MEDITERRANEAN LIFESTYLE, GUT MICROBIOTA, AND CARDIOVASCULAR RISK: MATCH MADE IN HEAVEN

Jananee Muralidharan

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JANANEE MURALIDHARAN

DOCTORAL THESIS

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Mediterranean Lifestyle, Gut Microbiota and Cardiovascular Risk: Match Made in Heaven

DOCTORAL THESIS

Thesis supervised by

Dr. Monica Bulló

&

Prof. Jordi Salas-Salvadó

Human Nutrition Unit
Department of Biochemistry and Biotechnology
Rovira i Virgili University
Reus, Tarragona
2021

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UNIVERSITAT ROVIRA i VIRGILI

Monica Bulló, Associate Professor at the Department of Biochemistry and Biotechnology of Universitat Rovira i Virgili

I STATE

That the present study, entitled "*Mediterranean lifestyle, gut microbiota, and cardiovascular risk: Match made in heaven*", presented by Jananee Muralidharan for the award of the degree of Doctor, has been carried out under my supervision at the Department of Biochemistry and Biotechnology of this University and it satisfies the requirements for an International Mention.

Reus, 27 April 2021

Doctoral Thesis Supervisor/s

Monica Bulló, PhD

Nutrition and Metabolic Disorders Research Group
Nutrition and Mental Health-SGR
Universitat Rovira i Virgili

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Dr. Jordi Salas-Salvadó

Human Nutrition Unit
Department of Biochemistry and Biotechnology
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கற்றது கைமண் அளவு, கல்லாதது

உலகளவு

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Abstract

ENGLISH

Obesity and metabolic syndrome are major public health issues increasing worldwide. Gut microbiota has established to play an important role in obesity, host energy metabolism and understanding its role in the context of health is essential.

As the primary objective of this thesis, we evaluated the effect of 1-year intensive weight-loss intervention in the context of PREDIMED-Plus study on gut microbiota composition. We observed that weight loss mediated by the intervention induces changes in gut microbiota and some of these microbial genera were associated with changes in adiposity parameters.

Secondly, we explored the differences in microbial composition with respect to various sources of protein intake, we observed that consuming animal-based proteins may have a stronger influence than plant-based proteins on gut microbiota. Finally, from the narrative review conducted on plant-based fats and gut microbiota, it can be concluded that replacement of saturated fats with plant sources of unsaturated fats could help in positive modulation of gut microbiota. We remark that there is a great need for human studies in the context of understanding the effects of different fat and protein sources on gut microbiota and consequently on health.

Overall, we conclude from this Doctoral thesis that hypocaloric Mediterranean diet, along with physical activity and behavioral changes can have beneficial effects on the host, potentially modulated via gut microbiota. Plant based protein or fat sources may have positive effects on gut microbial composition and functionality. Huge amount of challenges and opportunities awaits in front of us to better understand diet-host-microbiome interactions.

Abstract

SPANISH

La obesidad y el síndrome metabólico son importantes problemas de salud pública que están en aumento en todo el mundo. Se ha establecido que la microbiota intestinal juega un papel importante en la obesidad, el metabolismo energético del huésped y es esencial comprender su papel en el contexto de la salud.
Como objetivo principal de esta tesis, evaluamos el efecto que tuvo 1 año de intervención intensiva de pérdida de peso, en el contexto del estudio PREDIMED-Plus, en la composición de la microbiota intestinal. Observamos que la pérdida de peso mediada por la intervención induce cambios en la microbiota intestinal y que estos géneros microbianos se asociaron con cambios en los parámetros de adiposidad.

En segundo lugar, exploramos las diferencias en la composición microbiana con respecto a varias fuentes de ingesta de proteínas y observamos que el consumo de proteínas de origen animal puede tener una influencia superior que la de las proteínas de origen vegetal. Por último, a partir de la revisión descriptiva realizada sobre las grasas de origen vegetal y la microbiota intestinal, se concluye que el reemplazo de grasas saturadas por fuentes vegetales de grasas insaturadas podría ayudar en la modulación positiva de la microbiota intestinal. Remarcamos que existe una gran necesidad de estudios en humanos para comprender los efectos de diferentes fuentes de grasa y proteínas sobre la microbiota intestinal y, en consecuencia, sobre la salud.

En conclusión, una dieta mediterránea hipocalórica, junto con la actividad física y cambios comportamentales, puede tener efectos beneficiosos en el huésped, potencialmente modulados a través del microbiota intestinal. Las fuentes de proteínas o grasas vegetales pueden tener efectos positivos sobre la composición y funcionalidad microbiana intestinal. Nos esperan en el futuro una gran cantidad de desafíos y oportunidades para comprender mejor las interacciones dieta-huésped-microbioma.

Abstract

CATALAN

L'obesitat i la síndrome metabòlica són problemes importants de salut pública que estan en augment a tot el món. S'ha establert que la microbiota intestinal juga un paper important en l'obesitat, el metabolisme energètic de l'hoste i és essencial comprendre el seu paper en el context de la salut.

Com a objectiu principal d'aquesta tesi, avaluem l'efecte que va tenir 1 any d'intervenció intensiva de pèrdua de pes, en el context de l'estudi PREDIMED-Plus, en la composició de la microbiota intestinal. Vàrem observar que la pèrdua de pes mediada per la intervenció indueix canvis en la microbiota intestinal i alguns d'aquests gèneres microbians es van associar amb canvis en els paràmetres d'adipositat.

En segon lloc, vàrem explorar les diferències en la composició microbiana pel que fa a diverses fonts d'ingesta de proteïnes, i vàrem observar que el consum de proteïnes d'origen animal pot tenir una influència superior a la de les proteïnes d'origen vegetal. Finalment, a partir de la revisió narrativa realitzada sobre els greixos d'origen vegetal i la microbiota intestinal, es conclou que el reemplaçament de greixos saturats per fonts vegetals de greixos insaturats podria ajudar en la modulació positiva de la microbiota intestinal. Remarquem que es necessiten més estudis en humans per comprendre els efectes de diferents fonts de greixos i proteïnes sobre la microbiota intestinal i, en conseqüència, sobre la salut.

En conclusió, la dieta mediterrània hipocalòrica, juntament amb l'activitat física i canvis de comportament, poden tenir efectes beneficiosos en l'hoste, potencialment modulats a través de la microbiota intestinal. Les fonts de proteïnes o greixos vegetals poden tenir efectes positius sobre la composició i funcionalitat microbiana intestinal. Ens esperen en el futur una gran quantitat de desafiaments i oportunitats per a comprendre millor les interaccions dieta-hoste-microbioma.

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Abbreviations

SCFA	Short chain fatty acids
BCFA	Branched chain fatty acids
HMP	Human Microbiome Project
T2D	Type 2 Diabetes
TMAO	Trimethylamine-N-oxide
LPS	Lipopolysaccharides
CVD	Cardiovascular disease
MetS	Metabolic syndrome
IR	Insulin resistance
FFA	Free fatty acids
HDL	High density lipoproteins
VLDL	Very low-density lipoproteins
F/B	Firmicutes to Bacteroidetes ratio
BA	Bile acids
FXR	Farsenoid X receptor
TGR5	G-protein coupled receptor membrane type receptor
PUFA	Poly unsaturated fatty acids
GI	Gastro-intestine
GUDCA	glycoursodeoxycholic acid
BCAA	Branched chain amino acid
HFD	High fat diets

GLP-1	Glucagon like peptide-1
PYY	Peptide YY
GIP	Glucose dependent insulinotropoc polypeptide
HPD	High protein diets
NPD	Normal protein diets
SFA	Saturated fatty acids
MUFA	Monounsaturated fatty acids
TLR	Toll like receptors
COMIT	**C**anola multicenter intervention trial

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I. INTRODUCTION

Introduction

1. Intestinal microbiota: General overview

Development of sequencing technology from Sangers to 16S rRNA and shotgun metagenome has opened the door to identifying astounding variety of microorganism that cohabit with humans. Microbes (bacteria, fungi, archaea, and viruses) inhabit within and outside the human body at various sites such as the skin, nasal, oral and intestinal tracts. Together they play an integral part in host nutrient metabolism, immunomodulation, protection against pathogens amongst several other roles (1). In this thesis, we focus on the bacterial ecosystem present in the large intestine henceforth mentioned as "gut microbiota" or "intestinal microbiota" alternatively. Further on, we refer to "microbiome" in cases when referring to microbes and their genomes, whereas "microbiota" while referring to only the microbes itself.

In simple terms, large intestine can be considered as a chemostat where several microbial metabolic processes occur simultaneously giving out metabolites that are absorbed in the bloodstream which travel to various organs and begin an array of physiological reactions. The gut utilizes the undigested food components arriving to intestine as a substrate for their energy. Microbial metabolism yields in metabolites such as **short chain fatty acids** (SCFA), branched chain fatty acids (BCFA), amino acids, vitamins, gasses such as hydrogen, ammonia etc. Some of these fermentation products are used as substrate by other intestinal microbiota which is referred to as cross-feeding and some microbial genera are strongly dependent on cross-feeding for their survival. For example, *Faecalibacterium prausnitzii* would not grow in culture medium without the presence of acetate (2).

Large amount of research has indicated that several human disease conditions are associated with "dysbiosis" of intestinal microbiome. **Dysbiosis** is broadly defined as an imbalance or change in commensal microbial constituents of the intestine relative to the microbial constituents from a healthy intestine (3). Dysbiosis remains as a simple and central concept in understanding health and disease associations with gut microbiota. Even though widely used, it remains unclear as to what marks dysbiosis. Two major concerns with this definition are: 1) despite several landmark

studies we still do not have a complete comprehension of what constitutes to a healthy microbiota 2) dysbiosis necessarily does not talk about the functional abilities of different species at different conditions (4).

In order to identify what marks a healthy intestinal community, the Human Microbiome Project (HMP) accumulated over 42 terabytes of information cataloging genes and microbes associated to the core microbiome of human (5). Despite the remarkable amount of data collected, HMP was not able to conclude on what constitutes a healthy microbiome (6). While we are still unclear of what constitutes to a healthy intestinal microbiota, there are indications on what could potentially be favorable and unfavorable for the host. For example, with obesity, the diversity of microbial taxa in the intestine decrease and with weight loss, this is partially recovered (7). Conditions such as type 2 diabetes (T2D), colon cancer, inflammatory bowel disease, neurological diseases have also shown to be associated with disturbed gut eco system. Additionally, metabolites of gut microbial metabolism such trimethylamine-N-oxide (TMAO) or lipopolysaccharides (LPS) have been linked to an increased risk of atherosclerosis and inflammation respectively (8).

Being recognized as a key driver in host metabolism, gut microbiota provides a great opportunity to explore, manipulate and utilize it for the benefit of the host. As with solving any problem, understanding the causation and the role of mitigators behind the problem is essential. In the next section, we will go deeper into understanding the global epidemic, obesity and how gut microbiota plays a role in modulating obesity and associated metabolic processes.

2. Obesity and metabolic syndrome: A gut microbiota perspective (Can we blame our microbes?)

2.1 Obesity and metabolic syndrome: Need of the hour

Increasing rates of obesity all over the world are a cause of concern as it reduces the quality of life and decreases the life expectancy by 3.3-18.7 years (9). By current trends, global obesity prevalence is estimated to reach 20% of worlds adult population in 2030 (10). Obesity and associated health issues impose a large health care expenditure and a burden to the society. Excess weight gain and accumulation of fat causes an increased risk of several diseases such as cardiovascular disease (CVD), T2D and cancer. Obesity often lead to development of Metabolic syndrome (MetS) (11). The American Heart Association/ National Heart, Lund and Blood Institute defines MetS as the presence of at least 3 of the 5 factors listed below in the Figure 1 (12). Amongst these five factors, **abdominal obesity** and **insulin resistance** (IR) play a key role in the MetS pathophysiology. Expansion of fat mass, especially in the abdominal region, is associated with release of free fatty acids (FFA) (13). Circulating FFA in elevated levels, contribute directly to IR. Once IR starts to develop, it suppresses lipolysis via insulin leading to increased circulating FFA and ultimately the beginning of a vicious circle (13). Increased adiposity and IR can lead to impaired lipid metabolism via modifying lipoprotein lipase activity, resulting in **dyslipidemia** (14). Dyslipidemia is characterized by low circulating concentrations of high-density lipoprotein (HDL), high concentrations of triglycerides and very low-density lipoproteins (VLDL) and chylomicrons (14).

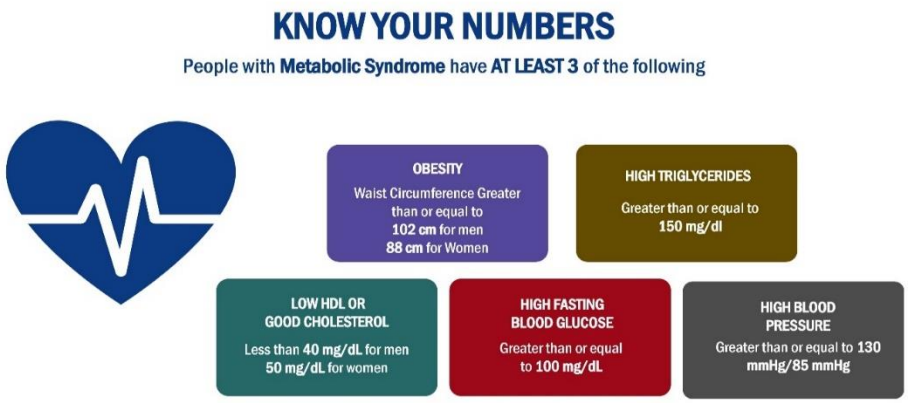

Figure 1: Criteria for identifying Metabolic syndrome (12)

Insulin is an important hormone involved in metabolism, synthesis and storage of carbohydrate, fat and proteins. Alterations in insulin action leads to deleterious metabolic consequences. IR is clinically described as the inability of normal concentrations of insulin to increase glucose uptake and utilization in the peripheral tissues (15). Another task of insulin during normal conditions is the stimulation of nitric oxide production from the endothelial cells (16). The nitric oxide produced are involved in vasorelaxation and increase capillarity of skeletal muscle which plays a role in maintaining blood pressure (17). In individuals with IR, the production of nitric oxide is impaired, which might enhance vasoconstriction, thus leading to **hypertension**. Overall, it can be summarized that central adiposity, IR, and development of other MetS components (such as hypertension and atherogenic dyslipidemia) could lead to myriad of metabolic disturbances, increasing CVD risks and CVD associated mortality (18).

Avoiding the development of these metabolic disorders and managing them at earlier stages could provide better quality of life. Obesity is a multifactorial disease with various causal factors such as genetic predisposition, behavioral, socioeconomic, environmental and lifestyle choices (19). Lifestyle choices including high caloric diet, sedentary lifestyle, smoking habits are considered as important

contributors towards development and worsening of obesity. Recent years of research have shown another contributor to obesity and associated metabolic disorders: the INTESTINAL MICROBIOTA.

2.2 Gut microbial composition in obesity

Landmark study by Backhead et al., established the involvement of intestinal microbiota as an environmental factor that regulates fat storage in the body. This study observed a significantly greater body fat accumulated in conventionally raised mice compared to the germ-free mice (20). Consecutively, Turnbaugh et al., found a significantly greater increase in body fat of germ-free mice when intestinal microbiota was transplanted from obese mice compared to the ones transplanted from lean mice (21). This study also noted a higher Firmicutes to Bacteroidetes ratio (F/B) in the obese mice compared to the lean mice.

Two most dominant phylum of the gut microbiota are Firmicutes and Bacteroidetes. Several studies have explored the ratio of these two major phyla in association with obesity. Even though many studies have explored this, the results remain inconsistent with some studies reporting a higher F/B in obese state, whereas an inverse association in others. Systematic review by Crovesy et al (22), remarked that in individuals with obesity, there is a higher F/B, Proteobacteria and lower prevalence of Bacteroidetes, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*. Firmicutes phylum contain more carbohydrate metabolizing enzymes contributing to metabolization of the carbohydrates and yield greater energy for the host (23). Systematic review by Castaner et al., reported the strong positive association between several members of Firmicutes (*Blautia hydrogenotorophica, Coprococcus catus, Eubacterium ventriosum, Ruminococcus bromii,* and *Ruminococcus obeum*) and obesity (24). It is interesting to note that some members of Firmicutes such as *Roseburia*, *Lactobacillus, Eubacterium* are known for their SCFA producing properties inducing beneficial effects to the host (25,26). An important family of Firmicutes, Lachnospiraceae including many SCFA producing members, is also recognized for its controversial role in human health (27). Members of Lachnospiraceae such as *Tyzzerella*, *Tyzzerella* 4, and *Coprococcus* 2

were observed to be enriched in high CVD individuals in the Bogalusa study (28). In the same study, some members of Bacteroidetes phylum (belonging to Prevotellaceae family) were associated with higher (*Prevotella* 2, *Prevotella* 7) and lower (*Alloprevotella*) CVD risk (28). Proteobacteria is another important phylum that has been positively associated with obesity. Increase in Proteobacteria abundance has also been associated with damage of intestinal barrier, thus contributing to low-grade inflammation (29). *Fusobacterium*, a member of this phylum is an opportunistic pathogen that has also noted to be increased in obese individuals (30,31). Differences in action of species or strains even within the same genera has also been reported. Increased abundance of *Lactobacillus* for example have been associated with obesity, whereas some specific species of this genera such as *Lactobacillus paracasei* and *Lactobacillus plantarum* have shown anti obese properties (32).

Even though many studies indicate an association between specific phylum or family with obesity, it is not possible to generalize the associations within all genera or species of the phylum or family. Thus, it is also important to evaluate the genus level or species level associations in order to achieve a better insight. Estimating the functional capabilities within the family or genera could also allow in understanding the potential conflicting results.

2.3 Gut Microbiota in obesity and metabolic syndrome: Mechanisms

Microbial LPS from gram-negative bacteria is a recognized player in microbiota mediated chronic low-grade inflammation. LPS also referred to as endotoxins consist of a lipid and a polysaccharide composed of O-antigen. LPS acts on the innate immune system and promotes release of inflammatory markers via toll like receptors (33). One of the initial studies in this context, have shown that LPS purified from *E. coli* can trigger obesity and IR in mice (34). In animals, two-to-three-fold increase in LPS has been shown after high fat diet (HFD) consumption in comparison to low-fat, low-carbohydrate or high carbohydrate diet (35). Higher peripheral concentration of LPS has been observed in individuals with MetS (36) and HFD

induced obesity (37); this trigger of inflammation by LPS is known as "**metabolic endotoxemia**". A large cohort study (n= 7169) by Pussinen et al., demonstrated the relationship between metabolic endotoxemia and T2D. In that study, circulating LPS levels were positively associated with an increased risk of T2D and negatively associated with HDL levels (38). Interestingly, HDL has shown to have an important role in regulating the effect of LPS on health. For individuals with low HDL, a higher inflammatory release of cytokines were noted (39), whereas with infusion of HDL, these effects were reduced (40).

The type and quantity of microbial metabolites that enters the host circulation system determines the changes in several processes such as energy homeostasis, lipogenesis, gluconeogenesis etc. Hence it is important to note the type of microbial metabolization and consequent metabolite formation in the intestine. The intestinal mucosal layer serves as a gatekeeper that allows a balanced flow of molecules from the lumen to the circulation. With improper functioning of intestinal barrier, generally referred to as "leaky gut", there is excess translocation of molecules such as LPS, that could induce inflammation in the host (41).

While certain composition of microbiota can cause dysbiosis and low-grade inflammation, on the other hand, the presence or growth of healthy bacterial ecosystem benefits the host. Butyrate, is a notable microbial metabolite, that serves as a source of energy for the colonocytes and have been noted to regulate gene expression in colon cancer cells by inhibiting histone deacetylases (42). Butyrate has also shown to impart beneficial effects on appetite control, host glucose, dyslipidemia and energy homeostasis (43). Circulating butyrate levels have also shown to be inversely associated to BMI, fasting glucose and FFAs (44). Indeed, few studies have attempted to supplement with oral butyrate to observe its anti-inflammatory and metabolic benefits in animals and humans (45,46). Beside butyrate, propionate and acetate have also shown some positive effects on markers of metabolic disease, however these results remain inconsistent (47,48). While higher circulating butyrate or SCFAs levels could induce beneficial effects, it has been questioned if this higher circulation levels could also indicate the presence of a leaky gut where there is a higher translocation of these molecules via the loose

intestinal barrier (49). This association also extends to bacterial genera, which produce these SCFAs. Commonly the genera producing the SCFAs are regarded as beneficial, however during obese state, this could become a double-edged sword as the production of SCFAs is a source of energy that in turn increase the energy harvest of the host (21,50).

Bile acids (BA) are other important class of metabolites that are modulated and metabolized by gut microbiota. BA are important signaling molecules that can act on lipid, glucose, energy metabolism. Two important receptors of BA are farnesoid X receptor (FXR) and G-protein coupled receptor membrane type receptor (TGR5). Mice studies have shown that BA involve in glucose homeostasis via activation of FXR (51,52). Some BA (cholic, deoxycholic acid) have also shown to have anti-microbial properties, thus having the ability to disturb the gut ecosystem (53). Almost 95% of the BA that are synthesized by liver (primary BA) are reabsorbed in the distal small intestine and reach the liver for enterohepatic circulation. Parts of BA, which enters the large intestine undergoes microbial deconjugation which results in secondary BA. Prior studies indicate the maintenance of BA pool (balance of primary to secondary BA ratio, levels of glycine to taurine conjugated BA ratio) in the circulation is important for proper metabolic functioning (54,55). Certain groups of gut microbiota (*Bilophila*, members of Clostridium, *Lachnoclostridium, Lactobacillus*) have reported to be involved in both increasing secondary BA as well shifting to a taurine-conjugated BA pool (56). Interestingly the proportion of BA that enter the intestine is also dependent on diet, which in case of Western diet has shown higher efflux of BA to the intestine (57). For example, in an animal study feeding, HFD with milk or polyunsaturated fatty acids (PUFA) increased the *Bilophila*, a bile loving bacterium abundance compared to that of normal chow fed diet, whereas supplementation of omega-3 fish oil suppressed the growth of *Bilophila* (58). Modulating the bile acid pool by remodeling the BA producing members of the intestine has also shown to induce anti-obesogenic effects (59). Thus, findings indicate an interplay amongst BA, diet, gut microbiota and host metabolism that may contribute to metabolic changes.

Figure 2 below summarizes the various potential pathways discussed in the above section on gut microbiota modulation in the development of obesity (adapted from (60). Overall, from the above-mentioned mechanisms and many more unexplored pathways it can be agreed that gut microbiota do play a major role in modulating obesity and MetS.

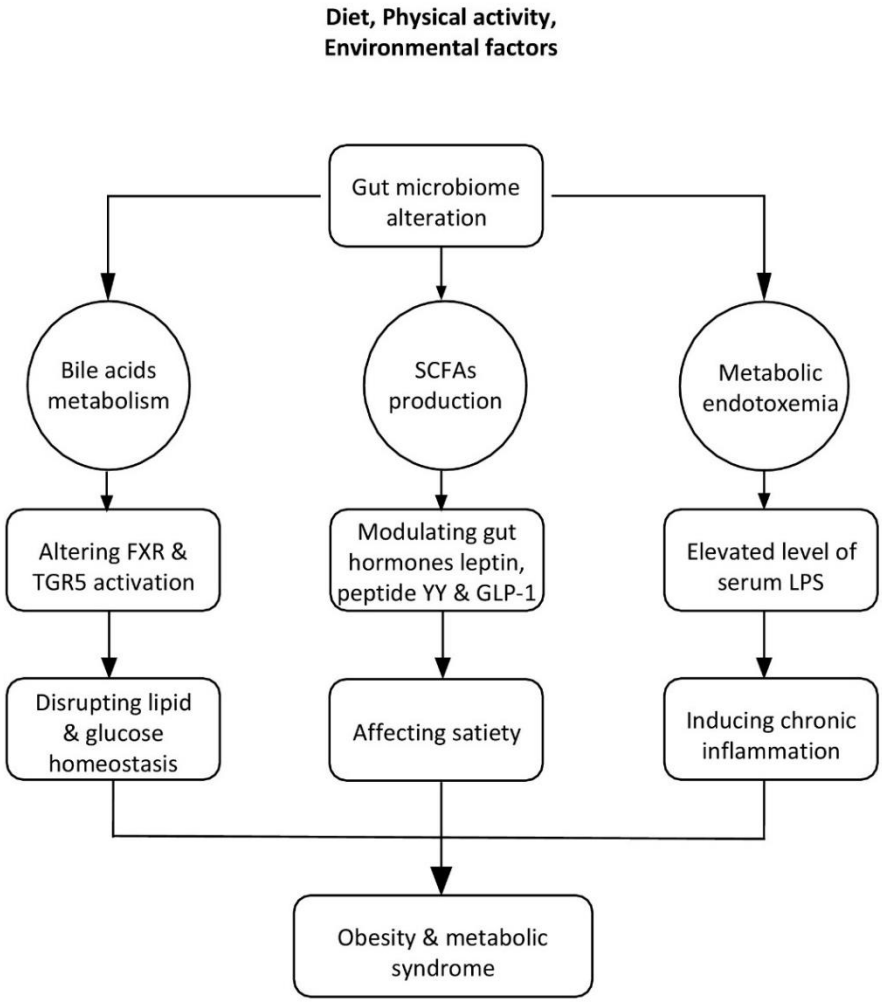

Figure 2: Overview of Diet-Gut-Metabolism interaction. Three important pathways in which gut microbiota modulates obesity and metabolic syndrome (adapted from (60))

3. Factors affecting intestinal microbiota: The Good, Bad and the Evil

The relationship between host and the microbes are bidirectional, complex and are driven by several factors both of modifiable and non-modifiable nature. Even though the direction of changes in the gut microbiota in disease conditions is not conclusive, it is certainly interesting to explore the possibility of altering the gut microbiota for a favorable health condition.

Colonization of gut microbiota begins as early as in infancy. While many factors influencing microbiome in adult life are modifiable, the infant gut microbiome is mostly influenced by maternal factors, which can be considered non-modifiable factors such as maternal health, mode of delivery, breastfeeding, antibiotics and early environmental exposures. Research has shown that pre-pregnancy overweight/obesity and excessive gestational weight gain can influence the maternal gut microbiota at time of delivery as well of their infants gut microbiota at early life (61). Possibility of early intestinal colonization influencing the shaping of gut microbiome post birth emphasizes the importance of understanding these factors. Even though infant microbiome and the factors that influence them are not in the scope of this thesis, we briefly go over these factors, in order to appreciate the complexity of intestinal ecosystem that co-exist with us.

3.1 Non-modifiable factors

3.1.1 Early life factors

3.1.1.1 Mode of delivery

Pioneering studies have shown that mode of delivery significantly changes the composition and richness of infant microbiome (62). Colonization of microbes at the initial months after birth gives an important window for their early development, and this could consequently influence disease susceptibility in long-term health (63). A systematic review evaluating the effects of birth mode on compositional differences of gut microbiota has shown that cesarean section is associated with lower abundance of Phyla Actinobacteria, Bacteroidetes up to 3 months of age.

Whereas for vaginally born infants, colonization of *Bacteroides* and *Bifidobacterium* were dominant (62). A recent longitudinal study reestablished these results of less abundance of *Bacteroides* and *Bifidobacterium*, along with higher abundance of potentially pathogenic *C.neonates, C.perfrigens* in cesarean born infants at 3 months of age (64). Members of *Bifidobacterium* have received attention due to its capability to metabolize human milk oligosaccharides and in turn reducing substrate for potential pathogens. Additionally, *Bifidobacterial* species host folate-synthesizing genes that are key for infant nutrition (65).

3.1.1.2 Feeding mode

One of the biggest sources of bacterial species to the infants is via breast milk. Breast milk not only also harbors several hundred bacterial species but also contains bioactive compounds such as immunoglobulins, cytokines, and growth factors (66). Breastfeeding unarguably is a promoted mode of feeding infants at least until 6 months of age and recommended until 24 months. Breastfeeding provides the infant with clean, safe food and confers protection against gastrointestinal (GI) infections, inflammatory diseases, such as atopy, asthma, obesity (67,68). Key bacterial genera such as *Bifidobacterium*, *Lactobacillus*, *Enterococcus* and *Staphylococcus* have been documented to transfer from breast milk to infant gut (69). Bacteria from breast milk play several important roles such as in the development of immunity, reduction of infections compared to bacteria developed from the consumption of formula milk fed infants (70). For example, Lactobacilli strains in breast milk have the ability to increase mucin expression in enterocytes of intestine that aids in better intestinal barrier formation (71). In addition, human oligosaccharide in breast milk, as mentioned in previous section, promotes growth of beneficial bacteria and aid in outcompeting potentially pathogenic organisms.

3.1.1.3 Antibiotics usage

Antibiotics at infancy and adulthood have important effects on intestinal microbiome. Depending on the dosage, duration and type of antibiotic use, the alterations in intestinal microbiome varies. In general, most antibiotics are administered to target particular bacterial infection, however unintentionally this could end up eliminating a broad range of gut inhabitants that might not necessarily

be pathogenic. This results in one of the most common observed effect of antibiotics as reduction in diversity. Consequently, the imbalance in ecosystem could increase the growth of pathogens leading to other infections and inflammation. Russel et al., (72) showed in mice, that vancomycin treatment led to elevated serum IgE and regulatory T cell populations. Another mice study with gestational and early life antibiotic exposure showed an earlier onset of inflammatory bowel disease because of enhanced CD4+T cells function (73).

Additional to the immunomodulating effects, early antibiotics exposure has also been associated to an increased risk of weight gain during childhood and later stages of life (74). Observational studies reporting treatment of broad and narrow spectrum antibiotics suggests that participants with antibiotic treatment are more prone to weight gain (75). Interestingly in adults, the microbiome modulating effects of antibiotics have been explored for regulating IR. Studies in animals and humans have shown the ability of antibiotics to potentially alter the glucose homeostasis via bile conjugating members of intestine (predominantly gram-positive members of Clostridium) (76). However, there are other studies suggesting no alteration in IR after antibiotic treatment (77,78). Irrespectively, it is clear that antibiotics are one of the key modulating factors for gut microbiome. This is one of the considerations in the design of studies: to exclude participants with any antibiotic treatment prior to at least 30 days before fecal sample collection in order to reduce the confounding factors.

3.1.2 Intrinsic factors

3.1.2.1 Host genetics

Compared to studies assessing environmental factors, studies understanding the relation between host genetics and gut microbiota are scarce. With the limited amount of research, it has been implied that host genetics do play a modest role in shaping gut microbiota and few members of the gut microbiota are heritable. Twin studies from Goodrich et al (79), has shown that bacterial the phyla Firmicutes, Actinobacteria, genus *Tenericutes*, genus *Turibacter* are more heritable, whereas Bacteroidetes phylum is least heritable. Also this study indicated that *Bifidobacterium* and LCT gene locus which encodes for lactase enzyme that

hydrolyses lactose, are closely associated (80,81). Results from Wang et al., analyzing gene-microbiome interaction in two large German cohorts (n=2183) showed that bacterial beta diversity is linked to 42 loci, including genes related to metabolism (such as VDR, POMC, GRID1) and genes related to immunity (CLEC16A, ILI1R2, MAP4K4) (82). Even though genes have modest interactions with intestinal microbiota, it might be interesting to explore these interactions in the context of autoimmune diseases with high genetic influences.

3.1.2.2 Sex

Differences in risk factors to develop cardiovascular, autoimmune, neurological diseases exist among different sexes (83,84). Previous research in humans have shown that gut microbiota compositions vary between male and female, being sex hormones associated with some of the genera (85). Mouse model study has demonstrated that with puberty, gut microbiota composition of male and female mice started to distinguish significantly in families such as Porphyromonadaceae, Veillonellaceae dominating in males. Interestingly this study also showed that in male, gut microbiome was able to modulate androgen concentrations. When conjugated androgen and estrogen escape the hepatic circulation, they reach large intestine and are broken down by gut bacterial Beta-glucuronidase (genes mainly encoded by Firmicutes), giving rise to free androgen/estrogen available for absorption (86). Given the fact that androgen has an impact on proliferation of adipose tissue, it would be interesting to understand if sex differences in gut microbial composition may play a role in regulating fat deposits via sex hormones. Report from the CORDIOPREV study has demonstrated that gut microbiota varies in a sex dependent manner while having the same BMI. This study reported that F/B was significantly higher amongst men at BMI <30 Kg/m^2, whereas became significantly lower than women at BMI >33 Kg/m^2 (87). Thus, it could be hypothesized that regional adiposity might be affected by sexual dimorphism of the detected microbial taxa in men and women.

3.1.2.3 Age

Shift in microbiota through age can be broadly classified into infancy, adults and elderly. Gut microbiota composition through adulthood is relatively stable and starts

to shift with ageing, usually defined at a threshold of 65-70 years. Co-evolving with us, the intestinal microbiota may contribute to progression of disease and frailty in the elderly. Not only age, but also the kind of community that the elders live in, has also shown to differ the gut microbiome, indicating an age and emotional interaction. For example, in elderly individuals living in community compared to long-term care, the Lachnospiraceae and the genes involved in producing SCFAs were enhanced (88). A wide population study elucidating the shifts in microbiota amongst adults showed that while younger adults and elderly had comparable diversity, the centenarians varied significantly from these groups. A decrease in Clostridium cluster and an increase in Proteobacteria was a characteristic of centenarian population. Centenarians also developed an inflammageing environment that supported opportunistic pathogens such as Fusobacterium, Bacillus, Staphylococcus (89).

Large trials such as ELDERMET (90) and NU-AGE (91) have tried to decipher microbiome and metabolites associated with ageing, cognitive decline and frailty. NU-AGE in particular, focused on the potential of a healthy dietary pattern such as Mediterranean diet in order to mitigate frailty via gut microbiota. This study identified group of "diet positive" (DP) and "diet negative" (DN) taxa that might have effect on frailty. DP taxa included *Faecalibacterium prausnitzii, Eubacterium* and *Roseburia*, and DN included *Ruminococcus torques*, *Collinsella aerofaciens*, *Coprococcus comes*, *Dorea formicigenerans*, *Clostridium ramosum*. While categorizing participants by their frailty level (non-frail, pre-frail, frail), a stepwise significant decrease in DP taxa as the frailty increased was reported. DN also positively associated with markers of inflammation and frailty (91).

Thus, we could infer that microbiome shifts through our biological and chronological age, potentially mitigated by factors such as environment, diet, other lifestyle factors and disease. Therefore, it is important to take into account the age when microbiota studies have to be evaluated and interpreted.

3.2 Modifiable factors

As we saw in the previous section, several intrinsic and extrinsic factors can influence microbiome. It is essential that gut microbiome stays balanced despite its exposure to several factors. Even though it is hard/impossible to modify the early life and intrinsic factors, a great amount of scope lies in ability to maintain the homeostasis via the factors that we can modify. This mainly includes diet, physical activity, smoking, environmental pollutants, alcohol intake and medications. In this section, we will go through the available literature, missing gaps and highlighting the importance of these modifiable factors that can influence gut microbiota.

3.2.1 Environmental factors

With growing pollution, humans are at constant risk of exposure to various exogenous chemical compounds, usually referred as xenobiotics. Public health concerns, especially in developing countries where there is not clear legislature on environmental guidelines, requires the scientific community to evaluate the risk of environmental exposure to health disorders. With exposure to xenobiotics, which are mostly non-polar, get absorbed in the GI tract and are metabolized by liver (92). In most cases, liver conjugates these chemicals and they are excreted via urine, with the exception of some, which are excreted by bile. The bile-associated xenobiotics reach the GI tract, which are metabolized by the bacteria. The ability to metabolize various xenobiotics lies in the presence of several enzyme families in our intestinal microbiota such as azoreductases, nitroreductases, β-glucuronidases and sulfatases (93). Results from HMP estimated human gut microbiota could harbor up to 3013 microbiome-encoded β-glucuronidases with potential to act on various glucuronide substrates (94). Kim et al., demonstrated that administration of *Lactobacillus reuteri* K8 and *Lactobacillus rhamnosus* K9 in mice modify the pharmacokinetics of acetaminophen by altering enzymes such as sulftase, arylsulfate sulfotransferase (95).

Other than the xenobiotics, geographical location is also an interesting environmental factor to be considered to modulate gut microbiota (96). Logically, it is expected that the habitat, culture, availability of food, even the presence of various xenobiotics, all dependent on the geographical location, may have an impact on

intestinal flora. "Bergmann's rule" suggests that at least to an extent, humans living at higher latitudes tend to have higher BMI, compared to lower latitudes (97). Exploratory study by Suzuki et al., conducted amongst 1020 healthy participants from 26 different populations (from 6 published studies) reported Firmicutes was highly positively associated with latitude and Bacteroides in a negative fashion (98). This is consistent with the consensus that Firmicutes contain members that are associated with energy harvest (99). Although further studies are required to validate this hypothesis, it can be speculated that patterns appear while assessing geographical location and differences in gut microbiota. One of the follow up question is which amount of distance do we start to observe these changes, is it is solely latitude dependent? With this regard, different centers in PREDIMED-Plus could potentially harvest differences in gut microbiota at baseline and could serve as an important factor to adjust.

3.2.2 Medication usage

Another important class of chemicals that reach the GI is the medications used by the host. The pharmacokinetics of the drugs consumed not only depends on the host, but also the millions of bacteria residing in the intestinal tract. While we have briefed about early life antibiotic intake in the previous section, the adult life antibiotic intake also have similar effects in depleting certain groups of bacteria and overall diversity (100). Apart from antibiotics, there are several other drugs prescribed in the adulthood and especially in the elderly to manage various conditions such as dyslipidemia, IR etc. Intake of drugs such as metformin for hyperglycemia is prevalent and several studies have established their mechanistic effects on lowering hepatic gluconeogenesis (101). In the last decade, interesting mechanism of metformin action via gut microbiota and its metabolites have identified. This drug could induce potential effects by decreasing gut *Bacteroides fragilis* and increasing glycoursodeoxycholic acid (GUDCA). These changes were accompanied with inhibition of FXR that has been reported to be involved in multiple metabolic diseases (102). Other medications such as proton-pump inhibitors, laxatives have also shown to be associated to various taxa in the gut microbiota. For example, in the TwinsUK study, positive associations between proton pump inhibitor users and

Stretococcaceae and Micrococcaceae abundances were noticed (103). Other associations in common drugs such as paracetamol or opioids were also identified with higher Streptococcaceae abundance. While drugs modulate gut microbiota composition, it is important to evaluate the resultant effects of these changes on drug response and patient outcomes. As the personalization of medication is growing, it might also be interesting in the future to explore this in a microbiota perspective.

3.2.3 Smoking habits

Smoking (tobacco) is unarguably a harmful habit and it accounts for 2363 disability adjusted life years per 100,000 people in Spain (2016) (104). One quarter of the Spanish population aged ≥ 15 years smoke (105). Worldwide tobacco smoking causes more than 7 million deaths per year. Smoking is highly positively associated to CVD risk mediated via vascular dysfunction, oxidative stress, and inflammation (106). Microbiota of oropharyngeal and tracheal sites in smokers and non-smokers differ, indicating potential alterations due to tobacco exposure. In addition, weight gain in people who quit smoking is a noticed observation (107). With gut microbiota's role in energy homeostasis, it is interesting to question if smoking could be an external factor that could affects its composition and function. Study by Biedermann et al., stated that smoking cessation induced changes in gut microbiota composition with an increase in alpha diversity, Firmicutes, Actinobacteria and a decrease in *Bacteroidetes* (108). However, the authors note that after 8 weeks of observation, the alpha diversity bounce back to baseline levels, showing no differences with non-smokers. Similarly, results from a Saudi population study reported that smokers were enriched in *Bacteroidetes* abundance (109). Whereas contradicting results were reported in a cross-sectional study, suggesting that F/B is higher in smokers compared to non-smokers, also genus *Prevotella* was significantly more abundant in smokers than non-smokers. *Bacteroides-Prevotella* ratio was also noticeably higher in smokers compared to non-smokers in patients with crohns disease. While there are studies exploring associations between smoking and microbiota composition, it is also important to account for confounding effects such as dietary intake, age, inflammatory status while assessing these effects.

3.2.4 Physical activity

Physical activity is a recommended tool to manage weight, improving cardiovascular health and ageing dysfunction (110). Compared to sedentary behaviors, moderate exercise (60-75 minutes per day) reduces the risk of mortality (111). It is startling and disappointing to mention that one-third of the world population do not achieve the recommended levels of physical activity (112). Among adults with diabetes, aerobic and resistance training has shown to reduce the peripheral levels of pro-inflammatory cytokines and increase anti-inflammatory cytokines (113). One of the interesting pathways via which physical activity imparts its benefits is via gut microbiota. Physical activity is considered as one of the important factors that can modify gut microbiota (114). The concept of muscle-microbiota axis has long been suggested by Bäckhed et al., but the evidences to support this has only been slowly developing (115). A recent landmark study identified *Vellionella atypica* species from elite athletes associating this species with performance enhancing role via its lactate metabolizing capability (116). This could potentially trigger the interest on developing probiotics adapted for this role of performance enhancement.

Past studies have displayed that periods of inactivity amongst people who exercise regularly results in changes in bowel movement, consistency of feces and gut microbiome composition (117). Cardiorespiratory fitness is also a factor that is correlated with fecal microbiota diversity in adult subjects (118). A recent systematic review summarizing the influence of human gut microbiota on healthy adults, reported that higher level of physical activity is associated with higher concentration of fecal SCFAs. In turn, acetate and butyrate (two major SCFA), enhance oxidative status of muscle fibers by increasing the muscle fat oxidation (119). Butyrate in specific also inhibits histone deacetylase, thus protecting the age related muscle mass loss (120). Formation of SCFA is dependent on the substrate type and availability along with a composition of gut microbiota that are able to utilize the substrates. Munukka et al, conducted a nutritional intake adjusted analysis and reported that 6-week of endurance intervention in women, resulted in the increase of Family Verrucomicrobiaceae and genus *Akkermansia*, whereas Proteobacteria phylum decreased (121). Consistently, in three other studies,

members of Verrucomicrobioa have reported to be higher in athletes compared to sedentary controls (122). Christensenellaceae family is another taxon that have been associated negatively with sedentary behaviors (123).

Amongst elderly, sarcopenia (i.e., loss of muscle mass and function occurring with ageing), is a key issue reducing the mobility, functions of muscles and eventually quality of life. Sarcopenia is represented by changes in anabolic-catabolic balance of muscle protein synthesis, which is in turn dependent on various mitochondrial function (124). As we have seen in previous section, ageing does strongly correlate with changes in gut microbiota, and it is tempting to hypothesize gut microbiota modulation may influence the progress of sarcopenia. Number of studies evaluating the effects of gut microbiota modulation on sarcopenia in human subjects is limited. Sarcopenia can be included in the bigger umbrella of frailty that involves failure of multiple physiological systems and presence of chronic low-grade inflammation (i.e., inflammageing, as seen in previous section of Ageing) (125,126). The NU-AGE study, including participants from five European countries, has demonstrated that frailty is associated with distinct gut microbiota composition (91). Using animal models, it has been demonstrated that distinct fecal microbiota composition along with pro-anabolic actions on the host tissue is associated with age-related muscle mass wasting (127). Dysbiotic gut might influence sarcopenia by reducing the bioavailability of dietary proteins or other nutrients. Other proposed modes of action for the muscle-microbiota axis are via activation of Toll like Receptors (TLR)-4, 5 and NF-kB, which have been associated with reduced quadriceps muscle strength in humans and muscle atrophy in animal models. With limiting evidence, it is not possible to comment on further about sarcopenia associated microbiome changes; hence future studies focusing on enhancing physical activity amongst elderly can include intestinal microbiota composition in order to understand the muscle-microbiome axis.

3.2.5 Diet

Amongst all the factors studied to date, diet is the largest known factor that has shown to influence the intestinal microbiome. The non-digested parts of human food intake reach the gut to support bacterial growth and in turn, the gut microbiota

produces various byproducts. The nature of byproduct is dependent on the substrate that is available, and this is in turn determined by structural complexity of the biomolecule, the digestive capability of the host, transit time and co-occurring nutrients. For example, transit time is a factor that could alter fecal consistency and fecal microbial composition (128). Other important factors that influence the effect of diet on gut microbiota is the host genetics as briefed in the above section. Even with presence of identical environment and diet, studies have shown that host genetics influence gut microbiota composition, however to a lesser extent (81).

Previous studies support dietary interventions can alter microbial communities in a rapid manner (129). Other reports, convey that the change obtained by interventions move back to the original/baseline composition after the intervention (130). Hence, it is important to perform long term research, as well as longitudinal studies including several time points within the intervention in order to achieve a better picture of these changes. In the following sections, we will discuss about effect and fate of various nutrients on gut microbiota, as well the effect of complete dietary patterns.

4. Individual components of diet and interaction with gut microbiota

4.1 Dietary carbohydrates

Carbohydrates are major sources of energy and are involved in several metabolic processes. One gram of carbohydrate contributes to four Kcal of energy and humans derive about 45-65% of their energy from carbohydrate intake (131). Dietary carbohydrates are categorized as digestible and nondigestible carbohydrates based on their ability to be broken down by the digestive enzymes. High carbohydrate diets, including large amounts of digestible carbohydrates are usually high in glycemic index and are associated with the risk of metabolic disorders such as obesity, T2D, dyslipidemia, and CVD (132,133). A result from Korean population study indicates that independent of fat intake, carbohydrate intake is associated with higher risk of T2D in male. Also, highly digestible carbohydrate diets increase adipogenesis and fat accumulation (134). On the other hand, nondigestible carbohydrates, serve as the major source of substrate for the intestinal flora and are regarded as beneficial components of diet (135). Dietary fibers are defined as carbohydrate polymers with ten or more monomers which when ingested are not hydrolysed by endogenous enzymes in the small intestine of human beings (136). A section of dietary fibers classified as “prebiotic fibers” are exclusively utilized by selective beneficial microbiota for their growth. “Functional fibers” on the other hand are extracted and or synthesized to have similar properties to that of dietary fibers (137). Carbohydrate to dietary fiber ratio has also been associated positively to triglycerides and MetS, and inversely with high-density lipoprotein cholesterol (138). This emphasizes on the understanding of high carbohydrate diet studies with a focus on the type of carbohydrates. As this thesis does not evaluate directly the effects of dietary/functional fibers, we henceforth refer to various types of fibers as “fibers” or “dietary fibers” for ease of understanding.

The structural properties of fibers influence the type of fermentation, favoring certain groups of bacteria in the intestine (139). Fibers can be classified as soluble or insoluble or gel forming based on their structural assembly and resistance to hydration. The structural differences also drive the availability of substrate at different sites of colon, hence giving a different gradient of commensals at different sites. In the distal colon, where there is higher availability of insoluble substrates, there is higher prevalence of *Bacteroides* (140). Thus, throughout the intestine there are different proportions and groups of saccharolytic bacteria metabolizing various substrates. Major carbohydrate fermenters and their pathways are represented in the below Figure 3.

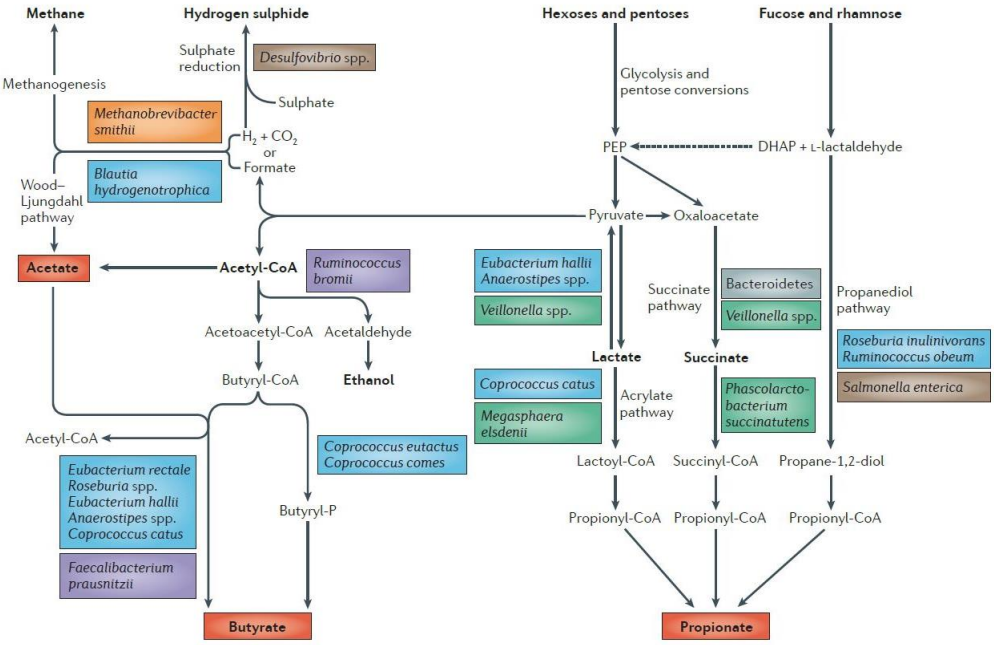

Figure 3 : Major carbohydrate metabolizing pathway in the large intestine (141)

Previous studies suggest that 10-60 g/day of non-digested carbohydrate arrive to the colon depending on the type of diet (139,142). Acetate, butyrate, propionate, succinate are the common SCFAs formed by microbial fermentation of fibers and they are absorbed by the host where a cascade of physiological process follows

(143). Amongst the studies exploring beneficial effects of nondigestible carbohydrates, butyrate stands out as this SCFA is associated with several health benefits.

A recent meta-analysis reporting the effect of various dietary fiber studies (no. of studies= 58) including 2999 healthy participants reported that *Bifidobacterium* and *Lactobacillus* spp. were found in higher abundance with consumption of prebiotic fibers (144) compared to placebo. Intervention studies with fiber intake also reported higher fecal butyrate concentration indicating the fermentation potential of various fibers (144). It is known that dietary interventions targeting gut microbiota results in different changes while comparing healthy and diseased adults. Ojo et al., recently performed a meta-analysis in the context of Randomized control trials (RCTs) of dietary fibers and gut microbiota in T2D individuals (145). Supporting the meta-analysis in healthy adults, this study also reported a significant increase in the relative abundances of *Bifidobacterium* in dietary fiber group compared with placebo. Significant reductions in body weight and glycated hemoglobin were also observed in the dietary fiber compared to placebo group. While targeted fiber or supplemented fiber intake induce beneficial effects, whole grains, fruits and vegetable intake have also shown to promote GI health and provide metabolic benefits (146–148).

While nondigestible carbohydrates or dietary fibers are known to induce positive effects to the host via intestinal microbiota; refined carbohydrates or simple sugars have shown to induce the opposite effects (132,149). Most digestible carbohydrates are absorbed in the small intestine. Fructose and glucose two of the most common sugars from diet, have shown detrimental effects on host by promoting bacterial genera associated with inflammation and affecting gut permeability (150,151). In animal studies comparing normal diet to high fat + high fructose diet (similar to Western diet), it was observed a decrease in *Bacteroides* and an increase in Firmicutes, along with weight gain (152,153).

A common strategy to evade the risk of high sugar induced weight gain, is the use of sweeteners such as sugar alcohols (polyols), natural sweeteners (stevia) and artificial sweeteners (aspartame, saccharine, cyclamates). These sweeteners are

non-nutritive, non-caloric or low caloric in nature. Even though substitution with sweeteners is a good strategy to reduce calorie intake, data from animal and human studies indicate their potential contribution to MetS and obesity, via gut microbiota(154). Suex et al., demonstrated that use of saccharin induced glucose intolerance via altering composition and functions of gut microbiota in mice (155). The health impact of switching from one sweetener to another or to another sugar is less explored. Several questions await to be answered within this field such as: 1) Are the effects similar while balancing natural sugars and artificial sweeteners, 2) Are there potential for new compounds such as alitame, neohesperidin dihydrochalcone (a flavanone glycoside) or any other candidates? 3) Are the effects of sweeteners similar amongst healthy, diabetic or elderly populations?

Overall, it can be agreed that intake of carbohydrate rich food high in fiber content and other nutrients (wholegrain, legumes, vegetables and fruits) should be increased. Whereas food containing refined and processed carbohydrates (white bread, sugar drinks, pastry, starchy vegetables) containing simple sugars must be decreased to maintain a healthy gut microbiota and metabolic profile.

4.2 Dietary proteins

Proteins are constituted by combination of various amino acids and serve as an important biomolecule in physiological and metabolic processes. Current protein requirements for adults are set at 0.80 g/Kg/day based on nitrogen balance (156). The amount of protein consumption vary drastically based on food availability, cultural habits, (157). Traditionally protein quality is evaluated by presence of essential amino acids, protein digestion and absorption capacity(158). Recently protein quality has also been assessed by their source (plant versus animal) and consequent impact on health. Most protein are efficiently absorbed after digestion and approximately only 10% of the undigested protein reach the large intestine (159). While the proximal region of the colon is predominantly saccharolytic, most of the protein fermentation occurs in the distal colon (160). The fermentation products of this substrate are similar to that of fibers (SCFAs, BCFAs) in addition to ammonia, phenol, hydrogen sulfide, and other putrefactive compounds (161).

Bacterial genera such as Bacteroides, Clostridium, Eubacterium, Lactobacillus, Fusobacterium and species such as Klebsitella spp., Escherichia coli, Streptococcus spp, Succinivibrio dextrinosolvens, Mitsuokella spp., and Anaerovibrio lipolytica have shown higher proteolytic capabilities (162).

High protein diets are recommended as a source for weight loss and has been reported in many studies that adults lose more fat mass when consuming a high protein diet(163). One of the proposed mechanisms by which protein may help weight loss is the appetite control. High protein preloads are associated with feeling of fullness and satiety (164). Hormones that are gut peptide secretion such as glucagon like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), peptide YY (PYY) are known to regulate satiety. Interestingly these peptides are reported to be increased in response to high protein intake, while comparing to high carbohydrate or high fat intake (164–166). A recent RCT comparing weight loss with calorie restricted high protein diet (HPD, 30% of energy) and calorie restricted normal protein diet (NPD, 15% of energy) showed that participants in the HPD lost more weight than those in the NPD (even though not statistically significant). These changes were related with changes in gut microbiota profile over the period of intervention (8 weeks), where HPD significantly increased the alpha diversity, and decreased *Prevotella_2*, *Faecalibaculum*, *Lachnospiraceae-UCG 004* as compared to the NPD (167).

While high protein intake certainly regulates metabolism, increasing number of studies have reported the importance of the source of protein (i.e., quality) and the balance with other nutrients (168). Animal and plant protein are consumed with other nutrients which make up the “protein package” and this package determines the effects on human health. For example, animal protein, most commonly derived from meat, contains also saturated fatty acids (SFA), whereas plant proteins tend to co-occur with fibers and polyphenols. Participants from the EPIC cohort who were classified as “meat eaters” (omnivorous) compared with “nonmeat eaters” (pesco-vegetarians) showed a higher energy intake, higher saturated fatty acid and lower fiber, PUFA intake (169). However, the omnivorous participants also had higher intake of vitamin B12, vitamin D, zinc and iodine compared to the vegetarians (169).

Previous meta-analysis has shown that red meat and processed meat consumption was associated with higher risk of T2D, whereas dairy products was associated with lower risk (170). In addition, replacing one portion of animal protein with (171) one portion of plant protein (from legumes, whole grains, nuts) was associated to a 21% lower diabetes risk.

With this regard, it is interesting to raise the question that, as with regulation of satiety via gut microbiota, could the sources of protein also modulate their effects based on gut microbiota? Few studies have explored this research question. One of the most known association is red meat consumption with high L-carnitine levels that induce TMAO production in the gut, which is associated with increased atherosclerosis incidence (8). Red meat and processed meat consumption introduces potentially harmful N-nitroso compounds, heterocyclic amines and heme compounds in the intestine, which is corresponded with significant abundance of *E. coli*, *Bacteroidetes fragilis* and these are positively associated with colorectal cancer risk (172,173). Comparison of HFD supplemented with protein sources from beef, casein and soy in mice for 90 days has shown that HFD with beef not only altered the gut microbiota, but also increased the levels of triglycerides, total cholesterol and inflammatory markers (IL1β, TNF-α, IL6) in serum (174).

On the other hand, soy protein, one of the most consumed plant proteins in Asia, is associated with lipogenesis via alterations in the microbial community, while comparing with milk protein isolate in golden Syrian hamsters (175). In rats, substitution of casein protein (~20 %) with soy protein in a cholesterol enriched diet for 6 weeks resulted in an increase in *Lactobacillus* species, and a decrease in *Parabacteroides*. *Lactobacillus* enrichment were also observed in studies evaluating various plant-protein sources (soy, mungbean, buckwheat) in addition to increase of *Bifidobacterium*, GLP-1 and reduction of Firmicutes (176,177). Another study conducted in rats showed that fishmeal compared to casein or soy protein significantly increased non beneficial indole, hydrogen sulphide and phenol in the caecal region, along with *Bacteroides and Parabacteroides* (by DGGE detection) (178). Seafood protein consumption characterised by high taurine content also induced a decrease in Proteobacteria levels in mice. Other plant protein sources such

as legumes and grains have also shown to impart effects on gut microbial composition (176,179). Pea proteins for example has shown to increase members of lactobacilli and bifidobacteria, correspondingly with an increase in SCFA (180). However, majority of the studies with legume and cereal consumptions attribute the observed gut microbial effects to the fiber component of legumes and cereals, compared to that of proteins.

Nuts are other important protein source from plants. Nuts contain between 10 and 20 g of protein/100 g of nuts, and the protein profile is considered suboptimal due to their amino acid content (181). Peanuts, walnuts, almonds and pistachio have high protein content, whereas macadamia and pecans have the lowest protein content (181). Nuts are also rich in other nutrients such as unsaturated fats, fibers, polyphenols and some minerals, and are considered good source of prebiotics. A recent meta-analysis summarizing the effects of nuts on gut microbiota showed that overall, nuts have the ability to shift the gut microbial community composition, with a preference towards bacterial taxon of *Clostridium*, *Roseburia*, *Lachnospira* and *Dialister* (182). Walnut protein derived peptide (PW5) has also shown to improve gut dysbiosis in transgenic mice and ameliorate Aβ plaques accumulation, which is a target in Alzheimer's disease treatment (183). Walnut and pistachio consumption has also been associated with increase in levels of SCFA forming members of Firmicutes and decrease in levels of *Bacteroides* (184,185). However, in nuts as there is also presence of other microbiota altering nutrients, hence it is unclear if these effects are solely derived from protein or a result of many components. Nonetheless, it can be established that nut consumption is beneficial for gut microbiota.

Despite evidence indicating harmful effect of consumption of animal-based protein compared to plant-based, few studies have reported no relation or even positive relation on gut microbial composition (186,187). With the growing demand for plant-based protein in the future, it is essential to conduct more studies focussing on understanding the microbiome modulated health implications of these proteins.

4.3 Dietary fats

Dietary fat is the macronutrient that provides maximum energy of 9 Kcal/g of intake. Due to this nature of fat, a low-fat diet is generally recommended to promote weight loss and reduce risks of metabolic diseases. However, research in the last decade has shown that the importance must be laid on the type of fat rather than the total fat consumption. Fat can be classified into 3 important groups depending on the saturated fatty acid content: SFA, Monounsaturated Fatty Acid (MUFA) and PUFA. Saturated fats are recognized to be higher in Western diet, whereas unsaturated fats (MUFA, PUFA) are higher in Mediterranean diet. The American Heart Association recommends a SFA intake of 5-6% of the total energy intake (in a 2000 Kcal diet) and to substitute SFA with PUFA or MUFA wherever possible.

Different types and sources of fat have been associated in the modulation of CVD, and one of the proposed mechanisms for this associations/effect is gut microbiota modulation. High amounts of dietary fats, as such in Western diet, likely reduce the saturation capacity of the small intestine to emulsify and absorb the fatty acids. Consequently, some portions of the unabsorbed lipids reach the large intestine. In vitro study conducted in a human GI simulator showed that a switch from carbohydrate-fat-protein balanced medium to a fat only medium, enhanced the microbial genera *Alistipes*, *Bilophila* and reduced the saccharolytic degraders such as *Bacteroides*, *Clostridium* and *Roseburia* (188), indicating certain preferential growth in presence of a fat substrate. Bacterial transplantation from HFD to germ-free mice has shown an increase in the intestinal *nfk1b* expression associated with inflammation and IR, displaying that diet (rich in fat) source could trigger inflammation via dysbiosis (189). Several mechanistic studies have shown HFD to disrupt the epithelial tight junctions in the intestine, which increases the translocation of LPS to stimulate proinflammatory responses in the host (190). It is interesting to note that one of the first studies demonstrating the increase in serum LPS level after HFD intake, enriched LPS containing bacterial genera in the gut. As well, this increase in LPS was only dependent on SFA levels and not the PUFAs (34). Another important pathway via which HFD alters gut microbiota composition is through bile acids. Bile acids are also signalling molecules, other than their

emulsifying capacity. For example, bile acid has shown to regulate genes involved in lipid (191), glucose (191) and energy homeostasis (192) by FXR and GPR, TGR5. Bile acids escaping the enterohepatic circulation, enters the colon where it could act as a substrate, preferentially selecting certain bacterial groups. Bacterial genera containing bile salt hydrolase enzyme (such as *Bacteroides, Bifidobacterium, Clostridium, Lactobacillus*) and deconjugating capacities (such as *Clostridium and Eubacterium*) play a key role in altering bile acid profile in circulation (56).

Mice study comparing HFD with various sources of fats such as palm oil ([polyunsaturated-to-saturated (P/S) ratio 0.4]), olive oil (P/S of 1.1) and safflower oil (P/S of 7.8) showed that palm oil intake increased body weight gain, triglyceride levels and upregulated lipid metabolizing genes in the small intestine. It was observed that these changes were mediated in a gut microbiota associated fashion, showing an increase in F/B (193). Prior reports suggest that differences exist even amongst different unsaturated fatty acids. A cross-over RCT comparing MUFA rich diet from various oil sources (canola oil, high oleic canola oil, high oleic-high DHA oil, corn-safflower oil and flax-safflower oil) and varying MUFA contents (from 18-72 %) showed no differences in the microbial *composition* after 30 days of consumption. However, MUFA diets in general increased Parabacteroides, *Prevotella*, Clastridiales, whereas an increase in *Faecalibacterium*, *Coprobacillus and Anaerostipes* were detected in the high oleic compared to high oleic-DHA oil consumption (194). Differences between n-3 and n-6 PUFA intake was noted in an observational study conducted amongst monozygotic twins. High n-3-PUFA ingestion was associated with an increase in the Lactobacillus group, whereas n-6-PUFA was negatively associated with Bifidobacterium abundances (195). Summarizing various studies, the systematic review conducted by the MyNewGut study group suggests that HFD, especially enriched in SFA could exert unfavourable effects on gut microbiota, whereas MUFA or PUFA intake shows no negative or inconsistent results, which needs further exploration (196).

Other than nuts, vegetable oils such as olive oil, sunflower oil, coconut oil, flaxseed oil, corn oil, safflower oil, palm oil and canola oil are some of the most consumed plant fat sources. Nuts and olive oil which are known for their health benefiting

properties deserves a special mention. As said in the above section (dietary protein), red meat intake increases circulating TMAO levels and this has shown to be mitigated via Mediterranean diet (MedDiet) in the PREDIMED study (197). The inhibition potential is attributed to the presence of 3, 3-dimethyl-1-butanol (DMB), commonly found in extra virgin olive oil (a major component of MedDiet). Virgin olive oil not only is rich in unsaturated fatty acids but is also rich in polyphenols. Hence, it makes the inferences on the effects difficult to be attributed only to unsaturated fatty acids. Hidalgo et al., explored this research question by comparing olive oil with equal fat content but varying polyphenol content and butter fat fed diet in mice for 12 weeks (198). Extra virgin olive oil and refined olive oil diets displayed similar abundances within each other, but clustered separately from butter fed diet, indicating predominant of the changes could be driven via fat. In this study DGGE was used and most genera identified were uncultured, hence future studies identifying these limitations must be conducted.

Compared to olive oil, studies investigating the effects of nuts are higher in humans. Ukhanova et al., conducted a RCT, where healthy participants underwent either pistachio (n= 16) or almond (n= 18) or no nuts intervention (control) for a period of 18 days. Pistachio and almonds enriched diets both were observed to modify the gut microbial composition (increasing butyrate producers) compared to control, with a stronger effect from pistachio. Similarly, pistachio consumption in pre-diabetic subjects also shifted their gut microbial composition indicated by decrease in three gut microbial metabolites (Hippurate, p-cresol sulfate and dimethylamine) identified in the 24-hour urine. Cross over RCT studying walnut consumption in overweight (n=18) men and women increased *Roseburia*, *Clostridium and Dialister,* and decreased *Ruminococcus*, *Oscillospira*, *Dorea* and *Bifidobacterium. This was observed to be associated to an improvement* in lipid profile compared to no nut consumption. These changes in the walnut group also corresponded with significant decrease in secondary bile acids compared to the control group, indicating the involvement of bile acid (199). Contrasting results with increase in Bifidobacterium, family Ruminococcaceae were noted in another cross over RCT with walnut consumption in 135 normal weight or overweight adults. While amount of walnut

consumption was almost similar (42g/day, 43g/day) in both the trials, the duration of trials (3 weeks, 8 weeks), washout periods and the geographical location of the two trials differed, indicating potential differences. Few studies evaluating the effects of other nuts and other vegetable oils have also been conducted, which are detailed more in the Result section of this thesis.

4.4 Dietary Polyphenols

Polyphenols are abundantly present in plants, fruits, and nuts with vast number of varying structures mainly grouped into flavonoids, stilbenes, lignans, and phenolic acids. Total polyphenol from dietary data of two different population studies averaged to 820 ± 323 mg/day (Spanish population) (200) and 863 ± 415 mg/day (Finnish population) (201). Majority of the dietary polyphenols are derived from products such as cocoa, soy products, red wine, berries, coloured vegetables (red cabbage, aubergines, and tomato), nuts, tea infusion etc. Existing evidence indicate their beneficial role in antioxidant, anti-inflammatory, anti-hyperglycaemic and cardio protective properties (202). Polyphenols are important section of compounds in the context of gut microbiota, as only 5-10% of the intake are absorbed in the small intestine, leaving the majority of these compounds to enter the large intestine (203). A recent observational study indicated that total polyphenol intake primarily from drinks (coffee, tea), fruits and plant-based protein sources (nuts, seed, legumes) correlated with faecal butyrate concentration indicating the prebiotic potential of dietary polyphenols (204). Abundant array of enzymes present in the intestinal microbiota serves as the key to metabolizing several types of polyphenols.

Absorption, metabolism and excretion of polyphenols have shown large inter-individual variations due to the differences in the gut microbiota. A classic example of this is the soy isoflavone daizdzein metabolism for which two metabolic fate awaits depending on the type of gut microbial composition (205).

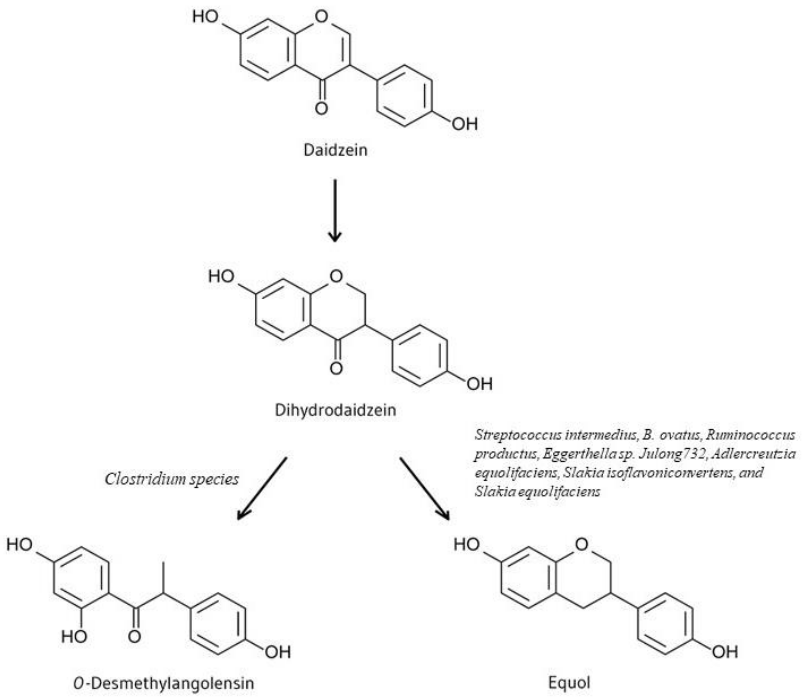

Figure 4: Two metabolic fates of soy isoflavone Daidzein via gut microbial metabolism (206)

As shown in Figure 4 above, daizein is converted into desmethylangolensin via *Clostridium* species in majority of subjects. Whereas about 30% of subjects convert daizein into equol via *Streptococcus intermedius, B. ovatus, Ruminococcus productus, Eggerthella sp. Julong732, Adlercreutzia equolifaciens, Slakia isoflavoniconvertens, and Slakia equolifaciens* (206). Also, with dietary ellagitannins and ellagic acid, the conversion to urolithin is dependent on the microbial composition. *Gordonibacter* species metabolize ellagic acids to urolithin which exert anti-inflammatory (207) activities, attenuate triglyceride accumulation in adipocyte, hepatocyte cell cultures (208). These differences in inter-individual subjects are important to be recognized as the metabolites of selective pathways are more bioactive compared to others. For example, comparing equol and non equol producers, benefits of equol production

have been linked to improvement of bone health and reduction in cardiovascular risk (209), although these results are not consistent (210–212).

A recent systematic review (202) of animal studies concluded that polyphenol intake consistently modified certain genera of the intestine. Genera that most consistently improved were *Akkermansia*, *Bacteroides*, *Blautia*, and *Roseburia*, and with less evidence for, *Bifidobacteria*, *Lactobacillus*, and *Alistipes*. Amongst these, *Roseburia*, *Blautia* increased in studies with treatment of proanthocyanidin (usually found in apples), reservatrol and tea polyphenols. Red wine (containing reservatrol) has also shown to increase *Bifidobacterium and Prevotella,* and correlate negatively with LPS concentrations (213).

Evidence from systematic reviews, meta-analyses of various cohorts indicate that rather than total polyphenol, the classes of polyphenols are more important factor to be studied (214). Age, ethnicity of population and baseline microbiota composition could potentially result in variations of results in studies. Also, differences in analytical methods, use of different phenol databases (from USDA or Phenol-Explorer) could potentially lead to inconsistent results.

4.4 Vitamins and minerals

Gut bacteria have clearly been recognized for their role in producing vitamin K and B groups for the host. This includes vitamins such as biotin, cobalamin, folates, nicotinic acid, riboflavin, thiamine, pyridoxine and panthotenic acid. Amongst the above vitamins, most commonly noted genomic pathway for synthesis are riboflavin and niacin, from bacterial phyla of Bacteroidetes, Proteobacteria and Fusobacteria (215). Production of these vitamins not only serve important for the host, but also are key in bacterial cross-feeding.

An 8-week randomized trial amongst 80 normal weight men and women consuming a whole grain rich or a refined grain rich diet quantified faecal menaquinone concentrations to understand the effect of diet mediated shift in vitamin K production (216). This study concluded that *Bacteroides* and *Prevotella* had the potential to distinguish the population into two groups of menaquinone producers

and this capacity to distinguish overpowered the treatment and time effect (216). In line, a recent mice study comparing vitamin K deficient versus vitamin K supplemented diet showed a clear difference in gut microbial community composition. Vitamin A, which has shown protection against infectious diseases, has also shown to modify gut microbiota (217). Increase in abundance of *Bifidobacterium* and *Akkermensia* two beneficial microbes were reported in supplementation of vitamin A in infants (218). Vitamin D, another fat-soluble vitamin is comparatively well researched for its beneficial health effects, especially in the context of diabetes. An ancillary study conducted to evaluate vitamin D supplementation for 12 months segregated the participants to glycaemic groups (based on changes in oral glucose tolerance) into group 1 (normal glucose tolerance, n=35), group 2 (prediabetic, n=28). Differences in Bacteroidetes to Firmicutes ratio (Group 1= 1.9, Group 2= 0.9), *Dialister* (% abundance, Group 1= 0.7, Group 2= 4.5), *Ruminococcus* (% abundance, Group 1= 1.6, Group 2= 3.0) were noted, along with changes in specific taxa associated with the lowest and highest quartiles of 25(OH)D (*Ruminococcus*, *Roseburia*, *Blautia*, *Dorea*) (219).

Minerals are also essential for human metabolism and interact with gut microbiota. Calcium which in epidemiological studies have shown to be associated with lower prevalence of obesity (220), has also shown to modulate gut microbiota. Dietary intervention in healthy participants with intake of 1000 mg calcium per day for eight weeks resulted in an increase in prevalence of Clostridium XVIII, a SCFA producer. Calcium intake (12 g/Kg) has also shown effect to increase *Bifidobacterium* spp., and increase *Bacteroides*/*Prevotella* ratio, corresponding with a decrease in LPS in HFD mouse model (221). Magnesium and iron intake has shown inconsistent results in both animal and human models, which requires further investigation. Interestingly, supplementation with iron has more often reported a decrease in beneficial bacteria such as *Bifidobacterium*. For example, in a RCT (222) conducted amongst Canadian infants with iron fortified cereals, a reduction in the mean abundance of family bidifobacteria were noted. Consistent results with reduction in *Bifidobacterium* levels were reported in another RCT conducted in Sweden infants after iron supplementation (223). Phosphorous, zinc, selenium and iodine are other minerals

that have shown to reshape the gut microbiota, however mostly experimented in animal studies (224). The supplementation and effect of minerals are dependent on various factors such as noted in one animal study where the effect of iodine depended on the fat content of the diet (225). Also, other factors such as the chemical form of supplementation, excipient of the supplements, and method of supplementation could also induce a change in the intestinal flora.

5. Dietary patterns altering gut microbiota

Our day-to-day food consumption involves intake of multiple food groups and nutrients that form a dietary pattern. Dietary pattern analysis in contrast to a single nutrient or single food intake analysis is closer to real life practice to understand the effect on human health. Dietary pattern-based studies could add a different perspective into the health effect based on the interactions and inhibitions occurring between the ingested compounds (226). Research from all around the world suggest that plant-based diets such as vegetarian or vegan or semi-vegetarian diets are beneficial to human health (227). Even though these diets are associated with health benefits, concerns over deficiency in certain nutrients (iron, vitamin B-12, protein) from these diets exists. According to the American Dietetic Association, the vegetarian diet features high intake of fruits, vegetables, legumes, nuts and whole grains that contribute to high intake of dietary fibers and polyphenols (228). Several studies reported a protective effect of vegetarian diets on chronic diseases such as T2D, hypertension, CVD, and different types of cancer (229–231). Vegan diet is very similar to the vegetarian diet but in complete devoid of any animal products, including dairy, which is commonly included in the vegetarian diet. It was only recently, since 1980´s that the vegan diets have emerged to be defined as specific subset of vegetarian diets worthy of study. The Adventist Health Study-2 showed, for the first time, that vegan diets conferred advantage in lowering risk for developing T2D compared to omnivorous diet. Similar to the Adventist study, the European Prospective Investigation into Cancer and Nutrition study (EPIC-study) also demonstrated that British vegetarians had a lower rate of CVD and risk factors compared to non-vegetarians in that study (232). Some reasons for this health protecting properties of vegetarian/vegan diets are attributed to high fiber, antioxidant, fruits and vegetables, wholegrains and low SFA consumption. One of the proposed pathway for the action of above mentioned nutrients/foods is via intestinal microbiota (233).

The presence of high fiber in the plant-based diet has shown to impart its beneficial effects, by the production of SCFA that has shown to have direct and indirect effects

on host metabolism (briefed in previous section). Other than the widely spoken dietary fibers, plant-based diets are also characterized by presence of polyphenols, which are majorly found in fruits, vegetables, tea, coffee, cocoa and wine. Polyphenolic components of tea such as epigallocatechin gallate, epicatechin gallate, epigallocatechin, gallocatechin, epicatechin and catechin has shown the ability to inhibit the growth of pathogens such as *Helicobacter pylori, Staphylococcus aureus, Listeria monocytogenes* and *Pseudomonas aeruginosa* (reviewed in (234)). Plant based diets have also been effective in reducing gut-derived TMAO levels and reduce chronic kidney disease, CVD risk (235).

Compared to a short-term vegetarian (3 months) to long-term vegetarians (> 3 months) have also shown to have effects on immune system modulated via gut microbiota (236). An observational study conducted in Slovenia, amongst long-term omnivores (n=29), vegetarian (n=31) participants noted an increased proportion of *Bacteroides/Prevotella group, Bacteroides thetaiotaomicron, Clostridium clostridioforme, and Faecalibacterium prausnitzii,* and lower proportion of *Clostridium* cluster XIVa in vegetarians compared to omnivores (237). Contrasting results with high abundances of *Prevotella* has been reported in studies comparing agrarian diet (plant based) to Western diets (animal based) (238,239). One of the few clinical trials following strict vegetarian diet amongst obese and T2D patients (n=6) for 30 days showed a decrease in BMI, triglycerides, LDL-cholesterol, and fasting and postprandial glucose concentrations (233). These changes corresponded with increase in members of Bacteroidetes phylum and decrease in Firmicutes, pathobionts of Enterobacteriaceae family. However, this study had a very low number of participants neither had a control group to compare the observed results. Higher abundance of Bacteroidetes was also reported in few studies amongst vegetarians and vegans compared to omniovores (240,241). Increase of BA pool in human is associated with meat and animal food consumption, this has also shown to increase bile acid resistant bacteria such as *Alistipes* and *Bilophila* which have been associated with harmful health effects (129,242).

Many of the studies conducted to understand the effect on gut microbiota from plant-based diet are observational in nature (243), hence doesn't allow us to

understand the causality of diet on gut microbiota. Future well controlled randomized trials, not only looking at the benefits, but also looking at the effects of overcoming the deficiency of certain nutrients from these diets on gut microbiota needs to be conducted. Plant-based diets may promote beneficial health effects via preferring microbes producing SCFAs and equol or decrease deleterious members such as BA and trimethylamine metabolizers.

5.1 Mediterranean diet and gut microbiota: Match made in heaven?

Mediterranean diet (MedDiet) is a dietary pattern emphasizing the consumption of plant food, that is regarded as one of the healthiest dietary patterns and it is established as a UNESCO cultural heritage. MedDiet is characterized by a high intake of whole grains, legumes, nuts, fruits, vegetables; moderate intake of seafood, white meat and dairy; low intake of red or processed meat and sweets, pastry and processed foods. Food groups mentioned above contribute to a diverse array of nutrients such as fibers, polyphenols, unsaturated fatty acids and micronutrients. These food groups and nutrients have all been associated with a better health status in several past studies. In a broader context, Mediterranean lifestyle includes high adherence to MedDiet, adequate hydration and the practice of physical activity contributing to a better health and mental status (244). A misconception around consumption of MedDiet is its high fat content potentially leading to weight gain or other metabolic disorders. Landmark PREDIMED study displayed that following MedDiet and increasing energy density characterized by olive oil or nuts, does not associate with weight gain (245).

Several components of MedDiet are capable to reach the large intestine and potentially mitigate some of their effects via gut microbial changes. For example, anti-carcinogenic capacities of MedDiet have been associated with their ability to increase Lactobacillus population in mouse models (246). MedDiet associated decrease in proinflammatory markers in overweight subjects have also been shown to be mediated via increase in intestinal lactic acid bacterial members (247). Possible links of modulating inflammation and IR via endocannabinoid system in a

gut microbiota dependent manner with consumption of MedDiet in humans have also been investigated (248). Assessing the associations between important components of MedDiet have shown that phenolic compounds from olive oil, red wine is associated to higher *Faecalibacterium*, legumes with *Coprococcus*, vegetables with *Dorea*, Rikenellaceae genera, most of them being a SCFA producers (249).

Interest in understanding the effects of MedDiet on intestinal flora have been increasing in the last decade since one of the first human studies from De Fillips et al., showed a high level adherence to MedDiet was associated with beneficial gut microbial composition and their corresponding metabolites (250). Gut microbial members such as *Lachnospira*, *Prevotella* were positively associated with high fat intake, plant-based diet, whereas these genera were negatively associated with omnivorous diet in this study. This study also showed that even within omnivores, the increase in MedDiet adherence was increasingly associated with SCFAs. Similar beneficial changes in MetS participants were noted in a RCT conducted by Haro et al. Consumption of MedDiet compared to a low-fat diet for 2 years resulted in an increase of *Prevotella distasonis*, *Bacteroides thetaiotaomicron*, *Faecalibacterium prausnitzii*, *Bifidobacterium longum* which are regarded as beneficial members of the intestinal flora (251). Transversal study conducted by the same research group showed that MedDiet adherence is associated with higher abundance of Bacteroidetes phylum, *Prevotella* genera and lower abundance of Firmicutes phylum and Lachnospiraceae family (252). As members of Firmicutes, especially family of Lachnospiraceae are characterized by carbohydrate metabolizing species, a consistent decrease is noted in MedDiet studies potentially due to their high unsaturated fat content in the MedDiet.

One of the largest MedDiet adherence study was conducted in the framework of NU-AGE study comprising elderly participants from 5 countries (UK, France, Italy, Netherlands and Poland). This study concluded that increasing MedDiet adherence can promote healthier ageing modulated via gut microbiota. Some taxa (*Faecalibacterium prausnitzii*, Eubacterium eligens, *Prevotella copri*, unclassified species of *Clostridium*, *Roseburia*) were positively associated to adherence to MedDiet in addition to negative association with markers of frailty and cognition

(91). Contrastingly other set of taxa including *Collinsella aerofaciens*, *Dorea formicigenerans*, *Coprococcus comes*, *Ruminococcus torques* showed negative associations with MedDiet. Overall, as mentioned in a recent commentary, MedDiet certainly offers a variety of nutrients important to maintain a balanced intestinal microbiome that could provide a protective effect on host health. Although considerable number of studies have investigated the MedDiet-microbiome interaction, the study size, duration and the MedDiet adherence measuring tools between various studies are not similar in order to draw a conclusive result. Moreover, as shown in the PREDIMED, NU-AGE study, elderly population especially at risk of various diseases might benefit from a particular "MedDiet", hence future long-term RCTs understand the MedDiet-microbiome interaction in this population is necessary. As mentioned above Mediterranean lifestyle includes not just MedDiet adherence but also performing physical activity, which could have additional beneficial effect on gut microbiota which should be explored in future studies.

II. JUSTIFICATION

Obesity is recognized as a global epidemic and it is rapidly growing worldwide posing a huge challenge for the society. Obesity is characterized by a chronic low-grade inflammation state, which can also increase the risk of IR, dyslipidaemia and cardiovascular diseases. Clustering of abdominal obesity, dyslipidaemia, hypertension and hyperglycaemia referred as MetS, results in a reduced quality of life and increased risk of mortality. Lifestyle changes are considered as the major contributors for obesity and metabolic disorder. A sedentary lifestyle with high caloric food consumption is a well-recognized driver of obesity.

Reduction of calorie intake and increasing calorie expenditure is a well-established method for weight reduction and improving metabolic profile. However, previous research indicates that even though various calorie restriction methods (such as low carbohydrate, high fat or low carbohydrate, high protein or low fat, high fiber) can aid in weight loss, they might not result in the same metabolic improvement, especially at long term. Landmark study such as PREDIMED, has established that with consumption of balanced nutrients, such as following a Mediterranean dietary pattern could result in improvement of MetS and consecutively reduce cardiovascular risk. Hence, it is interesting to explore the effect of an energy reduced Mediterranean diet, combined with physical activity on weight loss and risk factors of cardiovascular disease. PREDIMED-Plus study addresses this research question in an elderly Mediterranean population with overweight/obesity and MetS. Understanding the mechanisms driving the lifestyle modulated changes in obesity could provide better treatment opportunities and personalized care.

In the last few decades, research interests in understanding the mechanism behind metabolic disorders have led to identifying intestinal microbiota as a key driver in this global epidemic. Pioneering studies have noted that variation in gut microbiota can modulate the pathogenesis of obesity. In simple terms, gut microbiota can be considered as a chemostat, where undigested components of food reach the intestine and are metabolized by an array of microbial enzymes. The type of metabolization and the product formed depends on the composition of the microbial taxon present in the intestine and also the substrate available. Some of the microbial products formed are directly involved in important metabolic reactions such as

lipogenesis, glucogenesis and appetite regulation. Extensive research has gone into understanding the effect of individual components of diet on intestinal microbiota and their functionalities. It is also important to emphasize here that previous studies have shown variation in microbial profile even with changes in sources and quality of nutrients, thus indicating a factor of quality over quantity. However, gaps exist in literature to identify the effect of holistic lifestyle component on gut microbiota. Hence, it can be agreed that investigating the effects of lifestyle changes on health via intestinal bacteria could be of high value.

Considering all the aforementioned stated factors, this thesis aims to evaluate the effect of an energy-restricted Mediterranean diet and physical activity promotion on changes in gut microbial composition in the framework of PREDIMED-Plus study. This study provides a unique opportunity to evaluate the effect of a lifestyle intervention that could have a holistic impact on health, bringing it close to real life practice, adding value to the translational nature of this research. With the importance laid on quality of nutrients over quantity, we also aim to understand the differences in gut bacterial composition with varied protein and fat sources. Overall, this doctoral thesis would allow to add important piece of information to literature on gut microbiota.

III. HYPOTHESIS AND OBJECTIVES

Hypothesis 1: A 1-year lifestyle intervention using an energy reduced MedDiet and physical activity promotion would help in weight loss and improve cardiovascular risk factors via changes in gut microbiota

Objective 1.1: To investigate the effect of a 1-year intensive lifestyle weight loss intervention on gut microbiota composition and predicted functionality in the PREDIMED Plus study

Objective 1.2: To evaluate if the changes observed in objective 1.1 in microbial taxa are associated with changes in cardiovascular risk factors

Hypothesis 2: Differences in dietary protein quantity and sources are associated with differences in gut microbial composition and predicted functionality.

Objective 2.1: To cross-sectionally analyze the associations between the consumption of different sources of protein (plant and animal) and gut microbiota composition

Objective 2.2: To identify the 1-year associations between changes in protein intake (and its sources) and changes in gut bacterial composition and predicted functions

Hypothesis 3: Source and quality of fat can have different effects on health. Similarly, the gut microbiota could be modified with varying source and quality of fat.

Objective 3: To summarize the available literature in order to understand the effect of various plant-based fat sources and its impact on gut microbiota from various *in vivo* and *in vitro* studies.

IV. MATERIALS AND METHODS

1. PREDIMED Plus study

1.1 Objectives of PREDIMED Plus study

PREDIMED Plus (in Spanish: PREvención con DIeta MEDiterránea Plus) is an ongoing multicenter randomized parallel-group clinical trial conducted in Spain. The trial is conducted in health care centers from 23 different Universities and Health Research Institution from Spain. The primary aim of PREDIMED Plus is to evaluate the effectiveness of long-term intensive weight loss and maintenance based on lifestyle intervention on primary cardiovascular prevention. The key components of the lifestyle intervention include and energy reduced MedDiet, physical activity promotion and behavioral support (Intervention group). This intensive lifestyle intervention is compared to a calorie unrestricted MedDiet (Control group). This study was registered at the International Standard Randomized Controlled Trial (ISRCT; http://www.isrctn.com/ISRCTN89898870) with number 89898870 (Registration date: 24 July 2014).

With the immense amount of data collected from PREDIMED Plus, several intermediate and secondary end-points are being evaluated. Some examples of this include the evaluation of food intake and overall dietary pattern, kidney function, liver function, anti-hypertensive, anti-diabetic and lipid lowering medication needs. In this thesis, we focus on the first one-year intervention measurements from the PREDIMED Plus study.

1.2 Study population

Study participants were community dwelling adults in the age of 55-75 years (men) and 60-75 years (women) with overweight or obesity (BMI 27-40 Kg/m^2). Eligible participants also have to meet at least three components of MetS (issued by International Diabetes Federation, Nation Heart, Lung and Blood Institute and the American Heart Association (12)) as shown in Figure 1 (Introduction section).

Major exclusion criteria included:

- Inability to provide written informed consent or communicate with study staff.
- Documented history of previous CVD.
- Being permanent institutionalized or long-stay resident in a nursing home
- Have an active malignant cancer or history of malignancy within the last 5 years
- Inability to follow the recommended intervention diet or to perform physical activity.
- Food allergy to any component of the Mediterranean diet.
- Immunodeficiency or HIV-positive status.
- Cirrhosis or liver failure.
- Serious psychiatric disorders
- Alcohol abuse or addiction (or total daily alcohol intake >50g) or drug abuse within the past 6-months.
- Serious psychiatric disorders, including schizophrenia, bipolar disorder, eating disorders, and depression with hospitalization within the last 6 months.
- Any other condition that might interfere with adherence to the study protocol

Three screening visits were conducted during which the study protocol was explained and the participants signed the information consent. After the screening, participants willing to participate in the intervention and passed the inclusion criteria were randomly assigned to intervention or control group in each recruiting center based on computer-generated random number system. Married or unmarried couples were randomized together and participants were randomly assigned by stratification by center, sex and age group (<65, 65-70, 70 years).

1.3 Intervention group

Participants assigned to the Intervention group had six group and six individual sessions in the first 6 months of intervention. During this period, participants were encouraged to achieve a weight reduction of 10% and waist circumference

reduction of 5 to 10%. Participants were supported with various motivational sessions, alternative low calorie recipes and recommendations to increase physical activity on an individual basis. Additionally from 7-12 months, participants received telephone call from dietician to reinforce the trial objectives.

The energy restricted MedDiet aimed at energy reduction of 600 Kcal/day according to each particpants´ basal metabolic rate and physical activity level. Composition of macronutrient were determined by a distribution of 40-45 % carbohydrate, 35-40 % fat and 20 % protein. Dietary advices were designed in order to fit each persons projected and achieved monthly weight loss objectives. In general participants were advised to refrain from consumption of animal fats, sugar-sweetened beverages, pastry, processed foods, refined grains which are characteristics of Western diet. Adherence to the energy restricted MedDiet was evaluated by 17-item questionnaire (Table shown below). To increase the adherence to the MedDiet, the participants were supplemented with olive oil, mixed nuts. During the group-sessions, participants received weekly suggestive food shopping list adapted to the season.

After 6-months of intervention, participants were encouraged to nominally increase their physical activity to at least 45 minutes per day or 150 minutes per week. Various physical activities such as aerobic activities, biking, aqua-gym, flexibility training were suggested. In order to enhance the motivation and self-monitor, each participant were provided with a pedometer to monitor their steps. Every 4 months, the adherence to physical activity was monitored and participants were provided with solutions to overcome difficulties while accomplishing their physical activity goals.

Additional to the dietary and physical activity advises, participants were aided with behavioral support, which included problem-solving tools to facilitate self-control on emotional eating, or stress enhanced eating behaviors or sedentariness. This component of the intervention allowed participants to feel empowered and increased their long-term adherence to dietary and physical activity recommendations.

Table 1: List of questions in the 17-point Mediterranean diet adherence tool

	Questions	Criteria for 1 point
1	Do you use olive oil as main culinary fat?	Yes
2	How many fruit units (including natural fruit juices) do you consume per day?	≥3
3	How many vegetable servings do you consume per day? (1 serving = 200g [consider side dish as half serving])	≥2 (≥1 portion raw or as salad)
4	How many servings of white bread do you consume per day? (1 serving = 75g)	≤1
5	How many servings of cereals and whole grains (bread, rice, pasta) do you consume per week?	≥5
6	How many servings of red meat, hamburger or meat products (ham, sausage, etc) do you consume per week? (1 serving = 100–150g)	≤1
7	How many servings of butter, margarine, or cream do you consume per week? (1 serving = 12g)	<1
8	How many sweetened beverages (soft drinks, cola, bitter, juices without added sugars) do you drink per week?	<1
9	How many servings of legumes do you consume per week? (1 serving = 150g)	≥3
10	How many servings of fish or shellfish do you consume per week? (1 serving = 100-150g of fish or 4-5 units or 200g of shellfish)	≥3
11	How many times per week do you consume pastries, such as cookies, custard, sweets or cakes?	< 3

12	How many servings of nuts (including peanuts) do you consume per week? (1 serving = 30g)	≥3
13	Do you preferentially consume chicken, turkey, or rabbit meat instead of veal, pork, hamburger, or sausage?	Yes
14	How many times per week do you consume vegetables, pasta, rice, or other dishes seasoned with sofrito (sauce made with tomato and onion, leek, or garlic and simmered with olive oil)?	≥2
15	Do you preferentially add non-caloric artificial sweeteners to beverages (such as coffee or tea) instead of sugar?	Yes
16	How many servings of white bread, rice and/or pasta do you consume per week?	<3
17	How many glasses of wine do you drink per day?	2- for men 1-2 for women

1.4 Control group

Participants in the Control group received information on following MedDiet as suggested in PREDIMED study (253). General lifestyle recommendations for managing MetS were given to the participants in control group. Unlike the intervention group, dieticians did not recommend to lose weight in the control group. Dieticians emphasized on improving diet quality adhering to MedDiet and evaluated their adherence by 14-item validated questionnaire. No specific recommendations for increasing physical activity were given. Participants were offered a group, individual session in the beginning of the intervention, followed up by 6 months after their first visit. At the first and 6-month visit, participants were provided with free virgin olive oil and nuts in order to encourage compliance to the study. Participants in both groups were provided with an allotment of extra-virgin

olive oil (1 L/month) and almonds (125 g/month) for free. However, all participants were recommended to consume a total of 500 g/month of mixed nuts.

2. Measurements

2.1. Anthropometric and blood pressure measurements

Weight and height were measured with no shoes and light clothing with a calibrated scale and wall mounted stadiometer respectively. With the weight and height measurement, BMI was calculated as weight (Kg) divided by square of height (m). A validated semiautomatic oscillometer (Omron HEM-705CP, Netherlands) was used to measure blood pressure. Anthropometric measurements were taken in duplicate and blood pressure measurement in triplicates at baseline and 12 months visits by trained staff.

2.2 Biochemical measurements

Biological samples were collected and stored in freezers at -80°C. Blood samples were collected after an overnight fast to determine fasting plasma blood glucose, glycated hemoglobin, total cholestetol, LDL cholesterol, HDL cholesterol and triglycerides by routine enzymatic methods. For this purpose, blood samples were collected in two 10ml K2E EDTA tubes, one 4.5ml citrate tube and two 10 ml and 6 ml serum separator tubes. All biological samples were processed and stored at -80 ºC no later than one hour of extraction.

2.3 Fecal sample collection and processing

Five centers from the PREDIMED-Plus study collected fecal samples, out of which Reus and Malaga centers were the first to begin collection. For the feasibility of the study, these two centers were prioritized in the analysis of gut microbiota. Fecal samples were collected at baseline and 1-year in a sterile hermetic flask and they were instructed to bring it to the study center soonest possible (within 12 hours of extraction) in a cooled condition (with provided ice pack or stored at -20°C). Samples were aliquoted into approximately 250 g aliquots and stored at was -80°C.

Participants who were using antibiotics or pre/probiotics 15 days before sample collection were asked to do the collection 15 days after their use was stopped.

Frozen aliquots were used for fecal DNA extraction. Fecal DNA extraction was conducted using QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany) according to the manufacturers' protocol and an additional bead beating step of 5 minutes using FastPrep-24 5G Homogenizer (MP Biomedicals, California, USA) to the first lysing step. The quantity of DNA was evaluated using Qubit 2.0 Fluorometer-dsDNA (High Sensitivity kit, Invitrogen, Carlsbad, California, USA). After extraction, the DNA was stored at - 20°C until further processing.

Briefly, we used Ion Ribosomal 16S Kit (Thermo Fisher Scientific, Italy) that includes two primer sets selectively amplifying the corresponding hypervariable regions of the 16S region in bacteria: primer set V2–4–8 and primer set V3–6, 7–9. After sequencing, the individual sequence reads were filtered using Ion Reporter Software V4.0 to remove low quality and polyclonal sequences. Data were processed and separated into six hypervariable regions using an adapted script available from Mas Lloret J et al (16). Only variable region V4 was used for further analyses. These files were imported to QIIME2 and DADA2 pipeline was followed (further on Supplementary information 3). Taxonomy was assigned to the clustered sequences with SILVA 132 as 16S classifier database. Mitochondrial features and features unidentified at phylum level were removed in the pre-processing step in R (v 3.6) (17). Predicted metagenome functions were performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin (PICRUST2) (254) within QIIME2 (255) with q2-picrust2 plugin. MetaCyc, KEGG pathways were normalized within QIIME2 (256).

2.4 Dietary and physical activity measurements

Validated 143-item food frequency questionnaire used in the PREDIMED study was administered to evaluate the total food intake at baseline and 1-year of follow up (257,258). Spanish food composition tables were used to estimate total energy, macronutrients and micronutrients. Adherence to MedDiet and energy restricted

MedDiet were evaluated by 14-item questionnaire and 17-item questionnaire respectively.

Physical activity were assessed by rapid assessment of physical activity (259), Minnesota-REGICOR short physical activity questionnaire (260), and a questionnaire for sedentary behaviors of the Nurses´ Health study (261). The Minnesota-REGICOR questionnaire comprised of six questions on monthly frequency (number of days) and duration (min/day) in which six different types of physical activities was performed: Brisk walking, walking at a slow/normal pace, walking in the countryside, climbing stairs, working in the garden and do exercise or play sports at home, outdoors or in a gym. The metabolic equivalent of each activity expressed in metabolic equivalent task (MET) was calculated based on Compendium of Physical activity (262).

2.5 Study population for the analysis

Primary outcome of this thesis is gut microbiota composition hence the sample size were determined based on samples for which metagenomics were conducted. For this thesis, which is a sub-study from PREDIMED Plus. Samples were selected based on randomization conducted to balance age, sex, BMI and study center in a 1:1 composition. 400 participants were chosen based on the above randomization criteria.

Chapter 1: Effect of lifestyle intervention on gut microbiota composition

Amongst the 400 participants (at two time points), 62 samples were removed due to low DNA concentration or unavailability of sample. Following bioinformatic steps DADA2, 11 samples were removed due to low read quality. Out of the 727 samples processed downstream, 14 samples were removed due to low sequence quality. This led to a sample size of 362 participants at baseline (IG =183, CG= 179) and 351 participants at year-1 (IG = 173, CG=178). Amongst this, 1 sample in IG and 6 samples in CG did not have a baseline matched pair, hence were removed subsequently from the analysis.

Chapter 2: Associations between quality and quantity of protein intake and gut microbiota composition

From a total of 400 participants, we removed 77 participants due to filtering steps and excess energy intake at baseline or 1-year. This resulted in 323 participants for the analyses who had dietary, 16S rRNA data at both baseline and 1-year.

Chapter 3: Plant-Based Fat, Dietary Patterns Rich in Vegetable Fat and Gut Microbiota Modulation

Narrative review, no sample size available

3. Statistical analysis

Every chapter describes in detail the statistical methods performed with respect to each result. Here brief statistical methods are stated.

Baseline participants and their descriptive data, differences amongst the groups were presented as mean ± standard deviation or median ± 95% CI for continuous variables of normal and non-normal distribution respectively. Categorical variables are expressed as percentages (%). Differences between groups at baseline were tested using t-test (normal distribution) or wilcoxon test (non-normal distribution) or chi-square test (categorical variables). Normality of distributions was tested using shapiro-wilk test and or visualized by histograms. Differences in clinical measurements, dietary data and physical activity (1-year – baseline) were evaluated by Wilcoxon tests or t-test based on distribution. In cases where adjustments were required, a linear regression adjusted for covariates were used.

Microbiome data were normalized by cumulative sum scaling method using Metagenomeseq package(263). Alpha and beta diversity measures were estimated by phyloseq package. Other important measures such as B/F ratio, Prevotella to Bacteroides ratio, phylogenetic distance were computed at baseline and 1-year of intervention. For differential abundance analysis, we utilized Metagenomeseq package, which uses zero inflated negative binomial model, with possibility to adjust for covariates and include repeated measures. To evaluate if there are differences

between Intervention and Control group after intervention, we evaluated a sparse partial least square discriminant analysis from the package mixomics(264). Association analysis were conducted using various tools such as Maaslin2 or NBZIMM which incorporates negative binomial regression appropriate for the microbiome data, adjusted for the differences in reads between samples. PICRUST2 data were analyzed using the open-source software STAMP (Statistical Analysis of Metagenomics Profiles) with Welch's t-test option or using Maaslin2 regression model (in R software) (265,266).

Wherever appropriate, p-values were adjusted for false discovery rate in order to reduce type I error. Various tools for visualization of data were used from packages such as ggplot2, phyloseq, and microbiome(267,268).

3.1 A comment on microbiome data analysis

Data analysis with microbiome deserves a special note. Upstream processing of microbiome data could have significant effects on outcomes. However, the consensus on which is the right method for the processing of microbiome data is unsettled question. Starting from study design, sample collection, storage, and sequencing techniques, several factors play role in final microbiome dataset. Unlike well-established statistical tools for clinical data, microbiome data analysis is a highly evolving field with no clear consensus.

Main challenge of microbiome data comes from the fact that high-throughput sequencing (HTS) experiments are limited to deliver reads only up to a certain capacity. Review by Gloor GB et al (269), summarizes how in an ecological context it is not possible to obtain true abundances from HTS methods. This leads to considering the microbiome data as compositional in nature. Compositional data as described by Aitchison et al (270), Pawlowsky-Glahn et al (271) is data with *proportions or probabilities or with a constant or irrelevant sum, that contains information about the relationship between the parts of data*. The problem with existing methods of analysis is that compositional data might be examined using non-compositional data approach. In the last decade, several methods have been proposed to address the compositional nature of the data. Additional to the

compositional nature, the sparsity (presence of high number of zeroes) is a characteristic of microbiome data. Some amount of sparseness are due to true low abundance of certain taxa and low distribution in samples. However, there are also cases where sparsity occurs due to sequencing artifacts and highly variable sequencing depth between samples. This raises the issue of identifying a "true zero" or a "technical/methodological zero". Despite sophisticated tools available for statistical transformation, the problem might remain partially unsolvable limiting the inference from studies. Hence, it is suggested that along with HTS, it is important to explore quantitative approaches, mechanistic studies with tracing final metabolites to prove the hypothesis generated by HTS inferred data. Nevertheless, data obtained from HTS is essential, that allows us to hypothesize and test various outcomes, provided with the use of right methods.

V. RESULTS

Chapter 1

Effect on gut microbiota of a 1-year lifestyle intervention with Mediterranean Diet versus Energy-Reduced Mediterranean Diet and Physical Activity Promotion. PREDIMED-Plus Study

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Effect on gut microbiota of a 1-year lifestyle intervention with Mediterranean Diet versus Energy-Reduced Mediterranean Diet and Physical Activity Promotion. PREDIMED-Plus Study

Jananee Muralidharan[1,2], Isabel Moreno-Indias[#2,3], Mónica Bulló[#1,2], Jesús Vioque Lopez[4,5], Dolores Corella[2,6], Olga Castañer[2,7], Josep Vidal[8,9], Alessandro Atzeni[1,2], Jose Carlos Fernandez-García[2,3], Laura Torres-Collado[4,5], Rebeca Fernández-Carrión[2,6], Monsterrat Fito[2,7], Romina Olbeyra[9], Ana Maria Gomez-Perez[2,3], Serena Galiè[1,2], Maria Rosa Bernal-López[2,12], Miguel Angel Martinez-Gonzalez[2,10,11], Jordi Salas-Salvadó[&1,2], Francisco Jose Tinahones[&2,3]

[#] Corresponding authors, [&] Senior authors

1. Department of Biochemistry and Biotechnology, Human Nutrition Unit, IISPV, Hospital Universitari Sant Joan de Reus, Rovira i Virgili University, 43003 Reus, Spain

2. CIBER de Fisiopatología de la Obesidad y la Nutrición (CIBEROBN), Instituto de Salud Carlos III, 28029 Madrid, Spain

3. Unidad de Gestion Clínica de Endocrinología y Nutrición, Laboratorio del Instituto de Investigación Biomédica de Málaga (IBIMA), Hospital Universitario de Málaga (Virgen de la Victoria), Universidad de Málaga, Málaga, Spain

4. Instituto de Investigación Sanitaria y Biomédica de Alicante, ISABIAL-UMH, Alicante, Spain

5. CIBER Epidemiología y Salud Pública (CIBERESP), Instituto de Salud Carlos III (ISCIII), Madrid, Spain

6. Department of Preventive Medicine, University of Valencia, Valencia, Spain
7. Cardiovascular Risk and Nutrition (Regicor Study Group), Hospital del Mar Research Institute (IMIM), 08003 Barcelona, Spain

8. Endocrinology and Nutrition Department, Hospital Clinic Universitari, Barcelona, Spain

9. August Pi i Sunyer Biomedical Research Institute (IDIBAPS), Barcelona, Spain

10. University of Navarra, Department of Preventive Medicine and Public Health, IdiSNA, 31008 Pamplona, Spain

11. Department of Nutrition, Harvard T.H. Chan School of Public Health, Boston, MA 02115, USA

12. UGC Medicina Interna, Hospital Regional Universitario de Málaga, Spain

Corresponding authors' information

Dr. Isabel Moreno-Indias: isabel.moreno@ibima.eu

Dr. Monica Bullo: monica.bullo@urv.cat

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Running title: Mediterranean diet, weight loss and gut microbiota

Trial registration: ISRCT number 89898870

Data sharing: The datasets generated and analyzed during the current study are not expected to be made available outside the core research group, as neither participants' consent forms nor ethics approval included permission for open access. However, the researchers will follow a controlled data-sharing collaboration model, as in the informed consent participants agreed with a controlled collaboration with other investigators for research related to the project's aims. Therefore, investigators who are interested in this study can contact the PREDIMED Steering Committee by sending a request letter to predimed_scommittee@googlegroups.com. A data-sharing agreement indicating the characteristics of the collaboration and data management will be completed for the proposals that are approved by the Steering Committee.

Abbreviations:

MedDiet Mediterranean diet; IG Intervention group; CG Control group; MetS Metabolic syndrome; B/F Bacteroidetes-to-Firmicutes ratio; CVD Cardiovascular disease; MedScore Mediterranean diet adherence score; P/B Prevotella to Bacteroides ratio; PICRUST Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin; SCFA Short chain fatty acid; BCAA Branched chain amino acid

Abstract

Background: Mediterranean diet is a well-recognized healthy diet that has shown to induce positive changes in gut microbiota. Lifestyle changes such as diet along with physical activity could aid in weight loss and improve cardiovascular risk factors.

Objective: To investigate the effect of an intensive lifestyle weight loss intervention on gut microbiota.

Design: This is a sub-study of the PREDIMED-Plus, a randomized controlled trial conducted in overweight/obese male and female (55-75 years) with metabolic syndrome. Intervention group (IG) underwent an intensive weight-loss lifestyle intervention based on an energy-restricted Mediterranean diet (MedDiet) and physical activity promotion and the Control group (CG) underwent a non-energy restricted MedDiet for 1-year. Anthropometric, biochemical and gut microbial 16S rRNA sequencing data were analyzed at baseline (n=362) and 1-year follow-up (n=343).

Results: IG participants had a weight loss of 4.2 (IQR, -6.8, -2.5) kg vs. 0.2 (IQR, -2.1, 1.4) kg in the CG ($P < 0.001$). Reductions in BMI, fasting glucose, glycated hemoglobin, triglycerides, and increase in HDL were greater in the IG than in CG participants ($P < 0.05$). We observed a

decrease in *Butyricicoccus*, *Haemophilus*, *Ruminiclostridium 5* and *Eubacterium hallii* in the IG compared to the CG. Many genera shifted in the same direction within both intervention groups indicating an overall effect of MedDiet. Decreases in *Haemophilus*, *Coprococcus 3*, and few other genera were associated with a decrease in adiposity parameters in both intervention groups. Changes in *Lachnospiraceae NK4A136* was positively associated with changes in MedDiet adherence.

Conclusion: Weight loss induced by an energy-restricted MedDiet and physical activity induce changes in gut microbiota. The role of MedDiet induced changes on the host might be via short chain fatty acid producing bacteria, whereas, with energy restriction, these changes might be modulated with other mechanisms, which needs to be explored in future studies.

Keywords: Weight loss, Gut microbiota, Mediterranean diet, Energy restriction

Introduction

Microbiota colonizes the human gut during or shortly after birth and continues to grow and develop until it establishes a stable environment in adults. During adulthood, the variability and complexity of the human gut microbiome are influenced by several lifestyle choices, including dietary and non-dietary factors such as physical activity, stress, or smoking habits (1). Also, environmental factors, aging, medications, and diseases shift the composition and functionality of our microbes. Individuals with conditions such as diabetes, metabolic syndrome (MetS), cardiovascular risks have shown to have a dysbiotic gut with opportunistic pathogens (2). Obesity has been associated with lower diversity and richness of the microbiota, and with a decreased Bacteroidetes-to-Firmicutes ratio (B/F) (3), however, this remains inconclusive as some studies have failed to show this association (4,5). Different studies support gut microbiota as an environmental factor related to the progress of obesity and metabolic disturbances (2,6), even though the causal nature of this has not been completely understood.

Weight loss is an effective strategy for obese and overweight individuals to reduce the risk of developing metabolic disorders and cardiovascular diseases (CVD). Lifestyle changes using different dietary strategies and

increasing physical activity promotion have been recommended to lose weight (7). Diet is an important factor in modulating not only weight but also gut microbiota composition and function. Several studies have shown a change in the gut microbiota associated with specific dietary factors or patterns (8–10). A recent study conducted in the NU-AGE trial demonstrated that higher adherence to a Mediterranean diet (MedDiet) pattern for 1-year was associated with specific gut microbiome changes that were associated with improved health status and reduced frailty (11). Another recent study, conducted amongst overweight and obese participants adhering to MedDiet or an isocaloric control diet for 8 weeks, showed significant improvements in a decrease in circulating total cholesterol, insulin resistance, and fecal bile acids related to changes in gut microbiota (12). Combining the beneficial effects of energy-restricted MedDiet and physical activity in a weight-loss perspective could aid in the betterment of cardiometabolic risk factors through changing gut microbiota profile. In this sub-study conducted in the framework of the PREDIMED-Plus randomized trial, as the primary objective we evaluated the 1-year effect of energy-reduced MedDiet weight-loss lifestyle intervention program versus non-energy restricted MedDiet intake on gut microbiota composition in overweight/obese adults with MetS. As a

secondary objective, we explored the associations of the gut microbiota composition with respect to the components of the intervention.

Methods

Study design and participants

The present study was conducted in the frame of the PREDIMED-Plus study, further details on Supplementary method 1. The primary outcome of the parent study, PREDIMED-Plus is weight loss and a composite of CVD incidence. Evaluation of gut microbiota composition is an intermediate outcome of the PREDIMED-Plus study. Eligible participants were community-dwelling male and female of the age 55-75 years, 60-75 years respectively, without documented history of CVD at baseline with overweight/obesity (BMI ≥27 and ≤40 kg/m2), and with at least three components of MetS according to the American Heart Association and National Heart, Lung and Blood Institute. Details of the trial have been described elsewhere (13). Further details on the study can be found at https://www.predimedplus.com/. This study was registered at the International Standard Randomized Controlled Trial (ISRCT; http://www.isrctn.com/ISRCTN89898870) with number 89898870 (Registration date: 24 July 2014). Participants were not involved in the

design or conduct or reporting of the study, further information can be found in Supplementary method 1.

In this sub-study, a total of 400 participants matched for age, sex and BMI were randomly selected from the intervention group (IG, n=200) and control group (CG, n= 200) from two PREDIMED-Plus study centers (Reus and Malaga). Briefly, participants randomized to the IG were instructed to adhere to an energy-reduced MedDiet, accompanied by physical activity promotion to accomplish specific weight loss objectives. Trained dietitians conducted an individual motivational interview, a group session, and a phone call each month during the intervention follow-up (1-year). IG received an intensive intervention consisting of individualized behavioral support and participants in the CG received information on maintaining ad libitum unrestricted caloric MedDiet with no advice on weight loss strategies such as to increase physical activity. In the case of the CG, they received only 1 individual session and 1 group session every 6 months to motivate and adhere to the intervention. Trained dietitians and nurses conducted the intervention and collect baseline and 1-year measurements and biological samples.

Evaluation of food consumption, anthropometric and biochemical measurements

At baseline and 12-month follow-up visits, nurses measured waist circumference (midway between the lowest rib and the iliac crest, using an anthropometric tape), weight (using electronic calibrated scales), and height (using a wall-mounted stadiometer) twice. Dietary consumption was estimated by the dietitians using a validated food frequency questionnaire, energy and nutrient consumption were calculated using the Spanish food composition tables. Mediterranean diet adherence score (MedScore) was calculated from a modified version of a previously validated questionnaire(14) (17 points validated tool) and information on physical activity was collected using a validated questionnaire(15). Serum and plasma samples were collected at baseline and 1-year following the intervention after an overnight fast, and then aliquoted, stored at -80°C. Standard enzymatic methods were conducted to evaluate serum total cholesterol, high-density lipoprotein (HDL) cholesterol, and triglycerides concentrations. Low-density lipoprotein (LDL) cholesterol was calculated by the Friedwald formula whenever triglycerides were less than 300 mg/dL.

Fecal sample collection and processing

Supplementary method 2 describes fecal sample collection. Fecal DNA extraction was conducted using QIAamp PowerFecal DNA Kit (Qiagen,

Hilden, Germany) according to the manufacturer's protocol, and an additional bead-beating step of 5 minutes using FastPrep-24 5G Homogenizer (MP Biomedicals, California, USA) was added to the first lysing step. The quantity of DNA was evaluated using Qubit 2.0 Fluorometer-dsDNA (High Sensitivity kit, Invitrogen, Carlsbad, California, USA). After extraction, the DNA was stored at - 20°C until further processing.

16S rRNA sequencing and processing

Supplementary method 3 provides detail on 16S rRNA gene sequencing. Briefly, we used the Ion Ribosomal 16S Kit (Thermo Fisher Scientific, Italy) that includes two primer sets selectively amplifying the corresponding hypervariable regions of the 16S region in bacteria: primer set V2–4–8 and primer set V3–6, 7–9. After sequencing, the individual sequence reads were filtered using Ion Reporter Software V4.0 to remove low-quality and polyclonal sequences. Data were processed and separated into 6 hypervariable regions using an adapted script available from Mas Lloret J et al (16). Only variable region V4 was used for further analyses. These files were imported to QIIME2 and DADA2 pipeline was followed (further on Supplementary information 3). Taxonomy was assigned to the clustered sequences with SILVA 132 as the 16S classifier database.

Mitochondrial features and features unidentified at the phylum level were removed in the pre-processing step in R (v 3.6) (17). MetagenomeSeq package was used to normalize the samples using the cumulative sum scaling and log transformation method.

Bioinformatics and statistical analysis

Baseline characteristics of study participants were described as mean and standard deviations (SD) or median with 25% and 75% interquartile range (based on distribution) for quantitative variables, and as percentages for categorical variables. Differences in baseline characteristics were evaluated with chi-square tests for categorical variables, t-tests (for normally distributed variables), and Wilcoxon tests (for non-normally distributed variables). Effects of intervention on changes in different variables were evaluated using Wilcoxon tests, are shown appropriatelyaccording to its distribution. Above mentioned Wilcoxon and t-tests were evaluated using package MatrixTests in R (v 3.6.2) (18) and significance was determined at $p < 0.05$.

For the microbiome analysis, normalized data from the MetagenomeSeq package was used (19). Alpha diversity (chao1, Shannon index), B/F, log of *Prevotella* to *Bacteroides* ratio (P/B) (adapted from (20)), and phylogenetic distance were evaluated using packages microbiome and

picante (21,22). Effect of intervention (Time*Treatment) adjusted by baseline weight, sex, and study center was used to estimate the changes in alpha diversity, B/F, P/B, and phylogenetic distance by linear mixed model. Additionally, for the B/F, P/B we also adjusted by baseline ratio values. Principal coordinate analysis (PCoA) based beta diversity (Weighted UNIFRAC distance, Unweighted UNIFRAC, Bray-Curtis dissimilarity) was evaluated with the vegan package in R (v 3.6.2) and PERMANOVA was conducted with *adonis* function (999 permutations) using participants as *strata* and also adjusting for baseline weight, sex and study center (23). The condition for homogeneity was verified using the *betadisper* function.

To investigate the changes in microbial genera between the intervention groups, *fitZig* function from the MetagenomeSeq package which implements a zero-inflated Gaussian model was used. We accounted for the repeated measures with a mixed model and the analysis was carried out at the genus level. According to the author's recommendation (24), we calculated effective sample sizes and retained only the genera that had an effective sample size more than the median of all samples. To reduce the type I error rate in multiple testing, we used the false discovery (FDR) approach to correct p-values. A FDR of 10% was set for the between-group

analysis. For the within-group analysis, we used *fitfeature* function that uses a zero-inflated log-normal model. Log fold changes in *fitfeature* were calculated from the coefficients of the zero-inflated log-normal model. As well here, we calculated effective sample sizes and report the only genus that passes the threshold. For this analysis, FDR of 5% was set.

We also used a second approach using sparse partial least square discriminant analysis (sPLS-DA) to compare the results from those obtained in MetagenomeSeq. This supervised method from the mixOmics package selects features that can best discriminate the two intervention groups at the end of the intervention (25). The samples were center log-ratio transformed (using package Hotelling) and indexed with respect to their baseline samples, which accounts for within participant variations (adapted from (26)). The number of components and feature per component were calculated using *tune.splsda* function, based on minimum balanced error rate. Each feature selected has an associated loading representing the relative importance of that feature on the component for discriminating the groups. This is represented as variable importance in projection (VIP) and feature with VIP of > 1 is regarded as important for discrimination. Features having VIP > 1, were chosen to be compared with the results of MetagenomeSeq.

The associations between changes in measured biochemical variables and changes in microbial genera that significantly changed either in the IG or the CG (and VIP >1), were analyzed using a NBZIMM package in R, which uses a negative binomial mixed model and allows to adjust for covariates (27). Coefficients obtained from this along with adjusted p-values were visualized in R software using ggplots2 (28).To detect the associations in the overall population, we adjusted for group of intervention, study center, sex and baseline weight. p-values were corrected by FDR for multiple testing.

PICRUST analysis

Predicted metagenome functions were performed using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States plugin (PICRUST2) (29) within QIIME2 with q2-picrust2 plugin. MetaCyc pathways (30) were normalized within QIIME2, and analyzed using the open-source software STAMP (Statistical Analysis of Metagenomics Profiles) with Welch's t-test option (31). Those pathways with a $p<0.05$ were posteriorly analyzed in QIIME2 with the longitudinal plugin for paired sampled comparisons. For this analysis FDR of 10% was set.

Results

Characteristics of the study population

Flow-chart of selected participants is represented in Supplementary Figure 1. A total of 400 participants matched by age, sex, BMI were randomized to this study (200 per intervention group). After preprocessing steps (as mentioned in the Supplementary Figure 1, Supplementary method 3), data at baseline was available for 183 participants in the IG, and 179 participants in the CG and corresponding to 171 participants in IG, 172 participants in CG after 1-year. There were no significant differences in the measured baseline variables between groups (Table 1), except for higher body weight in the intervention group (p=0.03). Diet, food groups (Supplementary Table 1) and physical activity changes (Table 2) were in the expected direction, with significant improvements in IG versus CG.

After 1-year (Table 2), IG participants lost an average of 4.2 (IQR, -6.8, -2.5) Kg vs. 0.2 (IQR, -2.1, 1.4) Kg in the CG (P <0.001). Reductions in BMI, waist circumference, and levels of triglycerides, glucose, and glycated hemoglobin were greater in the IG than CG participants (all, P < 0.05) whereas a significant higher increase in HDL cholesterol was observed in the IG compared to the CG (P<0.05) (Table 2). Even though participants belonged to a Mediterranean region, the baseline MedDiet score was equal to or below the median MedDiet adherence score (low (≤

7), medium (8-10), and high (11-17)) in both arms of intervention (32). This adherence increased with 1-year of intervention in both groups.

Changes in alpha and beta diversity

No significant differences in alpha-diversity indices (Chao1, Shannon) adjusted for body weight at baseline between the two intervention groups or within groups were observed (Table 3). Time and treatment interaction did not vary significantly for weighted UNIFRAC, unweighted UNIFRAC, or Bray-Curtis dissimilarity (Table 3, Figure 1). Likewise, no differences were noted at baseline and 1-year time point (Supplementary Table 2). B/F increased significantly in the IG compared to the CG ($P < 0.05$), however, no changes in P/B were observed (Supplementary Table 3, Supplementary Figure 2 A-B). No differences in baseline alpha, beta diversity, B/F, and P/B were observed between the groups.

Effect of intervention on changes in gut microbiota

Differential abundance analysis between the two groups of intervention conducted at Genus level showed *Haemophillus*, *Butyricicoccus*, *Eubacterium hallii*, *Ruminiclostridium 5* were reduced and, *Coprobacter*, *uncultured bacterium* (from Rhodospirillales Order) increased in the IG compared to the CG (all FDR $p<0.1$) (Table 4, Supplementary Figure 3 (A-H)) while adjusting for sex, study center and baseline weight. LogFC

represents the coefficient of change in the Metagenomeseq model evaluated comparing IG vs CG. Some of the genera (*Haemophillus*, *Eubacterium halii*, *Ruminococcus NK4A214*) that were found to vary significantly between the groups in Metagenomeseq model, also contributed to characterizing the IG and CG in the sPLS-DA model (Supplementary Figure 4).

Figure 2 shows the Venn diagram of genera that shifted within both groups. Fifteen genera in the IG (Figure 2) and sixteen genera in the CG (Figure 2) were significantly different from baseline to 1-year within each intervention group and had a VIP >1 from the sPLS -DA model. Within IG analysis, seven amongst fifteen genera reducing in relative abundance belonged to the family *Lachnospiraceae*, whereas some of these such as *Roseburia*, *Dorea* increased in the CG (Supplementary Table 4, 5). Increase in some SCFA-producers such as *Lachnospira* and *Lachnospiraceae NK4A136* group were observed in both intervention groups (Supplementary Table 4, 5). Overall predominant changes in both groups belonged to genera from Lachnospiraceae and Ruminococacceae families.

Associations between changes in gut microbiota and measured variables

In the overall population, as well individually within groups, changes in *Eubacterium eligens* were negatively associated with changes in weight (FDR $p< 0.05$) waist circumference (insignificant FDR), glucose (insignificant FDR), HbA1c (insignificant FDR) (Figure 3, Supplementary Figure 5A, 6A). *Haemophilus*, which varied significantly between the groups of intervention, was positively associated with weight changes in the overall population (Figure 3). *Parabacteroides* was positively associated with triglyceride levels in the overall population, as well as in IG and CG (Figure 3, Supplementary Figure 5A, 6A). Interestingly, *Phascolarbacterium* that positively associated with energy intake also followed the same direction for weight, BMI, waist circumference and glucose, whereas negatively with physical activity. Changes in fiber intake were negatively associated with changes in *Haemophilus*, whereas positively with changes in *Eubacterium hallii* and *Ruminococcaceae UCG-003* (Figure 4). *Lachnospiraceae NK4A136* group was also positively associated with MedScore (Figure 4). Few other associations within IG and CG were observed (Supplementary Figure 5(A-B), 6(A-B)).

Changes in bacterial predicted metagenomics functions during intervention

Metabolic pathways specially belonging to the biosynthesis of nucleotide, nucleoside and amino acids and carbohydrates changed significantly between the two intervention groups (Figure 5). Compared to the CG, fermentation pathways leading to the generation of energy were reduced in the IG.

Discussion

We report for the first time the effect of a large long-term lifestyle-based weight loss intervention with energy-reduced MedDiet and increased physical activity on gut microbiota. Several changes in the relative abundance of genera have been observed within and between the intervention groups that can be attributed to weight loss, diet, and physical activity. Changes observed in the gut microbiota profile were also associated with changes in some CVD risk factors.

We observed a significant change in the relative abundance of members belonging to Firmicutes phylum (Decreasing: *Butyricoccus*, *Ruminiclostridium 5*, *Eubacterium hallii;* Increasing: *Ruminococcaceae* NK4A214, *Coprobacter*) and a significant increase in the B/F in the IG compared to CG, which could partly be explained by higher weight loss in IG compared to CG. Even though widely debated, it has been reported that during weight loss, the ratio B/F increase, suggesting that they may respond to energy restriction (3,33,34). An increase in B/F has also been reported with higher adherence to MedDiet as well as low animal protein intake (35).

Other results from the above MedDiet adherence study (35), indicating an increase in the relative abundance of *Dorea*, *Roseburia,* and *Coprococcus*

(all reported as SCFAs producers of the *Lachnospiraceace* family), also were in line with our results only in the non-energy restricted MedDiet group (CG). However, in the IG, we observed these taxa to reduce in 1-year of intervention. Correspondingly, we also observed a decrease in the predicted fermentation pathways in IG compared to CG. Although the reduction in these carbohydrate/fiber utilizing SCFAs producers could indicate contradictory findings, some studies have observed an increase in SCFAs gut production in obese compared to the normal weight individuals (36,37). Whether this increase in SCFAs producers may be the cause or the consequence of obesity remains to be elucidated. The high energy deriving capacities of carbohydrate/polysaccharide utilizing bacteria could create a net energy excess for the host, contributing to obesity. However, SCFAs, especially butyrate and their producers have been well associated with several beneficial health effects (38), hence a careful evaluation of their composition as well quantity is required to infer further.

Even though there were reductions in certain SCFAs producers in IG, we observed within the same group a selective increase in other SCFAs producers (39) such as *Lachnospiraceae NK4A136* group and *Ruminococcaceae* (*UCG-003, UCG-002*) which also associated positively with MedScore. We also observed that some SCFA producing genera

(*Lachnospira*, *Lachnospiraceae NK4A136* group and *Alistipes*) shift in the same direction within both intervention groups, reflecting overall the effect of MedDiet on gut microbiota. Increase of proteins, polyphenols, and unsaturated fats, have shown inhibitory activities to certain bacterial genera (40–42). In parallel in IG, participants consumed higher protein, polyphenols, unsaturated fats compared to CG, possibly leading to selective enrichment in certain SCFAs producers compared to others that might be inhibited by a synergy of the above-mentioned components. It has been demonstrated in a mice study, that calorie restriction could limit butyrogenic and promote propiogenic enzymes, which could lead to competition and selective growth of SCFA producers (43,44).

Changes in *Coprococcus 3* was positively associated with changes in weight, total cholesterol, triglyceride and negatively with HDL in the overall population. In line with our results, enrichment of *Coprococcus* genus has been associated with a high lifetime CVD risk profile in Bogalusa Heart study participants, as well as with obese phenotype (45).

Not only *Coprococcus*, but also other genera majorly belonging to Lachnospiraceae family (*Blautia*, *Dorea*, *Roseburia*, *Coprococcus 3*) and *Ruminococcus 1* were observed to be changing in opposite directions in the IG and the CG. We observed a positive association for changes in the

relative abundance of *Coprococcus 3*, *Dorea* with changes in weight significantly in the overall population and non-significantly in both the intervention groups, consistent with a Swedish study (46). This study also reported a positive association of these genera with plasma branched-chain amino acids (BCAA's), usually increased in T2D and MetS (47,48). Similar observations were made in the METSIM cohort, where *Blautia* was associated with higher BMI and also higher circulating BCAA's, whereas *Chistensenellaceae* R-7 group abundance was negatively associated with BCAA's (49). Consistently we found a negative association of *Chistensenellaceae* R-7 group with changes in weight, BMI, triglycerides, and plasma glucose. It has been demonstrated that following MedDiet enriched with extra virgin olive oil reduced circulating levels of BCAA's and was associated with a lower risk of T2D (50). Taking these findings into consideration, we suspect BCAA's as one of the pathways for glucose regulation in the IG via MedDiet associated weight loss and corresponding changes in gut microbiota (51,52). These results could also indicate that even with following the same dietary pattern, factors such as energy restriction and physical activity could play an additional beneficial role in overweight/obese individuals by altering glucose regulation via BCAA's (53).

In the IG, we also observed changes in some previously bile acid-associated bacteria such as *Lachnoclostridium* (containing members of 7α-dehydroxylating capacity) and *Bilophila* (deconjugator of taurine-bile acid), that have shown to control lipid and glucose metabolism in mice studies (54,55). Consistently, we observed a positive (non-significant) association between *Lachnoclostridium* and glucose. The observations we make above are specific to the IG, indicating that calorie restriction along with increase in physical activity could modulate bile related bacteria (56). Compared to dietary interventions, very few studies have been conducted studying the effect of physical activity on gut microbiota, and with contradictory results. *Haemophilus*, *Phascolarctobacterium* that overall had shown a positive association in this study with risk factors assessed, also negatively associated with changes in physical activity. We suspect the associations we observe here are not solely dependent on physical activity, rather a synergy between energy homeostasis and nutrient intake. Predicted metagenomics functions have been shown to differ between adults with different body weight and health status. In our study, we observed that predicted functions of the bacterial community in the gut of IG were trying to adapt to energy restriction by increasing biosynthesis pathways, especially carbohydrate and nucleotide biosynthesis. However,

as protein and fat intake increased in IG, we also observed a decrease in amino acid, lipid biosynthesis indicating an adaptation to diet. Many of the observations made in this study should also be interpreted in terms of calorie restriction as it has been reported that calorie restriction, could alter gut microbiota and their functionality independent of dietary regime (57,58).

With exception of few landmark studies (11,59), our study explores the effect of a healthy lifestyle intervention on gut microbiota in a comparatively large sample population and follow-up (35,44,60). The RCT design of our study allows us to establish causality when assessing the effect of the interventions, being one of the most important strengths, however, this does not apply when we assess associations as secondary analyses. Another strength of the present study is that we have observed significant differences between groups in all components of the intervention (weight loss, adherence to MedDiet, and physical activity) in the expected direction, allowing us to test for potential effects of the intervention on gut microbiota. The nature of the intervention comprising dietary intervention, behavioral therapy, and physical activity promotion, indicates the multilevel intervention strategy that promotes participants to follow the intervention and obtain clinical benefits.

As much as this multifaceted intervention strategy is beneficial, it implies a limitation on the inference of results that cannot be attributed solely to a single component of the intervention. Along with this, some limitations of this study also deserve to be mentioned. Firstly, our findings are limited to adults with high BMI who also meet the criteria for MetS and were living in a Mediterranean country. Therefore, they cannot be generalized to other populations or all individuals with MetS. Secondly, the lack of data on fecal metabolites and species level taxonomy, do not allow us to infer further the pathways associated with the associations we have observed. Thirdly, the dietary records were collected from a self-reported questionnaire which might over/ underestimate the intake of certain food groups. Future studies with a comprehensive set of metabolomics, metagenomics, along with intermediate time points would allow to better understand the transition of gut microbiota during the weight-loss period.

Overall, in this 1-year lifestyle-based intervention, we observed that an energy-restricted Mediterranean diet with physical activity and behavioral support, induced weight loss and improves CVD-associated risk factors. Decrease in several members of Firmicutes, especially belonging to the *Lachnospiraceae* and a selective increase in some SCFA producers were observed in IG. This work identifies that even with similar healthy dietary

patterns the addition of an intervention program enhancing calorie restriction and physical activity could have a significant benefit on the CVD risk factors potentially modulated via the gut microbiota.

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Conflicts of interest:

JS-S declares that he is a member of Danone S.A.'s Advisory Board, a member of the Danone Institute, and that he received payments from Danone S.A. for the purposes of scientific and technical consulting. JS-S reports serving on the board of and receiving grant support through his institution from the International Nut and Dried Fruit Council, and Eroski Foundation. JS-S reports receiving consulting fees or travel expenses from Nuts for Life and Australian Nut Industry Council. JS-S declares that Food companies Hojiblanca (Lucena, Spain) and Patrimonio Comunal Olivarero (Madrid, Spain) donated extra virgin olive oil; and the Almond Board of

California (Modesto, CA), American Pistachio Growers (Fresno, CA), and Paramount Farms (Wonderful Company, LLC, Los Angeles, CA) donated nuts for the PREDIMED-Pilot study.

Other authors have nothing to disclose.

Authors' contributions:

Study was designed by JSS, MB, MAMG, FT, DC, JVL, JV, MF, sample collection and processing was conducted at two centers by JM, SG, AA, IMI, JCFG, LTC, RFC, RO, AMPG and MRBL. Statistical analysis was conducted by JM, IMI, SG and AA with supervision of MB, FT and JSS. Manuscript was written by JM, IMI, JSS, MB and FT and accepted by all the authors.

Table 1: Baseline characteristics of study participants

	Intervention group (IG)	Control group (CG)
N	183	179
Age (years)	64.3 (5.1)	65.1 (4.9)
Sex (M/F)	97/86	77/102
Weight (kg)	89.7 (13.6)	86.7 (11.56) †
BMI (Kg/m2)	33.4 (30.8,36.0)	32.9 (30.5, 35.6)
Waist circumference (cm)	110.7 (9.8)	108.9 (9.55)
Diabetes (yes, %)	26.2 % (48/135)	20.6 % (37/142)
Hypercholesterimia (No/Yes)	94.5 % (10/173)	93.8 % (11/168)
Total Cholesterol (mg/dL)	203.0 (177.0,224.5)	197.0 (172.5,226.5)
LDL Cholesterol (mg/dl)	116.0 (94.5,140.8)	115.0 (97.0, 139.5)
HDL Cholesterol (mg/dL)	46.0 (40.0,57.0)	47.0 (42.0,54.0)
Triglycerides (mg/dL)	151.0 (55.3,246.8)	152.0 (68.5, 235.5)
Glucose (mg/dL)	104.0 (92.5,118.0)	103.0 (94.0,116.0)
Glycated Hemoglobin (%)	5.8 (5.6,6.3)	5.8 (5.5,6.3)
Physical activity (METs-min/week)	1627 (682, 3650)	1767 (839, 3308)
Energy intake (Kcal/day)	2546.5 (543.7)	2416.6 (514.7)
17-point Mediterranean adherence score	7.7 (2.1)	8 (2.4)
Smoking		
Current smoker	32	24
Former smoker	65	68
Never	85	87
No data	1	
Study center (Malaga/Reus)	66/117	73/106

Values expressed as Mean (SD) for normally distributed variables and Median (25% and 75% IQR) for non-normal distributions. † Significant difference <0.05. IG Intervention group, CG Control group. Chi-square, Wilcoxon and t-test were conducted for categorical, non-normal and normally distributed variables respectively

Table 2: Effects of intervention on anthropometric and biochemical variables measured

	Changes in IG (n= 171)	**Changes in CG (n=172)**	**p-value**
Weight (kg)	-4.2 (-6.8, -2.5)	-0.2 (-2.1, 1.4)	<0.001
BMI (Kg/m2)	-1.6 (-2.5, -0.9)	-0.05 (-0.8, 0.6)	<0.001
Waist circumference (cm)	-5 (-9.0, -1.8)	0.0 (-2.5,2.0)	<0.001
Total Cholesterol (mg/dL)	-1.0 (-17.5,14.0)	-2.0 (-22.0, 14.0)	0.767
LDL Cholesterol (mg/dl)	1.0 (-14.5,14.5)	-2.0 (-19.0, 13.0)	0.577
HDL Cholesterol (mg/dL)	3.0 (-0.5, 6.0)	2.0 (-2.3,6.0)	0.012
Triglycerides (mg/dL)	-19.0 (-52.5, 9.5)	-3.5 (-41.5,28.0)	0.028
Glucose (mg/dL)	-5.0 (-14.0, 2.0)	0.5 (-7.3, 8.0)	<0.001
Glycated Hemoglobin (%)	-0.1 (-0.3, 0.1)	0.0 (-0.1, 0.2)	0.002
Physical activity (METs-min/week)	1154 (0, 2633)	0 (-787, 743)	<0.001
17-point Mediterranean adherence score	6.0 (4.0, 8.5)	2.0 (1.0, 5.0)	<0.001
Energy intake (Kcal/day)	-318.2 (-655.6, 3.2)	44.3 (-329.3, 391.7)	<0.001

Values expressed as Median (25% and 75% IQR). IG Intervention group, CG Control group. Wilcoxon test was conducted for evaluating the differences between two groups of intervention

Table 3: Effects intervention on changes in alpha and beta diversity metrics

Diversity measures (n=343)	Treatment* Time (p-value)
Chao1[a]	0.16
Shannon diversity [a]	0.15
Phylogenetic distance [a]	0.21
Weighted Unifrac[b]	0.72
Unweighted Unifrac[b]	0.23
Bray Curtis dissimilarity[b]	0.33

[a] alpha diversity indexes, [b] beta diversity indexes. Effect of intervention (Treatment*Time) evaluated by linear mixed model adjusted for sex, study center and baseline weight for chao1, Shannon diversity and phylogenetic distance. Weighted Unifrac, Unweighted Unifrac, Bray Curtis dissimilarity were evaluated by PERMANOVA adjusted for sex, study center and baseline weight, participants as strata

Table 4: Differentially abundant genus between groups of intervention

Genus	logFC (ΔIG-ΔCG)	p-value	Adjusted p-value
Haemophilus	-7.6	<0.001	<0.001
Butyricicoccus	-4.2	<0.001	<0.001
Ruminiclostridium 5	-2.2	0.003	0.09
Eubacterium hallii	-2.2	0.006	0.08
O_Rhodospirillales_F_ uncultured_uncultured bacterium	4.3	0.006	0.05
Ruminococcaceae NK4A214	2.6	0.007	0.08
Coprobacter	2.3	0.030	0.08

IG Intervention group, CG Control group. Model adjusted for baseline weight, sex and study center. logFC is the beta estimate of the adjusted model. P-value adjusted by FDR for multiple testing

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covariates sex, study center, baseline body weight; adjusted p-value denoted by < 0.001 "***", < 0.05 "**", < 0.1 "*", <0.2 "º"

Figure 5: Heat map representing median values of significantly increasing or decreasing predicted metagenome pathways in the IG (n=171) and CG (n= 172). IG: Intervention group, CG: Control group

Figure 1: A) PCoA of weighted UniFrac distance showing two groups of intervention at two time points (CB, Control group at baseline; CO, Control group at year-1; TB, Intervention group at baseline; TO, Intervention group at year-1); B) PCoA of unweighted UniFrac distance; C) PCoA of Bray Curtis distance (n =343)

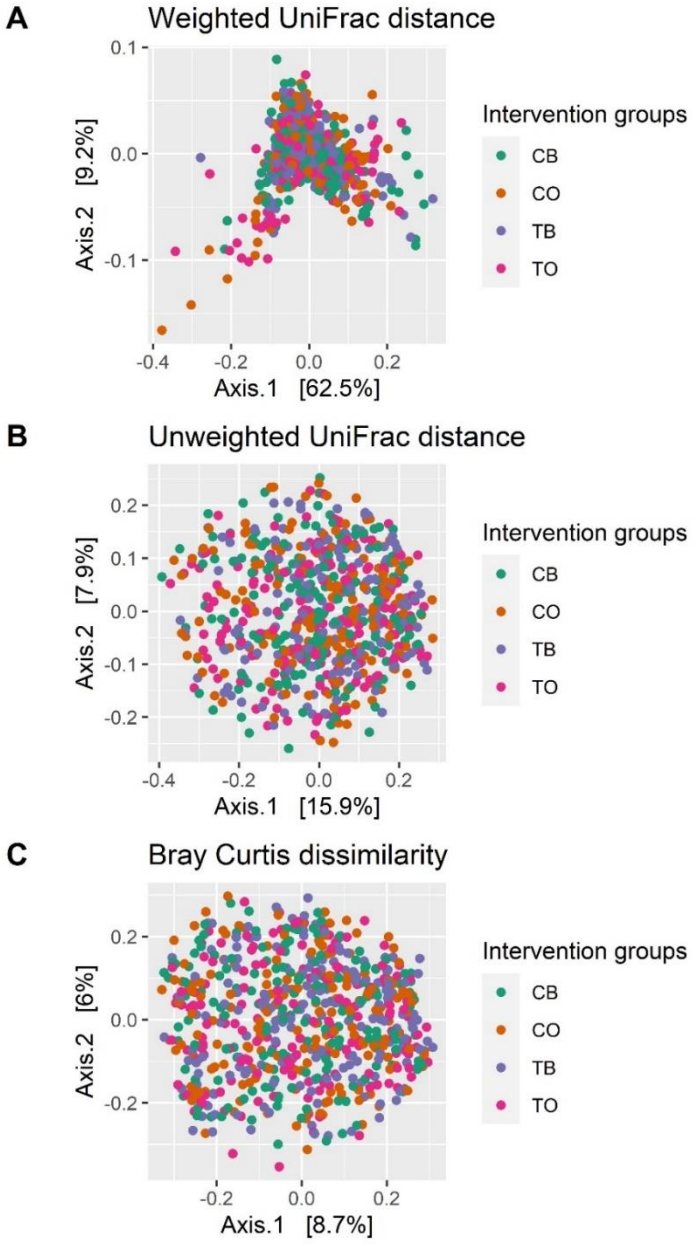


Figure 2: Venn diagram representing IG and CG by genera varying within groups evaluated with Metagenomeseq and having a VIP >1 from sPLS-DA model. Genera shifting in same direction as well in opposite directions within each intervention groups are shown in the figure. IG: Intervention group, CG: Control group

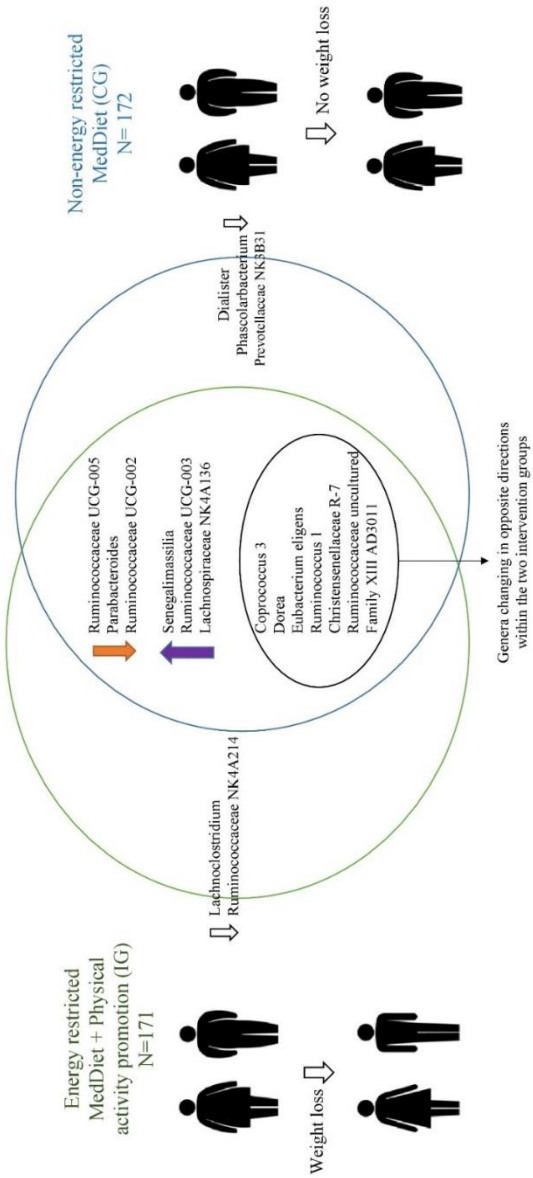


Figure 3: Heat plot showing associations in overall study population (n= 343) between changes in microbial genera and Clinical variables. Model evaluated by negative binomial mixed model, adjusting for covariates sex, study center, baseline body weight; adjusted p-value denoted by < 0.001 "***", < 0.05 "**", < 0.1 "*", <0.2 "o"

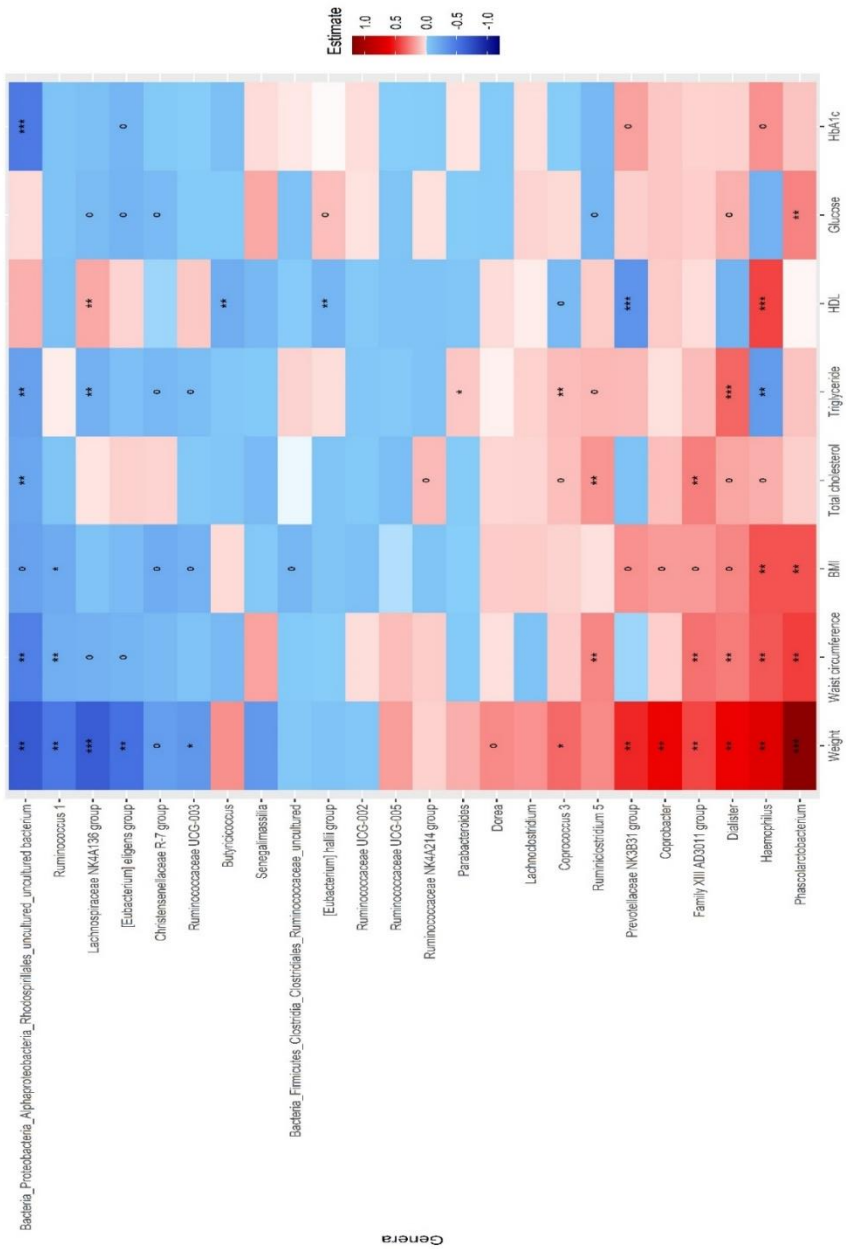
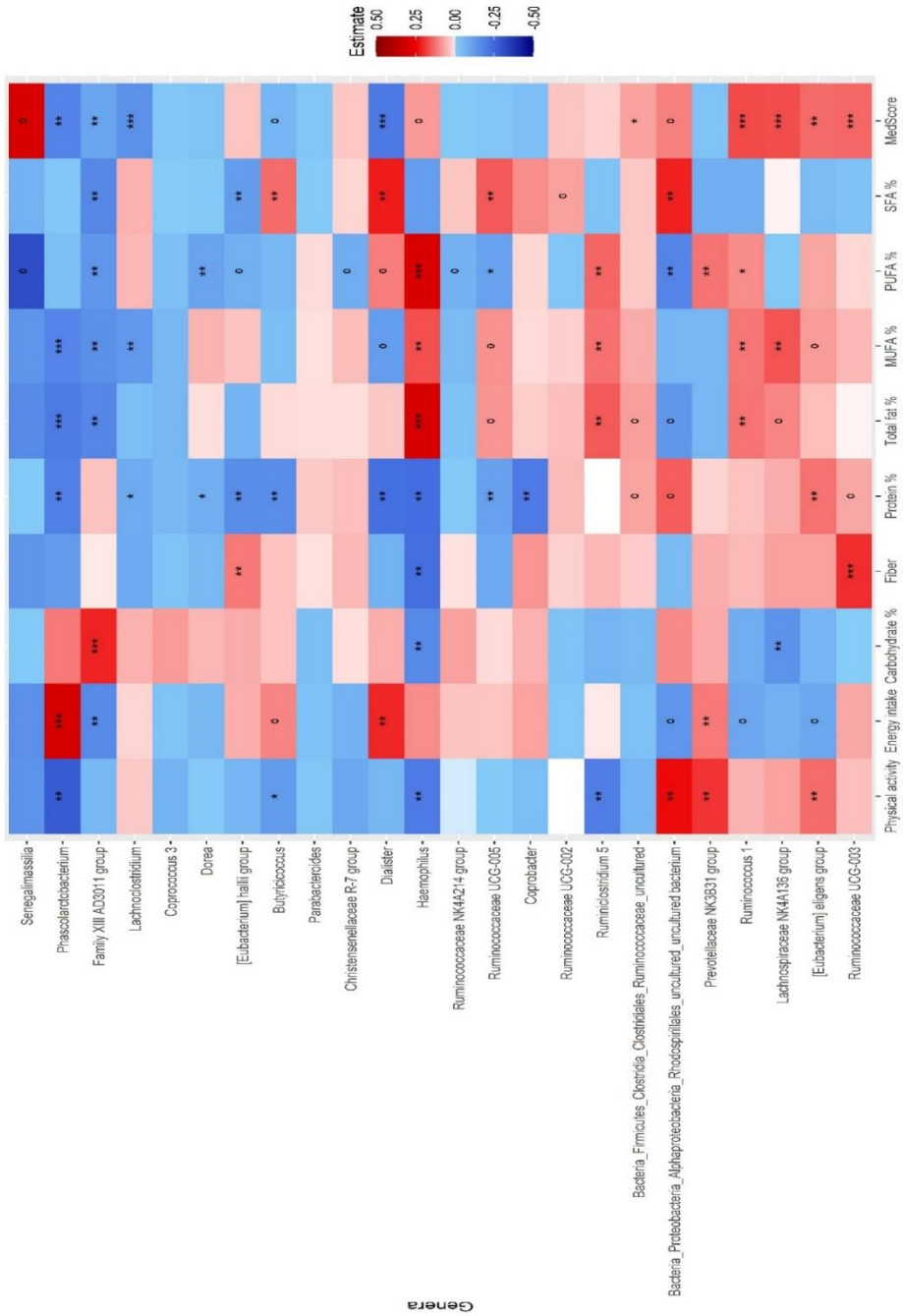


Figure 4: Heat plot showing associations in overall study population (n= 343) between changes in microbial genera and Energy intake variables. Model evaluated by negative binomial mixed model, adjusting for covariates sex, study center, baseline body weight; adjusted p-value denoted by < 0.001 "***", < 0.05 "**", < 0.1 "*", <0.2 "o"



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ONLINE SUPPLEMENTARY MATERIAL

Effect on gut microbiota of a 1-year lifestyle intervention with Mediterranean Diet versus Energy-Reduced Mediterranean Diet and Physical Activity Promotion. PREDIMED-Plus Study

Muralidharan J et al.

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Supplementary method 1: Study design and participants:

PREDIMED Plus (in Spanish: PREvención con DIeta MEDiterránea) study is a multicenter parallel randomized controlled study conducted in Spain to evaluate the effect on cardiovascular disease and mortality of an intensive weight-loss lifestyle intervention based on an energy-restricted MedDiet, physical activity promotion, and behavioural support (IG) or a control group receiving recommendations to adhere to a unrestricted caloric Mediterranean diet (CG). Primary endpoint of the intervention was to evaluate cardiovascular outcome, stroke or cardiovascular mortality. Study participants were randomized 1:1 into two equally sized groups and allocation was generated by computer based on stratification by sex, age and center. Complete description of the study and selection of participants have been described before [1]. Participants were not involved in the study design or conduct or report of the research. Medical doctors from primary care centers of the groups that belonged to the PREDIMED Plus study, were involved in the recruitment of participants. Four weeks of run in period including initial screening visit, a phone call at 2 weeks and final evaluation visit was arranged for each participant. Initial screening visit was arranged for the participants to understand the purpose and characteristics of the study after which a consent form was signed if they were interested to participate in the study.

Even though participants belonged to the Mediterranean region and assumed to consume a MedDiet already, the baseline MedDiet adherence score showed that overall participants were not following a strict traditional MedDiet pattern (mean(SD) IG= 7.7 (2.1), CG= 8 (2.4). With this in focus, participants in both arms of interventions were encouraged to follow a traditional MedDiet. In CG, participants received a written material with recommendations to follow the MedDiet, whereas participants in the IG were prescribed similar recommendations on MedDiet, however with an energy restriction on total energy intake, following a 17 point scale (described in [1]).Along with the explanation of the intervention diet, participants in the IG were given support with dietary materials, including general recommendations, a dietary plan, open menus and seasonal recipes, all according to the aimed energy restriction

calculated for each participant (energy restricted diets from 1200 to 3000 kcal/day were available). Dieticians delivered personalized and updated dietary counseling throughout the entire intervention by assessing the projected and achieved monthly weight-loss objectives and the accomplishment of the scores achieved in the 17-item questionnaire. In order to support the participants in IG with behavioral support, they were assisted with problem-solving strategies and practical tools to facilitate self-control on emotional eating or stress-driven behaviors, such as over intake, consumption of highly palatable foods or engaging in sedentary behaviors. Moreover, it included self-management approaches to improve participants' autonomy and empowerment in order to increase their long-term adherence to the dietary and physical activity recommendations. Participants in the CG received educational sessions with the same content to that used in the PREDIMED study (1). Accordingly, dietitians recommended an energy-unrestricted traditional MedDiet and individual visits and group sessions were programmed every 6 months during the first year. Participants in both intervention groups were provided with extra virgin olive oil (1L/month) and mixed nuts (125g/month) for free. Mediterranean diet adherence score (MedScore) was calculated from a 17 point validated tool [2]. Information on physical activity was collected using a validated questionnaire [3,4]. Five centers from PREDIMED-Plus study collected fecal samples, out of which Reus and Malaga, the two centers included in this study where the firsts to begin collection of sample. For the feasibility of study, we prioritized these two centers in the analysis of gut microbiota composition. " For this sub study we initially evaluated the sample size by previous literature on [5–8]. Further, we calculated effect size based on previous literature with available relative abundance of the common microbial taxa data at baseline and end-point. With an alpha of 0.05 and calculated effect size, we simulated various number of sample size ranging from 10-170 per group with the "powerAnalysis" package in R [9]. The minimum required number of samples per group ranged from 10 for *Lachnospira*, 30 for *Bacteroidetes*, 50 for *Bacteroides*, 110 for *Blautia*, 150 for *Bifidobacterium* and highest of 170 for Chao index.

For this sub study, samples from two of the centers of PREDIMED Plus were randomized based on age, sex, BMI and center criteria in a 1:1 composition. As shown in Supplementary figure 1, of the 400 participants randomized into IG and CG in a 1:1 manner, 62 samples were removed due to low DNA concentration after extraction or due to sample unavailability during processing. After the DADA2 filtering steps, 11 samples were removed due to low quality. Out of the 727 samples processed downstream, 14 samples were removed due to low sequence quality. This led to a sample size of 362 participants at baseline (IG =183, CG= 179), 351 participants at year-1 (IG = 173, CG=178). Amongst this, 1 sample in IG and 6 samples in CG did not have a baseline matched pair, hence were removed subsequently from the analysis.

Supplementary method 2: Fecal sample collection

Faecal samples were collected at baseline and 1-year in a sterile hermetic flask and they were instructed to bring it to the study center soonest possible (within 12 hours of excretion) in a cooled condition (with provided ice pack or stored at -20°C). Samples were aliquoted into approximately 250 g aliquots and stored at was -80°C. Participants who were using antibiotics or pre/probiotics 15 days before sample collection were asked to do the collection 15 days after their use was stopped.

Supplementary method 3: 16S rRNA sequencing and processing

Ribosomal 16S rRNA gene sequences were amplified from DNA using the Ion 16S Metagenomics Kit (Thermo Fisher Scientific, Italy). The kit includes two primer sets that selectively amplify the corresponding hypervariable regions of the 16S region in bacteria: primer set V2–4–8 and primer set V3–6, 7–9. Libraries were created using the Ion Plus Fragment Library Kit (Thermo Fisher Scientific, Italy). Barcodes were added to each sample using the Ion Xpress Barcode Adapters kit (Thermo Fisher Scientific, Italy). Emulsion PCR and sequencing of the amplicon libraries were performed on an Ion 530 chip (Ion 530TM Chip Kit) using the Ion Torrent S5TM system and the Ion 510/520TM/530TM Kit-Chef (Thermo Fisher Scientific, Italy) according to the manufacturer´s instructions. After sequencing, the individual sequence reads were filtered using Ion Reporter

Software V4.0 to remove low quality and polyclonal sequences. Data were processed and separated into 6 hypervariable regions using an in house adapted script available from Mas Lloret J et al [10]. Only variable region V4 was used for further analyses. These files were imported to QIIME2 and DADA2 pipeline was followed [11]. After 285 reads, the quality fell rapidly, hence sequences were trimmed at position 285. Chimeras were removed in the DADA2 pipeline. Taxonomy was assigned to the clustered sequences with SILVA 132 as 16S classifier database with 99% similarity. Mitochondrial features and features unidentified at phylum level were removed in the pre-processing step in R (v 3.6) [12]. MetagenomeSeq package was used to scale the samples using cumulative sum scaling and log transformation method was applied [13]. Prior to this step, we removed spurious features (constituting less than 10 counts) and samples with less than 500 reads.

Supplementary Table 1: Energy intake, nutritional composition at baseline and 1-year changes

	Baseline IG	Baseline CG	p-value[1]	Change in IG	Change in CG	p-value[2]
N	**183**	**179**		**171**	**172**	
Total Energy (Kcal/day)	2561.8 (2177.6,2841.9)	2416.6 (2105.4,2861.5)	>0.05	-318.2 (-655.6, 3.2)	44.3 (-329.3, 391.7)	<0.001
Carbohydrate (% TE)	40.1 (6.0)	40.6 (5.9)	>0.05	-5.2 (6.2)	-2.2 (6.9)	<0.001
Protein (% TE)	16.5 (14.9, 18.2)	16.7 (15.0,18.2)	>0.05	1.8 (2.6)	0.0 (2.2)	<0.001
Total fat (%TE)	40.2 (5.51)	39.9 (5.1)	>0.05	3.5 (-0.6,7.0)	1.7 (-1.4,7.2)	>0.05
Mono unsaturated fat (%TE)	20.8 (3.9)	20.3 (3.6)	>0.05	4.4 (1.3,7.4)	2.8 (-0.3, 6.8)	0.011
Poly unsaturated fat (%TE)	6.3 (6.4, 6.9)	6.1 (6.1, 6.6)	>0.05	1.3 (1, 1.7)	0.6 (0.3, 1.1)	<0.001
Saturated fat (%TE)	9.9 (1.7)	10.1 (1.7)	>0.05	-1.0 (-2.0,0.4)	-0.7 (-1.7, -0.4)	>0.05
Fiber(g/day)	25.4 (21.6,30.4)	25.9 (22.0,31.2)	>0.05	5.9 (8.9)	2.4 (8.0)	<0.001
Alcohol (g/day)	4.98 [1.28, 18.31]	4.38 [0.68, 12.33]	>0.05	-0.68 [-4.41, 0.40]	0.00 [-1.99, 2.32]	0.02
Total polyphenols (mg/day)	860.04 (283.56)	866.19 (265.09)	>0.05	-3.83 (288.38)	-28.20 (280.56)	>0.05
Food groups (g/day)						
Vegetables	328.57 (121.54)	360.36 (139.81)	0.021	65.66 (134.76)	-2.76 (144.33)	<0.001
Fruits	332.26 (161.22)	350.80 (181.32)	>0.05	64.51 [-28.23, 132.17]	32.60 [-75.45, 164.00]	>0.05
Legumes	20.56 [15.98, 29.70]	20.56 [15.98, 29.70]	>0.05	8.57 [0.00, 16.85]	0.00 [-4.57, 8.57]	<0.001
Cereals	168.55 (69.65)	156.06 (67.63)	>0.05	-51.29 (74.38)	0.28 (81.90)	<0.001
Dairy	310.16 (186.33)	334.93 (198.51)	>0.05	-21.80 (174.34)	-46.30 (180.71)	>0.05

	Baseline IG	Baseline CG	p-value[1]	Change in IG	Change in CG	p-value[2]
Meat	163.86 (61.62)	155.57 (57.51)	>0.05	-24.34 (55.81)	-7.25 (57.96)	< 0.05
Fish	106.90 [77.71, 130.67]	107.29 [82.14, 132.95]	>0.05	23.85 (52.06)	4.61 (49.45)	0.001
Nuts	12.85 [4.28, 26.64]	10.57 [5.99, 20.14]	>0.05	19.43 [6.57, 30.43]	12.85 [4.28, 25.71]	< 0.05
Olive oil	42.15 (14.96)	39.41 (14.15)	>0.05	2.93 (17.37)	9.75 (20.70)	0.001

IG Intervention group, CG Control group. p-value[1] for differences between IG and CG at baseline; p-value[2] differences between changes from baseline in two groups; TE, Total Energy. Wilcoxon and t-test were conducted for non-normal and normally distributed variables respectively

Supplementary Table 2: Beta-diversity metrics pre- and post-intervention

	Baseline (p-value)	1-yr (p-value)
Weighted Unifrac	0.615	0.91
Unweighted Unifrac	0.313	0.91
Bray Curtis dissimilarity	0.486	0.99

PERMANOVA conducted with adonis at baseline and 1 year time points

Supplementary Table 3: Bacteroidetes to Firmicutes and Prevotella to Bacteroides ratio

Other metrics	Treatment* Time (p-value)
Bacteroidetes-to- Firmicutes ratio	0.008 †
Log Prevotella-to-Bacteroides ratio	0.15

† Significant difference <0.05. IG Intervention group, CG Control group. Linear mixed model used to evaluate the effects adjusting for baseline ratio, baseline body weight, sex and study center.

Supplementary Table 4: Differentially abundant genus within Intervention group (IG)

Family	Genus	logFC	p	p.adj
Ruminococcaceae	*Ruminococcus 2*	-0.39	0.01	0.016
Ruminococcaceae	*Eubacterium coprostanoligenes*	-0.26	0.01	0.011
Lachnospiraceae	*Lachnoclostridium*[1]	-0.23	0.02	0.034
Barnesiellaceae	*Barnesiella*	-0.19	0.03	0.043
Lachnospiraceae	*Coprococcus 3*[1]	-0.17	0.01	0.016
Ruminococcaceae	*Ruminococcaceae UCG-005*[1]	-0.15	0.01	0.015
Lachnospiraceae	*Blautia*	-0.13	0.00	0.004
Lachnospiraceae	*Dorea*[1]	-0.12	0.00	0.002
Desulfovibrionaceae	*Bilophila*	-0.07	0.02	0.031
Ruminococcaceae	*Ruminococcus 1*[1]	-0.05	0.00	0.005
Lachnospiraceae	*Lachnospiraceae FCS020 group*	-0.05	0.00	0.000
Tannerellaceae	*Parabacteroides*[1]	-0.04	0.01	0.010
Ruminococcaceae	*Ruminococcaceae UCG-002*[1]	-0.03	0.00	0.004
Lachnospiraceae	*Roseburia*	-0.03	0.00	0.001
Peptostreptococcaceae	*Intestinibacter*	-0.03	0.01	0.010
Lachnospiraceae	*Ruminococcus gauvreauii group*	-0.03	0.00	0.006
Ruminococcaceae	*Ruminococcaceae NK4A214 group*[1]	-0.01	0.01	0.019
Marinifilaceae	*Butyricimonas*	0.02	0.00	0.004
Ruminococcaceae	*Ruminococcaceae_uncultured*[1]	0.03	0.00	0.001
Christensenellaceae	*Christensenellaceae R-7 group*[1]	0.03	0.02	0.027
Barnesiellaceae	*Coprobacter*	0.05	0.03	0.043
Rikenellaceae	*Alistipes*	0.11	0.01	0.010
Eggerthellaceae	*Senegalimassilia*[1]	0.11	0.00	0.001
Lachnospiraceae	*Eubacterium eligens group*[1]	0.15	0.00	0.008
Ruminococcaceae	*Ruminococcaceae UCG-003*[1]	0.16	0.00	0.006

Family	Genus	logFC	p	p.adj
Lachnospiraceae	*Lachnospiraceae NK4A136 group*[1]	0.17	0.00	0.004
Lachnospiraceae	*Lachnospira*	0.18	0.03	0.038
Ruminococcaceae	*Negativibacillus*	0.19	0.00	0.003
Family XIII	*Family XIII AD3011 group*[1]	0.24	0.01	0.014
Eggerthellaceae	*Slackia*	0.26	0.00	0.004

N=171 paired samples. logFc is the beta estimate from the MetagenomeSeq analysis. P.adj are the FDR adjusted p values for multiple testing. [1] Genera with VIP>1 in Spls-da model

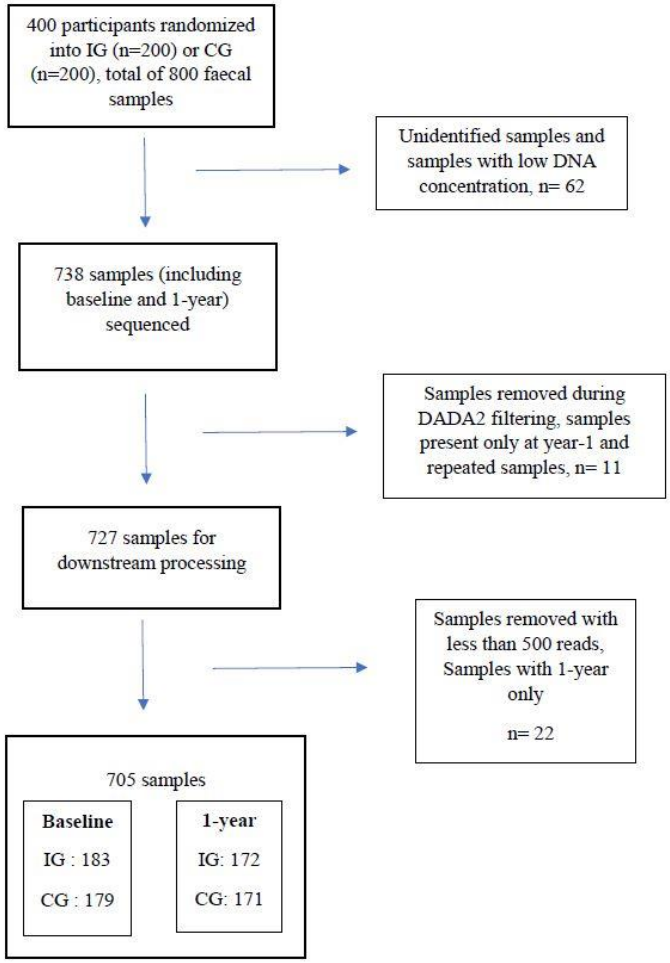
Supplementary Table 5: Differentially abundant genus within Control group (CG)

Family	Genus	logFC	p	p.adj
Veillonellaceae	*Dialister*[1]	-0.43	0.02	0.029
Barnesiellaceae	*Coprobacter*	-0.43	0.00	0.003
Family XIII	*Family XIII UCG-001*	-0.32	0.00	0.000
Acidaminococcaceae	*Phascolarctobacterium*[1]	-0.32	0.01	0.013
Lachnospiraceae	*Lachnospiraceae FCS020 group*	-0.31	0.00	0.000
Tannerellaceae	*Parabacteroides*[1]	-0.17	0.00	0.007
Christensenellaceae	*Christensenellaceae R-7 group*[1]	-0.16	0.01	0.021
Marinifilaceae	*Butyricimonas*	-0.16	0.00	0.003
Family XIII	*Family XIII AD3011 group*[1]	-0.11	0.00	0.000
Peptostreptococcaceae	*Intestinibacter*	-0.10	0.00	0.006
Ruminococcaceae	*Ruminococcaceae UCG-002*[1]	-0.10	0.00	0.004
Ruminococcaceae	*Ruminococcaceae UCG-005*[1]	-0.09	0.03	0.039
Ruminococcaceae	*Eubacterium coprostanoligenes group*	-0.06	0.01	0.017
Ruminococcaceae	*Ruminococcus 2*	-0.05	0.01	0.012
Victivallaceae	*Victivallis*	-0.02	0.01	0.019
Prevotellaceae	*Prevotellaceae NK3B31 group*	-0.02	0.02	0.033
Lachnospiraceae	*Eubacterium eligens group*[1]	-0.003	0.00	0.006
Ruminococcaceae	*uncultured*	-0.003	0.00	0.001
Lachnospiraceae	*Roseburia*	0.004	0.00	0.002
Rikenellaceae	*Alistipes*	0.02	0.02	0.024
Lachnospiraceae	*Lachnospira*[1]	0.03	0.01	0.017
Ruminococcaceae	*Ruminococcus 1*[1]	0.05	0.01	0.013
Ruminococcaceae	*Ruminococcaceae UCG-003*[1]	0.07	0.00	0.007
Ruminococcaceae	*Unidentified*	0.10	0.01	0.012
Lachnospiraceae	*Dorea*[1]	0.10	0.00	0.001

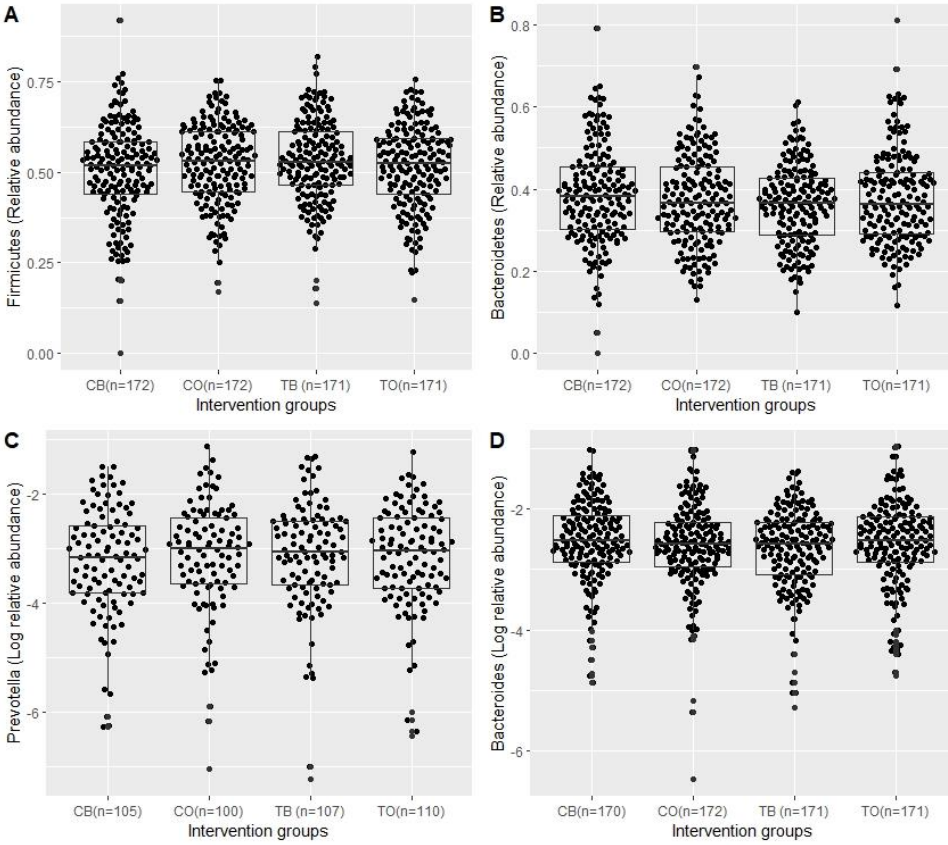
Family	Genus	logFC	p	p.adj
Lachnospiraceae	*Ruminococcus gauvreauii group*	0.11	0.00	0.007
Lachnospiraceae	*Eubacterium ruminantium group*	0.13	0.02	0.032
Lachnospiraceae	*Lachnospiraceae NK4A136 group*[1]	0.13	0.00	0.005
Lachnospiraceae	*Coprococcus 3*[1]	0.13	0.00	0.004
Ruminococcaceae	*Negativibacillus*	0.14	0.00	0.001
Ruminococcaceae	*Ruminiclostridium 5*	0.17	0.01	0.011
Eggerthellaceae	*Senegalimassilia*[1]	0.17	0.00	0.001
Lachnospiraceae	*Blautia*	0.21	0.01	0.013

N=172 paired samples. logFc is the beta estimate from the MetagenomeSeq analysis. P.adj are the FDR adjusted p values for multiple testing. [1] Genera with VIP>1 in Spls-da model

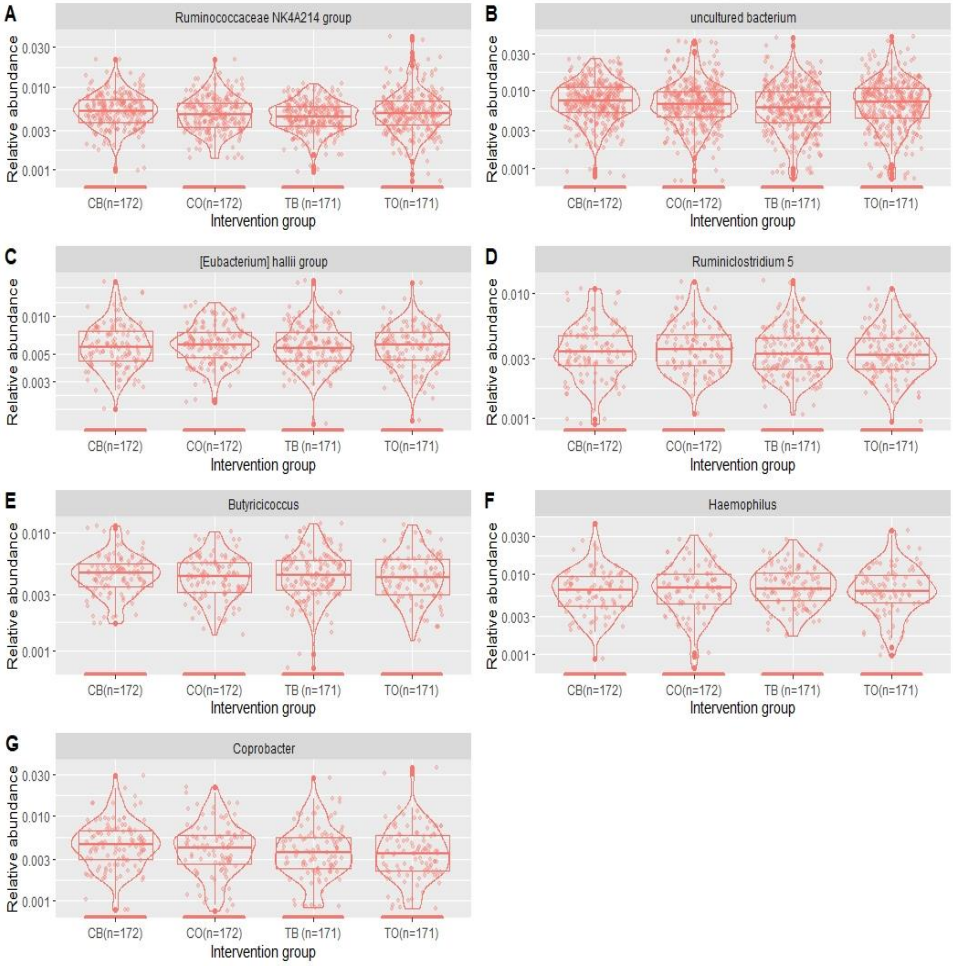
Supplementary Figure 1: Flow chart of participants included in the study



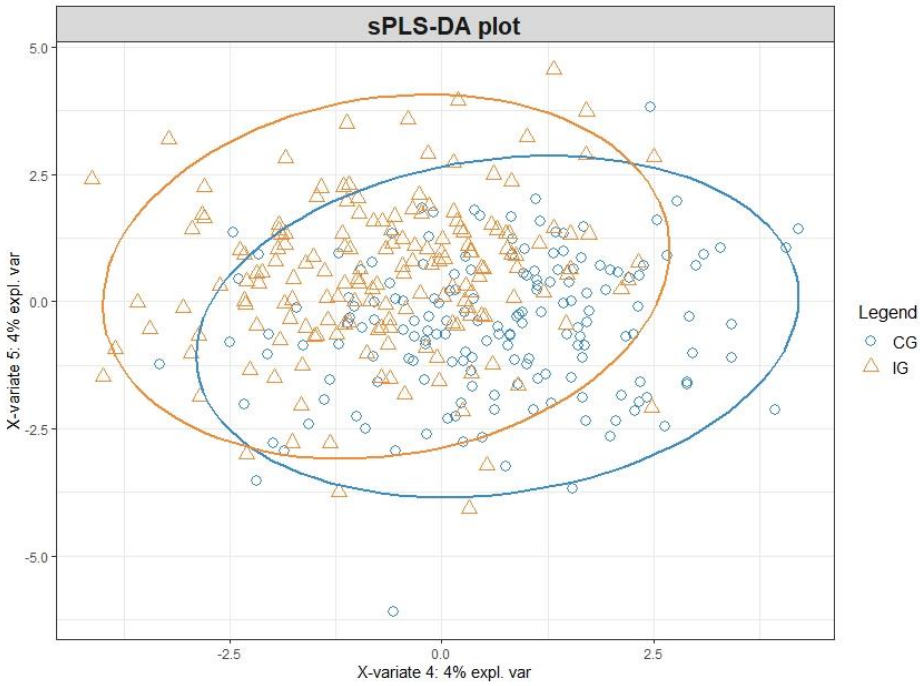
Supplementary Figure 2: Relative abundances plotted with boxplot and beeswarm plot of: A-B) phylum Firmicutes, Bacteroidetes; C-D) (log) of genera *Prevotella* and *Bacteroides*



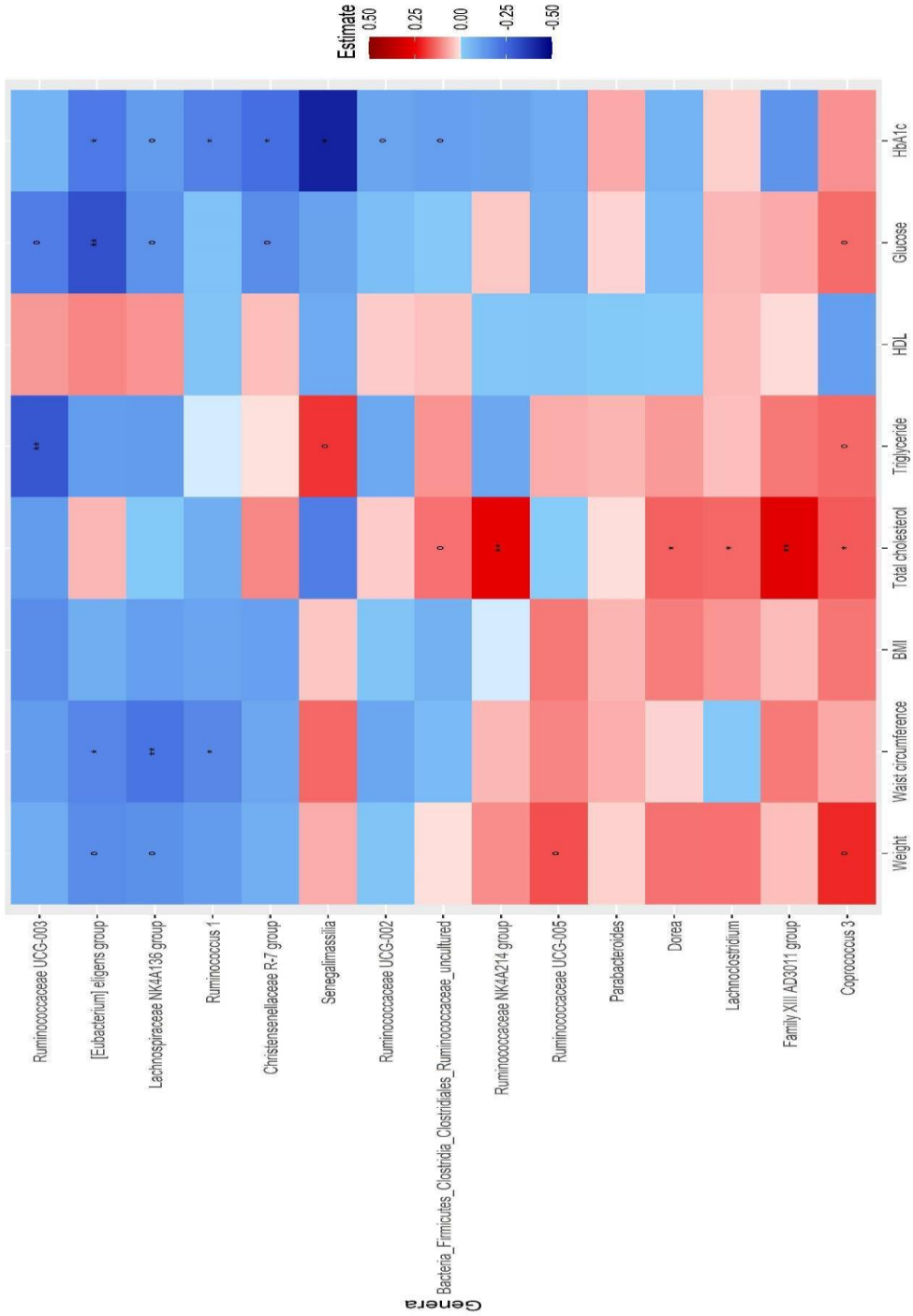
Supplementary Figure 3: Violin plots for genera that are differing significantly between both intervention groups. CB, Control group at baseline; CO, Control group at year-1; TB, Intervention group at baseline; TO, Intervention group at year-1



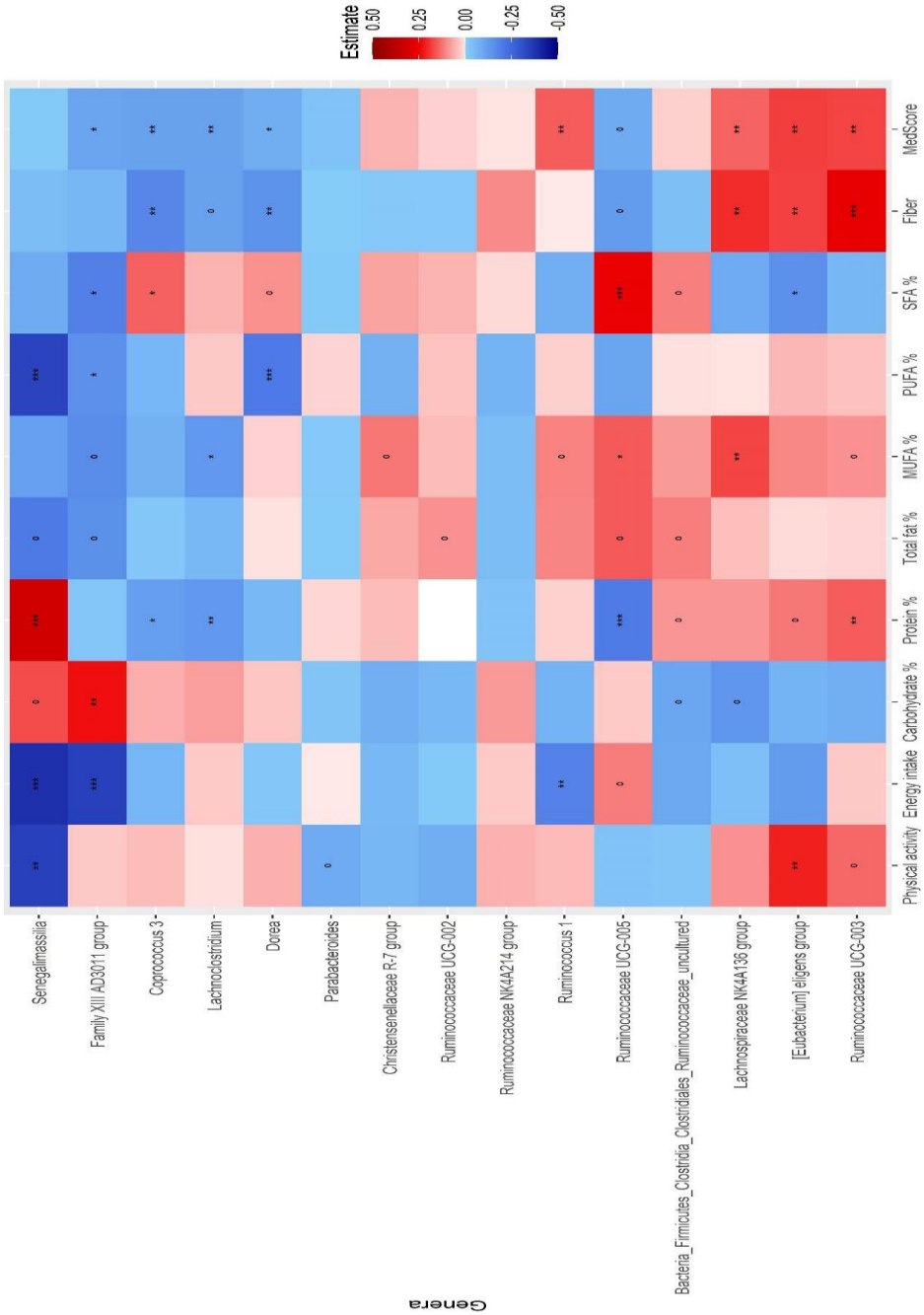
Supplementary Figure 4: Score plot of two-component sPLS-DA model showing samples clustering according to intervention group with 95% confidence level ellipses



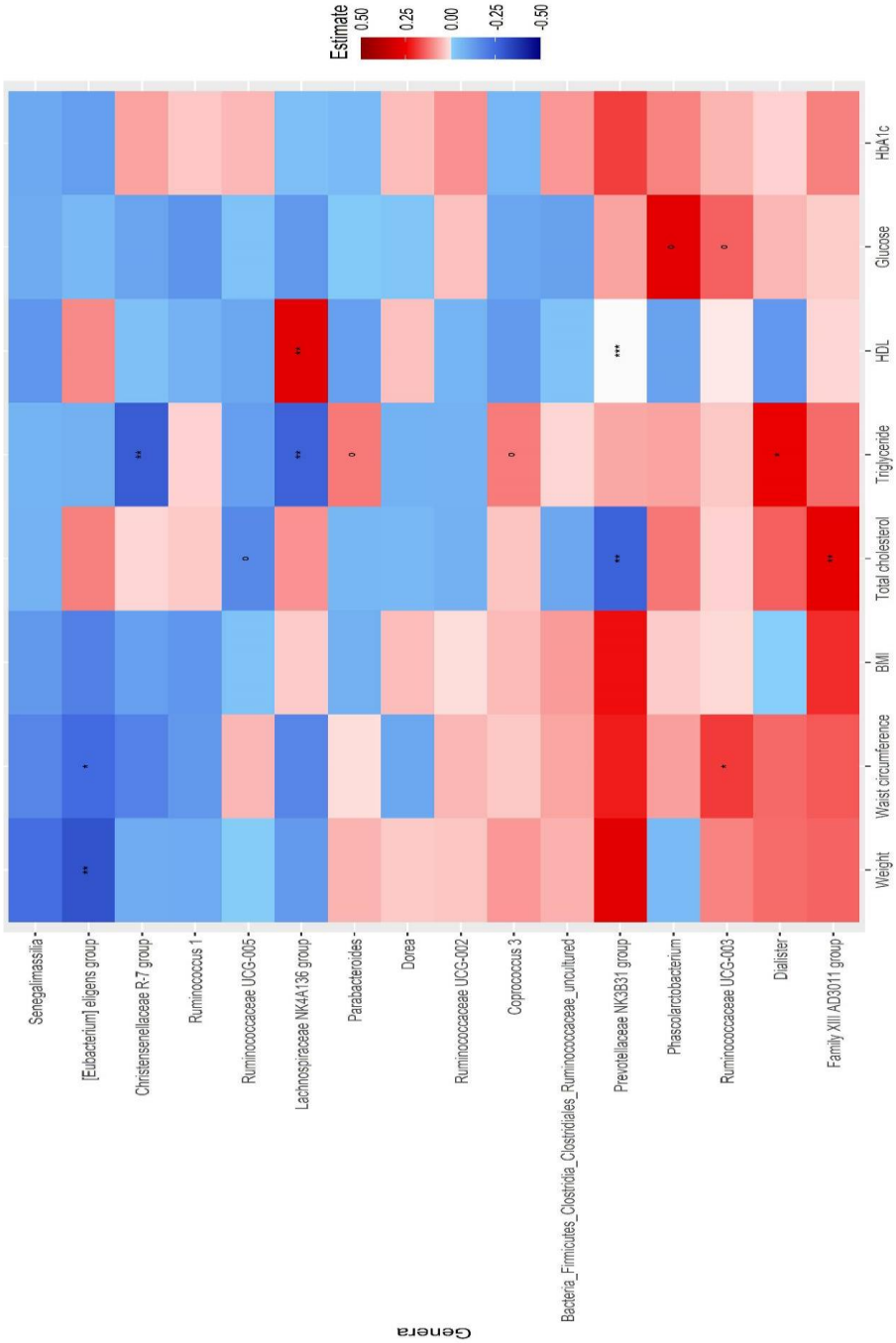
Supplementary Figure 5A: Heat plot showing associations of microbial genera and clinical variables in IG (n= 171); adjusted p-value denoted by < 0.001 "***", < 0.05 "**", < 0.1 "*", <0.2 "°"



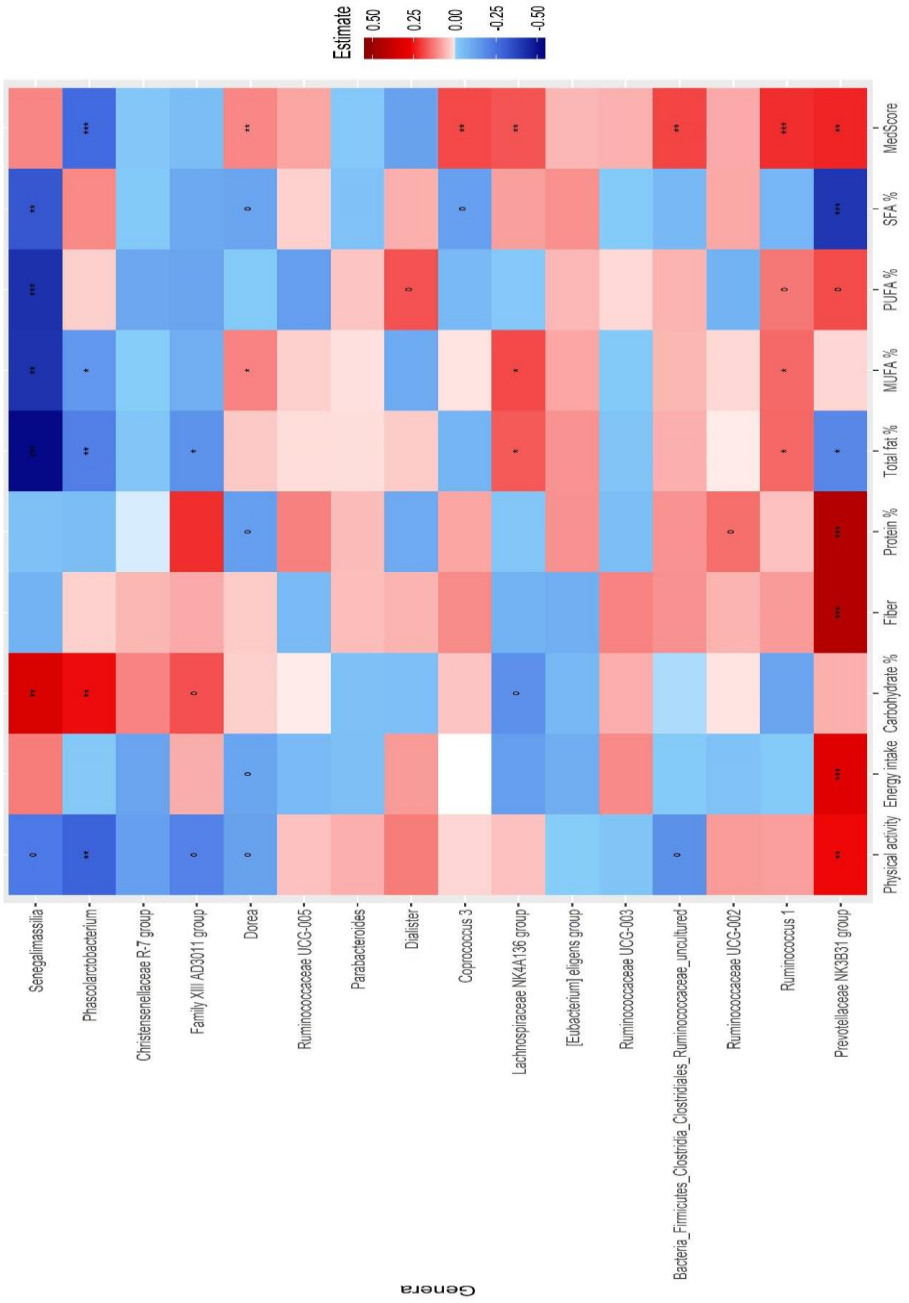
Supplementary Figure 5B: Heat plot showing associations of microbial genera and Energy intake variables within IG (n= 171); adjusted p-value denoted by < 0.001 "***", < 0.05 "**", < 0.1 "*", <0.2 "°"



Supplementary Figure 6A: Heat plot showing associations of microbial genera and anthropometric variables within CG (n= 172); adjusted p-value denoted by < 0.001 "***", < 0.05 "**", < 0.1 "*", <0.2 "o"



Supplementary Figure 6B: Heat plot showing associations of microbial genera and Energy intake variables within CG (n= 172); adjusted p-value denoted by < 0.001 "***", < 0.05 "**", < 0.1 "*", <0.2 "°"



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Chapter 2

Associations between the amount of dietary protein intake and protein sources with gut microbiota composition

Publication status: Under preparation

Associations between the amount of dietary protein intake and protein sources with gut microbiota compositions

Muralidharan J, Moreno-India I#, Bullo M#, Vioque J, Corella D, Fitó M, Vidal J, Hernández-Alonso P, Malaga-2, Alicante-2, Valencia-2, IMIM-2, Reus-4, IMIM-3, Pamplona-1, Tinahones F, Salas-Salvado J

Corresponding authors

Abstract

Background: Animal and plant protein vary by their co-nutrient cluster, amino acid composition and could have differential effects on health. This differential effect could be modulated via gut microbiota.

Objective: To evaluate the associations between the amount of dietary protein intake and protein sources on gut microbial composition.

Methods: This is a sub-study nested in the PREDIMED-Plus, a Spanish randomized controlled trial conducted in overweight/obese male and female (55-75 years) with metabolic syndrome. In 323 participants with available food frequency questionnaires and faecal 16S rRNA sequencing, we evaluated the associations between the intake of protein quantity and protein sources with gut microbiota profile at baseline and longitudinal changes.

Results: Overall dietary protein intake significantly increased over 1-year, with increases in both animal and plant protein sources, as well the ratio of plant to animal protein intake. Protein quantity at baseline was inversely associated with saccharolytic bacteria (*Anaerostipes* (r= -0.43, q-value =0.02), *Coprococcus* 2 (r= -0.36, q-value = 0.02)), whereas positively with *Escherichia-Shigella* (r= 0.63, q-value < 0.001) and *Ruminiclostridium 9* (r= 0.47, q-value < 0.001). At baseline, protein from red meat and processed meat were associated with predicted bacterial functions. 1-year changes in plant protein intake, ratio of plant to animal protein intake, legumes and nuts protein intake were consistently negatively

associated with changes in *Parasutterella*. Changes in various sources of animal protein had negative associations over changes in specific genera (*Butyricicoccus*, *Lachnospiraceae ND3007*, *Fusicatenibacter*).

Conclusion: Dietary protein intake differentially associates with various microbial genera, and especially with members of order Clostridiales. The associations between the total amount and different sources of animal protein and gut microbiota profile suggest that the source of protein may have a stronger influence on gut microbial functions compared to amount and sources of plant proteins.

Keywords: Protein, Gut microbiota, Animal protein, Plant protein

ISRTC number: 89898870 (Registration date: 24 July 2014)

Introduction

Protein is an important component of diet and it is involved in several metabolic processes. Protein rich diets have been increasingly popular in the context of weight loss as they are linked to increased satiation and energy expenditure (1). Importance on protein intake has not only been laid on the quantity but also on its amino acid content and the sources of protein consumed. Dietary protein can broadly be classified into animal (meat, fish, poultry, eggs and dairy) and plant (legumes, nuts and wholegrains) proteins based on their source of origin. Several epidemiological studies have shown inverse associations between plant protein intake and various cardio metabolic risk factors, whereas the reverse associations for some animal protein products (red, processed meat) were reported (2). This contrast between animal and plant-based protein is postulated due to the nutrient cluster along which they co-occur (3).

Besides the nutrient cluster in which the protein co-occurs, proteins could also affect human health in a gut dependent fashion. Approximately 12g of protein is shown to reach colon after consumption of a Western diet (4). Depending on the type and quantity of the protein reaching the colon, various proteolytic processes occur producing metabolites such as amino acids, short chain fatty acids (SCFA's), ammonia, hydrogen sulfide etc. One of the well-known gut microbial metabolite formed with consumption of red meat is trimethylamine N-oxide, which has shown to have atherogenic properties (5). Animal studies have shown that different protein sources varying in amino acid content can affect the mTOR signalling in the gut, which can play a role in immunity via regulating T cells (6). Thus, the protein and amino acid content of the diet has the potential to shift gut microbial composition and may induce physiological effects on the host.

Few animal and human studies have explored the effects of protein sources on gut microbiota. Animal studies with consumption of animal protein have consistently shown an increase in Proteobacteria, Firmicutes phyla, especially

members of Clostridiales and decrease in Bacteroidetes (7,8); on the other hand, human studies have not shown consistent results (9). It is also important to note that not all animal protein sources affect the intestinal microbiota in the same way. For example, red meat is associated with decrease/increase in beneficial/opportunistic pathogens respectively, whereas fish protein have been associated with improving insulin sensitivity modulated via gut microbiota in rats (10). Hence, there is also a need to investigate the effects of various animal sources on intestinal microbiota.

Review by the My New Gut study group suggests that even though high protein diets are effective in weight loss, their effect on gut microbiota might not necessarily be beneficial and this needs to be evaluated with caution (9). Therefore the aim of the present study is to evaluate the associations between the amount and sources of dietary protein intake sources with gut microbiota and predicted microbial functions using a cross-sectional and longitudinal approach in the context of the PREDIMED-Plus study.

Materials and methods

Study design and participants

This study was conducted in the framework of PREDIMED-Plus study. PREDIMED-Plus is an ongoing multicentre clinical trial aiming to evaluate the effects of intensive weight loss intervention based on Mediterranean diet, physical activity promotion and behavioural support on CVD events and mortality. The control group is given usual care advice without any advice for losing weight. Eligible participants were community dwelling men (55-75 years) and women (60-75 years) with overweight/obesity and at least three components of metabolic syndrome (according to American Heart Association and National Heart, Lung and Blood Institute) and without documented history of CVD at baseline. Details of the trial have been described elsewhere and further

information can be found at https://www.predimedplus.com/. This study was registered at the.

400 participants (200 in each group of intervention) with faecal samples processed at baseline and 1-year from two PREDIMED-Plus study centres were included in the study. Participants with extreme total energy intake (women < 500 and > 3500 kcal/day, and men < 800 and > 4000 kcal/day) were excluded (n= 12) (11). The 17-point questionnaire (P17 score), a modified version of a previously validated 14-point questionnaire, was used to evaluate Mediterranean adherence score (12).

Weight (using calibrated scales), height (using wall mounted stadiometer) and waist circumference (midway between lowest rib and the iliac crest, using an anthropometric tape) were measured in duplicates by nurses at baseline and 1-year visit. Body mass index (BMI) was calculated as weight divided by height squared (Kg/m^2). Serum and plasma samples were collected after an overnight fast, aliquoted and stored at -80°C. Serum total cholesterol, high density lipoprotein (HDL) cholesterol and triglycerides were measured with standard enzymatic methods. Low density lipoprotein (LDL) cholesterol was calculated by the Friedwald formula when the triglycerides were less than 300 mg/dl.

Variables for various dietary nutrients, protein sources were calculated using food groups estimated from Spanish Food composition tables (13,14). Food groups used for computing animal based protein sources included meat (unprocessed red meat, processed red meat, and white meat), dairy (non-fermented and fermented), and other animal sources (fish, shellfish, eggs). Plant based protein included grains, legumes, nuts and seeds, vegetables, mushrooms, fruits and berries. Individual food protein sources for example from red meat or nuts were considered in grams per day (g/d). Contribution of animal and plant protein to the total protein content were evaluated by dividing the g/d intake of animal or plant protein to total protein (g/day) and multiplied by 100 to obtain the percentage

contribution as adapted from protein calculations in the PREVIEW project (15). Description of various protein variables are shown in Supplementary information 2.

Faecal sample collection, 16S rRNA sequencing and processing

Faecal samples were collected from participants at baseline and 1-year in a hermetic flask. Participants were instructed to store the samples in freezing conditions before they bring it to the study centre (within 12 hours of excretion) in a cooled conditions (provided with ice pack to store at -20°C). Participants using probiotics or antibiotics were instructed to give samples only 15 days after the prebiotic or antibiotics use was stopped. Once the samples were collected, they were stored as aliquots at -80°C until further processing.

Faecal DNA extraction was conducted using QIAmp PowerFecal DNA kit (Qiagen, Hilden, Germany) along with a bead beating step of 5 minutes using FastPrep-24 5G Homogenizer (MP Biomedicals, California, USA). The extracted DNA was quantified using Qubit 2.0 Fluorometer-dsDNA (High Sensitivity kit, Invitrogen, Carlsbad, California, USA). Ion 16S Metagenomics kit from Thermo Fischer Scientific (Italy) was used to amplify the DNA. Supplementary Information provides details on 16S rRNA gene sequencing. Briefly, two primer sets of hypervariable regions of V2-4-8 and V3-6, 7-9 were used to amplify the bacterial genome. Sequenced samples were read with Ion Reported Software V4.0 and low quality, polyclonal sequences were removed. From the Mas Lloret J et al, an adapted script was used to process and separate the 6 hypervariable regions sequenced. Further on we used the V4 region for analyses. The sequenced files were imported to QIIME2 and quality control was performed using DADA2 using standard procedures (Supplementary information). Taxonomy was assigned to clustered sequences with SILVA 132 database. Features unidentified at phylum level, a minimum of 5% prevalence and mitochondrial features were removed in the pre-processing step in R (v3.6). Cumulative sum scaling, log

transformation from *Metagenomeseq* package (16) was used to normalize the samples for further use. Final sample used for the analyses included 323 participants.

Statistical and bioinformatics analyses

Data for continuous variables are presented as median (25%, 75% IQR) and quantitative variables are represented by percentage. Differences between baseline and 1-year anthropometric and clinical variables were evaluated using Wilcoxon tests. These were evaluated using *tableone* package in R.

Microbiome analysis using normalized data from *Metagenomeseq* package was used to evaluate the alpha diversity (chao1, Shannon index). Association of alpha diversity with various protein variables (total protein quantity (% of protein), P/A ratio and individual sources of protein (% animal protein, % plant protein, proteins from grains, legumes, nuts and seeds, dairy, poultry, fish, red meat, processed meat, total meat) were evaluated using linear model, adjusting for various variables (sex, BMI, carbohydrate, fat, fibre, alcohol intake and total P17 score) at baseline. For 1-year changes, we estimated the changes in alpha diversity, protein variables and adjusted by various confounders (sex, BMI, carbohydrate, fat, fibre, alcohol intake and total P17 score) including the intervention groups. All the associations between various microbial genera and protein variables were evaluated using (*Metagenomeseq* package in R software). We adjusted the associations for sex and baseline values of BMI, carbohydrate, fat, fibre, alcohol intake and total P17 score, and additionally with the intervention group for 1-year changes accounting for repeated measures. Significance was set at q-value < 0.10 after correcting for multiple testing (False Discovery Rate method) to avoid type I error.

Predicted metagenome functions were assigned using Phylogenetic Investigation of Communities by Reconstruction of Unobserved states plugin (PICRUST2) within QIIME2 using the q2-picrust2 plugin. Predicted bacterial metagenome

functions were expressed by KEGG orthologues. Associations between various KEGG orthologues and protein variables were evaluated in Maaslin2 package with linear models adjusted for the same factors mentioned above. Prior to conducting the associations, KEGG features not passing at least 10 % of relative abundance were removed and were transformed with centre log ratio.

Results

From a total of 400 participants we removed 77 participants due to filtering steps and excess energy intake at baseline or 1-year .This resulted in 323 participants for the analyses who had dietary, 16S rRNA data at both baseline and 1-year. Baseline and 1-year characteristics of the participants are presented in **Table 1** as median (25%, 75% IQR) for continuous variables and percentage for categorical variables. Weight, waist circumference, BMI, LDL-cholesterol and triglycerides significantly decreased after 1-year, while HDL-cholesterol and physical activity significantly increased. Total energy intake, and energy as protein, fat, monounsaturated fats and polyunsaturated fats (all as %), as well as total fibre, and the P17 score increased after 1-year, whereas and energy as carbohydrate and saturated fat (in %) decreased significantly.

Protein intake and sources of protein intake at baseline (Table 1, 2, Supplementary Table 1)

At baseline participants consumed 16.3 % (IQR 14.9, 18.1) protein out of which animal protein % and plant protein % contributed to 10.7 % (IQR 9.3, 12.3) and 5.5 % (IQR 4.9, 6.1) respectively. Grains contributed to maximum plant protein intake (15.4 g/day (IQR 7.7, 16.7)) followed by legumes. Animal proteins were majorly characterized by proteins from poultry, fish and dairy.

Baseline associations of protein quantity and sources with gut microbiota (Table 3)

Alpha diversity indexes (chao1 and Shannon diversity) did not associate between protein quantity, P/A ratio and various protein sources at baseline (not shown). At baseline, gut microbial composition was associated with all the protein sources except processed meat. Total protein intake was positively associated to *Escherichia-Shigella* (r= 0.63, q-value < 0.001) and *Ruminiclostridium* 9 (r= 0.47, q-value < 0.001). Genera from Clostridiales such as *Ruminococcus gauvreauii* (r= -0.34, q-value = 0.05), *Anaerostipes* (r= -0.43, q-value = 0.02) and *Coprococcus* 2 (r= -0.36, q-value= 0.02) were negatively associated to total protein intake. *Coprococcus* 2 was also negatively associated to plant protein, consecutively with P/A. Legume protein intake was positively associated with *Bifidobacterium* (r= 0.19, q-value = 0.02). Overall, some butyrate producers (*Barnesiella* and *Ruminococcacea* UCG 003) were positively associated to plant-based protein intake. On the other hand, *Ruminiclostridium* 9 that associated positively with total protein also associated positively with animal protein %. *Intestinibacter* was consistently positively associated with animal protein % and various animal protein sources (poultry, red meat, total meat).

Changes in protein quantity and sources of protein over 1-year (Table 1, 2, Supplementary Table 1)

Total protein intake increased from 16.3 % (IQR 14.9, 18.1) to 17.39 % (IQR 15.6, 19.2) at 1-year. Even though total protein increased significantly, protein expressed as g/kg body weight did not change significantly over 1-year of time. Animal protein %, plant protein % and P/A ratio increased significantly compared to baseline. Amongst the animal protein sources, all sources (protein from dairy (including fermented dairy), red meat, processed meat, poultry and total meat) decreased except fish protein intake. Plant protein sources coming from legumes and nuts increased while proteins from grain decreased. This decrease in grain proteins was potentially due to the decrease in the intake of refined grains as per Mediterranean diet adherence recommendations. However, we suspect this

reduction in refined grains would have been substituted with whole grains, as we also observed an increase in fibers.

Associations of 1-year changes in protein quantity and sources with changes in gut microbiota (Table 4, Supplementary figure 1)

Changes in P/A ratio and grain proteins were associated positively with changes in chao1 index (Supplementary Figure 1). Changes in total protein %, changes in the P/A ratio, and various components of plant protein (nuts, legumes, and grains) were consistently associated with changes in *Parasutterella*. Changes in total protein intake and various sources of protein (total animal sources, processed meat and dairy), were negatively associated with changes in *Butyricicoccus* and *Fusicatenibacter*.

Associations of baseline and 1-year changes in protein quantity and its sources with changes in predicted functions of gut microbiota (Table 5, Supplementary Table 2)

At baseline we observed 60 significant associations (q-value < 0.1) between various KEGG orthologs and protein sources, majorly dominated by red meat and processed meat protein. Amongst these associations, majority of the functions belonged to associations within the "metabolism family". At 1-year changes, no significant associations (q-value <0.1) were observed. For explorative purposes, while q-value at baseline and 1-year were extended to <0.25, we also observed nuts protein to contribute to many associations at baseline, whereas in 1-year changes, similar domination of red meat and processed meat proteins were observed.

Discussion

Corresponding to previous studies suggesting that protein intake could aid in weight loss, at baseline we observed a negative association between total protein intake and *Coprococcus 2*, a genera which has previously been associated positively with weight, BMI and triglycerides, as well as high CVD risk (17). On the other hand, total protein intake also positively associated with *Escherichia-Shigella*, some species of which are regarded as a gut pathobiont as observed in previous animal studies (18,19). At baseline, *Escherichia-Shigella*, was also positively associated with grain proteins but negatively with legumes. This interesting difference in association of legume versus grain protein was also observed for *Ruminococcus gauvreauii* and *Alistipes*. In the same line, an increase in *Alistipes* and a decrease in *Escherichia-Shigella* has shown after black bean consumption in mice (20). Majority of the beneficial effects of legumes and grains have been investigated in the aspect of dietary fibers and the protein modulation remains to be investigated. Proteins from legumes are rich in lysine, whereas grains are rich in tryptophan, methionine and cysteine, which could potentially differentiate the metabolizing capacities of the bacterial genera resulting the differences between observations. However, this hypothesis needs to be confirmed with *in vitro* studies. Interestingly it has also been shown in *in vitro* models that protein digestibility from cereals decrease in elders compared to legumes thus also varying the bio accessibility of the amino acids reaching the intestine (21). *Alistipes*, *Bifidobacterium* that positively associated with legume intake at baseline, are also SCFA producers and *Bifidobacterium* additionally is a ɤ-aminobutyrate producer which could contribute to the mechanism of beneficial action for legume consumption on health (22). However, the role of *Alistipes* is not clear as some studies report a protective effect on CVD (23), whereas a negative effect in others (24). At baseline, the plant protein %, P/A, and nuts protein were also positively associated with *Barnesiella*. It is interesting to note that lesser colorectal cancer risk in vegetarians (25) could be mitigated via

this genus as an *in vitro* study indicated anti-carcinogenic property for the members of Barnesiellaceae family (26).

Other result from our study such as the positive association between dairy protein intake and *Streptococcus*, was consistent with previous studies (27,28). Additionally dairy protein also positively associated with butyrate producers such as *Butyricicoccus*, *Butyricimonas*, *Odoribacter*, *Faecalibacterium* at baseline. Fermented dairy products have also shown beneficial role towards health which could potentially be mitigated via gut microbiota (29). However, the purported benefits of the fermented dairy via gut microbiota is predominantly attributed to its bacterial content (Lactic acid bacteria) (30) which we did not observe in our study.

With one-year changes of plant protein intake, we observed a negative association with *Parasutterella*, this was also consistent with negative association in the changes of P/A ratio, legumes, nuts, grains and dairy protein intake. This negative association with *Parasutterella* with predominantly plant-based proteins (except dairy) is important to remark, as *Parasutterella* at baseline was positively associated to nut and dairy protein intake. This genus is regarded as a protein degrader of the intestinal microbiota (31) and have shown increase with supplementation of mung beans in mice study (32). However, the contradictory negative association seen in our study could be due to two possible reasons: 1) selective increase of genera preferring certain protein sources and amino acid composition; 2) genera, such as *Escherichia* have shown competition for growth with other proteolytic degraders such as *Parasutterella* (32). Functionality of this genera however remains inconclusive with some human studies showing a beneficial effect (33), whereas others with a harmful association (34,35).

At 1-year, even though overall quantity of animal protein increased, we observed a decrease in protein contribution from dairy, poultry, red meat, processed meat and total meat, whereas fish protein was the only animal protein that increased.

These changes in the reducing part of animal proteins were consistently negatively associated mainly with changes in *Butyricicoccus*, *Fusicatenibacter*, *Lachnoclostridium* and *Lachnospiraceae ND3007*. However as *Butyricicoccus*, *Fusicatenibacter*, *Lachnospiraceae ND3007* were also negatively associated with the total protein intake, animal protein % and fish protein intake which increased over 1-year, it could be difficult to disentangle if there is an actual increase or decrease of this genera. At 1-year changes we observed that the changes in animal protein and its sources predominantly were associated in changes in targeted group of members of the order Clostridiales (*Butyricicoccus*, *Fusicatenibacter*, *Lachnoclostridium* and *Lachnospiraceae ND3007*, *Ruminococcus 1*) which is consistent with previous research in animals (36,37). We could postulate that the changes animal-based protein intake could have potentially stronger influence on gut microbial composition compared to the plant-based protein. However, the direction of this influence could potentially determine the implications on host health, therefore, more studies are needed to clarify the impact of these potential effects/associations.

Similarly, with the predicted functionality, associations were dominated with animal-based protein sources especially from red meat and processed meat, both at baseline and 1-year changes (considering a q-value < 0.1 and 0.25 respectively). At baseline out of the 60 significant associations we observed, 35% belonged to metabolic functions (amino sugar and nucleotides, peptidases etc.) followed by genetic information processing and environmental information processing functions. Amongst these associations, nine KEGG orthologues consistently associated at baseline and 1-year with meat protein intake. Considering plant-based associations below a q-value of 0.25 at baseline, protein from nuts dominated these associations providing further evidence supporting previous studies on nut consumption affecting the functional capabilities of gut microbiota (38,39). This emphasises the hypothesis above on the stronger influence of animal-based protein on intestinal microbiome. Even though

reductions in animal-based proteins are recommended, care must be paid on compensating the intake of other nutritive components obtained from the animal sources. Consuming high levels of plant-based proteins, medium levels of fish protein and low levels of red or processed meat such as recommended in Mediterranean diet could be a solution to balance nutrients (40).

The observations we make in this study should be perceived with care, as these associations are not causal in nature. Even though we adjusted the model with potential confounders, we cannot completely rule out the confounding effects. Our study also lacks faecal metabolomics data in order to validate the hypothesis and observations noted at the compositional level. Future well designed RCT with balancing other components of diet and varying protein sources considering intestinal microbial composition and metabolites are required to verify the observations we make here. This study also presents the observations in a Mediterranean population at high cardiovascular risk; hence directly extrapolating these results to other populations might not be possible. Another important limitation is that we collected the dietary data from FFQ, therefore over or under estimation of the food consumption is possible. Aside from these limitations, the strengths of this study also deserve to be mentioned. We report these results in a large population and a large array of important protein sources not just classifying at animal or plant-based levels. Unlike many studies focussing on protein rich diets we focus on diet with normal protein intake (15-20%), balanced with other nutrients which is closer to real life practice. This study provides information on an important section of population, who are more susceptible to sarcopenia and higher requirements of protein (41).

Overall, we conclude that the dietary protein quantity and their sources associate with various gut microbial genera majorly from the order of Clostridiales. The results of our study suggest that protein, especially red meat and processed meat protein may alter both the gut microbial composition and the predicted functionality compared to the plant-based protein sources. A normal protein diet

with balanced amount of plant-protein and animal-protein such as in Mediterranean diet could be promote beneficial gut microbiota. However future RCTs are needed in order to clarify if the associations observed in our study are the consequence of the effect of the amount and type of protein intake.

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Table 1: Baseline and 1-year characteristics of study population

	Baseline (n=323)	**One year (n=323)**	**p-value**
Sex (Male/Female)	163 /160		
Age (years)	64.63 (5.01)		
Hypercholesterimia (No/Yes)	20 / 303		
Diabetes prevalence (No/Yes)	246 /77		
Intervention groups (Control/Intervention)	162 / 161		
Weight (Kg)	87.80 (79.00, 97.20)	85.60 (77.00, 94.30)	0.009
Waist circumference (cm)	109.50 (103.50, 116.05)	107.20 (101.00, 113.00)	0.001
17-point Mediterranean adherence score	8.00 (6.00, 9.00)	13.00 (10.00, 15.00)	<0.001
BMI (Kg/m2)	33.04 (30.59, 35.97)	31.83 (29.69, 34.78)	0.001
Physical activity (METs-min/week)	1706.29 (762.24, 3388.35)	2419.58 (1083.92, 4314.68)	<0.001
Glucose (mg/dL)	103.00 (93.00, 117.00)	101.00 (92.00, 114.00)	0.306
Total cholesterol (mg/dL)	200.00 (174.00, 224.00)	194.00 (173.00, 222.75)	0.343
HDL (mg/dL)	46.00 (40.50, 55.00)	49.00 (42.00, 58.00)	0.004
LDL (mg/dL)	116.00 (96.00, 139.00)	113.00 (95.00, 135.75)	0.522
Triglycerides (mg/dL)	152.00 (116.00, 200.75)	139.00 (108.25, 183.75)	0.032
HbA1c (%)	5.80 (5.60, 6.30)	5.80 (5.50, 6.20)	0.334
Alcohol (g/day)	4.52 (0.69, 14.88)	4.23 (0.55, 13.04)	0.188
Total energy (Kcal/day)	2478.54 (2138.34, 2816.26)	2279.37 (2007.83, 2586.33)	<0.001
Carbohydrate %	40.83 (36.76, 43.87)	36.49 (32.54, 40.66)	<0.001

Protein %	16.30 (14.94, 18.11)	17.39 (15.63, 19.28)	<0.001
Total fat %	39.99 (37.23, 43.67)	43.30 (39.78, 46.58)	<0.001
Mono unsaturated fat %	20.63 (18.05, 22.91)	24.45 (21.87, 27.04)	<0.001
Poly unsaturated fat %	6.20 (5.35, 7.30)	7.61 (6.60, 8.50)	<0.001
Saturated fat %	9.97 (8.83, 11.29)	9.12 (8.26, 10.08)	<0.001
Fiber (g/day)	25.47 (21.36, 30.61)	30.57 (25.25, 34.99)	<0.001
Protein by body weight (g/Kg weight)	1.15 (0.97, 1.35)	1.16 (0.99, 1.39)	0.273

Table 2: Baseline and 1-year variables associated to protein intake

Protein variables	Baseline	One year	p-value
Total animal protein (g/d)	66.31 (56.16, 77.57)	64.15 (54.56, 74.93)	0.045
Total plant protein (g/d)	34.45 (29.23, 39.20)	34.96 (30.76, 40.10)	0.116
Animal Protein (% contribution to total protein)	66.29 (61.42, 70.63)	64.64 (60.25, 68.76)	0.002
Plant Protein (% contribution to total protein)	33.72 (29.40, 38.59)	35.40 (31.31, 39.84)	0.003
Animal protein (APE, % contribution to total energy)	10.70 (9.34, 12.36)	11.30 (9.73, 12.98)	0.03
Plant protein (PPE, % contribution to total energy)	5.54 (4.96, 6.11)	6.06 (5.57, 6.77)	<0.001
Ratio (PPE/APE)	0.51 (0.42, 0.63)	0.55 (0.46, 0.66)	0.003
Dairy protein (g/d)	14.27 (10.40, 19.52)	12.77 (8.95, 17.13)	0.003
Total meat protein (g/d)	29.81 (24.96, 37.39)	27.97 (22.64, 36.11)	0.008
Red meat protein (g/d)	7.93 (5.39, 11.57)	5.39 (3.60, 7.45)	<0.001
Processed meat protein (g/d)	7.67 (5.55, 10.35)	6.79 (4.33, 8.66)	<0.001
Poultry protein (g/d)	14.01 (12.86, 17.78)	15.15 (12.86, 17.78)	<0.001
Fish protein (g/d)	16.00 (12.28, 19.60)	17.62 (14.26, 22.55)	<0.001
Fermented dairy protein (g/d)	7.31 (4.59, 10.75)	6.99 (4.56, 9.78)	0.176
Beans protein (g/d)	4.44 (3.35, 6.19)	5.33 (3.70, 6.19)	<0.001
Grain protein (g/d)	15.42 (7.71, 16.76)	9.86 (7.56, 15.98)	<0.001
Nuts protein (g/d)	1.80 (0.84, 3.44)	5.11 (3.62, 6.81)	<0.001

Table 3: Associations between protein quantity and various protein sources and gut microbial composition at baseline

	Coefficient	**p-value**	**q-value**
Protein intake %			
Ruminococcus gauvreauii group	-0.34	0.01	0.05
Anaerostipes	-0.43	0.00	0.02
Coprococcus 2	-0.36	0.00	0.02
Escherichia-Shigella	0.63	0.01	0.06
Ruminiclostridium 9	0.47	0.00	0.00
Animal protein % (APE)			
Anaerostipes	-0.24	0.02	0.09
Intestinibacter	0.25	0.02	0.09
Ruminiclostridium 9	0.29	0.00	0.01
Plant protein % (PPE)			
Barnesiella	0.59	0.00	0.02
Coprococcus 2	-0.53	0.00	0.00
Ratio (PPE/APE)			
Barnesiella	2.31	0.01	0.02
Coprococcus 2	-1.88	0.00	0.02
Romboutsia	-2.25	0.02	0.05
Legumes protein (g/d)			
Ruminococcus gauvreauii group	-0.15	0.00	0.00
Ruminococcus torques group	-0.17	0.00	0.01
Alistipes	0.13	0.02	0.05
Anaerostipes	-0.24	0.00	0.00
Bifidobacterium	0.19	0.01	0.02
Escherichia-Shigella	-0.21	0.03	0.08
Lachnospiraceae NK4A136 grou p	-0.13	0.01	0.04
Streptococcus	0.20	0.01	0.02
Nuts protein (g/d)			
Barnesiella	0.17	0.01	0.02
Clostridium sensu stricto 1	-0.22	0.00	0.00
Oscillibacter	0.11	0.00	0.01
Parasutterella	0.19	0.01	0.02

	Coefficient	p-value	q-value
Romboutsia	-0.14	0.01	0.04
Ruminococcaceae UCG-003	0.08	0.03	0.10
Sutterella	0.14	0.01	0.03
Grains protein (g/d)			
Eubacterium hallii group	0.05	0.01	0.05
Ruminococcus gauvreauii group	0.07	0.00	0.01
Alistipes	-0.06	0.02	0.05
Escherichia-Shigella	0.17	0.00	0.00
Intestinibacter	0.08	0.00	0.02
Oscillibacter	-0.06	0.00	0.02
Romboutsia	0.08	0.00	0.01
Ruminiclostridium 5	0.04	0.02	0.07
Ruminiclostridium 6	-0.11	0.00	0.00
Ruminococcus 2	0.06	0.01	0.03
Poultry protein (g/d)			
Intestinibacter	0.05	0.01	0.08
Processed meat protein (g/d)			
NONE			
Red meat protein (g/d)			
Bilophila	-0.05	0.02	0.08
Intestinibacter	0.07	0.00	0.00
Odoribacter	-0.07	0.00	0.00
Parabacteroides	-0.04	0.01	0.04
Total meat protein (g/d)			
Intestinibacter	0.04	0.00	0.00
Odoribacter	-0.02	0.01	0.04
Ruminococcus 2	0.03	0.01	0.04
Dairy protein (g/d)			
Eubacterium hallii group	-0.03	0.03	0.08
Alistipes	0.04	0.01	0.05
Blautia	-0.03	0.02	0.05
Butyricicoccus	0.03	0.02	0.06
Butyricimonas	0.04	0.01	0.02
Faecalibacterium	0.05	0.00	0.00

	Coefficient	p-value	q-value
Odoribacter	0.03	0.02	0.05
Parasutterella	0.06	0.00	0.02
Ruminococcaceae UCG-002	0.03	0.02	0.05
Ruminococcaceae UCG-003	0.03	0.01	0.03
Streptococcus	0.06	0.00	0.01
Fish protein (g/d)			
Eubacterium eligens group	0.04	0.02	0.07
Coprococcus 2	-0.04	0.00	0.02
Oscillibacter	-0.03	0.02	0.09
Phascolarctobacterium	0.06	0.00	0.01
Ruminococcaceae UCG-002	-0.04	0.00	0.02

Associations were evaluated using Metagenomeseq and adjusted for baseline sex, BMI, carbohydrate, fat, fiber, alcohol intake and P17 score. Q-value represents the adjusted p-value < 0.1

Table 4: Associations between changes in protein quantity and various protein sources and changes in gut microbial composition over 1-year

	Coefficient	**p-value**	**q-value**
Protein intake %			
Butyricicoccus	-0.05	0.01	0.08
Fusicatenibacter	-0.05	0.01	0.08
Ruminococcaceae UCG-005	-0.06	0.02	0.09
Animal protein % (APE)			
Butyricicoccus	-0.08	0.01	0.04
Fusicatenibacter	-0.08	0.00	0.04
Lachnoclostridium	-0.07	0.02	0.09
Lachnospiraceae ND3007 group	-0.06	0.02	0.09
Ruminococcaceae UCG-005	-0.08	0.02	0.09
Plant protein % (PPE)			
Parasutterella	-0.31	0.00	0.02
Ratio (PPE/APE)			
Parasutterella	-2.94	0.00	0.03
Legumes protein (g/d)			
Eubacterium coprostanoligenes group	-0.20	0.00	0.03
Lachnoclostridium	-0.16	0.01	0.07
Parasutterella	-0.35	0.00	0.01
Nuts protein (g/d)			
Parasutterella	-0.43	0.00	0.00
Grains protein (g/d)			
Parasutterella	-0.12	0.01	0.06
Poultry protein (g/d)			
NONE			
Processed meat protein (g/d)			
Butyricicoccus	-0.09	0.02	0.08
Fusicatenibacter	-0.08	0.02	0.08
Lachnoclostridium	-0.10	0.00	0.02
Lachnospiraceae ND3007 group	-0.09	0.00	0.02
Red meat protein (g/d)			
Ruminococcus torques group	-0.10	0.01	0.06

	Coefficient	p-value	q-value
Fusicatenibacter	-0.11	0.01	0.05
Lachnospiraceae ND3007 group	-0.08	0.01	0.09
Total meat protein (g/d)			
Fusicatenibacter	-0.03	0.00	0.04
Lachnospiraceae ND3007 group	-0.02	0.01	0.06
Dairy protein (g/d)			
Ruminococcus torques group	-0.05	0.01	0.08
Butyricicoccus	-0.07	0.00	0.00
Lachnoclostridium	-0.05	0.02	0.08
Lachnospira	-0.05	0.02	0.08
Parasutterella	-0.08	0.01	0.08
Ruminococcus 1	-0.06	0.01	0.04
Fish protein (g/d)			
Fusicatenibacter	-0.05	0.01	0.06
Lachnospiraceae ND3007 group	-0.04	0.01	0.04
Ruminococcus 1	-0.05	0.02	0.08

Associations were evaluated using Metagenomeseq using repeated measures and adjusted for baseline sex, BMI, carbohydrate, fat, fiber, alcohol intake, P17 score and intervention group. Q-value represents the adjusted p-value < 0.1

Table 5: Associations between protein variables and bacterial predicted functions (KEGG orthologues)

KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
K02392	RMP	0.11	0.00	0.07	Bacterial motility proteins	Cellular Processes
K02406	RMP	0.10	0.01	0.10	Bacterial motility proteins	Cellular Processes
K01999	RMP	0.10	0.00	0.07	ABC transporters	Environmental Information Processing
K10112	RMP	0.10	0.00	0.07	ABC transporters	Environmental Information Processing
K02035	RMP	0.08	0.01	0.09	ABC transporters	Environmental Information Processing
K08483	RMP	0.09	0.00	0.07	Phosphotransferase system (PTS)	Environmental Information Processing
K03118	PMP	-0.09	0.01	0.09	Secretion system	Environmental Information Processing
K02057	RMP	0.08	0.01	0.08	Transporters	Environmental Information Processing
K02056	RMP	0.08	0.01	0.09	Transporters	Environmental Information Processing
K02429	RMP	-0.08	0.01	0.09	Transporters	Environmental Information Processing
K01990	RMP	0.03	0.01	0.10	Transporters	Environmental Information Processing

KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
K07713	PMP	-0.11	0.01	0.08	Two-component system	Environmental Information Processing
K04771	RMP	0.08	0.00	0.08	Chaperones and folding catalysts	Genetic Information Processing
K03530	RMP	-0.04	0.01	0.08	Chromosome	Genetic Information Processing
K03585	RMP	-0.08	0.01	0.09	Chromosome	Genetic Information Processing
K03574	RMP	0.07	0.00	0.08	DNA repair and recombination proteins	Genetic Information Processing
K03169	PMP	-0.03	0.01	0.08	DNA replication proteins	Genetic Information Processing
K03169	RMP	-0.03	0.01	0.09		Genetic Information Processing
K03655	PMP	-0.03	0.01	0.08	Homologous recombination	Genetic Information Processing
K03892	RMP	0.07	0.00	0.07	Transcription factors	Genetic Information Processing
K07729	RMP	0.08	0.00	0.08	Transcription factors	Genetic Information Processing
K03718	RMP	-0.08	0.01	0.08	Transcription factors	Genetic Information Processing
K02647	RMP	0.09	0.01	0.09	Transcription factors	Genetic Information Processing
K03704	RMP	0.08	0.01	0.09	Transcription factors	Genetic Information Processing
K07636	PMP	-0.04	0.00	0.07	Protein kinases	Metabolism
K01443	PMP	-0.06	0.01	0.07	Amino sugar and nucleo	Metabolism

KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
					tide sugar metabolism	
K00975	RMP	0.09	0.00	0.08	Amino sugar and nucleotide sugar metabolism	Metabolism
K00971	RMP	-0.07	0.01	0.08	Amino sugar and nucleotide sugar metabolism	Metabolism
K02564	PMP	-0.04	0.01	0.10	Amino sugar and nucleotide sugar metabolism	Metabolism
K05349	RMP	-0.04	0.01	0.10	Cyanoamino acid metabolism	Metabolism
K01126	PMP	-0.05	0.01	0.08	Glycerophospholipid metabolism	Metabolism
K01785	PMP	-0.04	0.00	0.07	Glycolysis / Gluconeogenesis	Metabolism
K03315	RMP	-0.08	0.01	0.09	Methane metabolism	Metabolism
K01190	PMP	-0.06	0.00	0.07	Other glycan degradation	Metabolism
K01190	RMP	-0.05	0.01	0.09	Other glycan degradation	Metabolism
K01686	PMP	-0.06	0.01	0.09	Pentose and glucuronate interconversions	Metabolism
K07258	RMP	0.10	0.00	0.07	Peptidases	Metabolism
K01284	RMP	-0.08	0.00	0.08	Peptidases	Metabolism
K03797	RMP	-0.04	0.01	0.08	Peptidases	Metabolism
K07258	PMP	0.09	0.01	0.09	Peptidases	Metabolism
K01278	RMP	-0.09	0.01	0.10	Peptidases	Metabolism

KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
K07636	RMP	-0.03	0.01	0.09	Protein kinases	Metabolism
K01187	PMP	-0.07	0.01	0.10	Starch and sucrose metabolism	Metabolism
K04487	RMP	0.08	0.01	0.08	Thiamine metabolism	Metabolism
K02551	RMP	-0.09	0.01	0.10	Ubiquinone and other terpenoid-quinone biosynthesis	Metabolism
K15532	PMP	-0.14	0.01	0.09	None	None
K01811	PMP	-0.06	0.01	0.09	Carbohydrate metabolism	Unclassified
K07322	RMP	-0.09	0.00	0.07	Cell division	Unclassified
K09955	PMP	-0.10	0.00	0.07	Function unknown	Unclassified
K07090	RMP	0.08	0.00	0.07	General function prediction only	Unclassified
K07053	RMP	0.06	0.00	0.08	General function prediction only	Unclassified
K07007	PMP	0.08	0.01	0.08	General function prediction only	Unclassified
K07025	PMP	-0.04	0.01	0.08	General function prediction only	Unclassified
K07148	PMP	-0.13	0.01	0.08	General function prediction only	Unclassified
K07007	RMP	0.07	0.01	0.08	General function prediction only	Unclassified
K03606	PMP	-0.11	0.01	0.08	Membrane and intracellular structural molecules	Unclassified
K01993	RMP	-0.08	0.01	0.08	Membrane and intracellu	Unclassified

KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
					lar structural molecules	
K07238	RMP	0.07	0.00	0.07	Other ion-coupled transporters	Unclassified
K04758	RMP	0.08	0.01	0.08	Other transporters	Unclassified
K01462	RMP	0.03	0.01	0.10	Others	Unclassified

RMP Red meat protein; PMP Processed meat protein (g/d); q-value < 0.1; L3- KEGG Name Level 3 name of KEGG ortholog; L1-KEGG Name Level 1 name of KEGG ortholog

Supplementary Information: Associations between the amount of dietary protein intake and protein sources with gut microbiota compositions

Supplementary method 1:

Calculation protein variables

$$\%\ Animal\ or\ plant\ protein\ to\ total\ protein\ content\ (\%AP\ or\ \%\ PP) = \frac{Animal\ or\ plant\ protein\ (\frac{g}{d})}{Total\ protein\ (\frac{g}{d})} \times 100$$

$$\%\ Animal\ or\ plant\ protein\ to\ total\ energy\ (\%\ APE\ or\ \%\ PPE) = \frac{\%\ AP\ or\ \%\ PP}{100} \times \%\ Energy\ of\ Protein$$

$$Ratio\ of\ Plant\ to\ animal\ protein\ = \frac{\%\ PPE}{\%\ APE}$$

Supplementary Table 1: Baseline and 1-year characteristics of various sources of protein

	Baseline (n= 323)	One year(n= 323)	p-value
Vegetables (g/d)	321.93 (243.83, 414.84)	360.58 (282.60, 446.43)	<0.001
Fruits (g/d)	310.36 (224.18, 433.73)	347.96 (270.37, 464.91)	0.001
Legumes (g/d)	20.56 (15.98, 29.70)	25.70 (17.14, 29.70)	<0.001
Grain intake (g/d)	191.50 (92.56, 208.92)	110.35 (92.14, 200.06)	<0.001
Dairy intake (g/d)	284.98 (183.10, 374.47)	248.78 (162.48, 346.18)	0.029
Total meat intake (g/d)	150.90 (123.56, 185.12)	135.44 (109.97, 174.24)	<0.001
Red meat intake (g/d)	42.84 (29.97, 64.26)	29.97 (19.98, 41.40)	<0.001
Processed meat intake (g/d)	36.18 (25.71, 47.14)	29.04 (19.51, 39.51)	<0.001
Poultry intake (g/d)	64.28 (64.26, 85.69)	74.26 (64.28, 85.69)	<0.001
Grain intake (g/d)	191.50 (92.56, 208.92)	110.35 (92.14, 200.06)	<0.001
Fermented dairy intake (g/d)	82.13 (46.42, 139.27)	83.79 (42.85, 144.21)	0.889
Nuts intake (g/d)	10.57 (4.28, 23.50)	29.99 (21.42, 39.56)	<0.001
Legumes intake (g/d)	20.56 (15.98, 29.70)	25.70 (17.14, 29.70)	<0.001

Supplementary Table 2: Associations between protein sources and predicted functional capabilities (KEGG) over 1-year changes

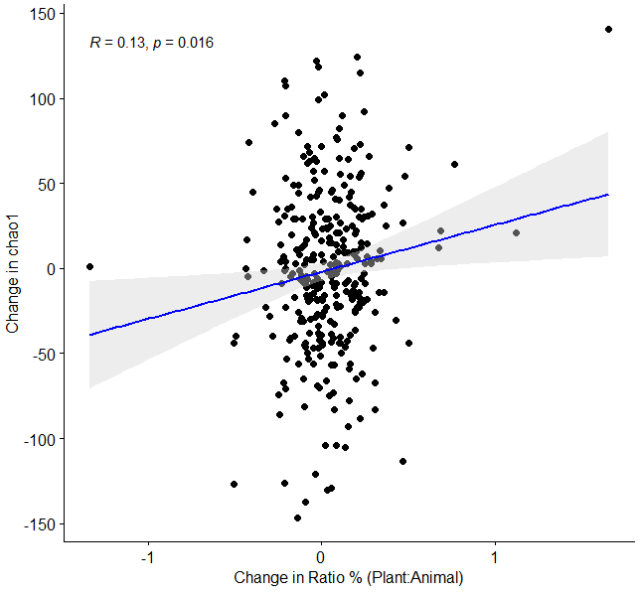
KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
K05989	PMP	-0.10	0.00	0.13	Others	Unclassified
K01192	PMP	-0.08	0.00	0.15	Lysosome	Cellular Processes
K01443	PMP	-0.04	0.00	0.17	Amino sugar and nucleotide sugar metabolism	Metabolism
K07007	PMP	0.05	0.00	0.17	General function prediction only	Unclassified
K15532	PMP	-0.10	0.00	0.17	None	None
K01586	PMP	0.02	0.01	0.18	Lysine biosynthesis	Metabolism
K09955	PMP	-0.06	0.01	0.19	Function unknown	Unclassified
K19302	PMP	0.02	0.01	0.19	None	None
K03569	PMP	0.02	0.01	0.20	Chromosome	Genetic Information Processing
K00945	PMP	0.02	0.01	0.20	Pyrimidine metabolism	Metabolism
K00558	PMP	0.02	0.01	0.20	Cysteine and methionine metabolism	Metabolism
K01462	PMP	0.02	0.01	0.20	Others	Unclassified
K01912	PMP	-0.07	0.01	0.20	Phenylalanine metabolism	Metabolism
K03768	PMP	0.03	0.01	0.20	Chaperones and folding catalysts	Genetic Information Processing
K18197	PMP	-0.13	0.01	0.20	None	None
K19271	PMP	-0.06	0.01	0.20	None	None
K03704	PMP	0.05	0.01	0.20	Transcription factors	Genetic Information Processing
K01802	PMP	-0.07	0.02	0.21	Protein folding and associated processing	Unclassified
K06969	PMP	0.02	0.02	0.21	Ribosome Biogenesis	Genetic Information Processing

KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
K07238	PMP	0.04	0.02	0.21	Other ion-coupled transporters	Unclassified
K00957	PMP	-0.04	0.02	0.21	Sulfur metabolism	Metabolism
K00833	PMP	-0.04	0.02	0.22	Biotin metabolism	Metabolism
K00891	PMP	0.02	0.02	0.22	Phenylalanine, tyrosine and tryptophan biosynthesis	Metabolism
K02372	PMP	0.03	0.02	0.22	Fatty acid biosynthesis	Metabolism
K15923	PMP	-0.06	0.02	0.22	None	None
K02470	PMP	0.02	0.03	0.23	DNA repair and recombination proteins	Genetic Information Processing
K00790	PMP	0.02	0.03	0.23	Amino sugar and nucleotide sugar metabolism	Metabolism
K01206	PMP	-0.06	0.03	0.23	Other glycan degradation	Metabolism
K02377	PMP	-0.04	0.03	0.23	Amino sugar and nucleotide sugar metabolism	Metabolism
K05515	PMP	0.02	0.03	0.23	Peptidoglycan biosynthesis	Metabolism
K00655	PMP	0.02	0.03	0.23	Glycerophospholipid metabolism	Metabolism
K01209	PMP	-0.05	0.03	0.24	Amino sugar and nucleotide sugar metabolism	Metabolism
K00014	PMP	0.02	0.03	0.24	Phenylalanine, tyrosine and tryptophan biosynthesis	Metabolism
K03624	PMP	0.02	0.03	0.24	Transcription machinery	Genetic Information Processing
K09458	PMP	0.02	0.03	0.24	Fatty acid biosynthesis	Metabolism
K02392	PMP	0.06	0.03	0.24	Bacterial motility proteins	Cellular Processes

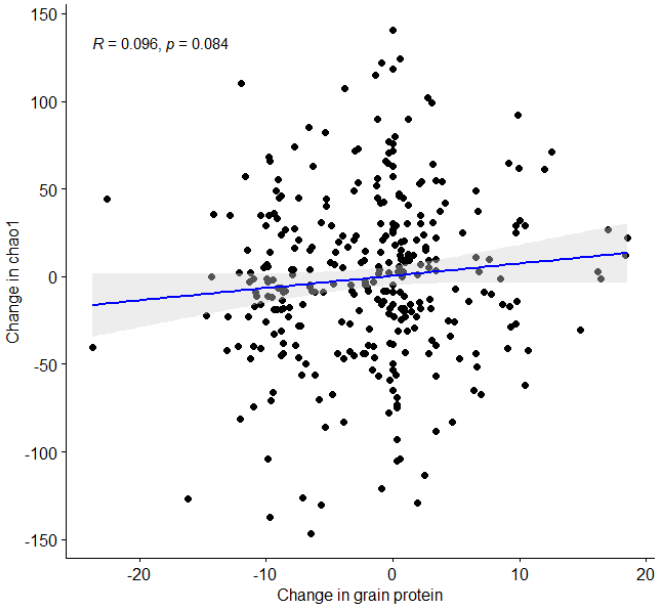
KEGG	PROTEIN DATA	COEFFICIENT	P-VALUE	Q-VALUE	L3-KEGG NAME	L1-KEGG NAME
K01872	PMP	0.01	0.03	0.24	Aminoacyl-tRNA biosynthesis	Genetic Information Processing
K02040	PMP	-0.01	0.03	0.25	ABC transporters	Environmental Information Processing
K00001	PMP	-0.06	0.03	0.25	Fatty acid metabolism	Metabolism
K01961	PMP	-0.02	0.03	0.25	Propanoate metabolism	Metabolism
K02469	PMP	0.02	0.03	0.25	DNA repair and recombination proteins	Genetic Information Processing
K02238	PMP	0.01	0.03	0.25	Secretion system	Environmental Information Processing
K03531	PMP	0.02	0.03	0.25	Chromosome	Genetic Information Processing

PMP Processed meat protein (g/d), q-value < 0.25, L3- KEGG Name Level 3 name of KEGG ortholog, L1-KEGG Name Level 1 name of KEGG ortholog

Supplementary Figure 1: Association between changes in ratio (PPE/APE) and chao1 index



Supplementary Figure 2: Association between changes in grain protein (g/d) and chao1 index



Chapter 3

Plant-based Fat, Dietary Patterns Rich in Vegetable Fat and Gut Microbiota Modulation

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REVIEW
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Plant-Based Fat, Dietary Patterns Rich in Vegetable Fat and Gut Microbiota Modulation

Jananee Muralidharan [1,2,3], Serena Galiè [1,2,3], Pablo Hernández-Alonso [1,2,3,4], Monica Bulló [1,2,3] and Jordi Salas-Salvadó [1,2,3]**

[1] Human Nutrition Unit, Department of Biochemistry and Biotechnology, Universitat Rovira i Virgili (URV), Reus, Spain, [2] Institut d'Investigació Sanitària Pere Virgili (IISPV), Reus, Spain, [3] Consorcio CIBER, M.P., Physiopathology of Obesity and Nutrition (CIBEROBN), Instituto de Salud Carlos III (ISCIII), Madrid, Spain, [4] Unidad de Gestión Clínica de Endocrinología y Nutrición del Hospital Virgen de la Victoria, Instituto de Investigación Biomédica de Málaga (IBIMA), Málaga, Spain

Diet is advocated as a key factor influencing gut microbiota. Several studies have focused on the effect of different carbohydrates, mainly fiber, on gut microbiota. However, what remains to be elucidated is the impact of a key component of diet that is widely debated upon: dietary fats. This review highlights the importance of understanding the source, quality, and type of fats that could differentially modify the intestinal microbiome. Fats from plant-based sources such as nuts, or vegetable oils have shown positive alterations in gut microbiota biodiversity both in *in vivo* and *in vitro* studies. Nuts and other plant-based fat sources, dietary patterns (e.g., Mediterranean diet) rich in polyunsaturated and monounsaturated fats and, in some cases, polyphenols, and other phytochemicals, have been associated with increased bacterial diversity, as well beneficial butyrate-producing bacteria imparting a positive metabolic influence. It is with this interest, this narrative review brings together evidences on different plant-based fat sources, dietary patterns rich in vegetable fats, and associated changes in gut microbiota.

Keywords: gut microbiota, plant-based fats, nuts, vegetable oils, Mediterranean diet

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Xia Xiao, Massachusetts General Hospital and Harvard Medical School, United States
Funmilola Abidemi Ayeni, University of Ibadan, Nigeria

****Correspondence:***
Monica Bulló
monica.bullo@urv.cat
Jordi Salas-Salvadó
jordi.salas@urv.cat

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INTRODUCTION

The significance of gut microbiota has grown from being just a hitchhiker to an active metabolic organ. The human gastrointestinal tract is composed of trillions of bacteria that play an important role in the host metabolism (1). This data directly suggest that the global microbiome potential is extremely high. Use of diet to alter gut microbiota as a potential therapeutic target is widely researched (2).

Dietary fibers are an important source for the fermentation of intestinal bacteria (3). An extensive amount of research has focused on understanding dietary fiber as a key part of plant-based diets (4, 5). However, other than dietary fibers, fractions of unabsorbed protein and dietary fat, reaches the large intestine and therefore can potentially be substrates that differentially influence the microbial system (6, 7).

Even though there are many studies in the context of high-fat diets and gut microbiota, studies differentiating them from plant and animal-based sources are relatively scarce. Irrespective of the type of fat, high fat diets (HFD) have frequently shown to induce an increase in the abundance of Firmicutes in comparison to the low fat diet (LFD) (8, 9). Linoleic acid, mainly coming from plant sources, are utilized by different gut microbial species to produce conjugated

linoleic acid (CLA) that has shown anti-inflammatory, anti-adipogenic, anti-diabetogenic, and anti-carcinogenic properties (10). Omega-3 fatty acids [ω-3 polyunsaturated fatty acids (PUFAs)] have received higher attention from scientific community due to its protective effects against inflammatory status both in *in vitro* and *in vivo* studies, compared to other types of fat (11), but its effects on microbiota regulation remain unclear [reviewed in (12)]. Unlike ω-3 PUFAs, monounsaturated fatty acids (MUFAs) have shown inconsistent results. In fact, a recent systematic review has shown that diets high in MUFA tend to decrease total bacterial numbers (13). Western diets rich in saturated fats and low in antioxidants, phytosterols, and other phytochemicals have shown to change gut microbiota favoring a pro-inflammatory state (14). Based on long-term dietary habits, gut microbial profile is divided broadly into two enterotypes: (i) *Prevotella* enterotype, found predominantly in the people consuming carbohydrate-based diets or the vegetarian diet; (ii) *Bacteroides* enterotype, found in high protein and/or animal products-derived diets (15).

With existing research, a diet with emphasis on plant-based foods and low consumption of red meat has been endorsed as a healthy dietary choice. Vegetarian or vegan diets (16–18) and the Mediterranean Diet (MedDiet) emphasizing the consumption of plant-based foods have shown to have beneficial impacts on gut microbiota (19), overall metabolism and health (20). Amongst these diets, MedDiet contains a high amount of plant-based fats (35–45% of total energy), sourced from olive oil [mainly extra virgin olive oil (EVOO)] and nuts. High-fat energy dense foods such as nuts or olive oil could be seen as foods contributing to weight gain that could lead to obesity or related morbidities. However, nuts and olive oil have not been associated with weight gain (21, 22), rather a direct association of these fat sources with healthy metabolic profiles has been shown (23), mainly ascribed to their specific fat composition and their bioactive molecule content.

Animal vs. Plant Fat

Prior animal studies have shown that the composition, and not the quantity of dietary fat, is important in modulating endotoxemia (24). Circulating endotoxins, majorly from the gram-negative bacteria, elicit inflammation. Serum endotoxins from human and animal studies depict that after ω-3 PUFA intake, the post-prandial serum endotoxin production is lower than that of saturated fatty acids (SFAs) (24, 25). The majority of these studies have considered the SFA source from vegetables (butter or palm oil), and fish oil as the major ω-3 PUFA source. As plant-based fats vary widely by composition, future studies comparing different plant-based fat sources will be of profound value. Interestingly, animal foods such as red meat and fish are not only sources of fats, but also sources of protein. In a study focusing on different protein sources, it was noted that soy-based protein had the highest circulating endotoxins compared to red or white meat sources (26). Even though plant protein in this study showed higher endotoxin levels, evaluation of animal sources should be considered skeptically due to the presence of heme, N-nitroso compounds, polycyclic aromatic hydrocarbons and heterocyclic amines in meat products that are involved in gut health-related problems (27).

With the growing popularity of vegetarianism, many studies have investigated the differences in gut microbiota with respect to plant-based diets (vegan or vegetarian) (5, 28, 29). Considering the wide range of fat sources available, only few studies have explored their effects on gut microbiota. The complex nature of food makes it difficult to determine the causal nature of a particular dietary component on gut homeostasis. Hence, when the synergistic effects of a food are considered, plant-based fat sources also rich in antioxidants and fibers would be a better substitute to animal-based fat sources carrying heme and/or nitroso-compounds.

Even though this is a growing area of research, the collection of literature in bringing together evidences keenly on the different fat sources from plant-based diets and their effects on gut microbiota is limited. Hence, the purpose of this narrative review is to summarize the relevant evidence (after reviewing in PubMed) linking the different plant-based fat sources and dietary patterns rich in vegetable fat sources and their impact on gut microbiota. We selected the articles by using a combination of search terms in PubMed for each section. The following keywords were included in each section: (i) nuts, pistachios, hazelnuts, cashews, walnuts, macadamia nuts, peanuts, almonds, brazil nuts, pine nuts, pecans, (ii) corn oil, castor oil, coconut oil, cottonseed oil, sunflower oil, olive oil, rapeseed oil, peanut oil, palm oil, rice bran oil, safflower oil, sesame oil, soybean oil, plant-based fat, (iii) Mediterranean diet. All the above-mentioned keywords were used in combination with an "AND" builder with the following keywords: gut microbiome, gut microbiota, intestinal microbiome. We included only human studies or those conducted on mice or rats. *In vitro* studies were included only in the appropriate places where there was not enough evidence from human or mouse/rat studies. Despite that, we cannot discard that some studies may not be included as this is not a systematic review.

NUTS AND GUT MICROBIOTA

Consumption of nuts has been shown to have protective effects against metabolic disorders such as type 2 diabetes (T2D), dyslipidemia, and cardiovascular disease (CVD). A recent prospective analysis conducted with 16,217 subjects with T2D showed that participants consuming ≥5 servings of nuts compared to ≤1 serving per month had a lower total CVD incidence, coronary heart disease incidence, CVD mortality and all-cause mortality (30). Previous meta-analysis reported a reduced risk for T2D, neurodegenerative disease, infectious diseases, with consumption of 28 g of nuts/day. Modulation of lipid metabolism, antioxidant activity and gut microbiota are some of the proposed mechanisms (31). Some of these benefits are driven by modulation in lipid metabolism, antioxidant activity, also via gut microbiota.

Nuts are a complex matrix of nutrients especially rich in fiber, unsaturated fatty acids (UNFAs) and different bioactive compounds such as tocopherols, phytosterols, phenolic

compounds, and minerals such as magnesium (32). Some of these nutrients can reach the colon intact, being able to change the gastrointestinal microbiota composition and function. Different nutrients and their metabolites, such as polyphenols have shown to aid in gut microbiota balance and growth of beneficial bacteria [reviewed in (33)]. The fermentation of fiber from nuts or other sources to beneficial end-products (e.g., butyric acid) and the biotransformation of phytochemicals have been reported to be associated with the transition to a healthier microbiota (34). Thus, nuts could exhibit prebiotic effects by enriching potentially beneficial microorganisms such as Bifidobacteria or lactic acid bacteria (35).

Fat from nuts may have also a major impact on gut microbiota because a considerable amount of fat present in nuts can arrive intact to the colon. Incomplete mastication or inaccessible fats inside cell structures remain unabsorbed during digestion and this small degree of fat moves to the intestine, serving as a prebiotic (36, 37). Atwater factors of almonds (38), pistachios (39), walnuts (40), and cashews (41) have indeed showed an overestimation of measured energy contents.

Among nuts, almonds, pistachios, and walnuts have showed to have different protective properties modulating, for example, insulin resistance, glucose metabolism, and lipid profile [reviewed in (42), (43), and (44)]. However, their prebiotic properties were not well-characterized until a few years ago. Different *in vitro* and *in vivo* studies have analyzed the prebiotic effect and fermentation properties of raw and roasted almonds, as well as almond skins. These studies have shown the ability of different components of almonds that could positively alter the composition of gut bacteria (45–47). In fact, a stimulatory effect on *Lactobacillus* spp., and *Bifidobacterium* spp., has been observed from raw and roasted almond consumption (47). Beyond almonds, several clinical feeding trials have demonstrated a modulatory effect of other types of nuts on gut microbiota. First in 2014, Ukhanova et al., performed two separated randomized, controlled, cross-over feeding studies with healthy subjects, giving them either almonds ($n = 18$) or pistachios ($n = 16$), in three interventions (no nuts, 42 or 84 g/day) each for 18 days (48). They showed that both types of nuts significantly affected microbiota. However, the prebiotic effect of pistachio intake on gut microbiota composition was much stronger than that of almond consumption. Moreover, pistachios increased the number of butyrate-producing bacteria, identified as potentially beneficial, whereas the numbers of *Bifidobacterium* were not affected by the consumption of either type of nut (48). Relevantly, a 4-month, crossover randomized clinical trial (RCT) conducted in 49 pre-diabetic subjects found a shift toward a healthier gut microbiota following pistachio consumption by assessing gut-derived metabolites in 24 h-urine (49). Three metabolites related with gut microbiota metabolism (i.e., hippurate, p-cresol sulfate and dimethylamine) decreased after pistachio diet compared with the nut-free control intervention.

In 2014, Liu et al., reported a 6-week study with 48 volunteers that were randomly assigned to three different intervention groups: (i) control group was supplied with 8 g/d of fructooligosaccharides; (ii) intervention group supplemented with 10 g/d of almond skins; and, (iii) intervention group with 56 g/d of roasted, unsalted, whole almonds (50). *Bifidobacterium* spp., and *Lactobacillus* spp., increased significantly in the almond and almond skin groups. The populations of *Escherichia coli* mildly changed, and the growth of *Clostridium perfringens* was significantly repressed in both almond intervention groups. The difference in the results of these two studies could be attributed to their duration, since Ukhanova et al. (48) administered nuts only for 18 days in contrast with 6 weeks in the case of Liu et al. (50). Another 3-week short-term nut crossover study was conducted in 29 parents and their respective children ($n = 29$). The parent-children duo consumed 42 and 14 g/d of almonds (including almond butter), respectively. Researchers reported significant changes at overall genus level after almond consumption vs. control intervention, especially in children (51).

A controlled-feeding randomized crossover study conducted in 18 healthy subjects assessed the beneficial effect of almond consumption on gut microbiota composition for periods of 3 weeks (52). This study compared the effect of consuming 1.5 servings of raw or processed (roasted or chopped) almonds or almond butter to a control almond-free intervention group. They showed that almond consumption increased the relative abundances of *Lachnospira*, *Roseburia*, and *Dialister*. Particularly, chopped almonds increased the abundance of *Lachnospira*, *Roseburia*, and *Oscillospira*, while whole almonds increased *Dialister*, compared to control. Overall, this study showed that almond consumption and its degree of processing differentially impact the relative abundances of bacteria genera in the gastrointestinal tract.

Two different trials were recently performed to assess the shift in the gut microbiota due to walnut consumption with a different length of intervention (53, 54). Holscher et al., evaluated using a 3 weeks crossover study design (1 week washout) the effect of 42 g of walnuts vs. no consumption, in 18 overweight but otherwise healthy men and women (53). Forty-nine to sixty percent higher relative abundance of *Faecalibacterium*, *Clostridium*, *Dialister*, and *Roseburia* and 16–38% lower relative abundances of *Ruminococcus*, *Dorea*, *Oscillospira*, and *Bifidobacterium* were observed in walnut consumption compared to the control period. Moreover, authors reported an improvement in the lipid profile in case of walnut supplementation. These results are supported by *in vivo* studies indicating that walnuts increased the relative abundances of *Firmicutes*, including the genera *Clostridium* (55) and *Roseburia* (56). In fact, walnuts showed mild protection to the colon against a potent carcinogenic reaction partially due to walnut-induced changes to the gut microbiome (55). Due to the negative association of *Faecalibacterium* and *Roseburia*, positive association of *Oscillospira* with age, it has been suggested that consumption of walnuts may help in age related changes in the gut microbiota (57, 58). Future studies assessing the aspects of walnut consumption on gut microbiota and aging would be of value.

In a similar—but of a longer duration—crossover RCT, 135 normo-weight or overweight healthy subjects consumed 43 g/d of walnuts or a nut-free diet for 8 weeks (54). Generalized UniFrac distance showed that walnut consumption significantly changed microbiome composition and diversity. By using multidimensional scaling approach,

authors reported dissimilarities of ∼5% between walnut and control diet interventions. Specifically, the abundance of the family *Ruminococcaceae* and genus *Bifidobacterium* increased significantly, while the genus *Blautia* and *Anaerostipes* decreased significantly during walnut consumption. A controlled feeding intervention study with roasted hazelnuts was conducted in hyperlipidemic (and age-matched normolipidemic) children and adolescents (7–17 years) for 8-weeks assessing the changes in gut microbiota. At baseline, the α- and β-diversity microbiota were significantly different between hyperlipidimic and normolipidemic participants. At baseline, subjects with hyperlipidemia had significantly lower concentrations of acetate, butyrate and propionate, whereas they had significantly higher levels of lactate, pyruvate and isobutyrate. The authors reported a non-significant difference in the microbial composition after the hazelnut intervention between the hyperlipidimic and control participants. In SCFAs' (measured in feces), only a significant increase in acetate concentrations was reported after the intervention in the hyperlipidimic group (59).

Taken together, although daily consumption of nuts (1–2 servings/d) have shown to impact gut microbiome by enhancing beneficial bacterial species, further studies are needed to determine whether: (i) these modulations are preserved during longer nut consumption periods; (ii) these modulations may also affect subjects with cardiometabolic diseases; and (iii) these modulations are associated with improvements in other disease-related parameters.

VEGETABLE OILS AND GUT MICROBIOTA

A common and popular plant-based fat source is vegetable oil. Consumption of vegetable oils rich in unsaturated fats has been associated with healthier metabolic conditions (low LDL (low density lipoprotein) cholesterol levels, and lower risk of T2D and CVD compared to other animal fat sources) (60, 61). This could be partly attributed to the type of fat but also to their high content in polyphenols and other phytochemicals in case of virgin olive oil (62). Vegetable oils are formed by a mixture of SFAs, UNFAs, MUFAs, or omega-6 polyunsaturated fatty acids (ω6 PUFAs), which can vary between different types of oils. Even though vegetable ω6 PUFAs have been considered pro-inflammatory in contrast to ω3 fatty acids, the interaction of omega-3 and omega-6 fatty acids in the context of inflammation is complex and still not properly understood (63–65).

Avocados are an important plant-based fat source that are also rich in dietary fibers. Only few studies have been conducted to explore the effects of avocado on gut microbiota. A recent RCT conducted amongst 160 adults (BMI $\geq$ 25 Kg/m^2) with parallel arms of treatment (iso-caloric meals, with or without avocado), evaluated the effect of Hass avocado consumption for 12 weeks. Compared to control, avocado consumption increased acetate ($p < 0.01$) and total SCFA's ($p = 0.02$) and the relative abundances of *Faecalibacterium* ($p = 0.01$) in feces (66). In a similar RCT with 51 healthy overweight/obese participants, the effect of avocado consumption on gut microbiota, biomarkers of inflammation, weight loss and body composition was tested. Participants either followed an avocado hypocaloric diet (1 Hass avocado- AVO) or a hypocaloric diet avoiding the consumption of avocados (CTRL) for 12 weeks. Relative proportions of genus *Bacteroides*, *Clostridium*, *Methanospaera*, and *Candidatus Soleaferrea* were altered significantly in the AVO group compared to CTRL group. Also a trend to decrease serum inflammatory markers IL-1β ($P = 0.07$) and C-reactive protein ($P = 0.074$) was observed in the AVO group compared with CTRL group (67).

Health benefits of olive oil, which is rich in MUFAs and polyphenols, has been largely related to a decrease in the incidence of CVDs and hypertension as well as being considered as a positive modulator in cognitive functions (68, 69). Olive oil can be categorized into four types based on the processing methods and its contents: extra-virgin olive oil, virgin olive oil, refined olive oil (ROO), and Orujo oil (68). Even though the main fatty acid composition remains the same, some polyphenolic components change in these four types of olive oil. Virgin olive oil has the highest polyphenol content (∼150–400 mg/kg), refined olive oil with the lowest polyphenol content (∼0–5 mg/Kg), and the common olive oil, pomace olive oil with intermediate polyphenol content (∼10–100 and ∼10–30 mg/Kg, respectively) (70). It is important to understand the difference in properties exerted by polyphenols in comparison to the fat profile of olive oils. With this regard, Hidalgo et al. (71) compared 12 week feeding of EVOO, ROO butter, and the standard chow diet in mice. Denaturing gradient gel electrophoresis (DGGE) and culture-dependent methods were used to analyze the microbiota in the feces. The family *Lactobacillaceae* appeared to increase in the butter group from baseline to week 12. Most of the species reported in all the diet groups were uncultured and no quantitative statistical evaluation was performed comparing the differences in microbiota composition. Hence, it is difficult to state specific differences among diets and/or time points. It was noted that most EVOO microbiota clustered with ROO, while microbiota cluster from butter was different. Also, butter diet induced changes closer to the gut microbiota of obese individuals, whereas the EVOO in the opposite direction and ROO with an intermediate behavior (71). Hence, it was observed that even though polyphenol content of the olive oil contributes to an extent to the changes in gut microbiota, the fat profiles also play a determining role.

Prieto et al. (72) compared the effects of a diet enriched in EVOO vs. butter (BT) in 26 Swiss Webster mice. They were fed with a standard diet (SD, $n = 8$) (3% of total energy from fats) or one of the two high fat isocaloric diets (35% of total energy from fats) enriched in EVOO ($n = 9$) or butter (BT, $n = 9$). Mice fed with BT diet, showed the highest systolic blood pressure (SBP), and SBP was positively correlated with *Desulfovibrio*. EVOO group had the lowest plasma insulin, which was correlated inversely with *Desulfovibrio*. Several other correlations were observed between the gut microbiota (at phylum, family, genus and species levels) and the measured metabolic syndrome (MetS) parameters. The authors concluded a positive metabolic impact of EVOO mediated by the gut microbiota (72). Similar result with reduction in SBP was reported in another mice study fed with EVOO (73), in which the taxonomic cluster of Clostridia cluster

XIVa was inversely correlated with SBP, and a significantly higher abundance of *Lactobacilli* was also seen in the EVOO group (73).

The quality of fats in terms of health is usually indicated by its levels of saturation or unsaturation. Recently a mice study was conducted to evaluate the differences amongst SFA, UNFAs on gut microbiota (8). Three different HFD (40% of total energy from olive oil, corn oil or milk fat) and a LFD were given to the mice for 12 weeks. This study not only evaluated the microbial changes in the gut, but also the host response to these changes, hence giving an overall picture on microbe-host homeostasis. All the HFD increased the abundance of Firmicutes. The following increased abundances were noted in each group: olive oil group (*Clostridiaceae*, *Peptostreptococcaceae*, *Ruminococcaceae*, and *Dorea* spp.); milk fat (*Erysipelotrichales* and several genera from *Ruminicoccus*); corn oil (*Turicibacteracea* and *Coprococcus* spp.). Acetic acid and propionic acid levels were decreased in the olive oil, corn oil group compared to the low-fat chow group, whereas milk fat had similar levels of SCFA to that of low fat chow group. Corn oil rich in ω6 PUFAs showed increase in risk factors for development of dysfunctional gut barrier, whereas the milk fat rich in SFA promoted host inflammation, and olive oil resulted in a less inflammatory environment compared to the other two diets (8).

Few studies have focused on the phenolic components of olive oil (74, 75) and their role in modulating gut microbiota. Phenolic compounds of olive oil in combination with thyme phenolic compounds have shown to increase in members of *Bifidobacterium* and decrease the oxidation of LDL in blood in hypercholesteremic participants (74). However, further research is required in elucidating the role of different components of olive oil on gut microbiota.

Flaxseed oil (FO), soybean oil, coconut oil, palm oil and canola oil are other types of vegetable fat sources that are usually consumed around the world. They vary from each other widely by fatty acids and bioactive components.

Palm oil and coconut oil are SFA rich vegetable oils. Comparing the vegetable fats based on their PUFA/SFA ratio, by supplementing either a HFD rich in palm oil, safflower oil or olive oil, had demonstrated that palm oil (having the lowest PUFA/SFA ratio) reduced the microbial diversity and increased the Firmicutes-to-Bacteroidetes ratio (9). Apart from palm oil, another vegetable fat rich in SFA source is coconut oil. Compared to palm oil, coconut oil is characterized by the presence of both medium- and long- chain fatty acids, which may have better implications for host energy balance than lipids rich in long-chain fatty acids. Coconut oil, in its virgin form (i.e., of higher quality) has shown to be associated with beneficial effects on secondary parameters of T2D in mice, along with an increased abundance of beneficial bacteria such as *Lactobacillus*, *Allobaculum*, and *Bifidobacterium* species (76). Recent results from animal studies comparing coconut oil vs. soybean oil based diets showed that soybean oil resulted in a detrimental metabolic health compared to coconut oil, however with no changes in cecal microbiota (77).

An interesting study compared gut microbiota composition after the consumption of either lard (rich in SFA), fish oil (rich in ω6-PUFA, MUFA) or soybean oil (rich in ω3-PUFA) as different source of fats in middle-aged rats (78). *In vitro* and *in vivo* studies showed a different gut microbiota structure in the fish oil group from soybean oil or lard groups. Fish oil group has the highest relative abundance of phylum Proteobacteria and genus *Desulfiovibrio*. Along with these observations, it was also noted that mRNA levels of inflammatory markers (IL-1β, IL-6, IL-17, IL-18, and TNF-α) were higher in the fish oil group. Both these results indicate that fish oil could potentially increase the risk of inflammation, contrary to the prior studies (79). In fact, a high content of PUFA in diet, even if recommended by public health, is considered to cause metabolic oxidative stress and inflammation (80), also high MUFA diet has been suggested to have less consistent effects on gut microbiota (13). Therefore, these results suggest a new insight into the potentially negative effect of fish oil on inflammation through changing the microbiota population, in contrast with a vegetable source of fats like soybean oil.

To better understand the role of different types of fats on metabolic health, four HFD enriched with either palm oil, olive oil, safflower oil, or a combination of both flaxseed oil and fish oil were fed to wild-type C57BL/6J male mice. The groups with high MUFA and PUFA contents (olive oil and flax plus fish oil) showed a lower plasma triglyceride and less weight gain. Also, different compositions in gut microbiota were found between groups. Especially, olive oil group was characterized by an increase in bacterial family of *Bacteroidaceae,* and flaxseed/fish oil group was the only one in which there was an increase in *Bifidobacteriacea* family. Both this bacterial family include commensal bacterial with beneficial effects on gut health (81).

Other than the soybean oil, FO is a plant-derived oil rich in ω3 PUFAs, mainly α-linolenic acid (ALA, 18:3 ω-3). Dietary FO has shown protection against acute alcoholic hepatic steatosis via ameliorating lipid homeostasis at adipose tissue-liver axis in mice (82). However, the impact of dietary FO on inflammation and gut microbiota in chronic alcoholic liver disease (ALD) remains unknown. In order to investigate this topic Zhang et al., evaluated the interplay among the diet, gut microbiota, inflammation and ALD in mice models of ALD (83). Sixty mice were randomly allocated into four groups: pair-fed (PF) with corn oil (CO) group (PF/CO); alcohol-fed (AF) with CO group (AF/CO); PF with FO group (PF/FO); AF with FO group (AF/FO). A reduction of *Porphyromonadaceae* and *Parasutterella*, and an increase in Firmicutes and *Parabacteroides*, were observed in AF group compared to the PF control. Supplementation of FO in the ethanol consumption group (AF/FO) reduced *Proteobacteria* and *Porphyromonadaceae* significantly compared with AF/CO group.

Canola Multicenter Intervention Trial (COMIT) evaluated the interactions between obesity status and dietary intake of mono- and poly-unsaturated oils on human gut microbiome with participants at MetS risk. The experimental diets used were: (1) conventional canola oil (Canola); (2) DHA-enriched high oleic canola oil (CanolaDHA); (3) high oleic canola oil (CanolaOleic); (4) blend of two PUFA-rich of corn/saffower oil (25:75, CornSaff); and (5) blend of flax/saffower oil (60:40, FlaxSaff) supplemented diets designed to maintain body weight during the treatment periods. Diets 1, 2, 3 were rich in MUFAs and diets 4, 5 rich in PUFAs. Clear differences were observed in the gut microbiota profiles of obese group vs. overweight and the normal weight participants, with Firmicutes dominating the obese group. The differences between MUFA and PUFA

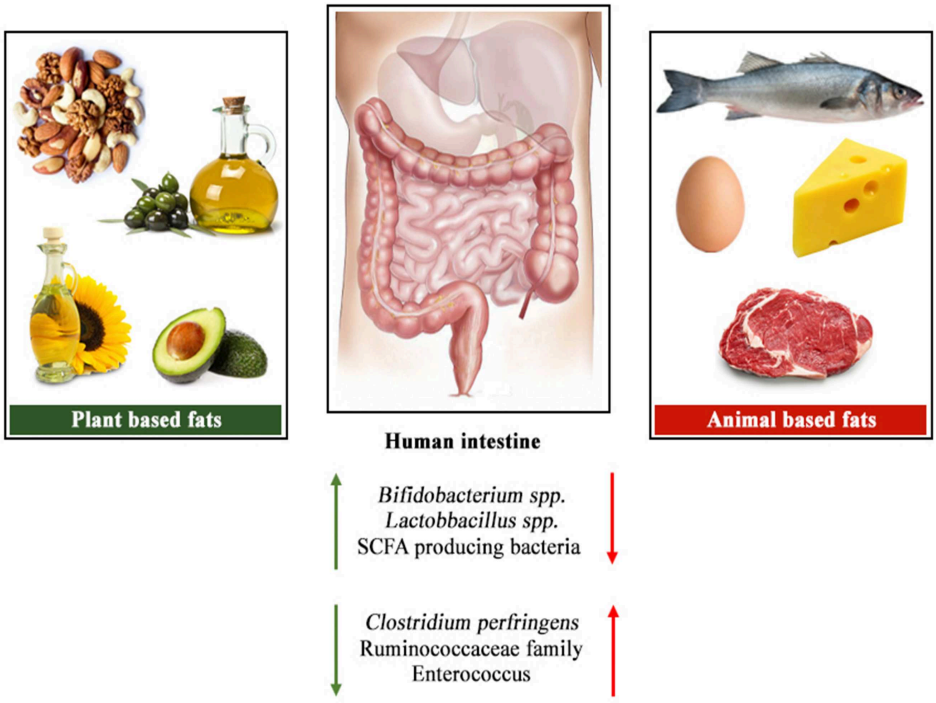

FIGURE 1 | Overview of the changes in the gut microbial profile with differences in animal and plant-based fat sources.

rich diets, continued to be segmented by the influence of BMI. Abundance of *Faecalibacterium* [which has shown anti-inflammatory properties (84)] differed across treatments, with highest abundance in CanolaOleic and lowest in CanolaDHA, indicating the potential of oleic acid with an anti-inflammatory property (85).

Figure 1 shows an overview of the changes in the gut microbial profile with differences in animal and plant-based fat sources. Even though plant-based oils have been part of our diet since many decades, the potential impacts of these oils on gut microbiota still remain relatively unknown. The ratio of different saturated or unsaturated fatty acids clearly impose different effects on gut microbiota, however the debate remains open on the levels that is most suitable for better gut health.

MEDITERRANEAN DIET AND GUT MICROBIOTA

The traditional view of single nutrient health effects has been shifting toward synergy of multiple food components and dietary patterns. The traditionall view of single nutrient health effects has been shifting toward synergy of multiple food components and dietary patterns. Understanding the effects of nutrient components and dietary patterns would be helpful to make lifestyle recommendations (86). Plant-based diets have been gaining acceptance and popularity due to the positive health benefits. Modulation of gut microbiota is one of the plausible mechanisms explaining these benefits. In terms of nutritional content, most of the plant-based diets are low in total and saturated fats compared to the omnivores diet (87, 88). However, MedDiet is an exception to this, with a high content of MUFA and PUFA from plant sources.

Several studies have emphasized the health effects of MedDiet since the seven countries study (89). MedDiet has been evaluated in terms of its effects on mortality, cardiovascular risks, mortality in several systematic reviews and meta-analysis (90, 91). The traditional MedDiet, is characterized by high consumption of vegetables, legumes, grains, fruits, nuts, and olive oil (plant-based foods), moderate consumption of fish and wine, and low consumption of red and processed meat and sugar. This dietary pattern rich in polyphenols, fiber and unsaturated fat, impart the above mentioned health benefits by various mechanisms including anti-oxidative potentials, anti-inflammatory properties and gut microbiota modulation (92) among others.

High-level adherence to MedDiet has shown to be positively associated with changes in beneficial gut microbiome and their metabolites (93). Contrary, a lower adherence to MedDiet was linked to higher urinary TMAO levels, a microbial metabolite that has been reported to be a marker for cardiovascular risk (94). Enhancement of fiber-degrading *Prevotella*, Firmicutes, and higher level of fecal short-chain fatty acids has been associated

with higher adherence to a MedDiet (19). Similarly, presence of fiber degrading *Prevotella* was seen higher in preadolescent Egyptian subjects ($n = 28$) following a MedDiet in comparison to preadolescents in Dayton, USA ($n = 14$) consuming a Western diet (95). An observational study conducted in Greece amongst 120 participants investigated the associations between adherence to MedDiet and gut microbiota pattern. In this study, a higher adherence to MedDiet was inversely associated with *E. coli* counts, higher *Bifidobacteria: E. coli* ratio. Within the SCFA's measured, acetate was present in highest proportions (i.e., higher molar ratio) in all the tertiles of MedDiet adherence score (low, medium, and high). Also, greater molar ratio of acetate was reported to be significantly associated with higher adherence to MedDiet (96). MedDiet score measured in another study as an indicator of adherence to diet showed similar results (97). It was observed that the higher MedDiet score was associated with abundance of phylum Bacteroidetes, family *Prevotellaceae* and genus *Prevotella*. Fecal propionate and butyrate were higher in participants with a higher MedDiet score. Also, the consumption of olive oil, the main source of MUFA of this diet, was associated with increasing proportions of taxa *Tenericutes* and *Dorea* (97). In another study conducted in the Mediterranean population, genus *Dorea* and *Lactobacillus* were over represented in those participants consuming a high PUFA/SFA ratio (93). Haro et al. (98) conducted an intervention study comparing MedDiet (35% fat: 22% monounsaturated; 6% polyunsaturated and 7% saturated) and a low fat high complex carbohydrates (LFHCC) diet (28% fat: 12% monounsaturated; 8% polyunsaturated and 8% saturated) for a period of 1 year amongst 20 obese men. Consumption of MedDiet showed an increase in beneficial *Roseburia* genus whereas consumption of LFHCC showed an increase in fiber degrading *Prevotella* and *F. prausnitzii* (98). Another interesting study compared the MedDiet and a vegan diet (*Ma-Pi 2*). The *Ma-Pi 2* diet is rich in seaweeds, wholegrains, legumes and fermented products. Both diets followed for 3 days in 12 reactive hypoglycemic participants, induced no changes in the gut microbial composition, however the SCFA's in the *Ma-Pi 2* diet group was increased significantly from baseline to the 4th day (99).

Compromised gut bacterial profile is observed amongst people with several metabolic disorders (100, 101) Exploring MedDiet as a nutritional therapy could help in the reestablishment of a beneficial gut ecosystem. In this regard, few studies have evaluated the effect of MedDiet on gut microbiota and health. A total of 239 participants (with and without MetS) from the CORDIOPREV study were randomly allocated in two groups: LFD (MetS, $n = 139$) and MedDiet group (MetS, $n = 101$). After 2 years of following the diets, participants in the MedDiet group showed a restoration of some species of gut microbiota (*P. distasonis, B. thetaiotaomicron, F. prausnitzii, B. adolescentis*, and *B. longum*) in only those with MetS (102). MedDiet has also been effective in betterment of gut microbial ecology amongst Crohn's disease patients by increasing the Bacteroidetes phylum and *Clostridium* genus after 6 weeks of MedDiet consumption (103).

Even though overall credits on the beneficial effects of MedDiet cannot only be given to the healthy fat profile, it cannot be discarded that other components of this dietary pattern (such us dietary fiber, some vitamins and minerals, polyphenols and other phytochemicals) may also exert effects on gut microbiota profile and activity. Therefore, future larger human intervention studies are required in order to understand the role of MedDiet and its components on gut microbiota alterations.

DISCUSSIONS

Increasing number of studies are focusing on the importance of plant-based diets, as well as on the components of this type of diet. Nuts, olive oil and other plant fat sources comes with a broad composition of fatty acids that has varied biological impacts. Investigating the potential role of PUFAs in inducing beneficial effects should be evaluated with care, as the enzymatic peroxidation products of PUFAs has shown carcinogenic potentials (104). Studies exploring the cumulative effects of the fat source (containing other non-fat components such fiber or antioxidants) could mask the isolated effects of oxidation products of PUFAs (105, 106). These studies could strengthen the importance in understanding the mechanism involved in the synergy of different dietary components and fat on gut microbiota.

Exploration of novel pathways such as for sterculic acid that has shown effects on insulin resistance and obesity via gut microbiota modulation could be of interest to develop nutritional therapies (107). A comprehensive systematic review conducted by Wolters et al., observed that a modulation of dietary fat—by quantity or quality—did not impose any effects on gut microbiota in interventional studies, whereas observational studies reported gut microbiota shifts (13). A key reason discussed by the authors of these studies was the low intervention follow-up time. Moreover, gut microbiota studies are subjected to inter-individual differences that complicates the analysis. Further interventional studies with dietary fats, focusing on the aspect of gut microbiota would aid in a better understanding and to establish nutritional recommendations.

Dietary fat is an essential component of diet that needs to be consumed in the right quantity and quality. Based on the studies included in this review, nuts, and other plant-based fats seem to exert a favorable effect on genus *Bifidobacterium*, *Roseburia*, and *Faecilibacterium*, which has been associated with positive health effects. High fat diets with SFA as the main fat component have consistently been correlated with negative modulation of gut microbiota such as decreasing relative diversity. Thus, replacement of SFAs with plant sources of PUFAs and MUFAs, especially those rich in polyphenol and other phytochemicals, would help in positive modulation of gut microbiota and the corresponding health implications.

AUTHOR CONTRIBUTIONS

JS-S and MB contributed to the design of the review and editing of the manuscript. JM, PH-A, SG, MB, and JS-S performed the bibliographical search and wrote the first draft. All the authors approved the final manuscript.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

VI. DISCUSSION

Discussion

This thesis is focused on understanding the effect of a lifestyle intervention on intestinal microbiota, further diving deep into elucidating the associations between some components of diet and the intestinal microbiota profile and its predicted function. This thesis was conducted in the framework of PREDIMED-Plus study, which targets on weight loss and associated CVD risk reductions in an elderly population with overweight/obesity and MetS. As it is well known that dietary interventions alter gut microbiota, we embarked on this research to find if an intensive weight loss intervention based on calorie restricted MedDiet, along with physical activity promotion and behavioral support could alter gut microbiome to a beneficial state for the host health. The results derived from this thesis provides many interesting findings that may be essential for future studies focused on lifestyle-based interventions as well adds value as it is conducted in an important section of population, who are at a high risk of CVD diseases.

It is well established that gut microbiota plays a role in host energy metabolism and metabolic regulation. Understanding the changes in gut microbiota in a lifestyle interventional setting is interesting and essential. Our study is the first to evaluate such multiple strategy based weight loss intervention on gut microbiota in a large scale population (**Chapter I**). After 1-year of intervention, we observed a significant decrease in weight (4.2 kg (IQR, -6.8, -2.5)) in the intervention group, accompanied by significant reductions in BMI, waist circumference, glucose, HbA1c and increase in HDL levels compared to the control group (similar effects that we have observed in the PREDIMED-Plus pilot study published in *Diabetes Care* (272)). These changes were accompanied with changes in the gut microbial composition and predicted functions. Even though widely debated, we noted a reduction in F/B ratio after 1-year in the intervention group compared to the control group. We also observed several changes in gut microbial composition that were consistent with previous studies conducted in the scope of MedDiet and gut microbiota (91,273). In specific, previous studies have shown increase in SCFA producers such as *Roseburia*, *Dorea*, *Coprococcus*, etc. following MedDiet, which was consistent with our observations in control group, who were suggested to adhere to a non-energy restricted MedDiet. However, these genera decreased in presence of calorie restriction in the

intervention group. This reduction in SCFA producers could be postulated by the fact that presence of carbohydrate/polysaccharide utilizing bacteria could increase the net energy absorption for the host contributing to obesity (274,275). Consecutively, in the intervention group we also observed reductions in several members of major saccharolytic phylum Firmicutes (*Butyricicoccus*, *Eubacterium hallii*, *Ruminiclostridium* 5) compared to the control group. Even though there were certain reductions in SCFA producers in the intervention group, there was also selective increase in some SCFA producers such as *Lachnospira*, *Lachnospiraceae* NK4A136 group and *Ruminococcaceae* (UCG-003, UCG-002), thus indicating that an energy restricted MedDiet could differentially influence specific SCFA producers compared to a non-calorie restricted MedDiet. This hypothesis is also supported by a previous published mice study where calorie restriction has shown to limit butyrogenic enzymes and promote propiogenic enzymes that could lead to competition and selective growth of certain SCFA producers (276).

Beyond SCFA producers, we also observed interesting changes in bacterial genera that have been noted to be involved in other health modulating mechanisms such as via BCAA and bile acid regulation. MedDiet adherence, especially when supplemented with extra virgin olive oil has demonstrated to decrease circulating levels of BCAA that is associated with lower T2D risk. Consistently, we observed a decrease in *Blautia*, *Dorea* in the intervention group, that were associated positively with circulating BCAA in other studies (277,278). *Chistensenellaceae* R-7 group that was found negatively associated with changes in weight, BMI, triglycerides and plasma glucose, has also shown to have negative association with BCAA, thus indicating a potential role of BCAA in the MedDiet mediated effects on health. We also observed, in the intervention group, a reduction in bile reducing members of intestine such as *Bilophila*, *Lachnoclostridium*, which has shown to regulate lipid, glucose metabolism in mice studies (59,279). With our results we would also like to insist that intestinal microbiota could impact host health via pathways beyond SCFAs such as bile-acid or BCAA pathways, which warrants being investigated in future studies.

We observed changes in predicted functional profiles of bacterial community in the intervention group. The biosynthesis pathways associated to carbohydrate and nucleotide biosynthesis increased in the intervention group probably to adapt to the energy restriction. On the other hand, there was a decrease in amino acid and lipid biosynthesis pathways potentially due to the higher direct availability of these biomolecules (from increasing dietary protein and fats) thus reducing their need to synthesize these biomolecules. However, future studies measuring fecal metabolites and circulating metabolites at the same time are required to confirm the observations we make in this study.

Diving further into this thesis in **chapter II** we investigated into understanding the associations of protein intake and gut microbial compositions. It has been established that protein could affect satiety hormones and also production of metabolites via gut microbiota in order to modulate weight loss (159). We explored these associations focusing on various protein sources (plant and animal protein) and quantity in the context of a normal protein diet (15-20 % of energy intake). Several epidemiological studies have shown negative association between plant protein intake and various cardio metabolic risk factors or cancer, whereas the reverse associations for some animal protein products (red, processed meat) (280). Digested/partially digested proteins in the form of various amino acids reach the intestine where they not only serve as substrate for microorganisms, but also some are metabolized by enterocytes (281). The composition and quantity of amino acids reaching the colon could vary the implications on host (282). Protein intake at baseline and 1-year changes during the PREDIMED-Plus intervention were consistently negatively associated with saccharolytic bacterial genera of the order Clostridiales (*Ruminococcus gauvreauii*, *Coprococcus* 2, *Ruminococcacea* UCG-005). At baseline, we observed Barnesiella to be associated positively with plant to animal protein ratio, plant protein (%) and nuts protein intake. Interestingly *Barnesiella* has been attributed to its anti-carcinogenic potential *in vitro* (283) that could potentially be a pathway via which vegetarians and vegans present a less colorectal cancer risk compared to omnivorous population (284).

In 1-year changes, we report that overall protein intake significantly increased from 16.3 % (IQR, 14.9, 18.1) to 17.4 % (IQR, 15.6, 19.3), however this increase did not result in significant increase of protein expressed as g/ Kg of body weight. The increase in total protein content was attributed with an increase in plant to animal protein ratio (< 0.05) even though both plant and animal protein intake increased. Increase in plant protein was characterized by increased consumption of legume and nuts, whereas animal protein was characterized by increase in fish intake and a decrease in all other sources such as dairy, red meat, processed meat and poultry. The reductions in these sources of animal protein consistently were associated negatively with changes in *Butyricicoccus*, *Fusicatenibacter*, *Lachnoclostridium* and *Lachnospiraceae ND3007*. The only consistent association between changes in plant protein (%) and its sources (legumes, nuts) were with changes in *Parasutterella*.

In the predicted metabolic functions evaluated with KEGG, we found significant associations (p-FDR < 0.1) at baseline dominated by animal protein sources (red meat, processed meat) and no associations with 1-year changes. Extending the p-FDR (up to < 0.25) to explore the associations at 1-year, we noted a similar domination of animal protein sources. This intrigues us to postulate that the animal-based protein could have potentially strong influence on gut microbial composition compared to the plant-based protein. However, as these observations are only associative in nature, we need future well designed RCTs to assess these effects of various protein sources on gut microbiome in order to establish causal effects. As suggested in the detailed review by MyNewGut group (285), even though high protein diets might prove effective for weight loss, their effects on gut microbiota might not necessarily be beneficial, and a careful balance of proteins with diversity of sources will be required to maintain a homeostasis in the gut. Thus, consuming proteins from majorly plant protein and reducing animal protein sources, such as in MedDiet could promote a favorable gut ecosystem.

Similar to chapter II, we have been interested in understanding the potential implications of dietary fats in a source based context on gut microbiota. Hence, in **chapter III**, we attempted to summarize the current literature on dietary fat mainly from plant-based sources (nuts and vegetable oils) and its effects on gut microbiota

(286). Even within a span of 1-year since the publishing of this review, a great range of literature has grown in this topic (287,288). Dietary fats are important class of nutrients that are attributed to modulate gut microbiota. The quantity and quality of dietary fat also determines changes in the gut microbiota. Many of the studies conducted in the past have focused on using saturated fats in terms of HFD which have shown to induce obesity via gut microbiota (289). HFD have shown to increase the phylum Firmicutes and its members such as *Coprococcus*, *Butyricicoccus* (290). However, the scope of dietary fats extends beyond saturated fats to other important classes of fats: monounsaturated fats and polyunsaturated fats. In our review we remark that literature comparing various fat sources on human studies is scarce and predominant of the studies we review are from animal models and *in vitro* studies. Nuts are an important group of foods that have a rich matrix of fibers, unsaturated fatty acids and polyphenols which serve as prebiotics for the gut bacteria (291). Interesting studies in the past have shown that even the processing methods of nuts could affect gut microbial changes differently (292–294). Various clinical trials with consumption of almonds, pistachios, and walnuts have shown an increase in major SCFA producers *Bifidobacterium*, *Lachnospira*, *Roseburia*, *Dialister,* and decrease in certain pathobionts such as *Clostridium perfringens* and *Escherichia coli* after its consumption. However, most of these studies were conducted amongst healthy individuals (295–298) with exception of few studies (298,299). Conducting future studies with populations with overweight or obesity who are qt risk of metabolic diseases could pave way to develop nutritional advices based on gut microbiota.

Vegetable oils are common sources of plant-based fats. Amongst the vegetable oils, extra virgin olive oil deserves a special mention due to its well-established health benefits. Animal studies by Patterson et al (300), Hidalgo et al (198), Priteo et al (301) has shown that olive oil could alter gut microbiota and may favor the host health by altering insulin and lipid profiles. The Canola Multicenter Intervention Trial (COMIT) reported that even while comparing oils from plant sources, changes in their MUFA and PUFA concentrations can significantly vary the gut microbiota profiles in overweight/obese humans (302). Interestingly, the influence of the MUFA

or PUFA based diets on gut microbiota were in a BMI dependent manner (302), thus emphasizing on requiring future trials in participants with overweight or obesity.

Taken together, our results support that a lifestyle intervention based on an energy restricted MedDiet and physical activity promotion, could aid in weight loss and reductions in cardiovascular risk factors mediated by changes in gut microbiota and their functions. Substituting animal-based protein and fat sources with plant-based sources could promote a beneficial gut ecosystem.

Strengths and limitations

The PREDIMED-Plus study aims to address an important public health concern: *Obesity*. Important merit of this study and consecutively this Doctoral thesis is that this intervention provides a holistic lifestyle-based approach that not only aids in weight loss but also long-term maintenance. This is achieved by the promotion of behavioral changes and providing calorie restriction with a local diet (MedDiet) approach that makes it sustainable for the participants to follow the diet for longer periods of time. With this regard, this thesis benefits from the strength of understanding the gut microbial changes from a multiple-intervention strategy. Large sample size and long time period of the intervention is another strength that deserves mention.

It is also necessary to underline the limitations of this thesis to carefully interpret the current results and improve future studies. This thesis was conducted in elderly participants at high cardiovascular risk living in the Mediterranean region, hence our findings may not be extrapolated to other general populations. As much as the lifestyle approach of this study is a strength, it could also be considered as a limitation as it could be difficult to disentangle the individual effects of each component of intervention. With respect to the observational results (chapter II), we cannot establish cause-effect relationships as these results can only be interpreted as associations. As well, the results obtained for nutritional intake data were evaluated based on food frequency questionnaire which could over or underestimate some food intake. Additionally, while evaluating associations, even though we adjust for potential confounders we cannot exclude residual

confounding. With respect to the microbial methodology, specific limitations need to be addressed. A major limitation of this thesis is the unavailability of fecal metabolomics that would have allowed us to strengthen our conclusions. The precision of 16S rRNA sequencing is limited to genus specific taxonomical level. Unlike epidemiology, the consensus for microbial statistical tests is not well established, and it is growing in a rapid rate, thus making it difficult to compare with various studies.

Discussion

VII. CONCLUSION

Conclusion

Hypothesis 1: A 1-year lifestyle intervention using an energy reduced MedDiet and physical activity promotion would help in weight loss and improve cardiovascular risk factors via changes in gut microbiota

Conclusion 1.1: A 1-year lifestyle intervention with calorie restricted MedDiet, physical activity and behavioral support, reduced weight and cardiometabolic risk factors corresponding with changes in microbial genera compared to control group. Many genera shifted in the same direction within both intervention groups indicating an overall effect of MedDiet.

Conclusion 1.2: Some of the genera that decreased in the intervention group (Haemophilus, Coprococcus 3) were associated directly with adiposity parameters. Changes in Lachnospiraceae NK4A136 was positively associated with changes in MedDiet adherence in overall population.

Associated article: Effect on gut microbiota of a 1-year lifestyle intervention with Mediterranean Diet versus Energy-Reduced Mediterranean Diet and Physical Activity Promotion. PREDIMED-Plus Study (American Journal of Clinical Nutrition, Accepted)

Hypothesis 2: Differences in dietary protein quantity and sources are associated with differences in gut microbial composition and predicted functionality.

Conclusion 2.1: At baseline total protein intake and various protein sources were associated majorly with saccharolytic members of Clostridales order. The predicted metabolic capacities of the gut bacteria were dominated by associations with animal-based protein sources.

Conclusion 2.2: After 1-year of intervention, we observed total protein, animal protein and plant protein increased. However the increase in animal protein was characterized by decrease in red meat, processed meat and increase in fish protein content. The increase in ratio of plant to animal protein was associated with reductions in Parasutterella.

Associated article: Associations between the amount of dietary protein intake and protein sources with gut microbiota composition (Manuscript in preparation)

Hypothesis 3: Source and quality of fat can have different effects on health. Similarly, the gut microbiota could be modified with varying source and quality of fat.

> Conclusion 3: We observe from previous in vivo and in vitro studies that compared to animal-based fats, plant-based fat especially from nuts could beneficially affect host health via increasing short chain fatty acid producing genera. The studies on vegetable oils are limited to animal studies and very few human studies thus creating a need in the future for studies to address this research gap. Substituting saturated fats (mainly from animal sources) with unsaturated fats from plant origin could benefit the host via alterations in gut microbiota.

Associated article: Plant-Based Fat, Dietary Patterns Rich in Vegetable Fat and Gut Microbiota Modulation (Frontiers in Nutrition, Published, https://doi.org/10.3389/fnut.2019.00157)

VIII. FUTURE OPPORTUNITIES

As with every scientific study, the limitations of this study could serve as the first opportunity of inspiration for future studies. Although intestinal microbiota studies pose certain challenges, it could take us a step closer to personalized nutrition and to provide better health care.

Microbiota aspects

Future interventions assessing intestinal microbiota could evaluate fecal, urine, circulating and tissue specific metabolomics in order to validate the hypothesis developed by the microbial taxa changes. Huge developments on sequencing technology could also allow future studies to dive deep into species or strain level taxonomical inferences. Along with the developments on the sequencing technology, there is also a need to set golden standard for the microbial processing and statistical tools. Few studies in the past have also shown that the gut microbiota returns back to its "normal" or basal condition after the completion of intervention, thus also evaluating post-interventional microbiota changes could serve useful. Evaluations of multiple time points within the interventional period could also provide insight into understanding the shifts during the intervention.

Even though microbiota studies are growing at an exponential rate, understanding their mechanisms of actions are still in its infancy, except for commercially available probiotics and therapeutics. *In vitro* gut models could serve as high importance for understanding these mechanisms. Recently it has also been reported that gut microbiota imparts its effect on host health by regulating microRNAs which opens a huge door for potential future research.

Study design, exposure, and outcome aspects

As mentioned in the chapter II, III, the human studies evaluating the varied effects of plant and animal-based nutrients on gut microbiota are scarce. Studies assessing plant protein sources in the context of gut microbiota are majorly based on soy protein and are comparatively meagre on sources such as legumes, nuts and grains. With growing demand and awareness of plant-based foods it would be essential to evaluate these effects in the future.

Certainly, MedDiet is a healthy diet, however this diet might not be sustainable for individuals living in other parts of the world. Thus, well designed trials evaluating the effects of local calorie restricted diets on gut microbiota in the future could serve useful for individuals from other parts of the world.

Within the PREDIMED-Plus consortium, it is also interesting to evaluate various other exposures and outcomes mediated by gut microbiota as this study collects a huge amount of clinical and biochemical data. One such proposal could be understanding the effects of drugs (such as metformin) on gut microbiota and host health that has been receiving high attention in the recent years. There are also opportunities to build prediction models for various diseases outcomes that could aid in early detection of diseases. In the near future as an extension of this thesis, we will also be working on the EAT2BENICE consortium that would evaluate the effect of MedDiet adherence on impulsive, compulsive behaviors mitigated by gut microbiota.

To summarize, although we are sitting on top of a gold mine (microbiota), we still must dig deeper…

IX. REFERENCES

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References

X. APPENDICES

Appendices

Scientific contributions belonging to this Doctoral thesis

Muralidharan J, Isabel Moreno-Indias, Mónica Bulló, Jesús Vioque Lopez, Dolores Corella, Olga Castañer, Josep Vidal, Alessandro Atzeni, Jose Carlos Fernandez-García, Laura Torres-Collado, Rebeca Fernández-Carrión, Monsterrat Fito, Romina Olbeyra, Ana Maria Gomez-Perez, Serena Galiè, Maria Rosa Bernal-López, Miguel Angel Martinez-Gonzalez, Jordi Salas-Salvadó, Francisco Jose Tinahones. Effect on gut microbiota of a 1-year lifestyle intervention with Mediterranean Diet versus Energy-Reduced Mediterranean Diet and Physical Activity Promotion. PREDIMED-Plus Study. American Journal of Clinical Nutrition. Accepted

Muralidharan J, [...], Mónica Bulló, Jordi Salas-Salvadó. Associations between the amount of dietary protein intake and protein sources with gut microbiota composition. Manuscript in draft

Muralidharan J, Serena Galiè, Pablo Hernández-Alonso, Monica Bulló, Jordi Salas-Salvadó. Plant-Based Fat, Dietary Patterns Rich in Vegetable Fat and Gut Microbiota Modulation. Frontiers in Nutrition 6 (2019): 157.

Other scientific contributions

Muralidharan, J., Papandreou, C., Sala-Vila, A., Rosique-Esteban, N., Fitó, M., Estruch, R., ... & Bulló, M. (2019). Fatty acids composition of blood cell membranes and peripheral inflammation in the predimed study: a cross-sectional analysis. Nutrients, 11(3), 576.

Papandreou, C., Sala-Vila, A., Galié, S., **Muralidharan, J**., Estruch, R., Fitó, M., ... & Bulló, M. (2019). Association Between Fatty Acids of Blood Cell Membranes and Incidence of Coronary Heart Disease: A Case-Control Study Nested in the PREDIMED Trial. Arteriosclerosis, thrombosis, and vascular biology, 39(4), 819-825.

Salas-Huetos, A., **Muralidharan, J**., Galiè, S., Salas-Salvadó, J., & Bulló, M. (2019). Effect of nut consumption on erectile and sexual function in healthy males: a secondary outcome analysis of the FERTINUTS randomized controlled trial. Nutrients, 11(6), 1372.

Canudas, S., Hernández-Alonso, P., Galié, S., **Muralidharan, J**., Morell-Azanza, L., Zalba, G., ... & Bulló, M. (2019). Pistachio consumption modulates DNA oxidation and genes related to telomere maintenance: a crossover randomized clinical trial. The American journal of clinical nutrition, 109(6), 1738-1745.

Galiè, S., Canudas, S., **Muralidharan, J**., García-Gavilán, J., Bulló, M., & Salas-Salvadó, J. (2020). Impact of nutrition on telomere health: systematic review of observational cohort studies and randomized clinical trials. Advances in Nutrition, 11(3), 576-601.

Atzeni, A., Galié, S., **Muralidharan, J**., Babio, N., Tinahones, F. J., Vioque, J., ... & Salas-Salvadó, J. (2021). Gut microbiota profile and changes in body weight in elderly subjects with overweight/obesity and metabolic syndrome. Microorganisms, 9(2), 346.

Book chapter:

Alasalvar, C., Salas-Salvado, J., Ros, E., & Sabate, J. (Eds.). (2020). Health benefits of nuts and dried fruits. CRC Press. (Chapter 13), Health benefits of nuts and dried fruits.

Muralidharan, J., Salas-Salvadó, J (2020) Growth of Plant-based Protein. Cracker (Magazine). International Nut council

Participation in national and international conferences

Congress: 26th European Congress on Obesity (ECO 2019), Apr 28 – May 01, 2019, Glasgow (UK)

Poster communication: Muralidharan J, Papandreou C, Sala Vila A, Rosique Esteban N, Fitó M, Estruch R, Martínez González MA, Corella D, Ros E, Razquín C, Castañer O, Salas-Salvadó J, Bulló M. Fatty acids composition of blood cell membranes and peripheral inflammation in the PREDIMED study: A cross sectional analysis

Scientific retreat: IISPV- IDBGI- IRBLleida Sant Hilari i Sacalam, November 7-9, 2018 (Girona)

Oral presentation: Primary analysis of changes in gut microbiota in the PREDIMED Plus study

International mobility

Mobility 1: Wageningen University, August 3- 15, 2019 (Netherlands)

Objective: To work on learning sample processing and data analysis in the framework of the European H2020 project Eat2BNice

Mobility 2: Bio-Me AS, August 29- 28 November, 2020 (Norway)

Objective: To learn practical laboratory testing of precision microbiome platform developed by Bio-Me AS. Mainly learn and work in bioinformatic, statistical tools related to gut microbiota processing.

National mobility and others

Summer school: EIT Health, INJOY Summer school, June 25- 4 July, 2018 (Barcelona)

Innovating the joy of healthy eating for elderly: Developing business projects with scientific knowledge

Summer school: 9th FISABIO Summer school, July 1-5 July, 2019 (Valencia)

Biomedical Research and Public health (Basic computational skills for Genomic Analysis)

Appendices

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