is to produce the low-friction surface necessary for cartilage function (Maroudas et al., 1968).

The cartilage ECM is composed of three main components: collagen, proteoglycan and tissue fluid. (Boushell et al., 2017; Mow et al., 1992). Type II collagen makes up 90% to 95% of the collagen in ECM and contributes to the formation of fibrils and fibres intertwined with proteoglycan aggregates. Other collagens are also identified in minor amounts (Sophia Fox et al., 2009). Proteoglycan is the second-largest group of macromolecules in the ECM that contributes to 10% to 15% of the cartilage wet weight (Sophia Fox et al., 2009). Proteoglycans represent a complex glycosylated protein composed of two main components: a protein core bound to one or several covalently unbranched glycosaminoglycan (GAG) side-chains (Boushell et al., 2017).

The GAG types commonly found in articular cartilage include chondroitin sulphate, dermatan sulphate, keratan sulphate, heparan sulphate, heparin and hyaluronan. Proteoglycans are attached to a hyaluronan backbone to form a proteoglycan complex. The largest and the most predominant proteoglycan in cartilage is aggrecan of which its core protein binds over a hundred of chondroitin sulphate and keratin sulphate side-chains (Gu & Chen, 2005). The negatively charged keratan and chondroitin sulphates in aggrecan are entrapped within the collagen network that causes the tissue to attract and retain water within the ECM in order to create a resisting applied pressure during compressive loading (Lindahl et al., 2015).

2.3 Epidemiology and disease impact

OA is the most common form of arthritis with a high prevalence worldwide (Cross et al., 2014). A recent report from the World Health Organization (WHO) in 2015 estimated that OA affects 3.7% of the global population (Briggs et al., 2016). Older people are commonly affected by this condition, reported by 10% of men and 18% of women aged over 60 years (Woolf & Pfleger, 2003).

According to the recent data, OA affects 10.2% of the adult population in New Zealand (NZ) and by gender is found to be higher in women (12.5%) compared to men (7.9%). In the NZ population over 55 years of age, 21.9% of women have physician-diagnosed OA compared to 13.9% of men. The prevalence of OA in NZ ethnic groups is found to be 12.2% in European, 6.8% in Māori, 3.5% in Pacific, and 2.9% in Asian (Ministry of Health, 2019).

OA is ranked as the 12th highest contributor to disability globally, and the 16th highest in NZ (Vos et al., 2017). With a significant economic burden, knee OA has been linked with large lifetime quality years loss in NZ (Abbott et al., 2017). The number of hip and knee replacement is projected to increase by 84% and 183%, respectively, by 2026. This equates to an additional 6,000 surgeries at a further expense of over $90 million annually (Hooper et al., 2014). Continuous and excruciating pain and disability are also accompanied with impaired mental health and limited social activities as the common sequelae of OA (Fautrel et al., 2005; Rosemann et al., 2007).

2.4 Pathophysiology, risk factors and classification of osteoarthritis

Cartilage erosion is the hallmark of OA, however it is considered as a whole joint disease affecting all components of joints (Loeser et al., 2012). Previously, OA was viewed as a wear-and-tear disease, but current evidence revealed the involvement of low-grade and chronic inflammation, catabolic enzymes, the complement system and cartilage metabolism during the initiation and progression of the disease (Felson, 2006; Q. Wang et al., 2011). Collagen degradation, mostly type II collagen, and proteoglycan depletion occur during OA leading to abnormal water retention or oedema in joint tissue in early OA (Buckwalter et al., 2005). Collagen synthesis seems to be upregulated in early OA, however there is no increase in collagen content. As a result, cartilage loses its mechanical

properties leading to cartilage erosion and chondrocyte apoptosis (Pearle et al., 2005). An increase in lymphocyte and macrophage infiltration and vascularity are involved in inflammation of the synovial membrane or synovitis (Benito et al., 2005). Increased subchondral bone remodelling occurs during OA which includes increased bone resorption and formation of new bone matrix that is more hydrated and have less density than normal matrix (Grynpas et al., 1991). The increased bone volume results in subchondral bone stiffness. Trabecular microfractures in subchondral bone are induced by overloading lead to further stiffness and cartilage erosion (Intema et al., 2010).

Pain is the dominant symptom of OA caused by inflammation of the synovial membrane or synovitis and the penetration of sensory nerves from subchondral bone into the articular cartilage (Walsh et al., 2010). Stiffness, change in joint shape, mobility restriction, muscle waste and weakness are the major clinical features of OA (Felson, 2006; Q. Wang et al., 2011).

OA is a multifactorial disease, with systemic or personal level factors including age, gender, oestrogen deficiency in women, race, genetics, and diet (Sharma et al., 2006), along with joint-level factors such as injury, repeated joint activity, labour occupation, and muscle weakness (Neogi & Zhang, 2013). Aging is a strong risk factor for OA (Chaganti & Lane, 2011). The female gender is more susceptible to develop OA and has a higher risk for hip and knee OA than male counterparts. The gender difference might be related to hormonal factors, reduced volume of knee cartilage, and higher reporting of pain and disability among the women compared with men (Hame & Alexander, 2013; Maleki-Fischbach & Jordan, 2010). With regards to race, prevalence of hip OA is very low in the Chinese population (Nevitt et al., 2002), whereas African-Americans have higher rates of symptomatic knee and hip OA compared to other races (Neogi & Zhang, 2013). These differences are partly related to genetic variation in joint structure (Y. Liu et al., 2017).

Based on the aetiology, OA can be classified into three major phenotypes: post-traumatic, age-related, and metabolic-associated osteoarthritis (MetOA). Post-traumatic OA may develop in people of age < 45 years after repetitive mechanical stresses or by a unique acute joint injury. Age-related OA occurs in patients > 65 years in the hip, knee, or hand without any history of trauma or metabolic syndrome. Patients aged between 45 and 65 years, overweight or obese, and with at least one of the components of metabolic

syndrome (diabetes mellitus, hypertension, dyslipidaemia) are affected with MetOA (Martel-Pelletier et al., 2014).

2.5 Biochemical markers of osteoarthritis

Radiographic imaging is gold standard for OA diagnosis; however, imaging techniques have limitations for early detection, predicting disease progression and structural assessment of cartilage has poor correlation with symptoms (Bauer et al., 2006; Kirwan & Elson, 2000). The main components of synovial joints derived from joint tissues have been used as biochemical markers of OA. These biomarkers are detectable in serum, plasma, urine, and synovial fluid, and have been widely applied for early detection, predicting OA progression, and monitoring intervention efficacy. The National Institutes of Health (NIH) proposed the BIPED classification of OA biomarkers indicated by burden of disease, investigative, prognostic, efficacy of intervention, or diagnostic (Bauer et al., 2006). So far, many biomarkers have been introduced, but all are still waiting validation for use in clinical practice. Despite much research in this area, there is no single golden biomarker of OA specific to the damaged tissue or sensitive to disease progression, therefore using a combination of biomarkers is recommended to provide more accurate information on joint health.

2.5.1 **Cartilage synthesis and degradation biomarkers**

Type II collagen is synthesized as a pre-propeptide with C- and N-terminal domains. During the aggregation and formation of type II collagen fibrils, these domains are cleaved. The procollagen II C-propeptide (PIICP) also referred as (CPII), is utilised as a marker of collagen synthesis (Shinmei et al., 1993). Cleaved C-propeptide has a short half-life, so it is considered a good marker of recent collagen synthesis (Poole, 2000). The N-terminal propeptide is produced in two isoforms; type IIA collagen (PIIANP) and type IIB (PIINP) (Sandell et al., 1991).

The proteolysis of collagen type II results in generation of 3/4 and 1/4 lengths. C-terminal telopeptides of type II collagen (CTX-II) is a linear six amino acid sequence cleaved from a 1/4 length. The urinary concentration of CTX-II has been extensively studied and utilized as biomarker of cartilage degradation and OA (Jayabalan & Sowa, 2014). Urinary CTX-II concentration has a strong correlation with radiographic OA and is proposed as a

good biomarker for measuring the OA progression in postmenopausal women (Garnero et al., 2003). The synovial fluid concentration of CTX-II was found to be higher in patients at the early stages of OA compared to healthy individuals (L. T. Nguyen et al., 2017). Elevated concentrations of urinary CTX-II have also been observed in rheumatoid arthritis (RA) (Krabben et al., 2015).

The 3/4 fragment from type II collagen undergoes further enzymatic degradation and releases several neo-epitopes including cleavage type II collagen (C2C), Coll2-1, nitrated form of Coll2-1 (Coll2-1NO2), and neoepitopes cleaved from collagen type I and II (C1, C2). These fragments provide additional information regarding turnover of collagen type II (Ameye et al., 2007).

The cartilage oligomeric matrix protein (COMP) is a non-collagenous cartilage matrix protein which binds to type I, II, and IX collagen fibres, fibronectin, and aggrecan (Tseng et al., 2009). Serum COMP concentrations have been associated with OA severity and radiographic grade (Henrotin, 2012; Lotz et al., 2013). A population-based study reported ethnic and sex differences in serum concentration of COMP, suggesting these factors need to be considered in the development of standards for this biomarker (Jordan et al., 2003).

Urine CTX-II and serum COMP are the most frequently studied biomarkers and have shown the best performance across all available biomarkers (Van Spil et al., 2010). Despite the variation in these biomarkers with age, sex, and ethnicity, these biomarkers have been known to be useful in the study of OA, particularly when used in combination with other data and biomarkers. The list of biomarkers of cartilage turnover and BIPED classification is presented in Table 2.1.

2.5.2 **Synovial and subchondral bone turnover biomarkers**

Many molecules related to the metabolism of synovium and subchondral bone have received attention as OA biomarkers.

- Serum hyaluronic acid (HA)
- Urinary glucosyl galactosyl- pyridinoline (Glc-Gal-PYD)
- Serum/ synovial fluid Glycoprotein 39 (YKL-40)

A summary list of subchondral bone turnover biomarkers includes:

- Serum N-terminal cross-linked telopeptides of type I collagen (NTX-I)
- Serum C-terminal cross-linked telopeptides of type I collagen (CTX-I)

Table 2.1 Biomarkers of cartilage turnover and BIPED classification.

Biomarker	Molecular process	Fluid	BIPED classification
CTX-II	Type II collagen degradation	Urine, SF	BPED
COMP	Non-aggrecan and non-collagenous proteins degradation	Serum, SF	BPD
Coll2-1	Triple helix type II collagen degradation	Urine, serum	P
Coll2-1NO_2	Nitrated triple helix type II collagen degradation	Urine, serum	P
C2C	Type II collagen degradation	Urine, serum	PE
C1C2	Collage type I and type II degradation	Urine, serum	PD
PIICP	Type II collagen synthesis	Serum, SF	PD
PIIANP	Type II collagen synthesis	Serum	P
Epitope 846	Aggrecan synthesis	SF	E

Abbreviation: CTX-II: C-terminal telopeptide of type II collagen; COMP: cartilage oligomeric protein; Coll2-1: triple helix collagen type II cleavage; Coll2-1 NO2: nitrated form of Coll2-1; C2C: C-terminal of 3/4 epitope of type II collagen; C1C2: C-terminal of 3/4 epitope of type I and type II collagen; PIICP: procollagen II C-propeptide; PIIANP: procollagen IIA N-propeptide; BIPED: Burden of disease, Investigative, Prognostic, Efficacy of intervention, Diagnostic; SF: synovial fluid. Adapted from (Mobasheri & Henrotin, 2011; Verttí, 2015)

2.5.3 **Metabolic biomarkers of osteoarthritis**

Metabolomics is an omics-based technique which allows comprehensive profiling of small molecular metabolites which provides a new array of tools for early diagnosis, monitoring intervention efficacy, and understanding the pathogenesis of diseases (Gowda et al., 2008).

Methods include nuclear magnetic resonance (NMR) and mass spectrometry (MS), along with gas and liquid chromatography (Patti et al., 2012). There are two metabolomic approaches; targeted and untargeted analysis which has been applied in the studies depending on the scope of the study. In a targeted approach, a particular number of known

metabolites are identified and quantified while other metabolites or mechanisms are disregarded (Mishur & Rea, 2012). In contrast, untargeted analysis detects and quantifies a broad set of metabolites in a biological system (Theodoridis et al., 2008).

Application of metabolomics are increasingly used in OA studies to understand the metabolism of disease and to identify new biomarkers (Jiang et al., 2013). Studies suggest that serum and synovial fluid lipidomic profiles are predictive biomarkers of the radiographic stage of OA associated with obesity (Lee et al., 2018). Using metabolomic analysis of synovial fluid, the metabolite profile of knee OA patients at early stages of disease was compared with patients at late stage and revealed that early-and late-stage OA had discriminative metabolite profiles as metabolites related to fatty acid metabolism, such as myristic acid, oleic acid and lanosterol, increased with the structural degeneration of OA. This finding also showed that metabolites in the early-stage OA were more related to glycolysis, while fatty acid metabolism was dominating in the late-stage OA (Kim et al., 2017). Chondrocytes, synovium and other cells of joint tissues become activated with increasing degrees of OA severity because of aberrant expression of pro-inflammatory and catabolic-related genes (Goldring & Otero, 2011), therefore, in the late-stages of OA, fatty acid biosynthesis dominates in order to maintain energy generation for catabolic processes. Taken together, these findings imply that metabolic processes could be robustly altered with progression of disease and may provide potential insight for interventions aimed at arresting the disease progression at an early stage. Therefore, metabolomics is considered a promising tool for investigating biomarkers for early OA diagnosis, stratification, and treatment (De Sousa et al., 2017).

2.6 Role of obesity in osteoarthritis

Obesity is considered the most influential risk factor for OA. A meta-analysis showed that overweight or obese individuals were approximately 2.5 and 4.6 times more likely to develop knee OA than normal weight individuals (Zheng & Chen, 2015). Obesity increases the risk of OA not only through excessive physical loading, but also through systemic factors associated with low-grade chronic inflammation (Cicuttini et al., 1996). Obesity is recognized as low-grade chronic inflammation in which adipose tissue secretes several pro-inflammatory cytokines such as IL-1ß, IL-6, TNF-α, inter-cellular adhesion

molecule 1 (ICAM1), and adipokines such as leptin and adiponectin (Tilg & Moschen, 2006).

The cytokines IL-1ß and TNF-α play a pivotal role in cartilage breakdown. They stimulate the production of type II collagen degrading enzymes, matrix metalloproteinase (MMPs), and prostaglandins E2, and suppress the synthesis of proteoglycan (Goldring & Goldring, 2004; X. Wang et al., 2011).

Leptin, an adipokine secreted from adipose tissue, acts as catabolic regulator of cartilage (Bao et al., 2010). Leptin up-regulates the gene expression of aggrecanase 1 and 2 (ADAMTS-4 and ADAMTS-5) and the combination of leptin with IL-1β increases production of MMP-1, MMP-3, and MMP-13 in human osteoarthritis cartilage (Koskinen et al., 2011).

Beside its role in OA pathogenesis, adiposity is a systemic actor for the OA pain process. A recent result from large cohort of knee and/or hip OA patients showed that the concentration of leptin to adiponectin ratio, a marker of adipose tissue dysfunction and visceral fat, was associated with pain intensity in postmenopausal women, but not in men (Sellam et al., 2021) .

Sarcopenia is defined as low skeletal muscle mass and is another complication of obesity that is associated with joint instability and malalignment (Roubenoff, 2004). It was reported that obese people who have sarcopenia have a 3-fold increased risk of knee OA in comparison to those with normal muscle mass, which leads to increased risk compared to either obesity or sarcopenia alone (Lee et al., 2012). Body composition phenotypes of low muscle mass and high adiposity have been reported in patients with knee and hip OA (Karlsson et al., 2015).

2.7 Relationship between the gut microbiota and obesity-associated osteoarthritis

There is a line of evidence that shows that obesity-associated OA is an inflammatory disorder potentially driven by gut microbial dysbiosis (Schott et al., 2018). Energy-dense food or western style diets can disrupt the intestinal commensal microbiota and increase intestinal permeability (Moreira et al., 2012). Due to disorganization of microbial balance and impairment of gut permeability, components derived from the gut microbiota such as lipopolysaccharide (LPS) and peptidoglycan (PGN) can penetrate internal sites and

initiate immune and inflammatory reactions (Hersoug et al., 2016; Wolf & Underhill, 2018). Synovial concentrations of LPS have been shown to be correlated with macrophage activity, osteophytes and the severity of joint space narrowing and the total Western Ontario and McMaster Universities Arthritis Index (WOMAC) score (Huang et al., 2016). These components from gut microbes can also stimulate the secretion of MMPs (Haglund et al., 2008) and exacerbate matrix degeneration of articular cartilage (MacDonald et al., 1994). Although the role of the gut microbiota in obesity-associated OA still needs to be further explored, some evidence was proposed that probiotics, prebiotics, and nutritional supplements can be used for normalizing the microbial communities and therefore may represent a therapeutic approach for obesity-associated OA treatment.

2.8 New Zealand Greenshell™ Mussel (*Perna canaliculus*), nutritional composition and bioactive lipid compounds

Perna canaliculus is a bivalve marine species commonly known as green-lipped mussel or Greenshell™ Mussel (GSM) and is endemic to New Zealand waters. GSM are distinguished from other species by the green colour around the lip of the shell which gives the mussel its name. Traditionally, interest in the beneficial health effect of GSM originates from observing that people living in coastal areas of New Zealand who frequently consumed GSM in their staple diet developed less arthritis than those residing inland.

The GSM industry has economic importance to New Zealand. In 1976, Seatone, a freeze-dried mussel extract was the first commercially anti-arthritic GSM product. Lyprinol, is an oil extract of GSM and was launched in 1998. The oil extract was obtained from stabilised, freeze-dried GSM powder by supercritical fluid extraction (CO2-SFE) (Singh et al., 2008; Whitehouse et al., 1997). Lyprinol capsules are composed of 50 mg mixture of GSM fatty acids (approximately 7.3 mg EPA and 5.5 mg DHA (14% and 11% of total fatty acids, respectively) formulated with 100 mg olive oil and 0.225 mg alpha-tocopherol as antioxidant (Singh et al., 2008).

Freeze-dried whole GSM extract is typically composed of a complex mixture of constituents mainly protein (55–60%), carbohydrates (5–15%), glycosaminoglycans e.g., chondroitin sulphate and heparin (5–15%), lipids (3–5%), mineral (5 %) and water (0.5–

4%). GSM contains micronutrients including iron, zinc, selenium, and antioxidants such as carotenoids, xanthophylls, and anthocyanins. The analysis of lipid content and fatty acid profile of GSM has shown that the lipid fraction contains a wide range of fatty acids, with approximately 90 fatty acids (Miller et al., 2011). The concentration of PUFA in GSM lipid ranged from 19 to 49.1 g/100 g, with 6–12 g/100 g DHA, and 8–24 g/100 g EPA as reported by a previous study (Miller et al., 2011). The lipid content and lipid classes of GSM are highly influenced by the season, lifecycle, diet, and habitat in which the mussels are grown. GSM gain omega-3 LC-PUFA from their diet, which is mainly zooplankton and phytoplankton. In addition, several minor lipid components including non-methylene-interrupted (NMI)-fatty acids, plasmalogen, phytosterols, and furan fatty acids are identified in GSM oil that are not present in most fish oil and have shown beneficial effects on human health (Miller et al., 2011; Wakimoto et al., 2011).

Evidence suggests that the anti-inflammatory activity of GSM predominantly resides in the lipid fraction. Novel anti-inflammatory omega-3 PUFAs including 5,9,12,15-octodecatetraenoic acid (OTA), 5,9,12,16-nonadecatertraenoic acid, 7,11,14,17-eicosatetraenoic acid (ETA), and 5,9,12,15,18-heneicosapentaenoic acid have been isolated from GSM oil (Singh et al., 2008; Treschow et al., 2007). Figure 2.2 illustrates the major and novel PUFA structures present in GSM.

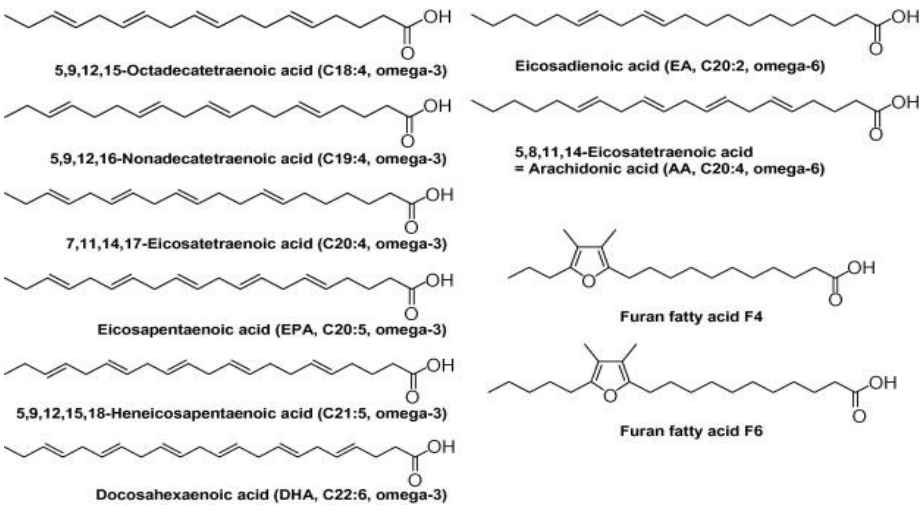

Figure 2.2 Structures of greenshell mussel omega-3 PUFAs

Omega-3 PUFA and novel anti-inflammatory furan fatty acids in comparison to omega-6 PUFAs eicosadienoic acid (EA) and arachidonic acid (AA). Produced from (Grienke et al., 2014) with permission from Elsevier.

2.9 Anti-inflammatory activity of New Zealand Greenshell^{TM} Mussel

The LC-PUFA have inhibitory effects on key enzymes in the arachidonic acid (AA) cascade. AA is metabolized by cyclooxygenase (COX) and lipoxygenase (LO) enzymes. As a result, metabolism of AA provides pro-inflammatory eicosanoids such as prostaglandins (PG), leukotrienes (LT), and 5-hydroxyeicosatetraenoic acid (5-HETE). Because of being structurally similar to AA, LC-PUFA from GSM competitively inhibit the COX and LOX enzymes by acting as alternative substrates (Bogatcheva et al., 2005).

Findings from *in vitro* studies have demonstrated the efficacy of the GSM lipid extract for inhibiting the 5-lipoxygenase (5-LOX) cascade in human monocytes stimulated with IL-4 (Dugas, 2000). Fatty acids sub-fractionated from the GSM lipid extract have shown strong inhibition of Leukotriene B4 (LTB4) and PG-E2 synthesis (Macrides et al., 1997). Further mechanisms of action of GSM oil include suppressing the expression of inducible nitric oxide synthase (iNOS), and COX-2 resulting in reduced nitric oxide (NO) and PGE2 synthesis; two mediators of the inflammatory response in macrophages. It also down-regulates the expression of pro-inflammatory cytokines, TNF-α, IL-6, and IL-1β, while suppressing the nuclear factor kappa B (NF-kB) signalling pathway through inhibiting the degradation of inhibitory kB and blocking the nuclear translocation of NF-kB (Chen et al., 2017). NF-kB is a transcription factor that plays a major role in the generation of cytokines, chemokines and other factors involved in the immune response (Baeuerle & Baichwal, 1997).

There is one *in vivo* study in which glycogen isolated from GSM showed anti-inflammatory effects in rats with induced footpad oedema. However, with further hydrolysis of protein the effect disappeared. This suggests that the protein component of glycogen was responsible for the anti-inflammatory effect (Miller et al., 1993). The majority of available studies on glucosamine and chondroitin sulphate are obtained from bovine and other marine sources. Because the origin of glucosamine or chondroitin sulphate has no impact on their bioactivity, it can be proposed that similar bioactivity can be found in GAG present in mussels. There is evidence from *in vitro* studies on chondrocytes that glucosamine and chondroitin exert anti-inflammatory effects through the inhibition of NF-kB pathways (Imagawa et al., 2011; Largo et al., 2003). Glucosamine and chondroitin supplementation resulted in lower systemic inflammation indicated by lower CRP levels in healthy individuals (Navarro et al., 2015). However, the effect of

whole meat GSM or protein and peptide fractions on inflammation has not yet been studied in humans.

2.10 Effect of Greenshell^{TM} Mussel extracts on cartilage metabolism and osteoarthritic symptoms

In addition to anti-inflammatory activity, the GSM extract prevents cartilage degradation. The chondroprotective effect of GSM oil in cytokine-induced canine chondrocytes has been investigated. GSM oils reduced the release of sulphated glycosaminoglycans (s-GAGs) (fragments derived from cartilage proteoglycan), down-regulated expression of catabolic genes including MMP1, MMP3, and MMP13 while up-regulating the expression of anabolic genes aggrecan (ACAN) and collagen type II alpha (COL2A1) (Buddhachat et al., 2017).

Subchondral bone remodelling plays a role in the early stages of the pathogenesis of MetOA pathogenesis (Goldring & Goldring, 2016), and regulating osteoclast activity is suggested as a therapeutic target related to OA subchondral bone remodelling (Zhu et al., 2021). A recent *in vitro* study revealed that the non-polar lipid fraction of GSM oil possesses potent anti-osteoclastogenic activity. Non-polar GSM lipids could inhibit osteoclast differentiation of the murine macrophage cell line, RAW 264.7 as shown by reduction of tartrate-resistant acid phosphatase (TRAP) activity and TRAP cell numbers (a marker of osteoclast activity and degree of bone resorption). It also diminished the formation of actin rings by osteoclasts which is an important element in bone resorption. In addition, mRNA expression of several genes related to osteoclast function and bone digestion including cathepsin K, carbonic anhydrase II (CA II), matrix metalloproteinase 9 (MMP-9), and nuclear factor of activated T-cells cytoplasmic 1 (NFATc1) a transcription factor of osteoclast differentiation was downregulated by the non-polar lipid fraction of GSM. The DHA and EPA in the form of free fatty acids were potentially responsible for this anti-osteoclastogenic effect (Siriarchavatana et al., 2021) .

In vitro studies on other components of GSM, glucosamine and chondroitin sulphate provided molecular mechanisms to support the effect of GSM in the prevention of cartilage loss. Similar to the lipid component of GSM, glucosamine sulphate can up-regulate mRNA and protein levels of aggrecan, and at the same time suppress the synthesis of collagen-degrading enzyme MMP-3 as shown in chondrocytes from OA

patients (Dodge & Jimenez, 2003) . The effect of omega-3 PUFA extracted from GSM (PCSO-524) on OA biomarkers was assessed in dogs diagnosed with OA. Treatment with GSM oil extract remarkably reduced cartilage degradation biomarkers measured by serum chondroitin sulphate WF6 epitope in contrast to the fish oil group. Further, clinical outcomes including movement and function were markedly improved (Soontornvipart et al., 2015).

The most recent pre-clinical trial investigated the cartilage-protective effect of whole meat GSM powder on early-stage metabolic OA in rats with diet-induced obesity. This study showed that rats fed with a high-fat/high-sugar (HFHS) diet enriched with whole GSM powder for 13 weeks had significantly lower serum concentrations of CTX-II than rats fed with just the HFHS diet. Furthermore, histopathological assessment (Mankin score) of cartilage in the knee joints was used to measure the severity of cartilage damage; with a trend for the HFHS rats to have a higher degree of surface irregularities than normal diet rats, and this was reduced in rats fed HFHS plus GSM (Siriarchavatana et al., 2019). The evidence suggested that dietary GSM can prevent or slow down the progression of metabolic OA at an early stage by preventing cartilage degradation.

Two studies used a high dose of GSM whole extract powder (3,000 mg/day) which was administered to patients with knee OA resulting in improvements in pain, stiffness, and mobility. The first study was an open label, single group pilot study, and was limited by the small sample size (n=21), and short duration (8 weeks) (Coulson et al., 2012). The second study was a randomised, comparator-controlled trial in which 40 participants were administrated either 3,000 mg/day of GSM whole extract powder or glucosamine sulphate for 12 weeks (Coulson et al., 2013). Both treatments reported significant improvements in OA outcomes with no significant differences between the two interventions in the second study.

Supplementation with stabilized GSM lipid extract (1,200 mg/day) containing 5.2% EPA and 3.4% DHA resulted in decreased pain and improvement in quality of life compared with a similar dosage of fish oil with higher EPA and DHA (18 and 12% of total fatty acids, respectively) in OA patients. This research implied that a stabilized GSM lipid extract is a well-tolerated and faster-acting complementary therapy than fish oil for OA treatment (Zawadzki et al., 2013). However, one study showed contradictory results. This study using a smaller dosage of a novel GSM lipid extract (600 mg/day), enriched in N-

acylethanolamine (NAE) and omega-3 LC-PUFA for 12 weeks, did not show any meaningful improvement in pain. However, joint stiffness improved in the lipid extract group and there was a reduction in paracetamol use in the GSM lipid extract-treated group compared to the placebo group during the study and after discontinuation of the intervention (Stebbings et al., 2017).

2.11 Effect of Greenshell^{TM} Mussel extract on body composition and metabolic parameters

There are a number of rodent studies that has shown an effect of GSM extract on modulating metabolism in obesity. A previous study showed increases in lean mass gain and decreases in fat mass gain in rats fed with HFHS plus GSM powder in comparison to rats only fed with HFHS (Siriarchavatana et al., 2019) . In line with this, another study reported reduced body weight gain, systemic inflammation and improved metabolic parameters in rats fed with a HFHS diet supplemented with freeze-dried powder of blue mussels for 12 weeks (Vaidya et al., 2017). Similarly, HFD enriched with GSM oil were able to completely prevent body weight gain compared to a HFD enriched in fish liver oil. The weight-reducing effect of the enriched diet was associated with decreased visceral fat mass (Loehfelm et al., 2021). In a recent study, the lipid lowering effect of GSM powder was reported. Adding GSM powder 25-45% in a basal diet of rats reduced the low-density lipoprotein cholesterol (LDL-C), and lipid peroxidation in liver tissue while increasing antioxidants such as glutathione and glutathione peroxidase activities (Aldairi et al., 2021). These findings suggest that GSM may modulate energy homeostasis and metabolism. Marine omega-3 PUFAs were demonstrated to reduce the size of visceral fat depots in diet-induced obese rats, without changing body weight and composition, emphasising a shift of adipose tissue (de Mello et al., 2019; Rokling-Andersen et al., 2009). This alteration in fat deposition might be due to modulation in lipogenesis and lipolysis mediated by PUFAs. A rodent study has shown that PUFAs increase lipolysis and fatty acid β-oxidation and supress lipogenesis in a tissue specific manner (Bargut et al., 2014). Therefore, consumption of New Zealand whole GSM extract could be advantageous for body composition and metabolic health in humans as well.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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Student name:	Maryam Abshirini
Name and title of main supervisor:	Professor Marlena C.Kruger
In which chapter is the manuscript/published work?	Chapter 3
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:

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Chapter 3 Green-lipped Greenshell™ Mussel (*Perna canaliculus*) extract supplementation in treatment of osteoarthritis: a systematic review

This systematic review helped to find gaps in the literature, to discover previous work in the area and identify their methodological strengths and limitations, and to investigate assessment questionnaires which can be employed for the purpose of this research.

This chapter is published as:

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Abstract

Objectives: Intervention studies using New Zealand green-lipped or Greenshell™ Mussel (GSM) (*Perna canaliculus*) extract in osteoarthritis (OA) patients have shown effective pain relief. This systematic review summarizes the efficacy of GSM extract in treatment of OA.

Methods: A literature search of the three databases EMBASE, MEDLINE, and Scopus was performed to identify relevant articles published up to March 2020. Inclusion criteria were clinical trials published in English measuring the effect of supplementation of whole or lipid extract of GSM on pain and mobility outcomes in OA patients.

Results: A total of nine clinical trials were included in systematic review, from which five studies were considered appropriate for inclusion in forest plot. Pooled results showed GSM extracts (lipid extract or whole powder) provide moderate and clinically significant treatment effect on visual analogue scale (VAS) pain score (effect size: -0.46; 95% confidence interval (CI): -0.82 to -0.10; P=0.01). The whole GSM extract improved gastrointestinal symptoms in OA patients taking anti-inflammatory medications as shown in one study. The GSM extract was considered generally well-tolerated in most of the studies.

Conclusion: The overall analysis showed that GSM provided moderate and clinically meaningful treatment effects on OA pain. However, the current evidence is limited by the number and quality of studies, and further larger and high-quality studies are needed to confirm the effectiveness and to identify the optimal GSM format. Nevertheless, it is worth considering using GSM extracts especially for patients seeking alternative pain relief treatments with fewer side effects compared to conventional treatments.

3.1 Introduction

Osteoarthritis (OA) is the most common degenerative joint disease involving cartilage and surrounding tissues. It is a leading cause of disability worldwide, particularly among the elderly. A rise in OA incidence has correlated with growing populations of elderly and obese people (Cross et al., 2014; Litwic et al., 2013). The economic cost of OA is substantial; this includes not only medical-related expenses but also losses in work productivity (Altman, 2010). The Osteoarthritis Research Society International (OARSI) guidelines recommend using analgesics and non-steroidal anti-inflammatory drugs (NSAIDs) to treat pain and inflammation (Bannuru et al., 2019). However, frequent and long-term use of these conventional therapies are often associated with adverse gastric events (Lamarque, 2004), which has prompted OA patients to seek alternative and complementary medicines for managing their symptoms (Ramsey et al., 2001). Further studies for identifying effective and safer treatments are required. A range of nutritional supplements, including glucosamine and chondroitin sulphate have been widely used by OA patients. However, meta-analyses and reviews have revealed contradictory results. A recent meta-analysis concluded that dietary supplements glucosamine and chondroitin were ineffective and provided only a small treatment effect on pain at medium- and long-term follow-ups. The lack of effect was explained by small number of studies included in meta-analysis and unavailability of long-term data. Moreover, approximately 50% of the included trials were at high risk according to the Cochrane risk of bias tool highlighting some limitations including inadequate description of or inappropriate methods of randomisation (Liu et al., 2018). In contrast, a second meta-analysis assessing 29 studies found oral supplementation with glucosamine or chondroitin sulphate significantly reduced pain in knee OA (Simental-Mendia et al., 2018).

Perna canaliculus, the green-lipped mussel, is endemic to New Zealand. It is grown for aquaculture only in New Zealand, where it is trademarked as the Greenshell™ Mussel (GSM). Various therapeutics such as Lyprinol® are produced from GSM. When taken orally in whole powder or oil extract formats, GSM has been found to be beneficial for pain relief, reducing inflammation and ameliorating other debilitating symptoms associated with inflammatory diseases such as rheumatoid arthritis (RA) and OA without causing the adverse side effects of NSAIDs. The underlying mechanisms explaining these effects are in the anti-inflammatory activity of bioactive lipids in mussels including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These lipids mediate the

inflammatory response by inhibiting both the cyclo-oxygenase (COX) and lipo-oxygenase (LOX) cascades of arachidonic acid (AA) metabolism, which results in a decrease in the synthesis of pro-inflammatory prostaglandins and leukotrienes (Halpern, 2000; McPhee et al., 2007). Further, GSM is reported to contain novel bioactive lipids such as pro-resolving lipid mediators which resolve inflammation through counter-regulating pro-inflammatory cytokines, clearing apoptotic neutrophils, and inducing wound healing and tissue regeneration (Wakimoto et al., 2011).

There have been only three systematic reviews evaluating results of clinical studies assessing the effects of GSM (whole or lipid extract) on joint symptoms of OA (Brien et al., 2008; Cobb & Ernst, 2006; Ulbricht et al., 2009), and none published in the last decade. These systematic reviews were conducted on only four or five randomised controlled trials (RCTs). All three concluded that GSM extracts were beneficial as adjuvants to conventional therapies for arthritic conditions. However, these systematic reviews were limited by the number of studies and included studies with methodological deficiencies which prevented them from reaching comprehensive and reliable conclusions. Hence a rigorous systematic review on this subject is needed to present updated and more conclusive evidence to evaluate the efficacy and safety of GSM supplements in clinical practice. In the present systematic review, we included a larger number of studies (n=9) and larger sample size to provide more representative results. The aim of this analysis was to systematically review the existing clinical trials in the literature in order to evaluate the effectiveness of GSM supplementation in the treatment of OA symptoms.

3.2 Material and methods

3.2.1 **Data sources and searches**

This systematic review was performed in accordance with the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (Moher et al., 2009). A systematic electronic search was conducted in the following databases from inception to March 2020: MEDLINE, Scopus, and EMBASE. A combination of relevant keywords including osteoarthritis, degenerative joint disorder, green-lipped mussel, *Perna canaliculus*, greenshell mussel, Seatone, Lyprinol, PCSO-524 and randomised clinical trial was applied to construct the search strategy. Initial screening of potentially relevant

records was conducted based on title and abstract, and then a final selection of included trials based on full-text evaluation was performed. In addition, the reference lists of included studies were reviewed for potential studies that were missed by the algorithm.

3.2.2 **Study selection and data extraction**

Clinical trials published in the English language were evaluated against the pre-defined criteria to be included in review. The eligibility criteria were randomised controlled or non-randomised controlled clinical trials; the GSM supplement was used as intervention in the form of lipid extract or freeze-dried whole powder (not in combination with other constituents with anti-inflammatory or anti-arthritic activity); the patients/participants were diagnosed with OA by radiography; and outcomes of interest relevant to OA were assessed. There are no published reports identifying significant differences in treatment outcomes based on joint location, and a study of gene expression comparing OA versus normal cartilage found that the genes associated with OA were not correlated with joint-site (Ramos et al., 2014). Therefore, specifying the site of OA was not a requirement for inclusion in this review; this factor also enabled the inclusion of more available trials for assessment. A summary of each trial including the general study design; number of participants; dose and duration of intervention; concomitant medication; and outcome measures and adverse effects, is outlined in Table 3.1.

3.2.3 **Risk of bias assessment**

The five-point Jadad scale was applied to assess the quality of the included trials (Jadad et al., 1996). In this scale, the likelihood of bias is measured based on the description of randomisation, blinding, and withdrawals from the trial on a scale of 0 (minimum) to 5 (maximum). Based on the total awarded score, studies with 0-2.5 scores were categorised as low quality and studies with 3-5 scores were defined as high quality presented in Table 3.1.

3.3 Results

3.3.1 **Initial search and result**

A flowchart of the search strategy and selection process is displayed in Figure 3.1. A total of 38 citations were retrieved from the searched databases and, after removal of duplicates, 30 publications were examined by title and abstract. Of these, 19 were

discarded due to including non-OA patients, or not RCT. The remaining 11 studies were selected for full text reading and were also examined to ensure that the researchers had obtained ethical approval for the study. Of these 11 studies, 2 did not meet the inclusion criteria and were excluded: one study administrated GSM in both the intervention and comparator group (placebo) (Puente et al., 2014), and in the second study GSM was in combination with other bioactive ingredients (Qu et al., 2015). This resulted in a final total of 9 studies to be included in this review.

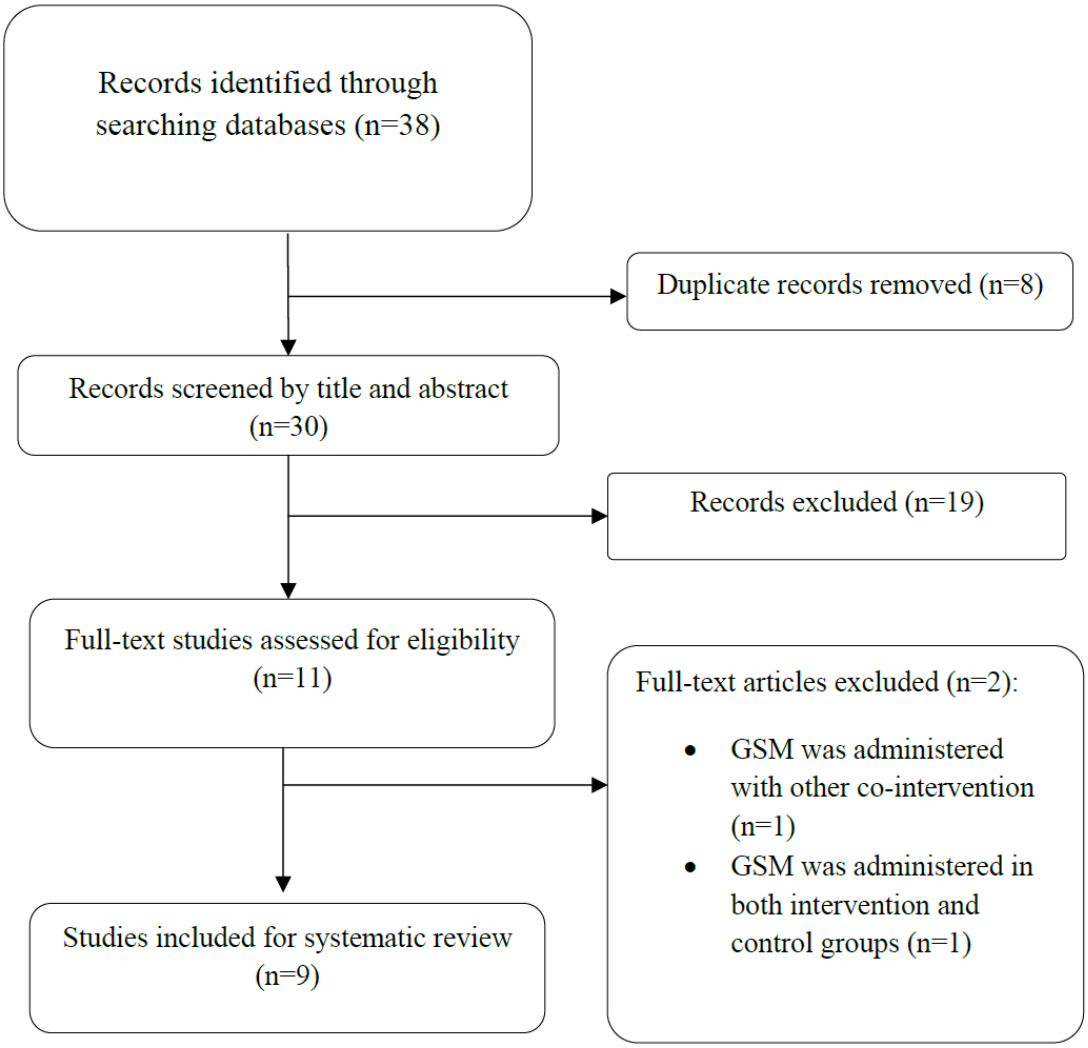

Figure 3.1 Flow diagram of study selection process

3.3.2 **Study characteristics**

Nine selected studies were published between 1980 and 2017. All studies involved both sexes with sample sizes ranging from 21 to 80 participants (Table 3.1). Across the studies, the average age of subjects ranged from 52.8 to 69.6 years. Four studies investigated only patients with knee OA (Coulson et al., 2013; Coulson et al., 2012; Gibson & Gibson, 1998; Lau et al., 2004). Three studies included patients with knee and/or hip OA (Cho et al., 2003; Stebbings et al., 2017; Zawadzki et al., 2013). Two studies did not specify the joint location (Gibson & Gibson, 1998; Gibson et al., 1980).

The treatment and follow-up duration varied among studies from 8 weeks to 6 months. A variety of product preparations and different dosages were evaluated. GSM was used in the form of a whole extract powder in four studies, with doses ranging from 1,050 to 3,000 mg/day (Audeval & Bouchacourt, 1986; Coulson et al., 2013; Coulson et al., 2012; Gibson et al., 1980). Four studies used a lipid extract in doses ranging 210 and 1,200 mg/day (Cho et al., 2003; Lau et al., 2004; Stebbings et al., 2017; Zawadzki et al., 2013). A single study compared a GSM lipid extract versus a whole GSM powder (Gibson & Gibson, 1998). The included studies used olive oil (Lau et al., 2004), glucosamine sulphate (Coulson et al., 2013), corn oil (Stebbings et al., 2017), whole fish powder (Gibson et al., 1980) or fish oil (Zawadzki et al., 2013) as controls; one study used a non-specified placebo (Audeval & Bouchacourt, 1986) and two studies lacked a comparator group (Cho et al., 2003; Coulson et al., 2012).

Seven of the nine included trials were parallel and randomised controlled trials (Audeval & Bouchacourt, 1986; Coulson et al., 2013; Gibson & Gibson, 1998; Gibson et al., 1980; Lau et al., 2004; Stebbings et al., 2017; Zawadzki et al., 2013), and the remaining two were one-arm open-label trials (Cho et al., 2003; Coulson et al., 2012). Two studies included patients with both OA and RA, but their results were analysed and presented separately (Gibson & Gibson, 1998; Gibson et al., 1980). Five of the nine studies were assessed to be of low quality (Jadad score of 1 – 2.5), and four studies were assessed to be of high quality (Jadad score of 3 – 5). According to the study quality assessment using the Jadad scale, only one study fulfilled all the criteria required to reach a score of 5 (Stebbings et al., 2017). Most of the studies had inclusion criteria restricted to the patients clinically diagnosed with OA, and some studies reported the data on severity of disease.

A range of outcomes were measured using standardized assessments, the most prevalent of which were the self-reported Visual Analogue Scale (VAS) for pain and the Western Ontario and McMasters OA Index (WOMAC) for OA outcome measurements (Coulson et al., 2013; Coulson et al., 2012; Stebbings et al., 2017). Other standardized tests included the patient's and/or physician's global assessment, the Lequesne Algofunctional Index for OA severity, quality of life assessed by Health Assessment Questionnaire (HAQ), short form version 12 heath survey (SF-12), and the Osteoarthritis Quality of Life (OAQol) assessment. Individualized or customized assessments were also performed including duration of morning stiffness, time taken to walk a fixed distance, grip strength, and assessment of gastrointestinal function or microbiota.

Table 3.1 General characteristic of the studies included in systematic review.

Study, year	**Study design**	**Sample size (female/male) Mean age**	**Participants [1]**	**Intervention and dose**	**Control**	**Concomitant medication**	**Duration of study**	**OA outcomes Measures**	**Results [2]**	**Jadad score**
Clinical studies assessing the effect of GSM whole extract powder										
Gibson, 1980	Double-blind, randomized placebo-controlled	I=16 C=22 (37F /1 M) 68.6 years	OA	Whole extract powder 1050 mg/day	Inactive fish capsule	NSAIDs	3-6 months	1. VAS 2. Morning stiffness 3. Functional index 4. Time taken to walk 50 feet 5. ROM of hip and knee joints Patient's global assessment	The GSM extract improved VAS and stiffness. No significant improvement in in ROM or grip strength was observed.	1.5
Audeval, 1986	Randomized, double-blind, placebo-controlled trial	I=27 C=26 (37 F/16 M) 65 y	Knee OA	6 capsules of whole extract powder/day Dosage not Specified	Placebo	NSAIDs	6 months	1. ARA functional classification 2. Duration of morning Stiffness 3. Intensity of pain 4. Joint mobility 5. Distance from heel to cheek 6. Utilizing walking sticks	The whole GSM extracts improved ARA function, pain intensity and patients and physicians global assessment.	2

								Patient's and physician's global assessment		
Coulson, 2012	Non-blinded, Non-randomized pilot	I=21 (13 F/8 M) 61.1 y	Knee OA	Whole extract powder (3,000 mg/day)	None	NSAID, Paracetamol	8 weeks	Primary outcome: 1. WOMAC to assess pain, stiffness and limitation of physical function. 2. Lequesne algofunctional index to measure pain, walking distance, and activities of daily living. Secondary outcome: 3. SF-12 to assess general quality of life GSRS to assess gastrointestinal function	All outcomes were improved; however, SF-12 showed improvement for physical but not mental components of quality of life.	1
Coulson, 2013	Randomized, non-blinded, Comparative controlled	I=21 (16 F/5 M) 56.7 y C=17 (12 F/5 M) 60 y	Knee OA	Whole extract powder (3,000mg/day)	Glucosamine sulphate	NSAIDs, paracetamol	3 months	Primary outcome: 1. Gut microbiota profile Secondary outcome: 2. WOMAC	Both treatment group significantly improved all the OA outcome measures and GSRS score.	3

								3. Lequesne Algofunctional index 4. SF-12 GSRS	In mussel group *Bifidobacterium* tended to increase and *Enterococcus* and yeast species to decrease. The *Clostridia species* tended to decrease from baseline to week 12 in both groups.	
Clinical studies assessing the effect of GSM lipid extract										
Gibson, 1998	Randomized, double-blind, comparative controlled for phase I, None-blind, non-controlled for phase II	I=15 (10 F/5 M) 57.3 y C=15 (12 F/3M) 52.8 y	OA	Lipid extract 210 mg/day	Whole extract powder 1,150 mg/day	3-6 months	NSAIDs	Assessment of pain and function: 1. VAS 2. Articular index 3. Morning stiffness 4. Grip strength 5. Night pain 6. Patient and physician's global assessments	Both GSM preparations improved all outcome measures, except for the VAS that improved only in GSM extract powder group.	2.5

Cho, 2003	Non-randomized, non-blind, non-controlled trial	I=54 (52 F/2 M) 61 y	Knee and/or hip OA	4 capsules of lipid extract /day Dosage not Specified	None	8 weeks	Use of NSAID was discontinued Use of rescue medication was not specified	Assessment of pain and function: 1. VAS, 2. Lequesne algofunctional index 3. Patient and physician's global assessment	Significantly improved all the outcomes	1
Lau, 2004	Randomized, double-blind, placebo-controlled trial	I=40 (35 F/5 M) 62.1 y C=40 (34 F/ 6 M) 62.9 y	Knee OA	Lipid extract 4 capsules/day for 2 months and then 2 capsules /day for 4 months Dosage not Specified	Olive oil	6 months	Paracetamol, additional rescue medication	Assessment of pain and function: 1. VAS 2. Patient's and Physician's global assessment 3. COKS 4. CAIMS2-SF	Significantly improved all outcomes	3
Zawadzki, 2013	Randomized, blinded, comparative controlled in stage I, non-blinded in stage II	Phase I I=25 (22 F/3 M) 65.58 y C=25 (22 F/3 M) 66.72 y Phase II I=22 (19 F/3 M) 67.23 y	Knee and/ or hip OA	Lipid extract 4 capsules 1,200 mg/day	Fish oil 1,200 mg/day	3-6 months	Paracetamol	Assessment of pain: 1. VAS (100 mm) Assessment of quality of life: 1. HAQ which cover eight categories of daily physical activities. These	In phase I and II treatment with the lipid extract showed a significant improvement of VAS, HAQ all categories and health and disease condition.	4.5

								included dressing, arising, hygiene, walking, heating, eating, grip and daily activities. Health and Disease Condition	Lipid extract did not show notable side effects while fish oil reported adverse side effect.	
Stebbings, 2017	Randomized, double-blind, placebo-controlled trial	I=39 (22 F/17 M) 66.4 y C=41 (22 F/19 M) 66.5 y	Knee or hip OA	Lipid extract enriched in NAE and long-chain omega-3 fatty acids 4 capsules 600 mg/day	Corn oil	3 months	NSAID, analgesic	Assessment of pain and function: 1. WOMAC pain scale 2. VAS (100 m) 3. Patient global assessment, 4. Total WOMAC score 5. WOMAC −20 responder 6. Physician global assessment Assessment quality of life 7. HAQ 8. OAQol Change in analgesic use recorded in diary	No improvement on pain and quality of life (VAS, OAQol, WOMAC pain subscale, Physician and patient global assessment, HAQ, WOMAC −20 responder, P >0.05). Joint stiffness significantly improved WOMAC stiffness subscale (P = 0.04).	5

									Significant difference in intake of paracetamol between the two groups (P=0.001).	

ARA: American Rheumatism Association, CRP: C-reactive protein, COKS: Chinese version of the Oxford Knee Score, CAIMS2-SF: Chinese version of the Arthritis Impact Measurement Scale 2-short form, C: control, ESR: erythrocyte sedimentation rate, GSRS: Gastrointestinal symptom rating scale, HAQ: Health assessment questionnaire, I: intervention, ROM: range of movement, SF-12: short from version 12 heath survey, OAQol: quality of life, VAS: Visual analogue scale, NAE: N-acylethanloamine, WOMAC: Western Ontario and McMasters OA Index.

[1] Number of participants enrolled. [2] For controlled trials, the effect of GSM was compared to control, and for the non-controlled trials, the baseline of the treatment group was compared to the endpoint.

3.3.3 **Data synthesis and analysis**

To enhance a visual inspection of favourable GSM extract results over placebo or a comparator, data from controlled trials that included the VAS pain scale as a primary outcome were pooled using a random-effects model. The mean difference (MD) of VAS between control and intervention groups was applied to calculate the overall effect size of the intervention. The effect size was expressed as the standardized mean difference (SMD) and 95% confidence interval (CI) from the random effects model. We applied the following formula to calculate the standard deviation (SD) of the MD for studies that did not report this parameter: $SD^2 = [(SD\ baseline^2 + SD\ final^2) - (2 \times 0.8 \times SD\ baseline \times SD\ final)]$. The heterogeneity across studies were evaluated by the I^2 statistic. Heterogeneity was considered low if $I^2 < 30\%$, moderate if $I^2 = 30–75\%$, and high if $I^2 > 75\%$. The random-effect model was used in the presence of high heterogeneity between studies (Higgins & Thompson, 2002). We considered the treatment effect small if the effect size was <0.3, moderate if the effect size was between 0.3 and 0.8, and large if the effect size was > 0.8. To interpret whether the effect size was clinically important, a threshold of 0.37 standardized units was considered as the minimum clinically important difference (MCID); this was based on previous reviews (da Costa et al., 2017; Liu et al., 2018).

The data included in analysis were based on the per-protocol data. Sensitivity analysis using influence analysis was performed to test the impact of each study on the overall effect size by the leave-one-out method (when one study is removed at a time and the analysis is repeated). Funnel plots were not carried out to indicate the presence of publication bias as it would be unreliable because this review contains less than 10 studies (Higgins et al., 2019). The analyses were performed using STATA version 14 (StataCorp, College Station, TX, USA). A p-value < 0.05 was considered statistically significant.

3.3.4 **Forest plot analysis**

From nine included studies, five were considered appropriate for inclusion into forest plot analysis. Four studies excluded because did not assess the VAS as outcome of our interest or were non-controlled clinical trial. The pooled analysis identified moderate and clinically important effects of GSM supplementation in reducing the VAS pain score (SMD: −0.46, 95% CI -0.82 to -0.10, P= 0.01), as shown in Figure 3.2. However, this

result was collated from a limited number of studies (n=5) with participant numbers ranging from 30 to 80 (278 cumulative participants). There was substantial heterogenicity among the included studies (I^2=53.7%, P=0.07). We were unable to explore potential sources of heterogeneity or the influence of different factors on the treatment effect due to the limited number of studies available for assessment.

The sensitivity analysis showed after removing each single study and reanalysing the effect sizes of the other studies, two studies by Zawadzki, et al.,2013 and Coulson et al., 2013 had significant effects on the pooled effect size (Appendix 1; Table S3.1).

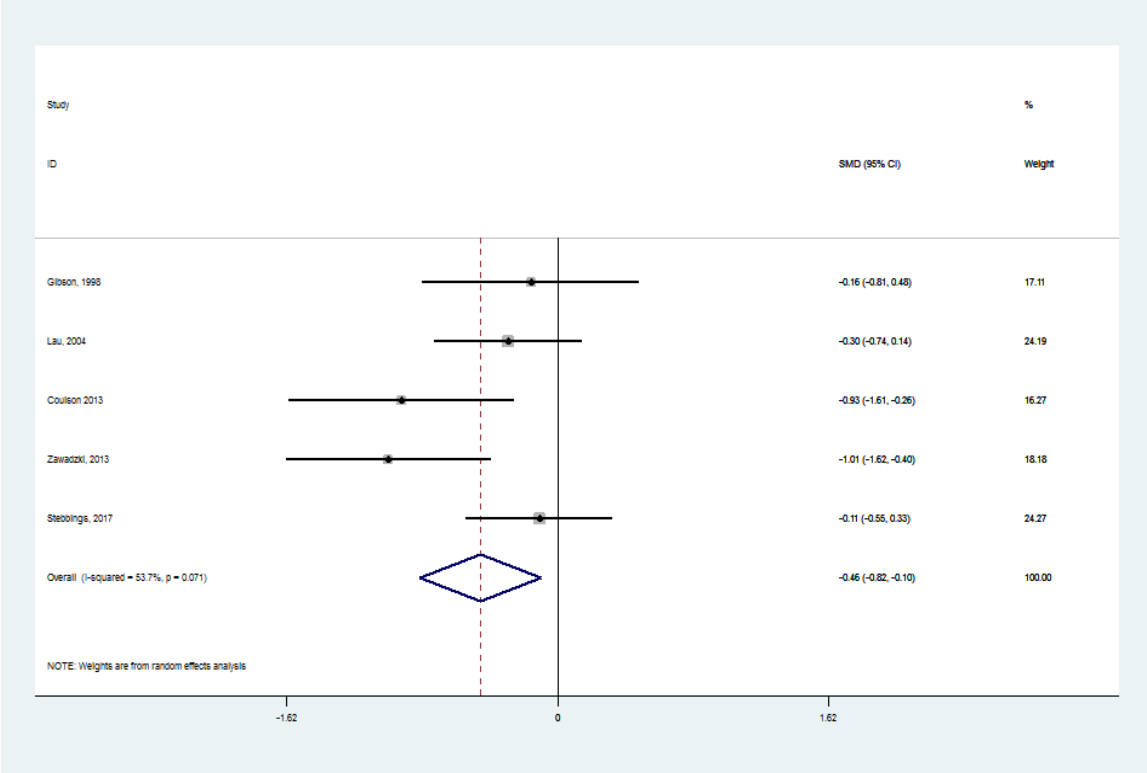

Figure 3.2 Forest plot displaying standard mean difference (SMD) and 95% confidence interval (CI) measuring the impact of GSM supplementation on VAS pain score.

The black dots estimate the study result and give a representation of the size of the study. A horizontal line representing the 95% confidence intervals of the study result, with each end of the line representing the boundaries of the confidence interval. The diamond represents the effect size and confidence intervals that combines all the individual studies together.

3.4 Discussion

We performed a comprehensive systematic review on the efficacy of both types of GSM preparations (whole stabilized powder and lipid extract) in treating knee and hip OA

symptoms, including 9 clinical trials and 452 participants. The OA signs, symptoms and physical function of each patient were assessed by the research groups using various parameters. VAS, the most prevalent measure for pain assessment, revealed a significant reduction in pain favouring the intervention group. Improvements in stiffness, physical function, and physical aspects of quality of life due to GSM intervention were also observed in most of the studies. However, the mental component of quality of life did not change meaningfully. Overall, our review demonstrates GSM products are moderately effective in reducing the VAS pain score in OA patients, however caution is required in interpretation of this finding due to the various study limitations. With the exception of some minor gastrointestinal adverse side effects reported by one research group (Coulson et al., 2013; Coulson et al., 2012), both lipid and powdered whole extract products were generally well-tolerated and safe to use.

The earliest studies using freeze-dried GSM powder reported a trend for improvement in pain and stiffness compared to a placebo (Audeval & Bouchacourt, 1986; Gibson et al., 1980). However, a notable weakness of these earlier studies was the lack of stability of the mussel products, which strongly affected their anti-inflammatory potency. Moreover, these studies did not provide information on the dose of NSAID medications used by the participants, and generally were of low quality and lacking in rigorous methodology (Whitehouse et al., 1997). There was only one RCT identified in the literature which compared the efficacy of a lipid extract and stabilized whole GSM powder in OA patients (Gibson & Gibson, 1998). In this randomised, double-blinded, controlled clinical trial with a parallel design, patients received the comparatively low dose of 210 mg/day lipid extract, or 1150 mg/day stabilized GSM powder for three months, after which all patients received the lipid extract for a further three months. The evaluation by patient and physician revealed a significant improvement in stiffness and function in both intervention groups reported by 70% of patients. The results of this study showed no difference between the degree of efficacy and the required time to show efficacy in lipid extract versus stabilized powder groups (Gibson & Gibson, 1998). However, a significant improvement in the VAS pain scale was observed only in the whole GSM powder group as 70% of patients benefited from it. This intriguing finding suggests that non-lipid components contributed to the health benefits, given that GSM has been shown to contain <2% lipids by wet weight (Miller et al., 2014), and approximately 8% lipids by dry weight (Siriarchavatana et al., 2019); thus, 1,150 mg GSM powder would contain <100 mg lipid,

which is less than half of the dose given to the lipid-group participants. Further studies are clearly needed to determine the relative contributions of GSM lipids, proteins, and other components to its beneficial effects in reducing OA symptoms.

In two other studies in which a high dose of GSM whole extract powder (3,000 mg/day) was administered to patients with knee OA, subjects in the intervention group exhibited improvements in OA outcome measures (Coulson et al., 2013; Coulson et al., 2012). The first study was an open label, single group pilot study, and the subsequent one was a non-blinded randomised, comparator-controlled trial. It is worth noting that the first study was limited by a small sample size (n = 21) and short duration (8 weeks) (Coulson et al., 2012). The first study showed that the WOMAC subscales and Lequesne algofunctional index as outcome measures for pain and function were improved; however, the general quality of life assessed through 12-item short form health survey (SF-12) showed improvement only in terms of physical components and not mental components. The follow-up study included a larger sample size (n=40) and compared 3,000 mg/day of GSM whole extract powder with 3,000 mg/day of glucosamine sulphate for a longer period of 12 weeks (Coulson et al., 2013). In this study, both groups reported significant improvements in the same outcomes as the first study, but no significant differences between the two interventions were observed. In must be noted that this study was not blinded, and allocation concealment is important particularly when reporting the patients' reported outcomes. However, it was not considered as exclusion criteria to enable the inclusion of more trials (Coulson et al., 2013). Interestingly, both studies showed that GSM whole extract powder significantly attenuated the detrimental gastrointestinal aspects assessed by GSRS scores, which are linked to the long-term use of NSAID medications. In addition to measuring gastrointestinal function, the second study assessed the gut microbiota profile. Both whole GSM extract and glucosamine sulphate supplementation resulted in slight changes in the microbiota profiles from baseline, with the most notable being a decrease in *Clostridia* species. These bacteria can induce a T-cell driven gastrointestinal inflammatory response (Stepankova et al., 2007), and the decrease in *Clostridia* occurred concurrently with both a decrease in inflammation and improvement in gastrointestinal and joint symptoms. These results further suggest that alteration in gut microbiota profiles should be regarded as a critical factor in determining the therapeutic efficacy of nutraceutical interventions such as GSM and glucosamine for

OA treatment (Coulson et al., 2013). Additional studies are warranted to confirm the interaction of GSM bioactive compounds with intestinal microbiota.

A number of studies have assessed the efficacy of GSM lipid extracts in treating OA. The Lau study (Lau et al., 2004) involving knee OA patients identified a positive benefit of GSM lipid extract versus olive oil control over 6 months. In this study all the arthritis assessment parameters including VAS, patient's, and physician's global assessment of arthritis, validated Chinese version of the Oxford Knee Score (COKS), and a validated Chinese version of the Arthritis Impact Measurement Scale 2-short form (CAIMS2-SF) that assessed physical function and psychological status, were significantly improved in the lipid extract group compared to olive oil. The greatest improvements from week 4 associated with GSM lipid were in the VAS pain perception and patients' global assessment of arthritis. Consistent with this study, an RCT by Zawadzki, et al. (Zawadzki et al., 2013) compared equal doses (1,200 mg/day) of GSM extract containing 5.2% EPA and 3.4% DHA versus fish oil containing a standardized amount of 18% EPA and 12% DHA in patients with knee and/or hip OA. Significant and positive effects on VAS pain scale quality of life and overall health condition were reported in the GSM group compared to fish oil. There was an 89% decrease in VAS pain score and 91% of patients reported an improvement in quality of life. No apparent adverse effects were observed in lipid extract group, while patients treated with fish oil showed less meaningful reduction in VAS pain score, and insignificant quality of life accompanied by some adverse effects. The researchers suggested that larger doses and a longer duration were required for fish oil to exhibit efficacy. Overall, this study concluded that GSM lipid extract was a safer alternative and resulted in faster and more notable treatment effects with smaller dosages compared with fish oil. It is of interest to note that the GSM resulted in significantly better health outcomes in this study even though the fish oil intervention contained three-fold higher amounts of EPA and DHA, suggesting that other components in GSM provided the bioactivity. This result is consistent with evidence that the GSM lipid fraction contains various bioactive lipids and unique fatty acids such as tetraenoic acids with great anti-inflammatory activity (Sinclair et al., 2000). These fatty acids have been demonstrated to compete much more efficiently than AA for the COX and LOX pathways (Treschow et al., 2007). Furthermore, furan fatty acids are unstable anti-inflammatory and antioxidant components detected in GSM in minor amounts. In a study by Wakimoto et al that employed the semisynthetic route from the furan dicarboxylic acid (the shark metabolite

of furan fatty acids), furan fatty acid ethyl esters showed more potent anti-inflammatory effect than EPA in a rat model of adjuvant-induced arthritis (Wakimoto et al., 2011).

In the most recent RCT reported by Stebbings (Stebbings et al., 2017), 80 patients with moderate to severe OA were randomly provided with a novel GSM lipid extract enriched in N-acylethanloamine (NAE) and long-chain omega-3 fatty acids (600 mg/day) versus corn oil as placebo over 12 weeks. In contrast to previous findings, the results showed no statistically significant difference between the intervention and placebo groups in pain or quality of life outcomes measured by various assessments including VAS, WOMAC-pain subscale, patient's and physician's global assessment, OAQol, and HAQ. However, joint stiffness measured by WOMAC-stiffness subscale improved in the lipid extract group compared to the placebo. In addition, patients in the lipid extract arm reduced their intake of paracetamol in comparison to the placebo group and this effect continued for 3 weeks after the intervention ceased at week 12. The researchers suggested that significant pain improvement was not detected due to the severity of disease in this cohort, as half of the patients had moderate to severe OA. This suggests that GSM may have greater efficacy on stiffness rather than pain perception in severe OA. Alternatively, the GSM product used in this study may have lacked specific bioactive lipids; in previous studies that obtained positive results with high doses of freeze-dried powdered products or lipid extracts the test products are likely to have contained different profiles of lipids compared to the product used by the Stebbings study. It would be interesting for future RCTs to assess pain and stiffness improvements in patients with differing stages of OA severity, and to identify the precise components that provide the bioactivity.

One limitation in all the studies included in this review was that of participant sex. All recruits were middle-aged or elderly, and all studies included participants of both sexes, but females predominated (nearly 80%). Because of this, the findings may not extrapolate to the male population. A second limitation was the absence of data on the menopausal status or use of hormone therapy by female participants. After reaching menopause, women have a higher risk and present more advanced stages of disease and more debilitating OA pain compared to age-matched males (Hame & Alexander, 2013; Tanamas et al., 2011). There is also evidence suggesting that oestrogen therapy reduces risks of joint symptoms (Chlebowski et al., 2013). Therefore, future studies should specifically include the menopausal and hormonal status of female participants. A third

limitation is duration of study: half of the included studies in this review were carried out for ≤ 3 months. It cannot be determined whether GSM supplements would have retained their efficacy for a longer period, or conversely whether additional health benefits would have been observed.

Moreover, it is worth noting that the number of studies available for this review was limited, which emphasises the need for further RCTs in order to establish optimal treatments and doses. These studies should consider controlling for gender differences, monitor adverse effects, measure changes in gut microbiota and employ standardized measurements for assessing OA symptoms. Furthermore, the included studies did not report the data on the omega-3 intake through dietary consumption or presence of omega-3 in supplements or placebo, which may have caused variability in efficiency across the studies, and this needs to be addressed in future trials.

This review expands on previous reports of GSM supplementation as a treatment for OA, as it has assessed a larger number of studies. Although most of the included studies were funded by pharmaceutical companies, the companies did not play roles in data analysis or interpretation, reducing the likelihood of bias. The forest plot for VAS pain outcome in this review was able to clearly demonstrate moderate efficacy of GSM over placebos or other interventions. From the evidence currently available, we conclude that use of GSM for patients with OA may provide benefit on pain and does not cause significant negative side effects. GSM could be of use, especially for those patients who seek alternative options for pain improvement with fewer gastrointestinal side effects. However further studies are needed to provide additional data.

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Student name:	Maryam Abshirini
Name and title of main supervisor:	Professor Marlena C.Kruger
In which chapter is the manuscript/published work?	Chapter 4
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:
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Chapter 4 Mass spectrometry-based metabolomic and lipidomic analysis of the effect of high fat/high sugar diet and Greenshell™ Mussel feeding on plasma of ovariectomized rats

The objective of this chapter was to examine the alteration in lipid and metabolite profiles of ovariectomized (OVX) rats with diet-induced obesity as a model for metabolic syndrome-associated osteoarthritis (MetOA) in postmenopausal women and to assess the effect of Greenshell™ Mussel (GSM) supplementation on this model using an untargeted liquid chromatography-mass spectrometry (LC-MS) metabolomics approach.

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Abstract

Background: The studies on animal model of obesity have provided evidence for beneficial effects of whole GSM powder on cartilage metabolism as well as metabolic parameters such as reduction in body weight gain, the however impact of GSM on metabolomic profile has not been investigated. This study aimed to examine the metabolomic changes caused by diet-induced obesity in ovariectomized (OVX) rats as a model for metabolic syndrome-associated osteoarthritis (MetOA) and effect of supplementation with Greenshell^{TM} Mussel (GSM) powder on this model using an untargeted liquid chromatography-mass spectrometry (LC-MS) metabolomic approach.

Methods: Ninety-six rats were fed with one of the four diets: control, control supplemented with GSM (control + GSM), high fat/high sugar (HFHS), or high fat/high sugar enriched with GSM (HFHS + GSM). After 8 weeks on experimental diets, half of the rats in each group were OVX and the other half were sham operated. After being fed for an additional 28 weeks, blood samples were collected for the metabolomics analysis. Lipid and polar metabolites were extracted from plasma and analysed by LC-MS. Multivariate analysis including principal component analysis (PCA), and orthogonal partial least squares discriminant-analysis (OPLS-DA) were performed to obtain information on differences in lipid and polar metabolite profiles between experimental diet groups. The features with values of variable important in projection (VIP) > 1 and false discovery rate (FDR) < 0.05 were selected as potential biomarkers. Subsequently, the relative intensity of these features was used to calculate the fold change (FC) to measure the magnitude of change between the treatment and their control groups.

Results: Twenty-nine lipid species were identified from four lipid subclasses including phosphatidylcholine (PC), lysophosphatidylcholine (LPC), diacylglycerol (DG), triacylglycerol (TG). In OVX rats, the majority of lipids including six DG, one LPC, fifteen PC, and four TG were down-regulated under HFHS diet, while two TG (56:10, 60:12) containing long chain polyunsaturated fatty acids (LC-PUFA) were up-regulated by HFHS diet compared to control diet. Adding GSM to HFHS diet increased the relative intensity of ten lipids that had been down-regulated by HFHS diet indicated by FC >1 including DG (47:10, 49:10), LPC (20:5), PC (34:3, 36:5, 36:6, 38:8), and TG (50:3, 54:7, 54:8). In sham operated rats, 29 lipids were identified of which 22 lipids were common

with the lipid identified in OVX rats. Similar to OVX rats, the relative intensity of the majority of lipids were decreased under HFHS diet when compared to control diet group, however inclusion of GSM to HFHS diet increased the relative intensity of eight lipids showed by FC >1. These include DG (49:10), LPC (20:5), PC (32:2, 34:3, 35:2, 36:6, 38:8), and TG (49:2, 51:3). A similar trend was noted in control diet; however, GSM treatment resulted in more lipids with significant alteration in both OVX and sham rats. Lipid pathway analysis showed HFHS diet activated the metabolism leading toward sphingomyelin (SM) synthesis, while this was supressed following inclusion of GMS powder.

A set of eight metabolites including five amino acids such as serine, threonine, lysine, valine, histidine, and three metabolites such as pipecolic acid, 3-methylcytidine, cholic acid were identified as potential biomarkers for the effect of HFHS diet and GSM supplementation in OVX rats. The relative intensity of all these metabolites increased with HFHS diet with exception of threonine, histidine, and methylcytidine that decreased under HFHS diet. Adding GSM powder elevated the relative intensity of threonine, while it decreased the lysine, valine, and pipecolic acid. In sham rats, four metabolites including two amino acids such as valine and tryptophan, and two metabolites 3-hydroxybutryric acid and cholic acid were identified as potential biomarkers. Valine and cholic acid were up-regulated under HFHS diet while 3-hydroxybutryric acid and tryptophan down-regulated. GSM treatment induced an increase in relative intensity of tryptophan, while it decreased the relative intensity in valine and cholic acid.

Conclusion: HFHS diet significantly altered the plasma lipidomic and metabolite profile of OVX and sham rats, while inclusion of GMS powder regulated some these alterations. Several lipids and metabolites were identified as potential biomarkers for the effect of HFHS diet and GSM supplementation in animal model of MetOA. Further studies are required to validate these findings.

4.1 Introduction

Osteoarthritis (OA) has been updated from being defined as a mechanical "wear and tear" disease to a metabolic disorder (Felson, 2013). Emerging evidence has shown the involvement of metabolic components in OA pathogenesis (Berenbaum et al., 2013). Metabolic syndrome, which is characterized by hypertension, dyslipidaemia, and diabetes, has been closely associated with low-grade systemic inflammation. Obesity and metabolic syndrome leads to a particular phenotype of OA known as metabolic syndrome-associated osteoarthritis (MetOA), which results in development of microstructural damage in the joints, articular cartilage deterioration and synovial endothelium dysfunction (Courties et al., 2019; Zhuo et al., 2012).

In order to understand and explore the MetOA, various diet-induced obesity models have been developed and validated *in vivo*. A high-fat diet has been shown to accelerate the progression of OA in both spontaneous and surgically-induced OA models (Datta et al., 2017). It has been demonstrated that feeding rats with a high fat, high sucrose (HFHS) diet for 12 weeks establishes OA-like cartilage alteration, with systemic and local synovial inflammation along with visceral fat accumulation (Collins et al., 2016). Furthermore, sex is a crucial factor in OA development as males are more predisposed to OA than females due to the effect of testosterone, while females are at higher risk following menopause due to oestrogen deficiency (Ma et al., 2007). A high energy diet and lack of oestrogen can have additive or synergistic effects, as we have previously shown that the combination of both factors caused a more severe pathological lesion in knee cartilage of rats than each factor individually (Siriarchavatana et al., 2020b).

Metabolomics approaches allow the profiling of small molecular metabolites from several metabolic pathways, providing a powerful tool for early diagnosis, monitoring intervention efficacy, and understanding the pathogenesis of disease (Gertsman & Barshop, 2018). The application of metabolomics is increasingly used in OA studies to understand the metabolism of disease and to identify new biomarkers. Some biomarkers have been identified by previous studies; for example, a pre-clinical study using an obese mouse model of OA demonstrated that serum levels of n-3 polyunsaturated fatty acids (PUFAs) were inversely associated with OA and wound size, while most n-6 PUFAs were positively associated with OA. Moreover, serum and synovial fluid levels of pentadecylic acid (C15:0, an odd-chain SFA) and palmitoleic acid (16:1, n-7) in this study were negatively correlated with joint degradation (Wu et al., 2017). Another study identified

that mice fed a high-fat diet to induce OA showed a distinct and sustained plasma metabolite signature high in phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) (Datta et al., 2017). However, metabolic changes induced by HFHS diet combined with ovariectomy that contribute to OA are not well understood.

Green-lipped mussel (*Perna canaliculus*) or Greenshell^{TM} Mussel (GSM) is a commercial marine species endemic to New Zealand (NZ) water. GSM is a rich source of omega-3 LC-PUFAs (Treschow et al., 2007), which are proven to have potent anti-inflammatory and anti-arthritic properties (Coulson et al., 2012). Our previous study has shown that flash-dried powder from whole GSM could prevent MetOA induction or slow its progression in a rat model of MetOA. The histological findings of this study demonstrated moderate to severe pathological changes in the knee joints of rats fed with the HFHS diet and who also underwent OVX as described in detail in two published studies (Siriarchavatana et al., 2020a; Siriarchavatana et al., 2019). The finding showed that inclusion of GSM in the diet of rats fed with HFHS for 13 weeks significantly reduced the C-terminal cross-linked telopeptide of type II collagen (CTX-II), a biomarker of cartilage degradation. After 36 weeks, GSM supplementation significantly attenuated the pathological cartilage lesion scores in the knee joints of OVX rats (Siriarchavatana et al., 2020b). The same study also identified an increase in lean mass gain and decrease in fat mass gain in rats fed with HFHS when GSM was incorporated in the diet. In line with this, Vaidya et al. reported reduced body weight gain, a decrease in systemic inflammation and improved metabolic parameters in rats fed with HFHS diet supplemented with freeze-dried powder of blue mussel for 12 weeks (Vaidya et al., 2017). Although these recent studies have provided evidence for advantageous effects of GSM on body weight, fat deposition and metabolism, it remains unclear what broader impact GSM has on metabolic regulation.

In the present study, liquid chromatography coupled to mass spectrometry (LC-MS) untargeted metabolomics was applied to reveal the impact of HFHS diet and GSM feeding on lipid and metabolite profiles in rats subjected to ovariectomy or sham surgery, to gain a better understanding of the beneficial effect of GSM in HFHS-induced obesity as a model of MetOA.

4.2 Methods

4.2.1 **Experimental methods**

The methodology used for the animal study was as described elsewhere (Siriarchavatana et al., 2020b), and the overall experimental design is presented in Figure 4.1. In brief, 96 female Sprague-Dawley rats were divided into four experimental diets: control, control supplemented with GSM (control + GSM), high fat/high sugar (HFHS), or high fat/high sugar enriched with GSM (HFHS + GSM). The proximal composition of diets can be found in Figure 4.1 and Appendix 2; Table S4.1. After eight weeks on an experimental diet, half of the rats underwent bilateral ovariectomy (OVX) and the rest of the animals underwent a sham surgical procedure. The test diet regimen continued through the end of the experiment. After 36 weeks, blood samples were collected from all rats by cardiac puncture into EDTA-anticoagulated tubes. The EDTA tubes were centrifuged at 1050 g, the plasma removed, and samples kept at -80 C until analysis. This study was performed in full compliance with the Massey University Animal Ethics committee (approval number 16/112). The findings on the pathological assessment and biochemical assay have been presented in a previous study (Siriarchavatana et al., 2020a).

4.2.2 **Metabolomic analysis**

4.2.2.1 **Chemicals and reagents**

Solvents and chemicals used for sample preparation, mobile phase, and LC–MS analysis (chloroform, methanol, acetonitrile isopropanol and formic acid) were purchased from Thermo Fisher Scientific (Auckland, New Zealand). Milli-Q® ultrapure water was purchased from Merck Millipore (Bedford, MA, USA). Ammonium formate (Fluka™, HPLC grade) was obtained from Sigma-Aldrich (Auckland, New Zealand). Lipid internal standard 1-palmitoyl(D31)-2-oleoyl-sn-glycero-3-phosphoethanolamine (16:0 d31-18:1-PE) was obtained from Avanti® (Avanti Polar Lipids, Alabaster, AL, USA). All chemicals were of LC–MS grade except chloroform, which was of analytical grade.

4.2.2.2 **Sample preparation**

Plasma samples were thawed at 4°C overnight and then vortexed. For lipid extraction, 100 µL of plasma was transferred into a 2-mL Eppendorf tube and mixed with 800 µL of -20°C extraction buffer ($CHCl_3$:MeOH 1:1, containing approximately 1.6 mg/L internal standards D_5-L-tryptophan, D_{10}-leucine, D_2-tyrosine and D_7-alanine), vortexed for 30 s

and stored at -20°C for 60 min. Subsequently, 400 µL of water was added, the mixture was vortexed 30 s and centrifuged for 10 minutes at 13663 g at 4 Celsius (Eppendorf Centrifuge 5427 R, Germany). Blank samples were prepared using the same procedure except that the plasma was replaced with 100 µL of MilliQ water. The pooled lipid QC samples were prepared by combining 30 µL of the lower phase from each sample in a new tube. The pooled QC samples were vortexed, aliquoted into multiple Eppendorf tubes, and evaporated to dryness under a stream of nitrogen at room temperature (Techne® Sample Concentrator) in the same manner as the rest of the samples. The dried samples were stored at -80 °C until further analysis.

Polar metabolites were extracted from the plasma samples following a monophasic extraction which has been validated for untargeted analysis (Fraser et al., 2020). Briefly, 50 µL of plasma was placed into an Eppendorf tube, to which was added 450 µL of pre-chilled acetonitrile:water (9:1 v/v). The mixture was vortexed (60 sec at 30 Hz) and then centrifuged for10 min at 13663 g at 4 °C (Eppendorf Centrifuge 5427 R, Germany) and 200 µL of extract placed into a high-performance liquid chromatography (HPLC) vial for analysis. All samples were stored at -80 °C for subsequent LCMS analysis of polar metabolites using hydrophilic interaction liquid chromatography (HILIC) chromatography. For QC samples, 100 µL of each extract was taken to form a pooled QC and added to HPLC vials for analysis.

4.2.2.3 **Instruments and conditions**

Lipid extracts were analysed using a Shimadzu LCMS-9030 mass spectrometer equipped with a Shimadzu Nexera-x2 Ultra Performance Liquid Chromatography® (UPLC) system by injecting 2 µL onto a Waters CSH-C18 column (2.1 x 100 mm, 1.7 µm particle size). Samples were held in the autosampler at 20 °C and the column oven held at 60 °C. Lipids were eluted over a 15 min gradient with a flow rate of 400 µL/min. The mobile phase was a mixture of water:acetonitrile:isopropanol (5:3:2 v/v containing 10 mM ammonium formate) (solvent A) and water:acetonitrile:isopropanol (1:9:90 v/v containing 10 mM ammonium formate) (solvent B). The gradient elution programme was as follows: 10–45% B (0–2.7 min), 45–53% B (2.7–2.8 min), 53–65% B (2.8–9 min), 65–89% B (9–9.1 min), 89–92% B (9.1–11 min), and finally to 100% B (11–11.1 min) and held for 0.8 min (11.1–11.9 min) before returning to 10% B (11.9–12 min) and held to re-equilibrate until 15 min (Huynh et al., 2019). The mass spectrometer was operated in positive ionisation mode, measuring full MS1 spectra from 250-1250 *m/z* across the entire

chromatogram, and also collecting Data Independent Acquisition (DIA) data in 20 *m/z* windows from 300-1100 *m/z*, with a 0.6 sec cycle time and collision energy of 25 normalised collision energy units. The source voltage was +4.0 kV with a nebulising gas flow of 2.0 L/min, heater gas flow of 10 L/min, interface temperature of 300 °C, drying gas flow of 10 L/min, desolvation line temperature of 250 °C and a heater block temperature of 400 °C. All drying and collision gasses used were nitrogen.

Polar metabolite extracts were analysed using a Shimadzu LCMS-9030 mass spectrometer equipped with a Shimadzu Nexera-x2 UHPLC system. Polar metabolites were measured by injecting 5 µL onto a Thermo Accucore HILIC column (2.1 x 100 mm, 2.6 µm particle size). Samples were held in the autosampler at 4 °C and the column oven held at 30 °C. Metabolites were eluted over a 23 min linear gradient with a flow rate of 400 µL/min. The mobile phases were water containing 10 mM ammonium formate (solvent A) and acetonitrile containing 0.1% formic acid (solvent B). The mass spectrometer was operated in both positive and negative ionisation modes, measuring full MS1 spectra from 70-1000 *m/z* across the entire chromatogram and, also collecting DIA data in 20 *m/z* windows from 70-900 *m/z*, with a 0.6 sec cycle time and collision energy of 25 normalised collision energy units. The source voltage was +4.0kV with a nebulising gas flow of 2.0 L/min, heater gas flow of 10 L/min, interface temperature of 300 °C, drying gas flow of 10 L/min, desolvation line temperature of 250 °C and a heater block temperature of 400 °C. All drying and collision gasses used were nitrogen.
The LC-MS underwent optimisation as part of the setup of the new Quadrupole Time-of-Flight (QTOF) Mass Spectrometry instruments. This included chromatographic column comparisons, gradients, solvents, and mass spec settings. Sample concentration and injection solvents were optimised from the experimental QC for every experiment run on the LC-MS.

4.2.3 **Data processing and statistical analysis**

Data were captured and converted to centroid MZML format using the Shimadzu file converter and imported into MS-Dial software for peak detection, gap filling, alignment, and noise elimination (Tsugawa et al., 2015). The resultant peak intensity table underwent run-order correction and normalization using pooled QC samples and Locally weighted Scatterplot Smoother (LOWESS) regression model. Ultimately, features with an average of QC-to-blank sample ratio of < 5 and CV of > 30% within the pooled QC samples were removed. In total, the full datasets for 96 rat samples were included in the lipidomic and

95 for metabolomic analysis as data from polar metabolites were not available for one sample.

Statistical analysis of the metabolite and lipid data were conducted using SIMCA version 16.0.1. software (Umetrics, Umea, Sweden). Multivariate analysis including PCA (principal component analysis), and OPLS-DA (orthogonal partial least squares discriminant-analysis) were conducted to obtain information on differences in lipid and polar metabolite profiles between experimental diet groups. The cross-validation was used to evaluate the robustness of the model. The features with values of variable important in projection (VIP) > 1 and using Benjamini–Hochberg false discovery rate (FDR) < 0.05 were considered to select the relevant lipid and polar metabolites. Subsequently, the relative intensity of the features was used to calculate the fold changes (FC) in lipids and polar metabolites between the treatment groups and their control by using Metaboanalyst version 4.0 (Chong et al., 2019). The metabolomic data were input into the Metaboanalyst for pathway enrichment analysis utilizing the Kyoto Encyclopedia of Genes and Genomes (KEGG) platform to analyse the topological characteristics of highly correlated metabolic pathways (Xia & Wishart, 2010).

Further, to visualise quantitative lipidomic data from the perspective of biosynthetic pathways, lipidomic data were input into the BioPAN platform available at https://lipidmaps.org/biopan/ to search the highly correlated metabolic pathways. BioPAN is a web-based tool available at the LIPID MAPS® Lipidomics Gateway, which combines lipid metabolism with a statistical analysis functionality. Nguyen et al has originally described the statistical model used in BioPAN (A. Nguyen et al., 2017). Briefly, BioPAN uses quantitative lipidomic data from two experiments (e.g a condition of interest treated and a control condition). The workflow was based on the calculation of the Z-score, which takes into account the mean and the standard deviation of the experiment considering a normal distribution of lipid subclass data (Gaud et al., 2021). The Z-score is used to predict whether a particular pathway is activated or suppressed between control and treated conditions. By default, Z-score > 1.645 corresponds to P < 0.05 indicating that a reaction or a pathway has been significantly modified in the treatment group compared to the control.

4.3 Results

This is the first study to investigate the effects of including whole meat GSM in the diet on plasma lipid and polar metabolites. Female rats fed with HFHS diet and subjected to OVX were used to model MetOA. The lipid and metabolite profiles obtained from 96 female rats divided into four diet groups and subjected to surgical procedures as described previously (Siriarchavatana et al., 2020b) were characterized and compared. HFHS diet markedly changed the lipidomic and metabolomic profile of rats subjected to OVX or sham, while treatment with GSM regulated some of these changes and reverted them to the levels observed in control diet groups.

4.3.1 **Lipidomic profile change in rats fed with HFHS diet and GSM**

The lipidomic data were analysed in positive mode and a total of 721 features was detected. After removal of noise and unstable molecules, 168 different lipid species consisting of 52 phosphatidylcholines (PC), and 42 triglycerides (TG) and other lipid classes were identified (Figure 4.2). To obtain the global information regarding lipidomic change, diet groups were analysed for OVX and sham rats separately. PCA was performed to visualize the grouping trends and detect outliers (Appendix 2; Figure S4.1); PCA plots of diet groups for OVX rats demonstrated a separation between HFHS and control diet groups with PC1 explaining 50.2% of the variance, while PCA for sham rats did not show a defined distinction. In OVX rats, two outliers from HFHS and control+ GSM diet, and in sham rats three outliers (two from control diet and one from control+ GSM), were observed. These samples were retained for the analyses as they could represent part of natural inter-animal variation among the samples.

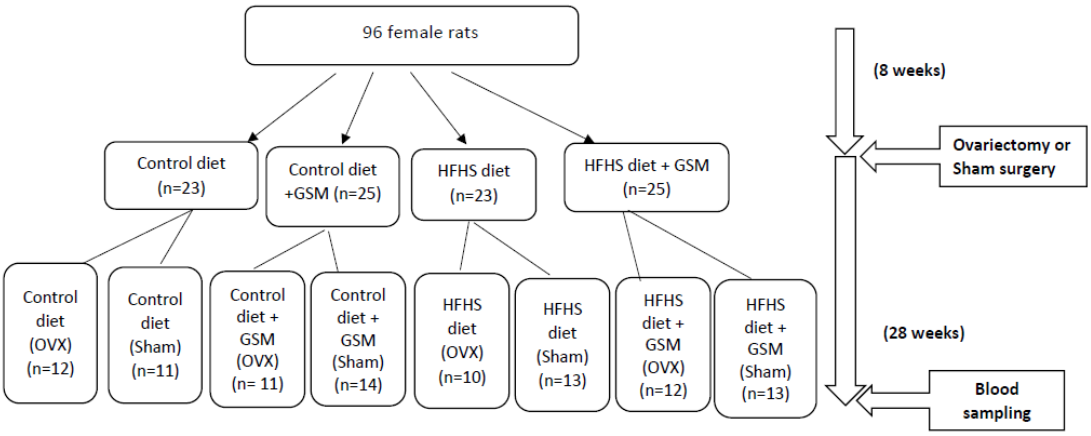


Figure 4.1 Overview of the rat study design.

Rats were randomised by maternal parentage, bone mineral density and body weight into groups of control diet (5% sugar/5% fat/15% protein from casein), control + GSM diet (5% sugar/5% fat/15% protein from 2:1 casein: GSM), HFHS diet (30% sugar/30% fat/15% protein from casein) or HFHS + GSM diet (30% sugar/30% fat/15% protein from 2:1 casein: GSM) diet. After 8 weeks into the study, approximately half of the rats in each group underwent sham surgery (ovaries left intact) while the other half underwent ovariectomy (OVX), after again being randomised by bone mineral density and body weight. Blood samples were collected after 36 weeks.

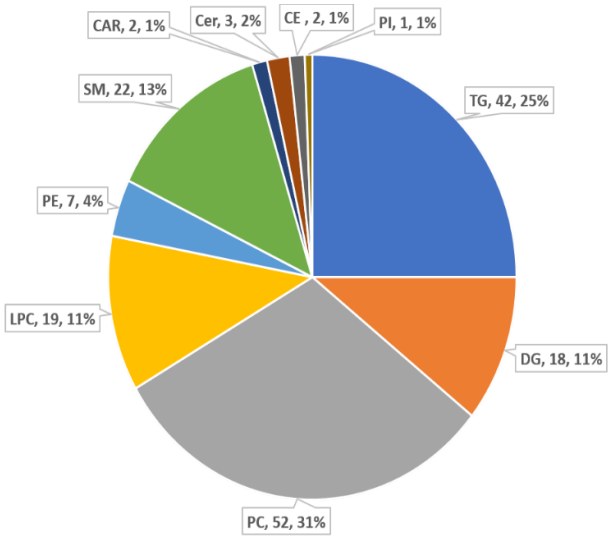
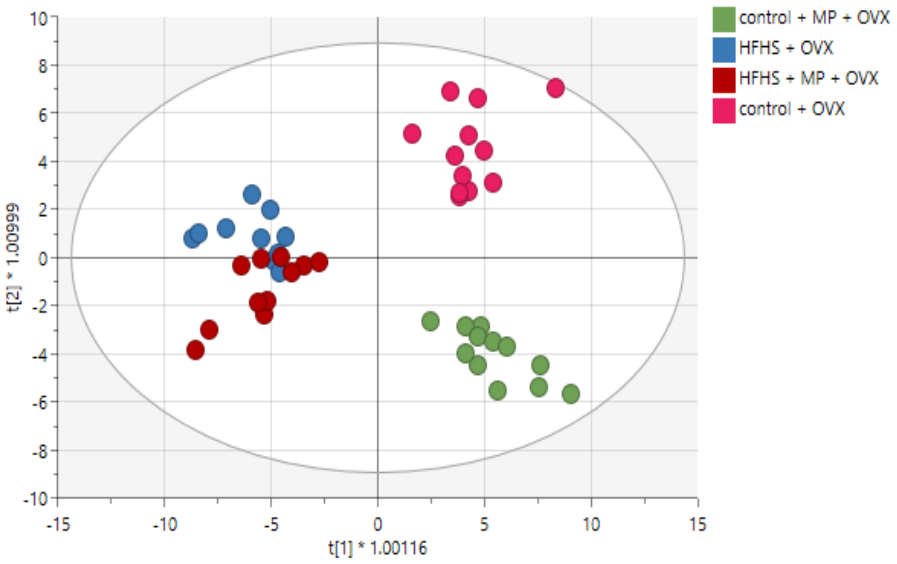


Figure 4.2 Composition of lipid classes that were considered for subsequent analysis in rat plasma samples detected by LC-MS.

The result of the OPLS-DA revealed clear differences among the four treatment groups in OVX and sham rats. The HFHS and control diet groups discriminated from each other, while the HFHS diet treated with GSM was located near to or with minor overlap with the HFHS diet (Figure 4.3A, B). OPLS-DA models were used to select the discriminating variables between the four experimental diet groups. Those lipids that satisfied both VIP >1.0 and FDR < 0.05 were further investigated. In OVX rats, 29 lipids were tentatively identified as biomarker candidate for HFHS-induced obesity and protective effect of whole meat GSM powder.

(A)



(B)

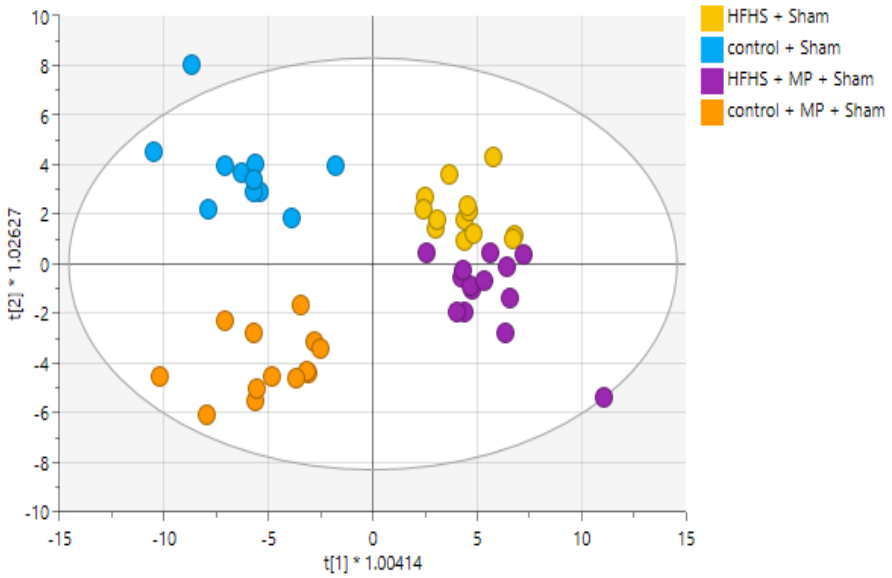


Figure 4.3 OPLS-DA analysis for lipidomic differences between diet groups showing good separation and robust model for sham rats and OVX rats.

Each circle represents lipid profile of single rat. (A) OPLS-DA scoring plot showing discrimination between diets groups in OVX rats (N=45); R2X (cum)=0.811, R2Y(cum)=0.608, Q2(cum)=0.498. Cross-validated ANOVA P-value = 4.38326e-05. (B) OPLS-DA scoring plot showing discrimination between diets groups in sham rats (N=51); R2X (cum)=0.755, R2Y(cum)=0.572, Q2(cum)=0.480. Cross-validated ANOVA P-value = 8.77889e-06. (D). HFHS: high-fat/high-sugar diet. MP = greenshell mussel powder.

Given the potential therapeutic value of GSM for MetOA, GSM supplementation may have regulated the lipid level changes affected by HFHS. To identify the magnitude of the changes in lipids after treatment with GSM in OVX rats, the FC in HGHS+GSM diet group with respect to HFHS diet and control+ GSM with respect to control diet were calculated (Table 4.1). Majority of lipids were more affected by HFHS diets than GSM feeding. Of the 29 lipids, only 2 increased in relative intensity, while the remaining 27 decreased under HFHS diet. The up-regulated lipids include two TG (56:10, 60:12) containing long polyunsaturated fatty acids, and down-regulated lipids include six diglyceride (DG), two lysophosphatidylcholine (LPC), fifteen PC and four TG. In OVX rats fed with HFHS+GSM, eleven lipids that had been down-regulated by HFHS were increased in relative intensity by >1-fold when compared to HFHS diet. Treatment with GSM in rats fed with HFHS diet resulted in significant increases of FC value for LPC (20:5) and TG (54:7), while PC (40:4) significantly decreased. Similarly, GSM treatment in control group induced an increase in relative intensities of fourteen lipids by >1-fold when compared with control. A significant increase of FC was noticed in two DG (47:10,49:10), two LPC (16:1, 20:5), four PC (32:2, 34:3, 36:6, 38:8) and all TG with exception of TG (48:1) when compared with control diet. Conversely, GSM treatment resulted in observation of significant decrease in relative intensity of four DG (38:5, 38:6, 47:9, 49:12), and four PC (34:4, 37:4, 38:7, 40:4).

Table 4.1 Plasma lipid affected by HFHS diet or GSM treatment in OVX rats

Lipids	Average Rt (min)	Adduct type	Fold Change		
			HFHS/ Control	HFHS+GSM /HFHS	Control+ GSM/ Control
DG 38:5	8.227	[M+Na]+	0.54*	0.76	0.49*
DG 38:6	7.444	[M+Na]+	0.76	0.83	0.61*
DG 47:10	6.276	[M+Na]+	0.74*	1.09	1.2*
DG 47:9	6.188	[M+Na]+	0.56*	0.78	0.66*
DG 49:10	7.184	[M+Na]+	0.89	1.12	1.34*
DG 49:12	6.177	[M+Na]+	0.69*	0.88	0.76*
LPC 16:1	2.228	[M+H]+	0.33*	0.96	1.19*
LPC 20:5	1.994	[M+H]+	0.28*	2.6 *	4.38*
PC 32:1	6.065	[M+H]+	0.19*	0.79	0.87
PC 32:2	5.506	[M+H]+	0.46*	1.00	1.14
PC 34:3	5.647	[M+H]+	0.42*	1.01	1.34*
PC 34:4	5.442	[M+H]+	0.37*	0.79	0.69*
PC 36:5	6.268	[M+H]+	0.73*	1.1	1.19
PC 36:6	5.639	[M+H]+	0.31*	1.24	1.32*
PC 37:4	6.613	[M+H]+	0.65	0.74	0.6*
PC 38:5	6.264	[M+H]+	0.41*	0.81	0.69
PC 38:7	6.181	[M+H]+	0.7*	0.87	0.76*
PC 38:8	5.685	[M+H]+	0.24*	1.29	2.26*
PC 40:4	7.786	[M+H]+	0.92	0.54*	0.32*
PC O-36:4	6.702	[M+H]+	0.66*	0.8	0.59*
PC O-36:5	6.567	[M+H]+	0.69	0.83	0.59*
PC O-39:10	6.912	[M+H]+	0.24*	0.76	0.87
PC O-39:4	7.076	[M+H]+	0.97	0.81	0.62*
TG 48:1	10.944	[M+NH4]+	0.2*	0.97	0.84
TG 50:3	10.844	[M+NH4]+	0.25*	1.08	1.03
TG 54:7	10.732	[M+NH4]+	0.62*	1.4 *	1.88*
TG 54:8	10.638	[M+NH4]+	0.66*	1.36	2.03*
TG 56:10	10.589	[M+NH4]+	1.07	1.36	2.22*
TG 60:12	10.677	[M+NH4]+	1.46*	1.16	1.94*

Lipids were determined by using the VIP value >1 and FDR (< 0.05) from the OPLS-DA model. Fold change was calculated by dividing the mean of the peak intensity of each lipid from each of the two groups. * Lipids showing significant differences (p-value < 0.05) between groups as determined by Student's t-test.

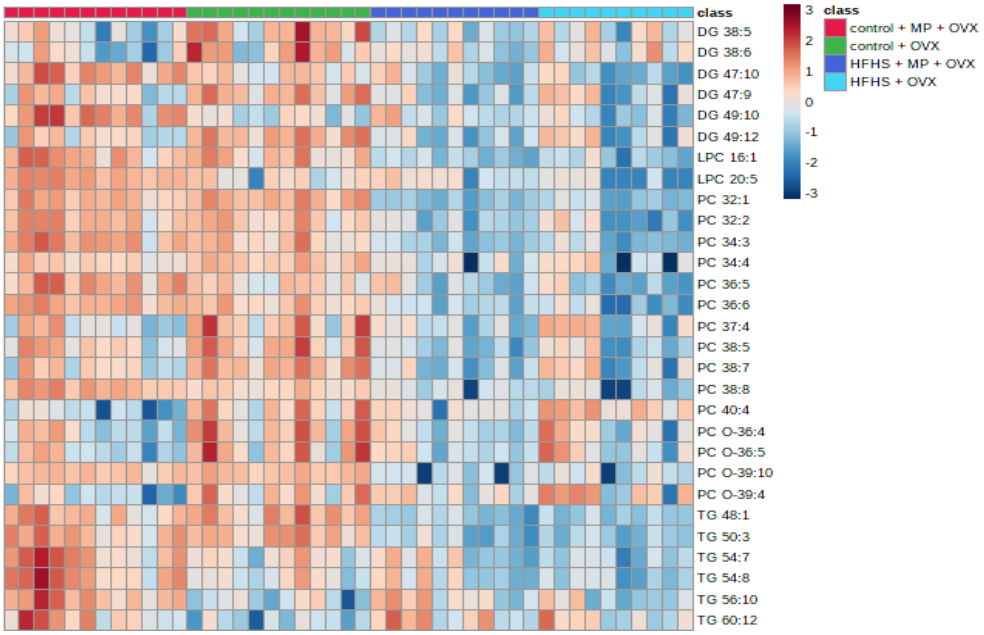
The OPLS-DA was also performed on the dataset for identifying lipids affected by HFHS and GSM in sham rats. Finally, 29 lipids were identified based on VIP > 1.0 and FDR < 0.05 as displayed in Table 4.2. Of these 29 lipid molecules, 22 lipids were commonly changed among OVX and sham rats. To identify the individual changes of the identified lipids, the FC of these lipids were calculated for each diet group with respect to their control group. Similar to OVX rats, the relative intensity of all lipids with exception of three TG (54:7, 54:8, 56:10) were decreased under HFHS diet when compared to control diet group. In respect of down-regulated lipids, GSM treatment induced increases in relative intensity of nine species with FC above 1 among which LPC 20:5 showed a significant increase. In contrast, two DG (38:5, 38:6) and two PC (40:4, O-40:8) showed significant decreases by GSM feeding when compared HFHS diet group. A similar trend was observed in control diet; however, GSM treatment yielded more lipids with significant differences. In addition, the lipid biomarkers of the four diet groups are shown as a clustering heatmap in Figures 4.4A and B.

Table 4.2 Plasma lipid affected by HFHS diet or GSM treatment in sham rats.

Lipids	Average Rt (min)	Adduct type	Fold Change		
			HFHS/ Control	HFHS+GSM/ HFHS	Control+ GSM/ Control
Cer 41:1	10.315	[M+H-H2O]+	0.54*	0.63	0.69*
DG 38:5	8.227	[M+Na]+	0.62*	0.69*	0.54*
DG 38:6	7.444	[M+Na]+	0.91	0.74*	0.61*
DG 47:9	6.188	[M+Na]+	0.47*	0.77	0.71*
DG 49:10	7.184	[M+Na]+	0.91	1.02	1.2*
DG 49:12	6.177	[M+Na]+	0.65*	0.8	0.78*
LPC 20:5	1.994	[M+H]+	0.41*	1.58*	3.14*
PC 32:1	6.065	[M+H]+	0.21*	0.97	0.89
PC 32:2	5.506	[M+H]+	0.4*	1.18	1.05
PC 33:2	5.87	[M+H]+	0.47*	0.88	1.27
PC 34:3	5.647	[M+H]+	0.44*	1.01	1.19
PC 34:4	5.442	[M+H]+	0.27*	0.74	0.68*
PC 35:2	6.695	[M+H]+	0.68*	1.03	1.34*
PC 36:4	6.186	[M+H]+	0.57*	0.79	0.78*
PC 36:6	5.639	[M+H]+	0.28*	1.06	1.08
PC 37:4	6.613	[M+H]+	0.49*	0.83	0.8
PC 38:5	6.264	[M+H]+	0.38*	0.86	0.77
PC 38:7	6.181	[M+H]+	0.65*	0.84	0.78*
PC 38:8	5.685	[M+H]+	0.26*	1.43	2.16*
PC 40:4	7.786	[M+H]+	0.71	0.63*	0.32*
PC O-36:4	6.702	[M+H]+	0.5*	0.82	0.62*
PC O-36:5	6.567	[M+H]+	0.49*	0.84	0.63*
PC O-39:4	7.076	[M+H]+	0.75*	0.82	0.7*
PC O-40:8	6.79	[M+H]+	0.71*	0.77*	0.63*
TG 49:2	10.894	[M+NH4]+	0.52*	1.4	1.35*
TG 51:3	10.905	[M+NH4]+	0.96	1.05	1.47*
TG 54:7	10.732	[M+NH4]+	1.1	1.11	2*
TG 54:8	10.638	[M+NH4]+	1.12	1.04	1.8*
TG 56:10	10.589	[M+NH4]+	1.09	1.3	1.87*

Lipids were determined by using the VIP value >1 and FDR (< 0.05) from the OPLS-DA model. Fold change was calculated by dividing the mean of the peak intensity of each lipid from each of the two groups. * Lipids showing significant differences (p-value < 0.05) between groups as determined by Student's t-test.

(A)



(B)

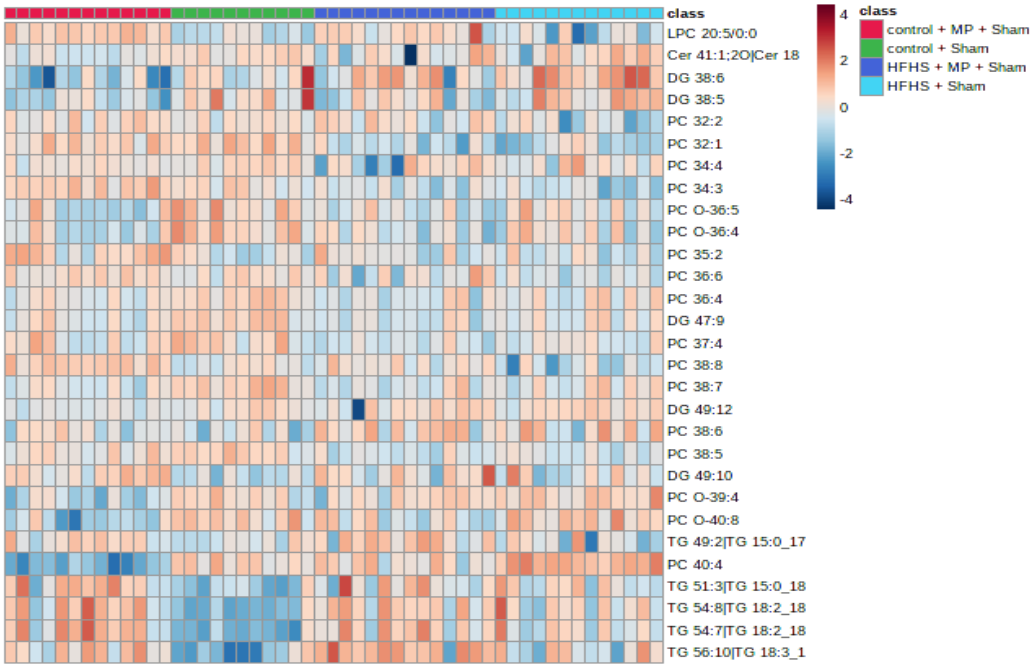


Figure 4.4 The heatmap showed the relationship between comparison of contents of lipids in OVX (A) and sham (B).

Each colour blocks corresponds to the value of relative content of each lipid. HFHS: high-fat/high-sugar diet, MP = greenshell mussel powder, OVX: ovariectomized.

The lipid network at the lipid subclass level for the OVX rats is presented in Figure 4.5A for the HFHS diet group and Figure 4.5B for the HFHS+GSM diet group. Biological alterations usually occur in a complex set of changes over hundreds of lipid molecular species, rather than at the level of single lipid molecular species. Therefore, the lipidomic datasets including all the detected lipids species were uploaded in the BioPAN. The result showed that reactions using diacylglycerol (DG) as a substrate were suppressed in both HFHS vs control and HFHS+GSM vs HFHS diets as shown in Figure 4.5. Diet-specific differences in lipid metabolism were shown by BioPAN, with the reaction pathway results of HFHS diet indicating active metabolism leading to accumulation of sphingomyelin (SM) (Z-score = 2.547), opposite to the catabolic metabolism of SM observed in the HFHS+GSM diet (Z-score = 0.576). Similar findings were observed on the lipidomic profiles of sham rats.

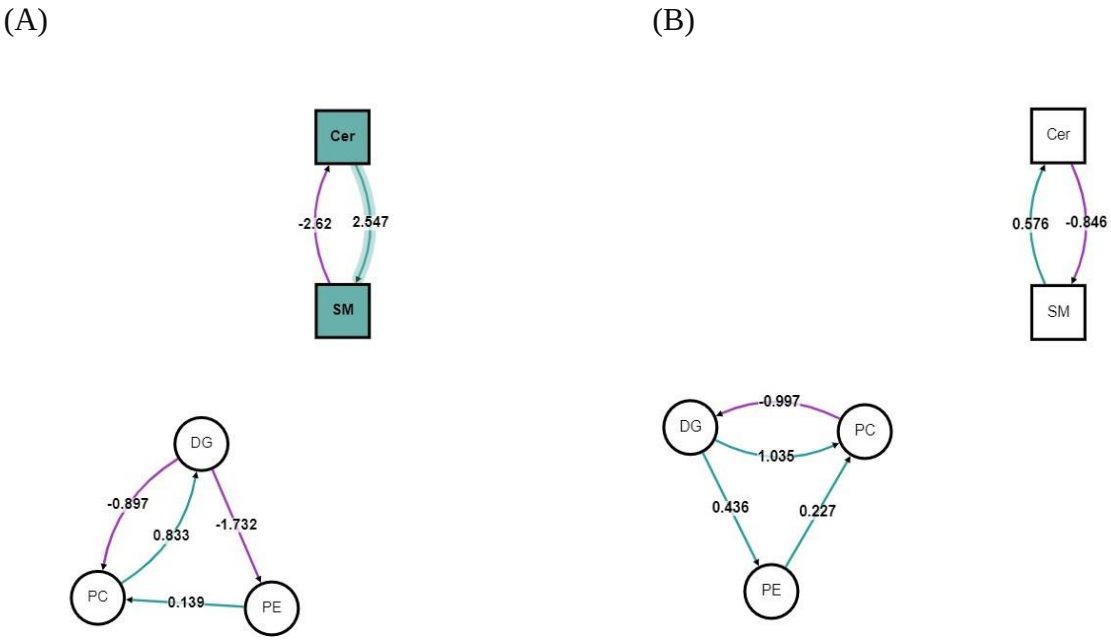


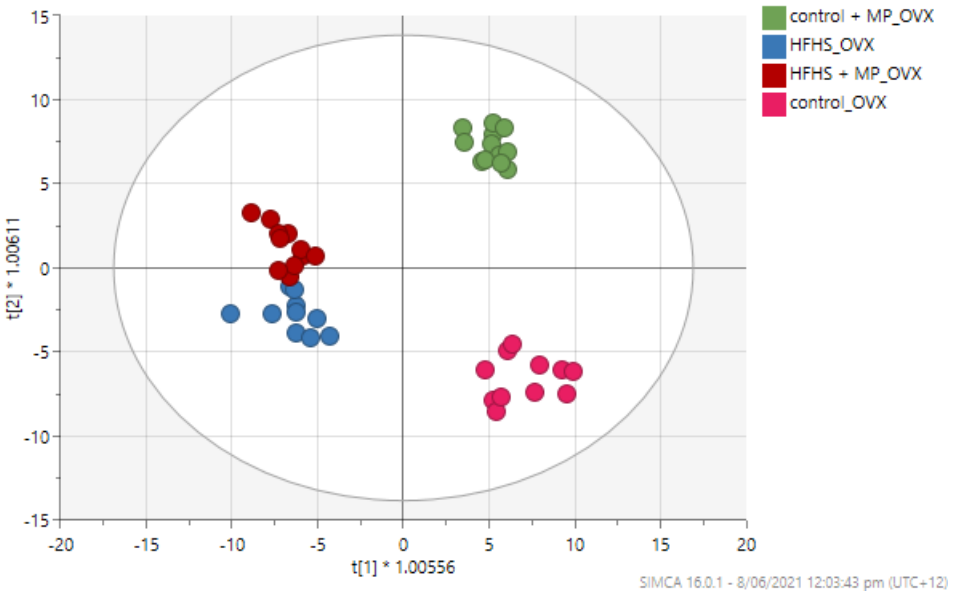
Figure 4.5 BioPAN lipid networks.

Lipid network graphs exported from BioPAN for the HFHS diet vs control (A) and the HFHS+GSM diet vs HFHS diet (B) in OVX rats. Green nodes correspond to active lipids and green shaded arrows to active pathways. Reactions with a positive Z score have green arrows while negative Z scores are coloured purple.

4.3.2 **Metabolomic profile change in rats fed with HFHS diet and GSM**

525 features in negative ionisation and 604 in positive ionisation were detected by metabolomics. After filtration and removal of noise and unstable compounds, 422 features (including 383 unknown and 39 known metabolites) in positive and negative ions were combined into a single table for statistical analysis. PCA was applied to analyse the distribution of the polar metabolites among the four diet groups in OVX and sham rats (Appendix 2; Figure S4.2). Overall, PCA showed separation between HFHS and control diet in OVX rats, although it was less clear in sham rats. The OPLS-DA score plots between the diet groups are shown in Figures 4.6A and 4.6B. The model was validated with fitness R2X cum and R2Y cum values of 0.445 and 0.891 respectively, and with a predictability Q2 cum value of 0.572 in OVX rats. The R2X and R2Y cum in sham rats were 0.307 and 0.624, respectively, with predictability Q2 cum values of 0.401.

(A)



(B)

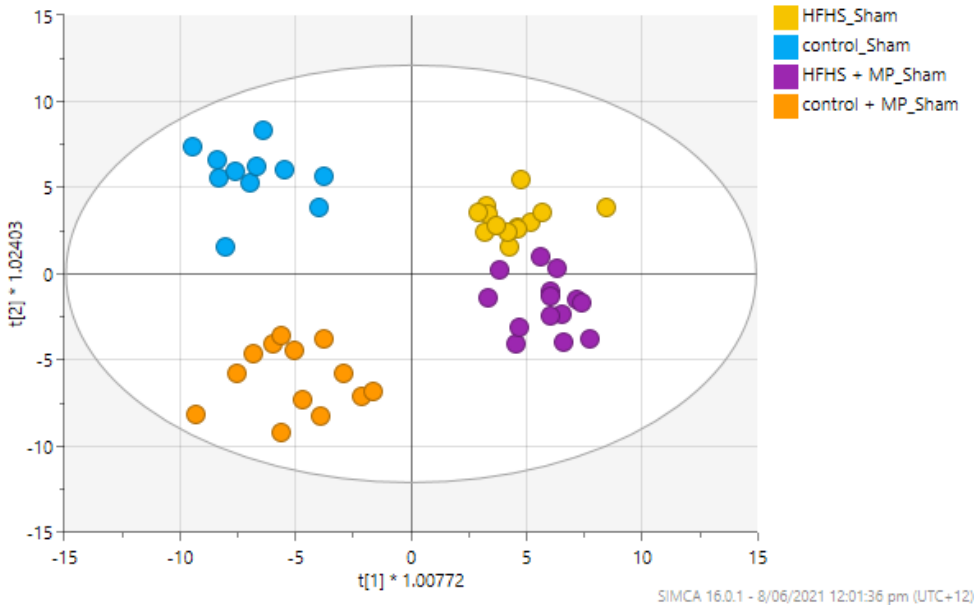


Figure 4.6 OPLS-DA analysis for metabolomic differences between diet groups showing good separation and robust model for sham and OVX rats.

Each circle represents metabolite profile of single rat. (A) OPLS-DA scoring plot showing discrimination between diets groups in OVX rats (N=44); R2X (cum)=0.445, R2Y(cum)=0.891, Q2(cum)=0.572. Cross-validated ANOVA P-value = 5.30302e-05, (B) OPLS-DA scoring plot showing discrimination between diets groups in sham rats (n = 51); R2X (cum) = 0.307, R2Y (cum) = 0.624, Q2 (cum) = 0.401. Cross-validated ANOVA p-value = 1.32627e-4. HFHS: high-fat/ high-sugar diet, MP = greenshell mussel powder, OVX: ovariectomized.

According to OPLS-DA model, major metabolites significantly contributing to the discrimination between the diet groups were selected based on both the VIP value > 1.0 and FDR < 0.05. In OVX rats, we identified 125 features fitting these criteria, of which eight were identifiable: cholic acid, serine, threonine, lysine, valine, pipecolic acid, histidine, 3- methylcytidine. The identified metabolites are described in Table 4.3. Five of them showed increased levels under HFHS diet. However, levels of three of them (lysine, valine and pipecolic acid) were regulated by GSM treatment with FC less than 1. Further, all the metabolites were decreased by GSM treatment in control diet except for 3-methylcytidine.

In sham rats, we observed a total of 80 features based on VIP >1 and FDR < 0.05, of which 4 were known metabolites: 3- hydroxybutyric acid, valine, cholic acid and tryptophan (Table 4.4). Two metabolites, 3-hydroxybutric acid and tryptophan,

significantly decreased under HFHS diet as compared to control diet, while the level of cholic acid significantly increased. However, GSM treatment decreased the relative intensity of up-regulated metabolites valine and cholic acid when compared to HFHS, while down-regulated tryptophan indicated a slightly higher intensity with FC >1 after GSM treatment, although these changes were not statistically significant. In respect of control diet, GSM treatment induced an increase in relative intensity of 3-hydroxybutric acid and cholic acid with FC value 1. Similar to OVX rats, a significant decrease of FC value below 1 was noted for valine.

Table 4.3 Plasma metabolite affected by HFHS diet or GSM treatment in OVX rats

Metabolites	Average Rt(min)	Adduct type	Fold Change		
			HFHS/Control	HFHS+GSM/ HFHS	Control+ GSM/ Control
Cholic acid	2.572	[M+H]+	1.42 *	1.02	0.59
Serine	11.141	[M+H]+	1.28	1.06	0.65 *
Threonine	10.263	[M+H]+	0.94	1.21	0.58 *
Lysine	9.533	[M+H]+	1.37 *	0.95	0.90 *
Valine	6.481	[M+H]+	1.19	0.82 *	0.71 *
Pipecolic acid	9.534	[M+H]+	1.69 *	0.91	0.33 *
Histidine	8.167	[M-H]-	0.97	0.87 *	0.93
3-Methylcytidine	6.516	[M+H]+	0.83	0.99	1.45 *

Metabolites were determined by using the VIP value >1.0 and FDR (< 0.05) from the OPLS-DA model. Fold change was calculated by dividing the mean of the peak intensity of each lipid from each of the two groups. * Metabolites showing significant differences (p-value < 0.05) between groups as determined by Student's t-test.

Table 4.4 Plasma metabolite affected by HFHS diet or GSM treatment in sham rats

Metabolites	Average Rt(min)	Adduct type	Fold Change		
			HFHS/Control	HFHS+GSM/ HFHS	Control+ GSM/ Control
3- hydroxybutyric acid	6.863	[M-H]-	0.55*	0.98	1.16
Valine	6.481	[M+H]+	1.03	0.80	0.63*
Cholic acid	2.572	[M+H]+	1.38*	0.42	1.23
Tryptophan	7.968	[M+H]+	0.80*	1.06	0.91

Metabolites were determined by using the VIP value >1.0 and FDR (< 0.05) from the OPLS-DA model. Fold change was calculated by dividing the mean of the peak intensity of each lipid from each of the two groups. * Metabolites showing significant differences (p-value < 0.05) between groups as determined by Student's t-test.

A summary of pathways highlighted as being affected during feeding with HFHS diet and GSM in our metabolomic analysis is given in Appendix 2; Table S4.2. The pathway enrichment showed the number of the hits or number of compound matches from the uploaded data, p-value, and impact factors. In OVX rats fed with HFHS diet, the primary bile acid biosynthesis had the largest -log (p-value) and the aminoacyl -t-RNA biosynthesis pathway had the highest impact value and a significant p-value indicating the most enriched pathways. Furthermore, GSM treatment in the HFHS diet in the OVX rats significantly impacted the histidine metabolism as shown based on -log (p-value) and impact value.

Analysing all eight treatment groups simultaneously was challenging in selection of biomarker and interpretation of the result. Furthermore, the main objective of the study was identifying biomarkers in the OVX rat as model for postmenopausal women. thereby the data of OVX and sham rat were analysed separately. It must be mentioned that metabolomic data were analysed using univariate analysis, two-way ANOVA to identify the metabolites affected by diet, OVX and GSM treatment. It was found that more than 70% of the results were the same for both OPLS-DA and the univariate method.

The relationships between inflammatory and OA markers with identified lipid and metabolites were assessed. In this study, no significant association was observed for lipids, but tryptophan that decreased under HFHS diet showed a positive association with

CTX-1 and CTX-II and was negatively associated with the inflammatory markers; TNF-α and Monocyte Chemoattractant Protein-1 (MCP-1) (data not shown).

4.4 Discussion

In this study, we revealed the effects of HFHS diet and GSM supplementation on plasma lipid and polar metabolites of rat subjected to OVX or sham surgary using MS-based metabolomic techniques. Twenty-nine plasma lipids were indentified and closely associated with the HFHS diet and/or GSM powder treatment. Most of identified lipids were DG, PC and TG, which were decreased by HFHS diet, while TG containing long chain and highly unsaturated fatty acids such as TG (56:10, 60:12) were increased. Some of the lipids that were significantly altered by the HFHS diet were recovered to normal diet levels by inclusion of GSM in the HFHS diet, although it was not an absolute recovery pattern. The metabolomic findings mainly centred on amino acid metabolism. Eight and four metabolites were identified in the OVX and sham rats, respectively. Feeding HFHS diet to OVX rats resulted in an increase of a subset of two essential amino acids, lysine, valine, and one metabolite pipecolic acid; these were downregulated by adding GSM to HFHS diet. Threonine was slightly decreased by HFHS diet and then increased with GSM inclusion. In sham rats, 3-hydroxybutric acid and tryptophan were significantly reduced by HFHS diet while cholic acid increased. Among these altered metabolites, cholic acid and tryptophan were restored to normal levels by GSM.

Although this is the first study that investigated the changes in the plasma lipidome and metabolite profiles of HFHS diet-induced obesity in OVX rats, previous metabolomic analyses have shown altered metabolism in rodents fed with high-fat diet as model of MetOA. Mice fed high-fat diet for 18 weeks exhibited a distinct lipidomic profile, in which LPC (20:4,17:0) and PC (36:2) were longitudinally and exclusively upregulated, and these biomarkers were able to predict the risk of OA induced by high-fat diet (Datta et al., 2017). Another study (Gowda et al., 2020) recently reported the alteration of plasma lipids in rats with high fat diet-induced obesity. After 8 weeks of high-fat diet feeding, PC and TG containing PUFA such as PC (32:1, 43:4, 32:4, 36:5) and TG (52:6, 54:7) were significantly decreased in rats. In line with these studies, we observed a decline in PC (32:1, 34:4, 36:5) and TG (54:7) in both OVX and sham rats fed with HFHS diet. GSM treatment increased the level of some PC and LPC species that had been down-

regulated by HFHS diet. As phospholipids are a major lipid class in the GSM lipid fraction (Miller et al., 2014), this indicates a potential biochemical role for GSM phospholipids that may regulate the concentration or synthesis of PC and LPC under HFHS diet conditions. However, the effect was not robust for most of the lipids; this is likely because the lipid proportion of the whole GSM powder was too low in quantity in the diet to demonstrate the effect.

The TG levels showed different patterns of changes depending on length and degree of saturation of acyl chain. An increase in TG (56:10, 60:12) but decrease of TG (48:1, 50:3, 54:7, 54:8) was observed in OVX rat fed with HFHS diet. Liu et al. demonstrated an increase in TG containing PUFA in rat with obesity induced by high-fat diet for 3 weeks (Liu et al., 2015). A prolonged high-fat diet induces adaptive adjustments in lipid metabolism in the liver such as compensatory synthesis of endogenous PUFA while suppressing the formation of lipoproteins that transport the *de novo* synthesized PUFA to peripheral organs. This leads to accumulation of PUFA in liver and plasma (Zhukova et al., 2014). The decline in DG presented in the current study has not been observed elsewhere, with one study reporting increased circulating DG levels in female mice fed with a high-fat diet (Pati et al., 2018). These contradictory findings may be due to the differences in animal species, feeding duration, age and particularly diet composition. In addition, endogenous lipid biosynthesis related to bio-conversion mechanisms may impact the resultant circulating lipid profile.

Sphingolipids play an important role in cell growth, differentiation, apoptosis, and vital signal transduction pathways. A high-fat diet promotes *de novo* sphingolipid synthesis, resulting in the elevation of sphingomyelin (SM) and ceramide (Cer) (Choi & Snider, 2015) in plasma, adipose tissue and liver (Turner et al., 2013). We detected 22 SM and 3 Cer in the lipidomic analysis, although these lipids with exception of Cer (41:1) were not among the major lipids contributing to discrimination between the diet groups. Most of the detected SM showed increases with HFHS diet, which agrees with the lipid pathway analysis showing a shift towards the formation of SM. *In vitro* evidence using chondrocytes or explants from rabbit articular cartilage demonstrated that a high dose of C2-Cer induced apoptosis and up-regulated matrix metalloproteinase activity (Sabatini et al., 2000). Higher concentrations of Cer species were observed in synovial fluid of late-stage OA patients, suggesting the potential involvement of Cer species in OA progression or as a response to OA (Kosinska et al., 2014). The exact function of SM and Cer species

in the pathophysiology of OA induced by obesity is still unknown; however, the reaction pathway result showed GSM treatment suppressed the SM synthesis in the current study and also significantly reduced cartilage degradation in the rat trial (Siriarchavatana et al., 2020b). This may suggest a potential role for GSM as a therapeutic intervention or preventative for high-fat-induced pathologic conditions by controlling and normalizing abnormal SM metabolisms. However, further mechanistic studies need to be conducted to dissect the mechanisms underlying high fat diet-mediated sphingolipid metabolism in OA and the impact of GSM treatment.

Additionally, some metabolites were identified as potential biomarkers explaining the impact of HFHS diet and GSM treatment in our study. In OVX rats, most of the metabolites including cholic acid, serine, lysine, valine and pipecolic acid increased in HFHS diet while threonine, histidine and 3-methylcytidine were down-regulated. In sham rats, 3-hydroxybutric acid and tryptophan decreased under HFHS diet, while valine and cholic acid showed the same trend as OVX rats under HFHS diet. Consistent with the previous reports (Lai et al., 2015; Liao et al., 2019), our results showed that the levels of the branched-chain amino acids (BCAAs), lysine, and particularly valine were elevated by HFHS diet. Meanwhile, 3-hydroxybutric acid, a catabolic intermediate of valine declined by HFHS diet. Higher levels of BCAAs are directly involved in insulin resistance via inhibiting the activity of AMP-activated protein kinase and subsequently contribute to metabolic dysregulation (Saha et al., 2010). Additionally, liver dysfunction caused by high-fat diet was found to result in accumulation of pipecolic acid, which is a minor metabolite of lysine, in the serum of obese mice; this is consistent with our findings (Kim et al., 2011). The data from this study showed that serine and threonine were increased by the HFHS diet in OVX rats, in whom myocardial hypertrophy and renal tubular epithelium degeneration occurred at $\geq$2X the incidence rate of OVX rats on a normal diet (Siriarchavatana, 2021). These data verify findings of a previous study (Lai et al., 2020), which demonstrated abnormal changes in serine and threonine in conditions such as myocardial injury. Further, alteration in amino acids such as tryptophan, alanine, valine, and histidine along with increases in relative concentration of n-butyrate, α-hydroxy-n-butyrate has been demonstrated in the urine of type 2 diabetes mellites animal models and human patients. Moreover, increased urinary β-hydroxybutyrate was also identified to be increased with age of diabetic rats (Salek et al., 2007). The rats used in current study were aged and oestrogen-deficient in addition to eating a chronic HFHS

diet, resulting in significant metabolic disorder. Thus, these metabolites may eventuate as biomarkers for detection or prediction of other disease states.

Elevated bile acids are caused by hepatic dysfunction. Cholic acid as a major primary bile acid is synthesised from cholesterol in the liver and has a role in lipolysis, cholesterol catabolism, and overall regulation of lipid metabolism (Dawson & Karpen, 2015). High fat diet accumulates TG and cholesterol in rat livers, which upregulate the bile acid synthetic enzymes, while downregulates its export and transportation. These changes result in bile acid deposition in the liver leading to steatohepatitis (Jia et al., 2013). Cholic acid was elevated by HFHS diet in our study, and these rats in both OVX and sham groups had approximately double the incidence of periportal bile ductular hyperplasia in the liver compared to their normal diet counterparts; this is supported by previous findings (Wu et al., 2014). Therefore, the increased cholic acids may be partly explained by prolonged consumption of a high fat diet causing dysregulation in bile acid hepatic homeostasis.

The levels of some of up-regulated amino acids including lysine, valine, pipecolic acid decreased and partially recovered with GSM treatment. From these results, it may be suggested that GSM treatment might partially improve the impaired amino acid metabolism induced by HFHS feeding. The GSM components that contributed to the potential efficacy observed in the OA disease model are yet unknown; nevertheless, numerous bioactive compounds have been identified within GSM that possess anti-inflammatory, antioxidant, anti-hypertensive, antibacterial, and antithrombin effects, mainly derived from its peptides, carbohydrates and lipids (Grienke et al., 2014). Analysis of bioactive peptides revealed high level of amino acids, such as glycine, valine, lysine, isoleucine, and alanine, which might have provided the observed effects.

The metabolites that were detected in this study were not found to be involved in inflammation as the main pathway that is assumed to be the effect of GSM on cartilage. This is supported by the data on inflammatory cytokines which did not reveal any effect by GSM on the inflammatory markers suggesting that an anti-inflammatory effect of GSM is on a local level rather than systemic (Siriarchavatana et al., 2020a). Moreover, this study did not assess the correlation between the data for knee cartilage assessment, such as the Mankin score, with the identified metabolites. The result was having difficulty in interpretation of data and revealing the metabolism of cartilage perturbed by the HFHG diet and the GMS supplement.

This study identified a small number of metabolites which is one of the inherent disadvantages of untargeted metabolomics as many identifications that only had MS1 level annotation and those without retention time confirmation; thus, identification heavily relied on annotations from the MS2 data.

Regarding the oestrogen-deficient condition, it was noted that majority of lipids commonly changed among OVX, and sham rats shared similar trends, suggesting that the plasma lipid profiles in rats were predominantly influenced by feeding conditions than oestrogen deficiency. With respect to GSM treatment, the control diet group presented accentuated lipidomic responses to GSM consumption while HFHS diet group showed a more tapered shift, possibly due to homeostatic mechanisms in response to prolonged feeding of HFHS. Although the results presented here are not sufficient to establish a direct contribution of the altered metabolites to OA pathogenesis, most of these lipids and metabolites have been shown to be significantly altered in plasma in other obesity-related disorders (Gowda et al., 2020; Liu et al., 2015). Moreover, the data were collected at only one time point, and in this study no strong correlation was observed between these lipids and polar metabolites and biomarkers of cartilage degradation or bone resorption, indicating these metabolites might be more closely connected to HFHS-induced obesity rather than to OA. The metabolomic profile of synovial fluid or cartilage tissue during disease-related alterations, as well as the metabolomic profile of plasma during the early stages of OA when the biomarkers of cartilage degradation are more prominent, will provide more precise information regarding the molecular changes of the joint microenvironment in MetOA and possible mechanisms of GSM to treat or prevent joint diseases. Regardless, this is the first study to report the combined effect of HFHS diet, OVX and GSM treatment on plasma lipid and metabolites of rats and provides important novel information about the individual and combined effects of these factors. A previous large cohort study on plasma has shown that very few extra lipids are gained from running samples in both positive and negative ionisation, as ~80-90% of the lipids from both modes are detected in positive mode alone (Huynh et al., 2019). Thus, we sought to perform the lipidomic profiling in positive ion mode so that it would be cost and time efficient and with high throughput.

In summary, untargeted lipidomic and metabolomic analysis identified several lipids in glycerophospholipids, glycerolipids and sphingolipids and amino acids altered by HFHS diet and GSM supplementation. Adding GSM recovered some of the metabolomic

alterations under HFHS diet with particularly robust responses in rats fed a control diet. Subsequent evaluation of lipid pathways revealed sphingolipid metabolism may be affected by HFHS diet, suggesting that increases in SM and Cer levels are potential causes of metabolic dysregulation under HFHS feeding, an effect which may be repaired by adding GSM to diet. These results help provide a basis for the metabolic changes that occur in female-specific obesity and MetOA induced by HFHS diet and ovariectomy, which highlight the need for additional tracking clinical studies on postmenopausal women.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Student name:	Maryam Abshirini
Name and title of main supervisor:	Professor Marlena C.Kruger
In which chapter is the manuscript/published work?	Chapter 5
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:

Involved in the study design, ethic application, participant recruitment, running of the study and data collection, analysis and interpretation and writing of the first draft of manuscript.

Please select one of the following three options:

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  Abshirini, M., J. Coad, F.M. Wolber, P. Von Hurst, M.R. Miller, H.S. Tian, et al., Effects of GreenshellTM mussel intervention on biomarkers of cartilage metabolism, inflammatory markers and joint symptoms in overweight/obese postmenopausal women: a randomized, double-blind, placebo-controlled trial. Frontiers in Me

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Chapter 5 Effects of Greenshell™ Mussel supplementation on biomarkers of cartilage metabolism, inflammatory markers, and joint symptoms in overweight/obese postmenopausal women

The objective of this study was to determine the effect of supplementation of Greenshell™ Mussel (GSM) on biomarkers of cartilage metabolism, inflammatory cytokines, and joint symptoms in overweight/obese postmenopausal women

This chapter was published in part in Frontiers in Medicine.

Abshirini, M., J. Coad, F.M. Wolber, P. Von Hurst, M.R. Miller, H.S. Tian, et al., (2022). Effects of Greenshell™ mussel intervention on biomarkers of cartilage metabolism, inflammatory markers and joint symptoms in overweight/obese postmenopausal women: a randomized, double-blind, placebo-controlled trial. Frontiers in Medicine: p. 3587.

Abstract

Background: Recent evidence showed that whole meat powder from the New Zealand Greenshell™ Mussel (*Perna canaliculus;* GSM) decreased an early cartilage degradation biomarker and late knee joint histological damage in rats with diet- and ovariectomy-induced obesity as a model of metabolic osteoarthritis (MetOA). To investigate the cartilage-protective potential of whole GSM powder in human subjects, a randomised double-blind placebo-controlled trial was conducted to determine the effect of whole meat GSM powder supplementation on biomarkers of cartilage metabolism, inflammatory cytokines, and joint symptoms in postmenopausal women with overweight/obesity and joint discomfort.

Methods: Fifty-five overweight/obese postmenopausal women living in New Zealand (BMI 25-35 kg/m^2, aged 55-75 years) were randomly assigned to receive 3 g/day whole meat GSM powder or placebo for 12 weeks. Primary outcomes included cartilage turnover biomarkers urinary C-telopeptide of type II collagen (CTX-II) and serum cartilage oligomeric matrix protein (COMP), which were measured in urine and blood samples at baseline, week 6 and week 12. Secondary outcomes assessed at baseline and end of the study included plasma inflammatory cytokines, along with joint pain and knee-related problems using a 100 mm Visual Analogue Scale (VAS) and the Knee injury and Osteoarthritis Outcome Score (KOOS) questionnaire, respectively.

Results: Forty-nine participants completed the study (GSM n=25, placebo n=24). The data were analysed as per-protocol. After 12 weeks, urinary CTX-II showed no significant change over time or between the groups (interaction effect P=0.1). However, in women with symptomatic knees, a significant difference was noted between the group (treatment effect P=0.04), as it was lower in the GSM group compared to placebo group at week 6 (P=0.04) and week 12 (P=0.03). Serum COMP and plasma cytokines were not affected. GSM supplementation showed greater reduction in the VAS pain score than placebo (-13.2±20.3 vs -2.9±15.9; P=0.04). No significant change in KOOS domains between the two groups was observed.

Conclusion: Oral supplementation of whole GSM powder at 3 g/day may slow down the degradation of type II collagen in postmenopausal women. GSM treatment conferred clinical benefit on overall joint pain. No significant effect was noted for inflammatory

cytokines, suggesting that GSM may act within the joint microenvironment rather than at the systemic level.

5.1 Introduction

Osteoarthritis (OA) is characterized by progressive degradation of articular cartilage and loss of joint function and is considered the most common type of joint disease and a leading cause of disability among the elderly (Lawrence et al., 2008). OA prevalence is higher among women compared to men and its incidence rises following menopause (Srikanth et al., 2005). Women also tend to have a greater severity of knee OA (O'Connor, 2007). This drastic increase in OA incidence among postmenopausal women is linked to oestrogen, which declines after menopause. The presence of both alpha and beta oestrogen receptors (ERα and ERβ) in cartilage indicates that chondrocytes may respond to oestrogen and therefore reduction in oestrogen likely influences the chondrocytes metabolism (Claassen et al., 2001). Furthermore, menopause is associated with weight gain and increased body mass index (BMI) which is highly correlated with increased risk of knee and hip OA (Holliday et al., 2011).

A report on the association between obesity and OA incidence for non-weight bearing joints indicates the involvement of obesity-related metabolic factors such as adipokines and pro-inflammatory cytokines (Yoshimura et al., 2011). Interestingly, the roles of mechanical loading and inflammation in development of radiographic knee OA were found to be more relevant in overweight and obese women than men (Roemer et al., 2021). Excessive fat tissue induces production and release of the adipokines and pro-inflammatory cytokines resulting in low-grade systemic inflammation (Hauner, 2005).

In addition, it is well-documented that inflammatory immune cells are recruited into the synovial joint and are involved in initiation of pathological changes in the synovial joint and initiation of obesity-associated OA (Nedunchezhiyan et al., 2022). Tumour necrosis factor-α (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) are involved in cartilage degradation and bone resorption. These cytokines stimulate the expression of the cartilage-degrading enzymes, matrix metalloproteinases (MMPs), while inhibiting the formation of collagen type II and other cartilage matrix components (Wang & He, 2018).

Molecules derived from synovial joint tissue, particularly cartilage, have been used as biochemical markers of OA to detect the early change in metabolic and chemical properties of cartilage or predict disease progression and treatment monitoring (Henrotin et al., 2016). Type II collagen is the main component of cartilage and makes up 90-95% of the total collagen in cartilage (Bauer et al., 2006). Proteolysis of type II collagen results

in fragments of C-terminal telopeptides of type II collagen (CTX-II), which is measured as a biomarker of cartilage degradation. Cartilage oligomeric matrix protein (COMP) is a non-collagen structural protein involved in stabilization of extracellular matrix through interaction with collagen fibrils (Mann et al., 2004). Urine CTX-II and serum COMP are the most frequently studied biomarkers and have shown the best performance across all available biomarkers for OA. Both markers have been shown to be elevated in patients with OA and are correlated with radiographic severity of OA (Sofat et al., 2019; Wang et al., 2019). Despite extensive research and development of various markers, no single gold standard biomarker that is specific and sensitive to the damaged tissue and OA progression has been identified; therefore measuring a panel of biomarkers is necessary to provide an accurate picture on joint tissue metabolism (Henrotin et al., 2016).

An extract from New Zealand green-lipped mussel (*Perna canaliculus*) known as Greenshell^{TM} Mussel (GSM) was found to be beneficial for joint health and symptom-relieving of OA in animal (Pollard et al., 2006) and human clinical trials (Coulson et al., 2013).

The inhibitory effect of omega-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in GSM on cyclooxygenase-2 (COX-2) and the 5-lipoxygenase (5-LOX) cascade suppress synthesis of Prostaglandin E2 (PGE2) and reduce the inflammatory response (McPhee et al., 2007). Furthermore, these fatty acids can resolve the existing inflammation through specialised pro-resolving mediators (SPMs) or derived compounds from EPA and DHA metabolised by LOXs. These novel anti-inflammatory molecules promote resolution of inflammation, tissue healing and relief of the chronic pain in rheumatic diseases (Chávez-Castillo et al., 2021; Chiang & Serhan, 2020).

Recently, the cartilage protective activity of GSM has been demonstrated in a rat model of metabolic OA (Siriarchavatana et al., 2019). The short-term pre-clinical study revealed that feeding with whole GSM powder decreased plasma concentration of CTX-II, in rats fed a high-fat/high-sugar (HFHS) diet, indicating a preventive effect of GSM on cartilage degradation (Siriarchavatana et al., 2019). Interestingly, in a matching long-term trial, rats were ovariectomized (OVX) to establish a model of post-menopausal OA combined with diet-induced obesity. Histopathological assessment of cartilage in knee joints demonstrated the Mankin score, a standard indicator of cartilage damage severity, was

reduced in GSM-fed rats (Siriarchavatana et al., 2020b). Based on these observations, whole GSM powder has the potential to reduce type II collagen degradation and thereby attenuate the progression of OA in human subjects.

Previous studies have mainly focused on symptom-modifying effects of GSM extracts among OA patients and there is a lack of clinical studies using cartilage metabolism biomarkers to measure the GSM chondroprotective efficacy.

The clinical diagnosis of OA is usually made once disease is at late stage and most likely irreversible. Thus, this study targeted postmenopausal women with overweight/obesity with joint discomfort who are at risk or with early stage of OA, when an intervention is more likely to be beneficial. The current study a randomised, assessor and patient blinded, placebo-controlled trial aimed to investigate whether 12 weeks of supplementation with whole meat GSM powder supplementation affects the levels of cartilage metabolism biomarkers (primary outcome) and inflammatory cytokines along with joint pain and knee-related symptoms and function (secondary outcome) in overweight/obese postmenopausal women with joint discomfort. The placebo group was included for control. This study hypothesised that supplementation with GSM powder will decrease cartilage degradation biomarkers; urinary CTX-II and serum COMP and result in reduction of inflammatory cytokine levels, joint pain score and knee-related symptoms compared to placebo.

5.2 Method and materials

5.2.1 **Study participants**

A total of 55 New Zealand women aged 55-75 years ≥5 years post-menopause (based on the natural cessation of menstruation), with self-reported body mass index (BMI) between 25 and 35 kg/m^2 (weight status was evaluated according to definition provided by Centre of Disease Control and Prevention: BMI 25 to <30 and 30 to 35 fell under overweight and obese, respectively), and living in the Manawatū-Whanganui area were included. Participants reporting joint pain or discomfort for at least 3 months prior to study commencement without daily use of analgesic medicine were included. Subjects were excluded if they had a formal diagnosis of clinical OA, inflammatory arthritis or rheumatoid arthritis (RA), diabetes mellitus, or atherosclerosis, having chronic liver or renal disorder detected based on the screening blood test, having allergy to mussels or

seafood, history of recent joint injury or trauma, smoking or having alcohol intake of more than two units per day, being on hormone replacement therapy for <6 months prior to the beginning of the trial, or taking anti-inflammatory drugs orally (glucocorticoids or NSAIDs) on a daily basis.

Massey University Human Ethics Committee approved this study: Southern A, Application 20/03. The patients/participants provided written informed consent to participate in this study. This study was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) with the number ACTRN12620000413921p. (Date of first registration: 27/03/2020).

5.2.2 **Study design**

A 12-week randomised double-blinded, placebo-controlled study design was conducted. Women who met the initial inclusion criteria were screened by a routine non-fasted blood test for liver and kidney function, blood glucose (HbA1c), and lipid profile including triglycerides, total cholesterol, HDL-cholesterol, LDL-cholesterol at MedLab Central, Palmerston North, New Zealand. The purpose of routine blood tests was to screen the potential participants for liver and kidney disease, and these were repeated at the end of study to assess the safety of supplement. Prior to the baseline visit, participants who were regularly consuming oily fish (more than one meal per week) or taking fish oil or other joint health supplements were required to undergo a four-week washout period. Then participants were randomly allocated into two groups, each consuming six capsules per day for 12 weeks: whole meat GSM powder (3 gram/day) or placebo (sunflower seed protein). The subjects were instructed to consume the capsules with or after their meals.

The flash-dried whole meat GSM powder used in this study was comprised of 41.4% protein, 30.8% carbohydrate, 10.1% fat (EPA and DHA was 20.7% and 8% total fatty acids, respectively), 10.7% ash, and 7% moisture. The dose of 3 g/day was selected as it is achievable through diet (equivalent to 1-2 mussels) and this dose and duration were comparable to previous studies using whole GSM extracts in knee OA patients which resulted in pain improvement without any major adverse side effects (Coulson et al., 2013). Flash-dried whole meat GSM powder was produced by Sanford Ltd (PernaUltra ^{TM}, Sanford, Blenheim, NZ) using standard manufacturing processes. Sunflower seed protein (BP Bulk powders, Braeside, Melbourne Australia) was used as placebo as a neutral source of protein and was selected to be relatively similar to GSM powder in

respect to macronutrient composition (66.6% carbohydrate, 24.3% protein, 3% fat, 3.4% moisture, 2.7% ash) and to be as inert and non-bioactive (not containing EPA and DHA) as possible. The main fatty acid present in sunflower seed was omega-6 linoleic acid which is not anti-inflammatory and could be pro-inflammatory. The amount of vitamin E for this small dose of placebo was negligible.

Both GSM powder and placebo were encapsulated in hard-shell capsules by a commercial facility (Alaron, Nelson NZ) and stored under nitrogen in the dark at room temperature or lower until use. The GSM and placebo capsules were matched in the shape, size, and colour of hard-shell encapsulant. Activated carbon sachets for absorbing moisture and odour were put in bottles to conceal any 'fishy' odour.

The nutritional composition and fatty acids profile of GSM powder and placebo used in the study is presented in Appendix 3; Table S5.1. Both supplements were assessed for proximate composition in a commercial testing laboratory (Food Testing Laboratory of Cawthron Analytical Services; Nelson, New Zealand). The Association of Official Analytical Chemists (AOAC) methods for crude protein (AOAC 981.10), total fat (AOAC 948.15), moisture at 105 ºC (AOAC 950.46) and ash (AOAC 920.153) were applied, and carbohydrate content was measured by calculation (100% -% crude protein -% total fat -% moisture -% ash). An aliquot of the total lipid extract from the GSM powder was trans-methylated in methanol/chloroform/hydrochloric acid (10:1:1, v/v/v) for 1 h at 100 ºC. Water was added, and the mixture was extracted three times with hexane/chloroform (4:1, v/v) to obtain fatty acids methyl ester (FAME). Sample were made up to 1 mL with an internal injection standard (19:0 FAME) and analysed by gas chromatography mass spectrometry (CG-MS) according to AOAC 963.22.

5.2.3 **Randomisation**

A randomisation list was generated by Excel and maintained by the project's supervising investigator, who did not interact with the study subjects or conduct the primary data analysis. Randomisation was stratified based on BMI (overweight: 25-29.9 kg/m^2 and obese: 30-35 kg/m^2) and age (55-64, 65-75 years) distribution. The primary researcher who was blinded to treatment codes allocated participants to two supplements (A and B). Participants were blinded to treatment group until all analyses were completed. Data were collected during participants' visit at baseline, follow-up (week 6) and end of the study (week 12) as shown in Figure.5.1. Recruitment, screening, and data collection took place

at the Human Nutrition Research Unit (HNRU) at Massey University, Palmerston North, New Zealand from August 2020 to September 2021.

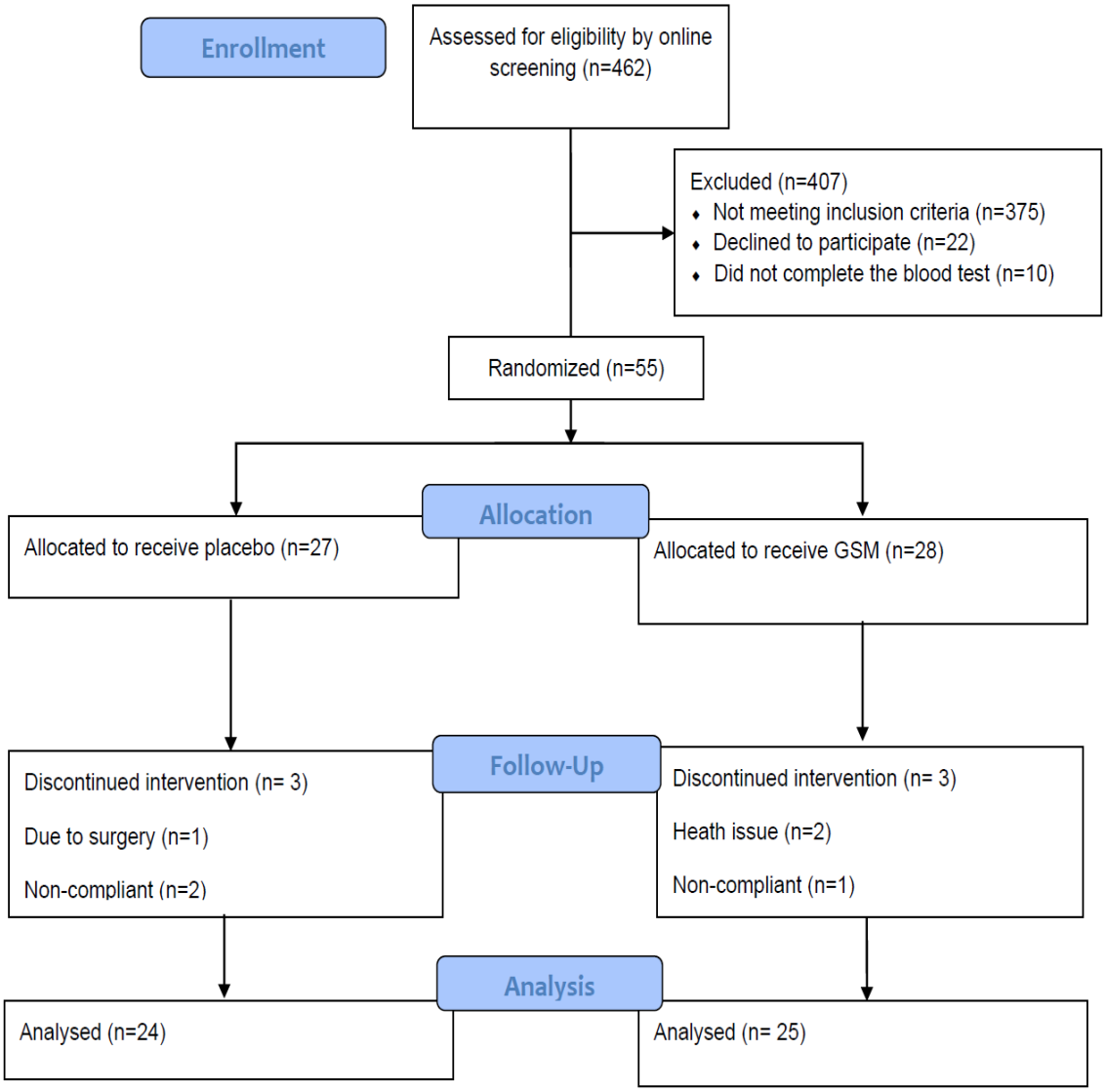


Figure 5.1 Schematic diagram of study design.

5.2.4 **Demographic, anthropometric, and physical activity measurement**

At baseline, participants completed a demographic questionnaire as well as anthropometric measurements including body weight and standing height measured using a beam balance to the nearest 0.2 kg and stadiometer to the nearest 0.1 cm, respectively. Body mass index (BMI) was calculated as weight (Kg) divided by height squared (m^2). Physical activity was assessed by the New Zealand Physical Activity Questionnaire – Short Form (NZPAQ-SF) (McLean & Tobias, 2004). The NZPAQ has been validated by Boon et al. (Boon et al., 2010), and physical activities were computed by metabolic equivalent of task (METs)-min/week, which was calculated by the scoring protocol of International Physical Activity Questionnaire (IPAQ) for continuous score ("IPAQ, R.C. Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire (IPAQ)-Short and Long Forms," 2005).

MET values and formula for calculation of MET-minutes were assessed and used as below:

- Walking MET-minutes/week at work = 3.3 × walking minutes × walking days at work
- Moderate MET-minutes/week at work = 4.0 × moderate-intensity activity minutes × moderate intensity days at work.
- Vigorous MET-minutes/week at work = 8.0 × vigorous-intensity activity minutes × vigorous
- Total Work MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores at work

5.2.5 **Biochemical analyses**

According to the assay protocol, second void morning urine specimens were collected. In human, the first void morning urine is the most concentrated urine which interferes with the analysis; therefore, the second void morning urine was collected after overnight fasting at baseline, week 6 and week 12 for assessment of CTX-II. The overnight fasting blood samples were collected by a certified phlebotomist at baseline, week 6 and week 12 to measure COMP. Blood samples were collected into serum and EDTA-anticoagulated plasma vacutainer tubes. The serum tubes were incubated for 1 hr at room temperature, followed by centrifugation at 2264 g for 10 min at 4˚C (Gyrozen 1248R

Multi-Purpose High-Speed, Korea) to isolate the serum. The EDTA tubes were centrifuged immediately after blood drawing in the same manner to collect the plasma. Serum, plasma, and urine samples were aliquoted and stored at -80˚C until use.

Measurement of serum biomarkers was performed using commercially available enzyme linked immunoassay (ELISA) kits. Assays for serum cartilage oligomeric matrix protein (COMP) were performed with BioVendor Research and Diagnostic Products (Karasek, Czech Republic). The detection limit was 0.4 ng/mL. The intra-assay precision co-efficient of variation (CV) was 4.0-8.0% and the inter-assay precision CV was 3.1–6.6%.

Urinary CTX-II concentrations were determined using an enzyme immunoassay (EIA) kit (Urine CartiLaps® EIA; Immunodiagnostic systems, Herlev, Denmark). Urinary creatinine (Cr) was measured by colorimetric method (RX Daytona+; Randox Laboratories Ltd). Concentrations of urinary CTX-II were corrected by urinary Cr using the following formula: corrected CTX-II value (ng/mmol Cr) = 1000 x urine CartiLaps (μg/L) / creatinine (mmol/L). The detection limit was 0.2 ng/mL. The intra-assay precision coefficient of variation (CV) was 5.2%, 4.6%, and 7.8% for high, medium and low ranges of measurement. The inter-assay precision co-efficient of variation (CV) were 6.9%, 10.8%, 12.2% for high, medium, and low ranges of measurement, determined by the manufacturer. Serum COMP and urinary CTX-II were assessed in duplicate. The kits were not checked for validity as it has been frequently used in previous studies which took place in our laboratory.

Plasma C-terminal telopeptide of type I collagen (CTX-1) at all time points and parathyroid hormone (PTH) at baseline were analysed by electrochemiluminescence immunoassays using the Roche COBAS® e411 system (Roche Diagnostics, Indianapolis, IN, USA). Cytokine assays were performed using BioLegend® LEGENDplex™ Multi-Analyte Flow Assay following the kit instructions and measured using a Beckman Coulter Gallios flow cytometer. The concentration of cytokines including TNF-α, IL-1β, IL-6, IL-4, IL-10, IL-15, and IL-18 were quantified in plasma at baseline and the end of the study. Baseline level of plasma 25(OH) vitamin D were analysed using isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MSMS) by Canterbury Health, Christchurch, New Zealand. Serum 25(OH)D ≥ 50 nmol/L at the end of winter, and 10 to 20 nmol/L higher at the end of summer to allow for seasonal variation, has been considered optimal for musculoskeletal health for people residing in Australia and New

Zealand (Nowson et al., 2012). Vitamin D insufficiency or deficiency in this study was considered as plasma 25(OH)D < 50 nmol/L.

5.2.6 **Self-assessment of pain visual analogue scale (VAS) and knee injury and osteoarthritis outcome score (KOOS)**

Secondary outcome measures including pain visual analogue scale (VAS) and Knee Injury and Osteoarthritis Outcome Score (KOOS) were recorded at baseline and week 12. The pain levels were reported by participants using a 100 mm linear measure of pain status scored from 0 to 100 mm where 0 was defined as having no pain and 100 the worst pain ever experienced within the past week. Pain rated at ≥30 was regarded as having a moderate to high level of pain. This cut-off was selected based on the required entry criteria of a previous clinical trial (Stebbings et al., 2017). Participants with more than one joint site with pain completed the VAS for overall joint pain.

KOOS is commonly utilized in research and clinical practice to measure short- and long-term consequences of knee problems (Braham et al., 2003). The previous week is the period included when answering the questions about the knee problem. It consists of 42 items which cover five domains: knee pain (Pain), other symptoms (Symptoms), activities of daily living (ADL), function in sport and recreation (Sport/Rec) and knee related quality of life (QOL). All items are scored on a 5-point Likert scale (0–4), and each domain is scored separately as the sum of all corresponding items. A total score has not been validated and is not recommended. Scores are then converted to a 0–100 scale (percentage of total possible score obtained), where 0 represents extreme knee problems and 100 represents no knee problems (Roos & Lohmander, 2003). This questionnaire was completed by those who reported knee pain and established cut-off score of ≤86 for any of the domains is used to classify individuals with symptomatic knees (Baldwin et al., 2017). The validity of KOOS has previously been demonstrated by construct and content and good to excellent test-retest reliability (Collins et al., 2011; Roos et al., 1998).

5.2.7 **Compliance assessment**

To assess subjects' compliance, diaries were provided to participants at baseline to record their daily intake of study supplement and analgesic medications. Participants were allowed to continue taking paracetamol or any supplements that did not contain omega-3 fatty acids or chondroprotective bioactive compounds. Compliance assessment was performed using cumulative capsule counts at the completion of the study, and adherence

was measured as a percentage: [(number of capsules provided minus number of unused capsules)/number of capsules provided] × 100. Adherence below 80% was considered a protocol violation.

Moreover, at the baseline and end of the study, the plasma and red blood cell membrane n-3 PUFA, EPA, DHA and total n-3 L-C PUFA (including alpha-linolenic acid (ALA, 18:3 n-3), stearidonic acid (SDA, 18:4 n-3), eicosatetraenoic acid (ETA 20:4 n-3), EPA, docosapentaenoic acid (DPA,22:5 n-3) and DHA) were measured to assess the adherence to study protocol. The plasma and red blood cell n-3 PUFA were analysed by gas chromatography (GC, Agilent Technologies Australia, Victoria, Australia). Fatty acids were identified to an external commercial fatty acid standard. The analysis were done at the Cawthron Institute, Nelson, New Zealand and methodologies are published elsewhere (Miller et al., 2020) .

5.2.8 **Safety assessment**

Any adverse side effect was recorded by participants in their diaries. Participants documented the events by rating the severity (mild, moderate, and severe) and medications required to treat the events. Moreover, routine laboratory blood test including liver and kidney function tests, blood glucose (HbA1c) and lipid profile (triglyceride, total cholesterol, HDL-cholesterol, LDL-cholesterol) were assessed from non-fasted venous blood samples at baseline and end of trial at MedLab Central Palmerston North, New Zealand.

5.2.9 **Statistical methods**

The sample size was based on urine CTX-II/ creatinine and serum COMP as the primary outcomes of the study. Sample size was calculated to detect 20% difference between the groups using the standard deviation from an unpublished report. For urine CTX-II/ creatinine a sample size of 24 was required to detect a 20% relative difference from baseline with 80% power. For serum COMP a sample size of 17 was required to detect a 20% difference between groups with a power of 95%. The sample size of 48 (n=24 per group) was required as a manageable sample size. Finally, a total sample size of 55 was needed to allow for at ~ 10% potential dropout rate (n=27-28 per group).

Statistical analysis was performed using IBM SPSS version 26.0 (Armonk, NY). Analysis was conducted on the dataset from participants who completed assessment at both timepoints (baseline and endpoint). Variables were checked for normality using the Kolmogorov-Smirnov, Shapiro-Wilk tests and data that were not normally distributed were log-transformed. The data were reported as mean ± standard deviation (SD) for normally distributed data, and as median (25^{th}, 75^{th} percentiles) for non-normally distributed data, and as frequencies for categorical data. The baseline characteristics of subjects between two treatment groups were compared using Student's t-test for parametric, and the Mann-Whitney U test for nonparametric data. Regarding the categorical variables, the distribution of participants was analysed using the Chi-square tests or Fisher's exact test where more than 20% of data cells had expected count below 5.

Missing data points were imputed with mean values of each group (the mean value of each group was assigned to those with missing data) to include all the data in the analysis. Outcome analyses were performed according to per-protocol and were conducted and with and without imputed missing values. Two-way repeated measures ANOVA was used to examine differences within each group over time (pre- vs post-intervention) and between the groups (GSM vs placebo). In case of a significant effect, analysis was followed by post hoc analysis adjusted with Bonferroni. The data were checked to meet all the assumptions of two-way repeated measure ANOVA prior to analysis. These included detecting no significant outliers, approximate normal distribution of dependant variables, and equal variances of the differences between groups checked with sphericity tests.

For the analysis of the cartilage markers, the data were corrected for the baseline in order to reduce the variability and achieve normal distribution. In order to control the effect of the main potential confounding factors, age and BMI on outcome measures, particularly cartilage markers, the treatment groups were stratified for these factors (Pourhoseingholi et al., 2012).

The interactions between treatments and time indicate differences in efficacy. For VAS pain and KOOS score analysis, covariates including baseline level of VAS pain or KOOS domain score, compliance, paracetamol use (yes or no), and season of enrolment were adjusted in models. The relationships between cartilage degradation markers, plasma

25(OH)D, VAS and KOOS domain score were assessed using Pearson correlations. Statistical significance was considered by two-sided $P<0.05$.

5.3 Results

5.3.1 **Baseline characteristics of participants**

The flow diagram of study is presented in Figure 5.1. Initially 462 women filled a pre-screening online questionnaire, and most of them were excluded due to distance from the location of research or being diagnosed with health condition mentioned in the exclusion criteria. Finally, 66 women passed the online screening and were phone interviewed and invited for a blood test screen. From this group, 55 completed the blood test and were eligible for trial entry. Of the 55 enrolled participants, 6 subjects dropped out from the trial. Finally, a total of 49 participants (GSM, n= 25 and placebo, n=24) completed the study. The baseline characteristics of the participants who completed the study are shown in Table 5.1. It is important to note that due to COVID-19 restrictions, one subject from the GSM group missed a follow-up visit. For blood markers analysis, blood samples were not available from two participants at week 6 from GSM group (one due to missing the visit due to COVID-19 lockdown and one due to phlebotomy issues). Urine samples were provided by all participants from both groups at all time points, except for one participant from the GSM group due to missing the visit at week 6.

The two groups were similar at baseline with respect to most demographic characteristics. For the overall study population, the mean age was 63.5±5.4 years; 67.4% of women were overweight (BMI between 25-29.9 kg/m^2) and 32.6% were obese (BMI $\geq$30 kg/m^2). The median (25th, 75th percentile) of physical activity level was 751 (318, 2373.7) MET-minutes/week and 764 (287, 1483) MET-minutes/week in placebo and GSM group, respectively, with no significant difference between the groups.

Majority of participants (89.1%) were of European-New Zealand ethnicity. Paracetamol use during the study was reported by 14 (28.5%) of the participants and did not differ between groups. Some significant differences were observed. Out of the 49 women, 21 (42.9%) were characterised as having moderate to high levels of joint pain (VAS pain score $\geq$ 30) and this was significantly different between the groups with a higher proportion in the GSM group (56% vs 29.2%, P=0.05). In term of knee related problems,

39 (79.6%) women had knee symptoms (KOOS domain score $\leq$ 86). With respect to joint pain location, 18 (36.7%) had only knee pain, 21 (42.8%) had pain at knee and hip or other joints, and 10 (20.4%) reported pain at hand and/or back or shoulder.

The baseline level of plasma CTX-I was comparable between the two groups, while the baseline level of plasma 25(OH)D and PTH were significantly different between the groups, as vitamin D level was higher and PTH was lower in the GSM group compared to the placebo group (P=0.007). However, the percentage of participants with vitamin D insufficiency or deficiency (25(OH)D below 50 nmol/L) was not significantly different between the groups with 2 (8%) in the GSM group and 6 (25%) in the placebo having vitamin D levels below the normal range (P=0.1).

Table 5.1 General characteristics of participants who completed the study.

General characteristics	Overall (n=49)	Placebo(n=24)	GSM (n=25)	P-value
Age (years), mean±SD	63.5±5.4	62.9±5.4	64.2±5.1	0.3
Height (cm), mean±SD	164.8+6.7	164.7±6.4	164.8±7.1	0.8
Weight (kg), median (25^{th}, 75^{th} percentiles)	75.7 (68.3, 86.2)	73.8 (68.2, 88.6)	77.2 (68.5, 86.2)	0.9
BMI categories, n (%)				0.6
Overweight	33 (67.4)	17(70.8)	16 (64)	
Obese	16 (32.6)	7 (29.2)	9 (36)	
Physical activity (MET-minutes/week), median (25^{th}, 75^{th} percentiles)	764 (307.5, 1794)	751 (318, 2373.7)	764 (287, 1483)	0.1
Ethnicity, n (%)				0.1
NZ European	44 (89.1)	20 (83.3)	24 (96)	
Māori/Other	5 (10.2)	4 (16.7)	1 (4)	
Season of enrolment, n (%)				
Spring	7 (14.3)	6 (25)	1 (4)	
Summer	17 (34.7)	6 (25)	11 (44)	0.1
Autumn	22 (44.9)	10 (41.7)	12 (48)	
Winter	3 (6.1)	2 (8.3)	1 (4)	
Whole body T-score < -2.5, n (%)	12 (24.5)	6 (25)	6 (24)	0.9
VAS pain score $\geq$ 30, n (%)	21 (42.9)	7 (29.2)	14 (56)	0.05
KOOS score $\leq$ 86, n (%)	39 (79.6)	18 (75)	21 (84)	0.4
Joint pain location, n (%)				
Knee	18 (36.7)	12 (50)	6 (24)	0.1
Knee and hip	3 (6.1)	1 (4.2)	2 (8)	
Others	28 (57.1)	11 (45.8)	17 (68)	
Paracetamol use, n (%)				
Yes	14 (28.5)	5 (20.8)	9 (36)	0.2
No	35 (71.4)	19 (79.2)	16 (64)	
Plasma CTX-I (ug/L), mean±SD	0.44±0.14	0.46±0.13	0.43±0.16	0.4
Plasma 25(OH)D, nmol/L, median (25^{th}, 75^{th} percentiles)	73 (56, 83)	69.5 (46, 79.7)	78 (64, 87)	0.007*
Plasma PTH, picomol/L, median (25^{th}, 75^{th} percentiles)	4.3 (3.5, 5.3)	4.7 (4.2, 6.4)	3.8 (3.5, 4.8)	0.007*

BMI: body mass index (kg/m^2); MET: metabolic equivalent of task; VAS: visual analogue scale; KOOS: knee injury and osteoarthritis outcome score; CTX-I: C-terminal telopeptides of type I collagen. Values are presented as mean ± standard deviation or median (25^{th} and 75^{th} percentile) for normally distributed and non-normally distributed variables, and n (%) for categorical variables for which the percentage within each treatment group is reported.

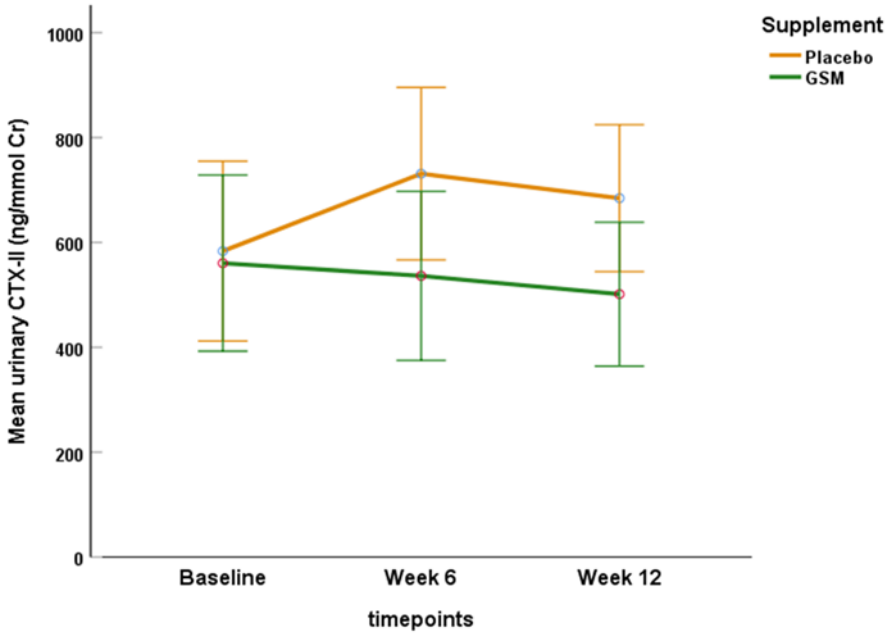
Student's t-test was used for variables with normal distribution, and the Mann-Whitney U test for non-normally distributed variables, and for categorical variables, the distribution of participants was analysed using the Chi-square tests. * Indicates significance at $P<0.05$.

5.3.2 **Evaluation of treatment on cartilage degradation markers**

At baseline, the mean urinary CTX-II concentration was 560.4±428 ng/mmol Cr in GSM group and 583.3±411 ng/mmol Cr in placebo group and there were no significant differences between the groups (P=0.8). As demonstrated in Figure 5.2A, the urinary CTX-II concentration slightly decreased from the baseline during the intervention in the GSM group, while it notably increased from baseline and peaked at week 6 and then slightly reduced at week 12 in the placebo group however, the overall change was not significant between the groups (interaction effect P=0.3).

As shown in Figure 5.2B, the baseline concentration of serum COMP was 972.3±272 and 1040.4±402 ng/ml in the GSM and placebo groups, respectively. As shown in Figure 2.B, serum COMP trended to slightly decrease in placebo and increase in GSM group. Overall, it remained stable and did not change meaningfully over the study period or between group (interaction effect P=0.1).

(A)



(B)

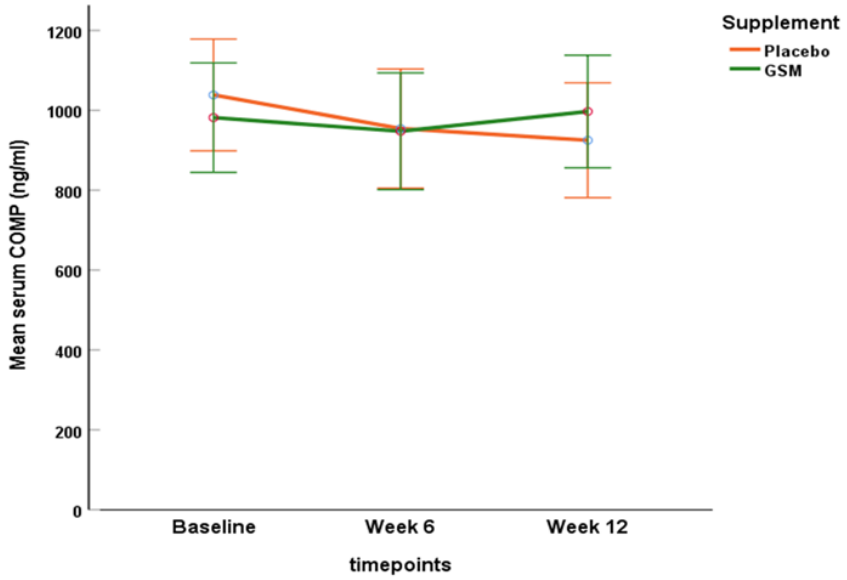


Figure 5.2 Patterns of change in urinary CTX-II (A) and serum COMP level (B) over the study period (baseline, follow-up, and endpoint) within each of the treatment groups.

No significant effects neither over time nor between the groups for urine CTX-II and serum COMP (interaction effect P=0.3, P=0.1, respectively). Data are expressed as the mean ± standard error. Placebo= orange, GSM= green.

To further evaluate the effect of GSM supplementation on urinary CTX-II concentrations, subjects with a KOOS domain score 86 or below were included in a further analysis (GSM, n=21 and placebo, n=18). The baseline characteristics of these subjects and concentration of cartilage degradation biomarkers were not statistically significant between the treatment groups (data not shown). As shown in Figure 5.3, in subjects with symptomatic knees, the urinary CTX-II showed similar pattern of change as for the overall population. The result of analysis on participants with KOOS below 86 showed urine CTX-II concentrations were significantly different among the treatment groups during the intervention (treatment effect P=0.04) with significantly lower levels in the GSM group compared to placebo at week 6 (534.6±255.4 vs 824.7±570.4 ng/mmol Cr, P= 0.04) and end of the study (496.6±204.2 vs 757.4±493.2 ng/mmol Cr, P=0.03). However, there was no significant change over time within groups (time effect P=0.9) and between groups (interaction effect P=0.3).

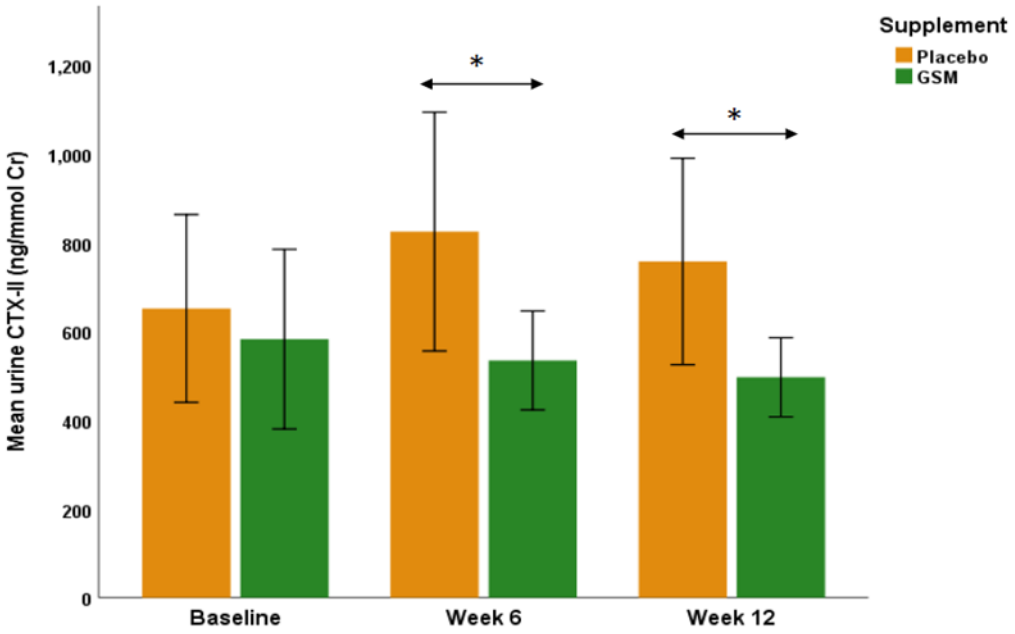


Figure 5.3 Patterns of change in urinary CTX-II levels over the study period (baseline, follow-up, and endpoint) within each of the treatment groups in those subjects with KOOS domain score of 86 or below.

There was a significant difference between groups as urinary CTX-II was significantly lower in GSM compared to placebo at week 6 (P= 0.04) and week 12 (P=0.03). The values are expressed at mean± standard error and compared by Student's t-test at each time point.

The analysis for urine CTX-II and serum COMP was conducted on the data corrected for baseline as shown in (Appendix 3; Figure S5.1). There was a significant change overtime (time effect P=0.03), although the overall change was not significant between the groups (interaction effect P=0.1). The result for serum COMP corrected for the baseline was similar to uncorrected as no significant effect was noted. The result of analysis for data, without imputing missing values, were similar to the imputed data (data not shown).

Furthermore, the mean value of plasma CTX-I was within the range reported by a previous study (0.45±0.1 ug/L) (Kruger et al., 2012), and remained stable throughout the study as no effect of GSM supplementation was evident (Appendix 3; Figure S5.2).

5.3.3 **Evaluation of treatment on VAS pain and KOOS domains score**

The baseline VAS pain score in the GSM group was 21.6±15.9 and in the placebo group was 29.4±21 and there was no significant difference between the two groups (P=0.07). The pattern of change in VAS pain score over the study period is presented in Figure 5.4. There was a significant change in VAS pain score between the groups. Both unadjusted and adjusted analysis of the VAS pain score showed a greater reduction from baseline in

the GSM group compared with placebo (-13.2±20.3 vs -2.9±15.9, P=0.03 unadjusted, and P=0.04 adjusted for covariates). A significant time effect (the difference between baseline and endpoint) was found for the VAS pain score (P=0.002 unadjusted). The rate of positive response in VAS pain score (at least 10 mm reduction) was 56% in GSM group as compared with 29% in placebo group (P=0.05), as shown in Figure 5.5.

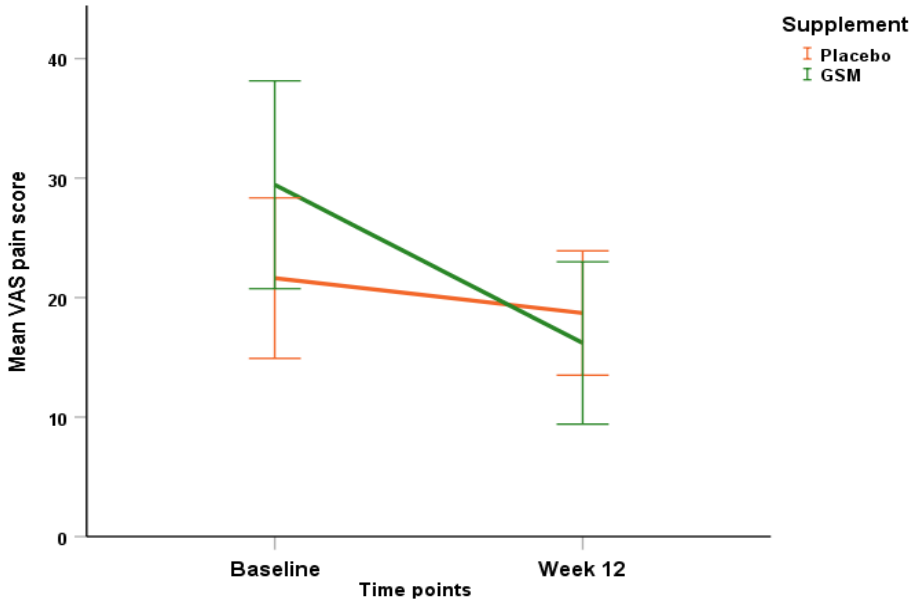


Figure 5.4 Pattern of change in VAS pain score over the study period (baseline and endpoint) within each of the treatment groups

Significant effect of time (P=0.002), and greater reduction in VAS pain score in GSM supplement compared to placebo (interaction effect P=0.03 unadjusted and P=0.04 adjusted for baseline level, compliance, use of paracetamol, and season of enrolment). Placebo = orange, GSM=green. Values are expressed as mean (95% confidence interval).

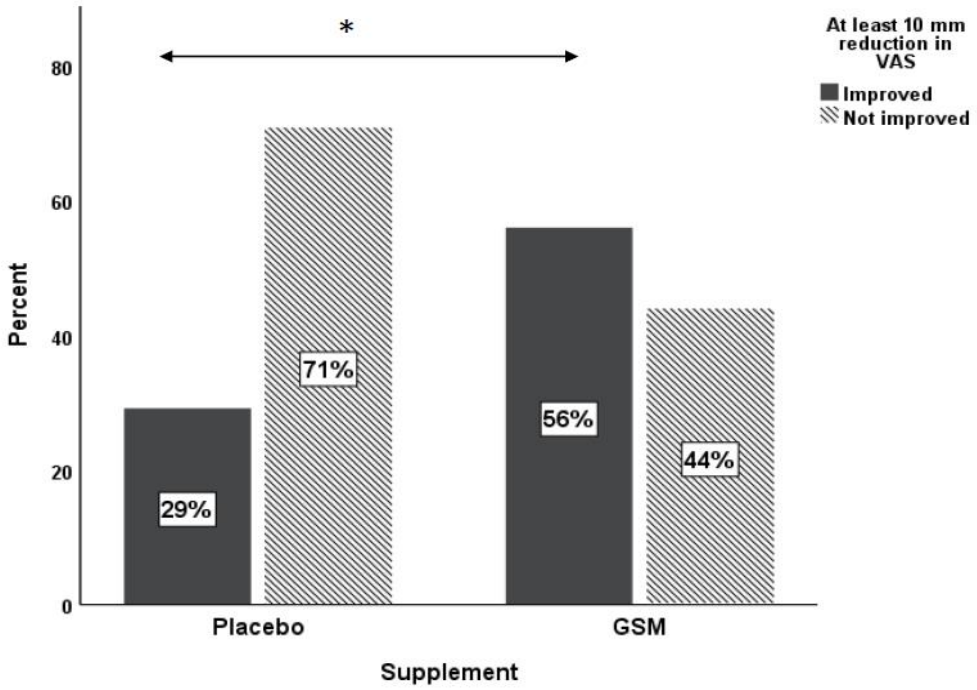


Figure 5.5 Proportion of responders (those who had >10 mm reduction in baseline VAS) versus non-responders in placebo and GSM supplement groups.
Y-axis indicating the percentage of responder and non-responder participants. *Pearson's chi-square test P= 0.05.

Further assessments of the KOOS domains focused on subjects with symptomatic knees (those with a score $\leq$86). The mean±SD of baseline, endpoint, and change in KOOS domains score across treatment groups are presented in Table 5.2. The baseline level of KOOS for any of domains was not different between the groups. There was no significant change between the two groups for any KOOS domains. However, a significant time effect was found for the KOOS pain domain (P=0.002 unadjusted); however, it lost its significance after adjustment for covariates (P=0.3 adjusted). Both unadjusted and adjusted analysis of KOOS symptoms domain revealed a significant time effect (P=0.02 unadjusted, and P=0.03 adjusted), although this change was not statistically significant between the groups (interaction effect P=0.6). There were no significant differences over time or between groups for other KOOS domains in both unadjusted and adjusted analyses.

Table 5.2 Mean±SD of KOOS domain scores over the 12 weeks of study across treatment groups in participants with cut-off scores of 86 or below.

	Placebo (n=18)	GSM (n=21)	P-value ŧ		
KOOS domains			Time effect	Treatment effect	Interaction effect
Pain					
Baseline	73.0±14.2	79.5±16.1	0.3	0.1	0.8
Endpoint	79.0±16.1	86.4±12.7			
Change	6.5±14.6	7.4±11.7			
Difference in change		0.912 (-4.4, 6.2)			
Symptoms					
Baseline	67.4±14.7	74.0±15.8	0.03*	0.09	0.6
Endpoint	71.4±17.8	81.2±17.9			
Change	4.0±18.7	7.2±11.6			
Difference in change		3.2 (-2.0, 8.4)			
Activities of daily living					
Baseline	77.2±13.6	81.8±17.2	0.2	0.3	0.8
Endpoint	84.5±15.0	88.6±15.1			
Change	7.2±13.1	6.8±14.8			
Difference in change		-0.33 (-7.0, 6.3)			
Sport/ recreation					
Baseline	59.8±27.8	70.3±26.5			
Endpoint	67.8±24.7	69.0±32.5	0.7	0.3	0.1
Change	8.0±25.6	-1.2± 31.0			
Difference in change		-9.2 (-23.4, 4.8)			
Quality of life					
Baseline	49.8±15.2	59.8±22.7			
Endpoint	61.9±22.3	70.3±22.2	0.1	0.1	0.8
Change	12.1±22.1	10.4±18.3			
Difference in change		-1.6 (-9.9, 6.7)			

Standardised scores for each of KOOS domain ranged from 0–100, with higher scores representing lower pain levels and a better KOOS response.

Mean (95% confidence interval) for difference in change between the groups (GSM vs placebo).

ŧ The two-way repeated measure ANOVA analyses were adjusted for baseline level, compliance, paracetamol use, and season of enrolment. * Indicates significance at $P \leq 0.05$

5.3.4 **Evaluation of treatment on plasma cytokines**

The plasma cytokines measured were present at low concentrations (picogram/mL) and in some of the samples were not detectable, which were not replaced with the Limit of Quantification (LOQ) in order to avoid overestimating the cytokine level. The levels of cytokines showed high variability and distribution was skewed. The medians (25^{th} and 75^{th} percentile) of plasma cytokines at baseline, end of the study and mean±SD of % change from baseline are presented in Table 5.3. There were no significant differences between the groups at each time point (baseline and endpoint) or over the time within the groups for any of the cytokines assessed by Mann-Whitney U test and paired t-test, respectively.

Table 5.3 Median (25th, 75th percentile) and mean±SD of plasma cytokines at baseline, end of the study and % change from baseline and number of participants with measurement above the detection limit in each group.

Cytokines (pg/mL)	Placebo		GSM		P-value
TNF-α		N= 20		N=20	
Baseline	26.8 (16.0, 98.2)		30.6 (9.0, 66.0)		0.4
Endpoint	37.0 (8.7, 104.3)		26.4 (14.9, 51.8)		0.5
% Change	32.6±158		40.3±131		0.8
IL-1β		N=20		N=17	
Baseline	15.3 (7.0, 49.6)		9.0 (4.1, 24.2)		0.3
Endpoint	8.5 (6.4, 92)		9.3 (4.9, 14.4)		0.3
% Change	70.7±268		46.6±162.2		0.7
IL-6		N=22		N=25	
Baseline	7.0 (3.9, 15.8)		4.6 (2.3, 10.8)		0.2
Endpoint	7.9 (4.2, 18.7)		5.1 (3.4, 9.7)		0.1
% Change	59.5±183		45.8±129.9		0.7
IL-15		N=19		N=18	
Baseline	344.9 (298.8, 706.5)		336.5 (241.4, 443.3)		0.2
Endpoint	376.3 (274.3, 822.7)		382.8 (288.6, 425.2)		0.5
% Change	7.7±289.8		18.9±231.6		0.3
IL-18					
Baseline	152.4 (88.7, 168.7)	N=22	114.8 (68.6, 199.9)	N=23	0.6
Endpoint	116.4 (104.2, 185.6)		105.0 (78.6, 159.7)		0.2
% Change	10.0±54.4		6.6±52.1		0.8

IL-4		N=21	N=24	
Baseline	47.6 (29.9, 86.0)		45.8 (20.2, 83.9)	0.4
Endpoint	41.4 (23.2, 149.7)		46.7 (20.2, 97.0)	0.4
% Change	49.2±167.2		38.5±137.0	0.8
IL-10		N=20	N=19	
Baseline	4.7 (2.5, 12.2)		3.4 (2.1, 7.3)	0.4
Endpoint	4.9 (2.3, 13.5)		4.3 (1.9, 6.3)	0.4
% Change	23.9±91.2		17.4±81.9	0.8

No significant difference between GSM vs placebo at baseline and endpoint using Mann-Whitney U test. No significant difference was observed overtime (baseline vs endpoint) within the group using paired t-test.

5.3.5 **Correlation between the baseline level of cartilage degradation markers, plasma 25(OH)D and self-reported KOOS outcomes**

As shown in Table 5.4, no correlation could be found between serum COMP and urinary CTX-II levels (r= 0.103, P=0.4) at the baseline. However, a significant negative correlation was found between urinary CTX-II levels and KOOS pain score (r= -0.292, P=0.02), symptoms score (r= -0.276, P=0.01), and ADL score (r= -0.443, P=0.002). Serum COMP did not show a correlation with any of the KOOS domain scores.

Correlations between baseline plasma 25(OH)D and cartilage markers and KOOS domain scores were also evaluated. Plasma 25(OH)D levels did not show any significant correlation with urinary CTX-II or serum COMP levels. Moreover, an insignificant correlation was found between plasma 25(OH)D and KOOS pain (r= 0.240, P=0.09), ADL score (r= 0.253, P=0.07) and quality of life (r= 0.252, P=0.08).

As expected, the baseline plasma CTX-I level was shown to be negatively correlated with whole body bone mineral density (r=-0.355, P=0.01) and not with any of the cartilage markers (data not shown).

Table 5.4 Correlation between baseline level of urinary CTXII, serum COMP, and plasma 25(OH)D with VAS pain and KOOS domain scores at baseline (n=49).

	Urine CTXII	Serum COMP	Plasma 25(OH)D
VAS pain score	0.132	0.01	0.150
KOOS			
Pain	-0.292*	-0.053	0.240
Symptoms	-0.276*	-0.006	0.201
ADL	-0.390*	-0.04	0.253
Sport/recreation	-0.222	-0.240	0.136
Quality of life	-0.318*	0.078	0.252
Urine CTXII	-	0.103	-0.236
Serum COMP	0.103	-	0.101
Plasma 25(OH)D	-0.236	0.101	-

Values represent Pearson correlation coefficients. * Indicates significance at $P \leq 0.05$

5.3.6 **Medication and analgesic use over the study period**

Participants continued their current medications prescribed to them by their physician for management of chronic diseases throughout the trial. The type of medications included cholesterol-lowering agents, anti-hypertensive medications, proton pump inhibitors, anti-depressants, and thyroid medications. The majority of subjects received COVID-19 vaccinations during the study. In the GSM group, 36% (n=9) of subjects used analgesic medication (Paracetamol) for joint symptoms, compared with 20.8% (n=5) in the placebo group. During the study, 8% (n=2) of subjects in the GSM and 16.6% (n=4) in the placebo group took NSAIDs (diclofenac sodium and ibuprofen) for headaches or migraine.

5.3.7 **Safety and adverse events**

Baseline blood analyses indicated that total cholesterol and LDL were above the normal range and elevated in both groups. HbA1c was also close to the upper cut-off of the normal range. Cholesterol and blood glucose may be elevated with obesity and menopause. There was no difference between the groups at the end of the study for the lipid profile other than HDL, liver enzymes and kidney function tests (Appendix 3; Table S5.2). Of all 49 subjects who completed the study, 20% (n=5) of subjects in the GSM group and 8.3% (n=2) in the placebo group reported adverse events that occurred on a few occasions during the intervention. The most frequent adverse event reported was mild to moderate indigestion and reflux (GSM, n=3 and placebo, n=1) for which two participants took omeprazole. Other adverse events include mild abdominal pain (GSM, n=1 and placebo n=1), and nausea (GSM, n=1).

5.3.8 **Compliance and adherence to study supplement**

A generally high adherence was observed (98%) in both groups. Two participants (one in GSM, and one in placebo) were not able to complete the final visit on week 12 due to COVID-19 restrictions and their final visit was postponed to week 16. Compliance was also confirmed by the analysis of omega-3 PUFA concentration in plasma and RBC membranes. The mean plasma and RBC concentrations of EPA, DHA, and total omega-3 L-C PUFA at baseline, end of the study and change from baseline are presented at Table 5.5. Plasma EPA and total n-3 L-C PUFA (g/L) increased by 0.57±1.4 and 0.32±5.0 g/L, respectively in the GSM group while they decreased in the placebo group by -0.31±1.2 and -2.07±5.1 g/L ($P \leq 0.05$). The plasma DHA concentration decreased in both groups although the decrease was greater in the placebo group compared to GSM (-1.6±2.3 vs -

0.27±1.8 g/L, P=0.03). Regarding the omega-3 PUFA in RBC, at the end of the study a higher level of DHA was shown in the GSM group compared to placebo (0.81±0.38 vs 0.62±0.32 g/L, P=0.07). In addition, RBC omega-3 index tended to increase in the GSM group while reduced in placebo (0.13±1.5 vs -0.02±2.8). However, there was no significant change between the groups or over the study period. Overall, the measurement of omega-3 PUFA in plasma indicated a good compliance rate at the time of sampling and confirmed the capsule count.

Table 5.5 Mean±SD in plasma and red blood cell (RBC) of n-3 long chain-polyunsaturated fatty acids (n-3 LC-PUFA) at baseline, end of study and change from baseline.

Plasma fatty acid (g/L)	Placebo (n=24)	GSM (n=25)	P-value[ł]
Eicosapentaenoic acid (EPA)			
Baseline	3.1±1.1	3.2±1.0	0.9
Endpoint	2.8±0.9	3.8±1.3	0.008 *
Change	-0.31±1.2	0.57±1.4	0.02 *
Docosahexaenoic acid (DHA)			
Baseline	7.2±2.9	6.5±2.0	0.3
Endpoint	5.6±1.6	6.2±2.0	0.2
Change	-1.6±2.3	-0.27±1.8	0.03 *
Total n-3 LC- PUFA			
Baseline	16.4+5.9	15.1±4.3	0.3
Endpoint	13.7±3.9	15.4±4.3	0.1
Change	-2.07±5.1	0.32±5.0	0.03 *
RBC fatty acid (g/L)	**Placebo (n=24)**	**GSM (n=23)**	**P-value[ł]**
Eicosapentaenoic acid (EPA)			
Baseline	0.39±0.3	0.51±0.34	0.2
Endpoint	0.62±0.32	0.81±0.38	0.07
Change	0.32±0.38	0.30±0.42	0.5
Docosahexaenoic acid (DHA)			
Baseline	1.9±1.1	2.31±2.2	0.3
Endpoint	2.7±1.1	3.1±1.6	0.3
Change	0.7±1.4	0.8±1.8	0.8
Total n-3 LC- PUFA			
Baseline	3.7±2.3	4.5±2.2	0.2
Endpoint	5.2±2.0	6.1±2.9	0.2
Change	1.4±3.0	1.6±3.3	0.8
Omega-3 index (%)			
Baseline	5.17±2.5	5.8±1.7	0.2
Endpoint	5.14±2.0	5.9±1.9	0.1
Change	-0.02±2.8	0.13±1.5	0.8

Values are reported as mean±SD. The total n-3 PUFA including alpha-linolenic acid (ALA, 18:3 n-3), stearidonic acid (SDA, 18:4 n-3), eicosatetraenoic acid (ETA 20:4 n-3), EPA, docosapentaenoic acid (DPA,22:5 n-3) and DHA. Omega-3 index is content of EPA + DHA in RBC membranes expressed as a percent of total fatty acids.

ł The difference between group at baseline, endpoint and change from baseline were determined by Student's t-test. * Indicates significance at $P \leq 0.05$

5.4 Discussion

This study was the first to evaluate the effect of whole meat GSM powder on cartilage metabolism in overweight/obese postmenopausal women with joint pain and discomfort using the biomarkers of type II collagen degradation (urinary CTX-II) and non-collagen cartilage degradation (serum COMP). The present study revealed that the change in the urinary CTX-II/Cr was not significantly different between the two treatment groups. The results showed it was moderately decreased following 12 weeks of GSM treatment but notably elevated in the placebo group. This effect was observed in subjects with symptomatic knees, as urinary CTX-II levels were significantly different between the treatment groups at week 6 and end of study. This study also showed benefits of GSM supplementation over placebo for secondary outcomes of VAS pain. The improvement for VAS pain in the GSM group was 13 mm which is considered as clinically meaningful (Kelly, 1998). However, GSM supplementation did not influence the level of circulating inflammatory cytokines.

The lack of effect of GSM on urinary CTX-II could be due to high levels of urinary CTX-II at baseline (571.6±415.6 ng/mmol Cr), which were higher than the values from a previous study (511.92 ± 486.21 ng/mmol Cr) using the same ELISA kit in elderly females with knee OA (age 64.45±10.6 years) (Arunrukthavon et al., 2020). Thus, due to high levels of urinary CTX-II, a notable reduction may not have been detected after 12 weeks of GSM treatment, and longer duration may result in a more significant effect. Of note, high levels of urinary CTX-II could be due to its high variability as a recent meta-analysis reported the mean levels were between 129 to 345 ng/mmol Cr in healthy adults (Hao et al., 2019). A significant difference in levels of urinary CTX-II between groups at follow-up and end of trial in participants with symptomatic knees was found, which may suggest these groups within the population obtain a larger cartilage-protective effect by GSM assessed through reduction of type II collagen degradation. However, the reason for elevation in level of urinary CTX-II observed in the placebo group during the intervention is not clear. This might be due to a withdrawal effect of chondroprotective supplements and dietary restrictions for omega-3 rich foods during the study by these participants. It worth mentioning that urine samples were collected following overnight fasting, although it is possible that it does not reflect the acute chondroprotective effect of GSM. However, it can be proposed that the chondroprotective effect of GSM is not

acute when consumed over a long period of time and could be reflected in general body fluids.

The effect of GSM on urinary CTX-II is consistent with a previous rat study which revealed a lower concentrations of serum CTX-II in diet-induced obese rats fed with GSM powder (Siriarchavatana et al., 2019). The concentration of serum CTX-II is in line with urine CTX-II in rats (Ishikawa et al., 2004); however, the serum CTX-II assay it is not the same as urine level in humans. Although the serum level has less analytical and biological variation than urine, we applied urine CTX-II in this study because it is known to have better clinical relevance than serum. Urine CTX-II has been used to discriminate OA patients from non-OA, and is strongly associated with clinical variables such as Kellgren-Lawrence grading (KLG) grade and knee OA symptoms (Luo et al., 2020).

In contrast, GSM supplementation did not affect the levels of serum COMP, a degradation marker from non-collagen components of cartilage. The baseline level of serum COMP was 1004±351 ng/ml in our study, which was in the expected range for women aged 60-69 as reported by kit manufacturer (1018±429 ng/ml) and by a previous study in elderly women (mean 872, range 256-1582 ng/ml) (Stabler et al., 2007). This lack of effect could be partly explained by the insignificant correlation between urinary CTX-II and serum COMP, suggesting that these markers are unlikely to change in parallel within the body after treatment. This is consistent with literature reporting a null association between the urinary CTX-II with serum COMP and other cartilage markers. Apparently, serum COMP levels have shown a different pattern from urine CTX-II across different age groups and gender. The levels of both urine CTX-II and serum COMP have shown a significant increase post-menopause; however, the increase tended to be less apparent for serum COMP and its level was generally lower in women than men within a similar age range (van Spil et al., 2013).

This study showed a clinically significant reduction of pain on VAS (over 10 mm reduction) in favour of GSM. To our knowledge there are only two recently published clinical trials of whole GSM powder (Coulson et al., 2013; Coulson et al., 2012); both showed improvement in pain and knee OA symptoms measured by VAS pain and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC). These studies used the freeze-dried whole GSM powder product GlycOmega^{TM} PLUS, which was administrated at the same dose (3 g/day) and duration as our study. The first trial was

a single arm with duration of 8 weeks resulting in a significant improvement in WOMAC total and sub-scores (pain, stiffness, and physical function) in knee OA patients (Coulson et al., 2012). In the second trial, GSM powder was compared to glucosamine sulphate in a 12 week intervention where both supplements showed equal effectiveness on the aforementioned outcome measures (Coulson et al., 2013). Our systematic review on the existing clinical trials concluded that both GSM lipid extract or whole meat powder products provide a clinically meaningful improvement in VAS pain for OA symptoms (presented in chapter 3). In terms of knee related symptoms, KOOS symptom domains showed change overtime, although the changes were not significant and did not reach the suggested minimal clinical improvement (at least 8 points improvement) (Roos & Lohmander, 2003).

In our study, the urinary CTX-II levels showed an inverse correlation with some of the KOOS domains scores, showing that a higher level of knee-related problems was reflected in a higher level of urinary CTX-II. This is in accordance with previous studies using the patient-reported outcome of WOMAC index (Arunrukthavon et al., 2020). The level of plasma CTX-I at baseline was comparable between the two groups and within the range reported by a previous study (0.45±0.1 ug/L) (Kruger et al., 2012).

The main compounds with bioactive properties in GSM are lipids PUFA, EPA and DHA that are known for their anti-inflammatory effect by inhibiting the COX enzyme, which most likely explain the analgesic and pain-reducing effect of GSM (McPhee et al., 2007). Matrix metalloproteinases (MMPs), particularly MMP-13, are primary enzymes involved in the degradation of type II collagen and inhibition of MMP-13 has been a target in OA treatment (Hu & Ecker, 2021). There is *in vitro* evidence showing that omega-3 from GSM oil extract down-regulates the expression of catabolic genes MMP-1, MMP-3 and MMP-13, while up-regulating the expression of anabolic genes that encode aggrecan and collagen type II-alpha (AGG and COL2A1) (Buddhachat et al., 2017). It must be noted that whole meat GSM powder also contains cartilage protective glycosaminoglycans such as glucosamine and chondroitin (3% of whole GSM powder extract) (Coulson et al., 2015). These compounds been shown to have inhibitory effects on MMP production *in vitro* (Derfoul et al., 2007). Three months supplementation with glucosamine (1.5 and 3 g/day) has been shown to reduce the urinary levels of CTX-II in athletes (Yoshimura et al., 2009). Although the glycosaminoglycan content in the dose provided in this study was ~ 90 mg/day which is less than the effective dose reported by the previously

mentioned study (Yoshimura et al., 2009), whole GSM powder is a blend of omega-3 PUFA, glucosamine and chondroitin and several other bioactive components; therefore, it can be speculated that GSM powder can provide additive chondroprotective effects through regulation of MMPs which result in suppression of type II collagen degradation. Further *in vitro* studies are required to elucidate detailed molecular mechanisms.

GSM supplementation did not significantly affect the inflammatory or anti-inflammatory cytokines as compared with placebo. Similarly, no significant change in circulating cytokine levels were observed in the previous study of obese rats fed with a GSM-enriched diet (Siriarchavatana et al., 2019). Changes in circulating markers of inflammation such as TNF-α, IL-6, CRP and adhesion molecules have not been observed in previous studies among healthy elders (Cornish et al., 2018), or healthy obese postmenopausal women supplemented with omega-3 PUFA or fish oil supplements (Holt et al., 2017). The median values for TNF-α, IL-6 and IL-1β at baseline were within minimum and maximum ranges reported for obese postmenopausal women from a previous study (Perry et al., 2008). It must be noted that this study assessed healthy obese postmenopausal women; thus, GSM treatment may have not been an effective means for ameliorating the markers of systemic inflammation for these individuals. Previous studies have shown centrally located fat mass is the main contributor to systemic inflammatory cytokines in obese postmenopausal women, thus loss of body fat through exercise or dietary interventions can reduce adipose inflammatory cytokines in healthy obese postmenopausal women (Perry et al., 2008). Further studies are warranted to determine if GSM treatment may ameliorate systemic inflammation in less healthy populations of obese postmenopausal women, and further to determine whether GSM exerts direct anti-inflammatory effects at the microenvironmental rather than systemic level.

One obvious strength of the current study is its novelty, assessing the effect of GSM supplementation on cartilage degradation markers in human subjects for the first time. Secondly, this study assessed EPA, DHA, and total omega-3 PUFA in plasma and RBC to confirm compliance of the study participants. GSM powders and products are a good source of EPA, DHA and total omega-3 PUFA as mussel derives these important fatty acids directly from the source, marine microalgae (Miller et al., 2014). The plasma concentration showed significant change in the level of omega-3 LCPUFA while the RBC did not. This could be due to the fact that RBC has longer half-life than plasma and reflects the long-term omega-3 PUFA intake while plasma reflects the shorter term (Harris & Von

Schacky, 2004). However, plasma levels of omega-3 LCPUFA were elevated in the GSM group, reflecting good compliance at the time of sampling study participants, and indicating that supplementation was tolerated and accepted.

Some limitations of our study must be acknowledged. Firstly, participants were not screened by radiographic evidence to detect OA due to a resource limitation. Observing the high concentration of urinary CTX-II raised the possibility of participants having established OA which would not be unexpected as most participants were older women with moderate to severe pain. Secondly, higher concentration of urinary CTX-II in women than men were reported in a previous study (Arunrukthavon et al., 2020), which was partly explained by the effects of menopause, and thus a male population may respond better to GSM supplementation. The effects of diurnal variation on biomarker levels should be taken into consideration. The urinary CTX-II has the highest concentration in the morning which decreases four hours after arising from bed and then remains stable till after 12 hours (Kong et al., 2006). The timing of urine sample collection in our study was in the morning between 8 to 9 a.m. which was most convenient for participants but is considered the highest phase of diurnal variation. Moreover, the urine sample was collected according to standard methods for CTX-II assessment, although a 24-hour urine sample collection was proposed to fully monitor the chondroprotective effect of active agents (Bihlet et al., 2021). However, in this study 24-hour samples were not collected due to the potential burden on participants.

Per-protocol analysis conducted in this study is subjected to bias including violation of principle of randomisation, and differential exclusion of subjects with severe disease in the treatment group which may not accurately reflect the efficacy that may be expected in clinical practice; therefore the results of this study should be interpreted with caution.

No minimum level of pain was set as inclusion criteria, and thus half of the study participants (53%) had mild symptoms (below 30 mm) at baseline. The study subjects were predominantly of New Zealand-European ethnicity. Previous research has reported significant ethnicity-based differences in the experience of pain and treatment response among OA patients (Herbert et al., 2017). Whether our findings are applicable to individuals with only severe symptoms or from other ethnicities is unclear and requires further research. This study used the KOOS questionnaire for outcome measure which is specifically for the knee joint because knee OA is dominant among postmenopausal

women (Cui et al., 2020), and the majority of the study participants was experiencing knee pain. This study was not limited to individuals with only knee pain in order to generalise the findings to individuals with pain at other joint sites. This also allowed to have the study population that represent the population of postmenopausal women with affected joints at different sites. Finally, this study focused on a limited number of cartilage degradation markers and lacked assessment of a cartilage synthesis marker. The ratio of type II collagen breakdown to synthesis has been proposed as a useful tool in predicting the progression of OA; therefore, we originally proposed to determine the concentration of C-terminal propeptide type II collagen (C-propeptide, also referred as CPII), a commonly measured collagen type II synthesis biomarker. However, delivery of the assay kits was severely delayed due to COVID-19 impacts on global transport systems, and the kits when received proved to be unusable. We acknowledge that the current results identifying effects of GSM supplement on urine CTX-II should be interpreted cautiously and need to be evaluated against other cartilage markers, specifically the ratio of CTX-II/CPII. However, these limitations do not negate our overall conclusions. It should be mentioned that a four-week washout period may have not been long enough to wash out supplements containing EPA and DHA from tissue. However, there were only a few participants consuming fish oil prior to the study and the baseline concentration of EPA and DHA were not different between the treatment groups.

In summary, the present study revealed that whole meat GSM powder did not decrease type II collagen degradation evaluated by urinary CTX-II concentration in overweight/obese postmenopausal women, however in those with knee symptoms, the urinary CTX-II was significantly decreased. GSM supplementation was effective in improving joint pain using VAS; however, it did not impact knee-related symptoms and the concentration of inflammatory cytokines.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Student name:	Maryam Abshirini
Name and title of main supervisor:	Professor Marlena C.Kruger
In which chapter is the manuscript/published work?	Chapter 6
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:

Involved in the study design, participant recruitment, running of the study, data collection, analysis and interpretation and writing of the first draft of manuscript

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Chapter 6 Effect of Greenshell™ Mussel supplementation on faecal microbiota, body composition and iron status markers in overweight/obese postmenopausal women

This chapter aimed to investigate the effect of Greenshell™ Mussel (GSM) powder on gut microbiota abundance, body composition and iron status markers in overweight or obese postmenopausal women.

Abstract:

Background: Intestinal bacteria perform important roles in maximizing energy extraction from food and regulating host immunity. Gut dysbiosis is described as an abnormality in microbiota homeostasis, which is correlated with inflammatory conditions such as obesity-related osteoarthritis (OA). Probiotics and prebiotics are potential novel therapeutic treatments for such conditions. There is evidence from animal and clinical studies reporting the ability of New Zealand green-lipped mussel (Greenshell^{TM} Mussel; GSM) in restoring gut microbial abundance and this is suggested to play a role in the efficacy of GSM in protecting against OA. In addition, recent data from animal studies reported a weight-reducing potential of GSM extract. GSM is a rich source of minerals, magnesium, zinc and particularly iron (both haem and non-haem) alongside iron absorption enhancers. The potential of GSM digestate on non-haem iron uptake in intestinal Caco-2 cell has been observed in one *in vitro* study. There have been limited investigations into the above-mentioned potential of GSM powder in human subjects. Therefore, the current study aimed to determine the effect of whole meat GSM powder on gut microbiota abundance, body composition and iron status markers in healthy overweight or obese postmenopausal women.

Methods: This study was a 3-month randomised, double-blind controlled trial involving 49 healthy postmenopausal women aged between 55 to 75 years old with body mass index (BMI) between 25-35kg/m^2. The women were randomly assigned to receive 3 gram/day of either GSM powder (n=25) or placebo (n=24). The faecal and blood samples for assessment of gut microbe abundance and the iron status marker were collected at baseline and the end of the study. Body composition measurements including whole body fat mass, lean mass and regional fat percentage were measured using Dual energy X-ray Absorptiometry (DXA) at baseline and end of the study.

Results: The participants had a higher abundance of *Clostridium* XIVa, *Lactobacillus*, and *Clostridium* IV than other species of bacteria at the baseline. The between-group comparison at baseline showed lower abundance of *Bacteroides*, *Clostridium* XIVa and *Akkermansia muciniphila* in the GSM group compared to placebo (*Bacteroides*: 2.8±0.75 vs 3.2±0.47 log ng/g faeces, P=0.04; *Clostridium* XIVa: 4.9±0.58 vs 5.3±0.57 log ng/g faeces, P=0.04; and *Akkermansia muciniphila*: 2.2+0.99 vs 2.7+0.6 log ng/g faeces,

P=0.08). After 12 weeks of intervention, the abundance of *Bacteroides* and *Akkermansia muciniphila* moderately increased in the GSM group while it decreased in the placebo group and the significant difference between two groups disappeared. *Bifidobacterium* abundance increased modestly in GSM group while it reduced in placebo group, however, none of these changes were statistically significant. Other bacteria including *Lactobacillus, Clostridium* IV and *Clostridium* XIVa remained at their baseline level during the intervention period.

The body composition parameters were not affected by the intervention. At baseline, the fat % was higher in the GSM group compared to placebo (44.9±4.1 vs 42.1±5.1, P=0.04), and this difference disappeared after 12 weeks of intervention. The body fat (BF)% did not change and iron marker levels did not change significantly over the study period among the groups.

Discussion: Supplementation with GSM over the 3 months tended to increase the abundance of some commensal bacteria such as *Bacteroides* and *Akkermansia muciniphila* and *Bifidobactria*. GSM supplemented women showed no change in body composition parameters, and iron status markers.

6.1 Introduction

The link between obesity and osteoarthritis (OA) onset and progression is well-documented. Metabolic disorders and inflammation are recognized as major risk factors that provide pathophysiological mechanisms that underpin this association (Visser et al., 2014). Furthermore, the role of the gut microbiota in obesity-associated OA has been documented. Restoring the gut microbiota by dietary interventions has been demonstrated to attenuate chronic inflammation in obese individuals and has been suggested as a new OA therapeutic (Schott et al., 2018; Xiao et al., 2014). Westernized diets characterized by high intake of saturated fat and low intake of fibre disrupt the microbial community and increase intestinal permeability (Moreira et al., 2012). Probiotics, prebiotics, and nutritional supplements that are used for regulating the microbial communities are proposed as plausible therapeutic options for OA treatment (Lei et al., 2017; Schott et al., 2018).

Perna canaliculus is a New Zealand marine species known as Greenshell^{TM} Mussel (GSM) which has been extensively studied for its anti-inflammatory compounds such as omega-3 polyunsaturated fatty acids (n-3 PUFA) (Coulson et al., 2015). A novel potential prebiotic effect of GSM has been documented in an animal (Siriarchavatana, 2021) and a human clinical trial (Coulson et al., 2013). For example, the experimental study of rats fed with a high-fat/high-sugar (HFHS) diet demonstrated that adding whole GSM powder to HFHS diet increased the cecum weight and caecal contents, which indicates enhancement in colonization of gut microbes. In addition, the production of short chain fatty acids (SCFA) such as propionic acid by gut microbes was significantly reduced in rats fed with the HFHS diet but increased when the rats were fed with GSM powder (Siriarchavatana, 2021). In a previous human study, supplementation with whole GSM extract powder or glucosamine sulphate extract (3 g/day) for 3 months improved the gastrointestinal symptoms along with OA symptoms and showed a reduction in the abundance of the *Clostridium* and *Staphylococcus* species and an increase in *Lactobacillus, Streptococcus* and *Eubacterium* species in the gut of patients with knee OA. Supplementation with GSM increased *Bifidobacterium* and *Enterococcus* in particular, and decreased yeast species (Coulson et al., 2013). The similar effect of GSM and glucosamine on gut microbes suggest that glucosamine and similar compounds present in GSM powder provide a substrate for gut bacteria and account for its prebiotic activity, although utilization of GSM by gut microbes has not been fully investigated.

There are studies showing that supplementation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) promoted the abundance of SCFAs producing bacteria such as *Bifidobacterium* and *Lactobacillus* (Watson et al., 2018).

Obesity predisposes individuals to subclinical inflammation and concurrently reduces iron absorption and systemic iron availability from cellular iron stores (Aigner et al., 2014). It was shown that obese postmenopausal women had a moderate degree of iron deficiency compared to non-obese women as obese women had higher levels of soluble transferrin receptor (sTfR), even though no difference between ferritin levels were observed. The sTfR marker is an indicator of cellular iron status and considered a useful clinical parameter for assessing iron status in these populations (Lecube et al., 2006). GSM contains high concentrations of both haem and non-haem iron along with iron absorption enhancers including cysteine-rich myofibrillar proteins, glycosaminoglycans (GAGs) and omega-3 polyunsaturated fatty acids (PUFA) (Coulson et al., 2015; Murphy et al., 2003). An *in vitro* study showed that GSM digestate enhanced non-haem iron uptake in model of human intestinal epithelial (Caco-2) cells to a similar extent as beef (Stewart et al., 2012). Therefore, we hypothesized that GSM powder may have the potential to improve iron status in overweight/obese postmenopausal women.

The growth of fat and muscle mass is in synchrony during the younger stages of life; however, the balance can be impaired with aging causing the state of high fat mass and relatively low muscle mass defined as sarcopenic obesity (Stenholm et al., 2008). The effect of menopause on body fat accumulation and distribution is well documented. It has been shown that postmenopausal women had 36% greater trunk fat, 49% more intra-abdominal fat, and 22% greater subcutaneous abdominal fat than premenopausal women (Toth et al., 2000). There are several studies that have shown associations of body composition-based obesity with joint instability and malalignment and knee OA, particularly in women. For example, a significant increased risk of radiographic knee OA was observed among obese women and men and sarcopenic obese women, but not among sarcopenic obese men (Misra et al., 2019; Suh et al., 2016). Thus, studying body composition in an intervention targeting OA in obese women allows for the identification of a novel clinical marker, given the implication of sarcopenic obesity in development of OA. Recently, the novel weight-reducing potential of GSM extract has been suggested from animal models (Loehfelm et al., 2021; Siriarchavatana et al., 2019). Supplementation with a high-fat diet (HFD) enriched with GSM oil prevented body

weight gain and was associated with decreased visceral fat mass in mice (Loehfelm et al., 2021). In a previous study of rats fed with HFHS diet, inclusion of GSM powder tended to increase the lean mass gain and decreased the fat mass gain in comparison to rats fed with only HFHS (Siriarchavatana et al., 2019).

This clinical intervention study aimed to determine whether GSM supplementation positively changes the faecal microbiota compared to placebo. A second objective of study was to assess the effect of GSM supplementation on body composition and an iron status marker compared with placebo in overweight or obese postmenopausal women.

6.2 Method and Materials

6.2.1 **Study participants**

A total of 55 New Zealand women aged 55-75 years old who were at least 5 years post-menopause were included in the study. A further inclusion criterion was having a body mass index (BMI) of between 25 to 35 kg/m^2. Exclusion criteria were formal diagnosis with OA or rheumatoid arthritis (RA), history of recent joint injury or trauma, having major chronic disease such as diabetes mellitus, or atherosclerosis. Participants with chronic liver or renal disorder detected based on the screening blood test were excluded as well as those with allergy to mussels or seafood; smoker or high intake of alcohol (>2 units per day); or use of any medication or antibiotics affecting the primary outcomes of the study within 3 months of beginning the trial. Other exclusion criteria were being on hormone replacement therapy or taking glucocorticoids drugs or non-steroidal anti-inflammatory drugs (NSAIDs) on a daily basis.

6.2.2 **Study design**

Participants who met the inclusion criteria were randomly assigned to either the GSM or placebo group, stratified by age (55-64, 65-75 years) and body mass index (BMI) (25-29.9 kg/m^2 and obese: 30-35 kg/m^2). The GSM group received six capsules of 500 mg each of whole GSM powder daily (equal to 3 gram per day) for 12 weeks, and the control group received the same amounts of identical capsules of sunflower seed protein as a placebo. The flash-dried whole meat GSM powder was produced by Sanford Ltd (ENZAQ facility, Blenheim, New Zealand) using standard manufacturing processes. The GSM powder contained 41.4% protein, 30.8% carbohydrate, 10.1% fat (EPA and DHA

was 20.7% and 8% total fatty acids, respectively), 10.7% ash, and 7% moisture. The dose of 3 g/day was selected as it is achievable through diet (equivalent to 1-2 mussels per day). Sunflower seed protein used as placebo (BP Bulk powders, Braeside, Melbourne Australia) contained 24.3% protein, 66.6% carbohydrate, 3% fat, 32.7% ash, and.4% moisture. Sunflower seed protein was used as placebo as a neutral source of protein and was selected to be relatively similar to GSM powder in respect to macronutrient composition and to be as inert and non-bioactive as possible. The amount of iron in GSM powder and placebo used in the study were 120 and 47 mg/kg for GSM powder and placebo, respectively. This equated to ~0.36 mg in 3 g GSM powder and ~0.14 mg in 3 g placebo.

Both GSM powder and placebo were encapsulated in hard-shell capsules by a commercial facility (Alaron, Nelson NZ) and stored under nitrogen in the dark at room temperature or lower until use. The GSM and placebo capsules were identically matched in the shape, size, and colour of the hard-shell encapsulant. Activated carbon sachets for absorbing moisture and odour were put in bottles to conceal any 'fishy' odour.

A randomisation list was generated by Excel and maintained by the project's supervising investigator, who did not interact with the study subjects or conduct the primary data analysis. Both the primary researcher and participants were blinded to treatment groups allocation until all analyses were completed. Data were collected during participants' visit at baseline, and end of the study (week 12). All participants self-reported an omnivorous diet before the study and maintained their normal diet throughout the trial. Recruitment, screening, and data collection took place at the Human Nutrition Research Unit (HNRU) at Massey University, Palmerston North, New Zealand from August 2020 to September 2021.

6.2.3 **Demographic, anthropometric, and body composition measurements**

The data on demographic characteristics were collected from participants at baseline. The anthropometric measurements including body weight and standing height were measured using a beam balance to the nearest 0.2 kg and a stadiometer to the nearest 0.1 cm, respectively, at baseline and end of the study. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Body composition measurements including whole and regional fat mass, lean mass and total body fat (BF) % were measured using a Hologic Horizon A, dual energy X-ray absorptiometry (DXA) at the baseline and week

12. With data provided by the DXA scan, appendicular lean mass (aLM) was calculated as the sum of lean mass in arms and legs, assuming that lean mass in these areas is skeletal muscle. For the purpose of detecting sarcopenia, aLM index was calculated by adjusting for height and fat mass. Linear regression was applied to model the relationship between aLM and height and fat mass, and the residual of regression was used to identify the sarcopenic participants. Individuals were classified as sarcopenic if their value fell into below the 20th percentile of residual distribution. Since this method considers the fat mass, it has been recommended for identifying sarcopenia in women and overweight and obese individuals (Newman et al., 2003).

6.2.4 **Dietary intake assessment**

Each participant's daily nutrient intake from the diet was measured using a three-day food record including two weekdays and one weekend day at the midpoint of the trial. The three-day food record has been recommended and considered as the 'gold standard' for dietary assessment. Instructions on how to accurately complete the food record was provided (Biro et al., 2002). The brand name of food products, recipes and food preparation were recorded. Each participant's nutrient intake was calculated using Foodworks 9 Professional, Xyris Software.

6.2.5 **Faecal sample collection, DNA isolation and gut microbe quantification**

Participants were provided with a stool sample collection kit at baseline and end of study. Each kit contained a container, an anaerobic bag with anaerobic sachet plus a freezer pack. Samples were delivered to the Human Nutrition Research Unit (Massey University, Palmerston North) and stored at -80 °C. Bacterial DNA was extracted using Isolate Faecal DNA kit (Bioline, NSW, Australia) at the completion of study. Briefly, 250 µL of each homogenised faecal sample was mixed with 750 µL of lysis buffer in a tube containing bashing beads and processed with a beater at maximum speed for 10 minutes to disrupt the cells following the manufacturer's instructions. The faecal DNA was eluted from column and stored at -80 °C for later analysis. The concentration and purity of extracted DNA were measured using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA). The quantitative real-time polymerase chain reaction (qRT-PCR), using SYBR™ Green Master Mix, was performed on a LightCycler® 480 Real-Time PCR instrument (Roche Applied Science). The specific primers for bacteria are presented in Table 6.1. The primers were published elsewhere (Bartosch et al., 2004; Earley et al.,

2019; Malinen et al., 2005). The primers were purchased from Invitrogen by Thermo Fisher Scientific. For each primer, a standard curve was generated and used for the calculation of each bacteria. The q-PCR were carried out in a total volume of 20 µL containing 10 µL 2× LightCycler® 480 SYBR Green I Master (Roche Diagnostic, Indianapolis, IN, USA), 1 µL of each primer (forward and reverse), and 3-8 µL of target DNA (due to high variability in concentration of extracted DNA); nuclease-free water was added to reach a final volume of 20 µL. For purposes of standardization, depending on DNA concentration of sample, 3, 5 and 8 µL of DNA was used for samples with concentrations >90, 90-10, and < 10 ng/µL, respectively.

Table 6.1 Primers used for quantitative real-time polymerase chain reaction (qRT-PCR)

Bacteria	Forward primer	Reverse primer
Bifidobacterium	TCGCGTCYGGTGTGAAAG	CCACATCCAGCRTCCAC
Lactobacillus	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG
Akkermansia muciniphila	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGAT
Bacteroides	GAAGGTCCCCCACATTG	CAATCGGAGTTCTTCGTG
Clostridium cluster IV	ACAATAAGTAATCCACCTGG	CTTCCTCCGTTTTGTCAA
Clostridium cluster XIVa	AAATGACGGTACCTGACTAA	CTTTGAGTTTCATTCTTGCGAA

6.2.6 **Biochemical analysis**

Non-fasted blood samples were collected at baseline and the end of the study to measure iron status markers including serum iron, transferrin, ferritin, sTfR, iron saturation, iron binding, as well as C-reactive protein (CRP) level as a marker of inflammation. All biomarkers were analysed at Medlab Central-Palmerston North, New Zealand. The serum-based iron indicators were measured by an automated clinical analyser available in the routine laboratory. Protein-based indicators such as serum ferritin, transferrin, and sTfR were measured by immunoassays. Transferrin saturation indicates the percentage of binding sites on all transferrin molecules bound to iron and is calculated as the ratio of serum iron to transferrin or serum iron to total iron-binding capacity (TIBC).

In this study participants were defined as iron-deficient or depleted if they had ferritin <30 µg/L or sTfR was >1.76 mg/L. Systemic inflammation was defined as a CRP concentration >5 mg/L. These values are according to hospital-based clinical laboratory reference, and previously published cut-offs (De Block et al., 2000; Thurnham et al., 2010).

6.2.7 **Compliance**

Compliance was checked at week 6 and end of study by examining the participant's records of their daily intake of study supplements. Compliance was assessed using cumulative capsule counts at the completion of the study, and adherence was measured as a percentage: (number of capsules provided minus number of unused capsules)/number of capsules provided, × 100. Adherence below 80% was considered a protocol violation.

6.2.8 **Statistical methods**

The microbial analyses were secondary objectives of the original trial, and the sample size calculation was not based on detecting significant changes in either gut microbiota abundance, body composition parameters or iron status markers. In brief, the sample size was based on urine CTX-II/ creatinine and serum COMP as the primary outcomes of the study. Sample size was calculated to detect 20% difference between the groups using the standard deviation from an unpublished report. For urine CTX-II/ creatinine a sample size of 24 was required to detect a 20% relative difference from baseline with 80% power. For serum COMP a sample size of 17 was required to detect a 20% difference between groups with a power of 95%. The sample size of 48 (n=24 per group) was required as a manageable sample size. Finally, a total sample size of 55 was needed to allow for at ~ 10% potential dropout rate (n=27-28 per group).

A previous study among knee OA patients investigating the effect of whole GSM powder on the gut microbiome using similar dose and duration, demonstrated changes in gut microbiota profile in relatively smaller samples sizes (n=38) (Coulson et al., 2013). Data analyses were performed with SPSS software IBM SPSS version 26.0 (Armonk, NY). Analyses were done on the dataset from those who completed at both timepoints (baseline and endpoint). Variables were checked for normality using the Kolmogorov-Smirnov and Shapiro-Wilk tests and data that were not normally distributed were log-transformed. The data were reported as mean ± standard deviation (SD) for normally distributed data, and as median (25^{th}, 75^{th} percentiles) for non-normally distributed data, and as frequencies for

categorical data. The between group differences were tested by using Student's t-test and the Mann-Whitney U test for parametric and nonparametric data, respectively. For categorical variables, the differences at baseline were assessed by Chi-square, and by Fisher's exact test where > 20% of cells had an expected count below 5. Two-way repeated measures ANOVA was used to examine differences within each group over time (pre- vs post-intervention) and between the groups (GSM vs placebo). The interactions between treatments and time indicate differences in efficacy. The level of significance was $P < 0.05$). In case of a significant effect, analysis was followed by post hoc analysis adjusted with Bonferroni. The data were checked to meet all the assumptions of two-way repeated measure ANOVA prior to analysis. These included detect no significant outlier, approximate normal distribution of dependant variable, and equal variances of the differences between groups checked with sphericity tests.

6.3 Results

6.3.1 **Baseline characteristics**

Out of 55 participants who enrolled in study, six participants withdrew during the trial. A total of 49 participants (GSM, n=25 and placebo, n=24) completed the study. The general characteristics and percentage of participants with iron depletion and sarcopenia are shown in Table 6.2. The prevalence of sarcopenia was 13% (n=3) in placebo and 24% (n=6) in the GSM group. At baseline, only one participant from the GSM group had serum ferritin <30 µg/L, and three participants (placebo, n=2 GSM, n=1) were detected with elevated sTfR >1.76 mg/L. Systemic inflammation assessed by CRP >5 mg/L was present in 6 women (12.2%) at baseline (GSM, n=3 and placebo, n=3). There were no differences between treatment groups for any of the baseline measurements.

Table 6.2 Baseline characteristics of participants who completed the study across the treatment groups.

Characteristic	Placebo (n=24)	GSM (n=25)	P-value
Age (year), mean±SD	62.9±5.4	64.2±5.1	0.3
Height (cm), mean±SD	164.7±6.4	164.8±7.1	0.8
Weight (kg), median (25^{th}, 75^{th} percentiles)	73.8 (68.2, 88.6)	77.2 (68.5, 86.2)	0.9
BMI (kg/m^2), mean±SD	29.1±4.2	29.1±3.3	0.9
MET-minutes/week, median (25^{th}, 75^{th} percentiles)	751 (318, 2373.7)	764 (287, 1483)	0.1
Having sarcopenia [1], n (%)	3 (13)	6 (24)	0.3
Serum ferritin [2] <30 µg/L, n (%)	0 (0)	1 (4)	0.5
Serum sTfR [2] >1.76 mg/L, n (%)	2 (8.3)	1 (4)	0.6
Serum CRP [2] >5 mg/L, n (%)	3 (12.5)	3 (12)	0.9

BMI: body mass index, MET: metabolic equivalent of task, sTfR: soluble transferrin receptors, CRP: C-reactive protein

[1] Data were available for 48 subjects (placebo n=23 and GSM n=25)

[2] Cut-off value according to hospital-based clinical laboratory reference range and previously published.

Values are presented as mean ± standard deviation or median (25^{th} and 75^{th} percentile) for normally distributed and non-normally distributed variables, respectively, and n (%) for categorical variables for which the percentage within each treatment group is reported.

Daily nutrient intake of participants is presented at Table 6.3. The daily intake of iron from the diet was 11.2±5 mg in the GSM and 16.2±17.8 mg in the placebo group, with no differences between the groups. Both groups were comparable in term of energy and macronutrient intakes.

Table 6.3 The daily energy and nutrients intake from diet (without supplement) of participants across treatment groups.

Daily energy and nutrients intake	Placebo (n=24)	GSM (n= 21)	Recommended daily intake (RDI) for women aged 51-70
Energy (kJ)	9339.1±3322.1	9030±4469.3	-
Protein (g)	99.9±52.3	93.04±31.2	46 g/day (0.75 g/kg)
Carbohydrate (g)	203.5±91.5	193.6±94.6	-
Fat (g) ŧ	101.1±44.5	100.9±68.8	-
Dietary fiber (g)			25 g/day*
SFA (g) ŧ	38.9±18	40.2±30.2	-
MUFA (g) ŧ	35.6±22	35.4±22.8	-
PUFA (g) ŧ	14.7±7.2	14.8±15.2	-
EPA (g) ŧ	0.04±0.05	0.03±0.04	-
DHA (g) ŧ	0.04±0.06	0.03+0.08	-
Calcium (mg)	1442.3±1797.1	1061.0±509.2	1300 mg/day
Sodium (mg)	2413.7±758	2282.0±910.4	460-920 mg/day*
Potassium (mg)	7524.0±14524	5357.4±1178.8	2800 mg/day*
Iron (mg)	16.2±17.8	11.2±5.0	8 mg/day
Zinc (mg)	13.8±9.5	11.5±5.9	8 mg/day
Magnesium (mg)	814.0±1636.1	732.2±171.9	320 mg/day

SFA: saturated fatty acids, MUFA: monounsaturated fatty acids, PUFA: polyunsaturated fatty acids, EPA: eicosapentaenoic acid, DHA: docosahexaenoic acid.

ŧ Relative to contribution of total fat to total energy intake.

*Based on adequate intake (AI) for adults.

6.3.2 **Evaluation of treatment on gut microbiota abundance**

The baseline abundance of each selected bacteria is demonstrated in Figure 6.1. The *Clostridium* XIVa and *Lactobacillus* had the highest abundance (mean±SE; 5.1±0.1 and 4.9±0.1 log ng/g faeces, respectively), followed by *Clostridium* IV and *Bacteroides* while *Akkermansia muciniphila* and *Bifidobacterium* had lowest abundance among all bacteria (2.5±0.1 and 2.3±0.3 log ng/g faeces). As demonstrated in Figure 6.2, between group comparison at baseline showed a significant difference in abundance of some of the bacteria which were lower in the GMS group than the placebo group. These bacteria include *Bacteroides* (2.8±0.75 vs 3.2±0.47 log ng/g faeces, P=0.04), *Clostridium* XIVa (4.9±0.58 vs 5.3±0.57 log ng/g faeces, P=0.04) and *Akkermansia muciniphila* (2.2+0.99 vs 2.7+0.6 log ng/g faeces, P=0.08). After 12 weeks of supplementation with GSM, there

was a relative increase in abundance of *Bacteroides* and *Akkermansia muciniphila*. The significant difference between the groups disappeared at the end of the intervention, while the abundance of these bacteria was maintained or decreased in placebo group. *Bifidobacterium* abundance tends to increase slightly after GSM supplementation while it reduced in the placebo group. The other bacteria including *Lactobacillus*, *Clostridium* IV and XIVa did not alter and remained at their baseline level during the intervention periods. Overall, no statistically significant changes in abundance of bacteria were noted during the intervention (time and treatment interaction effect) assessed by two-way repeated measure ANOVA.

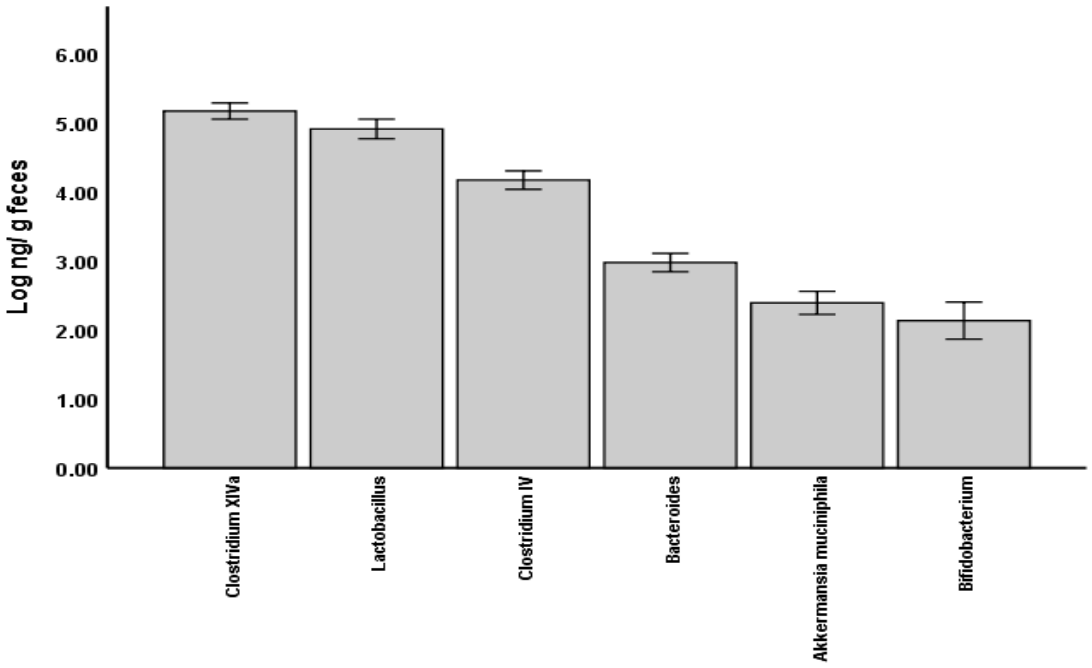
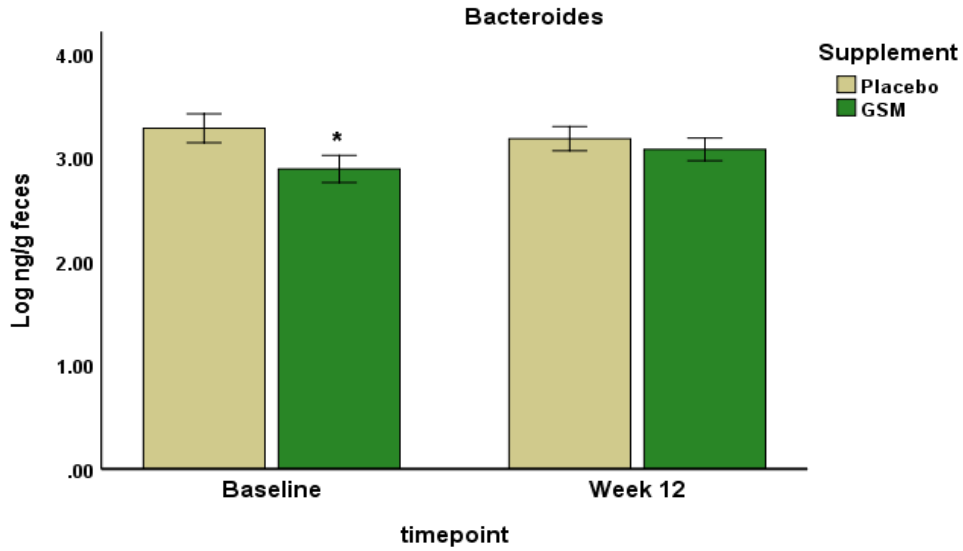
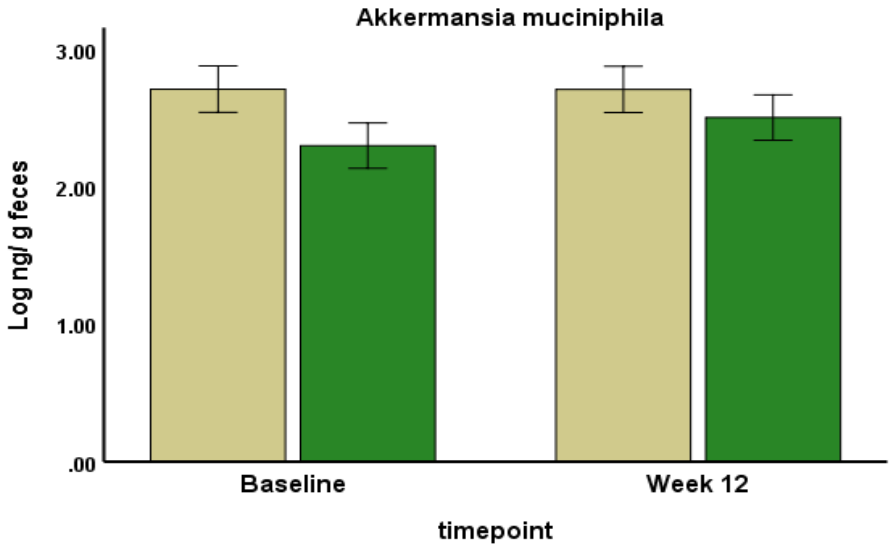
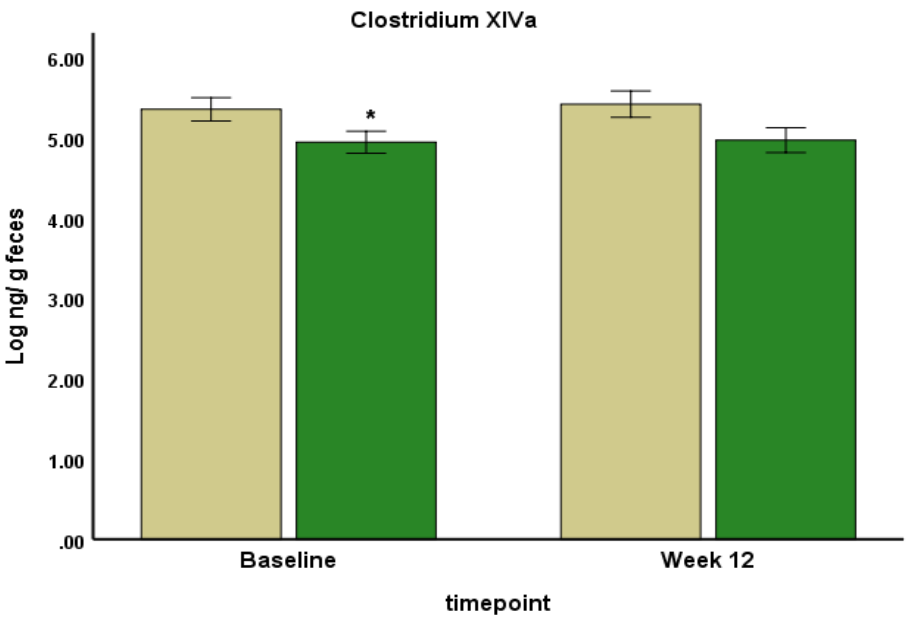
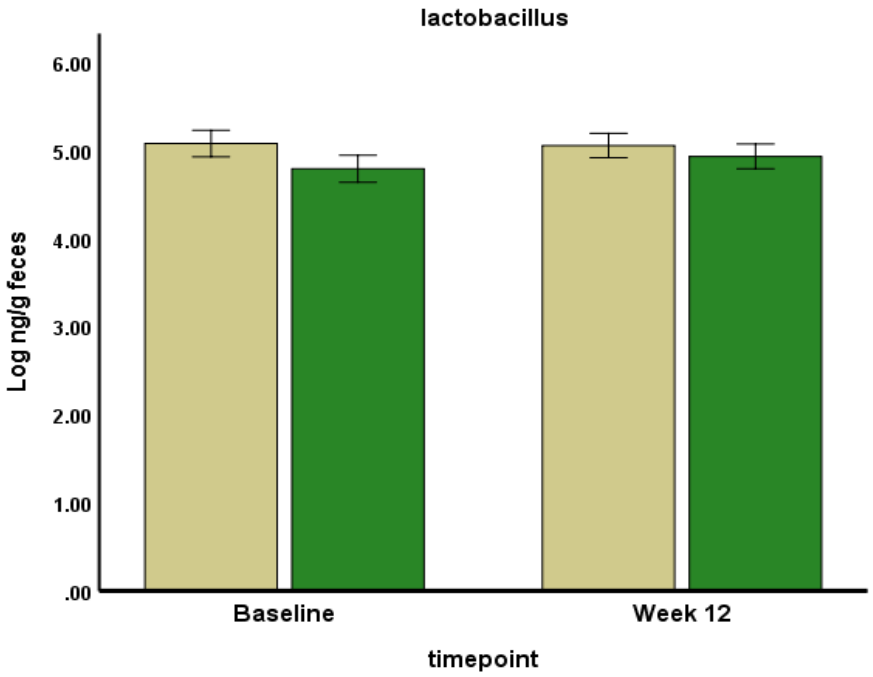


Figure 6.1 The abundance (mean±SE) of *Clostridium XIVa, Lactobacillus, Clostridium IV, Bacteroides, Akkermansia muciniphila, and Bifidobactrian* from the baseline faecal sample of overall study population


Bacteroides
Supplement
Placebo
GSM
4.00
3.00
2.00
1.00
.00
Log ng/g feces
*
Baseline
Week 12
timepoint


Akkermansia muciniphila
3.00
2.00
1.00
.00
Log ng/ g feces
Baseline
Week 12
timepoint


Clostridium XIVa
6.00
5.00
4.00
3.00
2.00
1.00
.00
Log ng/ g feces
*
Baseline
Week 12
timepoint


lactobacillus
6.00
5.00
4.00
3.00
2.00
1.00
.00
Log ng/g feces
Baseline
Week 12
timepoint

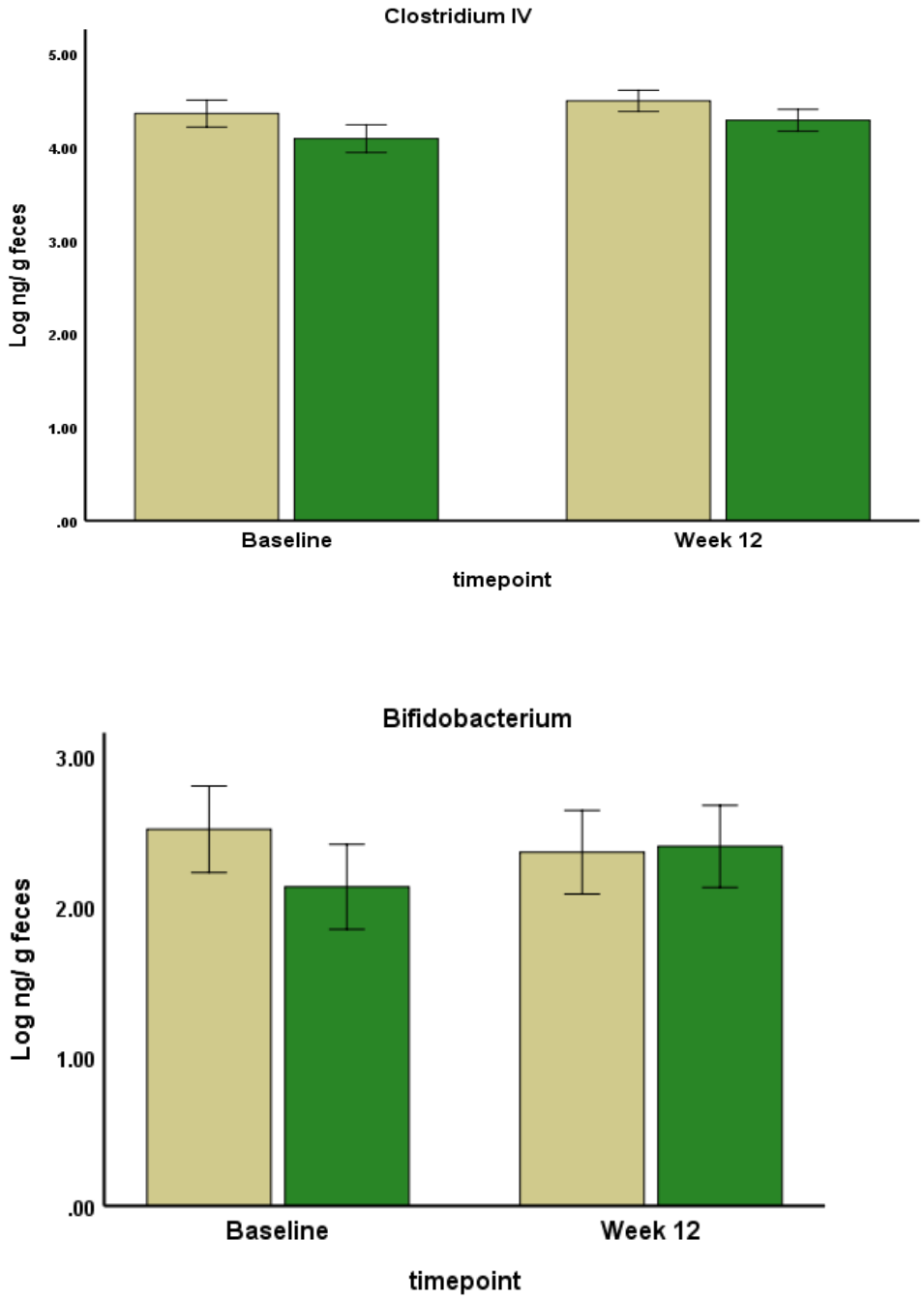


Figure 6.2 The abundance of bacteria at baseline and end of the study were measured by real time-PCR.
The concentration of bacteria is presented in Log_{10} scale ng/g of faeces (mean±SE). The two-way repeated measure ANOVA did not show any significant change within the groups (time effect) or any significant change from baseline between the groups (time and treatment interaction effect). Statistical differences between the groups at each timepoint were assessed using student t-test. *Indicates the significance at $P < 0.05$.

6.3.3 **Evaluation of treatment on iron markers and CRP level**

The baseline, endpoint and % change from baseline in iron markers of the study population across the treatment groups are presented in Table 6.4. Iron status markers and CRP level did not show any significant difference between the groups at the baseline, except for transferrin levels that were lower in GSM group compared to placebo (2.4±0.2 vs 2.6±0.3, P=0.06). The concentration of transferrin tended to increase over time in the GSM group while decreasing in the placebo group (2.7±6.0 vs -0.09±8.3). No significant interaction effect was noted for any of iron markers. A significant time effect was noted for ferritin levels (time effect, P=0.01).

The baseline mean±SD concentration of CRP was in the normal range (2.4±2.2 mg/L). The level of CRP increased during the study in both groups. The CRP >10 mg/L is defined as increased inflammation and used in iron studies as level of affecting ferritin level. Because none of the participants had CRP above this level, therefore correction of ferritin for inflammation was not necessary. Overall, there was no evidence of significant change in the iron status marker and CRP levels during the intervention using two-way repeated measure ANOVA.

Table 6.4 The baseline, endpoint and % change from baseline of iron markers and CRP level over the 12 weeks of study across treatment groups (n=47).

	Reference range	Placebo (n=23)	GSM (n=24)	P-value ǂ
Serum iron (µmol/L)	10-30			
Baseline		15.9±3.8	15.4±3.9	0.8
Endpoint		15.5±50.2	14.6±3.5	
% Change		-0.9±35.5	-0.1±31.3	
Transferrin (g/L)	2-3.2			
Baseline		2.6±0.3	2.4±0.2	0.1
Endpoint		2.5±0.3	2.5±0.2	
% Change		-0.09±8.3	2.7±6.0	
Ferritin (µg/L)	30-350			
Baseline		118.9±66.6	144.8±91.5	0.2
Endpoint		111.0±63.3	125.1±71.5	
% Change		-4.9±20.6	-6.5±30.5	
Iron saturation (%)	20-50			
Baseline		27.0±6.5	27.7±6.9	0.5
Endpoint		26.4±8.8	25.7±6.9	
% Change		1.1±34.1	-2.3±8.6	
sTfR (mg/L)	0.83-1.76			
Baseline		1.3±0.3	1.1±0.2	0.7
Endpoint		1.3±0.3	1.2±0.3	
% Change		0.2±8.6	1.9±16.0	
Iron binding (µmol/L)	50-80			
Baseline		59.4±7.01	56.0±6.4	0.8
Endpoint		15.9±3.8	15.4±3.9	
% Change		0.1±8.2	0.6±13.6	
CRP (mg/L)	< 5			
Baseline		2.1±2.3	2.8±2.1	0.9
Endpoint		2.5±3.1	3.3±2.7	
% Change		35.6±10	53.5±20	

ǂ The time and treatment interaction effect P-value. A significant time effect was noted for ferritin using two-way repeated measure ANOVA (P=0.01).

6.3.4 **Evaluation of treatment on body composition parameters**

The baseline, endpoint and % change from baseline in body composition parameters across the treatment groups are shown in Table 6.5. Body weight, lean mass, fat mass, trunk fat and android fat% were not different between the groups at the baseline and end of the study, while BF% and gynoid fat% were significantly different between the two groups at the baseline and were significantly higher in the GSM group compared to placebo (BF%: 44.9±4.1 vs 42.1±5.1, P=0.04, gynoid fat %: 46.7±4.2 vs 43.2±4.9, P=0.01, respectively). However, the difference for BF% at the end of the study was no longer significant (45.2±3.9 vs 42.9±5.3, P=0.09), while the difference for gynoid fat% between the two groups at end of the study remained significant (46.9±4.3 vs 43.7±5.0, P=0.03). However, no significant interaction effect for any of the parameters was observed.

Table 6.5 The baseline, endpoint and % change from baseline in body composition parameters over the 12 weeks of study across treatment groups (n=48).

	Placebo (n=23)	GSM (n=25)	P-value ŧ
Body weight (kg)			
Baseline	78.9±12.8	79.4±11.7	0.9
Endpoint	79.0±12.7	79.4±12.7	
% Change	0.1±1.9	0.12±1.0	
Lean mass (kg)			
Baseline	43.7±4.9	42.5±6.3	0.5
Endpoint	43.3±5.0	42.3±6.1	
% Change	-0.4±2.3	-0.1±0.9	
Fat mass (kg)			
Baseline	34.1±9.1	36.5±6.8	0.7
Endpoint	35.0±9.0	36.8±6.7	
% Change	1.2±3.4	0.8±4.2	
Body fat%			
Baseline	42.1±5.1	44.9±4.1	0.6
Endpoint	42.9±5.3	45.2±3.9	
% Change	1.0±2.6	0.6±3.0	
Trunk fat (kg)			
Baseline	16.1±2.4	17.2±4	0.4
Endpoint	16.3±4.2	17.3±3.8	
% Change	1.1±4.2	0.4±5.3	
Android fat%			
Baseline	40.8±5.7	42.1±5.3	0.8
Endpoint	41.2±5.6	42.5±5.0	
% Change	1.0±3.8	1.2±4.1	
Gynoid fat %			
Baseline	43.2±4.9	46.7±4.2	0.6
Endpoint	43.7±5.0	46.9±4.3	
% Change	0.99±2.9	0.52±3.2	

ŧ The time and treatment interaction effect P-value. The two-way repeated measure ANOVA was adjusted for baseline level of body fat % as a covariate.

6.4 Discussion

The results of our study showed that abundance of bacteria did not change following supplementation with GSM, however some of bacteria, particularly *Bacteroides*, *Akkermansia muciniphila* and *Bifidobacterium*, tended to moderately increase with whole GSM powder supplementation. With respect to iron status markers, no significant changes were noted except for ferritin levels which significantly decreased over time. No significant changes were observed in body composition.

So far, little is known about the effect of GSM on the gut microbiome. Presently, there is only one human and one rat study that directly evaluated the effect of GSM powder on gut microbiota. Our results revealed a slight increase in the abundance of *Bacteroides*, *Bifidobacterium* and *Akkermansia muciniphila* following GSM supplementation. *Bacteroides* is the most abundant bacteria in the human gut and uses glycan as its main source of energy (Zafar & Saier Jr, 2021). Chondroitin sulphate and glucosamine sulphate have very low absorption rates in the small intestine (5-15%), and reach the colon where more than 50% are degraded by the gut bacteria and then absorbed (Chourasia & Jain, 2004). This suggests that the gut microbiota play a crucial role in the bioavailability of chondroitin and glucosamine sulphate to the host (Ibrahim et al., 2012). Optimising gut microbiota may improve the therapeutic efficacy of GSM and help to ameliorate the OA condition (Ulmer et al., 2014). Previously it was shown that supplementation with glycosaminoglycans (GAG) such as chondroitin sulphate increased the abundance of *Bacteroides* (Shang et al., 2016). Similar to this result, a previous human study found increases in the abundance of *Bacteroides* and *Bifidobacterium* following supplementation with GSM powder (3 g/day) for 12 weeks (Coulson et al., 2013). Thus, it is possible that GSM powder which contains GAG provides substrates for these bacteria and supports their growth, which subsequently results in better absorption and enhanced protection of the gut barrier.

It should be noted that *Bifidobacterium* had the lowest abundance compared to other bacteria in our study. This could be because our study population was aged, and lowest abundance of *Bifidobacterium* (~5-10% relative abundance) has been confirmed among the elderly population. Other factors such as obesity, diabetes and allergies have been

associated with a lower number of this bacteria at various stages of life (Arboleya et al., 2016; Salazar et al., 2013).

Akkermansia muciniphila is another beneficial bacterial species which moderately increased after GSM supplementation in this study. These bacteria produce mucin-degrading enzymes and uses mucin in the mucus layer of the intestine as a source of nitrogen and carbon. It is known for its beneficial effect on obesity and improving insulin sensitivity and blood cholesterol (Depommier et al., 2019; Huang et al., 2015). Supplementation with omega-3 PUFA, which is the main type of fatty acid present in GSM (Miller et al., 2014), has shown to promote the abundance of this bacteria and to reduce gut inflammation (David et al., 2014). However, in a previous study, the abundance of this bacteria decreased in rats fed with a high-fat high sugar (HFHS) diet and adding GSM powder to diet did not change this pattern (Siriarchavatana, 2021). It should be taken into account that the relationship between this bacteria and the host is closely influenced by the energy intake from the diet and glucose and lipid metabolism (Everard et al., 2013). In the rat study (Siriarchavatana et al., 2019), the diet was abnormally high in both sugar and fat. In addition, the bacteria assessed in the rat study came from the caecum, which is a part of the gastrointestinal system that humans do not possess.

Clostridium IV, also called *C.leptum*, and cluster XIVa are the main cluster of *Clostridium* in healthy individuals, representing 10–40% of the total bacteria and contributing to regulation of intestinal homeostasis (Nagano et al., 2012). In our study, the abundance of *Clostridium* XIVa and IV remained stable in both the GSM and placebo groups over the study period. However, a previous study reported a notable reduction in *Clostridium* after GSM supplementation (Coulson et al., 2013). It must be noted that in that study, the majority of participants in both arms (GSM and glucosamine) had moderate to severe gastrointestinal complaints with a high proportion of pathogenic species of *Clostridium* such as *C. innocuum* and *C. tertium* (Coulson et al., 2013). Furthermore, the beneficial effect of *Clostridium* has mainly been related to the treatment of intestinal autoimmune disease such of colitis and allergic diarrhoea via inducing the expansion and differentiation of regulatory T lymphocytes (Treg cells) and producing SCFAs such as butyrate (Atarashi et al., 2013; Rivière et al., 2016). However, participants of the current study were healthy and without gastrointestinal symptoms; therefore, it

appears that the beneficial effect of GSM powder on *Clostridium* depends on the intestinal state of host and differs across species.

In this study, the diversity of gut microbiota was not assessed as it was unlikely to change based on previous data that showed supplementation with GSM (Coulson et al., 2013) or chondroitin sulphate (F. Liu et al., 2017) did not affect the biodiversity of gut microbiota, but it altered the abundance of individual genera of bacteria.

To our knowledge, this is the first study to investigate the effect of GSM powder on body composition in human subjects. Measurement of body composition is preferable to weight and body mass index, due to the fact that body composition accounts for changes in fat, water, and muscle mass as opposed to overall weight change (Nuttall, 2015). In the present study, increases in the BF% over the study period appeared to be less in GSM than placebo, suggesting that GSM may have ameliorated body fat gain. This is consistent with the study on rats fed with HFHS diet, in which adding whole GSM powder to the diet resulted in less body fat gain (Siriarchavatana et al., 2019). Similarly, reductions in body weight gain as well as inflammatory cytokines, serum triglycerides and mRNA expression of leptin were observed in rats fed with a high-fat diet enriched with freeze-dried blue mussel powder (Vaidya et al., 2017). Another study on male mice showed that a high fat diet containing 63% of fat from GSM oil was able to prevent body weight gain and decreased the visceral fat mass (Loehfelm et al., 2021). The current evidence on the weight-reducing effect of GSM is limited to animal models and the lack of effect on body composition in current study is not surprising and was anticipated, given that consuming 3 g GSM powder per day for 3 months may not be sufficient to result in changes in body weight or body composition. The recommended gold-standard for clinical practice in achieving weight loss and improving body composition includes several lifestyle-based interventions such as calorie restriction and regular physical activity for $\geq$6 months (Jensen et al., 2014). This approach has been found effective in a population of middle-aged, white women with obesity similar to our study population (Horne et al., 2020). Further, given that our participants were free living, and their dietary intake was assessed based on self-reported food intake and not controlled as in animal models, it could explain why the anti-obesity effects were not obvious in this study.

No significant changes were observed in iron status markers, except for a decrease in ferritin levels and an increase in transferrin level in the GSM group, although no

significant difference was observed between groups. This lack of change could be due to the fact the majority of participants had an adequate iron status and none of them were experiencing iron deficiency or anaemia with only 8% having a small or depleted body iron reserve at baseline. Moreover, the mean dietary iron intake of participants was above the 8 mg/day which is the recommended dietary allowance in women over 50 years (Russell et al., 2001). Further studies targeting participants with inadequate dietary iron intake or iron depletion, or iron deficiency, are required to assess the effect of GMS powder on iron status markers. It should be noted that the level of CRP did not change in both GSM and placebo groups during the study, and this effect is possibly confounded by the COVID-19 vaccination of study participants.

The results of this study should be interpreted with caution because of several limitations. Firstly, although randomisation was stratified based on BMI, a somewhat higher mean BF% was found in the GSM group compared to placebo, but as this was taken into consideration in data analysis, it is unlikely to have impacted the results. Secondly, it was impossible to consider the inclusion of a controlled diet to reduce inter-subject variation in dietary intake and microbiota over time. Moreover, it is important to note that some of the participants took medications prescribed to them by their physician for treating chronic conditions that had been administered to them for long periods of time before participating in the trial and were maintained as such during the trial. Thereby, it is likely that baseline abundance of microbiota was not influenced by these medications over the 12 weeks.

It is unlikely that small amount of fibre present in sunflower seed protein had modulatory effect on gut microbiota as most intervention studies with prebiotic dietary fibre have been conducted at relatively high doses (between 8 and 20 g) (Delzenne et al., 2020).

In conclusion, GSM supplementation had minor effects on the abundance of some of the commensal bacteria such as *Bacteroides*, *Akkermansia muciniphila* and *Bifidobacterium* which tended to increase over the 12 weeks study period. Iron status markers and body composition were not notably affected by GSM treatment; however, the results of this study do contribute to the knowledge surrounding the effects of whole GSM powder on these outcome measures in healthy postmenopausal women.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

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

Student name:	Maryam Abshirini
Name and title of main supervisor:	Professor Marlena C.Kruger

In which chapter is the manuscript/published work?	Chapter 7
What percentage of the manuscript/published work was contributed by the student?	85%

Describe the contribution that the student has made to the manuscript/published work:

Involved in the study design, participant recruitment, running of the study, data collection, analysis and interpretation and writing of the first draft of manuscript

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Student's signature:	Maryam Abshirini Digitally signed by Maryam Abshirini Date: 2022.07.29 10:28:22 +12'00'	Main supervisor's signature:	Marlena Kruger Digitally signed by Marlena Kruger Date: 2022.07.29 12:02:03 +12'00'

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Chapter 7 Impact of Greenshell^{TM} Mussel supplementation on plasma lipids and polar metabolites in overweight/obese postmenopausal women

This chapter evaluates the effect of Greenshell mussel supplementation on the lipid and metabolite profile of overweight/obese postmenopausal women using untargeted LC-MS metabolomic approach.

Abstract:

Background: A previous study showed adding Greenshell^{TM} mussel (GSM) powder to the diet reduced some of the metabolic changes that occurred in diet-induced obese and ovariectomized rats as a model of metabolic osteoarthritis (MetOA). This study aimed to investigate the effect of supplementation with GSM powder on the plasma lipid and metabolite profiles of overweight/obese postmenopausal women through using an untargeted liquid chromatography-mass spectrometry (LC-MS) metabolomic approach.

Methods: The dose of 3 g/day GSM powder or placebo was administrated to 49 overweight/obese postmenopausal women for a duration of 12 weeks. Plasma samples were collected at baseline and end of the study for metabolomic analysis. Orthogonal partial least squares discriminant analysis (OPLS-DA) was conducted to reveal metabolomic differences between treatment groups. ANOVA-simultaneous component analysis (ASCA) was performed to select the lipid and metabolites related to treatment, time, and their interaction.

Results: The OPLS-DA models were not robust and did not reveal any clear difference for metabolomic and lipidomic data. ASCA analysis identified 17 lipids including four ceramides (Cer), four triglyceride (TG), two phosphatidylcholine (PC), one alkenyl-acyl PC (plasmalogen), three lysophosphatidylcholine (LPC), one sphingomyelin (SM), one sterol (ST), and one cholesterol ester (CE) to be affected by the time, treatment, and their interaction. Most of the lipids were elevated following supplementation with GSM which include Cer (44:1, 42:1, 41:1), TG (56:4, 58:2), LPC (O-16:0), and LPC (18:3).

Eight metabolites were identified to have differences by ASCA analysis including three amino acids (L-glutamine, L-threonine, and 3-metthyl histidine), and five metabolites (taurine, biliverdin, urea, pipecolic acid, and 3-hydroxyanthrianilic acid). L-glutamine, taurine, pipecolic acid, and 3-hydroxyanthrianilic acid slightly up-regulated or maintained at the baseline level by GSM supplement, while they were significantly decreased by placebo supplement.

Conclusion: Lipid and metabolite profiles did not differ significantly between the GSM and placebo treatment groups over the 12 weeks of study in overweight/obese postmenopausal women through OPLS-DA analysis. However, some lipid and polar metabolites were identified as putative biomarkers related to time, treatment, and their

interaction. Some Cer, LPC, TG were found to increase due to GSM supplementation. In addition, the level of some unique glycerophospholipids such as plasmalogen PC (O-36:3), and LPC (O-16:2) were elevated following GSM supplementation. These lipid species most likely originated from fatty acids present in GSM which may indicate that consuming GSM powder can impact the lipidome in humans. In addition, majority of the metabolites such as threonine, histidine and pipecolic acid were maintained at the baseline level by the GSM supplement while they were significantly reduced in the placebo group. These potential biomarkers for the impact of GSM powder supplementation in MetOA model in postmenopausal women need to be confirmed by further targeted analysis. Further large-scale studies are required to control some of the factors that propagate the variation in metabolite profiles between individuals.

7.1 Introduction

Obesity is a strong risk factor for osteoarthritis (OA) and its role extends beyond imposing the excessive load on joints (Gabay et al., 2008). The new phenotype of OA associated with obesity and low-grade inflammation has gained attention in regards to pathophysiology, diagnosis, prognosis, treatment, and prevention and is defined as metabolic osteoarthritis (MetOA). (Bijlsma et al., 2011). Metabolomics is a comprehensive and sensitive tool to detect small molecules and metabolites, in cells, tissues and biological fluids by applying analytical methods such as mass spectrometry (MS) coupled with gas chromatography (GC) or liquid chromatography (LC) and nuclear magnetic resonance (NMR)) (Trivedi et al., 2017). There are a number of OA studies involving metabolomic analysis conducted to identify the biomarkers associated with the phenotype of OA and stage of disease. For example, metabolomic analysis of synovial fluid and plasma from OA patients with metabolic syndrome showed a different metabolite profile from those without metabolic syndrome and healthy controls. The phosphatidylcholine (PC) acyl-alkyl C34:3 and PC acyl-alkyl C36:3 were present at higher levels in both OA and metabolic syndrome (Zhang, Sun, Likhodii, et al., 2016). An increased lysophosphatidylcholine (LPC) to PC ratio in plasma was associated with knee OA, and this ratio is suggested as a potential biomarker for predicting late-stage knee OA (Zhang, Sun, Aitken, et al., 2016). This observation is likely to be caused by the overexpression of phospholipase A2, an enzyme that facilitates the conversion of PC to LPC, in response to pro-inflammatory cytokines (Leistad et al., 2011).

Exploring the effect of dietary and nutraceutical interventions on lipid and metabolite profiles in order to understand and identify the potential mechanisms of action and bioactive components is a growing area of research. In the previous metabolomic study, the impact of high-fat/high-sugar (HFHS) diet with and without inclusion of Greenshell^{TM} mussel (GSM) powder on lipid and metabolites profiles involving the untargeted metabolomic analysis of plasma from ovariectomized (OVX) rat was investigated (Abshirini et al., 2021). The results revealed differences in the lipidomic and metabolomic profiles of rats that received HFHS diet and GSM powder compared to their control groups. The result of that study showed several lipid species: PC, LPC, diacylglycerol (DG), triacylglycerol (TG); and amino acid metabolites; serine, threonine, lysine, valine, histidine, pipecolic acid, 3-methylcytidine, and cholic acid were altered due to the HFHS

diet, while inclusion of GSM powder to the diet tended to recover some of these alterations. These findings provide evidence that GSM powder has the potential to influence the metabolome, and this laid the foundation of the current investigation. Thus, the purpose of this study was to determine the effect of GSM powder supplement on the lipid and metabolite profile of overweight/obese postmenopausal women via untargeted metabolomic methods using LC-MS.

7.2 Method and materials

7.2.1 **Study participants**

Healthy, overweight postmenopausal women aged 55-75 years were recruited. All women were at least 5 years postmenopausal according to the natural cessation of menstruation and had body mass index (BMI) between 25 to 35 kg/m^2. Subjects did not have any chronic health conditions such as diabetes mellitus, atherosclerosis, and formal diagnosis of OA, inflammatory arthritis or rheumatoid arthritis (RA). Participants with chronic liver or renal disorder detected based on the screening blood test were excluded. Exclusion criteria were those having self-reported allergy to mussels or seafood, a history of recent joint injury or trauma, smoking, consuming alcohol of more than two units per day, being on hormone replacement therapy less than 6 months prior beginning the trial or taking anti-inflammatory drugs (glucocorticoids or non-steroidal anti-inflammatory drugs (NSAIDs) regularly or on the daily basis. The eligible participants did not consume any dietary supplements such as fish oil, GSM, or chondroitin sulphate for at least one month prior to participating in the study.

7.2.2 **Study design**

A detailed design of the study has been previously described in Chapter 5. In brief, participants were randomised into two groups stratified based on age (55-64 or 65-75 years) and body mass index (BMI) (overweight: 25-29.9 kg/m^2 or obese: 30-35 kg/m^2). Each group received 3 g/day of whole meat GSM powder or placebo (sunflower seed protein) for 12 weeks. The flash-dried whole GSM powder used in this study was comprised of 41.4% protein, 30.8% carbohydrate, 10.1% fat (EPA and DHA was 20.7% and 8% total fatty acids, respectively), 10.7% ash, and 7% moisture. Flash-dried whole meat GSM powder was produced by Sanford Ltd (ENZAQ facility, Blenheim, New

Zealand) using standard manufacturing processes. Sunflower seed protein was used as placebo (BP Bulk powders, Braeside, Melbourne Australia) as a neutral source of protein and was selected to be relatively similar to GSM powder in respect to macronutrient composition (24.3% protein, 66.6% carbohydrate, 3% fat, 2.7% ash, 3.4% moisture) and to be as inert and non-bioactive as possible.

Both GSM powder and placebo were encapsulated in hard-shell capsules by a commercial facility (Alaron, Nelson NZ) and stored under nitrogen in the dark at room temperature or lower until use. The GSM and placebo capsules were identical in the shape, size, and colour of the hard-shell encapsulant. To absorb moisture and odour, activated carbon sachets were put in bottles to conceal any 'fishy' odour.

A randomisation list was created by Excel and maintained by the project's supervising investigator, who was not involved with allocation and data analysis. Randomisation was stratified based on BMI (overweight: 25-29.9 kg/m^2 and obese: 30-35 kg/m^2) and age (55-64, 65-75 years) distribution. The primary researcher who was blinded to treatment codes allocated participants to two supplements (A and B). Participants were blinded to treatment group until all analyses were completed. Data were collected during participants' visit at baseline and end of the study (week 12). The study was conducted at the Human Nutrition Research Unit (HNRU) at Massey University, Palmerston North, New Zealand from August 2020 to September 2021.

7.2.3 **Plasma sample collection, lipid, and metabolite extraction**

Blood samples was collected after overnight fasting at baseline and at completion of the 12-week intervention. The blood samples were collected into plasma lithium heparin vacutainer tubes and were immediately centrifuged at 2264 g for 10 min at 4˚C (Gyrozen 1248R Multi-Purpose High-Speed, Korea) to isolate the plasma, then the obtained plasma samples were aliquoted and stored at -80˚C until use.

Lipid extraction was performed by adding 10 µL of plasma to 95 µL of butanol:methanol (1:1 v/v) containing 10 mM ammonium formate and then adding 5 µL of internal standard SPLASH mix (Avanti Lipids) based on work by Huynh et al (Huynh et al., 2019). The samples were then vortexed for 1 min, sonicated at room temperature for 60 min, centrifuged at 14,000g for 10 min at 20 °C and then 90 µL aliquot was transferred to a glass vial containing a 250 µL insert for LC-MS analysis, stored at -80 °C. Frozen samples

were thawed for 1 hr at room temperature, vortexed and sonicated for 15 min and kept at 25 °C prior to LC-MS analysis.

Polar metabolites were extracted from the plasma samples by a monophasic extraction according to the protocol described in rat study in chapter 4 (Fraser et al., 2020). Briefly, 50 µL of plasma was added to a tube, and 450 µL of pre-chilled acetonitrile/water (9:1 v/v) was added. The mixture was vortexed (60 s at 30 Hz) and then centrifuged for 10 min at 13,663× g at 4 °C (Eppendorf Centrifuge 5427 R, Germany), and 200 µL of extract was added into an HPLC vial for analysis. All samples were kept at -80 °C prior to LC-MS analysis of polar metabolites using hydrophilic interaction chromatography (HILIC) column. For QC samples, 20 µL of each lipid sample was taken to form a lipid pooled QC, while 100 µL of each polar sample was taken to form a polar pooled QC, both the lipid and polar pooled QCs were subsequently vortexed, and then multiple aliquots of the pooled QCs were transferred to new HPLC vials for analysis.

7.2.4 **Instrument and condition**

The analysis of lipid extract was performed using a Shimadzu LCMS-9030 mass spectrometer equipped with a Shimadzu Nexera-x2 Ultra Performance Liquid Chromatography® (UPLC) system by injecting 2 µL onto a Waters CSH-C18 column (2.1 × 100 mm, 1.7 µm particle size). Samples were held in the autosampler at 20 °C and temperature of the column oven was set at 60 °C. A mobile phase flow rate of 400 µL/min was employed for gradient elution at total 15-min run time for lipids. The mobile phase was a mixture of water/acetonitrile/isopropanol (5:3:2 v/v containing 10 mM ammonium formate) (solvent A) and water/acetonitrile/isopropanol (1:9:90 v/v containing 10 mM ammonium formate) (solvent B). The gradient elution programme was as follows: 10–45% B (0–2.7 min), 45–53% B (2.7–2.8 min), 53–65% B (2.8–9 min), 65–89% B (9–9.1 min), 89–92% B (9.1–11 min), and finally to 100% B (11–11.1 min) and held for 0.8 min (11.1–11.9 min) before returning to 10% B (11.9–12 min) and held to re-equilibrate until 15 min (Huynh et al., 2019). The positive ion mode, measuring full MS1 spectra ranged from 250 to 1250 m/z was applied the across the chromatogram, and collecting data independent acquisition (DIA) data in 20 m/z windows from 300 to 1100 m/z, with a 0.6 s cycle time and collision energy of 25 normalised collision energy units. The source voltage was + 4.0 kV, with a nebulising gas flow of 2.0 L/min, heater gas flow of 10 L/min, interface temperature of 300 °C, drying gas flow of 10 L/min, desolvation line

temperature of 250 °C, and heater block temperature of 400 °C. Nitrogen gas was used for all drying and collision.

Polar metabolite extracts were analysed using a Shimadzu LC-MS-9030 mass spectrometer equipped with a Shimadzu Nexera-x2 UHPLC system. Polar metabolites were measured by injecting 5 μL onto a Thermo Accucore HILIC column (2.1 × 100 mm, 2.6 μm particle size). Samples were held in the autosampler at 4 °C and the temperature of the column oven was set at 30 °C. A mobile phase flow rate of 400 μL/min was employed for linear gradient elution at total 23-min for polar metabolites. The mobile phases were water containing 10 mM ammonium formate (solvent A) and acetonitrile containing 0.1% formic acid (solvent B).

Both positive and negative ionisation modes, measuring full MS1 spectra from 70 to 1000 m/z were applied across the entire chromatogram, and collecting DIA data in 20 m/z windows from 70 to 900 m/z, with a 0.6 s cycle time and collision energy of 25 normalised collision energy units. The source voltage was +4.0 kV, with a nebulising gas flow of 2.0 L/min, heater gas flow of 10 L/min, interface temperature of 300 °C, drying gas flow of 10 L/min, desolvation line temperature of 250 °C, and heater block temperature of 400 °C. All drying and collision gases used were nitrogen.

7.2.5 **Data processing and statistical analysis**

Data processing included a series of procedures to convert the raw mass spectrometry data to data matrices adequate for statistical analyses. Peak detection, gap filling, alignment, and noise elimination were performed using the MS-Dial software version 4.8. Peak annotation was performed by using of retention times, accurate *m/z* and isotopic ratio, and full MS and MS/MS spectra were acquired for each feature. The data-independent acquisition (DIA) MS/MS spectral was used for identification of the lipidomic data. For lipidomic, features were searched against the built-in lipid library containing 257,000 in silico generated MS/MS lipid fragmentation spectra, while for metabolites, these were searched against the MS/MS public library containing 13,303 unique compounds (Tsugawa et al., 2015). The resultant peak annotations were manually checked for false positive annotations and some of the identified peaks were excluded from the final dataset as detailed below.

The resultant peak intensity table was subjected to run-order correction and normalization utilising pooled QC samples and using the locally weighted scatterplot smoother

(LOWESS) regression model. The identified features were filtered out based on the following criteria: features with an average of QC-to blank sample ratio of below 5 and coefficient variation (CV) of 30% within the pooled QC samples were removed.
Orthogonal projection to latent structure-discriminant analysis (OPLS-DA) using MetaboAnalyst 5.0 (Pang et al., 2021) was conducted to find the separation between the groups. The quality of model was assessed by predictive ability parameter (Q2). The $Q2 < 0$ indicates a model has no predictive ability, $0 < Q2 < 0.5$ with some predictive ability and $Q2 > 0.5$ suggests a good predictive ability (Llorach-Asunción et al., 2010). ANOVA-simultaneous component analysis (ASCA) was conducted to find metabolic change with time and treatment and their interaction using MetaboAnalyst 5.0. The permutation approach was used to validate the models. The significant variables were selected based on the leverage and the squared prediction errors (SPE) associated with each specific factor. The leverage and SPE were applied to reflect the importance of the metabolite to the model and its fitness of the model for each specific metabolite, respectively. Metabolites with high leverage and low SPE were well-modelled after each factor. This was followed by paired t-test to indicate the feature with significant change over time within each treatment group.

A linear mixed effect model is an efficient method for feature selection of metabolomic data obtained from time-course experiments (Mei et al., 2009). The linear-mixed effects model was used to test the treatment effect while accounting for subject and time effects. Then metabolite features that were significant were identified by using a multiple testing procedure that controls the false discovery rate (FDR).

Pathway analysis was performed on quantitative lipidomic data to identify the metabolic pathways that were affected by GSM and placebo supplement over the study period using the BioPAN platform available at https://lipidmaps.org/biopan/ (accessed on 7 Jul 2022). BioPAN calculated Z-score is a prediction on whether a particular reaction is significantly ($P< 0.05$) changing between conditions of interest and control, and by default a reaction is considered as significantly modified at a level of $P < 0.05$ or Z score > 1.645 (Gaud et al., 2021).

7.3 Results

7.3.1 **Lipidomics**

After data processing, 192 lipid species consisting of 92 TG, 25 PC that include 13 PC-O (plasmalogen), 12 DG, 16 ceramide (Cer), 8 sphingomyelin (SM), and the other lipid classes were identified and included in final dataset for statistical analysis (Figure 7.1).

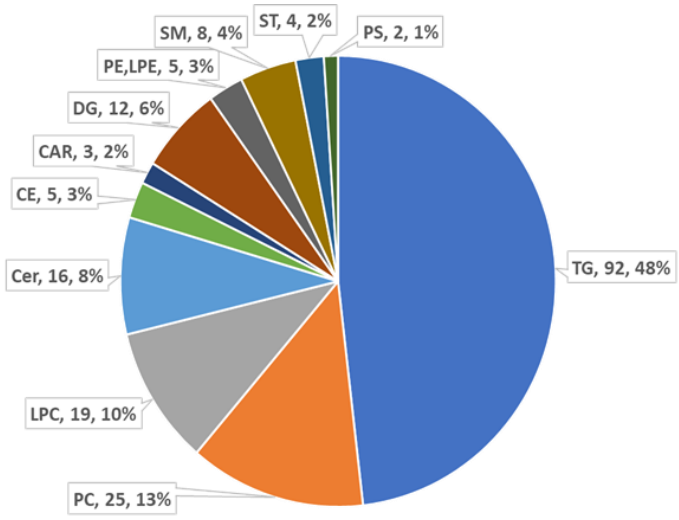


Figure 7.1 Composition of lipid classes that were included for statistical analysis in human plasma sample detected by LC-MS.

Abbreviation: CAR: Acyl carnitines, CE: Cholesteryl esters, Cer: Ceramide, DG: Diacylglycerol, ST: Sterols, PC: Phosphatidylcholine, PE: Phosphatidylethanolamine, PS: Phosphatidylserine, TG: Triacylglycerol.

The results of the OPLS-DA model of lipidomic data did not show clear separation among the treatment groups across the time points and Q2 value at -0.19 indicated the predictive ability of model was very low (Appendix 4; Figure S7.1). Similarly, the OPLS-DA analysis showed that the lipidomic profile did not differ in each treatment group GSM and placebo over time (baseline vs week 12), with poor models created having Q2=-0.101 and -0.293 for the GSM and placebo group, respectively (Appendix 4; Figure S7.2A, and B).

Leverage and SPE scatter plots of ASCA submodels for effects of group (treatment), time and their interaction are shown in Figure 7.2. Although this permutation approach showed the models had an effect over time, treatment and their interaction were not valid based on the P-value > 0.05. The details of the lipids identified in the leverage–SPE scatter plots

of ASCA submodels are presented in Table 7.1. Only one lipid species, TG (56:4) was well modelled by the effect of time, and four by the effect of treatment including PC (O-36:3), TG (54:7, 54:6), Cer (44:1); and thirteen lipids by their interaction between time and treatment including one HexCer (42:1) and three Cer (42:1, 44:1, 41:1), two LPC (O-16:2, O-16:0), LPC (18:3), two PC (36:6, 40:7), TG (58:2), SM (42:1), cholesterol ester (CE 20:5), and sterol (ST 29:1).

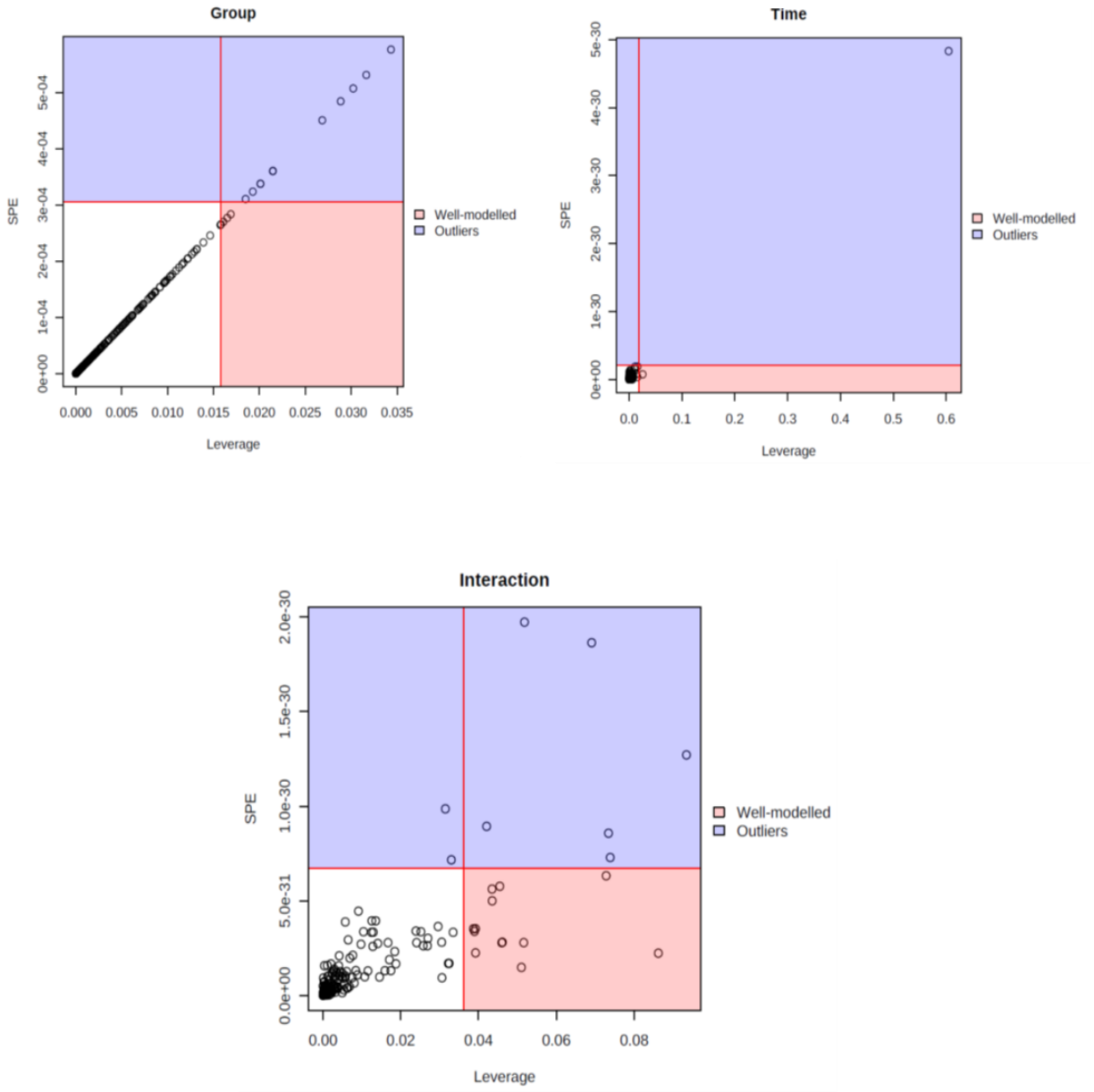


Figure 7.2 Leverage and squared prediction error (SPE) scatter plots of the ANOVA-simultaneous component analysis (ASCA) submodel for group (GSM and placebo) and time (baseline and endpoint), and their interactions in lipidomic data.
Vertical and horizontal lines represent cut-off leverage and SPE values, respectively. Lipid species with a high leverage value and a low SPE value were considered to be differential (the well-modeled group). Lipids appeared in blue area have patterns that are different from the major patterns.

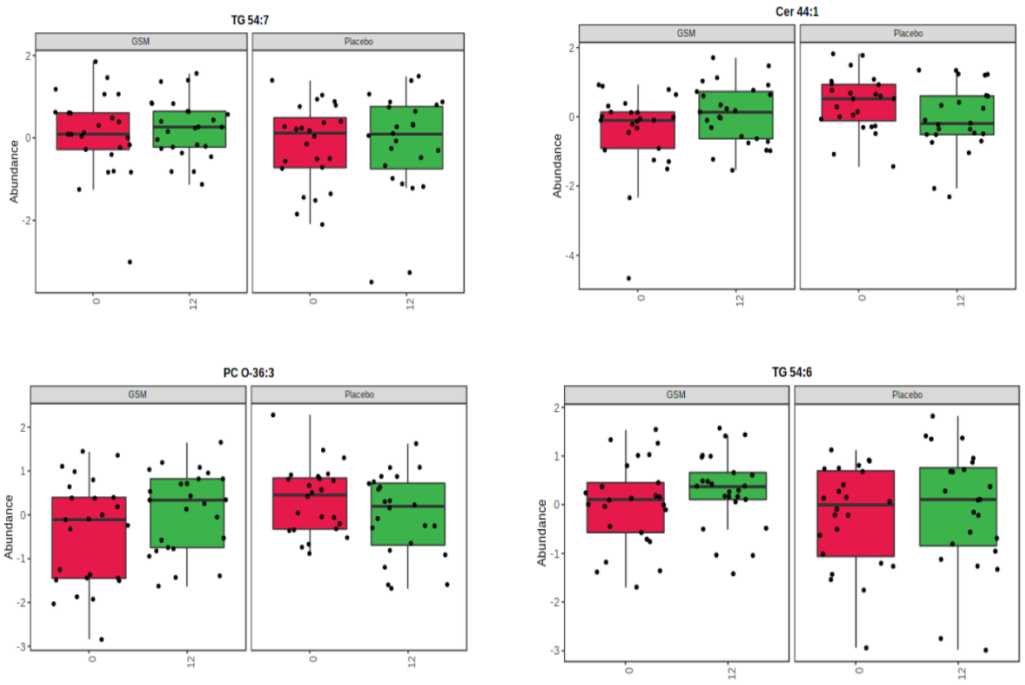
Table 7.1 Details of lipid species identified in the leverage-SPE scatter plots of the ASCA variables submodels

Treatment			Time			Treatment × Time (interaction)		
Compound	Leverage	SPE	Compound	Leverage	SPE	Compound	Leverage	SPE
PC O-36:3	0.0168	2.83E-4	TG 56:4	0.0255	7.55e-32	HexCer 42:1	0.0862	2.23E-31
TG 54:7	0.0164	2.77E-4				CE 20:5	0.0727	6.32E-31
TG 54:6|	0.0160	2.77E-4				LPC O-16:2	0.0516	2.79E-31
Cer 44:1	0.0158	2.65E-4				Cer 42:1	0.0510	1.48E-31
						SM 42:1	0.0461	2.81E-31
						ST 29:1	0.0459	2.79E-31
						LPC O-16:0	0.0454	5.77E-31
						TG 58:2	0.0434	5.00E-31
						Cer 44:1	0.0434	5.62E-31
						Cer 41:1	0.0392	2.25E-31
						PC 40:7	0.0392	3.53E-31
						PC 36:6	0.0389	3.39E-31
						LPC 18:3	0.0386	3.52E-31

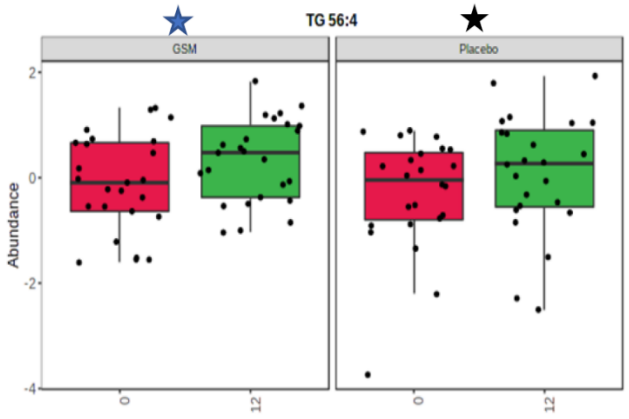
The normalized peak intensities of these lipids at baseline and end of the study within each group are shown in the box and whisker plots (Figure 7.3.A. B and C). Some the identified lipid species significantly changed over time within GSM and placebo groups determined by a paired t test. Lipid species TG (56:4) showed to significantly increase in both GSM and placebo groups over time. The abundance of Cer (42:1, 41:1), LPC (O-16:0), LPC (18:3), and TG (58:2) were notably elevated by GSM treatment. The Cer (44:1) and PC (36:6) were slightly increased or maintained in the GSM group while significantly reduced in placebo group.

Other lipids species including ST (29:1), SM (42:1), PC(O-36:3), TG (54:6), LPC (O-16:2), PC (40:7) and CE (20:5) demonstrated an increasing trend with GSM supplementation while decreasing or being maintained at baseline level in the placebo group. The details of the selected lipid species including retention time, adducts and the MS2 fragmentation of PC and TG lipids is presented in Appendix 4; Table S**7.**1.

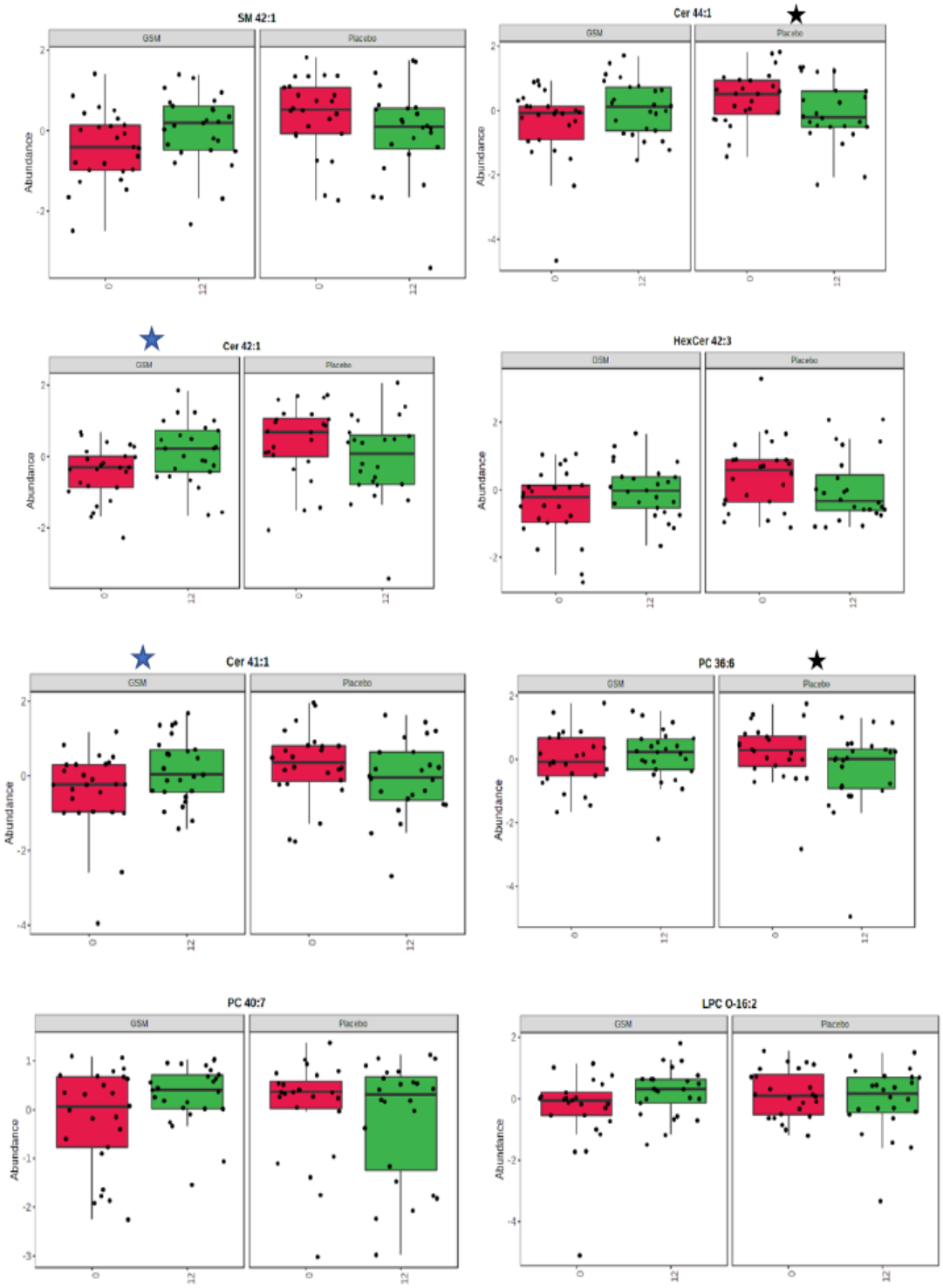
(A)


TG 54:7
Cer 44:1
PC O-36:3
TG 54:6
GSM
Placebo
Abundance
0
12

(B)


TG 56:4
GSM
Placebo
Abundance
0
12

(C)


SM 42:1
Cer 44:1
Cer 42:1
HexCer 42:3
Cer 41:1
PC 36:6
PC 40:7
LPC O-16:2
GSM
Placebo
Abundance
0
12

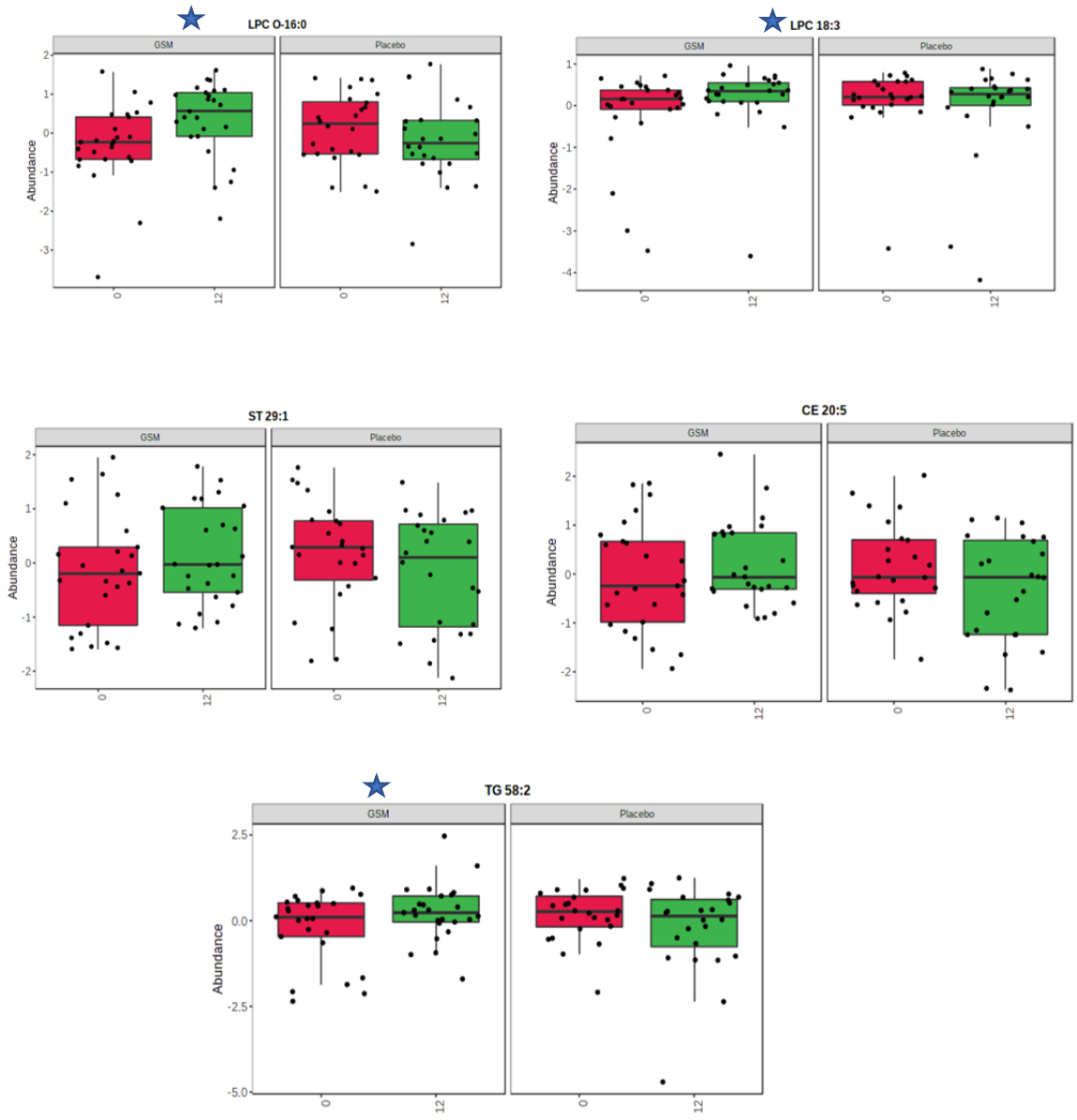


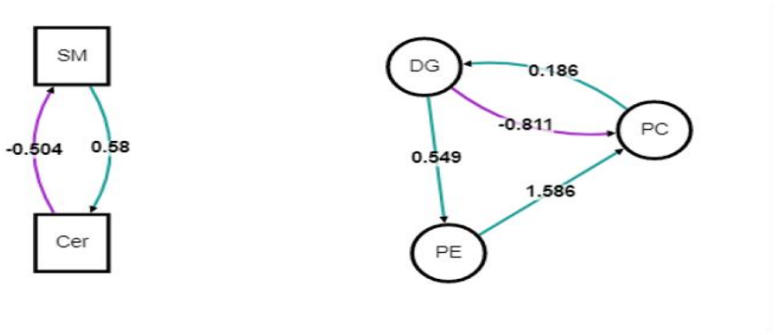
Figure 7.3. Boxplot of relative abundance for lipid species identified by ASCA submodel.

The relative abundance of lipids species that were affected by treatment (A), time (B) and their interaction (C) at the baseline and end of the study within each treatment. Y axes are represented as relative units. The bar plots show the normalised values (mean ± standard deviation). The boxes range from the 25% and the 75% percentiles and relative abundance of individual samples are indicated by dots. Medians are indicated by horizontal lines within each box. The lipids that changed significantly over time within each group were assessed by paired t test and indicated with blue and black star for GSM and placebo group, respectively.

Linear mixed models were performed to evaluate the consistency of the ASCA model, as it is more efficient with its interpretation of the difference. Five lipids were identified of which three of them were in the lipids class Hexosylceramide (HexCer 42:1, 40:1, 42:3) along with one PC (40:8) and one PE (P-38:5). Log of fold change (LogFC) was used to define the direction of change as upregulated and downregulated under active treatment compared with placebo when LogFC was positive and negative, respectively. Three HexCer (42:1, 40:1, 42:3) had an increasing trend while PC (40:8) and PE (P-38:5) had a decreasing trend under GSM treatment. However, none of the trends were significant according to FDR >0.05.

The lipid pathway graph is presented in Figure 7.4A and B. The result compared the lipid profile of two different treatments with the aim of detecting the lipid pathways that were activated or suppressed over time under each treatment. It was shown that pathways that synthesize the diacylglycerol (DG) from PC and pathways that synthesise PC from PE are activated under both GSM and placebo treatment. In contrast, the pathways that use DG as substate to synthesize PE were activated in the GMS group as opposed to placebo group. Interestingly, the BioPAN showed the difference in metabolism of sphingolipid. The SM synthesis from Cer was suppressed while the catabolism of SM to Cer was activated by GSM treatment; this suggests accumulation of Cer under GSM treatment. This was opposite to what observed in the placebo group as the SM synthesis was activated by placebo treatment.

(A)



(B)

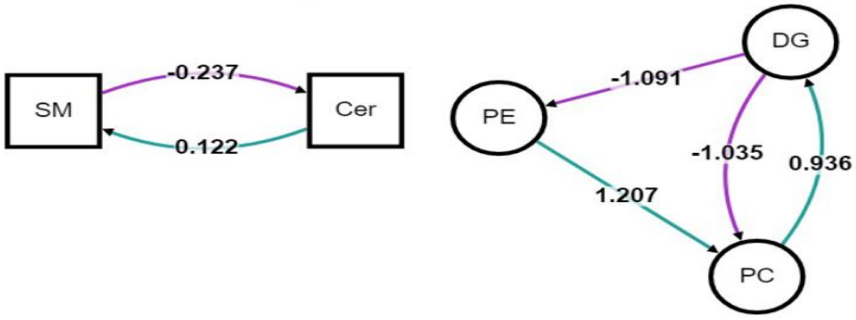


Figure 7.4. BioPAN lipid networks
Lipid network graphs exported from BioPAN for the GSM (A) and the placebo treatment (B). Reactions with a positive Z score have green arrows while negative Z scores are indicated with purple colour. End of the study was condition of interest, and baseline was control condition.

7.3.2 **Metabolomics**

After removal of the noise and unstable compounds, a total of 86 metabolites in negative and positive modes were included for statistical analysis. Similar to lipidomic data, OPLS-DA score plot did not reveal any clear separation among the treatment groups over time and the model demonstrated low predictive ability Q2=-0.02 (Appendix 4; Figure S7.3). The OPLS-DA analysis of metabolomic data for each GSM and placebo group is presented in Appendix 4; Figure S7.4A and B. Both models showed low predictive ability indicated by Q2=-0.570 and -0.189 for GSM and placebo, respectively. However, the pattern of metabolite profile differed slightly before and after supplementation in the GSM group.

Figure 7.5 shows important metabolites that were identified by ASCA and were well modelled by treatment, and interaction between time and treatment. Three metabolites were affected by the treatment which include taurine, biliverdin and urea, and five were altered by the interaction effect between time and treatment including L-glutamine, L-threonine, 3-methyl-histidine, pipecolic acid, and 3-hydroxyanthranilic acid presented in Table 7.2. No metabolite was identified to be affected by time.

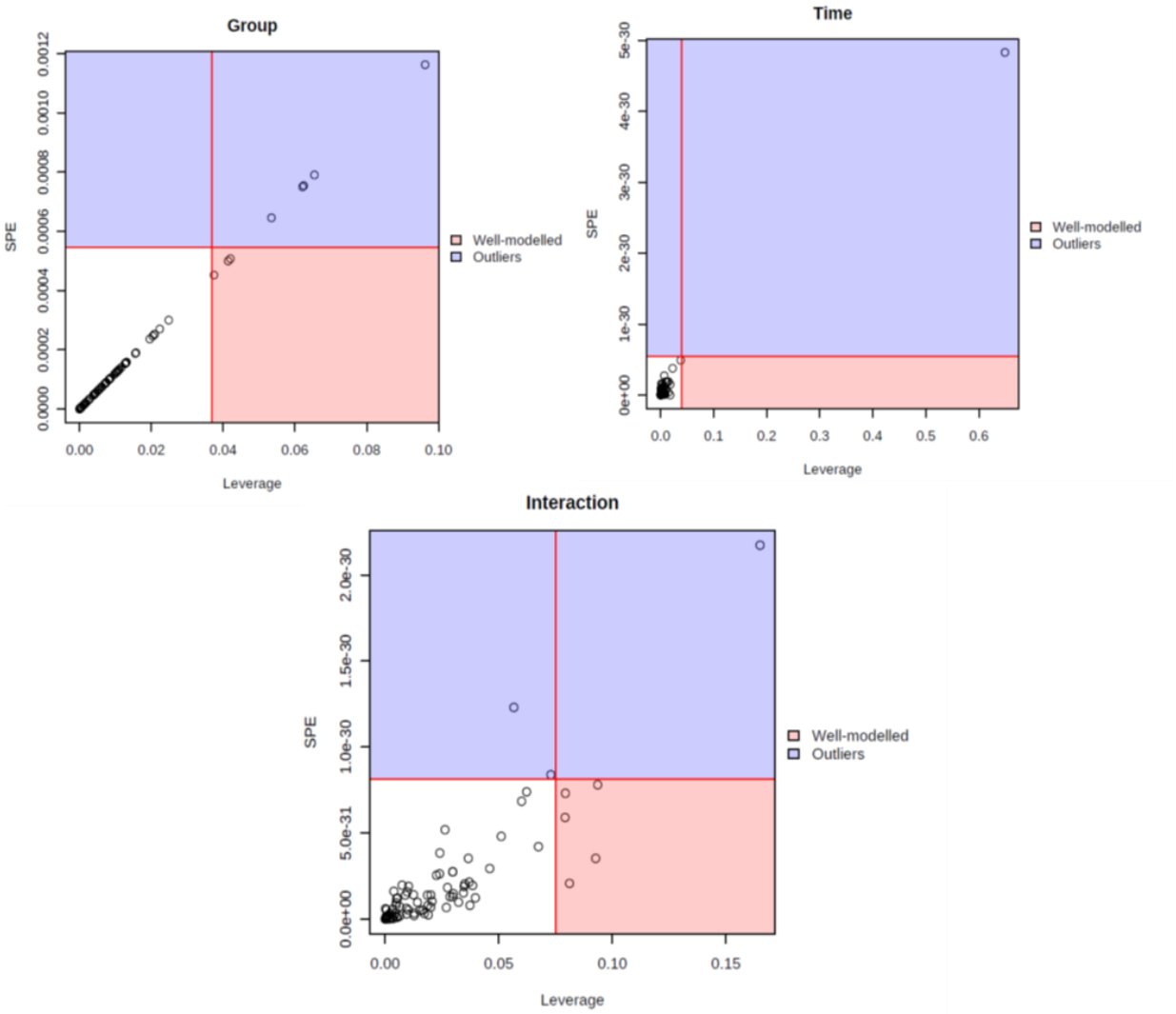


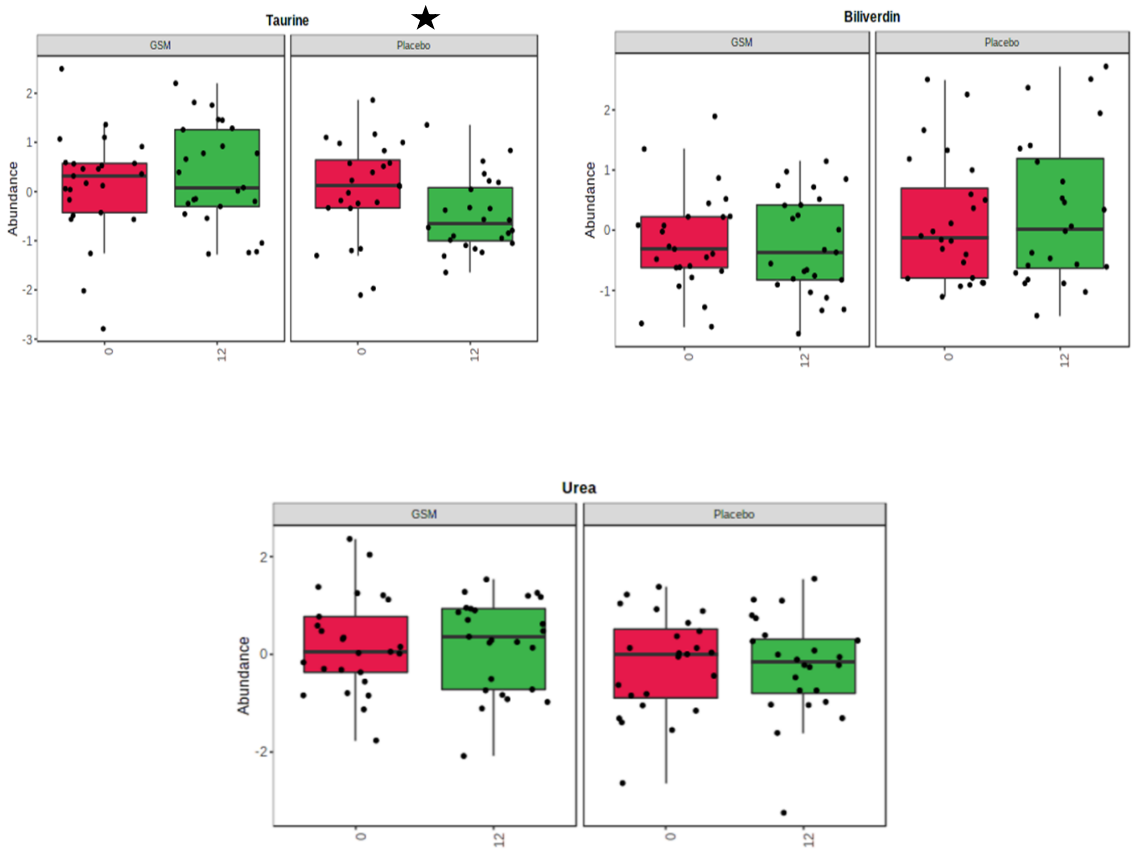
Figure 7.5. Leverage and squared prediction error (SPE) scatter plots of the ANOVA-simultaneous component analysis (ASCA) submodels for group (GSM and placebo) and time (baseline and endpoint), and their interactions in metabolomic data.
Vertical and horizontal lines represent cut-off leverage and SPE values, respectively. Metabolites species with a high leverage value and a low SPE value were considered to be differential (the well-modelled group). Metabolites appeared in blue area have patterns that are different from the major patterns.

Table 7.2. Details of metabolites identified in the leverage-SPE scatter plots of the ASCA submodels.

Treatment			Treatment × Time (interaction)		
Compound	Leverage	SPE	Compound	Leverage	SPE
Taurine	0.0420	0.0005	L-Glutamine	0.0935	7.8038e-41
Biliverdin	0.0413	0.0004	L-Threonine	0.0926	3.5283e-41
Urea	0.0374	0.0004	3-Methyl-histidine	0.0811	2.0723e-41
			Pipecolic acid	0.0792	7.3108e-41
			3-Hydroxyanthranilic acid	0.0791	5.9029e-41

Box and whisker plots showing the normalised peak intensities of important metabolites at baseline and end of the study within each group are presented in Figure 7.6.A and B. The level of taurine, pipecolic acid, 3-hydroxyanthranilic and L-glutamine were significantly reduced by the placebo while maintained at baseline level or slightly decreased by GSM treatment over the study period. Biliverdin and urea peak intensity was maintained at the baseline level at both GSM and placebo. The level of L-threonine showed a slight increase in GSM group while they decreased in placebo group. The other metabolites remained at the baseline in both GSM and placebo group.

(A)



(B)

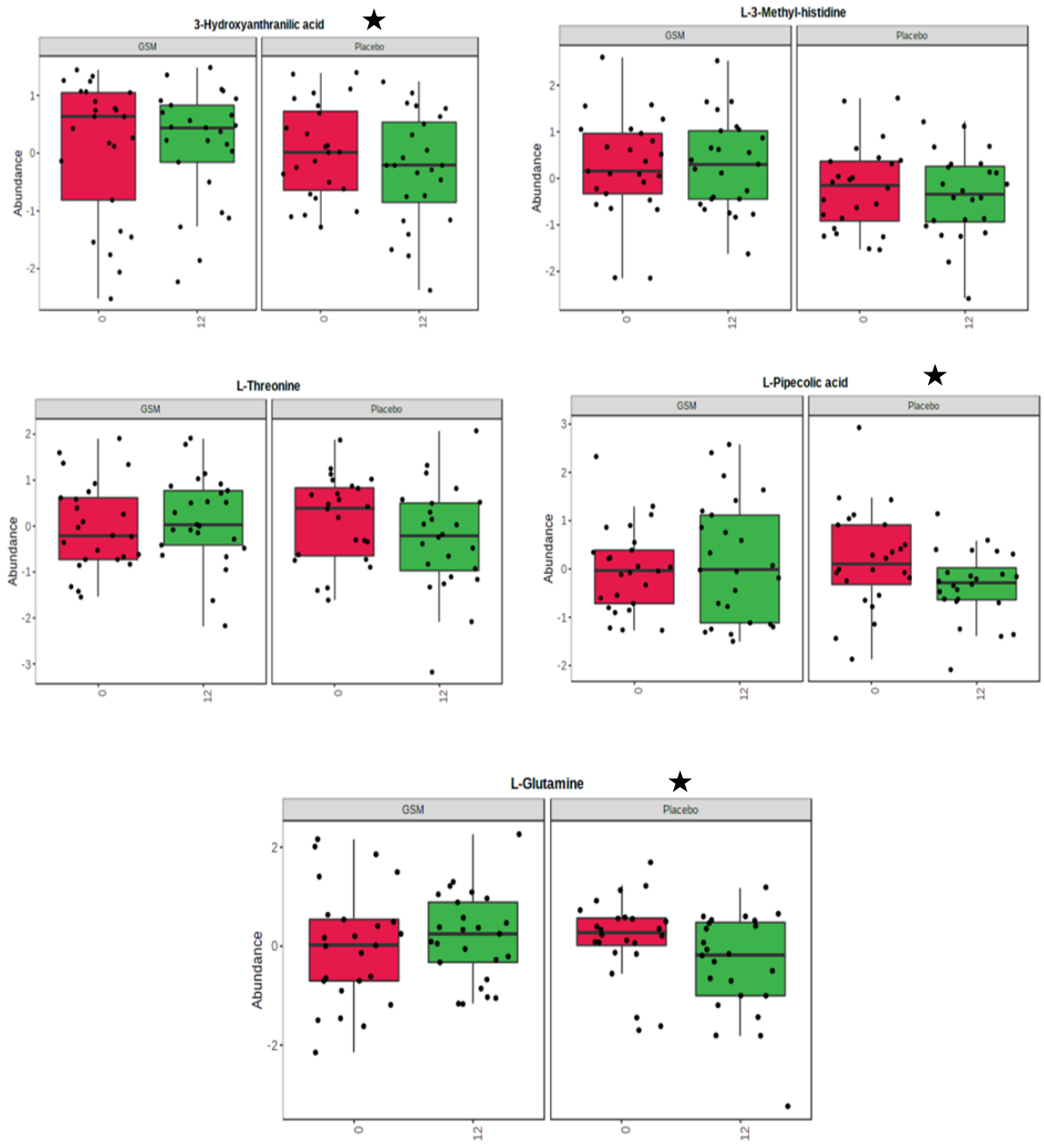


Figure 7.6. Boxplot of relative abundance for metabolites identified by ASCA submodels. Relative abundance of metabolites affected by treatment (A), interaction between time and treatment (B) at the baseline and end of the study within each treatment. Y axes are represented as relative units. The bar plots show the normalized values (mean ± standard deviation). The boxes range from the 25% and the 75% percentiles and relative abundance of individual sample are indicated by dots. Medians are indicated by horizontal lines within each box. The metabolites that changed significantly over time within each group assessed by paired t test is indicated with black star for placebo group.

Linear mixed models identified eight metabolites of which four were common with metabolites identified by ASCA. The identified metabolites include mevalonic acid lactone, 3-methyl-histidine, trimethyl-l-lysine, phenylacetylglutamine, 2-hydroxy-3-methylpentanoate, taurine, and urea had increasing trend under GSM treatment as indicated by positive LogFC. Only one metabolite biliverdin had a decreasing trend under GSM treatment as shown with negative LogFC. However, none of the trends were significant according to FDR >0.05.

7.4 Discussion

This study was conducted to enhance our current understanding of metabolic profiling in the plasma of an experimental model of metabolic OA treated with whole GSM powder as presented in an earlier chapter. In this study, an untargeted approach was used to identify the important lipid and metabolite differentiators between the GSM and placebo treatments over the 12 weeks study in overweight and obese postmenopausal women.

In summary, a panel of plasma lipids that were altered by the interaction effect between time and treatment effect includes HexCer (42:1), Cer (42:1, 44:1, 41:1), LPC (O-16:2, O-16:0), LPC (18:3), PC (36:6, 40:7), TG (56:4, 58:2), SM (42:1), ST (29:1), and CE (20:5). In general, the majority of these lipids tend to have higher relative abundance after GSM treatment while their level decreased or were maintained in the placebo group.

In this study, a set of eight metabolites were affected by the treatment or interaction between time and treatment which included three amino acids L-glutamine, L-threonine, 3-methyl-histidine and five metabolites including taurine, biliverdin, urea, pipecolic acid (a minor metabolite of lysine), and 3-hydroxyanthranilic acid (intermediate in metabolism of tryptophan). The two amino acids L-glutamine and L-threonine and one organic acid taurine tended to increase with GSM treatment while they tended to decrease in the placebo group. Pipecolic acid reduced under placebo conditions and other metabolites remained relatively unchanged under both treatments.

The lipidomic profiling identified several Cer molecules and one sphingomyelin (SM). The identified Cer mainly composed of d18:1 sphingoid base and with long chain saturated fatty acids (C24:0, 26:0). SM are composed of a Cer core linked to a phosphatidylethanolamine (PE) or PC (Floegel et al., 2013) . Conversion of SM to Cer is

up regulated in inflammation or stress (Zeidan & Hannun, 2010). Levels of circulating Cer have been positively associated with age, age-dependent disease and BMI (Huynh et al., 2019). A recent study showed a positive association between age and Cer species containing C24:0 and C24:1 in women and found that elevation in postmenopausal women was partially related to lower oestradiol levels (Vozella et al., 2019). Alterations in sphingolipid metabolism have been associated with joint damage in a model of OA as shown with suppression of sphingosine kinase-2, a key enzyme in the sphingolipid pathway, mitigating the histological damage and pain behaviour associated with OA in rats. The level of several SM in plasma and synovial fluid were at higher concentration in patients with OA (Kosinska et al., 2014). There have been a few studies on the effect of omega-3 PUFA on sphingolipid metabolism. One study in human subjects showed that a diet rich in omega-3 LC-PUFA decreased the Cer levels (Lankinen et al., 2009). Pathway analysis showed that the metabolism of sphingolipid shifted toward the synthesis of Cer from SM following GSM treatment which is in parallel with the up regulation of Cer level by GSM treatment. This is supported by the earlier finding from obese rat study which showed adding GSM powder suppressed the synthesis of the SM pathway. The increase in levels of Cer in plasma might reflect the increase in degradation of SM and the concomitant increase in Cer synthesis by GSM treatment. The sphingolipid metabolism and impact of GSM on Cer and SM species are worth further investigation to understand whether these data could explain some potential bioactivity of GSM powder.

Several glycerophospholipid including PC (O-36:3), PC (40:7, 36:6), LPC (O-16:2) and LPC (16:0, 18:3) were found to increase in plasma by GSM supplementation. Glycerophospholipids including PC and LPC are primarily synthesized in the liver and released into blood as part of lipoproteins, and the composition of fatty acids in these phospholipids can be modulated by dietary fat intake (Ranković et al., 2017). As reported previously from the rat study, plasma levels of phospholipids with long PUFAs such as PC (36:5, 36:6, 38:8), and LPC (20:5) increased to a greater level in rats fed with GSM than those in the control group (Abshirini et al., 2021). LPC containing fatty acids with 16 carbons (16:0, O-16:2) increased under GSM supplementation. These fatty acids are most likely originating from the GSM's diet from phytoplankton. The palmitoleic acid (C16: l, n-7) is specifically high in the fatty acid composition of phytoplankton such as diatoms, synthesized by desaturation of palmitic acid (C16:0), and are the precursors of the fatty acids (16:2, n-4) (Léveillé et al., 1997).

To the best of our knowledge, this is the first study to examine the effect of whole GSM supplementation on the metabolomic profile of humans. There is a study on female patients with rheumatoid arthritis (RA) who consumed a diet containing 75 g blue mussel meat daily (approximately 2.5 higher dose than our trial, estimating moisture content of 80%) as a cooked meal, 5 days a week for the duration of 11 weeks and this resulted in an improvement of the RA symptoms and perceived health. The analysis of plasma fatty acids revealed an increase in omega-3 PUFAs, EPA and DHA in plasma phospholipid and red blood cell following the mussel diet using a targeted approach (Lindqvist et al., 2019). We were not able to assess the fatty acids due to the untargeted approach of the study, however by characterization of PC and TG using MS2 fragmentation it seems that PC composed of omega-3 PUFA, DHA (22:6) and essential fatty acid linolenic acid (n-3, 18:3) increased after GSM supplementation. The main fraction of fatty acids in plasma are esterified to phospholipid, particularly PC. The increment in the PC containing omega-3 PUFA is supported by the earlier result that showed an increase in omega-3 PUFAs, EPA and DHA from the fatty acid analysis of plasma presented in chapter 5.

Diacyl PC (26:0), acyl-alkyl PC (34:2) has been found to be associated with MetOA (Chen et al., 2007). The altered metabolism of PC has been shared by both OA, metabolic syndrome and diabetes mellitus through a mechanism that is not fully understood. The normal cartilage tissue is covered by a thin layer of phospholipids, mostly PC, which contain unsaturated fatty acids that provide lubrication properties (Chen et al., 2007). In addition, PC (O-36:3) an alkenyl-acyl phospholipid (plasmalogen) was among the lipids increased by GSM diet. Plasmalogen has been found among the lipid components of GSM and marine bivalves mainly in the form of PE and phosphatidylserine (PS) accounting for 10-35% of total lipids (Hanuš et al., 2009; Miller et al., 2014). In humans a high proportion of plasmalogen are found in the brain and heart mostly in form of PE and to lesser extent PC (Nagan & Zoeller, 2001). The vinyl ether at sn-1 position are attached to saturated or monounsaturated fatty acids (C16:0, C18:0 and C18:1) (Wallner & Schmitz, 2011). In the sn-2 position, PUFA, specifically DHA (C22:6 omega-3) or arachidonic acid (C20:4 omega-6) are found (Fuchs, 2015). As part of cell membranes, plasmalogen plays a role as endogenous antioxidants due to their double bond vinyl ether, protecting the PUFA in the sn-2 position from oxidative stress (André et al., 2006). The reduced level of plasma ethanolamine plasmalogen has been associated with Alzheimer disease

and cognition deficits, and there is growing interest in a clinical intervention with plasmalogen as a potential therapeutic for Alzheimer disease (Su et al., 2019).

The PUFAs present in GSM might alter the composition and concertation of fatty acids in PC present in cartilage tissue, and this might explain the potential cause of cartilage protective properties observed by GSM supplementation, although other nutrients present in GSM can potentiate this effect. At this point, the data need to be verified by a targeted approach measuring the concentration of fatty acids using an authentic standard.

In the previously mentioned intervention study of feeding a blue mussel diet to RA female patients no significant change was found in serum metabolite profiles by Nuclear Magnetic Resonance (NMR) using an untargeted approach (Lindqvist et al., 2018). The current analysis showed the level of glutamine, a derivative of amino acid glutamic acid was increased by GSM supplementation. This could be due to the fact that glutamic acid is among the most abundant amino acids present in GSM. Some plasma metabolites were the same as those identified in the rat study, including two amino acids, threonine, histidine and one metabolite pipecolic acid which is an intermediate in the catabolism pathway of the essential amino acid L-lysine.

Pipecolic acid is an interesting metabolite that was up regulated in both the current study and rat study following GSM treatment. Previous studies have reported that circulating pipecolate levels were strongly associated with obesity and the metabolic syndrome (Libert et al., 2018). On the other hand, it has been shown to enhance insulin secretion in cell-based and animal models as a compensatory mechanism to regulate glucose homeostasis (Wang et al., 2013). A possible explanation for the observed increase in the level of pipecolic acid due to the GSM supplement could be that GSM might impact L-lysine metabolism and pipecolic acid up-regulation which have a beneficial effect on metabolism. Further investigations are needed to clarify this potential of GSM.

Apart from being two different species, the metabolomic results from the animal model and the human data differed possibly due to differences in dose and duration of GSM treatment as rats in the previous study were fed with a larger amount of GSM (diet containing 33% of protein and 16 or 1% of fat derived from GSM) for 36 weeks (presented in chapter 4) compared to 3 g per day for 12 weeks in the human study. Further, in the animal study the composition of the diet and physical activity were fully controlled

while in the present study, the participants continued their free-living regular diet with the advice to avoid omega-3 rich foods.

Using untargeted metabolomics enabled us to profile the small metabolites that can be used as putative biomarkers for the impact of GSM powder in human model of MetOA. However, the findings of this study need to be validated, and levels of lipids species should be measured on the same plasma samples using a targeted method with a similar analytical approach. In addition, the current lipidomic data did not detect any fatty acids and this could be because the analysis of the molecular lipids was done only in a positive ionization mode, and diacyl phospholipids lose their fatty acid side chain in a negative ionization mode (Sommer et al., 2006).

Further, the roles of other metabolites identified in this research, for example, taurine which was increased as the result of GSM supplementation, as well as other metabolites, 3-hydroxyanthranilic acid, urea, biliverdin are not understood at this point, and requires further investigation to elucidate the underlying causes.

In summary, metabolomic analysis revealed some lipid species including Cer, LPC, and TG with long chain PUFAs that were increased following GSM supplementation in overweight/obese postmenopausal women. A plasmalogen was among the lipids elevated by the GSM supplement. These membrane phospholipids are present in mussels acting as an endogenous antioxidant and are involved in disorders related to oxidative stress and inflammation. This finding is an interesting lead to determine how plasmalogens derived from GSM play role in cartilage protection and joint health. Some polar metabolites such as threonine, histidine and pipecolic acid were altered following GSM supplementation which were in common with the previous study on obese rats fed with GSM powder. These common polar metabolites can serve as novel potential metabolic biomarkers for the impact of GSM powder supplementation in a model of MetOA. Further targeted analysis is required to verify these findings. Finally, there is need for larger scale studies where metabolic status of individuals can be controlled to allow for a reduction in the variation caused by lifestyle associated factors such as diet, physical activity, and stress.

Chapter 8 Discussion, conclusion, and areas for future research

The main purpose of the research presented in this thesis was to investigate the chondroprotective effect of New Zealand Greenshell^{TM} Mussel (GSM) in overweight/obese postmenopausal women at risk of osteoarthritis (OA) through assessing changes in cartilage degradation biomarkers (Chapter 5).

The novel beneficial effects of GSM extract on gut microbiome and body weight gain prompted the second question of whether GSM powder can affect the gut microbial abundance and body composition which were addressed in chapter 6.

Additionally, this research included the study of ovariectomized (OVX), and diet-induced obese rats where the aim was to identify novel biomarkers/fingerprints associated with the GSM supplementation (Chapter 4), that were further explored in human samples from a clinical study through metabolomic analysis (Chapter 7).

8.1 Thesis discussion

8.1.1 Evaluation of the effect of GSM supplementation on cartilage biomarkers, inflammatory cytokines, and joint symptoms

This chapter sought to investigate the effect of whole GSM powder on cartilage degradation biomarkers, along with inflammatory cytokines, joint pain, and knee-related problems. This study reports that consumption of GSM powder at 3 g/day for a duration of 12 weeks did not have any significant effect on urinary C-telopeptides of type II collagen (CTX-II) in overweight/obese postmenopausal women, however a decreasing trend was observed in women in the GSM group. The women with symptomatic knee showed lower concentration of urine CTX-II at week 6 and week 12. No significant alteration was observed in the level of serum cartilage oligomeric matrix protein (COMP), a degradation marker from non-collagen components of cartilage after supplementation with GSM. The level of inflammatory cytokines did not change due to the intervention. The findings on the cartilage marker, CTX-II and inflammatory cytokines levels are in agreement with results from the study of obese rats fed with GSM powder (Siriarchavatana et al., 2019). In addition, the findings from this study revealed that GSM

supplementation successfully resulted in clinical improvement of joint pain compared to placebo with no major side effects. This finding is supported by a previously published study on patients with knee OA where GSM powder was administrated at the same dosage and duration used in the current study (Coulson et al., 2013). It is worth mentioning that the moderate reduction in joint pain and improvement in some of the knee-related symptoms observed in the placebo group of current study can be explained by a placebo effect which is expected in OA studies testing natural therapies as analgesic (Zhang, 2019). This also indicates that participants were properly blinded.

Although the exact mechanism by which GSM powder provides protection to cartilage remains to be clarified, however from the current findings it seems GSM powder might suppress the degradation of type II collagen while a non-collagen protein component of cartilage such as serum COMP is unlikely to be impacted.

The omega-3 PUFA from GSM oil were able to down-regulate the expression of catabolic genes matrix metalloproteinases (MMP)-1, MMP3, and MMP13 (the gene of collagen degrading enzymes), while up-regulating the anabolic genes AGG and COL2A1, the genes that express the aggrecan and collagen type II alpha 1 in chondrocytes. It also suppressed the release of sulphated glycosaminoglycans (s-GAGs) (Buddhachat et al., 2017).

Another important observation that confirmed the compliance to the study supplement was elevation in the concentration of omega-3 LC-PUFA, EPA and DHA in the plasma of the group that received GSM powder. This also highlights that an increase in intake of GSM can be promoted in New Zealand where fish and seafood are not major part of diet. The average consumption of fish and seafood among the participants prior to enrolment to the study was less than once a week (60%), once a week (35%) and more than once a week (5 %), and none were a regular mussel eater.

The level of plasma omega-3 LC-PUFA has been found to be a strong indicator of synovial fluid omega-3 LC-PUFA status in patients with inflammatory arthritis, and higher levels of these fatty acids in plasma and synovial fluid have been associated with less pain (Moghaddami et al., 2015). Thus, the elevation in plasma EPA and DHA observed in the current study can be a good reflection of an increase in these fatty acids in synovial fluid and synovial membranes where they may have anti-inflammatory effects.

Apart from determining the benefit of GSM powder on joint health, findings from this study contribute to knowledge of existing clinical studies regarding what the optimal and therapeutic dose of the GSM extract is. The current study concludes that GSM powder at the dose of 3 g/day for 12 weeks is sufficient for exerting a therapeutic effect in postmenopausal women with obesity. A previous study using the same dose and duration showed a high efficacy among knee OA patients of which mostly (89%) were overweight/obese (Coulson et al., 2013).

8.1.2 **Evaluation of the effect of GSM supplementation on faecal microbial abundance, body composition and iron status markers**

This chapter aimed to answer the question regarding the effect of GSM powder on abundance of gut microbes, body composition parameters, and iron status markers in overweight/obese postmenopausal women. The abundance of six species of bacteria which are important in gut homeostasis and regulating inflammation including *Clostridium* XIVa, *Clostridium* IV, *Akkermansia muciniphila, Lactobacillus, Bifidobacterium* and *Bacteroides* were investigated. Based on the results of this study, no significant changes were observed in the abundance of investigated gut microbes following the intervention, however treatment with GSM powder tended to moderately increase the abundance of *Bifidobacterium, Bacteroides* and *Akkermansia muciniphila*. This is consistent with data from a previous study that reported the beneficial effect of GSM powder and glucosamine sulphate on OA symptoms along with improvement of gastrointestinal symptoms and alteration in the gut microbiota profile in knee OA patients. In the GSM group, *Bacteroides, Lactobacillus Bifidobacterium, Eubacterium,* increased while *Enterococcus*, *Staphylococcus*, *Clostridium*. and yeasts decreased (Coulson et al., 2013). *Bacteroides* genus are most abundant in humans and can digest mucin (a type of glycan) in the mucosal layer in the large intestine in the absence of dietary glycan (Roth et al., 2016). This results in downstream inflammation in the host (Desai et al., 2016). Glycosaminoglycans (GAGs) such as chondroitin sulphate are found in GSM may serve as substrate for these bacteria and protect the gut barrier from degradation (Desai et al., 2016). Impaired gut permeability and mucosal degradation has been observed in an animal model (Collins et al., 2015) and humans with OA (Huang et al., 2016). Fish oil and hard-shell mussel oil has been shown to improve the intestinal integrity in mice as shown by a decrease in colonic myeloperoxidase activity and increase in diamine oxidase activity that indicate a decrease in inflammation and mucosal injury

(Wan et al., 2018). Apart from that, *Bacteroid* species secrete sulfatase, an enzyme that cleave the sulphate and increase the bioavailability of the glycan to the host (Ulmer et al., 2014). It seems the microbes of the host play an important role in degradation of glycan present in GSM and its subsequent therapeutic effects. Further research on the effect of GSM powder on gut microbe abundance is required.

With respect to body composition parameters, no significant change was observed. However, body fat (BF)% tended to increase to a lesser extent in the GSM group over the study period. Although this change was not significant, it was in line with data from the rat study (Siriarchavatana et al., 2019), and previous animal studies using a GSM oil extract. Enriching a high-fat diet (HFD) with GSM oil counteracted body weight gain in middle-aged obese mice, with significant reduction in visceral fat mass (Loehfelm et al., 2021). One human study found fish oil supplementation alone and then concomitantly with a very low-calorie diet, resulted in greater weight loss and decrease in body mass index (BMI) compared to the control group (Munro & Garg, 2013). The mechanisms by which omega-3 PUFA facilitates the reduction of body fat or body weight are yet to be understood. Omega-3 PUFA, particularly DHA promotes lipolysis and facilitates hepatic fatty acid oxidation, suppresses fatty acid synthesis and release of very low-density lipoprotein (VLDL) (Jump et al., 2008), which ultimately lead to a decrease in fat deposition. Furthermore, rats fed with GSM powder, at 25-45% of their basal diet had lower low density lipoprotein cholesterol (LDL-C), especially in the group with the highest mussel level in the diet (Aldairi et al., 2021). It should be noted that in these animal studies, the GSM was used at a very high dose and in a controlled feeding condition which is not representative of the relatively lower dose used by the human study.

The GSM powder did not appear to have a significant effect on iron status markers, except for ferritin levels that changed over the time. This change does not reflect a decrease in inflammation since CRP levels were slightly elevated in both treatment groups. It should be noted that participants of this study had normal levels of iron and sufficient iron intake from the diet which may explain the lack of effect. Further studies targeting the individuals with low iron stores might show improvement of iron markers.

8.1.3 **Evaluation of the effect of GSM supplementation on lipid and metabolite profile of animal model of metabolic OA and postmenopausal women**

Research conducted in this chapter reports metabolic changes in obese and ovariectomized (OVX) rats as a model of metabolic OA in response to adding GSM powder to the diet. This study was followed by a clinical trial assessing the effect of GSM powder supplementation on lipid and metabolite profiles of overweight/obese postmenopausal women through untargeted metabolomic analysis.

Data from the rat study revealed most lipid species including triglyceride (TG), diacylglyceride (DG), phosphatidylcholine (PC), and lysophosphatidylcholine (LPC) mainly containing long chain polyunsaturated fatty acids (PUFA) decreased under the high-fat high-sugar (HFHS) diet (Abshirini et al., 2021). Down-regulation in most of PC and TG including PC (32:1, 34:4, 32:4, 36:5) and TG (54:7, 54:8) observed under the HFHS diet in the current rat study were identical with results from a previous rat study which showed animals fed with high fat diet (HFD) had lower levels of TG, PC, and phosphatidylinositol (PI) containing monounsaturated fatty acid (MUFA) and PUFA while the same lipid classes with saturated and less unsaturated fatty acids increased (Gowda et al., 2020). However, there were inconsistencies with the results from some previous animal studies. For example, in a mouse study that used soybean oil as fat source some distinct phospholipids such as PC (38:4, 40:6) were raised by a HFD (Eisinger et al., 2014). The source of fat used in the HFD rodent studies strongly influenced the results of lipidomic analyses (An et al., 2022). The source of fat in the rat study (Abshirini et al., 2021), and in the earlier mentioned study (Gowda et al., 2020), was mainly soybean oil and lard which were low in PUFA, and may explain the decrease in lipids containing PUFA in these studies. However, inclusion of GSM powder to the HFHS diet, even though the fat content accounted only for 1% of diet total energy, was able to up-regulate some of the lipid species containing PUFA including PC (36:6, 38:8), LPC (20:5), TG (54:7, 54:8) that were down-regulated by HFHS diet.

In the rat study, the correlation between the identified lipids and severity of cartilage loss was not assessed and it is unclear if these lipids are involved in OA development (Abshirini et al., 2021). A previous study reported change in PC (36:2) and LPC (17:0, 20:4) which were associated with cartilage loss and OA severity in rats fed with HFD (Datta et al., 2017). The dysregulation in PC and LPC has been partly attributed to an increase in levels of leptin that are elevated in obese animal fed with HFD and it has been

linked to OA pathogenesis (Griffin et al., 2010). An *in vitro* study showed release of total LPCs into the media of human chondrocytes in response to leptin treatment. Leptin treatment of chondrocytes resulted in increases in the expression of autotaxin (ATX), an important metabolic enzyme that converts LPCs to the proinflammatory mediator lysophosphatidic acid (LPA), and the expression of matrix metalloproteinase 13 (MMP13), the main enzyme that degrades type II collagen in cartilage (Datta et al., 2017).

The lipidomic data from the study of postmenopausal women did not show any clear separation in treatment groups over the study period, and outcomes of corresponding multivariate analysis were weak, however some lipids were identified to be affected by time, treatment, and their interaction. The abundance of ceramide (Cer 42:1, 41:1), LPC (O-16:0), LPC (18:3), and TG (56:4, 58:2) was significantly elevated by GSM treatment. The increase in the levels of LPC and TG seemed to be in line with the observation from the rat study which showed adding GSM powder increased the relative abundance of the same class of lipids containing PUFA. LPC containing fatty acids with 16 carbons (16:0, O-16:2) were elevated by GSM supplementation. Palmitic acid (16:0) and to lesser extent palmitoleic acid (C16: l, n-7) are among the predominant fatty acids found in phospholipids and plasmalogens of mussels (Hanuš et al., 2009) which they obtain from their diet. The distinctive C16 fatty acids such as 16:1, and 16:2, 16:3 are exclusively produced by green algae and diatoms (Jónasdóttir, 2019).

Further, lipid pathway analysis in the rat and human studies revealed suppression in sphingomyelin (SM) synthesis by GSM treatment (Abshirini et al., 2021). At this point, we do not know the exact mechanism associated with the increased levels of distinct Cer species or their relationships with GSM supplementation or OA protection. Our hypothesis is that GSM may have activated the degradation of SM to Cer and subsequently increased Cer levels. Further work is required on the effect of GSM on sphingolipid metabolism.

Metabolite profiling from the rat study showed that the levels of some amino acids including serine, lysine, valine, threonine, histidine, and metabolites including cholic acid, pipecolic acid and 3-methylcytidine were altered under the HFHS diet in OVX rat. The alteration in branch chain amino acids such as valine has been associated with insulin resistance in obese animals (Horakova et al., 2016; Shearer et al., 2008). The elevation in serine and threonine and cholic acid in rats fed with HFHS diet is supported by a previous

study (Lai et al., 2020) and can be representative of other perturbations taking place with obesity such as myocardial, renal, and hepatic dysfunction (Lai et al., 2020; Siriarchavatana, 2021). Inclusion of GSM was able to partially regulate the HFHS diet-induced dysregulated metabolism of amino acids as level of some of amino acids including lysine, valine, and pipecolic acid that were up-regulated by the HFHS diet were down-regulated by GSM diet. Some of these amino acid such as lysine are involved in regeneration of type II collagen (Pauling & Corey, 1951). Threonine that was elevated by GSM supplementation in the human study, is crucial for the stability of the collagen triple helix (Jiravanichanun et al., 2006).

The underlying variation in human metabolism and differences between the rodent and human metabolomics has been a big challenge causing problems with interpretation of these studies. It should be noted that the biomarkers derived from a highly controlled rat experiment cannot be robust representation of human metabolites which are influenced by many factors causing variation (James & Parkinson, 2015), nevertheless, some common metabolites such as threonine, histidine and pipecolic acid identified in rat study and the human study could be novel potential metabolic biomarkers for the effect of GSM powder supplementation in a model of MetOA (Table 8.1).

Table 8.1.List of metabolites identified in obese and ovariectomized (OVX) rat model and postmenopausal women supplemented with GSM powder

Metabolites	Altered in obese and OVX rat fed with GSM	Altered in postmenopausal women in GSM group	Potential biomarkers
Cholic acid	Up-regulated		
Serine	Up-regulated		
Threonine	Up-regulated	Up-regulated	✓
Lysine	Up-regulated		
Valin	Down-regulated		
Pipecolic acid	Down-regulated	Up-regulated	✓
Histidine	Down-regulated		
3-Methylcytidine	Down-regulated		
Taurine		Down-regulated	
Biliverdin		Up-regulated	
Urea		Up-regulated	
L-glutamine		Up-regulated	
3-methly histidine		Up-regulated	
3-Hydroxyanthranilic acid		Down-regulated	

8.2 Conclusion

The results from cartilage biomarker analysis support the proposed application of whole meat GSM powder which may be a safe option for cartilage protection and OA prevention, along with exerting clinical benefits on joint pain in overweight/obese postmenopausal women. This research did not identify a significant effect on inflammatory cytokines. In addition, GSM relatively increased the abundance of some of the commensal gut bacteria such as *Bifidobacterium*, *Bacteroides* and *Akkermansia muciniphila* and resulted in a lesser increase in BF% compared to placebo. GSM meat contains a rich array of omega-3 PUFAs, several minor novel lipids, and cartilage protective compounds such as GAG which may impact a wide range of pathways from suppressing the degradation of type II collagen in cartilage to regulating the gut microbe population and improving body composition, all of which still need to be further investigated.

GSM treatment partially regulated the lipid and metabolite profiles that were perturbated by the HFHS diet and ovariectomy in rats. Data on overweight/obese postmenopausal women revealed some lipid species including Cer, LPC, and TG with long chain PUFAs that were elevated after GSM supplementation and some common polar metabolites such as threonine, histidine and pipecolic acid were found similar between the rat and the human study, although the direction of change following GSM supplementation was not different. Future targeted analysis is warranted to verify these polar metabolites to be suggested as novel potential metabolic biomarkers for the impact of GSM powder supplementation in MetOA.

8.3 Limitations, and areas of future research

Further randomised human intervention studies are required to investigate the benefit of GSM whole meat powder in human health, particularly in the area of joint health and OA. A study design that compares the effect of lipid to non-lipid fractions of a GSM extract on cartilage metabolism is recommended to give a more complete understanding of specific bioactive components of GSM responsible for the cartilage protective effects which will allow development of improved and more effective dietary supplements. This should also lead to a smaller dose (less capsules per day) and an increase in tolerability while providing more benefit, which ultimately will be preferable for consumers.

This study relied on only two cartilage degradation markers, and it was shown that degradation of type II collagen may be suppressed by the GSM powder, however whether GSM powder can stimulate or upregulate the synthesis of type II collagen needs to be evaluated using a cartilage synthesis marker. Further assessment of synovial inflammation markers; urinary glucosyl-galactosyl-pyridinoline and serum hyaluronan are suggested to ascertain whether GSM powder can prevent synovium inflammation. Additionally, the cartilage protective potential of GSM is suggested to be confirmed by morphological assessment of cartilage using magnetic resonance imaging (MRI) scans.

GSM powder has been investigated in early clinical studies among rheumatoid arthritis (RA) patients (Caughey et al., 1983; Huskisson et al., 1981; Larkin et al., 1985), and no significant symptomatic effect was noted. Given that severe cartilage degradation occurs during RA and high levels of urinary CTX-II have been reported as part of the progress of disease activity, the cartilage protective effect of GSM in this group of arthritis patients

is worth further evaluation (Landewé et al., 2006). This study targeted healthy overweight/obese postmenopausal women. As most of the women who participated had knee symptoms, findings may not be generalised to women with symptoms in other joint sites, nor men, nor those with clinical late-stage OA. Further studies are needed to focus on identifying the difference in response to GSM treatment among male and female genders, as well as individuals with different affected joint sites.

Finally, the observation from metabolomic analysis of the animal model and the human study provided a reference point for future targeted work. In the meantime, larger scale studies that control or consider the factors causing the variation in metabolite profile are needed to draw a more meaningful and accurate conclusion.

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Appendices

Appendix 1 Supplementary file of chapter 3

Table S3.1. Sensitivity analysis using leave-one-out method to assess the effect of GSM on visual analogue scale (VAS) pain score.

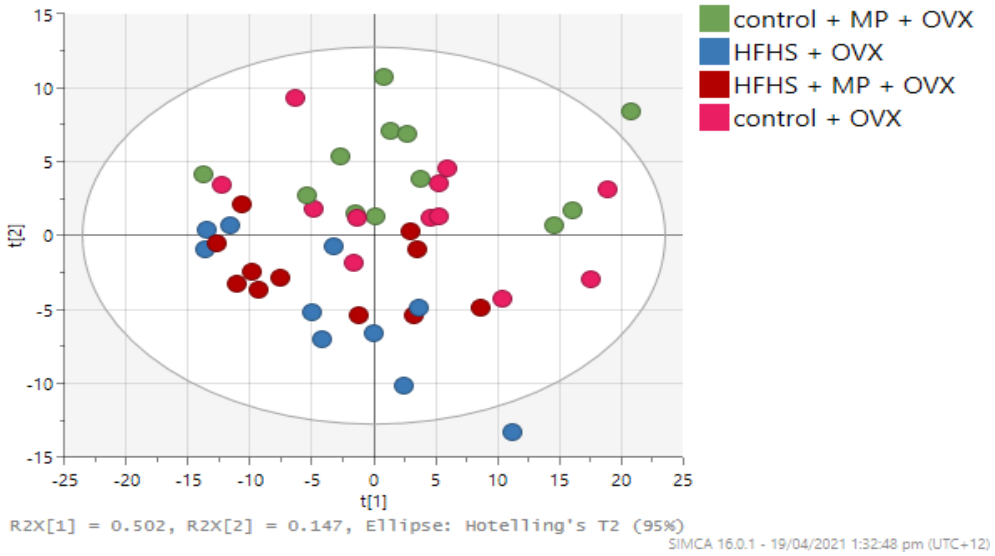
Trial excluded	Overall effect size, 95% CI	P-value	P for heterogeneity	I^2 (%)
Gibson, 1998	-2.722 (-5.146, -0.298)	0.02	0.003	78%
Coulson, 2013	-1.31 (-2.92, 0.3)	0.1	0.006	75.8%
Lau, 2004	-2.442 (-4.868, -0.017)	0.04	<0.001	83.7%
Zawadzki, 2013	-0.97 (-2.19,0.26)	0.1	0.055	60.6%
Stebbing, 2017	-2.534 (-4.815, -0.253)	0.02	<0.001	83.6%

Appendix 2 Supplementary file of chapter 4

Table S4.1. Composition of four experimental diet groups

Nutrient (%)	High fat/high sugar	High fat/high sugar+ GSM	Control	Control + GSM
Carbohydrate	30 (sucrose)	30 (sucrose)	5 (sucrose)	5 (sucrose)
Fat	30 (soy oil)	30 (49% from soy oil, 49% from lard, 1% from GSM)	5 (soy oil)	5 (84% from soy oil, 16% from GSM)
Protein	15 (casein)	15 (66% from casein, 33% from GSM)	15 (casein)	15 (66% from casein, 33% from GSM)

(A)



(B)

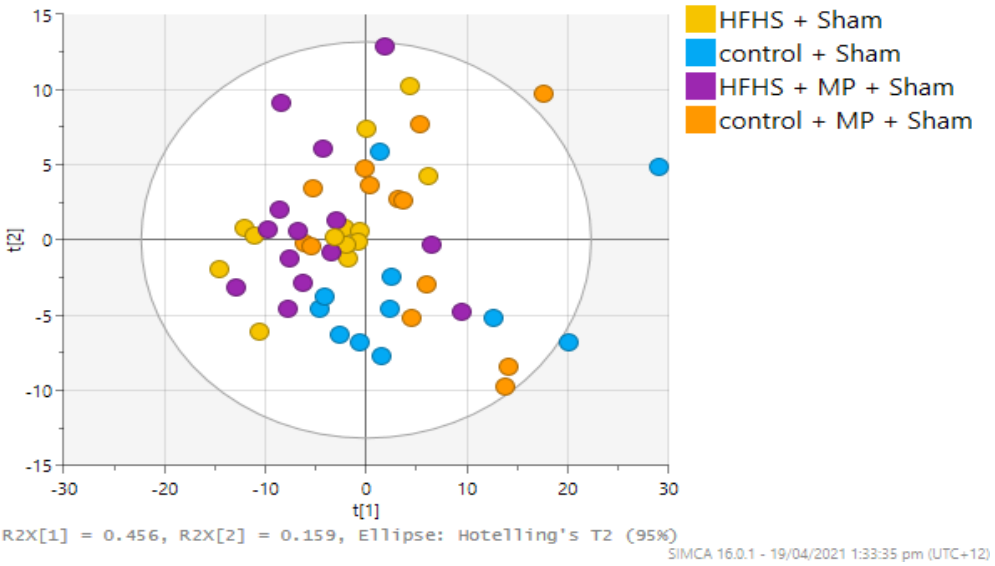
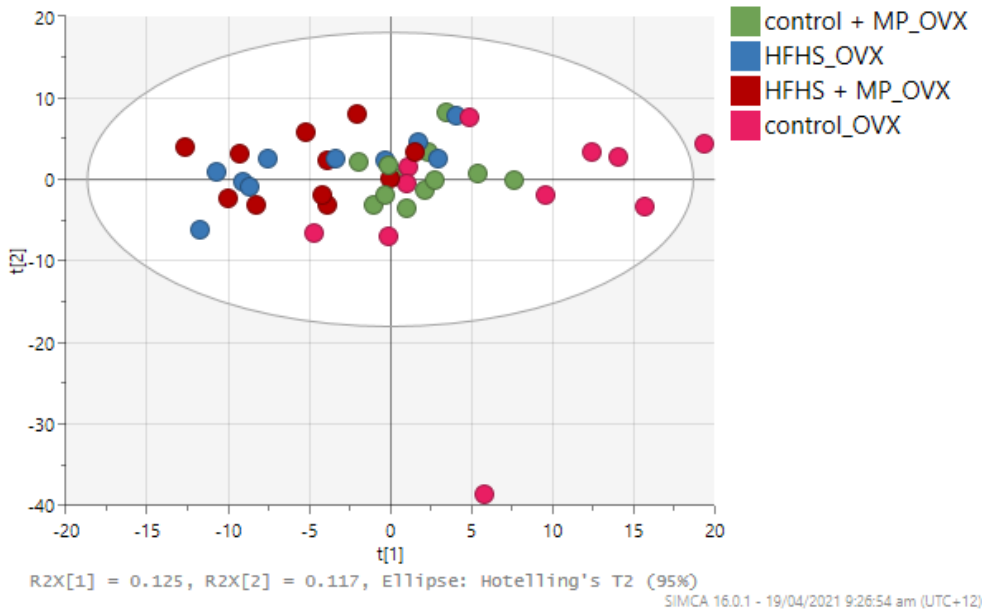


Figure S4.1. PCA scatter plot derived from lipidomic database. Each circle represents lipid profile of single rat. (A) PCA scoring plot showing variation of four diets groups in OVX rats (n=45), R2=0.869 and Q2=0.749. (B) PCA scoring plot showing variation of four diets groups in sham rats (n=51), R2=0.615 and Q2=0.544. HSHS: high-fat/high-sugar diet, MP: greenshell mussel powder, OVX: ovariectomized

(A)



(B)

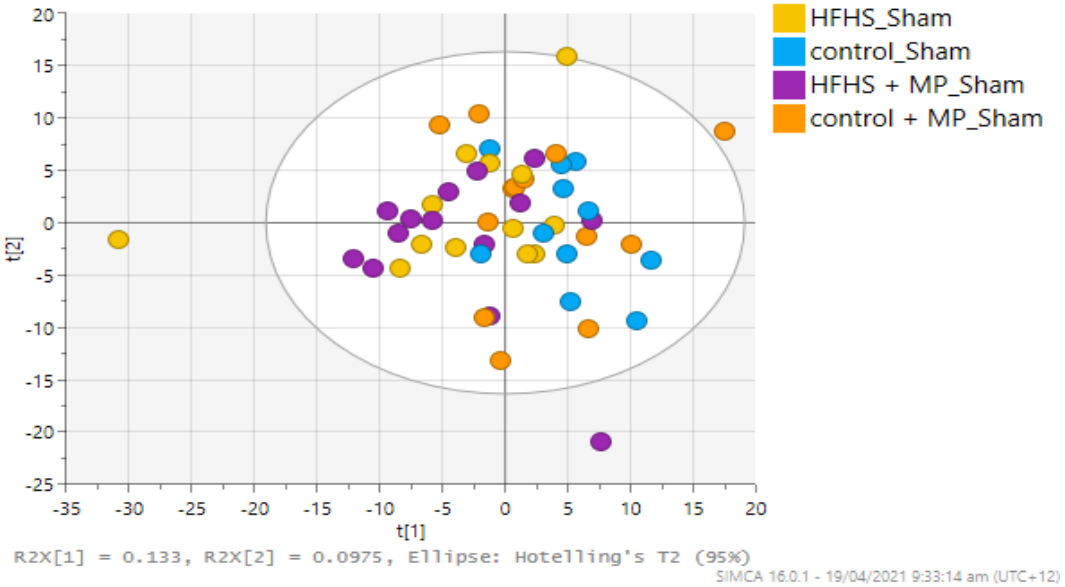


Figure S4.2. PCA scatter plot derived from metabolomic database. Each circle represents lipid profile of single rat. (A) PCA scoring plot showing variation of four diets groups in OVX rats (n=44), R2=0.65 and Q2=0.133. (B) PCA scoring plot showing variation of four diets groups in sham rats (n=51), R2=0.683 and Q2=0.232. HFHS: high-fat/high-sugar diet, MP: greenshell mussel powder

Table S4.2 Description of the total number of compounds in the pathway among diet groups.

HFHS vs control diet in OVX rats							
	Total Compound[1]	Hits[2]	Raw p[3]	-Log (p)	Holm adjust	FDR	Impact[4]
Primary bile acid biosynthesis	46	1	0.004733	2.3249	0.061526	0.061526	0
Biotin metabolism	10	1	0.024535	1.6102	0.29443	0.11663	0
Lysine degradation	25	2	0.026915	1.57	0.29606	0.11663	0
Aminoacyl-tRNA biosynthesis	48	5	0.038246	1.4174	0.38246	0.1243	0.16667
Valine, leucine and isoleucine degradation	40	1	0.058505	1.2328	0.52655	0.12676	0
Pantothenate and CoA biosynthesis	19	1	0.058505	1.2328	0.52655	0.12676	0
Glyoxylate and dicarboxylate metabolism	32	1	0.10461	0.98042	0.73227	0.1511	0.04233
Cysteine and methionine metabolism	33	1	0.10461	0.98042	0.73227	0.1511	0.02184
Sphingolipid metabolism	21	1	0.10461	0.98042	0.73227	0.1511	0
Valine, leucine and isoleucine biosynthesis	8	2	0.16819	0.77419	0.73227	0.21865	0
Glycine, serine and threonine metabolism	34	2	0.26919	0.56994	0.80757	0.31813	0.23069
Histidine metabolism	16	1	0.6455	0.1901	1	0.6455	0.22131
beta-Alanine metabolism	21	1	0.6455	0.1901	1	0.6455	0

HFHS+GSM vs HFHS diet in OVX rats							
	Total Compound[1]	Hits[2]	Raw p[3]	-Log (p)	Holm adjust	FDR	Impact[4]
Histidine metabolism	16	1	0.006228	2.2057	0.080959	0.040479	0.22131
beta-Alanine metabolism	21	1	0.006228	2.2057	0.080959	0.040479	0
Aminoacyl-tRNA biosynthesis	48	5	0.017514	1.7566	0.19265	0.063189	0.16667
Valine, leucine and isoleucine degradation	40	1	0.028826	1.5402	0.28826	0.063189	0
Pantothenate and CoA biosynthesis	19	1	0.028826	1.5402	0.28826	0.063189	0
Valine, leucine and isoleucine biosynthesis	8	2	0.029164	1.5352	0.28826	0.063189	0
Glycine, serine and threonine metabolism	34	2	0.25081	0.60066	1	0.46579	0.23069
Glyoxylate and dicarboxylate metabolism	32	1	0.41244	0.38464	1	0.53617	0.04233
Cysteine and methionine metabolism	33	1	0.41244	0.38464	1	0.53617	0.02184
Sphingolipid metabolism	21	1	0.41244	0.38464	1	0.53617	0
Primary bile acid biosynthesis	46	1	0.60804	0.21607	1	0.7186	0
Biotin metabolism	10	1	0.7441	0.12837	1	0.75483	0
Lysine degradation	25	2	0.75483	0.12215	1	0.75483	0

control+ GSM vs control diet in OVX rats							
	Total Compound[1]	Hits[2]	Raw p[3]	-Log (p)	Holm adjust	FDR	Impact[4]
Glycine, serine and threonine metabolism	34	2	0.001981	2.7031	0.025757	0.01429	0.23069
Aminoacyl-tRNA biosynthesis	48	5	0.002199	2.6579	0.026382	0.01429	0.16667
Valine, leucine and isoleucine biosynthesis	8	2	0.004022	2.3956	0.044238	0.017427	0
Primary bile acid biosynthesis	46	1	0.020969	1.6784	0.20969	0.068148	0
Histidine metabolism	16	1	0.038361	1.4161	0.34525	0.083116	0.22131
beta-Alanine metabolism	21	1	0.038361	1.4161	0.34525	0.083116	0
Biotin metabolism	10	1	0.11743	0.93022	0.82202	0.18591	0
Valine, leucine and isoleucine degradation	40	1	0.12871	0.8904	0.82202	0.18591	0
Pantothenate and CoA biosynthesis	19	1	0.12871	0.8904	0.82202	0.18591	0
Lysine degradation	25	2	0.16394	0.78532	0.82202	0.19433	0
Glyoxylate and dicarboxylate metabolism	32	1	0.19433	0.71147	0.82202	0.19433	0.04233
Cysteine and methionine metabolism	33	1	0.19433	0.71147	0.82202	0.19433	0.02184
Sphingolipid metabolism	21	1	0.19433	0.71147	0.82202	0.19433	0

HFHS vs control diet in sham rats							
	Total Compound[1]	Hits[2]	Raw p[3]	-Log (p)	Holm adjust	FDR	Impact[4]
Synthesis and degradation of ketone bodies	5	1	0.000273	3.564	0.002183	0.001092	0
Butanoate metabolism	15	1	0.000273	3.564	0.002183	0.001092	0
Tryptophan metabolism	41	1	0.004535	2.3434	0.027211	0.012094	0.14305
Primary bile acid biosynthesis	46	1	0.007022	2.1535	0.035111	0.014044	0
Aminoacyl-tRNA biosynthesis	48	2	0.032637	1.4863	0.13055	0.05222	0
Valine, leucine and isoleucine degradation	40	1	0.83955	0.075951	1	0.83955	0
Valine, leucine and isoleucine biosynthesis	8	1	0.83955	0.075951	1	0.83955	0
Pantothenate and CoA biosynthesis	19	1	0.83955	0.075951	1	0.83955	0

HFHS+ GSM vs HFHS diet in sham rats							
	Total Compound[1]	Hits[2]	Raw p[3]	-Log (p)	Holm adjust	FDR	Impact[4]
Valine, leucine, and isoleucine degradation	40	1	0.056828	1.2454	0.45462	0.15154	0
Valine, leucine, and isoleucine biosynthesis	8	1	0.056828	1.2454	0.45462	0.15154	0
Pantothenate and CoA biosynthesis	19	1	0.056828	1.2454	0.45462	0.15154	0
Aminoacyl-tRNA biosynthesis	48	2	0.088865	1.0513	0.45462	0.17773	0
Tryptophan metabolism	41	1	0.26192	0.58183	1	0.41907	0.14305
Primary bile acid biosynthesis	46	1	0.39651	0.40174	1	0.52868	0
Synthesis and degradation of ketone bodies	5	1	0.64199	0.19247	1	0.64199	0
Butanoate metabolism	15	1	0.64199	0.19247	1	0.64199	0

Control+ GSM vs Control diet in sham rats.							
	Total Compound[1]	Hits[2]	Raw p[3]	-Log (p)	Holm adjust	FDR	Impact[4]
Valine, leucine, and isoleucine degradation	40	1	0.000159	3.7997	0.001269	0.000423	0
Valine, leucine, and isoleucine biosynthesis	8	1	0.000159	3.7997	0.001269	0.000423	0
Pantothenate and CoA biosynthesis	19	1	0.000159	3.7997	0.001269	0.000423	0
Aminoacyl-tRNA biosynthesis	48	2	0.002781	2.5558	0.013904	0.005562	0
Primary bile acid biosynthesis	46	1	0.082918	1.0814	0.33167	0.13267	0
Tryptophan metabolism	41	1	0.13277	0.87691	0.3983	0.17702	0.14305
Synthesis and degradation of ketone bodies	5	1	0.2773	0.55705	0.5546	0.2773	0
Butanoate metabolism	15	1	0.2773	0.55705	0.5546	0.2773	0

[1] Total compound is the number of compounds involved in the pathway.

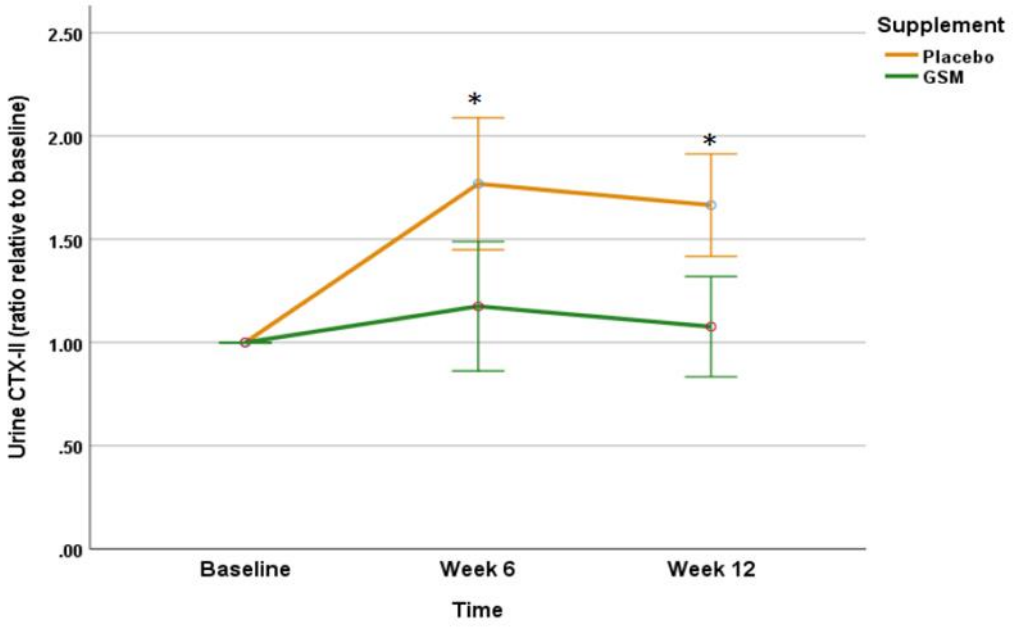
[2] Hits is the matched number from the user uploaded data.

[3] The raw p is the original p-value calculated from the enrichment analysis.

[4] Impact value is calculated from pathway topology analysis for comparison among different pathways. It represents the cumulative percentage of importance for the matched metabolite nodes involved in a pathway. The importance of each metabolite node is calculated from centrality measures and represents the percentage with regard to the total pathway importance.

Appendix 3 Supplementary file of chapter 5

(A)



(B)

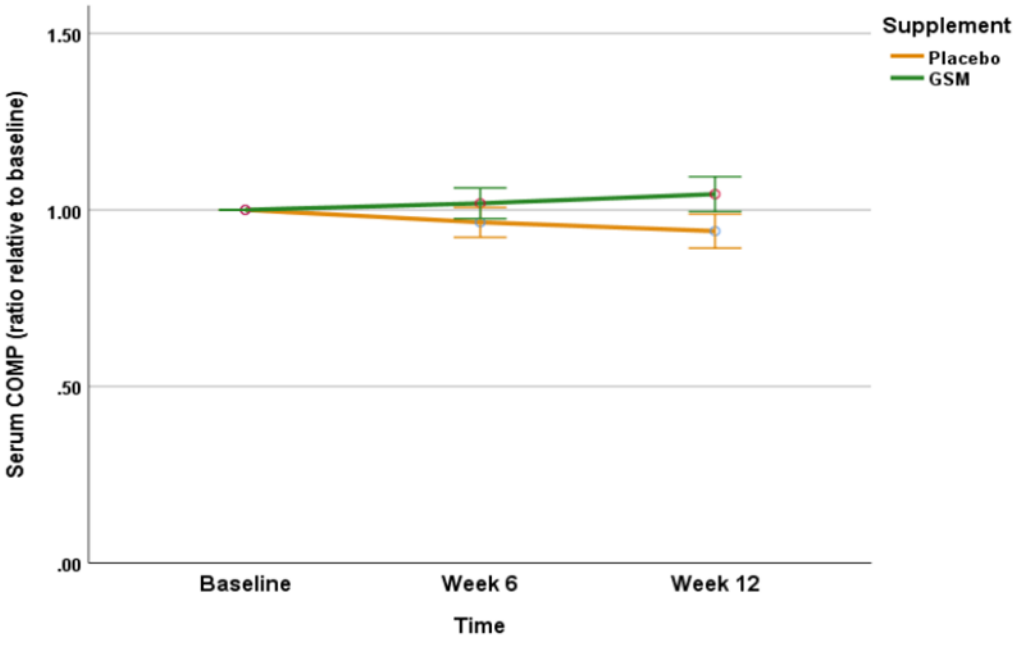


Figure S5.1. Pattern of change in urinary CTX-II level (A), and serum level of COMP (B) over the study period (baseline, follow-up, and endpoint) within each of the treatment groups. The levels are expressed as ratio relative to baseline. A significant time effect (P=0.03) for urine CTX-II (A). * Post-hoc comparison using the Tukey test showed difference between the baseline and week 6 and week 12. Placebo= orange, GSM= green. Data are expressed as mean ± standard error

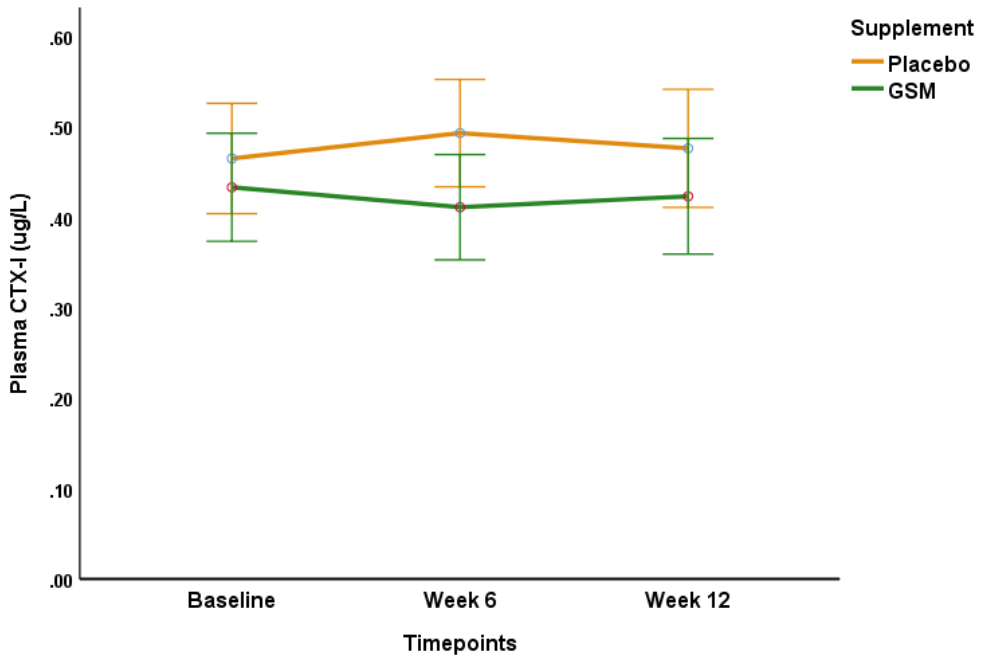


Figure S5.2. Pattern of change in plasma CTX-I level over the study period (baseline, follow-up, and endpoint) within each of the treatment groups. No significant neither over time nor between the groups.

Table S5.1. The proximate nutritional composition and fatty acids profile of whole greenshell mussel (GSM) powder and placebo (sunflower seed protein).

	GSM powder (g/100 g)	Placebo (g/100 g)
Crude protein	41.4	24.3
Carbohydrate	30.8	66.6
Ash (minerals)	10.7	2.7
Fat	10.1	3
Moisture	7	3.4
Fatty acid profile (% total fatty acids)		
C14:0 myristic acid	6.4	0
C16:0 palmitic acid	16.3	10
C16:1 palmitoleic acid	12.3	0
C18:0 stearic acid	4.3	5.8
C18:1n7 vaccenic acid	3.6	0.62
C18:1n9c oleic acid	1.3	34.4
C18:2n6c linoleic acid	1.7	33.1
C20:4n6 arachidonic acid (AA)	1.3	0
C20:5n3 eicosapentaenoic acid (EPA)	20.7	0
C22:5n3 docosapentaenoic acid (DPA)	1.1	0
C22:6n3 docosahexaenoic acid (DHA)	8	0
C20:4n3 eicosatetraenoic acid (ETA)	0.35	0

Table S5.2. mean ± SD of blood parameters for safety assessment among participants (n=47)

	Reference range ǂ	Placebo (n=23)	GSM (n=24)	P-value*
Total Cholesterol (mmol/L)	0-5.0			
Baseline		6.0±0.9	5.6±1.0	0.1
Endpoint		6.2±0.9	5.9±1.0	0.2
TG (mmol/L)	0-2.0			
Baseline		1.6±0.7	1.7±0.7	0.6
Endpoint		1.7±0.8	1.9±0.8	0.5
LDL (mmol/L)	< 2.5			
Baseline		3.6±0.8	3.3±1.0	0.2
Endpoint		3.6±0.8	3.4±1.0	0.4
HDL (mmol/L)	1.0-0.9			
Baseline		1.6±0.4	1.5±0.3	0.1
Endpoint		1.7±0.4	1.5±0.3	0.05
Total cholesterol/HDL	< 4.5			
Baseline		3.7±0.9	3.9±1.1	0.5
Endpoint		3.7±0.8	3.9±1.1	0.4
HbA1c (mmol/mol)	20-40			
Baseline		36.9±4.1	37.1±2.8	0.8
Endpoint		36.8±3.9	37.0±3.4	0.8
Bilirubin (µmol/L)	2-24			
Baseline		8.7±5.1	6.9±2.7	0.1
Endpoint		8.4±4.9	6.4±3.1	0.09
ALP (U/L)	20-110			
Baseline		83.8±22.3	85.8±18.4	0.7
Endpoint		85.1±21	89.9±21.4	0.4
GGT (U/L)	10-35			
Baseline		21.5±10.3	21.2±16.2	0.9
Endpoint		21.8±8.6	17.7±7.0	0.08
ALT (U/L)	0-45			
Baseline		18.4±7.4	19.8±8.2	0.5
Endpoint		18.7±5.0	18.5±7.4	0.9
AST (U/L)	10-45			
Baseline		19.4±3.2	22.0±6.1	0.08
Endpoint		20.5±3.8	21.2±6.0	0.6
Total protein (g/L)	65-80			
Baseline		70.0±4.3	69.8±2.9	0.8
Endpoint		70.1±4.0	70.5±4.2	0.7
Albumin (g/L)	32-48			
Baseline		38.5±2.0	39.2±2.3	0.2
Endpoint		38.1±2.4	39.0±2.0	0.1
Calcium (mmol/L)	2.10-2.55			
Baseline		2.3±0.09	2.3±0.06	0.8
Endpoint		2.4±0.15	2.3±0.08	0.4
Creatinine (µmol/L)	45-90			
Baseline		74.7±11.3	78.2±10.2	0.2
Endpoint		73.3±11.1	75.5±9.5	0.4
eGFR (ml/min/1.73 m^2)	60-89			
Baseline		75.8±12.1	70.1±11.1	0.1
Endpoint		76.3±11.3	72.9±11.0	0.2

ǂ Reference ranges provided by the Palmerston North hospital-based clinical laboratory

* The differences between group at baseline and endpoint were determined by Student's t-test.

Appendix 4 Supplementary file of chapter 7

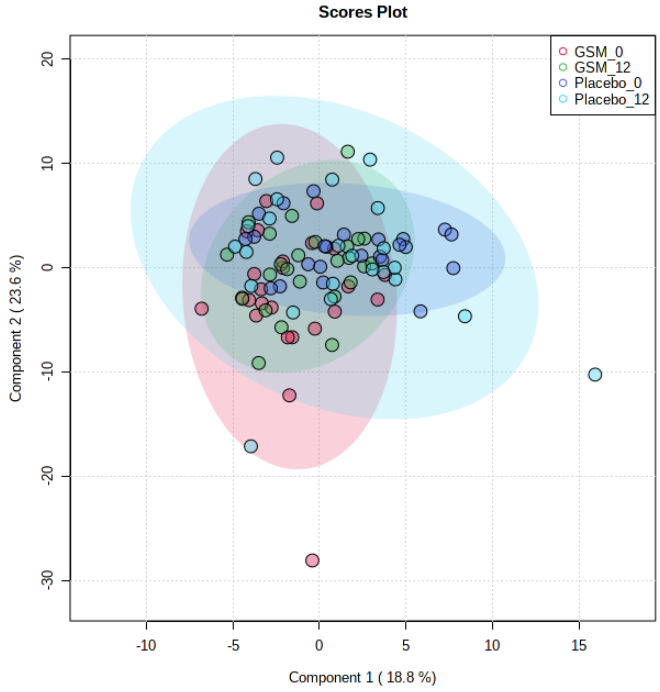
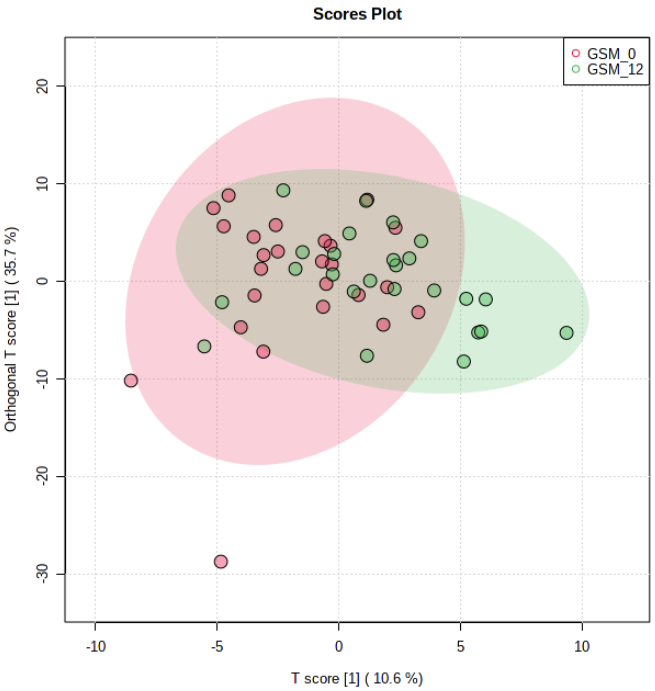


Figure S7.1. OPLS-DA analysis for lipidomic data between treatment group and their time points. Each circle represents the lipid profile of an individual. Red = greenshell mussel powder at week 0, Green: greenshell mussel powder at week 12, Purple: placebo at week 0, Blue: placebo at week 12. The data did not show good separation and robust modelling. The goodness of fit (R2) =0.09 and predictive ability of model (Q2) = -0.19.

(A)



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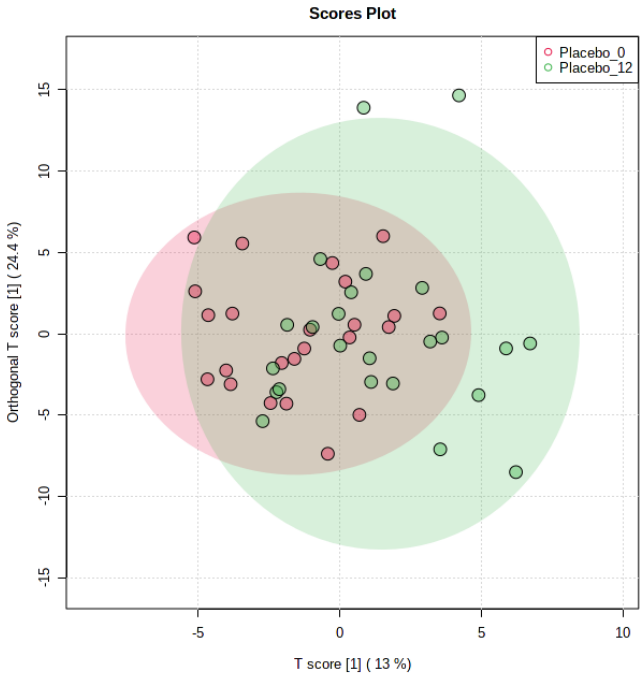


Figure S7.2. OPLS-DA analysis for lipidomic data of GSM (A) and placebo group (B) over their time points. Each circle represents the lipid profile of an individual. (A) Red = greenshell mussel powder at week 0, Green: greenshell mussel powder at week 12. The data did not show good separation and robust modelling. The goodness of fit (R2)=0.086 and predictive ability of model (Q2)= -0.101. (B) Red = placebo at week 0, Green: placebo at week 12. The data did not show good separation and robust modelling. The goodness of fit (R2)= 0.125 and predictive ability of model (Q2)= -0.239.

Table S7.1. The details of lipid species selected by the ASCA analysis.

Lipid name	Average Rt(min)	Average Mz	Adduct type	MS2 fragmentation
CE 20:5	11.367	688.60217	[M+NH4]+	
Cer 41:1 (d18:1/23:0)	10.716	636.62811	[M+H]+	
Cer 42:1 (d18:1/24:0)	10.792	632.63287	[M+H-H2O]+	
Cer 44:1(d18:1/26:0)	10.721	694.68036	[M+H]+	
HexCer 42:3 (d18:1/24:2)	10.488	806.64752	[M+H]+	
LPC 18:3	3.243	518.3208	[M+H]+	
LPC O-16:0	3.54	482.36008	[M+H]+	
LPC O-16:2	3.251	478.32782	[M+H]+	
PC 36:6	7.111	778.53473	[M+H]+	PC (18:3_18:3)
PC 40:7	8.374	832.58276	[M+H]+	PC (18:1_22:6)
PC O-36:3	8.602	770.60406	[M+H]+	
SM 42:1 (d18:1/24:0)	10.584	815.69897	[M+H]+	
ST 29:1	11.766	397.38181	[M+H-H2O]+	
TG 54:6	11.236	901.724	[M+Na]+	TG (18:2_18:2_18:2)
TG 54:7	11.156	899.70709	[M+Na]+	TG (15:2_18:2_21:3)
TG 56:4	11.576	928.83124	[M+NH4]+	TG (18:0_18:1_20:3)
TG 58:2	12.131	960.89307	[M+NH4]+	TG (22:0_18:1_18:1)

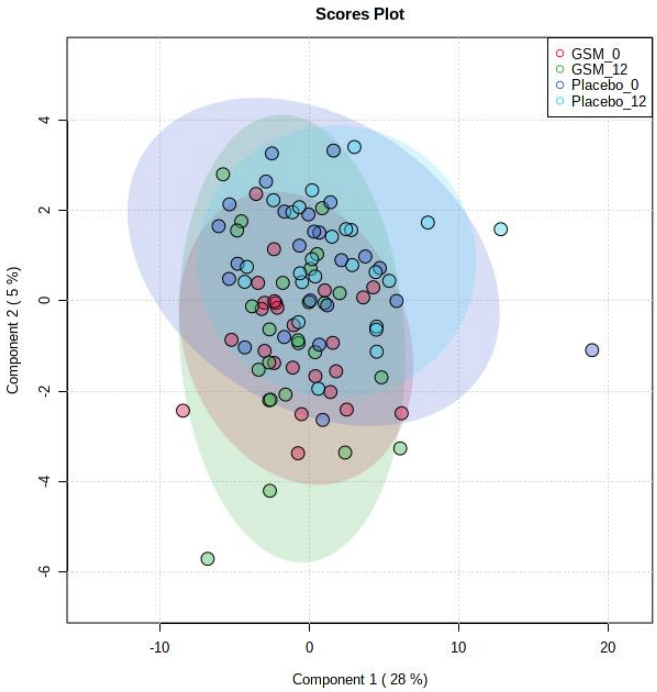
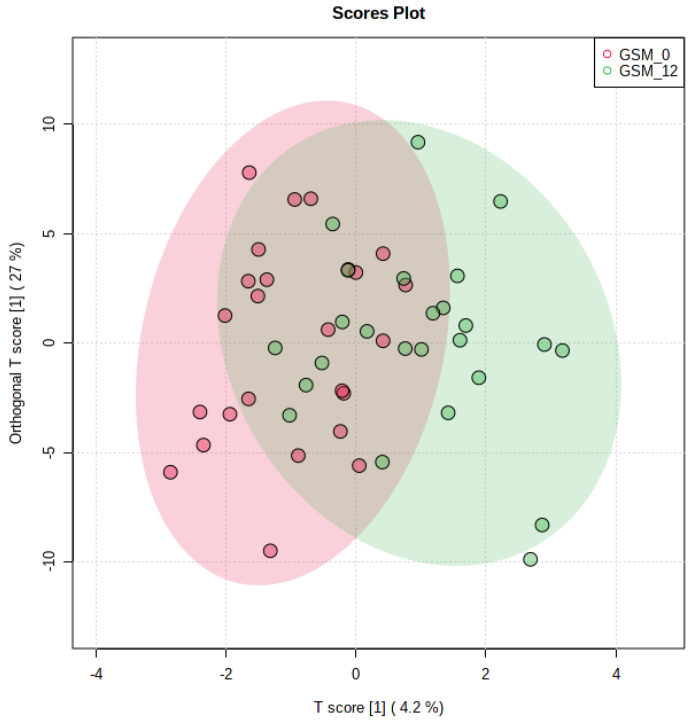


Figure S7.3. OPLS-DA analysis for metabolomic data between treatment group and their time points. Each circle represents the lipid profile of an individual. Red = greenshell mussel powder at week 0, Green: greenshell mussel powder at week 12, Purple: placebo at week 0, Blue: placebo at week 12. The data did not show good separation and robust modelling. The goodness of fit (R2) =0.07 and predictive ability of model (Q2) = -0.02.

(A)



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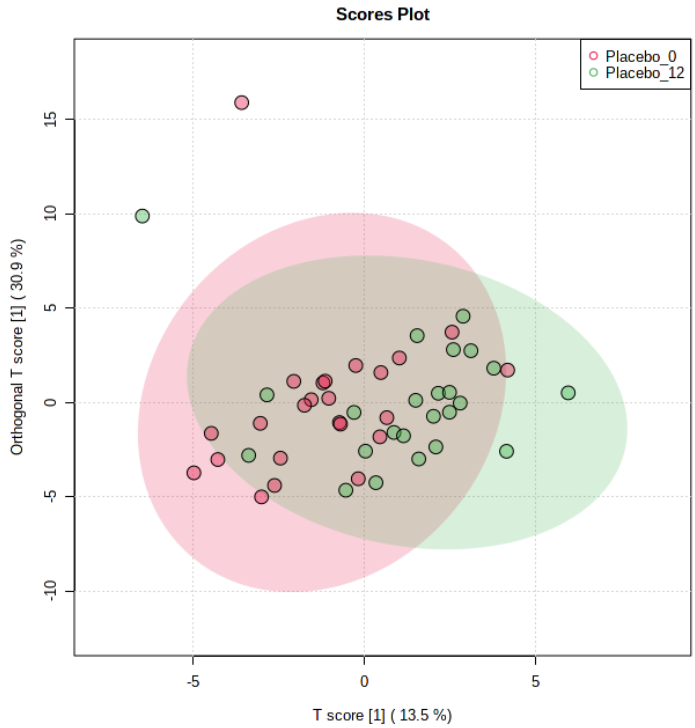


Figure S7.4. OPLS-DA analysis for metabolomic data of GSM (A) and placebo group (B) over their time points. Each circle represents the lipid profile of an individual. (A) Red = GSM at week 0, Green: GSM at week 12. The data did not show good separation and robust modelling. The goodness of fit (R2) = 0.322 and predictive ability of model (Q2) = -0.570. (B) Red = placebo at week 0, Green: placebo at week 12. The goodness of fit (R2) = 0.089 and predictive ability of model (Q2) = -0.189.

Appendix 5 Authors' contributions

Chapters and peer-reviewed papers	Authors	Contribution
Mass Spectrometry-Based Metabolomic and Lipidomic Analysis of the Effect of High Fat/High Sugar Diet and Greenshell™ Mussel Feeding on Plasma of Ovariectomized Rats (Chapter 4) Impact of Greenshell™ Mussel supplementation on plasma lipids and polar metabolites in postmenopausal women (Chapter 7)	Maryam Abshirini	The candidate was involved with methodology, data analysis, writing the original draft, editing.
	Diana Cabrera	Dr. Cabrera was involved in methodology and data analysis, reviewing, and revising the manuscript.
	Karl Fraser	Dr. Fraser was involved with methodology, reviewing, and revising the manuscript.
	Parkpoom Siriarchavatana	Dr. Siriarchavatana designed the methodology and collected the data (For chapter 4).
	Frances M. Wolber	Dr. Wolber was involved with conceptualization, providing the funding, supervision, and reviewing the manuscript
	Matthew R. Miller	Dr. Miller was involved with conceptualization, and funding acquisition, and reviewing the manuscript.
	Hong Sabrina Tian	Dr. Tian was involved in the funding acquisition and reviewing the manuscript.
	Marlena C. Kruger	Prof. Kruger was involved with conceptualization, providing the funding, research design, and

		reviewing the manuscript. She is the candidate's main supervisor.
Green-lipped Greenshell™ Mussel (*Perna canaliculus*) extract supplementation in treatment of osteoarthritis: a systematic review (Chapter 3)	Maryam Abshirini	The candidate was involved with data extraction, data analysis, and writing the original draft.
	Jane Coad	Prof. Coad was involved with reviewing and editing the manuscript.
	Frances M. Wolber	Dr. Wolber was involved with reviewing and editing the manuscript.
	Pamela von Hurst	Prof. von Hurst was involved with reviewing and editing the manuscript.
	Matthew R. Miller	Dr. Miller was involved with reviewing and editing the manuscript.
	Hong Sabrina Tian	Dr. Tian was involved with reviewing and editing the manuscript.
	Marlena C. Kruger	Prof. Kruger was involved with conceptualization, methodology, reviewing and editing the manuscript. She is the candidate's main supervisor.
Effects of Greenshell™ Mussel intervention on biomarkers of cartilage metabolism, inflammatory markers and joint symptoms in overweight/obese postmenopausal women (Chapter 5), Effect of Greenshell™ Mussel supplementation on faecal microbiota, body composition and iron status markers in overweight and obese postmenopausal women (Chapter 6)	Maryam Abshirini	The candidate was involved with ethics application, proposal writing, methodology, data collection, data analysis, writing the original draft, editing.
	Jane Coad	Prof. Coad was involved with conceptualization, methodology, supervision, reviewing and editing the manuscript.
	Frances M. Wolber	Dr. Wolber was involved with conceptualization, methodology, supervision, reviewing and editing the manuscript.
	Pamela von Hurst	Prof. von Hurst was involved with conceptualization, methodology,

		supervision, reviewing and editing the manuscript.
	Matthew R. Miller	Dr. Miller was involved with funding acquisition, reviewing and editing the manuscript.
	Hong Sabrina Tian	Dr. Tian was involved with funding acquisition, reviewing and editing the manuscript.
	Marlena C. Kruger	Prof. Kruger was involved with conceptualization, providing the funding, research design, reading and reviewing the manuscript. She is the candidate's main supervisor.

Appendix 6 Information sheet, consent form and questionnaires

Participants Information Sheet

MASSEY UNIVERSITY
COLLEGE OF HEALTH
TE KURA HAUORA TANGATA

The Effect of Green Shell Mussel Powder Versus Control on Cartilage Biomarker Responses and Inflammation in Older Women

You are invited to take part in in a dietary intervention study to determine the effect of supplementation with green shell mussel powder (3 gram per day) for 3 months on joint and inflammatory markers in healthy postmenopausal women.
The information sheet will inform you on why we are doing this trial, whether you meet the study inclusion criteria, what is involved in the study, the risks, and benefits of participating in the study and participants' rights.
If you agree to participate in our study, you will need to complete a Consent Form.
The contact's detail is as follow:

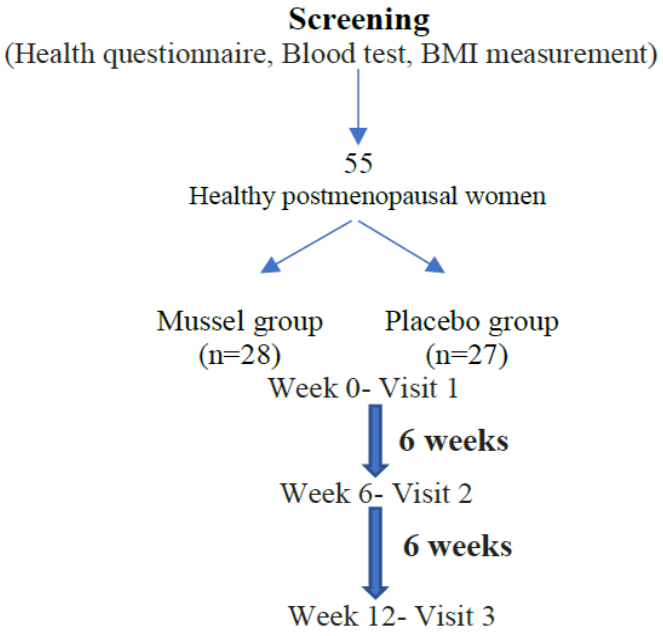
)octoral Candidate, Manager:
Maryam Abshirini
chool of Health Sciences, College of Health,
Massey University, Palmerston North, New
ealand
Mobile: [redacted]
mail: Maryam.Abshirini.1@uni.massey.ac.nz

Principal Investigator:
Professor Marlena Kruger
School of Health Sciences, College of Health, Massey University, Palmerston North, New Zealand
Phone: +6469517571
Email: M.C.Kruger@massey.ac.nz

Why are we doing this trial?

Women at postmenopausal years (55 years and older) are highly susceptible to develop joint /bone problems such as osteoarthritis. Decline in estrogen levels plays an important role in developing osteoarthritis. Moreover, many women experience weight gain at this stage, resulting in more stress on weight bearing joints like the knees and hips, causing joint tissue to get thinner and become very stiff and painful.
The green lipped or green shell mussel is a marine species native to New Zealand. It is rich in healthy fat such as long-chain omega-3 polyunsaturated fatty acids. A recent study conducted at Massey University showed whole meat green shell mussel powder has protective effect against the early stage of osteoarthritis associated with obesity. So, it is possible that green shell mussel powder has the same beneficial effect in human joints.
In this study we would like to investigate the effect of whole meat green shell mussel powder versus sunflower seed protein powder (control) on cartilage and biomarkers of inflammation in postmenopausal women.

Flow of our study:



Who are we looking for?

Fifty healthy postmenopausal women aged 55-75 years, to fit in to our study you should:

- Be post-menopause at least five year after the absence of menstruation.
- Have a body mass index (BMI) of 25-35 kg/m^2
- Non-smoker and not drinking the alcohol regularly
- No history of joint trauma or injury
- Not allergic to mussels or seafood
- Have no heart disease, diabetes
- Have no gastrointestinal diseases other than appendicitis
- Have no kidney or liver diseases or bleeding disorder and taking anticoagulants and antiplatelets e.g. warfarin ,aspirin
- Not have diagnosed with osteaoarhtitist or rheumatoid arthritis
- Not be taking joint health supplements e.g. Green Lipped mussel, Glucosamine, chondroitin, collagen or be willing to stop 4 weeks before the study begins
- Not taken antibiotics within the last 3 months
- Not be taking any vitamin, mineral or omega-3 supplements or be willing to stop 4 weeks before the study begins
- Not be on hormone replacement therapy or less than 6 months prior beginning the trial.
- Not continuously (everyday) be taking anti-inflammatory drugs; glucocorticoids, and NSAID drugs e.g. aspirin, ibuprofen, naproxen, diclofenac etc.

Will I get any financial compensation?

You will receive a $200 voucher to compensate you for your time and commitment involved in the study

What is involved?

Medical assessment before the study:

After signing a consent form and reading the information sheet, we will ask you to complete a form about your health and current medications/supplement intake. We will measure your height and current body weight. Based on that your body mass index (BMI) will be calculated as weight (kg) divided by height squared (m^2). Those with BMI 25-35 will be included. Then, you will be invited to attend an appointment at the MedLab Central Palmerston North to have a blood sample taken for general health parameters such as blood glucose and cholesterol. Those with abnormal healthy parameters will be excluded from the study at this point and referred to their health professional. After enrollment, you will be asked to complete the 3-day food diary. Those participants who eat oily fish and seafood more than once per week will be asked to avoid the consumption of oily fish (salmon, sardine, pilchard, and tuna) and seafood (mussels, oyster, crab e.g.) and fish oil, omega 3 supplement, joint health supplements such as Green Lipped mussel, glucosamine, chondroitin, collagen for 4 weeks before beginning the trial until the end of trial. Following the 4 weeks washout,

we will ask you to take 6 capsules daily each containing 0.5 gram powder of whole meat green shell mussel or identical placebo (sunflower seed protein) with your meal for 12-week periods.

Questionnaires:

Demography, 3-day food diary, KOOS, VAS and NZPAQ

We will ask you to answer questions about your demographics, diet, physical activity and medications. You will be asked to complete the New Zealand Physical Activity Questionnaire (NZPAQ), and knee injury and osteoarthritis outcome score (KOOS) and visual analogue scale (VAS) pain.

Urine and blood sample:

During the study, you will be invited to attend appointments at the Human Nutrition Lab at Massey University, Palmerston North at the beginning (week 0), week 6 and week 12 to have fasting blood and urine samples taken to assess cartilage degradation markers, bone degradation marker, vitamin D status, parathyroid hormone, inflammatory biomarker, iron status level, metabolites, and other markers of interest. You will be invited to attend an appointment at the MedLab Central Palmerston North after week 12 to have a blood sample to assess blood lipids, glucose levels, liver and kidney function markers.

Faecal samples

You will need to bring a faecal sample which was collected at home or research site. The faecal sample will need to be stored in your freezer. The sample will be triple contained which means it will be stored hygienically in your freezer. Faecal samples will be collected at baseline and week 12.

Dual X ray Absorptiometry scan (DXA)

You will be invited to have a full body dual X ray absorptiometry scan (DXA) scan at the Human Nutrition Lab at Massey University, Palmerston North at two occasions, at the beginning, and week 12 to measure body composition (fat/lean mass).

Before each visit you will receive an email or text messages confirming your appointment time

Time involved:

The total time for involvement in study will be approximately 5-6 hours over 12 weeks.

A schedule of measurements is given below:

Time	Time Involvement	Measurements
Before the study start (screening)	30 minutes	Questionnaire: Health screen, weight, and height measurements (through phone interview), screening blood sample at MedLab
Washout (-4 week to week 0)		
Week 0 –Visit 1	1.5-2 hours	Questionnaire: demographic detail, physical activity and knee function and joint pain. Fasting blood and DXA scan at Human Nutrition Lab, urine, and faecal sample collection
Week 6- Visit 2	30 minutes	Fasting blood sample at Human Nutrition Research Lab, Urine sample collection
Week 12, Visit 3	2 hours	Questionnaire: knee function and pain Fasting blood and, DXA scan at Human Nutrition Lab, Urine and faecal sample collection, 3-day food diary collection, Blood sample at MedLab

Benefit and Risk

A potential benefit of being involved in this study is that you will contribute to gaining

a better understanding of the beneficial effect of green shell mussel on joint health and development of new food products to prevent osteoarthritis.
You will also receive a summary of the main findings of the study which will either be posted or emailed to you.
As with all blood tests there may be some discomfort when the needle is inserted. You may also develop a bruise after the blood sample is taken, however this is unlikely. A certified phlebotomist will draw the blood. We will also use whole body DXA scan, on our Hologic DXA machine to assess your body composition. While no dose of radiation is harmless this dose is very low and unlikely to cause harm. The total effective dose of radiation to which you will be exposed to is 10 microsieverts (µSv), which is much lower than the range normally used in medical diagnostics. To place in perspective, the amount of radiation you are exposed to during a flight to the United Kingdom return is 100 µSv and from a dental Xray 50 µSv.
Should there be any abnormalities detected in your blood samples which MedLab and Human Nutrition Research Unit at Massey University will analyse, you will be referred to your General Practitioner.

Who will see the information about me?

When you join the trial, you will be given a number and thereafter all information will be filed with the code number and stored in a locked filing cabinet accessed by the research personnel only. The anonymous information will be used in academic presentations, publications, and reports to the funders. All personal data will be destroyed at the end of the trial. Scientific data, filed on paper, will be shredded and electronic data will be deleted from our computer records and databases after 10 years. For the first 5 years paper data will be stored in a locked filing cupboard within a locked office. For the last 5 years it will be stored in a secure archive where all data is stored in boxes labelled by barcode only. It is accessible by nominated staff only who require pin numbers for ID.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic, and your claim will be assessed by ACC in accordance with the Accident Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.
If your ACC claim is not accepted, you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

What are my rights?

You are under no obligation to accept this invitation to take part in this study. If you decide to participate, you have the right to:

- Decline to answer any particular question
- Withdraw from this study (at any time without having to give a reason);
- Ask any questions about this 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.

You will be given the opportunity to ask any questions you may have throughout the study.

Funding

This study is funded by the National Science Challenge, High Value Nutrition (HVN) "Musseling up: High-value Greenshell Mussel Foods", as a collaboration between Massey University, Cawthron Institute, Plant and Food Research Ltd and Sanford Ltd. HVN is underwritten by the Ministry of Business, Innovation and Employment (MBIE).

Committee Approval Statement

This project has been reviewed and approved by Massey University Human Ethics Committee: Southern A, Application 20/03. If you have any concerns about the conducts of this research, please contact Dr Negar Partow, Chair, Massey University Human Ethics Committee: Southern A, Telephone 04 801 5799 x 63363 or email: humanethicsoutha@massey.ac.nz

Please feel free to contact the researcher if you have any questions about this study Thank you for expressing interest in participating in this study

Screening questionnaire (Greenshell mussel study)

Contact Details

First Name*: _____
Last Name*: _____
Email Address*: _____
Mobile Number: _____
Postal Address:*_____
Post code*: _____

Please provide height*_____cm
Please provide weight* _____Kg
We will use these measurements to calculate your BMI to determine your eligibility.
Date of Birth (dd/mm/yyyy)*
_____

1. Please answer the following questions.*

	Yes	No	Unsure
Have you been postmenopausal for at least 5 years?			
In the last 3 months, have you been experiencing any joint discomfort when resting or going up and down the stairs?			
Are you willing to have urine and blood samples taken?			

2. Are you able to physically attend the research site for 3 study visits as well as comply with the following study requirements for over 12 weeks?
    - Follow a diet recommended by the study
    - Take 6 capsules of the dietary supplement daily
    - Complete questionnaires and food diary entries
    - Undergo a Dual X ray Absorptiometry scan (DXA)

    ( ) Yes

    ( ) No

    ( ) Unsure

3. Do you have any of the following medical conditions? Please check all that apply:

    ( ) Heart diseases

    ( ) Diabetes

    ( ) High Blood Fat (Lipids)

    ( ) Stomach (Gastrointestinal) Diseases or Disorders (except for appendicitis)

( ) Kidney diseases

( ) Liver diseases

( ) Bleeding disorders

( ) Osteoarthritis or rheumatoid arthritis

( ) None of the above

4. Are you currently taking any of the below supplements?
    - Joint health supplements (e.g. Green Lipped mussel, Glucosamine, Chondroitin, Collagen)
    - Fish oil or Omega-3 supplements
    - Willing to bring faecal samples collected at home

    ( ) Yes

    ( ) No

    ( ) Unsure

5. (If Yes) Are you willing to stop taking these supplements?

    ( ) Yes

    ( ) No

    ( ) Unsure

6. Are you currently taking any of the following medications? Please check all that apply:

    ( ) Anticoagulants or antiplatelets (e.g. warfarin, aspirin)

    ( ) Hormone replacement therapy

    ( ) Anti-inflammatory medication, glucocorticoids, and NSAID medication (e.g. aspirin, ibuprofen, naproxen, diclofenac etc.)

    ( ) None of the above

7. Do you take these Anti-inflammatory medication, glucocorticoids, and NSAID medication daily?

    ( ) Yes

    ( ) No

    ( ) Unsure

8. Please answer the following questions.

	Yes	No	Unsure
Have you taken any antibiotics within the past 3 months?			
Do you smoke and/or drink alcohol regularly? This means that you drink more than 2 standard drinks per day. Please note that 1 standard drink is equivalent to 1 of either the following: ● 330 ml of standard beer ● 100ml of wine ● 30ml of spirits			
Are you taking medication daily for your joint discomfort?			
Do you have a history of joint trauma or injury?			
Are you allergic to mussels or seafood?			

9. Are you committed to answering your phone when the research team reaches out to you at the appointed date and time that you choose?

   ( ) Yes

   ( ) No

10. If you are unable to attend an appointment at the research site, are you willing to inform the study staff?
    ( ) Yes, I will definitely let the study staff know if I can no longer attend or change my mind about attending

    ( ) No, I cannot be sure that I will let the study staff know if I can not or no longer wish to attend

The Effect of Green Shell Mussel Foods Versus Control Food on Cartilage Biomarker Responses and Inflammation In The postmenopausal Women

PARTICIPANT CONSENT FORM

This consent form will be held for a period of five (5) years

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

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

Signature:……..

Full Name: ……

Date: ……..

Demographics and Medical History Questionnaire

ID:

Date ……

Years of education: ……
Highest qualification: ……
Occupation: ……

Ethnic origin:

NZ European ☐ Maori ☐ Samoan ☐ Cook Island Maori ☐

Tongan ☐ Indian ☐ Chinese ☐ Other ☐ please specify: ……

Have you ever been diagnosed with any of the following conditions? (Please tick for the yes)

Osteoporosis	
Fractures	
Anaemia or iron deficiency	
High blood pressure	
Stroke	
Gastrointestinal bleeding (e.g. Gastric or peptic ulcers…)	
Food intolerance or allergies causing diarrhoea, bloating, cramping or constipation	
Long term diarrhoea or constipation	
Other please specify:	

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: _____

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)?

	Absent	Mild	Moderate	Severe
Abdominal pain				
Abdominal bloating				
Flatulence/wind				

- **Please list below if you are you taking medication or supplements, please specify how much and how often do you take them?**

  ……
  ……

  ……
  ……

  ……
  ……

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

New Zealand Physical Activity Questionnaire-Short Form

ID:

Date.....

I am going to ask you about the time you spent being physically active in the last 7 days. 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.'

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 excersice or leisure.

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

☐ .....days per week

☐ (GO TO 2) None (GO TO 3)

2. How much 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 **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 shown on this care (**Showcard 1- Moderate Physical Activity**). Do not include walking of any kind.

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

   ☐☐.....days per week

   ☐(GO TO 4) None (GO TO 5)

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

   ☐☐.....hours. ..... minutes

Vigorous physical activity

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

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

   ☐☐.....days per week

   ☐(GO TO 6) None (GO TO 7)

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

   ☐☐.....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

Notes:

NZPAQ - Short Form Showcards

Showcard 1: Moderate PhysicalActivity

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
Rubgy 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	

Visual Analogue Scale (VAS) for joint pain assessment

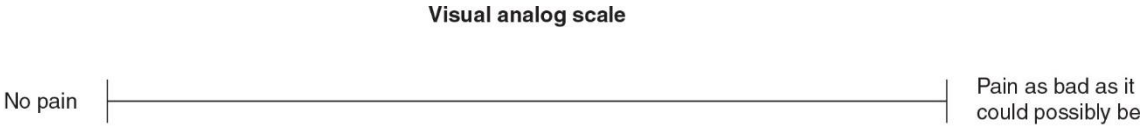
ID:

Date ……

Time point: …….

I. On scale of "No Pain" to the "Pain as Bad As it Could Possibly Be", how was your joint pain, on average, in the past week?

II.



III.

ID :

Date…….

3 Day Food Record

" The Effect of Green Shell Mussel Powder Versus Control on Cartilage Biomarker Responses and Inflammation In Elderly Women".

Thank you very much for taking part in our study We are extremely grateful for your time, and commitment!

(2 weekdays and one weekend)

If you have any questions, please contact ***Maryam Abshirini*** [redacted] *or email* Maryam.Abshirini.1@uni.massey.ac.nz

OR Professor Marlena Kruger during working hours on
(06) 951 7571
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 when you come for your next appointment

3-day food diary - What to do?

- Record all that you eat and drink on the 3 day over non-consecutive days (including one weekend day)

- 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	I 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

Time food was eaten	**Complete description of food (food and beverage name, brand, variety, preparation method)**	**Amount consumed (units, measures, weight)**
Example7: 55am	Sanitarium weetbix	2 weetbix
" "	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)
10.00 am	Raw Apple (gala)	Ate all of apple except the core, whole apple was 125g (core was ¼ of whole apple)
12.00 pm	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

Date_____ **DAY 1**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date_____ **DAY 1 continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recipes (Day 1)

Date_____ DAY 2

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date_____ **DAY 2 continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recepie (Day 2)

Date_____ **DAY 3**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date_____ **DAY 3 continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recipes (Day 3)

FASTING URINE AND FAECAL SAMPLE

COLLECTION INSTRUCTION

Please kindly read carefully

If you regularly have a bowel motion in the morning, please collect the fasting urine and faecal sample on the morning of your visit.

Once the faecal sample has been collected, please bring this sample to the Massey University Human Nutrition Research Unit or freeze. Thank you.

You have been provided with the following:

- A pair of disposable gloves
- A urine sample container
- A Styrofoam rectangular container
- A stool sample container
- Anaerobic bag
- Anaerobic sachet
- A cooler bag
- A freezer pack
- A brown paper bag

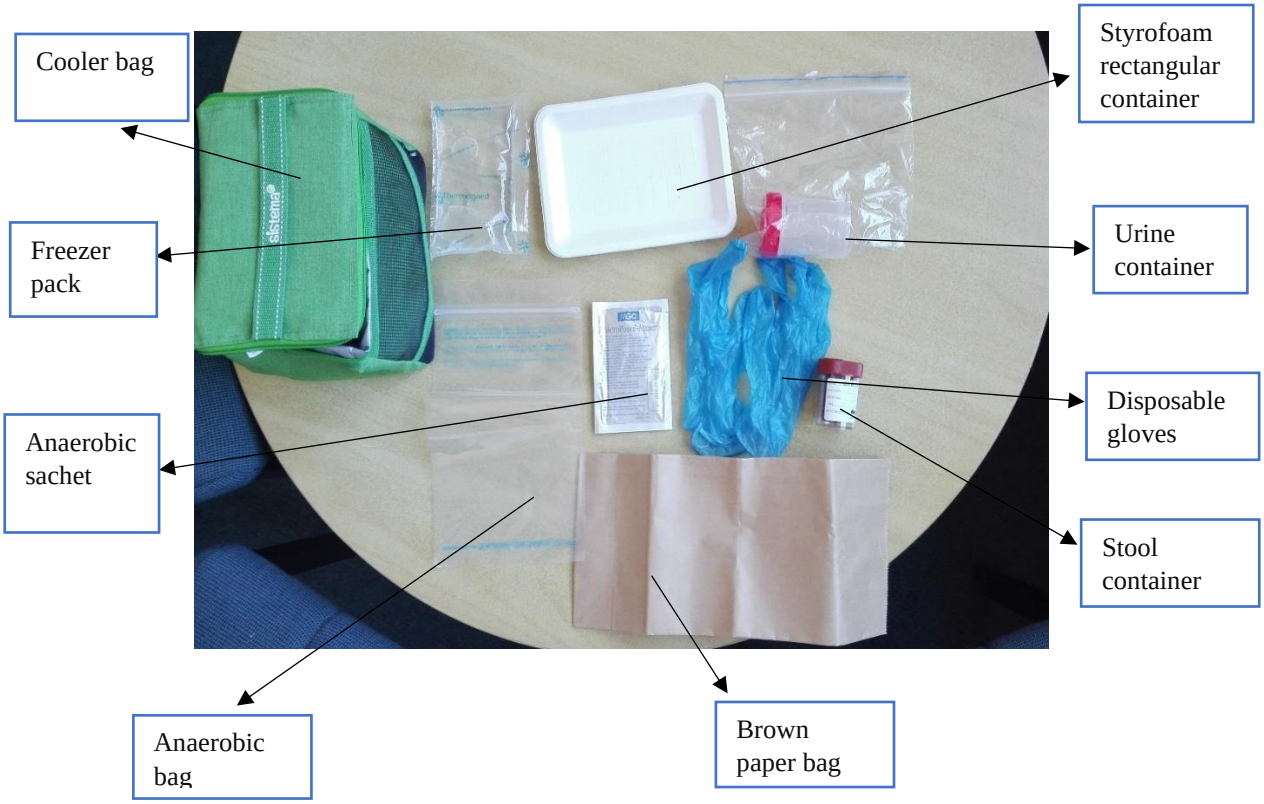
Instructions:

1. Use the **disposable gloves** while collecting the urine and faecal sample.
2. Please put the sterile **freezer pack** in the freezer to become frozen.
3. To obtain the urine sample please collect a clean-catch **midstream fasting urine sample** into the **urine sample container** to at least ½ full. Ensure the lid on the **urine sample container** is sealed and secure in its bag.
4. To obtain the faecal sample (please try to fill the **stool sample container** to at least a 1/3 full):

a. Catch the faecal sample using the **styrofoam rectangular container** (lined with toilet paper) before it reaches the toilet water. Use the **scoop on the lid** to transfer some of the faecal sample to the **stool sample container** (be careful not to contaminate the sample with any urine) OR

b. Void the faecal sample straight into the **stool sample container** (be careful not to contaminate the sample with any urine).

5. Ensure the lid on the **stool sample container** is secure and then seal the **stool sample container** in the **anaerobic bag** with the **anaerobic sachet** inside (remove the tinfoil outer packaging to reveal the white anaerobic sachet before putting the anaerobic sachet in the anaerobic bag). **Disposable gloves** can be removed at this point.
6. The **anaerobic bag** can then be put into the **cooler bag** prior to putting it into the freezer. The faecal sample is now triple contained and can be handled safely.
7. Excess faeces can be disposed of down the toilet and any used items can be put into the **brown paper bag** provided.
8. The **brown paper bag** can then be disposed of in the rubbish.
9. When transporting the urine and faecal sample to the Massey University Human Nutrition Research Unit, put the frozen **freezer pack** into the **cooler bag** with the contained urine and faecal sample to ensure it stays cool. Thank you



Compliance chart

ID	

Please record **the study supplements and all the medication and any vitamins, minerals or herbal supplements or antibiotic you took apart from the study supplements** since the start of study on the specified date, please specify the name of medication and how much you take (for example 1 paracetamol capsule/ tablets)

ID	day	date	Study capsules taken	Other Medication or supplement taken
Week 1	Monday			
Week 1	Tuesday			
Week 1	Wednesday			
Week 1	Thursday			
Week 1	Friday			
Week 1	Saturday			
Week 1	Sunday			
Week 2	Monday			
Week 2	Tuesday			
Week 2	Wednesday			
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Week 2	Saturday			
Week 2	Sunday			
Week 3	Monday			
Week 3	Tuesday			
Week 3	Wednesday			
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Week 3	Saturday			
Week 3	Sunday			
Week 4	Monday			
Week 4	Tuesday			
Week 4	Wednesday			
Week 4	Thursday			
Week 4	Friday			
Week 4	Saturday			
Week 4	Sunday			

ID	day	date	Study capsules taken	Other Medication or supplement taken
Week 5	Monday			
Week 5	Tuesday			
Week 5	Wednesday			
Week 5	Thursday			
Week 5	Friday			
Week 5	Saturday			
Week 5	Sunday			
Week 6	Monday			
Week 6	Tuesday			
Week 6	Wednesday			
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Week 6	Sunday			
Week 7	Monday			
Week 7	Tuesday			
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Week 7	Sunday			
Week 8	Monday			
Week 8	Tuesday			
Week 8	Wednesday			
Week 8	Thursday			
Week 8	Friday			
Week 8	Saturday			
Week 8	Sunday			

ID	day	date	Study capsules taken	Other Medication or supplement taken
Week 9	Monday			
Week 9	Tuesday			
Week 9	Wednesday			
Week 9	Thursday			
Week 9	Friday			
Week 9	Saturday			
Week 9	Sunday			
Week 10	Monday			
Week 10	Tuesday			
Week 10	Wednesday			
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Week 12	Monday			
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Week 12	Saturday			
Week 12	Sunday			