Master thesis

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Intake of beta-glucan and effect on lipid metabolism in relation to gut microbiota in healthy individuals.

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ABSTRACT

Background

Intake of beta-glucan, a soluble dietary fiber, for minimum 2 weeks may lower serum cholesterol. Soluble dietary fibers are subjected to colonic fermentation, resulting in the production of metabolites that can beneficially affect metabolic regulation in the host, including lipid metabolism. The aim of the current thesis was to investigate effects on lipid metabolism after intake of various amounts of beta-glucan in healthy adults.

Methods

Fourteen healthy participants were enrolled in this fixed-order crossover study. The intervention was given as three different test meals containing 0.5, 3.5 and 8 g beta-glucan, which were consumed for three consecutive evenings. Serum lipids were measured at baseline and on the following morning after the different interventions. Postprandial triglyceride response was measured at different time points after a glucose load (OGTT). Breath H_2 excretion was measured as an indicator of colonic fermentation. Lipoprotein subclasses were quantified from plasma using an NMR metabolomics platform. Data was processed with Microsoft Excel and analyzed with IBM SPSS statistics.

Results

Intake of 8 g beta-glucan significantly reduced the postprandial triglyceride response after an OGTT compared with baseline. Fasting breath H_2 excretion and fasting plasma concentration of acetate and butyrate was significantly increased after intake of all the test meals compared with baseline. In addition, there was a reduction in fasting particle concentration and total cholesterol in the HDL particles after all the test meals. The 0.5g beta-glucan test meal also reduced the triglyceride content in HDL particles. No effect was observed on fasting serum triglyceride and total cholesterol, or on LDL and VLDL subclasses, after intake of any of the test meals.

Conclusion

Three days of intervention with beta-glucan can reduce postprandial triglyceride response and modulate lipoprotein metabolism in healthy individuals. Whether these effects are due to beta-glucan alone or in combination with other fibers, and if the effects are related to colonic fermentation, is uncertain.

Appendix: Table of lipoprotein particle concentration, lipid constituents within the lipoproteins and mean diameter of the lipoproteins

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Abbreviations

μCRP	Micro C-reactive protein
Apo	Apolipoprotein
BG	Beta-glucan
BMI	Body mass index
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
CVD	Cardiovascular disease
CRF	Case Report Form
CRP	C-reactive protein
EFSA	European Food Safety Authority
FC	Free cholesterol
FFA	Free fatty acids
H_2	Hydrogen
HDL	High-density lipoprotein
HMW	High molecular weight
iAUC	incremental Area Under the Curve
IDL	Intermediate-density lipoprotein
IQR	Interquartile range
LCAT	Lecithin-cholesterol acyl transferase
LDL	Low-density lipoprotein
LMW	Low molecular weight
LPL	Lipoprotein lipase
MW	Molecular weight
NMR	Nuclear magnetic resonance
OGTT	Oral glucose tolerance test
OsloMet	Oslo Metropolitan University
PBMC	Peripheral blood mononuclear cells
PPM	Parts per million
PUFA	Polyunsaturated fatty acid
REK	Regional Committees for Medical and Health Research Ethics Sør-Øst
SFA	Saturated fatty acids

SCFA	Short chain fatty acids
TC	Total cholesterol
TG	Triglycerides
TRL	Triglyceride-rich lipoproteins
UiO	University of Oslo
VLDL	Very low-density lipoprotein

1.0 Introduction

1.1 Cardiovascular disease

Cardiovascular diseases (CVD) are the leading cause of death globally. In 2015 17.5 million people died from CVD, this accounted for 31% of all deaths worldwide (Othman, Moghadasian, & Jones, 2011; World Health Organization, 2017). CVD are a group of interrelated disorders that affect the heart and the blood vessels. This group of diseases includes, inter alia, coronary heart disease (CHD), atherosclerosis and heart failure. Some of these disorders are caused by a blockage in the blood vessels that prevents blood from delivering oxygen to the heart and brain. The most common reason for these blockages is accumulation of cholesterol in the inner wall of veins that oxygenate vital organs, such as the heart or the brain. This accumulation of cholesterol is a key pathological process in atherosclerosis. Atherosclerosis is an inflammatory response to risk factors, especially elevated levels of low-density lipoprotein (LDL) cholesterol (Mahan, Escott-Stump, & Raymond, 2012). LDL primary function is to deliver cholesterol to cells in the body. When there are excess levels of LDL-cholesterol in plasma, small LDL particles can penetrate the cell walls and enter the vascular lumen, where the LDL particle can be oxidized. This leads to the formation of plaque in the vascular lumen, narrowing the arteries and thus limiting the flow of oxygen (National Heart Lung and Blood Institute, 2016). Because of this, LDL-cholesterol is one of the major risk factors for developing CVD. Elevated levels of other lipids such as triglycerides (TG) and free fatty acids (FFA) are also considered independent risk factors for CVDs (World Health Organization, 2017).

1.2 Lipoproteins

Lipids are hydrophobic, meaning that they are water-insoluble and must therefore be transported in plasma by proteins such as lipoproteins (Ferrier, 2011). The most important plasma lipids are TG and cholesterol. Both of these hydrophobic molecules are transported by the lipoprotein system, thus the plasma metabolism of these two lipids is closely interrelated (Frayn, 2010). Lipoproteins are amphipathic and possess both lipophilic and hydrophilic properties, making them able to transport lipids in plasma. They have a lipid, hydrophobic core, which contains TG, cholesteryl esters (CE) and free cholesterol (FC) and are surrounded by an outer hydrophilic surface containing phospholipids and FC (Frayn, 2010). There are many different lipoproteins which have different functions, depending on their composition

(Hagve & Berg, 2011). VLDL and chylomicrons are rich in TG and are often referred to together as the triglyceride-rich lipoproteins (TRL), their primary function is delivering TG to tissues. LDL and high-density lipoprotein (HDL) are mainly involved in transporting cholesterol to and from cells. Chylomicrons transport TG from the enterocytes to the liver after meals (Frayn, 2010).

Each lipoprotein has a protein molecule bound to its surface, the apolipoproteins. These are important in lipid transport, binding to specific receptors or transport proteins on the surface of cells, thereby directing the lipoprotein to the proper site of metabolism. The apolipoproteins are grouped into four classes depending on function- apoA, apoB, apoC and apoE. ApoB is found in chylomicrons, VLDL and LDL. There are two isoforms, apoB100 and apoB48. The first is produced in the liver and is incorporated into VLDL and the latter is formed in intestinal cells and incorporated into chylomicrons. ApoA1 activates the enzyme lecithin-cholesterol acyl transferase (LCAT), which has a role in forming cholesteryl esters using fatty acids from HDL. Because of its amphipathic properties that enables it to bind strongly to various lipid classes, it may have a special role in interacting with cell membranes and collecting cholesterol from the cells. ApoC2 activates the enzyme lipoprotein lipase (LPL), which hydrolyzes TG in lipoproteins to release FFA. ApoE is found in the TRL chylomicrons and VLDL and also in HDL. ApoE is essential for normal catabolism of the TRL and mediates cholesterol catabolism (Frayn, 2010; Germana, Smilowitza, & Zivkovica, 2010).

1.3 Lipoprotein metabolism

Lipoprotein metabolism can generally be divided into two major pathways: the exogenous pathway and the endogenous pathway (Figure 1). The chylomicrons are involved in the exogenous pathway and VLDL, LDL and HDL are involved in the endogenous pathway. The exogenous pathway of lipoprotein metabolism refers to the distribution of dietary fat, which is mainly TG and cholesterol, from the intestine to peripheral tissues (Frayn, 2010). TG consist of glycerol and three fatty acids (Mahan et al., 2012). They are synthesized in the enterocytes from monoglycerides and fatty acids, which are absorbed from the intestinal lumen. TG are transported from the enterocytes via the lymphatics, into the circulation and finally to the liver in chylomicrons. The chylomicrons have a core consisting of TG and CE and a surface coat of phospholipids and unesterified cholesterol, and apoB48 and -A1. When

fat is ingested, plasma TG levels rise subsequently in the form of chylomicrons (Hagve & Berg, 2011).

Fatty acids are removed from the chylomicrons by lipoprotein lipase (LPL) in the capillaries of tissues which express this enzyme, mainly muscle tissue and adipose tissue. The chylomicrons interact with other particles in the circulation, such as HDL, which take up phospholipids, unesterified cholesterol and apolipoproteins shredded from the chylomicron's surface coat. HDL is responsible for removing cholesterol from peripheral tissues and transporting it to the liver for excretion, or for reuse in VLDL (Frayn, 2010). Apolipoproteins are passed on between lipoproteins in the circulation, causing them to become substrates for enzymes such as LPL. As TG are hydrolyzed from chylomicrons and surface material is degraded, the lipoproteins shrink in size and their density is increased. The reduced chylomicron particles are known as chylomicron remnants, which are rich in CE. These particles may be taken up by the liver through the LDL-receptor related protein (Frayn, 2010). Remnant cholesterol is the cholesterol content of TRL, composed of VLDL and intermediate-density lipoprotein (IDL) in the fasting state and of these two lipoproteins in addition to chylomicron remnants in the postprandial state. Elevated postprandial plasma TG is a marker of elevated non-fasting remnant cholesterol. These particles are also associated with ischemic heart disease and chronic inflammation (Nordestgaard & Varbo, 2014; Varbo & Nordestgaard, 2014).

The endogenous pathway of lipoprotein metabolism involves the distribution of TG and cholesterol from the liver to other tissues. VLDL is synthesized in the liver for the purpose of distributing TG and cholesterol to peripheral tissues. VLDL exports TG, apolipoproteins and other lipids from the liver to tissues such as adipocytes, where they are stored as the body's major energy reserve (Ferrier, 2011). Cholesterol is essential for cells as it is a component in the cell membrane and also a precursor for hormones and vitamins such as vitamin D. As VLDL passes through circulation, TG are hydrolyzed by LPL, causing an abundance of surface material, which is then passed on to other particles, mainly HDL. Loss of TG and surface substances causes the VLDL particles to become smaller and richer in CE. Eventually they become LDL particles, which are the most cholesterol-rich lipoproteins and also the most atherogenic. These smaller, denser particles then have two possible fates: they can either be removed by the LDL receptor in the liver or other tissues, delivering CE to the liver and peripheral tissues, or they may remain in the circulation (Frayn, 2010).

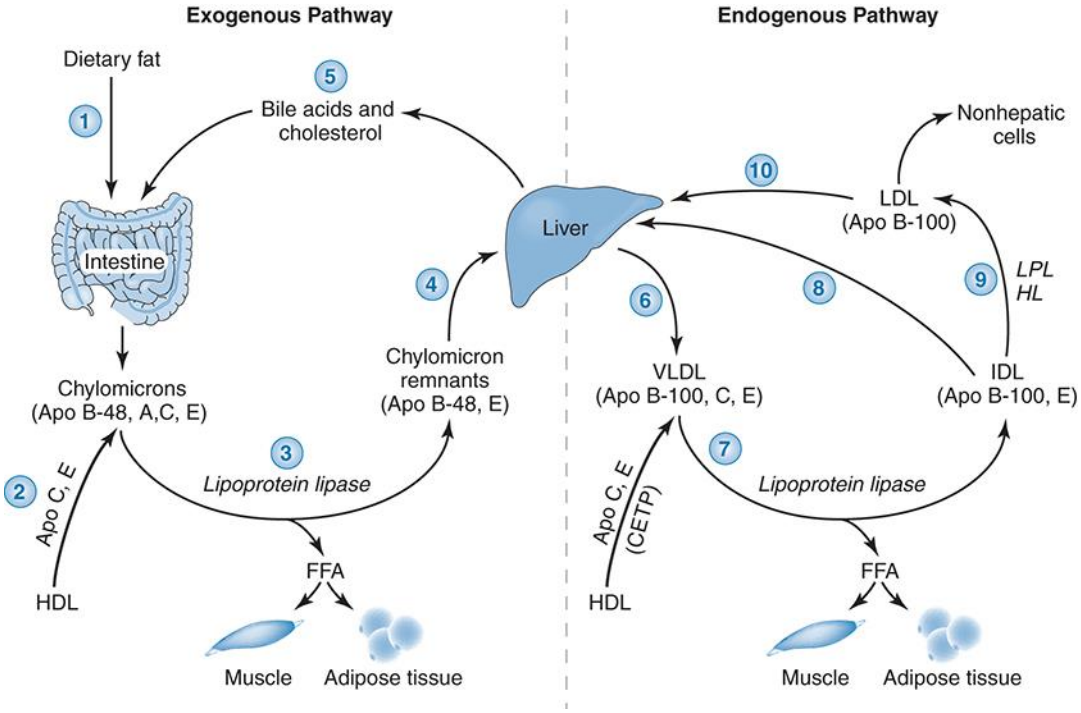

Figure 1. The exogenous and the endogenous pathway of lipoprotein metabolism. Apo, apolipoprotein; FFA, free fatty acids; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LPL, lipoprotein lipase; VLDL, very low-density lipoprotein. Adapted from Healthappointments (n.d).

LDL's primary function is, as previously mentioned, to deliver cholesterol to peripheral tissues. They do this by binding to cell surface LDL receptors, which recognize the apolipoproteins apoB100, and apoE. Macrophages express receptors, which can bind LDL, among these is a large family of receptors known as scavenger receptors. These receptors particularly have a high affinity for LDL that has been submitted to oxidative damage in the sub-endothelial space, but not for normal LDL. Furthermore, the scavenger receptors are not downregulated in response to increased intracellular cholesterol, causing cholesterol to accumulate in macrophages and create foam cells. This contributes to the formation of atherosclerotic plaque in the endothelial lumen (Ferrier, 2011; Frayn, 2010).

HDL particles are responsible for removing cholesterol from peripheral tissues by transporting it to the liver for excretion. Cholesterol may also be transferred from HDL to VLDL or chylomicrons via the action of cholesteryl ester transfer protein (CETP). The liver is the only organ which can remove cholesterol from the body, either through the formation of bile salts, by direct excretion in the bile or it can be reused in synthesizing VLDL. This pathway of removing cholesterol from peripheral tissues and excretion via the liver is known as reverse cholesterol transport (Frayn, 2010).

Lipid metabolism is also linked to carbohydrate metabolism. Fatty acids can be synthesized in the liver from non-lipid precursors such as amino acids and glucose under conditions of high carbohydrate availability. This pathway is stimulated by insulin, therefore, when glucose is in excess it is converted into lipids in the liver and stored in adipose tissue as TG. This metabolic pathway is a pathway for disposing of excess carbohydrate, it is also known as De novo lipogenesis (Frayn, 2010).

1.3.1 lipoprotein subclasses and particle size

Lipoproteins can be divided into subclasses based on density and particle size. Some lipoprotein subclasses are highly associated with increased risk of CVD, such as small dense LDL particles. Small HDL and large VLDL subclasses have been associated with the severity of coronary artery disease. With the use of nuclear magnetic resonance (NMR) spectroscopy, lipoprotein subclasses and their lipid constituents can be analyzed. In the present thesis we have analyzed fourteen different lipoprotein subclasses: XXL, XL, L, M, S and XS VLDL; XL, L, M, and S HDL; L, M and S LDL and IDL. This allows for a more detailed study of lipoprotein metabolism (A Rundblad, Holven, Bruheim, Myhrstad, & Ulven, 2018).

The TRL, such as VLDL and chylomicrons, begin as large particles that are progressively reduced in size as they pass through circulation. VLDL have been isolated from healthy males in previous studies and have been studied for their interaction with macrophages, immune cells involved in the atherosclerotic process. The larger, less dense VLDL particles induced significantly more TG accumulation within macrophages compared to smaller, denser VLDL (Germana et al., 2010).

It is well established in epidemiology that small, dense LDL particles lead to elevated risk of heart disease, much due to their increased ability to penetrate the arterial wall. Inside the sub-endothelial matrix, LDL is more submitted to oxidative damage, which causes increased uptake by the scavenger receptor and subsequently leads to the beginning of atherosclerosis (Frayn, 2010; Germana et al., 2010). LDL have a relatively long half-life in the circulation and they are continuously acted upon by catalytic and transfer activities. Thus, LDL particles that stay in the circulation longer get smaller and denser. In addition to this, LDL particles that remain in circulation longer also become more susceptible to oxidation. This may explain why smaller LDL particles are more atherogenic than larger LDL particles (Germana et al.,

2010). The long half-life of LDL is also the reason why it takes several weeks to lower serum TC.

The size of the HDL particle may also play a role in the development of heart disease. Dyslipidemic patients with insulin resistance and visceral obesity have an increased concentration of small, dense HDL and decreased concentration of large HDL particles. In addition, case control studies demonstrate an inverse relationship between coronary heart disease risk and large HDL particles, and a positive relationship with small, dense HDL. The size and composition of HDL also influences reverse cholesterol transport. It has been suggested that the HDL size is probably important for efficient cholesterol transport and may reflect HDL particles' ability to reach different intercellular spaces. In addition, the size and density of the HDL particle may affect its ability to accept cholesterol from cells (Germana et al., 2010; Maria Lankinen et al., 2014). Another factor that contributes to the antiatherogenic properties of HDL is that HDL contains an enzyme, paraoxonase, which is believed to protect LDL from oxidation in the artery wall, thereby preventing atherosclerosis (Barter, 2005).

1.4 Triglycerides and cardiovascular disease

The concentration of TG in plasma varies widely between different individuals, depending on factors such as genetics, fitness level and diet. A typical concentration of TG in serum is 1 mmol/L after an overnight fast (Frayn, 2010). After consumption of meals rich in fat, TG levels rise postprandially, in the form of chylomicron-TG. Unlike the absorption of glucose after a meal, the process of absorbing TG is slow and the peak in plasma TG concentration does not occur until 3-5 hours after the meal (Frayn, 2010). Diets rich in fat result in elevated postprandial lipid concentrations, which is associated with progression of atherosclerosis. There is evidence that prolonged elevated plasma TG concentrations is an independent risk factor for CVD due to increased VLDL synthesis and an increased amount of small dense LDL particles, which in turn are associated with progression of atherosclerosis and increased risk of CVD (Budoff, 2016; World Health Organization, 2003).

1.5 Diet and prevention of CVD

Lifestyle plays an important role when it comes to risk of developing CVD. Lifestyle factors such as tobacco smoking, physical inactivity or unhealthy diet may affect a person's risk profile. There is strong epidemiological evidence that intake of saturated fatty acids (SFA)

increases LDL-cholesterol and total cholesterol (TC). There are however different types of SFA and these elicit different effects. SFA that have between 12-16 carbons in their fatty acid chain exert the greatest cholesterol elevating effects. Among these are myristic acid (C14:0) and palmitic acid (C16:0), these SFA are abundant in diets rich in red meat and dairy products (Food and Agriculture Organization of the United Nations, 2010; World Health Organization, 2003)

Lifestyle modifications such as increased intake of fruit and vegetables, polyunsaturated fats and dietary fiber in addition to reduced intake of SFA, subsequently reduces the risk of CVD. Dietary fiber is found in wholegrain products, cereal, fruits and vegetables. It reduces risk of CVD through lowering plasma cholesterol values (AbuMweis, Jew, & Ames, 2010; Frayn, 2010; Gunness & Gidley, 2010). There is convincing evidence that polyunsaturated fatty acids (PUFA) such as n-3, found in oily fish, lower plasma TG concentrations. Fasting elevated concentrations of plasma TG can also be improved through lifestyle modifications such as physical activity (Folkehelseinstituttet, 2015; World Health Organization, 2003). In summary, to promote cardiovascular health, diets should provide a low intake of SFA, an adequate amount of PUFA and dietary fiber and at least 400 – 500 grams of fruits and vegetables (Food and Agriculture Organization of the United Nations, 2010).

1.6 Dietary fiber and beta-glucan

Dietary fiber is a heterogeneous group of chemical compounds found in plants, it is defined in literature as a group of carbohydrates which cannot be digested or absorbed in the small intestine due to lack of digestive enzymes in humans (Theuwissen & Mensink, 2008; Xu & Knight, 2015). Dietary fiber is mainly divided into two main groups; water soluble and water insoluble fiber. Whether a type of fiber is soluble or insoluble depends on the chemical structure. Insoluble fibers such as cellulose are mainly found in vegetables and cereal grains such as wheat. These types of fiber contribute to, inter alia, regulate bowel movement. Soluble fibers are characterized by their gel forming properties, they create a physical barrier in the gut, which inhibits absorption of macronutrients such as lipids and carbohydrates (Theuwissen & Mensink, 2008).

There is strong evidence supporting the link between TC and LDL-cholesterol lowering and reduced risk of CVD (Whitehead, Beck, Tosh, & Wolever, 2014). The European guidelines for the management of dyslipidemias recommend that lifestyle modifications, such as

consumption of 5-15 g/d soluble fiber from oat and barley products may be beneficial in reducing CVD risk. The main active component considered responsible for these positive effects is beta-glucan (BG). Food standards agencies worldwide support health claims regarding intake of BG from oat and barley and cholesterol lowering, including the European Food Safety Authority (EFSA). In order to attain positive health effects, the diet must contain at least 3 g BG/d for minimum 2 weeks (Nutrition & Allergies, 2011; Whitehead et al., 2014).

BG is a highly viscous, soluble fiber found in barley and oat and is composed by glucose molecules with mixed b-(1→4) and b-(1→3) bonds which are responsible for its physical properties (Othman et al., 2011).

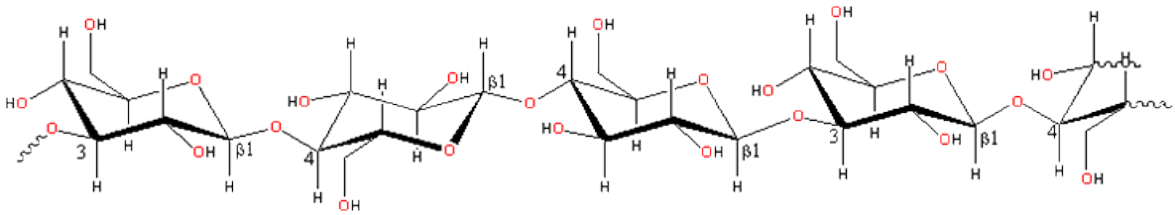

Figure 2. Beta-glucan structure

BG is associated with beneficial health effects including cholesterol lowering, weight management and reduction of postprandial glycemic responses (Gunness & Gidley, 2010; Hollænder, Ross, & Kristensen, 2015; Talati et al., 2009; Wang et al., 2016). Three biological mechanism have been suggested to be responsible for the cholesterol reducing effects of soluble dietary fiber: reduced re-absorption of bile salts from the small intestine, reduced glycemic response leading to lower insulin stimulated hepatic cholesterol synthesis and reduced absorption of cholesterol (Gunness & Gidley, 2010).

Bile acids are formed from cholesterol in the liver, secreted in bile and as much as 95% is reabsorbed in the ileum into the enterohepatic circulation. Studies have shown that intake of soluble dietary fiber increases fecal excretion of bile acids by inhibiting reabsorption. More plasma cholesterol therefore needs to be converted into bile acids and this consequently contributes to a reduction in plasma TC and LDL-cholesterol (Frayn, 2010; Gunness & Gidley, 2010; Talati et al., 2009).

Hepatic cholesterol synthesis is stimulated by insulin. Viscous fiber reduces the rate of intestinal absorption of glucose through increasing viscosity in the small intestine, creating a physical barrier. Reduced glucose absorption subsequently leads to reduced pancreatic

production and secretion of insulin, resulting in a lower rate of VLDL and cholesterol synthesis in the liver (Frayn, 2010; Gunness & Gidley, 2010; Talati et al., 2009).

The viscous properties of BG depend on molecular weight (MW) and solubility (Anttila, Sontag-Strohm, & Salovaara, 2004; Wolever et al., 2011). MW is important when it comes to the physiological benefits of BG. High molecular weight (HMW) BG is suggested to have a greater cholesterol lowering effect than low molecular weight (LMW) BG due to higher viscosity and greater gel-forming properties (Whitehead et al., 2014). In a randomized controlled trial conducted by Wang and colleagues they also found that HMW BG can alter the composition of the gut microbiota towards a reduced CVD risk (Wang et al., 2016).

1.7 Dietary fiber and gut microbiota

The metabolic benefits of dietary fiber and whole grain appear to be partly associated with colonic fermentation of indigestible carbohydrates by the gut microbiota (Nilsson, Johansson-Boll, Sandberg, & Björck, 2016). The human microbiota is a collection of microbes that inhabit our body, most of which inhabit the gut (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016). The amount of bacteria in the gut is in fact the same as the number of human cells in the body (Sender, Fuchs, & Milo, 2016). The understanding and appreciation of the gut microbiota's role in metabolic regulation and disease prevention has recently increased significantly. The microbiome strengthens the immune system, protects against pathogens, produce nutrients and vitamins and assist the host in digestion of complex dietary nutrients. Studies have shown that the composition of the gut microbiota plays a role when it comes to disease development such as diabetes and CVD (Koh et al., 2016; Sheflin, Melby, Carbonero, & Weir, 2017; Wang et al., 2016; Xu & Knight, 2015).

The gut microbiota can be altered as a response to several endogenous and exogenous factors, but diet has shown to be the single most important driver of altering the microbiota (Sheflin et al., 2017). Changes in the composition of the microbiota can be seen after 1-3 days in response to dietary changes. High-fiber diets are associated with increased richness and/or diversity of the gut microbiota which, in turn, is associated with lower risk of chronic diseases, such as coronary heart disease, dyslipidemia and stroke (Sheflin et al., 2017; Wang et al., 2016).

Dietary fiber cannot, as mentioned previously, be digested by enzymes in the human gut, we therefore rely on the microbiota to ferment these non-digestible carbohydrates (Xu & Knight, 2015). Fermentation results in multiple groups of metabolites which can affect human health and disease (den Besten et al., 2013). SCFA are the major group of metabolites derived from colonic fermentation, mainly acetate, propionate and butyrate.

A substantial part of the SCFA produced during colonic fermentation are used as energy substrates for the colonocytes but some enter the hepatic portal vein and are taken up by the liver where they function as energy sources and substrates for cholesterol synthesis (den Besten et al., 2013). Some of these may also enter the systemic circulation and exert positive metabolic effects (Koh et al., 2016; Nilsson, Östman, Knudsen, Holst, & Björck, 2010). SCFA have been shown to affect glucose, lipid and cholesterol metabolism in peripheral tissues, where they act as signal molecules or substrates for receptors. In addition, SCFA have been shown to reduce plasma cholesterol in rodents and humans, possibly by affecting enzymes involved in the regulation of cholesterol synthesis (den Besten et al., 2013; Gunness & Gidley, 2010). In addition to this, dietary fibers that lead to high amounts of SCFA lower the pH in the colon, lower pH-values prohibit overgrowth of pH-sensitive pathogenic bacteria (den Besten et al., 2013; Nilsson et al., 2010). SCFA also prohibit leaky gut and inflammation by maintaining a healthy colonic mucus barrier (Desai et al., 2016).

There are several studies investigating effects of BG on plasma lipids, especially LDL-cholesterol, but few studies have investigated the effect on plasma lipids in healthy individuals related to activity of gut microbiota. Studies that investigate cholesterol-lowering effect in relation to BG intake are usually conducted in intervention periods that last for several weeks. Few studies, if any, have explored what effects BG has on cholesterol and other plasma lipids after a short intervention period of three days.

1.8 Aim of the study

The present master thesis is a part of a project where the aim is to investigate effects of BG on blood glucose and satiety regulation related to gut microbiota. The aim of this thesis is to investigate how intake of different amounts of BG for three consecutive days affects circulating levels of cholesterol, lipoproteins, SCFA and TG in healthy individuals and whether the effects are related to changes in colonic fermentation.
The objectives of this master thesis are therefore:

1. To investigate fasting values of cholesterol, TG and lipoproteins after a short intervention with different doses of BG.
2. To investigate the postprandial response of TG after a short intervention with different doses of BG followed by a glucose challenge
3. To investigate fasting hydrogen (H_2) excretion and fasting plasma SCFA values, as indicators of colonic fermentation after a short intervention with different doses of BG.

2.0 Method

2.1 Subjects, recruitment and screening

This master thesis is part of a study that was completed in the spring of 2017. The aim of the study was to investigate effects of BG on blood glucose and satiety regulation related to gut microbiota. A previous master student investigated how intake of different doses of beta-glucan for three consecutive days affected the postprandial blood glucose after an oral glucose tolerance test (OGTT) in healthy individuals, and if effects were related to increased activity among gut microbiota.

The participants enrolled in the study where healthy adults between the age of 18-65 years, they were required to have a stable body weight and a BMI between 18.5 and 27 kg/m^2. Both genders were included in the study. In addition to this, subjects needed to have fasting blood glucose levels of ≤ 6 mmol/L. Subjects that were enrolled to participate in the study also needed to be inclined to limit their consumption of dietary products that are rich in BG (mainly oat and barley-based products) two weeks prior to and throughout the course of the study. In addition to these limitations, subjects also had to refrain from using any probiotic products and other dietary supplements four weeks prior to and during the study. Other exclusion criteria included food allergies, intolerances and treatment with antibiotics during the previous three months. There could be no use of medication during the study. Exclusion criteria are presented in table 1.

Table 1: Exclusion criteria

Exclusion criteria	
Chronic metabolic diseases	Any type of diabetes, CVD, cancer last six months
Inflammatory bowel diseases (IBD)	Chron's disease, ulcerative colitis, celiac disease and irritable bowel syndrome (IBS).
Food allergies and intolerances	
Pregnant or nursing women	
Smokers and people using snus	
Fasting blood glucose values	≥ 6.1 mmol/L
CRP	< 10 mg/L
BMI	< 18.5 and > 27 kg/m^2
Planned weight reduction and/or 5% weight change previous three months	
Antibiotics ≤ three months prior to and during the study period	
Blood donor last two months prior to and/or during the course of the study	
Not willing to stop using dietary supplements four weeks prior to the study start and during the whole study period	
Large alcohol consumption	> 40 g per day
Hormonal treatment	Except use of oral contraceptives

CRC, C-reactive protein; CVD, cardiovascular disease; BMI, body mass index; IBD, inflammatory bowel disease; IBS, irritable bowel syndrome

The study was conducted according to the guidelines in the Declaration of Helsinki and approved by the Regional Committees for Medical and Health Research Ethics Sør-Øst (REK). The study is registered at ClinicalTrials.org and written informed consent forms were obtained from all the subjects in the study.

The participants of the study were recruited from employees and students at Oslo Metropolitan University (OsloMet). The process of recruiting started in August 2016 and continued through February 2017 and was conducted through social media, presentations in classes and e-mails. It resulted in 238 inquiries and 63 appointments for screening. After the screening process was completed, 18 subjects eventually signed a consent form and thus were ready to receive the intervention. Four subjects dropped out after baseline, leaving 14 subjects that completed the study and were included in the data analysis.

Before baseline measurements, subjects were requested to fulfill a food frequency questionnaire in order to map their habitual diet during the past year. The subjects were encouraged not to change their habitual diet or their normal level of physical activity during

the study. Medical history was mapped during the screening process and the subjects also received further information about dietary restrictions and about the aim of the study. Clinical examinations including finger-prick capillary blood samples (to assess fasting blood glucose value), height, body weight, BMI, body composition and blood pressure measurements were conducted.

2.2 The evening test meals

The intervention was given in the form of three meals which consisted of 100 g cereal containing either a low, medium or high amount of BG (0.5, 3.5 and 8 g, respectively). It was consumed together with 200 ml of low-fat milk. The cereal was based on a commercial product provided by Mills- "Vita Hjertego' Flerkorn med Granola, Tranebær og Blåbær". In the test meal with the lowest amount of BG, BG was replaced with inulin. The 3.5 g BG evening test meal was identical to the commercial product provided by Mills. The evening test meal that contained 8 g of BG consisted of 60 g of the commercial product by Mills and 40 g of another commercial product from Oatwell- "Betavivo havrehjerter". The 8 g BG test meal both looked and tasted different from the two other test meals, which were similar to each other.

The energy content was similar between the test meals, but the macronutrient content differed slightly. The test meal with the highest amount of BG (8 g) also contained the highest amount of protein and fat, and the lowest amount of carbohydrate compared to the two other evening test meals. The test meal with the lowest amount of BG (0.5 g) contained the highest amount of carbohydrate and the lowest amount of protein and fat. The 8 g and 3.5 g BG test meals consisted of BG with HMW. MW in the 0.5 g test meal could not be decided due to insufficient amount. Total dietary fiber content ranged from 15.8 g, 18.5 g and 20.1 g in the 0.5, 3.5 and 8 g BG test meals, respectively. Composition of the evening test meals is presented in table 2. The content of BG in the test meals were estimated to be 0.5, 3.5 and 8 g BG and are therefore referred to by these amounts throughout the present master thesis, and not the analyzed amounts.

Inulin is another type of soluble fiber which was used to replace BG as a source of dietary fiber in the test meals. The amount of inulin differed between the test meals, with 8.4 g, 6.7 g and 4.4 g in the 0.5, 3.5 and 8 g BG test meals, respectively (Table 2).

Table 2. Evening test meal composition

Evening test meal composition			
	0.5 g beta-glucan	3.5 g beta-glucan	8 g beta-glucan
Energy (kcal)	350	360	347
Fat	2.26	3.79	4.42
Protein	8.32	9.75	13.2
Carbohydrate	69.7	65.5	54.6
Total dietary fiber	15.8 ± -0.3	18.5 ± -0.2	20.1 ± -0.6
Mean molecular weight (MW)[1]	Not detectable	890 080 ± 26 163	1 049 400 ± 12 728
Water content	9.4 ± 0.06	7.5 ± 0.21	6.9 ± 0.07
Amount of beta-glucan in % of dry weight	0.7 ± 0.02	3.5 ± 0.08	7.1 ± 0.03
Amount of inulin	8.4 ± 0.3	6.7 ± 0.1	4.4 ± 0.1
Amount of available starch[2]	58.3 ±1.6	54.6 ± 2.5	50.0 ± 2.2
Estimated amount of arabinoxylan[3]	4.1 ± -0.1	4.5 ± -0.1	4.7 ± -0.1
Estimated amount of cellulose[4]	1.1 ± -0.04	2.0 ± -0.0	1.9 ± -0.5

Evening test meal composition per 100 g. macronutrient analyses were performed by Eurofins and are presented as mean. Fiber composition was analyzed by Nofima and is presented as mean ± SD.
[1] MW of beta-glucan, presented in Dalton
[2] The available starch is the part of the starch which is digestible
[3] The sum of arabinose and xylose
[4] Measured amounts of glucose minus the amount of beta-glucan

2.3 Design

The study was a single blinded fixed-order crossover study. Increased amounts of BG (fixed order) was administered in favor of a randomly assigned BG dose to avoid any carryover effect by the previous dose. The participants were randomly assigned into three groups, each group starting with the test meal containing the lowest dose of BG (0.5 g), which was the control meal. The subjects participated in three consecutive three-day dietary interventions, each separated by a two-week washout to avoid carryover effect. There was also a two-week washout prior to the start of the study. They were given the test meals at day one of every test week, which were consumed for three consecutive evenings. Each intervention period included two visits at OsloMet, adding up to a total of six visits (V0 – V5) throughout the study. Blood samples were taken fasted at baseline (V0), and at every visit (V1-V5). In addition to this, urine samples and fecal samples were also collected at every visit.

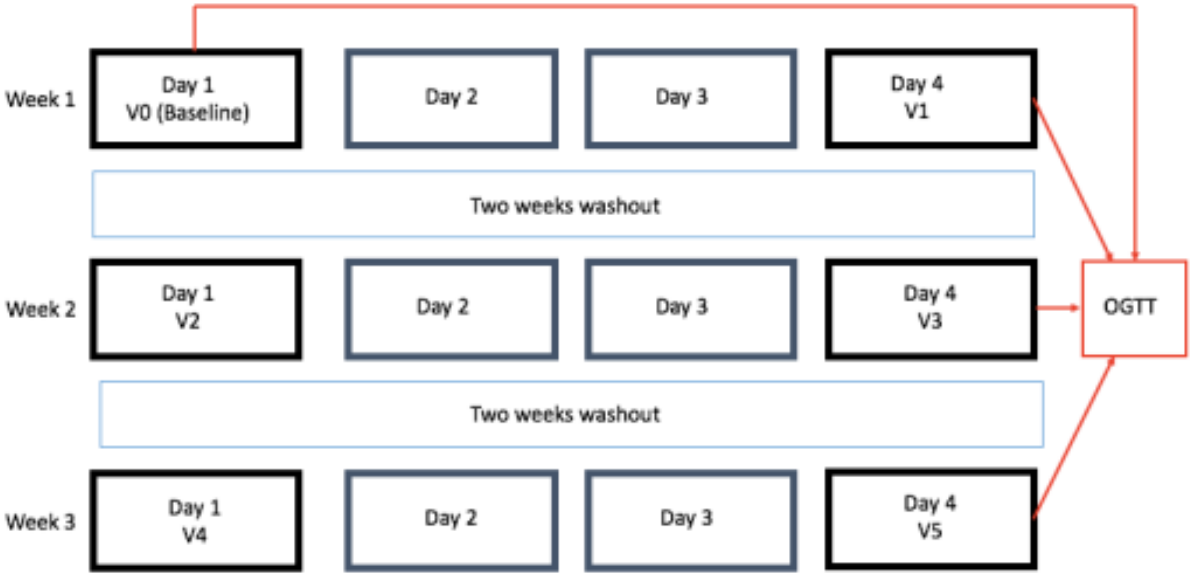

Figure 3. Study outline. OGTT, oral glucose tolerance test; V, visit.

During the first week of the study, an OGTT was included at V0 and V1. Week two and week three included an OGTT at V3 and V5, respectively. Before each visit, the participants had to refrain from heavy physical activity and alcohol for 24 hours. After each three-day intervention period (V0-V5) the participants met fasted at OsloMet from 8 pm the night before, they delivered spot samples of urine and feces, filled out a Case Report Form (CRF) and completed a body composition measurement. At every meeting, fasting measurements of venous blood samples and breath H_2 were collected.

The participants were given an OGTT containing 75 g of glucose at baseline, V1, V3 and V5, after the fasting measurements had been taken.

Venous blood samples were drawn fasted and 0, 30, 60 and 120 minutes after the OGTT, the blood samples were drawn after an overnight fast (minimum 12 hours) by a bioengineer. Serum was obtained from 8.5 ml serum gel tubes and turned 6-10 times before spindown after 30 minutes (1300 – 1500 g, 15 minutes), and kept in a refrigerator (4°C) until it was sent to Fürst Medical Laboratory, Oslo, Norway within 24 hours. Serum triglycerides were taken fasted and 30, 60 and 120 min after the OGTT. In addition, total cholesterol was measured fasted at every visit.

Dietary fiber is fermented by bacteria in the colon. The fermentation process generates production of H_2 and SCFA, breath H_2 and SCFA are therefore both used as biomarkers of colonic fermentation. Breath H_2 was measured fasted and 15, 30, 60, 90, 120, 150 and 180 minutes after the OGTT. It was measured using a Gastro^{+TM} gastrolyzer (Bedfont Gastrolyzer). SCFA were measured in plasma.

The participants were asked to deliver morning urine spot samples and fecal samples at every visit. Morning urine was collected in a urine container and placed in a 10 ml cryocane and stored at -80°C at OsloMet. The fecal samples were taken before measurements at baseline (V0) and from the first stool after consumption of the previous evening test meal. The fecal samples were stored at -20°C at OsloMet prior to shipment to Nofima for microbiota analyzes.

2.3.1 Variables analyzed in the present thesis

In the present thesis we have analyzed variables related to lipid metabolism, such as TG and cholesterol. We have chosen to mainly include fasting values, with the exception of TG which was also measured after an OGTT. The selected variables included in the present thesis is fasting cholesterol and TG values, postprandial TG values after an OGTT, fasting breath H_2 values and fasting values of SCFA. In addition, we have analyzed fourteen different lipoprotein subclasses of VLDL, LDL, HDL and IDL. We have analyzed particle concentration of the different lipoproteins and TC, FC, CE and TG content in the lipoprotein particles. Further, we have analyzed particle diameter of HDL, VLDL and LDL.

2.4 Lipoprotein subclass and metabolite analyses

Metabolic biomarkers were quantified from EDTA plasma using a commercial high-throughput proton NMR metabolomics platform (Brainshake Ltd; www.brainshake.fi). VLDL, LDL, IDL and HDL subclass levels and average particle size were quantified using proton NMR. This method quantifies routine lipids, lipoprotein subclass profiling with lipid concentrations within fourteen subclasses, fatty acid composition, abundant proteins and various LMW metabolites. The fourteen lipid subclass sizes were defined as follows: extremely large VLDL with particle diameters from 75 nm upwards and a possible contribution of chylomicrons, five VLDL subclasses (64·0, 53·6, 44·5, 36·8 and 31·3 nm), IDL (28·6 nm), three LDL subclasses (25·5, 23·0 and 18·7 nm) and four HDL subclasses

(14·3, 12·1, 10·9 and 8·7 nm). The following components of the lipoprotein subclasses were quantified: total lipids, phospholipids, cholesterol, cholesteryl esters, free cholesterol and TG. The mean size for VLDL, LDL and HDL particles were calculated by weighting the corresponding subclass diameters with their particle concentrations.

2.5 Statistical analyses

Data were processed using Microsoft Excel for Mac 2017 (version 15.32) and exported to IBM SPSS Statistics (version 24.0; SPSS, Inc., Chicago, IL, USA). Sample size was calculated based on a previous study by Nilsson and colleagues where effects of a barely-based evening test meal on blood glucose incremental Area Under the Curve (iAUC) were observed (Nilsson, Östman, Holst, & Björck, 2008). Due to small sample size, data were analyzed using non-parametric tests. Friedman's ANOVA was used to compare differences in postprandial response and fasting values between baseline and interventions and between the different interventions. When the findings were statistical significant, pairwise comparisons were further investigated with Wilcoxon Signed Rank Test to examine where the difference between baseline and the interventions, and between interventions occurred. With a strength of 80% and an acceptance of 5% of type 1 error, the total number of subjects required was set to approximately 13-17 subjects in the study. The significance level was set to 5 % $P \leq 0{,}05$. Data are presented as median (25-75 percentile).

The TG iAUC was calculated in Microsoft Excel for Mac 2017 (version 15.32). The iAUC for each subject was calculated by subtracting the fasting value (0 minute) at each visit from the corresponding values after 30, 60 and 120, minutes after the OGTT, using the trapezoidal rule with the following equation:

$$A = \frac{(y1+y2)x\ (x2-x1)}{2}$$

3.0 Results

3.1 Baseline characteristics

Participants were recruited from employees and students at OsloMet between August 2016 and February 2017. The study population consisted of 14 healthy subjects, two men and twelve women, aged between 18-65 years. Median age was 28 years and median BMI was 22.4 kg/m^2. The participants were healthy and had normal levels of fasting serum lipids. Clinical and biochemical baseline characteristics of the participants are shown in table 3.

Table 3. Baseline characteristics

Gender:	Female *(n)*	12
	Male *(n)*	2

Age	28 (24.0 – 39)
BMI (kg/m^2)	22.4 (20.7 – 24.6)
Insulin (pmol/L)	64.5 (58.8 – 83.8)
Blood glucose (mmol/L)	5.1 (4.8 – 5.7)
Total triglycerides (mmol/L)	0.74 (0.65 – 0.87)
Total cholesterol (mmol/L)	4.4 (3.8 – 4.5)
μCRP (mg/L)	0.4 (0.3 – 0.8)

BMI, body mass index; μCRP, micro C-reactive protein. Values are median (25 – 75 percentile). All variables are measured in the fasting state.

3.3 Postprandial triglyceride response

The participants consumed test meals containing 0.5, 3.5 and 8 g BG for three consecutive evenings before meeting fasted at OsloMet, campus Kjeller, the next morning. Serum TG was measured in venous blood samples fasted and 30, 60 and 120 minutes after an OGTT. There were no significant changes in fasting TG levels after intake of 0.5, 3.5 and 8 g BG for three days compared to baseline levels (data shown in table 4). TG iAUC was calculated in order to compare the effect of the test meals on TG response after the OGTT. Intake of 8 g of BG significantly reduced the postprandial TG response (iAUC 0-120 minutes) compared with baseline ($p = 0.01$). There was also a trend towards a lower TG response (iAUC 0-120 minutes) with an intake of 3.5 g of BG compared with baseline ($p = 0.06$) (data shown in table 5). The postprandial TG response is illustrated in Figure 4.

Table 4. Fasting triglyceride values

Intervention	Triglycerides
	Median (25 – 75 percentile)
Baseline	0.74 (0.64 – 0.92)
0.5 g beta-glucan	0.69 (0.62 – 0.89)
3.5 g beta-glucan	0.83 (0.69 – 0.95)
8 g beta-glucan	0.85 (0.75 – 0.96)

Fasting triglyceride values after three days of intervention with different amounts of beta-glucan, given as evening test meals for three days.

Table 5. Triglyceride incremental Area Under the Curve (iAUC) after an OGTT following three days of intervention with the test meals

Intervention	Triglyceride iAUC (0 -120 minutes)
	Median (25 – 75 percentile)
Baseline	-1.2 (-7.9 – 5.3)
0.5 g beta-glucan	-5.6 (-9.3 – -0.9)
3.5 g beta-glucan	-4.8 (-12.0 – -3.5)
8 g beta-glucan	-10.5 (-15.3 – -4.5) *

iAUC, incremental Area Under the Curve; OGTT, oral glucose tolerance test.

*Different from baseline (P = 0.01)

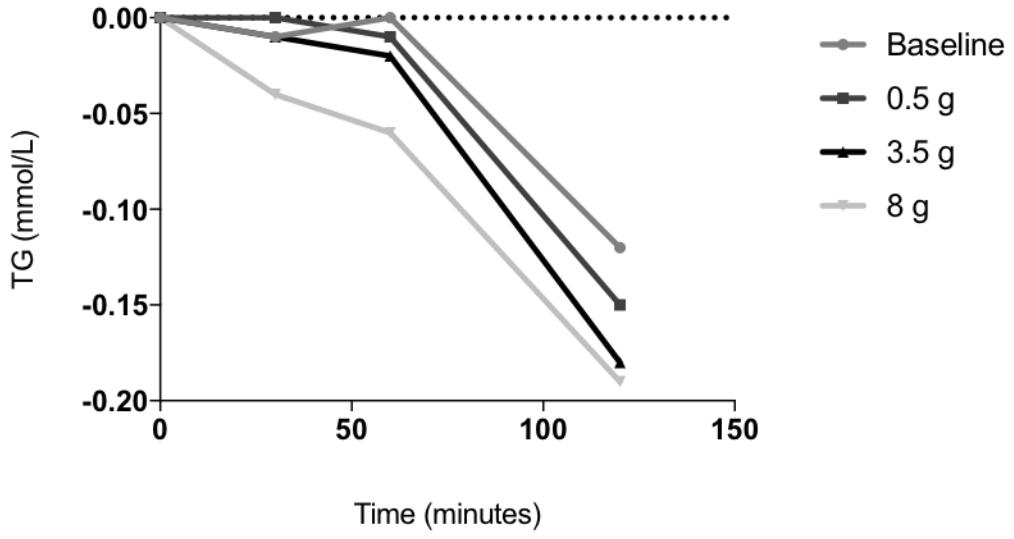

Figure 4. Postprandial serum triglyceride response after an OGTT following three days of intervention with different amounts of beta-glucan. Values are presented as Δ values. The values are median values (25- 75 percentile).

3.4 Cholesterol

Total cholesterol was measured fasted at baseline and after intake of the three test meals for three consecutive evenings. There were no significant changes in fasting cholesterol values after intake of different BG amounts compared to baseline (data shown in table 6).

Table 6. Fasting serum cholesterol values after an overnight fast following three days of intervention with different amounts of BG

Intervention	Fasting total cholesterol
	Median (25 – 75 percentile)
Baseline	4.4 (3.8 – 4.5)
0.5 g beta-glucan	4.2 (3.8 – 4.4)
3.5 g beta-glucan	4.4 (3.8 – 5.1)
8 g beta-glucan	4.3 (3.6 – 4.7)

3.4 Breath H_2 and SCFAs

Breath H_2 excretion was measured as a marker for colonic bacterial fermentation following intake of the test meals. Fasting breath H_2 excretion was significantly elevated with 329 % (p=0.009), 386 % (p=0.003) and with 571 % (p=0.003) compared with baseline after intake of the test meals consisting of 0.5, 3.5 and 8 g BG, respectively. Furthermore, intake of 8 g BG for three consecutive days increased breath H_2 excretion significantly with 38 % compared with intake of the 3.5 g BG intervention (p = 0.04) (Figure 5). There were no significant changes in fasting breath H_2 excretion between the 0.5 g intervention and the 3.5 g and 8 g BG interventions.

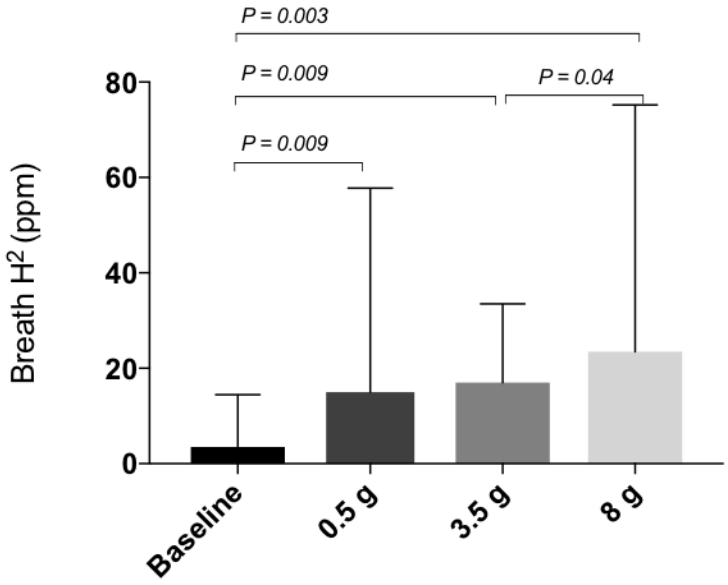

Figure 5. Breath H_2 values after an overnight fast following three days of intervention with test meals containing different amounts of BG. Overall difference between interventions was tested with Friedman's ANOVA. Pairwise comparisons were investigated with Wilcoxon Signed Rank Test. Values are expressed as median and interquartile range (IQR) represented by vertical bars.

Short chain fatty acids (SCFA), mainly acetate, butyrate and propionate, are products of bacterial fermentation in the large intestine. SCFA was measured in plasma after intake of BG for three consecutive days. Fasting butyrate was increased after intake of 0.5 g BG, 3.5 g BG and 8 g BG compared with baseline (P = 0.006, 0.006 and 0.002 respectively) (Figure 6). The plasma concentration of acetate was also increased after intake of 0.5 g, 3.5 g and 8 g BG compared with baseline (P = 0.02). Both SCFA increased the most after intake of 8 g BG. There were no observed significant changes in propionate concentration after intake of any of the different test meals (data presented in figure 6).

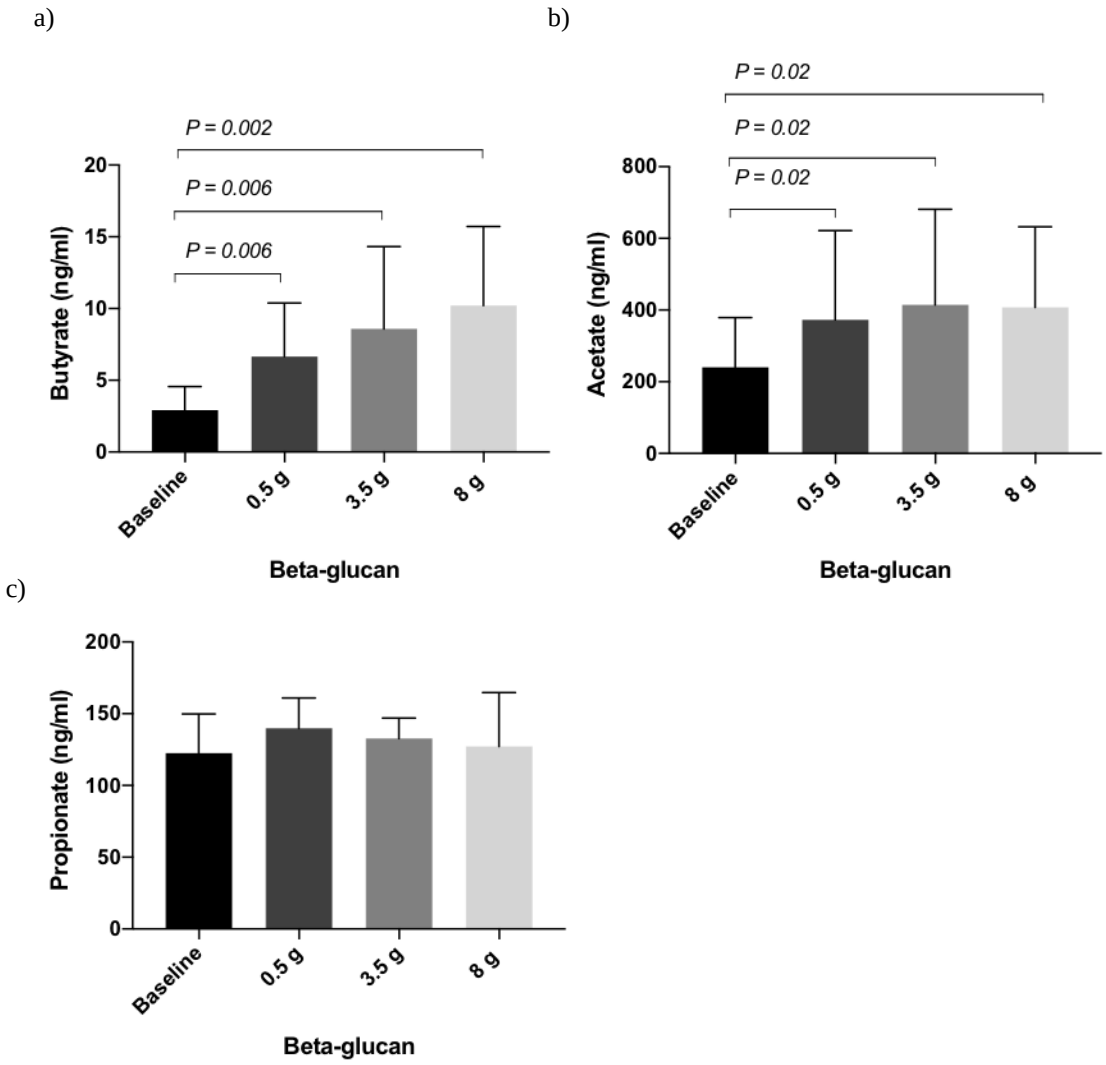

Figure 6. Fasting plasma concentrations of the SCFA (a) butyrate, (b) acetate and (c) propionate after intake of BG for three days. Overall difference between interventions was tested with Friedman's ANOVA. Pairwise comparisons were investigated with Wilcoxon Signed Rank Test. Values are expressed as median and IQR represented by vertical bars. N=14.

3.5 Lipoprotein subclasses

The particle concentrations and the concentrations of lipid constituents (TG, TC, FC, CE) in fourteen lipoprotein subclasses were analyzed to investigate the effect of different amounts of BG on lipoprotein metabolism (Appendix). There were no significant changes in particle concentration in any of the VLDL or LDL subclasses (Appendix). However, significant changes were found in the XL, L and M-HDL subclasses (Figure 7). In the XL-HDL subclass there was a significant reduction in particle concentration after intake of 0.5 g BG and 3.5 g BG compared to baseline (P = 0.006 and 0.02, respectively). Furthermore, particle concentration was significantly reduced with intake of 8 g compared with 3.5 g BG (P = 0.001). In the L-HDL subclass there was a significant reduction in particle concentration after intake of the 3.5 g and 8 g BG meal compared to baseline (P = 0.001). There was also a significant reduction in concentration of M-HDL particles after intake of the 0.5 g BG test meal compared to baseline (P = 0.01). In addition, particle concentration was also reduced after intake of 8 g compared to baseline and to intake of 3.5 g BG (P = 0.02 and 0.002, respectively).

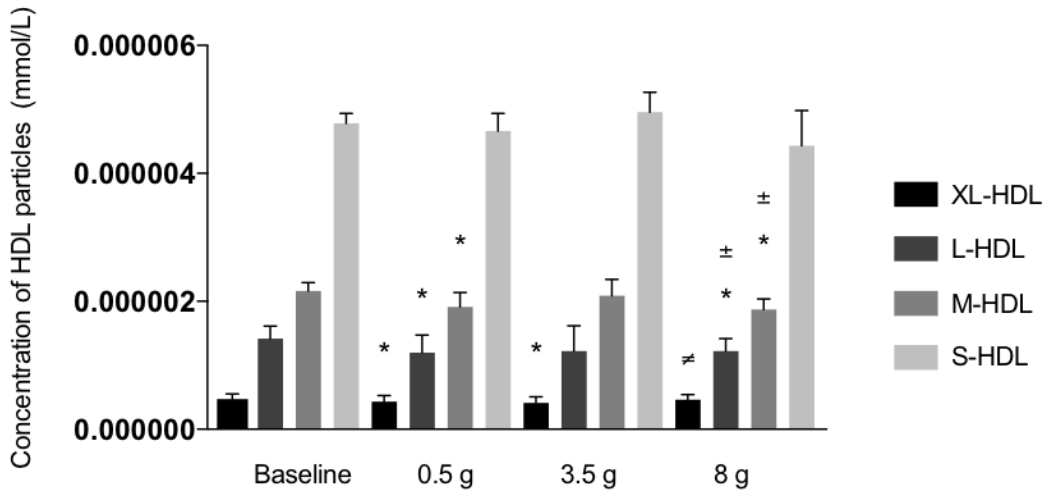

Figure 7. Concentration of XL, L, M and S HDL particles after intake of BG for three consecutive days. Overall difference between interventions was tested with Friedman's ANOVA. Pairwise comparisons were investigated with Wilcoxon Signed Rank Test. Values are expressed as median and IQR.
* Different from baseline (P < 0.05)
≠Different from 0.5 g beta-glucan (P < 0.05)
± Different from 3.5 g beta-glucan (P < 0.05)

Furthermore, the TG content in the XL-HDL subclass was significantly reduced with intake of the 0.5 g BG test meal compared to baseline (p = 0.002) (Figure 8). There were no significant changes in the TG content within the L, M or S-HDL subclasses after intake of the test meals containing 3.5 g or 8 g BG (Figure 8).

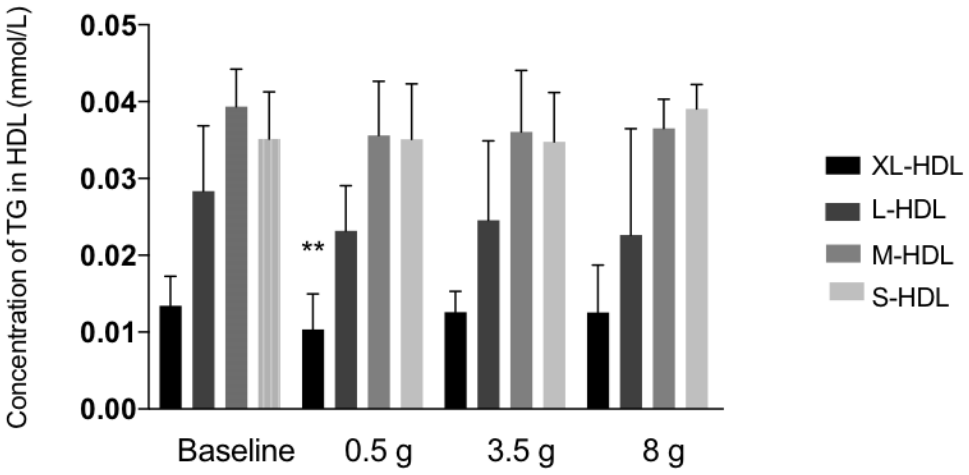

Figure 8. TG in XL, L, M and S-HDL particles. Measured fasting at every visit following intake of BG for three consecutive days. Overall difference between interventions was tested with Friedman's ANOVA. Pairwise comparisons were investigated with Wilcoxon Signed Rank Test. Values are expressed as median and IQR.
** Different from baseline (P < 0.005)

TC was significantly reduced in all the HDL subclasses (Figure 9). In the XL-HDL subclass, TC was reduced after intake of 0.5 g 3.5 g and 8 g BG compared to baseline (P = 0.01, 0.01 and 0.003, respectively). TC was also significantly reduced after intake of 0.5 g and 8 g BG meal compared to baseline in both the L-HDL and M-HDL subclasses (P = 0.006 and 0.02, and P = 0.02 and 0.002, respectively). In addition, TC was significantly reduced after intake of 8 g compared to the 3.5 g BG test meal in the M-HDL subclass (P = 0.02). In the smallest HDL subclass however, there was a significant increase in TC after consumption of 3.5 g BG compared to baseline (P = 0.01). However, there was a reduction in TC after consumption of 8 g BG compared to intake of 0.5 g and 3.5 g BG in the S-HDL subclass (P = 0.02 and 0.008, respectively).

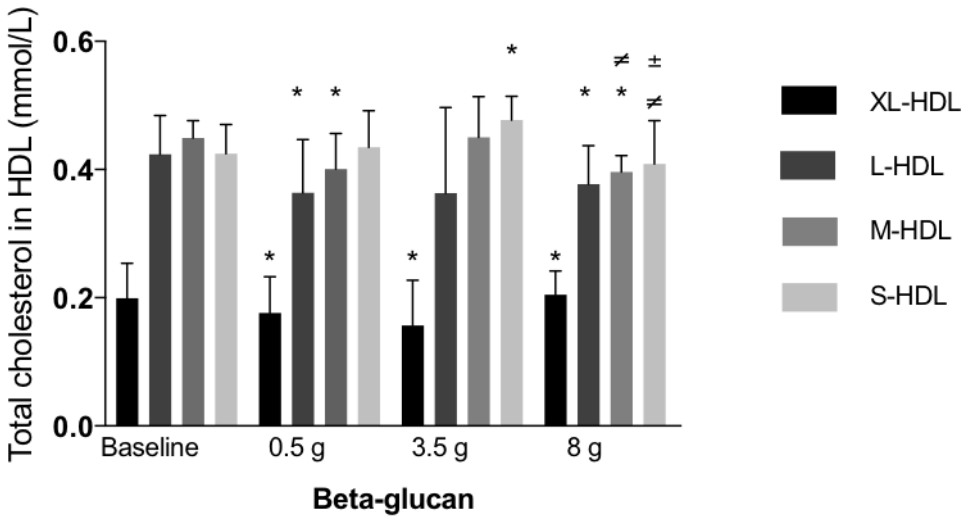

Figure 9. TC in XL, L, M and S-HDL (mmol/L). Measured fasting at every visit following intake of BG for three consecutive days. Overall difference between interventions was tested with Friedman's ANOVA. Pairwise comparisons were investigated with Wilcoxon Signed Rank Test. Values are expressed as median and interquartile range IQR.
* Different from baseline (P < 0.05)
≠ Different from 0.5 g beta-glucan (P < 0.05)
± Different from 3.5 g beta-glucan (P < 0.05)

Remnant cholesterol was significantly increased after intake of 3.5 g BG compared to baseline (P = 0.009). However, after intake of 8 g BG there was a significant reduction in remnant cholesterol compared to intake of 3.5 g BG (P = 0.03) (Figure 10).

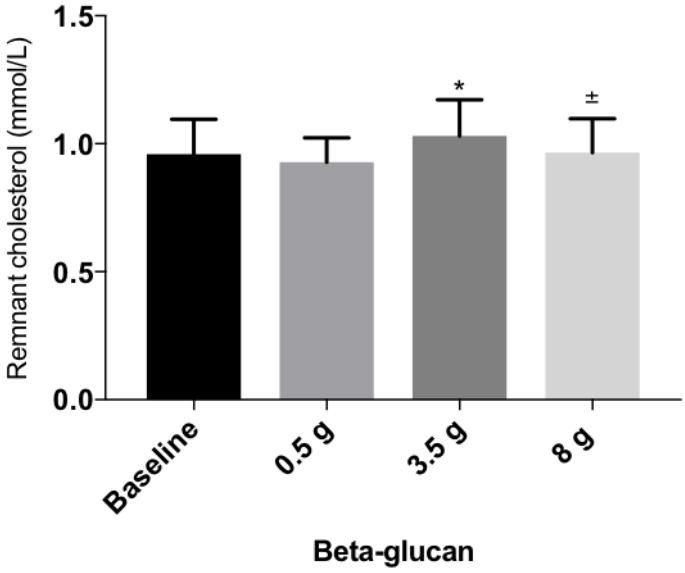

Figure 10. Remnant cholesterol particles (mmol/L) after intake of different amounts of BG. Measured fasting at every visit. Overall difference between interventions was tested with Friedman's ANOVA. Pairwise comparisons were investigated with Wilcoxon Signed Rank Test. Values are expressed as median and IQR.
* Different from baseline (P = 0.009)
± Different from 3.5 g beta-glucan (P = 0.03)

The mean diameter of the LDL particle was significantly reduced after intake of 3.5 g BG compared to baseline (p = 0.001) (Table 7), but not in any of the other lipoprotein subclasses (Appendix).

Table 7. Mean diameter for LDL particles

Intervention	LDL-D
	Median (25 – 75 percentile)
Baseline	23.6 (23.54 -23.59)
0.5 g beta-glucan	23.5 (23.52 – 23.59)
3.5 g beta-glucan	23.5 (23.48 – 23.61) **
8 g beta-glucan	23.5 (23.51 – 23.60)

** Different from baseline (p = 0.001)

4.0 Discussion

In this single-blinded crossover study, we investigated the effect of different amounts of BG for three consecutive days on lipid metabolism in relation to gut microbiota in healthy individuals.

We discovered that a short-term intake of BG did not significantly reduce total fasting cholesterol or fasting TG compared with baseline. However, postprandial TG response (iAUC 0-120 min) after an OGTT was significantly reduced compared with baseline after intake of 8 g BG. Furthermore, there was an increase in fasting breath H_2 excretion and plasma acetate and butyrate after intake of all the different amounts of BG, indicating increased activity by gut microbiota. In addition, a significant reduction in particle concentration in all of the HDL subclasses, with the exception of the smallest subclass was observed. TC was significantly reduced after intake of different amounts of BG in all of the HDL subclasses, compared with baseline. TG content in the XL-HDL particle was reduced after intake of 0.5 g BG.

To our knowledge, there are no other studies, which have investigated the effect on fasting lipids and lipoprotein subclasses after intake of different amounts of BG for a short three-day intervention period. Most studies investigating the lipid lowering effects of dietary fiber are conducted in periods of several weeks.

The present study detected no significant changes in fasting total cholesterol levels. This contradicts the findings of previous studies where consumption of BG has been shown to lower both TC and LDL-cholesterol. A review by Othman and coworkers investigated results from studies conducted in the last 13 years and concluded that intake of ≥3 g BG/day may reduce plasma TC and LDL-cholesterol levels by 5-10 % in normocholesterolemic and hypercholesterolemic subjects (Othman et al., 2011). These findings are also supported by other meta-analyses of randomized control trials (RCT) investigating effects of BG from oat and barley on plasma lipids. These concluded that BG consumption reduced LDL-cholesterol and TC compared to control, but no effect was seen on HDL (AbuMweis et al., 2010; Talati et al., 2009; Whitehead et al., 2014).

The reason for lack of effects observed on TC and LDL-cholesterol in the present study is most likely due to the short intervention period, based on previous studies it is plausible to

assume that if the intervention period had been extended, plasma TC and LDL-cholesterol levels might have been reduced. It usually takes minimum two weeks to achieve a cholesterol lowering effect with intake of dietary fiber due to the relatively long half-life of lipoproteins, such as LDL (Whitehead et al., 2014). Furthermore, the present study included healthy individuals with normal lipid values, which makes it difficult to demonstrate changes in TC and LDL-cholesterol.

We did not detect any changes in fasting plasma TG levels following three days of intervention with BG. These findings are consistent with the findings of a meta-analysis of 28 RCT investigating effects of oat BG on blood lipids (Whitehead et al., 2014). However, a systematic review of 8 RCTs detected a small TG lowering effect after intake of BG derived from barley (Talati et al., 2009). The concentration of beta-glucan in barley and oats is similar, hence it is plausible to assume that a similar reduction could be achieved with beta-glucan derived from oats. Beneficial effects on TG levels were also discovered in a meta-analysis of RCT conducted by Hollænder et al. (2015). They discovered that whole grain foods tended to lower plasma TG compared with control in healthy adults (Hollænder et al., 2015). The results from this study may indicate that other dietary fibers have beneficial effects on lipoprotein metabolism in healthy individuals. Perhaps changes in fasting plasma TG levels could have been detected in the present study if the intervention period was longer. Lack of effect might also be due to the healthy participants with normal lipid values.

In the present study, we detected a reduction in postprandial TG response after a glucose challenge following intake of 8 g BG compared with baseline. There is accumulating evidence showing a strong correlation between elevated postprandial TG levels and CVD risk (Dallinga-Thie, Kroon, Borén, & Chapman, 2016), which is why we consider these findings to be relevant. Our results are also similar to a sub-cohort analysis by Hitze and colleagues (2008) who discovered an inverse association between intake of total dietary fiber in the habitual diet and postprandial TG response. In the study by Hitze et al, the participants were given a fluid meal containing 29.6 % carbohydrate, 11.9% protein and 51.6% fat, after a 12 hour fast. Blood samples were then drawn between 0.5 hours up until 9 hours after consumption of the fluid meal to assess postprandial lipid metabolism (Hitze et al., 2008). These methods are similar to ours but are not fully comparable due to different intervention periods and different macronutrient content in the oral tests administered.

Participants in the study by Hitze et al. (2008) were middle-aged men who were all mildly overweight (mean BMI 27.3 ± 0.4) in contrary to the participants in the present study who were all healthy, normal-weight and mostly consisted of women. Taken together, this indicates that reduction in postprandial TG levels after intake of different dietary fibers can be achieved within different populations and that dietary fiber also exerts beneficial effects on TG response in healthy individuals.

Previous studies have reported that dietary fiber also elicit beneficial effects on TG response following a fat load intake. In a study by Tiihonen and colleagues (2015) they investigated effects of polydextrose, a soluble fiber that elicits similar effects as BG, on postprandial TG response induced by a high fat meal. Intake of polydextrose resulted in lower values for the postprandial TG response in healthy normolipidemic patients (Nilsson, Johansson-Boll, & Björck, 2015; Tiihonen et al., 2015). These results are similar to ours, suggesting that intake of dietary fiber may reduce the postprandial response of both lipids and glucose in healthy individuals.

The observed reduction in postprandial TG response in the present study might be due to several different mechanisms induced by dietary fiber, such as reduced glycemic response. In our intervention, a previous master student discovered that BG reduced the glycemic response and thereby insulin secretion (data not shown), which may have led to reduced TG synthesis in the liver and subsequently reduced TG response after the OGTT.

Other factors, such as SCFA produced during colonic fermentation, might affect postprandial TG response. In the present study we also observed an increase in plasma concentrations of acetate and butyrate after intake of BG. A study conducted by Tiihonen and colleagues (2015) suggests that intake of soluble fiber may contribute to a lower postprandial TG response by generation of acetate, propionate and butyrate through fermentation, which may modulate lipid metabolism. It is suggested that these can regulate energy metabolism and modify lipid digestion and absorption, possibly via G protein-coupled receptors. Taken together, it is possible that SCFA derived from colonic fermentation elicit beneficial effects on postprandial TG response, but we do not yet fully understand the mechanisms behind these effects (Canfora, Jocken, & Blaak, 2015; den Besten et al., 2013; Tiihonen et al., 2015).

SCFA may also affect cholesterol metabolism. SCFA have been shown to reduce plasma concentrations of cholesterol in rodents and humans (den Besten et al., 2013). In a study examining the effect of dietary acetic acid (acetate) on serum lipid values in rats fed a diet containing 1% cholesterol, a diet containing no cholesterol, or a diet containing 1% cholesterol with 0·3% acetic acid, the authors discovered that a diet containing acetate resulted in significantly lower values of serum TC and TG (Fushimi et al., 2006). This could imply that the reduction in postprandial TG response observed in the present study might also be linked to an increased concentration of acetate, induced by intake of BG or other mechanisms related to metabolites produced from colonic fermentation.

In addition, acetate has been reported to increase levels of the enzyme cholesterol 7α-hydroxylase (CYP7A1), which is involved in the conversion of cholesterol to bile acids and thus leading to increased excretion of cholesterol (den Besten et al., 2013; Fushimi et al., 2006). Even though we could not detect any changes in fasting serum TC in the present study, based on results from previous studies it is tempting to assume that the increased acetate concentration could have resulted in a reduction in serum TC values, if the intervention period in the study had been extended.

We discovered a dose-response relationship between intake of beta-glucan and breath H_2 excretion. H_2 is produced during colonic fermentation of dietary fiber. It is excreted in breath and is therefore frequently used as a marker for this process (Behall, Scholfield, van der Sluijs, & Hallfrisch, 1998). All the test meals significantly increased breath H_2 excretion compared with baseline and indicates increased amount of H_2 -producing bacteria in the gut after intake of increasing amounts of BG. These changes were also reflected in the increased plasma concentration of acetate and butyrate, which are also markers of colonic fermentation. This is in accordance with a previous study conducted by Nilsson et al. demonstrating increased gut-fermentation activity measured as breath H_2 excretion and SCFA concentration, after intake of barley-kernel based bread for three consecutive days compared to white wheat bread (Nilsson et al., 2015). In sum, increased breath H_2 excretion in addition to increased concentration of acetate and butyrate indicates increased activity among the gut microbiota, but if these metabolites are responsible for the effects we observed on different lipoproteins is currently uncertain.

In our study, no changes in fasting serum cholesterol levels were observed. However, significant reductions in particle concentrations and the concentrations of TC and TG in several of the lipoprotein subclasses were detected using NMR spectroscopy. Surprisingly, we detected a reduction in HDL particle concentration and reduced TC and TG content in the HDL subclasses. Previous studies have mainly discovered reduction in LDL-cholesterol following intake of BG and other soluble fibers but have found no effect on serum HDL-cholesterol levels (AbuMweis et al., 2010; Talati et al., 2009; Whitehead et al., 2014). However, these studies did not investigate effects on lipoprotein subclasses. Furthermore, the reduction we discovered in particle concentration and TC and TG in HDL are only a brief reflection of lipoprotein metabolism and the changes might have been temporary. The results from the present study demonstrate that even though reduction in serum TC values cannot be detected after a three-day intervention period, small significant changes in lipoprotein subclasses can still be discovered when lipids are measured with NMR spectroscopy. These changes could consequently lead to changes in lipoprotein metabolism and eventually a reduction in fasting serum cholesterol.

The reduction in HDL particle concentration in the XL, L and M subclasses in the present study could possibly be a result of decreased cholesterol in the cells and subsequently less cholesterol to be transported to the liver in HDL, and thus less particles. This is a probable scenario as we did not detect an increase in any of the other lipoprotein subclasses. This is also reflected by the reduced concentration of cholesterol in all the HDL subclasses, with the exception of the smallest subclass. The smallest HDL particle is cholesterol-depleted after delivering cholesterol to the liver or transferring it to TRL and thus ready to accept further cholesterol from cells (Frayn, 2010). Most of the reduction in cholesterol in HDL was observed after intake of 8 g BG. After intake of 3.5 g BG, CE and TC increased in the smallest HDL subclass but was reduced after intake of 8 g BG. Perhaps this could imply that 3.5 g is an ideal dose of BG intake and that the increase in TC and CE reflects an enhanced ability to accept cholesterol from peripheral tissues in response to this dose, resulting in improved reverse cholesterol transport. A meta-analysis by Whitehead et al also suggests that a minimum dose of 3 g BG is enough to lower cholesterol, and that consuming more may not have any additional effect (Whitehead et al., 2014). All in all, intake of 3 g of BG per day may eventually result in lower cholesterol values and is feasible for most people to incorporate into the habitual diet.

Excess cholesterol is, as mentioned previously, transported from cells to the liver by HDL where it can be excreted as cholesterol or as bile salts in the bile, the process which is formally known as reverse cholesterol transport (Frayn, 2010). It is well known that BG inhibits reabsorption of bile and thus more cholesterol is excreted, this mechanism could possibly have resulted in less cholesterol for HDL to gather from peripheral tissues and thus contributed to improved reverse cholesterol transport in the present study. This mechanism has also been suggested by the authors of an RCT investigating effects of fish oil on lipoprotein subclasses, they hypothesized that the lack of observed increase in HDL-cholesterol could indicate improved reverse cholesterol transport (Amanda Rundblad, Holven, Ottestad, Myhrstad, & Ulven, 2017). Perhaps this could also be linked to the increased production of acetate observed in the present study, possibly contributing to increased cholesterol excretion via CYP7A1, as previously mentioned. Previous studies have reported that cholesterol efflux capacity (CEC); the ability of HDL to accept cholesterol from macrophages and a key step in reverse cholesterol transport, is inversely associated with cardiovascular risk independent of HDL concentration (Qiu, Zhao, Zhou, & Zhang, 2017). Based on this, our results may indicate that a diet rich in beta-glucan may have other antiatherogenic properties than lowering LDL-cholesterol.

Changes in HDL subclasses have also been reported after intake of a diet consisting of wholegrain, fish and bilberries in a randomized trial by Lankinen and coworkers (2014). Their results suggest that a diet rich in wholegrain, bilberries and especially fatty fish causes changes in the distribution of HDL subclasses toward larger particles. The authors suggest that the changes may be attributed to protective functions of HDL such as reverse cholesterol transport (Lankinen, 2014), which is in accordance with our suspicion of increased excretion of cholesterol. Even though our intervention and results were slightly different from those discovered by Lankinen and coworkers, the fact that other studies have also detected changes in HDL subclasses implies that dietary interventions including dietary fiber may have beneficial effects on HDL and lipid metabolism in healthy individuals.

After consumption of 8 g BG we observed a significant reduction in fasting remnant cholesterol compared with 3.5 g BG. Remnant cholesterol mainly consists of VLDL and IDL, which are relatively rich in TG, so it may seem like a higher dose of BG has a lowering effect on TG levels. These effects can be regarded as beneficial because elevated remnant cholesterol is a known contributor to increased risk of developing atherosclerosis and

ischemic heart disease (Varbo & Nordestgaard, 2014). Based on these findings it may look as if BG also reduces remnant cholesterol, thereby contributing to a reduced risk of atherosclerosis and, in turn, CVD.

We discovered a small significant decrease in LDL particle diameter after intake of 3.5 g BG. Although it is unfavorable to have smaller denser LDL particles, the reduction in LDL diameter in the present study was very small and the median diameter was within normal range. Therefore, it is unlikely that this small reduction in LDL diameter has a negative impact on CVD risk in the participants in our study. This small reduction could possibly be a reflection of the increased cholesterol in the S-HDL subclass after intake of 3.5 g BG and changes in cholesterol distribution between lipoprotein particles. Consequently, this could possibly lead to a reduction in cholesterol as a result of increased excretion via the liver due to improved reverse cholesterol transport.

Further, the test meal with the lowest amount of BG (0.5 g) contained inulin. Inulin is a soluble fiber which is also fermented by the gut microbiota, resulting in increased production of beneficial SCFA and H^2 (Carlson, Erickson, Hess, Gould, & Slavin, 2017; Roberfroid, 1993) . There are also other factors in the diet that may be subjected to colonic fermentation and could have affected the function and composition of the gut microbiota in the subjects' in the present study, such as high fat diets, antibiotics, weight and age. In addition, whole grain foods are rich in other potentially bioactive phytochemicals such as plant sterols, vitamins, minerals and antioxidants. Consequently, the mechanisms for the beneficial effects of whole grain diets may be multifactorial and remain to be elucidated (Nilsson et al., 2010).

The reduction we observed in TG in the XL-HDL subclass after intake of 0.5 g BG could possibly be attributed to inulin, because inulin was used to replace BG in the 0.5 g test meal. Further, the reduction in TG in HDL could possibly be explained by reduced TG synthesis in the liver as a result of reduced glycemic response and reduced insulin secretion. This is supported by observations made on insulin in the same intervention. After BG intake, there was a reduction in fasting insulin concentration and increased insulin sensitivity (unpublished data). Overall, it may seem like different soluble fibers exert beneficial effects on TG and cholesterol values and that they may affect different lipoproteins, possibly because they might work through different mechanisms. It is however important to note that these are

speculations, and we do not fully understand the biological mechanisms which have led to our results.

4.1 Strengths and limitations in the study

The study was s single blinded fixed-order crossover study. The participants were given increasing amounts of BG to avoid carryover effect from the previous intervention. In addition, the two-week washout period was most likely sufficient in avoiding carryover effect. The crossover design is a strength in the study, reducing between-subject variability as they act as their own control. In addition, the same statistical power may be obtained with a crossover design with fewer participants in comparison with a parallel design (Louis, Lavori, Bailar, & Polansky, 1984). The blinding secured for systematic bias, as the participants were not aware of the amount of BG consumed.

The present study has certain limitations. The participants were mainly recruited from the student mass and employees at the faculty of health sciences at OsloMet and mostly consisted of women. One can assume that these participants are more interested in healthy eating than people in average and that these factors may threaten the generalizability and the external validity of the study. The fact that this population may be healthier than average could have been the reason for lack of effect on other variables measured. The small sampling size (n = 14) is also a limitation in this study. Power calculation was initially performed and revealed that a number of 13-17 participants were required to demonstrate effects. However, these calculations were based on glucose as the primary endpoint, not lipids. Due to this, the study might be underpowered. An increase in the number of participants may have led to significant changes in some of the other parameters which were measured, such as serum TC or LDL-cholesterol.

Nevertheless, NMR spectroscopy allows for a detailed study of the lipoprotein subclasses, which gave us to the opportunity to detect changes within the lipoprotein subclasses that we would not have been able to detect without using this method.

The uneven distribution of gender may also have affected the results in this study. In the current study there were twelve women and two men and it is of interest to note that women generally have higher concentrations of HDL than men and also a greater ability to remove postprandial lipids from circulation (Bloomer & Lee, 2013). Whether the effect observed on

HDL in the present study would still be visible with a more even distribution of gender is unknown, and it may be probable to think that if more men were included in the study our results could have been different.

Further, the present study was not a fully controlled dietary intervention and the diet and nutrients may have differed between subjects. In addition, the test meals were consumed in the participants' home, not allowing for controlling compliance. The participants were restricted from BG intake, but they may have consumed dietary fiber from other sources than cereals, which could have affected fermentation and the composition of the microbiota. However, the participants filled out a CRF to secure that they followed the dietary restrictions and that the test meals were consumed as requested. This, in addition to the increased breath H_2 excretion and the increased concentration of acetate and butyrate indicates that the test meals were consumed as instructed.

In regard to the test meals, the energy content was similar, but the macronutrient content differed slightly. The test meal with the highest amount of BG also contained the highest amount of protein. Dietary protein is also fermented by the gut microbiota, resulting in production of metabolites such as SCFA. Dietary protein may consequently have contributed to the generation of SCFA and H_2. However, the availability of fermentable carbohydrates in the gut seems to suppress protein fermentation, so it is probably unlikely that this has affected our results (Rist, Weiss, Eklund, & Mosenthin, 2013).

5.0 Conclusion

A short-term intake of BG can reduce postprandial TG response and modulate lipoprotein metabolism in healthy individuals. The effect observed on HDL particle concentration and cholesterol content after intake of BG may indicate improved reverse cholesterol transport. We cannot rule out that other soluble fibers present in the test meals such as inulin, may also elicit beneficial effects on lipoprotein metabolism. Whether the observed effects of BG are related to colonic fermentation is currently uncertain and needs to be further investigated.

5.1 Future perspectives

To further explore the metabolic changes after intake of BG, it would be interesting to investigate expression of genes related to lipoprotein metabolism after BG consumption using peripheral blood mononuclear cells (PBMC). Investigating the PBMC gene expression may reveal possible changes in genes related to regulation of lipid metabolism. Gene expression of enzymes involved in lipid regulation would also give us a more detailed image of the underlying mechanisms behind the changes in lipoprotein subclasses. In the present study, we only investigated effects on fasting SCFA, breath H^2 and lipoprotein values. It would be interesting to further investigate the postprandial response of these parameters at different times after the OGTT. Further, apolipoproteins were not measured. It would have been interesting to measure apolipoproteins as these may provide a better understanding of the distribution of the lipoproteins.

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Appendix: Table of lipoprotein particle concentration, lipid constituents within the lipoproteins and mean diameter of the lipoproteins

Particle concentration	Baseline	0.5 g	3.5 g	8 g	P-value*
XXL-VLDL	7.00E-11	4.43E-11	6.53E-11	6.84E-11	0.18
XL-VLDL	2.21E-10	5.78E-11	2.53E-10	2.58E-10	0.20
L-VLDL	1.52E-09	1.63E-09	2.02E-09	1.99E-09	0.35
M-VLDL	8.63E-09	8.64E-09	9.78E-09	1.06E-08	0.28
S-VLDL	1.68E-08	1.82E-08	1.84E-08	1.78E-08	0.41
XS-VLDL	2.61E-08	2.61E-08	2.69E-08	2.59E-08	0.36
XL-HDL	4.73E-07	4.33E-07	4.14E-07	4.63E-07	***0.02***
L-HDL	1.41E-06	1.20E-06	1.22E-06	1.22E-06	***0.01***
M-HDL	2.16E-06	1.91E-06	2.09E-06	1.87E-06	***0.00***
S-HDL	4.78E-06	4.66E-06	4.96E-06	4.43E-06	0.10
L-LDL	1.40E-07	1.37E-07	1.55E-07	1.41E-07	0.12
M-LDL	1.11E-07	1.09E-07	1.25E-07	1.16E-07	0.08
S-LDL	1.30E-07	1.27E-07	1.43E-07	1.35E-07	0.10
IDL	8.59E-08	8.40E-08	9.31E-08	8.39E-08	0.17
Total cholesterol	Baseline	0.5 g	3.5 g	8 g	
XXL-VLDL	1.89E-03	9.15E-04	1.61E-03	2.04E-03	0.24
XL-VLDL	3.87E-03	8.80E-04	3.43E-03	4.61E-03	0.36
L-VLDL	1.70E-02	1.49E-02	1.84E-02	2.29E-02	0.48
M-VLDL	8.46E-02	7.74E-02	8.42E-02	9.19E-02	0.17
S-VLDL	1.23E-01	1.15E-01	1.33E-01	1.20E-01	0.12
XS-VLDL	1.63E-01	1.62E-01	1.64E-01	1.56E-01	0.60
XL-HDL	1.99E-01	1.76E-01	1.57E-01	2.05E-01	***0.03***
L-HDL	4.24E-01	3.64E-01	3.64E-01	3.77E-01	***0.01***
M-HDL	4.49E-01	4.01E-01	4.50E-01	3.96E-01	***0.01***
S-HDL	4.25E-01	4.35E-01	4.77E-01	4.09E-01	***0.01***
L-LDL	6.69E-01	6.64E-01	7.56E-01	6.93E-01	0.16
M-LDL	3.79E-01	3.71E-01	4.34E-01	3.99E-01	0.75
S-LDL	2.22E-01	2.21E-01	2.55E-01	2.39E-01	0.11
Free cholesterol	Baseline	0.5 g	3.5 g	8 g	
XXL-VLDL	6.60E-04	1.75E-04	5.20E-04	6.67E-04	0.08
XL-VLDL	1.54E-03	3.85E-04	1.42E-03	1.84E-03	0.06
L-VLDL	6.38E-03	4.53E-03	7.81E-03	8.30E-03	0.11
M-VLDL	2.76E-02	2.83E-02	3.21E-02	3.53E-02	0.26
S-VLDL	4.06E-02	4.64E-02	4.68E-02	4.27E-02	0.17
XS-VLDL	4.83E-02	4.80E-02	5.19E-02	4.66E-02	0.45
XL-HDL	6.19E-02	5.81E-02	5.17E-02	6.11E-02	***0.01***
L-HDL	9.13E-02	7.99E-02	8.02E-02	8.24E-02	***0.04***
M-HDL	8.19E-02	6.86E-02	8.08E-02	6.71E-02	***0.00***
S-HDL	1.02E-01	1.00E-01	1.03E-01	9.39E-02	0.07
L-LDL	2.00E-01	1.99E-01	2.21E-01	2.03E-01	0.19
M-LDL	1.14E-01	1.12E-01	1.24E-01	1.18E-01	0.25
S-LDL	6.69E-02	6.51E-02	7.25E-02	6.99E-02	0.07

Cholesteryl esters	Baseline	0.5 g	3.5 g	8 g	
XXL-VLDL	1.23E-03	6.92E-04	8.75E-04	1.35E-03	0.43
XL-VLDL	2.09E-03	7.07E-04	2.13E-03	2.70E-03	0.65
L-VLDL	1.11E-02	9.51E-03	1.31E-02	1.47E-02	0.76
M-VLDL	5.56E-02	4.68E-02	5.45E-02	5.95E-02	0.09
S-VLDL	7.30E-02	6.76E-02	8.71E-02	7.75E-02	***0.04***
XS-VLDL	1.15E-01	1.12E-01	1.16E-01	1.11E-01	0.42
XL-HDL	1.38E-01	1.24E-01	1.11E-01	1.47E-01	0.06
L-HDL	3.33E-01	2.84E-01	2.84E-01	2.95E-01	***0.01***
M-HDL	3.66E-01	3.34E-01	3.70E-01	3.27E-01	***0.01***
S-HDL	3.21E-01	3.37E-01	3.67E-01	3.16E-01	***0.01***
L-LDL	4.69E-01	4.64E-01	5.37E-01	4.89E-01	0.07
M-LDL	2.67E-01	2.61E-01	3.13E-01	2.79E-01	0.08
S-LDL	1.59E-01	1.56E-01	1.83E-01	1.67E-01	0.12
Triglycerides	Baseline	0.5 g	3.5 g	8 g	
XXL-VLDL	1.12E-02	7.61E-03	1.08E-02	1.14E-02	0.18
XL-VLDL	1.35E-02	3.58E-03	1.66E-02	1.60E-02	0.30
L-VLDL	5.76E-02	5.77E-02	7.47E-02	7.03E-02	0.50
M-VLDL	1.45E-01	1.46E-01	1.73E-01	1.86E-01	0.63
S-VLDL	1.24E-01	1.35E-01	1.48E-01	1.52E-01	0.51
XS-VLDL	6.21E-02	6.36E-02	6.45E-02	6.37E-02	0.51
XL-HDL	1.35E-02	1.03E-02	1.26E-02	1.26E-02	***0.04***
L-HDL	2.83E-02	2.32E-02	2.45E-02	2.27E-02	0.09
M-HDL	3.94E-02	3.56E-02	3.60E-02	3.65E-02	0.09
S-HDL	3.52E-02	3.51E-02	3.48E-02	3.91E-02	0.69
L-LDL	6.52E-02	6.46E-02	6.44E-02	6.56E-02	0.65
M-LDL	3.58E-02	3.51E-02	3.55E-02	3.43E-02	0.53
S-LDL	2.12E-02	2.04E-02	2.09E-02	2.03E-02	0.37
IDL	7.04E-02	7.17E-02	6.97E-02	7.53E-02	0.58
Diameter	Baseline	0.5 g	3.5 g	8 g	
VLDL	3.60E+01	3.57E+01	3.63E+01	3.63E+01	0.80
HDL	1.01E+01	1.00E+01	1.00E+01	1.01E+01	0.22
LDL	2.36E+01	2.35E+01	2.35E+01	2.36E+01	***0.00***
Remnant cholesterol	Baseline	0.5 g	3.5 g	8 g	
	9.59E-01	9.28E-01	1.03E+00	9.66E-01	***0.02***
* Friedman's ANOVA					