EFFECT OF β-GLUCAN MOLECULAR WEIGHT AND VISCOSITY ON THE MECHANISM OF CHOLESTEROL LOWERING IN HUMANS

by

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A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

The cholesterol-lowering effect of mixed linkage (1→3) (1→4)-β-D-glucans (β-glucan) from barley has been documented, yet the underlying mechanism responsible for this action and factors influencing it, such as physicochemical properties of β-glucan and genetic background of an individual, remain unclear.As a component of dietary fibre, β-glucan also has the potential to shift the gut microbial community, however, whether alterations in the gut microbiota are associated with the physiological effects of β-glucan have yet to be determined.

This study was designed to assess the effects of β-glucan molecular weight (MW) and dose on loweringserum cholesterol levels and to elucidate its mechanism of action in human subjects. Additionally, this study examined gene-diet interactions as well as changes in the gut microbiota profile following consumption of barley foods. In a controlled four phase crossover trial, mildly hypercholesterolemic but otherwise healthy subjects (n =30) were randomly assigned to receive breakfasts containing 3g high MW (HMW), 5g low molecular weight (LMW), 3g LMW barley β-glucan or a control diet with wheat and rice (WR control), each for 5 weeks. The washout period between the phases was 4 weeks.

The consumption of 3g/d HMW diet lowered total cholesterol (TC) compared with WR control diet (P =0.0046), but not the LMW diet at either 3g/d or 5g/d. Individuals with the SNP rs3808607-G allele of *CYP7A1* had greater TC reduction in response to 3g/d HMW β-glucan diet compared to the individuals carrying homozygous TT alleles ($P<0.01$). Cholesterol absorption

and synthesis were not changed, but bile acid synthesis increased by 3g/d HMW diet compared to the control. Consuming 3g HMW/d β-glucan altered gut microbiota at the phylum and genus levels and the impacted microbial members was correlated with favorable shifts of cardiovascular disease risk factors.

In conclusion, physicochemical properties of β-glucan play critical roles in the cholesterol-lowering effect and gut microbiota alteration ability of β-glucan. The results suggest the increasing bile acid synthesis rather than inhibiting cholesterol absorption and synthesis is the mechanism responsible for the cholesterol reducing property of β-glucan.The altered microbiota profile by HMW β-glucan is associated with its physiological effect.

ACKNOWLEDGMENTS

FOREWORD

This thesis is written to satisfy the graduation requirements of the doctoral program at the Department of Human Nutritional Sciences at the University of Manitoba. The project "Effect of β-glucan molecular weight and viscosity on the mechanism of cholesterol lowering in humans" is funded by Agriculture Agri-Food Canada Growing Forward program. This thesis is written in manuscript format and it comprises one literature review and three individual manuscripts, which are either submitted to a peer-review journal or under preparation for publication. Chapter *3 High molecular weight β-glucan decreases serum cholesterol differentially based on the CYP7A1 rs3808607 polymorphism in mildly hypercholesterolemic adults* has been submitted to The Journal of Nutrition and currently under review. Chapter 5 *High molecular weight barley β-glucan promotes a favorable human gut microbiota associated with reduced cardiovascular disease risk* has been submitted to Frontiers in Microbiology and currently under review. Chapter 4 *Mechanisms of cholesterol-lowering effect of barley β-glucan* and will be submitted to The American Journal of Clinical Nutrition. Chapter 2 *Literature review* will be revised as a narrative review and the target journal for submission is Nutrition Reviews. The contributions of the first author and coauthors of each manuscript are listed at the end of chapters 3, 4 and 5 before the sections of references.

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7α-HC	7α-Hydroxy-4-cholesten-3-one
ABCG5	ATP binding cassette sub-family members G5
ABCG8	ATP binding cassette sub-family members G8
AHA	American Heart Association
APOA1	ApolipoproteinA1
APOB	Apolipoprotein B
APOCI	Apolipoprotein C I
APOCII	Apolipoprotein C II
APOE	Apolipoprotein E
AUC	Area under curve
BMI	Body mass index
BP	Blood pressure
CA	Cholic acid
CAZymes	Carbohydrate active enzymes
CDCA	Chenodeoxycholic acids
CDI	*Clostridium difficile* infection
CHD	Coronary heart disease
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7-alpha-hydroxylase
CYP27A1	Sterol 27-hydroxylase
D_2O	Deuterium oxide

DP	Degree of polymerization
FISH	Fluorescence *in situ* hybridization
FMT	Fecal microbiota transplantation
FSR	Fractional rate of synthesis
FTR	Fractional turnover rates
FXR	Farnesoid X receptor
β-Glucan	(1→3) (1→4)-β-D-glucan
GI	Gastrointestinal
HDL	High-density lipoprotein
HDL-C	HDL cholesterol
HMGCR	3-Hydroxy-3-methylglutaryl-coenzyme A reductase
HMGCS	3-Hydroxy-3-methylglutaryl-coenzyme A synthase
HMW	High molecular weight
LDL	Low-density lipoprotein
LDL-C	LDL cholesterol
LDL-R	LDL-receptor
LOD	Limit of detection
LWM	Low molecular weight
LXR	Liver X receptor
MAF	Minor allele frequency
MIDA	Mass isotopomer distribution analysis

MW	Molecular weight
NPC1L1	Niemann Pick Disease Type C1 Like-1
OUT	Operational taxonomic units
PCoA	Principal coordinate analysis
PDSIRM	Dual stable-isotope ratio method
PERMANOVA	Permutational multivariate analysis of variance
PICRUSt	Phylogenetic investigation of communities by reconstruction of unobserved states
PLS-DA	Partial least square discriminant analysis
qPCR	Quantitative real-time PCR
RBC	Red blood cells
SCFA	Short chain fatty acids
SNP	Single nucleotide polymorphism
SR-B1	Scavenger receptor class B, type 1
SREBF1(SREBP1)	Sterol response element binding protein 1
SREBF2(SREBP2)	Sterol response element binding protein 2
TC	Total cholesterol
TG	Triglyceride
UWL	Unstirred water layer
WR	Wheat and rice

CHAPTER 1: INTRODUCTION

1.1 Introduction

Cardiovascular disease (CVD) is the leading cause of death in Canada and worldwide (1, 2). About half of Canadians over 40 years old are at risk of developing CVD due to the elevated circulating cholesterol levels (3). Mediating cholesterol concentrations, especially LDL cholesterol (LDL-C) to the desirable levels (total cholesterol < 5.2 mmol/L and LDL-C < 2.6 mmol/L) via adopting a healthful lifestyle, is the primary strategy for preventing CVD (4). Consumption of cereal (1→ 3)(1 → 4) β-D-glucan (β-glucan) to reduce circulating cholesterol has been documented extensively (5, 6). Of the main commercial cereals, barley (*Hordeum vulgare* L.) is one thatcontains a higher level of β-glucan (7, 8). Canada is one of the world's largest barley producers and exporters (9, 10). However, the health benefits of barley, such as the cholesterol-lowering property, have not been well recognized by the public. In fact, the majority of barley is used for farm animal feed and alcohol production (beer and whisky) withminimal quantitiesaccounted for food production(10).

Although numerous studies support the health claim that a daily intake of a minimum of 3 g β-glucan soluble fibre from barley and oat grain products can actively reduce blood cholesterol concentrations (11-13), more and more researchers recognize that the physicochemical properties, in terms of molecular weight and viscosity, may play a role in the cholesterol-lowering effect of β-glucan (8, 14). Yet, it is not well demonstrated whether the cholesterol-lowering effect of β-

glucan depends on the daily dose only, or whether the physicochemical properties of β-glucan are also play critical roles in action.

Moreover, the mechanism responsible for the cholesterol-lowering effect of β-glucan is not well elucidated. Evidence suggests that β-glucan reduces circulating cholesterol via three different pathways: inhibiting cholesterol absorption by elevating the viscosity of intestinal content (15-17); suppressing cholesterol synthesis by the fermentation products short chain fatty acids (SCFA) (18, 19); and interrupting enterohepatic circulation of bile acids resulting in depletion of liver and circulating cholesterol (19-21). However, due to the limitations of the design and discrepant results of the available studies, these proposed mechanisms are currently more suggestive rather than definitive. Therefore, more investigations are necessary to confirm the proposed mechanisms of the cholesterol-reducing property of β-glucan.

It has been recognized that the effect of dietary intervention on changing plasma lipid concentrations differs significantly among individuals (22-24). Studies investigating whether individuals respond differently to β-glucan in lowering cholesterol are insufficient. Identifying the candidate genes for the potential gene-diet interactions is required for a better interpretation of clinical outcomes as well as for more precise dietary recommendations.

In addition, the degradation of β-glucan in humans depends on the carbohydrate active enzymes (CAZymes) produced by resident gut microorganisms referred to as gut microbiota (25, 26). As a result of fermentability, β-glucan possibly alters the gut microbiota. The products generated from the fermentation may contribute toward lowering cholesterol levels and improve other metabolic

parameters (27). The advances in sequencing techniques coupled with bioinformatic tools have accelerated our understanding of gut microbiota (28), which plays crucial roles in human health and diseases (29, 30). However, the influence of β-glucan consumption on the gut microbiota and how this potential influence is associated with the hypocholesterolemic property of β-glucan are insufficiently investigated. A comprehensive examination employing the advanced sequencing and bioinformatic tools is needed to elucidate the impact of β-glucan consumption on the gut microbial community.

In summary, although the cholesterol-lowering property of barley β-glucan has been well established, research gaps remain in the knowledge of the importance of the physicochemical properties of β-glucan in action and also the underlying mechanisms. Individuals' genetic variation may result in different responses to β-glucan intervention in lowering cholesterol concentrations. As a non-digestible but fermentable carbohydrate, β-glucan has potential to influence gut microbiota that has been associated with numerous metabolic disorders. Therefore, gene-diet-interactions and the gut microbiota alteration are two factors that should be considered when investigating the cholesterol-lowering effect of β-glucan.

1.2 Objectives and hypotheses

1.2.1 Objectives

The specific objectives of this study are:

1. To elucidate the importance of physicochemical properties of β-glucan in the cholesterol-lowering effect via an*in vivo* human study.

2. To investigate the mechanismsresponsible for the cholesterol-lowering effect of β-glucan at a physiological level, including cholesterol absorption and synthesis inhibition, bile acid enterohepatic circulation interruption.

3. To determine the potential gene-diet interactions which are associated with lowering cholesterol property of β-glucan.

4. To examine the influence of β-glucan consumption on gut microbiota.

1.2. 3 Hypotheses

Hypotheses to be tested in this study include:

1. Three grams high molecular weight β-glucan with high viscosity has greater efficacy in lowering circulating cholesterol levels than the low molecular weight β-glucan that possesses low viscosity.

2. β-glucan lowers cholesterol via three mechanisms: inhibiting cholesterol absorption, suppressing cholesterol biosynthesis and interrupting enterohepatic circulation of bile acids by decreasing bile acid reabsorption.

3. Single nucleotide polymorphism (SNP) of *CYP7A1* and *APOE* are associated with different responses to β-glucan intervention in lowering cholesterol levels.

4. β-glucan is able to alter the gut microbiota at the phylum and genus levels toward a pattern that benefits host's health in lowering cholesterol levels.

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CHAPTER 2: LITERATURE REVIEW

2.1 Health benefits of β-glucan: from dose to viscosity

2.1.1 Chemical structure of β-glucan

β-D-glucan (β-glucan) present in cereal endosperm cell walls is a family of unbranched polysaccharides, comprised of (1→4) and (1→3), linked β-D-glucopyranosyl units (approximately 70% are 1→4 and 30% are 1→3) (1, 2). Of the main cereal grains, barley and oats contain the largest amount of β-glucan (3~12% dwb, dry weight basis) (3). β-glucan has also been identified in other cereals such as wheat and rye, however, the amount is insignificant (3, 4). Some chemists consider β-glucan as a linear polymer (5, 6). The conformation of the β-glucan chain is determined by the relative number of (1→3)- and (1→4) -glycosidic bonds that connecting the glucose units (5). The random interposition of (1→3) -linked glucosyl residue results in irregularly spaced molecular kinks along the β-glucan molecule, making it soluble in water. In contrast, cellulose, made up of linear and ribbon-like (1→4)-linkages only, is insoluble in water (7). A different way of visioning the structure of (1→3), (1→4)- β-glucan is that it is composed mainly of cellotriosyl with a degree of polymerization (DP) of DP3 (n =number of average molecular weight/molecular weight of monomer unit), and cellotetraosyl (DP4) residues connected by single (1→3)-β-linkages (3, 7). β-glucans in cereals differ in their DP3: DP4 ratio,approximately 2:1, 3:1, 3:1, 4:1 for oats, barley, rye and wheat, respectively (3). A DP3: DP4 ratio of 2.0 -3.0 allows (1→3), (1→4)- β-glucan to be partially soluble in aqueous media (7).

2.1.2 Health benefits of β-glucan

The cholesterol-lowering effect of β-glucan found in oats and barley has been documented in numerous animal (8-10) and human studies (11-14). The claim (or similar claim) that a daily intake of a minimum of 3 g β-glucan soluble fibre from barley/oat grain products can actively reduce blood cholesterol concentrations, which is a risk factor for heart disease, has been allowed by various worldwide food regulatory agencies including U.S. Food and Drug Administration (FDA) (15), European Food Safety Authority (EFSA) (16) and Health Canada (17). Another well accepted health benefit of β-glucan is the reduction of the postprandial glycaemic response (18-24).In a recent review article, Tosh (2013) used the "dose response" (β-glucan dose to postprandial blood glucose responses) and β-glucan to available carbohydrate ratio in foods as indicators to assess the glycaemic response to β-glucan, and concluded that "intact grains as well as a variety of processed oat and barley foods containing at least 4 g of β-glucan and 30–80 g available carbohydrate can significantly reduce postprandial blood glucose" (25). Additionally, other health benefits of β-glucan that have been proposed but are less established(26)include increased satiety leading to a reduction in energy intake (27) and improved digestion and absorption of nutrients (28).

2.1.3 Physicochemical properties of β-glucan and its health benefits

The physicochemical properties of β-glucan linked to its physiological effects mainly include molecular weight (MW) and solubility (concentration), which together control β-glucan volume occupancy in solution, and thus determine its viscosity (3). Literature values for MW of cereal β-glucan have been reported to range from 6.5×10^4 to 3.1×10^6 g/mol (29). Discrepancies can

be attributed both to cultivar variations and environmental factors (29). Yet, the methods used to measure viscosity, as well as extraction and isolation methods, are also factors contributing to discrepancies. For example, Wood and Beer (1) described the MW of oat β-glucan extracted at 90˚C to be 30% higher than β-glucan extracted at 37 ˚C and that by using sodium hydroxide total extraction can be achieved, however MW can be decreased significantly. Moreover, whether the endogenous enzymes (β-glucanases) have been inactivated prior to the extraction is another critical factor for MW determination. Samples extracted without β-glucanase inactivation may have been depolymerized (1). The instrument that has been extensively used for determining MW is high performance size exclusion chromatography (HPSEC) (30).

According to Wood and Beer (1), "solubility refers to extractability under certain specified conditions of sample preparation, solvent, temperature, time, and liquid :solids ratio". Therefore, it is difficult to compare solubility from studies that utilize different extraction methods. For instance, the amount of β-glucan extracted from untreated flour is greater than from samples treated with ethanol (4). However, for nutritional and physiological evaluation purposes, an *in vitro* extraction system that mimics human digestion is considered to be a more appropriate method to extract β-glucan (1). Additionally, solubility is a function of MW (31) and the total amount of β-glucan available in the sample. Tosh and colleagues (2008) reported that in oat bran muffins when MW declined, the solubility (estimated as a % of total β-glucan) raised but subsequently decreased. Independent of MW, muffins containing 8g of β-glucan always released more soluble β-glucan than muffins containing 4g of β-glucan (32).

Viscosity can be defined as the resistance of a fluid to flow (33). Even at low concentrations, β-glucan is able to form highly viscous solutions(34). With concentrations < 0. 2%, β-glucan behaves like a Newtonian fluid and is shear rate independent; when the concentrations are above 0. 2%, β-glucan behaves like a non-Newtonian fluid and is shear rate sensitive (34). Thus, same shear rate is an important element when comparing the viscosity of β-glucan.Viscosity of β-glucan can be measured by a rheometer with controlled strain or a rotational viscometer (Rapid Visco Analyzer, RVA) (33). Viscosity is determined by both the concentration in solution and MW (35). Tosh et al. (36) showed that the viscosity of oat cereal extracts increased exponentially with increasing concentration and MW. Due to shear rate sensitivity, measuring the proportion of β-glucan solubilized (concentration) and the MW was thought to be a more reliable way to estimate physiological effect of β-glucan as these two physicochemical properties are relatively stable to extrinsic factors (37).

Physicochemical properties have been suggested to be responsible for the health benefits that resulting from not only consuming cereal β-glucan but also other non-starch polysaccharides, for instance guar gum (3). Evidence for supporting this suggestion is particularly solid for the glycemic response attenuating effect of β-glucan. As first reported by Jenkins and colleagues back in 1978 (38), viscosity is an important factor of soluble polysaccharides in flattening the postprandial blood glucose response. In the past few decades, the attenuating glycemic response effect of β-glucan has been well documented (18-21, 25, 34, 35, 39). Wood's group (18, 21, 35) provided sufficient evidence demonstrating the critical role of viscosity in this effect. In one of their randomized human studies with repeated measures, Regand et al.(18) showed an negative correlation between $\log_{10}$ (viscosity) and the mean peak blood glucose response with all the

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treatment foods except pastas that are often reported to have a lower glycemic response. This linear relationship has also been observed between changes of PBGR and peak MWtimes concentration of β-glucan (MW_p x C), which together determine viscosity. Additionally, depolymerized β-glucan in bread and pasta in this study was shown to have reduced bioactivity in reducing glycemic response.

Though the viscosity of β-glucan is considered to be the key factor for its health benefits (3), there is inadequate evidence from human studies supporting the importance of β-glucan physicochemical properties in lowering cholesterol. Previously, studies investigating the cholesterol-lowering effect of β-glucan focussed on the effective daily dose. Based on meta-analysis results, Ripsin et al. (13) hypothesized that "approximately 3 g/d of soluble fiber from oat products can lower the total cholesterol level from 0.13 to 0.16 mmol/L and that the reduction is greater in those with initially higher blood cholesterol levels". Three grams of β-glucan per day has been accepted as an efficacious dose for cholesterol reduction in health claims approved by food regulatory agencies worldwide(15-17). Nonetheless, the importance of viscosity in the cholesterol reduction ability of β-glucan is unclear. Most previous studies investigating the cholesterol-lowering efficacy of β-glucan failed to provide detailed characteristics of β-glucan in terms of MW and viscosity. A few studies have examined the cholesterol reduction effect of β-glucan with different MW levels (37, 40-42), but the generated conclusions remain contradictory. In a double-blind, parallel-design, multicenter clinical trial, Wolever et al. (37) reported that diets containing 3g β-glucan with a high MW (2, 210, 000 g/mol) or 3g or 4 g β-glucan with medium-MW (530, 000 g/mol) reduced LDL-C, while 4g/day of low MW (210, 000g/mol) β-glucan was ineffective. Yet, in a randomized, double-blind, crossover

study, Frank et al. (40) reported that consuming yeast-leavened oat breads with 6g β-glucan with

high (797, 000 g/mol) or low MW (217, 000 g/mol) per day, had the same effect on lowering

blood concentrations of lipids. The discrepancies of these two studies may be attributed to many

factors, including differences in sample size (n =345 vs. n =22), study design (parallel vs.

crossover with 2 weeks as washout period), treatment period (4 weeks vs. 3 weeks), and food

matrix (ready-to-eat breakfast cereals vs. yeast-leavened breads). These two studies all include

subjects with higher LDL-C (> 3.0 mmol/L), however, compared endpoint and changes from

baseline of blood lipid concentrations, respectively in statistical analysis. With respect to food

matrix, Frank et al.(40) in their conclusion also emphasized that MW of β-glucan may not play

critical roles in resulting the physiological benefits in moderately hypercholesterolemic subjects

when it is consumed in in the food format as oat bran breads and being incorporated in the

habitual diet. Moreover, β-glucan was extracted differently in these two studies (*in vitro*

digestion extraction vs. hot water extraction) and the MW were also measured and expressed in

different ways (peak MW vs. calcofluor average MW). Apparently, Wolever et al.'s study (37)

has advantages such as larger sample size, a more appropriate extraction method, and a longer

treatment period compared to Frank et al.'s study(40). However, to demonstrate the importance

of β-glucan physicochemical properties in its cholesterol-lowering effect, more human studies

that can confirm Wolever et al.'s findings (37) may be required.

2.1.4 Food processing affects physicochemical properties of β-glucan

Both MW and solubility of β-glucan can be affected during food processing (36). β-glucan is

tolerant to high temperature while enzymatic degradation can happen under prolonged treatment

and lower temperatures (43). Depolymerization of β-glucan may occur when β-glucanases are

present. For example, rye and barley breads containing wheat flour aresusceptible to β-glucan depolymerization (36, 44, 45), because of the existence of β-glucanases in wheat flour (36). It has been reported that freezing (30) and freeze-thaw cycling (43) were able to decrease solubility. Also, the declined solubility affected the bioactivity of β-glucan. Oat bran muffins treated with four freeze-thaw cycles were significantly less able to attenuate blood glucose compared to fresh oat bran muffins (43). Additionally, depolymerization may also result from oxidative-reductive reactions occurring in the food matrix. Ascorbic acid present in a food mixture is another factor that can cause β-glucan depolymerisation(46). Kivela et al. (46) reported that with the presence of iron sulphate, adding ascorbic acid (10 mM) or its oxidation product (dehydroascorbic acid) to β-glucan caused a significant reduction in solution viscosity and MW.

However, not all food processing causes depolymerization of β-glucan. Tosh et al. (36) reported that oat cereals processed in an industrial twin-screw extruder under certain conditions (extrusion temperature 181˚C, standard mechanical energy 135 Wh/kg) kept the MW at a level similar to the untreated oat cereals, however raised the solubility almost twice (from 38.7 to 66.8%). Moreover, Aman et al. (36, 43) in a study assessing the molecular weight distribution of β-glucan in oat-based foods, summarized that oats, rolled oats, oat bran, and oat bran concentrates all had high Calcofluor average MW (206×10^4 to 230×10^4 g/mol) and essentially monomodal distributions; for the oat-containing experimental foods, extruded flakes, macaroni, and muffins all had high average MW ($>180 \times 10^4$ g/mol) but pasteurized apple juice, fresh pasta, andteacake, contained degraded β-glucan ($< 60 \times 10^4$ g/mol).

Therefore, to obtain oat or barley products with high bioactivity, researchers and the food industry should consider avoiding any processing that can cause depolymerisation. On the other hand, due to the sensitivity of β-glucan to food processing, researchers are able to manipulate the level of MW and viscosity using food processing for research purposes.

2.2 β-glucan and cholesterol metabolism: cholesterol absorption, synthesis and elimination (CASE)

2.2.1 Cholesterol and cardiovascular disease (CVD)

Cardiovascular diseases (CVD) are the number one cause of death globally (47). An estimated 17.3 million people died from CVD in 2008, representing 30% of all global deaths (47). In Canada, CVD was ranked as the number two cause of death in 2011(48). Elevated circulating lipid levels such as total cholesterol (TC) and LDL-cholesterol (LDL-C) are predictive biomarkers for CVD (49, 50). LDL-C is defined as the primary target for the treatment of coronary heart disease (CHD) (51). 57% of Canadians aged 40-59 have unhealthy TC levels ($\geq$ 5.2 mmol/L) and 40% of Canadian adults in this age group have unhealthy LDL-C (≥ 3.5 mmol/L) (52).Hypercholesterolemia and especially elevated plasma concentrations of LDL-C is a key contributor for the initiation and progression of atherosclerosis, which is the underlying cause of many cardiovascular episodes (53, 54). Undesirable cholesterol levels place approximately half of Canadians at risk for developing CVD.

Cholesterol homeostasis is determined by cholesterol absorption, whole body *de novo* synthesis and elimination (55), which is referred as CASE. A typical western diet contains approximately 300 -500 mg of cholesterol per day (56, 57)and the human body synthesizes about 700-900 mg of cholesterol per day (58, 59).Normal daily cholesterol elimination (or turnover) mainly includes excretion into the gastrointestinal tract (600 mg/day) and conversion to bile acids (400 mg/day). A small amount of cholesterol is used for replacement of skin that is sloughed (85 mg/day), the biosynthesis of steroid hormones (50 mg/day), and as components for cell

membranes (58).The following sections will discuss the possible influence of β-glucan consumption to cholesterol metabolism.

2.2.2 β-Glucan and cholesterol absorption

Cholesterol absorption is process with numerous steps, which involves the activities of digestive enzymes (e.g. for de-esterification), the formation of micelles in the small intestinal lumen, and the activities of transport proteins at the enterocyte level (57, 60). The upper part of the small intestine contains the main sties that is responsible for cholesterol absorption (60). Niemann-Pick- C-1 like 1 (NPC1L1) protein (promoting sterols going through the brush border membrane of the enterocyte) and ATP-binding cassette G5 /G8 (ABCG5/G8) transporters (promoting active efflux of cholesterol from enterocytes back into the intestinal lumen for excretion) play main roles in the uptake of cholesterol at the enterocyte level (57). Before interacting with the transmembrane protein, cholesterol must pass a diffusion barrier called unstirred water layer (UWL) located at the intestinal lumen–membrane interface (57).

Viscous dietary fibre may be able to alter the cholesterol absorption through increasing the viscosity of intestinal content and lead to a decreasing rate of diffusion by increasing the thickness of UWL, and thus result in decreased cholesterol absorption. Inhibiting cholesterol absorption has been proposed as one of the underlying mechanisms for the cholesterol reduction effect of soluble fibre (61, 62).Gallaher et al. (63) addressed the connection between digesta viscosity and plasma cholesterol concentrations in an animal model. Hamsters fed hydroxypropyl methycellulose (HPMC) with a high viscosity had a more viscous intestinal content as well as a significantly lower plasma cholesterol concentration compared to the treatment with a low

viscosity HPMC. A similar finding has been observed in chicks. Wang et al. (64) reported that chicks fed barley had greater intestinal viscosity and lower total and LDL cholesterol compared with chicks fed β-glucanase treated β-glucan and corn-soybean. Yet, the results of β-glucan consumption influencing cholesterol absorption in humans remain inconsistent. Naumann et al. (65) reported that β-glucan consumption decreased cholesterol absorption by measuring the cholesterol absorption marker sitosterol. However, Lia et al. (66) reported no changes in the absorption of dietary cholesterol in ileostomy subjects after consuming an oat bran meal containing β-glucan, where a radioisotope method was used for determining the cholesterol absorption. Therefore, the available but inconsistent methods and results from human studies are insufficient to demonstrate whether β-glucan reduces circulating cholesterol levels through inhibiting dietary cholesterol absorption.

2.2.3 β-Glucan and cholesterol synthesis

In humans, the principle sites for cholesterol *de novo* synthesis are the liver and central nervous system (CNS) (67). Cholesterol biosynthesis is a highly complex process involving over 30 different reactions and more than 15 enzymes present in numerous different subcellular compartments (56). Acetyl-CoA derived from an oxidation reaction is the starting material for cholesterol synthesis (56). The formation of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) from one molecule of acetyl-CoA (2C) and one molecule of acetoacetyl-CoA is considered the initial step of cholesterol synthesis (56, 67). The HMG-CoA is subsequently converted to mevalonate by enzyme 3-hydroxy-3-methylglutaryl-Co-enzyme A reductase (HMG-CoA reductase or HMGCR), which is a reaction considered to be the committed step in the cholesterol biosynthesis pathway. Thus, HMG-CoA reductase is considered as the rate-limiting enzyme in cholesterol synthesis and inhibiting activity of HMG-CoA reductase isone of the strategies of the

statin class of cholesterol lowering medications (67). The rate of cholesterol synthesis is highly

responsive to the cellular level of cholesterol. This feedback regulation is principally mediated

by changes in the amount and activity of HMG-CoA reductase (68).

The products generated from β-glucan fermentation in the gastrointestinal (GI) tract, such as

short chain fatty acids (SCFA), are thought to be able to suppress cholesterol synthesis (69). The

SCFA are saturated aliphatic organic fatty acids with 1 to 6 carbon atoms. The major SCFAs ($\geq$

95%) are acetate (C2) buryrate (C3) and propionate (C4) (70). Besten et al. (70) in a review

mentioned that propionate was shown to be able to reduce the cholesterol synthesis rate by

decreasing the activity of HMG-CoA reductase. Moreover, the cholesterol synthesis inhibiting

effect of SCFA has also been revealed in rodent models (71, 72). Chen et al. (71) reported that

rats fed the cholesterol-propionate diet hadsignificantly lower serum and liver cholesterol levels

than rats fed the cholesterol diet without propionate. In another rodent study conducted by Hara

et al. (72), rats fed a SCFA diet had in a lower hepatic cholesterol synthesis rate (measured using

^3H_2O as a tracer) and reduced plasma cholesterol concentrations.

However, human studies on the cholesterol synthesis inhibiting effect of SCFA are inadequate.

No interventional study has directly proved that SCFA can inhibit cholesterol synthesis, but at

least one study revealed an association between serum SCFA levels and cholesterol

concentrations. Wolever et al. (73) reported that the serum acetate: propionate ratio was

positively correlated to serum cholesterol levels in men, although the correlation was not

significant in women.The production of SCFA is determined by various factors, such as the

numbers and types of microbiota present in the colon (74), substrate source (74, 75), as well as

gut transit time (74, 75). More details of SCFA production and bacterial fermentation will be discussed in 2.2.5.

Furthermore, the SFCA production ability of β-glucan has been studied *in vitro* and *in vivo*. Queenan et al. (11) conducted a batch model intestinal fermentation system using human fecal inoculums to yield representative microbiota, and found that concentrated oat β-glucan produced a high concentration of butyrate during fermentation, comparable to inulin and guar gum. Bird et al. (76) reported increased large bowel (caecal and proximal distal colon) SCFA levels along with decreased plasma cholesterol concentrations in pigs after 3 weeks of feeding a novel barley cultivar (Himalaya 292) containing less starch. Consumption of the same barley cultivar in humans also resulted in increased fecal SCFA excretion, especially high butyrate levels, compared with refined cereals. However cholesterol changes were not reported in this study (77).

Despite the examination of SCFA production from β-glucan consumption in animals or in humans, studies conflict as to whether or not reduced cholesterol synthesis is an underlying mechanism of cholesterol-lowering due to β-glucan. Lathosterol, an intermediate of cholesterol synthesis,is considered to be a reliable surrogate for whole-body cholesterol synthesis (78). A study of ileostomy subjects (n =6) showed "no marked" increase in postprandial serum lathosterol after consuming a meal containing oat bran, although the changes of serum lathosterol concentrations was strongly related to the change in bile acid excretion measured (66). On the other hand, in a parallel trial with healthy subjects (n =22), a fruit drink containing β-glucan lowered LDL-C levels and resulted in increased serum lathosterol levels (65), which was opposite to the assumption that β-glucan reduces cholesterol concentrations and cholesterol

synthesis. As yet, no study has proven that reducing cholesterol synthesis is a mechanism responsible for the cholesterol-lowering effect of β-glucan in humans.

2.2.4 β-Glucan and cholesterol elimination (bile acid formation)

Bile acid synthesis is the primary metabolic pathway for catabolism of cholesterol in humans (79). The amount of cholesterol used for producing bile salt molecules is approximately 400 mg per day (80). Bile acids are a group of sterol-derived compounds that function as detergents in the intestine to aid the digestion and absorption of fats and fat-soluble molecules (81). Once the physiological function is accomplished, approximately 95% of bile acids are reabsorbed in the terminal ileum (67, 81). A small amount of bile acids escape reabsorption and are excreted with the feces (81). In the liver, the conversation of bile acid from cholesterol occurs at a rate that precisely corresponds the loss of bile acids within the feces (80). The depletion of intrahepatic cholesterol subsequently up regulates the expression of LDL receptor (67) and recruits lipoprotein cholesterol from the circulation (67). Interrupting the enterohepatic circulation of bile acids that leads to depletion of liver cholesterol for replenishing the loss of fecal bile acids is one of the strategies for lowering cholesterol levels. Bile-acid sequestrating agents (e.g. Cholestyramine) were developed based on this pathway (67).

Cholesterol 7 α-hydroxylase pathway is referred to as the "neutral" or "classical" bile acid pathway (82). It is initiated by cholesterol 7 α –hydroxylase (CYP7A1), the rate-limiting enzyme of this pathway (79). The enzyme activity of CYP7A1 can be reflected by the serum/plasma concentration of 7α -hydroxy-4-cholesten-3-one (7 α- HC or C4), an intermediate of bile acids in the classical pathway (83, 84). The enterohepatic circulation of bile acids are under precise negative feedback control, which primarily regulated at the transcriptional level, such as via

transcriptional control of bile acid synthetic enzymes farnesoid X receptor (FXR) (82). Besides their function in fat absorption, bile acids are also important signalling molecules that coordinately regulate lipid, glucose, drug and energy metabolism, as well as their own synthesis. The primary bile acid chenodeoxycholic acid (CDCA) is the most efficacious endogenous FXR ligand and FXR is an inhibitor of enzyme CYP7A1 (79). The increased fecal bile acid excretion and decreased bile acid reabsorption leads to less active FXR and more active CYP7A which together lead to more bile acid production.

β-glucan was proposed to work like bile acid sequestrants, to interrupt enterohepatic circulation of bile acid thus reducing circulating cholesterol concentrations (85).The increase in bile acid excretion resulting from β-glucan consumption has been extensively reported in ileostomy patients (66, 86-88). In a controlled crossover study conducted by Ellegard el al. (2007), 9 ileostomy patients consuming a native β-glucan (11.6 g) diet for 3 days had 40% more ileal bile acid exeretion compared with consumption of hydrolysed β-glucan in the diet for 3 days. Moreover, native β-glucan in this study also increased serum 7 α- HC levels by 57% within 24 h of consumption (87). The evidence surrounding β-glucan reducing circulating cholesterol via increasing bile acid execration is less clear in healthy subjects. In a controlled but non-randomized study conducted by Marlett et al. (89) , 9 normolipidemic men consuming a diet containing oat bran with 5.4 g of soluble β-glucan had increased synthesis and factional turnover rates of two primary bile acids cholic acid (CA) and CDCA, along with decreased serum cholesterol levels compared to a low fibre diet without β-glucan. Yet, the washout period was not mentioned in this study. In another controlled crossover clinical trial, 8 healthy subjects were given either an oat bran breakfast containing 11 g of oat β-glucan or a wheat bran breakfast for 3

days, with an 11-day washout period in between. After 8 and 12 h of consuming an oat bran breakfast, 7 α- HC levels were 84% and 92% higher than before breakfast, respectively, but wheat bran failed in stimulating the rise of 7 α-HC. However, serum cholesterol levels were not changed in this study (88).

Overall, there is some existing evidence supporting a role for β-glucan in decreasing cholesterol levels through interrupting bile acid metabolism. Yet, due to the limitations of the design of previous studies, e. g. unhealthy subjects, small sample size, short treatment period and no washout period, more evidence from appropriate clinical trials with healthy subjects are required to solidify that interrupting the enterohepatic circulation of bile acids is the mechanism responsible for the cholesterol reducing effect of β-glucan.

2.2.5 Proposed mechanisms and assessing methodologies

Based on the aforementioned literature review, there is evidence suggesting that β-glucan reduces circulating cholesterol levels through the following mechanisms:

1) β-glucan increases intestinal viscosity, and thus raises the thickness of UWL and results in intestinal cholesterol absorption;

2) β-glucan interrupts the enterohepatic circulation of bile acids through a mechanism similar to bile acid sequestrants, inhibiting bile acid reabsorption and sequentially up-regulating bile acid synthesis from hepatic cholesterol, which further removes cholesterol from circulation; and

3) β-glucan inhibits cholesterol biosynthesis through SCFA produced from β-glucan fermentation by gut microbiota.

2.2.5 Assessing methodologies

Numerous methodologies have been established for assessing cholesterol absorption and synthesis, as well as bile acid kinetics in humans.

As summarized by MacKay et al. (90) and Pouteau et al. (91), methodologies for investigating cholesterol absorption include " direct methods", such as the intestinal perfusion technique that directly measures the net absorption of total cholesterol enters the upper intestine by incubating the subjects with lumen-tubes (92) and "indirect methods" that use sterol values measured in plasma or faeces to estimate cholesterol absorption. The "indirect methods" include cholesterol balance methods that measure the difference between dietary sterol intake and fecal sterol excretion (90, 93, 94), isotope ratio methods that calculate the cholesterol absorption percentage based on the ratio of radioactively labeled cholesterol to labeled β-sitosterol in faeces compared to this ratio given orally (95), or based on the ratio of intravenous administrated radioactive cholesterol to orally administrated radioactively labeled cholesterol (96), stable isotope methods that adopt the radioisotope methods but replace the radio isotope tracers with stable isotope tracers (97, 98), as well as absorption surrogate methods that use serum plant sterol (e.g. β-sitosterol) to predict cholesterol absorption (99).

The intestinal perfusion technique has a significant weakness of invasiveness although results generated from this method are objective in reflecting both exogenous and endogenous cholesterol absorption (90, 100). Methods employing radioisotope tracers, such as cholesterol balance methods and isotope ratio methods, are relatively simplistic to execute compared with "direct methods" but the exposure to radioactive isotope in these methods requires additional

safety considerations, such as dose and frequency of radioactive exposure. Surrogate methods without using isotope tracers are technically simple and suitable for large population studies; however, the results generated from surrogate methods are not quantitative and the accuracy can be influenced by factors including gender, genetics and dietary background (90). The previously developed plasma dual stable-isotope ratio method (PDSIRM) (101), is able to determine cholesterol absorption with good precision and accuracy and it has been used extensively in human studies. However, PDSIRM requires intravenous injection of labeled cholesterol, which makes the methods more complicated to perform. The more recently developed single stable-isotope method requires only one oral administration of stable isotope and has been shown as reliable as PDSIRM in determining cholesterol absorption (102). The single stable-isotope method measures the ^{13}C enrichment in red blood cells (RBC) over the period of 24-96 hours after oral administration of ^{13}C labeled cholesterol. Overall, the stable-isotope methods are reliable for comparing treatment differences for dietary and pharmacological intervention (90), yet the cost is relatively greater compared to the absorption surrogate methods .

Cholesterol synthesis can be determined by several methods (90)including balance methods that measure the difference between dietary intake of cholesterol and cholesterol excretion including both faecal neutral sterols and bile excretion (93), fractional conversion of squalene that determines cholesterol absorption rate based on the calculation of the fraction of intravenously administrated radiolabelled mevalonate converted to cholesterol (103, 104), mass isotopomer distribution analysis (MIDA) that measures biosynthesis and turnover of polymers *in vivo*(105), cholesterol synthesis precursors methods that predict cholesterol synthesis based on plasma cholesterol precursors levels (e.g. lathosterol) (78, 99), and deuterium incorporation methods that

measures the incorporation rate of deuterium derived from deuterium oxide (D_2O) within the body water pool into plasma or RBC cholesterol pool (106). The cholesterol balance methods measure cholesterol synthesis in a direct way, however these methods are vulnerable to errors as they requires metabolic steady state and rely on accurate records of dietary cholesterol intake and fecal and bile cholesterol excretion (90). The method of fractional conversion of squalene is quick to execute but it involves intravenous infusion of radiolabeled mevalonic acid (90). The MIDA only needs single point measurement yet relies on very complex mathematical model for calculation and it is also invasive (90). The cholesterol synthesis precursors method, similar to surrogate methods for assessing cholesterol absorption, is simple to execute but does not provide quantitative measurement (90) and the results can be influenced by many factors, such as genetic background and dietary sterol intake. The deuterium incorporation method, using body water as a precursor, only requires two blood samples over 48 hours after the oral administration of deuterium oxide (D_2O) (106). In this method, the cholesterol fractional synthesis rate (FSR) of free cholesterol is predicted by the incorporation rate of deuterium derived from D2O within body water pool into the circulatory cholesterol pool (106). Although the principle of the deuterium incorporation method is based on one assumption that free cholesterol synthesis in the main plasma pool accurately reflects whole body cholesterol synthesis, the validation of this method has been confirmed by the evaluations against other methods, including the balance method , MIDA and the cholesterol synthesis precursors method (90).

Since the hypothesis of this thesis focuses on the conversion from cholesterol to bile acid, this section will focus on methods for the determination of bile acid synthesis instead of total cholesterol elimination. Cholesterol 7α-hydroxylase is the rate-limiting enzyme in the classical

bile acid biosynthesis pathways (84, 107). Human studies are limited in indirectly detecting this hepatic hydroxylase activity by noninvasive means. Bertolotti et al. (108) summarized three methods for determining bile acid synthesis *in vivo*. These three methods have also been used in human studies and assay of cholesterol side chain degradation that measures [^{3}H] water or [^{14}C] CO_2 released from the side chain degradation from 27-carbon cholesterol to 25-carbon primary bile acids (109), assay of cholesterol-7α hydroxylation using [7α-^{3}H] cholesterol that measures tritium enrichment in the body water resulting from intravenous infusion of [7α-^{3}H] cholesterol, in which ^{3}H is replaced by a hydroxyl group during bile acid formation (110), and measurement of serum levels of 7α-hydroxycholesterol that measures serum concentration of the enzymatic activity indicator 7α-hydroxycholesterol. In addition, isotope dilution technique that determines bile acid pool and fractional turnover rates (FTR) after oral administration of stable isotope labeled bile acids has also been used for the prediction of bile acid synthesis in humans, in which synthesis rate is can be calculated by multiplying bile acid pool by FTR (111-113). Another method that has been widely used in detecting the treatment effect of dietary and pharmacological interventions on bile acid synthesis is the measurement of serum 7α-HC levels, which is a precursor of bile acids and also an indicator of enzymatic activity of 7 α-hydroxylase (83, 84). Methods that are developed based on cholesterol side chain degradation and cholesterol-7α hydroxylation cleverly used chemical reactions in bile acid biosynthesis pathway, yet, require intravenous infusion of radioactive isotopes and specific apparatus (e.g. breath-collecting apparatus) (108). Moreover, the difficulty in synthesizing the [7α-^{3}H] cholesterol adds additional limits to the cholesterol-7α hydroxylation assay (108). The isotope dilution technique uses stable isotope labeled bile acids instead of radioactive labeled tracers and oral administration in instead of intravenous infusion, but the utilization of 2-3 isotope tracers

increases the cost of this method. The method of measurement of serum levels of 7α-hydroxycholesterol is considered to be relatively simple and convenient because it only requires one blood sample and no other tracers. However, this method can only identify the increase of enzymatic activity not the decreased activity as it measures the "spillover" of excess 7α-hydroxycholesterol from the liver (108). Moreover, 7α-hydroxycholesterol can be possibly formed from the auto-oxidation of cholesterol during extraction and sampling at the expense of accuracy (83). On the other hand, measurement of serum 7 α-HC levels, despite the same simplicity as 7α-hydroxycholesterol method, is able to detect both increased and decreased bile acid synthesis (83). Also, 7 α-HC is relatively stable than 7α-hydroxycholesterol during sampling and analysis (83). Moreover, the 7 α-HC method has been successfully used to determine bile acid synthesis changes resulting from oat β-glucan consumption (87, 88), although the measurements are semi-quantitative (84).

In summary, the available approaches for assessing cholesterol absorption and synthesis, as well as bile acid formation, and their strengths and weaknesses have been discussed in this section. While determining the appropriate approach in research, investigators should consider the research budget, accessibility of facilities and technical support (90), as well as the primary objective of the research (e.g. quantitative vs. semi-quantitative). Additionally, for a research involving multiple investigations of cholesterol absorption, synthesis and bile acid kinetics, choosing methods that can be performed simultaneously and not interfere with each other is another important consideration.

2.3 Gene-diet interactions

2.3.1 Gene and cholesterol metabolism

Maintaining cholesterol hemostasis is a highly complex process involving regulation of a number of intracellular and extracellular (transporting) proteins. Key proteins playing roles in cholesterol metabolism include apolipoprotein B (APOB), apolipoproteinA1 (APOA1), apolipoprotein E (APOE), apolipoprotein C II (APOCII) and apolipoprotein C I (APOCI) for cholesterol transporting (56); LDL-receptor (LDL-R) and scavenger receptor class B, type 1 (SR-B1) for plasma LDL and HDL clearance (56); 3-hydroxy-3-methylglutaryl-Co-enzyme A synthase (HMGCS), 3-hydroxy-3-methylglutaryl-Co-enzyme A reductase (HMGCR), sterol response element binding proteins (*SREBF1* and *SREBF2,* also known as SREBP1 and SREBP2) for cholesterol synthesis (56); Niemann Pick Disease Type C1 Like-1 (NPC1L1) protein and the heterodimer formed by the ATP binding cassette sub-family members G5 and G8 *(*ABCG5 *and* ABCG8,respectively) for cholesterol absorption (57); cholesterol 7α-hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27A1), and transcription factors such as liver X receptor (LXR) for cholesterol biliary elimination (56).

Genetic variations, such as Single Nucleotide Polymorphisms (SNP) contribute in shaping intrinsic circulating cholesterol levels in individuals. For example, the genetic variants of *APOE* and their roles in determining cholesterol concentrations have been well established (114-116). The *APOE* encodes apolipoprotein E (APOE), which is the critical component in the formation of very low density lipoprotein (VLDL) and chylomicrons and it acts as the primary ligand for lipoprotein receptors (115). Three APOE isoforms have been identified (APOE2, APOE3 and APOE4) based one two SNP rs429358 and rs7412 (117). These isoforms differ in their

interaction with specific lipoprotein receptors, such as LDL-R and therefore vary in uptake of postprandial lipoprotein particles and further resulting in differences in total and LDL-C levels at a genotype level (115). It has been reported that subjects with the *E2/E2* genotype had about 31% lower mean LDL-C concentrations than those with the *E4/E4* genotype (114). As of April 2014, 155 distinct genetic variants including *APOE* variants have been reported to be associated with cholesterol metabolism by the genome-wide association studies (GWASs) conducted by a large international consortia (118-120).

2.3.2 Diet and cholesterol metabolism

The acceptance of the connection between diet and heart disease can be tracked back to the 1950s, when Professor Ancel Keys' was challenged by Sir George Pickering for his hypothesis of the connection between diet and heart disease (121). This challenge became one of the driving forces for the launch of the landmark Seven Countries Study (SCS) (121), which was the first important study that aimed to investigate how diet and lifestyle as well as other risk factors influence CVD in diverse countries and cultures for a prolonged period of time (122).Finding from the SCS demonstrated dietary saturates and cholesterol relate positively to serum cholesterol but polyunsaturated fatty acids relate inversely (123, 124). Furthermore, Keys and colleagues (125) showed that increased fibre intake contributed to lowering cholesterol. In the past six decades, thousands of studies have been conducted to investigate the diet-heart connection. Moreover, a number of bioactive compounds in foods including the β-glucan soluble fibre have been identified for preventing CVD by reducing related risk factors (126). Currently, medical institutions and government agencies have accepted the concept that nutrition plays a

major role in the epidemic of CVD and potentially in the control of the disease (121, 127),

although the dispute of the diet-heart connection still continues (128, 129).

2.3.3 Gene-diet interactions

It has been recognized that the impact of dietary intervention to plasma lipid concentrations

differs significantly among individuals (130-132). This may be due to an interaction between

gene and lifestyle. The term "nutrigenetics" refers to "the role of DNA sequence variation in the

responses to nutrients" (133). In a recent review, Abdullah et al. (120) summarized that variants

of 28 genes have been associated with responses of circulating cholesterol levels to different

diets:ATP-binding cassette sub-family (ABC) A1 (*ABCA1*), -G1 (*ABCG1*), -G5 (*ABCG5*), and -

G8 (*ABCG8*); adiponectin (*ADIPOQ*); apolipoprotein (APO) A1 (*APOA1*), -A4 (*APOA4*), -A5

(*APOA5*), -B (*APOB*), -C3 (*APOC3*), and -E (*APOE*); cluster of differentiation 36 (*CD36*);

cholesteryl ester transfer protein (*CETP*); cholesterol 7-alpha-hydroxylase (*CYP7A1*); fatty acid

binding protein 2 (*FABP2*); 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*HMGCR*); low-

density lipoprotein receptor (*LDLR*); hepatic lipase (*LIPC*); lipoprotein lipase (*LPL*); liver X

receptor alpha (*LXRA*); nuclear factor kappa B1 (*NFKB1*); Niemann-Pick C1 like 1 (*NPC1L1*);

peroxisome proliferator-activated receptor (PPAR) -alpha (*PPARA*), -delta (*PPARD*), and -

gamma (*PPARG*); tumor necrosis factor alpha (*TNFA*); and uncoupling proteins-1 (*UCP1*) and -

3 (*UCP3*).

Among the 28 genes listed by Abdullah et al.(120), *APOE* was the only one that has been

associated with different responses to β-glucan regarding serum cholesterol levels (134).In

parallel 2-week metabolic dietary studies, Jenkins et al. (134) reported that *E2* carriers of

APOE were more responsive in lowering LDL-C than *E3* and *E4* carriers to oat and wheat bran. Besides *APOE*, no other gene has been examined for the potential gene-diet interactions that are associated with different responses to the cholesterol-lowering effect of β-glucan.

2.3.4 Candidate gene selection

Identifying the candidate gene for the nutrient of interest is the first step in the investigation of the gene-diet interactions. As was suggested by Mutch et al. (135), the candidate genes often meet one or more of the following conditions

"1) Genes that are chronically activated during a disease state and have been previously demonstrated to be sensitive to dietary intervention.

2) Genes with functionally important variations.

3) Genes that have an important hierarchical role in biological cascades.

4) Polymorphisms that are highly prevalent in the population.

5) Genes with associated biomarkers, rendering clinical trials facile."

Experimental studies usually have smaller sample size than cohort observational studies. Therefore, based on the 3rd condition, to investigate the gene-diet interactions in an experimental study, the least common allele that occurs in the population, which is reflected by minor allele frequency (MAF), is an important factor that should be considered.

Considering all these factors together, the SNP -204 T >G (rs3808607) in the promoter region of the *CYP7A1* becomes the top candidate gene that may be associated with different responses to β-glucan consumption. The *CYP7A1* encodescholesterol 7 α-hydroxylase – the rate-limiting enzyme in the classical bile acid synthesis pathway (136, 137) that is the main route for

cholesterol elimination. The genetic variation of *CYP7A1* has been associated with intrinsic blood lipid profiles and also gene-diet or gene-drug interactions. The -204 T >G polymorphism in *CYP7A1* has been associated with variation in plasma LDL-C levels and triglyceride levels in the Framingham Offspring Study (138). Specifically, the G variant was associated with increased concentration of plasma LDL-C in men, and the homozygous GG variant had higher triglyceride concentrations in women (138). SNP rs3808607 has also been associated with varied responses to diet or drugs in lowering cholesterol levels. In an 8-year follow-up study, 131 Czech males had changed their food habits towards a lower consumption of red meat and animal fat and a higher consumption of fruits and vegetables. The results showed that homozygote carriers of the SNP rs3808607-G allele had greater reduction in TC and LDL-C compared to homozygous TT carriers (174). In accordance with this observational study, randomized, double-blind, placebo-controlled intervention studies revealed that G carriers have greater cholesterol reduction in response to plant sterols (2.0 and 3.2 g/day for 4 to 8 weeks) compared with homozygous TT carriers. Interestingly, in contrast, G carriers have been observed to be poor responders to the cholesterol-lowering drug atorvastatin in lowering LDL-C in a randomized, placebo-controlled, double-blind study consisting of parallel arms (140). These findings together shed light on the so-called "personalized nutrition"— therapy or diet recommendations that are given with the consideration of the intrinsic character of individuals.

To summarize, cholesterol metabolism can be affected by dietary factors, genetic factors and also the interaction between these two factors. Gene-diet interactions associated with the cholesterol-lowering effect of β-glucan consumption are poorly investigated. Based on previous studies, SNPs of *APOE* and *CYP7A1* might be legitimate gene variants to investigate due to their

important roles in cholesterol metabolism, their relative large MAF score, and their potential influence on the proposed mechanisms for the cholesterol-lowering effect of β-glucan.

2.4 Impact of β-glucan on gut microbiota

The human body itself cannot produce glucanases for breaking down β-glucan. Similar to other non-digestible carbohydrates, the degradation of β-glucan in humans relies on carbohydrate active enzymes (CAZymes) produced by resident gut microorganisms (141, 142). Therefore, the entry of β-glucan to the human gastrointestinal (GI) tract can possibly influence the microbial population. On the other hand, by-products generated from β-glucan degradation such as SCFA have been reported to affect cholesterol metabolism (143).

2.4.1 Gut microbiota

The human GI tract harbors 10^{14} bacterial cells with more than 1000 distinct bacterial species (144). This assortment of microorganisms inhabiting the length and width of the GI tract is referred to as the gut microbiota (145). The genes of the microbiota are referred to collectively as the microbiome (146).

Firmicutes and Bacteriodetes are usually the dominant bacterial phyla present in the adult gut (144). Actinobacteria, Proteobacteria, and Verrucomicrobia are commonly found phyla but generally minor constituents (144). In addition to bacteria, archaea also present in human GI tract (146, 147). Gut microbiota play a critical role in human health including early immune development, immune response development (148), energy metabolism (149) and vitamin

synthesis (150); however, aberrations in the gut microbiota lead to a number of diseases, such as allergy, colon cancer and inflammatory bowel disease (148). Moreover, a disrupted gut microbiota has also been linked to chronic disease and metabolic disorders (146, 147, 151, 152). Recent studies showed that the altered dominant phylum Firmicutes and Bacteroidetes are associated with obesity. Ley et al. (153) reported that the relative proportion of Bacteroidetes was lower in obese people when compared with lean people, and this proportion also increased with weight loss induced by a low-calorie diet.On the other hand, positive manipulation of gut microbiota may act as a potential therapy for treating or preventing diseases and disorders. Fecal microbiota transplantation (FMT) is referred to the transfer of gut microbiota from a healthy donor into a recipient for recoverying of the gut microbial community to a normal structure (154). The FMT has been successfully used in treating *Clostridium difficile* infection (CDI), which is an infectious disease can be caused by antibiotics induced microbiota dysbiosis (154). The accelerating understanding of the important roles of gut microbitoa in diseases warrants the microbiota manipulation to be used in treatingdisorders that are related with microbiota dysfunction (154, 155).

2.4.2 Carbohydrate degradation by gut microbiota

The main functional pathways of the gut microbiota are usually associated with carbohydrate fermentation and degradation pathways (156, 157). Fermentable food ingredients are defined as prebiotics if their consumption is able to "result in specific changes in the composition and/or activity of the gastrointestinal microbiota", and consequently confer benefit(s) upon host health (158).

On a daily basis, approximately 20- 60 g of dietary carbohydrates escape host digestion enzymes and reach the colon (142). The non-digestible carbohydrates are comprised of resistant starches, plant cell wall polysaccharides and non-digestible oligosaccharides, in spite the fact that some di- and mono-saccharides (e.g., sugar alcohols) also being digested and/or absorbed at a limited degree (142). Complex carbohydrate fermentation in the intestine is associated with interactions between bacterial community members that are restricted to one or a few nutrients (referred to as nutritionally specialized species) and members that target a number of glycans (widely adapted species) (67, 142, 159). Chassard et al. (67) summarized the predominant bacterial groups that are more effective in fermenting the major diet-derived carbohydrate: *Bacteroides* (for resistant starch, xylan), *Roseburia* (for resistant starch, xylan and oligosaccharides), *Ruminococcus* (for resistant starch, cellulose) and *Bifidobacterium* (for oligosaccharides). Nonetheless, non-digestible carbohydrate degradation is a complex process. Usually, a bacterial consortium works together in order to utilize the substrate effectively and economically. For example, sugar produced by fibre degradation is not only used for the growth of the main degraders (fibrolytic species), but can also be used by glycolytic bacteria that are not able to degrade complex fibers (67). In fact, the exact specificity of a CAZyme is difficult to be predicted based on family membership, especially when considered from an ecological perspective.

2.4.3 *β-Glucan and gut microbiota*

To date, the influence of β-glucan consumption on gut microbiota is not entirely clear. Both *in vitro* and *in vivo* studies have been conducted to investigat the gut microbiota altering capability of β-glucan (160-164), especially the potential probiotic effect, but the conclusions remain

obscure. Mitsou et al. (2010) reported daily consumption of a cake containing 0.75 g of β-glucan/serving (one serving per day) for 30 days induced a strong bifidogenic effect compared to baseline in elderly people (≥ 50 years old), whereas *Lactobacillus* spp. did not change (163). In contrast, Pieper et al. (2008) fed piglets barley and oat varieties with different levels of β-glucan for 15 days and reported that hulless barley with a high level of β-glucan (65 g/kg^{-1} and 84 g/kg^{-1} DM) decreased *Lactobacilli* in the ileum compared with oats containing a low level of β-glucan (32g /kg^{-1} and 23 g/kg^{-1} DM); however, *Bifidobacterium* was not examined in this study (164). These two studies were conducted in humans and animals, respectively, and they also differ in treatment period, the daily dose of β-glucan, as well as the methodologies used for bacterial enumeration. In the study by Mitsou et al. (2010), bacteria were enumerated by culture-dependent agar culture (163), whereas in Pieper et al. (2008)'s study, bacteria were quantified using culture-independent quantitative real-time PCR (qPCR) (164). Different microbiota characterization methodologies across similar studiesmay have played a role in causing inconsistent conclusions(165). Culture-dependent enumeration has obvious limitations, as the vast majority of microbial species are non-cultivable (166). Culture-independent methods that rely on specific oligonucleotide probes, such as qPCR and fluorescence *in situ* hybridization (FISH) usually enumerate with specific but limited microbial taxonomic groups (165). Fortunately, advances in sequencing techniques (i.e. 16S ribosomal DNA sequencing, wholegenome shotgun or metagenomic sequencing) coupled with bioinformatics tools (i.e. mothur or QIIME) allows researchers to collect genomic information of all microorganisms in a given environment or community (146, 167). Accordingly, the availability of the data surrounding the gut microbiome has advanced our understanding of the relationship between gut microbiota and many diseases. However, no study has employed these advanced investigation

tools to investigate the influence of β-glucan consumption on the gut microbiota, and no data regarding the changes in microbiota, in terms of diversity, richness and specific members, due to β-glucan consumption is available.

2.4.4 Short chain fatty acids (SCFA)

Host physiology can be influenced by both the presence of the microbiota and their fermentation metabolites. Products resulting from complex carbohydrate fermentation are gases (CO_2 and H_2) and short chain fatty acids (SCFA). The major SCFA have a combined concentration of 50 –150 mM in the colon(168) and are composed of acetate (C2), butyrate (C3) and propionate (C4) (70). Most SCFA can be rapidly absorbed in the cecum and colon; only 5% to 10% is excreted in the feces (169). SCFA serve as an energy source and also as signalling molecules (70). Energy harvested from SCFA accounts for approximately 10% of the totally daily caloric requirements (70, 170). The three major SCFA have different metabolic fates after being absorbed. Acetate is metabolized to a lower degree in the colon but transported to the liver and utilized by peripheral tissues (169), where it can be used for lipogenesis, and it is also the primary substrate for cholesterol synthesis (74). Butyate is the preferred energy source of colonocytes (74), therefore it plays an important role in the regulation of epithelial cell proliferation and differentiation (74, 169). Lastly, propionate is the primary precursor for gluconeogenesis in ruminants; however, propionate metabolism in humans is not fully understood. Propionate metabolism in humans has been suggested to be associated with a lipid lowering effect (169), as discussed in 2.2.3.

2.4.5 MW and β-glucan fermentability

Physicochemical properties of β-glucan, such as MW are thought to be the key factor in its cholesterol-lowering effect. Yet, whether β-glucans with high and low MW possess different capabilities in altering the gut micrbiota is unknown. The only report on this subject was from Hugh et al. (139) but the conclusion is questionable. In an *in vitro* study conducted by Hugh et al. the fermentation ability of β-glucan fractions from barley (average MW of 243,000 172,000 and 137,000 g/mol) and oats (average MW of 230, 000 and 150, 000 g/mol) was investigated by incubating the substrate with human fecal microbiota. Several microbial groups or subgroups including *Bifidobacterium* genus, *Bacteroides-Prevotella* group, and the *Lactobacillus-Enterococcus* group were enumerated with FISH. Although the author concluded that the *Bacteroides-Prevotella* group increased with all but the 243, 000 g/mol barley and 230, 000 g/mol oat substrates after a 24 hour incubation period, their data showed that *Bacteroides–Prevotella* also increased in the "no treatment" control group at 10 hours' incubation thereby nullifying the intended effect. Thus, this study failed to validate the difference in fermentability of β-glucan with different MW. In addition, the MW reported in this study are not ideal representatives of "high" and "low" of MW-- the assigned range of 150, 000 to 243, 000 g/mol belonging to the low MW range that failed to reduce serum cholesterol concentrations in clinical studies (37). Therefore, low and high MW ranges need to be standardized in the field, and studies with valid levels of low and high MW are needed to examine the question of whether the MW of β-glucan affects its capability in altering gut micrbiota.

In summary, β-glucan consumption has the potential to influence the gut microbiota, and the altered gut microbiota together with their metabolites (such as SCFA) may influence cholesterol

metabolism. However, no comprehensive examination of how β-glucan consumption shifts the

gut microbiota has been conducted. Whether β-glucan reduces cholesterol levels through

affecting the composition of gut microbiota and their metabolites remains unclear and

necessitates further investigation.

2.5 Summary

β-glucan is a soluble fibre found in oat and barley. The cholesterol-lowering effect of β-glucan

has been well documented (12, 171), and this has resulted in the approval of the health claim that

a daily intake of a minimum of 3 g per day of β-glucan soluble fibre from barley/oat grain

products can actively reduce blood cholesterol (15-17). Recent research reveals that this effect

may not be solely dose dependent but may vary depending on certain physicochemical properties

(37), which necessitates further investigation.

The literature suggests that β-glucan reduces circulating cholesterol via inhibiting cholesterol

absorption by increasing the viscosity of intestinal content, suppressing cholesterol biosynthesis

by the fermentation product SCFA, and interrupting entrohepatic circulation of bile acids (85).

However, so far these proposed mechanisms have not been well validated in humans. Therefore,

well- designed human studies with valid methods that are able to detect the influence of β-glucan

consumption on cholesterol metabolism are needed to elucidate the underlying mechanism of

action.

Furthermore, cholesterol metabolism is affected by both dietary and genetic factors and the

interaction between the two (172). The examination of gene-diet interactions associated with β-

glucan consumption and cholesterol reduction is limited. Available evidence suggests that genetic variants of *APOE*(134) and *CYP7A1* (173-175)are valid candidates to begin the investigation of the gene-diet interaction related to the cholesterol-lowering effect of β-glucan.

In addition, degradation of β-glucan relies on the microbiota residing in the colon. β-glucan has the potential to influence gut microbita (163, 164) and fermentation products (e.g. SCFA) have been associated with cholesterol metabolism (73). However, more evidence from human studies is needed to elucidate the gut microbiota changes following β-glucan consumption and whether the potentially altered microbiota is related to its cholesterol-lowering property.

In conclusion, more studies are needed to identify the role of β-glucan physicochemical properties in cholesterol-lowering, to clarify the mechanisms responsible for this effect, to assess the potential gene-diet interactions and to investigate the influence of β-glucan consumption on gut microbiota, which may be associated with the cholesterol-lowering effect of β-glucan.

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CHAPTER 3: HIGH MOLECULAR WEIGHT β-GLUCAN DECREASES SERUM CHOLESTEROL DIFFERENTIALLY BASED ON THE *CYP7A1* RS3808607 POLYMORPHISMIN MILDLY HYPERCHOLESTEROLEMIC ADULTS

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3.1 Abstract

Background: A cholesterol-lowering effect of β-glucan, a soluble fiber with viscous property, has been documented. The molecular weight (MW) of β-glucan, which contributes to viscosity, and an individual's genotype might influence the cholesterol-lowering efficacy of β-glucan.

Objectives: This study was designed to determine whether the cholesterol-lowering efficacy of barley β- glucan varied as a function of MW and thedaily dose consumed. Our second aim was to determine if any gene-diet interactions are associated with cholesterol-lowering efficacy of β-glucan.

Methods: In a randomized controlled crossover trial, 30 mildly hypercholesterolemic adults (12 men and 18 women, aged 27-78 y, BMI: 20-40 kg/m^2,total cholesterol (TC): 5.0-8.0 mmol/L, LDL cholesterol (LDL-C): 2.7-5.0 mmol/L)were randomly assigned to receive breakfast containing 3 g high MW(HMW)/d, 5g low MW (LMW)/d, 3g LMW/d barley β- glucan or a control diet, each for 5-wk. The washout period between the phases was 4-wk. Fasting blood samples were collected at the start and end of each phase for blood lipids analysis and genotyping.

Results: Consumption of 3 g HMW/d β-glucan lowered TC by -0.12 mmol/L when compared to the control (P =0.0046) but the LMW β-glucan, at either 3g/d or 5g/d, did not change serum cholesterol concentrations. This effect of HMW β-glucan was associated with genotype-by- diet interaction, where individuals with the SNPs rs3808607-G allele of *CYP7A1* compared to TT carriers had greater responses to 3g HMW/d β-glucan in lowering TC (-0.26 vs. 0.15 mmol/L, P =0.0006).

Conclusions: The HMW β-glucan rather than LMW β-glucan reduced circulating TC effectively in mildly hypercholesterolemic adults. The cholesterol-lowering effect of β-glucan may also be determined by the genetic characteristics of an individual. These data show that individuals carrying the *CYP7A1*SNPs rs3808607-G allele are more responsive to the cholesterol-lowering effect of β-glucan with HMW than TT carriers.

This trial was registered at ClinicalTrials.gov with ID NCT01408719 and the manuscript was written following Consolidated Standards of Reporting Trials (CONSORT) guidelines.

Keywords: Barley,β-glucan, cholesterol,*CYP7A1,*molecular weight, polymorphism,viscosity

3.2 Introduction

Various food regulatory agencies have approved health claims for β-glucan in barley or oat products for reducing blood cholesterol concentrations (1-4). However some of the previous clinical trials have exhibited heterogeneity in the degree of cholesterol-lowering (5-8). Physiochemical properties of β-glucan, such as viscosity, molecular weight (MW) and solubility (concentration), have been suggested in mediating physiological outcomes in humans(9). However, there are inconsistent data available as to the role of these physicochemical properties in the cholesterol-lowering effects of β-glucan (10-12). Lack of careful characterization of β-glucan in terms of MW and viscosity may be one of the factors resulting in the discrepant observations found in these intervention studies. In this study, barley food containing carefully characterized high MW β-glucan (3g HMW/d) and low MW β-glucan (3g LMW/d) were used for testing the hypothesis that physiochemical properties of β-glucan rather than dose alone are responsible for its cholesterol-lowering effect. The LMW β-glucan with high dose (5g LMW/d) was used for answering whether LMW with increased daily consumed amount can achieve the same cholesterol-lowering efficacy as 3g HMW/d.

Significant inter-individual variability exists for cholesterol lowering in response to various dietary interventions(13-15). Besides the varied physicochemical characteristics of β-glucan across intervention studies, the genetic characteristics of subjects might be an additional factor that is responsible for the heterogeneity. Besides the varied physiochemical characteristics of β-glucan across intervention studies, the genetic characteristics of participants might be an additional factor that is responsible for the heterogeneity. Genetic variants of key enzymes involved in cholesterol metabolism can potentially influence an

individual's response to a dietary intervention (13). For example, genetic variants of *APOE* have been associated with different responses to soluble fiber in lowering circulating LDL-C concentrations(16). Moreover, the single nucleotide polymorphism (SNPs) rs3808607-T >G in the promoter region of the *CYP7A1* gene has been associated with significant variability in circulating cholesterol concentrations in response to various dietary interventions and cholesterol-lowering medications(17-21). *CYP7A1* encodescholesterol 7 α-hydroxylase, the rate-limiting enzyme in the classical bile acid synthesis pathway(22, 23). The function of CYP7A1 in mediating cholesterol metabolism can be isoform specific (24). Results from Castro-Orós et al.'s study suggested that G allele of rs3808607 compared with homozygous T alleles showed a greater cholesterol reduction in response to plant sterol (21). Interrupting bile acid metabolism is the main proposed underlying mechanism for the cholesterol-lowering effect of β-glucan. However, to date, no study has addressed whether individuals carrying varied *CYP7A1* genotypes will respond to β-glucan intervention differently.

Accordingly, the primary objective of this study was to determine the roles of MW and daily dose of β-glucan on its cholesterol-lowering effect. Our second objective was to investigate if genetic variations in *CYP7A1* and/or *APOE* influence the responsiveness of cholesterol biomarkers to dietary β-glucan.

3.3 Subjects and Methods

3.3.1 Subjects

Male and female participants aged 27-78 years with a BMI >20 and <40 kg/m^2 were selected on the basis of fasting serum TC between 5.0 and 8.0 mmol/L and fasting serum LDL cholesterol (LDL-C) between 2.7 and 5.0 mmol/L. Exclusion criteria included any history of heart, liver or kidney disease, hypertension and diabetes (type 1 and 2) and taking prescription medication for lowering blood lipids or glucose. Those with thyroid conditions were permitted to take part in the study provided their medication/treatment had been stable for the preceding 3 months and did not change during the course of the study. Volunteers self-reported themselves as "healthy", which was confirmed by the study physician following a physical examination. Written informed consent was obtained from all participants. This study was conducted according to the principles expressed in the Declaration of Helsinki and all research procedures were approved by the University of Manitoba's Biomedical Research Ethics Board (Ethic reference no. B2010:057).

3.3.2 Diet intervention

A randomized, single-blinded, controlled-diet, crossover trial was conducted at the Clinical Nutrition Research Unit at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba, Winnipeg. The study consisted of 4 phases. Each phase was 5 weeks (35 days) and separated by a ≥ 4-week washout period.The length of intervention period was chosen based on the study of Behall et al. (7)that was able to detect the cholesterol-lowering effect of barley β-glucan in 5 weeks.The washout period of 4 weeks was the standard length for eliminating the dietary effects on affecting cholesterol metabolism and has been used as effective time for eliminating the effect of β-glucan (5).Energy needs of each subject were estimated based on age, gender, body weight and

physical activity levels and adjusted proportionately in 300-kcal increments. Breakfast foods in the format of crepes, tortillas, porridge and chips were formulated from barley to contain β-glucan varying in MW and dose:3g HMW/d, 3g LMW/d and 5g/d LMW. The 3g HMW/d and 3g LMW/d treatments had the same amount of barley ingredients but differed with respect to level of β-glucan MW. The 5g LMW/d treatment had a similar level of β-glucan MW to the 3g LMW/d treatment but contained a higher total amount of β-glucan. High and low levels of β-glucan MW were achieved by altering food processing protocols. The LMW β-glucan in the experimental barley food treatments was strategically attained using unconventional food processing techniques, such as prolonged dough resting for 120 min at room temperature or soaking coarsely milled native barley grain in apple juice containing ascorbic acid overnight at 4°C. The HMW β-glucan treatment was made from barley that was heat treated using micronizing or boiling and toasting.Wheat and rice were used to replace barley in the control breakfast. Lunch and dinner were designed with a 7-day rotating menu. Overall, participants received study diets containing approximately 30% of energy as fat, 55% as carbohydrate and 15% as protein.The nutrition information and fiber content of the experimental diets are listed in **Table 3.1** using an energy level of 2800 kcal as an example.

Table3.1 Nutrient content of the experimental diets[1].

	WR Control	3g LMW	5g LMW	3g HMW
Energy, kcal/d	2821	2843	2853	2843
Carbohydrate, % energy	55.7	55.7	55.6	55.7
Fiber, g/d	31.2	36.6	41.5	36.6
Soluble fiber, g/d	5.7	8.7	10.7	8.7
β-glucan, g/d	LOD	3	5	3
Protein, % energy	15.9	15.9	15.9	15.9
Fat, % energy	30.8	30.5	30.4	30.5
Saturated, % energy	9.0	8.9	8.9	8.9
Cholesterol, mg/d	312.7	312.7	312.7	312.7

[1] Fiber, total dietary fiber. LMW, low molecular weight. LOD, limit of detection (value < 0.6%). HMW, high molecular weight. WR, wheat and rice.

3.3.3 Characterization of β-glucan

High and low levels of β-glucan MW were achieved by altering food processing protocols. The LMW β-glucan treatment in experimental barley foods was strategically attained using unconventional food processing techniques, such as prolonged dough resting for 120 min at room temperature or soaking coarsely milled native barley grain in apple juice containing ascorbic acids overnight at 4°C. The HMW β-glucan treatment was made from barley that was heat treated using micronizing or boiling and toasting. Wheat and rice containing insignificant levels of β-glucan were used to replace barley in the breakfast food items as a control. A modified *in vitro* digestion method using a shaking water bath (25) adapted from the protocol developed by Beer et al. (26) was conducted for the enzymatic extraction of β-glucan. Total β-glucan content was determined by the enzymatic method (AOAC 995.16). The methods for determining the physicochemical properties of β-glucan were performed as described by Tosh et al. (27); the amount of soluble (extractable) β-glucan was measured using flow-injection analysis (FIA) and expressed as % (soluble β-glucan /total β-glucan x100); the weight average MW was determined by size-exclusion HPLC and the viscosity of β-glucan was measured by controlled strain rheometer (ARES, TA Instruments, New Castle, DE) over a shear rate range of 0.1 - 400 s^{-1} at 37 °C. The physicochemical properties of β-glucan are illustrated in **Table 3.2**. The intervention breakfasts were consumed at the clinical unit at RCFFN under supervision from Monday to Friday. Intervention breakfasts for the weekend, as well as daily lunches and dinners were packed for home consumption. Subjects agreed to limit caffeinated beverage intake to 2 servings/day (237 ml per serving), and alcohol intake to no more than 1 drink per day(355 ml of beer; 148 ml of wine; 45 ml of spirits). Physical activities were monitored by a biweekly questionnaire.

Table 3.2 Molecularweight, solubility and viscosity of experimental breakfasts[1].

	WR Control	3g LMW	5g LMW	3g HMW
Average MW, *g/mole*	68,000	288,000	292,000	1,349,000
Average Solubility, *%*	LOD	31.4	30.25	36.9
Average Viscosity,mPa·s	2.5	30.9	31.2	182.3

[1]HMW, high molecular weight; LMW, low molecular weight; LOD, limit of detection; WR, wheat and rice. MW, solubility and viscosity are the means of 4 types of food items: crepes, tortillas, porridge and chips.

3.3.4 Serum lipid concentrations

Serum cholesterol concentrations (TC and LDL-C) were the primary outcomes of this study. Gene-diet interactions were the secondary outcomes.Twelve hour fasted serum and plasma samples were collected on days 1, 2, 34 and 35. Blood samples were centrifuged at 2675 xg for 20 min at 4 °C, separated to serum, plasma, buffy coat and red blood cells, and stored at -80 °C until analysis. Serum TC, HDL cholesterol (HDL-C), and triglycerides (TG) were measured using the automated enzymatic methods on a Vitros-350 chemistry analyzer (Ortho-Clinical Diagnostics, Markham, ON, Canada). Serum LDL-C was estimated using the Friedewald equation(28). Body weight was recorded every Monday through Friday at the clinical unit. Waist circumference was measured at the beginning and end of each phase.

3.3.5 DNA extraction and genotyping

DNA was extracted from the buffy coat of heparinized blood samples following the instructions of the DNeasy® Blood & Tissue kit (QIAGEN, Germantown, MD, USA). The concentration and purity of DNA were measured by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

SNP of rs3808607 for *CYP7A1* as well asrs429358 and rs7412 for *APOE*, were genotyped using TaqMan® GTXpress™ Master Mix with allele-specific probes on the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Inc., Foster City, CA, USA).

3.3.6 Sample size and randomization

The sample size (n =35) was calculated based on an estimated 6.5% decrease in serum LDL-C over 35 days, reflecting treatment against control based on reduction from baseline, with the alpha (type I error) of 0.05, power of 80%, and a within subject standard deviation of 0.4

mmol/L. The 6.5% LDL-C reduction was chosen as a realistically achievable effect in mildly hypercholesterolemic adults based on published studies on oat, barley and extracted β-glucan that were included in the health claim application in Canada (2).

The 24 permutations of the treatment phase order were randomly assigned a number between 1-24. Upon providing informed written consent each subject was randomly assigned by the clinical coordinators to 1 of the 24 sequence orders using the random number generating function of Microsoft Excel.

3.3.7 Statistical analysis

Blood lipids and anthropometrics were analyzed using linear mixed-models (PROC MIXED, version 9.2; SAS Institute Inc, Cary, NC). Log transformations were performed for data with model residuals that were not normally distributed (HDL cholesterol, triglyceride and total: HDL cholesterol). Diet, genotype and genotype by diet interaction were modeled as fixed factors for analyzing the start, end and change of blood lipid concentrations. Two genotype factors, *CYP7A1* and *APOE*, were examined each with three levels. Each genotype factor was evaluated separately in the model described above because there were not enough subjects to allow a joint evaluation of their effects. Blood lipid levels at the end of the intervention (average of lipid concentrations on days 34 and 35) of the intervention and change from baseline were adjusted using three covariates: the baseline concentration of the lipid (average of lipid concentrations on days 1 and 2)being analyzed, as well as changes in body weight and waist circumference (as these two parameters showed strong association with the Ge outcomes of TC and LDL-C concentrations in the potential covariate test, $P<0.05$). Subjects were treated as a random factor and the correlation between repeated measures of these individuals was modeled using the first-order autoregressive (AR (1)) option in the PROC

MIXED procedure. Fisher's Least Significant Difference tests were used for multiple comparisons.

Hypotheses concerning genotype-by-diet interaction were tested using preplanned contrasts. These tests were used to compare genotype differences in a given diet to that same difference in the control diet for *CYP7A1* and *APOE* separately.

For addressing the correction between viscosity and cholesterol-lowering efficacy of the diet, linear regression of cholesterol changes on log(viscosity) was performed using SAS (PROC REG, version 9.2; SAS Institute Inc, Cary, NC) and plotted with GraphPad Prism (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla CA, USA). A value of $P< 0.05$ was considered to be significant.

3.4 Results

3.4.1 Baseline characteristics and subject completion

After screening 278 volunteers, 45 subjects were selected for the study. Eight subjects

dropped out due to reasons not related to the study; 2 subjects were excluded during the study

because of low tolerance to the study diet and missing the study diet for at least 2 days; and 1

was excluded from all analyses due to a later discovery of cholesterol-lowering medication

use. Another 4 subjects were excluded from the statistical analysis because of a later

discovery of ineligibility (e.g. met inclusion criteria at screening but did not meet criteria

when day 1 blood samples were analyzed). Baseline characteristics of anthropometry, blood

pressure, concentrations of TC, LDL-C, HDL-C and TG for the 30 subjects eligible for

analysis are presented in **Table 3.3** (Baseline characteristics of subjects based on genotypes

of *CYP7A1* are presented in Supplemental Table 3.1). Participant flow is listed in **Figure 3.1**.

No adverse effects were observed in the study.

Table 3.3 Baseline characteristics of the subjects (n =30).

	Value	SD
Age, *year*	59	11
Gender, *% for men*	40	
Body weight, *kg*	80.3	19.3
BMI, *kg/m^2*	28.5	6.5
Waist circumference, *cm*	95.9	16.7
Blood pressure, *mm HG*		
Systolic	126.7	19.7
Diastolic	74.3	9.6
Serum lipid measurement, *mmol/L*		
TC	5.98	0.72
LDL-C	3.67	0.54
HDL-C	1.53	0.43
TG	1.71	0.78

278 Screened for eligibility

5 Excluded for analysis
233 Excluded
1 Excluded due to a later discovery of
Not meeting inclusion criteria
cholesterol lowering medicine intake
Selected by other clinical trials
4 Excluded due to a later discovery of
conducted simultaneously at RGFEN
3 be originally included

.

45 Selected for the study and proceeded
to randomization

10 Withdrew
1 Excluded due to low intolerance to
the study diet
1 Excluded due to missing the study diet
for 2 days
8 Dropped-out with reasons not related to
the study

35 Completed the study

30Eligible for analysis
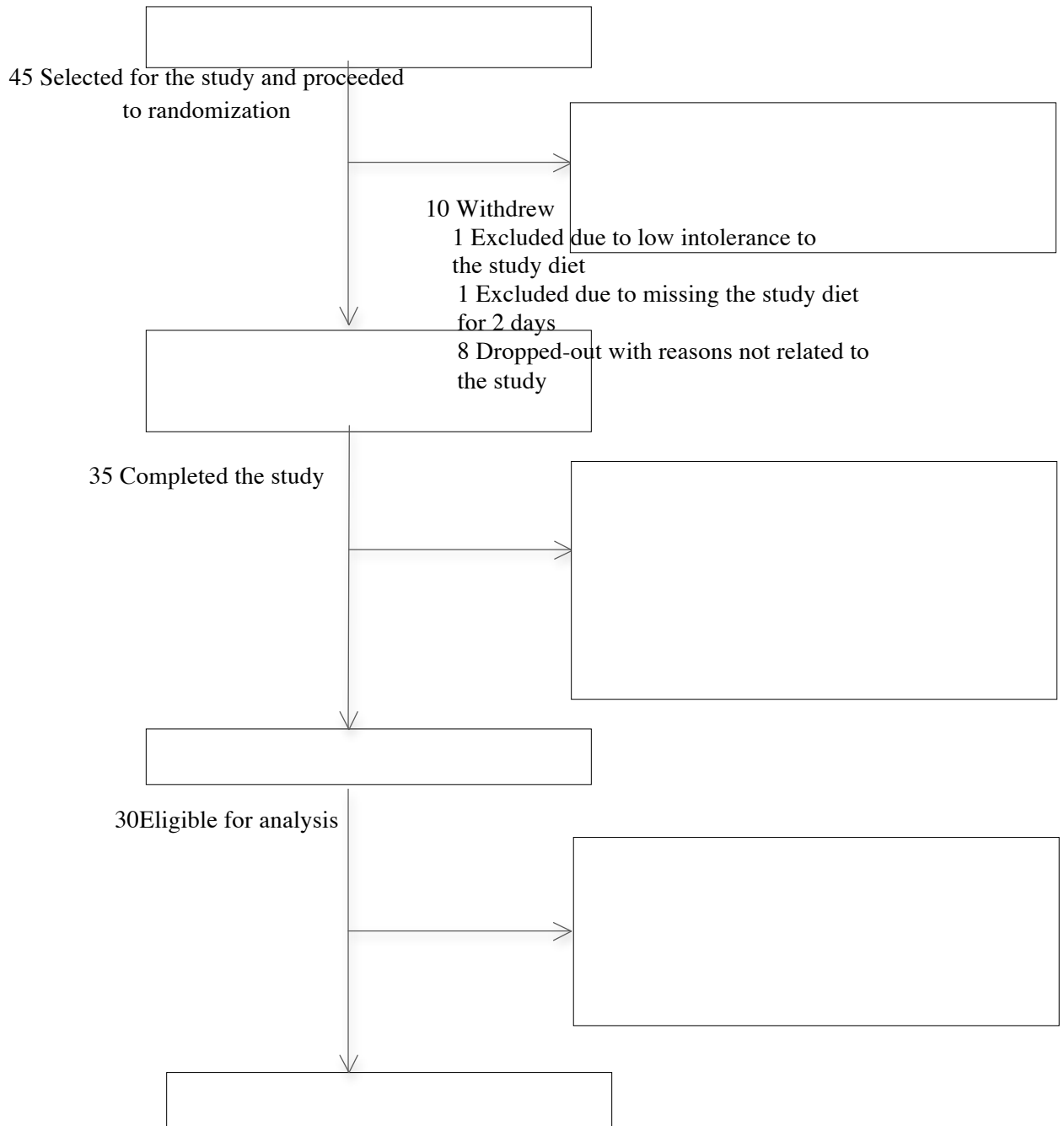
3.4.2 Changes in body weight and waist circumference

No difference in the weight loss was observed among experimental diets overall (P =0.20), although a weight loss within the treatment was observed across all experimental diets. No significant difference in waist circumference change was observed among experimental diets but 3g/d LMW and 3g/d HMW β-glucan reduced waist circumference from baseline. Overall, β-glucan consumption in this study did not affect body weight and waist circumference (data of changes of body weight and are shown in Supplemental Fig.3.1). Physical activities were not changed based on the biweekly questionnaire for monitoring energy expenditure.

3.4.3 Diet effect on blood lipid profile

After the 5-wk's intervention, TC was reduced by 3g/d HMW β-glucan compared to the WR Control (P =0.0046), but not by either 3g/d LMW or 5g/d LMW β-glucan. β-glucan did not change LDL-C compared to the control diet (P =0.08). Serum concentrations of HDL-C and TG were not changed by β-glucan (P = 0.28 and 0.75, respectively, **Table 3.4**)

Despite the randomized crossover study design, TC and LDL-C concentrations at the start of each experimental diet were not as consistent as one might expect. In the original randomization plan, only diet was considered. When diet alone was included in the mixed model, we observed a trend showing differences in the start levels of TC and LDL-C across the experimental diets (P =0.072 and 0.071 for TC and LDL-C, respectively,**Supplemental Table 3.3**). When diet, *CYP7A1* genotype and their interaction were included in the mixed model, baseline concentrations of TC and LDL-C were different among the experimental diets(Table 3.4). To reduce variability in outcome of blood lipid reduction that is due to the starting levels, we used baseline lipid concentrations as covariates in the analysis of the endpoint and absolute changes in blood lipids.

Table 3.4Changes in circulating lipids for mildly hypercholesterolemic adults in response to β-glucan diets andWR control for 5 weeks[1,2].

	Diet				SEM	P-value[3]		
	WR Control	3g LMW	5g LMW	3g HMW		Diet	Genotype	Genotype x Diet
TC, *mmol/L*								
Start	5.75[b]	5.94[ab]	5.98[b]	6.03[a]	0.14	0.039	0.93	0.12
End	5.49[a]	5.44[ab]	5.54[a]	5.37[b]	0.096	0.029	0.9	0.013
Change	-0.41[a]	-0.46[ab]	-0.36[a]	-0.53[b]	0.096	0.0046	0.9	0.013
LDL-C, *mmol/L*								
Start	3.51[b]	3.63[ab]	3.72[a]	3.73[a]	0.11	0.046	0.38	0.38
End	3.4	3.39	3.46	3.31	0.078	0.077	0.93	0.045
Change	-0.23	-0.23	-0.17	-0.31	0.077	0.077	0.045	0.023
HDL-C, *mmol/L*								
Start	1.46	1.52	1.49	1.49	0.07	0.52	0.24	0.89
End	1.38	1.38	1.37	1.37	0.07	0.19	0.98	0.73
Change	-0.08	-0.14	-0.12	-0.13	0.02	0.28	0.43	0.65
TG, *mmol/L*								

Start	1.69	1.73	1.72	1.77	0.13	0.36	0.41	0.041
End	1.49	1.5	1.59	1.56	0.12	0.95	0.19	0.19
Change	-0.2	-0.22	-0.13	-0.21	0.07	0.75	0.7	0.11

[1] Change, lipid concentrations of End-Start; End, lipid concentrations at the end of each dietary intervention; HMW, high molecular weight; LMW, low molecular weight; Start, lipid concentrations at baseline; WR, wheat and rice.
[2]Values are least square mean ± SEM for TC and LDL-C. Vaules are mean ± SEM for HDL-C and TG as log transformed data of these two parameters were used for statistical analysis. n =30. Labeled means in a row without a common letter differ, $P<0.05$ (Fisher's Least Significant Difference test).
[3]P-values are shown for the main effects of Diet, Genotype effect of *CYP7A1* SNPs 3808607, and the Genotype x Diet interaction. End and Change of lipid concentrations were adjusted by the baseline values, changes in body weight and waist circumference.

3.4.4 Genotype by diet effect on blood lipid profile

Genotype-by-diet interactions were observed for *CYP7A1* SNP rs3808607 in response to β-glucan changing TC (P =0.013) and LDL-C (P =0.023) (T).

To further analyze the genotype-by-diet interactions, the changes of blood lipids for different genotype groups of *CYP7A1* SNP rs3808607 in responding to 3g LMW, 5g LMW, and 3g HMW β-glucan compared to the control were illustrated in **Table 3.5**. Lowering of TC with 3g HMW β-glucan was more pronounced for G allele carriers compared to the TT homozygous carriers (P =0.0006 for G allele vs. TT and P = 0.0011 for GG vs. TT). G allele carriers did not differ from TT carriers in reducing LDL-C (P =0.16). GG carriers showed a tendency towards greater LDL-C reduction; however, the difference did not reach statistical significance compared with TT carriers (P =0.051).

Genotype differences for TG change were observed in all β-glucan treatments compared to WR Control. G allele carriers had greater TG reduction after 3g LMW, 5g LMW, and 3g HMW β-glucan diet interventions compared to the TT homozygous carriers (P <0.05).

No genotype-by-diet interaction was observed for *APOE* in this study(**Supplemental Table 3.2**).

Table 3.5 Changes in circulating lipids in response to genotype by diet interaction between *CYP7A1 and* β-glucan diets compared to WR control[1,2].

	Genotype			SEM	*P*- value for contrasts	
	TT (n=11)	GT (n=11)	GG (n=8)		G allele vs.TT[3]	GG vs. TT[4]
WR Control						
TC, *mmol/L*	-0.46	-0.38	-0.38	0.17	--	--
LDL-C, *mmol/L*	-0.28	-0.25	-0.15	0.13	--	--
HDL-C, *mmol/L*	-0.053	-0.083	-0.13	0.035	--	--
TG, *mmol/L*	-0.44	0.0063	-0.17	0.12	--	--
3g LMW						
TC, *mmol/L*	-0.49	-0.4	-0.49	0.17	0.76	0.61
LDL-C, *mmol/L*	-0.37	-0.12	-0.2	0.13	0.27	0.78
HDL-C, *mmol/L*	-0.15	-0.14	-0.12	0.034	0.18	0.12
TG, *mmol/L*	-0.2	-0.13	-0.29	0.12	0.041	0.069
5g LMW						
TC, *mmol/L*	-0.28	-0.4	-0.39	0.17	0.12	0.22
LDL-C, *mmol/L*	-0.17	-0.24	-0.094	0.13	0.49	0.67
HDL-C, *mmol/L*	-0.11	-0.11	-0.14	0.034	0.52	0.47
TG, *mmol/L*	-0.16	-0.049	-0.32	0.2	0.023	0.036
3g HMW						
TC, *mmol/L*	-0.31	-0.58	-0.7	0.17	0.0006	0.0011
LDL-C, *mmol/L*	-0.26	-0.29	-0.38	0.13	0.16	0.051
HDL-C, *mmol/L*	-0.085	-0.14	-0.17	0.035	0.63	0.84
TG, *mmol/L*	-0.11	-0.21	-0.25	0.12	0.0039	0.036

[1] HMW, high molecular weight; LMW, low molecular weight; WR, wheat and rice.
[2] Value are least square mean ± SEM.

3.4.5 Viscosity and cholesterol-lowering efficacy

To test the relationship between β-glucan viscosity and the cholesterol-lowering efficacy, we performed the linear regression of cholesterol changes (TC and LDL-C) on log (viscosity) for individuals based on their *CYP7A1* SNP rs3808607 genotype. Linear relations of TC changes on log (viscosity) ($P = 0.0053$, $R^2 = 0.10$) and LDL-C on log (viscosity) ($P = 0.058$, $R^2 = 0.07$) were observed in *CYP7A1* SNP rs3808607-G allele (G/T and G/G) carriers. However, no linear relationship of cholesterol changes on log (viscosity) was observed in carriers of *CYP7A1* SNP rs3808607 T-homozygotes (T/T, $P = 0.46$ $R^2 = 0.01$ for TC; $P = 0.98$ $R^2 = 0$ for LDL-C) (**Figure 3.2**).

ΔTC, *mmol/L*

a) carriers of the *CYP7A1* SNP rs3808607-G allele (GT and GG); b) carriers of *CYP7A1* SNP rs3808607 T-homozygot(T/T). *P* values were from linear regression test.

P =0.0053, R^2 =0.10

ΔLDL-C, *mmol/L*

log (viscosity)

P =0.026 R^2 =0.07

log (viscosity)

b) TT carriers of *CTP /AT*
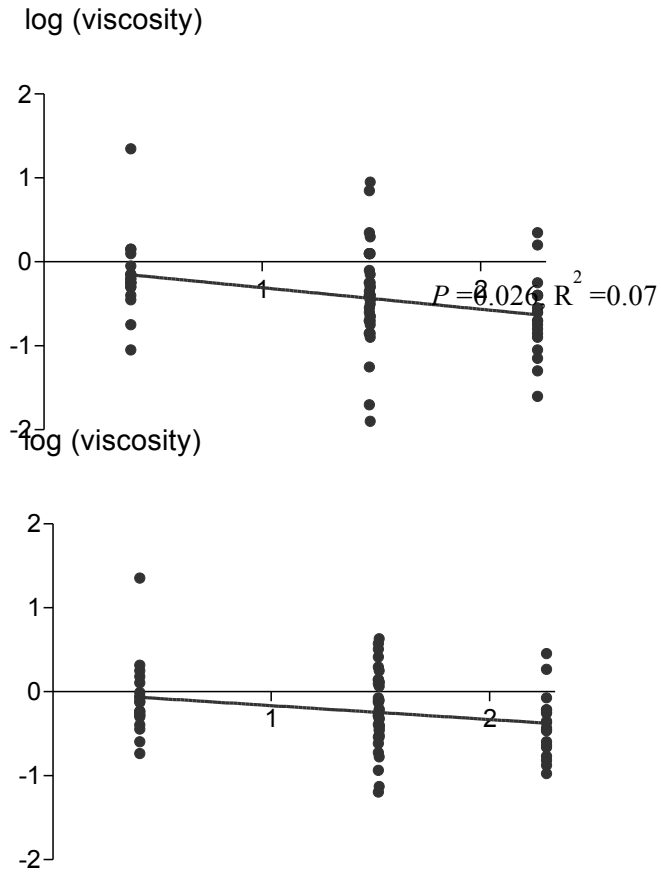
ATC *mmol/l*

AI DI C *mmol/l*

Control*P* =0.46, R^2 =0.01

log (viscosity)

P =0.98, R^2 =0

log (viscosity)

82
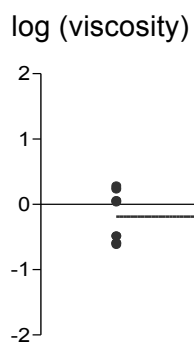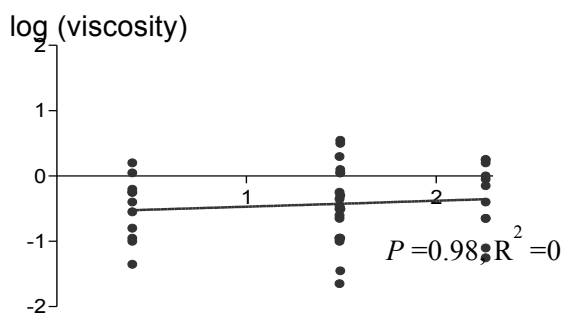
3.5 Discussion

Results from this study confirmed the critical roles of HMW of β-glucan in lowering TC. Consuming 3g HMW/d β-glucan with high viscosity showed greater efficacy in lowering TC compared to the control. However, diets containing 3g and 5g LMW/d β-glucan both possessing low viscosity did not alter TC compared to the control. The efficacy of HMW β-glucan demonstrated by the present study is consistent with a previous study by Wolever et al. (10) that showed β-glucan with MW greater than 530,000 g/mol reduced TC and LDL-C while LMW (210, 000g/mol) β-glucan was ineffective. Also, in our study, when the MW was low (292,000 g/mol), increasing the dose to 5g/d did not result in a further reduction of cholesterol. The results of the current study provide additional evidence to support the hypothesis that physiochemical properties of β-glucan, rather than daily intake amount alone are determining factors for the cholesterol-lowering effect of β-glucan.

Serum LDL-C tended to decrease after the consumption of 3g HMW/d β-glucan (P =0.08); however, this reduction was not statistically significant. Yet, we would interpret our results for the change in LDL-C with caution because the WR control coupled with a low fat and high fiber diet also decreased cholesterol levels, thus, increasing the difficulties in detecting the statistical differences due to β-glucan diets.In the present study, a low fat and high fiber diet was employed as a background diet (intervention diet subtracts barley or wheat and rice ingredients); a diet previously demonstrated to be effective in reducing circulating cholesterol levels(29, 30). Our control diet incorporating wheat and rice into the background diet reduced TC significantly from baseline (P =0.0028, paired student t test for within treatment difference).

Genotype-by-diet interactions were observed for *CYP7A1* SNPs rs3808607 in the TC and LDL-C response to diets. The efficacy of the 3g HMW β-glucan diet in lowering cholesterol was associated with *CYP7A1* SNPs rs3808607-G allele acting in a dominant fashion.To our knowledge this is the first gene-diet interaction determined for *CYP7A1* SNPs rs3808607 in response to β-glucan ingestion in lowering blood cholesterol concentrations. A recent review by Abdullah et al. (15) summarized numerous gene-by-diet interactions showing between-individual variability in response to similar dietary interventions for lowering circulating cholesterol. This epidemiological and intervention evidence warrants a deeper look into the need for considering an individual's genetic background when investigating dietary strategy in preventing cardiovascular disease. SNPs rs3808607 located at the promoter region of the *CYP7A1* gene might influence the negative control of gene expression of *CYP7A1* (18). Although the biological difference between G and T alleles in regulating bile acids and cholesterol metabolism are not entirely clear, our results indicate that the rs3808607-G allele carriers are more responsive to the interruption of bile acids enterohepatic circulation that potentially induced by β-glucan (31-35). Through longer time exposure this depletes cholesterol stores effectively lowering circulating cholesterol concentrations. Here, the potential bile acid metabolism interruption occurred in SNPs rs3808607-T homozygotes seems not to be sufficient enough to cause an effect and therefore these individuals did not to lower circulating TC and LDL-C in response to the β-glucan intervention.

The critical role of the viscosity of β-glucan in its cholesterol-lowering effect and the pronounced responses by G allele carriers to the high viscous β-glucan are further demonstrated by the linear relationship between cholesterol changes and log (viscosity) (Figure 3.2). We therefore postulate that that high MW that is responsible for resulting high viscosity of β-glucan and possession of the G allele of rs3808607 are two key factors

responsible for the cholesterol-lowering efficacy of β-glucan. Without including the effect of genotype and genotype-by-diet interaction as covariates, diets containing β-glucan showed no differences in lowering cholesterol compared to control (P =0.13, Table-S3.3). Therefore, genetic variants of *CYP7A1* and MW of β-glucan together may provide a better explanation to the heterogeneity of responsiveness of lipid levels observed in previous studies (36).

No association between *APOE* variants and different responses to the β-glucan intervention was presently observed. Although it has been suggested by Jenkins et al. (16) that carriers of E2 are more responsive than E3 andE4 to diets with increased soluble fiber intake, after they adjusted for unequal baseline values, *APOE* variants did not show difference in response to oat bran compared to wheat treatment. The receptor-binding function of *APOE* is remarkably isoform specific (37), thus *APOE* variants can result in varied cholesterol up-take and metabolizing abilities. The observed decreases in cholesterol concentrations in this study do not appear to be a result of enhanced lipoprotein clearance by the favorable *APOE* genotype. However, limitations such as the unevenly distributed variant frequencies, as well as the relatively small subgroup sample size cannot be ignored. For instance, only 3 subjects carried the E2 allele of APOE among 30 subjects in our study. The *CYP7A1* variants were distributed almost evenly among our subjects, which are similar to the frequency found in the general population (38). Considering the study size calculation and randomization was not for detecting gene-by-diet interaction, we suggest that a future study with larger sample size for the specific SNPs is needed to confirm the current finding.

The changes in serum TG did not differ among the experimental diets, consistent with results reported by previous studies(7, 10). However, in the analysis of genotype-by-diet interaction, G allele carriers of rs3808607 showed greater responses in lowering TG concentrations than

TT homozygous carriers in responding to all β-glucan diets. Correlation between bile acid synthesis and serum TG concentrations has been reported (39-41). The activity of CYP7A1 may affect the TG concentrations. This G allele-fashioned TG-lowering effect was observed in both LMW and HMW β-glucan diets compared to control. Therefore, high viscosity might not be the determining factor for the TG-lowering effect of β-glucan. However, these findings need to be confirmed by future studies.

Most barley foods are made from pearled barley or flour. The traditional cooking such as boiling and baking that inactivates β-glucanase allows maintenance of medium to high MW and viscosity levels. Therefore, health claims regarding the cholesterol-lowering effect of β-glucan will be valid for barley foods most commonly encountered by consumers.

In conclusion, our results indicate that HMW β-glucan, but not LMW β-glucan, is efficacious in lowering circulating total cholesterol concentrations. This study provides evidence that individuals carrying the G allele of *CYP7A1* SNPs rs3808607 are more responsive to HMW β-glucan's effect of lowering circulating cholesterol concentrations than those homozygous for the TT allele. Validation of this finding in subsequent studies with larger sample size will provide more insight to the effect of genotypes on the efficacy of a dietary intervention and plausible physiological mechanism of action.

Contributions and Acknowledgments

The authors' responsibilities were as follows:

YW conducted the clinical trial from phase 1 to 4 (assigning experimental diets, supervising breakfast, scheduling blood draw and all related measurements, communicate with subjects, study physician, phlebotomists, metabolic kitchen manager to ensure a smooth running of the trial),collected and managedall clinical data and samples from the clinical trial, processed, aliquoted and stored blood samples and fecal samples, analyzed blood lipids, genotyped SNPs, performed statistical analysis, wrote and finalized the manuscript. YW also contributed in recruiting and screening potential subjects and updating the ethical reports.

SVH was responsible for ethical report preparation, recruiting and screening potential subjects. SVH was also responsible forsupervising YW during trial conduction, conducting the clinical trial and collecting samples and data from the trial from phase 1 to 2. SVH conducted the analysis of the nutrient content of experimental foods, contributed in manuscript revision. SVH wrote the section randomization in Method and exclusion criteria in the section of subjects.

PE provided facilities for genotyping analysis and supervisedYW for the SNP concept, contributed in manuscript revision.

SJT contributed to body weight data preparation and manuscript revision.

THG was responsible for the analysis of molecular weight, viscosity and solubility of β-glucan.

EMA contributed in the scientific discussions of physicochemical property measurement and editing.

GHC was responsible for supervising first author for statistical analysis and contributed in editing the manuscript.

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ıre-S 3.1 Changes in body weight and waist circumference in response to lucan diets and WR control. $P = 0.62^{\S}$

Values are presented as means with their standard errors.
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Mean values were significantly different when compared within treatment groups from endpoint to baseline using a two-tailed paired t-test.
§Diet means were compared using ANOVA
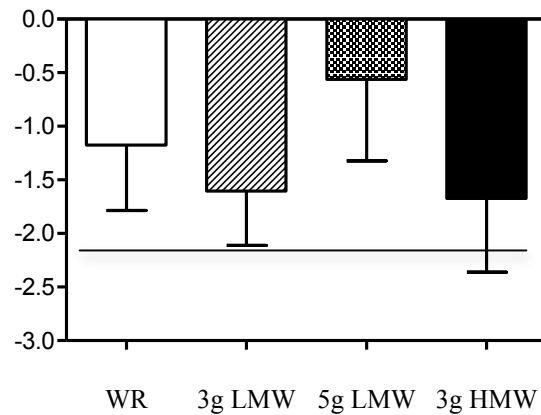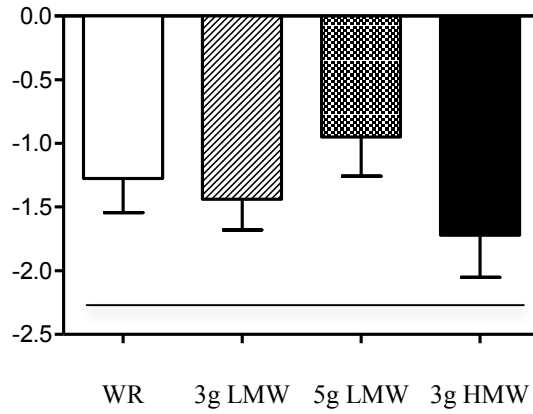
Table S-3.1 Differences of baseline characteristics of subjects based on *CYP7A1* genotypes.

n=30	All subjects [1]	SD	T/T(n=11)	G/T (n=11)	G/G (n=8)	SEM	*P*-value
			Subgroup by *CYP7A1* genotype[2]				
Age, *year*	59	11	57	61	59	4	0.76
Body weight, *kg*	80.3	19.3	81.2	82.8	75.5	6.3	0.72
BMI, *kg/m²*	28.5	6.5	29.1	28.3	28.1	2.1	0.94
Waist circumference, *cm*	95.9	16.7	96.9	98.6	90.9	5.4	0.60
Blood pressure , *mm HG*							
Systolic	127	20	123	133	123	6	0.42
Diastolic	74	10	74	74	75	3	0.97
Sex							
Male, %	40%		27%	64%	25%		0.13
Serum lipid measurement, *mmol/L*							
TC	5.98	0.72	6.13	5.86	5.95	0.24	0.7
LDL-C	3.67	0.54	3.59	3.65	3.83	0.17	0.62
HDL-C	1.53	0.43	1.72	1.35	1.51	0.13	0.14
TG	1.71	0.78	1.81	1.89	1.33	0.24	0.27

[1] Value presented as means and standard deviation (SD).
[2] Value presented as least square mean and standard errors of means (SEM). Comparison for baseline of different genotype group was analyzed by ANOVA

Table-S3.2 Genotype by diet interaction of *APOE* on changing circulating lipids in response to β-glucan diets compared to WR control[1,2].

	Diet				SEM	*P*-value[3]		
	WR Control	3g LMW	5g LMW	3g HMW		Diet	Genotype	Genotype x Diet
TC, *mmol/L*								
Start	5.75	5.87	5.91	5.92	0.18	0.6	0.41	0.4
End	5.51	5.35	5.51	5.38	0.12	0.26	0.14	0.11
Change	-0.39	-0.55	-0.39	-0.52	0.11	0.27	0.14	0.11
LDL-C, *mmol/L*								
Start	3.49	3.53	3.63	3.58	0.14	0.6	0.33	0.33
End	3.39	3.33	3.38	3.27	0.087	0.44	0.071	0.099
Change	-0.23	-0.34	-0.24	-0.36	0.086	0.44	0.071	0.099
HDL-C, *mmol/L*								
Start	1.46	1.5	1.46	1.47	0.093	0.33	0.79	0.02
End	1.39	1.33	1.39	1.36	0.025	0.25	0.91	0.31
Change	-0.1	-0.16	-0.1	-0.13	0.026	0.37	0.8	0.5
TG, *mmol/L*								
Start	1.78	1.84	1.78	1.94	0.13	0.66	0.51	0.44

End	1.65	1.61	1.63	1.68	0.12	0.93	0.17	0.99
Change	-0.081	-0.11	-0.096	-0.05	0.094	0.87	0.12	0.99

[1] Start, lipid concentrations at baseline; End, lipid concentrations at the end of each dietary intervention; Change, lipid concentrations of End-Start; WR, wheat and rice; LMW, low molecular weight; HMW, high molecular weight.

[2] Values are least square mean ± SEM for TC and LDL-C. Vaules are mean ± SEM for HDL-C and TG as log transformed data of these two parameters were used for statistical analysis. n =30. Labeled means in a row without a common letter differ, $P<0.05$ (Fisher's Least Significant Difference test).

[3] P-values are shown for the main effects of Diet, Genotype effect of *APOE*, and the Genotype x Diet interaction. End and Change of lipid concentrations were adjusted using three covariates: the baseline concentration of the blood lipid being analysed, as well as changes in body weight and waist circumference. Use of these covariates can change the apparent mathematical relationship between Start, End and Change.

Table-S3.3 Changes incirculating lipids wihout and with main effects of genotype and genotype by diet interations [1,2].

	WR Control	3g LMW	5g LMW	3g HMW	SEM	*P* for diet effect[3]	*P* adjusted by *CYP7A1*[4]	*P* adjusted by *APOE*[5]
Total cholesterol, *mmol/L*								
Start	5.74	5.90	5.96	5.95	0.14	0.072	0.039	0.6
End	5.45 [a]	5.41 [ab]	5.59 [a]	5.42 [b]	0.13	0.13	0.029	0.26
Change	-0.29 [a]	-0.48 [ab]	-0.37 [a]	-0.53 [b]	0.09	0.13	0.0046	0.27
LDL cholesterol, *mmol/L*								
Start	3.51 [a]	3.59 [ab]	3.69 [b]	3.66 [b]	0.10	0.071	0.046	0.6
End	3.39	3.35	3.50	3.35	0.11	0.14	0.077	0.44
Change	-0.12	-0.24	-0.20	0.31	0.07	0.14	0.077	0.44
HDL cholesterol, *mmol/L*								
Start	1.46 [a]	1.52 [b]	1.49 [ab]	1.49 [ab]	0.07	0.021	0.52	0.33
End	1.38	1.38	1.37	1.37	0.07	0.12	0.19	0.25
Change	-0.08	-0.14	-0.12	-0.13	0.02	0.19	0.28	0.37
Triglyceride, *mmol/L*								
Start	1.69	1.73	1.72	1.77	0.13	0.31	0.36	0.66
End	1.49	1.50	1.59	1.56	0.11	0.91	0.95	0.93

Change	-0.20	-0.22	-0.13	-0.21	0.09	0.51	0.75	0.87

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[1] Start, lipid concentrations at baseline; End, lipid concentrations at the end of each dietary intervention; Change, lipid concentrations of End-Start; WR, wheat and rice; LMW, low molecular weight; HMW, high molecular weight.

[2] Values are mean ± SEM. Means in the same row with different superscript letters are significantly different among treatments (Fisher's Least Significant Difference test for multiple comparison, $P < 0.05$).

[3] P-values are shown for the main effect of Diet without geneotype and genotype x diet interaction effects.End and Change of lipid concentrations were adjusted using three covariates: the baseline concentration of the blood lipid being analysed, as well as changes in body weight and waist circumference.

[4] P-values are shown for the main effects of Diet with the adjustment of Genotype effect of *CYP7A1*, and the Genotype x Diet interaction. End and Change of lipid concentrations were adjusted using three covariates: the baseline concentration of the blood lipid being analysed, as well as changes in body weight and waist circumference.

[4] P-values are shown for the main effects of Diet with the adjustment of Genotype effect of *APOE*, and the Genotype x Diet interaction. End and Change of lipid concentrations were adjusted using three covariates: the baseline concentration of the blood lipid being analysed, as well as changes in body weight and waist circumference. Use of these covariates can change the apparent mathematical relationship between Start, End and Chang.

CHAPTER 4: MECHANISMS OF CHOLESTEROL- LOWERING EFFECT OF BARLEY β-GLUAN

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This trial was registered at ClinicaTrials.gov with ID NCT01408719.

4.1 Abstract

Background: The cholesterol-lowering effect of β-glucan has been well documented. However, the underlying mechanism(s) responsible for its action is not fully understood.

Objectives: The primary objective of this study was to determine whether the consumption of barley β-glucan lowers cholesterol by affecting the cholesterol absorption, synthesis or bile acid synthesis. Our additional objective was to determine whether the underlying mechanisms are related to the genotype of *CYP7A1* SNP rs3808607.

Methods: In a controlled, randomized crossover study, subjects with serum total cholesterol (TC) between 5.0 and 8.0 mmol/L, LDL cholesterol (LDL-C) between 2.7 and 5.0 mmol/L (n =30) were randomly assigned to receivebreakfasts containing 3g high MW(HMW), 5g low MW (LMW), 3g LMW barley β- glucan or a wheat-rice control,each for 5 weeks. American Heart Association (AHA) diet as used as background diets for all treatment groups.The washout period between the study phases was 4 weeks. Cholesterol absorption was determined by assessing the enrichment of ^{13}C-cholesterol over 96 h following oral administration; fractional rate of synthesis (FSR) for cholesterol was assessed by measuring the incorporation rate of deuterium derived from deuterium oxide (D_2O) within the body water pool into the erythrocyte cholesterol pool over 24 h; bile acid synthesis was determined by measuring the serum 7α-hydroxy-4- cholesten-3-one (7αHC) concentrations. Single nucleotide polymorphism (SNP) rs3808607 for *CYP7A1* was genotyped using TaqMan® Assay.

Results: Consumption of 3g/d HMW β-glucan did not affect cholesterol absorption or cholesterol synthesis. Increased bile acid synthesis after consumption of 3g/d HMW β-glucan compared with the control(14.3 ng/ml vs.11.3 ng/ml, *P* =0.047), was observed in all subjects,

and more pronounced in individuals carrying homozygous-G of rs3808607 (12.6 ng/ml vs. 8.1 ng/ml, $P =0.033$).

Conclusions: Increased bile acid synthesis rather than inhibition of cholesterol absorption or synthesis may be the mechanism responsible for the cholesterol-lowering effect of barley β-glucan. Accordingly, the pronouncedly increased bile acid synthesis of G allele carriers of rs3808607 explains the pronounced TC reduction in this genotype.

Keywords: absorption,bile acids, β-glucan, cholesterol, mechanism synthesis

4.2 Introduction

The cholesterol-lowering effect of β-glucan found in oats and barley have been well documented (1, 2). The statement that a daily intake of a minimum of 3 g β-glucan soluble fibre from barley/oat grain products can reduce blood cholesterol concentrations has been approved as a health claim by several food regulatory agencies worldwide (3-5). Previous studies showed that the physicochemical properties of β-glucan play important roles in this cholesterol-lowering effect (6). However, to date, the precise underlying mechanisms responsible for its action are not well elucidated.

Cholesterol hemostasis is mainly determined by cholesterol absorption from diet (~45% of total cholesterol input), *de novo* synthesis (~65% - 75% of total cholesterol input), and hepatobiliary elimination (7-10). It has been proposed that β-glucan could influence cholesterol metabolism from any of these three cholesterol input or output pathways (11).

Since β-glucan is not absorbed, its physicochemical properties may alter the luminal environment of the small intestine such that the transit time increases and thickness of the unstirred water layer (UWL) increases, consequently reducing intestinal cholesterol absorption (12). The degradation of β-glucan in humans mainly relies on gut microbiota in the colon (13, 14). Short chain fatty acids (SCFA) generated from β-glucan fermentation in the colon might be able to affect cholesterol synthesis (15-17). However, the evidence supporting these two speculations, especially in humans is insufficient.

Bile acid biosynthesis accounts for approximately 40% of the daily elimination of cholesterol (18). Approximately 95% of the bile acids are reabsorbed in the terminal ileum (19, 20) and a small amount of bile acids are excreted with the feces (19). The conversion of bile acid from cholesterol occurs at a rate that precisely corresponds to the loss of bile acids within the feces (21). β-glucan has been thought to work as a bile acid sequestrant reducing cholesterol by decreasing bile acid reabsorption and further up-regulating bile acid synthesis (11, 22). The interruption of bile acid enterohepatic circulation resulting from β-glucan consumption has been observed in ileostomy patients by measuring bile acids in the ileostomy effluents (23); however, there is insufficient evidence in healthy subjects supporting this mechanism. 7α-hydroxy-4-cholesten-3-one (7α-HC) is a bile acid intermediate of the classical synthetic pathway (24), which is strongly correlated with the activity of the key enzyme cholesterol 7 α-hydroxylase (25). Thus, measuring blood 7α-HC levels is considered to be a reliable method for revealing bile acid synthesis in healthy humans (24).

In addition, genetic variation of *CYP7A1* SNP rs3808607, the gene encoding 7 α-hydroxylase, has been associated with different responses to β-glucan in lowering cholesterol levels. G allele carriers showed more pronounced responses in reducing total cholesterol (TC) than T/T allele carriers (6). G allele was speculated to be more responsive to dietary intervention in elevating the bile acid synthesis in our previous study (6). Yet, no evidence of phenotypes at the physiological level, in terms of bile acid synthesis, has been collected to confirm this speculation.

Collectively, our hypotheses are 1) β-glucan lowers blood cholesterol levels via suppressing cholesterol absorption, decreasing cholesterol biosynthesis and /or interrupting the enterohepatic

circulation of bile acids; 2) G allele carriers of rs3808607 have higher level of bile acid synthesis in responding to β-glucan intervention. Accordingly, the primary objective of this study was to assess whether the lowered serum cholesterol levels resulting from β-glucan consumption are due to the inhibition of cholesterol absorption, cholesterol synthesis, or increased bile acid synthesis. The additional objective was to assess the phenotype of *CYP7A1* SNP rs3808607 in responding to β-glucan intervention at a physiological level.

4.3 Methods

4.3.1 Procedure for clinical trial

The details of subject selection and clinical trial protocol were descried previously (6). Briefly, in a controlled, four phase crossover trial, subjects with serum TC between 5.0 and 8.0 mmol/L, LDL-C) between 2.7 and 5.0 mmol/L (n =30) were randomly assigned to receive AHA Step1 diet as background diets and different breakfasts containing 3g HMW, 3g low LMW, 5g LMW barley β- glucan or a control diet, each for 5 weeks. The washout period between the study phases was 4 weeks. The blood sample for cholesterol synthesis and bile acid synthesis determination were collected in days 34 and 35 of each phase.

4.3.2 Determination of cholesterol absorption

The cholesterol absorption in response to the intervention was determined by stable isotope methodology (26, 27). On day 30 of each study phase, subjects ingested 5g of margarine with 75 mg of [3, 4-^{13}C] cholesterol (99% APE; Cambridge Isotope Laboratories, Andover, MA, USA) spread on half English muffin. Fasting blood samples obtained prior to oral administration of isotope tracers (0 h) and during the following 96 hours on day 31 (24 h), day 32 (48 h), day 33 (72 h), and day 34 (96 h). Free cholesterol was extracted from red blood cells (RBC) using a method modified based on Folch's procedure (28). The isotopic enrichment of [3,4- ^{13}C] cholesterol was determined using gas chromatography isotope ratio mass spectrometry (GC-IRMS, ThermoFinnigan, Bremen, Germany) where samples were run through a GC unit, a combustion reactor and a mass spectrometer (26, 27). The cholesterol extracts were combusted to ^{13}C-enriched CO_2 and the combusted CO_2 gas was analyzed for ^{13}C enrichment against the international standard Vienna Pee Dee Belemnite (V-PDB) limestone. The area under curve

(AUC) of ^{13}C enrichment from 0 h to 96 h was calculated for determining cholesterol absorption.

4.3.3 Determination of cholesterol biosynthesis

The fractional cholesterol synthesis rate in response to the intervention diets was determined by stable isotope method using deuterated water (29). Specifically, cholesterol synthesis was assessed by measuring the rate of deuterium derived from D_2O within the body water pool into the erythrocyte cholesterol pool (29). On day 34 of each study phase, subjects ingested 0.7 g of D_2O /kg estimated body water (60% of body weight). Fasting blood samples were collected prior to D_2O oral administration on day 34 (0 h) and day 35 after D_2O ingestion (24 h). Blood samples were centrifuged and separated as plasma, buffy coat and RBC, and stored at -80° C until analysis. The lipid extracts were separated using GC and isolated cholesterol was submitted into a pyrolysis reactor to release H_2gas. Plasma water samples were run through a high temperature conversion elemental analyzer (TC-EA). Deuterium enrichments for both RBC and plasma water were measured by IRMS relative to the reference gas. Normalization to Vienna standard mean ocean water (V-SMOW) was performed using a regression equation between the online and offline method with data from the offline method expressed relative to V-SMOW. The fractional rate of synthesis (FSR) for cholesterol was calculated using the following equation:

FSR (pools/day) $= \frac{\Delta\, D-Cholesterol}{\Delta\, D-PW\, \times 0.478} \times 100\%$

Where:

ΔD-Cholesterol = difference in deuterium enrichment between 0-h and 24-h for cholesterol

ΔD-PW = difference in deuterium enrichment between 0-h and 24-h for plasma water

0.478 = ratio of labeled H atoms replaced by deuterium during *in vivo* biosynthesis (26).

4.3.4 Determination of bile acid biosynthesis

Bile acid biosynthesis was determined by measuring the serum concentration of 7α-HC using the Ultra performance liquid chromatography (ACQUITY UPLC System, Waters, Mississauga, ON, CA) coupled with a tandem mass spectrometer (Quattro micro™ API, Waters, Mississauga, ON, CA). 7α-HC was extracted by C18-E solid phase extraction (SPE) (Phenomenex®,Torrance, CA ,USA) mounted in a pump (KNF lab pump, KNF NEUBERGER, Trenton, NJ, USA) connected SPE processor (Agilent Technologies , Santa Clara, CA, USA) following the method modified based on Burkard et al. (30). 7α-Hydroxy-4-cholesten-3-one-d$_7$ (Medical Isotopes. Inc. Pelham, NH, USA) was used as an internal standard. Briefly, SPE cartridges were pre-conditioned with 2 x 2 ml methanol, 2 x 2 ml water and 2 x 2 ml 100 mM ammonium carbonate buffer pH 9.3. Serum (750 μl) mixed with ammonium carbonate buffer (1:1 v: v) and 150 μl of internal standard (40 ng/ml) were applied to the activated SPE cartridge. The speed for the mixture passing the cartridge was controlled within one drop per second passing through the cartridge using a vacuum pump. Subsequently the cartridges were washed with 2 x 2 ml of water and dried under N$_2$. Bile acids were desorbed with 3 ml of methanol. The eluted substances were dried under N$_2$, and dissolved in 150 μl of methanol before injecting into the UPLC. A reserved phase C18 column (Kinetex™ 1.7 μm XB-C18 100 Å, LC Column 100 x 2.1 mm, Phenomenex®Torrance, CA,USA) with mobile phase water with 0.1% formic acid (A) and methanol (B) was used for the separation. The ions used for the tracing of unlabelled 7α-HC were m/z 401 > 177 and the ion for tracing the 7α-Hydroxy-4-cholesten-3-one-d$_7$ were 407 > 177 (31).

4.3.5 Statistical analysis

Cholesterol absorption indicated by AUC of ^{13}C enrichment, cholesterol synthesis indicated by FSR and bile acid synthesis indicated by serum concentration of 7α-HC were analyzed using linear mixed-models (PROC MIXED, version 9.2; SAS Institute Inc, Cary, NC). Log transformations were used for data that were not normally distributed (7α-HC concentrations). Diet was modeled as fixed factors. Subjects were random effects in the model and the correlation between repeated measures of these individuals was modeled using the first-order autoregressive (AR (1)) option in the PROC MIXED procedure. Fisher's Least Significant Difference tests were used for multiple comparisons.

Linear regression of the means of 7α-HC concentration on log(viscosity) was performed using SAS (PROC REG, version 9.2; SAS Institute Inc, Cary, NC) and plotted with GraphPad Prism (GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla CA, USA). Nonparametric correlation was performed by using Spearman's rank correlation (JMP[®], Version *10*. SAS Institute Inc., Cary, NC). A value of *P*< 0.05 was considered to be significant.

4.4 Results

4.4.1 Changes in cholesterol absorption

Cholesterol absorption was not affected by β-glucan ingestion ($P = 0.35$). The AUC of 96 h enrichment of ^{13}C were 533.32, 542.66, 546.77 and 577.36 d ‰ ·h for the control, 3g LMW, 5g LMW and 3g HMW β-glucan, respectively (Table 4.1).

4.4.2 Changes in FSR for cholesterol

The FSR for cholesterol was not affected by β-glucan ingestion ($P = 0.35$). FSR resulting from the experimental diets assessed in a 24-h time window were 7.74%, 7.76%, 8.35% and 9.39% for control, 3g LMW, 5g LMW and 3g HMW β-glucan, respectively (Table 4.1).

4.4.3 Changes in serum 7α-HC concentrations

Consumption of 3g HMW β-glucan resulted in higher 7α-HC levels compared to control (14.26 ng/ml vs. 11.3 ng/ml, $P = 0.047$, Table 4.1). 7α-HC levels were not affected by LMW β-glucan, which is consistent with the results for lack of changes in suerm cholesterol. 7α -HC concentrations were positively correlated with the viscosity of β-glucan ($P = 0.049$, $R^2 = 0.91$, Figure 4.1).

4.4.4 Changes in cholesterol kinetics and serum 7α-HC in responding to CYP7A1 SNP

When the subjects were sub-grouped based on their genotypes of *CYP7A1* SNP rs3808607, 3g/d HMW β-glucan resulted in higher level of 7 α-HC levels compared to control (P =0.033) in GG genotype group, but not in TT and GT genotype groups.

AUC of RBC ^{13}C enrichment in G/G group showed a trend (P =0.07) for responding to 3g/d HMW β-glucan but not in GT or TT groups.

FSR for cholesterol in responding to β-glucan intervention did not show any association with *CYP7A1* SNP rs3808607.

Table 4.1 Changes of ^{13}C enrichment in RBC, cholesterol fractional synthesis rate (FSR) and serum concentrations of 7α-HC in response to β-glucan intervention[1,2].

	Diet				SEM	P -value[3]
	WR Control	3g LMW	5g LMW	3g HMW		
AUC-^{13}C enrichment in RBC, d ‰						
All subjects	533.32	542.66	546.77	577.36	29.02	0.35
TT	581.65	541.96	558.85	556.92	47.02	0.50
GT	493.25	563.53	554.82	533.48	40.09	0.39
GG	544.57	519.55	523.8	673.75	72.12	0.07
Cholesterol FSR, %						
All subjects	7.74	7.76	8.35	9.39	0.76	0.12
TT	6.08	7.96	8.63	9.18	1.1	0.12
GT	8.90	8.7	7.92	10.4	1.21	0.24
GG	8.23	6.13	8.54	8.07	1.78	0.49
7α-HC, ng/ml						
All subjects	11.30[a]	12.88[ab]	12.02[ab]	14.26[b]	1.16	0.047
TT	10.23	12.88	9.77	11.75	1.26	0.27
GT	16.6	15.49	15.85	18.62	1.26	0.57
GG	8.13[a]	10.00[ab]	10.96[ab]	12.59[b]	1.32	0.033

[1] WR, wheat and rice; LMW, low molecular weight; HMW, high molecular weight.
[2] Least squares means (LSM) and standard errors of means (SEM) are presented. LSMs in the same row with different superscript letters are significantly different among treatments (Fisher's Least Significant Difference test for multiple comparison, $P <0.05$).
[3] P-values of AUC were for the AUC normalized by the dose of ^{13}C-cholesteorl.

Figure 4.1 Linear relationship between 7 α- HC concentration and log (viscosity) of β-glucan.

Values are means ± SEMs for the four treatments following the order of WR control, 3g LMW, 5g LMW and 3g HMW from left to right. *P*-value was from linear regression test.

$P = 0.049$, $r^2 = 0.91$
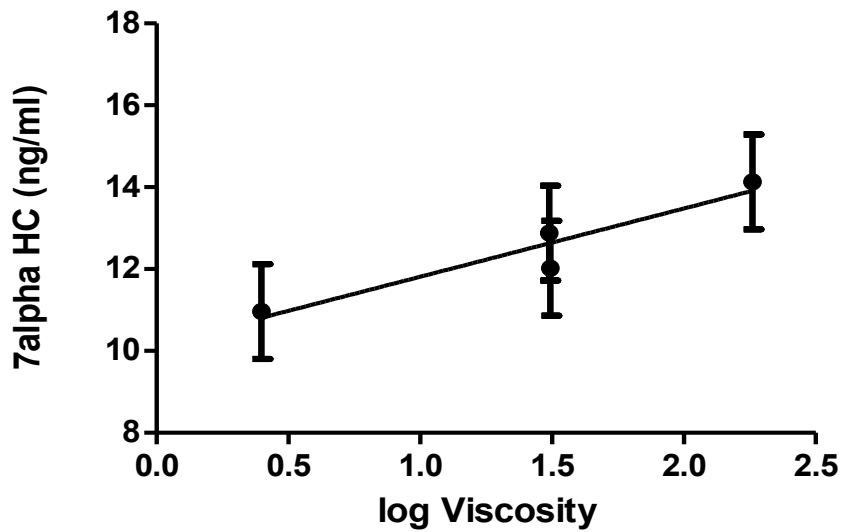
4.4.5 Correlation between kinetic parameters and blood lipids

In the nonparametric correlation (Table 4.2), FSR for cholesterol was negatively related to the blood TC, LDL-C and HDL-C levels (P = 0.0021, 0.0007, 0.020, respectively), though the coefficient was low (Spearman's ρ =-0.28, -0.31, -0.21, respectively). On the other hand, ^{13}C-enrichment in RBC (AUC) showed a positive relationship with blood TC, LDL-C and HDL-C levels (P= 0.0031, 0.010, <0.01, respectively; Spearman's ρ =0.27, 0.24, 0.41, respectively), but a negative relationship with TG (P<0.001, Spearman's ρ =-0.35). 7α-HC levels was not related with TC or LDL-C, but positively related with TG and TC/HDL-C (P<0.0001, 0.0005, respectively, Spearman's ρ =0.47, 0.32, respectively).

BMI and waist circumference were strongly related with AUC (P <0.0001, Spearman's ρ = -0.64, -0.67, respectively), and weakly related with FSR for cholesterol (P =0.0005, 0.0001, respectively, Spearman's ρ =0.32, 0.35, respectively).

AUC and FSR were negatively related with each other (P =0.0008, Spearman's ρ =- 0.30). 7α-HC was not found to be related with either AUC or cholesterol FSR.

Table 4.2 Nonparametric correlation of kinetic parameters, anthropometrics and blood lipids.[1]

Variable	By variable	Spearman's ρ	*P*-value
FSR			
	AUC	-0.30	0.0008
	7α- HC	0.15	0.13
	BMI	0.32	0.0005
	Waist circumference	0.35	0.0001
	TC	-0.28	0.0021
	LDL-C	-0.31	0.0007
	TG	0.14	0.13
	HDL-C	-0.21	0.012
	TC/HDL-C	0.11	0.24
AUC			
	FSR	-0.30	0.0008
	7α- HC	-0.074	0.44
	BMI	-0.64	<.0001
	Waist circumference	-0.67	<.0001
	TC	0.27	0.0031
	LDL-C	0.24	0.0101
	TG	-0.35	<.0001
	HDL-C	0.41	<.0001
	TC/HDL-C	-0.29	0.0015
7α- HC	FSR	0.15	0.13
	AUC	-0.074	0.44

BMI	0.18	0.064
Waist circumference	0.26	0.0055
TC	0.026	0.78
LDL-C	-0.026	0.79
TG	0.47	<.0001
HDL-C	-0.26	0.0065
TC/HDL-C	0.32	0.0005

[1] Spearman's ρ**,**Spearman's correlation coefficient; FSR, fractional rate of synthesis for cholesterol; AUC, area under curve of ^{13}C enrichment; 7-α HC, 7α-hydroxy-4- cholesten-3-one.

4.5 Discussion

The significance of the present study is clarifying the underlying mechanisms responsible for the cholesterol-lowering effect of β-glucan. This study employed comprehensive methodologies covering three metabolic pathways that might be affected by β-glucan consumption: cholesterol absorption, cholesterol synthesis, and bile acid synthesis.

The cholesterol-lowering efficacy of β-glucan has been demonstrated in our previous study, where consumption of 3g/d high MW β-glucan diet lowered TC significantly but neither the consumption of 3g/d or 5g/d low MW β-glucan. Consistently, in the present study, we found that consuming 3g/day high MW β-glucan resulted in higher serum 7α-HC levels compared to the control, but not any of the LMW β-glucan. However, cholesterol absorption measured by the area under the [3,4]-^{13}C cholesterol RBC enrichment curve for 96 h and FSR for cholesterol in 24-h time window were not changed by any of the β-glucan diets. These results indicate that increasing bile acid synthesis rather than inhibiting cholesterol synthesis or absorption is the mechanism responsible for the cholesterol-lowering effect of β-glucan.

Inhibition of cholesterol absorption has been speculated widely as the mechanism responsible for the cholesterol lowering effect of β-glucan (32). Dietary cholesterol absorption, occurring in the upper intestine (33), is a multistep process involving the activities of digestive enzymes and the formation of micelles in the lumen, as well as the transport proteins at enterocyte level (34). Before interacting with the transmembrane protein, cholesterol must pass the UWL toward the brush border of the small intestine (34). Hypothetically, with the viscous property, β-glucan

might be able to affect the lipid absorption by altering the luminal environment such as slowing

the transit time and increasing the thickness of UWL, or affecting activities of digestive enzymes

and the formation of micelles (12, 35). Naumann et al. (2006) reported that serum sitosterol, as a

cholesterol absorption marker, was decreased with consumption a fruit drink containing β-glucan

(36); however, in an ileostomy study, Lia et al. (1997) reported no changes in the absorption of

dietary cholesterol after an oat bran meal containing β-glucan, where a radioisotope method with

$[β$ -4-^{14}C]-sitosterol and $[1α, 2α$-n-^{3}H]-cholesterol as tracers was used (37). This discrepancy

may be due to the different methodologies used for measuring cholesterol absorption. As an

inhibitor of intestinal cholesterol uptake, the use of sitosterol may lead to an underestimation of

the actual capacity of cholesterol absorption (38). In agreement with Lia et al's observation, our

96 h-^{13}C-cholesterol enrichment from consumption of β-glucan diet did not differ from the

control diet. Therefore, our results provide more evidence suggesting the cholesterol-lowering

effect of β-glucan is not mediated through the inhibition of cholesterol absorption but alternative

pathways.

Deuterium incorporation method utilized in this study is a reliable means to assess

cholesterogenesis (39). In this isotopic method, heavy water (D_2O) was used as a tracer to

determine the synthesis of free cholesterol. The fractional synthesis rate (FSR, in pools/day) of

free cholesterol was calculated from the rate of incorporation of heavy water into *de novo*

synthesized erythrocyte cholesterol across four experimental diets. Ellegard et al. (2007)

reported in ileostomy patients that within 24 hours of consumption, native β-glucan increased

bile acid excretion and also increased serum lathosterol concentrations, a marker for cholesterol

synthesis (40). Cholesterol homeostasis is regulated by both cholesterol input and output. The

loss of bile acid in excretion and the subsequent demand of cholesterol for compensating the bile acid pool can possibly up-regulate the synthesis of cholesterol (11). However, in our study, changes of cholesterol FSR failed in reaching statistical significance. Despite the discrepancy in increasing and not changing cholesterol-synthesis, both Ellegard et al. (2007)'s study and our present study reject the hypothesis that consumption of β-glucan inhibits the cholesterol synthesis.

Our results demonstrated that consumption of HMW β-glucan is able to increase bile acid synthesis. Moreover, our data also revealed that the high viscosity resulting from HMW of β-glucan is the factor that drove this mechanism: serum 7 α-HC concentrations were positively correlated with the viscosity of the treatment diets (Figure 4.1). After ingestion, high viscous β-glucan enters the lumen of small intestine, where it may act similarly as a bile-acid sequestrating agent to interact with bile acids and lead to bile acid being entrapped instead of being reabsorbed. The expected consequences of these actions would increase fecal bile acid loss and enhanced hepatic bile acid synthesis from cholesterol to replenish the bile acid pool (11). The interruption of entrohepatic circulation of bile acid caused by β-glucan has been reported in ileostomy patients (37, 40-43). In a short-term interventional crossover study, 9 subjects with conventional ileostomies received diet containing either native or hydrolyzed β-glucan (11. 6g), each for 3 days. The native β-glucan consumption resulted in 40% more ileal bile acid excretion compared with consuming the hydrolysed β-glucan diet. Moreover, native β-glucan in this study also increased serum 7 α- HC levels by 57% within 24 h of consumption (40). A study from Marlett et al. (22) investigated whether oats lower cholesterol levels by decreasing bile acid and fat absorption, and increasing bile acid synthesis in 9 normolipidemic men. Subjects consumed a

low fibre diet for 28 days and followed by oat bran diet containing 5.4 g of soluble β-glucan for another 28 days without a washout interval. The oat bran diet resulted in increased synthesis and factional turnover rates of two primary bile acids cholic acid (CA) and chenodeoxycholic acids (CDCA), along with decreased serum cholesterol levels compared to a low fiber diet without β-glucan. These previous studies observed bile acid metabolism being interrupted by β-glucan, but are all with limitations such as small sample size (n <10). Moreover, ileostomy studies usually have short intervention period for just a few days.Marlett et al. 's study(22)had a longer term for intervention, but lacked a washout period. The results from the present study with 30 healthy subjects with a controlled, randomized and crossover design, therefore provide stronger evidence that β-glucan reduces circulating cholesterol concentration through increasing bile acid synthesis.

The interaction between β-glucan and bile acids is not fully understood. Studies for elucidating how β-glucan can "bind" or "entrap" bile acids are inadequate. In a ^{13}C NMR titration study, with a model bile salt (taurochenodeoxycholic -TCDC) in micelles, Gunness et al. (2010) suggested that β-glucan interacts directly on a molecular length scale (44). However the MW of β-glucan was not considered in this study and only one type of conjugated bile acid was used in the micelle model. Therefore, to investigate the interaction between bile acid and β-glucan, future studies with a complete bile acid/micelle model and with the consideration of the special role of high MW in the action are requested.

Cholesterol-lowering effects were associated with the genetic variation of *CYP7A1* rs3808607, and specifically, individuals carrying SNP rs3808607-G allele *CYP7A1* were more responsive to HMW β-glucan in lowering cholesterol levels than the individuals who were homozygous for the

rs3808607-T allele (6). In the present study, the subjects were sub-grouped based on genotypes of *CYP7A1* rs3808607, and we found that individuals homozygote for the G allele (G/G) showed significant response to 3g HMW β-glucan in increasing 7 α-HC concentrations compared to the control, but not for subjects who carry the TT and GT genotype of CYP7A1 rs3808607. This result reveals the important role of CYP7A1 in the bile acid interruption mechanism and the influence of the genetic variation in this action. As reported previously, the G-allele mediates greater gene expression than homozygote T based on their increased transcriptional activity; this was suggested to lead to increased bile acid synthesis in the liver since CYP7A1 is the key enzyme in the classical pathway (45). This confirmed our previous speculation that rs3808607-G allele carriers are more responsive to the elimination of bile acids from the enterohepatic circulation caused by β-glucan (37, 40, 41, 46, 47). As a consequence, under the stimulation of 3g HMW β-glucan that "bound" or "entrapped" the greatest amount of bile acid, GG carriers responded the "elimination signal" given by the decreased level of primary bile acid (resulting from the suppression of bile acid reabsorption) in the liver, to produce a greater amount of bile acid for complementing the fecal loss. On the other hand, T/T and carriers were insensitive to this negative feedback and unresponsive to enhance the bile acid synthesis.

Nonparametric correlation revealed the internal relationship among the metabolites and the relationship of kinetics with BMI and body fat distribution (Table 4.2). Cholesterol homeostasis in humans is determined by intestinal cholesterol absorption, *de novo* synthesis, and the elimination such as using cholesterol for bile acid production in the liver, which regulates each other. Alteration of any of these three perspectives may affect the other. AUC of ^{13}C-enrichment and FSR were negatively correlated with each other (Spearman's ρ=- 0.30, P = 0.0008), which

agrees with the hypothesis that when more free cholesterol enters the liver from the diet, synthesis of cholesterol will be suppressed, and vice versa. Moreover, a strong negative relationship between BMI and cholesterol absorption and also a strong negative relationship between waist circumference and cholesterol absorption were observed (Spearman's ρ=-0.64, P <0.001 and Spearman's ρ=-0.67, P <0.001, respectively). However, this correlation is invalid to reflect the relationship between body size and cholesterol absorption as the ^{13}C-cholesterol in this study was given at one consistent dose (0.75 mg) despite the body size to all the subjects. Subjects who had a smaller BMI obtained more ^{13}C-cholesterol/kg body weight than the subjects who had a larger BMI. Therefore, more ^{13}C-cholesterol might be accumulated in smaller body size subjects than larger body size subjects. However, including body weight and WC in the PROC MIXED procedure did not change the statistical results (data not shown). On the other hand, D$_2$O was given based on the size of the subject's body water pool. BMI and waist circumference showed a weak positive relationship with cholesterol synthesis (Spearman's ρ =0.32, P =0.0005 and Spearman's ρ =0.36, P =0.0001, respectively). These results suggest individuals having larger body size might have greater cholesterol synthesis ability.

Our results also indicate that serum TG concentrations have a positive correlation with 7 α-HC (Spearman's ρ= 0.47, P<0.001). The correlation between bile acid and TG metabolism has been observed clinically (48). For example, individuals with decreased bile acid synthesis due to a CYP7A1 deficiency showed increased serum TG concentrations (48, 49). Farnesoid X receptor (FXR) is a nuclear receptor playing critical roles in bile acid synthesis and also TG metabolism. Bile acids such as CDCA are endogenous ligands for FXR (50), which mediate the suppression of bile acid synthesis via a negative feedback mechanism (51). The activation of FXR also

modulates free fatty acids oxidation and TG clearance (48). Therefore, it is not surprising to see that bile acid synthesis and TG concentrations are positively correlated with each other. Overall, the results in current study showed that consumption of 3g HMW β-glucan increased serum 7 α-HC but did not affect the AUC of ^{13}C-cholesterol enrichment in RBC and cholesterol FSR. Secondarily, in agreement with our previous finding, homozygous rs3808607-G allele carriers had in greater responses in lowering serum cholesterol levels and higher level of bile acid synthesis after ingesting 3g HMW β-glucan. In conclusion, the present study suggests that interrupting enterohepatic circulation of bile acids rather than inhibiting cholesterol absorption and synthesis is the mechanism responsible for the cholesterol-lowering effect of β-glucan; the association between varied genotypes of *CYP7A1*rs3808607 and different responses to the cholesterol-lowering effect of β-glucan is attributed to the different responsiveness in producing bile acids of the three genotypes, TT, GT and GG.

Contributions and Acknowledgements

The authors' responsibilities were as follows:

YW responsible for conducting the clinical trial (detailed responsibilities were listed at the end of Chapter 3) and isotopic tracer administration, collecting data and samples from the clinical trial, preparing samples for measuring ^{13}C-cholesterol enrichment from RBC and deuterium enrichment from RBC and plasma water, measuring ^{13}C-cholesterol and deuterium on IRMS with the assistants of the technician, preparing isotopic data for statistical analysis from raw data generated from IRMS, performing statistical analysis. YW development the protocol of 7α-HC extraction based on the published literature and measured 7α-HC on UPLC with the assistants of the technician Haifeng Yang. YW analyzed the blood lipids, genotyped SNPs and wrote the manuscript.

SVH was responsible for supervising YW during trial conduction, conducting the clinical trial and collecting samples and data from the trial from phase 1 to 2. SVH also trained YW for isotopic tracer administration and was responsible for tracer administration from phase 1 to 2. SVH analyzed ^{13}C-cholesterol and deuterium enrichment for 8 subjects.

SJT contributed to sample reparation for ^{13}C-cholesterol and deuterium RBC enrichment analysis and manuscript revision.

SMT was responsible for physicochemical property measurements of the treatments, and scientific discussion regarding design and execution of the clinical trial and food preparation techniques.

PJHJ was responsible for scientific co-supervising the first author for the clinical trial, the blood lipid and cholesterol kinetic concept.

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CHAPTER 5: HIGH MOLECULAR WEIGHT BARLEY β-GLUCAN PROMOTES A FAVORABLE HUMAN GUT MICROBIOTA ASSOCIATED WITH REDUCED CARDIOVASCULAR DISEASE RISK

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5.1 Abstract

Background: The physiological benefits of β-glucan, such as cholesterol-lowering effect have been documented. However, whether active modulation of gut microbiota by β-glucan is associated with its physiological effects is unknown.

Objectives: The objectives of this study were to determine the impact of β-glucan on gut microbiota composition of mildly hypercholesterolemic individuals and to identify if the altered microbiota is associated with bioactivity of β-glucan in improving risk factors of cardiovascular disease (CVD).

Methods: In a randomized, controlled crossover study, individuals received either a treatment breakfast containing 3g high molecular weight (HMW), 3g low molecular weight (LMW), 5g LMW barley β-glucan or wheat and rice (WR) as control for 5 weeks. American Heart Association (AHA) diet as used as background diets for all treatment groups. Phases were separated by 4-week washout periods. Fecal samples were collected at the end of each intervention phase and subjected to illumina sequencing of 16S rRNA genes.

Results: Results revealed that at the phylum level, supplementation of 3g/d HMW β-glucan increased Bacteroidetes and decreased Firmicutes abundance compared to control ($P < 0.001$). At the genus level, consumption of 3g/d HMW β-glucan increased *Bacteroides* ($P = 0.002$), tended to increase *Prevotella* ($P < 0.1$) but decreased *Dorea* ($P < 0.1$), whereas diets containing 5g LMW β-glucan and 3g LMW β-glucan failed to alter gut microbiota composition. The impacted *Bacteroides, Prevotella,* and *Dorea* composition were correlated with favorable shifts of CVD risk factors, including body mass index (BMI), waist circumference, blood pressure, HDL cholesterol (HDL-C) and triglyceride (TG) levels.

Conclusions: Results suggest that consumption of HMW β-glucan favorably alters the composition of the gut microbiota and this altered microbiota profile as a result of HMW β-glucan consumption is associated with a reduction of CVD risk factors. Together, our study suggests that β-glucan shift gut microbiota in a MW-dependent manner and altering gut microbiota may be one of the underlying mechanisms responsible for the physiological benefits of β-glucan.

5.2 Introduction

Mixed-linkage $(1\rightarrow3)(1\rightarrow4)$- β-D-glucan (β-glucan) is a linear polysaccharide composed of D-glucose monomers joined by $(1\rightarrow4)$-glycosidic bonds (~ 70%) with an occasional $(1\rightarrow3)$-glycosidic bond (~30%) (1). It is the major soluble fiber in oats and barley (2). The documented physiological benefits resulting from consuming products containing β-glucan include cholesterol lowering (3, 4), reduction of postprandial glycaemic responses (5-7), and weight management via increasing satiety (8, 9). Previous investigations into the mechanisms underlying these beneficial effects are limited to the action of β-glucan in the upper gastrointestinal (GI) tract; however, the fermentation of β-glucan occurring in the lower GI tract has been insufficiently studied. Since humans lack enzymes for breaking down dietary fibre, degradation of β-glucan relies on the microbes residing the lower GI tract(10, 11). The assortment of the microbes inhabiting the GI tract refers to gut mcirobiota (12), which plays an important role in human health and diseases (12, 13). Altered gut microbiota has been associated with metabolic disorders, including obesity (14-17), diabetes (17, 18) and cardiovascular disease (CVD) (19-21). Current reports suggest manipulation of gut microbiota toward enhancing the proportion of beneficial members of the microbial community is a promising strategy to treat or prevent metabolic diseases(13, 18, 22).Mixed-linkage β-glucan as fermentable dietary fiber (23) could actively impact the microbiota as exhibited from *in vitro* fermentation (24) and *in vivo* intervention studies (25, 26). However, conclusions regarding the changes of bacterial taxa resulting from β-glucan consumption or fermentation remain inconsistent and a well-designed study is needed to provide comprehensive information of the shifts in the gut microbiota following β-glucan consumption in humans.

Physicochemical properties of β-glucan, such as molecular weight (MW), are considered to be critical factors in its physiological properties (27, 28) and the extent of β-glucan fermentation in the distal GI tract may also depend on its physicochemical structure (24). Yet, whether β-glucan with low or high MW alters gut microbiota in a differential manner is unknown.

In this study we aimed to investigate 1) whether consumption of barley β-glucan alters the composition of gut microbiota; 2) whether the shift in gut microbiota is dependent on the MW of β-glucan; and 3) whether shifts in gut microbiota are associated with physiological effects resulting from β-glucan consumption.

5.3 Materials and Methods

A randomized, single-blinded, controlled crossover trial was conducted at the Clinical Nutrition Research Unit at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN), University of Manitoba (Winnipeg, MB, Canada) in accordance with the principles expressed in the Declaration of Helsinki. All research procedures were approved by the University of Manitoba Biomedical Research Ethics Board (Ethic reference no. B2010:057). The clinical trial was registered with ClinicalTrials.gov (ID NCT01408719).

5.3.1 Subjects

Male and female subjects aged 27-78 years (n =30) with a body mass index (BMI; in kg/m^2) between 20 and 40 were selected on the basis of fasting serum total cholesterol (TC) between 5.0 and 8.0 mmol/L and LDL cholesterol (LDL-C) between 2.7 and 5.0 mmol/L. Potential Volunteers were excluded using the following criteria: intake of blood lipid lowering medications, herbal or nutritional supplements which are known to affect blood lipids and any dietary restrictions which would affect trial completion. No antibiotics intake was reported at the time of screening. A study physician evaluated the health history before the volunteers were accepted as subjects. The physician also provided medical supervision throughout the study. Written informed consent was obtained from all subjects. Of the 30 subjects in this study, a subset of 19 subjects who completed the study and provided fecal samples for all 4 study phases were selected for the microbiota profile analysis.

5.3.2 Study design and experimental diets

As described in the parallel study (29), the human clinical trial consisted of four 5-week study phases separated by 4-week washout periods.Subjects were randomized to one of four experimental diet in each phase: a) a wheat and rice (WR) based control; b) 3g LWM β-glucan (288 kDa); c) 5g LMW barley β-glucan (292 kDa) and d) 3g high molecular weight (HMW) barley β-glucan (1,349 kDa). Each participant received all four treatments during the study in randomized orders. The barley treatment diets were incorporated into breakfast in the format of crepes, tortillas, porridge, and chips. Wheat and rice were used to replace barley in breakfast as control. Lunch and dinner were designed using a 7-day rotating menu to provide daily energy needs. Energy requirements of each subject were estimated based on age, gender, body weight and physical activity levels and adjusted proportionately in 300 kcal increments.Macronutrient profile of treatment diets provided approximately 30% of energy as fat, 55% as carbohydrate and 15% as protein. Subjects consumed their intervention breakfast meals under supervision at the clinical unit at the RCFFN on weekdays to ensure compliance. Remaining weekday meals and weekend meals including intervention breakfast were packed for home consumption and empty containers were returned to ensure compliance.

Body weight was monitored everyday on weekdays at the clinical unit prior to breakfast. Waist circumference was measured at the beginning and the end of each intervention phase. Fasting serum and plasma samples were collected on days 1, 2, 34 and 35. Serum TC, HDL cholesterol (HDL-C), LDL-C and triglycerides (TG) were measured.

Approximately 15 g of stool samples were collected at the end of each phase, between days 30 and 35. Subjects were advised to collect stool samples from three locations of one bowel

movement and immediately store the collected samples in their household -20 °C freezer or in a

consistent location with low temperature. Upon transport, samples were transferred to chilled

coolers and then stored for future analysis at -80 °C.

5.3.3 DNA extraction and quality check

Approximately 200 mg of each fecal sample was used for DNA extraction using ZR Fecal DNA

MiniPrep^{TM} (Zymo Reseach, Irvine, CA, USA), which included a bead-beating step for the

mechanical lysis of the microbial cells. DNA was quantified using a NanoDrop 2000

spectrophotometer (Thermo Scientific, DE, USA). DNA samples were normalized to 20 ng/μl,

and quality checked by PCR amplification of the 16S rRNA gene using universal primers 27F

(5'-GAAGAGTTTGATCATGGCTCAG-3') and 342R (5'-CTGCTGCCTCCCGTAG-3') as

described by Khafipour et al. (30). Amplicons were verified by agarose gel electrophoresis.

5.3.4 Library construction and illumina sequencing

Library construction and illumina sequencing were performed as described by Derakhshani et al.

(31). In brief, the V4 region of 16S rRNA gene was targeted for PCR amplification using

modified F515/R806 primers (32). The reverse PCR primer was indexed with 12-base Golay

barcodes allowing for multiplexing of samples. PCR reactions were performed in duplicate for

each sample and contained 1.0 µl of pre-normalized DNA, 1.0 µl of each forward and reverse

primers (10 µM), 12 µl HPLC grade water (Fisher Scientific, ON, Canada) and 10 µl 5 Prime

Hot MasterMix® (5 Prime, Inc., Gaithersburg, USA). Reactions consisted of an initial

denaturing step at 94°C for 3 minutes followed by 35 amplification cycles at 94°C for 45

seconds, 50°C for 60 seconds, and 72°C for 90 seconds; finalized by an extension step at 72°C

for 10 minutes in an Eppendorf Mastercycler® pro (Eppendorf, Hamburg, Germany). PCR products were then purified using a ZR-96 DNA Clean-up Kit™ (ZYMO Research, CA, USA) to remove primers, dNTPs and reaction components. The V4 library was then generated by pooling 200 ng of each sample, quantified by Picogreen dsDNA (Invitrogen, NY, USA). This was followed by multiple dilution steps using pre-chilled hybridization buffer (HT1) (Illumina, CA, USA) to bring the pooled amplicons to a final concentration of 5 pM, measured by an Qubit® 2.0 Fluorometer (Life technologies, ON, Canada). Finally, 15% of PhiX control library was spiked into the amplicon pool to improve the unbalanced and biased base composition, a known characteristic of low diversity 16S rRNA libraries. Customized sequencing primers for read1 (5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′), read2 (5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) and index read (5′-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3′) were synthesized and purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, IA, USA) and added to the MiSeq Reagent Kit V2 (300-cycle) (Illumina, CA, USA). The 150 paired-end sequencing reaction was performed on a MiSeq platform (Illumina, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, Department of Animal Science, University of Manitoba, Canada.

5.3.5 Bioinformatic analyses

Bioinformatic analyses were performed as described by Derakhshani et al. (31). In brief, the PANDAseq assembler (33) was used to merge overlapping paired-end Illumina fastq files. Sequences with mismatches or ambiguous calls in the overlapping region were discarded. The output fastq file was then analyzed by downstream computational pipelines of the open source

software package QIIME (34). Assembled reads were demultiplexed according to the barcode

sequences and exposed to additional quality-filters so that reads with more than 3 consecutive

bases with quality scores below 1e-5 were truncated, and those with a read length shorter than 75

bases were removed from the downstream analysis. Chimeric reads were filtered using UCHIME

(35) and sequences were assigned to Operational Taxonomic Units (OTU) using the QIIME

implementation of UCLUST (36) at 97% pairwise identity threshold. Taxonomies were assigned

to the representative sequence of each OTU using RDP classifier (37) and aligned with the

Greengenes Core reference database (38) using PyNAST algorithms (39). The phylogenetic tree

was built with FastTree 2.1.3. (40) for further comparisons between microbial communities.

5.3.6 Alpha-diversity and β-diversity measurement

Within community diversity (α-diversity) was calculated using QIIME. Alpha rarefaction curve

was generated using Chao 1 estimator of species richness (41) with 10 sampling repetitions at

each sampling depth. An even depth of approximately 3984 sequences per sample was used for

calculation of richness and diversity indices. To compare microbial composition between

samples, β-diversity was measured by calculating the weighted and unweighted Unifrac

distances (42) using QIIME default scripts. Principal coordinate analysis (PCoA) generated two-

dimensional plots using PRIMER v6 software using resulting distance matrices (43).

Permutational multivariate analysis of variance (PERMANOVA) (44) was used to calculate P-

values and test for significant differences of β-diversity among treatment groups. The PICRUSt

(Phylogenetic Investigation of Communities by Reconstruction of Unobserved States, version

1.0.0) was used to predict metagenome function based on 16S rRNA sequences (45). For the

PICRUSt analysis, closed-reference OTUs were picked at 97% similarity against the Greengenes

database. The OTUs were normalized for predicted 16S rRNA copy number before predicting

gene family abundance for each metagenome based on KEGG orthology groups (Kos) using the

Kyoto Encyclopedia of Genes and Genomes (45).

5.3.7 Statistical analysis

Statistical analyses were performed as described by Li et al. (46) and Derakhshani et al. (31).

Non-normally distributed data were Box-Cox transformed and then used to assess the effect of

experimental diet using MIXED procedure of SAS version 9.2 (SAS Institute Inc., Cary, NC,

USA). Changes of α-diversity measurements (Chao1, Good's coverage, Shannon and Simpson,

Observed species) were tested using MIXED procedure of SAS. Differential abundances

in bacterial phyla and genera were tested using MIXED and GLIMMIX procedures of SAS, with

treatment, phase and phase ×treatment as fixed factors. Subjects were treated as a random factor

and the correlation between repeated measures of these individuals was modeled using the first-

order autoregressive (AR (1)) option.Pairwise comparisons among the groups were tested

using Tukey studentized range adjustment. The differences between groups were considered

significant at $P< 0.05$ while trends were observed at $P< 0.1$.

Permutational multivariate analysis of variance (PERMANOVA) (44) was used to calculate P-

values and test for significant differences of β-diversity among treatment groups. The effects of

treatment, period, and treatment by period were considered fixed factors and subject as the

random factor.

Partial least square discriminant analysis (PLS-DA; SIMCA P+ 13.0, Umetrics, Umea, Sweden) was performed on genus data to identify the effects of treatments. The PLS-DA is a particular case of partial least square regression analysis in which Y is a set of variables describing the categories of a categorical variable on X. In this case, X variables were bacterial genera and Y variables were experimental diets. For this analysis, data were scaled using Unit Variance in SIMCA. Cross-validation was performed to determine the number of significant PLS components and a permutation testing was conducted to validate the model. To avoid over-parameterization of the model, variable influence on projection value (VIP) was estimated for each genus and genera with VIP < 0.50 were removed from the final model (47, 48). R^2 estimate then was used to evaluate the goodness of fit and Q^2 estimate was used to evaluate the predictive value of the model. The PLS-regression coefficients were used to identify genera that were most characteristics of each treatment group. The significant shifts of taxa were determined when the error bars of each component above or below x axis of coefficient plot. The results of PLS-DA were visualized by PLS-DA loading scatter plots.

Welch's t-test was applied on gene function from PICRUSt outputs, percentage of the predicted metagenome made by a given KEGG functional module, to compare differences in predicted metagenomic functions between treatment groups.

Correlations between bacterial taxa and clinical parameters were performed using nonparametric Spearman's rank correlation (JMP®, Version *10.* SAS Institute Inc., Cary, NC, USA). The clinical parameters and bacterial taxa defined as response (Y) variables and a multivariate platform was used to examine how many variables relate to each other. For each correlation, the

correlation coefficient (Spearman's ρ) and *P*-value were obtained. The correlation coefficient values ranged from -1 to +1 with larger absolute values indicating stronger relationship while positive and negative values indicating the direction of association. Alpha value for the correlation confidence intervals was set up as 0.05.

5. 4 Results

5.4.1 Changes in metabolic biomarkers

The results of blood glucose, blood pressure, body weight, waist circumference, and BMI were consistent with the results of parallel study that included 30 subjects and focused on examining the blood cholesterol lowering effects of β-glucan (Table-S5.1). The only exception was the TC levels, which was reduced by 3g HMW β-glucan in the parallel study but not in the sub-set of 19 individuals subjected to microbiome analysis.

5.4.2 Changes in α- and β-diversity

Alpha-diversity indices of fecal microbiota, or the diversity within a community at one site, did not change in response to consumption of β-glucan (Table 5.1). In contrast, β-diversity of the fecal microbial community, or the dissimilarity between communities of many sites, measured by weighted UniFrac was significantly affected by β-glucan supplementation (P = 0.002 PERMANOVA, Figure 5.1). In the pairwise comparison, significant differences were observed among microbial communities between 3g HMW and 3g LMW β-glucan (P =0. 014), 3g HMW β-glucan and the control (P = 0. 011), and 5g LMW β-glucan and the control (P =0. 044). The community composition profile of the above-mentioned three paired comparisons, which showed significant differences in their β-diversity, were further compared using PLS-DA as discussed below (Figure 5.2, 5.3,5.4).

Table 5.1 Biodiversity of microbial communities in feces affected by β-glucan diets.

Alpha Diversity[1]	Diet[2]				SEM[3]	*P*-value[4]
	WR Control	3g LMW	5g LMW	3g HMW		
Chao1	2502[5]	2594	2548	2730	122	0.22
Good's coverage	0.85	0.84	0.84	0.83	0.01	0.14
Observed species	865	893	881	931	33	0.16
Shannon	7.45	7.54	7.51	7.68	0.19	0.36
Simpson	0.98	0.98	0.98	0.98	0.003	0.58

[1]Chao1 and Good's coverage are the richness indices; Shannon and Simpson are diversity estimators.
[2]Diets include: Wheat and rice control (WR Control), 3g/d low molecular weight β-glucan (3g LMW), 5g/d low molecular weight β-glucan (5g LMW), 3g/d high molecular weight β-glucan (3g HMW).
[3]SEM, standard error of means.
[4]*P*-values are shown for the diet effect between groups analyzed by mixed model ANCOVA with period and the interaction of period×diet as fixed factors.
[5]Data are presented as mean value.

etween microbial communities of stool samples in responses to β-glucan and control iets.

a) comparison among four experimental diets; b to g) pairwise comparison between any two Weighted UniFrac was used to generate a matrix of pairwise distances between communities, then a scatterplot was generated from the matrix of distances using PCoA analysis. P-value for each comparison was obtained from PERMANOVA and considered significant at $P < 0.05$.

3g HMW

3g LMW

P =0.014

3g LMW
3g HMW

P =0.53

3g LMW
Control

P =0.011

3g HMW
Control

P =0.044

5g LMW
Control
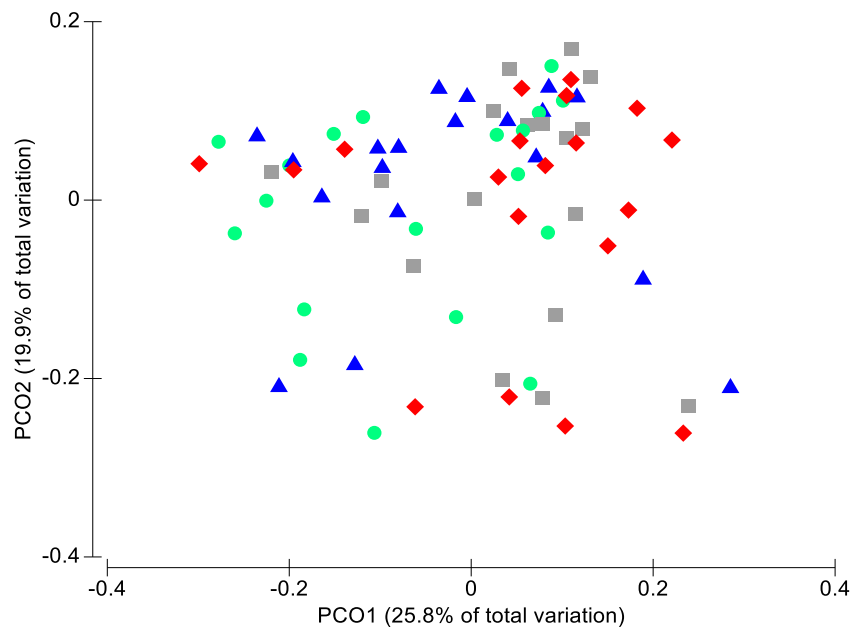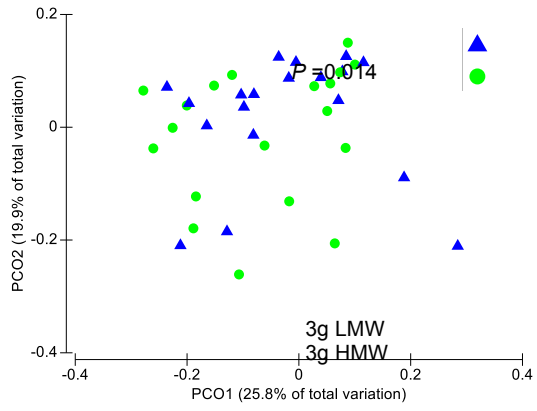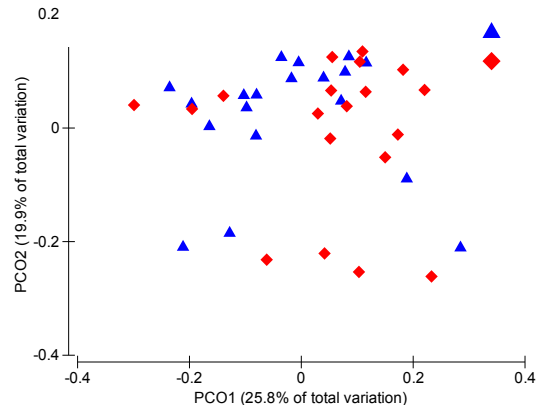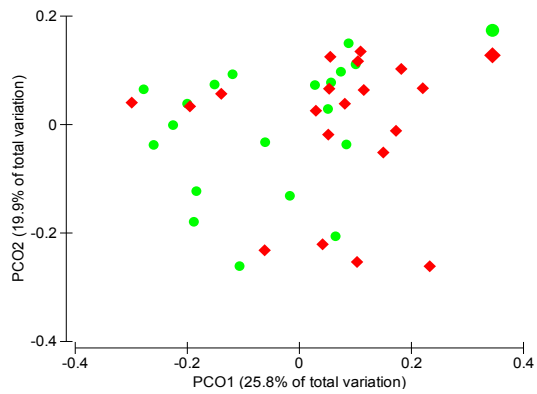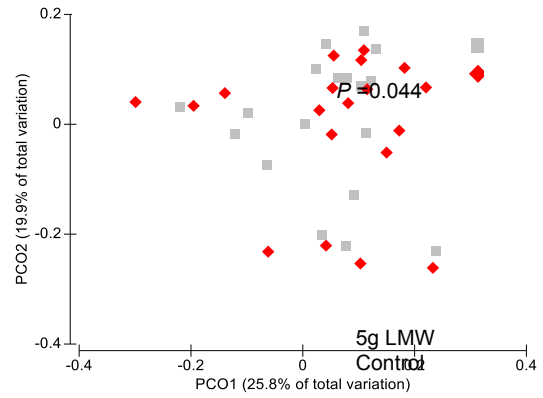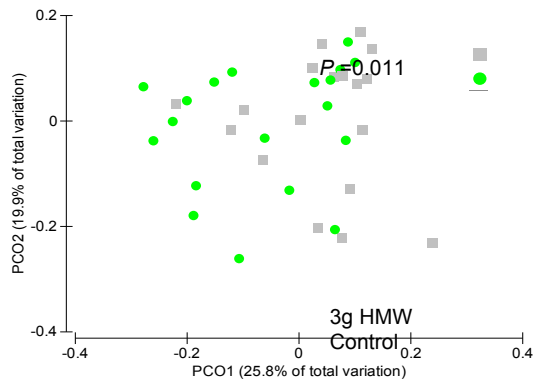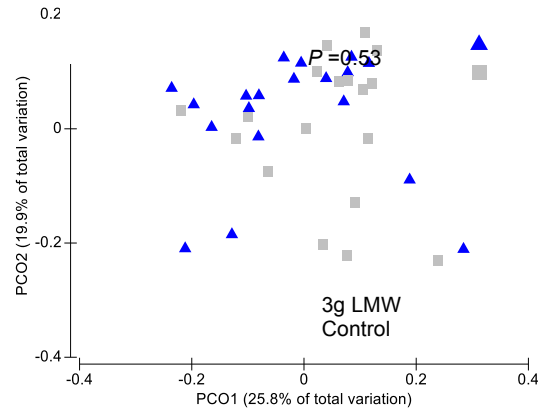
5.4.2 Changes in bacterial community composition changes

Due to low similarity with the reference sequences, some sequences could only be affiliated to phylum (P), class (C), order (O), or family (F) levels. Overall, 12 bacterial phyla were identified, of which two phyla Firmicutes and Bacteroidetes were abundant (above 1% of population), accounting for approximately 84% and 9% of the entire bacterial community, respectively (Table 5.2). Another 5 phyla, including Actinobacteria, Verrucomicrobia, Proteobacteria, Euryarchaeota and Tenericute were present at abundances between 0.1 and 1% of the total population(Table 5.2). The remaining 5 phyla, Cyanobacteria, Synergistetes, Lentisphaerae, Deferribacteres, Elusimicrobia were in low abundance (< 0. 1%). Populations of Firmicutes and Bacteroidetes were significantly impacted in response to 3g/d HMW β-glucan consumption, with Firmicutes significantly decreased (77.91% for 3g HMWβ-glucan vs. 89.69% for control, $P <$ 0.001)andBacteroidetes significantly increased (14.22% for 3g HMWβ-glucan vs. 4.99% for control, $P < 0.001$) compared to the control. However, Firmicutes and Bacteroidetes populations did not change in response to 5g/d LMW and 3g/d LMW β-glucan consumption and all remaining phyla did not change across all treatments.

Table 5.2 Relative abundances of phyla of microbial communities in feces afterβ-glucan intervention.

Phylum	Diet[1]				SEM[2]	*P*-value
	Control	3g LMW	5g LMW	3g HMW		
Above 1 % of population						
Bacteroidetes	4.99[a3]	7.59[a]	9.95[ab]	14.22[b]	1.67	<0.001[4]
Firmicutes	89.69[a]	87.50[a]	82.36[ab]	77.91 [b]	2.16	<0.001[4]
Between 0.1% and 1% of the population						
Actinobacteria	0.64	0.71	1.07	0.55	0.25	0.33[5]
Euryachaeota*	0.40	0.21	1.05	0.13	0.29	0.099[5]
Proteobacteria	0.18	0.10	0.14	0.32	0.13	0.66[5]
Tenericutes	0.086	0.066	0.093	0.19	0.06	0.47[5]
Verrucomicrobia	0.14	0.39	0.19	0.39	0.17	0.28[5]

[1] Diets include: Wheat and rice control (Control), 3g/d low molecular weight β-glucan (3g LMW), 5g/d low molecular weight β-glucan (5g LMW), 3g/d high molecular weight β-glucan (3g HMW).
[2]SEM, Standard Error of Mean.
[3] Data are presented as mean values.a,b,c Means for the diet effect are significantly different at *P*< 0.05.
[4]*P*-values are shown for the diet effect between groups analyzed by mixed model ANCOVA with diet, period, period×dietas fixed factors.
[5]*P*-values are shown for the diet effect between groups analyzed by GLIMMIX procedure of SAS with diet, period, period×diet as fixed factors.
* Euryachaeota is a phylum of the Archaea.

At the lower taxonomical level and across all samples, 169 distinct bacterial taxa were detected of which 44 had a relative abundance above 0.1% of community (Table 5.3). Of these 44 taxa, 24 were identified at the genus level, 14 at the family level, 4 at the order level, and 2 at the class level. Irrespective of treatment group, *Bacteroides* was the most abundant genera in p. Bacteroidetes, whereas Firmicutes were dominated by *Blautia*. Compared to control, consumption of 3g/d HMW β-glucan increased the abundances of *Bacteroides*(12.26% for 3g HMW β-glucan vs. 5.96% for control, *P* = 0.002, Table 5.3). Consumption of 3g/d HMW β-glucan also increased the relative abundance of Bacteroidales compared to the 3g LMW β-glucan treatment (0.24 vs. 0.12%, *P* = 0.04). Moreover, Rikenellaceae and *Prevotella*, showed increasing trends (*P*< 0.1) following 3g/d HMW β-glucan consumption compared to control . In contrast, *Streptococcus* (p. Firmicutes) was significantly decreased by 3g/d HMW β-glucan compared to control (0.035% vs. 0.27%, *P* = 0.02). Additionally, *Dorea* showed a decreasing trend (*P*< 0.1) in response to 3g/d HMW β-glucan consumption.

Table 5.3Relative abundances of genera of microbial communities in feces afterβ-glucan intervention.

Phylum	Genus	Diet[1]				SEM[2]	
		Control	3gLMW	5g LMW	3g HMW		*P*- value
Actinobacteria							
	Bifidobacterium	1.03[3]	1.00	1.24	0.67	0.3	0.57[4]
Bacteroidetes							
	Bacteroides	5.96^a	6.31^{ab}	8.30^{ab}	12.26^b	1.60	0.0029[4]
	Rikenellaceae(f)	0.75	1.15	1.24	1.76	0.41	0.053[5]
	Prevotella	0.48	0.54	1.46	1.84	0.71	0.095[5]
	[Barnesiellaceae](f)	0.11	0.2	0.31	0.57	0.19	0.37[5]
	Parabacteroides	0.17	0.14	0.18	0.23	0.054	0.62[5]
	Bacteroidales(o)	0.17^{ab}	0.12^a	0.21^{ab}	0.24^b	0.065	0.045[5]
	Bacteroidaceae (f)	0.079	0.13	0.12	0.15	0.14	0.32[5]
Euryarchaeota*							
	Methanobrevibacter	0.39	0.21	1.05	0.13	0.29	0.099[5]
Firmicutes							

Blautia	11.43	9.31	11.55	8.5	1.78	0.35[4]
Coprococcus	9.11	9.17	8.21	7.95	1.51	0.83[4]
Ruminococcus	6.18	6.83	5.82	4.8	0.92	0.21[4]
Lachnospiraceae(f) unclassified	6.77	6.48	7.22	7.29	0.84	0.72[4]
Ruminococcaceae(f)	4.63	4.32	3.34	4.04	0.78	0.41[4]
Faecalibacterium	5.34	4.71	3.92	4.32	1.57	0.17[5]
Ruminococcaceae(f)	3.12	2.85	1.97	2.98	0.82	0.50[4]
Coprobacillaceae(f)	2.45	2.91	3.31	2.91	0.66	0.55[4]
Roseburia	1.75	2.73	2.74	2.26	0.55	0.28[4]
[Ruminococcus]	3.43	2.86	2.99	2.76	0.58	0.49[4]
Oscillospira	1.56	1.62	1.29	1.48	0.48	0.82[4]
Clostridia(c) unclaasified	2.91	2.64	2.87	2.58	0.3	0.63[4]
Clostridiales(o)	1.28	1.24	1.06	1.3	0.2	0.70[4]
Dialister	0.46	0.3	0.23	0.24	0.21	0.53[5]
Christensenellaceae(f)	0.05	0.33	0.14	0.17	0.17	0.77[5]
Dorea	1.01	0.81	0.77	0.65	0.14	0.070[4]
Lachnospira	0.4	0.46	0.36	1.06	0.22	0.18[5]

Peptostreptococcaceae(f)	0.34	0.67	0.47	0.26	0.19	0.47[4]
Lachnospiraceae(f)	0.43	0.43	0.31	0.69	0.19	0.54[4]
Catabacteriaceae(f)	0.42	0.61	0.35	0.81	0.5	0.27[4]
Catenibacterium	0.3	0.56	0.73	0.35	0.37	0.23[4]
Clostridia(c)	0.14	0.17	0.13	0.17	0.04	0.63[4]
Clostridiales(o) unclassified	0.098	0.27	0.28	0.11	0.14	0.86[4]
[*Eubacterium*]	0.15	0.12	0.11	0.11	0.12	0.98[5]
Firmicutes(p) unclassified	0.25	0.24	0.26	0.21	0.032	0.39[4]
[Coprobacillaceae](f) unlassified	0.2	0.29	0.32	0.23	0.12	0.90[5]
Clostridiaceae(f) unclassied	0.11	0.12	0.091	0.15	0.09	0.97[5]
Clostridium	0.097	0.14	0.084	0.12	0.08	0.96[5]
Turicibacter	0.056	0.079	0.043	0.066	0.07	0.98[5]
Streptococcus	0.27[a]	0.14[ab]	0.043[ab]	0.035[b]	0.07	0.025[5]
Proteobacteria						
Enterobacteriaceae(f) unclassfied	0.12	0.011	0.18	0.54	0.19	0.095[5]
Tenericutes						
RF39(o)	0.082	0.062	0.085	0.17	0.058	0.52[5]

Verrucomicrobia						
Akkermansia	0.14	0.38	0.19	0.39	0.17	0.28[5]

[1] Diets include: Wheat and rice control (Control), 3g/d low molecular weight β-glucan (3g LMW), 5g/d low molecular weight β-glucan (5g LMW), 3g/d high molecular weight β-glucan (3g HMW).

[2]SEM, Standard Error of Mean.

[3] Data are presented as mean values.a,b,c Means for the diet effect are significantly different at $P < 0.05$.

[4]P-values are shown for the diet effect between groups analyzed by mixed model ANCOVA with diet, period, period×dietas fixed factors.

[5]P-values are shown for the diet effect between groups analyzed by GLIMMIX procedure of SAS with diet, period, period×diet as fixed factors.

* Euryachaeota is a phylum of the Archaea.

The PLS-DA results (given for the cut-off value of 0.5) supported SAS output, however, due to the higher sensitivity of PLS-DA methodology, additional significant differences among treatments, especially in low abundance taxa, were identified. For instance, when comparing 3g HMW β-glucan vs. control, two additional low abundance taxa, *Collinsella* and Lactobacillales (O), were identified to be positively associated with the control.

Figure 5.2 Partial least square discriminant analysis (PLS-DA) loading plot based on the relative abundance of the putative bacterial genera in stool samples and their association with 3g HMW or control diet.

The presenting genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to the treatments indicate positive association with either treatment group. The goodness of fit and R^2 =0.94 and predictive value of the model Q^2 =0.24 were estimated based on three components. Some sequences could only be assigned to class (c), order (o), or family (f) levels.

The presenting genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to the treatments indicate positive association with either treatment groups. The goodness of fit and $R^2 = 0.94$ and predictive value of the model $Q^2 = 0.22$ were estimated based on three components. Some sequences could only be assigned to class (c), order (o), or family (f) levels.

Coriobacteriaceae (f) unclassified +-

Catabacteriaceae(f)
Clostridia(c) unclassified Ruminococcaceae(f)
Parabacteroides +- Oscillospira

Actinobacteria
Bacteroidetes

Bacteroides +-
Ruminococcaceae(f) unclassified
Ruminococcus
Streptococcus

Proteobacteria
Synergistetes
Tenericutes
Verrucomicrobia

Other
Prevotella
[Ruminococus]

Coprococcus
Roseburia
Dorea Bifidobacterium [Coprobacillaceae(f)]

Enterobacteriaceae (f) unclassified +-

3g

Blautia

Clostridia(c)
Lachnospiraceae(f)

PC 1

PC 2

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The presenting genera are colored according to their corresponding phyla. The size of circles is indicative of taxa abundance. Taxa closer to the treatments indicate positive association with either treatment group. The goodness of fit and R^2 =0.94 and predictive value of the model Q^2 = 0.22 were estimated based on three components. Some sequences could only be assigned to class (c), order (o), or family (f) levels.

5.4.3 Correlations between the altered bacterial taxa and CVD risk factors

Spearman's rank correlation was conducted to test the association between bacterial taxa and risk factors of CVD (Table 5.4). Weak (Spearman'sρ between 0.20 and 0.39) and moderate correlations (Spearman'sρ between 0.40 and 0.59) were observed between parameters associated with CVD including BMI, waist circumference, total cholesterol levels, LDL-C, HDL-C, TG, glucose, systolic and diastolic blood pressure and specific members of bacterial community (Table 5.4).). Bacteria, which were affected by 3g HMW β-glucan and also showed correlation with parameters associated with CVD, were *Bacteroides* which was negative correlated withwaist circumference (P =0.031, Spearman'sρ =-0.25) and diastolic BP (P =0.019, Spearman'sρ =-0.28); *Prevotella* which showed negative correlation with TG (P=0.029, Spearman'sρ =-0.25); and *Dorea* which was positively associated with BMI (P=0.019, Spearman'sρ = 0.27), waist circumference (P=0.0082, Spearman'sρ =0.31) and diastolic BP (P =0.019, Spearman'sρ =0.28).

Table 5.4 Nonparametric correlation between metabolic parameters and bacterial taxa.

Metabolic parameters[1]	Taxon	Phylum	Abundance[2]	Spearman's ρ[3]	P-value[4]
BMI	*Turicibacter*	Firmicutes	0.16	-0.46	<0.0001
	Catenibacterium	Firmicutes	0.39	0.31	0.0077
	[Barnesiellaceae]	Bacteroidetes	0.46	0.26	0.023
	Lachnospiraceae	Firmicutes	0.59	-0.21	0.075
	Dorea	Firmicutes	0.90	0.27	0.019
	Ruminococcaceae	Firmicutes	6.56	-0.28	0.015
	Faecalibacterium	Firmicutes	6.84	0.23	0.047
	Ruminococcus	Firmicutes	7.48	-0.27	0.017
	Bacteroides	Bacteroidetes	8.59	-0.2	0.082
Waist circumference	*Turicibacter*	Firmicutes	0.16	-0.47	<0.0001
	Parabacteroides	Bacteroidetes	0.19	-0.21	0.074
	[Eubacterium]	Firmicutes	0.27	0.27	0.025
	Catenibacterium	Firmicutes	0.39	0.25	0.033
	Lachnospiraceae	Firmicutes	0.59	-0.24	0.044

Metabolic parameters[1]	Taxon	Phylum	Abundance[2]	Spearman's ρ[3]	P-value[4]
	Dorea	Firmicutes	0.90	0.31	0.0082
	Faecalibacterium	Firmicutes	6.84	0.21	0.074
	Bacteroides	Bacteroidetes	8.59	-0.25	0.031
Total cholesterol	[Ruminococcus]	Firmicutes	3.38	-0.29	0.013
	Roseburia	Firmicutes	3.43	-0.24	0.041
	Ruminococcaceae	Firmicutes	6.56	0.24	0.038
	Ruminococcus	Firmicutes	7.48	0.32	0.0055
LDL cholesterol	[Eubacterium]	Firmicutes	0.27	0.2	0.086
	[Ruminococcus]	Firmicutes	3.38	-0.33	0.0043
	Ruminococcaceae	Firmicutes	6.56	0.29	0.013
	Lachnospiraceae unlcassified	Firmicutes	7.37	-0.3	0.0098
	Ruminococcus	Firmicutes	7.48	0.3	0.0098
	Blautia	Firmicutes	11.27	-0.24	0.037
Triglyceride	Enterobacteriaceae Unclassified	Proteobacteria	0.18	0.25	0.031

Metabolic parameters[1]	Taxon	Phylum	Abundance[2]	Spearman's ρ[3]	*P*-value[4]
Triglyceride	*Parabacteroides*	Bacteroidetes	0.19	-0.25	0.029
	Clostridiaceae unclassified	Firmicutes	0.24	0.34	0.0028
	Catabacteriaceae	Firmicutes	0.43	-0.24	0.042
	Peptostreptococcaceae	Firmicutes	0.66	-0.28	0.016
	Christensenellaceae	Firmicutes	0.96	-0.38	0.0007
	Dialister	Firmicutes	1.06	0.25	0.03
	Prevotella	Bacteroidetes	1.09	-0.25	0.029
	Oscillospira	Firmicutes	3.02	-0.33	0.0043
	Ruminococcaceae	Firmicutes	6.56	-0.26	0.025
	Lachnospiraceae unclassifed	Firmicutes	7.37	0.24	0.037
HDL cholesterol	*Turicibacter*	Firmicutes	0.16	0.37	0.0012
	[Eubacterium]	Firmicutes	0.27	-0.26	0.024
	Dorea	Firmicutes	0.90	-0.22	0.057
	Dialister	Firmicutes	1.06	-0.26	0.023

Metabolic parameters[1]	Taxon	Phylum	Abundance[2]	Spearman's ρ[3]	P-value[4]
HDL cholesterol	*Roseburia*	Firmicutes	3.43	-0.26	0.025
	Faecalibacterium	Firmicutes	6.84	-0.31	0.0065
	Ruminococcus	Firmicutes	7.48	0.26	0.023
Glucose	Peptostreptococcaceae	Firmicutes	0.66	-0.25	0.034
	Dialister	Firmicutes	1.06	-0.21	0.073
	Clostridia(c) unclassified	Firmicutes	2.95	-0.21	0.065
Systolic BP	Catabacteriaceae	Firmicutes	0.43	0.36	0.0019
	Peptostreptococcaceae	Firmicutes	0.66	-0.28	0.017
	Bifidobacterium	Actinobacteria	0.95	-0.24	0.04
	Roseburia	Firmicutes	3.43	-0.34	0.0035
	Bacteroides	Bacteroidetes	8.59	-0.24	0.045
Diastolic BP	*Turicibacter*	Firmicutes	0.16	-0.34	0.0035
	Firmicutes unclassified	Firmicutes	0.27	0.27	0.025
	[Eubacterium]	Firmicutes	0.27	0.29	0.014
	Catabacteriaceae	Firmicutes	0.43	0.32	0.0074

Lachnospiraceae	Firmicutes	0.59	-0.32	0.0065
Dorea	Firmicutes	0.90	0.28	0.019
Bacteroides	Bacteroidetes	8.59	-0.28	0.019

[1] Anthropometrics, biochemical markers and blood pressure collected at the end of the intervention: BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BP, blood pressure.
[2] Abundance is shown for the composition of bacterial taxa in percentage (%).
[3]Spearman's correlation coefficient.
[4]*P*-values are shown for the probability of the Spearman's correlation.

5.4.4 Predicted metagenome function

Predicted metagenome functions between treatment groups measured by PICRUSt were shown to be consistent with treatment differences observed with weighted Unifrac (Figure-S 5.1-5.5). Predicted shifts in glycerolipid metabolism and other glycan degradation were increased by 3g HMW β-glucan compared with control (P = 0.026 and P = 0.021, respectively; Figure S 5.1). Same results for glycerolipid metabolism and other glycan degradation were observed when compare 3g HMW vs. 3g LMW β-glucan (P = 0.024 and P = 0.002, respectively; Figure S 5.2). However, although 5g LMW β-glucan showed differences compared with control in weighted Unifrac, only three metabolisms (phenylpropanoid biosynthesis, cyanoamino acids metabolism, glycosaminoglycan degradation) were predicted to be different using PICRUSt (Figure-S 5.5), whereas no differences were observed between 3g LMW β-glucan and control with both weighted Unifrac or PICRUSt(no difference to be graphed).

5.5 Discussion

The main finding of this controlled feeding study was that gut microbiota was modulated in response to barley β-glucan consumption on a molecular weight-dependent manner, and that the shifts in gut microbiota was correlated with reduced CVD risk factors. Specifically, consumption of 3g/d HMW β-glucan, resulted in elevated Bacteroidetes and decreased Firmicutes as did several of their respective bacterial taxa (Table 5.2). The shifts in *Bacteroides, Prevotella,* and *Dorea* composition were shown to favorably correlate with an improved CVD risk factor profile, such as BMI, waist circumference, TG, blood pressure and HDL-C (Table 5.4). Consumption of 5g/d LMW β-glucan only impacted the β-diversity but not the composition of gut microbiota

compared to control (Figure 5.1g, Tables 5.32, 5.3). Additionally, the consumption of 3g/d LMW β-glucan failed to alter either the β-diversity or the composition of gut microbiota.

Previous *in vitro* fermentation and *in vivo* intervention studies have been conducted examining the ability of β-glucan to alter microbiota, however, conclusions are obscure leaving the breadth of the research inadequate (24-26, 49, 50). This was partially due to the inconsistencies in study design, experimental models and methodologies used for bacterial community analyses, i.e. quantitative PCR and fluorescence in site hybridization (FISH) that can only monitor selected members of the bacterial community. In this research, we used a randomized controlled human trial that is considered gold standard for a clinical study. Furthermore, we employed 16S rRNA sequencing for bacterial community profiling, which is superior over previously used methods(51, 52).

Our results support that modulation in gut microbiota following barley β-glucan consumption is dependent on β-glucan MW as bacterial communities more profoundly shifted in response to diet containing 3g HMW β-glucan. To date, there are few studies that have investigated the impact of physicochemical properties of β-glucan on microbiota composition. In an *in vitro* fermentation study, Hughes et al. (24)assessed the impact of barley and oat derived β-glucan with different MW levels (average MW 130, 172, 243 kDa for barley β-glucan and 150, 230 kDa for oat β-glucan) on fecal microbiota using FISH and concluded that the *Bacteroides-Prevotella* group increased from baseline with all but the 243 kDa barley and 230 kDa oat substrates. However, the β-glucan MW levels tested in the abovementioned study did not include ideal representatives of "high" MW β-glucan that could result in physiological benefits attributed to this compound

(28, 53). For instance, it has been shown that β-glucan with MW lower than 210 kDa fails to lower cholesterol levels(28). Thus, the tested range of 150 kDa to 243 kDa in the Hughes et al. study is considered very low MW and no true "high" MW level was examined. In our study, we selected the low and high MW β-glucan (288 kDa and 1349 kDa, respectively) on the basis of cholesterol-lowering capability of this compound (28).

The high and low MW β-glucan utilized in our study was rationalized on the basis of cholesterol-lowering capability where MW lower than 210 kDa failed to lower cholesterol levels (28).The MW β-glucan contributes to high viscosity (54) in the intestinal lumen, which is believed to slow transit time following ingestion (55, 56). Consequently, the slower transit time allows for prolonged exposure to fermentable food compounds by larger numbers of bacteria (57), possibly accounting for the observed effect of HMW β-glucan in altering microbiota. Additionally, glycosaminoglycan degradation and other glycan degradation were increased in the predicted metagenome (genes related with these functions were detected at a higher level) with diet containing 3g HMW β-glucan compared to the control (Figure-S 5.1)indicating that HMW β-glucan may be a preferred fermentable substrate over LMW β-glucan for the bacterial group Bacteroidetes.

The observedchanges in microbial community at the phylum level in response to 3g/d HMW β-glucan have been associated with a "lean" microbiota pattern. Ley et al. (15) described that the relative proportion of Bacteroidetes is decreased in obese people compared with lean people, and this proportion also increased with diet-induced weight loss, indicating that there might be a unique microbiota profile for lean and obese individuals. The observed increase in Bacteroidetes

and decrease in Firmicutes resulting from consumption of 3g/d HMW β-glucan in our study is consistent with the "lean" microbial pattern described by Ley et al.(15). In the present study, weight loss was not observed in the present study that partially due to the controlled study design which intended to maintain body weight. To examine and interpret the potential weight loss effects of β-glucan consumption, further research with a specific study design aiming at the correlation between β-glucan consumption, microbiota shifts, and weight management is required.

Gut microbiota in humans have been classified into three clusters dominated by *Bacteroides, Prevotella and Ruminococcus,*respectively*,* and termed enterotypes(58). Enterotypes are reported to be independent of nationality, gender, age or BMI (58) but can be modified by dietary patterns. For instance, children of rural Africa who eat high-carbohydrate/high-fibre diets have a higher Bacteroidetes/Firmicutes ratio than European children who eat typical Western diets, as well as a unique abundance of the *Prevotella* enterotype, whereas European children have a higher abundance of *Bacteroides*, indicating that *Prevotella* and *Bacteroides* are highly modifiable based on carbohydrate and animal protein intake, respectively (59). Following this study, Wu et al. (60)conducted a dietary intervention study (intervention period for 10 days) with high/fat/low-fibre or low-fat/high-fibre diet but failed to observe alteration of enterotype, thus, concluded that alternative enterotype status are associated with long-term diet. Interestingly, the present intervention study showed that consuming 3g/d of HMW β-glucan was able to significantly increase *Bacteroides* and moderately increase *Prevotella* in a short-term intervention of 35 days. Different enterotypes mentioned above may use various routes to generate energy from fermentable substrates available in the colon, which suggests a potential

specialization in ecological niches or guilds (58). Accordingly, the altered enterotypes by 3g/d HMW β-glucan may have a different fermentation product profile, such as SCFA composition that is strongly associated with host metabolism (61-63).

In the present study, the increased *Bacteroides* by 3g/d HMW β-glucan showed a negative correlation with BMI, waist circumferences and blood pressure (Table 5.4). *Prevotella* that tended to be increased showed a negative correlation with TG. Additionally, *Dorea* that tended to be decreased by 3g/d HMW β-glucan was negatively associated with BMI, waist circumferences and diastolic blood pressure but positively associated with HDL-C. Consumption of 3g/d HMW β-glucan also decreased *Streptococcus* compared with the control. But these two groups of genera were not correlated with biochemical markers, anthropometric markers or blood pressure that were measured in the clinical trial. Altogether, these results suggest that consumption of 3g/d HMW β-glucan for 35 days is able to alter the gut microbiota and the altered microbiota are correlated with a favourable shift in parameters associated with CVD. However, since these metabolic parameters were not changed significantly in the sampled subjects (n=19), studies with appropriate sample size are required to further investigate the causality—whether the altered gut microbiota causes the improvement of the CVD risk factors.

In addition, predicted metagenome bacterial functions exhibited increased glycerolipid metabolism with HMW β-glucan diet compared to control and LMW β-glucan diet, which has been associated with obesity and type 2 diabetes via energy homeostasis, appetite control and insulin sensitivity (64). These observations indicated that HMW β-glucan-induced gut microbial shifts may lead to reduced CVD risk and shifts the glycerolipid metabolism via an increased

Bacteroidetes: Firmicutes ratio. That being said, we should take into account that PICRUSt

predictions are based on known functions of the microbial communities present in the human GI

tract obtained from the metagenomic studies. These predictions need to be confirmed using

whole genome shotgun DNA (metagenomics) or RNA (metatranscriptomics) sequencing of

analyzed samples.

Alpha-diversity of microbiota did not change by β-glucan consumption in this study. Beta-

diversity of microbiota, however, was impacted. The differences in β-diversity among treatments

were significant when determined using weighted UniFrac distances rather than unweighted

UniFrac distances, suggesting that β-glucan's impact was through changing the abundance of

certain members of the microbial community but not their presence and/or absence.

Overall, our results suggested that β-glucan with HMW was able to alter the gut microbiotaand

the shifts observed were positively associated with an improved CVD risk factor profile.

Although studies with large samples size are needed to confirm the relationship between the

altered gut microbiota by β-glucan and the improved CVD health benefits, results from current

study are in line with of the hypothesis that altering gut microbiota is responsible for the

physiological benefits of HMW β-glucan consumption.

Contributions and Acknowledgements

YW conducted the clinical trial, collected and managed fecal and blood samples, extracted fecal

DNA, prepared DNA samples for illumina sequencing, performed bioinformatics and statistical

analysis. YW wrote the manuscript except the methodologies of Library construction and illumina sequencing and Bioinformatic analyses and PICRUSt.

HMT conducted PICRUSt analysis, prepared graphs generated from PICRUSt analysis and wrote the methodology related to PICRUSt analysis. HMT also contributed to manuscript revision.

EK was responsible for the illumine sequencing and wrote the methodologies of Library construction and illumina sequencing and Bioinformatic analyses.

NPA, PJJ and EK conceived and designed the experiment. YW, HMT and EK developed the bioinformatics and statistical models and analyzed the data.

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Supplementary Material

Table-S 5.1 Comparison of metabolic parameter changes responding to β-glucanintervention in 19 subjects.

Metabolic parameter[1]	Diet[2]				SEM[3]	*P*-value[4]
	Control	3g LMW	5g LMW	3g HMW		
Body weight (kg)	77.9	77.7	78.1	77.7	0.26	0.6
BMI (kg/m^2)	27.5	27.3	27.6	27.4	0.11	0.34
Waist circumference (cm)	95.6	90.6	91.4	90.9	0.8	0.34
Total cholesterol (mmol/L)	5.44	5.37	5.48	5.32	0.16	0.19
LDL cholesterol (mmol/L)	3.43	3.37	3.44	3.33	0.12	0.49
HDL cholesterol (mmol/L)	1.39	1.35	1.4	1.36	0.025	0.32
Triglyceride (mmol/L)	1.45	1.4	1.43	1.4	0.081	0.87
Glucose (mmol/L)	4.68	4.7	4.72	4.68	0.073	0.9
Systolic BP (mm Hg)	119	118	120	114	4.22	0.31
Diastolic BP (mm Hg)	70	68	69	69	2.05	0.65

[1] Anthropometrics, biochemical markers and blood pressure collected at the end of the intervention: BMI, body mass index; LDL, low-density lipoprotein; HDL, high-density lipoprotein; BP, blood pressure.
[2] Diets include: Wheat and rice control (Control), 3g/d low molecular weight b-glucan (3g LMW), 5g/d low molecular weight b-glucan (5g LMW), 3g/d high molecular weight (3g HMW)
[3] Standard Error of Mean
[4] P-values are shown for the treatment effect between groups analyzed by mixed model ANCOVA with treatment, period, period×treatment as fixed factor.

**5.1 Comparison of function changes of the microbiota resuling from 3g HMW β-glucan
:rol diet (predicted).**

3g HMW and 3g LMW β-glucan diet(predicted).

95% confidence intervals

Valine, leucine and isoleucine biosynthesis		7.10e-3
Valine, leucine and isoleucine degradation		9.35e-3
Peptidoglycan biosynthesis		9.77e-3
Phenylalanine, tyrosine and tryptophan biosynthesis		0.015
Methane metabolism		0.027
C5-Branched dibasic acid metabolism		0.035
Lysine biosynthesis		0.036
Glutathione metabolism		0.037
Lipopolysaccharide biosynthesis proteins		0.039
D-Arginine and D-ornithine metabolism		0.040
Isoquinoline alkaloid biosynthesis		0.041
Lipopolysaccharide biosynthesis		0.044
Chloroalkane and chloroalkene degradation		0.047
Toluene degradation		0.049

p-value (corrected)

0.0   1.5
Mean proportion (%)   Difference in mean proportions (%)

■ 3g HMW   ■ 5g LMW

180

β-glucan and control diet (predicted).

95% confidence intervals

		p-value (corrected)
Phenylpropanoid biosynthesis		0.011
Cyanoamino acid metabolism		0.013
Glycosaminoglycan degradation		0.047

Mean proportion (%)   Difference in mean proportions (%)

■ 5g LMW   ■ Control
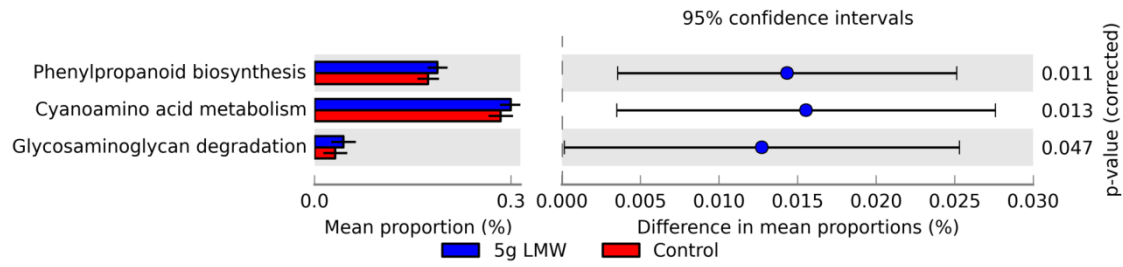
CHAPTER 6: DISCUSSION AND CONCLUSION

6.1 Overall discussion and implications

The present study demonstrated that two factors are important for the cholesterol-lowering effect of β-glucan: the high viscosity of β-glucan associated with HMW and possession of the G allele of rs3808607 in individuals. Secondly, this study clarified that the mechanism responsible for the cholesterol-lowering action of β-glucan is increasing bile acid synthesis rather than inhibiting cholesterol absorption and synthesis. Thirdly, this study for the first time revealed the gut microbiota alteration ability of β-glucan in humans and suggested an association between the altered gut microbiota and the physiological benefits resulting from β-glucan consumption.

The cholesterol-lowering outcomes from this study confirmed the health benefits stated in the health claims related to the cholesterol reduction effect of β-glucan and the outcomes are clinically relevant. Consumption of a diet containing 3g of HMW β-glucan per day lowered TC by 0.53 mmol/L (8.7 % reduction from baseline).Clinical evidence indicates that a 1% reduction in TC or LDL-C is associated with 2-3% or 1% decreased risk, respectively, of CHD (1). The cholesterol reduction that resulted from consuming 3g/d HMW β-glucan coupled with a Step 1 diet in the present study could reduce the risk of CHD events by 16.6 to 24.9%. Even with the calculation in a more conservative but simplistic way—subtracting the effect of Step 1 background diet (TC reduction is established as 7.1% from baseline for the control diet),

additional consumption of 3g HMW β-glucan per day can still lead to 3.2 -4.8% reduction of CHD events.

More importantly, the present study revealed that the efficacy of β-glucan in lowering cholesterol is determined by factors beyond the total amount of daily intake, including physicochemical properties of β-glucan and genetic characteristics of consumers, which were not always addressed in previous studies.

Physicochemical properties,such as MW, viscosity and solubility, play important roles in the cholesterol-lowering action of β-glucan. We found daily consumption of 3g/d of HMW β-glucan with high viscosity lowered TC significantly compared with WR control, but the 3g/d LMW or 5 g/d LMW β-glucan failed to change blood cholesterol concentration compared to the control. The present study using a randomized and crossover design confirmed Wolever et al. (2).'s finding that diets containing oat β-glucan with a HMW (2, 210,000 g/mol) and medium-MW (530,000 g/mol) reduced LDL-C, while LMW (210, 000g/mol) β-glucan was ineffective, thus, providing additional evidence that high viscosity and high MW are the key factors for the cholesterol-reducing effect of β-glucan. This is an important message to consumers and the food industry because food preparation and processing can affect the physicochemical characteristics of β-glucanand subsequently impact its physicochemical benefits. In this study, the LMW β-glucan treatment in experimental barley foods was strategically attained using unconventional food processing techniques, such as prolonged dough resting for 120 min at room temperature or soaking coarsely milled native barley grain in apple juice containing ascorbic acid overnight. Hence, β-glucan was depolymerized by theendogenous enzyme β-glucanases and oxidative-

reductive reactions, respectively. The HMW β-glucan was prepared by heat treatment including micronizing, boiling and toasting so that the solubility of β-glucan increased, thus, increasing viscosity.To obtain the most effective β-glucan for reducing blood cholesterol levels, food processing leading to depolymerisation of β-glucan should be avoided and food processing that retains the high viscosity should be employed. Most barley foods are made from pearled barley or flour and undergo some type of cooking which inactivates β-glucanase and allows maintenance of medium to high MW and viscosity levels. Therefore, health claims will be valid for barley foods most commonly encountered by consumers.

An individual's genetic variation (*CYP7A1* SNP rs3808607) is associated with different degrees of responses to β-glucan intervention in lowering blood cholesterol levels. The present study showed that 3g/d HMW β-glucan reduced TC greater in G allele carriers than homozygous TT carriers when compared to the control. Therefore, the efficacy of the 3g HMW β-glucan in lowering cholesterol was associated with *CYP7A1* SNP rs3808607-G allele acting in a dominant fashion. Additionally, the linear relationship between log (viscosity) of β-glucan and cholesterol changes was observed in G allele carriers only but not in homozygous TT carriers, which further confirmed the interaction between *CYP7A1* SNP rs3808607 and β-glucan in its cholesterol-lowering action. To date, studies aimed at investigating the interactions between genotypes and β-glucan in lowering cholesterol are limited. The present study is the first ~~study~~ that identified *CYP7A1* SNP rs3808607 to be associated with varied responses to the hypocholesterolemic effect of β-glucan. Interestingly, Kajinmi et al. (3) reported that *CYP7A1* SNP rs3808607-G allele carriers were associated with poor LDL-C reduction in response to the cholesterol-lowering drug atorvastatin. Our finding along with Kajinmi et al.'s results is reminiscent of the

proposal of personalized therapy of hypercholesterolemia. Indeed, dietary recommendations or therapeutic strategies based on an individual's genetic characteristics can lead to more effective management of diseases. Although the cholesterol-lowering effect of β-glucan is well recognized by researchers (4, 5) and accepted by many worldwide food authorities (6-8), there are studies that failed to detect β-glucan lowering blood cholesterol (9, 10). Besides the insufficient level of MW of β-glucan utilized in these studies, genetic characteristics of subjects might be another factor responsible for the heterogeneity of β-glucan effects observed in a previous meta-analysis (5). Overall, the findings of this study provide an alternative interpretation for the studies that failed to demonstrate the cholesterol-lowering effect of β-glucan and it will contribute to a development of personalized dietary recommendation for lowering blood cholesterol levels.

Secondly, results from this present study contribute to clarifying the underlying mechanisms responsible for the cholesterol-lowering effect of β-glucan. The mechanisms responsible for the action of lowering cholesterol by β-glucan have been proposed but not validated sufficiently in humans. The hypothesized mechanisms include inhibiting cholesterol absorption(11-13), suppressing cholesterol synthesis (14, 15) and decreasing bile acid reabsorption (15-17). The present study covered the validation of all three proposed mechanisms by utilizing isotopic methods in assessing cholesterol absorption and synthesis as well as measuring the serum surrogate for bile acid synthesis. The results showed that 3g HMW β-glucan significantly reduced serum cholesterol levels and enhanced bile acid synthesis without affecting cholesterol absorption and synthesis. Therefore, we conclude that β-glucan lowers cholesterol through interrupting the enterohepatic circulation of bile acid and enhancing bile acid synthesis. Moreover, this phenomenon is more pronounced in individuals carrying homozygous-G of

rs3808607, which further confirmed the speculation that rs3808607-G allele carriers are more responsive to the elimination of bile acids from the enterohepatic circulation caused by β-glucan. However, genetic variants of *APOE* were not associated to different responses to cholesterol-lowering effect of β-glucan as hypothesized.

Furthermore, this is the first controlled and randomized crossover study employing the broad 16S rRNA sequencing to assess the changes of composition of microbial communities following β-glucan consumption. The reported changes of 7 phylum and 45 family and genus members, as well as the changes of ecological parameters, provide a comprehensive understanding of how β-glucan alters the gut microbiota. Again, HMW was noticed to be the determining factor in shifting gut microbiota. Ingestion of 3g/d HMW β-glucan for 35 days resulted in increased Bacteroidetes and decreased Firmicutes. At the genus level, 3g/d HMW β-glucan increased *Bacteroides*, tended to increase *Prevotella* but decrease *Dorea*, whereas diets containing 5g/d LMW β-glucan and 3g/d LMW β-glucan failed to alter gut microbiota composition. Moreover, the altered gut microbiota by 3g HMW β-glucan was related to alternative enterotypes that potentially produce different fermentation product profiles, such as SCFA (18). The composition of SCFA has been associated with cholesterol metabolism (19). Importantly, the modified bacterial taxa, *Bacteroides, Prevotella,* and *Dorea* were correlated with favorable shifts of CVD risk factors including BMI, waist circumference, blood pressure, HDL-C and TG levels. Thus, the results from the current study provided evidence in response to the hypothesis that β-glucan is able to alter the gut microbiota. The altered microbiota is not directly associated with the cholesterol-lowering effect of β-glucan but correlated with reduction of other risk factors related with CVD.

6.2 Limitations and future directions

The controlled, randomized and crossover design employed in this study is considered to be the "gold standard" for evaluating treatment intervention (4). This design together with a full-feeding strategy advanced the present study in avoiding the influence of food habit and other confounding variables to the targeting effect.

The control diet used in this study contained approximately 30% of energy as fat, 55% as carbohydrate and 15% as protein. It was low in saturated fatty acids and high in dietary fibre (11.1 g/1000kcal). This diet is close to the Step 1 diet which is recommended by American Heart Association (AHA) for treating hyperlipidemia (20). The treatment diets used in this study were designed based on Step 1 diet but replace wheat and rice ingredients with barley in breakfast food items, therefore, containing additional β-glucan soluble fibre. Within the control diet, TC was reduced by approximately 7% from baseline. The efficacious control diet increased the difficulties in detecting the statistical significance of a greater cholesterol-lowering effect of β-glucan, because health benefits of β-glucan and Step 1 diet do not necessarily work in a "1+1=2" pattern. Therefore, the future controlled clinical trials aiming to investigate functional food or food ingredients in disease risk prevention should consider using a control diet that is similar to subjects' dietary habit rather than an effective healthy diet.

Gene-diet interaction between *CYP7A1* SNP rs3808607 and β-glucan in lowering cholesterol was identified for the first time by this study. No association between *APOE* variants and

different responses to β-glucan intervention has been observed. However, the sample size of the present study was not determined for investigating gene-diet interactions. The genotypes of subjects were unknown prior to the study and the current sample size (n =30) might be too small to detect potential gene-diet interactions (21). For instance, only 3 *E2* carriers of *APOE* were identified among 30 subjects in this study, and *E2* was previously reported to be more responsive than *E3* and *E4* carrier to oat bran in lowering LDL-C (22). The non-association of *APOE* variantsand cholesterol-lowering effect of β-glucan revealed in this study might be attributed to the small size of subgroups. The three *CYP7A1* variants were distributed almost evenly in this study (TT =11, GT =11, GG =8), which is not dissimilar to the minor genotype frequency in the general population (23). But again, due to the small sample size of each the genotype subgroups, current results may need to be confirmed in a clinical trial with a larger sample size.

The exact differences of the major (TT) and minor (GT and GG) genotypes of rs3808607 with respect to their functions in cholesterol and bile acid metabolisms are unclear. Results from Castro-Orós et al.'s study suggested that the G allele, compared with the T alleles, has lower binding affinity to the inhibitory nuclear transcription factors of bile acid biosynthesis and, thus, greater promoter activity for bile acid syntehsis(24). Bile acid biosynthesis is believed to occur mainly in pericentral hepatocytes (25) under precise negative feedback control, and is mainly achieved through transcriptional control of bile acid synthetic enzymes CYP7A1 (26). The large bile acid flux through the peripotal cells is one of the important factors that can down-regulate the bile acid production (25). Decreased bile acid reabsorption caused by viscous β-glucan ingestion can lead to an attenuation of down-regulating bile acid synthesis and correspondingly enhance bile acid production. In agreement with this speculation, we observed that G allele

carriers responded better to 3g HMW β-glucan in lowering TC than T homozygotes. Moreover, G homozygotes showed greater bile acid synthesis under the intervention of 3g HMW β-glucan compared to the control. Based on our data and study from Abrahamsson et al. (2005), G allele carriers are not necessarily greater bile acid producers (27). However, following introduction to a dietary compound or medical agent that interrupts enterohepatic bile acid circulation, G allele carriers (especially GG) may be more responsive than TT to the stimulation of up-regulating bile acid synthesis from cholesterol. However, results of the current study were obtained at a physiological level. To test the hypothesis that G allele carriers of rs3808607 are more sensitive to bile acid interrupting agents in enhancing bile acid production, more studies at a molecular level are required.

This is the first study that provided a comprehensive examination of the influence of β-glucan to gut microbiota. However the functions of altered bacterial members are unclear. The functions of the altered microbiota in the current study were predicted based on 16S rRNA sequences rather than bacterial genome. For a more precise interpretation of the function of gut microbiota, the investigation of the bacterial functions by a more advanced technology, e.g. shotgun, as well as bacterial metabolites is required. In addition, fecal samples used for microbiome analysis were collected at the end of each phase, but baseline data of microbiome were missing. The 4 - weekwashout period was designed to avoid the carryover of cholesterol-lowering effect. Whether 4 weeks is sufficient for microbiota to be recovered from β-glucan intervention is unknown. Therefore, before knowing the legitimate wash-out period for β-glucan altering gut microbiota, future studies are suggested to collect both baseline and endpoint samples for microbiome analysis to detect the potential carryover effect.

In addition, determination of fecal bile acid excretion and SCFA levels in feces and blood are leftfor future investigation. Although increasing bile acid synthesis by HMW β-glucan has been demonstrated by the current study, the assessment of fecal bile acid excretion will complete the test of the hypothesis that the increased bile acid synthesis IS due to the fecal loss of bile acids. Moreover, the determination of SCFA in feces and blood will provide further information to interpret the altered gut microbiota by β-glucan.

6.3 Final conclusion

In conclusion, this study demonstrated that the physicochemical properties of β-glucan, MW and viscosity, are critical factors for determining the cholesterol-lowering efficacy of β-glucan. Moreover, individuals' intrinsic characteristics, specifically *CYP7A1* SNP rs3808607, are associated with different responses to β-glucan intervention. Individuals carrying G alleles are more responsive than T homozygotes allele carries in lowering their cholesterol levels. Furthermore, results from this study clarified that enhancing bile acid synthesis, rather than influencing cholesterol synthesis and absorption, is the mechanism responsible for the cholesterol-lowering effect of β-glucan. In addition, β-glucan with HMW is able to alter gut microbiota. The altered microbiota is related to a favorable shift of metabolic parameters associated with CVD. Therefore, altering the gut microbiota might be another underlying mechanism for the physiological effects of β-glucan.

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APPENDICES

Appendix I: Ethics approval for studiescorresponding to chapter 3, 4 and 5

BANNATYNE CAMPUS
Research Ethics Boards

UNIVERSITY OF MANITOBA

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

April 26, 2010

Dr. N. Ames
c/o Dr. S. Harding
Richardson Centre for Functional Foods and Nutraceuticals
196 Innovation Drive
Winnipeg, MB

Dear Dr. Ames:

Re: B2010:057
Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans

In response to your submission dated April 13, 2010 the above-named study was reviewed by the full board at the meeting of the Biomedical Research Ethics Board on April 26, 2010 and will be considered for approval conditional to the following:

- Please make a small administrative change to the genetic consent form by adding a spot for the participant's initials in the footer of each page.
- Please clarify the amount of blood collected in Tsp or Tbsp in the genetic consent form.
- When you are ready to register your trial on Clinical Trials.gov please contact Shelly Rempel-Rossum, Research Ethics Board Coordinator at remross@cc.umanitoba.ca or 789-3389 to obtain a password and instructions. In your e-mail please provide the title and ethics reference number of the study, e-mail and telephone number of the individual responsible for processing the registration.

The issues outlined above must be met to the satisfaction of the chair of the Board prior to providing a certificate of final approval. When you have completed the requested revisions, **one (1) copy** of the revised portions of your project must be submitted to the Bannatyne Campus Research Ethics office. **Please summarize your revisions and address the concerns of the board in a cover letter and make sure your revision is highlighted so they can be identified in the text of the revised document. Also note, the Biomedical Research Ethics Board deadlines do not apply to the review of these revisions therefore you may submit them as soon as they are complete.**

Yours sincerely

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

NA/SR/lw

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Coordinator **Telephone**: (204) 789-3389/ **Fax:** (204) 789-3414

www.umanitoba.ca/faculties/medicine/research/ethics

BANNATYNE CAMPUS
Research Ethics Boards

UNIVERSITY
OF MANITOBA

P126-770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Tel: (204) 789-3255
Fax: (204) 789-3414

APPROVAL FORM

Principal Investigator: Dr. N. Ames
Sponsor: Agriculture and Agri-Foods Canada

Ethics Reference Number: B2010:057
Date of Approval: November 15, 2010

Protocol Title: Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans

The following is/are approved for use:

- **Research Subject Information and Consent Form, Version 2 dated 11/1/2010**
- **Food Survey submitted November 15, 2010**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, University of Manitoba on behalf of the committee as per your submission dated October 28 and electronic mail dated November 15, 2010. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations of Canada*.

A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes. The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only. For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone:** (204) 789-3255/ **Fax:** (204) 789-3414

www.umanitoba.ca/medicine/ethics

BANNATYNE CAMPUS
Research Ethics Boards

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Winnipeg, Manitoba
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Fax: (204) 789-3414

UNIVERSITY
OF MANITOBA

APPROVAL FORM

Principal Investigator: Dr. N. Ames
Sponsor: Agriculture and Agri-Foods Canada

Ethics Reference Number: B2010:057
Date of Approval: April 26, 2011
Date of Expiry: April 26, 2012

Protocol Title: Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans

The following is/are approved for use:

- **Annual Approval**
- **Research Subject Information and Consent Form, Version 2 dated 11/5/2010**
- **Additional Research Subject Information and Consent Form for Genetic Analysis, Version 1 dated 11/5/2010**

The above was approved by Dr. Nicholas Anthonisen, Chair, Biomedical Research Board, Bannatyne Campus, and University of Manitoba on behalf of the committee per your submission dated April 5, 2011. The Research Ethics Board is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement, and the applicable laws and regulations of Manitoba.   The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the *Food and Drug Regulations of Canada*.

This approval is valid until the expiry date only.   A study status report must be submitted annually and must accompany your request for re-approval. Any significant changes of the protocol and informed consent form should be reported to the Chair for consideration in advance of implementation of such changes.   The REB must be notified regarding discontinuation or study closure.

This approval is for the ethics of human use only.   For the logistics of performing the study, approval should be sought from the relevant institution, if required.

Sincerely yours,

Nicholas Anthonisen, MD, Ph.D
Chair,
Biomedical Research Ethics Board
Bannatyne Campus

Please quote the above Ethics Reference Number on all correspondence.
Inquiries should be directed to the REB Secretary **Telephone**: (204) 789-3255/ **Fax**: (204) 789-3414

www.umanitoba.ca/medicine/ethics

P126 - 770 Bannatyne Avenue
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UNIVERSITY | **BANNATYNE CAMPUS**
OF MANITOBA | Research Ethics Boards

BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF FINAL APPROVAL FOR AMENDMENTS AND ADDENDUMS

PRINCIPAL INVESTIGATOR: Dr. N. Ames	INSTITUTION/DEPARTMENT: UofM / Richardson Centre for Functional Foods and Nutraceuticals	ETHICS #: B2010:057
BREB MEETING DATE (If applicable):	**APPROVAL DATE:** December 13, 2012	
STUDENT PRINICIPAL INVESTIGATOR SUPERVISOR (If applicable):		

PROTOCOL NUMBER: NA	PROJECT OR PROTOCOL TITLE: Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: Agriculture and Agri-Foods Canada	

REMINDER: THE CURRENT BREB APPROVAL FOR THIS STUDY EXPIRES: April 26, 2013

REVIEW CATEGORY OF AMENDMENT:	Full Board Review ☐	Delegated Review ☒
Submission Date of Investigator Documents: December 7, 2012	**BREB receipt date of Documents:** December 11, 2012	

THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:

Document Name	Version(if applicable)	Date

Protocol:

Amendment per report received December 11, 2012

Consent and Assent Form(s):

Other:

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the amendment to the research study/project named on this *Certificate of Approval* as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. The amendment and documents listed above were granted final approval by the Chair or Acting Chair, UM BREB.
BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulation of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

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QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:
1. This amendment is acceptable on scientific and ethical grounds for the ethics of human use only. *For logistics of performing the study, approval must be sought from the relevant institution(s).*
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. This approval is valid until the expiry date noted on this certificate of approval. A Bannatyne Campus Annual Study Status Report must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the BREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form.**
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM BREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report.**

Sincerely,

Lindsay Nicolle, MD, FRCPC
Chair, Biomedical Research Ethics Board
Bannatyne Campus

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P126 - 770 Bannatyne Avenue
Winnipeg, Manitoba
Canada R3E 0W3
Telephone 204-789-3255
Fax 204-789-3414

UNIVERSITY OF MANITOBA | **BANNATYNE CAMPUS**
Research Ethics Boards

BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF ANNUAL APPROVAL

PRINCIPAL INVESTIGATOR: Dr. N. Ames	INSTITUTION/DEPARTMENT: UofM / Richardson Centre for Functional Foods and Nutraceuticals	ETHICS #: B2010:057
BREB MEETING DATE (If applicable):	APPROVAL DATE: April 24, 2013	EXPIRY DATE: April 26, 2014
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable):		

PROTOCOL NUMBER: NA	PROJECT OR PROTOCOL TITLE: Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: Agriculture and Agri-Foods Canada	

Submission Date of Investigator Documents: April 10, 2013	BREB Receipt Date of Documents: April 12, 2013

REVIEW CATEGORY OF ANNUAL REVIEW: Full Board Review ☐   Delegated Review ☒

THE FOLLOWING AMENDMENT(S) and DOCUMENTS ARE APPROVED FOR USE:

Document Name(if applicable)	Version(if applicable)	Date

Annual approval
Annual approval implies that the most recent **BREB approved** versions of the protocol, Investigator Brochures, advertisements, letters of initial contact or questionnaires, and recruitment methods, etc. are approved.

Consent and Assent Form(s):

CERTIFICATION
The University of Manitoba (UM) Biomedical Research Board (BREB) has reviewed the annual study status report for the research study/project named on this **Certificate of Annual Approval** as per the category of review listed above and was found to be acceptable on ethical grounds for research involving human participants. Annual approval was granted by the Chair or Acting Chair, UM BREB, per the response to the conditions of approval outlined during the initial review (full board or delegated) of the annual study status report.

BREB ATTESTATION
The University of Manitoba (UM) Biomedical Research Board (BREB) is organized and operates according to Health Canada/ICH Good Clinical Practices, Tri-Council Policy Statement 2, and the applicable laws and regulations of Manitoba. In respect to clinical trials, the BREB complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations of Canada and carries out its functions in a manner consistent with Good Clinical Practices.

QUALITY ASSURANCE
The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

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www.umanitoba.ca/faculties/medicine/ethics

QUALITY ASSURANCE

The University of Manitoba Research Quality Management Office may request to review research documentation from this research study/project to demonstrate compliance with this approved protocol and the University of Manitoba Policy on the Ethics of Research Involving Humans.

CONDITIONS OF APPROVAL:

1. This amendment is acceptable on scientific and ethical grounds for the ethics of human use only. *For logistics of performing the study, approval must be sought from the relevant institution(s).*
2. This research study/project is to be conducted by the local principal investigator listed on this certificate of approval.
3. The principal investigator has the responsibility for any other administrative or regulatory approvals that may pertain to the research study/project, and for ensuring that the authorized research is carried out according to governing law.
4. <u>This approval is valid until the expiry date noted on this certificate of approval.</u> A **Bannatyne Campus Annual Study Status Report** must be submitted to the REB within 15-30 days of this expiry date.
5. Any changes of the protocol (including recruitment procedures, etc.), informed consent form(s) or documents must be reported to the BREB for consideration in advance of implementation of such changes on the **Bannatyne Campus Research Amendment Form.**
6. Adverse events and unanticipated problems must be reported to the REB as per Bannatyne Campus Research Boards Standard Operating procedures.
7. The UM BREB must be notified regarding discontinuation or study/project closure on the **Bannatyne Campus Final Study Status Report.**

Sincerely,

Lindsay Nicolle, MD, FRCPC
Chair, Biomedical Research Ethics Board
Bannatyne Campus

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Appendix II: Study advertisement

Appendix III: Forms corresponding to clinical trial studies description in Chapter 3, 4, 5

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-8883
Fax (204) 474-7552
peter_jones@umanitoba.ca

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

EST.1877

RCFFN preliminary trial screening consent

You have expressed an interest in participating in a study at the Richardson Center for Functional Foods and Nutraceuticals. You have been invited to have your health assessed to determine if you meet the requirements of the study.

The clinical coordinator team will assess your cholesterol level, medical history, body measurements, and your availability over the next 2 years. Depending on your results you will be offered the opportunity to participate in a study.

To allow the necessary information to be obtained, you agree to provide fasting blood samples (approximately 10 ml or 2 teaspoons) for the measurement of blood cholesterol, blood count, and iron levels.

Prior to taking part in any study, you will be given the specific study consent form to read and sign if you are still interested in participating.

The blood is taken from a vein in the forearm, as is usually done during a blood test. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site.

I understand I can withdraw from this process at any time at my discretion.

_____   _____   _____
Participant's Signature   Participant's Name (please print)   Date

_____   _____   _____
Investigator's Signature   Investigator's Name (please print)   Date
(or Clinical Coordinator)   (position)

Page 1 of 1
08/17/2010

Consent from for future contact

Room 106
196 Innovation Drive
Winnipeg, Manitoba
Canada R3T 2N2
Telephone (204) 474-8883
Fax (204) 474-7552
peter_jones@umanitoba.ca

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

EST. 1877

Study Participant Consent for Future Contact

I have completed a research trial at the Richardson Centre for Functional Foods and Nutraceuticals and give permission for the clinical staff to archive my contact information such that I may be contacted about future studies. It is understood that this information will be kept on file for 10 years under lock and key.

I understand I can withdraw my information from this list at any time at my discretion.

_____   _____   _____
Participant's Signature   Participant's Name (please print)   Date

_____   _____   _____
Investigator's Signature   Investigator's Name (please print)   Date
(or signature of person who   (position)
conducted consent discussion)

Page 1 of 1
07/12/2010

General screening form

General Subject Screening Form

To be filled out by participant:

Circle appropriate YES/NO responses

Name:				
Date of Birth:	Month	Day	Year	
Sex:	Male:	Female:	Postmenopausal: YES	NO
Caucasian	YES	NO	for Spice trial only	

Contact Information	
Street Address	
Postal Code	
City	
Home Phone:	
Cell Phone:	
Email:	

Medical History

Diabetes mellitus	YES	NO	If Yes to Other, please specify:
Thyroid disease	YES	NO	
Kidney disease	YES	NO	
Liver disease	YES	NO	
Heart disease	YES	NO	
Hypertension	YES	NO	
Other	YES	NO	

Cholesterol lowering medication? *(in the last 3 months)*		YES	NO
Other medications	YES NO	If Yes, specify: Are the doses of these medications stable? YES...NO	
Vitamin, Mineral supplement	YES NO	If Yes, specify:	
Herbal, food supplement	YES NO	If Yes, specify:	
Laxatives	YES NO		
Fiber	YES NO		
Allergies (food such as corn)	YES NO	If Yes, specify:	
Vegetarian	YES NO		
Any metallic bone components	YES NO		

Lifestyle		
Smoker?	YES	NO
If Yes, how many per day?		
Drink Alcohol?	YES	NO
If Yes, how many drinks/week		

To be filled out by a study coordinator:

Screening Information		
Weight	lbs:	kg:
Height	' "	m:
BMI (kg/m^2)		
Waist circumference (inches)		
Hip circumference (inches)		
Blood pressure	Systolic	Diastolic
Screening code (initials:mm:day:year; eg TR:07:22:10)		

Is subject fasted for blood sampling?   YES   NO

Do you have a nutrition study preference?

(a) Spice/Herb (free-living)

(b) Plant sterol cholesterol lowering (1 meal/day)

(c) Healthy oils (full-feeding)

(d) Barley fiber (full-feeding)

Screening medical examination form

Barley Study 2010
Screening Medical Examination Form

Phase	Study Physician	Subject Code
One	Dr. Edward Kesselman	
		Subject Number
Date of Visit	Investigator	Treatment Code
___11___ / __2010_____ MM DD YR	Dr. Nancy Ames	

COMPLETE PHYSICAL EXAMINATION

A. Vital Signs

Body Weight:_____ lbs _____ kg   **Height**: _____cm

Respirations:_____

Blood Pressure (seated): _____/_____ mmHg   **Heart Rate**:_____bpm
                                    systolic   diastolic

Race/Ethnic Origin:
    Caucasian   African-American/Canadian   Asian   Other:_____

B. Body Systems (Check the appropriate box if organ system was examined. If not done, write N/D in the box)

	Normal	Abnormal	*Details of abnormal finding
1) Ears, Nose, Throat			
2) Eyes			
3) Dermatological			
4) Musculoskeletal			
5) Lymph Nodes			
6) Neurological			
7) Cardiovascular			
8) Respiratory			
9) Endocrine			
10) Urogenital			
11) Gastrointestinal (complete section C)			

C. Gastrointestinal Cont...

Bowel Habits: Frequency_____/Day   **Urination**: Frequency_____/Day

                    Consistency_____   Nocturia_____/Night

Screening medical examination form-2

Barley Study 2010
Screening Medical Examination Form

Medications:
Hospitalizations:
Family History:

D. Inclusion Criteria Screening		
(All of the following must be 'YES' for valid entry into the study)	YES	NO
Have you been Diagnosed with Psoriasis		
Subject, aged 18-60 years old who has given informed written consent AGE:_____		
LDL-cholesterol 2.1-4.9 mmol/L VALUE:_____		
BMI within the range of 22-32 kg/m^2 VALUE:_____		
Complete blood count (CBC) within normal ranges.		
Blood biochemistry within normal ranges.		
Electrocardiogram normal.		

E. Medical History **Exclusion** Criteria Screening Questionnaire		
(All of the following must be answered 'NO' for valid entry into the study)	YES	NO
Have you taken a medication affecting lipid metabolism (cholestyramine, colestipol, niacin, colfibrate, gemfibrozil, probucol, HMG-CoA reductase inhibitors, and high-dose dietary supplements, plant sterols or fish oil capsules) within the past 3 months?		
Do you take systemic antibodies, corticosteroids, androgens or phenytoin within the past 3 months?		
Are you on anticoagulant therapy?		
Do you smoke?		
Do you consume large amounts of alcohol? (more than 2 drinks per day or 12 drinks per week) Do you follow a specific diet?		

Screening medical examination form-3

Barley Study 2010
Screening Medical Examination Form

Do you have major food allergy?		
Do you have lactose intolerance?		
Have you had major surgery in the last 6 months?		
Do you have diabetes mellitus?		
Do you have kidney disease?		
Do you have liver disease?		
Do you have heart disease?		
Do you have gastrointestinal, pancreatitis or biliary disease (onset within past three months)?		
Have you had cancer? If yes, occurrence of therapy within past 1 year?		
Do you have anemia, bleeding disorder or significant blood loss/donation?		
Do you have uncontrolled thyroid disease or hypertension? (Subject will be accepted if she is on a stable dose of a thyroid or blood pressure medication that has no known effects on blood lipid metabolism.)		
Do you have a history of eating disorders?		
F. Additional Physician Notes		
Based on the inclusion and exclusion criteria above, and the medical exam is the subject eligible to participate in the study protocol (circle one):		
YES	NO	
Physician's Signature:_____		
Date:_____		

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DXA requisition form

Richardson Centre
for Functional Foods
and Nutraceuticals

196 Innovation Drive
Winnipeg, Manitoba R3T 6C5

(204)474-9989 (phone)
(204)474-7552 (fax)

Requisition for DXA Scan

Study name: Effect of Beta Glucan Molecular Weight and Viscosity on Mechanism of Cholesterol Lowering In Humans

Ethics #: H2010:057

Scans Ordered by:

      Name: Dr. Nancy Ames

      Phone Number: 474-7187

Primary Investigator:

Name: Dr. Nancy Ames   Signature:_____

Physician co-signer:

Name: Dr. Edward Kesselman   Signature:_____

_____

Subjects to be studied: __Human   __ Animal (Specify)_____

Subjects Name: _____

Subjects Study ID #: _____

Measurements: Length/Height_____cm   DOB(DD/MM/YY):___/____/____

                  Weight_____kg

Total Number of Scans Approved in REB protocol for each individual:_____

Pregnancy Test conducted   __ Yes __ No __ Not Applicable

Scans Required:
Scan ID#
_____   __ Whole Body   __ Body Part (Specify)_____
_____   __ Infant   Pediatric   Adult   Small Animal (circle one)

DATE OF Scan (DD/MM/YY) # __ : __/__/__   **DATE OF Scan** (DD/MM/YY) # __ : __/__/__
DATE OF Scan (DD/MM/YY) # __ : __/__/__   **DATE OF Scan** (DD/MM/YY) # __ : __/__/__
DATE OF Scan (DD/MM/YY) # __ : __/__/__   **DATE OF Scan** (DD/MM/YY) # __ : __/__/__

Required Format: (check all the apply)   __ Hard Copy   __ CD-ROM

Charge Information- Office Use Only

DXA data collection from

DEXA DATA COLLECTION FORM   Subject Code:_____ Phase: _____

Study:_____   Date (DD/MMM/YY): _____

┌───┐
│ **To be completed by study staff:**   │
│   │
│   **DAY: 1   2   29   30** (please circle one)   │
│   │
└───┘

Since screening has the volunteer had any radiation exposure

(e.g. x-rays, radiotherapy, CT scan)?   Yes __ / No __

Date & details: _____

FEMALES ONLY:

Possibility of being pregnant   Yes __ / No __

If there is a possibility and/or they are pre-menstrual and not using contraceptives, has a

pregnancy test been offered?   Yes __ / No __

Has a test been taken? Yes __ / No* __

*If no, why? _____   ┌────────────────────────────────────┐
                                           │ If yes, what was the result? **- / +** │
                                           │   │
                                           │ Subject's Authorization _____ │
If pregnant DO NOT undergo DEXA.   └────────────────────────────────────┘

Check:

      Jewelry removed (including glasses):   Yes __ / No __

      Spine straight: Yes __ / No __

Time of scan (HH:MM): _____

Comments: _____

Investigator Initials:_____

1

Consent form

RESEARCH SUBJECT INFORMATION AND CONSENT FORM

Title of Study: Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans

Investigators:
Dr. Nancy Ames, PhD and Dr. Peter Jones, PhD
Richardson Centre for Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, SmartPark
Winnipeg, Manitoba R3T 6C5
Phone:(204) 474-9787

You are being asked to participate in a research study. Please take your time to review this Information and Consent Form and discuss any questions you may have with the study staff. You may take your time to make your decision about participating in this clinical trial and you may discuss it with your regular doctor, friends and family. This consent form may contain words that you do not understand. Please ask the study doctor or study staff to explain any words or information that you do not clearly understand.

Purpose of Study

Barley is a major western Canadian agricultural crop, primarily for the brewing and livestock feed industries, but relatively little barley is consumed as food in Canada. Barley is an excellent source of the soluble fibre known as beta-glucan. Consuming 3 or more grams of beta-glucan a day can help an individual lower their blood cholesterol levels and help to maintain a healthy body weight. However, the characteristics of the beta-glucan can change when barley is cooked and/or processed to produce food products. Therefore, the purpose of this study is to determine if these changes to the beta-glucan effect the cholesterol lowering effect of dietary barley.

Study procedures

If you agree to take part in this study, as part of a pre-screening visit, you will be asked to have a fasting (nothing to eat or drink 12 hours before the test) blood sample of approximately two teaspoons taken to measure your blood fat levels. If you meet eligibility requirements, you will be invited back for further screening where a fasting blood sample of four teaspoons will be taken to do a complete blood count, and biochemistry profile. All baseline values must be normal as verified by the study physician prior to enrollment in the study and any abnormality in tests performed at screening will result in exclusion. An electrocardiogram (EKG) may be performed at the discretion of the physician in charge. Prior to beginning the study, you will undergo a physical examination by a physician to ensure that you are in good health. During the physical examination, the physician will measure your vital signs examine the normality of body systems and ask you some questions regarding your medical history.

It is a controlled-diet, randomized, crossover trial. This trial is modeled after previously successful functional food trials at our clinic, consistent with current recommendations to decrease cardiovascular risk and improvement of circulating lipid profiles. We will provide a

Consent form -2

comprehensive "lifestyle approach" to all volunteers. Metabolic diets of precisely known composition will be provided to you under strict supervision at the clinical research facility at the Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba. Diets will be nutritionally adequate and provide 100% of energy requirements. Our proposed study will include four treatment phases of 35 days each, separated by at least a 4 week washout interval where subjects will consume their habitual diets:

Phase A: Control phase diet – minimal barley and minimal beta-glucan.

Phase B: Low molecular weight/low viscosity beta-glucan – 3 grams of beta-glucan with low viscosity per day as barley food products (i.e. muffins, tortillas, pilafs, bread).

Phase C: Low molecular weight/low viscosity beta-glucan – 5-6 grams of beta-glucan with low viscosity per day as barley food products (i.e. muffins, tortillas, pilafs, bread).

Phase D: High molecular weight/high viscosity beta-glucan – 3 grams of beta-glucan with high viscosity per day as barley food products (i.e. muffins, tortillas, pilafs, bread).

You will undergo 4 controlled dietary periods of 35 days each, separated by at least a 4 week washout phase. During each treatment period subjects will consume prepared nutritionally adequate solid foods diets as three meals per day plus snacks, prepared at the Richardson Clinical Nutrition Research Unit (RCNRU), where at least one meal per day will be consumed under supervision. The remaining meals will be prepared and packaged for take out. Diets will be consistent in composition across each 35 day period, designed using nutrient composition databases, to contain 35% of energy as fat, 50% carbohydrate and 15% protein. Upon completing the study you will have consumed all 4 treatments, however, you will not know which treatment you are consuming during each phase.

Micronutrient composition of the diet will be tailored to meet individual requirements. The diet will be given as 7 day rotating meal cycles to provide you some variety in foods. Daily cycles will be as similar as possible in terms of macro and micronutrient composition. Each day's intake will be divided into breakfast, lunch and supper plus snacks, the total energy intake each day being equal.

Twelve-hour fasting blood samples will be collected on day 1 (30.5 ml or 2 tablespoons), day 2 (28 ml or less than 2 tablespoons), day 30 (14 ml or 1 tablespoon), day 31 (14 ml or 1 tablespoon), day 32 (14 ml or 1 tablespoon), day 33 (14 ml or 1 tablespoon), day 34 (28 ml or less than 2 tablespoons) and day 35 (30.5 ml or 2 tablespoons) of each of the 4 phases of the trial. Blood samples obtained on day 1 and 2 will be used to measure baseline values for different study measurements, whereas blood samples obtained at the end of the treatment phase will be used for determining if there was an effect of the diet treatment. Blood samples collected on days 31-35 will also be used for cholesterol absorption measurements (please see below).

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Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans

Each blood test will take approximately 5 minutes. The total blood volume required for this trial will be approximately 730 ml (2 ½ cups). Fecal samples will be collected over a 24 hour period using a fecal collection kit provided between days 30 – 35 of each phase.

On day 30 of the study, a fasting blood sample (14 ml or 1 tablespoon) will be obtained and you will be requested to consume a small quantity of labeled cholesterol (stable carbon isotope $[3,4]^{13}$C-cholesterol) mixed into margarine on half an English muffin. This labeled cholesterol is nearly identical to regular cholesterol but can be distinguished from normal cholesterol as it contains a heavier form of carbon. Following the consumption of this labeled cholesterol, a fasting blood sample (14 ml or 1 tablespoon) will be collected each morning for the next 3 days (day 31-33). On day 34 of the study, you will also be requested to provide a fasted blood sample (28 ml or less than 2 tablespoons) followed by the consumption of three tablespoons of tagged water (known as deuterium). The movement of these tagged materials will permit assessment of how much dietary cholesterol your body absorbs as well as how much cholesterol is produced inside your body. All of the above tagged materials are non-radioactive, non-toxic, and do not pose any health risk to you. A final fasted blood sample (30.5 ml or 2 tablespoons) will be required on day 35 of the study.

You may be asked to complete a survey/questionnaire on the taste and quality of barley foods you have consumed in each phase of the study. You may also be asked to comment on your feeling of fullness following during each study phase. These questionnaires will be short and administered by one of our nutrition study staff.

Risks and Discomforts
As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part.
The soy milk enhanced with plant sterols formulated with the proposed preparation procedures, has been shown to have no known direct negative side effects on health in several dozen existing animal and human experiments. Some known risks, although rare, are associated with placing a needle into a vein. These include the possibility of infection, perforation or penetration of the needle through the vein, and bleeding, pain, or bruising at the site. In case you feel any discomfort during the experimental trial a physician, Dr. Kesselman, will be available to contact at any time. Dr. Kesselman can be reached at (204) 954-4486.

Benefits
You may not benefit from participation in this research; however, the study should contribute to a better understanding how barley beta-glucan lowers cholesterol levels and whether food processing techniques impact the cholesterol lowering effects. Other benefits of participating in the study include: 1) access to your test results when they become available; 2) fully prepared and portioned healthy diet for 140 days.

Effect of Beta-Glucan Molecular Weight and Viscosity on the Mechanism of Cholesterol Lowering in Humans

Costs

All clinic and professional fees, diagnostic and laboratory tests that will be performed as part of this study are provided at no cost to you. There will be no cost for the study treatment that you will receive.

Payment for participation

Along with all your meals and snacks during each treatment phase you will receive up to a maximum (total dollar value) of $900 at completion of this study for your time and inconvenience of the study schedule. Following the completion of each dietary phase volunteers will be remunerated with $125 and upon completing all 4 dietary treatment phases $400 cash payment (per volunteer total cash value of $900). During the final three phases there will be good will gestures offered to the volunteers for entertainment in and around Winnipeg (Goldeye baseball game tickets, Manitoba Moose hockey tickets, etc) at no cost to you.

Alternatives

You do not have to participate in this study. The study coordinators, physician and principal investigator will answer any questions you have about the experimental group of this study. You should be aware that lipid lowering medications exist as an alternative to lowering blood cholesterol levels.

Confidentiality

Records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Only the RCFFN staff directly involved with your participation in the study may review/copy medical information that may reveal your identity. With your permission, the study doctor will also write to your Family Doctor to tell him/her that you are taking part in a study or to obtain further medical information. The Biomedical Research Ethics Board at the University of Manitoba may also review your research-related records for quality assurance purposes. If the results of the trial are published, your identity will remain confidential. Personal information such as your name, address, telephone number and/or any other identifying information will not leave the Richardson Centre for Functional Foods and Nutraceuticals.

Study samples will be stored in a freezer at the RCFFN. Only the study coordinators and the principal investigator will have access to the samples. Stored samples will be coded in a manner that does not reveal you identity and only the principal investigator and study coordinator will be able to link these samples to you. Your samples will only be used for the analyses outlined in this form and not stored for any longer than 5 years, not shared with any other group, unless indicated in the protocol, without your specific consent.

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Consent form -6

Voluntary Participation/Withdrawal From the Study
Your decision to take part in this study is voluntary. You may refuse to participate or you may withdraw from the study at any time. Your decision to not participate or to withdraw from the study will not affect your other medical care.

Your participation in this study may be terminated without your consent by the study coordinators, physician or principal investigator. The study staff will withdraw you if he/she feels that participation is no longer in your best interest, or if you fail to follow the directions of the study staff.

If you decide to participate, you will agree to cooperate fully with the study visit schedule, and will follow the study staff's instructions.

We will tell you about new information that may affect your health, welfare, or willingness to stay in this study.

Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially close.

Medical Care for Injury Related to the Study
In the event of an injury that occurs to you as a direct result of participating in this study, or undergoing study procedures you should immediately notify the study physician, Dr. Kesselman at (204) 954-4486 or go to your nearest emergency room to receive necessary medical treatment. You are not waiving any of your legal rights by signing this consent form nor releasing the investigator or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Kesselman will be contacted, who will inform you of the results.

Questions
You are free to ask any questions that you may have about your treatment and your rights as a research subject. If any questions come up during or after the study or if you have a research-related injury, contact the study doctor and the study staff.

Investigator:	Dr. Nancy Ames	Tel No.	204-474-7187
Co-investigator:	Dr. Peter Jones	Tel No.	204-474-8883
Study Coordinator:	Dr. Scott Harding	Tel No.	204-474-7842
Study Physician	Dr. Edward Kesselman	Tel No.	██████████

For questions about your rights as a research subject, you may contact:
The Biomedical Research Ethics Board, University of Manitoba at 789-3389

Do not sign this consent form unless you have a chance to ask questions and have received satisfactory answers to all of your questions.

Stool sample collection instruction

Consent

I agree to allow the study doctor to inform my family doctor that I am participating in this study or to obtain information regarding my medical history.

Yes ☐   No ☐

1. I have read and understood this Information and Consent Form, and I freely and voluntarily agree to take part in the clinical trial (research study) described above.

2. I understand that I will be given a copy of the signed and dated Information and Consent Form. I have received an explanation of the purpose and duration of the trial, and the potential risks and benefits that I might expect. I was given sufficient time and opportunity to ask questions and to reflect back my understanding of the study to study personnel. My questions were answered to my satisfaction.

3. I agree to cooperate fully with the study doctor and will tell him if I experience any side effects, symptoms or changes in my health.

4. I am free to withdraw from the study at any time, for any reason, and without prejudice to my future medical treatment.

5. I have been assured that my name, address and telephone number will be kept confidential to the extent permitted by applicable laws and/or regulations.

6. By signing and dating this document, I am aware that none of my legal rights are being waived.

Signature:_____ Date/Time: _____

Printed name of above:_____

I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the subject whose name and signature appears above. I confirm that I believe that the subject has understood and has knowingly given their consent to participate by his/her personally dated signature.

Signature:_____ Date/Time: _____

Printed name of above:_____ Study role:_____

ALL SIGNATORIES MUST DATE THEIR OWN SIGNATURE

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11/5/2010   Initials of Subject: _____

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1. Freeze the ice pack provided by the study once you get home.

2. **Check your study ID and study phase** on the collection tube (the plastic tube with blue cap). If the study ID and study phase on the collection tube is not correct, please correct it by yourself, and inform clinical coordinator when you visit Richardson Centre the next time.

3. Empty your bladder. Flush toilet. Place the collection unit under the rear part of the toilet seat with the round side pointing toward the back.

4. Have a bowel movement. **Obtain the sample from 3 randomly different places of the stool** using the spoon attached to the cap of the collection tube. **Fill in about one third of the collection tube with stool sample**.

5. Close the tube tightly. **Write down the date and time of the bowel movement** on the plastic bag provided by the study. Discard the used collection unit.

6. Wrap the collection tube with the frozen ice pack, and keep them in the plastic bag. Keep the collected sample in a cold place (as cold as you can find at home). **Please keep the sample at the same place for all the phases to make sure the sample is treated in consistent condition**.

7. Return the stool sample to the study as soon as you can. Please place the sample in the

   stool collection box in the blood draw room at Richardson Centre.

Data collection form

Study Phase Information:

Subject Code: _____

Study Phase: _____   Treatment: _____

Start Date: _____   End Date: _____

SECTION 1: START AND END WEIGHT

Day 1: Weight (kg)_____   Staff Initials: _____

Day 2: Weight (kg)_____   Staff Initials: _____

Day 34: Weight (kg) _____   Staff Initials: _____

Day 35: Weight (kg) _____   Staff Initials: _____

SECTION 2: WAIST CIRCUMFERENCE

Day 1: First measurement (cm) _____   Second measurement (cm)   _____

Day 35: First measurement (cm) _____   Second measurement (cm)   _____

SECTION 3: SEATED BLOOD PRESSURE

Has the subject consumed caffeine, used any medication or eaten any food in the last 12 hours

and/or exercised in the past 2 hours?   **Yes No**

If yes, subject needs to be rescheduled for a blood pressure measurement.

Instructions: Apply cuff to left arm. After applying the cuff, the subject must be quiet and remain continuously seated without legs crossed for 5 minutes. Instruct the subject not to talk during the reading. Wait 1 minute after each reading before taking the next reading.

Day 1:

a. First blood pressure measurements: __ __ __ / __ __ __

b. Second blood pressure measurement: __ __ __ / __ __ __

c. Third blood pressure measurement: __ __ __ / __ __ __

d. Average (2nd and 3rd) blood pressure measurement: __ __ __ / __ __ __

Day 8:

a. First blood pressure measurements: __ __ __ / __ __ __

b. Second blood pressure measurement: __ __ __ / __ __ __

c. Third blood pressure measurement: __ __ __ / __ __ __

d. Average (2nd and 3rd) blood pressure measurement: __ __ __ / __ __ __

Day 15:

a. First blood pressure measurements: __ __ __ / __ __ __

b. Second blood pressure measurement: __ __ __ / __ __ __

c. Third blood pressure measurement: __ __ __ / __ __ __

d. Average (2nd and 3rd) blood pressure measurement: __ __ __ / __ __ __

Day 22:

a. First blood pressure measurements: __ __ __ / __ __ __

b. Second blood pressure measurement: __ __ __ / __ __ __

c. Third blood pressure measurement: __ __ __ / __ __ __

d. Average (2nd and 3rd) blood pressure measurement: __ __ __ / __ __ __

Day 29:

a. First blood pressure measurements: __ __ __ / __ __ __

b. Second blood pressure measurement: __ __ __ / __ __ __

c. Third blood pressure measurement: __ __ __ / __ __ __

d. Average (2nd and 3rd) blood pressure measurement: __ __ __ / __ __ __

Day 35:

a. First blood pressure measurements: __ __ __ / __ __ __

b. Second blood pressure measurement: __ __ __ / __ __ __

c. Third blood pressure measurement: __ __ __ / __ __ __

d. Average (2nd and 3rd) blood pressure measurement: __ __ __ / __ __ __

SECTION 4: Stable Isotope Tracers

Day 30

Tracer: _[3,4]^{13}C-cholesterol premixed in margarine_

___   _½ English muffin toasted?_

___   _10g of isotope enriched Becel on muffin?_

___   _All isotope/muffin consumed?_

Day 34

Tracer: _D$_2$O – Deuterium Oxide_

___   _Weigh out 0.7g per kg body water?_

___   _Provide D$_2$O following fasted blood sample?_

___   _Add 100-200 ml of orange juice to cup which contained D$_2$O?_

___   _Orange juice consumed?_

SECTION 5: BLOOD COLLECTION

DAY 1

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right _____ /Left_____

Collect: 1 X 8 ml serum (red/grey SST tube)   _____

2 X 4 ml plasma heparin (green top)   _____

2 X 6 ml plasma EDTA (purple top)   _____

1 X 2.5 ml whole blood (Paxgene tube)   _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why:* _____

Blood Draw: Excellent _____   Good _____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

DAY 2

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right _____ /Left_____

Collect: 1 X 8 ml serum (red/grey SST tube)   _____

2 X 4 ml plasma heparin (green top)   _____

2 X 6 ml plasma EDTA (purple top)   _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why:* _____

Blood Draw: Excellent ____   Good ____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

DAY 30

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right _____ /Left_____

Collect: 1 X 8 ml serum (red/grey SST tube)   _____

                       1 X 6 ml plasma EDTA (purple top)   _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why: _____*

Blood Draw: Excellent _____   Good _____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

DAY 31

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right _____ /Left_____

Collect: 1 X 8 ml serum (red/grey SST tube)   _____

                       1 X 6 ml plasma EDTA (purple top)   _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why: _____*

Blood Draw: Excellent _____   Good _____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

DAY 32

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right ____ /Left____

Collect: 1 X 8ml serum (red/grey SST tube)   _____

1 X 6 ml plasma EDTA (purple top)   _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why: _____*

Blood Draw: Excellent ____   Good ____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

DAY 33

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right ____ /Left____

Collect: 1 X 8 ml serum (red/grey SST tube)   _____

1 X 6 ml plasma EDTA (purple top)   _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why: _____*

Blood Draw: Excellent ____   Good ____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

DAY 34

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right _____ /Left_____

Collect: 1 X 8 ml serum (red/grey SST tube)   _____

                 2 X 4 ml plasma heparin (green top)   _____

                 2 X 4 ml plasma EDTA (purple top)   _____

                 1 X 2.5 ml whole blood (Paxgene tube) _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why:* _____

Blood Draw: Excellent _____   Good _____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

DAY 35

Date (DD/MM/YY) _____   Time (HH:MM) _____

Did the subject fast?   Yes___/No ___   Feeling ill? _____

Caffeine in last 24hrs?   Yes___/No ___   If yes, when _____

Alcohol in last 24hrs?   Yes ____ / No ____   If yes, when _____

Arm: Right _____ /Left_____

Collect: 1 X 6 ml serum (red/grey SST tube)   _____

                 2 X 4 ml plasma heparin (green top)   _____

2 X 6 ml plasma EDTA (purple top)   _____

Have all blood samples been obtained?   Yes ___ / No** ____

*** If No, please state reasons why:* _____

Blood Draw: Excellent _____   Good _____   Difficult _____   Very Difficult _____

Comments:_____

Blood Drawn By:_____

SECTION 6: DXA

DAY 1 or 2

Date (DD/MM/YY): _____   Time of scan (HH:MM): _____

Since screening has the volunteer had any radiation exposure

(e.g. x-rays, radiotherapy, CT scan)?   Yes ____ / No ____

Date & details: _____

FEMALES ONLY:

Possibility of being pregnant   Yes ____ / No ____

Has a pregnancy test been offered?   Yes ____ / No ____

Has a test been taken? Yes ____ / No* ____   Test Results (circle):**Negative / Positive**

Result Confirmation:

Volunteer signature:_____ Study staff signature:_____

*If no test please explain why?

_____

_____

Check:

      Jewelry removed (including glasses):   Yes ____ / No ____

      Spine straight: Yes ____ / No ____

Comments:

_____

_____

Staff Initials:_____

DAY 34 or 35

Date (DD/MM/YY): _____   Time of scan (HH:MM): _____

Since screening has the volunteer had any radiation exposure

(e.g. x-rays, radiotherapy, CT scan)?   Yes ____ / No ____

Date & details: _____

FEMALES ONLY:

Possibility of being pregnant   Yes ____ / No ____

Has a pregnancy test been offered?   Yes ____ / No ____

Has a test been taken? Yes ____ / No* ____   Test Results (circle):**Negative / Positive**

Result Confirmation:

Volunteer signature:_____ Study staff signature:_____

*If no test please explain why?

_____

_____

Check:

     Jewelry removed (including glasses):   Yes ____ / No ____

     Spine straight: Yes ____ / No ____

Comments:

_____

_____

Staff Initials:_____

SECTION 7: PHYSICAL ACTIVITY AND LIFESTYLE MONITORING

Week 2

In the past week has your exercise level changed?   Yes   No

    If Yes, was it:

    More Active   Less Active   No Exercise

Please remind to keep your exercise level constant throughout the study.

1. Have you taken any prescription or non-prescription drugs in the past week?

    Yes   No

    If Yes, specify: description   amount

    _____   _____

    _____   _____

2. Have you taken any vitamins, minerals or other supplements in the past week?

    Yes   No

    If Yes, specify: description   amount

    _____   _____

    _____   _____

3. Have you been ill in the past week?

    Yes   No

    If Yes, describe illness: _____

4. If you were ill in the past week, did your eating change as a result?

    Yes   No

    If Yes, describe: _____

Week 5

In the past week has your exercise level changed?   Yes   No

   If Yes, was it:

   More Active   Less Active   No Exercise

Please remind to keep your exercise level constant throughout the study.

5. Have you taken any prescription or non-prescription drugs in the past week?

   Yes   No

   If Yes, specify: description   amount

   _____   _____

   _____   _____

6. Have you taken any vitamins, minerals or other supplements in the past week?

   Yes   No

   If Yes, specify: description   amount

   _____   _____

   _____   _____

7. Have you been ill in the past week?

   Yes   No

   If Yes, describe illness: _____

8. If you were ill in the past week, did your eating change as a result?

Yes   No

If Yes, describe: _____

ADVERSE EVENTS

Description	Start Date	Intensity*	Outcome	Date Ended	Diet Related?

*Mild: An event that is easily tolerated by the volunteer, causing minimal discomfort and not interfering with everyday activities

Moderate: An event that is sufficiently discomforting to interfere with normal everyday activities

Severe: An event which is incapacitating and prevents normal everyday activities

STUDY COMMENTS & PROTOCOL DEVIATIONS

Date (DD/MM/YY)	Comments	Study Personnel Initials

STATUS SUMMARY

Volunteer completed the study Yes or No

Volunteer withdrew from the study   Date of withdrawal: _____

_____

REASON FOR WITHDRAWAL

___   Informed consent withdrawn by the volunteer

___   Failure to comply with study requirements

___   Investigator decision to withdraw volunteer

Provide rationale for withdrawal (if applicable):

_____

_____

_____

_____

___   Volunteer's final results sent

___   Statement supplier form completed

Volunteer received compensation (DD/MM/YY):_____

Volunteer's Signature_____   Staff Initials _____