GUT MICROBIOME AND GENETIC HETEROGENEITY IN THE IMPACT OF DIETARY FAT CONSUMPTION FROM DIFFERENT SOURCES ON CHOLESTEROL METABOLISM IN HUMANS

by

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ABSTRACT

The extent to which consumption of dairy products influences the risk of cardiovascular disease (CVD) is not resolved and remains as a challenging area of research. In a multicenter randomized crossover trial, participants consumed five isoenergetic diets, including cheese, butter, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) and low-fat carbohydrate (CHO) for 4-weeks. First, the effect of dairy fat on gut microbiome and the potential link with CVD risk was examined. Post-intervention stool samples were analysed for changes in gut microbiome. Significant differences in phylum level were observed for Firmicutes between PUFA (74.40 ± 3.81) vs. CHO (65.90 ± 3.48), and for Verrucomicrobia between cheese (0.10 ± 1.52) vs. butter (4.64 ± 1.52) containing diets. Further, the participants were categorized into two groups, overweight (OW) and obese (OB); no effects were observed in the OW group with respect to β-diversity whereas significant differences were observed between PUFA vs. MUFA ($p=0.007$), PUFA vs. butter ($p=0.039$), PUFA vs. CHO ($p=0.018$), PUFA vs. cheese ($p=0.002$), MUFA vs. CHO ($p=0.004$), MUFA vs. cheese ($p=0.003$), butter vs. CHO ($p=0.014$) and butter vs. cheese ($p=0.002$) in the OB group. Second, the genetic associations with selected single nucleotide polymorphisms (SNPs) were investigated. Few SNPs show genotype-diet interaction on serum lipids. In addition, partial least squares (PLS) analysis for DF (cheese + butter) vs. UFA (MUFA + PUFA) suggested that combinations of various SNPs explain the variance in HDL-C, LDL-C, total cholesterol and triglyceride levels.

Overall, this study indicated that the dietary fat from various sources impact the gut microbial β-diversity, and BMI exerts major influences in altering gut microbiome. Predictive analysis showed that a group of SNPs potentially provide opportunities for

personalized dietary recommendations partly based on their genetic characteristics, in order to lower their CVD risk.

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DEDICATION

To my amma, appa, wife, sisters and brothers, and all my nephew niece and cousins……

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ABBREVIATIONS

APO B	Apolipoprotein B
APOE	Apolipoprotein E
ABCA1	ATP-binding cassette subfamily A, member1
ABCA5	ATP-binding cassette subfamily A, member 5
ABCG5	ATP-binding cassette subfamily G, member 5
BMI	Body mass index
CHO	Carbohydrates
CVD	Cardiovascular disease
CYP7A1	Cholesterol 7α-hydroxylase
CVD	Cardiovascular disease
DHCR7	7-dehydocholesterol reductase
DNA	Deoxyribonucleic acid
HDL-C	High-density lipoprotein cholesterol
INAF	Institute of Nutrition and Functional Foods
LDL-C	Low-density lipoprotein cholesterol
LDL-R	LDL receptor
LIPA	Lipase A, lysosomal acid type
LIPC	Lipase C, hepatic type
LIPG	Lipase G, endothelial type
LPL	Lipoprotein lipase
MTTP	Microsomal triglyceride transfer protein

MCFA	Medium chain fatty acid
NPC1L1	Niemann-Pick C1 like intracellular cholesterol transporter 1
PPARA	Peroxisome proliferator activated receptor alpha
PCSK9	Proprotein convertase subtilisin/kexin type 9
PUFA	Polyunsaturated fatty acid
RBC	Red blood cells
RCFFN	Richardson Centre for Functional Foods and Nutraceuticals
SCAP	Sterol regulatory element binding transcription factor chaperone
SFA	Saturated fat acids
SNP	Single nucleotide polymorphism
SREBF	Sterol regulatory element binding transcription factor 2
TC	Total cholesterol
TG	Triglyceride

CHAPTER I

GENERAL INTRODUCTION

1.1 INTRODUCTION

Current evidence suggests high-fat diets are associated with metabolic disorders, including obesity, cardiovascular disease (CVD), high blood pressure and diabetes (1). High-fat intake is predominant in the Western diet and the type and quality of dietary fats have a significant effect on the progression and expansion of CVD (2). Thus, the American Heart Association recommends diets with low saturated fat acids (SFA) intake and high intake of vegetables, fruits and whole grains to maintain cardiovascular health. CVD risks have been associated with circulating lipoprotein levels, including low high-density lipoprotein cholesterol (HDL-C) and high low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC) and triglycerides (TG). Moreover, low-grade systemic inflammation (3, 4), dyslipidemia and dysfunctional lipid metabolism are also indicated as critical mediators of CVD progression. First, results from a large clinical study, the lipid research clinics coronary primary prevention trial, indicated that CVD mortality and morbidity incidence was lower with a reduced level of TC and LDL-C in blood (5). Further, 1-2% of CVD risk reduction was observed with 1% of the LDL-C reduction, and this was observed by several studies conducted with a lipid-lowering agent (6, 7). Increased concentrations of TC and LDL-C present in the circulation is an indicator of the CVD events and these circulatory lipids are the primary targets for the CVD prevention therapy.

Cardiovascular health depends not only on dietary fats but also other factors, such as genetics, diet, lifestyle and other diseases that may have a continuous influence on disease progression and mortality (8). As of now, evidence is pointing to gut microbiome and host genetics as

being associated with cardiovascular health (9). The gut microbiome is one of the emerging underestimated factors, and has been associated with numerous diseases including CVD (10, 11). Circulating lipids, on the other hand, are well established markers for CVD related events. Common genetic variants in the human population are linked to modifications in cardiometabolic risk factors (12). However, the effect of dietary fat intake on the cardiometabolic risk parameters remains unclear. Thus, considering the role of common genetic variants and gut microbiome as potential factors impacting cardiovascular health may assist in understanding the linkage to dietary fat consumption from various sources.

1.2 DIETARY FATS AND CARDIOVASCULAR HEALTH

1.2.1 DIETARY SATURATED FATS

SFAs were the prevalent fats in the Western diet, and they have gradually been replaced by PUFA due to the industrialization of vegetable oils (13). Dairy products containing milk fats are rich in SFA; dairy products are the main SFA source for US population, and they remain highly controversial in the research area of dairy intake and cardiovascular health. Evidence suggests that CVD risk is associated with an increased level of LDL-C which is increased upon SFA consumption (14, 15). The World Health Organization (WHO) recommends people to get <10% of their total energy from SFA, and the 2010 dietary guidelines for Americans from the American Heart Association (16) recommends consuming <7% of one's energy from SFA to reduce CVD risk. In contrast, consumption of milk and dairy products have been associated with increased levels of HDL-C (14, 17) and reductions in blood pressure (18, 19), both linked to lowered CVD risk. High milk intake, (one pint or more per day) has also been shown to reduced CVD risk (20-22).

Long-chain SFA (>12 C) induce pro-inflammatory signals and increase the level of circulating pro-inflammatory markers, which are associated with CVD risk events (23, 24). Evidence suggests that the long chain SFA, such as myristic acid and palmitic acid, might induce apoptosis through nuclear factor κB (NFκB) in human coronary artery endothelial cells (25). Also, intercellular adhesion molecule-1 was up-regulated by stearic acid in human endothelial cell lines and pro-inflammatory responses via phosphorylation of NF-κB in a dose-dependent manner (26). However, short (C6:0) and medium (C12:0) SFA do not cause lipotoxicity. According to the published evidence, the role of SFA from dairy foods and related products in cardiovascular health is highly debatable, but at the same time consumption of larger amounts of milk reduced CVD risk, regardless of the component of the milk (27). A study showed no association between intakes of dairy, yoghurt, and cheese products and CVD risk (28). Also, results from a multi-ethnic study of atherosclerosis (MESA) showed that higher SFA consumption from dairy products reduces CVD risk by 29% (29). Furthermore, results from a meta-analysis showed that replacement of PUFA for SFA resulted in no change in CVD risk (30). In contrast, mortality of women with CVD in the Netherlands was associated with consumption of butter (31). Interestingly, cheese has no similar association with CVD as butter does (32), and other meta-analysis studies have suggested that dietary SFA replacement with PUFA (Linoleic acid) reduces the risk of CVD (33). At the same time, fermented milk foods and low-fat dairy foods have been shown to possess potential cardioprotective effects (27, 34). Dietary lowering of the level of HDL-C has not been considered a risk factor for cardiac events. However, various observational studies, controlled clinical trials and mechanistic studies strongly suggest that high concentrations of HDL-C in the circulatory system promotes cardiovascular health (35-37). Instead of overlooking diet effects on HDL-C,

the additional explanation was given by examining the ratio between TC and HDL-C. This TC: HDL-C ratio includes the amounts of cholesterol in the triacylglycerol-rich VLDL fraction, which also positively correlates with CVD risk. Hence, the TC: HDL-C ratio is considered a precise risk predictor for CVD events (38, 39). Further, Mensink et al., conducted a meta-analysis with 60 published studies between 1970 and 1998 examining the effects of the amount and type of fat on TC: HDL-C and levels of other lipids. This study has shown that SFA compared with carbohydrates increases plasma HDL-C concentrations in the same proportion as LDL-C (14), with no influence on the ratio of TC/HDL-C, another influential CVD risk factor (40). Moreover, Mensink *et al.* showed that myristic acid and palmitic acid intake raises both plasma LDL-C and HDL-C levels compared to carbohydrates intake, so their impact on the TC/HDL-C ratio is neutral. Stearic acid (18:0) intake compared to carbohydrates has a neutral effect on the ratio of TC/HDL in plasma (14). Also, consumption of lauric acid (12:0) dramatically increases TC compared to carbohydrates (14). Finally, SFA also raises plasma HDL-C concentrations compared to MUFA and PUFA (41).

In general, dietary SFA increases plasma LDL-C levels through down regulation of liver LDL-receptor and as well as modulation of sterol regulatory element-binding protein 2 (SREBP2)(42). The exact mechanism by which SFA regulates SREBP2 activity remains to be established. However, evidence concerning the influence of SFA on gene expression in humans is limited, and the interaction and physiological mechanism of SFA with lipid metabolism remains unclear. Also, a reverse cholesterol transport mechanism is the way in which peripheral lipids are entered and cleared in the liver and HDL-C levels play a crucial role in this process (43). HDL-C also possesses beneficial properties such as anti-

inflammatory, antithrombic and antioxidant effects, thereby exerting favorable impacts on the endothelium (43).

In summary, SFA such as lauric, myristic, and palmitic acids have been shown to increase the levels of LDL-C and HDL-C in the circulation. High intake of these fatty acids have been linked to increased risk for CVD and type 2 diabetes. Additionally, lower CVD risk has been associated with dairy-derived medium chain fatty acids such as pentadecanoic and heptadecanoic acid (17). The effect of SFA from dairy foods on cardiovascular health is unclear, but at the same time, not all SFA consumption is linked to increased CVD risk because different types of SFA have different impact on various cells. Most studies have attributed the connection between dairy SFA intake and CVD risk, which continues to represent the leading CVD risk worldwide. Besides a wide range of diverse nutritional compositions between different dairy products, other factors such as genetics and lifestyle have a major influence on CVD risk events (27, 30, 31). Thus, a specific focus on fatty acids and their relation to cardiovascular health may be more important rather than a focus on general statements such as "effect of SFA consumption of on cardiovascular health". Most of the previous research has not considered the concept of diet-host genetic interactions that can promote cardiovascular health.

1.2.2 DIETARY MONOUNSATURATED FATTY ACID

The mediterranean diet contains a high level of olive oil which is rich in MUFA. Olive oil is rich in oleic acid and plant sterols; however, the use of olive oil is not that common in the Western diet. A few decades ago, MUFA-rich canola oil consumption is increased in the USA (44). MUFA has a higher melting point than PUFA, as MUFA contains one double bond

whereas PUFA possesses two or more double bonds. Structurally, oleic acid and palmitoleic acid are cis-isomers of MUFA. A diet rich in MUFA contains a high level of oleic acid, representing 92% of cis-MUFA (45). Published evidence shows that the antioxidant properties of oleic acid present in the olive oil may have a beneficial effect on blood lipid levels. Thus, olive oil consumption within the Mediterranean diet may partially explain the reductions in the incidence of CVD (46-48). Results from human clinical studies have shown that the effect of MUFA intake has either a neutral (49) or a hypotensive effect (50) when compared to a carbohydate-based diet. Olive oil, which contains high oleic acid, leads to significant reductions in total blood pressure, which has been observed in hypertensive patients who have consumed a diet rich in MUFA (51). Also, consumption of olive oil reduces inflammatory markers, enhances antioxidants in serum (47) and reduces NFκB mediated inflammatory response in peripheral blood mononuclear cells (47). However, the anti-inflammatory properties of oleic acid are modest on inflammatory processes, however polyphenolic compounds present in the olive oil also possess anti-inflammatory properties (52). In addition, there was no significant association with plasma phospholipid and MUFA or oleic acid on CVD risk (53, 54). In summary, dietary intake of MUFA showed improvement in cardiovascular events. Olive oil consumption is considered healthy as it is a component of the Mediterranean diet which is accepted as a healthy dietary pattern in terms of reductions in CVD risk. However, the whole intake of MUFA should be considered in relation to cardiovascular health benefits rather than focusing on oleic acid alone.

1.2.3 DIETARY POLYUNSATURATED FATTY ACIDS

PUFAs are present in vegetable and plant oils including corn, sunflower, and soybean which are rich in linoleic acid (LA), whereas alpha-linolenic acid (ALA) is found in green vegetables and oils including soybean, rapeseed, flaxseed, and fish oils (55-57). Dietary PUFA contains essential fatty acids, such as LA and ALA, which play an essential role in inflammation associated with CVD (13). Desaturase (Δ6) and elongase (Δ5) are the enzymes which play crucial role in the metabolism of these essential fatty acids. Activities of the enzymes, Δ6 and Δ5 desaturases are influenced by metabolic diseases such as diabetes mellitus, hypertension and hyperlipidemia (57, 58) *FADS1* and *FADS2* are the genes responsible for encoding Δ5 and Δ6 desaturase, respectively, in human (58) and they are involved in the conversion of LA and ALA into their long chain fatty acids. Hence, mammals have to depend on their diet for this long chain fatty acid synthesis (13). Cell membranes contain LA and ALA which are important energy sources (59) and studies have demonstrated that LA and ALA may alter cellular lipid metabolism (60, 61), intracellular signalling pathways (62), and cytokine secretion (63). After consumption of PUFA-rich diets, LA and ALA undergo several processes including generation of energy through beta-oxidation, energy storage as TG, incorporation into the structural component of the cell and production of long-chain PUFA via elongation and desaturation processes. Intake of PUFA, specifically LA, has distinctive effects on the size of lipoproteins, and an inverse association has been observed with LDL-C particle sizes, including LDL-C, small LDL-C, large VLDL-C and total LDL-C, while a positive association has been observed with large HDL-C levels (64, 65).

In general, evidence suggests that LA has a beneficial effect on circulating healthy lipoprotein profiles and a non-beneficial effect on plasma TG concentrations (66, 67). Further, LA

promotes hepatic clearance of LDL-C via reducing cholesterol generation and increasing LDL receptor expression (68). Additionally, LA also plays a significant role in hepatic cholesterol metabolism, increasing bile acid synthesis from cholesterol by inducing the rate-limiting enzyme 7 α-hydroxylase and farnesoid X receptor (LXR) (69). These are the primary proposed mechanisms by which a high intake of LA lowers circulating lipid levels. Thus, LA has a different impact on both circulating concentrations of HDL-C and LDL-C (70). The long-chain fatty acids such as EPA and DHA, which are derived from ALA, have an effect on the membrane-associated cellular signalling through transcription factors such as PPAR-α, - γ, NFκB and SREBP. The latter factors play a major role in controlling inflammation processes, fatty acid and TG metabolism, and differentiation of adipocytes (71).

In summary, LA has a significant impact on lowering LDL-C, and ALA-derived long-chain fatty acids influence inflammation and other transcription factors (60, 62). LA and ALA are the essential fatty acids that humans and other higher animals acquire from the diet. In general, PUFA-rich diets are neutral or beneficial with respect to CVD risk when compared to MUFA, but the impact of host genetics needs to be considered from a cardiovascular health perspective.

1.3 LIPID METABOLISM AND GENETIC DETERMINANTS

Lipid metabolism mainly consists of three pathways, including dietary cholesterol absorption, cholesterol biosynthesis and reverse transport of cholesterol. These pathways are interrelated and impact the genetic predisposition of an individual to have higher risk for CVD (72, 73). Triacylglycerol (TAG) is a main exogenous source of fat intake in Western diet. In the

digestive system, TAG is digested by pancreatic lipase into fatty acids and gets into the enterocyte and is esterified to cholesterol esters (CE) with the help of intestinal acyl-CoA: cholesterol acyl-transferase 2 (ACAT2). Finally, TAG is resynthesized in the endoplasmic reticulum and assembled with chylomicrons by the microsomal TAG transport protein (MTP). Moreover, cholesterol equilibrium is maintained by cholesterol intake (74), *de novo* synthesis and fecal excretion of cholesterol. Cholesterol absorption is complex, but a few sterol carriers are responsible for absorption of cholesterol, such as Niemann-Pick C1-like 1 (NPC1L1) and the ABC transporters, including ABCG5 and ABCG8. Further, ABC transporters, such as ABCG1 and ABCA1, are responsible for retaining peripheral cholesterol to the liver (75). The Generation of HDL-C in blood occurs through ABCA1 transporter which transfers cholesterol and phospholipid to apolipoprotein receptors (76). For cholesterol homeostasis, endogenous cholesterol synthesis contributes around 70% of total cholesterol in humans. The rate-limiting enzyme, HMG-CoA reductase (HMGR) is involved in cholesterol bio-synthesis from acetyl CoA to mevalonate to isoprenes to squalene and finally to cholesterol. Whenever, hepatic cholesterol is low, cholesterol equilibrium is maintained by HMGR enzyme which upregulate the SREBP 2 (77). Further, liver LDL- receptors are also important to retrieve cholesterol and LDL-C from plasma and peripheral tissues, and LDL-R is highly expressed due to low hepatic cholesterol levels with activation of SREBP 2 (78).

Bile acid (BA) secretion is another important way to control cholesterol levels, for example, BA biosynthesis from cholesterol is mediated by cholesterol 7α-hydroxylase (CYP7A1) in the liver. Alternative synthesis of BA in macrophages, as well as in the liver, is controlled by sterol 27-hydrolase (CYP27A1) and oxysterol 7a-hydroxylase (CYP7B1). BA is critical to

activate FXR in the liver which is inhibited by CYP7A1 expression (79). When hepatic BA concentration is low, circulating cholesterol and hepatic cholesterol is removed by inhibition of CYP27A1. Circulating cholesterol contains different type of apolipoprotein which is most important for cholesterol clearance. Both HDL-C and LDL-C have different apolipoproteins, HDL-C contains antiatherogenic apolipoprotein A1, and LDL-C contains apo B lipoprotein. Plasma HDL-C/LDL-C ratio is regulated by both apo A1 and apo B lipoprotein concentrations (80). HDL-C plays a crucial role in cholesterol equilibrium via reverse cholesterol transport, which clears cholesterol from the peripheral tissues (80). HDL-C gives one unit of cholesterol ester to LDL-C with the exchange of one unit triacylglycerol by the action CE transport protein (CETP). Suppressing the action of CETP could alter the plasma HDL-C/LDL-C ratio and potentially reduce CVD risk (81). Current research knowledge indicates that cholesterol homeostasis is maintained and regulated by several factors such as enzymes, transcription factors, transporters and bile acids. Also, the gene diet interaction and genetic variants could explain the effect of dietary fat consumption on the host lipid metabolism.

Before, the human genome project, most researchers were focused only on the health benefits of dietary fats and other nutrient intakes in various human populations (17, 82, 83). Dietary fat has a direct impact on the metabolic markers associated with CVD risk events (17). However, lipid metabolism is complex and numerous functional and non-functional genes and proteins play a vital role in cholesterol homeostasis. Various mutations in the gene may alter the function of the corresponding gene and lead to increased risk for related diseases (84). Recently, numerous methods have been used to identify the common genetic traits with broad range conditions, including various disease states to nutrition intake (85). Currently, human genetic studies have identified various genes and loci that control blood lipid levels in

different populations by genome-wide association studies (GWAS), pedigree, epidemiological and candidate gene studies (84). Therefore, assessment of the genetic variants that are associated with dietary fat metabolism is important to cardiovascular health. The first step is identifying the primary contributing genes (72, 73), as well as their relevant genetic variants, that influence lipid metabolism (12). Notably, gut microbiome also influences the lipid metabolism through different mechanisms such as trimethylamine N-oxide (TMAO) generation, primary and secondary bile acid metabolism pathways, and short-chain fatty acids (SCFA) production. In addition, results of metagenomics analyses has shown that the human intestinal microbiome has lipid-associated genes, which are involved in biosynthesis and bioconversion of lipids.

1.4 THE INTESTINAL MICROBIOME

The gastrointestinal tract (GI) of all higher animals is diverse and complex. Many studies have analyzed gut microbiome diversity and functions in various disease conditions (89-92). The term dysbiosis is defined as an imbalance or alteration of gut microbiome ecology in the gut. Evidence suggested that age (86, 87), diet (88-90), and diseases (91-93) are associated with gut microbiome dysbiosis. Many studies have suggested alterations in the gut microbial ecology with different diets (95-97). Furthermore, Bacteroides enterotype was associated with a diet rich in protein and animal fat, while Prevotella enterotype was associated with carbohydrate-enriched diets (94). Hence, gut microbiome with specific enterotypes has been associated to particular diversity and dietary habits of people (95). In addition, altered microbiome was observed in a single day in mice when the diets were switched from low fat to high fat, and from polysaccharide-rich diets to those high in simple sugars (96). Specific

bacterial population with increased Oscillibacter group and decreased level of *Eubacterium rectale* were observed when overweight men were fed a reduced carbohydrate diet for ten weeks (97). Recent findings suggested that >10-fold increases in plasma trimethylamine N-oxide (TMAO) in the blood is associated with CVD risk and the gut microbiome could be responsible with the generation of TMAO upon oral supplementation of choline (98). Thus, diet might have an impact in the alteration of gut microbiome which can be considered as a "relatively unstable organ" of the GI tract.

Various disease conditions such as inflammatory bowel disease and diabetes associated with alterations in the intestinal physiology and host defenses, may result in changes in the GI tract microbiome population (90,91). It is possible that studying the gut microbiome could lead to an increased understanding of how to maintain or control the overall health status of the host. Furthermore, very limited research has been conducted to correlate alterations in the gut microbiome with cardiovascular health. Therefore, understanding the role of the gut microbiome on modifying CVD risk could lead to knowledge of how microbiome shifts maintain or control the overall CVD status of the host.

1.5 GENETICS

Genes involved in the lipid metabolism and loss of function mutations alter circulating lipid levels (84). Loss of function mutations in *APOA1, ABCA1* and *LCAT* genes are associated with low levels of HDL-C (10 to 34 mg/dl) in the circulation and mutations in *CETP* (0.83 mg/dl), *LIPG* (15.5 mg/dL) and *SCARB*1 (27.36 mg/dL) genes are associated with high levels of HDL-C in the circulation (99, 100). Further, high level of circulating TC has been associated with a mutation in genes involved in LDL-C metabolism, including *APOB*,

LDLRAP1 and *PCSK* genes. Further, meta-analysis and GWAS have identified that 190 genetic variants are associated with TC, LDL-C and HDL-C levels in nearly 60 genes. The coding region of the genes has only 9% variants, which was identified in databases such as Ensembl and RefSeq database. Based on GWAS analyses, 7% of the variants are present in the 10 lipid-related genes, including *APOB, APOE, APOH, LPL, CETP, ABCG5/8, PCSK9, GCKR, HNF1A* and *HNF4A* (101). A high level of TG is known as hypertriglyceridemia or chylomicronemia, and the presence of a genetic variant within TG-related genes have been associated with circulating TG levels. More than 30 SNPs have an important connection with circulating TG concentrations (102). SNPs in *LPL* and *APOA5* gene loci have been associated with increase in TG metabolism (103). Further, a bi-allelic genetic mutation in *MTTP* and *APOB* genes resulted in low or no TG levels in the blood (104). Very low TG concentration is associated with an allelic mutation in *APOC3,* and *ANGPTL3* genes resulted in lack of apoC-III and angiopoietin-like protein 3 (105, 106). Also, *LPL* p.S474X variant shows beneficial effect in cardiovascular health by increasing HDL-C and reducing TG levels (107), and the inverse association with HDL-C and TG level was found with *LPL*p.D36N. The joint effect of these two LPL variants is an elevated level of TG and a lowered level of HDL-C, as confirmed in a couple of studies (108, 109). Another epidemiology study reported that LPL p.S474X, p.D36N and p.N318S have an alternative response on circulating HDL-C levels (110). Thus, mid to moderately high level of TG has been associated with multiple genetic variants within TG-related genes in the lipid metabolism, which increased CVD risk in a genetically susceptible individual.

In summary, human genetic study approaches may yield tools to investigate circulating TC, HDL-C and LDL-C concentrations and their associations with cardiovascular risk. Genetic variants identified so far have an impact on circulating lipids, but the combination of several genetic variants and their effects with dietary fat consumptions on lipid levels are more important to investigate further. In addition, the relationship between gut microbiome functions and energy harvesting from the host's diet in conjunction with host lipid microbiome on cardiac health is still unclear. Furthermore, no human study specifically investigated the dairy SFA compared to other fats with comprehensive approach to examine the effects of dairy SFA on CVD risk factors.

1.6 RATIONALE

Given the diversity of nutritional composition among different dairy SFA and their effects on circulating blood lipids are controversial. The primary focus of the current research addresses the impact of dairy SFA on gut microbiome and common genetic variant responses to cholesterol metabolism. Thus, investigating the role of dietary fats, particularly SFA from dairy products on circulating lipid levels, as well as the genetic and dietary factors that modulate the specific pathway would help to understand the relationship between dairy SFA intake and CVD. Also, an examination of effects of MUFA, PUFA and carbohydrates on lipids will help to understand their respective roles in CVD. In addition, the role of dietary fat-mediated gut microbiome changes and its impact on cardiac health is still unclear. Furthermore, no human intervention study specifically investigated the dairy SFA compared with MUFA, PUFA and CHO using a comprehensive approach to study the effects of dairy SFA intakes on CVD. Therefore, the primary focus of this research is to define the role of

dairy SFA intake on CVD prevention using a precise full feeding diet-controlled human intervention trial design.

1.7 STUDY DESIGN

This clinical trial was designed as a multicenter trial, involving two centers: the Richardson Centre for Functional Food and Nutraceutical (RCFFN), University of Manitoba and Institute of Nutrition and Functional Foods (INAF), University of Laval. This study consists of five dietary interventional phases including butter, cheese, CHO, MUFA and PUFA diets. The SFA content in the butter and cheese diets were be similar. In the other diets, CHO, MUFA and PUFA were replaced for SFA of the butter and cheese diets by dietary manipulations. Cheddar and mozzarella were used in the "cheese" arm of the study (2x50g per day, which corresponds to 2 servings of cheese, contributing to approximately half of the total daily intake in SFA). CHO was a mix of simple and complex carbohydrates that reflect current intakes in the North American diet. MUFA was mostly from olive oil and PUFA was mostly from sunflower oil to achieve a ratio of n6/n3 of approximately 10, which also reflected current intakes in the North American diet. The following table shows the major macronutrient compositions of intervention diets (**Table 1.1**)**.** Eligible participants' usual energy intakes were estimated at the beginning of the study using a web-based food frequency questionnaire tool (111). Experimental diets were provided as part of a full feeding protocol under carefully controlled isocaloric conditions to maintain body weight constant. All meals and foods were provided to participants to control for energy and macronutrient intake. On weekdays, participants came to the Clinical Investigation Units of RCFFN and INAF to consume their breakfast under the supervision of at least one member of the staff, at which

time they were given their meal for rest of day in ice cooled packaged bags. Weekend meals were prepared, packaged and delivered to their home. All take home meals were provided in containers that could be heated in the microwave oven when necessary.

Participants were instructed to consume their entire experimental diets and participants had no restriction to water and caffeine-free beverages over the course of the isocaloric phases of the study. Alcohol consumption was not allowed during the study as it is a key determinant of HDL concentrations. Vitamin supplements and natural health products were strictly forbidden during the entire experimental period. Consumption of tea and coffee (black) was allowed with a limit of 2 servings per day. On weekdays, just before breakfast, body weight was recorded with subjects standing with their back to the scale. Subjects were instructed to maintain their usual physical activity which was recorded for each week by filling the activity form. Blood pressure at rest (following a 10-minute rest) was measured thrice at an interval of three minutes at the beginning (day 1& 2) and at the end (day 28 & 29) of each 4-week intervention period. At the start and end of each intervention phase, fasting blood samples were collected, and serum, plasma, RBC and WBC were separated and stored in -80°C for analysis. For gut microbial diversity and functional analysis, stool samples were collected from the participants and stored in -80°C for analysis. The following figure (**Figure 1.1**) shows the schematic representation of the study design.

Table 1. 1 The major nutrient composition of experimental diets

	CHEESE	BUTTER	CHO	MUFA	PUFA
PROTEIN, %	15	15	15	15	15
CHO, %	53	53	60	53	53
FAT, %	32	32	25	32	32
SFA, %	13	13	6	6	6
MUFA, %	14	14	14	21	14
PUFA, %	5	5	5	5	12

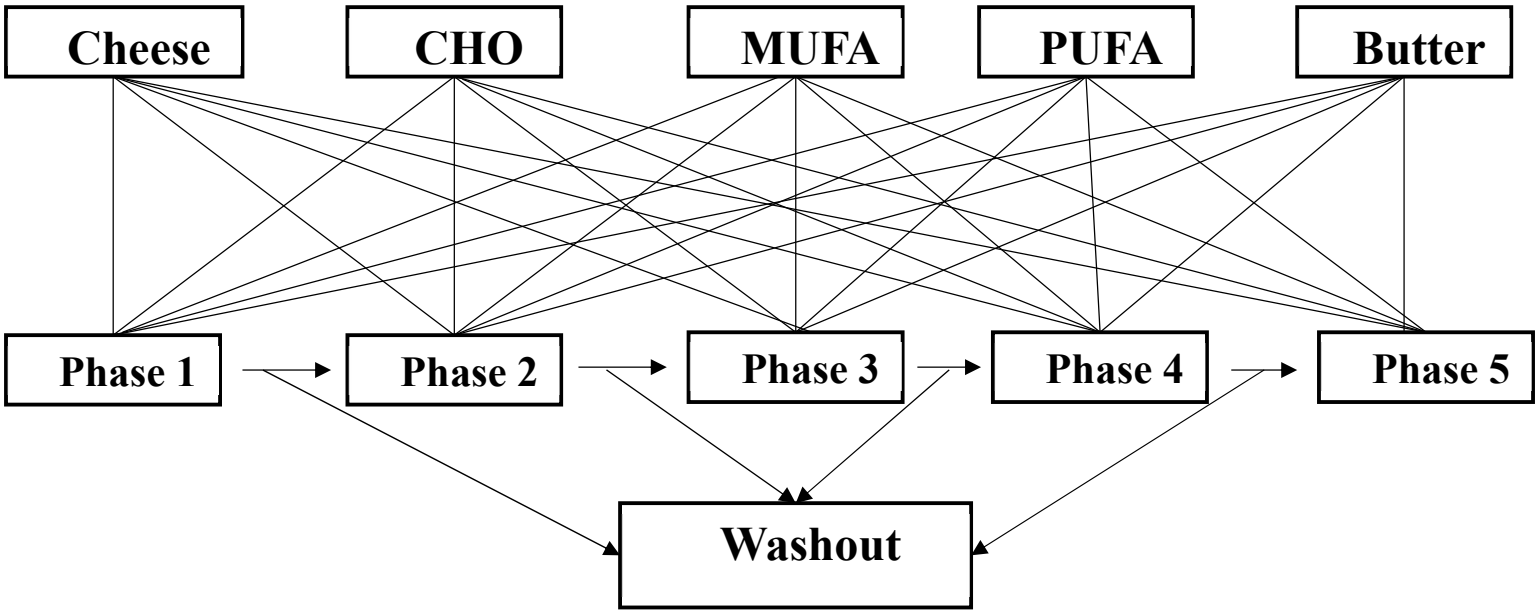

Figure 1. 1 Randomized crossover design of the study

1.8 HYPOTHESIS

The hypotheses to be tested include:

1. Consumption of a diet rich in dairy products and other dietary fat will modify the β-diversity (dissimilarity) of gut microbiome and gut microbiome associated function related CVD risk biomarkers

2. Genetic variants in candidate genes with functional links to cholesterol will associate with the degree of circulating cholesterol responsiveness following the dairy intake and other dietary fat

1.9 OBJECTIVES

The overarching purpose of this research programme is to extend knowledge on the recommended level of dairy consumption in Canada in relation to gut microbial changes and circulating cholesterol and inflammatory biomarker concentrations, with particular focus on identifying a genetic basis for any heterogeneity in responsiveness of cholesterol metabolism to dairy intake. Specific objectives include:

1. Consumption of SFA from dairy, and its impact on the gut bacterial community, and the link with CVD, have not been fully explored. The main objective of the study is to assess how dairy fat consumption modulates the gut microbiome

2. We aimed to analyse the differences in serum lipid concentration and their association with single SNP or a combination or further, we aimed at explaining the variability in the serum lipid response to SFA vs unsaturated fatty acids (UFA) using partial least squares regression analysis.

1.10 OUTLINE OF THIS THESIS

This thesis is comprised of three manuscripts, and includes a general Introduction (chapter I), that includes a summary of recent literature on impact of dietary fat intake from different source on cholesterol metabolism, cardiovascular risk, genetics and general introduction of gut microbiome (chapter II). Chapter II comprises a literature review on understanding connections and roles of gut microbiome to cardiovascular diseases. Specifically, the literature review focusses on the question, "how the altered gut environment and microbiome-derived metabolites impact on cardiovascular events". Chapter III summarizes the clinical trial methods, results and discussion on impact of dairy fats on gut microbiome and its role in cardiovascular health. The chapter IV covers the clinical trial findings on responsiveness of circulating serum cholesterol based on with genetic heterogeneity. Chapter II is submitted to Canadian Journal of Microbiology and rest of the chapters i.e. III and IV are currently under the revision process with collaborators and anticipating submission to scientific journals in next two months. Chapter V provides a general discussion and overall summary of the thesis with implications, remarks, and limitation and future directions.

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BRIDGE TO CHAPTER II

The following chapter supports the literature-based association of gut microbiome and its connection to CVD. Also, the possible mechanism, how the gut microbiome communicates with host physiology and its implication close to identify and understand the gut microbiome-host interaction in cardiovascular health perspectives are also explained. In general, this chapter is an introduction to the topic of gut microbiome mediated mechanism which relates to the CVD risk factors.

CHAPTER II

MINI-LITERATURE REVIEW

UNDERSTANDING CONNECTIONS AND ROLES OF GUT MICROBIOME TO CARDIOVASCULAR DISEASES

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Abbreviations: CVD: cardiovascular disease; BA: bile acid; FMT: fecal microbial transplantation; IBD: inflammatory bowel disease; IBS: inflammatory bowel syndrome; SCFAs; short-chain fatty acids; TMAO: trimethylamine N-oxide.

Keywords: Gut microbiome, Cardiovascular disease, trimethylamine N-oxide, Bile acid, Short-chain fatty acids

2.1 ABSTRACT

The gut microbiome encompasses trillions of residing microbes, mainly bacteria, which play a crucial role in maintaining the physiological and metabolic health of the host. Gut microbiome has been associated with several diseases including cardiovascular disease (CVD). A growing body of evidence suggests that an altered gut environment and gut microbiome-derived metabolites are associated with CVD events. Gut microbiome communicates with host physiology using different mechanisms, including trimethylamine N-oxide (TMAO) generation, primary and secondary bile acid metabolism pathways, and short-chain fatty acids (SCFA) production. The main concept of this review is the association of gut microbiome to CVD and its implications towards understanding the gut microbiome-host interaction. Manipulation of gut microbiome through specific dietary intervention is a simple approach to identify a novel metabolic pathway or a novel therapy to advocate new dietary recommendations, and new preventive measures or screening biomarkers to reduce CVD risk in humans.

2.2 INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of deaths worldwide (1). CVD risks have been associated with several factors including dyslipidemia and dysfunctional lipid metabolism (2), smoking (3), obesity (4), physical activity (5), diabetes mellitus (6), and hypertension (7). Recently, the role of gut microbiome in the development of CVD risk events has been identified and this potentially increases research attention towards the linking between gut microbiome and CVD (8-10). The gut microbiome is more diverse and complex microbial populations, which is being described as an organ in its own (11). Gut microbiome acts as a strong trigger to host's circulating molecules, which in-turn plays pivotal role in various processes including immunological, metabolic and physiologic pathways (12, 13). Hence, there is no doubt that changes in the gut microbiome have been associated with wide range of conditions include obesity (14, 15), CVD (9, 16) and diabetes (17, 18). This short review mainly focuses on the role of gut microbiome in association with CVD risk.

2.3 GUT MICROBIOME

Higher animal gastrointestinal tract contains complex and diverse of gut microbiome. Published evidence suggested that various disease conditions and diets have altered the gut microbiome diversity and functions (19-23). The composition of gut microbiome varies from person to person (24); however, stable microbiome can be observed over the period of time (25). Major dominant phyla of the gut microbiome are Firmicutes, and Bacteroidetes, other less dominant phyla are Actinobacteria, Proteobacteria, Fusobacterial and Verrucomicrobia (12). In addition, methanogenic archaea and eukaryotic microorganisms are present in the gut along with viruses (12). Healthy gut microbiome comprises of over 90% of species belonging

to Firmicutes and Bacteroidetes (26) and the relative abundance of species can vary due to multiple factors such as gender and age (27) dietary practices (28), BMI (29), geography, physical activity, socio-economic status, housing pets, antibiotic intake and diseases (30-34).

Modern humans have gut microbiome profiles which coevolved in parallel with their dietary practices. Certainly, we may not exactly know or predict the extent of physical activity and exact dietary habit of our hunter-gatherer ancestors; however, based on clues about the human dietary evolution close to apes, whose genetic difference is approximately 2-3% compared to humans (35). Our ancestors' diets were mainly based on plant derived foods (36, 37) which composed of complex carbohydrates and indigestable fiber that would be fermented by the gut microbiome for energy. These indigestable macromolecule can be converted to short chain fatty acids (SCFAs), These SCFAs exhibit wide range of beneficial physiological effects on the host (38, 39). Some microbes in the gut do not have the capacity to ferment dietary fibers and however, these microbes are able to utilize the SCFA produced by other microbes (40). SCFAs have been observed to reduce the levels of cytokine via inhibiting of nuclear factor kappa B (41). In addition, SCFAs are energy sources for both gut microbiome and the host. Also, they act as signalling molecules by binding with G-protein-coupled receptors expressed in immune cells, adipose and epithelial cells (42, 43) and regulate the inflammatory process (44).

The gut microbiome communicates with immune system via epithelial cells. Toll like receptor and other pattern recognition receptors of microbes and their products are detected on the epithelial cells; activation of these receptors eventually activates immune cells as well as other signalling cascade to bolster the the gut barrier (45). During the cascade of immunological

process, activated epithelial cell's metabolic functions are reduced and leptin and fat absorption are also reduced (46). Thus, the gut epithelial cells rely on residing gut microbiome to maitaing the metabolics and immune functions.

Gut microbiome also affects the host metabolism through altering bile acid composition. Escaped primary bile acids (BA) from reabsorption can be converted to secondary bile acid by the gut microbiome. These microbiome derived secondary BA enter into circulation and act as signalling molecules to influence the host metabolism and inflammation process (47, 48).

2.4 GUT MICROBIOME AND CVD

A large number of biological events and factors have been associated with CVD, for example, atherosclerosis is a chronic inflammatory event which consists of plaque formation in the arteries, and accumulation of lipids in the endothelium and leucocytes (49, 50). Initial studies found that the pathogens such as *Porphyromonas gingivalis*, *Helicobacter pylori* and *Chlamydia pneumonia* were associated with atherosclerosis (51-53). The mechanism behind these findings suggest that *Chlamydia pneumonia* species are responsible for the pathogenesis of atherosclerosis via invading macrophages to form foam cells and leading to recruitment of leucocytes to expand the atherosclerotic lesions (54, 55). Apart from this finding, link between gut microbes and atherosclerosis is not well understood. It leads us to an obvious question on what could be the connection and roles of the residing gut microbes to trigger CVD and cause disease progression. However, current research evidence suggests that gut microbiome has been established as a contributing factor in CVD risk.

In specific, CVD risk is associated with high levels of trimethylamine N-oxide (TMAO) in the blood. The gut microbiome could be responsible for the generation of TMAO from the dietary trimethylamine (8, 56). In addition, patients with atherosclerosis have increased *Collinsella* species when compared to healthy counterparts, while their gut is enriched with butyrate-producing bacteria *Roseburia* and *Eubacterium* species (57). Patients with atherosclerotic plaque have a microbiome patterns with high levels of Proteobacteria and low levels of Firmicutes (58). Gut microbiome derived lipopolysaccharide (LPS) is responsible for disrupted intestinal barriers and its association with systemic inflammation (59, 60). Accumulation of LPS further damages intestinal barrier which results in metabolic endotoxemia (21). Obese and CVD patients have a high level of inflammatory markers and gut microbiome derived metabolites could have an influence on the host physiology (61, 62). Interestingly, administration of probiotic *Lactobacillus plantarum* reduces infarct size in the myocardial infarction in an animal model via a change in the gut environment (63). In another myocardial infarction study, reduced left ventricular hypertrophy was observed with administration of probiotic strain, *Lactobacillus rhamnosus* GR-1 (64). This study revealed that the modulation of the gut microbial environment by ingestion of probiotic could benefit in the reduction of CVD risk. Hypertension is another important risk factor for CVD. In hypertensive rats, significantly increased ratio of Firmicutes/Bacteroidetes, and reduced level of the gut microbiome richness, diversity, evenness were observed (65). Interestingly, low blood pressure was observed in treatment-resistant hypertensive patients when administrated with different antibiotics, which tend to alter the gut environment (66).

Gut microbiome association with some parameters of metabolic syndrome were studied in Finnish men from the Metabolic Syndrome in Men (METSIM) study (67). Phylum

Tenericutes and Family Christensenellacea have been associated with increased level of acetate, HDL-C and lower BMI and Triglyceride (TG) levels. Methanobrevibacter and Peptococcaceae were enriched and correlated with reduced levels of TG. Further, TMAO level was associated with abundance of Peptococcaceae, Prevotella, and negative TMAO association with *Faecalibacterium prausnitzii* (67). Host energy and lipid metabolism were altered by the gut microbiome in both conventional and germ-free mice (68).

The gut microbiome diversity has been associated with circulating lipid levels. The LifeLines population study by Fu et al. used fecal samples from 893 participants for bacterial analysis based on the 16rRNA genes approach (69). They identified that the gut microbiome diversity and richness was associated with circulating lipid levels. A positive correlation was observed between gut microbiome diversity with HDL-C, and a negative correlation was observed between bacterial richness with BMI and TG levels (69). This result provides more insights into gut microbiome and its association with cardiometabolic parameters. Some previous studies found that bacterial taxa including *Akkermanisa, Tenericute* and *Christensenellaceae* were associated with obesity (70) which is another condition associated to CVD risk (71, 72). Bacteria and eukaryotes produce sphingolipids and share the same enzymatic steps at initial pathway. Serine palmitoyltransferase enzyme (SPT) is responsible for the initial condensation of amino acid and fatty acid, and SPT highly conserved in both bacteria and eukaryotes (73). Human GI tract has 30%-50% of *Bacteroides* and *Prevotella* species (74, 75). The Bacteroidetes phylum species, including *Bacteroides*, *Prevotella* and *Porphyromonas*, synthesizes sphingophospholipids and glycosphingolipids (48, 76, 77). Further, *Odoribacter* species has been associated with the production of the bacterial sulfonolipids (SL) (78). The bacteria derived SL possesses anti-inflammatory effects and also shows the maturation of the

immune system in an animal model (79). However, the functional and other biochemical characterization of SL is not well understood. In a mice study, oral administration of bacterial-derived sphingolipids (BS) was readily taken up and metabolized by the liver (80). In enterocyte, BS is hydrolyzed and incorporated into triglycerides and then enters into the circulatory system as chylomicron (81). Therefore, *Bacteroides* and *Prevotella* species provides endogenous bacterial lipids which are similar to eukaryotic sphingolipid, and this may be the possible mechanism for gut microbiome influence on mammalian host lipid metabolism (81). Further research on this is rquired to understand how the gut microbiome is involved in the production of various type of sphingolipids.

Given the role of gut microbiome in host metabolism, modulation of the gut microbiome in the gut may have a connection to CVD risk parameters and lower the blood pressure but further investigation is required. Also, low blood pressure was associated with butyrate producing *Odoribacter* in obese and overweight pregnant women (82). Therefore, evidence suggest that alteration in the gut-associated communities have major contribution in alteration of the CVD risk parameters.

2.5 TMAO AND CVD

Recently, a novel pathway linking TMAO and atherosclerosis have been identified (16). Based on the metabolomics analysis of human plasma sample that identified the TMAO and choline were associated with CVD risk. L-carnitine contains phosphatidylcholine, and trimethylamine, which generates TMAO and choline molecules that have been associated with CVD (16, 65). Enzymes from Gut microbiome, TMA lyases uses L-carnitine to produce the gas trimethylamine which is then metabolized by the action of liver enzyme flavin

monooxygenase (FMO) to TMAO (8). Increased atherosclerotic effects were observed with gut microbiome mediated phosphatidylcholine metabolism whereas these effects did not occur in a germ-free mouse model (16). Dietary supplementation of choline increases plasma TMAO, which enhances foam cell and atherosclerotic formation and L-carnitine supplementation also showed the same effects (16, 83). Phosphatidylcholine ingestion increases plasma TMAO levels whereas phosphatidylcholine along with oral antibiotic suppresses the TMAO levels. Platelet hyperresponsiveness and thrombosis risk have been observed with TMAO generation. Choline and TMAO supplemented diets fed mice showed increased thrombosis compared to germ free mice fed with same diets (84). Platelet hyperresponsiveness explains the CVD risk events in human study, in which dose dependent correlation has observed between platelet aggregation and TMAO levels in plasma (85)

Obesity and diabetes status are associated with CVD risk, considering the role of altered gut microbiome along with host metabolism in diabetes and obesity will give more insights in CVD risk as well (14). An elevated level of TMAO was observed in diabetic animal models as well as patients with various other metabolic diseases (86, 87). Higher CVD risk has been associated with an increased level of TMAO in patients with diabetes. TMAO and diabetic connection also observed in mice model; the flavin containing mono oxygenase 3 (FMO3) and TMAO expression is suppressed by insulin in liver and glucagon reverses this effect (88). In diabetes induced mice model, elevated TMAO level has been correlated with high FMO3 expression. Improved insulin tolerance and development of atherosclerosis was observed in mice with knockdown of FMO3 (89). The specific type of microbiome profile is associated with high TMAO synthesis which identified with *Prevotella* enterotype compared to other

enterotypes (83). Thus, microbiome mediated TMAO levels were identified with protein and fat-rich diets. Hence, dietary modulation alters some taxa in the gut microbial population thereby influence the level of microbiome-mediated TMAO generation.

2.6 BILE ACID SIGNALLING

The liver is the primary organ for synthesising the primary BA from cholesterol oxidation and the gut microbiome has a role in secondary BA production (90). High level of primary BA and low or absence of secondary BA have been observed in the germ-free mice model with enlargement of the gallbladder (91, 92). BA is most important in the lipid metabolism because cholesterol elimination from the body occurs through BA secretion. Cholic acid and chenodeoxycholic acid are conjugated to glycine in humans, and gut microbiome is responsible to de-conjugate the primary BA into secondary BA such as deoxycholic and lithocholic acid (93). Diet mediated change in the gut microbiome is responsible for the altered production of BA (12). For example, manipulating cholic acid via diet induced increase in Firmicutes phylum, which is associated with excretion of deoxy-cholic acid levels in the faeces (94). Therefore, microbiota derived BA may act as signalling molecule and have implications not only on gut microbiome content but also on the host. In this context, various forms of BA interact with receptors such as farnesoid X receptor (FXR) and a G protein-coupled receptor (TGR5)(95-97). Lipid and glucose metabolisms are regulated via FXR, and it has been demonstrated that FXR deficient mice fed with normal diet show impaired reverse cholesterol transportation which leads to developing hypercholesterolemia (98). Therefore, the gut microbiome may interplay lipid and glucose metabolism through primary/secondary BA signalling as an effect of the dietary change.

2.7 SHORT-CHAIN FATTY ACIDS (SCFAS) AND CVD

Short-chain fatty acids (SCFAs) are volatile acids that are important for maintaining intestinal health in humans. Gut microbiome have shown to produce SCFAs from resistant starch and undigested carbohydrates in the colon (99). SCFAs are a subset of saturated fatty acids containing six or fewer carbon molecules that include acetate, propionate, butyrate, pentanoic (valeric) acid, and hexanoic (caproic) acid (100). The gut microbiome mainly relies on the host for nutrients, such as undigested food material, non-digestible fibers, resistant starch, arabinoxylan, beta-glucan, and mucosal cells (100, 101). In the proximal colon, gut microbiome utilizes carbohydrate as the preferred substrate for fermentation over the proteins (102). Differential effect of SCFAs was observed on serum lipid levels. In a human study, rectal infusion of acetate and propionate has shown to increased total cholesterol (TC) and triglyceride (TG) concentrations in blood (103). Further, the same author indicated that propionate infusion does not affect blood TC and TG levels, whereas acetate infusion increased blood TC concentrations. Thus, it is clear that the mixture of acetate and propionate rectal infusion has some or considerable impact on blood cholesterol levels. Further, it was observed, cholesterol synthesis from acetate was inhibited by propionate (104), and an animal study confirmed the mechanism of action; the propionate tends to inhibit HMG CoA Reductase (105). However, ingestion of various forms of SCFAs provided inconsistent results on blood lipid levels. In addition, blood lipid profile were not altered by consumption of sodium propionate (2.7 g) with bread and as sodium propionate capsule (7.5 g) (106, 107). Therefore, the health effect of SCFAs has been inconsistent on blood lipid levels because of the difference between the individuals and the administration route and the formulation types.

Overweight or obesity has been associated with CVD and diabetes risk events (4), these are significant problems in the developed world. Investigating the association between gut microbiome and SCFA production and their health implications still remains an active and promising area to explore. Reports suggest that obesity has been associated with a high-fat diet and calorie dense food intake (108), and supplementation with butyrate prevents high-fat-diet-induced obesity in mice model (109). Moreover, oral acetate administration also diminished weight gain when compared to the oral injection of water in rats (110). Studies showed that SCFAs such as acetate, propionate, and butyrate are individually protective against obesity. Overall, the above results indicate that SCFAs protect against obesity, but the anti-obesity mechanism of SCFA remains unclear. It could be that propionate and butyrate both suppress appetite, whereas acetate may act via another mechanism. In summary, the systemic health benefit of microbiome can be achieved by dietary approach and recommendation. As per evolution, our ancestral diet was plant-based, and the gut microbiome has co-evolved simultaneously, and the Western diet deviates more from our ancestral diet.

2.8 MICROBIOME BASED THERAPEUTIC APPROACH

Identification of TMAO pathway and its association with microbiome opens a new venture for microbiome-based CVD therapeutic intervention. Resilience of gut ecology can be achieved by the supplementation or an intervention modification. For example, high fiber or prebiotic produces SCFA and increases beneficial bacteria which impact on the gut homeostasis. Another approach, fecal microbial transplantation (FMT) from a healthy donor to patient has shown displacement of harmful bacteria. This was studied in *Clostridioides difficile* infection

in humans, and this is the new approach for treating gastrointestinal diseases such as inflammatory bowel disease (IBD) and inflammatory bowel syndrome (IBS). This FMT method can be beneficial also in patients with CVD related events. Improved insulin sensitivity was shown in allogenic fecal transplant compared to autologous transplant in humans (111). Thus, FMT could be a feasible treatment approach for cardiometabolic disorders and also useful to study the link with CVD.

Interventions targeting gut microbiome mediated host interaction pathway such as TMAO pathway, SCFA and primary/secondary bile acid signalling are the most promising approach for the CVD prevention and treatment. A structural analog of choline 3,3-dimethyl-1-butanol has shown to block TMA production and reduce TMAO levels in mice on high choline or L-carnitine diets and also reduces the process of foam cell formation and atherosclerosis (112). Moreover, 3,3-dimethyl-1-butanol is a small molecule with specific target to CVD, and it does not significantly impact the gut microbiome in developing resistance.

2.9 CONCLUDING REMARKS

Diet type and quality have a significant impact on the circulating lipid levels which are major contributors for CVD (113, 114). Additionally, evidence from observational studies suggested that long-term diet can modify the gut microbiome thereby change the gut physiochemical environment (115). Moreover, diverse dietary practices are sources for complex nutrients and these in-turn enhance microbial diversity. However, which component of the nutrient has an effect on certain bacterial taxa is not well understood (116). For example, a single substrate such as xylan- degrading capacity varies among different species from the same genus (117). Thus, any dietary component and gut microbial-derived health-promoting effect may not be

the same for all individuals. Hence, the dietary or supplementation based microbial therapy should necessarily be personalised and targeted to manage or prevent CVD risk. Therefore, Personalised therapy or advice targeting gut microbiome based FMT, supplementation of prebiotic and probiotics, TMAO inhibitors or dietary based recommendation is mandatory to manage or prevent CVD in humans.

Author contribution

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Conflict of interest statement

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BRIDGE TO CHAPTER III

Gut microbiome and gut microbiome mediated compounds have directly or indirectly contributed to several diseases. Also, various dietary intakes have been associated with gut microbiome alteration which play major role in human health and disease. Using the controlled dietary intervention study i.e. various dietary fat from different sources, effects on gut microbiome changes and its association to cardiovascular related parameters have examined. Chapter III comprises gut microbiome data originated after various dietary fat consumption and link to cardio metabolic risk factors.

CHAPTER III

CHEESE AND BUTTER INTAKE MODULATES GUT MICROBIOME IN HEALTHY HUMANS: A RANDOMIZED CONTROLLED TRIAL

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Benoit Lamarche was involved in the primary experiment design. Peter Jones, Ethendhar Rajendiran, and Ehsan Khafipour were responsible for the subsequent experiment design and sample collections. Ethendhar Rajendiran, Zhengxiao Zhang and Ehsan Khafipour were involved in the data analysis. All authors contributed to writing the manuscripts. Final version of manuscript was approved by all authors.

Conflict of interest

The authors declare there is no conflict of interest.

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Abbreviations:

Clinical Trial Registry number and website: http://www.clinicaltrials.gov (NCT02106208).

3.1 ABSTRACT

A growing body of evidence suggests that diet alters the human gut microbiome. Further, gut microbiome-derived metabolites have been associated with cardiovascular (CV) health. Consumption of saturated fatty acids (SFA) from dairy source, their impact on the gut microbiome, and their link with CV health, have not been fully explored. The main objective of the study is to assess how SFA consumption modulates the gut microbiome. A subset of participants from a multicenter randomized crossover trial, consumed five isoenergetic diets, including those rich in cheese, butter, monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA) or low-fat high carbohydrate (CHO) each for 4-weeks. Post-intervention stool samples were collected from participants and bacterial DNA was extracted and subjected to V4 16S rRNA gene in Illumina MiSeq sequencing. Results suggest significant differences in relative abundances of Firmicutes between PUFA (74.40 ± 3.81) vs. CHO (65.90 ± 3.48) and Verrucomicrobia between cheese (0.10 ± 1.52) vs. butter (4.64 ± 1.52) containing diets, but not with other phyla. Differences in β-diversity were only observed between CHO vs. PUFA rich diets (p=0.008), MUFA vs. PUFA (p=0.014) and PUFA vs. butter (p=0.015) regardless of BMI. Further, BMI also showed differences in β-diversity between obese (OB) vs. overweight (OW), ($p < 0.05$) group regardless of the dietary intake. Further, we found there were no effects in OW group in β-diversity whereas significant differences were observed between PUFA vs. MUFA (p=0.007), PUFA vs. butter (p=0.039), PUFA vs. CHO (p=0.018), PUFA vs. cheese (p=0.002), MUFA vs. CHO (p=0.004), MUFA vs. cheese (p=0.003), butter vs. CHO (p=0.014) and butter vs. cheese (p=0.002) in OB group. Our results suggest that the BMI of an individual, specifically OB, exerts a major impact on the response of gut microbiome to dietary compositions. However,

the specific clinical significance of dissimilarity at genus level and their metabolites on CV health has yet to be explored.

3.2 INTRODUCTION

Cardiovascular disease (CVD) is one of the leading causes of death world wide. Dairy food is a rich source of SFA, and its health benefit remain unresolved in the research area of cardiovascular health (1, 2). Elevated levels of blood lipids are main risk factors for CVD. In most cases, CVD risk has been evaluated based on the cardiometabolic biomarkers in the blood to predict the disease risk and management. Several factors such as genetic makeup and dietary intake, and interaction between diet and genetics affects the blood lipid levels (3, 4). However, certain portion of the variation in blood lipids remains still unknown. Notably, the human body harbors a trillion of microbial cell with a remarkable ecosystem and functions (5). Current evidence suggests that diet-induced changes in the gut microbial communities are the contributing factor for chronic diseases including CVD (6, 7), obesity (8, 9) and diabetes (10, 11). Recent findings suggest that gut microbiome derived trimethylamine N-oxide (TMAO) in the blood is associated with CVD risk (12, 13). Further, a dysbiotic gut microbiome was observed in individuals with other cardiovascular risk events including atherosclerosis (7) and diabetes (11, 14). A reduced level of butyrate-producing bacteria, *Roseburia* species has been observed in atherosclerosis and type 2 diabetic patients (15). Also, bacterial DNA which are similar to high levels of Proteobacteria and low levels of Firmicutes have been identified in atherosclerotic plaques of patients (16). Thus, emerging evidence suggest that gut microbiome may have direct or indirect role in the development and progression of some diseases.

Elevated levels of inflammatory markers have been identified in obesity and diabetes, and CVD patients (17). From a gut microbiome point of view, a disrupted intestinal barrier associated with composition of harmful microbiome results in systemic inflammation due to translocation of bacterial lipopolysaccharide (LPS) (18, 19). Further, high levels of LPS through a disrupted intestinal barrier leads to metabolic endotoxemia (20).

A body of evidence suggests that the different dietary consumptions alters the human gut microbiome. However, the relation between CVD risk and gut microbiome dysbiosis remains unexplained. With considering the diversity of nutrition composition of different dairy products, beside other confounding factors, data on the link between dairy intake and CVD risk factors are controversial. In addition, in terms of consumption of dairy SFA specifically, the impact of cheese and butter on the gut microbiome remains unknown. Indeed, a gap remains regarding the role of dairy intake in gut microbiome dysbiosis from a cardiovascular health perspective. Hence, in the above context, the current objective was, using a well-controlled human intervention trial, to specifically investigate the action of SFA compared with monounsaturated fatty acids (MUFA), poly-unsaturated fatty acids (PUFA) and carbohydrate (CHO) on the gut microbiome.

3.3 MATERIALS AND METHODS

A randomized, single-blind, cross-over, full-feeding multicenter trial, involved Richardson Centre for Functional Foods and Nutraceuticals (RCFFN) at the University of Manitoba, and the Institute of Nutrition and Functional Foods (INAF), University of Laval (21). Only the subgroup of participants from RCFFN participated in the microbiome analysis. Twenty-five participants

were considered for the analysis, in which six completed all five intervention phases, nine participants completed four phases, four participants completed three phases, and five participants completed two intervention phases (**Figure 3.1**). All research protocols were approved by Biomedical Research Ethics Board, University of Manitoba. Endpoint measurement of lipid levels, glucose, insulin, C-reactive protein (CRP), apolipoprotein B, and adiponectin were measured and correlated with gut microbiome in this controlled feeding intervention study.

3.3.1 STUDY PARTICIPANTS

Healthy men and women volunteers between 18-65 y, with a waist circumference greater than 80 cm for women and greater than 94 cm for men, as well as with blood HDL-cholesterol levels of ≤1.53 mmol/L for women and ≤1.34 mmol/L for men, were included in the study. Individuals with a history of CVD or type 2 diabetes, those on cholesterol-lowering or hypertension medications, and smokers were not eligible for the study. Participants consumed five diets each for 4-wk with >4-wk washout periods between intervention diets. The five isocaloric diets (35 % energy from fats) were: (i) a diet rich in SFA from cheese, (ii) a diet rich in SFA from butter, (iii) a diet rich in MUFA, (iv) a diet rich in PUFA and (v) a low-fat (25 % energy from fats), high carbohydrate diet (CHO). Eligible participants' usual energy intake was estimated at the beginning of the study using web-based food frequency questionnaire tool. Content of SFA were matched in cheese and butter diets whereas in others experimental diets, SFA contents were replaced and manipulated by MUFA, PUFA and CHO. Experimental diets were identical for energy and macronutrient content whereas calcium was higher in cheese diet compared to other four experimental diets. Seven-day rotation menu were used, which included three meals and one

snack in a day across five diets. More details about the participants' recruitment and dietary interventions were previously described in Brassard et al., 2017 (21).

3.3.2 FECAL SAMPLE COLLECTION AND DNA EXTRACTION

Two-gram stool samples were collected from each participant at the beginning and end of each intervention phase (Days 2 and 30). Participants were provided with Feces Collection Paper Fe-Col (Alpha laboratories, Hampshire, UK) to accommodate sample collection and were advised to collect the sample from one bowel movement. Stool samples were collected using stool nucleic acid collection and preservation tubes (NORGEN Biotek Corp, Thorold, ON, Canada) which preserve DNA at room temperature for up to 21wk. Upon transfer to the lab, samples were stored at - 80°C until the analyses were performed. Samples were homogenized, and a 200-300 mg of sample was used for DNA extraction using ZR Fecal DNA MiniPrep kit. DNA quantity was determined using a NanoDrop 2000 (Thermo Fisher Scientific Inc, MA, USA). All DNA samples were normalized to 20 ng/µl concentration, and the quality of the DNA was checked using PCR with 16S rRNA gene primers 27F 95′-GAAGAGTTTGATCATGGCTCAG-3′) and 342R (5′-CTGCTGCCTCCCGTAG-3′) as described (22).

3.3.3 CONSTRUCTION OF LIBRARY AND SEQUENCING BY ILLUMINA

Library construction was performed using V4 region of 16S rRNA gene by PCR amplification of F515/R806 primers (23) as described previously (24). PCR reaction was carried out in duplicate for each sample and reaction mixtures contained 1.0 µL of normalized DNA, 1.0 µL of both forward and reverse primers of HPLC grade water (Fisher Scientific, Ottawa, ON, Canada), and 10 µl of 5 Prime Hot Master Mix (5 Prime, Inc., Gaitherburg, MD, USA). PCR reactions were performed in an Eppendorf Mastercycler Pro (Eppendorf, Hamburg, Germany) using the

following steps; an initial denaturation at 94°C for 3 min, then 35 amplification cycles at 94°C for 45 sec, 50°C for 60 sec, 72°C for 90 sec, and a final extension at 72°C for 10 min. PCR amplicons were purified using ZR-96 DNA Clean-up Kit (ZYMO Research, Irvine, CA, USA). Further, 200 ng of purified amplicon each was used to construct the V4 library; the quantification was carried out using Picogreen dsDNA (Invitrogen, Burlington, ON, Canada). Pre-chilled hybridization buffer (HT1; Illumina, San Diego, CA, USA) was used to dilute the amplicons at 5 pM as the final concentration, which was measured using Qubit 2.0 Fluorometer (Life Technologies, Burlington, ON, Canada). In each amplicon pool, 15% of PhiX control library was added to improve the unbalanced and biased base composition of 16SrRNA libraries. The customized sequence was synthesized, purified by polyacrylamide gel electrophoresis (Integrated DNA Technologies, Coralville, IA, USA) and added to the MiSeq Reagent Kit v3 (600-cycle; Illumina, San Diego, CA, USA). For customized sequencing primers are read1 (5′-TATGGTAATTGTGTGCCAGCMGCCGCGGTAA-3′), read2 (5′-AGTCAGTCAGCCGGACTACHVGGGTWTCTAAT-3′) and index read (5′-ATTAGAWACCCBDGTAGTCCGGCTGACTGACT-3′). Sequencing reaction was carried out on a MiSeq platform (Illumina, San Diego, CA, USA) at the Gut Microbiome and Large Animal Biosecurity Laboratories, University of Manitoba, Canada.

3.3.4 BIOINFORMATIC APPROACHES

Overlapping paired-end Illumina fastq files were merged by using the FLASH assembler (25). Mismatch and ambiguous sequences in the overlapping region were eliminated. The open source software package QIIME version 1.9.1 was used to analyze the fastq files (24). Ambiguous sequences with below 20 phred quality scores (Q-scores) were discarded and based on the

barcode, sequences assembled reads were demultiplexed, and exposed to additional filters. Operational Taxonomic Units (OTU) sequences were selected by using the QIIME implementation of UCLUST at 97% pairwise identity threshold. Before that UCHIME was used to filter chimeric reads (26). RDP classifier (27), Greengenes Core reference database (28) and PyNAST algorithms (29) were used to classify the taxonomies of each OUT sequences. FastTree 2.1.3. (30) was used to build the phylogenetic tree as well as further comparisons between microbial communities. Fecal microbiome richness and diversity indices were calculated with the depth of 11,000 sequences per sample. The open source software QIIME was used to calculate alpha-diversity (within-community diversity) and species richness and evenness, which including Shannon, Simpson, Chao1, observed the number of species, goods coverage and PD whole tree.

Bray Curtis dissimilarity (31) was calculated using QIIME default scripts to measure beta-diversity. Two-dimensional plots of Principal coordinate analysis (PCoA) was generated by using PRIMER 7 software. Hierarchical clustering analysis and a visual interpretation heat map of the bacterial taxa, genus similarity among diets were performed using R software (32). Predicted metagenome function based on 16S rRNA gene sequence (Langille et al., 2013) was performed by using the open source software, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, version 1.0.0). The Greengenes database was used to select closed-reference OTU with 97% similarity for the PICRUSt analysis. The Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology groups (KOs) database was used predict gene family abundance for each metagenome (33). Correlation plots were generated by R software (3.3.1 version) with the corrplot package (34).

3.3.5 STATISTICAL CONSIDERATIONS

GLIMMIX procedure (Bonferroni correction adjustment) was used to analyze the alpha-diversity indices differences among dietary interventions, considering diet, sex and BMI effects as fixed factors, and subjects as random factors (SAS version 9.4). Permutational multivariate analysis of variance (PERMANOVA, Primer 7) was performed and beta-diversity was calculated by using Bray curtis *p*-value of among the dietary interventions, considering diet, sex and BMI as fixed factors, and subject as random factors (35). For alpha-diversity and beta-diversity within BMI (OW or OB) were analysed using GLIMMIX procedure (Bonferroni correction adjustment), considering diet and sex as a fixed effect, and subject as random effects (SAS version 9.4). Relative abundance of bacterial taxa at the genus levels was compared using Multivariate Association with Linear Models (MaAsLin) analysis, considering BMI, sex and diet as fixed effects, and subject as random effects. Functional microbiome analysis between the dietary interventions was generated using a KEGG functional module, using STAMP 2.0 with the predicted metagenome percentage. Welch's t-test was performend on PICRUSt outputs of the gene. Non-parametric Spearman's rank correlation was used to correlate between bacterial taxa, and other metabolic parameters using Prism 7. Differences between treatment groups were considered significant at $P < 0.05$.

3.4 RESULTS

Dietary fat impacts on the bacterial community were analysed across all dietary interventions, including dairy cheese (n=19) and butter (n=17), MUFA (n=18), PUFA (n=16) and CHO (n=21). Baseline characteristics of the participants were showed in the supplementary **Table 3.4**. Moreover, β-diversity was used to test the carryover effect of the gut microbiome between the

washout period and the intervention phases, and there was no significant difference among the day1 sample (baseline) of each intervention phase as per randomization (diet effect, p=0.991; phase effect, p=0.892; diet x phase interaction, p=0.177).

3.4.1 ALPHA AND BETA DIVERSITY

Alpha-diversity indices were not altered upon consumption of any of the dietary interventions, but the overall diet interaction was significant for Shannon (p=0.02) and PD whole tree (p=0.004) diversity indices (**Table 3.1**). β-diversity measured by weighted UniFrac was significantly altered between CHO vs. PUFA (p=0.008, **Figure 3.3, panel A**), MUFA vs. PUFA (p=0.014, **Figure 3.3, panel A**) and PUFA vs. butter (p=0.015**, Figure 3.3, panel A**). Further, BMI also showed significant differences in β-diversity between OB vs. OW ($p < 0.05$, **Figure 3.3, panel B**). In addition, BMI had a significant effect on β-diversity after different dietary intakes, for example, cheese (p=0.03, **Figure 3.3, panel C**), CHO (p=0.01, **Figure 3.3, panel D**) and MUFA (p=0.03, **Figure 3.3, panel E**) intake altered β-diversity between OW and OB people, but not with butter and PUFA intake. Interestingly, regardless of the dietary intevention, β-diversity was not altered in the OW group (p=0.99, **Figure 3.3, panel G**) whereas in OB group, it was significant altered by diet (p=0.0001, **Figure 3.3, panel H**). Further, we carried out pair-wise analyses for β-diversity between the diets in OB group (**Figure 3.3, panel I)** and observed significant differences between PUFA vs. MUFA (p=0.007), PUFA vs. butter (p=0.039), PUFA vs. CHO (p=0.018), PUFA vs. cheese (p=0.002), MUFA vs. CHO (p=0.004), MUFA vs. cheese (p=0.003), butter vs. CHO (p=0.014), butter vs. cheese (p=0.002) and CHO vs. cheese (p=0.002).

3.4.2 SHIFTS OF MICROBIAL COMMUNITIES AS EFFECT OF DIET AND BMI

Dietary fat impacts on the bacterial community were analysed across all dietary interventions. From all fecal samples, five bacterial phyla including Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Verrucomicrobia were identified at above 1% of the community level (**Table 3.2)**. Firmicutes phylum was more abundant in the PUFA group (74.40 ± 3.81) compared to the CHO group (65.90 ± 3.48), and Verrucomicrobia phylum was higher in butter intake group (4.64 ± 1.52) compared to cheese intake group (0.10 ± 1.52). Firmicutes/ Bacteriodetes ratio (F/B ratio) was higher in PUFA (9.36 ± 1.78) intake compared to other diets but not statistically significant. The overall diet interactions with Firmicutes (p=0.05) and Verrucomicrobia (p=0.02) were significant. The abundance across other phyla did not change between dietary interventions (**Table 3.2**), nor were BMI, nor BMI and diet interactions significant (**Table 3.2**). However, relative abundance of bacterial phyla within BMI showed (Supplementary **Table 3.5**) significant differences in Firmicutes phylum between butter (0.55 ± 0.06) and PUFA (0.74 ± 0.06) specifically within the OW group. Further, this difference was not observed within the OB group at the phylum level (Supplementary **Table 3.6**). Moreover, our results suggest that regardless of the BMI, g_*Peptoniphilus* was lower after cheese (-0.0533 ± 0.0126, p=0.0126, **Figure 3.2**), MUFA (-0.0613 ± 0.0212, p=0.004, **Figure 3.2**) and PUFA (-0.0497 ± 0.0216, p=0.0239, **Figure 3.2**) containing diets compared to butter intake. Further, g_*Streptococcus* was significantly low in MUFA intake compared to butter (-0.155 ± 0.0592, p=0.0105, **Figure 3.2**), but not with CHO, cheese and PUFA containing diets. In addition, g_*Turicibacter* was also significantly high after cheese (0.133 ± 0.0526, p=0.0134, **Figure 3.2**) consumption compared to butter, but not after CHO, MUFA and PUFA intakes.

3.4.3 CORRELATION OF BACTERIAL TAXA WITH CARDIOMETABOLIC RISK FACTORS

Correlation analyses were conducted using Spearman non-parametric analyses, in which associations have been carried out among bacterial taxa and cardiometabolic risk factors. Significant Spearman correlations were observed between specific groups of bacterial communities, including family, genus and order level, with cardiometabolic risk factors and other parameters, including TC, HDL-C, LDL-C and TG levels and HDL-C/TC ratio, CRP, APOb, adiponectin, glucose and insulin levels (Supplementary, **Table 3.7**).

In our Spearman non-parametric analysis, we observed positive correlations with cardiometabolic parameters (**Table 3.3**) suggesting that bacterial taxa belong to the Proteobacteria phylum, *g_Bilophila* , which was associated with TC (ρ=0.253; *p*=0.012), HDL-C/TC (ρ=0.2007; *p*=0.0499), APOb (ρ=0.3638; *p*=0.0003) and glucose (ρ=0.2572; *p*=0.0114), while *g_Sutterella* was associated with TG (ρ=0.2876; *p*=0.0045), HDL-C/TC (ρ=0.2217; *p*=0.0299), CRP (ρ=0.2023; *p*=0.0481), glucose (ρ=0.3499; *p* = 0.0005), and *g_Desulfovibrio* was associated with LDL-C (ρ=0.225; *p*=0.0275). Further, the bacterial taxa residing in the Firmicutes phylum, *g_Epulopiscium*, was associated with TC (ρ=0.2206; *p*=0.0308), TG (ρ=0.3204; *p*=0.0015), HDL-C/TC (ρ=0.203; *p*=0.0473), CRP (ρ=0.2139; *p*=0.0364) and glucose (ρ=0.2158; *p*=0.0347) levels, while *g_Megamonas* was associated with HDL-C/TC (ρ=0.2791; *p*=0.0059), CRP (ρ=0.2881; p=0.0044), glucose (ρ=0.3131; *p*=0.0019), insulin (ρ=0.4829; *p*=<0.0001). In addition, *g_Ruminococcus* species was associated with TG (ρ=0.3858; *p*=0.0001), HDL-C/TC (ρ=0.383; *p*=0.0001) and insulin (ρ=0.2055; *p*=0.0445) levels, while *g_Phascolarctobacterium* was associated with TG (ρ=0.2757; *p*=0.0066), HDL-C/TC (ρ=0.274; *p*=0.0069) and Apob (ρ=0.2448; *p*=0.0162). Also, *g_Collinsella* of the Actinobacteria phylum

was positively associated with HDL-C/TC (ρ=0.3117; *p*=0.002), APOb ρ=0.2181; *p*=0.0328) and insulin ρ=0.23; *p*=0.0209) levels (**Table 3.3**).

3.4.5 PREDICTED METABOLIC FUNCTION OF GUT MICROBIOME

Predicted metabolic function of the gut microbiome between treatments carried out based on differences in bacterial metabolic role was observed with weighted UniFrac indices of OTU. Pairwise analysis of dietary intervention was also performed based on the16S rRNA sequences annotated to KEGG database. Our results suggest that glycosphingolipid synthesis was higher in cheese intake compared to CHO intake (**Figure 3.4 A**, *p* =0.043). In addition, butter consumption increased lysine degradation (**Figure 3.4 E,** *p*=0.036) and lipid biosynthesis proteins (**Figure 3.4 E**, *p*=0.042), compared to CHO intake. Also, propanoate metabolism (**Figure 3.4 F,** *p*=0.041), pyruvate metabolism (**Figure 3.4 F,** *p*=0.041) and phosphatidylinositol signaling (**Figure 3.4 F,** *p*=0.042) were higher following butter intake compared to MUFA intake. G-protein-coupled receptors (**Figure 3.4 C,** *p*=0.029) and sphingolipid metabolism (**Figure 3.4 C**, *p*=0.036) were higher with cheese intake compared to PUFA intake. Further, butter intake increased glycosphingolipid biosynthesis-ganglio series (**Figure 3.4 G,** *p*=8.23e-3), sphingolipid metabolism (**Figure 3.4 G,** *p*=0.012), glycosphingolipid biosynthesis-globo series (**Figure 3.4 G,** *p*=0.013), glycosaminoglycan degradation (**Figure 3.4 G,** *p*=0.017), oxidative phosphorylation (**Figure 3.4 G,** *p*=0.018), ABC transporters (**Figure 3.4 G,** *p*=0.019), phosphatidylinositol signaling system (**Figure 3.4 G,** *p*=0.020), inorganic ion transport and metabolism (**Figure 3.4 G,** *p*=0.040) and steroid hormone biosynthesis (**Figure 3.4 G,** *p*=0.045) compared to PUFA intake. In addition, significant differences were observed between butter and

cheese intake for lipid biosynthesis proteins (**Figure 3.4 D,** p=0.021) and citrate cycle and fatty acid biosynthesis indices (**Figure 3.4 D,** p=0.049).

3.5 DISCUSSION

Our well-controlled cross over feeding trial findings suggest that consumption of various dietary fats from different sources such as cheese and butter, other fats, MUFA rich, and PUFA rich diets and low-fat CHO modulated the intestinal gut microbiome. Different diet consumption patterns have been shown by others to deeply alter the gut microbial structure in animal models (3, 36). In our study, Firmicutes levels were high in PUFA diet consumption and low with CHO diet intake. High-fat diets from animal-based diet, including cheese and meat consumption have been shown to shift the bile-tolerant microbes (*Alistipes*, *Bilophila* and *Bacteroides*) and decreased the levels of Firmicutes which metabolize plant based polysaccharides (*Roseburia*, *Eubacterium rectale* and *Ruminococcus bromii*) structure in human populations also (37). Specifically, higher abundance of *Akkermansia muciniphila* showed better improvement in fasting glucose and triglyceride and body fat distribution in OW and OB people with caloric restriction diet (1200 to 1500 kcal/day) for 6 weeks (38). BMI has a major impact on the gut microbiome and decreased microbial diversity has been observed in obese condition, in which profound changes have noticed in incresed level of Firmicutes and decreased level of Bacteriodetes phylum, and increased pathogenic microbes (39-41). Regardless of BMI in our study, PUFA consumption increased the Firmicutes phylum (**Table 3.2**) and butter intake increased the Verrucomicrobia phylum. In addition, Firmicutes/ Bacteriodetes ratio (F/B ratio) is also commonly used to represent the gut microbiome in both human and animal model studies (42), and significant reduction in F/B ratio and improving the growth of *Bacteriodes*, *Roseburia*,

Faecalibacterium and Clostridium XIVa have observed with obese with calroic restcion diet (43). Further, high saturated fat that is palmitic and an oleic acid-rich diet increased the F/B ratio (44) there were no differences in the F/B ratio among the dietary interventions in our study.

A recent study found that a strong geographical effect on the gut micriobiome diversity has been observed between Mediterranean diet and the modern Paleolithic diet (45-47). Moreover, the sudden decrease in the gut microbiome diversity in Western populations seems to modify the overall gut microbiome state, which may contibute to increase prevelance of some diseases including diabetes, obesity, asthma and inflammatory bowel disaese (10, 48-50). Further, gut microbiome diversity shrinkage has been observed with processed food intake, which have food additives and emulsifies and xenobiotics (51). Diet and BMI alone has significant impact on β-diversity in our study (**Figure 3.3A & 3.3B**). Further, intake of cheese, MUFA and CHO diet has impact on β-diversity between OB vs.OW participants (**Figure 3.3, C**, **E** and **G**). Further, there was no significant impact of different dietary fats intake on the β-diversity within OW group (**Figure 3.3H**), whereas in OB group, different dietary fat consumption alters the β-diversity (**Figure 3.3I**) which clearly reveals that dietary fats from different sources have a major impact on β-diversity. In contrast, a recent study found that there is no difference in β-diversity in healthy men with BMI (23.0 ± 2.1 kg/m^2) consumed high fat diet rich in saturated fat (48% of energy from fat), with an additional 1000 kcal/day as whipping cream (52). Therefore, our human study suggest different dietary fats from differenct sources have vast effect on the gut microbiome diversity in OB rather than in the OW group in a short time feeding but in long term effects yet to determined.

As a diet impact on certain gut microbial species levels was identified in our study, a lower abundance of g_*Peptoniphilus* was observed after cheese, MUFA, and PUFA feeding, whereas g_*Streptococcus* was low abundance only in the MUFA feeding. g_*Peptoniphilus* and g_*Streptococcus* are commensal bacteria belonging to the gram-positive anaerobic cocci, which are isolated from the clinical specimens (53). These gram-positive anaerobic cocci have been understudied, and their precise functions have yet to be determined (54). Our correlation analysis suggested a lower level of g_*Peptoniphilus* which were negatively associated with TG levels and a low abundance of g_*Streptococcus* which were negatively associated with HDL-C/TC (Supplementary, **Table 3.7**). However, the cheese diet was linked to a high level of g_*Turicibacter,* which showed an association with high HDL-C and low CRP levels in our correlation analysis (**Table 3.3** and supplementary **Table 3.7**). A positive correlation of g_*Turicibacter* with HDL-C levels were also observed in a human intervention study (55). An increased level of g_*Turicibacter* has been correlated with production of butyrate when fed a barley-based diet with a high fat content. Also, a lower level of g_*Turicibacter* has been observed after consumption of a high-fat diet (56). Thus, our result suggesting that g_*Turicibacter* may be beneficial effect as a response to cheese intake but the beneficial role of g_*Turicibacter* is yet to explored.

Interestingly, *g_Bilophila* and *g_Sutterella* of the Proteobacteria phylum correlated with increased cardiometabolic parameters including TC, TG, LDL-C, ApoB, CRP and glucose (**Table 3.3**) levels. A study supports the concept that short-time animal fat feeding increases sulphate reducing bacteria and *Bilophila* species, which increases inflammatory responses (57). Hence, bacteria belong to Proteobacteria are able to produce LPS which is responsible for increasing gut permeability and systemic inflammatory responses (58). In addition, hydrogen gas

(H_2) is produced by Desulfovibrio species in the colon during fermentation process (59). However, in our study, *g_Desulfovibrio* was positively associated with LDL-C levels (**Table 3.3**). Further, butyrate producing anaerobes also can produce or use H_2 through the reversible hydrogenases (59, 60). Therefore, *g_Bilophila* species have been generally associated with an increased inflammatory response and may enhance inflammatory disease prevalence in genetic susceptible animals (57). Moreover, *g_Desulfovibrio* has been found in gut inflammatory patients' faeces and gut biopsy samples (61). This evidence suggests that these similar microbial changes can be observed in genetic susceptible humans as well. However, the pathogenicity of *g_Bilophila*, *g_Sutterella* and *g_Desulfovibrio* in human remains to be fully explored.

Our Spearman correlation result shows, *g_Bacteriodes* was positively associated with TC, while f_[Barnesiellaceae] and *g_Butyricimonas* were also positively associated with HDL-C/TC. Also, the predicted metabolic function of the gut microbiome shows cheese and butter intake increases biosynthesis of sphingolipids and glycosphingolipids (**Figure 3.4 A, C** and **G**), which are recognized by G-protein-coupled receptors of the host epithelial cells. Therefore, *Bacteroides* and *Provotella* species provide endogenous bacterial lipids which are physically similar to eukaryotic sphingolipids. This is the possible mechanism through which gut bacteria influence mammalian host lipid metabolism (62). However, the biochemical and functional roles of sphingolipids are yet to determined, and more research is needed to understand how the gut microbiome is involved in the production various type of sphingolipids.

Strength of current study: The study was designed as a crossover and controlled full feeding design. Limitations of the study include smaller sample size longer period of commitment for participants and difficulty to control the environmental factor (weather and climate) impact on

gut microbiome. In addition, fecal microbiome richness and diversity indices were calculated with the depth of 11,000 sequences per sample and if the depth of sequence reads were increased per sample, it would provide more details about microbiome richness and diversity and addressed some unknown scientific questions.

Overall, this study's highlight is that it used a crossover, randomized design to investigate the impact of SFA from dairy and from other dietary source on gut microbiome to improve CV health. Moreover, BMI has a significant impact on the β-diversity of the gut microbiome. Thus, diet-induced changes have a role in shifting the gut microbiome to some extent, leading to reduced CVD risk through the predicated metabolic roles of gut bacteria (PICRUST). Further, metagenomics, or meta-transcriptomics sequencing approaches are required to confirm the PICRUST predictions. In general, gut health and gut homeostasis are maintained by the correct proportion of gut microbiome, such as richness, stability, and diversity of gut microbes. Individuals who are low in microbial richness are more susceptible to obesity, dyslipidemia, and insulin resistance than individuals with high microbial richness (18, 19, 63). In the present study, the β-microbial diversity significantly changed between BMI groups, suggesting that a dietary component or the food matrix of these diets may be altering certain microbial community. Thus, our study reveals that intake of various dietary fats, including cheese, butter, MUFA, PUFA and CHO shifts in certain microbial species, which may be associated with physiologically important downstream CVD related health effects.

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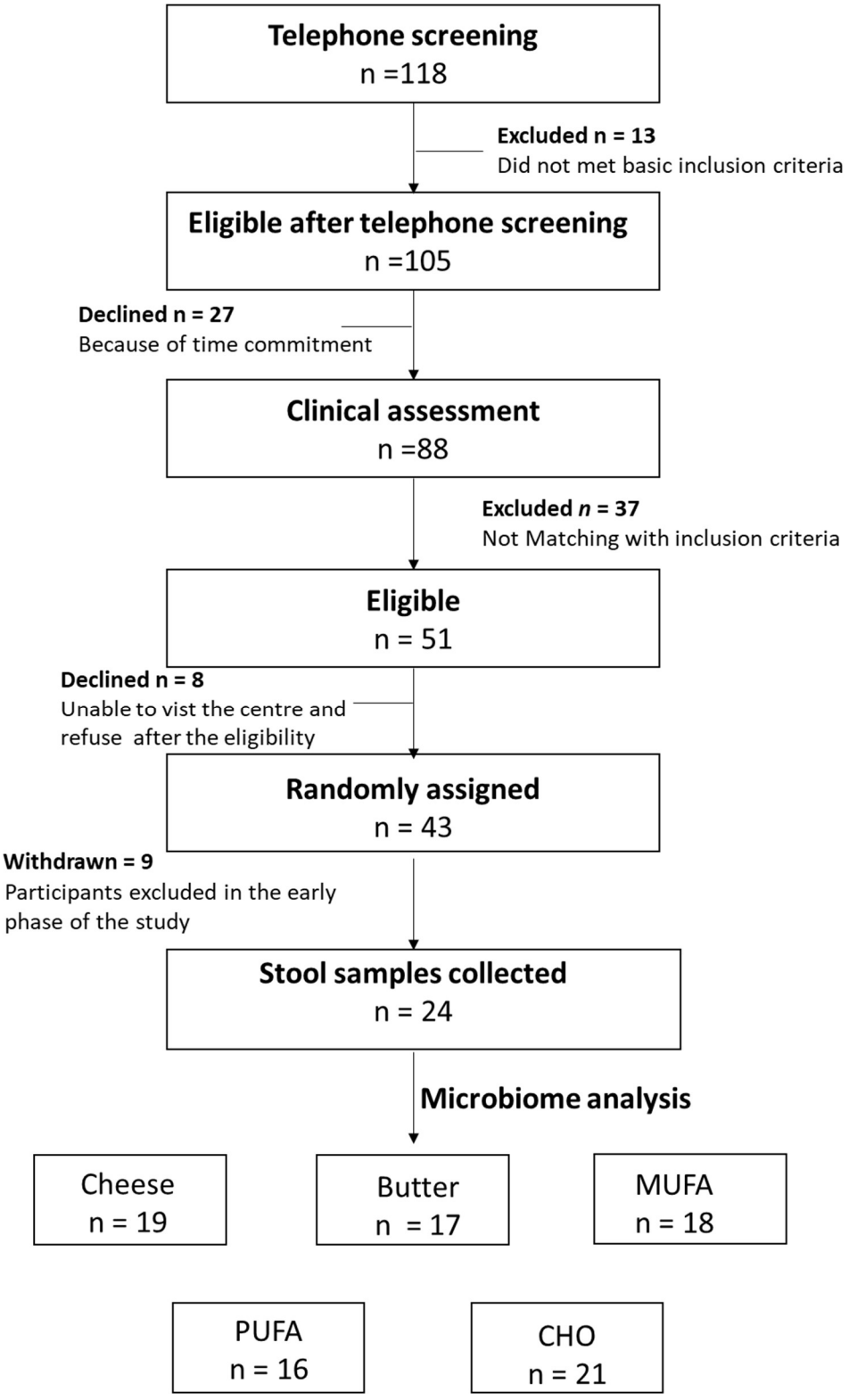


Figure 3. 1 Flow chart

Table 3. 1 Consumption of cheese and butter form dairy source and other dietary intervention on gut microbial diversity

Diversity indices[1]	Diets[2]					Interaction *P* value[3]		
	Cheese (n=19)	Butter (n=17)	MUFA (n=18)	PUFA (n=16)	CHO (n=21)	Diet	BMI	Diet x BMI
Shannon	6.66 ± 0.19	6.10 ± 0.20	6.10 ± 0.19	6.20 ± 0.20	6.47 ± 0.18	0.02	0.29	0.41
Simpson	0.96 ± 0.01	0.93 ± 0.02	0.94 ± 0.01	0.93 ± 0.01	0.96 ± 0.01	0.25	0.36	0.38
Observed species	955.4 ± 44.2	870.3 ± 46.4	816.6 ± 44.9	848.4 ± 46.9	897.9 ± 42.5	0.10	0.25	0.32
Chao1	2044.6 ± 110.7	1904.5 ± 116.9	1701.9 ± 112.7	1757.6 ± 118.6	1945.7 ± 105.5	0.13	0.48	0.33
Goods coverage	0.955 ± 0.002	0.958 ± 0.002	0.962 ± 0.002	0.961 ± 0.002	0.958 ± 0.002	0.12	0.38	0.25
PD whole tree	38.48 ± 1.33	35.16 ± 1.36	35.13 ± 1.33	35.28 ± 1.37	36.93 ± 1.30	0.004	0.21	0.36

[1]Shannon, Simpson and PD whole tree are diversity estimators a; Chao1 and Good's coverage are the richness indices.

[2]Five isocaloric diets (35 % energy from fats) included; (i) cheese, (ii) butter, (iii) monounsaturated fatty acids (MUFA), (iv) polyunsaturated fatty acids (PUFA) and (v) a low-fat, high-carbohydrate (CHO).

[3]*P* values are shown as a diet effect among the diet group estimated by GLIMMIX procedure (SAS version 9.4), Bonferroni correction adjustment was used analysis the change in relative abundance of phylum between interventions. Diet, sex and BMI considered as fixed effect, subject as random effect.

[3]*P* value ≤ 0.05 consider as significant, [a,b]*superscripts were significantly different.*

Table 3. 2 Relative abundances of bacterial phyla in human gut after consumption cheese and butter from dairy and other dietary sources

	Diets[1]					Interaction *P* value[3]		
Phylum[2]	Cheese (n=19)	Butter (n=17)	MUFA (n=18)	PUFA (n=16)	CHO (n=21)	Diet	BMI	Diet x BMI
Firmicutes	70.77 ± 3.675^{ab}	60.85 ± 3.78^{ab}	67.03 ± 3.66^{ab}	74.40 ± 3.81^{a}	65.90 ± 3.48^{b}	0.05	0.24	0.79
Bacteroidetes	22.56 ± 3.13	27.63 ± 3.28	27.56 ± 3.17	18.74 ± 3.32	26.84 ± 3.00	0.11	0.18	0.55
Actinobacteria	4.41 ± 0.63	2.54 ± 0.66	2.56 ± 0.64	3.93 ± 0.67	3.55 ± 0.60	0.07	0.34	0.51
Proteobacteria	1.42 ± 0.28	1.54 ± 0.28	1.27 ± 0.28	1.13 ± 0.29	1.95 ± 0.26	0.06	0.13	0.92
Verrucomicrobia	0.10 ± 1.52^{a}	4.64 ± 1.52^{b}	0.83 ± 1.48^{ab}	0.77 ± 1.54^{ab}	1.16 ± 1.45^{ab}	0.02	0.77	0.37
Firmicutes/ Bacteroidetes ratio	3.70 ± 1.74	5.45 ± 1.75	4.83 ± 1.70	9.36 ± 1.78	3.24 ± 1.58	0.09	0.34	0.59

[1]Five isocaloric diets (35 % energy from fats) included; (i) cheese, (ii) butter, (iii) monounsaturated fatty acids (MUFA), (iv) polyunsaturated fatty acids (PUFA) and (v) a low-fat, high-carbohydrate (CHO).

[2]Bacteria phylum abundance (OTU, level 2) was identified from the sample after the intervention, all values were represented as least square mean with standard error of mean and >1% of the community. GLIMMIX procedure (SAS version 9.4)., Bonferroni correction adjustment was used analysis the change in relative abundance of phylum between interventions. Diet, sex and BMI considered as fixed effect, subject as random effect.

[3]*P* value ≤ 0.05 considered as significant, *[a,b]superscripts were significantly different.*

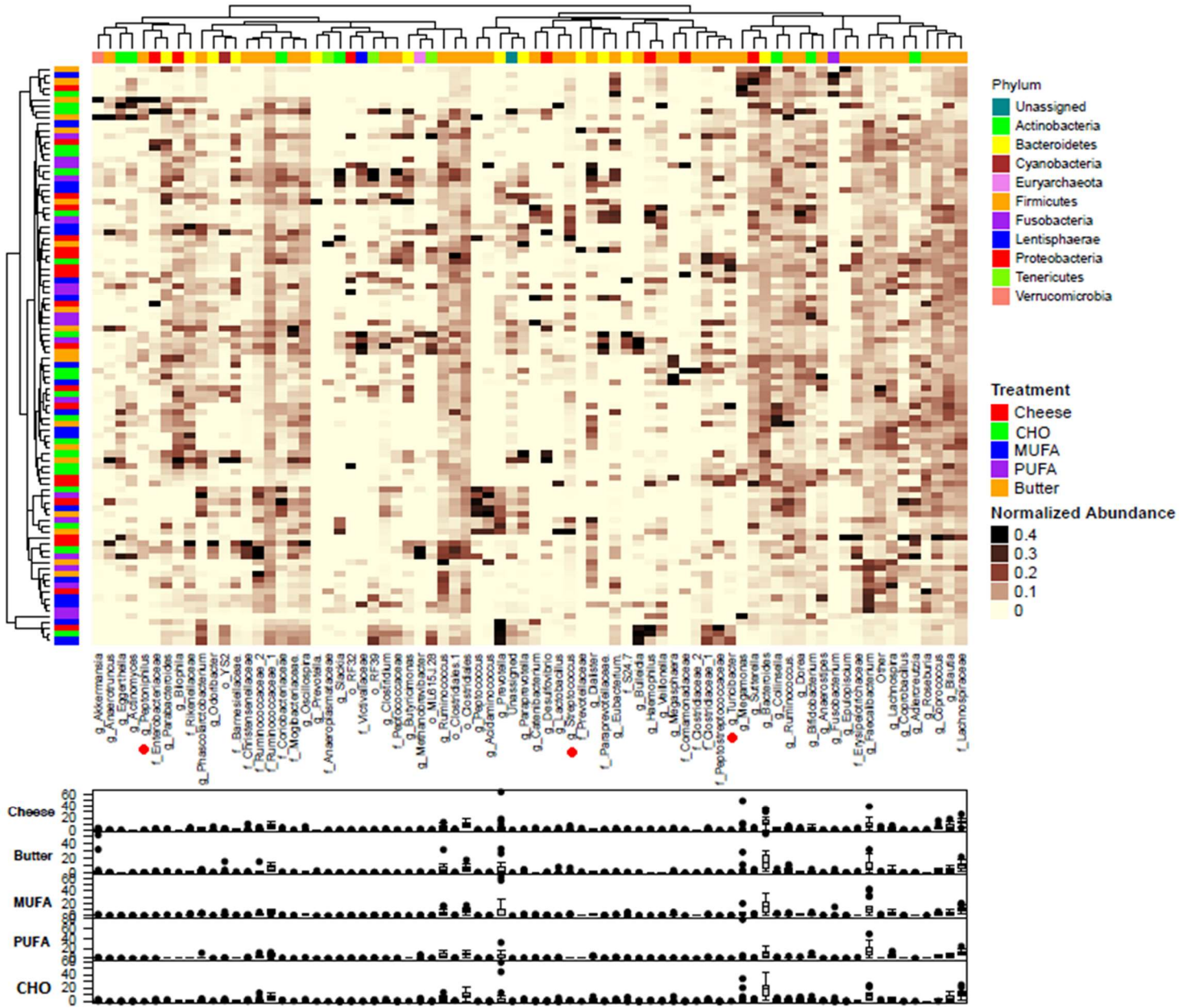

Phylum
Unassigned
Actinobacteria
Bacteroidetes
Cyanobacteria
Euryarchaeota
Firmicutes
Fusobacteria
Lentisphaerae
Proteobacteria
Tenericutes
Verrucomicrobia
Treatment
Cheese
CHO
MUFA
PUFA
Butter
Normalized Abundance
0.4
0.3
0.2
0.1
0
g_Akkermansia
g_Anaerotruncus
g_Eggerthella
g_Actinomyces
g_Peptoniphilus
f_Enterobacteriaceae
g_Parabacteroides
g_Blautia
f_Rikenellaceae
g_Phascolarctobacterium
g_Odoribacter
o_YS2
f_Barnesiellaceae
f_Christensenellaceae
f_Ruminococcaceae_2
f_Ruminococcaceae_1
f_Coriobacteriaceae
f_Mogibacteriaceae
g_Oscillospira
g_Prevotella
f_Anaeroplasmataceae
g_Sarcina
g_RF32
f_Victivallaceae
o_RF39
g_Clostridium
f_Peptococcaceae
g_Butyricimonas
g_Methanobrevibacter
o_MB15J28
g_Ruminococcus
o_Clostridiales_1
o_Clostridiales
g_Peptococcus
g_Acidaminococcus
g_Prevotella
Unassigned
g_Parapreevotella
g_Catenibacterium
g_Desulfovibrio
g_Lactobacillus
g_Streptococcus
f_Prevotellaceae
g_Dialister
f_Paraprevotellaceae
g_Eubacterium
f_S24_7
g_Bilieida
g_Haemophilus
g_Veillonella
g_Megasphaera
f_Comamonadaceae
f_Clostridiaceae_2
f_Clostridiaceae_1
f_Peptostreptococcaceae
g_Turicibacter
g_Megamonas
g_Sutterella
g_Bacteroides
g_Colinsella
g_Ruminococcus
g_Dorea
g_Bifidobacterium
g_Anaerostipes
g_Fusobacterium
g_Epulopiscium
f_Erysipelotrichaceae
g_Faecalibacterium
Other
g_Lachnospira
g_Coprobacillus
g_Adlercreutzia
g_Roseburia
g_Coprococcus
g_Blautia
f_Lachnospiraceae
Cheese
Butter
MUFA
PUFA
CHO

Figure 3. 2 Heatmap of phylum and genus level of microbiome abundance with dietary interventions in humans.

Genus *Peptoniphilus* was significantly low in cheese (-0.0533 ± 0.020, $p = 0.012$), MUFA (-0.0613 ± 0.021, $p = 0.004$) and PUFA (-0.0497 ± 0.021, $p = 0.023$) intake compared to butter, but not with CHO. Further, genus *Streptoccous* was significantly low in MUFA intake (-0.155 ± 0.059, $p = 0.010$) compared to butter, but not CHO, cheese and PUFA intake. Also, genus *Turcibacter* was high abundance in cheese consumption (0.133 ± 0.052, $p = 0.013$) compared to butter, but not with CHO, MUFA and PUFA consumption (shown in red dot).

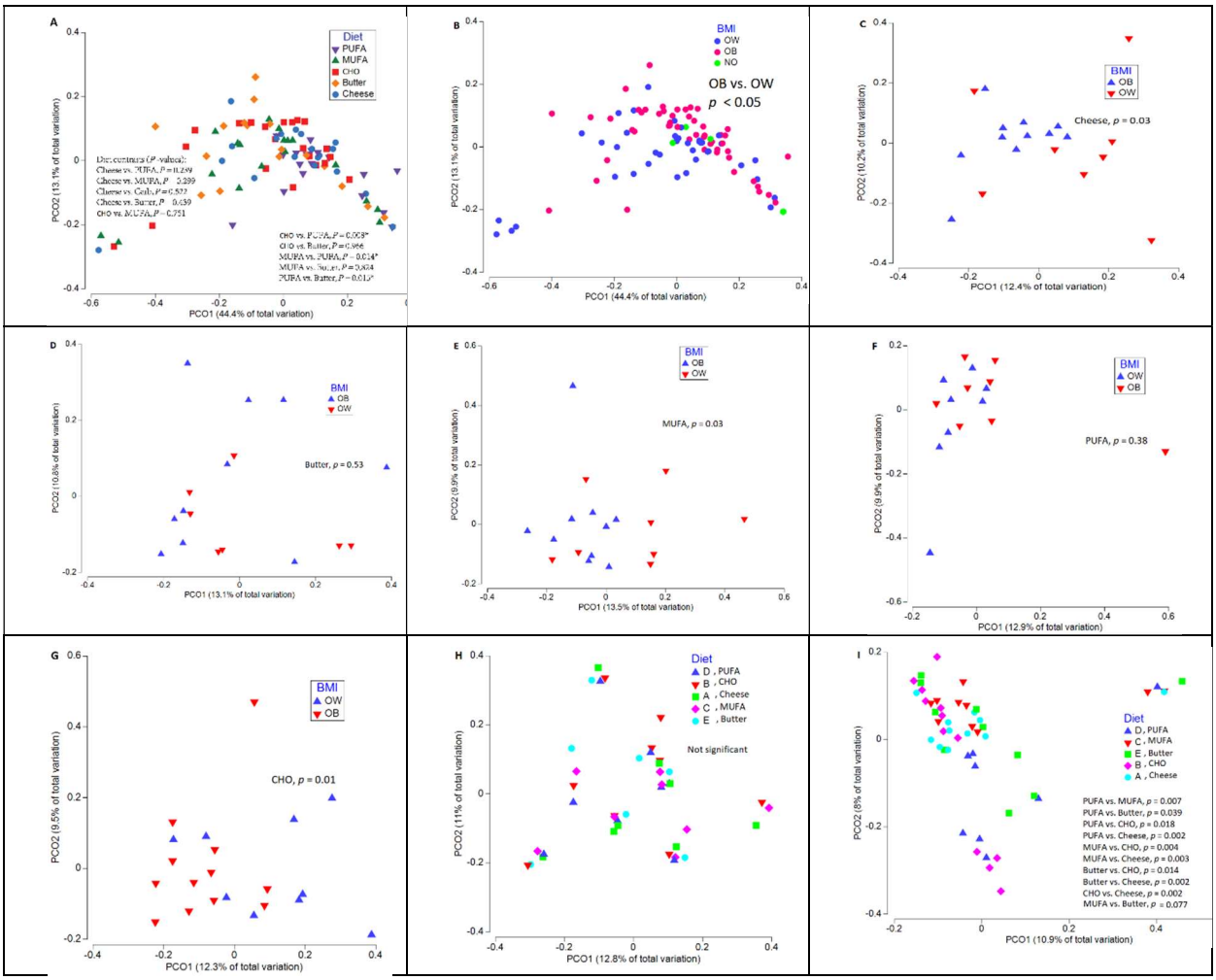


Figure 3. 3 Principal coordinates analysis (PCoA) of weighted UniFrac distances.

Consumption of isocaloric diets, 35% energy from fats with different dietary sources were tested on the human gut microbiome (Figure 3A). The effect of body mass index, BMI (Figure 3B), [Overweight BMI range =25–29.9 kg /m^2 (OW), obese BMI range = >30 kg/ m^2 (OB) and Normal weight BMI range =18.5–24.9 kg /m^2 (ON); OB vs. OW, $p < 0.05$]. The effect of dietary intervention and BMI impact on the human gut microbiome, cheese (OB vs. OW, $p = 0.03$, Figure 3C), butter (OB vs. OW, $p = 0.53$, Figure 3D), CHO (OB vs. OW, $p = 0.01$, Figure 3E), MUFA (OB vs. OW, $p = 0.03$, Figure 3F) and PUFA (OB vs. OW, $p = 0.38$, Figure 3G). The effect of different dietary intervention within BMI, such as OW people (Figure 3H) or OB participants (Figure 3I) on the human gut microbiome. For pairwise comparison, Bray Curtis p-value of among the dietary intervention, BMI, specific diet intervention with OB vs. OW and all dietary interventions within OB or OW were calculated using PERMANOVA, Primer 7 and considered significant for $p \leq 0.05$.

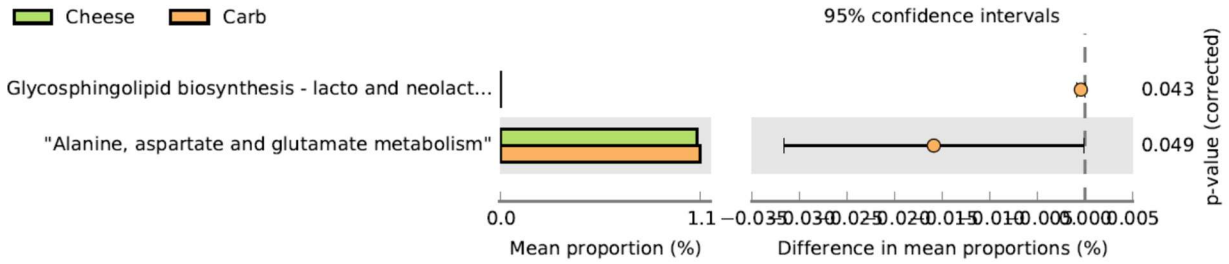
Table 3. 3 Non-parametric correlation analysis of bacterial taxa and positive association with metabolic parameters

Phylum	Taxa[1]	Metabolic parameters[2]	Spearman's ρ[3]	p value[4]
Proteobacteria	g_Bilophila	TC	0.2534	0.0127
	,,	HDL-C/TC	0.2007	0.0499
	,,	APOb	0.3638	0.0003
	,,	Glucose	0.2572	0.0114
	g_Desulfovibrio	LDL-C	0.225	0.0275
	g_Sutterella	TG	0.2876	0.0045
	,,	HDL-C/TC	0.2217	0.0299
	,,	CRP	0.2023	0.0481
Firmicutes	g_Epulopiscium	TC	0.2206	0.0308
	,,	TG	0.3204	0.0015
	,,	HDL-C/TC	0.203	0.0473
	,,	CRP	0.2139	0.0364
	,,	Glucose	0.2158	0.0347
	g_[Ruminococcus]	TG	0.3858	0.0001
	,,	HDL-C/TC	0.383	0.0001
	,,	Insulin	0.2055	0.0445
	g_Phascolarctobacterium	TG	0.2757	0.0066
	,,	HDL-C/TC	0.274	0.0069
	,,	ApoB	0.2448	0.0162
	g_Megamonas	HDL-C/TC	0.2791	0.0059
	,,	CRP	0.2881	0.0044
	,,	Glucose	0.3131	0.0019
	,,	Insulin	0.4829	<0.0001
	g_Dorea	Insulin	0.2156	0.0349
	g_Coprobacillus	TG	0.2443	0.0165
	g_Bulleidia	Adiponectin	0.3483	0.0005
	g_Clostridium	,,	0.2684	0.0082
	g_Veillonella	Glucose	0.2747	0.0068
	g_Anaerostipes	,,	0.2444	0.0164
	g_Catenibacterium	,,	0.205	0.0451
	g_[Eubacterium]	Insulin	0.2153	0.0351
	g_Lactobacillus	,,	0.2452	0.016
	g_Megasphaera	,,	0.2449	0.0162
	g_Peptoniphilus	,,	0.3211	0.0014

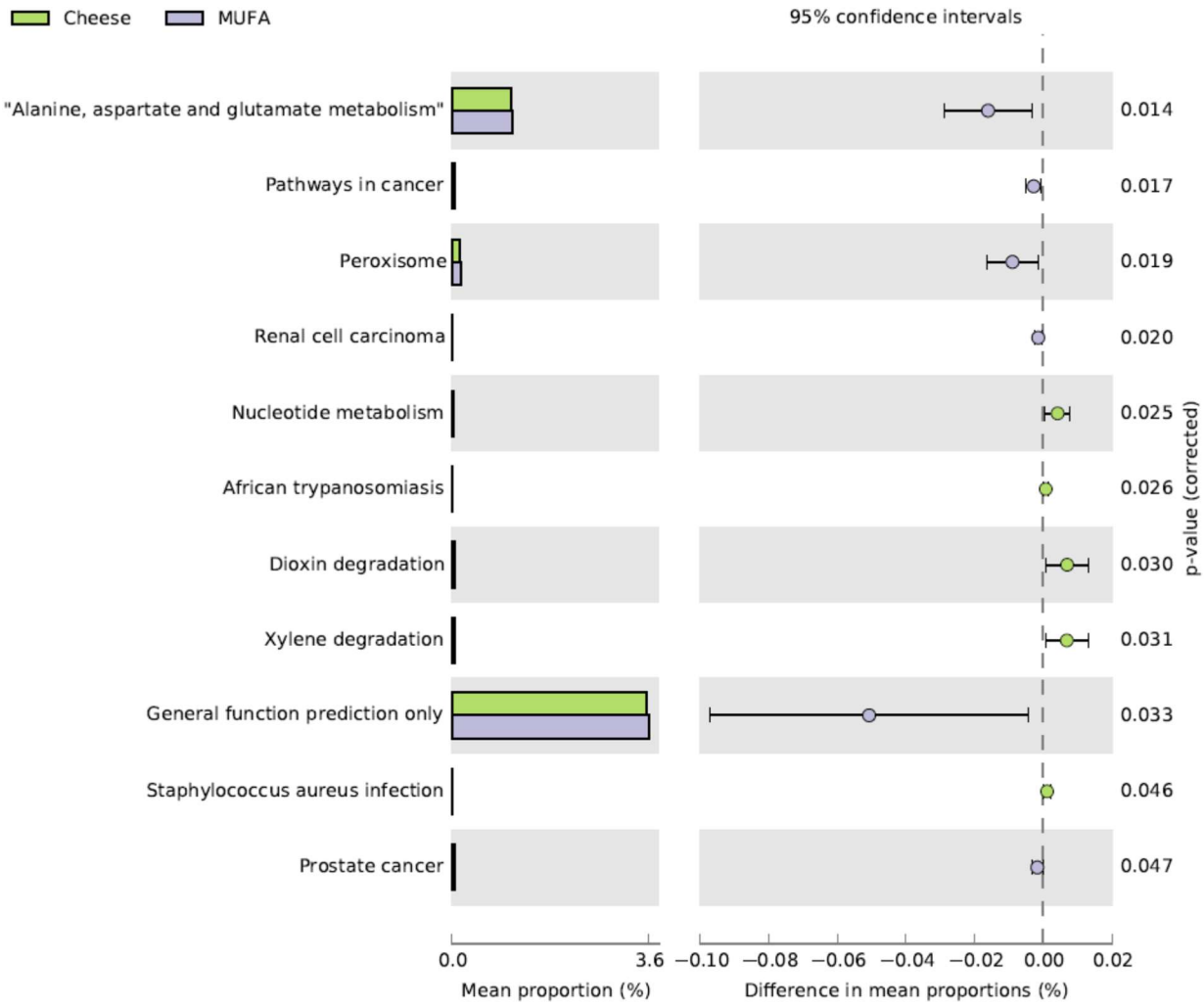
Bacteroidetes	g_Bacteroides	TC	0.2348	0.0213
	f_[Barnesiellaceae]	HDL-C/TC	0.2593	0.0107
	g_Butyricimonas	,,	0.2171	0.0336
	,,	APOb	0.3098	0.0021
	g_Paraprevotella	Adiponectin	0.2475	0.015
Actinobacteria	g_Eggerthella	TG	0.2036	0.0466
	,,	Insulin	0.3277	0.0011
	g_Collinsella	HDL-C/TC	0.3117	0.002
	,,	APOb	0.2181	0.0328
	,,	Insulin	0.2354	0.0209

[1]Taxa were denoted as c, class; o, order; f, family and g, genus levels; [2]Anthropometrics, metabolic parameters and blood pressure measurements recorded at the end of the dietary interventions: TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein; LDL-C, low-density lipoprotein; HDL-C/TC, ratio of high density lipoprotein/ total cholesterol; CRP, C-reactive protein; APOb, Apolipoprotein; [3]Non-parametric Spearman's rank correlation coefficient; [4]Spearmen's correlation probability values.

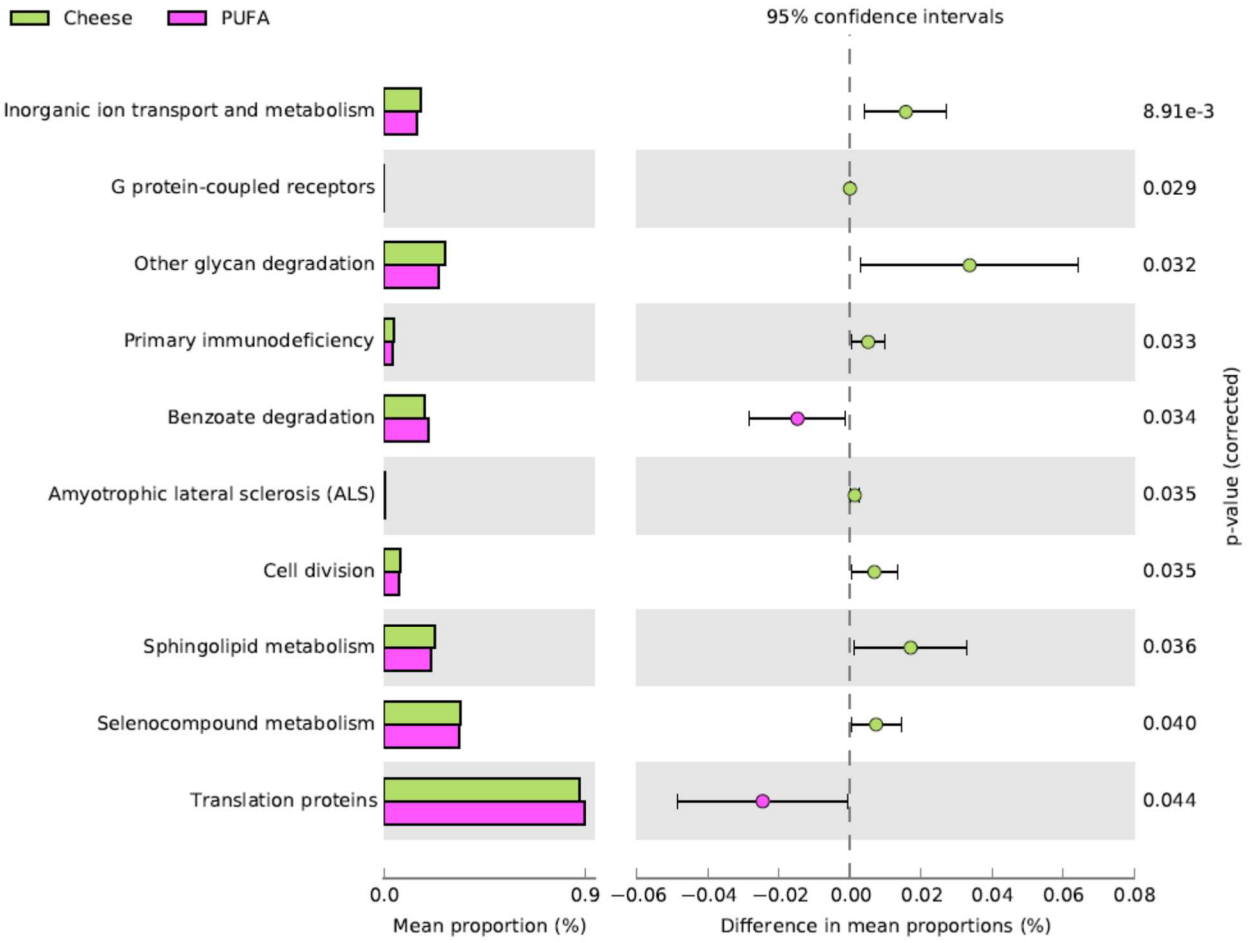
A


Cheese
Carb
95% confidence intervals
Glycosphingolipid biosynthesis - lacto and neolact...
"Alanine, aspartate and glutamate metabolism"
0.043
0.049
p-value (corrected)
0.0
1.1
−0.035
−0.030
−0.025
−0.020
−0.015
−0.010
−0.005
0.000
0.005
Mean proportion (%)
Difference in mean proportions (%)

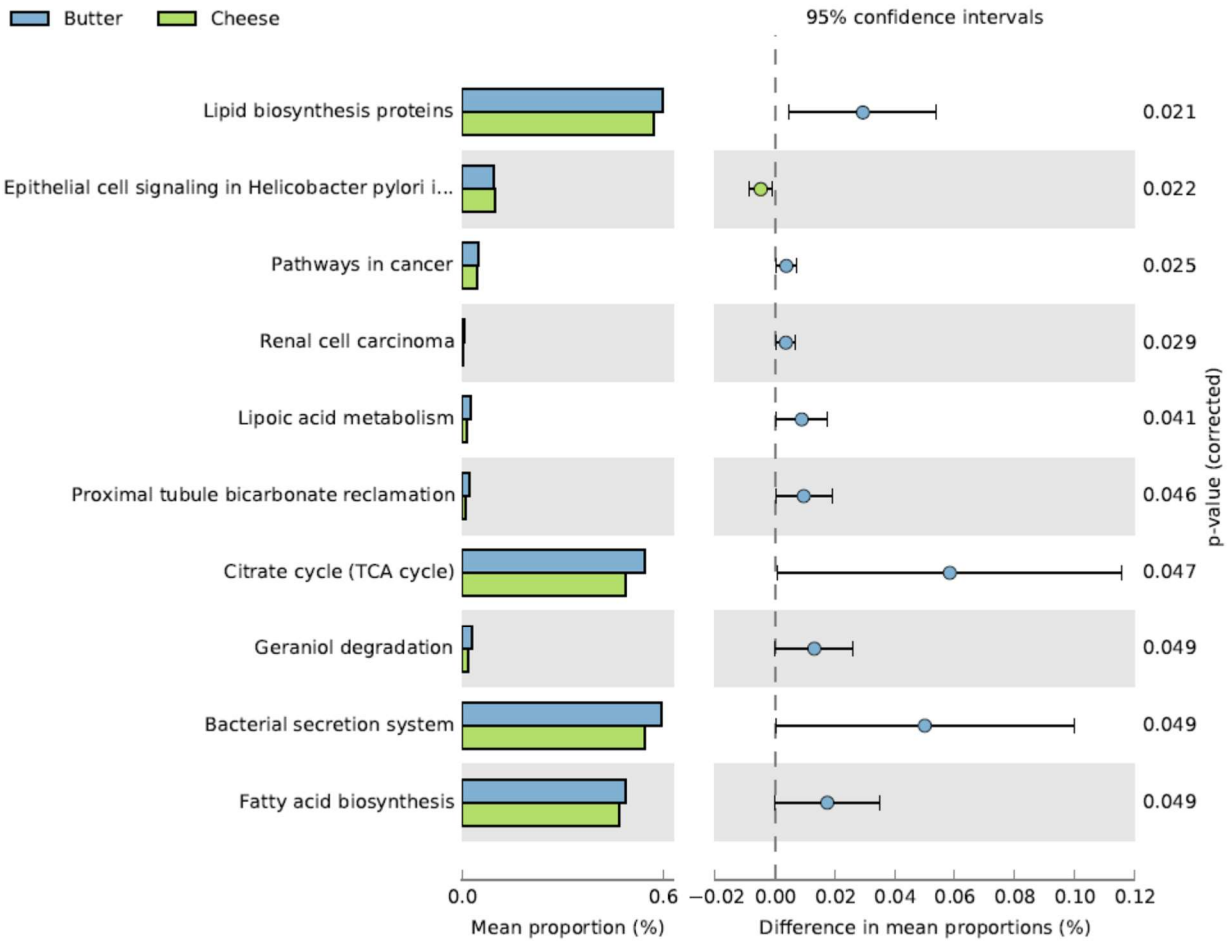
B


Cheese
MUFA
95% confidence intervals
"Alanine, aspartate and glutamate metabolism"
Pathways in cancer
Peroxisome
Renal cell carcinoma
Nucleotide metabolism
African trypanosomiasis
Dioxin degradation
Xylene degradation
General function prediction only
Staphylococcus aureus infection
Prostate cancer
0.014
0.017
0.019
0.020
0.025
0.026
0.030
0.031
0.033
0.046
0.047
p-value (corrected)
0.0
3.6
−0.10
−0.08
−0.06
−0.04
−0.02
0.00
0.02
Mean proportion (%)
Difference in mean proportions (%)

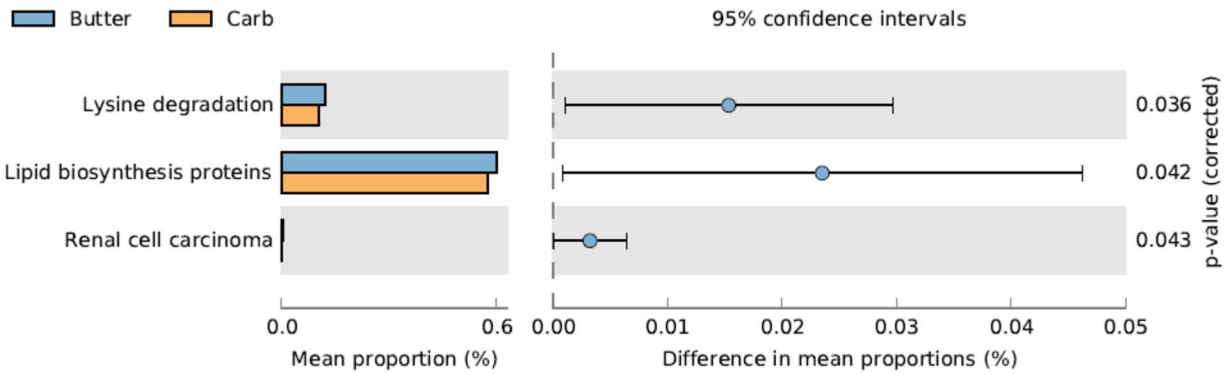
C


Cheese
PUFA
95% confidence intervals
Inorganic ion transport and metabolism
G protein-coupled receptors
Other glycan degradation
Primary immunodeficiency
Benzoate degradation
Amyotrophic lateral sclerosis (ALS)
Cell division
Sphingolipid metabolism
Selenocompound metabolism
Translation proteins
8.91e-3
0.029
0.032
0.033
0.034
0.035
0.035
0.036
0.040
0.044
p-value (corrected)
0.0
0.9
Mean proportion (%)
−0.06
−0.04
−0.02
0.00
0.02
0.04
0.06
0.08
Difference in mean proportions (%)

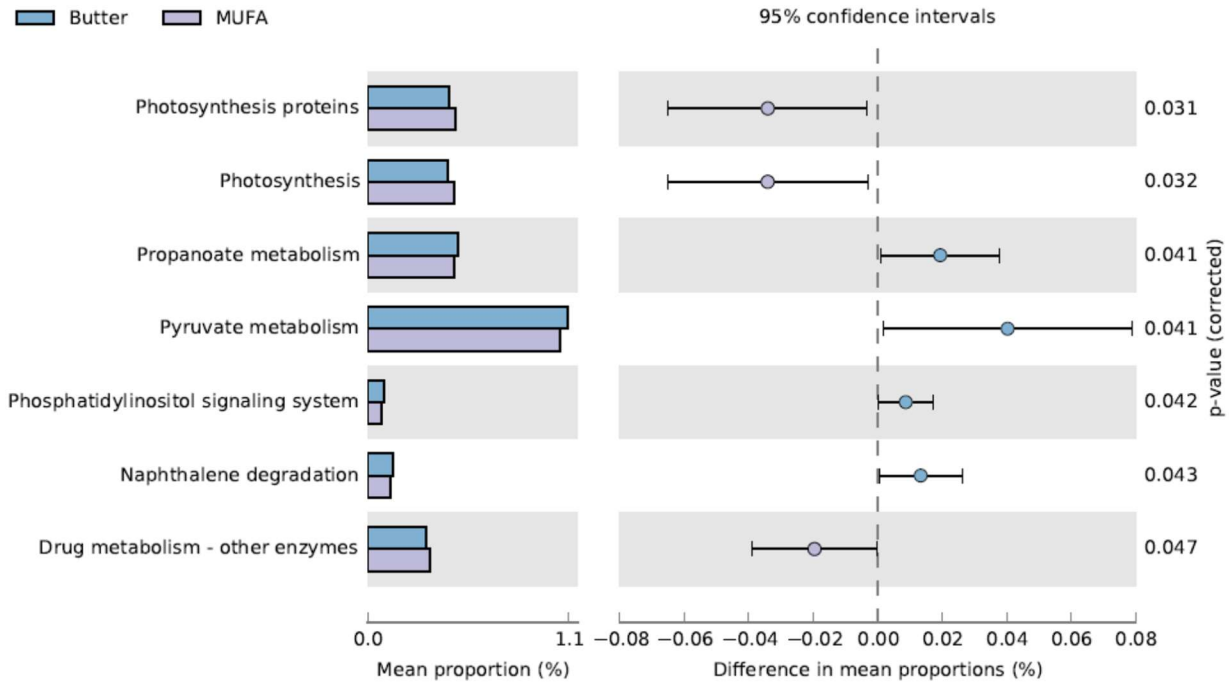
D


Butter
Cheese
95% confidence intervals
Lipid biosynthesis proteins
Epithelial cell signaling in Helicobacter pylori i...
Pathways in cancer
Renal cell carcinoma
Lipoic acid metabolism
Proximal tubule bicarbonate reclamation
Citrate cycle (TCA cycle)
Geraniol degradation
Bacterial secretion system
Fatty acid biosynthesis
0.021
0.022
0.025
0.029
0.041
0.046
0.047
0.049
0.049
0.049
p-value (corrected)
0.0
0.6
Mean proportion (%)
−0.02
0.00
0.02
0.04
0.06
0.08
0.10
0.12
Difference in mean proportions (%)

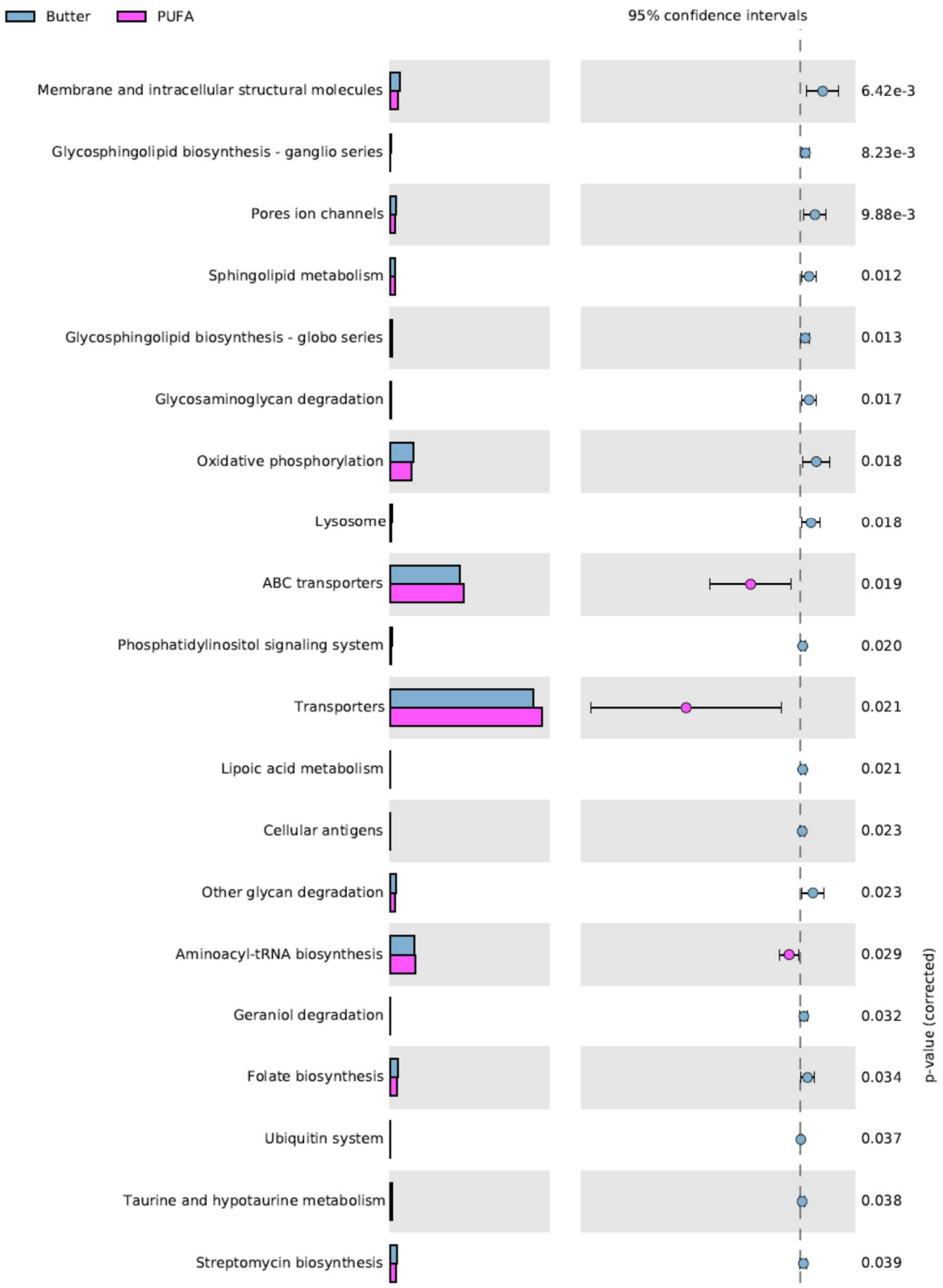
E


Butter
Carb
95% confidence intervals
Lysine degradation
Lipid biosynthesis proteins
Renal cell carcinoma
0.036
0.042
0.043
p-value (corrected)
0.0
0.6
Mean proportion (%)
0.00
0.01
0.02
0.03
0.04
0.05
Difference in mean proportions (%)

F


Butter
MUFA
95% confidence intervals
Photosynthesis proteins
Photosynthesis
Propanoate metabolism
Pyruvate metabolism
Phosphatidylinositol signaling system
Naphthalene degradation
Drug metabolism - other enzymes
0.031
0.032
0.041
0.041
0.042
0.043
0.047
p-value (corrected)
0.0
1.1
Mean proportion (%)
−0.08
−0.06
−0.04
−0.02
0.00
0.02
0.04
0.06
0.08
Difference in mean proportions (%)

G


Butter
PUFA
95% confidence intervals
Membrane and intracellular structural molecules
Glycosphingolipid biosynthesis - ganglio series
Pores ion channels
Sphingolipid metabolism
Glycosphingolipid biosynthesis - globo series
Glycosaminoglycan degradation
Oxidative phosphorylation
Lysosome
ABC transporters
Phosphatidylinositol signaling system
Transporters
Lipoic acid metabolism
Cellular antigens
Other glycan degradation
Aminoacyl-tRNA biosynthesis
Geraniol degradation
Folate biosynthesis
Ubiquitin system
Taurine and hypotaurine metabolism
Streptomycin biosynthesis
6.42e-3
8.23e-3
9.88e-3
0.012
0.013
0.017
0.018
0.018
0.019
0.020
0.021
0.021
0.023
0.023
0.029
0.032
0.034
0.037
0.038
0.039
p-value (corrected)

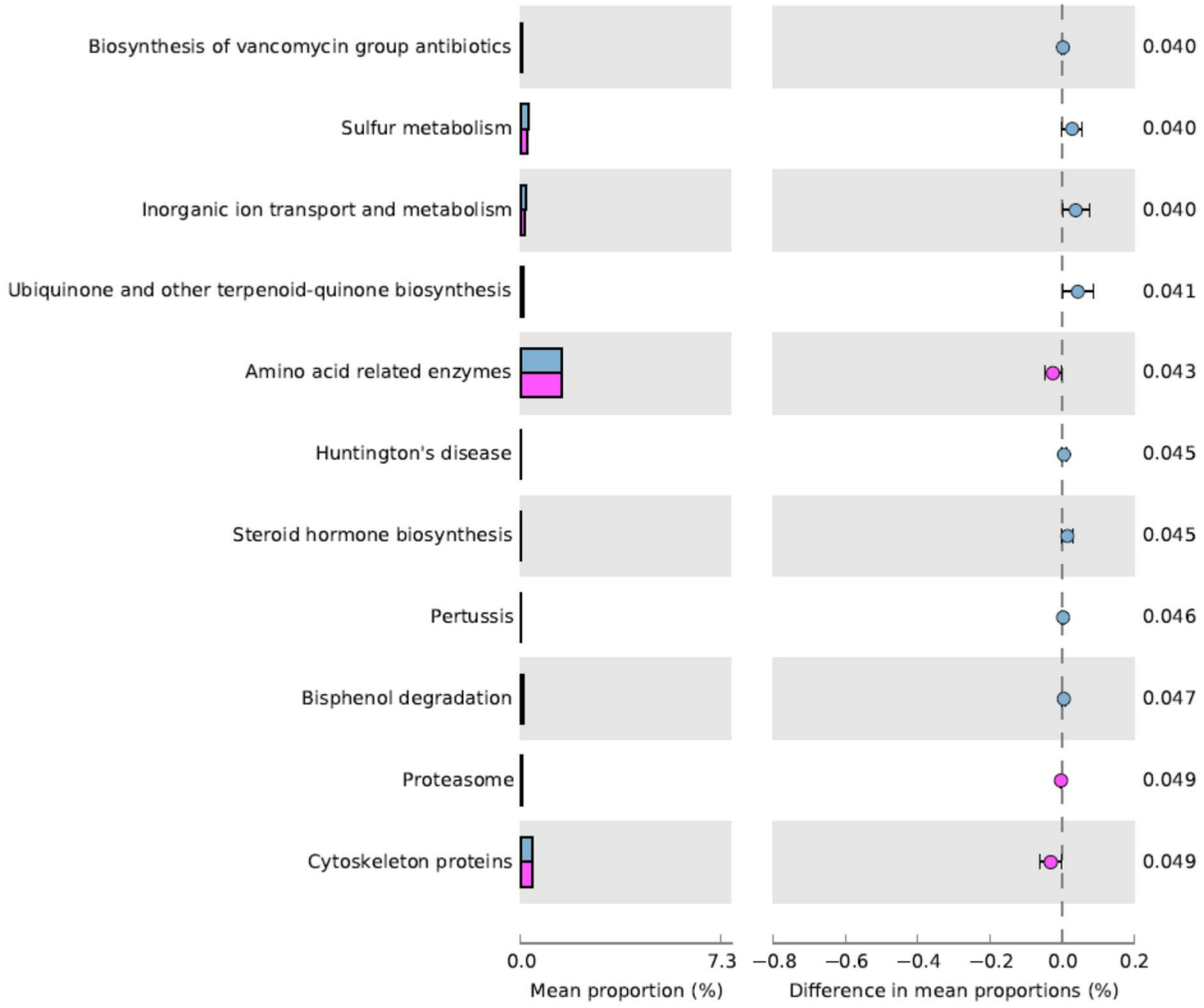


Figure 3. 4 Predicted metabolic function of the gut microbiome based on the 16rRNA sequences annotated to KEGG database.

(A) cheese vs. CHO, (B) cheese vs. MUFA, (C) cheese vs. PUFA, (D) butter vs. cheese, (E) butter vs. CHO, (F) butter vs. MUFA, (G) butter vs. PUFA

Supplementary data

Table 3. 4 Baseline characteristics for participants in microbiome analysis

	RCFFN (*n*=25)
Ethnicity, n (%)	
Caucasian	10 (40)
Asian	5 (20)
African/African American	5 (20)
Hispanic	4 (16)
Other	1 (4)
Women, n (%)	8 (32)
Men, n (%)	17 (68)
Age[1], y	38.9 ± 13.8
Body[1] w, kg	89.5 ± 19.8
BMI[1], kg/m^2	32.6 ± 5.2
Waist circumference[1], cm	101.2 ± 9.3

[1]Values are represented as means ± SDs

Table 3. 5 Relative abundances of bacterial phyla in human gut after consumption cheese and butter from dairy source and other dietary sources in overweight people (OW)

	Diets[2]					Interaction *P* value[3]
Phylum[2]	Cheese (n=8)	Butter (n=7)	MUFA (n=8)	PUFA(n=8)	CHO (n=9)	Diet
Firmicutes	67 ± 5.7	55 ± 6.0[a]	62 ± 6.0	74 ± 6.0[b]	65 ± 6.0	0.03
Bacteroidetes	29 ± 6.0	33 ± 6.0	31 ± 6.0	19 ± 6.0	28 ± 5.0	0.16
Actinobacteria	3.0 ± 0.1	2.0 ± 1.0	2.0 ± 1.0	4.0 ± 1.0	3.0 ± 0.9	0.71
Proteobacteria	1.3 ± 0.3	1.4 ± 0.3	1.2 ± 0.3	0.7 ± 0.3	2.1 ± 0.3	0.23
Firmicutes/ Bacteroidetes ratio	2.36 ± 2.65	2.27 ± 2.65	3.90 ± 2.48	10.34 ± 2.48	3.81 ± 2.34	0.17
Verrucomicrobia	-	-	-	-	-	-

[1]Five isocaloric diets (35 % energy from fats) included; (i) cheese, (ii) butter, (iii) monounsaturated fatty acids (MUFA), (iv) polyunsaturated fatty acids (PUFA) and (v) a low-fat, high-carbohydrate (CHO).

[2]Bacterila phylum abundance (OTU, level 2) was identified from the sample after the intervention, all values were represented as least square mean with standard error of mean and >1% of the community. GLIMMIX procedure (SAS version 9.4)., Bonferroni correction adjustment was used analysis the change in relative abundance of phylum between interventions. Diet and sex as fixed effect, subject as random effect.

[3]*P* value ≤ 0.05 consider as significant, [a,b]*superscripts were significantly different.*

Table 3. 6 Relative abundances of bacterial phyla in human gut after consumption cheese and butter from dairy source and other dietary sources in obese people (OB)

	Diets[1]					Interaction *P* value[3]
Phylum[2]	Cheese (n=11)	Butter (n=10)	MUFA (n=10)	PUFA(n=8)	CHO (n=12)	Diet
Firmicutes	75 ± 4.4	65 ± 4.5	71 ± 4.5	76 ± 5.0	67 ± 4.2	0.35
Bacteroidetes	17 ± 2.0	24 ± 4.0	24 ± 4.0	19 ± 4.0	26 ± 3.0	0.36
Actinobacteria	5.6 ± 0.8	2.7 ± 0.8	3.0 ± 0.8	4.0 ± 0.9	3.7 ± 0.9	0.05
Proteobacteria	1.7 ± 0.4	1.8 ± 0.4	1.7 ± 0.4	1.3 ± 0.4	2.3 ± 0.3	0.23
Firmicutes/ Bacteroidetes ratio	5.22 ± 2.23	7.90 ± 2.25	6.23 ± 2.23	8.50 ± 2.48	2.35 ± 2.11	0.19
Verrucomicrobia	0.7 ± 2.4	0.2 ± 2.4	5.5 ± 2.4	0.2 ± 2.7	1.3 ± 2.2	0.42

[1]Five isocaloric diets (35 % energy from fats) included; (i) cheese, (ii) butter, (iii) monounsaturated fatty acids (MUFA), (iv) polyunsaturated fatty acids (PUFA) and (v) a low-fat, high-carbohydrate (CHO).

[2]Bacterila phylum abundance (OTU, level 2) was identified from the sample after the intervention, all values were represented as least square mean with standard error of mean and >1% of the community. GLIMMIX procedure (SAS version 9.4)., Bonferroni correction adjustment was used analysis the change in relative abundance of phylum between interventions. Diet and sex as fixed effect, subject as random effect.

[3]*P* value ≤ 0.05 consider as significant.

Table 3. 7 Non-parametric correlation analysis of bacterial taxa and metabolic parameters

Metabolic parameters[1]	Phylum	Taxa[2]	Spearman's ρ[3]	p value[4]
TC	Proteobacteria	g_Bilophila	0.2534	0.0127
	Firmicutes	g_Epulopiscium	0.2206	0.0308
	Firmicutes	g_Peptococcus	-0.318	0.0016
	Firmicutes	g_Ruminococcus	-0.2484	0.0147
	Firmicutes	f_Ruminococcaceae_2	-0.3447	0.0006
	Firmicutes	g_[Eubacterium]	-0.2132	0.0371
	Bacteroidetes	g_Bacteroides	0.2348	0.0213
	Bacteroidetes	f_Prevotellaceae	-0.2798	0.0058
	Bacteroidetes	g_Prevotella	-0.2381	0.0195
	Actinobacteria	g_Slackia	-0.2815	0.0055
LDL-C	Proteobacteria	g_Desulfovibrio	0.225	0.0275
	Firmicutes	g_Fusobacterium	0.2047	0.0455
	Firmicutes	g_Oscillospira	0.3079	0.0023
	Firmicutes	g_Peptococcus	-0.2617	0.01
	Firmicutes	f_Ruminococcaceae_2	-0.2078	0.0422
	Firmicutes	g_Ruminococcus	-0.2267	0.0264
	Bacteroidetes	f_Prevotellaceae	-0.2578	0.0112
TG	Proteobacteria	g_Sutterella	0.2876	0.0045
	Proteobacteria	f_Comamonadaceae	-0.2307	0.0237
	Firmicutes	g_[Ruminococcus]	0.3858	0.0001
	Firmicutes	g_Epulopiscium	0.3204	0.0015
	Firmicutes	g_Megamonas	0.3373	0.0008
	Firmicutes	g_Phascolarctobacterium	0.2757	0.0066
	Firmicutes	f_Ruminococcaceae_1	-0.3209	0.0014
	Firmicutes	f_Ruminococcaceae_2	-0.3447	0.0006
	Firmicutes	g_[Eubacterium]	-0.2381	0.0195
	Firmicutes	g_Bulleidia	-0.2014	0.0491
	Firmicutes	g_Dialister	-0.3329	0.0009
	Firmicutes	g_Faecalibacterium	-0.2553	0.0121
	Firmicutes	g_Lachnospira	-0.238	0.0195
	Firmicutes	g_Megasphaera	-0.2734	0.007
	Firmicutes	g_Peptoniphilus	-0.2016	0.0489
	Firmicutes	g_Ruminococcus	-0.2845	0.005

	Bacteroidetes	f_[Paraprevotellaceae]	-0.3804	0.0001
	Bacteroidetes	g_[Prevotella]	-0.2135	0.0367
	Bacteroidetes	g_Parabacteroides	-0.2174	0.0333
	Actinobacteria	g_Eggerthella	0.2036	0.0466
	Actinobacteria	g_Slackia	-0.356	0.0004
	Lentisphare	f_Victivallaceae	-0.3598	0.0003
	Firmicutes	g_Coprobacillus	0.2443	0.0165
	Tenericutes	f_Anaeroplasmataceae	-0.3275	0.0011
	Euryarchaeota	g_Methanobrevibacter	-0.2793	0.0059
HDL-C	Proteobacteria	f_Comamonadaceae	0.3204	0.0015
	Firmicutes	f_Lachnospiraceae	0.233	0.0224
	Firmicutes	f_Peptococcaceae	0.2047	0.0455
	Firmicutes	f_Peptostreptococcaceae	0.2561	0.0118
	Firmicutes	g_Dialister	0.2053	0.0448
	Firmicutes	g_Lactobacillus	0.2014	0.0491
	Firmicutes	g_Turicibacter	0.2405	0.0183
	Firmicutes	g_Veillonella	0.2263	0.0266
	Firmicutes	g_Peptococcus	-0.3674	0.0002
	Firmicutes	g_Megamonas	-0.2318	0.0231
	Firmicutes	g_Phascolarctobacterium	-0.2336	0.022
	Firmicutes	f_Ruminococcaceae_2	-0.215	0.0354
	Firmicutes	g_[Ruminococcus]	-0.3235	0.0013
	Bacteroidetes	f_[Barnesiellaceae]	-0.3142	0.0018
	Actinobacteria	g_Collinsella	-0.2397	0.0187
	Actinobacteria	g_Odoribacter	-0.2373	0.0199
HDL-C/TC	Proteobacteria	g_Bilophila	0.2007	0.0499
	Proteobacteria	g_Sutterella	0.2217	0.0299
	Proteobacteria	f_Comamonadaceae	-0.3062	0.0024
	Firmicutes	g_Megamonas	0.2791	0.0059
	Firmicutes	g_Phascolarctobacterium	0.274	0.0069
	Firmicutes	g_[Ruminococcus]	0.383	0.0001
	Firmicutes	g_Epulopiscium	0.203	0.0473
	Firmicutes	g_Faecalibacterium	-0.273	0.0071
	Firmicutes	g_Lachnospira	-0.2157	0.0348
	Firmicutes	g_Streptococcus	-0.253	0.0129
	Firmicutes	g_[Eubacterium]	-0.2333	0.0222
	Bacteroidetes	f_[Barnesiellaceae]	0.2593	0.0107
	Bacteroidetes	g_Butyricimonas	0.2171	0.0336

	Bacteroidetes	f_[Paraprevotellaceae]	-0.2153	0.0352
	Actinobacteria	g_Collinsella	0.3117	0.002
	Actinobacteria	g_Slackia	-0.205	0.0451
	Firmicutes	g_Coprobacillus	0.2106	0.0395
	Tenericutes	f_Anaeroplasmataceae	-0.2573	0.0114
CRP	Proteobacteria	g_Sutterella	0.2023	0.0481
	Proteobacteria	g_Desulfovibrio	-0.3964	<.0001
	Firmicutes	g_Epulopiscium	0.2139	0.0364
	Firmicutes	g_Megamonas	0.2881	0.0044
	Firmicutes	g_Catenibacterium	-0.3692	0.0002
	Firmicutes	g_Clostridium	-0.2958	0.0034
	Firmicutes	f_Peptococcaceae	-0.3486	0.0005
	Firmicutes	g_Oscillospira	-0.2603	0.0104
	Firmicutes	g_Phascolarctobacterium	-0.2118	0.0383
	Firmicutes	g_Turicibacter	-0.2031	0.0472
	Firmicutes	o_Clostridiales	-0.2693	0.008
	Bacteroidetes	f_[Barnesiellaceae]	-0.2016	0.0488
	Bacteroidetes	g_[Prevotella]	-0.2474	0.0151
	Bacteroidetes	g_Butyricimonas	-0.2566	0.0116
	Bacteroidetes	g_Odoribacter	-0.3266	0.0012
	Bacteroidetes	g_Prevotella	-0.2089	0.0411
	Actinobacteria	f_Coriobacteriaceae	-0.2322	0.0228
	Tenericutes	f_Anaeroplasmataceae	-0.4223	<.0001
APOb	Euryarchaeota	g_Methanobrevibacter	-0.2967	0.0033
	Proteobacteria	g_Bilophila	0.3638	0.0003
	Proteobacteria	f_Comamonadaceae	-0.2058	0.0443
	Firmicutes	g_Catenibacterium	0.2094	0.0406
	Firmicutes	g_Phascolarctobacterium	0.2448	0.0162
	Firmicutes	f_Ruminococcaceae_2	-0.2258	0.027
	Firmicutes	g_[Eubacterium]	-0.262	0.0099
	Firmicutes	g_Acidaminococcus	-0.2339	0.0218
	Firmicutes	g_Peptococcus	-0.2973	0.0033
	Bacteroidetes	g_Butyricimonas	0.3098	0.0021
	Bacteroidetes	f_Prevotellaceae	-0.2652	0.009
	Actinobacteria	g_Collinsella	0.2181	0.0328
Adiponectin	Firmicutes	g_Bulleidia	0.3483	0.0005
	Firmicutes	g_Clostridium	0.2684	0.0082
	Firmicutes	g_Dorea	-0.3473	0.0005

	Firmicutes	g_Megamonas	-0.3935	<.0001
	Firmicutes	g_Megasphaera	-0.2524	0.0131
	Firmicutes	g_Ruminococcus	-0.3148	0.0018
	Firmicutes	f_Clostridiaceae_2	-0.2011	0.0494
	Firmicutes	f_Erysipelotrichaceae	-0.2224	0.0294
	Firmicutes	g_Anaerostipes	-0.2447	0.0163
	Bacteroidetes	g_Paraprevotella	0.2475	0.015
	Bacteroidetes	g_Bacteroides	-0.3066	0.0024
	Bacteroidetes	f_[Barnesiellaceae]	-0.231	0.0236
	Bacteroidetes	g_Butyricimonas	-0.3748	0.0002
	Bacteroidetes	g_Odoribacter	-0.2776	0.0062
	Actinobacteria	g_Bifidobacterium	-0.3952	<.0001
	Actinobacteria	g_Collinsella	-0.3539	0.0004
Glucose	Proteobacteria	g_Bilophila	0.2572	0.0114
	Proteobacteria	g_Sutterella	0.3499	0.0005
	Firmicutes	g_Veillonella	0.2747	0.0068
	Firmicutes	g_Epulopiscium	0.2158	0.0347
	Firmicutes	g_Megamonas	0.3131	0.0019
	Firmicutes	g_Anaerostipes	0.2444	0.0164
	Firmicutes	g_Catenibacterium	0.205	0.0451
	Firmicutes	f_Christensenellaceae	-0.301	0.0029
	Firmicutes	f_Peptococcaceae	-0.233	0.0223
	Firmicutes	g_Coprobacillus	-0.2308	0.0237
	Firmicutes	f_Ruminococcaceae_1	-0.2623	0.0098
	Firmicutes	f_Ruminococcaceae_2	-0.2773	0.0062
	Firmicutes	g_Oscillospira	-0.2112	0.0389
	Bacteroidetes	f_Prevotellaceae	-0.2167	0.0339
	Bacteroidetes	g_Paraprevotella	-0.2793	0.0059
Insulin	Firmicutes	g_[Eubacterium]	0.2153	0.0351
	Firmicutes	g_[Ruminococcus]	0.2055	0.0445
	Firmicutes	g_Dorea	0.2156	0.0349
	Firmicutes	g_Lactobacillus	0.2452	0.016
	Firmicutes	g_Megamonas	0.4829	<.0001
	Firmicutes	g_Megasphaera	0.2449	0.0162
	Firmicutes	g_Peptoniphilus	0.3211	0.0014
	Firmicutes	f_Peptococcaceae	-0.3611	0.0003
	Firmicutes	f_Ruminococcaceae_1	-0.3629	0.0003
	Firmicutes	g_Clostridium	-0.3452	0.0006

	Firmicutes	g_Lachnospira	-0.2631	0.0096
	Firmicutes	g_Oscillospira	-0.3082	0.0023
	Bacteroidetes	g_Paraprevotella	-0.3122	0.002
	Actinobacteria	g_Bifidobacterium	0.3277	0.0011
	Actinobacteria	g_Collinsella	0.2354	0.0209
	Actinobacteria	f_Coriobacteriaceae	-0.2706	0.0077
	Tenericutes	f_Anaeroplasmataceae	-0.2865	0.0047
		f_[Mogibacteriaceae]	-0.201	0.0496

[1]Anthropometrics, metabolic parameters and blood pressure measurements recorded at the end of the dietary interventions: TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein; LDL-C, low-density lipoprotein; HDL-C/TC, ratio of high-density lipoprotein/ total cholesterol; CRP, C-reactive protein; APOb, Apolipoprotein

[2]Taxa were denoted as p, phylum; c, class; o, order; f, family and g, genus levels

[3]Non-parametric Spearman's rank correlation coefficient

[4]Spearmen's correlation probability values

BRIDGE TO CHAPTER IV

Most of the published evidence suggested that gut microbiome have a role in health and disease, Chapter III has indicated that dietary fat from different sources have different effects on gut microbiome and are associated to cardiometabolic risk factors. Existing data establish that the genetic make up of the individuals determine the circulating cardiometabolic factors upon intake of nutrients or food from numerous sources. Impact of different diets based on genetic basis of individuals on their circulating cholesterol has not been investigated in controlled feeding design. Chapter IV investigates the role of common genetic variants present in the cholesterol metabolic pathway genes and their effect on the responsiveness of blood lipid levels in a recommended full controlled dietary consumption.

CHAPTER IV

A COMBINATION OF SINGLE NUCLEOTIDE POLYMORPHISMS IS ASSOCIATED WITH THE INTERINDIVIDUAL VARIABILITY IN BLOOD LIPID CONCENTRATIONS IN HUMANS UPON CONSUMPTION OF DAIRY FATS

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Short running head: Common genetic variants and response to dairy fat

Abbreviations:

APOB, apolipoprotein B; APOE, apolipoprotein E; ABCA1, ATP-binding cassette subfamily A, member1; ABCA5, ATP-binding cassette subfamily A , member 5; ABCG5, ATP-binding cassette subfamily G, member 5; CHO, carbohydrates; CYP7A1, cholesterol 7α-hydroxylase; CVD, cardiovascular disease; DHCR7, 7-dehydocholesterol reductase; LDL-R, LDL receptor; LIPA, lipase A, lysosomal acid type; LIPC, lipase C, hepatic type; LIPG, lipase G, endothelial

type; LPL, lipoprotein lipase; MTTP, microsomal triglyceride transfer protein; NPC1L1, Niemann-Pick C1 like intracellular cholesterol transporter 1; PPARA, peroxisome proliferator activated receptor alpha; PCSK9, proprotein convertase subtilisin/kexin type 9; SCAP, sterol regulatory element binding transcription factor chaperone; SREBF2, sterol regulatory element binding transcription factor 2; TC, total cholesterol; TG, triglyceride.

Clinical Trial Registry number and website: http://www.clinicaltrials.gov (NCT02106208).

4.1 ABSTRACT

Background: Inter-individual variability in blood lipid response to dairy fats (DF) has been reported and may be partially explained by genetic variation. Effect of DF intake and the contribution of single-nucleotide polymorphisms (SNPs) alone or in combination to variability in blood response is unknown.

Objective: We aimed to analyze the responses of blood lipid to the feeding with alternative lipid sources from dairy products. The genetic associations with selected SNPs were investigated.

Design: In a multicenter randomized crossover trial, 92 participants consumed five isoenergetic diets, including cheese, butter, (MUFA), polyunsaturated fatty acids (PUFA) and low-fat carbohydrate (CHO) for 4-weeks. The genetic association was performed with endpoint to endpoint serum lipids after genotyped 21 selected SNPs.

Results: SNP *ABCA1*-rs2066714 (p = 0.009), *DHCR7*-rs1044482 (p = 0.035) and *NPC1L1*-rs2073547 (p = 0.038) show genotype-diet interaction on serum HDL-C responsiveness to dietary treatment consumption. In addition, genotype-diet interactions were observed with LDL-C responsiveness to dietary treatment for *PPAR*-rs6008259 (p = 0.025) and *LIPG*-rs2000813 (p = 0.024) in a linear mixed model. In addition, partial least squares regression (PLS) analysis for DF vs. UFA suggested that a combination of 6 SNPs (*ABCA1*-rs2066714, *NPC1L1*-rs2073547, SREBF2-rs2228313, *DHCR7*-rs1044482, *DHCR7*-rs760241 and *PPAR*-rs6008259) explained 32.8% of the variance in HDL-cholesterol response to dairy feeding. Two SNPs (*LIPG*-rs2000813 and *ABCA1*-rs2066714) explained 16.5% of the variance in LDL-cholesterol, while a combination of 3 SNPs (*ABCA1*-rs2066714, *PPAR*-rs6008259,

LIPG-rs2000813) explained 18.8% of the variance in total cholesterol levels. Furthermore, 19.8% of the variance of the concentration of triglyceride levels in response to dairy feeding was explained by a combination of 5 SNPs (*LIPG*-rs2000813, *SCAP*-rs12487736, *ABCA1*-rs2230808, *DHCR7*-rs1044482, *LIPC*-rs6083).

Conclusions: SNPs *ABCA1*-rs20066714, *DHCR7*-rs1044482, *NPC1L1*-rs2073547 and *LIPG*-rs2000813, *PPAR*-rs6008259 variants associate with serum HDL-C and LDL-C concentrations, respectively. Also, a combination of SNPs associated with a variability in serum lipids following intake of DF vs UFA, will warrant predictive algorithms for personalized dietary recommendations.

Keywords: Cardiovascular disease, randomized controlled trial, nutrigenetics, gene diet interaction, cholesterol, genetic variations, personalized nutrition. single-nucleotide polymorphisms

4.2 INTRODUCTION

Cardiovascular diseases (CVD) remain the leading cause of morbidity and mortality worldwide, representing 31% of all global deaths (1). Although a healthy diet is widely acknowledged as a cornerstone of disease prevention and management, its optimal composition, in terms of specific foods or isolated nutrients, is still a matter of great debate (2). The role of dietary fat consumption in the etiology of CVD has been investigated since the studies of Ancel Keys in the 1950s (3) and yet, to date, its implication remains controversial (3-6). Indeed, although all dietary sources of saturated fats (SFA) were undiscriminatingly considered to have rather harmful effects on blood lipids and cardiovascular health, a growing body of evidence now suggests these effects differ based on specific food or dietary patterns (2, 4, 5) and several recent intervention studies failed to observe negative associations between dairy product consumption and parameters of cardiovascular health (4, 7-9). In addition to SFA, dairy products also contain cholesterol, which can potentially raise blood lipid concentrations, therefore fueling debate on their role as part of a healthy diet (5, 10, 11).

Moreover, health effects may vary across dairy products. For example, based on studies with free living participants and controlled feeding trials, it has been shown that the intake of fat from cheese resulted in lower LDL-C concentrations compared to a matched intake of fat from butter (4, 10-12), although both dairy products contributed to similar SFA intake. These results suggest that fat from butter has a different effect compared to fat released from cheese. The discrepancy between these results has been suggested as originating from the variability within the food matrices used (4, 13, 14), as well as differences in lifestyle and genetic background of study participants (15, 16), as has been discussed in several meta-analyses and reviews (17-20).

Blood lipid concentrations, which are widely used as biomarkers for CVD risk events, exhibit high interindividual variability in response to dietary fat intake (21-23). Host genetics partly explain this variability (22, 24, 25) and studies exploring gene-diet interactions thus provide crucial information in order to establish personalized dietary recommendations aiming at lowering CVD risk. The effect of single nucleotide polymorphisms (SNPs) on the response of blood lipid levels to dairy product intake has only been recently investigated (15, 16, 24, 26). For example, carriers of the TT genotype at rs135549 in *peroxisome proliferator-activated receptor α* (*PPARA*) displayed lower total cholesterol/HDL-C and LDL/HDL ratios after consumption of skimmed *vs* semi-skimmed milk for 12 months (15). In a Mexican population, homozygotes for the A allele at rs9282541 in *ATP-binding cassette subfamily A1* (*ABCA1*) had higher serum HDL-C concentrations compared to carriers of the G allele following consumption of a diet low in SFA for 12 weeks (27). Moreover, a combination of 3 SNPs, i.e. *ABCG5*-rs6720173, *CYP7A1*-rs3808607 and *DHCR7*-rs76024, was found to partly explain the variability in serum lipid concentration response following dairy intake, i.e. 3 servings/d of dairy including 375 mL of 1% fat milk, 175 g of 1.5% milk fat and 30 g of 34% milk fat from cheese (16). However, no study has yet investigated the combinatory effects of more SNPs using newer advanced statistical approach for the prediction model on blood lipid concentrations in response to dairy consumption (cheese and butter) in a controlled full feeding trial.

The aim of the current study was thus to further explore how the genetic makeup of an individual contributes qualitatively to the effects of cheese and butter on blood lipid concentrations compared to other diets. Since blood lipid concentration is a complex phenotype that involves numerous genes in lipid metabolism and transport, we assessed the

effect of a combination of SNPs in order to increase the amount of explained variability, a first step in the establishment of personalized dietary recommendations.

4.3 METHODS

4.3.1 STUDY DESIGN AND PARTICIPANTS

The study was a randomized, single-blind, cross-over, full-feeding multicenter trial carried out at the Richardson Centre for Functional Foods and Nutraceuticals (University of Manitoba) and at the Institute of Nutrition and Functional Foods (University of Laval) as described elsewhere (28). Ninety-two healthy male (n=43) and female (n=49) participants aged 18-65 y, with a waist circumference greater than 80 cm for women and greater than 94 cm for men, as well as with blood HDL-C concentrations ≤1.53 mmol/L for women and ≤1.34 mmol/L for men, were recruited for the study. Individuals with a history of CVD or type 2 diabetes, those on cholesterol-lowering or antihypertensive medications, and smokers were not eligible for the study. Participants consumed five diets, each for 4 weeks, with 4-week washout periods between intervention diets. The isoenergic diets included four diets with 35% energy from fat, namely (i) a diet rich in SFA from cheese, (ii) a diet rich in SFA from butter, (iii) a diet rich in n-9 MUFA, (iv) a diet rich in n-6 PUFA, and (v) a low fat, high CHO diet (25% energy from fat). Details about the interventions were previously described in Brassard et al., 2017 (**Supplementary Table 4.5**) (28). Eligible participants' usual energy intake was estimated at the beginning of the study using a web-based food frequency questionnaire tool. SFA contents were matched in cheese and butter diets whereas in other experimental diets, SFA contents were replaced and manipulated by MUFA, PUFA and CHO levels. All research protocols

were approved by the Biomedical Research Ethics Board (University of Manitoba and Université Laval) and all participants received study informed consent.

4.3.2 BLOOD COLLECTION AND LIPID ANALYSES

Twelve-hour fasting blood was collected at the beginning and the end of the intervention periods. Blood samples were centrifuged (3000 *g* for 20 min at 4 °C) within 1-2 hr of collection, and aliquots of blood fractions were prepared and stored at -80°C before further analysis. Endpoint serum concentrations of TC, HDL-C and TG were measured by Roche/Hitachi Modular (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. Serum LDL-C concentrations were calculated using the Friedewald equation.

4.3.3 DNA EXTRACTION AND GENOTYPING

Genomic DNA was extracted from buffy coat of blood samples using DNeasy Blood and Tissue Kit (Qiagen). DNA concentrations and purity were measured by a NanoDrop (Thermo Fisher Scientific). Genotyping was carried out by using TaqMan GTXpress Master Mix with specific allele probes on a Real-Time PCR system (StepOnePlus, Applied Biosystem (**Supplementary Table 4.7**). SNPs in genes involved in the metabolism and transport of lipids and bile acids were selected based on previously published studies (16, 29) (**Supplementary Table 4.8**). Allele frequency of the examined SNPs are shown in **Supplementary Table 4.9**. Ninety-two participants were considered for the genotyping analysis, of which sixty-five completed all five intervention phases, seven completed four phases, five completed three phases, eight completed two phases and seven completed one phase.

4.3.4 STATISTICS

Linear mixed model

Endpoint serum lipid concentrations were analyzed using a mixed model that included fixed categorical effects of diet, sex and centre, and the continuous covariates of age, BMI and baseline serum lipid concentration (treatment-specific baseline) and subject as random factors. The analysis was carried out as a repeated measure analysis considering the five periods, and the variance-covariance structure among periods was assumed to be compound symmetry. The MIXED procedure in SAS (version 9.4, SAS Institute Inc.) was used for the analysis. A dominant genetic model was applied and multiple comparisions were tested among diets using a Tukey-Kramer adjustment. SNPs that showed a significant SNP × diet interaction effect on serum lipids were evaluated with the "Slice" option. Slice is also well-known as an analysis of simple effects in the Mixed procedure to compare genotype effects within each diet. For all tests, the bilateral alpha risk was α=0.05.

Partial least squares regression

In a second analysis, individual endpoint serum lipid concentrations of participants who consumed cheese and butter diets were averaged and these two diets grouped together (henceforth referred as dairy fat (DF) diets) while individual endpoint blood lipid concentrations of the participants who consumed MUFA and PUFA diets were averaged and these two diets were grouped together (henceforth referred as unsaturated fat (UFA) diets) (n=79). Before grouping individuals into DF and UFA, Pearson's correlations (r) were calculated between end point serum lipid (TC, LDL-C, TG and LDL-C) concentrations following intake of cheese and butter, and for MUFA and PUFA. Linear mixed model was

first applied to identify which SNPs among the 21 candidate SNPs exhibited a significant SNP × diet interaction with the endpoint serum lipid response upon consumption of DF vs UFA diets, considering diet, sex, age, BMI, centre and baseline serum lipid concentration as fixed factors and subjects as random factors.

Following comparison of serum lipid concentrations in participants after consumption of DF vs UFA with the linear mixed model, SNPs for which the SNP × diet interaction p-value was <0.2 were selected for partial least squares (PLS) regression analysis, which is the multivariate regression extension of principal component analysis (30). When a SNP exhibited a p-value <0.2 in both the additive or dominant genetic models, the genetic model yielding the lowest p-value was kept. Additionally, for all four serum lipid concentrations tested, BMI, age, sex and the concentration of the respective blood lipid concentration at day 1 of the feeding period were tested in linear mixed models not including SNPs. When one of these variables displayed a significant diet interaction, it was selected for PLS regression analysis. First, a PLS regression model including all thus selected variables coded in units of variance was built. Variables were ranked according to their variable importance in the projection (VIP) value and several PLS regression models were then built using increasing VIP threshold values. For each of the outcome measure, i.e. TC, HDL-C, LDL-C and TG, the model maximizing the explained variance (R^2) and the R^2 following cross-validation was selected (31). SIMCA-P13 software (Umetrics) was used for all PLS regression analyses.

4.4 RESULTS

4.4.1 SERUM LIPID CONCENTRATION RESPONSE TO THE INTERVENTION DIETS

Serum lipid concentrations were significantly different following the 5 dietary interventions, as previously described in Brassard et al., 2017 (for TC, p=<0.0001; for LDL-C, p=<0.0001; for TG, p=0.0007; for HDL-C, p=0.0051); and baseline characteristics of the participants were shown in **Supplementary Table 4.6.** Nonetheless, there was relatively high interindividual variabilities in serum TC, LDL-C, TG and HDL-C concentrations in our study participants following each dietary interventions (**Figure 4.1 A, B, C, D** and **E**). The average CV values for serum lipids after all 5 dietary interventions in were for TC, 18.98%; for LDL-C, 34.50%; for TG, 55.79%; and for HDL-C, 19.59%. A portion of these variabilities in serum lipids could be explained by the SNPs present in our study individuals. The specifics of these SNP-diet interaction are described in the following sections.

4.4.2 EFFECTS OF SNP × DIET INTERACTIONS ON SERUM LIPID CONCENTRATIONS

The SNP × diet interaction exhibited a significant effect on HDL-C concentrations for *ABCA1*-rs2066714 (p=0.009), *DHCR7*-rs10444824 (p=0.035) and *NPC1L1*-rs2073547 (p=0.038) (**Supplementary Table 4.10**). Homozygous carriers of the major T allele at *ABCA1*-rs2066714 had significantly lower HDL-C concentrations compared to carriers of the C allele following the MUFA diet (1.13 ± 0.01 vs 1.20 ± 0.02; p=0.0165; **Figure 4.2**). Homozygous carriers of the major T allele at *DHCR7*-rs10444824 had significantly higher HDL-C concentrations compared to carriers of the heterozygous upon intake of PUFA diet (1.18 ± 0.02 vs 1.09 ± 0.02; p=0.0124; **Figure 4.3**). Further, homozygous carriers of the major A allele at *NPC1L1*-rs2073547 had lower HDL-C concentrations compared to carriers of the G allele after consumption of MUFA diet (1.13 ± 0.01 vs 1.19 ± 0.02; p=0.0139; **Figure 4.4**).

Furthermore, there was a significant effect of SNP × diet interaction on LDL-C concentrations for *PPAR*-rs6008259 (p=0.0207) and *LIPG*-rs2000813 (p=0.024) (**Supplementary Table 4.10**). Homozygous carriers of the major G allele at *PPAR*-rs6008259 showed a tendency towards higher LDL-C concentrations compared to carriers of the A allele, but this trend was not statistically significant following diet rich in PUFA (2.88 ± 0.07 vs 2.74 ± 0.11, p=0.3013; **Figure 4.5**) and CHO (3.12 ± 0.07 vs 2.96 ± 0.11; p=0.2260; **Figure 4.5**). In addition, homozygous carriers of the major C allele at *LIPG*-rs2000813 showed lower LDL-C levels compared to carriers of the T allele but this trend was not statistically significant after intake of cheese (4.94 ± 0.09 vs 5.05 ± 0.09, p=0.3094; **Figure 4.6**) and butter (4.95± 0.09 vs 5.18± 0.09, p=0.1145; **Figure 4.6**).

Moreover, carriers of the C allele at *ABCA1*-rs2066714 consistently displayed significantly elevated TC and LDL-C concentrations compared to carriers of the TT genotype following the five dietary interventions (**Supplementary Table 4.10**). Likewise, carriers of the E2 isoform of APOE exhibited marginally significant (p=0.0514) lower LDL-C concentrations and significantly (p=0.029) higher HDL-C concentrations, compared to carriers of the E3 and E4 isoforms following the five dietary interventions.

4.4.3 COMBINATIONS OF SNPS ASSOCIATED WITH THE SERUM LIPID RESPONSES TO THE DIETARY INTERVENTIONS

Since endpoint serum lipid concentrations following consumption of diets with similar SFA amounts, i.e. cheese and butter, were highly correlated and since endpoint serum lipid concentrations following consumption of diets with similar unsaturated fatty acids (UFA) amounts, i.e. MUFA and PUFA, were also highly correlated (**Table 4.1 & 4.2**), endpoint

serum lipid concentrations following cheese and butter (DF) interventions were averaged and hereafter referred to as DF, and endpoint serum lipid concentrations following MUFA and PUFA (UFA) interventions were averaged and hereafter referred to as USF (n=79).

Analysis of HDL-C concentrations in participants after consumption of DF vs UFA with the linear mixed model showed that 8 SNPs exhibited SNP × diet interactions with p-values lower than <0.2 (**Table 4.3**): *ABCA1*-rs2066714, *APOB*-rs676210, *DHCR7*-rs760241, *DHCR7*-rs1044482, *LPL*-rs3200218d, *NPC1L1*-rs2073547, *PPAR*-rs6008259 and *SREBF2*-rs2228313. These SNPs were selected for further PLS regression analysis. Additionally, since we also observed a significant (p=0.046) BMI × diet interaction, this variable was also entered in the PLS regression analysis. The first PLS regression model including the 8 selected SNPs and BMI was found to explain 32.6% of the variance and 23.6% of the variance following cross-validation. To improve the model and find an association of variables more predictive of the HDL-C concentrations, we sequentially filtered out those that displayed the lowest VIP value (i.e. those that made a less important contribution to the PLS regression model). After application of several increasing VIP value thresholds, we selected a significant model (p-value after cross-validation ANOVA= $3.31.10^{-5}$) that included 6 SNPs and BMI and explained 32.8% of the variance (R^2), with a R^2 after cross-validation of 23.8% (**Table 4.4**). We repeated the same procedure for TG, LDL-C and TC level responses and the corresponding results can be found in **Table 4.4**.

4.5 DISCUSSION

We here report that a combination of genetic polymorphisms explains the variability of serum lipid response following consumption for 4 weeks of diets differing in their fatty acid profile.

Although several systematic review and meta analysis studies have shown that the replacement of dietary SFA with MUFA or PUFA has beneficial effects on CVD risk (32-34), the effect of dairy products, which are usually relatively high in SFA, on CVD risk is still a matter of great debate (10, 11, 18, 35, 36). The high interindividual variability of blood lipid concentrations in response to dairy consumption may explain the discrepancies between existing studies (37, 38). Since this variability is partly due to variations in genes involved in lipid and bile acid metabolism and transport (16, 39, 40), it is of paramount importance to identify these in order to provide more personalized dietary recommendations aimed at improving cardiovascular health. In this well-controlled crossover full feeding study, we carried out an original statistical approach, i.e. applying linear mixed models to identify SNPs significantly associated with the serum lipid response to dietary interventions followed by PLS regression, a multivariate approach, aimed at increasing the explained variability.

The first noteworthy observation is that dietary fat from different sources differentially alters blood lipid concentrations, as we published previously (28). Since we observed a relatively high interindividual variability of serum blood lipid concentrations following consumption of the different diets, we then sought to identify some SNPs associated with this variability. Indeed, several factors are assumed to be involved, e.g. sex, age, microbiota, and genetics have been shown to make a crucial contribution to this variability (41-44). Our analysis shows that the effect of the interaction between the dietary intervention and *ABCA1*-rs2066714, *DHCR7*-rs10444824 and *NPC1L1*-rs2073547 significantly affected serum HDL-C concentrations and that *PPAR*-rs6008259 and *LIPG*-rs2000813 exhibited significant SNP × diet interaction on serum LDL-C concentrations. We did not observe any significant SNP × diet interaction on TC and TG concentrations, but only evidenced a significant genotype

interaction for *ABCA1*-rs2066714 on TC and LDL-C concentrations and for APOE isoform on LDL-C and HDL-C concentrations.

ABCA1 is a membrane protein involved in the efflux of cholesterol and phospholipids from peripheral cells to lipid-poor apolipoproteins (45, 46), a major route to remove excess cholesterol from peripheral cells to the liver via generation of HDL-C (47, 48). It has also been suggested to contribute to the secretion of intestinal HDL within the circulation (49, 50). *ABCA1*-rs2066714 is a missense mutation that has been associated with a significant reduction in HDL-C concentrations and cholesterol efflux (51, 52). *7-dehydrocholesterol reductase* (*DHCR7*) encodes the enzyme responsible for the conversion of 7-dehydrocholesterol to cholesterol (53), with the former molecule also serving as a precursor of cholecalciferol in keratinocytes upon UVB exposure (54). Abdullah et al. have previously shown that *DHCR7*-rs760241 was associated with reduction in LDL-C following recommended dairy intake (55). In spite of this, carriers of the T allele at rs1044482 exhibited higher in HDL-C concentrations following consumption of PUFA-rich diet. This is the first study report that rich PUFA intake in association with *DHCR7-rs1044482* SNP impacts HDL-C levels (**Figure 3).** *NPC1L1* encodes for a membrane protein that plays a key role in the intestinal absorption of dietary and biliary cholesterol (56-58). Pharmacological inhibition of this protein with ezetimibe and studies of rare mutations or SNPs in its encoding gene have shown that NPC1L1 critically alters serum lipid concentrations and alters CVD risk (59-61). Moreover, SNPs in *NPC1L1* have been associated with the interindividual variability in the LDL-C response to ezetimibe treatment (57, 62). It has previously been shown that the interaction of a synonymous SNP in *NPC1L1* with dietary cholesterol had a significant effect on plasma cholesterol (63). Further, studies have suggested that targeting genetic variants at *NPC1L1* could be an ideal target for

CVD prevention (64, 65). Results from a meta analysis, showed that genetic variants at NPC1L1 were associated with reduction in circulating LDL-C levels in type 2 diabetics (66). In the present work, SNP rs2073547 was not associated with LDL-C whereas consumption of MUFA-rich diet attenuated this effect on HDL-C response.

Since the effects of the consumption of fat from dairy products vs unsaturated fatty acids on cardiovascular health have not been fully elucidated, we decided to analyse the contribution of SNPs in the variability of the response to diets similar in their SFA vs USF content, i.e. grouping cheese and butter diets compared to MUFA and PUFA diets. Our original statistical approach, i.e. first identifying SNPs significantly associated with the variability in the serum lipid response to dietary interventions and then finding the best combination of these SNPs associated with this variability. Thus, allowed us to show that a combination of 6 SNPs, namely *ABCA1-rs2066714, NPC1L1-rs2073547, SREBF2-rs2228313, DHCR7-rs1044482, DHCR7-rs760241* and *PPAR-rs6008259*, explained 32.8% of the variance in HDL-C concentrations. Further, a combination of 3 SNPs, *ABCA1-rs2066714, PPAR-rs6008259* and *LIPG-rs2000813,* could explain 18.8% of the variance in TC. *LIPG-rs2000813* and *ABCA1-rs2066714* SNPs were explained 16.5% of the variance in LDL-C, while a combination of *LIPG-rs2000813, SCAP-rs12487736, ABCA1-rs2230808, DHCR7-rs1044482,* and *LIPC-rs6083* SNPs explained 19.8% of the variance in TG.

Our results suggest that several SNPs in genes encoding proteins involved with lipid and bile acid metabolism and transport interact with dietary fat from different sources, namely cheese and butter from dairy versus MUFA and PUFA-rich diets, and thus differentially alter serum lipid concentrations. Moreover, by combining some of these SNPs together, we were able to

explain a significant part of the variability in serum lipid concentrations following diets high in SFA vs UFA. Although a large part of this variability remains unexplained, we believe this approach could constitute a first step in the establishment of models aiming at predicting serum lipid concentrations in specific individuals following dietary interventions. Other SNPs or kinds of variations, e.g. copy number variants or epigenetic modifications, are likely involved in explaining this variability. Future trials will allow their identification and improve the quality of the predictive models. This information will contribute to building better algorithms in order to provide patients or individuals at risk for CVD with more personalized dietary recommendations, partly based on their genetic characteristics, in order to lower their CVD risk.

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Author contribution: Benoît Lamarche designed the primary experiment. Peter Jones, Ethendhar Rajendiran, and Peter Eck were responsible for the subsequent experiment design and sample collections. Ethendhar Rajendiran, Peter Eck and Charles Desmarchelier analyzed data. Charles Desmarchelier carried out PLS regression analysis. Ethendhar Rajendiran and Charles Desmarchelier wrote the manuscript with subsequent revisions from Peter Eck and Peter Jones. All authors contributed to writing the manuscript by providing their comments and suggestions. Final version of the manuscript was approved by all authors.

Disclosures

ER, YS, VR, PJ, CD, MTG, DB, IG, EL, AT, PC and BL have no conflict of interest.

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Nutritional Fundamentals for Health Inc, which selling nutraceutical and functional foods. AT received funds from Dairy Farmers of Canada and he is also a member of the board of the Yogurt In Nutrition Initiative (YINI). BL and PC have received funds from Dairy Farmers of Canada (completed in 2017), Agriculture and Agri-Food Canada (completed in 2017), Canola Council of Canada (completed in 2017) to study the impact of dietary fats on cardiometabolic health. Their research on dietary fats is also currently funded by the Canadian Institutes for Health Research.

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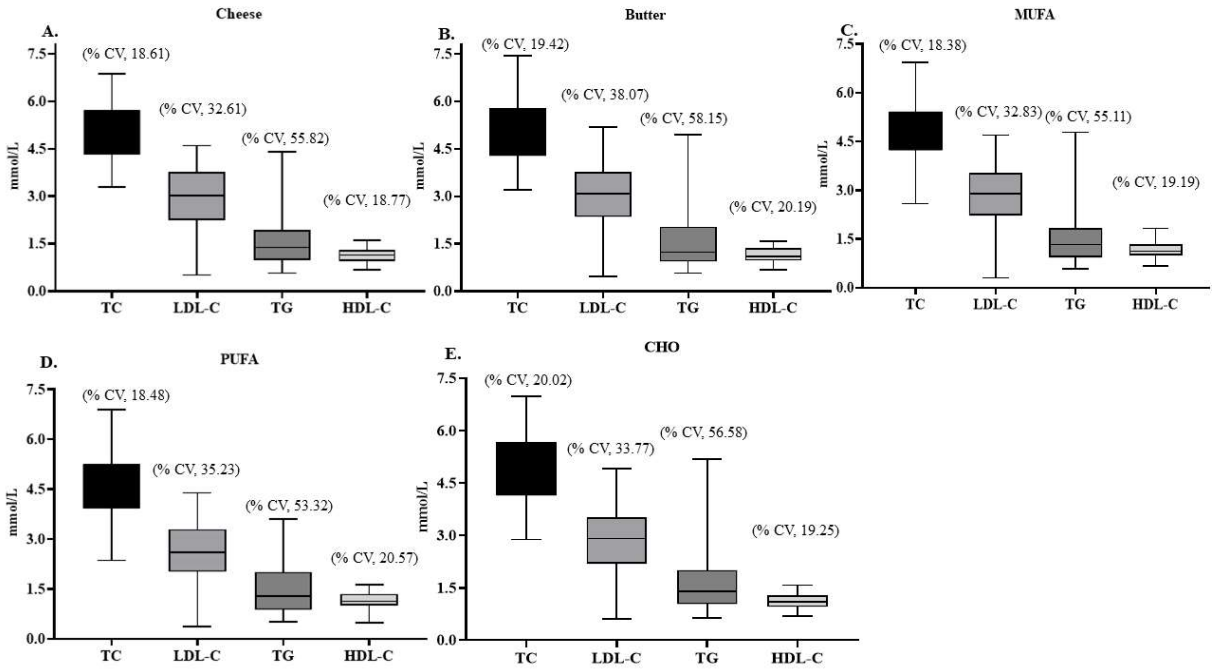

Figure 4. 1 Interindividual variability in serum lipids response after 4 weeks dietary interventions.

Variability in serum lipids (TC, LDL-C, TG and LDL-C) response after intake of dietary interventions were representing as coefficient of variation (CV %), Cheese diet (panel A, n=79), butter diet (panel B, n=80), MUFA diet (panel C, n=78), PUFA diet (panel D, n=79) and CHO diet (panel E, n=76). Raw data distributions are shown in box plot graph.

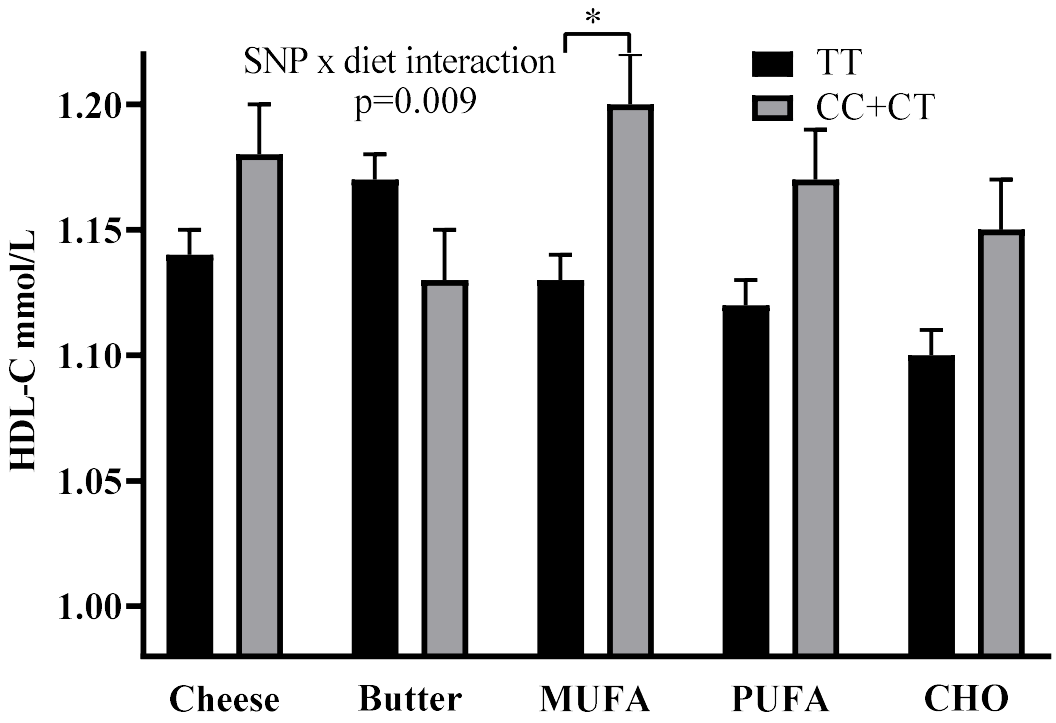

Figure 4. 2 Effect of *ABCA1*-rs2066714 genotype on HDL-C concentration after five 4-week-long dietary interventions.

The slices effect shows the differences in HDL-C between the genotype with MUFA intake (TT vs. CC+CT, p = 0.0165). For each diet, genotype groups were distributed as follows: cheese (TT=50, CT= 23 and CC=6), butter (TT=52, CT= 22 and CC=6), MUFA (TT=51, CT= 21 and CC=6), PUFA (TT=52, CT= 23 and CC=4) and CHO (TT=47, CT= 23 and CC=6). Data are presented as least squares means ± SEM.

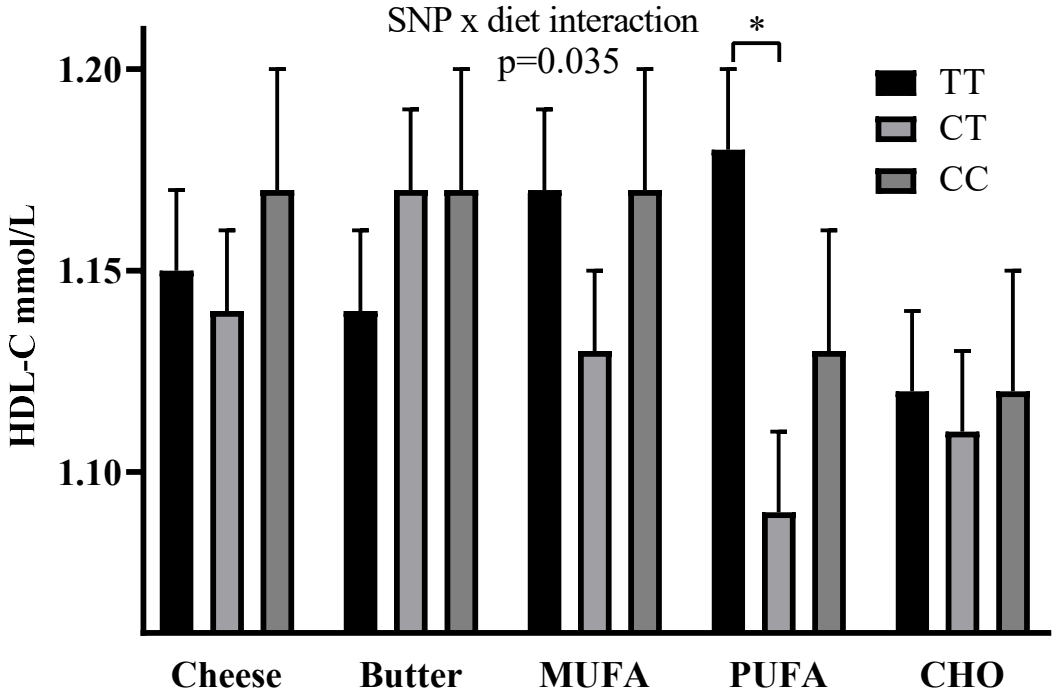

Figure 4. 3 Effect of *DHCR7-rs1044482* genotype on HDL-C concentration after 4-week-long dietary interventions.

The slices effect shows the differences in HDL-C between the genotype with PUFA intake (TT vs. CT, p = 0.0124). For each diet, genotype groups were distributed as follows: cheese (TT=38, CT= 29 and CC=12), butter (TT=35, CT= 31 and CC=14), MUFA (TT=38, CT= 29 and CC=11), PUFA (TT=38, CT= 29 and CC=12) and CHO (TT=36, CT= 28 and CC=12). Data are presented as least squares means ± SEM.

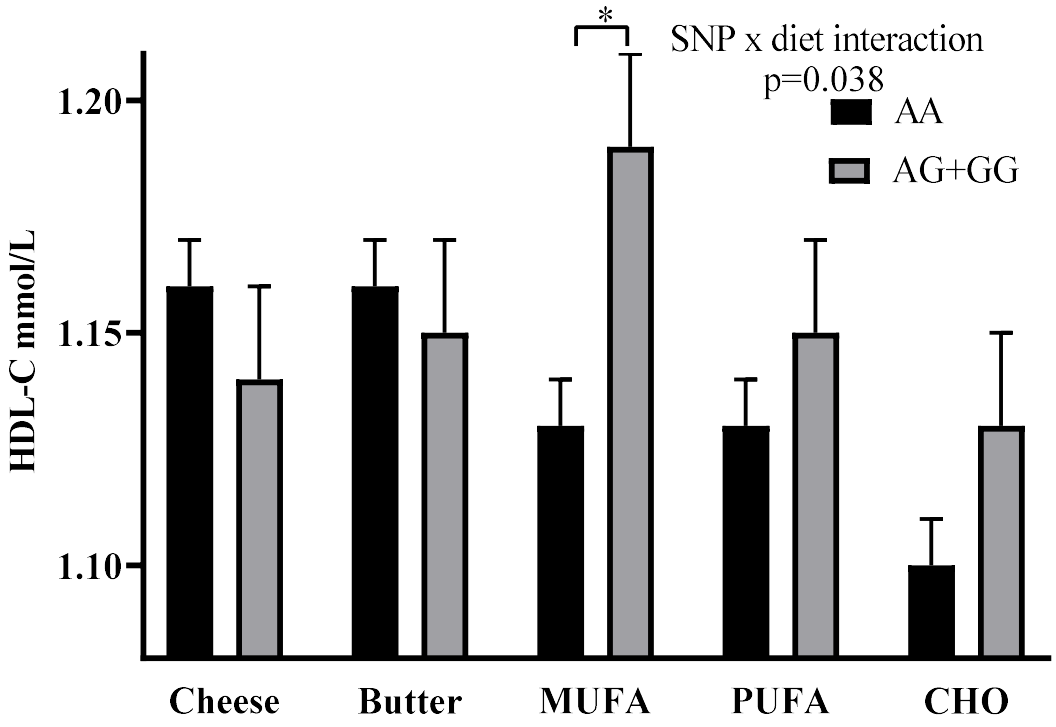

Figure 4. 4 Effect of *NPC1L1-rs2073547* genotype on HDL-C concentration after 4-week-long dietary interventions.

The slices effect shows the differences in HDL-C between the genotype with MUFA intake (AA vs. GG+AG, p = 0.0139). For each diet, genotype groups were distributed as follows: cheese (AA=42, AG= 31 and GG=6), butter (AA=44, AG= 30 and GG=6), MUFA (AA=44, AG= 28 and GG=6), PUFA (AA=43, AG= 30 and GG=6) and CHO (AA=41, AG= 29 and GG=6). Data are presented as least squares means ± SEM.

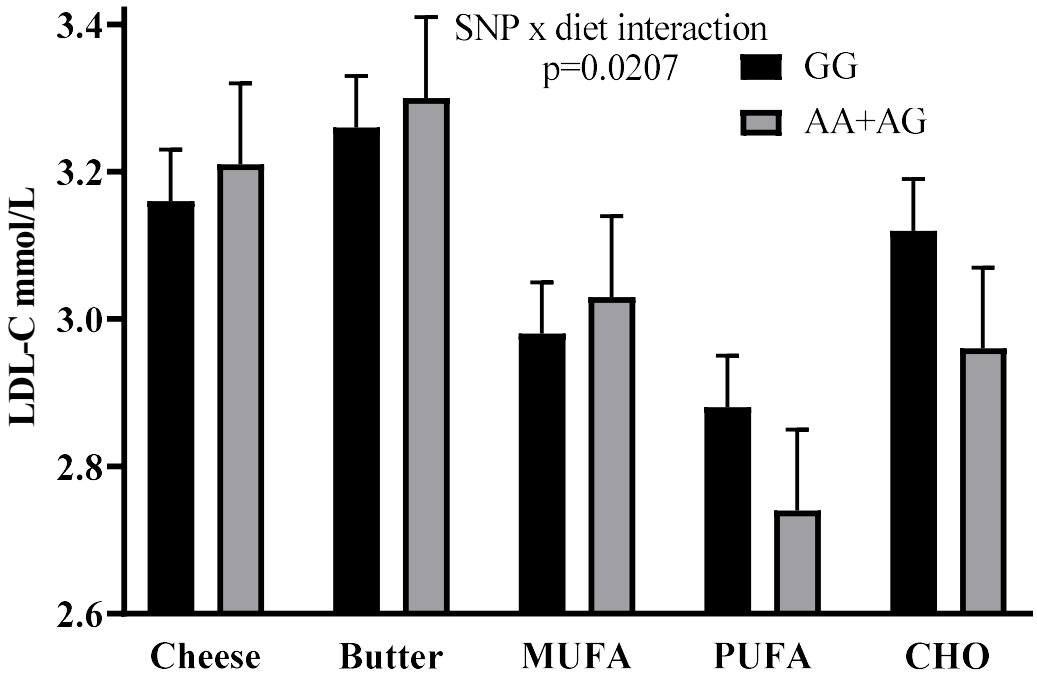

Figure 4. 5 Effect of *PPAR-rs6008259* genotype on LDL-C concentration after4-week-long dietary interventions.

For each diet, genotype groups were distributed as follows: cheese (GG=55, AG= 7 and AA=17), butter (GG=52, AG= 9 and AA=18), MUFA (GG=53, AG= 9 and AA=16), PUFA (GG=54, AG= 8 and AA=17) and CHO (GG=53, AG= 7 and AA= 16). Data are presented as least squares means ± SEM.

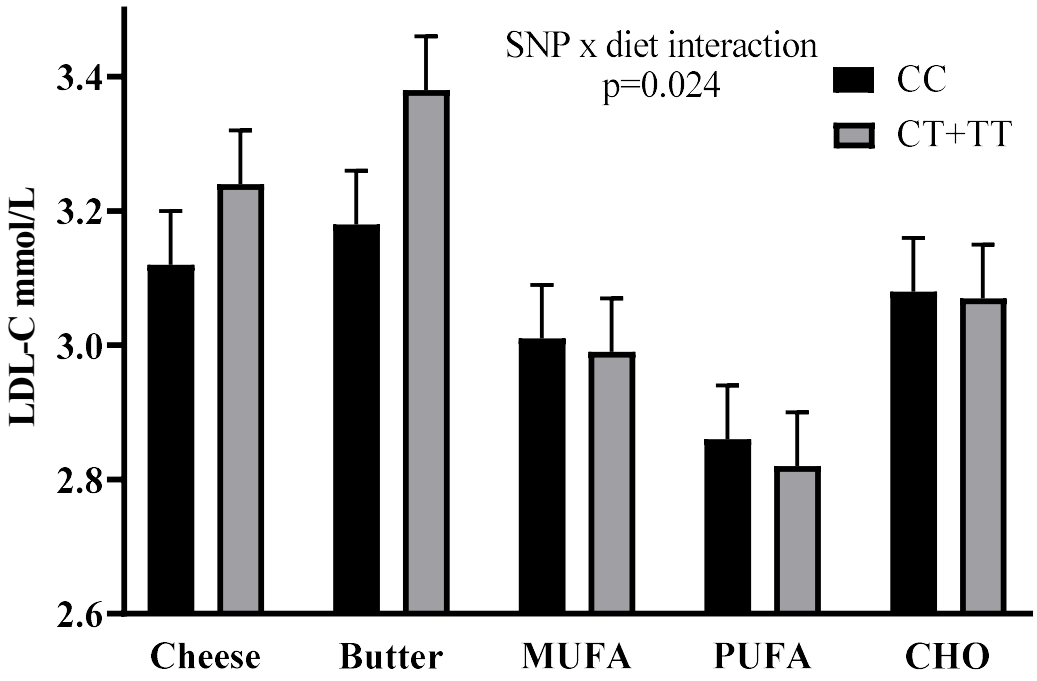

Figure 4. 6 Effect of *LIPG-rs2000813* genotype on LDL-C concentrations after 4-week-long dietary interventions.

For each diet, genotype groups were distributed as follows: cheese (CC=40, CT=30 and TT=9), butter (CC=41, CT= 30 and TT=9), MUFA (CC=37, CT= 30 and TT=9), PUFA (CC=38, CT=32 and TT=9) and CHO (CC=38, CT= 29 and TT=9). Data are presented as least squares means ± SEM.

Table 4. 1 Correlations of serum lipid concentrations of individuals following intake of two dairy diets (cheese and butter)

	Butter TC[1]	Butter LDL-C	Butter TG	Butter HDL-C
Cheese TC	r = 0.8936 P <0.0001	-	-	-
Cheese LDL-C	-	r =0.9082 P <0.0001	-	-
Cheese TG	-	-	r =0.8285 P <0.0001	
Cheese HDL-C	-	-	-	r =0.8905 P <0.0001

[1]End point serum lipid (TC, LDL-C, TG and LDL-C) concentrations following 4-week-long intake of cheese (n=79) and butter (n=79) were used to calculate Pearson's *r*.

Table 4. 2 Correlations of serum lipid concentrations of individuals following intake of two unsaturated diets (MUFA and PUFA)

	MUFA TC[1]	MUFA LDL-C	MUFA TG	MUFA HDL-C
PUFA TC	*r* =0.8914 P <0.0001	-	-	-
PUFA LDL-C	-	*r* =0.8924 P <0.0001	-	-
PUFA TG	-	-	*r* =0.8947 P <0.0001	-
PUFA HDL-C	-	-	-	*r* =0.7949 P <0.0001

[1]End point serum lipids (TC, LDL-C, TG and LDL-C) concentrations following 4-weel-long intake of PUFA (n=79) and MUFA (n=79) were used to calculate Pearson's *r*.

Table 4. 3 List of SNPs entered in partial least squares regression analysis following selection by linear mixed model analysis[1]

HDL-C		LDL-C		TC		TG	
Gene and SNP rs no.	**p-value**	**Gene and SNP rs no.**	**p-value**	**Gene and SNP rs no.**	**p-value**	**Gene and SNP rs no.**	**p-value**
NPC1L1-rs2073547[2]	0.012	*ABCA1*-rs2066714	<0.001	*ABCA1*-rs2066714	0.002	*LIPG*-rs2000813[2]	0.030
SREBF2-rs2228313	0.015	*LIPG*-rs2000813[2]	0.028	*PPAR*-rs6008259[2]	0.106	*SCAP*-rs12487736[2]	0.053
DHCR7-rs1044482[2]	0.021	*LIPC*-rs6083	0.049	*LIPC*-rs6083	0.145	*ABCA1*-rs2230808[2]	0.059
ABCA1-rs2066714	0.025			*LIPG*-rs2000813[2]	0.187	*DHCR7*-rs1044482[2]	0.110
DHCR7-rs760241	0.037					*CYP7A1*-rs3805607	0.147
APOB-rs676210[2]	0.109					*LIPC*-rs6083[2]	0.152
PPAR-rs6008259	0.150						
LPL-rs3200218[2]	0.197						

[1] SNPs were ranked by increasing p-values.

[2] These SNPs were entered in the dominant genetic model.

[3] *ATP-binding cassette subfamily A, member1 (ABCA1;* rs2066714*) cholesterol 7α-hydroxylase (CYP7A1;* rs3808607*); 7-dehydocholesterol reductase (DHCR7;* rs760241, rs1044482*); sterol regulatory element binding transcription factor chaperone (SCAP;* rs12487736*); sterol regulatory element binding transcription factor 2 (SREBF2;* rs2228313*); APOB (*rs676210*); Niemann-Pick C1 like intracellular cholesterol transporter 1 (NPC1L1;* rs2073547*); peroxisome proliferator activated receptor alpha (PPARA;* rs6008259*); lipase C, hepatic type (LIPC;* rs6083*); lipoprotein lipase (LPL;* rs3200218*); and lipase G, endothelial type (LIPG;* rs2000813). See Supplemental Table 1 for a complete list of gene names and symbols.

Table 4. 4 SNPs entered in partial least squares regression analysis of blood lipid concentrations following selection by linear mixed model analysis[1]

HDL-C		LDL-C		TC		TG	
R^2	32.8	R^2	16.5	R^2	18.8	R^2	19.8
Q^2	23.8	Q^2	8.6	Q^2	11.2	Q^2	11.1
Regression coefficients		**Regression coefficients**		**Regression coefficients**		**Regression coefficients**	
Constant	0.090	Constant	0.233	Constant	0.456	Constant	0.063
BMI	-0.003	Age	-0.003	Age	-0.005	Age	-0.003
DHCR7-rs760241	-0.054	BMI	-0.008	BMI	0.012	*DHCR7*-rs1044482[2]	0.074
DHCR7-rs1044482[2]	-0.040	D1-LDL-C	-0.053	*PPAR*-rs6008259[2]	-0.102	*LIPChep*-rs6083[2]	-0.069
NPC1L1-rs2073547[2]	-0.043	*ABCA1*-rs2066714	-0.058	*ABCA1*-rs2066714	0.108	*SCAP*-rs12487736[2]	0.099
PPAR-rs6008259	-0.018	*LIPG*-rs2000813[2]	-0.092	*LIPG*-rs2000813[2]	-0.078	*ABCA1*-rs2230808[2]	0.087
SREBF2-rs2228313	-0.056					*LIPG*-rs2000813[2]	0.099
ABCA1-rs2066714	0.036						

[1] Variables were ranked by decreasing variable importance in the projection values.

[2] These SNPs were entered in the dominant genetic model.

[3] See Table 6 for a gene names and symbols used in this analysis.

Genotypes were coded as follows: major allele homozygotes, 0; heterozygotes, 1; minor allele homozygotes, 2 in the additive genetic model; major allele homozygotes, 0; heterozygotes and minor allele homozygotes, 1 in the dominant genetic model. Hence,

a positive regression coefficient indicates that subjects possessing minor alleles display an increase in the corresponding blood lipid concentration.

Supplementary Table

Table 4. 5 Mean nutritional composition of the five experimental diets

	Cheese	**Butter**	**MUFA**	**PUFA**	**CHO**
Energy1, kcal	2654±567	2615±537	2647±550	2649±576	2618±561
Cheese, g/2500 kcal	90.0	0	0	0	0
Butter, g/2500 kcal	0	48.9	0	0	0
Lipids, %	32.0	32.0	32.0	32.0	25.0
SFA	12.6	12.4	5.8	5.8	5.8
MUFA	12.5	12.3	19.6	12.6	12.6
PUFA	4.8	4.8	4.8	11.5	4.8
Carbohydrates, %	51.9	52.0	51.9	51.9	58.9
Proteins, %	16.0	16.0	16.0	16.0	16.0
Calcium, mg/ 2500 kcal	1261.0	811.1	812.2	811.7	841.6
Total fibers, mg/2500 kcal	30.7	30.6	30.6	30.6	30.5
Cholesterol, mg/2500 kcal	272.1	272.4	271.5	272.2	272.4
Sodium, mg/2500 kcal	2482	2480	2479	2479	2485

All values represented as mean ± SD[1] (adopted from previously described by Brassard et al., 2017)

Table 4. 6 Characteristics at screening of subjects having completed at least one diet (n=92)[1]

	INAF (*n*=57)	RCFFN (*n*=35)	*P*
Ethnicity, n (%)			< 0.0001
Caucasian	55 (96.5)	11 (31.4)	--
Asian	0 (0)	10 (20.0)	--
African/African American	0 (0)	7 (28.6)	--
Hispanic	1 (1.75)	6 (17.1)	--
Other	1 (1.75)	1 (2.9)	--
Women, n (%)	32 (56.1)	17 (48.6)	0.48
Age, y	40.6 ± 13.6[2]	36.8 ± 13.3	0.19
Body weight, kg	86.5 ± 21.0	89.5 ± 19.8	0.51
BMI[3], kg/m^2	30.3 ± 6.3	31.6 ± 5.6	0.23
Waist circumference, cm	100.6 ± 14.1	103.8 ± 13.9	0.29
Plasma lipids, mmol/L			
Total C	5.18 ± 1.00	4.70 ± 0.81	0.02
LDL-C[4]	3.22 ± 0.84	2.79 ± 0.73	0.01
HDL-C	1.21 ± 0.20	1.15 ± 0.22	0.15
TG[3]	1.50 ± 0.83	1.64 ± 1.17	0.68
Total cholesterol:HDL-C ratio	4.37 ± 1.01	4.28 ± 0.89	0.67
Glucose[3], mmol/L	5.21 ± 0.47	5.09 ± 0.54	0.21
Blood pressure, mm Hg			
Systolic	113.1 ± 12.0	115.6 ± 17.0	0.45
Diastolic	69.4 ± 10.1	77.1 ± 10.8	0.0008
10-year Framingham risk score, %	3.9 ± 4.3	3.5 ± 3.7	0.53

[1] P values were determined with the use of Chi-Square test for categorical variables and a Student's t-test for continuous variables. INAF: Institute of Nutrition and Functional Foods; RCFFN: Richardson Center on Functional Foods and Nutraceuticals; BMI: body mass index; C: cholesterol; HDL: high-density lipoprotein; LDL: low-density lipoprotein; TG: triglyceride

[2] Values are means ± SDs unless otherwise indicated.

[3] Analyses were performed on log-transformed data because original values were not normally distributed

[4] n=56 for the INAF because of one missing value (adopted from previously described by Brassard et al., 2017)

Table 4. 7 SNPs ID corresponding to ABI genotyping assays catalogue numbers described in this study

	SNP ID	ABI assay ID #
1	rs6720173	C__29001998_10
2	rs3808607	C__27492121_20
3	rs760241	C___1828603_20
4	rs676210	C___3216558_10
5	rs1044482	C___7472254_10
6	rs2073547	C__15951156_10
7	rs6008259	C__30475324_10
8	rs7421	C_189341819_10
9	rs429358	C___3084793_20
10	rs6083	C____305149_10
11	rs1051338	C___8870360_20
12	rs3200218	C___9639440_10
13	rs2228313	C__16170982_10
14	rs2228314	C___2189943_10
15	rs12487736	C__33912364_10
16	rs562556	C____998751_10
17	rs2306986	C__16195230_10
18	rs2066714	C___2741083_1_
19	rs2230808	C___2741104_1_
20	rs11887534	C__26135643_10
21	rs688	C___2804264_20
22	rs2000813	C___1519458_20

Table 4. 8 List of SNPs in genes involved in the metabolism and transport of cholesterol and bile acids

Gene	SNPs	Type of SNP	Variation	MAF	Gene function
1.*ABCA**1***	*ATP-binding cassette subfamily A, member 1*				Cholesterol efflux pump in the cellular lipid removal pathway.
	rs2230808	Missense	C to T	T=0.2907	
	rs2066714	Missense	T to C	C=0.2083	
2. *ABCG5*	*ATP-binding cassette subfamily G, member 5*				Half-transporter (with ABCG8) that promotes intestinal and biliary excretion of sterols
	rs6720173	Missense	G to C	C=0.2073	
3. *ABCA5*	*ATP-binding cassette subfamily A, member 5*				
	rs11887534	Near gene-5'	G to C	C=0.0670	
4. *APOB*	*Apolipoprotein B*				Apolipoprotein B is the primary apolipoprotein of chylomicrons, VLDL, IDL, and LDL particles. Acts as a ligand for LDL receptors in various cells throughout the body
	rs676210	Missense	G to A	A=0.2929	
5. *ApoE*	*Apolipoprotein E*				Apolipoprotein E is a glycoprotein present in human plasma; ApoE is associated with triglyceride-rich lipoproteins (chylomicrons and VLDLs) and HDL

	Two SNPs, i.e. rs7412 and rs429358, were combined for determination of APOE isoforms, E2 (7.9%), E3 (78.6%) and E4 (13.5%)				
6. C*YP7A1*	*cholesterol 7α-hydroxylase*				The rate-limiting enzyme in the synthesis of bile acid in the classic pathway
	rs3808607	Near gene-5'	T to G	G=0.4526	
7. *DHCR7*	*7-dehydrocholesterol reductase*				Enzyme which catalyzes the conversion of 7-dehydrocholesterol to cholesterol
	rs760241	Missense	G to A	A=0.1472	
	rs1044482	Missense	T to C	C=0.4351	
8. *LDLR*	*LDL receptor*				A cell surface protein involved in receptor-mediated endocytosis
	rs688	Synonymous	C to T	T=0.3908	
9.*LIPC*	*lipase C*, hepatic type				Regulation of plasma triglycerides. Function of the enzyme is controlled by HDL.
	rs6083	Missense	A to G	A=0.5277	
10. *LIPG*	*lipase G, endothelial type*				Involved in lipoprotein and possibly HDL metabolism
	rs2000813	Missense	C to T	T=0.2291	
11. *LPL*	*lipoprotein lipase*				Lipoprotein lipase functions as a homodimer, and has the dual functions of triglyceride hydrolase and
	rs3200218	3'-UTR	A to G	G=0.1886	

					ligand/bridging factor for receptor-mediated lipoprotein uptake.
12. *MTTP*	*Microsomal triglyceride transfer protein*				A protein that plays a central role in lipoprotein assembly
	rs2306986	Missense	G to C	C=0.0582	
13. *NPC1L1*	*Niemann-Pick C1 like intracellular cholesterol transporter 1*				A protein that controls the influx of free cholesterol into cells through vesicular endocytosis and plays a critical role in intestinal cholesterol absorption.
	rs2073547	Near gene-5'	A to G	G=0.2369	
14. *PCSK9*	*Proprotein convertase subtilisin/kexin type 9*				
	rs562556	Missense	A to G	G=0.1436	A convertase belonging to the proteinase K subfamily which induces LDLR degradation
15. *PPARA*	*Peroxisome proliferator activated receptor alpha*				Nuclear transcription factor that regulates lipid metabolism
	rs6008259	3'-UTR	A to G	A=0.3210	
16. *SCAP*	*Sterol regulatory element binding transcription factor chaperone*				A protein with a sterol sensing domain which is involved in SREBFs regulation.
	rs12487736	Missense	C to T	T=0.5431	
17. *SREBF2*	Sterol regulatory element binding transcription factor 2				
	rs2228314	Missense	G to C	C=0.3411	

	rs2228313	Missense	G to C	C=0.0681	Transcription factor that controls cholesterol homeostasis by stimulating transcription of sterol-regulated genes.

List of 21 SNPs in 17 genes involved in cholesterol metabolism were tested in this assocation study, MAF, minor allele frequency based on global population at NCBI

Table 4. 9 Allele frequency of SNPs corresponding to this genetic association study

	ABCG5 -rs6720173	CY7A1 -rs3808607	DHCR7 -rs760241	APOB -rs676210	DHCR7 -rs1044482	NPC1L1 -rs2073547
Genotype 1	66 (GG)	29 (TT)	81 (GG)	53 (GG)	45 (TT)	50 (AA)
Genotype 2	26 (GC)	45 (GT)	11 (AG)	31 (AG)	32 (CT)	36 (AG)
Genotype 3	0 (CC)	18 (GG)	0 (AA)	8 (AA)	15 (CC)	6 (GG)
Sum	92	92	92	92	92	92
Frequency allele 2	0.14	0.44	0.06	0.26	0.34	0.26
Expected 1	68 (GG)	29 (TT)	81 (GG)	51 (GG)	40 (TT)	50 (AA)
Expected 2	22 (GC)	45 (GT)	10 (AG)	35 (AG)	41 (CT)	35 (AG)
Expected 3	2 (CC)	18 (GG)	0 (AA)	6 (AA)	10 (CC)	6 (GG)
Chi-square	2.49	0.01	0.37	1.2	4.52	0.02
p-value	0.11	0.94	0.54	0.27	0.03	0.89

Cont.

	PPAR -rs6008259	APOE -rs7421	APOE -rs429358	LIPC -rs6083	LIPA -rs1051338	SREBF2 - rs2228313	SREBF2 -rs2228314	SCAP -rs12487736
Genotype 1	64 (GG)	71 (CC)	59 (TT)	33 (AA)	41 (TT)	78 (GG)	50 (GG)	27 (TT)
Genotype 2	10 (AG)	10 (CT)	20 (CT)	35 (AG)	38 (GT)	14 (GC)	30 (CG)	45 (CT)
Genotype 3	18 (AA)	1 (TT)	3 (CC)	24 (GG)	13 (GG)	0 (CC)	12 (CC)	20 (CC)
Sum	92	82	82	92	92	92	92	92
Frequency allele 2	0.25	0.07	0.16	0.45	0.35	0.08	0.29	0.46
Expected 1	52 (GG)	70 (CC)	58 (TT)	28 (AA)	39 (TT)	79 (GG)	46 (GG)	27 (TT)
Expected 2	35 (AG)	11 (CT)	22 (CT)	46 (AG)	42 (GT)	13 (GC)	38 (CG)	46 (CT)
Expected 3	6 (AA)	0 (TT)	2 CC)	19 (GG)	11 (GG)	1 (CC)	8 (CC)	20 (CC)
Chi-square	46.4	0.83	0.6	4.94	0.074	0.62	4.2	0.02
p-value	0	0.36	0.44	0.03	0.39	0.43	0.04	0.88

Cont.

	PCSK9 -rs562556	MTTP - rs2306986	ABCA1 - rs2066714	ABCA1 - rs2230808	ABCA5 - rs11887534	LDLR -rs688	LIPG - rs2000813
Genotype 1	69 (AA)	77 (GG)	58 (TT)	51 (CC)	81 (GG)	29 (CC)	45 (CC)
Genotype 2	23 (AG)	15 (CG)	27 (CT)	31 (CT)	11 (CG)	50 (CT)	37 (CT)
Genotype 3	0 (GG)	0 (CC)	7 (CC)	10 (TT)	0 (CC)	13 (TT)	10 (TT)
Sum	92	92	92	92	92	92	92
Frequency allele 2	0.13	0.08	0.22	0.28	0.06	0.41	0.31
Expected 1	70 (AA)	78 (GG)	56 (TT)	48 (CC)	81 (GG)	32 (CC)	44 (CC)
Expected 2	20 (AG)	14 (CG)	32 (CT)	37 (CT)	10 (CG)	45 (CT)	39 (CT)
Expected 3	1 (GG)	1 (CC)	5 (CC)	7 (TT)	0 (CC)	16 (TT)	9 (TT)
Chi-square	1.88	0.72	2.14	2.33	0.37	1.34	0.33
p-value	0.17	0.39	0.14	0.13	0.54	0.25	0.57

Table 4. 10 The effect of single nucleotide polymorphisms in genes involved in cholesterol metabolism and transport and the response of serum lipids levels at the end of each dietary intervention in healthy adults

Gene, SNP, genotype	n	TC[1], mmol/L	LDL[1], mmol/L	TG[1], mmol/L	HDL[1], mmol/L
***ABCG5*-rs6720173**					
Cheese					
GG	56	4.96 ± 0.07	3.18 ± 0.07	1.38 ± 0.05	1.15 ± 0.01
CG	23	5.08 ± 0.12	3.18 ± 0.11	1.55 ± 0.08	1.16 ± 0.02
Butter					
GG	56	5.05 ± 0.07	3.29 ± 0.07	1.31 ± 0.05	1.15 ± 0.01
CG	24	5.09 ± 0.12	3.24 ± 0.11	1.44 ± 0.08	1.16 ± 0.02
MUFA					
GG	57	4.82 ± 0.07	3.04 ± 0.07	1.37± 0.05	1.15 ± 0.01
CG	21	4.68 ± 0.12	2.87 ± 0.11	1.33 ± 0.09	1.17 ± 0.02
PUFA					
GG	55	4.59 ± 0.07	2.87 ± 0.07	1.29 ± 0.05	1.13 ± 0.01
CG	24	4.52 ± 0.12	2.76 ± 0.11	1.28 ± 0.08	1.17 ± 0.02
CHO					
GG	55	4.87 ± 0.08	3.10 ± 0.07	1.40 ± 0.05	1.11 ± 0.01
CG	21	4.82 ± 0.12	3.00 ± 0.11	1.49 ± 0.09	1.12 ± 0.02
P[2]					
Diet		<.0001	<.0001	0.0030	0.0553
Genotype		0.8641	0.4773	0.4127	0.3285
Interaction		0.2308	0.4026	0.2215	0.7890
***ABCA1*-rs2066714**					
Cheese					
TT	50	4.92 ± 0.07	3.12 ± 0.07	1.41 ± 0.06	1.14 ± 0.01
CC+CT	6+23	5.13 ± 0.10	3.28 ± 0.09	1.46 ± 0.07	1.18 ± 0.02
Butter					
TT	52	4.98 ± 0.07	3.21 ± 0.07	1.29 ± 0.05	1.17 ± 0.01
CC+CT	6+22	5.20 ± 0.10	3.34 ± 0.09	1.45 ± 0.08	1.13 ± 0.02
MUFA					

TT	51	4.65 ± 0.07	2.92 ± 0.07	1.28 ± 0.05	1.13 ± 0.01[a]
CC+CT	6+21	5.02 ± 0.10	3.12 ± 0.10	1.51 ± 0.08	1.20 ± 0.02[b]
PUFA					
TT	52	4.43 ± 0.07	2.74 ± 0.07	1.23 ± 0.05	1.12 ± 0.01
CC+CT	4+23	4.83 ± 0.10	3.03 ± 0.10	1.38 ± 0.08	1.17 ± 0.02
CHO					
TT	47	4.71 ± 0.08[a]	2.98 ± 0.07[a]	1.42 ± 0.06	1.10 ± 0.01
CC+CT	6+23	5.10 ± 0.10[b]	3.21 ± 0.10[b]	1.45 ± 0.08	1.15 ± 0.02
P[2]					
Diet		<.0001	<.0001	0.0491	0.0844
Genotype		**0.0044**	**0.0378**	0.1157	0.0854
Interaction		0.2651	0.3587	0.1846	**0.0093**
***ABCA1*-rs2230808**					
Cheese					
CC	44	4.93 ± 0.08	3.12 ± 0.08	1.45 ± 0.06	1.13 ± 0.01
TT+CT	9+27	5.07 ± 0.09	3.25 ± 0.09	1.40 ± 0.07	1.18 ± 0.02
Butter					
CC	44	5.00 ± 0.08	3.22 ± 0.08	1.33 ± 0.06	1.16 ± 0.01
TT+CT	9+27	5.13 ± 0.09	3.35 ± 0.09	1.36 ± 0.07	1.15 ± 0.02
MUFA					
CC	44	4.68 ± 0.08	2.92 ± 0.08	1.31 ± 0.06	1.15 ± 0.01
TT+CT	8+26	4.92 ± 0.10	3.10 ± 0.09	1.42 ± 0.07	1.17 ± 0.02
PUFA					
CC	46	4.46 ± 0.08	2.75 ± 0.08	1.26 ± 0.06	1.12 ± 0.01
TT+CT	8+25	4.72 ± 0.10	2.96 ± 0.09	1.30 ± 0.07	1.16 ± 0.02
CHO					
CC	43	4.78 ± 0.08	2.99 ± 0.08	1.48 ± 0.06	1.09 ± 0.01
TT+CT	8+25	4.95 ± 0.10	3.18 ± 0.09	1.35 ± 0.07	1.14 ± 0.02
P[2]					
Diet		<.0001	<.0001	0.0191	0.0780
Genotype		0.1047	0.1215	0.9974	0.1730
Interaction		0.6797	0.8235	0.1024	0.2307

***ABCA5*-rs11887534**					
Cheese					
GG	68	4.99 ± 0.07	3.17 ± 0.06	1.42 ± 0.05	1.15 ± 0.01
CG	11	5.06 ± 0.18	3.23 ± 0.17	1.50 ± 0.13	1.15 ± 0.03
Butter					
GG	70	5.03 ± 0.07	3.26 ± 0.06	1.33 ± 0.05	1.15 ± 0.01
CG	10	5.28 ± 0.18	3.40 ± 0.17	1.48 ± 0.13	1.20 ± 0.04
MUFA					
GG	68	4.76 ± 0.07	2.98 ± 0.06	1.33 ± 0.05	1.16 ± 0.01
CG	10	4.99 ± 0.19	3.10 ± 0.17	1.56 ± 0.13	1.16 ± 0.04
PUFA					
GG	69	4.56 ± 0.07	2.83 ± 0.06	1.25 ± 0.05	1.14 ± 0.01
CG	10	4.66 ± 0.18	2.89 ± 0.17	1.50 ± 0.13	1.10 ± 0.04
CHO					
GG	65	4.83 ± 0.07	3.05 ± 0.06	1.41 ± 0.05	1.15 ± 0.01
CG	11	5.02 ± 0.18	3.23 ± 0.17	1.53 ± 0.13	1.15 ± 0.03
P^2					
Diet		<.0001	<.0001	0.6413	0.0243
Genotype		0.3318	0.5143	0.1450	0.9394
Interaction		0.7859	0.8231	0.6756	0.2788
***DHCR7*-rs760241**					
Cheese					
GG	71	5.01 ± 0.07	3.19 ± 0.06	1.44 ± 0.05	1.15 ± 0.01
AG	8	4.88 ± 0.20	3.10 ± 0.18	1.29 ± 0.14	1.16 ± 0.04
Butter					
GG	69	5.09 ± 0.07	3.29 ± 0.06	1.37 ± 0.05	1.16 ± 0.01
AG	11	4.90 ± 0.18	3.21 ± 0.17	1.11 ± 0.13	1.16 ± 0.03
MUFA					
GG	69	4.78 ± 0.07	3.01 ± 0.06	1.35 ± 0.05	1.14 ± 0.01
AG	9	4.86 ± 0.19	2.92 ± 0.18	1.41 ± 0.14	1.27 ± 0.04
PUFA					
GG	70	4.56 ± 0.07	2.83 ± 0.06	1.28 ± 0.05	1.13 ± 0.01
AG	9	4.67 ± 0.19	2.89 ± 0.18	1.27 ± 0.14	1.19 ± 0.04

CHO					
GG	68	4.86 ± 0.07	3.08 ± 0.06	1.43 ± 0.05	1.11 ± 0.01
AG	8	4.83 ± 0.20	3.04 ± 0.18	1.34 ± 0.14	1.15 ± 0.04
P^2					
Diet		<.0001	<.0001	0.1553	0.0431
Genotype		0.8676	0.7778	0.4376	0.1530
Interaction		0.3041	0.8116	0.1568	0.0633
***DHCR7*-rs1044482**					
Cheese					
TT	38	4.94 ± 0.09	3.11 ± 0.08	1.45 ± 0.07	1.15 ± 0.02
CT	29	5.03 ± 0.11	3.24 ± 0.10	1.40 ± 0.08	1.14 ± 0.02
CC	12	5.08 ± 0.16	3.25 ± 0.15	1.42 ± 0.12	1.17 ± 0.03
Butter					
TT	35	5.03 ± 0.09	3.23 ± 0.08	1.41 ± 0.07	1.14 ± 0.02
CT	31	5.07 ± 0.11	3.28 ± 0.10	1.33 ± 0.07	1.17 ± 0.02
CC	14	5.13 ± 0.16	3.40 ± 0.15	1.22 ± 0.11	1.17 ± 0.03
MUFA					
TT	38	4.75 ± 0.09	2.96 ± 0.08	1.34 ± 0.07	1.17 ± 0.02
CT	29	4.80 ± 0.11	3.02 ± 0.10	1.39 ± 0.08	1.13 ± 0.02
CC	11	4.84 ± 0.17	3.05 ± 0.15	1.35 ± 0.12	1.17 ± 0.03
PUFA					
TT	38	4.57 ± 0.09	2.81 ± 0.08	1.25 ± 0.07	1.18 ± 0.02[a]
CT	29	4.54 ± 0.11	2.85 ± 0.10	1.31 ± 0.08	1.09 ± 0.02[b]
CC	12	4.63 ± 0.17	2.91 ± 0.15	1.31 ± 0.12	1.13 ± 0.03[b]
CHO					
TT	36	4.83 ± 0.09	3.04 ± 0.08	1.41 ± 0.07	1.12 ± 0.02
CT	28	4.90 ± 0.11	3.10 ± 0.10	1.47 ± 0.08	1.11 ± 0.02
CC	12	4.84 ± 0.17	3.09 ± 0.15	1.35 ± 0.12	1.12 ± 0.03
P^2					
Diet		<.0001	<.0001	0.0414	0.0388
Genotype		0.8786	0.7465	0.9095	0.5967
Interaction		0.9799	0.9832	0.6339	**0.0353**
***CYP7A1*-rs3808607**					

Cheese					
TT	26	5.00 ± 0.11	3.22 ± 0.10	1.34 ± 0.08	1.14 ± 0.02
GT	38	5.03 ± 0.09	3.32 ± 0.08	1.50 ± 0.07	1.16 ± 0.02
GG	15	4.92 ± 0.15	3.15 ± 0.13	1.27 ± 0.11	1.16 ± 0.03
Butter					
TT	26	5.11 ± 0.11	3.32 ± 0.10	1.30 ± 0.08	1.17 ± 0.02
GT	38	5.11 ± 0.09	3.32 ± 0.08	1.32 ± 0.07	1.16 ± 0.02
GG	16	4.89 ± 0.15	3.12 ± 0.13	1.35 ± 0.11	1.13 ± 0.03
MUFA					
TT	25	4.88 ± 0.12	3.09 ± 0.10	1.30 ± 0.08	1.16 ± 0.02
GT	37	4.81 ± 0.10	2.98 ± 0.08	1.40 ± 0.07	1.16 ± 0.02
GG	16	4.60 ± 0.15	2.89 ± 0.14	1.23 ± 0.11	1.14 ± 0.03
PUFA					
TT	27	4.61 ± 0.11	2.88 ± 0.10	1.24 ± 0.08	1.14 ± 0.02
GT	38	4.59 ± 0.09	2.82 ± 0.08	1.35 ± 0.07	1.13 ± 0.02
GG	14	4.48 ± 0.15	2.82 ± 0.13	1.06 ± 0.11	1.14 ± 0.03
CHO					
TT	28	4.85 ± 0.11	3.08 ± 0.10	1.44 ± 0.08	1.09 ± 0.02
GT	34	4.93 ± 0.10	3.11 ± 0.08	1.47 ± 0.07	1.12 ± 0.02
GG	14	4.72 ± 0.15	2.99 ± 0.14	1.17 ± 0.11	1.16 ± 0.03
P^2					
Diet		<.0001	<.0001	0.0218	0.2585
Genotype		0.4928	0.7169	0.1712	0.9282
Interaction		0.9304	0.6621	0.0735	0.5163
***APOB*-rs676210**					
Cheese					
GG	47	4.99 ± 0.08	3.20 ± 0.08	1.36 ± 0.06	1.16 ± 0.01
AA+AG	7+25	4.99 ± 0.10	3.15 ± 0.09	1.52 ± 0.07	1.14 ± 0.02
Butter					
GG	47	5.07 ± 0.08	3.29 ± 0.08	1.29 ± 0.06	1.17 ± 0.01
AA+AG	6+27	5.05 ± 0.10	3.26 ± 0.09	1.43 ± 0.07	1.13 ± 0.02
MUFA					
GG	47	4.81 ± 0.08	3.04 ± 0.08	1.30 ± 0.06	1.16 ± 0.01
AA+AG	6+25	4.75 ± 0.10	2.93 ± 0.09	1.45 ± 0.07	1.15 ± 0.02

PUFA					
GG	49	4.55 ± 0.08	2.85 ± 0.08	1.22 ± 0.06	1.14 ± 0.01
AA+AG	6+24	4.61 ± 0.10	2.83 ± 0.09	1.37 ± 0.07	1.14 ± 0.02
CHO					
GG	45	4.85 ± 0.08	3.07 ± 0.08	1.42 ± 0.06	1.11 ± 0.01
AA+AG	8+23	4.87 ± 0.10	3.07 ± 0.09	1.43 ± 0.07	1.12 ± 0.02
P[2]					
Diet		<.0001	<.0001	0.0243	0.1111
Genotype		0.9809	0.7232	0.1296	0.6098
Interaction		0.8712	0.7543	0.4847	0.4787
***NPC1L1*-rs2073547**					
Cheese					
AA	42	4.95 ± 0.09	3.13 ± 0.08	1.42 ± 0.06	1.16 ± 0.01
AG+GG	31+6	5.04 ± 0.09	3.23 ± 0.09	1.44 ± 0.07	1.14 ± 0.02
Butter					
AA	44	5.08 ± 0.09	3.27 ± 0.08	1.38 ± 0.06	1.16 ± 0.01
AG+GG	30+6	5.04 ± 0.09	3.28 ± 0.09	1.30 ± 0.07	1.15 ± 0.02
MUFA					
AA	44	4.71 ± 0.09	2.96 ± 0.08	1.33 ± 0.06	1.13 ± 0.01[a]
AG+GG	28+6	4.88 ± 0.10	3.04 ± 0.09	1.40 ± 0.07	1.19 ± 0.02[b]
PUFA					
AA	43	4.50 ± 0.09	2.77 ± 0.08	1.31 ± 0.06	1.13 ± 0.01
AG+GG	30+6	4.66 ± 0.09	2.92 ± 0.09	1.25 ± 0.07	1.15 ± 0.02
CHO					
AA	41	4.82 ± 0.09	3.04 ± 0.08	1.45 ± 0.06	1.10 ± 0.01
AG+GG	29+6	4.90 ± 0.10	3.11 ± 0.09	1.40 ± 0.07	1.13 ± 0.02
P[2]					
Diet		<.0001	<.0001	0.0086	0.0416
Genotype		0.4537	0.4612	0.7640	0.4145
Interaction		0.3548	0.6420	0.4962	**0.0380**
***PPAR*-rs6008259**					
Cheese					
GG	55	4.97 ± 0.08	3.16 ± 0.07	1.42 ± 0.05	1.15 ± 0.01
AA+AG	17+7	5.04 ± 0.12	3.21 ± 0.11	1.46 ± 0.08	1.16 ± 0.02

Butter					
GG	52	5.04 ± 0.08	3.26 ± 0.07	1.39 ± 0.05	1.14 ± 0.01
AA+AG	18+9	5.09 ± 0.12	3.30 ± 0.10	1.26 ± 0.08	1.19 ± 0.02
MUFA					
GG	53	4.79 ± 0.08	2.98 ± 0.07	1.40 ± 0.05	1.16 ± 0.01
AA+AG	16+9	4.77 ± 0.12	3.03 ± 0.11	1.27 ± 0.08	1.15 ± 0.02
PUFA					
GG	54	4.62 ± 0.08	2.88 ± 0.07	1.30 ± 0.05	1.13 ± 0.01
AA+AG	17+8	4.46 ± 0.12	2.74 ± 0.11	1.24 ± 0.08	1.15 ± 0.02
CHO					
GG	53	4.91 ± 0.08	3.12 ± 0.07	1.43 ± 0.05	1.12 ± 0.01
AA+AG	16+7	4.73 ± 0.12	2.96 ± 0.11	1.41 ± 0.09	1.11 ± 0.02
P^2					
Diet		<.0001	<.0001	0.0042	0.0403
Genotype		0.6931	0.7953	0.4698	0.7048
Interaction		0.1039	**0.0207**	0.3735	0.4125
***LIPC*-rs6083**					
Cheese					
AA	26	4.73 ± 0.11	3.00 ± 0.10	1.34 ± 0.08	1.10 ± 0.02
AG	31	5.13 ± 0.10	3.29 ± 0.09	1.42 ± 0.07	1.19 ± 0.02
GG	22	5.13 ± 0.12	3.25 ± 0.11	1.54 ± 0.09	1.16 ± 0.02
Butter					
AA	28	4.97 ± 0.10	3.20 ± 0.10	1.33 ± 0.08	1.15 ± 0.02
AG	28	5.14 ± 0.10	3.38 ± 0.09	1.22 ± 0.07	1.18 ± 0.02
GG	24	5.11 ± 0.12	3.25 ± 0.11	1.53 ± 0.08	1.14 ± 0.02
MUFA					
AA	26	4.69 ± 0.11	2.92 ± 0.10	1.34 ± 0.08	1.12 ± 0.02
AG	30	4.78 ± 0.10	3.01 ± 0.09	1.32 ± 0.07	1.16 ± 0.02
GG	22	4.91 ± 0.12	3.08 ± 0.11	1.43 ± 0.09	1.19 ± 0.02
PUFA					
AA	28	4.47 ± 0.11	2.72 ± 0.10	1.34 ± 0.08	1.13 ± 0.02
AG	30	4.53 ± 0.10	2.84 ± 0.09	1.20 ± 0.07	1.13 ± 0.02
GG	21	4.78 ± 0.12	3.00 ± 0.11	1.32 ± 0.09	1.16 ± 0.02
CHO					

AA	22	4.64 ± 0.11	2.91 ± 0.10	1.37 ± 0.08	1.09 ± 0.02
AG	31	4.93 ± 0.10	3.14 ± 0.09	1.42 ± 0.07	1.12 ± 0.02
GG	23	5.02 ± 0.12	3.17 ± 0.11	1.50 ± 0.09	1.14 ± 0.02
P^2					
Diet		<.0001	<.0001	0.0120	0.0556
Genotype		0.1003	0.2501	0.2907	0.2128
Interaction		0.1155	0.1275	0.2108	0.1979
***LIPA*- rs1051338**					
Cheese					
TT	36	4.99 ± 0.10	3.17 ± 0.09	1.47 ± 0.07	1.14 ± 0.02
GT+GG	33+10	4.99 ± 0.09	3.19 ± 0.08	1.39 ± 0.06	1.16 ± 0.01
Butter					
TT	37	4.93 ± 0.09	3.17 ± 0.09	1.34 ± 0.07	1.14 ± 0.02
GT+GG	34+9	5.17 ± 0.09	3.37 ± 0.08	1.35 ± 0.06	1.17 ± 0.01
MUFA					
TT	36	4.73 ± 0.10	2.93 ± 0.09	1.39 ± 0.07	1.15 ± 0.02
GT+GG	36+6	4.83 ± 0.09	3.05 ± 0.08	1.33 ± 0.06	1.16 ± 0.01
PUFA					
TT	38	4.54 ± 0.09	2.81 ± 0.09	1.31 ± 0.07	1.12 ± 0.02
GT+GG	33+8	4.60 ± 0.09	2.86 ± 0.08	1.33 ± 0.06	1.15 ± 0.01
CHO					
TT	35	4.80 ± 0.10	3.00 ± 0.09	1.46 ± 0.07	1.12 ± 0.02
GT+GG	33+8	4.90 ± 0.09	3.05 ± 0.08	1.40 ± 0.06	1.11 ± 0.02
P^2					
Diet		<.0001	<.0001	0.0091	0.0525
Genotype		0.4054	0.3474	0.5107	0.4079
Interaction		0.2793	0.2643	0.9088	0.7328
***LPL*-rs3200218**					
Cheese					
AA	48	5.06 ± 0.08	3.23 ± 0.07	1.46 ± 0.06	1.15 ± 0.01
AG+GG	26+5	4.89 ± 0.10	3.10 ± 0.09	1.37 ± 0.07	1.16 ± 0.02
Butter					
AA	50	5.08 ± 0.08	3.30 ± 0.07	1.34 ± 0.06	1.16 ± 0.01
AG+GG	26+4	5.03 ± 0.10	3.24 ± 0.09	1.36 ± 0.07	1.15 ± 0.02

MUFA					
AA	49	4.82 ± 0.08	3.02 ± 0.07	1.36 ± 0.06	1.16 ± 0.01
AG+GG	26+3	4.74 ± 0.10	2.95 ± 0.10	1.36 ± 0.07	1.14 ± 0.02
PUFA					
AA	48	4.64 ± 0.08	2.88 ± 0.07	1.31 ± 0.06	1.16 ± 0.01
AG+GG	26+5	4.47 ± 0.10	2.79 ± 0.09	1.24 ± 0.07	1.10 ± 0.02
CHO					
AA	48	4.87 ± 0.08	3.07 ± 0.07	1.45 ± 0.06	1.13 ± 0.01
AG+GG	26+2	4.83 ± 0.10	3.08 ± 0.10	1.40 ± 0.08	1.09 ± 0.02
P[2]					
Diet		<.0001	<.0001	0.0156	0.0175
Genotype		0.3691	0.5461	0.6282	0.2656
Interaction		0.6586	0.5513	0.7245	0.2293
***SREBF2*-rs2228313**					
Cheese					
GG	68	5.00 ± 0.07	3.17 ± 0.06	1.45 ± 0.05	1.15 ± 0.01
CG	11	4.99 ± 0.17	3.21 ± 0.15	1.30 ± 0.12	1.17 ± 0.03
Butter					
GG	68	5.04 ± 0.07	3.25 ± 0.06	1.37 ± 0.05	1.15 ± 0.01
CG	12	5.18 ± 0.17	3.42 ± 0.15	1.22 ± 0.12	1.19 ± 0.03
MUFA					
GG	66	4.80 ± 0.07	2.99 ± 0.06	1.39 ± 0.05	1.16 ± 0.01
CG	12	4.73 ± 0.17	3.04 ± 0.15	1.19 ± 0.12	1.13 ± 0.03
PUFA					
GG	67	4.57 ± 0.07	2.83 ± 0.06	1.31 ± 0.05	1.14 ± 0.01
CG	12	4.58 ± 0.17	2.90 ± 0.15	1.16 ± 0.12	1.15 ± 0.03
CHO					
GG	64	4.84 ± 0.07	3.04 ± 0.06	1.46 ± 0.05	1.12 ± 0.01
CG	12	4.93 ± 0.17	3.23 ± 0.15	1.25 ± 0.12	1.13 ± 0.03
P[2]					
Diet		<.0001	<.0001	0.2124	0.0574
Genotype		0.8390	0.4953	0.1013	0.9208
Interaction		0.6865	0.6337	0.9782	0.4776

***SREBF2*-rs2228314**					
Cheese					
GG	41	4.93 ± 0.09	3.11 ± 0.08	1.48 ± 0.06	1.13 ± 0.02
CC+CG	9+29	5.06 ± 0.09	3.26 ± 0.08	1.36 ± 0.06	1.17 ± 0.02
Butter					
GG	40	5.03 ± 0.09	3.24 ± 0.08	1.37 ± 0.06	1.14 ± 0.02
CC+CG	10+30	5.10 ± 0.09	3.32 ± 0.08	1.32 ± 0.06	1.17 ± 0.02
MUFA					
GG	39	4.73 ± 0.09	2.95 ± 0.08	1.37 ± 0.06	1.14 ± 0.02
CC+CG	8+31	4.85 ± 0.09	3.05 ± 0.08	1.34 ± 0.06	1.17 ± 0.02
PUFA					
GG	40	4.55 ± 0.09	2.82 ± 0.08	1.26 ± 0.06	1.14 ± 0.02
CC+CG	9+30	4.60 ± 0.09	2.87 ± 0.08	1.30 ± 0.06	1.13 ± 0.02
CHO					
GG	38	4.80 ± 0.09	3.02 ± 0.08	1.44 ± 0.06	1.10 ± 0.02
CC+CG	9+29	4.91 ± 0.09	3.13 ± 0.08	1.41 ± 0.06	1.13 ± 0.02
P^2					
Diet		<.0001	<.0001	0.0104	0.0449
Genotype		0.3962	0.3812	0.6021	0.2775
Interaction		0.9613	0.8183	0.5014	0.6135
***SCAP*-rs12487736**					
Cheese					
TT	23	5.04 ± 0.12	3.19 ± 0.11	1.48 ± 0.09	1.15 ± 0.02
CT	39	4.90 ± 009	3.09 ± 0.09	1.40 ± 0.06	1.15 ± 0.02
CC	17	5.14 ± 0.14	3.37 ± 0.13	1.43 ± 0.10	1.14 ± 0.03
Butter					
TT	20	5.00 ± 0.13	3.23 ± 0.11	1.26 ± 0.09	1.17 ± 0.02
CT	42	4.96 ± 0.09	3.17 ± 0.08	1.36 ± 0.06	1.16 ± 0.01
CC	18	5.33 ± 0.14	3.59 ± 0.13	1.42 ± 0.10	1.12 ± 0.03
MUFA					
TT	23	4.72 ± 0.12	2.92 ± 0.11	1.32 ± 0.09	1.18 ± 0.02
CT	39	4.73 ± 0.09	2.93 ± 0.09	1.38 ± 0.07	1.14 ± 0.02
CC	16	4.98 ± 0.14	3.24 ± 0.13	1.35 ± 0.10	1.15 ± 0.03

PUFA					
TT	22	4.50 ± 0.12	2.78 ± 0.11	1.20 ± 0.09	1.15 ± 0.02
CT	41	4.57 ± 0.09	2.80 ± 0.08	1.35 ± 0.06	1.14 ± 0.02
CC	16	4.65 ± 0.14	3.00 ± 0.13	1.22 ± 0.10	1.11 ± 0.03
CHO					
TT	21	4.80 ± 0.12	2.97 ± 0.11	1.54 ± 0.09	1.10 ± 0.02
CT	38	4.76 ± 0.09	2.97 ± 0.09	1.41 ± 0.07	1.12 ± 0.02
CC	17	5.13 ± 0.14	3.41 ± 0.13	1.31 ± 0.10	1.14 ± 0.03
P^2					
Diet		<.0001	<.0001	0.0026	0.0689
Genotype		0.1940	0.0603	0.9418	0.8359
Interaction		0.3751	0.3138	0.0679	0.5614
***PCSK9*-rs5622556**					
Cheese					
AA	61	5.02 ± 0.07	3.19 ± 0.07	1.46 ± 0.05	1.15 ± 0.01
AG	18	4.90 ± 0.13	3.13 ± 0.12	1.32 ± 0.09	1.16 ± 0.03
Butter					
AA	61	5.06 ± 0.08	3.27 ± 0.07	1.32 ± 0.05	1.17 ± 0.01
AG	19	5.06 ± 0.13	3.27 ± 0.12	1.42 ± 0.09	1.12 ± 0.02
MUFA					
AA	61	4.79 ± 0.07	3.00 ± 0.07	1.36 ± 0.05	1.16 ± 0.01
AG	17	4.75 ± 0.14	2.97 ± 0.12	1.34 ± 0.10	1.15 ± 0.03
PUFA					
AA	59	4.54 ± 0.07	2.81 ± 0.07	1.27 ± 0.05	1.14 ± 0.01
AG	20	4.68 ± 0.13	2.94 ± 0.12	1.32 ± 0.09	1.13 ± 0.02
CHO					
AA	60	4.85 ± 0.07	3.06 ± 0.07	1.43 ± 0.05	1.12 ± 0.01
AG	16	4.84 ± 0.14	3.10 ± 0.12	1.42 ± 0.10	1.09 ± 0.03
P^2					
Diet		<.0001	<.0001	0.2304	0.0677
Genotype		0.9632	0.8844	0.9917	0.4282
Interaction		0.3727	0.3672	0.2525	0.6974
***MTTP*-rs2306986**					
Cheese					

GG	68	4.99 ± 0.07	3.18 ± 0.06	1.42 ± 0.05	1.14 ± 0.01
CG	11	4.99 ± 0.17	3.14 ± 0.15	1.33 ±0.13	1.22 ± 0.03
Butter					
GG	69	5.09 ± 0.07	3.30 ± 0.06	1.33 ± 0.05	1.15 ± 0.01
CG	11	4.89 ± 0.17	3.11 ± 0.15	1.30 ± 0.13	1.16 ± 0.03
MUFA					
GG	68	4.80 ± 0.07	3.01 ± 0.06	1.34 ± 0.05	1.15 ± 0.01
CG	10	4.73 ± 0.17	2.92 ± 0.16	1.33 ± 0.13	1.18 ± 0.03
PUFA					
GG	67	4.58 ± 0.07	2.83 ± 0.06	1.30 ± 0.05	1.14 ± 0.01
CG	12	4.52 ± 0.17	2.87 ± 0.15	1.04 ± 0.13	1.14 ± 0.03
CHO					
GG	64	4.86 ± 0.07	3.07 ± 0.06	1.44 ± 0.05	1.10 ± 0.01
CG	12	4.82 ± 0.17	3.08 ± 0.15	1.18 ± 0.13	1.18 ± 0.03
P^2					
Diet		<.0001	<.0001	0.0323	0.2693
Genotype		0.6562	0.7101	0.2280	0.2360
Interaction		0.8017	0.3439	0.1497	0.3441
***LDLR*-rs688**					
Cheese					
CC	24	4.90 ± 0.11	3.06 ± 0.11	1.50 ± 0.08	1.14 ± 0.02
CT	43	5.01 ± 0.08	3.21 ± 0.08	1.39 ± 0.06	1.15 ± 0.01
TT	12	5.14 ± 0.17	3.30 ± 0.16	1.42 ± 0.12	1.20 ± 0.03
Butter					
CC	25	4.99 ± 0.11	3.21 ± 0.10	1.43 ± 0.08	1.11 ± 0.02
CT	44	5.07 ± 0.08	3.29 ± 0.08	1.31 ± 0.06	1.17 ± 0.01
TT	11	5.23 ± 0.17	3.40 ± 0.16	1.29 ± 0.12	1.20 ± 0.03
MUFA					
CC	22	4.66 ± 0.12	2.89 ± 0.11	1.38 ± 0.08	1.13 ± 0.02
CT	44	4.83 ± 0.08	3.03 ± 0.08	1.35 ± 0.06	1.17 ± 0.01
TT	12	4.85 ± 0.17	3.08 ± 0.16	1.33 ± 0.12	1.16 ± 0.03
PUFA					
CC	20	4.53 ± 0.12	2.75 ± 0.11	1.40 ± 0.09	1.12 ± 0.02
CT	46	4.65 ± 0.08	2.92 ± 0.08	1.27 ± 0.06	1.15 ± 0.01

TT	13	4.38 ± 0.17	2.76 ± 0.15	1.11 ± 0.12	1.13 ± 0.03
CHO					
CC	23	4.69 ± 0.12	2.90 ± 0.11	1.48 ± 0.08	1.10 ± 0.02
CT	41	4.91 ± 0.09	3.15 ± 0.08	1.38 ± 0.06	1.12 ± 0.02
TT	12	4.99 ± 0.17	3.15 ± 0.16	1.49 ± 0.12	1.15 ± 0.03
P[2]					
Diet		<.0001	<.0001	0.0033	0.1378
Genotype		0.5018	0.3961	0.4756	0.2831
Interaction		0.1562	0.3856	0.5662	0.6429
***LIPG*- rs2000813**					
Cheese					
CC	40	4.94 ± 0.09	3.12 ± 0.08	1.40 ± 0.07	1.16 ± 0.02
TT+CT	9+30	5.05 ± 0.09	3.24 ± 0.08	1.43 ± 0.07	1.14 ± 0.02
Butter					
CC	41	4.95 ± 0.09	3.18 ± 0.08	1.33 ± 0.07	1.15 ± 0.02
TT+CT	9+30	5.18 ± 0.09	3.38 ± 0.08	1.34 ± 0.07	1.17 ± 0.02
MUFA					
CC	37	4.79 ± 0.09	3.01 ± 0.09	1.29 ± 0.07	1.17 ± 0.02
TT+CT	10+31	4.79 ± 0.09	2.99 ± 0.08	1.41 ± 0.06	1.14 ± 0.02
PUFA					
CC	38	4.58 ± 0.09	2.86 ± 0.08	1.23 ± 0.07	1.15 ± 0.02
TT+CT	9+32	4.57 ± 0.09	2.82 ± 0.08	1.32 ± 0.06	1.13 ± 0.01
CHO					
CC	38	4.88 ± 0.09	3.08 ± 0.08	1.41 ± 0.07	1.14 ± 0.02
TT+CT	9+29	4.84 ± 0.09	3.07 ± 0.08	1.43 ± 0.07	1.10 ± 0.02
P[2]					
Diet		<.0001	<.0001	0.0095	0.0446
Genotype		0.6157	0.6405	0.4604	0.4087
Interaction		0.0858	**0.0245**	0.7102	0.2908
APOE[3]					
rs7412 + rs429358					
Cheese					
E2	10	4.91 ± 0.17	2.91 ± 0.15	1.62 ± 0.12	1.21 ± 0.03

E3	44	5.02 ± 0.08	3.22 ± 0.07	1.43 ± 0.06	1.14 ± 0.01
E4	23	5.00 ± 0.12	3.25 ± 0.11	1.33 ± 0.08	1.15 ± 0.02
Butter					
E2	10	4.85 ± 0.17	3.00 ± 0.15	1.20 ± 0.12	1.24 ± 0.03
E3	47	5.07 ± 0.08	3.27 ± 0.07	1.44 ± 0.06	1.14 ± 0.01
E4	21	5.19 ± 0.12	3.47 ± 0.11	1.20 ± 0.09	1.16 ± 0.02
MUFA					
E2	9	4.55 ± 0.17	2.69 ± 0.15	1.25 ± 0.12	1.26 ± 0.03
E3	45	4.83 ± 0.08	3.02 ± 0.07	1.45 ± 0.06	1.14 ± 0.01
E4	22	4.82 ± 0.12	3.13 ± 0.11	1.21 ± 0.08	1.14 ± 0.02
PUFA					
E2	10	4.23 ± 0.17	2.43 ± 0.15	1.29 ± 0.12	1.18 ± 0.03
E3	42	4.69 ± 0.08	2.94 ± 0.07	1.33 ± 0.06	1.14 ± 0.01
E4	23	4.51 ± 0.12	2.85 ± 0.11	1.18 ± 0.08	1.12 ± 0.02
CHO					
E2	8	4.74 ± 0.18	2.82 ± 0.16	1.52 ± 0.13	1.18 ± 0.04
E3	45	4.93 ± 0.08	3.13 ± 0.07	1.47 ± 0.06	1.11 ± 0.01
E4	22	4.78 ± 0.12	3.10 ± 0.11	1.30 ± 0.08	1.15 ± 0.02
P[2]					
Diet		<.0001	<.0001	<.0001	0.0243
Genotype		0.3021	**0.0514**	0.1034	**0.0290**
Interaction		0.1954	0.1054	0.1098	0.7827

[1]Values are least-squares means ± SEM at end of each dietary intervention, ATP-binding cassette subfamily A, member1 (*ABCA1*; rs2230808, rs2066714) and member 5(*ABCA5*; rs11887534); ATP-binding cassette subfamily G, member 5 (*ABCG5*; rs6720173); APOE (rs7412, rs429358); cholesterol 7α-hydroxylase (*CYP7A1*; rs3808607) 7-dehydocholesterol reductase (*DHCR7*; rs760241, rs1044482); LDL receptor (*LDL-R*; rs688); proprotein convertase subtilisin/kexin type 9 (*PCSK9*; rs562556); sterol regulatory element binding transcription factor chaperone (*SCAP*; rs12487736); sterol regulatory element binding transcription factor 2 (*SREBF2*; rs2228313, rs2228314); *APOB* (rs676210); Niemann-Pick C1 like intracellular cholesterol transporter 1 (*NPC1L1*; rs2073547); peroxisome proliferator activated receptor alpha (*PPARA*; rs6008259); lipase C, hepatic type (*LIPC*; rs6083); lipase A, lysosomal acid type (*LIPA*; rs1051338); lipoprotein lipase (*LPL*;

rs3200218); microsomal triglyceride transfer protein (*MTTP*; rs2306986); and lipase G, endothelial type (*LIPG*; rs2000813) were tested.

[2] Labeled means in each row representing according to diet, SNPs and diet x SNPs interaction analysed using SAS mixed procedure with sex, BMI, age, centre, baseline lipids (day 1value of TC, LDL-C, TG and HDL-C) as a fixed effect and participant as a random effect, and Tukey-Kramer adjustment was used to test the significant and the p value < 0.05 considered as significant.

[3]APOE was presented and compared such as sum of Ɛ2Ɛ3 and Ɛ2 as E2, sum of Ɛ1Ɛ3 and Ɛ3 as E3 and sum of Ɛ3Ɛ4 as E4 isoforms of APOE

n is stands for number of participants distributed based on the dietary intervention and the genotype or combination of genotype in the specific gene of interest

Superscripts shows the significant slice effect of the concern SNPs genotype effects within diet for those SNPs have shown significant genotype or diet genotype interaction.

CHAPTER V

GENERAL DISCUSSION

5.1 SUMMARY AND IMPLICATIONS

CVD is the leading cause of death worldwide. The quality and type of dietary fat plays an important role in influencing the CVD progression (1). High fat intakes are predominant in Western diets, where the average intake of dietary fats in the Western diet ranges from 50-100 g per day, contributing about 35-40 % of the total energy. Dairy food is a rich source of SFA and it remains highly controversial in the research area of dairy intake and cardiovascular health. Dairy products containing milk fats are rich in SFA; indeed, ~21% of SFA derive mainly come from dairy products in the North American diet. Evidence suggests that high intake of SFA increases plasma LDL-C which is associated with increased risk for CVD (2, 3). WHO recommends less than ten percentage of total energy from SFA and AHA and 2010 the American dietary guidelines (5) recommends to consume <7% energy from SFA to reduce CVD risk. In contrast, increased HDL-C (3, 4) and a reduction in blood pressure were observed upon consumption of milk and dairy products (5, 6). Furthermore, higher intakes of milk, that is one pint or more per day, shows a reduction in the CVD risk (7-9). The debate over the impact of SFA on CVD risk is unresolved and highly confusing for the consumer, with direct consequences on the consumption of products with a high SFA content such as cheese and butter. In addition, health effects may vary across dairy products. For example, the published evidence shows that that the intake of fat from cheese resulted in lower LDL-C concentrations compared to a matched intake of fat from butter (4, 38, 39), although both dairy products contributed to similar SFA intake. A growing body of evidence now suggests these effects differ based on specific food matrices or dietary patterns. However, positive

results regarding the impact of dairy fat on HDL-C, blood pressure and other cardiometabolic risk factors will provide convincing evidence supporting the importance to revisit the SFA-CVD risk association. Most of the dietary guidelines are based on the LDL-raising effects of SFA, and yet the fact that SFA in dairy products are consumed along with several other nutrients or food matrices effects that have been almost ignored. Our primary result suggested (**Appendix III**, page # 241) that cheese- and butter-based dietary interventions had similar effects on the levels of circulating total cholesterol and LDL-C. Baseline LDL-C significantly modified the LDL-C response to treatment (P for interaction=0.02), the increase in LDL-C being significantly greater with butter than with cheese only among individuals with high baseline LDL-C levels. SFA from cheese and butter had no significant effect on several other non-lipid cardiometabolic risk factors, including inflammation markers, blood pressure and insulin-glucose homeostasis compared with CHO, MUFA and PUFA (10).

Recent evidence suggested that the gut microbiome is linked with CVD risk through production of TMAO in the gut. As a result of metagenomics analyses, the human intestinal microbiome has lipid-associated genes, which are involved in the biosynthesis and bioconversion of lipids (11, 12). A mouse model study suggests that the gut bacteria mainly affect the dietary fat digestion process such as emulsification, absorption, transportation, storage and lipid peroxidation via the metabolic and bile acid signalling (13, 14). Also, lipid hydrolysis, isomerization and biohydrogenation process has been carried out by the ruminant gut bacteria (15), and human gut bacteria have similar function but not yet characterized. Also, the gut bacterial diversity has been associated with circulating lipid levels. The LifeLines population study by Fu et al. found that the gut microbial diversity and richness was associated with circulating lipid levels. A positive correlation was observed with HDL-C, and

an inverse correlation was observed with BMI and TG levels. Hence, result of this study provides more insights into gut microbiome and its association with CVD risk parameters. Many studies have suggested alterations in the gut microbial ecology with effect of different diets (16-18). Furthermore, dietary protein and fat intakes were associated with Bacteroides enterotype, and rich carbohydrate diet intake was associated with Prevotella enterotype (19). Hence, the gut bacteria with a specific enterotype was associated with particular diversity and dietary habits of the people. Also, increased energy intake from MUFA, n3 PUFA, n6 PUFA and soluble fiber had a significant association with reduced level of Bacteroides spp. (20). An abundance of Bacteroides spp. was identified with consumption of plant-derived diet (21). The impact of dietary fat in mouse model studies have suggested, reduced level of Bacteroidetes and increased level of Firmicutes with high-fat diet intake (17, 22, 23). Present microbiome results suggested significant differences in Firmicutes Phylum between PUFA vs, CHO and Verrucomicrobia between cheese vs. butter containing diets, but not with other phyla. Differences in β-diversity were only observed between CHO vs. PUFA rich diets, MUFA vs. PUFA and PUFA vs. butter regardless of BMI. Further, BMI also showed differences in β-diversity between OB vs. OW group. Further, we found that OB status of an individual has major impact on β-diversity compared to OW individauls. In addition, intake of cheese, MUFA and PUFA diets lowers *g_Peptoniphilus*, and lower level of *g_Streptococcus*. was found only in MUFA feeding. However, the cheese diet was linked to a high level of *g_Turicibacter* and the clinical importance,and the association of circulating lipids with these species yet to explored. Finally, the BMI of an individual, specifically OB, exerts a major impact on the response of gut microbiome to consumption of dietary fats from different sources.

For few decades, human genetic studies have been investigating the biological importance of impact of genes on the cholesterol metabolism and explore their associations to CVD risk. Several factors are contributing differences in the circulating blood lipid levels such as age (24), sex (25), genetics (26) and gut microbiome (27). Moreover, we observed a relatively high interindividual variability in blood lipids response after intake of dietary fats from different sources. Hence, we genotyped 92 participants for 21 common SNPs in genes involved in lipid metabolism and transport and that have been previously associated with the interindividual variability in blood lipid concentrations. Few SNPs, *ABCA1-rs*2066714, *DHCR7*-rs10444824 and *NPC1L1*-rs2073547 showed significant SNP × diet interaction on serum HDL-C and *PPAR*-rs6008259 and *LIPG*-rs2000813 showed significant SNP × diet interaction on serum LDL-C. In addition, predictive analysis for DF vs. UFA suggested that 32.8% of the variance in HDL-C was observed with a combination of 6 SNPs (*ABCA1*-rs2066714*, NPC1L1*-rs2073547*, SREBF2*-rs2228313*, DHCR7*-rs1044482*, DHCR7*-rs760241 and *PPAR*-rs6008259). Two SNPs (*LIPG*-rs2000813 and *ABCA1*-rs2066714) explained 16.5% of the variance in LDL-C, while a combination of 3 SNPs (*ABCA1*-rs2066714, *PPAR*-rs6008259, *LIPG*-rs2000813) explained 18.8% of the variance in total cholesterol levels. Furthermore, 19.8% of the variance of the TG levels in response to dairy feeding was explained by a combination of 5 SNPs (*LIPG*-rs2000813, *SCAP*-rs12487736, *ABCA1*-rs2230808, *DHCR7*-rs1044482, *LIPC*-rs6083).

In general, gut health is maintained by the right proportion of gut microbiome. Individuals who are low in microbial richness are more susceptible to certain disease condition than individuals with high microbial richness (28). In addition, cheese- and butter-based dietary interventions had similar effects on the levels of circulating total cholesterol and LDL-C based

on our published evidence (Brassard et al. 2017). Also, based on our predicted metabolic function of the gut microbiome shows cheese and butter intake increases the biosynthesis of sphingolipids and glycosphingolipids (29). As a result, the potential exists for gut microbial-mediated endogenous lipids, which are physically similar to eukaryotic sphingolipids, to have an influence on metabolic pathways, thus potentially explaining why cheese and butter have similar effects on total cholesterol and LDL-C. In this study, α- microbial diversity was not altered by different dietary intake in our dietary interventions. However, significance changes in the β-microbial diversity was observed in obese people upon intake of different dietary fat, suggesting that a dietary component or the food matrix of these diets may be altering certain microbial community abundances. Further, genetic analysis has shown the there was little impact of the genetic make-up in determining the response in the circulating lipids levels upon consumption of dietary fat from various sources. The difference in observed lipids levels are too small and it seems the difference does not have any clinical significance related dietary fat intake and CVD risk factors in our study.

5.2 STRENGTH, LIMITATIONS AND FUTURE DIRECTIONS

Firstly, current study was a well-controlled, randomized, single blind study with cross-over and full-feeding design. All intervention diets (seven days rotation menu, **appendix II**, page # 232) were prepared at the metabolic kitchen under the supervision of kitchen manager. Experimental diets were provided as part of a full feeding protocol under carefully controlled isocaloric conditions to maintain body weight constant. All meals and foods were provided to participants so that control for energy and macronutrient intake was optimized. Fatty acid profile was measured after the intervention to ensure the more accurate association between

dietary intake and plasma fatty acid levels (data not shown in this thesis). Some limitations are acknowledged to occur in the current research study, and likely could have resulted in type I (false positive) and type II (false negative)

Secondly, the gut microbiome analysis was carried out only from RCFFN-participants, 25 participants were considered for the gut microbiome analysis, in which six completed all five intervention phases, nine participants completed four phases, four participants completed three phases, and five participants completed two intervention phases. Microbiome analyses for human intervention trials represent a relatively new field and thus sample size calculations have yet to be empirically derived. Similar studies have found significant differences in gut microbiome between dietary treatments using relatively small sample sizes (30, 31). One of the limitations for microbiome analysis, our sample size is relatively small, we found some interesting result such as BMI status of an individual play major role in altering β-diversity. Additional metagenomics, or meta-transcriptomics sequencing approaches are required to confirm the predicted metabolic function of the gut microbiome, using studies designed with a larger sample size.

Thirdly, limited number of SNPs within certain genes were studied in this current study based on candidate gene approach. However, in this research we failed to detect an association of other SNPs in the candidate genes apart from selected SNPs or the combination of SNPs in the candidate genes related to cholesterol metabolism upon different dietary fat intake. This leads a potential type II error which can be rectified through use of next-generation DNA sequencing in future research, by which the whole set of genes of interest could be tested completely. Also, in future dietary intervention study considering genetic heterogeneity in

circulating lipid responsiveness to dietary fats must select carries of genotype which have previously been linked with phenotypic responses to dietary fats (example, hypo vs. hyper-responder). Even though not neither with type I errors, the use of whole genome sequencing is great method where, apart from the candidate SNP approach, it would exclude bias in the genes and SNPs selection. In addition, the major limitation in predictive model (PLS regression) analysis, we grouped two diets such a cheese and butter (referred as dairy fat (DF) diets), and MUFA and PUFA (referred as unsaturated fat (UFA) diets) because these diets are highly correlated with blood lipid levels (Chapter IV, Table 4.1 & 4.2). Moreover, the fat contents are similar between cheese and butter; MUFA and PUFA. Further, for the PLS analysis, only one dependent Y variable is required, so we performed PLS among DF vs. UFA. Pairwise PLS regression analysis is underway will be included in the chapter IV as well as in publication process.

5.3 FINAL CONCLUSIONS

The current knowledge of published evidence suggests that cholesterol homeostasis has been controlled by various genes that encode proteins, including lipoprotein receptors proteins, enzymes, lipid transfer and transporters and regulating proteins (32, 33). Dietary fats type and diet quality, dietary patterns and food matrices have a major impact on the circulating lipid levels including TC, LDL-C and TG which are major contributors for CVD (1, 34,38,39). Furthermore, the common genetic variants in the genes that associated with cholesterol metabolism also determine the circulating blood lipid levels. Identification of common genetic variants behind the lipid metabolism as the effect of different dietary fats consumption provided more insights in preventing CVD risk. Additionally, based on observational study,

long-term diet intake can modify the gut microbiome thereby alter the gut physiochemical environment (35). Moreover, dietary food contains complex nutrients, and which nutrient has as effect on certain bacteria taxa is not clear. However, intervention study might address some extent about microbiome and their effects on host physiology. Further, it is hard to choose or avoid specific dietary component from food those have effects on various bacterial taxa in a intervention study (36). For example, degrading capacity of xylan varies from species to species under the same genus (37). However, the food choices have been made by an individual, and each of them has a specific genetic makeup, so both have influence on promoting health benefit or risk for certain diseases. In conclusion, CVD association with dietary fats intake alone is well established from intervention and observational studies. Both dietary choice and the host genetic of individuals have a critical role in contributing CVD. Future research must consider dietary fats intake on gut microbiome, host genetics or microbial derived metabolite and non-targeted metabolomics on the physiological effects. Therefore, immense information about host genetics, novel biomarker and gut microbiome in future nutritional research can be useful to modify or advice new dietary recommendation to management of CVD risk in humans.

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APPENDICES

APPENDIX I

ETHICS APPROVAL FOR STUDIES CORRESPONDING TO CHAPTER III AND IV

UNIVERSITY OF MANITOBA | Research Ethics - Bannatyne
Office of the Vice-President (Research and International)

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BIOMEDICAL RESEARCH ETHICS BOARD (BREB)
CERTIFICATE OF ANNUAL APPROVAL

PRINCIPAL INVESTIGATOR:	INSTITUTION/DEPARTMENT:	ETHICS #:
Dr. P. Jones	Richardson Centre for Functional Foods and Nutraceuticals/Food Sciences	HS18188 (B2014:018)
BREB MEETING DATE (If applicable):	**APPROVAL DATE:** February 9, 2016	**EXPIRY DATE:** February 24, 2017
STUDENT PRINCIPAL INVESTIGATOR SUPERVISOR (If applicable):		

PROTOCOL NUMBER:	PROJECT OR PROTOCOL TITLE:
2013-251	2013-251 Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors
SPONSORING AGENCIES AND/OR COORDINATING GROUPS: Laval University and Dairy Farmers of Canada	

Submission Date of Investigator Documents:	BREB Receipt Date of Documents:
January 28, 2016	January 29, 2016

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.

CONDITIONS OF APPROVAL:

1. The study 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 annual 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,

[redacted]

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

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

APPENDIX II

FORMS CORRESPONDING TO STUDIES DESCRIBED IN CHAPTER III AND IV

PARTICIPANT CONSENT FORM

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RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

Title of Study: Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors.

Protocol #: 2013-251

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Dairy Farmers of Canada
1801 McGill College Avenue
Suite # 700
Montreal, QC H3A 2N4
Phone: [redacted]

Title of the study: Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors

You are being asked to participate in a Clinical Trial (a human research study). Please take your time to review this 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 before you make your decision. 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. The study doctor (and or/ institution) is (are) receiving professional fees and financial support to conduct this study.

Purpose of Study

This Clinical Trial is being conducted to study the effects of consumption of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other risk factors for heart disease. You are being asked to take part in this study because you are aged between 18-65 yrs and you have a waist girth and blood triglycerides moderately elevated. A total of 70 (35 from Winnipeg area and 35 from Québec City area) participants will participate in this study.

This research is being done because market trends depicted by Agriculture and Agri-Food Canada suggest stagnation in cheese consumption, with potentially important impact on this key industry in Canada. This is in part due to the commonly accepted notion that saturated fat in the diet, of which cheese contributes significantly, increases the risk of heart disease. Yet, a rather large body of recent evidence suggests that although saturated fat may have been projected to be unhealthy its impact on the risk of heart disease may in fact be less important than originally thought. This concept that dairy fat increases the risk of heart attacks therefore needs to be revisited, and this is one of the key objectives of this proposed research program.

Study procedures

If you take part in this study, you will have the following tests and procedures:

Screening: If you agree to take part in this study, as part of a 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. During screening, we will also measure your body weight, height, waist circumference and blood pressure as well as your record your age. In order to be eligible for the study, your age should be between 18 and 65 with waist circumference greater than 80 cm for women and greater than 94 cm for men, as well as with blood HDL-Cholesterol levels $\leq$1.53mmol/L for women and $\leq$1.34 mmol/L for men. Individuals with a previous history of cardiovascular disease, type 2 diabetes, on cholesterol lowering or hypertension medications and smokers will not be eligible to participate. If you are eligible to participate based on your screening result you will begin the trial. Pregnancy tests will be performed for all pre-menopausal female participants at screening visits and at the beginning of each phase, if the test is positive at screening or during the study they will be asked to stop participation in the study. Any change in your health status at any point during the study needs to be reported to the study investigators.

Dietary study: The study will consist of 5 phases of 4 weeks each during which you will consume morning breakfast along with your assigned treatment under supervision. The five treatment phases will be interrupted by 4 weeks washout phases during which you follow your habitual diets. We will ask that you limit your consumption of alcohol and caffeinated beverages throughout the phases.

Stable isotope tracer intake: To assess fatty acid oxidation and synthesis, you will be asked to consume ^{13}C-palmitate and D_2O (heavy water) at the end of each phase. On day 29 of each phase, you will receive blended liquid meal with ^{13}C-palmitate (15 mg/kg). Hot liquid meal blended palmitate shows high recovery of $^{13}CO_2$ in breath samples (Delany et al.,2000). On the same day you will be provided with containers for breath test and you will be requested to collect breath samples at 1 hour intervals up to 9 hours. Breath filled containers will be collected on next day from you and will be used to measure fatty oxidation. The abundance of $^{13}CO_2$ will be measured by mass spectrometer. In addition, on day 29, 0.7 g of D_2O /kg estimated body water will be given orally prior to breakfast as a tracer to measure fractional fatty acid synthesis rate over 24 hours in RBC.

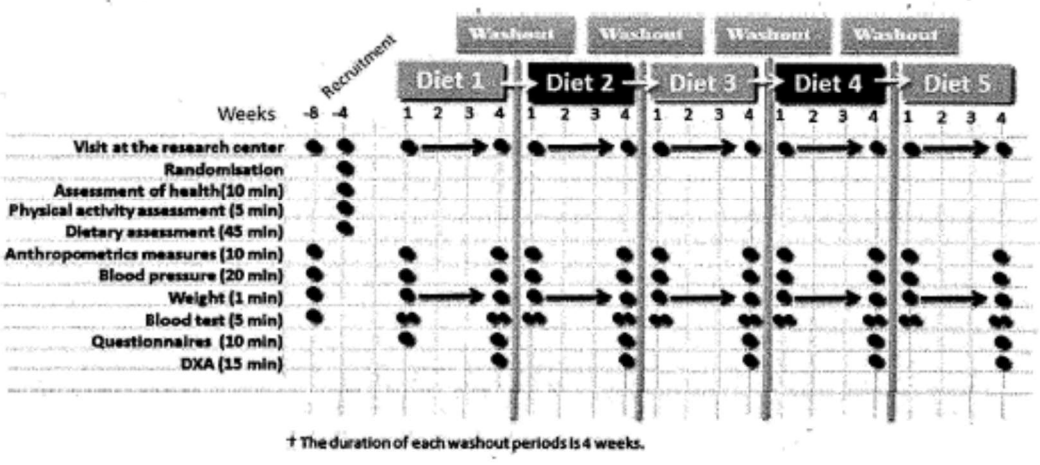
Microbial analysis: In addition, we would also study the influence of these diets on gastrointestinal microbial diversity in the current study population. For this analysis, you will be asked to provide your stool samples (4 or 5 scoops - 4g) 2 days at the beginning (days 2 and 3) and 1 day end of each phase (day 30) of the trial. Stool sample collection kits including containers will be provided to collect stool samples. However, it is optional for you to provide the stool sample. It will not affect your study participation even if you do not select the option to provide stool samples

Description of tests

Overall health status for eligibility: During recruitment, an overall assessment of your health will be done to ensure your eligibility for the study. A first screening visit of 30 minutes will be done to assess your eligibility. During this visit, we will check blood pressure, anthropometric (body weight and height) measurements and a blood test. At a second visit, we will review your medical history and the medications you take and we will clearly explain the instructions of the study. These visits will be conducted by a nutritionist, under the supervision of our medical research team.

The following is a schematic representation of the study plan

Figure 1. Schematic representation of the research protocol and tests provided



† The duration of each washout periods is 4 weeks.

Dietary and physical activity assessment: Before the beginning of the study, you must complete a food frequency questionnaire on the Web (duration of about 45 minutes) to assess the energy content (calories) and the proportions of protein, fat and carbohydrates (sugars) of your regular diet. Habits of physical activity will also be assessed using a standardized questionnaire.

Blood samples: At the screening visit, fasting blood sample of 2 teaspoons will be collected to check your blood counts, your lipid profile and to ensure the proper functioning of your liver, your kidneys and your thyroid gland. During days 1, 2 of each phase fasting blood samples of approximately 22 mL will be collected during each of these 2 days. During days 29 and 30 of each phase fasting blood samples of approximately 42 ml will be collected during each of these 2 days. These blood samples will be used to assess your lipid profile and other risk factors for heart disease. The total amount of blood collected for all samples in the study will be about 650 ml (about 2 ½ cups) spread over a period of about nine months. Generally a person can give up to 450 ml of blood every 2 months safely. The amount of blood collected in the context of this project is lower than that. However, you must refrain from donating blood during the study and for a period of two months following your participation in this project. Finally, you should refrain from vigorous physical activity 3 days before each blood sampling.

Resting blood pressure: Blood pressures at rest (following a 10-minute rest) will be taken early in the morning after a 12 hours fasting and will be repeated twice at an interval of three minutes. These measures will be taken at the first screening visit and at the beginning and at the end of each 4-weeks period.

Anthropometric measurement: Measures of your weight, your height (once), your waist and your hip circumference will be done at the screening visit and at the beginning and at the end of each 4-week period. Taking waist and hip circumference is done using a measuring tape and involves no pain. You will be weighed every day of the week during each 4-weeks period to monitor changes in your weight.

Body composition measurement (DXA): Body composition (fat mass and lean mass) will be measured with a procedure called dual energy x-ray absorptiometry (DXA). The examination takes place in a room specially designed for this test. A radiology technician or other person authorized to operate the device will perform the test. The test does not require any special preparation for the subject. You will be asked not to wear anything metal. The procedure takes about 15 minutes and the dose of radiation is very low (0.037 to 0.074 mrem according to the exposure time required to obtain results of high precision). The total exposure in this project with 5 measures of body composition with DXA will be a maximum of 0.185 mrem, which is 54 times less than a dental x-ray exposure which is estimated at 10 mrem. Exposure to cosmic rays during a 6 hours flight from Montreal to Vancouver is estimated at 3 mrem. The exposure in this project level is therefore quite safe.

Experimental diets

The 5 phases of treatments will include:

1- A diet containing saturated fat from cheese (13% of calories as saturated fat);
2- A diet containing saturated fat from butter (13% of calories as saturated fat);
3- A carbohydrate-rich diet low in saturated fat (6% of calories from saturated fat);

4- A diet rich in monounsaturated fat, mainly from olive oil, and low in saturated fat (21% of calories from monounsaturated fat, 6% saturated fat);
5- A diet rich in polyunsaturated fat, largely from sunflower oil, and low in saturated fat (12% of calories as polyunsaturated fat, 6% saturated fat).

The nutrient composition of the diets will be as follows:

	Diets				
	CHEESE	BUTTER	CHO	MUFA	PUFA
Protein, %	15	15	15	15	15
CHO, %	53	53	60	53	53
FAT, %	32	32	25	32	32
SFA, %	13	13	6	6	6
MUFA, %	14	14	14	21	14
PUFA, %	5	5	5	5	12

CHO-Carbohydrates; SFA-Saturated fatty acids; MUFA-Monounsaturated fatty acids; PUFA-Polyunsaturated fatty acids.

The experimental diets were based on the Canada's Food Guide recommendations. No known risk of short term deficiency or excess in energy, protein, fat or carbohydrate is present with the experimental diets. Two of the 5 experimental diets contained 13% of calories from saturated fat, which is slightly above the average consumption for Canadian adults, which is about 11%. However, for 3 of the 5 experimental diets, the relative content of saturated fat is 6% of calories. So on average over the experimental period of 20 weeks, your average intake of saturated fat is 8.8%, which meets the target of the Canadian recommendations.

This study is with a double blinded design which means neither you nor the clinical staff will know which diets you will be receiving. In an emergency, this information will be made available. You will receive all 5 diets. These blood samples will be obtained for assessment of blood fat levels and fat metabolism. Each blood test will take approximately 5 minutes.

For the purpose of subsequent analysis, we will store plasma and serum samples. At the end of the study, your records will be stored in accordance with Health Canada's regulations for 25 years-at RCFFN. During the course of the study, processed blood will be stored at the RCFFN in -80°C freezers, for a maximum of 5 years from the end of the study where possible. At this point the investigators will re-apply for a further extension of storage to accommodate new analysis indicated at that time. In the absence of such an application, all samples will be destroyed by autoclaving. During the storage period if you should change your mind about your samples being stored you have the opportunity to withdraw your consent. Simply call the centre at [redacted] or any of the investigators listed on page 1 of this consent and inform them that you wish to withdraw your consent. The stored blood will be available for re-analysis of samples if values are called into question. In which case we may attempt to contact you by phone or letter requesting that you attend an information session where the new information will be discussed.

Participation in the study will be for 36 weeks. The researcher may decide to take you off this study if it is in the participant's medical best interest, participant's condition worsens, failure to follow the study protocol. You can stop participating at any time. However, if you decide to stop participating in the study, we encourage you to talk to the study staff and your regular doctor first.

There are no serious health consequences of sudden withdrawal from the study for you. Your participation in this research project is entirely on a volunteer basis. You can refuse to participate or you can interrupt your participation anytime, throughout the study period, without any penalty or loss of benefits to which you would otherwise be entitled. Participants will receive a sealed and confidential letter which states their individual results of their blood tests, global physical health and dietary evaluation along with the mean values obtained from the entire study population. The letter will be sent by the principal investigator at RCFFN to the mailing address on the personal information form that participants fill out prior to enrolment to the study.

Risks and Discomforts

As with any clinical trial, there may be as yet unknown or unforeseen risks of taking part in the study. 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. Shahrokh Nejad Ghaffar, will be available to contact at any time. Dr. Shahrokh Nejad Ghaffar can be reached at [redacted]

Benefits

There may or may not be direct medical benefit to you from participating in this study. We hope the information learned from this study will benefit other participants with increased risk for cardiovascular disease in the future. In addition to the above, you will also receive your results when they become available.

Costs

All clinic and professional fees, diagnostic and laboratory tests which 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. The study cost and honorariums will be covered by Dairy Farmers of Canada, the study sponsor.

Payment for participation

You will receive up to a maximum of $1500 at completion of this study for your time and inconvenience of the study schedule. This amount will be divided into 5 portions. You will receive $200 after the completion of each phase until the end of phase 4 and $450 after the completion of phase 5. You would also get an additional $50 per phase for fecal collection. If you withdraw early from the study, you will receive an appropriate pro-rated fraction of this amount.

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.

Confidentiality

Information gathered in this research study may be published or presented in public forums, however your name and other identifying information will not be used or revealed. Medical records that contain your identity will be treated as confidential in accordance with the Personal Health Information Act of Manitoba. Despite efforts to keep your personal information confidential, absolute confidentiality cannot be guaranteed. Your personal information may be disclosed if required by law. All study documents related to you will bear only your assigned patient number (or code) and /or initials.

All data pertaining to the measurements that will be done in this multicenter study will be shared with the partner institution (INAF, Laval University). These data will be entered into the private website (http://inaf.fsaa.ulaval.ca/fani/index.php?langue=en) developed by Dr. Benoit Lamarche and team at the Laval University protected with username and password. Study coordinators at RCFFN and INAF only will have access to this website. Your name and personal information (name, address, and phone number) will be kept confidential and won't be transmitted to the partner or entered in the above mentioned private website. Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: Study coordinator of INAF will be accessing research study records of participants. The University of Manitoba Biomedical Research Ethics Board may review research-related records for quality assurance purposes.

All records will be kept in a locked secure area and only those persons identified will have access to these records. If any of your medical/research records need to be copied to any of the above, your name and all identifying information will be removed. No information revealing any personal information such as your name, address or telephone number will leave the Richardson Centre for Functional Foods and Nutraceuticals. With your permission your Family Physician (GP) will be notified about your participation in this study.

The study is registered on a publicly available registry databank at clinicaltrials.gov. ClinicalTrials.gov is a website that provides information about federally and privately supported clinical trials. A description of this clinical trial will be available on http:/ClinicalTrials.gov. This website will not include information that can identify you. At most, the website will include a summary of the results. You can search this website at any time.

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 not to participate or to withdraw from the study will not affect your other medical care at this site. If your study doctor feels that it is in your best interest to withdraw you from the study, your study doctor will remove you without your consent. We will tell you about any new information that may affect your health, welfare, or willingness to stay in this study.

If you decide to participate, you will agree to co-operate fully with the study visit schedule, and will follow the study staff's instructions. If you are an employee of University of Manitoba, be sure that your performance evaluation will not be affected by your decision not to participate. Should you wish to withdraw your participation from the study, you must inform the study coordinators so that your file can be officially closed.

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. Shahrokh Nejad Ghaffar at [redacted] 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 or releasing the investigator(s) or the sponsor from their legal and professional responsibilities. If any health abnormalities are identified in the clinical tests conducted during this experiment, Dr. Shahrokh Nejad Ghaffar 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 participant. 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 listed in page 1. For questions about your rights as a research participant, you may contact The University of Manitoba Biomedical Research Ethics Board at [redacted] Do not sign this consent form unless you have had a chance to ask questions and have received satisfactory answers to all of your questions.

Statement of Consent

I have read this consent form. I have had the opportunity to discuss this research study with Peter Jones and or his/her study staff. I have had my questions answered by them in language I understand. The risks and benefits have been explained to me. I believe that I have not been unduly influenced by any study team member to participate in the research study by any statement or implied statements. Any relationship (such as employee, student or family member) I may have with the study team has not affected my decision to participate. I understand that I will be given a copy of this consent form after signing it. I understand that my participation in this clinical trial is voluntary and that I may choose to withdraw at any time. I freely agree to participate in this research study.

I understand that information regarding my personal identity will be kept confidential, but that confidentiality is not guaranteed. I authorize the inspection of my medical records by Benoit Lamarche and his team at Laval University, Dairy Farmers of Canada, the Food and Drug Administration, the Health Protection Branch, government agencies in other countries, and The University of Manitoba Biomedical Research Ethics Board. By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

Title of the study: Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors

I agree to being contacted in relation to this study. **Yes ☐ No ☐**

I agree to my family physician being notified of my participation in this study. Yes ☐ No ☐

I agree to being contacted for future studies at the RCFFN. **Yes ☐ No ☐**

I agree to participate in the fecal sample collection **Yes ☐ No ☐**

Participant signature_____ **Date** _____
(day/month/year)

Participant printed name: _____

I, the undersigned, have fully explained the relevant details of this research study to the participant named above and believe that the participant has understood and has knowingly given their consent

Printed Name: _____**Date** _____
(day/month/year)

Signature: _____

Role in the study: _____

Appendix II

PARTICIPANT GENETIC ANALYSIS CONSENT FORM

RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM

ADDITIONAL RESEARCH PARTICIPANT INFORMATION AND CONSENT FORM FOR GENETIC ANALYSIS

Title of Study: Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors

Investigator: Peter Jones, PhD

Richardson Centre for Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, SmartPark
Winnipeg, Manitoba R3T 2N2
Phone: (1[redacted]

Vanu Ramprasath, PhD
Richardson Centre for Functional Foods and Nutraceuticals
University of Manitoba
196 Innovation Drive, SmartPark
Winnipeg, Manitoba R3T 2N2

Sponsor: Benoit Lamarche,
Institute on Nutrition and Functional Foods
Laval University
Pavillion des Services,
2440 Hochelaga Blvd,
Quebec, QC, G1V 0A6
Phone (4[redacted]

Dairy Farmers of Canada
1801 McGill College Avenue
Suite # 700
Montreal, QC H3A 2N4
Phone: (1[redacted]

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

Initials of participant: _____

you do not understand. Please ask the study staff to explain any words or information that you do not clearly understand.

Nature and Duration of Procedure

From the blood drawn during the clinical study entitled "Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors", we would like to extract DNA and perform genetic analyses using a laboratory technique that augments and recognizes specific genes. This will allow us to determine why some people respond differently than others when consuming different fats as shown by their plasma lipid levels. DNA is a molecule found in the cells of your body that is organized into genes that contain all of the information needed to make the proteins, which perform specific biological functions in your body.

Confidentiality and Safekeeping of DNA Samples

All the information obtained about you and the results of the research will be treated confidentially. We will protect your confidentiality by assigning your DNA sample a specific code. This code will link you to your DNA sample and can only be decoded by the principal researcher or an individual authorized by the latter. Samples of your DNA will be kept at the Richardson Centre for Functional Foods and Nutraceuticals, University of Manitoba, under the supervision of Dr. Peter Jones for a 10-year period following the end of the research project. After this time, all samples will be destroyed. Your DNA samples will only be used for the purpose of this research project. Your samples will not be used for any additional analyses, nor stored for any longer than 10 years, nor shared with any other group, other than is indicated in the protocol, without your specific consent.

Your participation and the results of the research will not appear in your medical record. Although the results of this study may be published or communicated in other ways, it will be impossible to identify you. Unless you have provided specific authorization or where the law permits or a court order has been obtained, your personal results will not be made available to third parties such as employers, government organizations, insurance companies, or educational institutions. This also applies to your spouse, other members of your family and your physician. However, for the purposes of ensuring the proper management of research, it is possible that a member of an ethics committee, a Health Canada representative, or a representative from the Richardson Center for Functional Foods and Nutraceuticals may consult your research data and record. You can communicate with the research team to obtain information on the general progress or the results of the research project. Project updates will be mailed at the end of the project. However, we will not communicate any individual results to you.

Potential Risks and/or Benefits

As the DNA will be extracted from blood samples that have already been taken, there are no additional invasive procedures to undergo and no physical risks to you. While there may be no direct benefits to you for taking part in these additional analyses, we hope that the results will

Initials of participant: _____

provide novel information on the influence of genetic characteristics of people and their response to dietary fat consumption during the study time, particularly with regard to plasma lipid levels.

Signature of Participant

The content of the procedure and procedures associated with it have been fully explained to me, and all experimental procedures have been identified. I have had the opportunity to ask questions concerning any and all aspects of the project and procedures involved, and may continue in the future to ask further questions at any time, as it is my right to do so. I am aware that I may refuse to participate as well as withdraw my consent at any time. I acknowledge that no guarantee or assurance has been given by anyone as to the results to be obtained and that my participation in this study is completely voluntary. Confidentiality of records concerning my involvement in this project will be maintained in an appropriate manner. Samples will neither be utilized for any additional analyses, nor stored for any prolonged period, or shared with any other group other than is indicated in the protocol without my specific consent.

I, _____, have read the above description. I have been made aware of all the procedures, advantages and disadvantages of the study, which have been explained to me. By signing this consent form, I have not waived any of the legal rights that I have as a participant in a research study.

_____ _____

Signature of Participant Date

For Clinical Study Coordinator: I confirm that I have explained the purpose, duration etc of this clinical trial, as well as any potential risks and benefits, to the participant whose name and signature appears above. I confirm that I believe that the participant 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

Initials of participant: _____

Appendix II

PARTICIPANT SCREENING CONSENT FORM

UNIVERSITY OF MANITOBA | Richardson Centre for Functional Foods and Nutraceuticals

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

Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors

Screening Consent

I have expressed an interest in participating in the above named study. I have been invited to have my health assessed to determine if I meet the requirements of the study.

Depending on my cholesterol level and other health indicators, and my availability over the next 10 months, I will be offered the opportunity to participate in this study.

To allow the necessary information to be obtained, I agree to provide fasting blood samples (approximately 10 ml or 2 teaspoons) for the measurement of blood cholesterol and other biochemical and hematological tests. I also agree to have a physical assessment done (blood pressure, weight, height, waist and hip measurement etc.), to complete a health history form.

Prior to taking part in the study, I shall be given the specific study consent form to read and sign if I am still interested in participating.

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

Appendix II

PARTICIPANT GENERAL INFORMATION FORM

WEEK	DATE OF OBSERVATION			INITIALS OF SUBJECT		# SUBJET(CODIFICATION)	
	DAY	MONTH	YEAR	NAME	FAMILY NAME	HDL	

General information

PROJECT HDL #2013-251

Name :_____ Family Name: _____

Gender : ❑ W ❑ M

Date of birth : ___/___/_____ Age : _____

Coordinates:

Address : _____ Postal Code: _____

Telephone : _____ *(home)* _____ *(office)*

_____ *(cell phone)* _____ *(other)*

Email : _____

Coordinates of another person to join (in case) :

Family Name :_____ Name : _____

Relationship with the subject : _____ Telephone : _____

Email : _____

Interest and motivation :

What is your interest in participating in this research project?

_____

How did you get information about this research project?

_____

Availability for the meetings at the research center? :

Availability in the morning: ❑ Yes ❑ No *Precision :* _____

Availability at lunchtime: ❑ Yes ❑ No *Precision :* _____

Preferences :

❑Monday ❑Tuesday ❑Wednesday ❑Thursday ❑Friday ❑Saturday* ❑Sunday*

** If there is no other alternative*

Are you planning a trip or travel outside Manitoba over the next few weeks or months?

❑ Yes ❑ No

If yes, precise: _____

COMMENTS

_____

_____

Appendix II

PARTICIPANT MEDICAL QUESTIONNAIRE SCREENING-1 FORM

UNIVERSITY OF MANITOBA

PROJECT HDL #2013-251

WEEK	DATE OF OBSERVATION			INITIALS OF SUBJECT		# SUBJET(CODIFICATION)	
	DAY	MONTH	YEAR	NAME	FAMILY NAME	HDL	

Medical Questionnaire
SCREENING S1

Sex : ☐ **M** ☐ **W**

Date of birth: _____ **Age:** _____

BLOOD PRESSURE AT REST (AFTER 10 MINUTES OF REST)

ARM: ☐ **LEFT** ☐ **RIGHT**

TYPE OF DEVICE: ☐ **Mercury** ☐ **Automatic**

10 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

13 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

16 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

Avg. Systolic BP (mmHg) = _____ **Avg. Diastolic BP (mmHg) =** _____

Heart rate / min = HR(1):_____ HR(2): _____ HR(3): _____ HR(mean):_____

ANTHROPOMETRIC MEASURMENTS

Body weight (kg): _____ **Height (m):** _____ **BMI (kg/m^2):** _____

Waist girth (cm) : WG(1): _____ **WG(2):** _____ **WG(3):** _____

Hip girth (cm) : HG(1): _____ **HG(2):** _____ **HG(3):** _____

Completed by: _____

ETHNICITY

- ❑ Caucasian (eg North America, Europe, Middle East, North Africa, etc.)
- ❑ African and Afro-American (eg Jamaica, Caribbean, Niger, Haiti, etc.)
- ❑ Autochthon
- ❑ Inuit (eg Eskimo)
- ❑ Asian (eg Far East, Southeast Asia, Cambodia, China, etc.)
- ❑ Hispanic (eg Mexico, Cuba, Dominican Republic, etc.)
- ❑ Other: _____

BLOOD SAMPLES: CHECKLIST

Did you eat or drink anything except water in the last 12 hours? ☐ **Yes** ☐ **NO**

Have you consumed alcohol or a product containing alcohol in the last 48 hours? ☐ **Yes** ☐ **NO**

In the last 24 hours, have you done intensive exercise? ☐ **Yes** ☐ **NO**

BLOOD SAMPLES: ☐ LEFT ARM ☐ RIGHT ARM

BLOOD SAMPLES: ☐ **Yes** ☐ **NO**

EXTRA BLOOD SAMPLE (Keep frozen) ☐ **Yes** ☐ **NO**

Nurse's initials: _____

Comments:

_____

_____

Appendix II

PARTICIPANT MEDICAL QUESTIONNAIRE/LIFE HABITS SCREENING-2 FORM

UNIVERSITY OF MANITOBA

PROJECT HDL # 2013-251

WEEK	DATE OF OBSERVATION			INITIALS OF SUBJECT		# SUBJET(CODIFICATION)	
	DAY	MONTH	YEAR	NAME	FAMILY NAME	HDL	

Medical Questionnaire/Life habits
SCREENING S2

Sex : ☐ **M** ☐ **W**

MEDICAL HISTORY

Family history	**YES**	**NO**	**Don't Know**	**If Yes, specify** F=father, M=mother, S=siblings, GP=grand parents, U=unknown (ex : adopted)
Type 1 Diabetes	❑	❑	❑	
Type 2 Diabetes	❑	❑	❑	
Cardiovascular diseases (Class 1)**	❑	❑	❑	
Cardiovascular diseases (Class 2)***	❑	❑	❑	
Hypertension	❑	❑	❑	
Dyslipidemia	❑	❑	❑	
Endocrine disorders	❑	❑	❑	
Cancers	❑	❑	❑	
Others	❑	❑	❑	

Personal history	**YES**	**NO**	**Don't Know**	**If Yes, specify**	**Age (dx) ***
Type 1 Diabetes	❑	❑	❑		
Type 2 Diabetes	❑	❑	❑		
Cardiovascular diseases (Class 1)**	❑	❑	❑		
Cardiovascular diseases (Class 2)***	❑	❑	❑		
Hypertension	❑	❑	❑		
Dyslipidemia	❑	❑	❑		
Endocrine disorders	❑	❑	❑		
Gastrointestinal disorders	❑	❑	❑		
Hepatic diseases	❑	❑	❑		
Cancer	❑	❑	❑		
Surgeries (past or future)	❑	❑	❑		
Other(s)	❑	❑	❑		
Kidney diseases	❑	❑	❑		

* *Age at diagnosis*
** *Class 1: heart attack, Ischemic cardiomyopathy, angina, "bypass"…*
*** *Class 2: stroke, aneurysm, thrombophlebitis, cerebral hemorrhage, peripheral vascular disease …*

MEDICATION: **Yes ❑** **No ❑**

Name of drug	Dose	Freq.	Indication	Start	Stop	*
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑

* *Check this box if the participant has not stopped using the drug.*

Drug Allergies: Yes ❑ No ❑

If yes, specify: _____

Natural health products

Do you consume dietary supplements, vitamins and / or minerals, homeopathic remedies and other natural products (probiotics, medicinal plants, omega-3 …): **Yes ❑** **No ❑**

Would you be willing to stop taking these supplements at least 6 weeks before the beginning of the study? **Yes ❑** **No ❑** **Not applicable ❑**

Name of product	Dose	Freq.	Indication	Start	Stop	*
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑
				___/___/____	___/___/___	❑

* *Check this box if the participant has not stopped using the natural health product*

WOMEN ONLY (PREGNACY-GYNECOLOGY)

Are you postmenopausal? Yes ❑ No ❑

Premenopausal women

Are you currently taking oral contraceptives or other forms of hormonal contraception?
Yes ❑ No ❑

Do you have a regular menstrual cycle? Yes ❑ No ❑ Not applicable ❑

Average length of your menstrual cycle: _____
Date of your last periods (1st day) : _____
Next expected menstruation date: _____

Are you pregnant? Yes ❑ No ❑

Are you planning a pregnancy soon? Yes ❑ No ❑
If yes, how long ? _____

Postmenopausal women

Age at menopause: _____ Yrs Don't know ❑

Type of menopause:
Natural ❑ *Hysterectomy* ❑ *Hysterectomy + partial oophorectomy (1 ovary)* ❑
Hysterectomy + total oophorectomy (2 ovaries) ❑ *Tubal ligation* ❑
Caused by chemotherapy or other treatment ❑ *Don't know* ❑

Are you currently taking hormone replacement? Yes ❑ No ❑
If yes, start date: ___/___/___
If yes, type of hormones: _____

Have you ever taken hormone replacement in the past? Yes ❑ No ❑
If yes, stop date : ___/___/___
If yes, type of hormones: _____

LIFE HABITS

Alcohol **Yes ❑ No ❑** Type(s) of alcohol: _____
Quantity consumed: _____
How often : _____

Tobacco **Yes ❑ No ❑** Date of arrest (if any) : _____

If yes, how often: _____

Street drug Yes ❑ No ❑ Date of arrest (if any): _____
If yes, how often: _____

NUTRITION / EATING HABITS

	YES	NO	Don't know	If yes, specify
Allergies:				
Foods	❑	❑	❑	_____
Others	❑	❑	❑	_____
Intolerances	❑	❑	❑	_____
Special dietary habits (Ex: vegetarianism)	❑	❑		_____
Consumption of:				
Soft drinks	❑	❑	❑	_____
Energy drinks	❑	❑	❑	_____
Coffee	❑	❑	❑	_____
Tea	❑	❑	❑	_____

Number of meal /day : _____

Number of snack (s)/day: _____

PHYSICAL ACTIVITY

Type	*Frequency*	*Duration*
_____	_____	_____
_____	_____	_____
_____	_____	_____
_____	_____	_____

COMMENTS

Appendix II

PARTICIPANT MEDICAL QUESTIONNAIRE DURING THE INTERVENTION FORM (DAY 1 &DAY29)

UNIVERSITY OF MANITOBA

PROJECT HDL #2013-251

WEEK	DATE OF OBSERVATION			INITIALS OF SUBJECT		# SUBJET(CODIFICATION)	
	DAY	MONTH	YEAR	NAME	FAMILY NAME	HDL	

Medical Questionnaire D1-D29

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐ Diet 5 ☐

BLOOD PRESSURE AT REST (AFTER 10 MINUTES OF REST)

ARM : ☐ **LEFT** ☐ **RIGHT**

TYPE OF DEVICE : ☐ **Mercury** ☐ **Automatic**

10 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

13 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

16 min Systolic BP (mmHg) = _____ Diastolic BP (mmHg) = _____

Avg. Systolic BP (mmHg) = _____ **Avg. Diastolic BP (mmHg) =** _____

Heart rate / min = HR(1):_____ HR(2): _____ HR(3): _____ HR(mean):_____

Side effects questionnaires completed: **Yes** ☐ **No** ☐

ANTHROPOMETRIC MEASURMENTS

Body weight (kg): _____

Waist girth (cm) : WG(1): _____ **WG(2):** _____ **WG(3):** _____

Hip girth (cm) : HG(1): _____ **HG(2):** _____ **HG(3):** _____

Completed by: _____

Date of your last periods (1st day) : _____

Not applicable: ☐

BLOOD SAMPLES: CHECKLIST

Did you eat or drink anything except water in the last 12 hours? ☐ Yes ☐ NO

Have you consumed alcohol or a product containing alcohol in the last 48 hours? ☐ Yes ☐ NO

In the last 24 hours, have you done intensive exercise? ☐ Yes ☐ NO

BLOOD SAMPLES: ☐ LEFT ARM ☐ RIGHT ARM

BLOOD SAMPLES: ☐ Yes ☐ NO

EXTRA BLOOD SAMPLE (Keep frozen) ☐ Yes ☐ NO

Nurse's initials: _____

For visit D1 of diet 2, diet 3, diet 4, diet 5 :

Washout duration before this diet: _____ **days**

☐ **Not applicable (D1 of the first diet)**

☐ **Not applicable (visit D29)**

STABLE ISOTOPE TRACER INTAKE: CHECKLIST

Intake of ^{13}C-palmitate on D29 ☐ Yes ☐ NO

***Intake of Deuterium on* D29** ☐ Yes ☐ NO

☐ **Not applicable (visit D1)**

COMMENTS

_____

_____

_____

Appendix II

PARTICIPANT MEDICAL QUESTIONNAIRE FORM (DAY 1 &DAY29)

UNIVERSITY OF MANITOBA

PROJECT HDL #2013-251

WEEK	DATE OF OBSERVATION			INITIALS OF SUBJECT		# SUBJET(CODIFICATION)	
	DAY	MONTH	YEAR	NAME	FAMILY NAME	HDL	

Medical Questionnaire D2-D30

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐ Diet 5 ☐

ANTHROPOMETRIC MEASURMENTS

Body weight (kg): _____

BLOOD SAMPLES : CHECKLIST

Did you eat or drink anything except water in the last 12 hours? ☐ Yes ☐ NO

Have you consumed alcohol or a product containing alcohol in the last 48 hours? ☐ Yes ☐ NO

In the last 24 hours, have you done intensive exercise? ☐ Yes ☐ NO

BLOOD SAMPLES: ☐ LEFT ARM ☐ RIGHT ARM

BLOOD SAMPLES: ☐ Yes ☐ NO

EXTRA BLOOD SAMPLE (Keep frozen) ☐ Yes ☐ NO

Nurse's initials: _____

For visit D30 of all diets:

Number of days on the diet: _____ days

☐ **Not applicable (visit D2**

CHANGE IN MEDICATION Yes ☐ No ☐

Name of drug	Dose	Freq.	Indication	Start	Stop
				__/__/__	__/__/__
				__/__/__	__/__/__
				__/__/__	__/__/__

				__/__/__	__/__/__
				__/__/__	__/__/__

CHANGE IN NATURAL HEALTH PRODUCTS Yes ❑ No ❑

Name of drug	Dose	Freq.	Indication	Start	Stop
				__/__/__	__/__/__
				__/__/__	__/__/__
				__/__/__	__/__/__
				__/__/__	__/__/__
				__/__/__	__/__/__
				__/__/__	__/__/__

PHYSICAL ACTIVITY

During the last month, is that your level of physical activity has changed?

- ❑ **Yes, my level increase**
- ❑ **Yes, my level decrease**
- ❑ **Yes, I didn't exercise**
- ❑ **No**

FECAL SAMPLES: CHECKLIST

Fecal sample collected on D2	☐ **Yes**	☐ **NO**
Fecal sample collected on D3	☐ **Yes**	☐ **NO**
Fecal sample collected on D30	☐ **Yes**	☐ **NO**

BREATH SAMPLES: CHECKLIST

Breath sample collected on D30	☐ **Yes**	☐ **NO**

COMMENTS

_____

_____

Appendix II

PARTICIPANT SIDE EFFECT-INTENSITY QUESTIONNAIRE FORM (DAY 1 &DAY30)

UNIVERSITY OF MANITOBA

PROJECT HDL #2013-251

DATE OF OBSERVATION			INITIALS OF SUBJECT		# SUBJET(CODIFICATION)	
DAY	MONTH	YEAR	NAME	LAST NAME	HDL	

QUESTIONNAIRE ON SIDE EFFECTS-INTENSITY

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐ Diet 5 ☐

Day 1 ☐ Day 30 ☐

Indicate if you experienced side effects listed below over the **last four weeks** and if so, how hard was the intensity.

Side effects	Intensity			
	None[0]	Mild[1]	Moderate[2]	Severe[3]
1. Headache	☐	☐	☐	☐
2. Anxiety	☐	☐	☐	☐
3. Fatigue / exhaustion	☐	☐	☐	☐
4. Lack of energy	☐	☐	☐	☐
5. Tend to become exhausted quickly	☐	☐	☐	☐
6. Decreased appetite	☐	☐	☐	☐
7. Increased appetite	☐	☐	☐	☐
8. Hiccup	☐	☐	☐	☐
9. Nausea	☐	☐	☐	☐
10. Vomiting	☐	☐	☐	☐
11. Indigestion	☐	☐	☐	☐
12. Stomach or abdominal pain	☐	☐	☐	☐
13. Constipation	☐	☐	☐	☐
14. Diarrhea	☐	☐	☐	☐
15. Flatulence	☐	☐	☐	☐
16. Abdominal bloating	☐	☐	☐	☐
17. Palpitations	☐	☐	☐	☐
18. Balance disorders	☐	☐	☐	☐
19. Decreased ability to concentrate	☐	☐	☐	☐
20. Flushing	☐	☐	☐	☐
21. Feeling cold	☐	☐	☐	☐
22. Joint or members pain	☐	☐	☐	☐
23. Numbness, burning or itching (feet/hands)	☐	☐	☐	☐
24. Dark or depressing thoughts	☐	☐	☐	☐
25. Other	☐	☐	☐	☐

Appendix II

PARTICIPANT SIDE EFFECT-FREQUENCY QUESTIONNAIRE FORM (DAY 1 &DAY30)

PROJECT HDL #2013-251

UNIVERSITY OF MANITOBA

DATE OF OBSERVATION			INITIALS OF SUBJECT		# SUBJET(CODIFICATION)	
DAY	MONTH	YEAR	NAME	LAST NAME	HDL	

QUESTIONNAIRE ON SIDE EFFECTS-FREQUENCY

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐ Diet 5 ☐

Day 1 ☐ Day 30 ☐

Indicate if you experienced side effects listed below over the **last four weeks** and if so, how hard was the intensity.

Side effects	Frequency			
	Never[0]	Rarely[1]	Sometimes[2]	Often[3]
1. Headache	☐	☐	☐	☐
2. Anxiety	☐	☐	☐	☐
3. Fatigue / exhaustion	☐	☐	☐	☐
4. Lack of energy	☐	☐	☐	☐
5. Tend to become exhausted quickly	☐	☐	☐	☐
6. Decreased appetite	☐	☐	☐	☐
7. Increased appetite	☐	☐	☐	☐
8. Hiccup	☐	☐	☐	☐
9. Nausea	☐	☐	☐	☐
10. Vomiting	☐	☐	☐	☐
11. Indigestion	☐	☐	☐	☐
12. Stomach or abdominal pain	☐	☐	☐	☐
13. Constipation	☐	☐	☐	☐
14. Diarrhea	☐	☐	☐	☐
15. Flatulence	☐	☐	☐	☐
16. Abdominal bloating	☐	☐	☐	☐
17. Palpitations	☐	☐	☐	☐
18. Balance disorders	☐	☐	☐	☐
19. Decreased ability to concentrate	☐	☐	☐	☐
20. Flushing	☐	☐	☐	☐
21. Feeling cold	☐	☐	☐	☐
22. Joint or members pain	☐	☐	☐	☐
23. Numbness, burning or itching (feet/hands)	☐	☐	☐	☐
24. Dark or depressing thoughts	☐	☐	☐	☐
25. Other	☐	☐	☐	☐

Appendix II

PARTICIPANT OSTEODENSITOMETRY QUESTIONNAIRE FORM

UNIVERSITY OF MANITOBA

HDL PROJECT
2013-251

DAY	OBSERVATION DATE			SUBJECT'S INITIAL		# SUBJECT (CODIFICATION)	
	DAY	MONTH	YEAR	FIRST NAME	LAST NAME		
D30						HDL	

Diet 1 ☐ Diet 2 ☐ Diet 3 ☐ Diet 4 ☐ Diet 5 ☐

Short osteodensitometry questionnaire

Sex: ☐ Women ☐ Men

1. Have you already had surgical intervention that needed screw, metal plate or prosthesis? Precise : _____	☐ Yes ☐ No
2. Do you have any "piercings"?	☐ Yes ☐ No
3. Have you ever had a scoliosis diagnosis?	☐ Yes ☐ No
4. In the past 2 weeks, have you had an exam involving any radioactive medicine? (nuclear medicine, barium) Why?_____ ***If yes, don't do the test***	☐ Yes ☐ No
5. If you are woman, do you have the possibility of being pregnant? ***If yes, don't do the test***	☐ Yes ☐ No
6. Would you like a pregnancy test? If No, Why? _____	☐ Yes ☐ No

Comments:

_____

_____

_____

Appendix II

PARTICIPANT OSTEODENSITOMETRY SCAN REQUISITION FORM

Richardson Centre
for Functional Foods
and Nutraceuticals

196 Innovation Drive
Winnipeg, Manitoba R3T 6C5

[redacted] (phone)
[redacted] (fax)

Requisition for DXA Scan

Study name: Integrated research program on dairy, dairy fat and cardiovascular health. Part 1: Impact of dairy fat compared with carbohydrates, mono and poly unsaturated fatty acids on plasma lipids and other cardiometabolic risk factors

Ethics #:_____ **B2014:018**_____

Scans Ordered by:

Name:

Phone Number:

Account to be billed:

Primary Investigator:

Name:_____Dr. Peter Jones_____ **Signature:**_____

Physician co-signer:

Name:___ Dr. Shahrokh Nejad Ghaffar _____ **Signature:**_____

Subjects to be studied: __Human __X__ **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:___5_____

Pregnancy Test conducted __ **Yes** __ **No** __ **Not Applicable**

Scans Required:

Scan ID#

_____ _X_ 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** X__ **CD-ROM**

Charge Information- Office Use Only

Appendix II: ***PARTICIPANT FOLLOW-UP FORM***

UNIVERSITY OF MANITOBA

HDL PROJECT # 2013-251

DATE OF OBSERVATION			INITIALS OF SUBJET		# SUBJECT (CODIFICATION)	
DAY	MONTH	YEAR	NAME	FAMILY NAME	HDL	

Follow-up HDL

Period

Baseline ☐
Washout Phase 1 ☐
Washout Phase 2 ☐
Washout Phase 3 ☐
Washout Phase 4 ☐

1- During the specified period, how much alcohol weekly have you consumed?
(One drink = 341 ml bottle of beer, 150 ml of wine or 1-5 oz of liquor)

_____ No. of drink(s)/week No alcohol ❑

2- During the last week, how much alcohol have you consumed?
(One drink = 341 ml bottle of beer, 150 ml of wine or 1,5 oz of liquor)

_____ No. of drink No alcohol ❑

3- During the washout period, have you had particular health problems?

_____
_____
_____
_____

Comments:

_____
_____
_____

Appendix II

PARTICIPANT CHECKLIST AND MEAL QUESTIONNAIRE FORM

PROJET HDL # 2013-251 / 20-01-2014

Date: _____ Week:____ ID: _____ Diet: D

CHECKLIST HDL PROJECT

Day	Meal	Foods	Check if consume	Coffee, tea	Medication
Monday	**Breakfast**	Orange and raisins muffins Berries smoothies	( ) ( )		
	Snack	Pineapple	( )		
	Lunch	Salmon bread Bechamel sauce Parsnip and carrots Bread Oat and dates cookies	( ) ( ) ( ) ( ) ( )		
	Dinner	Leek soup Thai noodle with pork Sugar pie	( ) ( ) ( )		
Tuesday	**Breakfast**	Granola Vanilla yogurt Raspberries and blueberries	( ) ( ) ( )		
	Snack	Oat muffins	( )		
	Lunch	Carrot soup Chili con carne Basmati rice Cranberry bread	( ) ( ) ( ) ( )		
	Dinner	Curry chicken Couscous Broccoli and cauliflower Chocolate cake	( ) ( ) ( ) ( )		
Wednesday	**Breakfast**	Bagel Peanut butter Pineapple Orange juice	( ) ( ) ( ) ( )		
	Snack	Apple bread	( )		
	Lunch	Vegetables soup Mexican tortilla Chocolate pudding Orange	( ) ( ) ( ) ( )		
	Dinner	Pork with orange sauce Potatoes Green beans Bread Baklava	( ) ( ) ( ) ( ) ( )		
Thursday	**Breakfast**	Berries yogurt Oatmeal muffins	( ) ( )		
	Snack	Applesauce	( )		
	Lunch	Spinach, chicken and cranberry salad Balsamic dressing Bread Maple syrup pudding	( ) ( ) ( ) ( )		

Day	Meal	Foods	Check if consume	Coffee, tea	Medication*
Thursday	**Dinner**	Asian chicken Green pees Brown rice Apple crisp	( ) ( ) ( ) ( )		
Friday	**Breakfast**	Yogurt and raspberry muffin Berries smoothie	( ) ( )		
	Snack	Red grapes	( )		
	Lunch	Lentils bread Mashed sweet potatoes Blueberries and yogurt	( ) ( ) ( )		
	Dinner	Butternut squash soup Jambalaya Orange and almond cake	( ) ( ) ( )		
Saturday	**Breakfast**	Banana bread Peanuts Apple	( ) ( ) ( )		
	Snack	Fruits Rice Krispies bar	( )		
	Lunch	Rice salad with chicken Carrots and celery Dip Oat and raisin cookies	( ) ( ) ( ) ( )		
	Dinner	Fin trout with raspberries sauce Potatoes with herbs Green beans Brownies Milk	( ) ( ) ( ) ( ) ( )		
Sunday	**Breakfast**	Bread Homemade cretons Applesauce Orange juice	( ) ( ) ( ) ( )		
	Snack	Molasses muffin	( )		
	Lunch	Quiche Tabouleh salad Vanilla pudding	( ) ( ) ( )		
	Dinner	Lemon chicken Garlic pasta Pepper Carrots cake	( ) ( ) ( ) ( )		

*Medication : Usually only Tylenol is allowed in addition to medications you take and which have been approved by the coordinator of the study. Consult the study coordinator before taking any other medication or supplement.

Any health problem:

_____

_____

Food eaten outside of the study diet:

_____

_____

Appendix II

PARTICIPANT WEEKLY PHYSICAL ACTIVITY FORM

HDL PROJECT # 2013-251

UNIVERSITY OF MANITOBA

Date: _____ Week:_____ Subject ID: HDL-_____ Diet:_____

WEEKLY PHYSICAL ACTIVITY FORM

During the week, what sports or physical activities have you done (including walking, gardening, shoveling)

Sport or physical activity	How many time a week	How long each time (in minutes)	Travel distance (walk or bicycle)	Intensity*	Comments

* You can describe the intensity of your physical activity as follows:

- **Low** (I sweat very little)
- **Moderated** (I sweat and I feel slightly out of breath)
- **High** (I am very breathless)

Appendix II

PARTICIPANT WEIGHT AND ENERGY LEVEL FORM

Project HDL 2013-251/20-0102014

WEIGHT AND ENERGY LEVEL FORM

Diet:____[redacted]____ Subject :ID[redacted]

Week	Day	Date	Weight (Kg)	Energy level (Kcal)
1	1	19/08/2015		
	2	20/08/2015		
	3	21/08/2015		
	4	22/08/2015	Saturday	
	5	23/08/2015	Sunday	
	6	24/08/2015		
	7	25/08/2015		
2	8	26/08/2015		
	9	27/08/2015		
	10	28/08/2015		
	11	29/08/2015	Saturday	
	12	30/08/2015	Sunday	
	13	31/08/2015		
	14	01/09/2015		
3	15	02/09/2015		
	16	03/08/2015		
	17	04/08/2015		
	18	05/08/2015	Saturday	
	19	06/08/2015	Sunday	
	20	07/08/2015		
	21	08/09/2015		
4	22	09/09/2015		
	23	10/09/2015		
	24	11/09/2015		
	25	12/09/2015	Saturday	
	26	13/09/2015	Sunday	
	27	14/09/2015		
	28	15/09/2015		

Appendix II

PARTICIPANT TERMINATION FORM

UNIVERSITY OF MANITOBA

HDL Project (no 2013-251)

WEEK/VISIT	DATE OF OBSERVATION			INITIALS OF SUBJET		# SUBJET(CODIFICATION)	
	DAY	MONTH	YEAR	NAME	FAMILY NAME		
[redacted]							

TERMINATION

Reason for termination:

- ☐ 1 Completion of treatment period, as defined by protocol.
- ☑ Early termination (choose primary reason):
  - ☐ 2 Adverse event, specify: _____
    INAF: *Complete Rapport d'effets indésirables*
    RCFFN *: Complete Local Serious Adverse Event (SAE) Form*
  - ☑ 3 Dislike of study product.
  - ☐ 4 At the request of the investigator or sponsor.
  - ☐ 5 At the request of patients family physician or study physician.
  - ☐ 6 Protocol violation.
  - ☑ 7 Illness or health problem, specify: _____
  - ☐ 8 Patient lost to follow-up.

Investigator's statement:

I certify that all information entered in this case report form by myself, my associates or designates is complete and accurate to the best of my knowledge.

_____ [redacted]

Investigator signature Date (Day/Month/Year)

Appendix II

PARTICIPANT FOOD FREQUENCY QUESTIONNAIRE INSTRUCTIONS

Food frequency questionnaire self-administered via Internet

Self-administered food frequency questionnaire via the Internet from the Institute of Nutrition and Functional Foods (INAF) is a questionnaire assessing dietary intake of respondents during the last month.

This questionnaire is available on the website:

http://inaf.fsaa.ulaval.ca/ffq/.

On the home page, users must enter their username and password to log in private. The user name assigned to each participant is a coded name taking into account the project name and the participant number, determined by the project coordinator (i.e. for a project on yogurt, users may be appointed YOG1 , YOG2, YOG3 and so on). The password is generated automatically by the system when the coordinator registered users in the project.

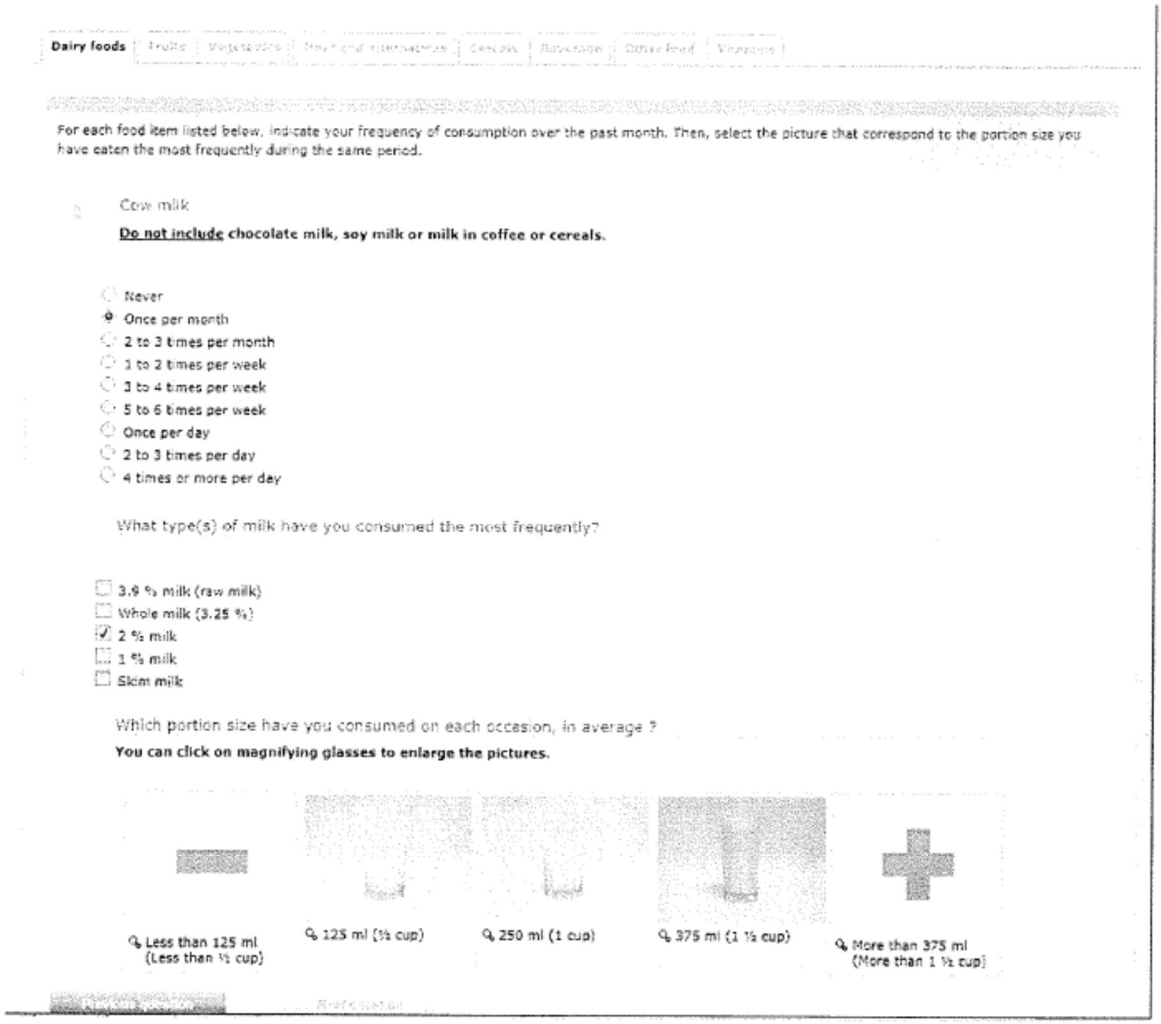
The questionnaire includes 136 questions divided into eight food groups, which are:

1 - Dairy Products
2 - Fruits
3 - Vegetables
4 - Meat and Alternatives
5 - Bread and cereals
6 - Beverages
7 - Other Food
8 - Supplements

Each question is built on the "Frequency - Food - Serving (FFS)" principle. As can be seen in the example shown on the next page, first we ask users to indicate the frequency with which each food or food group is consumed, the choices can go extremes from "never" to "4 or more times a day."

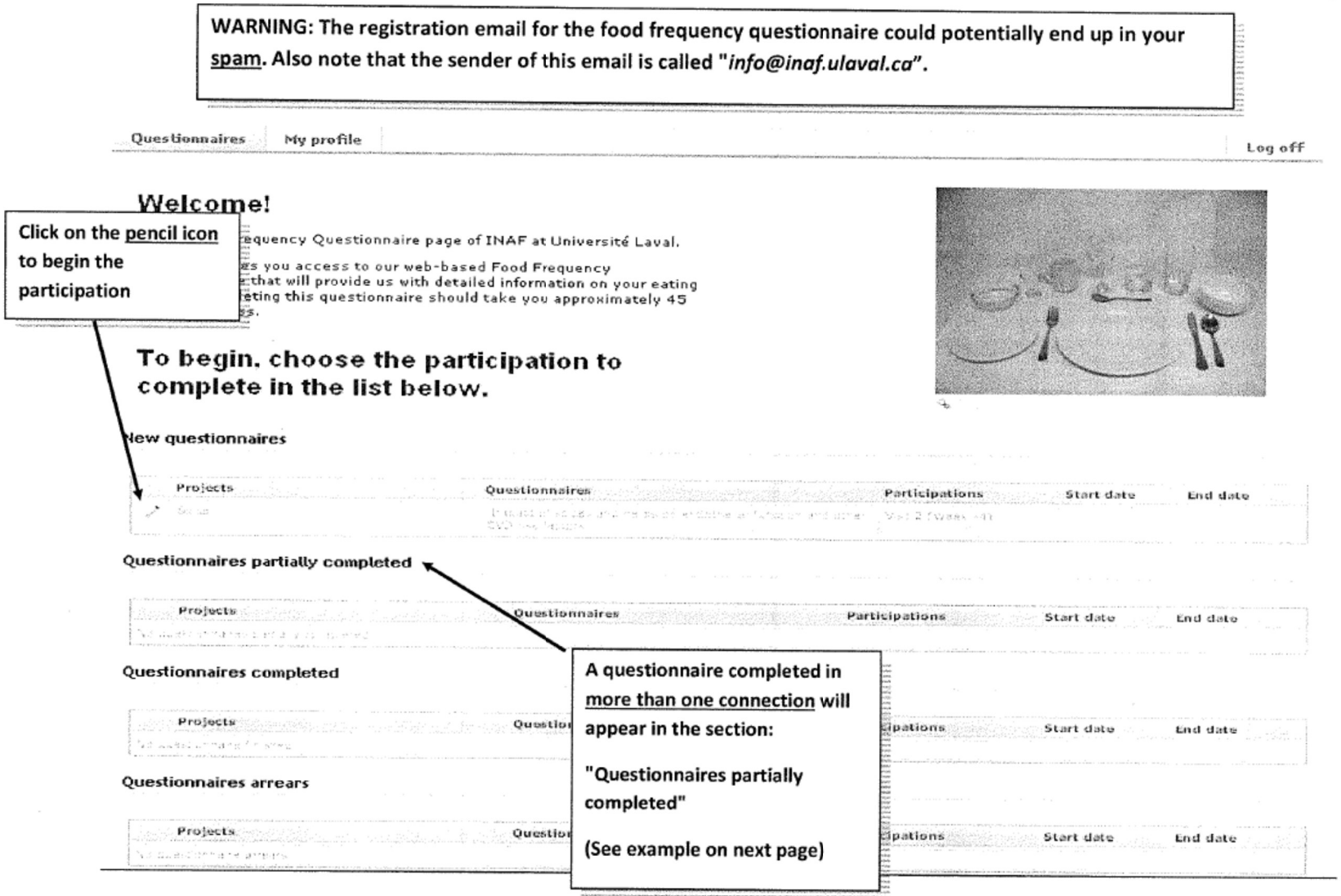
Then we ask, if necessary, any specific type of food consumed (i.e. 3.25% milk, 2% milk, 1% milk, skim milk). Multiple choices are available most of the time as well as the choice "I do not know."

Finally, we ask the user on the size of the portion consumed. The user must click on the image corresponding to the portion that was most frequently consumed during the last month. To help the user to choose the right portion, the volume or weight of each portion is indicated at the bottom of each image. In terms of volume, the portions are indicated both in international measures (ml) and imperial measures (cup). The same principle applies in weight (the portions are indicated in grams as well as in ounces). Finally, users can view larger images by clicking on the magnifying glass located in the lower left corner of each image.



It takes about 45 minutes to complete the questionnaire and the user has the choice to complete its participation in more than one connection. The data is automatically saved when the user logs out from the site.

Finally, coordinators of the project can extract a wide range of information from this questionnaire, such as total daily nutrient intakes of each subject, nutrient intakes provided by one or more specific foods (i.e. junk food ("fast food")) or the number of servings of different groups of Canada's Food Guide for each user.

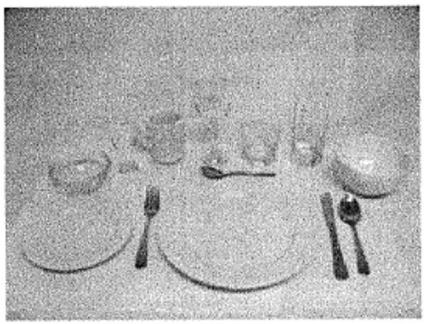

WARNING: The registration email for the food frequency questionnaire could potentially end up in your spam. Also note that the sender of this email is called "info@inaf.ulaval.ca".
Questionnaires
My profile
Log off
Welcome!
Click on the pencil icon to begin the participation
To begin, choose the participation to complete in the list below.
New questionnaires
Projects
Questionnaires
Participations
Start date
End date
Questionnaires partially completed
Questionnaires completed
Questionnaires arrears
A questionnaire completed in more than one connection will appear in the section:
"Questionnaires partially completed"
(See example on next page)

Questionnaires | My profile | Log off

Welcome!

to the Food Frequency Questionnaire page of INAF at Université Laval.

This page gives you access to our web-based Food Frequency Questionnaire that will provide us with detailed information on your eating habits. Completing this questionnaire should take you approximately 45 minutes or less.



To begin, choose the participation to complete in the list below.

New questionnaires

Projects	Questionnaires	Participations	Start date	End date
[illegible]	[illegible]	[illegible]		

Questionnaires partially completed

Projects	Questionnaires	Participations	Start date	End date
[illegible]	[illegible]	[illegible]	[illegible]	

Questionnaires completed

Projects	Questionnaires	Participations	Start date	End date
[illegible]				

Questionnaires arrears

APPENDIX III

PRIMARY RESULTS AND PUBLICATION

Comparison of the impact of SFAs from cheese and butter on cardiometabolic risk factors: a randomized controlled trial[1–3]

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ABSTRACT

Background: Controversies persist concerning the association between intake of dietary saturated fatty acids (SFAs) and cardiovascular disease risk.

Objective: We compared the impact of consuming equal amounts of SFAs from cheese and butter on cardiometabolic risk factors.

Design: In a multicenter, crossover, randomized controlled trial, 92 men and women with abdominal obesity and relatively low HDL-cholesterol concentrations were assigned to sequences of 5 predetermined isoenergetic diets of 4 wk each separated by 4-wk washouts: 2 diets rich in SFAs (12.4–12.6% of calories) from either cheese or butter; a monounsaturated fatty acid (MUFA)–rich diet (SFAs: 5.8%, MUFAs: 19.6%); a polyunsaturated fatty acid (PUFA)–rich diet (SFAs: 5.8%, PUFAs: 11.5%); and a low-fat, high-carbohydrate diet (fat: 25%, SFAs: 5.8%).

Results: Serum HDL-cholesterol concentrations were similar after the cheese and butter diets but were significantly higher than after the carbohydrate diet (+3.8% and +4.7%, respectively; $P < 0.05$ for both). LDL-cholesterol concentrations after the cheese diet were lower than after the butter diet (−3.3%, $P < 0.05$) but were higher than after the carbohydrate (+2.6%), MUFA (+5.3%), and PUFA (+12.3%) diets ($P < 0.05$ for all). LDL-cholesterol concentrations after the butter diet also increased significantly (from +6.1% to +16.2%, $P < 0.05$) compared with the carbohydrate, MUFA, and PUFA diets. The LDL-cholesterol response to treatment was significantly modified by baseline values (P-interaction = 0.02), with the increase in LDL cholesterol being significantly greater with butter than with cheese only among individuals with high baseline LDL-cholesterol concentrations. There was no significant difference between all diets on inflammation markers, blood pressure, and insulin-glucose homeostasis.

Conclusions: The results of our study suggest that the consumption of SFAs from cheese and butter has similar effects on HDL cholesterol but differentially modifies LDL-cholesterol concentrations compared with the effects of carbohydrates, MUFAs, and PUFAs, particularly in individuals with high LDL cholesterol. In contrast, SFAs from either cheese or butter have no significant effects on several other nonlipid cardiometabolic risk factors. This trial was registered at clinicaltrials.gov as NCT02106208. *Am J Clin Nutr* 2017;105:800–9.

Keywords: cardiovascular risk factors, CVD, dairy products, men and women, randomized crossover controlled trial, SFA

INTRODUCTION

Most dietary guidelines have advocated for a restriction of dietary SFAs for the optimal management of cardiovascular health (1, 2). However, the association between SFAs and risk of cardiovascular disease (CVD)[12] remains controversial. On the one hand, several meta-analyses of observational studies have shown that self-reported intakes of SFAs were not associated with increased risk of all-cause mortality, CVD, coronary artery disease (CAD), or ischemic stroke (3, 4). In contrast, a systematic review of early randomized controlled trials (RCT) has shown a small but significant reduction in CVD risk when dietary SFAs have been substituted with PUFAs (5).

The controversy surrounding SFAs and CVD risk has been further fueled by data from studies that have shown that dietary SFAs may have different associations with CVD risk depending on the food source of SFA. For example, a 5% increase in energy from dairy SFAs in the Multi-Ethnic Study of Atherosclerosis has been associated with a 38% lower CVD risk, whereas a 5% increase in energy from meat SFAs was shown to predict a 69%

[1]Supported by grants from the Dairy Research Cluster Initiative (Agriculture and Agri-Food Canada, Dairy Farmers of Canada, the Canadian Dairy Network, and the Canadian Dairy Commission).

[2]Funders were not involved in the design and protocol development; conduct of the trial; collection, management, analysis, or interpretation of the data; preparation, review, or approval of the manuscript; or decision to submit the manuscript for publication. The project was funded as part of a competitive funding initiative with a scientific review performed by an independent expert panel.

[3]Supplemental Tables 1 and 2 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://ajcn.nutrition.org.

[11]These authors contributed equally to this work.

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[12]Abbreviations used: apo B, apolipoprotein B; CAD, coronary artery disease; CVD, cardiovascular disease; INAF, Institute of Nutrition and Functional Foods; ITT, intent-to-treat; RCFFN, Richardson Center for Functional Foods and Nutraceuticals; RCT, randomized controlled trial.

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higher CVD risk (6). The food matrix may also modify the impact of SFAs on CVD risk factors. In a meta-analysis of RCTs, de Goede et al. (7) have shown that, for similar SFA intakes and ratios of PUFAs to SFAs, the consumption of hard cheese reduced LDL cholesterol and HDL cholesterol compared with the effect of butter.

Results from the meta-analysis by de Goede et al. (7) were limited in scope because they were based on data from only 5 RCTs, each of which had relatively small sample sizes (n = 14–49). All studies were conducted in Europe (8–11) or in Australia (12). To our knowledge, no previous study has compared the effects of SFAs from cheese and butter on cardiometabolic risk to those of MUFAs or PUFAs in a North American context. Finally, the majority of the available RCTs have focused on lipid risk factors. Therefore, there has been limited information regarding the impact of SFAs from different sources on nonlipid cardiometabolic risk factors.

The primary objective of this multicenter, randomized, crossover, controlled-consumption study was to compare the impact of SFAs from different dairy food sources, namely cheese and butter, on plasma lipid concentrations, blood pressure, and other cardiometabolic risk factors including factors that are related to glucose-insulin homeostasis and inflammation. As a secondary objective, we compared the impact of consuming SFAs from different dairy sources to that of other fat sources, including MUFAs and PUFAs, on cardiometabolic risk. On the basis of the available evidence, we hypothesized that the cheese matrix would attenuate the cardiometabolic effects associated with the consumption of SFAs.

METHODS

Participants

This study was undertaken as a multicenter RCT that involved the following 2 Canadian research centers: the Institute of Nutrition and Functional Foods (INAF) in Quebec City and the Richardson Center for Functional Foods and Nutraceuticals (RCFFN) in Winnipeg. Recruitment took place between May 2014 and May 2015. Advertisements were published in newspapers, circulated in local hospitals, and displayed on notice boards at companies near the university campuses. Invitations were also sent via mailing lists that were available in both centers. To be eligible, participants had to be 18–65 y old, have a waist circumference ≥94 and ≥80 cm for men and women, respectively, and have HDL-cholesterol concentrations below the age- and sex-specific 75th percentiles (≤1.34 and ≤1.53 mmol/L for men and women, respectively) to exclude individuals with high HDL-cholesterol concentrations, which was the primary study outcome. The study originally set out to recruit individuals on the basis of a high waist circumference and serum triglyceride concentration >1.7 mmol/L. However, this combination of inclusion criteria yielded an extremely low recruitment rate. The triglyceride criterion was modified to the aforementioned HDL-cholesterol criterion 3 mo into the study to facilitate recruitment. Participants had to have stable body weight (±2.5 kg) ≥6 mo before their inclusion in the trial. Menopausal status was defined as being without regular menses ≥1 y. Exclusion criteria were as follows: a history of CVD, type 2 diabetes, or monogenic dyslipidemia; the use of medications for hypertension, hyperlipidemia, or glycemic control; uncontrolled endocrine disorder such as hypothyroidism or hyperthyroidism; smoking; a Framingham-calculated CAD risk score >20%; any food allergies or aversion to foods that were included in the menu; particular nutritional habits such as vegetarianism; and women with menstrual irregularities including those who were experiencing perimenopause. The use of an anti-inflammatory drug was prohibited during the entire study period including the 4-wk preintervention period and washout periods. Other medications were allowed as long as the use and dosage had been stable over the 1 y that preceded the recruitment of the participants. The study protocol was thoroughly explained during the screening process, and written consent was obtained from all participants before undertaking the dietary phases. The study protocol was approved by local ethical boards and was registered on 4 April 2014 at clinicaltrials.gov at NCT02106208.

Experimental diet composition and study design

We used a single-blind crossover study design in which participants were randomly assigned to 8 predetermined sequences of the following 5 treatments: *1*) a diet that was rich in SFAs from cheese, *2*) a diet that was rich in SFAs from butter, *3*) a diet that was rich in MUFAs, *4*) a diet that was rich in PUFAs, and *5*) a high-carbohydrate, low-fat diet. The SFA content was matched in the cheese and butter diets. In the other diets, MUFAs, PUFAs, and carbohydrates replaced SFAs from the cheese and butter diets through dietary manipulations. Specifically, proteins from cheese in the cheese diet were replaced by increasing the serving sizes of meat and eggs in the other 4 diets. The grams of fat from cheese in the cheese diet were replaced by corresponding amounts of butter fat, refined olive oil, and corn oil in the butter, MUFA, and PUFA diets respectively. Carbohydrates from foods (vegetables, fruit, and grains) as well from added sugars (honey, sugar and brown sugar, jam, maple syrup, and 100% fruit juice) were substituted for fats in the 4 high-fat diets (cheese, butter, MUFA, and PUFA diets). Vegetables, fruit, and grain products with low-fiber contents were chosen in the carbohydrate diet to balance fiber intake across all diets. The identified foods and added sugars represented, on average, 77% and 23% of added carbohydrates, respectively, in the carbohydrate diet. More specifically, the carbohydrate diet provided a mean of 10.0 g added sugars (per 2500 kcal/d) compared with the amount in the cheese diet, whereas the remaining extra carbohydrates came from foods.

Diets were identical in terms of energy, protein, cholesterol, sodium, and fiber contents. Calcium and potassium contents were higher in the cheese diet than in the other 4 experimental diets (**Table 1**). Each of the 5 treatment phases had a 4-wk duration and was followed by a washout period ≥24 d. Three meals and 1 snack were provided each day to participants on the basis of a 7-d rotating menu, which was reproducible for the 5 diets and similar across the 2 participating research centers. The experimental diets were developed with the use of the Nutritional Database System for Research (2011; Nutrition Coordinating Center). Dietetic technicians prepared all recipes and meals in the metabolic kitchen of participating centers. Diets were provided under isoenergetic conditions to maintain a constant body weight. Energy needs for each participant were estimated with the use of validated equations (13) as well as from values that

TABLE 1
Nutritional composition of the 5 predetermined experimental diets[1]

	Cheese	Butter	MUFA	PUFA	CHO
Energy,[2] kcal	2654 ± 567	2615 ± 537	2647 ± 550	2649 ± 576	2618 ± 561
Cheese, g/2500 kcal	90.0[3]	0	0	0	0
Butter, g/2500 kcal	0	48.9	0	0	0
Lipids, %	32.0	32.0	32.0	32.0	25.0
SFAs	12.6	12.4	5.8	5.8	5.8
MUFAs	12.5	12.3	19.6	12.6	12.6
PUFAs	4.8	4.8	4.8	11.5	4.8
CHOs, %	51.9	52.0	51.9	51.9	58.9
Protein, %	16.0	16.0	16.0	16.0	16.0
Calcium, mg/2500 kcal	1261.0	811.1	812.2	811.7	841.6
Total fibers, g/2500 kcal	30.7	30.6	30.6	30.6	30.5
Cholesterol, mg/2500 kcal	272.1	272.4	271.5	272.2	272.4
Sodium, mg/2500 kcal	2482	2480	2479	2479	2485

[1] For the comparison between treatments, $P = 0.82$ (mixed models). Butter and cheese: $n = 77$; MUFAs: $n = 74$; PUFAs: $n = 76$; and CHO: $n = 72$. CHO, carbohydrate.
[2] All values are means ± SDs.
[3] Mean (all such values).

were estimated with the use of a validated, quantitative, web-based food-frequency questionnaire that was completed before the beginning of the trial (14). During each dietary phase, participants were asked to come to the INAF or RCHFN ≥3 times/wk to pick up the meals and snacks. Subjects were also encouraged, whenever possible, to consume either breakfast or lunch on site every weekday under staff supervision. Subjects were instructed to consume all of the food provided, and only that food, while limiting the consumption of caffeinated beverages and sugar-free beverages to 2 beverages/d. A discretionary amount of 0% fat fluid milk (105 g) was provided to subjects weekly if requested. Alcohol intake was not allowed 2 d before the beginning of the study and during each dietary phase. Body weight was monitored continuously throughout each dietary phase, and food provision was adjusted when subjects body weight fluctuated >2 kg over 1 wk. Subjects were instructed to maintain their usual physical activities except for the 4 d that preceded blood sampling at the various stages of the study, during which subjects were asked not to engage in any form of vigorous physical activity. Subjects could not be blinded to the cheese diet but were blinded to the other 4 diets.

Compliance

Compliance to treatments was assessed via checklists that were filled out by participants on a weekly basis, which allowed for the identification of foods that were consumed and foods that were not consumed. Checklists provided information on beverage intake as well as on current medication use. Participants were asked to notify the coordinator who was in charge of the project from both centers before starting any new medication. Compliance was assessed during each dietary phase. Thus, a subject may have been compliant in some phases but not in others. It was decided a priori that we would exclude from the analyses all data points that were collected during a treatment phase for which the self-reported compliance was <80%.

Risk-factor assessment

Body weight, together with waist and hip circumferences, were measured according to standardized procedures throughout the study (15). The mean of the 2 postdiet values was used for the calculation of postdiet BMI. Body fat and composition were assessed with the use of dual-energy X-ray absorptiometry (GE Healthcare) at the end of each dietary phase. Systolic and diastolic blood pressures were determined at screening, beginning, and the end of each dietary phase from the mean of 3 consecutive measurements that were taken 10 min apart in the sitting position with the use of an automated blood pressure monitor (Digital BPM HEM-907XL model; Omron).

Analyses of cardiometabolic risk factors were performed on 12-h fasting blood samples that were collected from the antecubital vein. All cardiometabolic risk factors were measured twice on consecutive days at the end of each dietary phase, and the mean of the 2 measurements was used in all analyses. Treatment-specific baseline values were measured once. Laboratory analyses were carried out with staff blinded to study treatments.

Serum total cholesterol, triglyceride, and HDL-cholesterol concentrations were assessed with the use of a Roche/Hitachi Modular system (Roche Diagnostics) according to the manufacturer's specifications and with the use of proprietary reagents. Serum LDL-cholesterol concentrations were calculated with the use of the Friedewald equation except in 2 subjects who had serum triglyceride concentrations >4.5 mmol/L on 4 occasions, in which cases, LDL cholesterol was considered to be missing. Plasma total apolipoprotein B (apo B) concentrations were measured with the use of the a commercial ELISA kit (A70102; Alerchek). Serum high-sensitivity C-reactive protein concentrations were determined with the use of the Behring Latex-Enhanced highly sensitive assay on a Behring Nephelometer BN-100 (Behring Diagnostic) and the calibrators (N Rheumatology Standard SL) that were provided by the manufacturer as described previously (16). High-sensitivity C-reactive protein concentrations were considered to be missing when the mean of the 2 consecutive postdiet values was >10 mg/L. Adiponectin was measured with the use of a commercial ELISA kit for the human form (K1001-1; B-Bridge International). Fasting blood glucose concentrations were examined with the use of colorimetry, whereas insulin concentrations were tested with the use of electrochemiluminescence (Roche Diagnostics). Finally, the HOMA-IR was calculated with the use of the formula that was developed by Matthews et al. (17).

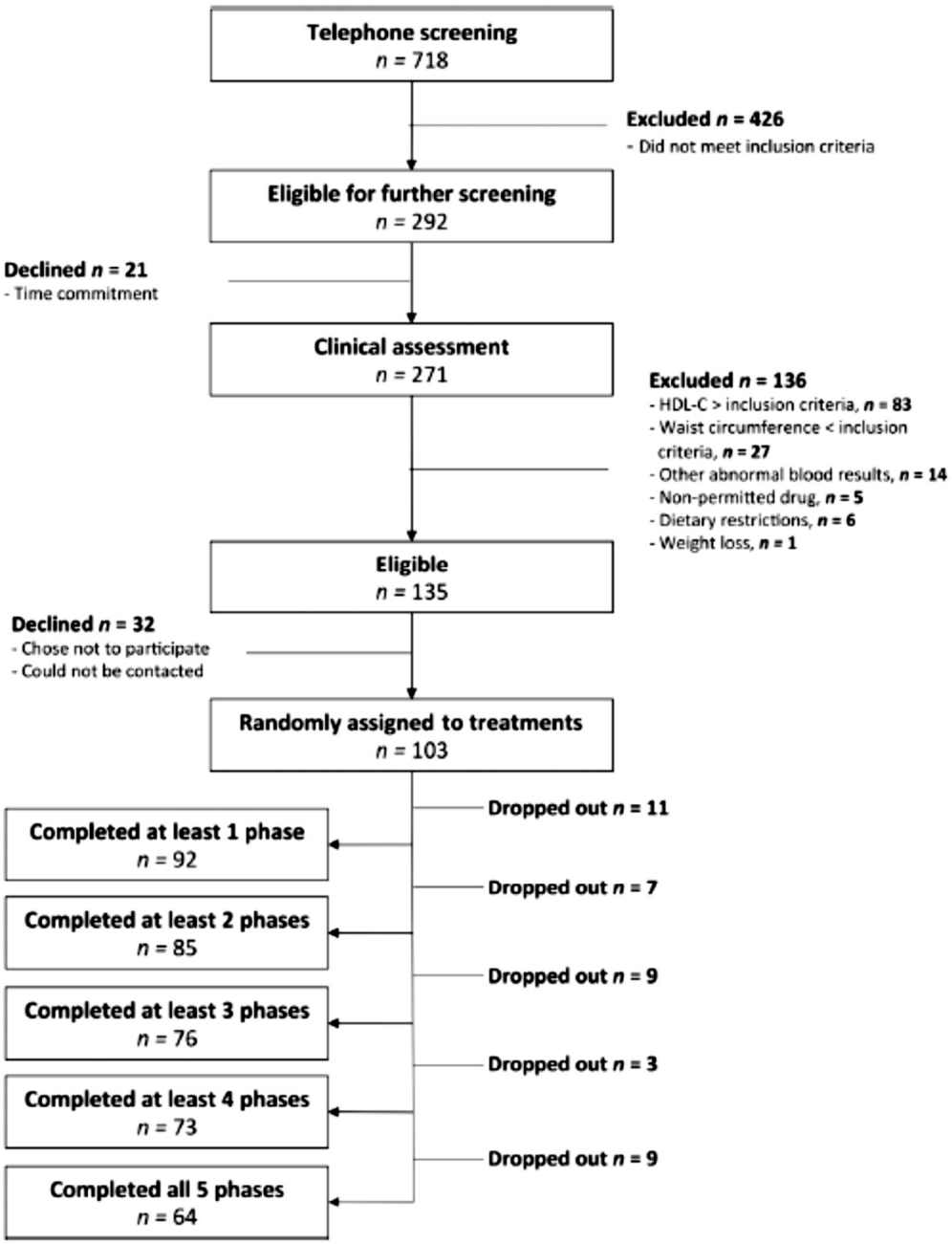

FIGURE 1 Flowchart diagram. Of 135 eligible subjects, 103 individuals were randomly assigned. The dropout rate was 37.9% (39 of 103 subjects who were randomly assigned). Reasons for dropping out were as follows: job, study, family, or travel constraints ($n = 12$); lost to follow-up ($n = 6$); loss of interest ($n = 5$); the protocol was too demanding ($n = 5$); diet issues (did not like the food; $n = 5$); health problems that were unrelated to the study protocol ($n = 3$); pregnancies ($n = 2$); and medical constraint ($n = 1$). A total of 92 subjects completed ≥1 phase, whereas 64 subjects completed all 5 phases. HDL-C, HDL cholesterol.

Sample-size calculations

The change in HDL cholesterol after cheese compared with after butter was considered as the primary analysis for a priori sample-size calculations. Accordingly, it was determined that a sample size of $n = 70$ would allow for the detection of a 6.3% between-diet difference in plasma HDL-cholesterol concentrations with a power of 90% ($P = 0.05$). A dropout rate of 20% was projected on the basis of our recent experience in a similarly

designed, multicenter, crossover consumption study that comprised 5 diets, each of which were separated by a 4-wk washout (18). The required sample size of $n = 90$ was slightly exceeded during recruitment.

Statistical analyses

Differences in study outcomes in treatments (postdiet values) were assessed with the use of mixed models for repeated measures in SAS software (v9.4; SAS Institute Inc.) with the treatment, sex, center, and sequence of treatments as fixed effects and subjects as a random effect. A pairwise comparison of treatments was examined only when the overall P value for the main treatment effect in the mixed models was <0.05. The Holm-Bonferroni procedure was used to adjust for multiple comparisons of the various treatments (19). This sequential step-down approach is considered one of the best-known techniques to control for a family-wise error rate. The method is similar to the classical Bonferroni correction. Specifically, the method ensures that the probability of ≥1 false discovery under the null hypothesis is fixed at the specified α level. However, the Holm-Bonferroni correction offers more statistical power without requiring any further assumption. Briefly, P values of the tests under consideration are rank ordered so that $P_1 \leq P_2 \leq \ldots \leq P_k$, where k is the number of tests. Original P values are sequentially adjusted; e.g., the adjusted value of P_1 is equal to k times the original value of P_1, and the adjusted value of P_2 is the maximum between the adjusted value of P_1 and $(k - 1)$ times the original value of P_2. Adjusted P values are evaluated against the criterion of $\alpha = 0.05$. The multiple comparisons under consideration were set a priori as follows: cheese compared with butter; cheese compared with carbohydrates, MUFAs, and PUFAs; and butter compared with carbohydrates, MUFAs, and PUFAs. MUFA, PUFA, and carbohydrate comparisons were not considered because they were not part of the primary aim of the study. With the use of a most parsimonious modeling approach, potential confounders of the changes in cardiometabolic risk factors with treatment, including baseline values of the selected outcome, center, sequence of treatments, sex, age, BMI, waist:hip ratio, and ethnicity and their interaction with treatment, were included in the final mixed models only when they were shown to be significant at $P < 0.05$. The normality in the distribution of all study outcomes was considered, and data were log transformed when required. In the primary analyses, multiple imputations of missing data were not used because mixed models have been shown to be robust even when a significant proportion of data are missing at random (20). However, data were also analyzed with the use of an intent-to-treat (ITT) approach with multiple imputation of missing data. More detailed information on how this analysis was conducted and the results are presented in **Supplemental Table 1**.

RESULTS

Figure 1 presents the study flowchart. Consumption by participants was initiated on 9 July 2014, and all participants completed the intervention on 18 February 2016. Of 135 eligible men and women, a total of 103 individuals were randomly assigned to the treatment sequences, 92 subjects completed ≥1 treatment phase, and 64 subjects completed all 5 treatments. Of 39 participants who dropped out, 20 individuals were from the INAF, and 19 individuals were from the RCFFN. The main reasons for dropping out were as follows: job, study, family, or travel constraints ($n = 12$); lost to follow-up or a loss of interest ($n = 11$); the dietary protocol was too demanding or because of diet issues ($n = 10$); and medical constraints, pregnancies, or other health problems ($n = 6$). A total of 14 treatment-specific data from 8 participants at the RCFFN with compliance <80% were excluded as per the eligibility criteria for analyses. The self-reported compliance of all participants at the INAF was >80% for all dietary phases. Characteristics at the screening of the 92 subjects who were included in the analyses are shown in **Table 2**. Participants from the INAF and RCFFN had a similarly low 10-y CVD Framingham risk score at baseline.

The mean ± SD self-reported compliance to diets during each treatment phase on the basis of food-consumption checklists was high (98.7% ± 2.4%) and was similar between treatments ($P = 0.83$; Kruskal-Wallis test; data not shown) after the exclusion of data from noncompliant subjects. Self-reported compliance was significantly different between centers, although the difference was marginal [INAF: 99.4% ± 1.2%; RCFFN: 97.5% ± 3.4% ($P < 0.0001$; Kruskal-Wallis test)]. The mean duration of each dietary phase was 27.9 ± 0.9 d and was similar in treatments ($P = 0.60$; Kruskal-Wallis test). The median washout time between consecutive treatments was 33 d. No difference was observed between diets in terms of the frequency of self-reported, nonserious adverse events (**Supplemental Table 2**).

Table 3 presents the anthropometric and cardiometabolic risk profiles of participants after each diet. Waist circumference, BMI, and body fat were stable throughout the experiment, which reflected the isoenergetic nature of the trial. Serum HDL-cholesterol concentrations were similar after the cheese and butter diets but were significantly higher (+3.8% and +4.7%, respectively; $P < 0.05$ for both) than after the carbohydrate diet. After the cheese diet, LDL-cholesterol concentrations were significantly lower (−3.3%; $P < 0.05$) than after the butter diet but were higher (+2.6%, +5.3%, and +12.3%; $P < 0.05$ for all) than after carbohydrate, MUFA, and PUFA diets, respectively. LDL-cholesterol concentrations after the butter diet were significantly higher (+6.1%, +8.9%, and +16.2%; $P < 0.05$ for all) than after the carbohydrate, MUFA, and PUFA diets, respectively. The baseline LDL-cholesterol concentration significantly modified the LDL-cholesterol response to treatment (P-interaction = 0.02). As shown in **Figure 2**, the difference in LDL cholesterol between cheese and butter diets was significant in subjects with high baseline LDL cholesterol but not in those with lower baseline LDL cholesterol irrespective of the comparator nutrient. No such interaction was observed for other cardiometabolic risk factors.

Cheese led to higher serum triglyceride concentrations (+5.1% and +10.0%; $P < 0.05$ for both) compared with the effects of butter and PUFAs, respectively, but not compared with the effects of MUFAs or carbohydrates. Butter was associated with reduced serum triglycerides (−6.8%; $P < 0.05$) compared with the effect of carbohydrates but not compared with the effects of MUFAs and PUFAs. There was no difference between cheese and butter in terms of apo B concentrations and the cholesterol:HDL-cholesterol

TABLE 2
Characteristics at screening of subjects who completed ≥1 diet (n = 92)[1]

	INAF (n = 57)	RCFFN (n = 35)	P
Ethnicity, n (%)			<0.0001
Caucasian	55 (96.5)	11 (31.4)	—
Asian	0 (0)	10 (20.0)	—
African/African American	0 (0)	7 (28.6)	—
Hispanic	1 (1.8)	6 (17.1)	—
Other	1 (1.8)	1 (2.9)	—
Women, n (%)	32 (56.1)	17 (48.6)	0.48
Age, y	40.6 ± 13.6[2]	36.8 ± 13.3	0.19
Body weight, kg	86.5 ± 21.0	89.5 ± 19.8	0.51
BMI,[3] kg/m^2	30.3 ± 6.3	31.6 ± 5.6	0.23
Waist circumference, cm	100.6 ± 14.1	103.8 ± 13.9	0.29
Plasma lipids, mmol/L			
Total cholesterol	5.18 ± 1.00	4.70 ± 0.81	0.02
LDL cholesterol[4]	3.22 ± 0.84	2.79 ± 0.73	0.01
HDL cholesterol			
Women	1.30 ± 0.17	1.26 ± 0.21	0.56
Men	1.10 ± 0.19	1.04 ± 0.15	0.24
TG[3]	1.50 ± 0.83	1.64 ± 1.17	0.68
Total cholesterol:HDL cholesterol	4.37 ± 1.01	4.28 ± 0.89	0.67
Glucose,[3] mmol/L	5.21 ± 0.47	5.09 ± 0.54	0.21
Blood pressure, mm Hg			
Systolic	113.1 ± 12.0	115.6 ± 17.0	0.45
Diastolic	69.4 ± 10.1	77.1 ± 10.8	0.0008
10-y Framingham risk score, %	3.9 ± 4.3	3.5 ± 3.7	0.53

[1] P values were determined with the use of a chi-square test for categorical variables and a Student's t test for continuous variables. INAF, Institute of Nutrition and Functional Foods; RCFFN, Richardson Center on Functional Foods and Nutraceuticals; TG, triglyceride.
[2] Mean ± SD (all such values).
[3] Analyses were performed on log-transformed data.
[4] n = 56 for the INAF because of one missing value.

ratio. However, both diets significantly increased apo B and the cholesterol:HDL-cholesterol ratio compared with the effects of the MUFA and PUFA diets. Finally, there was no significant difference in blood pressure, inflammation markers, and indexes of glucose-insulin homeostasis across the experimental diets. There was also no significant interaction between treatment and sex or adiposity in the prediction of the response to diets for any cardiometabolic risk factors. As shown in Supplemental Table 1, results from the ITT and multiple imputation analysis and those from the per protocol analysis were similar with a few minor exceptions. Specifically, the ITT analysis revealed no significant treatment effect on postintervention HDL cholesterol, and there was no significant difference in postintervention LDL-cholesterol concentrations between cheese and carbohydrate diets.

Figure 3 shows differences in observed values compared with values that were predicted on the basis of accepted equations (21) for changes in blood lipids and apo B when carbohydrates were replaced isoenergetically by SFAs from either cheese or butter. In the absence of a food-matrix effect, differences between observed and predicted changes should have been equal to zero. Observed LDL-cholesterol and HDL-cholesterol concentrations were significantly lower than predicted values when carbohydrates (6.8% of energy) were replaced by SFAs from cheese. This was not the case for butter. The replacement of carbohydrates by SFAs from cheese led to higher serum triglyceride concentrations than were predicted, whereas the replacement of carbohydrates by SFAs from butter led to higher-than-predicted apo B concentrations.

DISCUSSION

To our knowledge, this large, randomized, and carefully controlled consumption study provides new perspectives relative to the effects of dietary SFAs on cardiometabolic risk. One unique methodologic aspect of this investigation was that SFAs from cheese and butter were compared with carbohydrates but also with other control nutrients (i.e., MUFAs and PUFAs). First, the isocaloric replacement of carbohydrates by SFAs from both cheese and butter increased serum HDL-cholesterol concentrations. Although the changes in HDL cholesterol with cheese and butter were similar in magnitude, values that were recorded after the cheese diet were significantly lower than those that were predicted with the use of established equations (21). The different effects of SFAs from cheese and butter on LDL cholesterol were amplified in men and women with high baseline LDL-cholesterol concentrations irrespective of the comparator nutrient. Finally, SFAs from cheese and butter had no effect on several nonlipid cardiometabolic risk factors compared with the effects of carbohydrates, MUFAs, and PUFAs.

Only a few RCTs have compared the effects of SFAs from cheese and butter on LDL-cholesterol and HDL-cholesterol concentrations (8–12). All of the studies have used a crossover design with sample sizes that ranged from n = 14 to n = 49, and

TABLE 3
Anthropometric measures, plasma lipid profiles, and nonlipid cardiovascular disease risk factors at the end of each dietary intervention in 92 subjects[1]

	Cheese	Butter	MUFA	PUFA	CHO	*P*-between diets
Waist circumference, cm	100.8 ± 14.4	101.1 ± 14.0	100.3 ± 14.0	100.7 ± 14.5	100.6 ± 13.0	0.29
BMI,[2] kg/m^2	30.6 ± 6.2	30.6 ± 6.2	30.4 ± 6.1	30.6 ± 6.3	30.3 ± 5.5	0.93
Body fat, kg	32.6 ± 11.7	33.2 ± 11.6	32.5 ± 11.4	33.2 ± 11.9	31.9 ± 10.6	0.14
Total cholesterol, mmol/L	5.00 ± 0.94	5.10 ± 0.95	4.82 ± 0.89[3,4]	4.60 ± 0.81[3,4]	4.89 ± 0.92[3,4]	<0.0001
LDL cholesterol, mmol/L	3.19 ± 0.81	3.30 ± 0.84[3]	3.03 ± 0.78[3,4]	2.84 ± 0.69[3,4]	3.11 ± 0.79[3,4]	<0.0001
HDL cholesterol, mmol/L	1.10 ± 0.19	1.11 ± 0.21	1.10 ± 0.19	1.10 ± 0.20	1.06 ± 0.19[3,4]	0.0051
TG,[2] mmol/L	1.43 ± 0.70	1.36 ± 0.73[3]	1.38 ± 0.67	1.30 ± 0.62[3]	1.46 ± 0.71[4]	0.0007
Cholesterol:HDL cholesterol	4.67 ± 1.04	4.73 ± 1.18	4.50 ± 1.08[3,4]	4.28 ± 1.01[3,4]	4.71 ± 1.08	<0.0001
apo B, g/L	1.72 ± 0.50	1.74 ± 0.58	1.65 ± 0.50[3,4]	1.53 ± 0.50[3,4]	1.68 ± 0.50[4]	<0.0001
hs-CRP,[2] mg/L	2.82 ± 2.82	2.48 ± 2.40	2.15 ± 2.03	2.56 ± 2.53	2.53 ± 2.38	0.82
Adiponectin,[2] μg/L	7.01 ± 3.14	7.07 ± 2.91	7.05 ± 3.00	6.95 ± 2.89	6.86 ± 2.83	0.14
SBP, mm Hg	109.9 ± 13.4	109.0 ± 12.4	111.4 ± 12.9	109.9 ± 13.0	109.8 ± 12.6	0.20
DBP, mm Hg	70.0 ± 10.4	68.9 ± 9.8	68.7 ± 9.8	68.8 ± 11.3	69.9 ± 10.0	0.46
Fasting glucose, mmol/L	4.99 ± 0.57	4.96 ± 0.54	4.97 ± 0.51	4.99 ± 0.55	4.94 ± 0.55	0.85
Fasting insulin,[2] pmol/L	118 ± 70	118 ± 60	120 ± 81	118 ± 64	115 ± 55	0.83
HOMA-IR[2]	3.85 ± 2.57	3.78 ± 2.03	3.89 ± 2.88	3.82 ± 2.38	3.65 ± 1.88	0.82

[1] All values are means ± SDs. For all variables (except body fat and hs-CRP): cheese and butter, n = 77; MUFAs, n = 74; PUFAs, n = 76; and CHOs, n = 72; for body fat: cheese, n = 73; butter, n = 74; MUFAs, n = 71; PUFAs, n = 68; CHOs, n = 67; and for hs-CRP: cheese, n = 71; butter and PUFAs, n = 68; MUFAs, n = 66; and CHO, n = 64. P values were for the main treatment effects in mixed models. Pairwise comparisons of treatments were examined only when the P value of the main treatment effect was <0.05. Covariates (baseline values of the selected variable, sex, age, BMI, center, sequence, waist:hip ratio or waist circumference, and ethnicity) were included in the mixed models only when they were shown to be significant at P < 0.05. CHO, MUFA, and PUFA diets were not compared specifically because they were not part of the primary objectives of the study. apo B, apolipoprotein B; CHO, carbohydrate; DBP, diastolic blood pressure; hs-CRP, high-sensitivity C-reactive protein; SBP, systolic blood pressure; TG, triglyceride.
[2] Analyses were performed on log-transformed data.
[3] Significantly different from cheese, P < 0.05.
[4] Significantly different from butter, P < 0.05.

3 of the 5 studies provided all foods to the participants (9–11). Results, which were very consistent between studies, have been summarized in a recent meta-analysis (7). For a similar ratio of PUFAs to SFAs, the consumption of cheese compared with that of butter significantly reduced HDL cholesterol by 0.05 mmol/L (95% CI: 0.02, 0.09 mmol/L) and LDL-cholesterol concentrations by 0.22 mmol/L (95% CI: 0.14, 0.29 mmol/L) (7). In our study, the consumption of SFAs from cheese and butter led to similar HDL-cholesterol concentrations, which were higher than those after consumption of the low-fat, high-carbohydrate diet, but were similar to the values after consumption of the MUFA and PUFA diets. In contrast, after consumption of the cheese diet, serum HDL-cholesterol concentrations were lower than those that were predicted on the basis of established predictive equations (21), which was a result that was consistent with a small food-matrix effect that modulated the impact of SFAs on

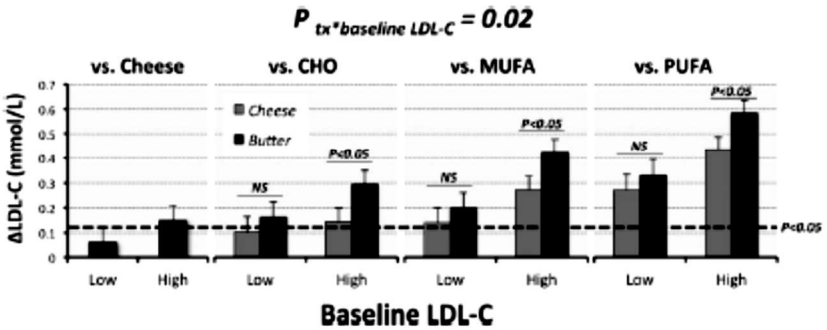

FIGURE 2 Interaction between baseline LDL-C concentrations and diet-induced changes in LDL-C. Values are presented as means ± SEMs. Subjects were classified as having relatively high or low LDL-C at baseline with the use of the median (3.1 mmol/L) LDL-C concentrations in all subjects at screening. The dotted line identifies the value above which the change in LDL-C concentrations with SFAs from cheese or butter was significant compared with that of other nutrients as determined with the use of mixed models. The tx*baseline LDL-C interaction was significant whether LDL-C was analyzed as a continuous variable or as a categorical variable (high compared with low). CHO, carbohydrates; LDL-C, LDL cholesterol; tx*baseline, treatment × baseline.

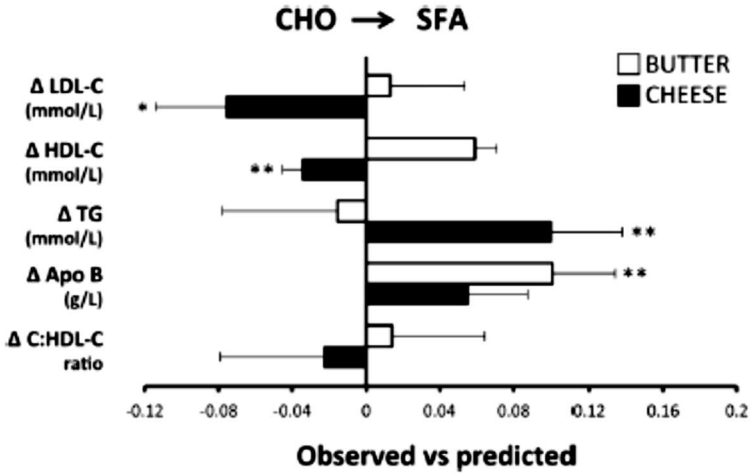

FIGURE 3 Mean ± SEM predicted compared with observed changes in blood lipids with SFAs from butter ($n = 66$) and cheese ($n = 70$) as determined on the basis of the equations of Mensink et al. (21). A paired t test was used to determine P values for the difference in observed compared with predicted changes in blood lipids when SFAs from cheese (6.8% of energy) or from butter (6.6% of energy) replaced CHO. *,**For the difference between observed and predicted changes in blood lipids: *$P < 0.05$, **$P < 0.01$. Apo B, apolipoprotein B; C, cholesterol; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; TG, triglycerides.

HDL cholesterol. In previous RCTs, Tholstrup et al. (8) reported the smallest difference in HDL cholesterol between butter and cheese (mean difference: 0.03 mmol/L; NS), and the study participants had the lowest baseline HDL-cholesterol concentrations (mean: 1.23 mmol/L). In our study, participants also had relatively low HDL cholesterol as per our recruitment criteria (HDL cholesterol below age- and sex-specific 75th percentiles), which suggested that the food-matrix effects that mediate the impact of SFAs on HDL cholesterol may be attenuated when baseline HDL cholesterol is lower. It has become clear that HDL-cholesterol concentrations may poorly reflect the antiatherogenic properties of HDL particles (22). More studies are needed to investigate whether SFAs from different sources have an effect on the cholesterol efflux capacity as well as on HDL anti-inflammatory and antioxidant properties.

In our study, the mean reduction in LDL cholesterol after consumption of the cheese diet compared with after consumption of the butter diet was 0.11 mmol/L ($P < 0.05$), which was one-half of what was observed (−0.22 mmol/L; 95% CI: −0.29, −0.14 mmol/L) in the meta-analysis by de Goede et al. (7). The food-matrix effect in our study was further evidenced by the fact that the substitution of SFAs from cheese by carbohydrates led to lower LDL cholesterol than was predicted on the basis of the established predictive equations (21). This outcome was not the case for SFAs from butter. Our data also suggest that the LDL-cholesterol–raising effect of SFAs from butter compared with that of SFAs from cheese is amplified in individuals with higher LDL-cholesterol concentrations. The extent to which baseline LDL cholesterol modifies the response to dietary changes has been documented in the past, and our results further support this notion (23). Some of the variability in the LDL-cholesterol response to dietary SFAs has been attributed to interindividual differences in the rate of catabolism of LDL particles. Individuals in whom LDL-cholesterol concentrations are high because of a reduced fractional catabolic rate of LDL particles may be particularly sensitive to changes in dietary SFAs, which are known to downregulate the LDL-receptor activity (24). Single nucleotide polymorphisms in the apolipoprotein E gene and other genes that are involved in cholesterol metabolism may also influence the LDL-cholesterol response to changes in dairy SFAs (25), but it is unclear how different sources of SFAs may modify these effects. Finally, it has been suggested that the effects of dietary SFAs and cholesterol on the lipid profile may be attenuated in obese and insulin-resistant individuals and those with metabolic syndrome (26, 27). In our study, interindividual variations in body weight, waist circumference, or the HOMA index did not modify the impact of SFAs (irrespective of source) on LDL cholesterol compared with that of the substitute nutrients.

Very few studies have compared the impact of cheese and butter on nonlipid risk factors. The current study revealed no differences in nonlipid cardiometabolic risk factors between the cheese and butter diets. Thus, the food-matrix effects appeared to be very specific to cholesterol metabolism. It has been shown that the calcium content in cheese may alter the whole-body cholesterol pool by reducing lipid absorption in the intestine, thereby enhancing the excretion of SFAs and cholesterol through feces and by suppressing endogenous cholesterol synthesis in the liver (28–30). The difference in calcium intake between butter and cheese diets (~400 mg/2500 kcal) was less than one-half that in previous RCTs (8–12), but this reduction may have been enough to induce different LDL cholesterol responses to the cheese and butter. The phospholipids that are present in milk-fat globule membranes of all dairies except butter as well as the bacterial content of cheese have also been evoked as potential mechanisms that may underlie the differential effects of SFAs from cheese and butter on LDL-cholesterol concentrations (31–33). Finally, the extent to which differences in the relative contents of specific SFAs in cheese and butter are responsible for the food-matrix effect is unclear (7).

As expected, the replacement of SFAs from either cheese or butter by MUFAs and PUFAs reduced serum LDL-cholesterol and apo B concentrations (21, 34). In contrast, SFAs, irrespective of the dietary source, had no effects on HDL cholesterol,

inflammation markers, indexes of insulin-glucose homeostasis, or blood pressure compared with the effect of MUFAs and PUFAs. In general, cheese consumption has shown no association with risk of CAD or hypertension and may even be associated with reduced risks of stroke and type 2 diabetes (35). Recent data from a systematic review and meta-analysis also suggested that butter shows relatively small or neutral overall associations with mortality, CVD, and diabetes (36). The reconciliation of data from RCTs and observational studies is challenging. Further studies are needed to explore how the neutral effects of SFAs from cheese and butter on several cardiometabolic risk factors, compared with the effects of MUFAs and PUFAs, possibly abrogate their LDL-cholesterol raising effects on CAD-related outcomes.

This study has several strengths. First, to our knowledge, it is the largest trial thus far to have compared SFAs from butter and cheese. The large sample size combined with the controlled-consumption conditions and crossover nature of the study provided statistical power to detect very small effects in treatments. To our knowledge, this is the first study to compare effects of SFAs from both cheese and butter with those of carbohydrate-, MUFA-, and PUFA-rich diets, simultaneously. Weaknesses include a limited capacity to assess true compliance, which was based on self-reporting. The high dropout rate is also a limitation, although it was not entirely unexpected considering the duration and the commitment that were needed to complete the protocol. However, mixed models are robust when data are missing at random, which was very probable in this study. Sensitivity analyses with the use of data from the center with the lowest dropout rate yielded similar results (data not shown). Further ITT analyses also supported the notion that there is a food-matrix effect that modulates the impact of SFAs on LDL-cholesterol concentrations as well as no effect of SFAs, irrespective of the food source, on nonlipid cardiometabolic risk factors (Supplemental Table 1).

In conclusion, data from this large, carefully controlled RCT suggest that there is a significant food-matrix effect that modulates the impact of SFAs on blood lipids. This food-matrix effect on LDL cholesterol may be exacerbated in individuals with high baseline LDL cholesterol and appears to be independent of whether SFAs from both cheese and butter are substituted for carbohydrates, MUFAs, or PUFAs. These findings reinforce the importance to consider whole foods and food sources as opposed to single nutrients when assessing the impact of diet on health. Finally, data indicate that SFAs from cheese have no significant effect on several nonlipid cardiometabolic risk factors, which may partly explain why cheese intake has not been associated with increased risk of CAD in observational studies (35).

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