UNIVERSITAT DE BARCELONA

Effect and Metabolism of lignans on gut microbiota and their impact on health

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EFFECT AND METABOLISM OF LIGNANS ON GUT MICROBIOTA AND THEIR IMPACT ON HEALTH

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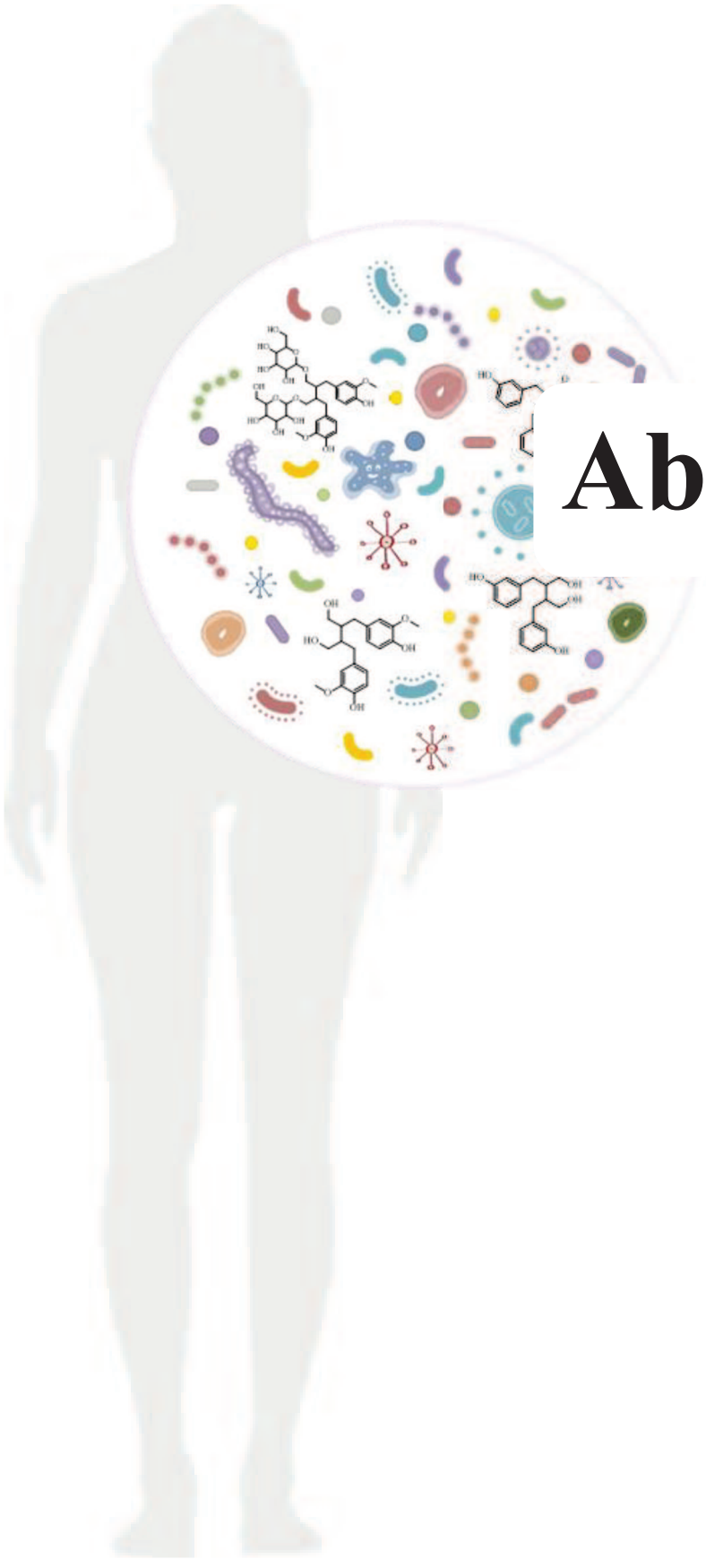


Abstract

Abstract

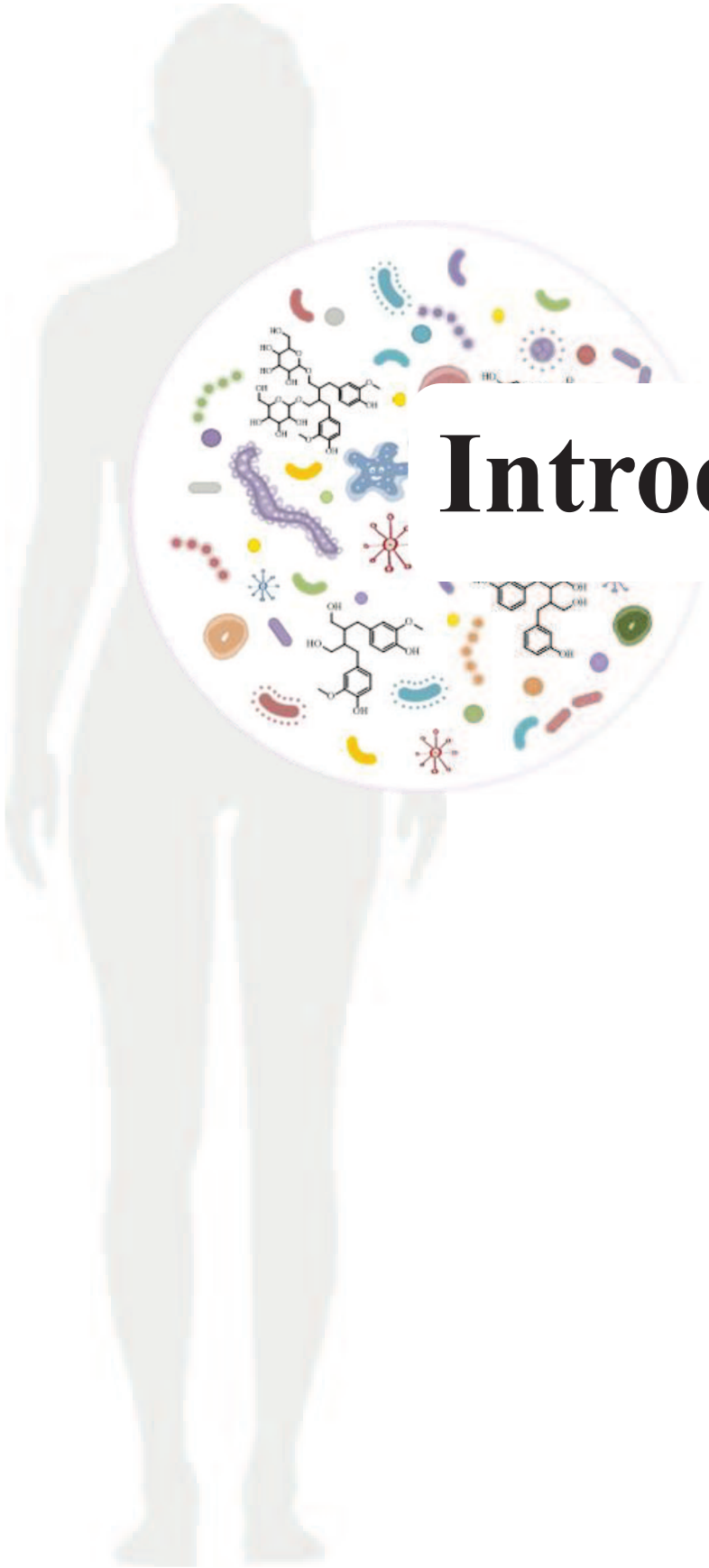
The World Health Organization (WHO) [1] says that CVDs are number one cause of death globally. An estimated 17.7 million people died from CVDs in 2015, representing 31% of all global deaths. Over three quarters of CVD deaths take place in low-and middle-income countries. Some of the risk factors are raised blood pressure, raised blood glucose, raised blood lipids, and overweight and obesity. A large number of epidemiological studies have associated the consumption of polyphenols with a decreased risk of CV or coronary heart disease. Lignans are a class of polyphenols formed by 2 phenylpropane units, also referred as plant phytoestrogens. When ingested, they can be metabolized by the gastrointestinal microbiota to their bioactive forms, the enterolignans enterodiol (ED) and enterolactone (EL)[2,3] . The presence of ED and EL in the body has been correlated with the prevention of some chronic disease like cardiovascular disease, osteoporosis, and hyperlipidemia, and some cancers like colon cancer, breast cancer, prostate cancer and menopausal syndrome[2,4,5]. Moreover, since in the last consensus International Scientific Association for Probiotics and Prebiotics (ISAPP) concluded that plant polyphenols can also meet the criteria of prebiotics; lignans could act as a prebiotic. Although, still more studies in the target host are required[6].

Malnutrition is estimated to contribute to more than one third of all child deaths, although it is rarely listed as the direct cause[7]. From WHO sources[7] we know that in 2014, approximately 462 million adults worldwide were underweight, while 1.9 billion were either overweight or obese. In 2016, an estimated 155 million children under the age of 5 years were suffering from stunting, while 41 million were overweight or obese. Around 45% of deaths among children under 5 years of age are linked to undernutrition.

These mostly occur in low- and middle-income countries. At the same time, in these same countries, rates of childhood overweight and obesity are rising.

It is becoming increasingly apparent that gut microbiota play a pivotal role in the development and etiology of malnutrition[8–10]. The microbiota influence host metabolism, nutrient absorption, inflammation and even hormonal signaling, leading to changes in linear growth and weight gain in mice and humans[11,12].

In this thesis I aim to study the impact of dietary lignans and yogurts on cardiovascular risk parameters, deepen on knowledge of lignans metabolism by gut microbiota and evaluate the impact of lignans in health and malnourishment.



Introduction

Introduction

0.1 Polyphenols

0.1.1 Definition and occurrence

Polyphenols comprises a large family of more than 8000 different compounds naturally occurring secondary metabolites of plants derived from the phenylpropanoid and polyketide biosynthetic pathways[13]. They play an important role in the ecology of most plants, including UV screens to protect against ionizing radiation and provide coloration, plant resistance against microbial pathogens (phytoalexins), deterrence of herbivores such as insects as well as reproduction, nutrition, and growth, notably through interactions with other organisms above and below ground (insects, symbiotic fungi, and bacteria)[14,15].

Chemically, phenolics are characterized by having at least one aromatic ring, with one or more hydroxyl groups attached. A general accepted classification divides polyphenols in flavonoids and non-flavonoids. Flavonoids have a C6-C3-C6 structure and the main sub-classes of dietary flavonoids are flavonols, flavones, flavan-3-ols, anthocyanidins, flavanones and isoflavones, while those that are comparatively minor components of the diet are dihydroflavonols, flavan-3,4-diols, coumarins, chalcones, dihydrochalcones and aurones. The non-flavonoids group is classified according to the number of carbons that possess and comprises phenolic acids, lignans, stilbens and others[15,16]. In **Figure 0.1** are represented the main classification with examples of each compound.

Figure 0.1. Main classification of polyphenols with an example.

0.1.2 Polyphenol metabolism

The metabolism of polyphenols (**Figure 0.2**) starts in the lumen of the small intestine where they can undergo post-absorptional modifications (e.g. methylation, sulfatation, and glucuronidation). Those that are not absorbed through the gut barrier of the small intestine can pass to the large intestine. In the colon, they can be metabolized via esterase, glucosidase, demethylation, dihydroxylation and decarboxylation activities of bacteria, resulting in smaller metabolites, some of which can be absorbed through colonocytes[17].

Metabolism (digestion) starts in the oral cavity, where we can find the amylase enzyme. Thus the time the food is in the mouth is short; the function of this enzyme is limited. The reduction in the particle size of food, helps to ameliorate the access of the enzymes in the next steps of the digestion[18]. When the food reaches the intestine after being in the stomach, the pH arises from 2 to 7, allowing the activation of the enzymes secreted by pancreas or bile and starting to form water-soluble micelles[18]. Most of polyphenols need to be hydrolyzed before they can be absorbed through the enterocyte. There are two different mechanisms, first one is by the action of the lactase-phloricin hydrolase (LPH) normally present in the brush border of the enterocyte or through β-glycosidase cytosolic (CBG) found in the cytosol [16,18]. After the deglycosilation of LPH, aglycones are released and they can enter inside the enterocyte by passive diffusion, as a result of its lipofility[16,18–20]. In the other side CBG, acts inside the enterocyte transporting, through glycose transporter associated to sodium (SGLTS), the most polar glucosides[18,19]. But not all the polyphenols can be hydrolyzed in the small intestine, this compounds will reach the colon where they can be hydrolyzed and metabolized through colon microbiota and after they can be absorbed [20].

Once polyphenols are transformed to simple aglycones, in the enterocyte, they can undergo further structural modifications such as methylation, sulfatation and/or glucuronidation, giving the second phase metabolites. The enzymes capable to metabolize aglycones are: catechols-*O*-methyltransferase (COMT), which catalyzes the transference of a group methyl from adenosylmethionin to the aglicones with a diphenolic end", sulfotransferases (SULT) which are able to produce a sulfate group from the phosphoadenosine-phosphosulfate to a hydroxyl of the aglycone, and uridine-5'-diphosphate glucuronosyltransferases (UGT) which catalyzes the transference of the glucuronic acid from UD-glucuronic acid to the phenolic compounds. Polyphenols which are able to go through the basolateral barrier of the enterocyte, go to the blood stream, and through the portal vein arrive to the liver where they can undergo new conjugations of the phase II metabolism[18–20].

Through the blood stream transport, polyphenol can be distributed in the tissues and it has been shown that they can also overpass the hematoencephalic barrier, which normally can be just crossed by lipidic compounds[18]. Some of the conjugated phenols, that cannot cross the basolateral membrane, are transported through transporters again to the intestinal lumen of the small intestine. At the same time, the conjugation of phenols in the liver makes easier the excretion through bile's into the enterohepàtic circulation where they can be reabsorbed by the small intestine. Polyphenols which have not been absorbed through the small intestine arrive to the colon, as the ones that has been absorbed, metabolized in the liver and excreted through the bile or directly excreted from the enterocyte to the gut[19]. Colonic microbiota hydrolyzes glycosides to aglicones and can metabolize them to more simple phenolic acids. These compounds can be absorbed and metabolized by the liver before being excreted to the urine. Compounds that are not absorbed are eliminated through the feces.

Phase II metabolites can vary depending on the substrate nature, intake dosage, the specie and sex. The administered dose plays a crucial role to determine the first place of metabolism. Generally, high doses allow the liver to be the first metabolism, while with smaller amounts the metabolism occurs in the enterocyte, being the liver the second mechanism to undergo further modifications[19]. Conjugation mechanisms are highly efficient and in plasma aglycones can be found in very low concentrations if they are detected[16,19,21]. **Figure 0.2** is a schematic representation of the absorption and biotransformation of phenolic compounds.

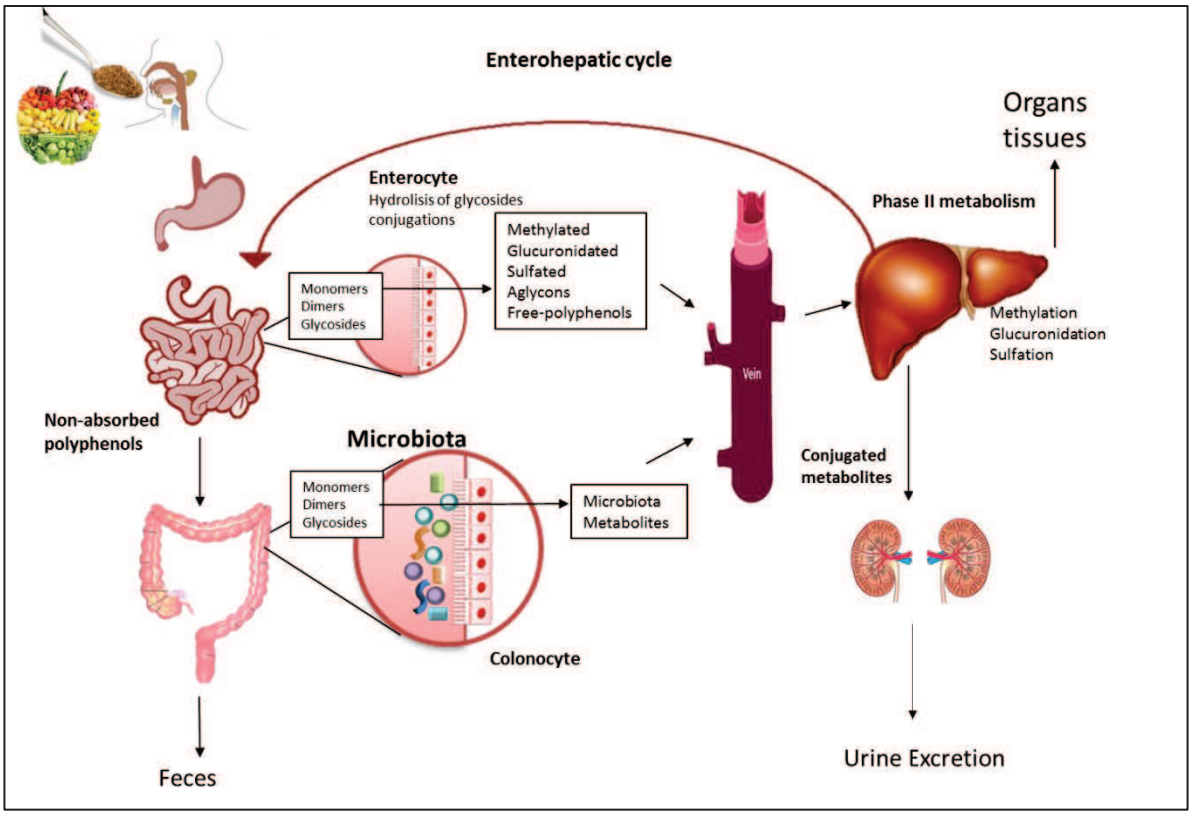


Figure 0.2. Schematic representation of polyphenol metabolism and excretion

0.1.3 Lignans

This thesis has been focused on the role of lignans in human health as well as its absorption and metabolism. Lignans include a number of diphenolic compounds with 1,4-diarylbutane structure such as pinoresinol (PIN), matairesinol (MAT), secoisolariciresinol diglucoside (SDG), 1-acetoxypinoresinol, lariciresinol (LAR), syringaresinol, sesamin and isolariciresinol. Biological activity of lignans is related to the activation of these compounds by gut microbiota to enterolactone (EL) and enterodiol (ED) which are phytoestrogens in mammals. Phytoestrogens are weak estrogens found in plants or derived from plant precursors. In addition, lignan metabolites ED and ELhave been shown to aid in the prevention of several chronic diseases like cardiovascular disease, colon cancer, and breast cancer among others. In **Figure 0.3** are shown the main structures of lignans and their metabolites ED and EL.

Lignans were first detected in man in 1979, and were identified independently by two groups who described their work in the same issue on Nature Journal [22,23].

The gut microbiota is the responsible for the conversion of plant lignans to mammalian lignans, also called enterolignans. “The production of enterolignans from lignans contained in food requires the interaction of anaerobic bacteria related functionally and also distantly related phylogenetically”[24]. Several authors demonstrated how both rat intestinal and human fecal microbiota produce ED and EL.

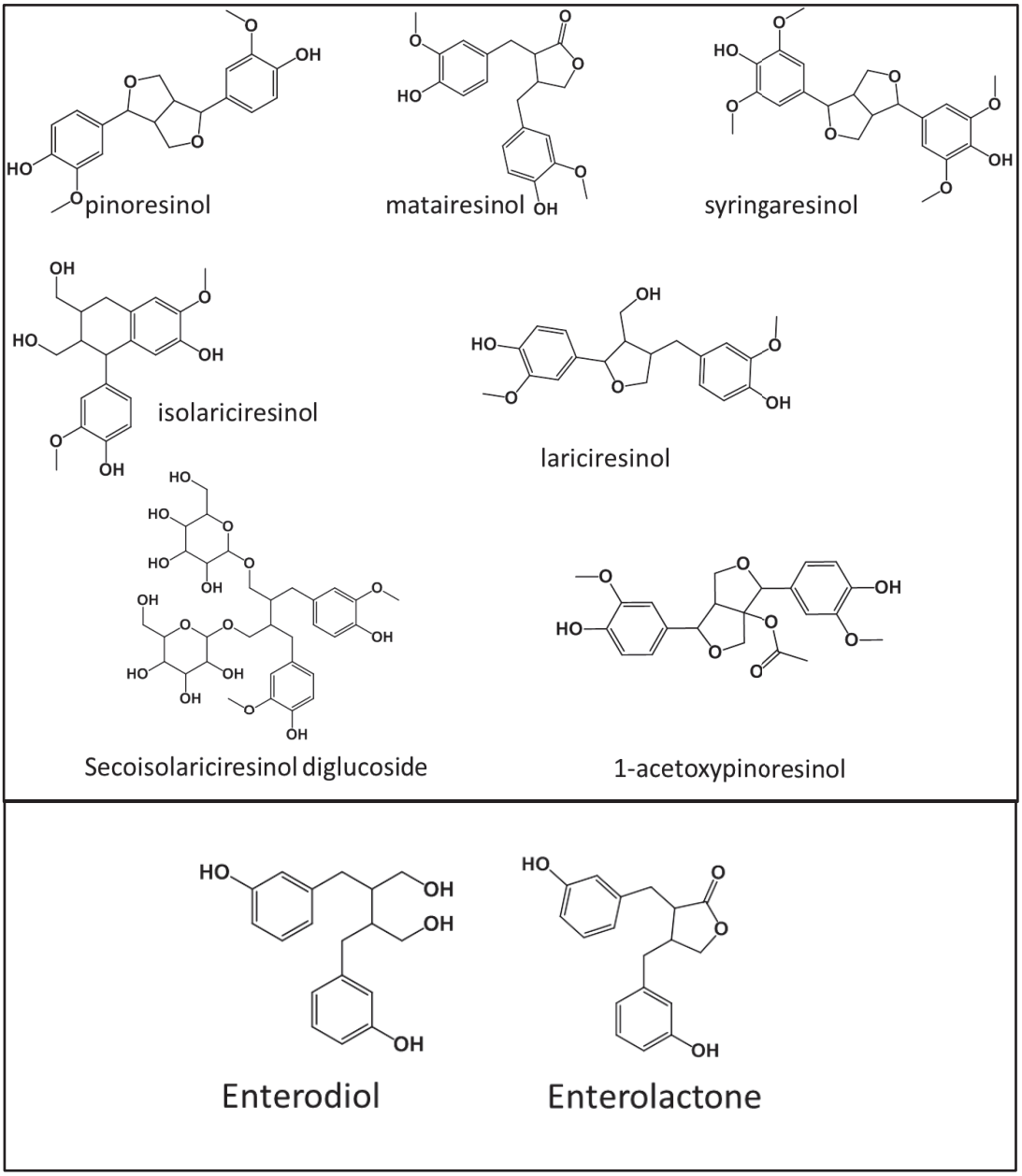


Figure 0.3 Main structures of lignans and their enterolignans.

0.1.3.1 Food sources

Dietary lignans are fiber-related polyphenols, which are abundantly available in fiber-rich foods, e.g., cereals, legumes, vegetables, fruit berries but also in non-fiber foods such as tea and alcoholic beverages[25].

Regarding oil seeds and nuts the richest source of lignans is flaxseed[26]. Flaxseed contains about 75-800 times more lignans than cereal grains, legumes, fruits and vegetables[26,27]. secoisolariciresinol diglycoside (SDG) is the major lignan of flaxseed, along with minor contents of MAT, PIN, LAR and ILAR. SDG ranges from 11.7 to 24.1mg/g in defatted flour and 6.1 to 13.3mg/g in whole flaxseed flour [26,28].

The second highest lignan concentration has been described in sesame seeds, with PIN as the main constituent after sesamin. Total lignan concentrations in sunflower seeds, and cashew nuts have also been found to be relatively high[25]. From cereals, rye, wheat, triticale, oat and spelt has shown the highest total amount of lignans[25]. Brassica vegetables (cabbages, Brussel sprouts, kale) also contain high levels of lignans (185-2321 μg/100g) mainly in form of PIN and lariciresinol[29].

PIN and 1-acetoxypinoresinol are the major phenolic compounds in virgin olive oil and olives[30]. They were identified for the first time in the 2000's, for two different studies, the health benefits of the consumption of virgin olive oil of these beneficial properties could be explained in part for the presence of these lignans[31–33].

In a study of Tetens et al.[34] mean dietary lignan intakes estimated using the Dutch database ranged from 1 to 2 mg/day. They studied the lignan intake in five different European countries, Denmark, Finland, Italy, Sweden and United Kingdom. When lariciresinol and PIN were included in the estimation of the total lignan intakes,

cereals, grain products, vegetables, fruits and berries were the most important dietary sources of lignans[34].

0.1.3.2 Metabolism of lignans

As I mentioned before, the bioavailability of dietary lignans influences the bioactivity of these compounds. Lignans derived from food occur mainly as glycosides and their absorption requires deglycosylation as a first step. β-glucosidases from gut microbiota are the responsible of this hydrolysis. Bioactive aglycones are extensively conjugated during and after absorption through the gut barrier (colonocyte). They are conjugated to form *O*-glucuronides and sulphate esters. This conjugation first occurs in the gut barrier and then reaches the liver, where it can be further metabolized[4]. The formation of anionic derivatives by conjugation with glucuronide and sulphate groups facilitates their urinary and biliary excretion, and explains their rapid elimination. Microbiota can deconjugate the glucuronide and sulphate groups excreted in the bile. Later on, in the colon, the glycosylated, sulfated and glucuronidated forms of phytoestrogens are deconjugated by bacterial enzymes, consequently the reuptake of phytoestrogens is enhanced, and then subjected to further metabolism by the intestinal microbiota[4] .

The transformation of plant lignans to the enterolignans: ED and EL, by bacterial community has been previously described by several authors [4,24,35,36]. Microbiota hydrolyzes the sugar moiety of diglycoside lignans. The deglycosilation is followed by demethylation and dehydroxylation to form dihydroxyenterodiol and ED respectively. Dehydroxylation is followed by dehydrogenation from ED to produce EL[24]. Some interindividual differences has been observed in terms of enterolignans

producers, some of the factors influencing the bioactivation of lignans in the large intestine are diet, transit time, intestinal redox state and, the composition and activity of the colonic microbiota. Thus, a subdivision of enterolignans producers to weak, moderate and strong phenotypes has been proposed [37].

Unlike other lignans, sesamin is converted into enterolignans at a lower rate (1.1%) compared to SDG (57.2%)[38]. Sesamin is only partially metabolized in the colon, it is absorbed and metabolized in the liver to hydroxylated metabolites, excreted in bile, undergo enterohepatic circulation, and are further metabolized to mammalian lignans by the microbiota. Hence, the site of metabolism differ from that of the more hydrophilic lignan precursors as SDG[2,39]. This work is focused on the metabolism of lignans by the action of gut microbiota.

0.1.3.3 Functions and health

The incident and mortality of many chronic diseases, such as breast, prostate, and colorectal cancer, as well as cardiovascular diseases are high in Western countries compared with those in Asia. Moreover, phytoestrogens are abundant in plasma and urine of subjects living in areas with low cancer incidence[3]. Lignans and their metabolites have been reported to exert protective effects against diet-related chronic diseases through a variety of mechanisms including phytoestrogenic and antioxidant effects[25]. The latter effect is of particular interest as many chronic diseases are characterized by an oxidative stress component in the disease etiology, from initiation of carcinogenesis, damage to pancreatic islet cells and LDL lipid peroxidation in atherosclerosis[40]. Other functions of lignans include their influence on hormone

metabolism but also on their enzyme activity or gene expression[25,40]. Main lignan functions and the potential health benefits are summarized in **Figure 0.4.**

Antioxidant activity

It has been shown that SDG could have beneficial effects because of its ability of scavenge hydroxyl radicals in cancer and lupus nephritis, showing SDG to exert powerful antioxidant activity[25,41]. Comparisons of antioxidant activity between SDG, SECO, and vitamin E, showed a highest effectivity of SECO and ED, and the lowest for vitamin E[42]. In another study ED and EL exert higher antioxidant activity than SDG[43].

Thus, they can act as antioxidant against DNA damage and lipid peroxidation. And they would have been suggested to contribute to reduction of hypercholesterolemia, hyperglycemia, and atherosclerosis[42].

Estrogenic-Antiestrogenic functions

Lignans and their derived ED and EL act either as estrogen agonists or antagonists. The chemical structure of these biphenolic compounds resemble that of 17-b-estradiol and they exert biphasic agonistic estrogenic and antiestrogenic activities in vitro and in vivo[25]. The plant lignans are presently thought to have no hormonal effects, but both enterolignans have a very weak estrogenic activity and bind with low affinity to the estrogen receptor.

In a study with 28 postmenopausal women by Hutchins et al. [44], they observed that giving 5 g or 10g of flaxseed per day, they influenced endogenous hormone metabolism by a decrease of estrone sulphate and estradiol in plasma. The decreases in 17-estradiol and estrone sulfate concentrations reported in this study suggested that consuming flaxseed may offer protection against breast cancer. And also, due to its

estrogenic activity, they can mitigate menopausal symptoms and decrease the risk for osteoporosis

The estrogen antagonistic effect of ED and EL may be useful for conventional hormonal replacement therapy in postmenopausal women[25]. In addition, lignans and their derived metabolites have also been associated with a reduction on the risk of breast cancer through the modulation of the estrogen receptors[25]. However, Piajing et al.[45], conclude that the oral supplementation of enterolignans should be prescribed with caution in particular to postmenopausal women and hormone-dependent breast cancer patients due to a potential tumor growth stimulation.

The transformation of plant lignans by intestinal microbiota might be essential for the estrogenic/antiestrogenic activity.

Hormone metabolism and availability

ED and EL act as estrogen agonist or antagonist, as well as in hormone metabolism and availability[25]. They might regulate estrogen receptors expression and degradation, so they are able to influence the hormonal status of both normal tissues and tumors.

Diets with flaxseed increase serum concentrations of prolactin and decrease 17-b-estradiol and estrone sulphate[44]. EL is inhibitor of human estrogen synthetasa and the concentrations of lignans inhibited aromatase in peripheral and or cancer cells and decreased estrogen levels. EL appeared to be the highest inhibitor of 5-alpha-reductase (that converts testosterone to 5-alpha-dihydrotestosterone, biologically the most active androgen) and 17-b-hydroxysteroid dehydrogenase in human genital skin fibroblasts monolayers and homogenates, and in benign prostatic hyperplasia tissue homogenats[46]. Thus, long-life dietary with lignans may influence the development of hormone-

dependent tumours[25] and the conversion of androgens to estrogens in breast cells is thought to be important in the etiology of breast cancer[3].

Lignans on gene expression and/or enzyme activity

EL and ED or their plant sources might modulate estrogen receptors protein expression and degradation and therefore influence hormonal status. Enterolignans can modify gene expression and the activity of enzymes involved in health and the metabolism of both normal tissues and tumours[25].

In a study of Prasad et al.[47] SDG suppressed PEPCK gene expression at a concentration of 100μM, this fact might be the explanation in the prevention of type-2 diabetes.

A potential mechanism of dietary anticarcinogenenesis involves the induction of detoxifying phase II enzymes, such as NADPH:quinone reductase. The results of Wang et al.[48] demonstrated that dietary lignans are capable of activate NADPH:quinone reductase induction in Colo205 cells by promoting NADPH:quinone reductase mRNA expression, suggesting a mechanism involved in colorectal cancer chemoprevention.
Plant lignans have shown to exert anti-platelet activating factor activity. Platelet activating factor has been related to aggregation and degranulation of platelets, and is an important mediator in inflammation and asthma[25].

Hemmings et al.[49] found hepatobeneficial effect of increased levels of gamma-glutamyltranspeptidase associated to lignans in flaxseed supplemented diet in the livers of rats. Chen et al.[48] found that ENL inhibited insulin-like growth factor-1 receptors signaling in human prostatic carcinoma a PC-3 cells and also cyclin D1 expression, which resulted in the inhibition of proliferation and migration of prostate cancer cells.

Lignans decreased extracellular levels of vascular endothelial growth factor, which is a key factor in promotion of breast cancer angiogenesis[48]. That fact might be the explanation for the observed decrease in tumor growth and metastasis.

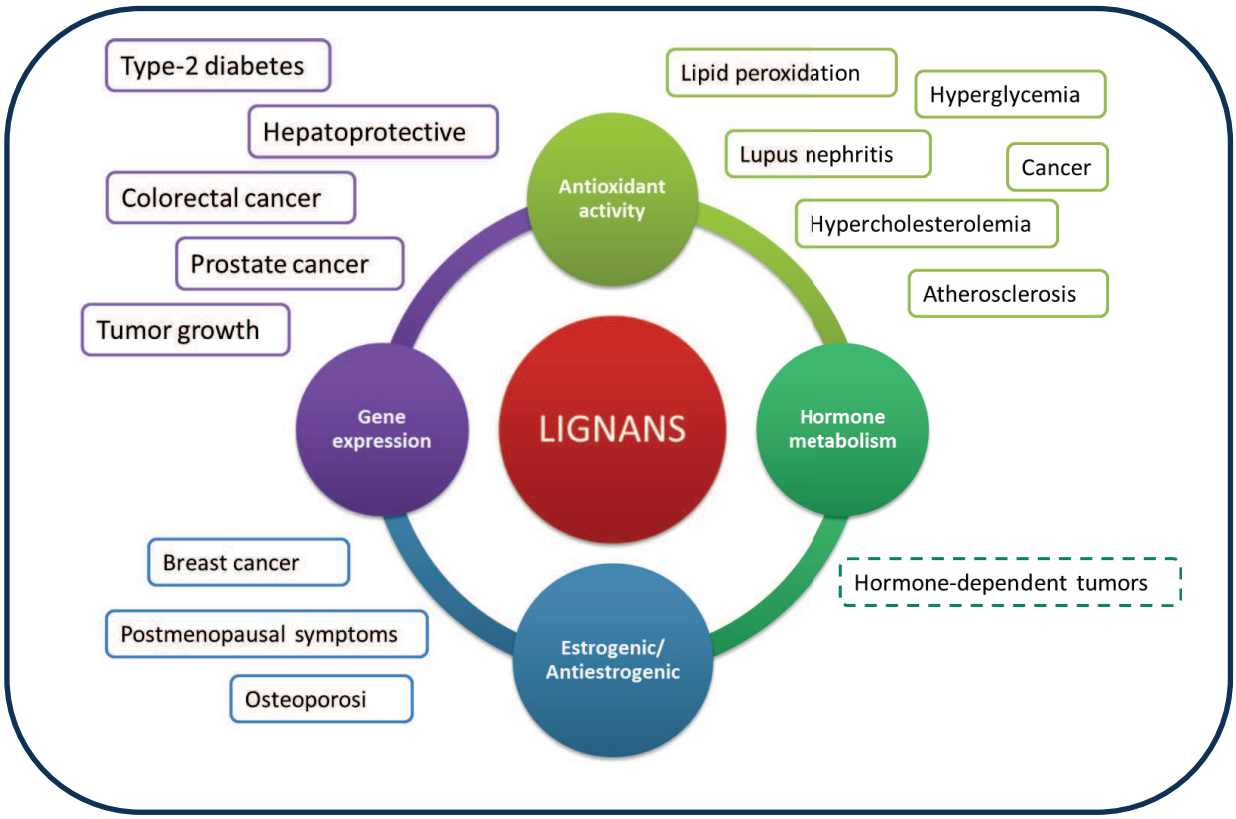


Figure 0.4. Summary of main functions of lignans and the beneficial health effects associated to them. Controversial beneficial effects are marked in broken line.

0.1.3.4 Lignans and Cardiovascular Disease

The major heart diseases are resulted from oxidative stress, inflammation, obesity, diabetes, dyslipidemia and hypertension are interrelated and contribute to an atherogenic environment that promotes the development of cardiovascular diseases, leading the causes of mortality among industrialized nations[50]. Animal and human studies have suggested that SDG and its metabolites possess antioxidant properties and are capable of reducing oxidative stress. Evidence suggest that SDG is capable to

mediate the serum total cholesterol, low density lipoprotein, total cholesterol and high density lipoprotein ratio which lead to less androgenic complication and antioxidative prevention[50].

Evidence from animal studies

Several studies have shown a beneficial effect of lignans on cardiovascular health in animal models. A series of studies conducted in a laboratory investigated the effects of flax lignans on atherosclerosis in rabbits [51–53]. Flaxseed diet resulted to improve in the lipid profile and showed to be effective in the reduction of the development of aortic atherosclerosis.

Two studies by Penumathsa et al.[54,55] and a study by Felmlee et al.[56] in rats also showed cardioprotective effects of SDG. Specifically, the results showed that SDG and SECO cause similar dose-dependent reductions in serum and hepatic cholesterol levels. And both lignans also decreased the rate of weight gain and accumulation of hepatic parenchymal fat.

Although most animal model studies shown cardiovascular effects of lignans, not all the studies gave positive results. In a study of Sano et al.[57] SDG had no effect on atherosclerosis and/or thrombus formation. Overall, most of studies show improvements in the biomarkers of CVD with SDG.

Evidence from human studies

Similar to animal studies, several human studies have shown beneficial effects on cardiovascular biomarkers.

A prospective study in 167 middle–aged men living in Northeast Finland, the Kuopio Ischaemic Heart Disease Risk Factor Study, significant associations were found between elevated serum EL concentration and CHD-related and CVD-related mortality[58].

It has been demonstrated that EL at lower concentrations stimulates the production of sex hormone binding globulin (SHBG) in liver cancer cells[59]. Vegetarians have higher plasma and urinary levels of lignans and also higher SHBG, which might be due to increased EL production in the colon from higher fiber-related food intake. Low SHBG was a risk factor for CHD mortality in a female study, and was correlated with HDL-c in men[49].

In a study Erkkila et al.[60] observed that whole-grain cereals was associated with reduced progression of coronary atherosclerosis in postmenopausal women with coronary heart disease, supporting that may protect against atherosclerosis.

A research among American men relating phytoestrogen intake and CVD risk factors showed that high lignan intake may be associated with an increase of apoB-containing lipoproteins and a decrease in fasting insulin and C-peptide[61].

In a review on flaxseed consumption and CVD risk[62], where nine intervention studies were included, concluded that daily consumption of ground flaxseed meal may reduce total cholesterol and low density cholesterol without significantly altering triglycerides or high density lipoprotein cholesterol.

Two studies of Hallund et al. [63,64] suggest that SDG may not improve markers of CVD in healthy individuals in comparison with those with hyperlipidemia or CVD. Overall, the majority of studies that used purified SDG found improvements in markers of CVD. Hence, lignan intake may have an important protective effect against vascular disease in humans.

0.1.3.5 Gut bacteria responsible for the conversion of plant lignans to enterolignans

Studies on the intestinal bacteria involved in the formation of EL from secoisolriciresinol diglucoside (SDG) arrived to the conclusion that the activation of SDG involved the interaction of anaerobic bacteria related functionally and also distantly related phylogenetically, most of which are members of the dominant human intestinal microbiota. The bioactivity of lignans depends on their transformation by gut bacteria. Several authors have demonstrated how rat intestinal microbiota and human fecal microbiota produce EL and ED, but still it is not well-known since there can be many different bacteria responsible of this conversion.

Clavel et al.[65] focused on the isolation of bacteria strains and was able to correlate high concentrations of Peptrosteptococcus productus and *Clostridium coccoides* with high concentrations of EL. In addition, *Eggertella lenta* was also correlated with the production of EL. Deglycosilation, demethylation dehydroxylation and dehydrogenation reactions are required for lignan transformation into EL and ED, and some intestinal bacteria were identified to as the responsible of each part of the reaction, all of them are summarized in **Table 0.1**. The deglycosylation of SDG into SECO is the first step for the formation of ED and EL from SDG. Many bacterial strains have been identified to catalyze this reaction, most of them from *Bacteroides, Bifidobacterium, Lactobacillus* and *Clostridium* phylum, but also *Enterococcus* and some *Lactococcus*[24,35,66–68], indicating that might not be a limiting step in the production of enterolignans in the human gut[66,69]. The second step in the transformation of SDG to EL is the demethylation of SECO. *E limosum* and *Blautia producta* catalyse this reaction. Subsequently dihydroxyenterodiol is dehydroxylated to ED and Eggertella

lenta is one of the strains capable for it. Some bacterial strains have been correlated to the oxidation of ED to EL, *Lactonifactor longoviformis, Ruminococcus sp. END-1, Strain END-2, ED-Mt61and PYG-S6*[67,70,71]. The recent study of Gaya et al.[35], found that *Bifidobacterium adolescentis* INIA P784 was able to produce ED from lignan extract. A community formed by Clos *Clostridium saccharogumia ,Eggerthela lenta, Blautia product* and *Lactonifactor longoviformis was also able to transform SDG to EL and ED.*

The bacterial strain *Eggerthela lenta* did the conversion of PIN and Lariciresinol to SECO and *Ruminococcus productus* was able to do the desmethylation and dehydroxylation of Matairesinol to ED[38,67].

Table 0.1. Bacterial Strains involved in the conversion of lignans to enterolignans.

STEP	**Especies involved**	**References**
SDG to SECO	*Bacteroides spp* *Clostridium spp* *Bifidobacterium pseudocatenalum WC 401* *Bacteroides distasonis* *Bacteroides fragilis* *Bacteroides ovatus* *Clostridium cocleatum* *Clostridium sp. SDG-Mt85-3Db* *Bifidobacterium adolescentis* *B. animalis* *B. bifidum* *B. breve* *B. catenulatum* *B.dentium* *B. infantis* *B. longum* *Enterococcus faecalis* *Enterococcus faecium*	24,35,66–68

	lactococcus lactis cremoris *l.lactis diacetylactis* *l. lactis* *l. lactis lactis* *Lactobacillus paracasei* *Lactobacillus reuteri* *Lactobacillus rhamnosus*	
SDG to ED and EL	*Clostridium saccharogumia ,Eggerthela lenta, Blautia product and Lactonifactor longoviformis* *B. adolescentis INIA P784*	35,69,72
SECO to dehydroxyenterodiol	*Butybacterium methylotrophicum* *Eubacterium callanderi* *Eubacterium limosum* *Ruminococcus productos* *Peptostreptococcus productus*	66
SECO to ED and EL	*Peptostreptococcus productus sp. SDG-1* *Eubacterium sp. SDG-2*	48
SECO to ED	*Clostridium scindens* *Eggerthella lenta* *Peptostreptococcus productus SECO-Mt75m3* *Eggerthella lenta SECO-Mt 75m2*	65,67
Dehydroxyenterodiol to ED	*Clostridium scindens* *Eggertella spp* *Strain ARC-1*	24,65,73
ED to EL	*Lactonifactor longoviformis* *Ruminococcus sp. END-1* *Strain END-2* *ED-Mt61* *PYG-S6*	67,70,71
Arctigenin and SDG to	*Eggerthela sp. SDG-2* *Ruminococcus sp. END-1*	70

EL and ED		
Desmethylation of MAT	*Ruminococcus productus*	[67]
Matairesinol to dehydroxyenterolactone	*Ruminococcus productus*	[66]
PIN AND LAR TO SECO	*Eggerthella lenta*	[67]
PIN to LAR	*Enterococcus faecalis strain PDG-1*	[74]

0.2 Microbiota

0.2.1 Defining a healthy gut microbiota

Of the 100 trillion cells that human body have, only the ten percent are actually human[75]. The rest belong to bacteria, funghi, and other microbes. Our body is fully inhabited by bacteria and other microorganisms in all surfaces and cavities that are in contact with the outside. Many physiological functions necessary for our health are covered by these organisms that live in symbiosis with us and it has been shown that alterations in the balance of these species affect different areas of our metabolism and even in areas as surprising as our behavior. Latest discoveries about the microbiota highlight the importance of this symbiosis to the point that the microbiota can be considered as another organ. Life without this symbiosis would not exist. Moreover, there is good evidence that humans co-evolved a requirement for their microbiota[76].

Our intestinal system harbors an enormous number of non-pathogenic bacteria, eukaryotic microorganisms, archae, viruses and bacteriophages, comprising a community collectively referred to as the intestinal microbiota. Bacteria account for the

majority of these microorganisms. Over 500 different bacterial species build a dynamic community consisting of both persistent and transient members. The structure of the community is influenced by genotype, age, immune status, diet and environmental factors. Also changes in oxygen concentration along the gastrointestinal tract are reflected in regional differences in bacterial concentrations and community composition [76]. The three most dominant bacterial phylum in the colon are the Firmicutes, Bacteroidetes, and Actinobacteria, with Proteobacteria and Verrucomicrobia generally less abundant[77]. The association of bacteria with the intestinal tracts is far older than human life itself, and apparently bacterial communities have co-evolved with their animal and human hosts[78].

Although there is a general agreement in the fact that microbes are important to human health, with the exception of the pathogens, the roles that these microbes play in health and disease remain to be fully elucidated. Some different compositions between healthy and disease states have been observed, but still a causal relationship has not been established. Definition of a healthy human microbiome is not yet defined[79], but in an attempt of defining it from a ecologistic standpoint, the stability of a community can be thought of as a functional property of the health of a community. To measure the stability of a community there are two key concepts related to it the resistance (ability of a community to resist change in the setting of an ecologic stress) and the resilience (return to an equilibrium state following a stress-related perturbation)[79]. And so, it has been correlated that a healthy, diverse diet promotes a more diverse gut microbiota[80].

In fact, five phyla represent the majority of bacteria that comprise the gut microbiota. There are approximately 160 species in the large intestine of any individual[76].

Some of the diseases that have been related to different patterns of microbial colonization are obesity, inflammatory bowel diseases like Crohn's disease or ulcerative colitis, irritable bowel syndrome, type 1 diabetes, autism, allergy, asthma, celiac disease and/or metabolic syndrome among others[81–85]. It has been shown that some microbial distributions may make a person more susceptible to infection or disease. For example there are some microbes that convert luminal compounds into potential carcinogens causing higher risk for cancer and can lead to adverse response to chemotherapeutic agents[86]. The comparison of healthy individuals with others with a disease has arisen to the concept of dysbiosis[79].

Some of the factors that can influence in the shape of the gut microbiota are diet, antibiotics, host genotype, geographic origin, birth mode, age and stress. To alleviate the conditions linked to an altered microbiota some treatments are being developed, in which the goal is to increase beneficial bacteria. The main approaches are: prebiotics, probiotics and symbiotics, which is a combination of these.

0.2.2 Probiotics

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits on the host [87].Yogurts can be considered as probiotic according to Guarner et al.[75] and some of the attributed benefits are prevention/management of diarrhea, enhance the immune response, improve lactose digestion and absorption among others. Nowadays, most of the probiotics consumed by humans come from fermented dairy products as yoghurt (produced using a culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*).

0.2.3 Prebiotics

Is considered a prebiotic a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health[88]. Gibson et al.[89] first introduced the concept of prebiotics in 1995.

As we learn more about the ecology of the gut microbiota, it is increasingly clear that the prebiotic concept has been used as a primarily saccharolytic and fermentative microbes community evolved to work in partnership with its host's digestive system to derive energy and carbon from complex plant polysaccharides which would otherwise be lost in feces[76].

According to the definition of prebiotic the following are considered prebiotics: Inulin, fructooligosaccharides (FOS), galactooligosaccharides (GOS), lactulose, xylooligosaccharides (XOS), resistant starch (RS), human milk oligosaccharides (HMOs), beta-glucan, other dietary fibers and non-digestible oligosaccharides, but also is starting to realize that others could be considered as non-carbohydrate compounds, including polyphenols, minerals and vitamins[90]. In this work the role of polyphenols, specifically lignans, as possible prebiotics has been explored.

0.2.4 Polyphenols and microbiota

As I aforementioned, most polyphenol must undergo intestinal transformations by microbiota and enterocyte enzymes in order to be absorbed through the enterocyte and colonocyte. In most cases, a complex network of different intestinal microbiota species is necessary for full biotransformation. The individual variability with respect to

richness and biodiversity of own intestinal microbiota taxa are determinants for the ability of a person to get the most fully bioactive derivatives from ingested polyphenols.

So, consumption of food with high levels of polyphenols, together with having appropriate gut microbiota diversity, are important to help in the fight against infectious diseases and maintain a good health status. Parkar et al.[91] demonstrated that some pure phenolic acids metabolites have the ability to influence the microbial balance, in vitro, in order to promote gut health. Increasing bifidobacterium with concentrations as low as 10 μg/mL in comparison with insulin (a prebiotic who needs concentrations of the order of mg/mL) they can increase the ratio Bacteroides/Firmicutes.

There is growing evidence that phenolic catabolites and untransformed substrates, may function as prebiotics capable of shaping the human gut microbiota composition. In addition, some catabolites after absorption may have further beneficial effects provided that sufficient concentrations are achieved for a sufficient time in the relevant tissue[77]. In this work the modulation of the microbiota with lignans has been explored.

0.3 Cardiovascular Disease

0.3.1 The global burden of cardiovascular disease

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels and they include coronary heart disease, cerebrovascular disease, peripheral arterial disease, rheumatic heart disease, congenital heart disease and deep vein thrombosis and pulmonary embolism. The World Health Organization (WHO) [1] says that more people die annually from CVDs than from any other cause which means that

CVDs are number one cause of death globally. An estimated 17.7 million people died from CVDs in 2015, representing 31% of all global deaths. Over three quarters of CVD deaths take place in low-and middle-income countries.

The prevention of behavioral risk factors as tobacco use, unhealthy diet, obesity, physical inactivity and harmful use of alcohol are key strategies for most cardiovascular diseases. Some risk factors, such as age, sex and genetic predisposition, are not modifiable, but others depend on lifestyle and are also strongly related to each other. Some of the risk factors that may show up in individuals as raised blood pressure, raised blood glucose, raised blood lipids, and overweight and obesity.

A large number of epidemiological studies have associated the consumption of polyphenols with a decreased risk of CV or coronary heart disease. In a meta-analysis of the relation of tea with these diseases, the authors concluded that consumption of tea had a cardioprotective effect[92]. A literature review on moderate wine consumption showed similar results[92]. More recently, a meta-analysis of randomized intervention studies related flavan-3-ols with a reduction in biomarkers of cardiovascular risk[92]. In addition, several epidemiological studies have shown a potential protective effect of enterolignans against cardiovascular diseases[3,50,51,62,92,93].

0.3.2 The PREDIMED Study

PREDIMED stands for *Prevención con Dieta Mediterránea* (Prevention with Mediterranean Diet). This was a large Spanish primary prevention trial which included 7,447 Spanish participants (55–80 years, 58% women) who were at high risk for cardiovascular disease, but otherwise healthy (initially free of cardiovascular disease). It was a prospective, randomized, multicentric and controlled trial, ISRCTN35739639.

Volunteers were recruited through primary health care centers from 8 different Spanish regions and they were randomized to one of the following nutritional intervention groups: a) Mediterranean Diet (MD) supplemented with extra virgin olive oil, b) MD supplemented with nuts and c) Control group: low-fat diet according to the recommendations of the American Heart Association, AHA[94]. Once the informed consent was signed, several personal, anthropometric and health-related data were taken: blood pressure (triplicate), height, weight, waist circumference, etc. and the participants were asked to fill out an inclusion questionnaire, a general questionnaire about demographic and sociological data, a follow-up questionnaire, a food frequency questionnaire[95,96], a questionnaire of adherence to MD[96] and a physical activity questionnaire[97].

The inclusion criteria were:

- Free of cardiovascular disease.
- Diagnosed with Type-2 Diabetes Mellitus or having three or more of the following CV risk factors:
    - Smokers (>1 cigarette/day during the last month)
    - Arterial hypertension (SBP≥140 mm Hg and/or DBP≥ 90 mm Hg, or antihypertensive medication)
    - Hypercholesterolemia (LDL cholesterol ≥160 mg/dL, HDL cholesterol ≤40 mg/dL for men or ≤50 mg/dL for women, or anticholesterolemic medication)
    - Overweight or obese (BMI ≥ 25 kg/m2)
    - Family history of early ischemic cardiopathy
    - Ability and willingness to change eating habits.

- Not suffering any serious illness that impedes participation in a dietary intervention study.
- Not having or having had alcohol or drug addiction.

Moreover, biochemical determinations were performed with biological samples (blood, urine and toenails). Blood pressure and electrocardiograms were also performed. Patients were visited once a year to repeat the questionnaires and take biological samples.

Within the PREDIMED study, numerous substudies have demonstrated, for example, that both types of MD, with olive oil or nuts, reduced LDL cholesterol, glucose, BP and biomarkers of inflammation after only 3 months of intervention[98]. A substudy was also performed with 1224 participants, comparing both MD groups with the control group after one year to demonstrate that the MD could significantly revert the metabolic syndrome[99]. Other research papers were focused on the beneficial effects of the MD on obesity [100,101] or type-2 diabetes[102], among others. Hence, these results indicate that among persons at high cardiovascular risk, a MD supplemented with extra-virgin olive oil or nuts reduced the incidence of major cardiovascular events and the biomarkers associated.

0.4 Malnutrition

0.4.1 Definitions and prevalence

Malnutrition is estimated to contribute to more than one third of all child deaths, although it is rarely listed as the direct cause[7]. Lack of access to highly nutritious foods, especially in the present context of rising food prices, is a common cause of malnutrition. Poor feeding practices, such as inadequate breastfeeding, offering the wrong foods, and not ensuring that the child gets enough nutritious food, contribute to malnutrition. Infection – particularly frequent or persistent diarrhea, pneumonia, measles and malaria – also undermines a child's nutritional status. Malnutrition refers to deficiencies, excesses, or imbalances in a person's intake of energy and/or nutrients. The term malnutrition addresses 3 broad groups of conditions:

- undernutrition, which includes wasting (low weight-for-height), stunting (low height-for-age) and underweight (low weight-for-age);
- micronutrient-related malnutrition, which includes micronutrient deficiencies (a lack of important vitamins and minerals) or micronutrient excess; and
- overweight, obesity and diet-related noncommunicable diseases (such as heart disease, stroke, diabetes and some cancers).

There are 4 broad sub-forms of undernutrition: wasting, stunting, underweight, and deficiencies in vitamins and minerals. Undernutrition makes children in particular much more vulnerable to disease and death. Low weight-for-height is known as wasting. It usually indicates recent and severe weight loss, because a person has not had enough food to eat and/or they have had an infectious disease, such as diarrhea, which has caused them to lose weight. A young child who is moderately or severely wasted has an increased risk of death, but treatment is possible. Low height-for-age is known as

stunting. It is the result of chronic or recurrent undernutrition, usually associated with poor socioeconomic conditions, poor maternal health and nutrition, frequent illness, and/or inappropriate infant and young child feeding and care in early life. Stunting holds children back from reaching their physical and cognitive potential. Children with low weight-for-age are known as underweight. A child who is underweight may be stunted, wasted, or both.

From WHO sources[7] we know that in 2014, approximately 462 million adults worldwide were underweight, while 1.9 billion were either overweight or obese. In 2016, an estimated 155 million children under the age of 5 years were suffering from stunting, while 41 million were overweight or obese. Around 45% of deaths among children under 5 years of age are linked to undernutrition. These mostly occur in low- and middle-income countries. At the same time, in these same countries, rates of childhood overweight and obesity are rising.

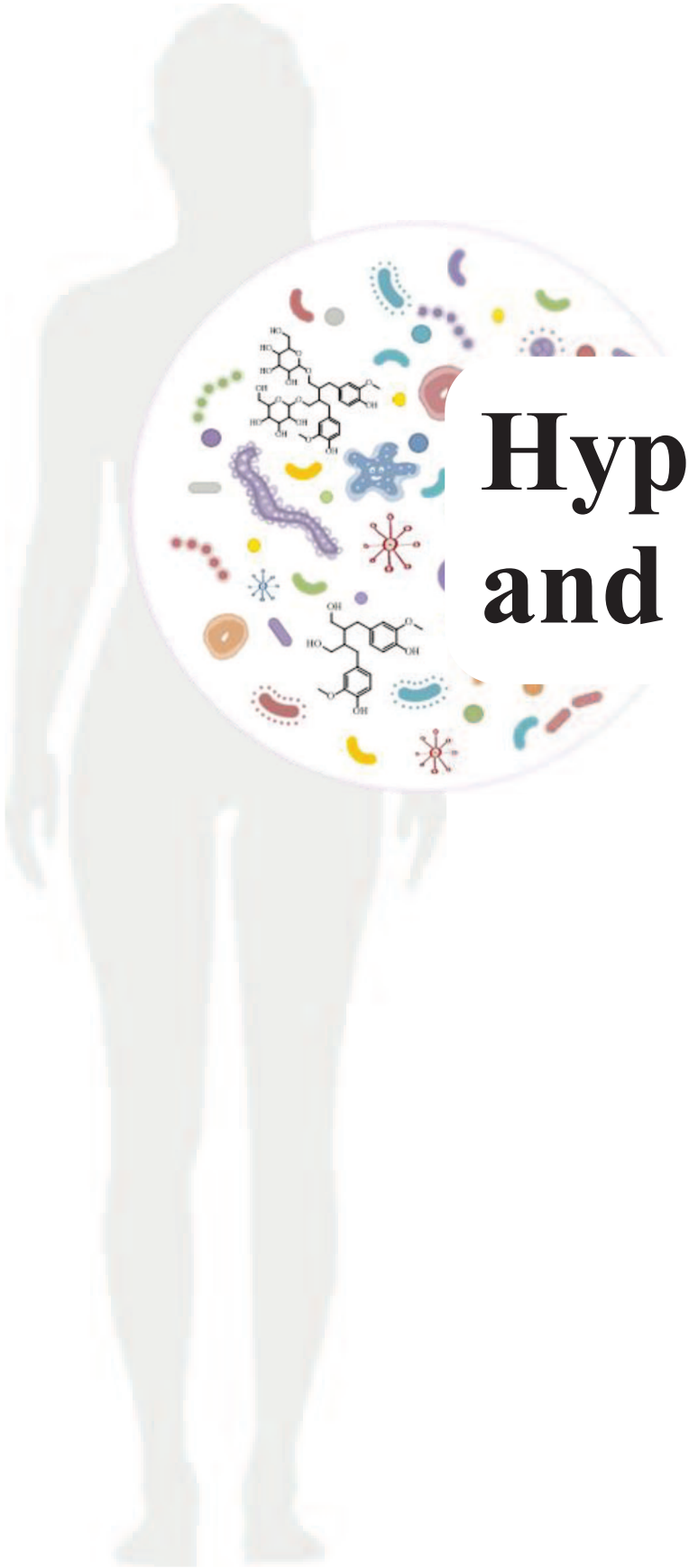
It is becoming increasingly apparent that gut microbiota play a pivotal role in the development and etiology of malnutrition[8–10]. The microbiota influence host metabolism, nutrient absorption, inflammation and even hormonal signaling, leading to changes in linear growth and weight gain in mice and humans[11,12].

0.4.2 The double-burden

Worldwide human health is extremely compromised by the double-burden of obesity and malnutrition. Undernutrition is a serious health issue in developing countries most for women and children. However, the situation is not exclusively undernutrtion for the poor and overnutrtion for the better off. Obesity is shifting progressively from the wealthier to the poorer groups with rising country income[103,104].

Interestingly, undernutrtion and obesity can exist concurrently[105]. The typical pattern is an overweight mother with a nutritionally stunted child. Studies show that early-life malnutrition can result in obesity among adults[103]. Diet and nutrition early in life play an important role in these metabolic disorders. However, the prevalence and severity of obesity and malnutrition cannot be attributed to overeating or food insecurity alone[106]. Dietary intake in early life is a strong driver of intestinal microbial composition[105]. Another interesting fact of the double-burden is that malnutrition and obesity share some features including a fatty liver, microbial dysbiosis, increased intestinal permeability, systemic inflammation, high-blood pressure and increased risk of chronic infections[105].

Early life microbiota is less stable and resilient, resulting in higher risk of obesity and its disease associations by being more sensitive to alterations[107]. Thus, it is clear that early life changes in microbiota composition can alter susceptibility to developing obesity later in life and consequently more susceptibility to cardiovascular diseases. By understanding the differing energy harvest and metabolic capabilities of each child's microbiota, we may be able to create microbiota-based interventions to reverse susceptibility to obesity early in life.



Hypothesis and aims

Hypothesis and aims

Some polyphenols, such as lignans, can be absorbed just through the colon after the gut microbiota action and metabolism. The increase of their metabolites in the human body results in decrease of cardiovascular risk parameters. Moreover, because of their potential beneficial effect on gut microbiota, lignans can be considered as prebiotics. In order to develop this hypothesis, the following aims have been set:

(1) Evaluate the principal food sources of lignans intake in the PREDIMED Study

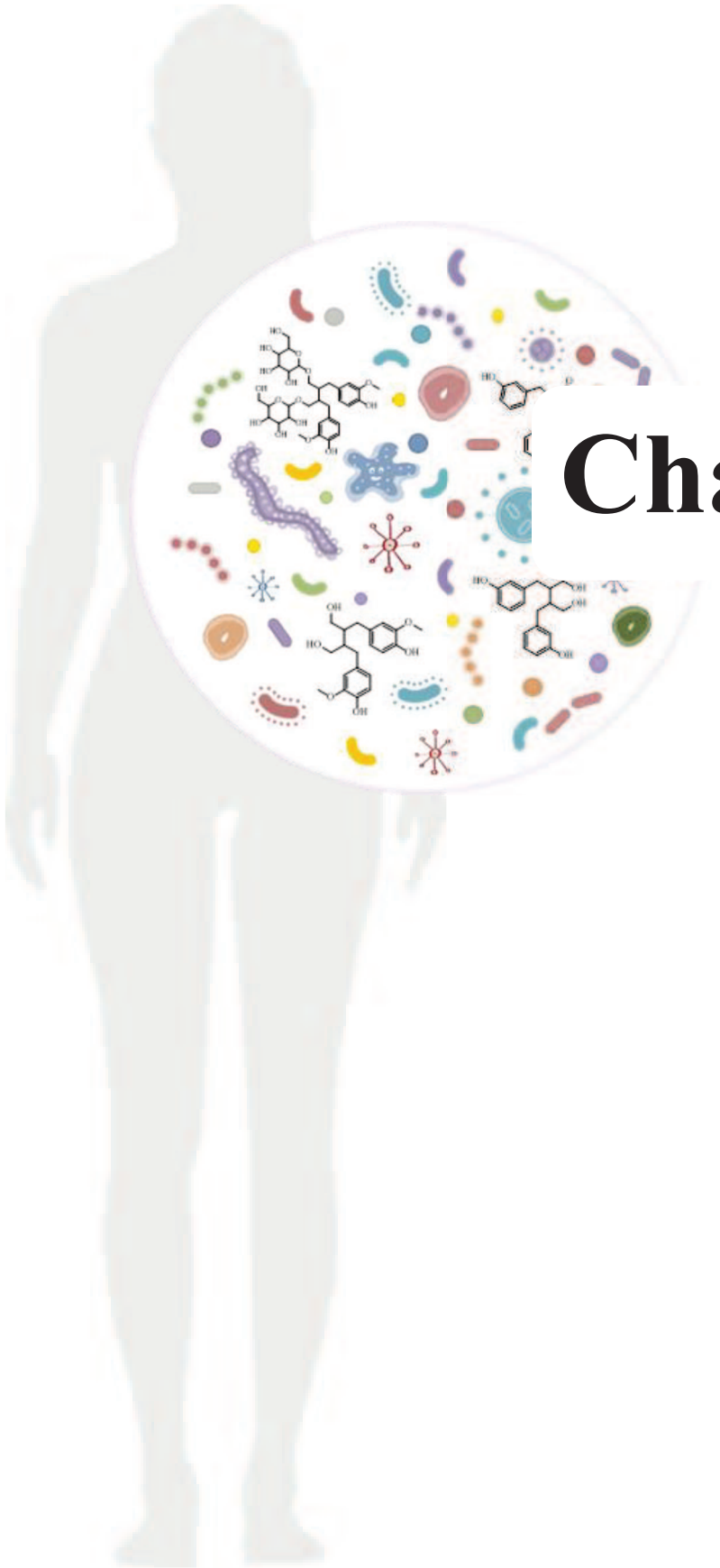
(2) Evaluate the decrease in cardiovascular risk parameters of the body based on synergism between probiotics and lignans in the PREDIMED Study.

(3) To study the microorganisms involved in the metabolism of phenolic compounds, specifically lignans.

(4) Evaluate the impact of phenolic compounds on an artificial microbial community *in vitro*.

(5) Carry out a study in healthy and malnourished mice to study the impact of lignans in both situations on gut microbiota.

(6) To evaluate the enterolignans formation in healthy and malnourished mice after a flaxseed supplemented diet.



Chapter 1

Chapter 1: Lignan and yogurt consumption on cardiovascular risk parameters in the PREDIMED Study

1.1 Introduction

Polyphenols such as lignans[108] are metabolized by microbiota in the colon, and their metabolites have shown to have health benefits. Some polyphenols have even been considered as prebiotics because of their ability to alter the microbiota profile and/or levels[109]. The main lignan polyphenols are pinoresinol (PIN), matairesinol (MAT), secoisolariciresinol (SECO), 1-acetoxypinoresinol, lariciresinol (LAR), arctigenin (ARC), syringaresinol and isolariciresinol (ILAR). Lignan intake has been related to beneficial health effects including the prevention of cancer and cardiovascular diseases (CVDs) [3]. Flaxseed and other seeds have high lignan concentrations, as do some fruits and vegetables as well as beverages such as wine, coffee and tea[25].

Probiotics are live microorganisms that confer health benefits on the host when administered in adequate amounts [87]. Most of the probiotics currently consumed by humans come from fermented dairy products such as yogurt produced using cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* [75]. The benefits attributed to probiotics include the prevention/management of diarrhea, enhancement of immune response and improved lactose digestion and absorption[110].

Since nutrients and foods are consumed in combination, nutritional epidemiology recognizes the importance of studying the effect of dietary patterns on health[111]. Food synergy is defined as additive or more than additive influences of foods and food constituents on health, linking dietary patterns and foods with disease prevention [112].

Previous studies carried out within the PREDIMED framework have shown an association between yogurt consumption and a decrease in the incidence of the metabolic syndrome and type 2 diabetes[113,114]. However, the associative effects of yogurt and lignan consumption have not been studied to date. The aim of this work is to assess the health benefits of lignans and yogurt consumption on cardiovascular risk parameters (CVR-P) such as the lipid profile, glycemic profile, body mass index and blood pressure in a well-characterized elderly population.

1.2 Material and Methods

1.2.1 Study design

A cross-sectional study was performed using baseline data from the PREDIMED cohort. A detailed description of the study has been published elsewhere [115,116]. Baseline data collection was carried out in Spain from June 2003 to June 2009. Briefly, the PREDIMED study was a large prospective, multicenter, randomized, controlled trial aimed to assess the effect of the traditional Mediterranean diet (MD) on the primary prevention of clinical cardiovascular events in elderly participants at high risk and was undertaken from October 2003 to December 2010. The 7447 eligible participants were randomized to one of the following intervention groups: MD supplemented with extra virgin olive oil, MD supplemented with nuts, or a control diet (low-fat diet) group. The trial was stopped after a median follow-up of 4.8 years after determining the benefits of the MD in the prevention of major cardiovascular events (myocardial infarction, stroke, or cardiovascular death) compared to the low-fat group [117].

This study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving human participants/patients were approved by the

Institutional Review Boards of the participating centers (Clinical Trial Registration: ISRCTN of London, England: 35739639). Written informed consent was obtained from all the participants.

1.2.2 Population characteristics, cardiovascular risk parameters, anthropometric measures and diet

Of the 7447 participants, 278 were excluded: 275 because they had not completed the food frequency questionnaire (FFQ) at baseline, and in 3 participants the HDL-cholesterol (HDL-c) values were missing. The final number of participants included was 7169.

To assess the diet and lifestyle characteristics of the study population, the participants filled out the following validated questionnaires: a 137-item semi-quantitative FFQ[118], a 14-point questionnaire on adherence to the traditional MedDiet [119], and the Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire [120]. The participants also completed a general questionnaire to provide information about lifestyle habits, concurrent diseases and medication use.

Body weight and height were measured with minimum clothing and no shoes, using calibrated scales and wall-mounted stadiometers, respectively. Blood pressure was measured in triplicate in a sitting position, using a semiautomatic sphygmomanometer (Omron HEM-705CP, Hoofddorp, The Netherlands), with a 5-min interval between each measurement, and recording the mean of the 3 values according to the procedure recommended by the European Hypertension Society[121]. Biochemical analyses were performed in local laboratories. Glucose was measured by the glucose-oxidase method, cholesterol by esterase-oxidase-peroxidase, triglycerides (TGs) by

glycerol-phosphate oxidase-peroxidase, and HDL-c by direct measurement. All the local laboratories fulfilled the external quality control requirements. When TGs were <300 mg/dL, LDL-cholesterol (LDL-c) was calculated with the Friedewald formula[122]. An inter-laboratory comparison trial of nine laboratories was conducted. A mean of 200 samples from each laboratory was analyzed for total cholesterol, HDL-c, and TGs. The Medical Research Institute of the Mar laboratory, which uses ABX-Horiba commercial kits in a PENTRA-400 autoanalyzer (ABX-Horiba), was used as the reference. One center was unable to provide samples for the compliance study.

1.2.3 Categories of lignan and yogurt consumption

Total energy and nutrient intake were calculated using Spanish food composition tables[123]. Lignan intake was calculated by multiplying the content of lignans in a particular food item (mg/g) by the daily consumption of this food (g/day). Data regarding the lignan content in foods were obtained from the Phenol-Explorer database[124].Values of lignan intake were divided into low or high with the median being the cut-point, or into tertiles, depending on the analysis.

The food frequency questionnaire (FFQ) included questions concerning the consumption of dairy products. In the validation study, the intra-class correlation coefficient between dairy product consumption from the FFQ and repeated food records was 0.84 [118]. Responses to individual dairy items of the FFQ were converted to average daily consumption (g/day) and categorized into total yogurt (including full-fat and low-fat) and total dairy intake without yogurt (including all types of milk, cheeses, custard, whipped cream and ice cream). The consumptions were then divided into the following categories: 0 yogurts/day, from 0 to <1 yogurts/day and ≥1 yogurts/day, or tertiles, depending on the analysis. The total dairy consumption was divided in tertiles.

Lignan, dairy and other nutrient intake were adjusted for total energy intake since it is associated with disease risk and is usually proportional to nutrient intake.

1.2.4 Statistical analysis

Descriptive analyses were conducted to compare the baseline characteristics across categories of yogurt consumption at baseline. Values are presented as mean±standard deviation (SD) for continuous variables and frequencies (and percentages) for categorical variables. For continuous variables, the differences between groups were analyzed using an ANOVA test. The chi square test was used for categorical data.

General linear models (GLMs) were used to assess the relationship between categorical exposure variables (lignans, yogurt, total dairy products and yogurt plus lignans) and cholesterol, TGs, blood pressure, glucose and weight. Multivariate models were adjusted for recruitment center, sex, age, smoking, soft drinks, carbohydrates, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, n-3 fatty acids, family history of heart disease, diabetes, hypertension, and dairy, fiber and energy intake. A test for linear trend was performed with the use of the resulting variable as a continuous variable.

Given the prebiotic nature of lignans, it is plausible that yogurt consumption may have differential effects on CVR-P depending on the intake of these polyphenolic compounds. Therefore, to test for statistical interactions between lignans and yogurt in different CVR-P, stratified analyses were performed and interaction p-values were calculated.

All statistical analyses were conducted using SAS software[125].All t- tests were 2-sided and P-values less than 0.05 were considered significant.

1.3 Results

The baseline characteristics of the study participants are summarized in **Table 1.1**. Around 23 % of the population did not consume any yogurt (1631 participants), 54% consumed <1 yogurt per day (3840 participants) and 24% consumed ≥1 yogurt per day (1698 participants). The distribution of sex, smoking, level of education, energy expenditure during leisure time, age, participants with hypertension and cholesterol was significantly different between groups. In contrast, participants with diabetes were equally distributed among the three yogurt groups. Participants with the highest yogurt intake also had the highest intake of carbohydrates, protein and fiber, but the lowest cholesterol intake. Non-consumers had higher blood pressure, glucose and TGs but a lower body mass index (BMI) and HDL-c levels. There were no significant differences between yogurt consumption and MD adherence (MedDiet Score).

Table 1.1. Baseline characteristics of 7169 elderly Spanish participants at high cardiovascular risk from the PREDIMED cohort according to categories of yogurt consumption assessed by food frequency questionnaire adjusted for energy.

	Non consumers	**< 1 yogurt/day**	**≥1 yogurt/day**	
Characteristics	**n (%)**	**n (%)**	**n (%)**	***P value*[a]**
No. of participants (n=7169)	1631 (22.7)	3840(53.6)	1698 (23.7)	
Sex, women	663 (40.6)	2201 (57.3)	1216 (71.6)	<.001
Smoking				<.001
Never	784 (48.0)	2382 (62.0)	1217 (71.7)	
Current	393 (24.1)	622 (16.2)	187 (11.0)	
Former	454 (27.8)	836 (21.8)	294 (17.3)	
Education				0.01
University	68 (4.2)	148 (3.8)	55 (3.2)	
Secondary	309 (18.9)	754 (19.6)	271 (15.9)	
Elementary	1254 (76.9)	2938 (76.5)	1372 (80.8)	
Arterial Hypertension[b]	1310 (80.3)	3197 (83.3)	1420 (83.6)	0.01
Diabetes[c]	813 (49.8)	1808 (47.08)	848 (49.9)	0.06
Hypercholesterolemia[d]	1134 (69.5)	2804 (73.0)	1247 (73.4)	0.01
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	***P value*[e]**
Age (years)	67.3 ± 6.2	66.8 ± 6.1	67.5 ± 6.0	<0.001
Energy expenditure[f] (MET-h/d)	4.0 ± 4.2	3.9 ± 4.0	3.7 ± 3.7	0.04
Dietary pattern (g/day)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	***P value*[e]**
Mediterranean diet adherence score	8.5 ± 1.9	8.7 ± 1.9	8.7 ± 1.9	0.61
Total dairy	279.0 ± 207.1	378.9 ± 204.1	494.9 ± 228.2	<0.001
Yogurt, total	0.0 ± 0.0	65.3 ± 38.6	196.6 ± 94.6	<0.001
Low-fat yogurt	0.0 ± 0.0	44.5 ± 44.2	142.3 ± 116.1	<0.001
Milk, total	235.5 ± 194.1	266.7 ± 185.8	271.6 ± 184.0	0.37
Low-fat milk	174.8 ± 194.9	221.1 ± 198.5	234.9 ± 196.1	0.02
Cream and whipped cream	0.39 ± 3.39	0.65 ± 5.79	0.30 ± 2.0	0.01
Cheese	15.1 ± 17.1	14.6 ± 15.6	12.6 ± 15.7	<0.001
Low-fat cheese	11.2 ± 21.5	13.7 ± 18.9	17.2 ± 23.0	<0.001
Dairy desserts	13.7 ± 43.3	10.5 ± 26.1	9.1 ± 28.5	0.07
Other dairy[g]	1.75 ± 6.7	1.76 ± 5.3	1.97 ± 6.7	0.23

Soft drinks	21.7 ± 72.6	18.7 ± 63.8	14.1 ± 47.5	0.008
Nutrient intake [h]				
Total energy (Kcal/day)	2300 ± 600	2351.6 ± 581.6	2046.6 ± 483.5	<0.001
Carbohydrates (g/day)	234.7 ± 46.3	238.0 ± 42.9	242.6 ± 36.0	<0.001
Protein (g/day)	87.8 ± 14.4	92.3 ± 13.9	96.6 ± 13.4	<0.001
SFA[i] (g/day)	25.8 ± 6.4	25.3 ± 6.0	24.4 ± 5.1	<0.001
MUFA[j] (g/day)	50.1 ± 11.6	48.8 ± 11.3	47.2± 10.4	<0.001
PUFA[k] (g/day)	16.2 ± 5.5	15.8 ± 5.3	15.2 ± 4.7	<0.001
Fiber (g/day)	24.6 ± 7.6	25.5 ± 7.8	26.3 ± 7.0	<0.001
Total cholesterol (mg/day)	368.9 ± 111.1	368.9 ± 116.5	357.5 ± 91.9	<0.001
n-3 fatty acids (g/day)	2.2 ± 0.79	2.2 ± 0.80	2.2 ± 0.73	0.07
Lignan intake (mg/day)	0.59 ± 0.2	0.60 ± 0.2	0.61 ± 0.2	0.04
Cardiovascular risk parameters	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	***P value*** [e]
Body Mass Index (Kg/m^2)	29.6 ± 3.5	29.9 ± 3.7	30.0 ± 3.7	0.50
Systolic blood presure (mmHg)	149.7 ± 19.1	148.6 ± 19.0	148.2 ± 19.1	0.40
Diastolic blood pressure (mmHg)	83.2 ± 10.2	82.9 ± 10.0	82.2 ± 10.5	0.01
Glucose (mg/dL)	123.5 ± 39.8	121.1 ± 42.0	122.1 ± 41.3	0.44
Lipid profile (mg/dL)	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	***P value*** [e]
Total cholesterol	210.7 ± 38.3	210.7 ± 38.1	212.2 ± 38.2	0.22
HDL-cholesterol	52.8 ± 13.0	53.8 ± 14.3	55.6 ± 13.9	<0.001
LDL-cholesterol	130.3 ± 33.5	130.2 ± 33.4	130.2 ± 34.4	0.99
Triglycerides	142.1 ± 79.1	136.8 ± 83.7	132.5 ± 67.3	0.08

[a] χ^2 tests; [b] Arterial hypertension was defined as systolic blood pressure (SBP) ≥ 140 mmHg, diastolic blood pressure (DBP) ≥ 90 mmHg, or taking antihypertensive medication); [c] Diabetes was diagnosed when fasting plasma glucose concentrations of ≥7.0 mmol/L (≥126.1 mg/dL), 2-h plasma glucose concentrations of ≥11.1 mmol/L (≥200.0 mg/dL) after an oral dose of 75 g glucose, or insulin treatment.; [d] Hypercholesterolemia was defined as LDL-cholesterol ≥ 160 mg/dL, HDL-cholesterol≤ 40 mg/dL, or antihyperlipidemic medication.; [e] One-way ANOVA tests; [f] In physical activity at leisure time.; [g] Cream cheese and condensed milk.; [h] FFQ was used to estimate the dietary pattern by multiplying the frequency of consumption of all food items by the average portion size using Spanish food composition tables and was carried out by trained dietitians.; [i] SFA: Saturated Fatty Acids ; [j] MUFA: Monounsaturated Fatty Acids; [k] PUFA: Polyunsaturated Fatty Acids.

1.3.1 Lignans intake and food sources

Figure 1.1 shows the individual lignan intake and the chemical structures of the different lignans as well as the main lignan food sources ingested by the PREDIMED cohort. The lignan most frequently consumed was PIN (0.31±0.25 mg/day), followed by 1-acetoxypinoresinol (0.25±0.12 mg/day), lariciresinol (0.12±0.06 mg/day), syringaresinol (0.07±0.09 mg/day), secoisolariciresinol (0.06±0.06 mg/day), isolariciresinol (0.03±0.07mg/day), medioresinol (0.01±0.01 mg/day) and matairesinol (0.004±0.002 mg/day). The main lignan food sources were olive oil (over 60%), wheat products (about 15%), tomatoes and derivatives (8%), red wine (5%), asparagus (4%), kiwis (3%) and other fruits and vegetables. **Table 2.2** shows the main food sources of each lignan.

1.3.2 Lignan intake and CVR-P

Table 1.3 shows the relationship between lignan intake and CVR-P. Participants with the highest (>0.67 mg/day) and medium (0.46-0.67 mg/day) lignan intakes had significantly lower plasma glucose levels (estimated beta-coefficients β=-6.08, $P<0.001$ and β=-4.16, $P=0.002$, respectively) compared to those with the lowest lignan intake (P-trend=0.02). No significant associations were observed for other CVR-P across the lignan groups.

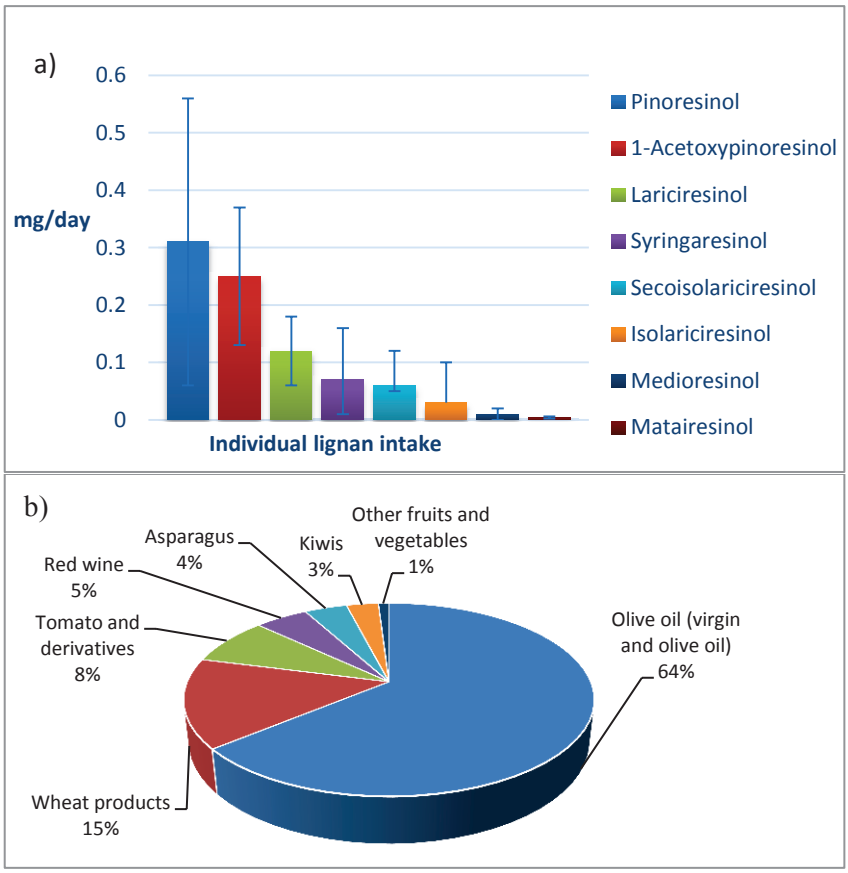


Figure 1.1 Lignan intake of 7169 elderly Spanish participants at high cardiovascular risk from the PREDIMED cohort at baseline. a) Average intake of each individual lignan: pinoresinol, 1-acetoxypinoresinol, lariciresinol, syringaresinol, secoisolariciresinol, isolariciresinol, medioresinol and matairesinol. b) Percentage of the lignan food sources.

Table 1.2. Mean intake of lignan compounds and their food sources of 7169 elderly Spanish participants at high cardiovascular risk from the PREDIMED study.

Lignan	Intake (mg/day)	SD[a]	Food sources
Pinoresinol	0.31	0.25	Olive oil (96%), asparagus (0.7%), refined wheat (0.6%), whole-grain wheat (0.6%)
1-Acetoxypinoresinol	0.25	0.12	Olive oil (100%)
Lariciresinol	0.12	0.06	Wheat (67%), whole-grain wheat (11%), tomato (6.5%), asparagus (4%)
Secoisolariciresinol	0.06	0.06	Kiwi (37%), asparagus (31%), red wine (19%), whole-grain wheat (6%)
Syringaresinol	0.07	0.09	Whole-grain wheat (81%), asparagus (10%), kiwis (3%), red wine (3%)
Isolariciresinol	0.03	0.07	Red wine (100%)
Medioresinol	0.01	0.01	Whole-grain wheat (53%), tomato (21%), kiwi (15%), asparagus (8%)
Matairesinol	0.004	0.002	Red wine (74%), asparagus (8%), tea (6%), whole-grain wheat (6%)

[a] Standard Desviation

Table 1.3. Association between yogurt, dairy or lignans consumption and CVR-P of 7169 elderly Spanish participants at high cardiovascular risk from the PREDIMED cohort.

General Linear Models		Model[a]	Group 1 vs Group 0[b]		Group 2 vs Group 0[b]		P-trend
			β (95%CI)	P-value	β (95%CI)	P-value	
Lignans intake	Total cholesterol (mg/dL)	**1**	1.08 (-1.26, 3.42)	0.37	1.50 (-0.83, 3.84)	0.21	
		2	1.19 (-1.20, -3.60)	0.73	2.42 (-0.20, 5.05)	0.07	0.60
	HDL-cholesterol (mg/dL)	**1**	0.34 (-0.49, 1.19)	0.42	1.04 (0.20, 1.88)	0.01	
		2	-0.15 (-1.01, 0.71)	0.20	0.05 (-0.89, 0.99)	0.92	0.81
	LDL-cholesterol (mg/dL)	**1**	-2.46 (-7.42, 2.49)	0.33	-5.51 (-10.45, -0.57)	0.03	
		2	0.96 (-1.25, 3.18)	0.20	2.38 (-0.03, 4.80)	0.05	0.29
	Triglycerides (mg/dL)	**1**	-2.46 (-7.42, 2.49)	0.33	-5.51 (-10.45, -0.57)	0.03	
		2	-1.68 (-6.77, 3.40)	0.51	-2.57 (-8.14, 2.98)	0.36	0.03
	Glucose (mg/dL)	**1**	-2.93 (-5.51, -0.34)	0.03	-2.46 (-5.04, 0.12)	0.62	
		2	-4.16 (-6.78, 1.54)	0.002	-6.08 (-8.95, -3.21)	<.001	0.02
	BMI[c] (kg/m^2)	**1**	-0.10 (-0.31, 0.10)	0.32	-0.17 (-0.37, 0.037)	0.11	
		2	0.01 (-0.20, 0.22)	0.92	0.11 (-0.12, 0.34)	0.36	0.29
	Weight (kg)	**1**	-0.12 (0.72, 0.47)	0.68	-0.086 (-0.68, 0.51)	0.78	
		2	0.45 (-0.17, 1.08)	0.53	0.65 (-0.03, 1.32)	0.06	0.57
	Waist circumference (cm)	**1**	-0.49 (-1.06, 0.09)	0.10	-0.99 (-1.57, -0.41)	0.001	
		2	-0.22 (-0.82, 0.37)	0.46	-0.26 (-0.92, 0.40)	0.44	0.005
	SBP[d] (mmHg)	**1**	0.94 (-0.25, 2.14)	0.12	0.67 (-0.53, 1.88)	0.27	
		2	0.97 (-0.26, 2.19)	0.12	1.31 (-2.06, 2.67)	0.06	0.90
	DBP[e] (mmHg)	**1**	0.06 (-0.58, 0.71)	0.35	-0.26 (-0.91, 0.38)	0.42	

		2	0.18 (-0.48, 0.84)	0.59	0.27 (-0.46, 1.01)	0.46	0.09
General Linear Models		**Model[a*]**	**<1 yogurt/day vs non consumers**		**≥1 yogurt/day vs non consumers**		**P-trend**
			β (95%CI)	**P-value**	**β (95%CI)**	**P-value**	
Total Yogurt	Total cholesterol (mg/dL)	**1**	-3.00 (-5.40, 0.62)	0.01	-3.34 (-6.17, -0.51)	0.02	
		2	-2.92 (-5.30, -0.53)	0.02	-3.33 (-6.20, -0.48)	0.02	0.03
	HDL-cholesterol (mg/dL)	**1**	-0.31 (-1.17, 0.54)	0.48	0.08 (-0.93, 1.10)	0.87	
		2	-0.41 (-1.27, 0.44)	0.34	-0.14 (-1.16, 0.88)	0.78	0.81
	LDL-cholesterol (mg/dL)	**1**	-1.74 (-3.94, 0.46)	0.12	-2.45 (-5.05, 0.15)	0.06	
		2	-1.65 (-3.87, 0.55)	0.14	-2.39 (-5.04, 0.23)	0.07	0.06
	Triglycerides (mg/dL)	**1**	-5.10 (-10.16, -0.06)	0.05	-8.47 (-14.46, -2.48)	0.005	
		2	-4.14 (-9.19, 0.91)	0.11	-6.94 (-12.97, -0.91)	0.02	0.07
	Glucose (mg/dL)	**1**	-0.93 (-3.56, 1.71)	0.49	1.33 (-1.79, 4.45)	0.40	
		2	-0.87 (-3.51, 1.72)	0.50	1.82 (-1.30, 4.94)	0.25	0.50
	BMI[c] (kg/m^2)	**1**	0.14 (-0.06, 0.35)	0.18	0.11 (-0.14, 0.36)	0.39	
		2	0.14 (-0.06, 0.35)	0.18	0.13 (-0.12, 0.38)	0.32	0.11
	Weight (kg)	**1**	0.88 (0.26, 1.49)	0.005	0.75 (0.02, 1.48)	0.04	
		2	0.90 (0.29, 1.52)	0.004	0.88 (0.15, 1.69)	0.02	0.007
	Waist circumference (cm)	**1**	0.51 (-0.088, 1.11)	0.09	-0.17 (-0.88, 0.54)	0.63	
		2	0.59 (-0.01, 1.19)	0.055	0.04 (-0.67, 0.76)	0.90	0.42
	SBP[d] (mmHg)	**1**	-0.33 (1.57, 0.91)	0.60	-0.42 (-1.89, 1.06)	0.58	
		2	-0.37 (-1.61, 0.87)	0.55	-0.48 (-1.96, 0.99)	0.52	0.62
	DBP[e] (mmHg)	**1**	-0.04 (-0.71, 0.62)	0.89	-0.25 (-1.04, 0.54)	0.53	
		2	-0.04 (-0.71, 0.62)	0.90	-0.22 (-1.01, 0.58)	0.59	0.65

Full-fat Yogurt	Total cholesterol (mg/dL)	**1**	1.70 (-0.63, 4.04)	0.15	-1.72 (-5.65, 2.22)	0.39	
		2	1.19 (-1.28, 3.66)	0.34	-2.43 (-6.47, 1.61)	0.24	0.047
	HDL-cholesterol (mg/dL)	**1**	0.36 (-0.47, 1.20)	0.39	0.95 (-0.46, 2.36)	0.19	
		2	0.56 (-0.32, 1.44)	0.21	0.99 (-0.45, 2.44)	0.18	0.12
	LDL-cholesterol (mg/dL)	**1**	2.11 (-0.03, 4.25)	0.05	-1.95 (-5.58, 1.67)	0.29	
		2	1.81 (-0.45, 4.08)	0.12	-2.38 (-6.10, 1.34)	0.21	0.047
	Triglycerides (mg/dL)	**1**	0.03 (-4.91, 4.97)	0.99	-7.60 (-15.94, 0.73)	0.07	
		2	-1.90 (-7.12, 3.31)	0.47	-9.33 (-17.87, -0.79)	0.03	0.02
	Glucose (mg/dL)	**1**	-2.27 (-4.84, 0.30)	0.08	-0.81 (-5.14, 3.51)	0.71	
		2	-1.85 (-4.55, 0.84)	0.18	0.63 (-3.77, 5.03)	0.78	0.98
	BMI[c] (kg/m^2)	**1**	0.23 (0.03, 0.44)	0.03	0.24 (-0.11, 0.58)	0.18	
		2	0.19 (-0.03, 0.40)	0.08	0.22 (-0.13, 0.58)	0.22	0.06
	Weight (kg)	**1**	0.72 (0.13, 1.32)	0.02	0.51 (-0.50, 1.52)	0.32	
		2	0.78 (0.15, 1.41)	0.01	0.70 (-0.33,1.74)	0.18	0.037
	Waist circumference (cm)	**1**	0.70 (0.12, 1.28)	0.02	0.25 (-0.74, 1.24)	0.61	
		2	0.48 (-0.14, 1.09)	0.13	0.16 (-0.85, 1.17)	0.75	0.30
	SBP[d] (mmHg)	**1**	0.82 (-0.38, 2.02)	0.18	1.84 (-0.22, 3.91)	0.08	
		2	0.43 (-0.83, 1.70)	0.50	1.33 (-0.77, 3.44)	0.21	0.42
	DBP[e] (mmHg)	**1**	1.06 (0.41, 1.70)	0.001	0.63 (-0.48, 1.76)	0.27	
		2	0.81 (0.13, 1.49)	0.02	0.36 (-0.76, 1.50)	0.52	0.31
Low-fat Yogurt	Total cholesterol (mg/dL)	**1**	-3.51 (-5.62, -1.40)	0.001	-1.79 (-4.51, 0.93)	0.20	
		2	-4.40 (-6.65, -2.15)	<.001	-2.87 (-5.75, 0.01)	0.05	0.08
	HDL-cholesterol (mg/dL)	**1**	-0.89 (-1.64, -0.13)	0.02	-0.38 (-1.35, 0.59)	0.44	
		2	-1.05 (-1.85, -0.24)	0.01	-0.63 (-1.65, 0.40)	0.23	0.57

	LDL-cholesterol (mg/dL)	**1**	-3.00 (-4.93, -1.06)	0.002	-1.17 (-3.65, 1.31)	0.36	
		2	-3.80 (-5.87, -1.72)	<.001	-2.11 (-4.75, 0.52)	0.11	0.20
	Triglycerides (mg/dL)	**1**	-0.43 (-4.89, 4.03)	0.85	-4.36 (-10.13, 1.40)	0.14	
		2	-0.82 (-5.59, 3.95)	0.73	-4.53 (-10.60,1.56)	0.14	0.34
	Glucose (mg/dL)	**1**	1.56 (-0.76, 3.88)	0.19	2.69 (-0.33, 5.70)	0.08	
		2	1.29 (-1.18, 3.76)	0.31	2.80 (-0.36, 5.97)	0.08	0.39
	BMI[c] (kg/m^2)	**1**	0.0007 (-0.18, 0.18)	0.99	-0.104 (-0.35, 0.14)	0.40	
		2	0.12 (-0.08, 0.32)	0.23	0.04 (-0.21, 0.30)	0.72	0.35
	Weight (kg)	**1**	0.24 (-0.29, 0.78)	0.38	0.058 (-0.64, 0.76)	0.87	
		2	0.64 (0.06, 1.21)	0.03	0.55 (-0.18, 1.30)	0.14	0.025
	Waist circumference (cm)	**1**	0.18 (-0.35, 0.71)	0.51	-0.80 (-1.49, -0.11)	0.02	
		2	0.55 (-0.02, 1.12)	0.06	-0.30 (-1.02, 0.42)	0.42	0.67
	SBP[d] (mmHg)	**1**	-0.87 (-1.96, 0.22)	0.12	-1.37 (-2.79, 0.04)	0.06	
		2	-0.62 (-1.79, 0.55)	0.30	-1.12 (-2.62, 0.37)	0.14	0.28
	DBP[e] (mmHg)	**1**	-0.94 (-1.52, -0.35)	0.001	-0.96 (-1.72, -0.20)	0.01	
		2	-0.076 (-1.38, -0.13)	0.02	-0.76 (-1.56, 0.04)	0.06	0.25
General Linear Models		**Model[a*]**	**Group 1 vs Group 0[f]**		**Group 2 vs Group 0[f]**		**P-trend**
			β (95%CI)	**P value**	**β (95%CI)**	**P value**	
Total dairy[g]	Total cholesterol (mg/dL)	**1**	-4.34 (-6.70, -2.17)	<.001	-4.52 (-7.18, -1.86)	<.001	
		2	-4.30 (-6.60, -2.01)	<.001	-4.36 (-7.09, -1.62)	0.002	<.001
	HDL-cholesterol (mg/dL)	**1**	-1.06 (-1.87, -0.25)	0.01	-1.07 (-2.02, -0.11)	0.03	
		2	-0.87 (-1.69, -0.005)	0.04	-0.65 (-1.63, 0.33)	0.19	0.005
	LDL-cholesterol	**1**	-2.41 (-4.50, -0.32)	0.02	-2.23 (-4.68, 0.22)	0.07	

	(mg/dL)	**2**	-2.34 (-4.45, -0.22)	0.03	-2.18 (-4.70, 0.33)	0.09	0.048
	Triglycerides (mg/dL)	**1**	-4.35 (-9.15, 0.45)	0.07	-2.93 (-8.58, 2.71)	0.31	
		2	-4.74 (-9.58, 0.11)	0.06	-4.13 (-9.93, 1.65)	0.16	0.37
	Glucose (mg/dL)	**1**	3.35 (0.85, 5.85)	0.008	8.93 (5.99, 11.87)	<.001	
		2	2.64 (0.14, 5.14)	0.04	7.89 (4.89, 10.88)	<.001	<.001
	BMI[c] (kg/m^2)	**1**	0.15 (-0.05, 0.35)	0.15	0.11 (-0.12, 0.35)	0.34	
		2	0.075 (-0.13, 0.28)	0.46	-0.011 (-0.25, 0.23)	0.93	0.68
	Weight (kg)	**1**	0.12 (-0.46, 0.71)	0.67	-0.14 (-0.83, 0.56)	0.70	
		2	-0.08 (-0.67, 0.50)	0.78	-0.53 (-1.24, 0.18)	0.15	0.82
	Waist circumference (cm)	**1**	0.18 (-0.38, 0.75)	0.52	-0.22 (-0.90, 0.46)	0.52	
		2	-0.05 (-0.62, 0.52)	0.86	0.70 (-1.39, -0.003)	0.05	0.16
	SBP[d] (mmHg)	**1**	-0.25 (-1.43, 0.92)	0.67	-0.82 (-2.22, 0.59)	0.25	
		2	-0.28 (-1.46, 0.90)	0.64	-0.77 (-2.21, 0.67)	0.29	0.025
	DBP[e] (mmHg)	**1**	-0.14 (-0.77, 0.48)	0.65	-0.57 (-1.32, 0.19)	0.14	
		2	-0.22 (-0.85, 0.41)	0.50	-0.78 (-1.53, -0.019)	0.04	0.02

[a] Model 1: adjusted for recruitment center, sex and age; Model 2: adjusted for recruitment center, sex, age, smoking, soft drinks, carbohydrates, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, n-3 fatty acids, dairies. [*]Model 2 replacing dairies by fiber.; [b] Lignans groups were formed according to tertiles, group 0: <0.46 mg/day, group 1: 0.46-0.67 mg/day, and group 2: >0.67 mg/day ; [c] BMI: Body Mass Index.; [d] SBP: Systolic Blood Pressure.; [e] DBP: Diastolic Blood Pressure.; [f] Total dairy groups were formed according to tertiles, group 0: < 200g/day, group 1: 200-500 g/day, and group 2: >500g/day.; [g] Total dairy comprises whole/low-fat/skim milk, condensed milk, ice-cream, custard and all types of cheeses (ricotta, cured cheeses…).

1.3.3 Total yogurt, full-fat yogurt, low-fat yogurt or dairy intake and CVR-P

The associations between the intake of yogurt, full-fat yogurt, low-fat yogurt or other dairy products and CVR-P are presented in **Table 1.3**. Participants consuming any kind of yogurt had significantly lower total cholesterol levels (β=-2.92, P=0.02 for <1 yogurt/day, and β=-3.33, P=0.03 for ≥1 yogurt/day, P-trend=0.03) compared to non-consumers. Those with the highest intake (≥1 yogurt/day) also had lower TG levels (β=-6.94, P=0.02) compared to non-consumers. In addition, in both groups, yogurt consumption was associated with higher weight (β=0.90, P=0.004 and β=0.88, P=0.02 for 1 yogurt/day and ≥1 yogurt/day, respectively, P-trend=0.007).

Low intake of full-fat yogurt was associated with higher weight and higher diastolic blood pressure (β=0.78, P=0.01 and β=0.81, P=0.02 respectively). An intake of ≥1 full-fat yogurt/day was correlated with a decrease in TG levels (β=-9.33, P=0.03). However, there were no significant differences in the other CVR-P. Regarding low-fat yogurt, consumers of 1 yogurt/day had lower total cholesterol values (β=-4.40, P<0.001), HDL-c (β=-1.05, P=0.01), LDL-c (β=-3.80, P<0.001) and diastolic blood pressure (β=-0.076, P=0.02) but a higher weight (β=0.64, P=0.03) compared to non-consumers.

Finally, association between total dairy intake and CVR-P was examined. A total dairy intake of more than 500g/day was associated with lower total cholesterol (β=-4.36, P=0.002), and diastolic blood pressure (β=-0.78, P=0.04), and higher plasma glucose levels (β=7.89, P<0.001). Total dairy intake of 200-500g/day was associated with lower total cholesterol (β=-4.30, P<0.001), HDL-c (β=-0.87, P=0.04), LDL-c (β=-2.34, P=0.03), and higher glucose levels (β=2.64, P=0.04). Significant linear associations were found for total dairy intake and total cholesterol (P-trend<0.001),

HDL-c (P-trend=0.005), LDL-c (P-trend=0.048), glucose (P-trend<0.001), systolic blood pressure (P-trend=0.025), and diastolic blood pressure (P-trend=0.02).

1.3.4 Joint analysis of lignans and yogurt consumption

Table 1.4 shows the results of the GLMs used to assess the association between yogurt consumption and different CVR-P stratified by lignan intake. The participants with the highest consumption of lignans (>0.6 mg/day) and total yogurt had significantly lower levels of total cholesterol (β=-6.18, P=0.001, P-interaction=0.01) and LDL-c (β=-4.92, P=0.005, P-interaction=0.05), and TGs (β=-7.98, P=0.049, P-interaction=0.21), although the P for interaction was not significant in the latter. Participants with a higher consumption of yogurt but a lower intake of lignans (<0.6 mg/day) had a significantly higher BMI (β=0.51, P=0.006) and weight (β=1.35, P=0.01), while those with high lignan intake showed no differences in BMI and weight (β=-0.04, P=0.81, P-interaction=0.41 and β=0.391, P=0.45, P-interaction=0.42, respectively).

Table 1.4. General linear models for the association between cardiovascular risk parameters and the joint intake of yogurt and lignans of 7169 elderly Spanish participants at high cardiovascular risk from the PREDIMED cohort.

GLM		Low lignan intake (<0.6mg/day) (n = 3525)		High lignan intake (>0.6mg/day) (n = 3644)		P-interaction
	Model[a]	β (95%CI)	P	β (95%CI)	P	
Total cholesterol (mg/dl)	**Model 1**					0.05
	<1 yogurt/day vs non consumers	-2.22 (-5.74, 1.29)	0.21	-3.71 (-6.96, -0.46)	0.02	
	≥1 yogurt/day vs non consumers	0.63 (-3.61, 4.88)	0.77	-6.48 (-10.28, -2.67)	<0.001	
	Model 2					0.01
	<1 yogurt/day vs non consumers	-2.57 (-6.03, 0.89)	0.14	-3.83 (-7.05, -0.62)	0.02	
	≥1 yogurt/day vs non consumers	-0.59 (-3.63, 4.81)	0.78	-6.18 (-9.97, -2.40)	0.001	
HDL-cholesterol (mg/dl)	**Model 1**					0.27
	<1 yogurt/day vs non consumers	0.59 (-0.65, 1.83)	0.35	-1.17 (-2.36, 0.01)	0.05	
	≥1 yogurt/day vs non consumers	0.62 (-0.87, 2.12)	0.41	-0.49 (-1.87, 0.89)	0.49	
	Model 2					0.79
	<1 yogurt/day vs non consumers	0.07 (-1.18, 1.32)	0.91	-1.14 (-2.32, 0.04)	0.06	
	≥1 yogurt/day vs non consumers	-0.21 (-1.74, 1.32)	0.79	-0.48 (-1.87, 0.91)	0.50	
LDL-cholesterol (mg/dl)	**Model 1**					0.16
	<1 yogurt/day vs non consumers	-0.96 (-4.18, 2.25)	0.55	-2.45 (-5.48, 0.57)	0.11	
	≥1 yogurt/day vs non consumers	1.39 (-2.49, 5.28)	0.48	-5.54 (-9.06, -2.03)	0.002	
	Model 2					0.05

	<1 yogurt/day vs non consumers	-1.09 (-4.25, 2.08)	0.50	-2.74 (-5.72, 0.24)	0.07	
	≥1 yogurt/day vs non consumers	1.91 (-1.95, 5.76)	0.33	-4.92 (-8.41, -1.43)	0.005	
Triglycerides	**Model 1**					0.05
(mg/dl)	<1 yogurt/day vs non consumers	-9.41(-16.97, -1.86)	0.01	-1.07 (-7.86, 5.72	0.76	
	≥1 yogurt/day vs non consumers	-8.76 (-1.79, 0.38)	0.06	-7.36 (-15.30, 0.58)	0.07	
	Model 2					0.21
	<1 yogurt/day vs non consumers	-7.53 (-15.18, 0.11)	0.05	-1.31 (-8.15, 5.53)	0.71	
	≥1 yogurt/day vs non consumers	-5.93 (-15.19, 3.33)	0.21	-7.98 (-15.94, -0.015)	0.049	
Glucose	**Model 1**					0.08
(mg/dl)	<1 yogurt/day vs non consumers	-0.55 (-4.40, 3.30)	0.78	-1.19 (-4.80, 2.43)	0.52	
	≥1 yogurt/day vs non consumers	1.91 (-2.73, 6.56)	0.42	0.98 (-3.25, 5.22)	0.65	
	Model 2					0.07
	<1 yogurt/day vs non consumers	-0.47 (-3.66, 2.71)	0.77	-0.89 (-4.76, 2.99)	0.65	
	≥1 yogurt/day vs non consumers	-2.20 (-5.15, 0.75)	0.14	-1.92 (-5.41, 1.57)	0.28	
BMI[b] (kg/m^2)	**Model 1**					0.11
	<1 yogurt/day vs non consumers	0.22 (-0.08, 0.52)	0.14	0.07 (-0.22, 0.37)	0.63	
	≥1 yogurt/day vs non consumers	0.25 (-0.11, 0.61)	0.18	-0.003 (-0.35, 0.34)	0.98	
	Model 2					0.44
	<1 yogurt/day vs non consumers	0.28 (-0.02, 0.58)	0.007	0.06 (-0.23, 0.36)	0.65	
	≥1 yogurt/day vs non consumers	0.51 (0.15, 0.88)	0.006	-0.04 (-0.39, 0.31)	0.81	
Weight (kg)	**Model 1**					0.94

	<1 yogurt/day vs non consumers	1.21 (0.33, 2.09)	0.006	0.56 (-0.30, 1.42)	0.20	
	≥1 yogurt/day vs non consumers	1.05 (-0.01, 2.10)	0.05	0.47 (-0.53, 1.48)	0.35	
	Model 2					0.42
	<1 yogurt/day vs non consumers	1.20 (0.32, 2.09)	0.008	0.57 (-0.29, 1.43)	0.20	
	≥1 yogurt/day vs non consumers	1.35 (0.27, 2.43)	0.01	0.39 (-0.62, 1.41)	0.45	
Waist circumference (cm)	**Model 1**					0.05
	<1 yogurt/day vs non consumers	0.70 (-0.14, 1.54)	0.10	0.36 (-0.49, 1.22)	0.40	
	≥1 yogurt/day vs non consumers	0.25 (-0.76, 1.26)	0.63	-0.42 (-1.42, 0.58)	0.41	
	Model 2					0.37
	<1 yogurt/day vs non consumers	0.70 (-0.15, 1.55)	0.11	0.35 (-0.51, 1.21)	0.42	
	≥1 yogurt/day vs non consumers	0.42 (-0.61, 1.46)	0.42	-0.54 (-1.55, 0.47)	0.29	
SBP[c] (mmHg)	**Model 1**					0.96
	<1 yogurt/day vs non consumers	0.26 (-1.50, 2.02)	0.77	-0.88 (-2.63, 0.87)	0.32	
	≥1 yogurt/day vs non consumers	-0.05 (-2.18, 2.08)	0.96	-0.72 (-2.77, 1.34)	0.49	
	Model 2					0.69
	<1 yogurt/day vs non consumers	-0.26 (-2.03, 1.50)	0.77	-1.14 (-2.86, 0.59)	0.20	
	≥1 yogurt/day vs non consumers	-0.23 (-2.38, 1.93)	0.84	-1.09 (-3.13, 0.95)	0.30	
DBP[d] (mmHg)	**Model 1**					0.08
	<1 yogurt/day vs non consumers	0.09 (-0.85, 1.04)	0.85	-0.15 (-1.09, 0.79)	0.76	
	≥1 yogurt/day vs non consumers	-0.02 (-1.16, 1.12)	0.97	-0.34 (-1.45, 0.76)	0.54	
	Model 2					0.08

	<1 yogurt/day vs non consumers	-0.08 (-1.03, 0.86)	0.86	-0.12 (-1.05, 0.82)	0.80	
	≥1 yogurt/day vs non consumers	0.16 (-0.99, 1.32)	0.78	-0.12 (-1.23, 0.99)	0.83	

[a] Model 1: adjusted for recruitment center, sex and age; Model 2: additionally adjusted for smoking, soft drinks, carbohydrates, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, family history of heart disease, diabetes and hypertension.

[b] BMI: Body Mass Index.

[c] SBP: Systolic Blood Pressure.

[d] DBP: Diastolic Blood Pressure.

1.4 Discussion

The present cross-sectional study, evaluated the ameliorative effects of lignans, yogurts and the joint consumption of lignans and probiotics on CVR-P in humans. Previous studies on yogurt and lignan consumption have shown beneficial effects on human health, but as shown in the present study joint consumption of these foods had a stronger impact on CVR-P and was associated with lower cholesterol and LDL-c levels and a trend to lower TGs levels. To our knowledge, this is the first study to suggest that polyphenol and yogurt intake can improve CVR-P and particularly the lipid profile.

Some polyphenols can be metabolized and absorbed through the gut barrier, but they usually reach the colon where they are metabolized by the microbiota and absorbed[17]. Lignans are metabolized by the intestinal microbiota to ED and EL [108,126].There is some evidence indicating that lignan-rich foods are protective against cardiovascular disease and some cancers, including breast, colon, and prostate cancer[3,127,128]. In this study, a higher lignan intake was associated with a decrease in glucose levels. In addition, stratified analyses related to sex showed lower glucose levels in both men and women (data not shown). Pinoresinol was the lignan most frequently ingested, mainly (96%) from olive oil. In a study of plant lignans by During et al.[129], pinoresinol showed the strongest anti-inflammatory effect in the human intestine. In a cross-sectional study including 242 males and females in northern Italy, matairesinol was associated with lower vascular inflammation and endothelial dysfunction[130]. In a prospective cohort study including 570 men[131], the evaluation of 4 lignans (lariciresinol,pinoresinol, secoisolariciresinol, and matairesinol) showed that the intake of matairesinol was inversely associated with mortality due to a reduction in cardiovascular disease and cancer. In this population, matairesinol was the lignan least

consumed, and the main food sources of lignans were olive oil, wheat, tomato, red wine, asparagus and kiwis (**Table 1.2**).

The gut microbiota can also be influenced by the diet, which has a direct impact on the gut environment, including transit time and pH [132]. The prebiotic effect of polyphenols has been studied previously [109], and it has been suggested that polyphenols may affect the relative viability of beneficial bacterial groups such as *Firmicute*s and *Bacteroides*[133–135]. The polyphenol-microbiota interaction is evident[136,137], but more holistic approaches involving the use of high-throughput "omics" tools are needed to shed light on the physiological relevance of this interaction in humans.

As a probiotic yogurt has benefits for consumer health. Its functional properties have been confirmed by studies on the metabolic activity of yogurt bacteria in the human intestine[75,138–140]. As a functional food, yogurt has been associated with benefits for cardiovascular and gastrointestinal health, weight management, and type 2 diabetes, among others [141,142]. In the present study, total and low-fat yogurt intake were correlated with higher weight, but yogurt intake together with a high lignan diet did not produce any increase in weight. Obesity is a CVR-P and is related to increased levels of TGs, LDL-c, and cholesterol, and decreased HDL-c levels. In accordance with Cormier et al. [143],yogurt consumption was associated with lower levels of cholesterol and TGs. Stratified analyses on sex showed some differences between men and women, with more than one yogurt per day being associated with lower cholesterol and TG levels in men but a higher BMI and weight in women. Full-fat yogurt was correlated with higher LDL-c and lower TG levels, while low-fat yogurt was correlated with lower total cholesterol, HDL-c and LDL-c, but with no impact on TGs. It has been suggested that the potential underlying mechanisms for weight loss or the prevention of weight gain could be stimulatory effects on the growth of beneficial intestinal bacteria [144]. An

alternative mechanism of action is that yogurt consumption induces higher satiety, and therefore, a reduction in appetite [141]. This latter effect could also involve microbiota, with microbial manipulation of eating behavior via the nervous system and the gut-brain axis [145,146]. A study by H. Zapata et al. [147] concluded that manipulation of the intestinal microbiota may be beneficial for maintaining health in older adults.

High lignan and yogurt consumption was associated with lower levels of total cholesterol, LDL-c and TGs, while HDL-c values did not decrease, indicating an improved lipid profile. Yogurt consumption did not affect serum glucose levels, but these levels significantly increased when the total dairy intake was considered. On one hand, it seems that microbiota associated with yogurt intake could metabolize lignans more efficiently and, on the other hand, lignans may help to modulate gut microbiota by increasing the beneficial strains [90,135].

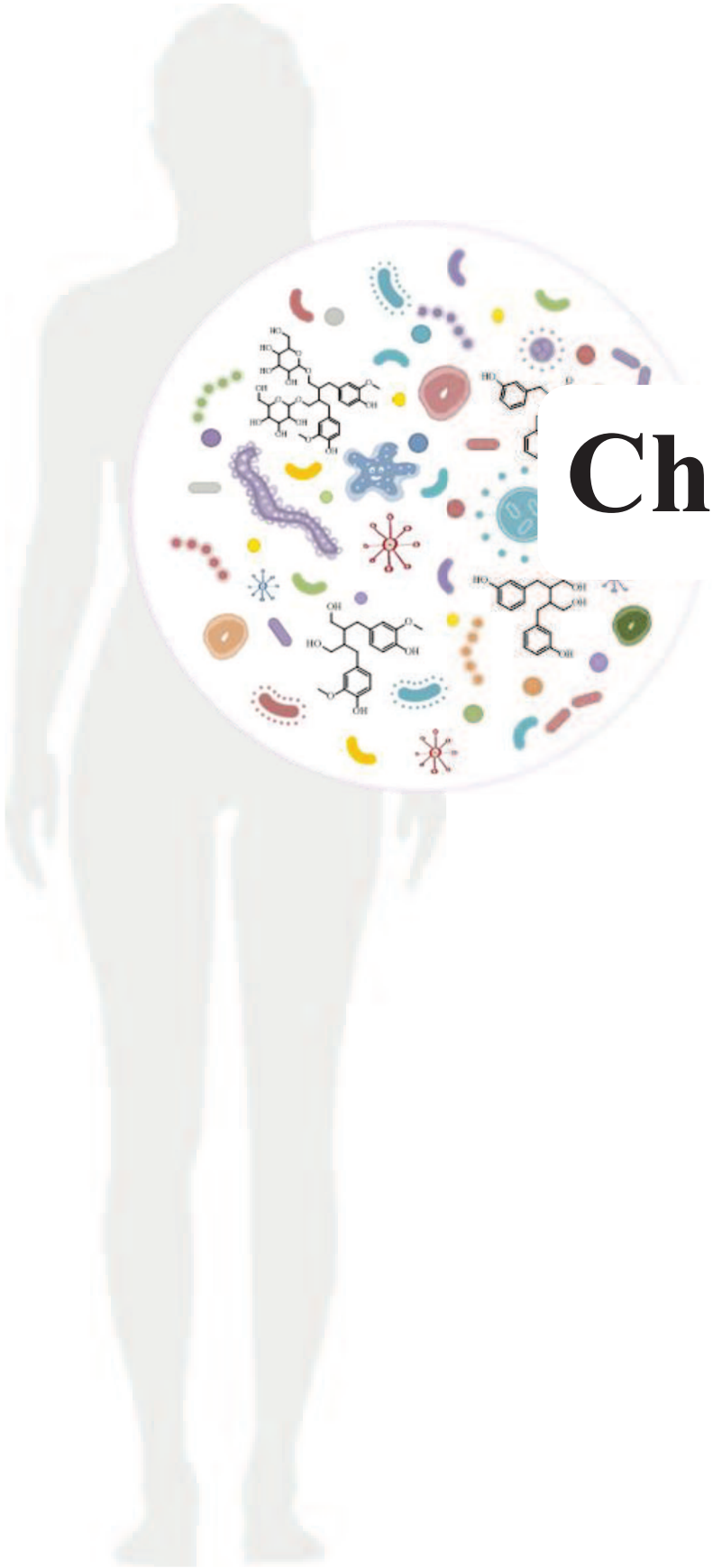
Studying the role of diet nutrients in chronic conditions such as cardiovascular diseases is complex since "we don't eat nutrients, we eat foods"[148]. Moreover, limiting analysis to individual nutrients may fail to take into account many potential interactions between dietary components and requires a large sample size and adjustment for other nutrients. Therefore, study of the synergy between foods and bioactive compounds could be a useful approach in the prevention of disease [149,150].

This study has taken a novel approach by focusing on the potential health benefits of lignans, yogurt and their joint consumption; nevertheless, some limitations should be noted. Firstly, the data obtained was from an elderly population at high cardiovascular risk, which may limit the generalization of the results. Secondly, lignan intake was calculated with FFQs and Phenol-Explorer, which is the most comprehensive polyphenol database available, although information about some foods is still limited. It should also be considered that polyphenol content in foods can differ according to the

preparation method, maturity at harvesting, environmental factors or storage conditions[108,151]. It is important to be aware the fact that some confounding variables such as lifestyle or stress, among others, could be ignored since they were not recorded in the questionnaires. Finally, since this was an observational study a cause-effect relationship cannot be established from the results, and therefore, the hypothesis should be confirmed in future clinical trials.

1.5 Summary

These findings suggest that an associative effect of lignans and yogurt may ameliorate CVR-P in humans. Therefore, daily low-fat yogurt consumption in a healthy, well-balanced diet with a high content of lignan-rich foods, such as flaxseed or extra virgin olive oil, may be recommended to enhance the beneficial effects of these two foods when ingested separately, at least in elderly populations. Further clinical trials focusing on the differences in lignan metabolites in yogurt consumers and non-consumers are needed. Moreover, the development of modifications in microbiota communities following yogurt and lignan intake should be studied, and how these modification affect human health should be evaluated.



Chapter 2

Chapter 2: Study in vitro of lignan metabolism by intestinal bacteria

2.1 Introduction

Lignans are a class of polyphenols formed by 2 phenylpropane units, also referred as plant phytoestrogens. When ingested, they can be metabolized by the gastrointestinal microbiota to their bioactive forms, the enterolignans enterodiol (ED) and enterolactone (EL)[2,3]. These enterolignans can produce estrogenic and antiestrogenic effects when bound to estrogen receptors[152]. Flaxseed (*Linum usitatissimum*) is one of the major sources of lignans in diet, and its main lignan compound is secoisolariciresinol diglucoside (SDG). The content of SDG in flaxseed ranges from 11.7 to 24.1 mg/g defatted flour and 6.1 to 13.3 mg/g in whole flaxseed flour[26]. The presence of ED and EL in the body has been correlated with the prevention of some chronic disease like cardiovascular disease, osteoporosis, and hyperlipidemia, and some cancers like colon cancer, breast cancer, prostate cancer and menopausal syndrome[2,4,5].

In the last years, there has been an increasing interest to study the gut microbiota and its impact on health, since these microbes help to maintain homeostasis and have been correlated with host health. For example, it has been shown that the ratio between Bacteroidetes and Firmicutes can be good indicator of the stability and diversity of the gut microbiome. A low ratio of Bacteroidetes:Firmicutes has been linked to obesity, the consumption of high caloric diets, and/or sedentary life[80,153,154]. Studies examining dysbiosis or microbiota imbalances have associated some illnesses like inflammatory bowel diseases, irritable bowel syndrome, or Crohn's disease, with lower bacterial diversity[155]. On the other hand some strains have been correlated with a positive impact

in the human health, known as probiotics, mainly *Lactobacillus* and *Bifidobacteria*[6,89,140].

Gut microbiota may play a crucial role in the potential health benefits of polyphenols[156], since intestinal bacteria are responsible for metabolizing lignans to enterolignans. This conversion involves the interaction of anaerobic bacteria that are related functionally, and also distantly related phylogenetically[66]. Some of the bacterial strains involved in the metabolism of SDG are from the phylum *Bacteroides, Bifidobacterium, Lactobacillus* and *Clostridium*[2,35,69,72]. However, there is still a knowledge gap about all specific microbial strains involved in the metabolism of lignans. Moreover, lignans could act as a prebiotic: the last consensus International Scientific Association for Probiotics and Prebiotics (ISAPP) concluded that plant polyphenols can also meet the criteria of prebiotics, but still more studies in the target host are required[6]. Evidence suggests that health benefits associated to polyphenol consumption depend on microbial utilization and the metabolites produced[6].

In this project, I aim to expand our knowledge of which bacterial strains are involved in the metabolism of the lignans individually. This knowledge will help us to understand why some people are and some people are not ED or EL producers, and to start exploring the possibility of new bacterial probiotic targets since it has been associated with different health benefits on the host. And secondly, I aim to study the ability of intestinal bacterial community to metabolize or co-metabolize lignans and study the impact of lignans in a complex bacterial community. Those will allow us to better assess the fate of polyphenols, the effects of polyphenol microbe interactions and improve our understanding of the impact of polyphenols on host.

2.2 Material and Methods

2.2.1 Chemicals and reagents

Standards of SDG, EL and ED were purchased from Sigma Aldrich (St Louise, MO., USA) and secoisolariciresinol (SECO) was purchased from Toronto Research Chemicals (Toronto, ON, Canada): all of these were used to identify chromatographic peaks. HPLC-grade Acetonitrile and Formic Acid were purchased from Merck KGaA Co. Ltd (Darmstadt, Germany), analytical grade of n-hexane, sulfuric acid, hydroxide sodium were purchased from Sigma Aldrich (St Louise, MO., USA). Ultrapure water (Milli-Q) was obtained from a Millipore system (Bedford, MA, USA).

2.2.2 Individually bacterial strains challenge

All bacterial strains used in this study were human isolated commensals. Strains were obtained from Dr. Emma Allen-Vercoe (University of Guelph), Dr. G. Reid (University of Western Ontario), isolated from human biopsis and fecal samples from Xoxocotla (Mexico), ordered from the DSMZ culture collection (Leibniz Institute, Germany), or ordered from ATCC (American Type Culture Collection). Commensal bacteria strains were all grown on fastidious anaerobe agar (FAA) (LabM) in anaerobic conditions. All strains are listed below in **Table 2.1**.

Each bacterial strain was inoculated separately in 5mL on fastidious anaerobe broth (FAB) (LabM) anaerobically with 200 ng/mL SDG or 200 ng/mL SECO and/or lignans extracted from the flaxseed to study the ability of each bacteria to metabolize lignans during 5 days at 37 ºC. Also two replicates of negative controls were incubated.

Table 2.1. Source and provider of the bacterial strains challenged with flaxseed extract, 200 ng/mL of its main lignan secoisolariciresinol diglucoside (SDG), and 200ng/mL of Secoisolariciresinol (SECO).

#	Name	Phylum	Family	Genus	Source	Provided by[a]
1	*Akkermansia muciniphila*	Verrucomicrobia	Akkermansiaceae	Akkermansia		ATCC [a]
2	*Akkermansia muciniphila CC51001 Hb*	Verrucomicrobia	Akkermansiaceae	Akkermansia	Biopsies and feces	E.A.V[b]
3	*Anaerotruncus colihominis*	Firmicutes	Ruminococcaceae	Anaerotruncus	Feces	DSMZ[c]
4	*Bacteroides dorei 5/1/36 (D4)*	Bacteroidetes	Bacteroidaceae	Bacteroides	Biopsies and feces	E.A.V
5	*Bacteroides dorei 9/1/42 FAA*	Bacteroidetes	Bacteroidaceae	Bacteroides	Biopsies and feces	E.A.V
6	*Bacteroides ovatus 3/8/47 FAA*	Bacteroidetes	Bacteroidaceae	Bacteroides	Biopsies and feces	E.A.V
7	*Bacteroides sp. 2/2/4*	Bacteroidetes	Bacteroidaceae	Bacteroides	Feces	E.A.V
8	*Bacteroides vulgatus 3/1/40A*	Bacteroidetes	Bacteroidaceae	Bacteroides	Biopsies and feces	E.A.V
9	*Clostridium paraputrificum*	Firmicutes	Clostridiaceae	Clostridium	Feces and biopsies	E.A.V
10	*Clostridium subterminale*	Firmicutes	Clostridiaceae	Clostridium	Feces and biopsies	E.A.V
11	*Clostridium tyrobutyricum*	Firmicutes	Clostridiaceae	Clostridium	Feces	DSMZ
12	*Clostridium peptostreptococcus*	Firmicutes	Peptostreptococcaceae	Peptostreptococcus	Feces	DSMZ
13	*Escherichia coli 3/2/53 FAA*	Proteobacteria	Enterobacteriaceae	Escherichia	Biopsies and feces	E.A.V E.A.V
14	*Escherichia coli 4/1/47 A FAA*	Proteobacteria	Enterobacteriaceae	Escherichia	Biopsies and	E.A.V

					feces	
15	*Klebsiella pneumoniae 4/1/44*	Proteobacteria	Enterobacteriaceae	Klebsiella	Biopsies and feces	E.A.V
16	*Klebsiella pneumoniae 1/1/55*	Proteobacteria	Enterobacteriaceae	Klebsiella	Feces and biopsies	E.A.V
17	*Parabacteroides distasonis 2/1/33B*	Bacteroidetes	Porphyromonadaceae	Parabacteroides	Feces and biopsies	E.A.V
18	*Parabacteroides distasonis 31/2*	Bacteroidetes	Porphyromonadaceae	Parabacteroides	Feces and biopsies	E.A.V
19	*Peptostreptococcus russellii*	Firmicutes	Peptostreptococcaceae	Peptostreptococcus		Isolated
20	*Bacteroides fragilis 3/1/12*	Bacteroidetes	Bacteroidaceae	Bacteroides	Feces and biopsies	E.A.V
21	*Faecalibacterium prausnitzii*	Firmicutes	Clostridiaceae	Faecalibacterium		ATCC
22	*Lachnospira multipara*	Firmicutes	Lachnospiraceae	Lachnospira		DSMZ
23	*Veillonella parvula*	Firmicutes	Veillonellaceae	Veillonella		ATCC
24	*Prevotella copri*	Bacteroidetes	Prevotellaceae	Prevotella	Feces and biopsies	DSMZ
25	*Ruminococcus callidus*	Firmicutes	Ruminococcaceae	Ruminococcus	Feces and biopsies	ATCC
26	*Clostridium citroniae*	Firmicutes	Clostridiaceae	Clostridium	Feces and biopsies	ATCC
27	*Bacillus licheniformis [4]*	Firmicutes	Bacillaceae	Bacillus	Feces	Isolated
28	*Bacillus licheniformis [5]*	Firmicutes	Bacillaceae	Bacillus	Feces	Isolated
27	*Clostridium bifermentans [18]*	Firmicutes	Clostridiaceae	Clostridium	Feces	Isolated
28	*Clostridium bifermentans [19]*	Firmicutes	Clostridiaceae	Clostridium	Feces	Isolated
29	*Clostridium perfringens*	Firmicutes	Clostridiaceae	Clostridium	Feces	Isolated

30	*Clostridium symbiosum*	Firmicutes	Lachnospiraceae	Lachnoclostridium	Feces	Isolated
31	*Enterococcus faecium*	Firmicutes	Enterococcaceae	Enterococcus	Feces	Isolated
32	*Sarcina ventriculi*	Firmicutes	Clostridiaceae	Clostridium	Feces	Isolated
33	*Streptococcus pasteurianus*	Firmicutes	Streptococcaceae	Streptococcus	Feces	Isolated
34	*Bifidobacterium adolescentis OB21 BHI 8*	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	Feces and biopsies	E.A.V
35	*Bifidobacterium adolescentis F16 #17*	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	Feces and biopsies	E.A.V
36	*Bifidobacterium longum OB21 D5 12*	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	Feces and biopsies	E.A.V
37	*Bifidobacterium longum OB EAV1 7 FAA*	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	Feces and biopsies	E.A.V
38	*Bifidobacterium longum AB8 #7*	Actinobacteria	Bifidobacteriaceae	Bifidobacterium	Feces and biopsies	E.A.V
39	*Lactobacillus gasseri ATCC 33323*	Firmicutes	Lactobacillaceae	Lactobacillus		G.R.
40	*Lactobacillus johnsonii DSM 20553*	Firmicutes	Lactobacillaceae	Lactobacillus		G.R.
41	*Lactobacillus rhamnosus GG*	Firmicutes	Lactobacillaceae	Lactobacillus		G.R.
42	*Lactobacillus mucosae LM1*	Firmicutes	Lactobacillaceae	Lactobacillus		G.R.
43	*Lactobacillus reuteri RC14*	Firmicutes	Lactobacillaceae	Lactobacillus		G.R.
44	*Bacteroides thetaiotaomicron*	Bacteroidetes	Bacteroidaceae	Bacteroides	Feces and biopsies	ATCC

a) ATCC : American Type Culture Collection; E.A.V: Emma Allen-Vercoe (University of Guelph); DSMZ: culture collection (Leibniz Institute, Germany); Isolated: from Xoxocotla community (Mexico); G.R.: Dr. G. Reid (University of Western Ontario).

2.2.3 Bacterial community preparation and inoculation

In an anaerobic chamber, bacterial cultures from frozen stocks were first plated on FAA, and then individually inoculated in 5 mL with FAB. After growing them for 48hr, all strains were normalized to an O.D. of 0.40 and the 16S rRNA gene was sequenced to ensure their purity. Once the purity of each strain was ensured, they were inoculated in 10mL with FAB, and they were inoculated in a defined ratio as shown in **Figure 2.1.** The composition of each phylum is shown in **Figure 2.2** in the phylogenetical tree. Four replicates of the complex microbial community were challenged with 200ng/mL of SDG and both the microbiome-modulating properties and the ability to metabolize SDG of these communities were evaluated at different time points (24h, 48h and 5 days). The shifts in the bacterial composition were evaluated using Illumina MiSeq analysis using the 16S V4 region. To study the action of the lignans on the community I also had four control replicates consisting in the community itself without SDG.

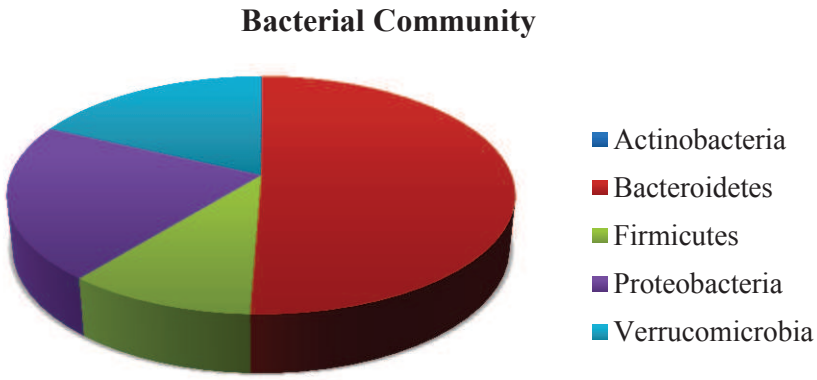


Fig 2.1. Distribution in percentage of relative abundance of the main phyla in vitro microbiota community. 50.56% of the community belonged to the phyla Bacteroidetes, 21.21% of the phyla corresponded to Proteobacteria, 17.94% corresponded to Verrucomicrobia, 10.25% corresponded to Firmicutes and 0.04% was Actinobacteria.

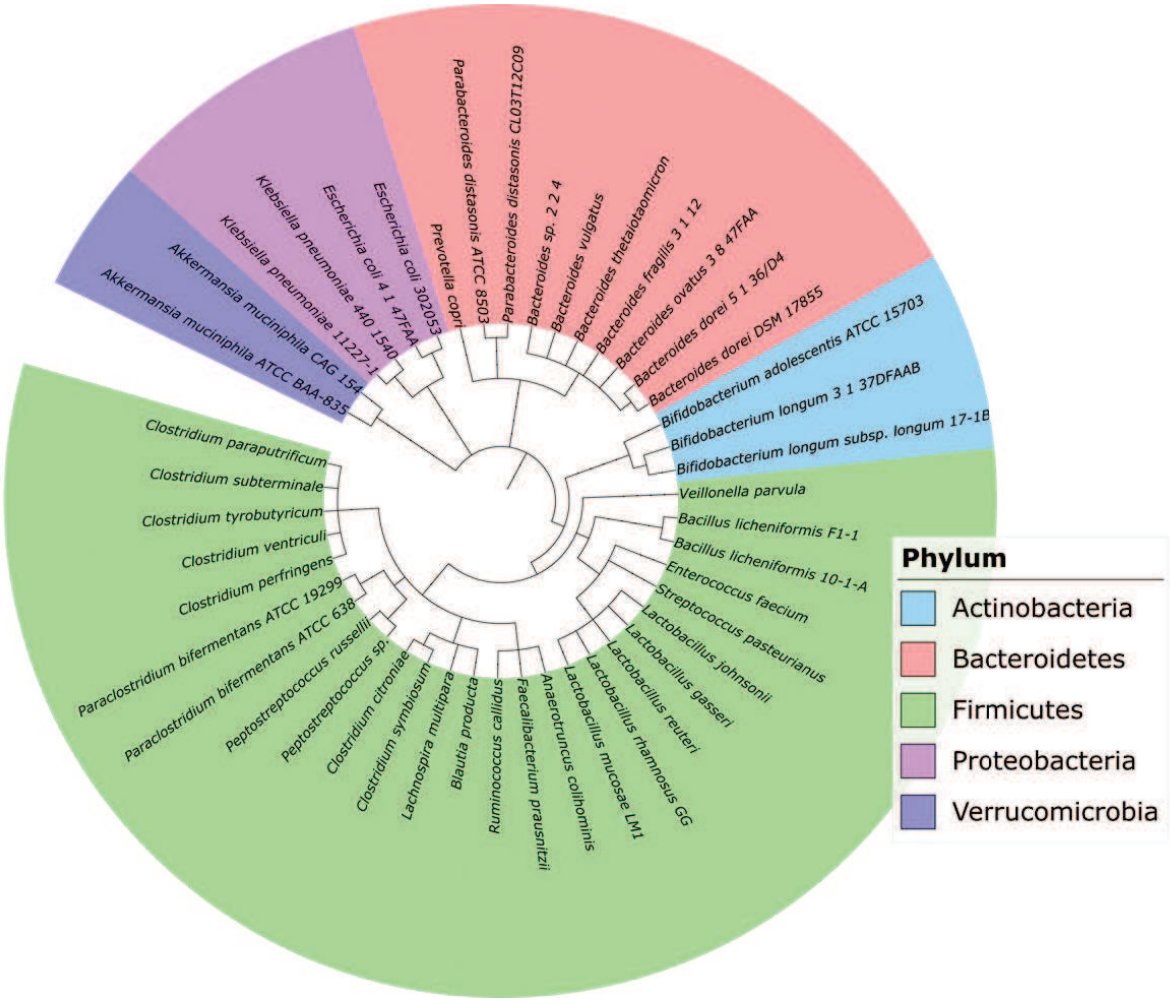


Fig 2.2. Phylogenetic analysis of the bacterial community. Interactive Tree Of Life (iTOL) tool has been used to generate the phylogenetic tree[157].

2.2.4 Extraction of lignans from flaxseed

For the extraction of flaxseed lignans the method described for C. Eliasson et al.[158] was followed with minor modifications. The first part consisted of defatting the flaxseed: 1 g of flaxseed was milled and defatted with n-hexane (5 mL) in continuous shaking for 1 hour. Then, the defatted flaxseed flour was filtered and washed with 5mL of n-hexane. Defatted flaxseed was dried at 105ºC till constant weight. The second part consisted of alkaline hydrolysis: briefly, 4 mL of water were added to 100mg of defatted flaxseed flour; once hydrated, 5 mL of 2M NaOH were added to the mixture, in continuous shaking for 1 h at 20 ºC. Then, pH was adjusted at 3 with sulfuric acid 2M and centrifuged (1700 g, 10 min). Supernatant was taken and a second centrifuge (11000 g, 5 min) was carried out. 0.8mL of the supernatant were mixed with 1.2 mL 95% EtOH to remove free polysaccharides for 10 min and centrifuge (11000 g, 5min). Samples were filtered through 0.45 um syringe filter. The extraction was done by triplicate and it was injected in the MS.

2.2.5 Lignan and enterolignan extraction from the cultures

Solid Phase Extraction StageTips were done and used as described before by Rappsilber et al[159] with an optimized extraction for lignans. StageTips were conditioned by 100 µL of methanol and equilibrated using 100 µL of water (0.1% formic acid). Then 100 uL of sample was loaded in the StageTips and it was forced to pass through the C18 column. The tips were then washed using 100 µL of 98% water with 1% formic acid and 2% methanol. After that, analytes were eluted with 100 µL of methanol. Samples were SpeedVac till dryness and reconstituted with 20 µL of water (0.05 % formic acid). Samples were stored at -20ºC till its analysis in the MS.

2.2.6 High performance nano-liquid chromatography multiple reaction monitoring tandem mass spectrometry method for the determination of lignans

LC−MRM−MS/MS analysis. An Agilent triple quadruple 6460 (Agilent) with the ChipCube nanospray ion source coupled with an Agilent 1200 nanoflow HPLC was used for MRM analysis in positive mode. For optimization and MRM sample analysis, 43 mm 75 μm i.d. C18 chip was employed. Solvent A (10% acetonitrile + 0.1% formic acid) and solvent B (90% acetonitrile + 0.1% formic acid) were used at a flow-rate of 0.4 μl/min and the injection volume was 4μl. A non-linear gradient was applied: 0 min, 90% A; 0-3 min, 90% A; 3-3.50 min, 40% A; 3.5-6 min, 60% A; 6-6.50 min, 60% A; 6.50-8 min, 100% A; 8-9.50 min, 100% A and then returned to initial conditions over 1 min and re-equilibrated for 5 min. The resolution of both MS1 and MS2 was set to unit, and for each compound the two highest transition peaks were analyzed, the highest peak was used to quantify, and the second transition was used to confirm the analyte of interest together with the comparison with the standard. Optimized MRM parameters for each compound are shown in **Table 2.2**. Data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00).

Table 2.2. Multiple reaction monitoring (MRM) parameters optimized for the identification and quantification of the flaxseed lignans secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO) and the enterolignans enterodiol (ED) and enterolactone (EL) in the LC-MS.

Compound	MF	MW	$[MW+H]^+$	Rt (min)	Fragmentor (V)	CE (V)	Quantification transition	Confirmation transition
ED	$C_{18}H_{22}O_4$	302.37	303.16	8.2	135	5	303/267	303/107
EL	$C_{18}H_{18}O_4$	298.34	299.13	9.0	170	15	299/133	299/263
SDG	$C_{32}H_{46}O_{16}$	686.71	687.29	6.8	150	7	687/345	687/327
SECO	$C_{20}H_{26}O_6$	362.17	363.18	7.8	100	7	363/163	363/137

MF: Molecular formula; MW: Molecular weight; Rt: Retention time: CE: Collision Energy.

2.2.7 16s RNA extraction from the cultures

In order to evaluate the changes in microbial community composition, total DNA was extracted by the standard method phenol–chloroform extraction [160,161]. The 16S rRNA gene V4 region was amplified and sequenced at the Integrated Microbiome Resource lab (IMR) at Dalhousie University (Halifax, Canada) using an Illumina MiSeq platform and following the procedure previously described[162]. Raw sequencing was processed using a standardized qiime1 pipeline (Microbiome Helper, Morgan Langille[163]) to obtain the OTU table and taxonomy. Measures of principle component analysis (PCA) plots, were also generated in qiime2 using this pipeline.

2.2.8 Statistical analysis

Statistical significance was calculated by using a two-tailed Student's t-test unless otherwise stated, with assistance from GraphPad Prism Software Version 4.00 (GraphPad Software, San Diego California USA, www.graphpad.com). If not otherwise specified statistical significance was given as *** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05; ns (not significant) p-value> 0.05. The results are expressed as the mean value with standard error of the mean (SD), unless otherwise indicated.

2.3 Results

2.3.1 Concentration of lignans in the flaxseed extract

The SDG concentration in the flaxseed was calculated. Thus, a Mass Spectrometry method was developed as mentioned before. **Figure 2.3** shows the chromatogram of the analytes SDG, SECO, ED and EL and **Table 2.3** shows the Limits of Quantification (LOQ) and Limits of Detection (LOD) of each standard.

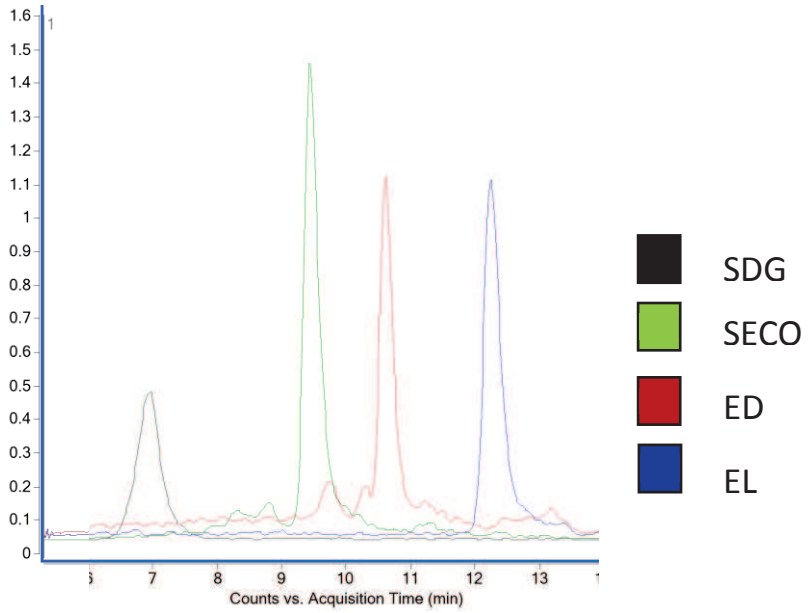


Figure 2.3. LC-MS/MS Multiple Reaction Monitoring (MRM) chromatogram of secoisolariciresinol diglucoside (SDG) (m/z 687.0 > 261.1); secoisolariciresinol (SECO) (m/z 321.1 > 271.1), enterodiol (ED) (m/z 407.0 > 261.1) and enterolactone (EL) (m/z 407.0 > 261.1).

Table 2.3. Limits of Detection (LOD) and Limits of Quantification (LOQ) of the method used to quantify lignans secoisolariciresinol dyglucoside (SDG), secoisolariciresinol (SECO) and enterolignans enterodiol (ED) and enterolactone (EL).

Compound	LOD[a] (ng/mL)	LOQ[b] (ng/mL)
ED	0.43	1
EL	0.15	1
SDG	0.56	5
SECO	0.50	2

a)LOD: Limit of Detection; b) LOQ) Limit of Quantification.

The average concentration of SDG and the standard deviation obtained from the whole flaxseed was 9.06 mg/g ± 0.6. The reaction yield of the defatting process was of 48 %.

2.3.2 Bacteria involved in the conversion of lignans to enterolignans

The schema reaction of the metabolism of lignans by microbiota is represented in **Figure 2.4**. Each strain was inoculated with flaxseed lignan extract, SDG and SECO individually to study the ability of each species to metabolize lignans. This experiment revealed that fifteen of the forty-four different bacteria tested were capable of metabolizing Secoisolariciresinol diglucoside (SDG), the main lignan compound in flaxseed. From Bacteroidetes phylum, the species responsible to break the two sugar moieties to produce SECO were *Bacteroides ovatus 3_8_47FAA*, *Bacteroides dorei 5_1_36 (D4)*, *Bacteroides dorei 9_1_42 FAA*, and *Bacteroides thetaiotaomicron*. Furthermore, four species of Bacteroidetes (*Bacteroides fragilis 3_1_2*,

Parabacteroides distasonis 2_1_33B, *Parabacteroides distasonis 31_2* and *Bacteroides 2_2_4*) were able to break the sugar moieties but also to do the demethylation and dehydroxylation, so after inoculating with SECO, ED and EL were detected. From the Firmicutes phyla *Enterococcus faecium*, *Sarcina ventriculi*, *Bacillus licheniformis*, *Faecalibacterium prausnitzii* and *Peptostreptoccus russellii* were able to metabolize SDG to SECO. And *P.russellii* and *F. prausnitzii* were also able to continue the metabolism to enterodiol and enterolactone. There were six Firmicutes species that were able to metabolize SECO to EL or ED, but they couldn't metabolize SDG: *Lactobacillus johnsonii*, *Lactobacillus reuteri*, *Lactobacillus gasseri*, *Lactobacillus rhamnosus*, *Lachnospira multipara*, and *Veillonella parvula*. From Actinobacteria phyla *Bifidobacterium adolescentis OB_ 21_BHI8* and *Bifidobacterium longum OB21_D5_12* metabolized SDG and SECO to enterodiol and enterolactone. Finally, from Verrucomicrobia phyla, *Akkermansia muciniphila* was able to desmethylate and dehydroxylate SECO to ED and EL, but was not able to metabolize SDG.

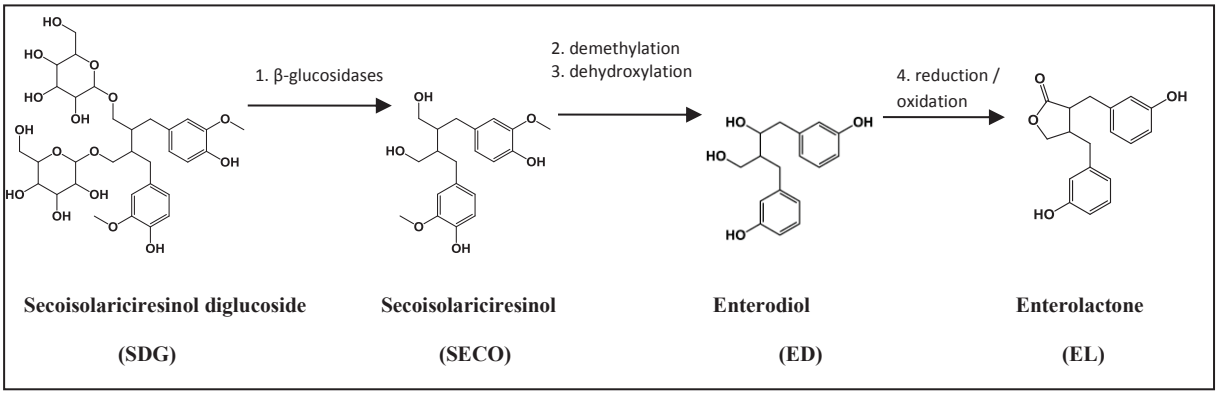


Fig 2.4. Degradation pathway of lignans by microbiota. Microbiota hydrolyze the sugar moiety of diglycoside lignans by the action of glucosidases enzymes. The deglycosilation is followed by demethylation and dehydroxylation to form enterodiol (ED). Dehydroxylation is followed by dehydrogenation from ED to produce enterolactone (EL)[24].

2.3.3 Conversion of lignan to enterolignan by an artificial community

To study the metabolism of lignans by an entire microbial community I measured the concentration of the lignans SDG, SECO and their metabolites ED and EL (**Table 2.4**) in the mixed community shown in Figures 3.1 and 3.2. After 48 hours of incubation, the community had metabolized almost all the SDG into SECO, ED and EL. The highest concentration of SECO was achieved at 24 hours of our experiment (37.51±9.43). ED concentration was maximal at 48 hours with a concentration of 53.10±6.18 and EL was at maximum concentration at 5 days after the experiment started, with a concentration of 37.21±11.26. After 5 days I couldn't find any SDG left and SECO was under LOQ.

Table 2.4. Concentration of lignan secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO) and the enterolignans enterodiol (ED) and enterolactone (EL) at 24h, 48h and 5 days in the bacterial community expressed in ng mL^{-1}.

	24H		**48H**		**5 DAYS**	
	mean	**SD[1]**	**mean**	**SD[1]**	**mean**	**SD[1]**
SDG	23.92	10.50	<LOQ[2]		<LOD[3]	
SECO	37.51	9.43	21.60	8.55	<LOQ[2]	
ED	11.17	10.56	53.10	6.18	50.41	8.41
EL	10.75	2.33	26.19	18.22	37.21	11.26

1-SD: Standard Desviation; 2- LOQ: Limit of Quantification; 3- LOD: Limit of Detection

2.3.4 Main changes in the microbiota profile

To understand the effect of the lignan SDG on the bacterial community, I looked for changes in the community at 24h, 48h and 5 days. Basically, PCoA plots and bar plots together with its statistics comparing both communities were used to achieve this aim.

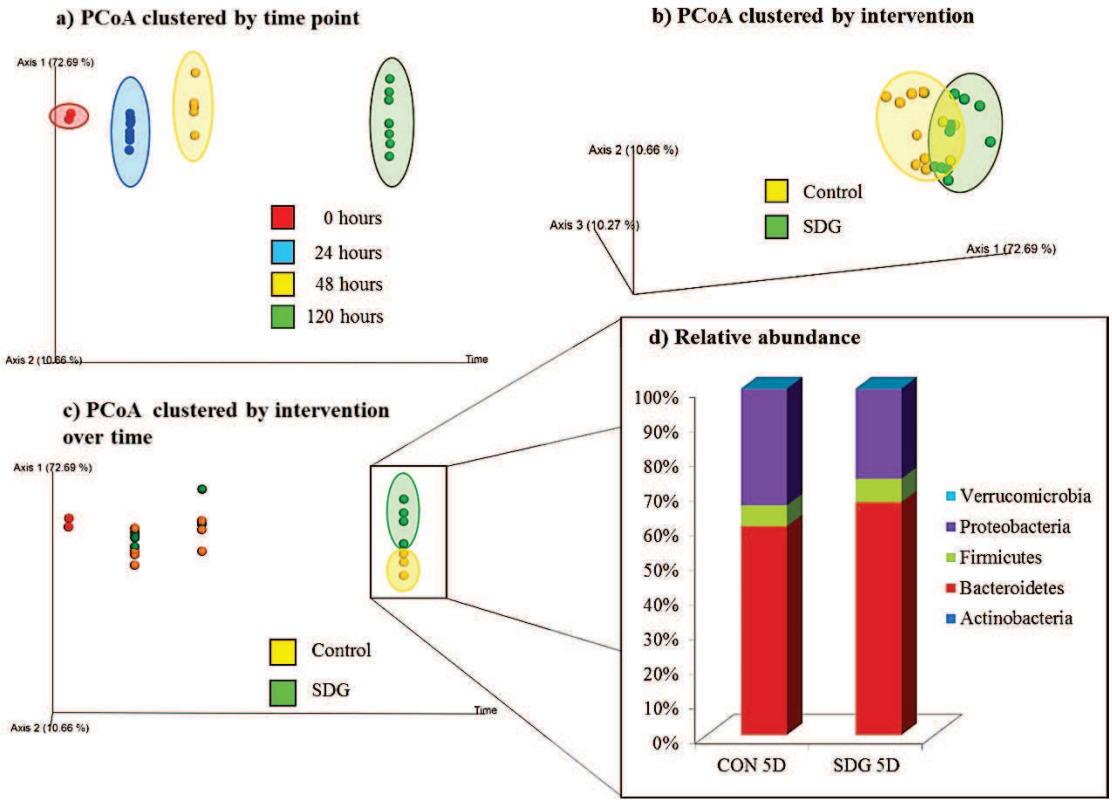


Figure 2.5. PCoA cluster plots of the microbial community at different timepoints. **a)** The scores plot obtained from PCA of microbiome at the different time points of the experiment; **b)** PCoA obtained in the overall experiment clustered by the presence or absence of secoisolariciresinol diglucoside (SDG); **c)** PCoA overtime clustered by intervention. It can be clearly differenciate at 120 hours of inoculation between Control and lignan SDG; **d)** Bacterial distribution at phyla level at five days after inoculation.

PCoA score plots were used to compare the microbial profiles, as it is shown in **Figure 2.5.** Changes in the community over time were considerable, and they clustered 100% depending on the time point we were looking. **Figure 2.5.b)** showed that when the time was not taken as an axis the bacterial community clustered pretty well in the absence or presence of SDG. Principal component 1 (PC1) captured 72.69% of the total variance. Principal component 2 (PC 2) captured 10.66% of the total variance. Principal component 3 (PC 3) captured 10.27% of the total variance. In **Figure 2.5 c)** the PCoA shows both time and intervention (Control or SDG). At early time points (the first cluster on the left, at 24 hours) we cannot differentiate between absence or presence of SDG; at 48 h we start to differentiate the two clusters; and after 5 days of inoculation we can differentiate between the two clusters. The results suggest that the artificial bacterial community shifts with the presence or absence of lignans after 5 days of inoculation.

The main differences in the phylum level after 5 days of inoculation were that the presence of SDG led to a higher relative abundance of Bacteroidetes ($p=0.03$), and lower proportion of Proteobacteria ($p= 0.03$). No significant differences were found between Actinobacteria, Firmicutes and/or Verrucomicrobia.

Figure 2.6 shows the community composition at the species level after 24 hours, 48 hours and 120 hours of incubation in anaerobic conditions with or without the lignan SDG. There was an increase of the *Escherichia coli* over time as well as the *Bacteroides sp*. and *Bacteroides ovatus*. On the other hand *Lachnospiraceae, Clostridium bifermentans* and Akkermansia muciniphila decreased over time.

The comparison in detail of the main differences between the presence or absence of SDG in the community are shown in **Figure 2.7**. After five days of incubation the presence of SDG allowed higher proportion of *Bacteroides* ($p=0.03$),

corresponding in an increase of *B. fragilis, P. distasonis, Bacteroides sp.* and *B. vulgatus* in the community. Conversely, *E. coli* was less abundant in the presence of SDG ($p=0.008$). However, *A. muciniphila* showed no differences between treatments.

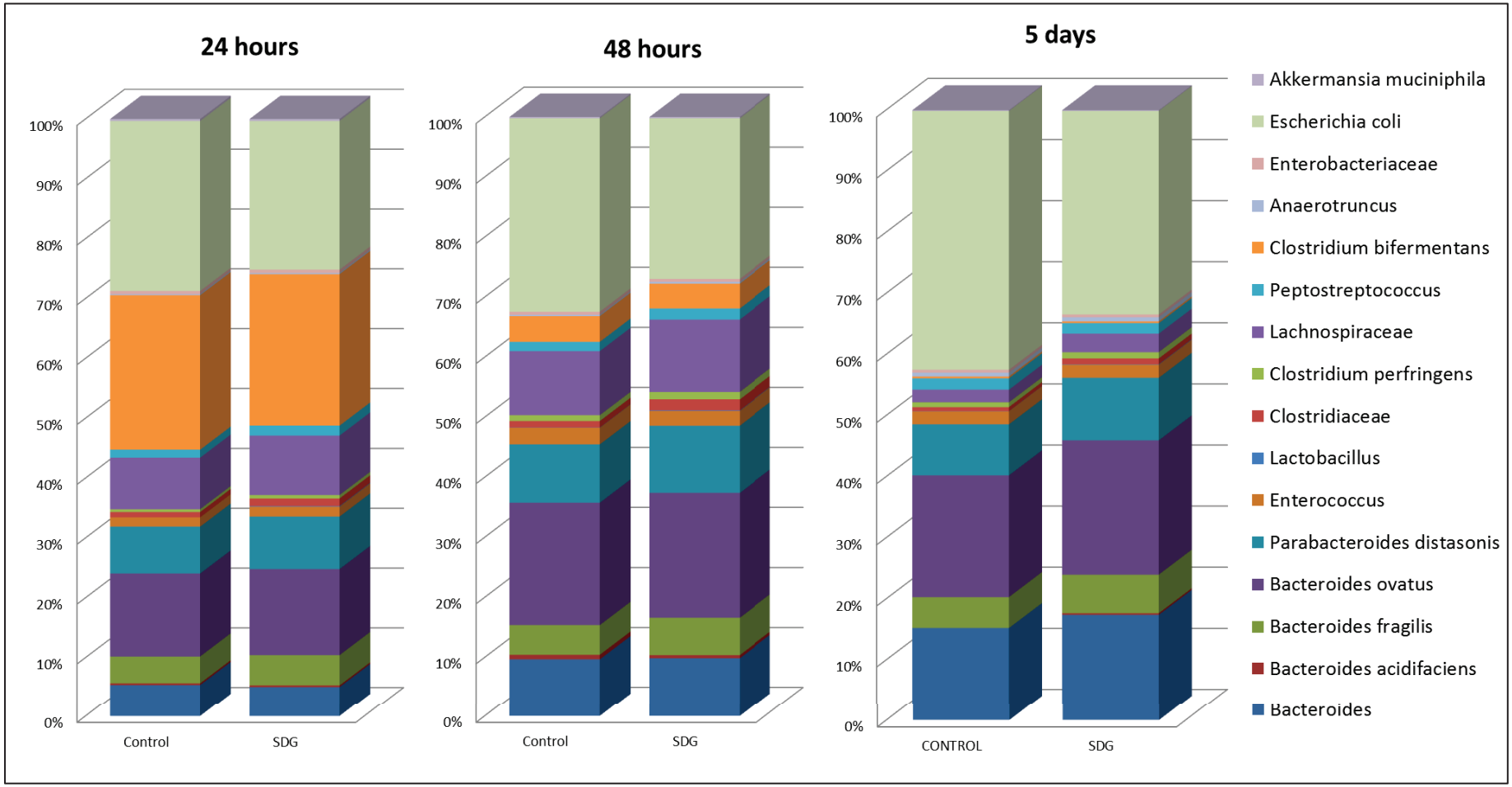


Figure 2.6 Relative abundance shifts in the microbial community between treatments and over time.

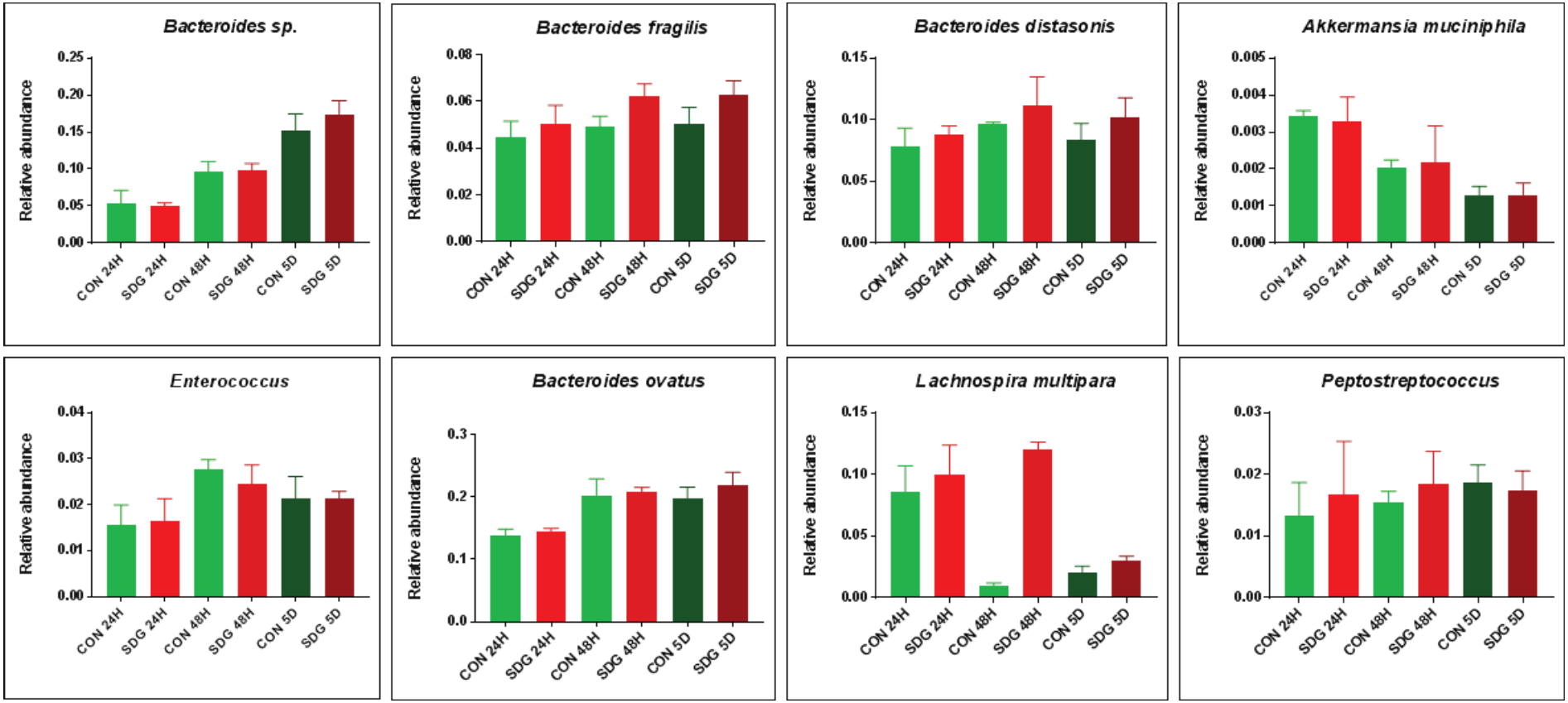


Figure 2.7 Relative abundance of microbial bacteria in the intervention groups overtime. *Bacteroides sp., B. fragilis, B. ovatus, P. distasonis* and *Lachnospira multipara* increased with the presence of the lignan SDG. *Escherichia coli* abundance decreased with the presence of SDG. *A. muciniphila* and *Enterococcus* had no differences between presence/absence of SDG after five days.

2.4 Discussion

The role of gut microbiota in health and disease is of increasing interest to scientists. The microbiota seems to be involved in, and have a deep impact on, human health[76,81,164]. There is still much to explore, but the principal efforts need to be addressed in the direction to answer how it can be shaped, what are the main probiotics for each health issue and what we can consider a prebiotic for those probiotics. Flaxseed lignans could be considered as a potential prebiotic[6], since they need to be metabolized by gut microbiota, have a positive impact on the "good bacteria" and their metabolites are linked to health[128,165].

In this study I have expanded the knowledge about the strains capable of metabolizing lignans, specifically SDG. Ten species has been described for the first time as responsible for its metabolism. The simulation of a bacterial community allowed us to study how SDG is converted to SECO, ED and EL, but also to study the impact of SDG on the bacterial community. *Escherichia coli* was significantly less abundant with the presence of SDG, and *Bacteroides* were significantly more abundant. Reasonably it correlates with the fact that almost all *Bacteroides* are able to metabolize flaxseed lignans, while *E. coli* is not able to metabolize it.

SDG was extracted from flaxseed and also was acquired commercially to study both scenarios. As the SDG from flaxseed could have others lignans naturally present in flaxseed SDG commercially results are the once described in this chapter. For the metabolism of SDG just when both gave positive results was taken as a positive result. The contents of SDG in the flaxseed that I obtained agreed with other published before

and hence the optimization of the extraction SDG from flaxseed procedure was successful[25,29].

In this work 10 new species were found to be able to metabolize SDG and/or SECO that had never been associated with this ability before up to our knowledge. From Bacteroidetes phyla *B. thetaiotaomicron* was the first time found to be responsible to metabolize SDG into SECO. *Bacteroides fragilis, B. ovatus, B. dorei* and P. *distasonis* have been described before with this ability[24,66–68]. Four species from Firmicutes phyla were found to be responsible for the metabolism of SDG into SECO for the first time, Fa*ecalibacterium prausnitzii, Bacillus licheniformis, Peptostreptococcus russellii* and *Sarcina ventriculi*. *F. prausnitzii* has gained interest as a potential probiotic strain, since it has been negatively correlated in diseases like Parkinson's disease, Crohn's disease, ulcerative colitis and obesity[166,167]. Recently, has been linked to healthy human microbiota, and changes in its abundance have been linked to dysbiosis[167]. Some *Lactobacillus* have been described before as responsible for the metabolism of SECO into EL or ED, but for the first time I found *L. johnsonii* and *gasseri* to be also part of this group. Surprisingly *Lachnospira multipara* and *Veillonella* were also able to demethylate and dehydroxylate SECO into ED. Actinobacteria, such as *B. adolescentis* and *B. longum*, have been previously studied by Gaya et al.[35], and according to them exhibited the ability to produce SECO, in this work same results were found. *Akkermansia muciniphila* was able convert SECO into EL; this is very interesting since *Akkermansia* has been inversely associated with cardiovascular diseases, diabetes, obesity and low-grade inflammation[168]. Evidence suggests that some strains of *A. muciniphila* and *F. prausnitzii* are good candidates as next-generation probiotics [168,169].

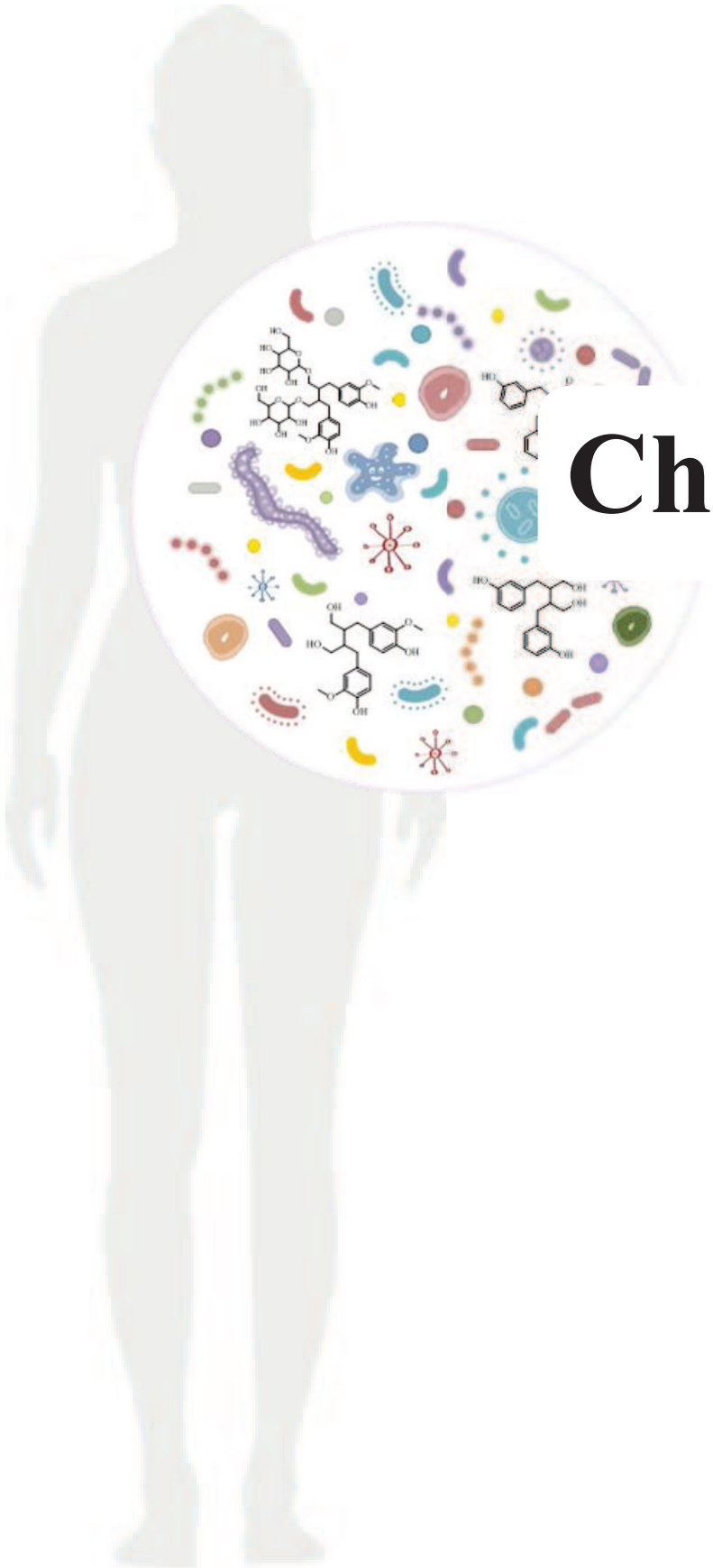
MRM-MS in positive mode was used to identify and quantify the metabolites from the biological samples. Every parameter was optimized individually for each compound. Two different transitions were used to quantify and confirm each analyte comparing with the standard, and they agreed with the ones predicted by the Human Metabolome Database[170]. This allowed us to have the same MS method for different matrices, for the flaxseed extract and also the lignans extracted from the broth. The artificial community that I created metabolized almost all the SDG in 48 hours. And in the short period of 24 hours the community had already produced the enterolignans EL and ED. The inoculation was done in a still growing phase for the culture, so I could observe the shifts in the community as it was growing. Bacteroidetes increased significantly their abundance with the presence of SDG. These findings correlate with other studies of *in vitro* incubations with tea and wine polyphenols[171,172]. A higher ratio Bacteroidetes:Firmicutes has been linked to a healthy diet and lifestyle and consequently a better health on the host. Another interesting difference between the communities was that the presence of SDG had a negative impact in *Escherichia coli* relative abundance. That could be as a result of a worse growth of itself in comparison of the whole community. *E. coli* is a Gram-negative, facultatively anaerobic and nonsporulating bacterium. Most *E. coli* strains do not cause disease, but virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis, and Crohn's disease[173–175]. In the past some studies have shown that the presence of polyphenols caused an increase of the beneficial *Akkermansia muciniphila*[165], although I didn't observe any difference in this experiment.

In vitro experiments have some limitations, as the number of bacteria that can be grown is limited when trying to recreate a human gut. But it has to be seen as a first step to understand the networks that bacteria create and how they shift, first individually, and

then inside in a community. As it has been shown in this work, lignans and flaxseed could act as a prebiotic: it can be metabolized by some beneficial bacteria such as Bacteroidetes, and increase their relative abundance in a community. However, more studies needs to be done to fully understand its role in health and disease. The full description and characterization of each strain involved, and the translation to animal and humans in health and disease, needs to be assessed.

2.5 Summary

In this work, 10 new strains have been described for the first time as responsible for the metabolism of SDG, like *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* which are already potential probiotics. Also the study of an artificial community with SDG increased Bacteroides and decreased *Escherichia coli.* Further investigation of the impact of flaxseed lignans on gut microbiota and health is needed, as they could potentially be considered as prebiotics.



Chapter 3

Chapter 3: Study of how lignans impact on gut microbiota in health and malnutrition.

3.1 Introduction

Worldwide human health is compromised by the double-burden of obesity and malnutrition[103,104]. Interestingly, undernutrition and obesity can exist concurrently[105]. Obesity is a modifiable risk factor for cardiovascular disease (CVD) and the World Health Organization (WHO) [1] says that more people die annually from CVDs than from any other cause. Diet and active lifestyle are the main strategies to treat obesity and reduce cardiovascular incidence in people. Interestingly, malnutrition and obesity share some characteristics like systemic inflammation, fatty liver, high blood pressure and microbial dysbiosis[176106]. In addition, undernourished mothers have higher rates of morbidity and mortality, but also to give birth to low-weight children, and those children have an increased risk of developing cardiovascular disease as adults[177]. It has been hypothesized that the gut microbiota plays an important role in metabolic health and may contribute to both undernutrition and obesity[106]. Thus, undernutrition in childhood could affect the development of metabolic capacities of the gut microbiome in ways that result in inadequate function or contribute to the sequelae of malnutrition, like increased risk of cardiovascular diseases later in life[177]. Thus, it is important to study how to get a healthy gut microbiome and if it can be a shared goal in both malnutrition and obesity.

Flaxseed is emerging as a functional food ingredient because of its potential health benefits such as in reduction of cardiovascular disease, atherosclerosis, diabetes, cancer and neurological disorders[62,178]. It is the richest source of lignans; specifically its

main lignan compound is secoisolariciresinol diglucoside (SDG)[26,27]. Evidence suggests that SDG is capable to mediate the serum total cholesterol, low density lipoprotein, total cholesterol and high density lipoprotein ratio which lead to less androgenic complication and antioxidative prevention[50]. Furthermore, flaxseed contains other beneficial nutrients like α-linolenic acid (ALA, omega-3 fatty acid) and fiber. Since lignans need the gut microbiota to be metabolized into their bioactive form enterodiol and enterolactone, and since polyphenols can shape the gut microbiota, it has been suggested that polyphenols could act as prebiotics[90]. The impact of dietary flaxseed on the intestinal microbiota and host health during early-life undernutrition has never been explored.

We aim to investigate the early-life impact of lignans on microbial metabolism during healthy development and malnutrition in a mouse model. This knowledge will allow us to understand how dietary components can be used to affect specific organisms in the microbiota, and to explore the role of microbiota in health and disease. Moreover, it may provide clues for the creation of new generations of probiotics and prebiotics.

3.2 Materials and Methods

3.2.1 Animal studies

All animal work was done according to the Canadian Council on Animal Care guidelines, utilizing protocols that were approved for use by the Animal Care Committee at the University of British Columbia (Certificate A14-0164). Three-week-old, female C57BL/6 mice were ordered for the experiment (Jackson Laboratory, Bar Harbor, ME) and housed in a barrier animal facility at the University of British Columbia (UBC) with a 12 hour light-dark cycle. Upon arrival, mice were randomized and housed into separate groups (2 cages per group, 4 mice per cage) which were either fed a malnourished diet (MD) moderately low in protein (7%) and fat (5%) or an isocaloric control diet (CD) with 20% protein and 15% fat, similar to one used in previous studies to induce protein malnutrition[179]. Another subset of mice were fed with the same malnourished diet containing 8% flaxseed (MDFS) or with the control diet also containing 8% flaxseed (CDFS) (Research Diets, New Brunswick, NJ, **Table 3.1**). The chow was irradiated before use and mice were given the diet *ad libitum* throughout experiments.

During 3 weeks mice were assessed by measuring the food intake and the weight gain. In addition, after three weeks of diet tail-length (a proxy for linear growth) was measured, gastrointestinal (GI) physiology was assessed histologically and inflammation was assessed via cytokine profiling.

Table 3.1. Nutritional composition of mice diets

Ingredient	Control diet (CD)	Control diet +8%flaxseed (CDFS)	Malnourished diet (MD)	Malnourished diet +8%flaxseed (MDFS)
g				
CASEIN	200	176.7	71	47.7
L-Cysteine	3	3	1.07	1.07
corn starch	346	341	557	552
maltodextrin 10	45	45	70	70
dextrose	250	250	250	25
sucrose	0	0	2.41	2.41
cellulose, bw200 (insoluble fiber)	75	54.8	75	54.8
inulin (soluble fiber)	25	18.3	25	18.3
Soybean	70	46	23.3	0
mineral mix s10026	10	10	0	0
mineral mix s10026A	0	0	5	5
dicalcium phosphate	13	13	11	11
calcium carbonate	5.5	5.5	0	0
potassium citrate	16.5	16.5	8.2	8.2
sodium chloride	0	0	1.9	1.9
vitamin mix	10	10	10	10
choline bitartrate	2	2	2	2
flaxseed	0	87.6	0	87.6
Total (g)	**1071.05**	**1080.15**	**1112.93**	**1122.03**
g				
protein	177	177	62.8	62.8
carbohydrate	647.3	647.3	883.3	883.3
fat	72.2	72.2	24.1	24.1
Fiber	100	100	100.0	100.1
Insoluble	75	75	75	75
Soluble	25	25	25	25
kcal				
protein	708	708	251	251
carbohydrate	2589	2589	3533	3533
fat	650	650	217	217
total	3947	3947	4001	4001
kcal %				
kprotein	18	18	6	6
carbohydrate	66	66	88	88
fat	16	16	5	5
kcal/g	**3.68**	**3.65**	**3.60**	**3.60**

3.2.2 Histology: Goblet cell and mucus layer preservation ex vivo

For visualizing the mucus layer and goblet cells, 1 cm sections of the colon were excised from mice, immediately submerged into methanol-Carnoy's fixative for 2 h at 4°C and then transferred to 100% ethanol. Paraffin-embedded tissues were cut into 5 μm slices and stained with Alcan blueperiodic acid (AB-PAS) using standard techniques. Thickness of the mucous layer was measured under light microscope, taking approximately 10 measurements per tissue section. Total goblet cell number was determined by enumerating all PAS+ goblet cells per 40x field with 5 fields counted per tissue section.

3.2.3 Microbiome analysis

In order to assess the composition of the microbiota, fecal pellets of malnourished or control-fed mice +/- flaxseed were homogenized using a bead-beating method (FastPrep instrument, MP Biomedicals, Solon, OH), and total DNA was extracted by the standard method phenol–chloroform extraction [160,161]. The 16S rRNA gene was amplified and sequenced at the Integrated Microbiome Resource lab (IMR) at Dalhousie University (Halifax, Canada) using an Illumina MiSeq platform and following the procedure previously described[162] . Raw sequencing was processed using a standardized qiime1 pipeline (Microbiome Helper, Morgan Langille[163]) to obtain the OTU table and taxonomy. Measures of alpha and beta diversity, and the associated rarefaction curves and principle component analysis (PCA) plots, were also generated in qiime1 using this pipeline. To discover taxa which differed significantly between the

groups, LEfSe analysis[180,181] was performed to compare flaxseed intervention within either the control or malnourished groups.

3.2.4 Flow cytometry

Cytometric Bead Array (CBA) Mouse Inflammation Kit (Countess, BD Biosciences) was used to quantitatively measure Interleukin-6 (IL-6), Interleukin- 10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Interferon-γ (IFN-γ), Tumor Necrosis Factor-α (TNF-α), and Interleukin-12p70 (IL-12) protein levels in tissue culture supernatants following the protocols[182]and their populations were analyzed by an LSR II flow cytometer (BD Biosciences) using software packages from CellQuest and FlowJo version 8.7.

3.2.5 Metabolite extraction from feces

Samples were thawed at room temperature and weighed in 2 mL sterile eppendorf tubes. For the targeted analysis of phenolic metabolites, feces were extracted with 10% weight/volume with 80% methanol, 19.5% water and 0.5% formic acid and homogenized under intense shaking for 5 minutes twice with steel balls. The fecal solution was centrifuged (10 min, 14000 rpm, 4ºC), filtered (0.22 μm) and evaporated till dry and stored at 4ªC till the analysis.

3.2.6 MS Analysis

LC−MRM−MS analysis. An Agilent triple quadruple 6460 (Agilent) with the ChipCube nanospray ion source coupled with an Agilent 1200 nanoflow HPLC was used for MRM analysis in positive mode. For optimization and MRM sample analysis, 43 mm 75 μm i.d. C18 chip was employed. Solvent A (10% acetonitrile + 0.1% formic acid) and solvent B (90% acetonitrile + 0.1% formic acid) were used at a flow rate of 0.4 ul/min and the injection volume was 4ul. A non-linear gradient was applied: 0 min, 90% A; 0-3 min, 90% A; 3-3.50 min, 40% A; 3.5-6 min, 60% A; 6-6.50 min, 60% A; 6.50-8 min, 100% A; 8-9.50 min, 100% A and then returned to initial conditions over 1 min and re-equilibrated for 5 min. The resolution of both MS1 and MS2 was set to unit, and for each compound the two highest transition peaks were analyzed, the highest peak was used to quantify, and the second transition was used to confirm the analyte of interest together with the comparison with the standard. Optimized MRM parameters for each compound are shown in **Table 3.2**. Data were processed using the Mass Hunter Qualitative Analysis software (version B.06.00).

Table 3.2. **Multiple reaction monitoring (MRM) parameters** optimized for the identification and quantification of the flaxseed lignans secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO) and the enterolignans enterodiol (ED) and enterolactone (EL) in the LC-MS.

Compound	MF	MW	$[MW+H]^+$	Rt (min)	Fragmentor (V)	CE (V)	Quantification transition	Confirmation transition
ED	$C_{18}H_{22}O_4$	302.37	303.16	8.2	135	5	303/267	303/107
EL	$C_{18}H_{18}O_4$	298.34	299.13	9.0	170	15	299/133	299/263
SDG	$C_{32}H_{46}O_{16}$	686.71	687.29	6.8	150	7	687/345	687/327
SECO	$C_{20}H_{26}O_6$	362.17	363.18	7.8	100	7	363/163	363/137

MF: Molecular formula; MW: Molecular weight; Rt: Retention time: CE: Collision Energy.

3.2.7 Statistical analysis

Statistical significance was calculated by using a two-tailed Student's t-test or the Mann–Whitney U-test (for non-parametric data) unless otherwise stated, with assistance from GraphPad Prism Software Version 4.00 (GraphPad Software, San Diego California USA, www.graphpad.com). If not otherwise specified statistical significance was given as *** p-value < 0.001; ** p-value < 0.01; * p-value < 0.05; ns (not significant) p-value> 0.05. The results are expressed as the mean value with standard error of the mean (SD), unless otherwise indicated.

3.3Results

3.3.1 Weight gain, tail length and average food intake

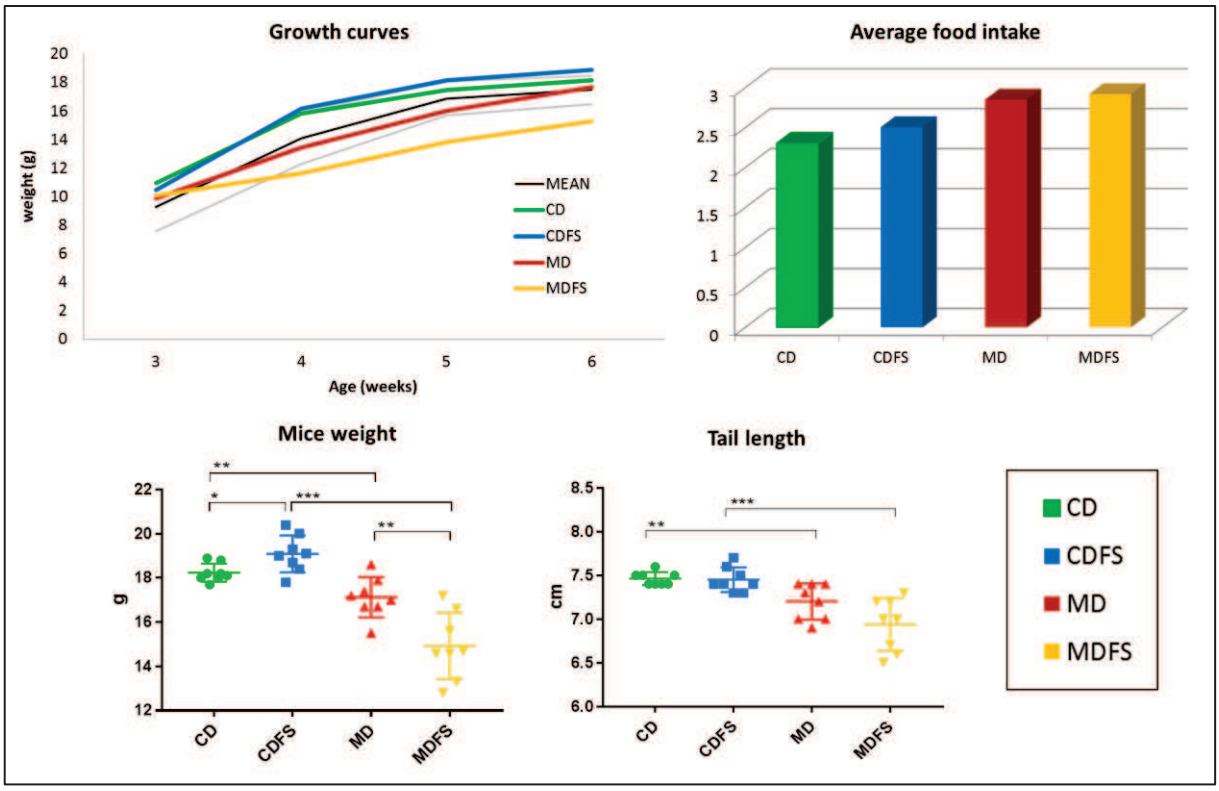


Figure 3.1. Comparison of the tail length and weight of mice at third week between interventions. Althought the food intake was the same between the four groups, control mice with flaxseed (CDFS) had more weight than control diet group (CD) and malnourished mice with flaxseed (MDFS) gained less weight than malnourished mice (MD).

Growth curve, weight gain, tail length and average food intake are shown in **Figure 3.1**. The average food intake was similar in the 4 groups of mice. We did not see any significant difference in the food intake or the calories intake per day and mice. The growth curves and the weight gain at the end of the experiment showed some differences between the groups, as did the tail length. Malnourished and malnourished mice with flaxseed had smaller tail than control and control diet with flaxseed mice ($p<0.05$ and $p<0.005$, respectively). Surprisingly, while control mice given flaxseed

seemed to have similar or improved weight gain compared to control mice without flaxseed, malnourished mice given flaxseed grew substantially more slowly and were significantly smaller at endpoint compared to their malnourished counterparts, despite consuming the same amount of food.

3.3.2 Cytokines

Cytokine response was measured to assess the possible inflammation for the different diets. Flaxseed appeared to increase the anti-inflammatory cytokine IL-10, although just the increase of the IL-10 in the CDFS group in comparison to the CD group gave significant differences ($p=0.049$). The other cytokines studied (IL-6, IL-12, IFN-γ, MCP1 and TNF-α) gave similar results. See **Table 3.3**.

Table 3.3. Flow cytometry results for each intervention group.

CYTOKINE (pg/g)	CD median	CDFS median	p-value[1]	MD median	MDFS median	p-value[2]	p-value overall[3]
IFN-γ	**2.38**	**3.19**	**0.38**	**3.30**	**3.72**	**0.72**	**0.44**
IL-6	**8.08**	**15.12**	**0.11**	**4.28**	**7.63**	**0.67**	**0.09**
IL-10	**74.32**	**116.60**	**0.049***	**81.28**	**79.34**	**0.64**	**0.28**
IL-12	**16.49**	**23.67**	**0.13**	**19.84**	**13.23**	**0.39**	**0.24**
MCP1	**204.0**	**203.1**	**0.95**	**191.7**	**143.7**	**0.22**	**0.26**
TNF-α	**57.26**	**70.79**	**0.16**	**67.48**	**47.91**	**0.57**	**0.48**

Description of the groups:
CD- Control diet; CDFS- Control Diet + 8% Flaxseed; MD- Malnourished Diet; MDFS- Malnourished Diet +8% Flaxseed

1- t-Student test with Mann-Whitney correction to compare CD vs CDFS. p significance when $p<0.05$.

2- P-value calculated with t-Student test with Mann-Whitney correction to compare MD VS MDFS. P significance* when $p<0.05$.

3- P-value overall. Analisis of the variance of the 4 intervention groups. P significance when $p<0.05$.

3.3.3 Histology: mucus layer thickness and goblet cells

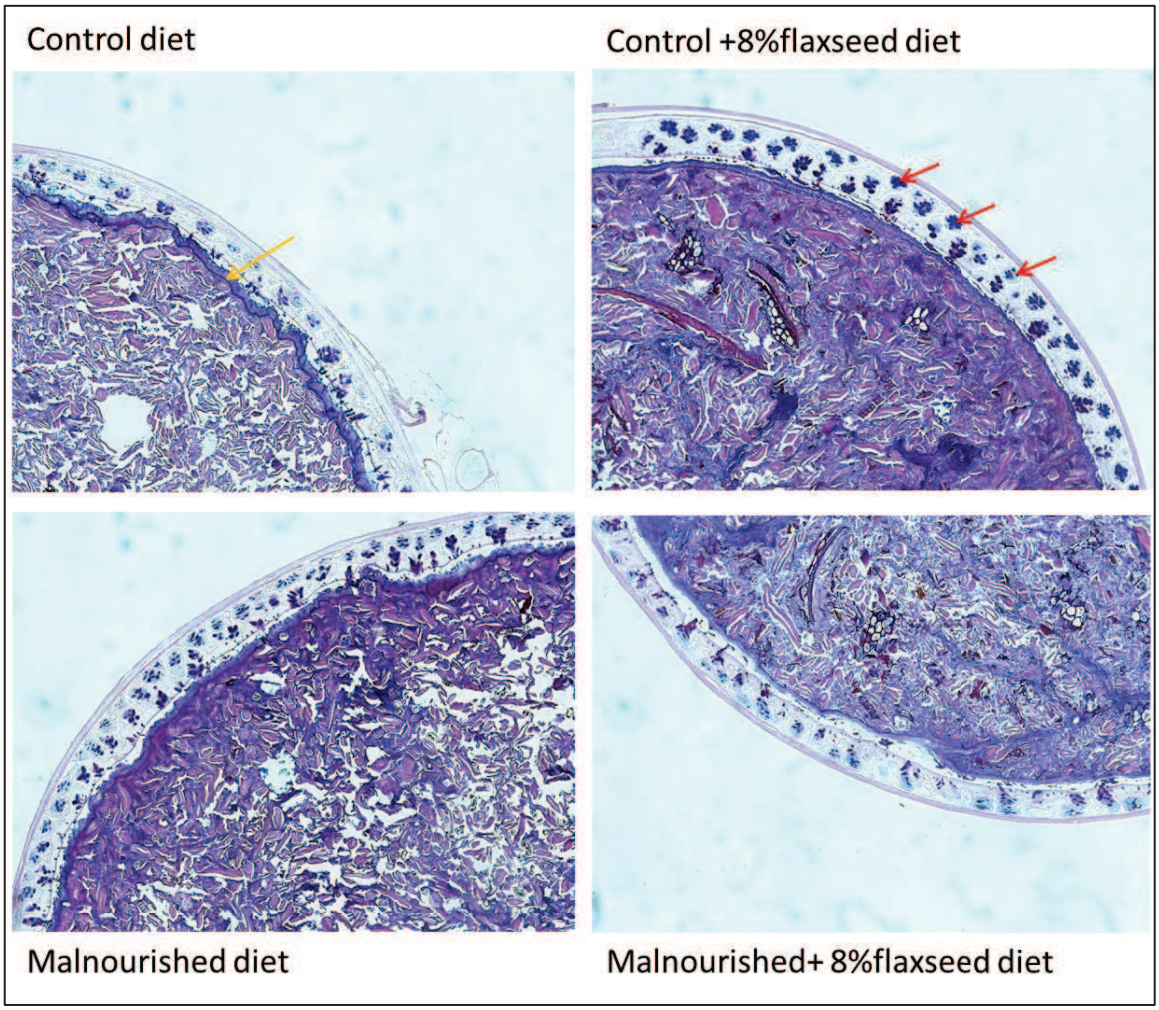


Fig 3.2. Dietary intervention does not cause histopathological changes in the colon. Histological slices of the large intestine from the 4 interventions. Red arrow points goblet cells and the yellow arrow points to the inner mucus layer.

The histological analysis of the mice showed no differences between diets with or without flaxseed (**Figure 3.2 and Table 3.4**). The only difference was shown in the inner mucus layer thickness when compare CDFS vs MDFS (p=0.004). CDFS mice had thicker mucus layer than MDFS, although the average goblet cells counts showed no differences.

Table 3.4. Comparison of inner mucus layer thickness and the average goblet cell counts per crypt in the different interventions (control diet (CD), control diet with flaxseed (CDFS), malnourished diet (MD) and malnourished diet with flaxseed (MDFS)).

	CD	CDFS	p-value	MD	MDFS	p-value	p-overall
Inner mucus layer thickness (µm)	12.98	12.11	0.96	11.60	8.70	0.09	0.04
Average goblet cell counts per crypt	7.7	9.2	0.44	7.6	8.4	0.61	0.54

3.3.4 Metabolites concentration in feces over time

Metabolic differences were shown between mice in the standard diet fortified with flaxseed and malnourished mice fortified with flaxseed. At first week of diet, malnourished mice had significantly increased enterodiol and secoisolariciresinol but no differences were shown in the produce of enterolactone. After three weeks of diet, MDFS mice had produced significantly less lignan metabolites than CDFS.

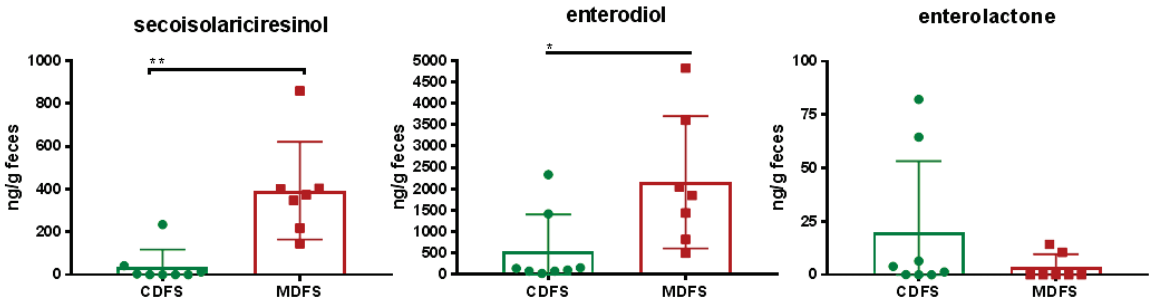


Fig 3.3. Metabolites concentration at first week of diet extracted from feces of the control group with flaxseed (CDFS) and the malnourished group with flaxseed (MDFS).

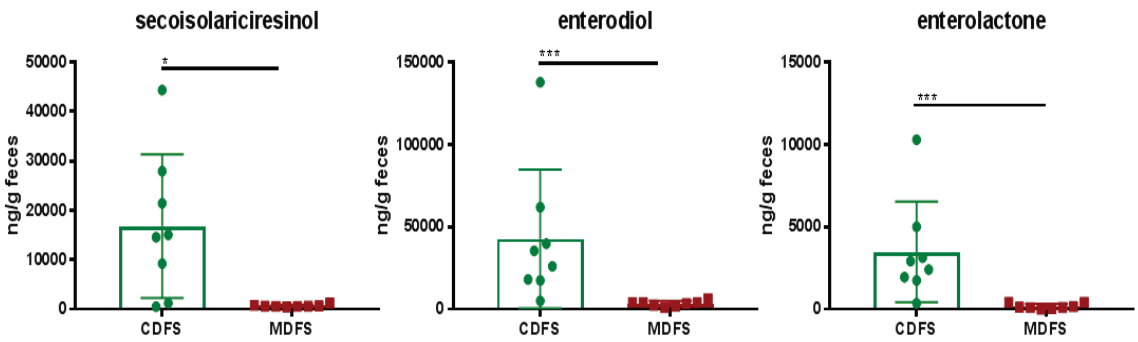


Fig 3.4. Metabolites concentration at third week of diet extracted from feces of the control group with flaxseed (CDFS) and the malnourished group with flaxseed (MDFS).

3.3.5 Gut microbiota analysis

The analyses of the sequencing results of 16S rRNA gene at region V4, allowed building the charts of bacterial distribution at phylum level (**Figure 3.5**) and family level (**Figure 3.6**). The main significant differences between interventions after three weeks of diet at phylum level were Bacteroidetes (p=0.007), Actinobacteria (p=0.0001), Proteobacteria (p=0.001) and Verrucomicrobia (p=0.002).

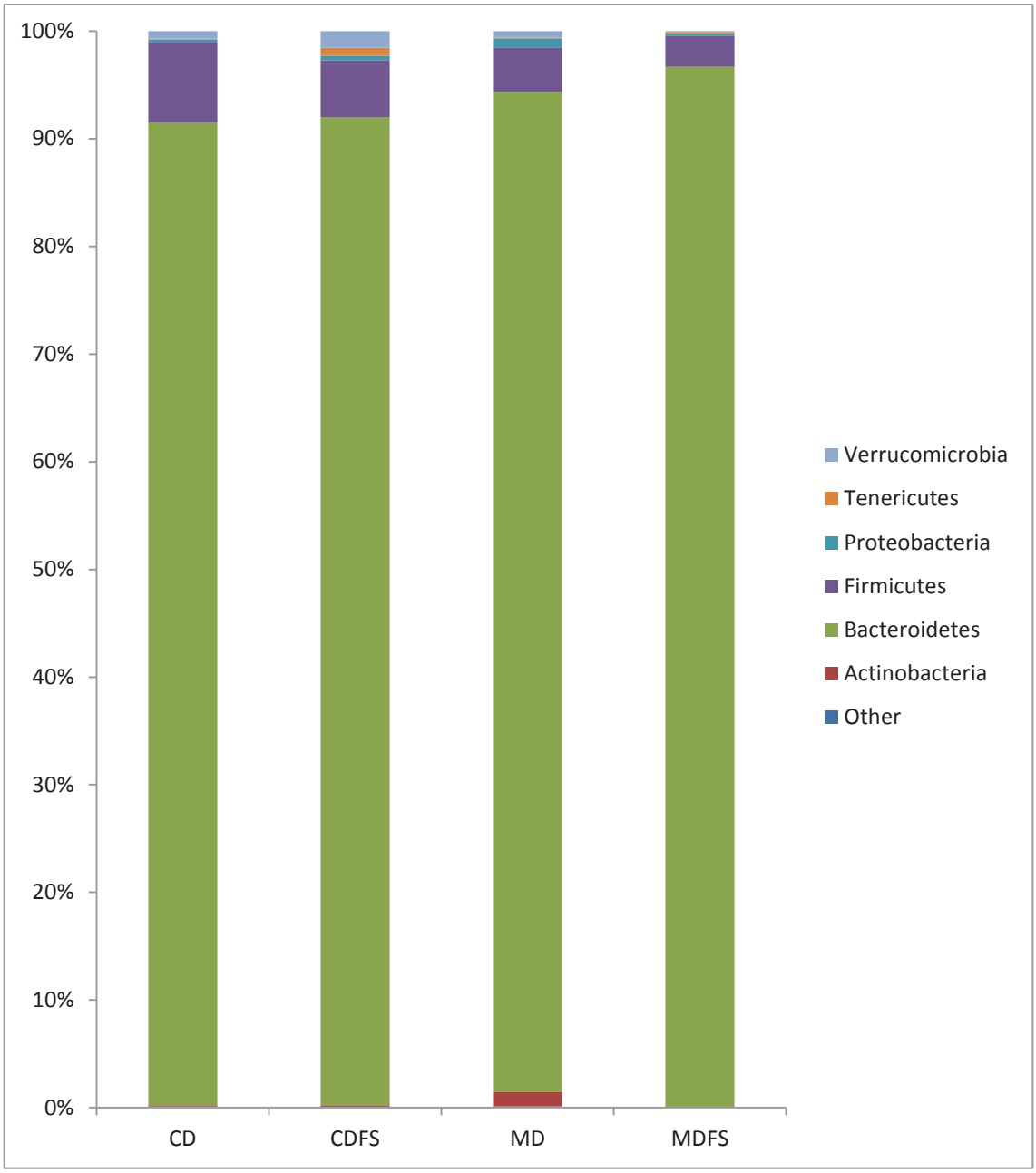


Figure 3.5. **Bacterial distribution at phylum level** in the different diet groups: control diet (CD), control diet with flaxseed (CDFS), malnourished diet (MD) and malnourished diet with flaxseed (MDFS).The main differences that can be appreciated are at phylum Bacteroidetes(p=0.007), Actinobacteria (p=0.0001), Proteobacteria (p=0.001) and Verrucomicrobia (p=0.002).

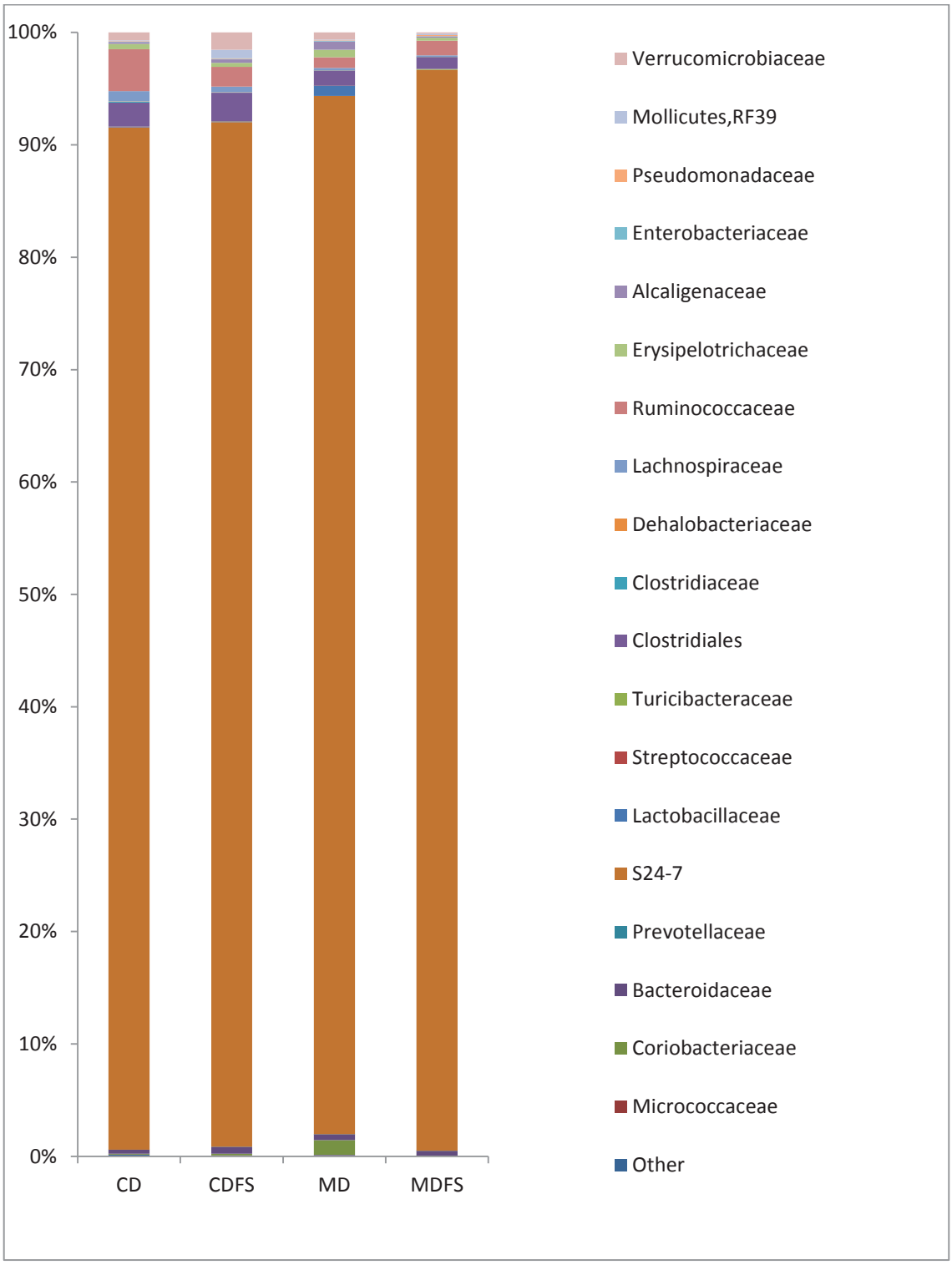


Figure 3.6. **Bacterial distribution at family level** found in gut microbiota after 3 weeks of dietetic intervention of control diet (CD), control diet +8%flaxseed (CDFS), malnourished diet (MD) and malnourished diet + 8% flaxseed (MDFS).

3.3.5.1 α-diversity and PCA scores

The analysis of α-diversity shows us some differences between dietary interventions (**Figure 3.7 a)**). The group which received the CDFS had higher diversity than the CD. The group who received the MDFS had significantly less diversity than the group under the MD.

PCA scores plots were used to objectively compare microbiome profiles. The scores plot obtained from submitting the 4 independent intervention groups (CD, CDFS, MD, MDFS) to PCA is shown in **Figure 3.7 b)**. The scores clustered in three groups according to the four types of dietetic interventions. Principal component 1 (PC 1) captured 34.8% of the total variance. Principal component 2 (PC 2) captured 23.6% of the total variance. Principal component 3 (PC 3) captured 20.8% of the total variance. The results showed that the cluster between interventions are clearly separated, suggesting that the bacterial community in the gut shifts with diet intervention and with the presence or absence of lignans.

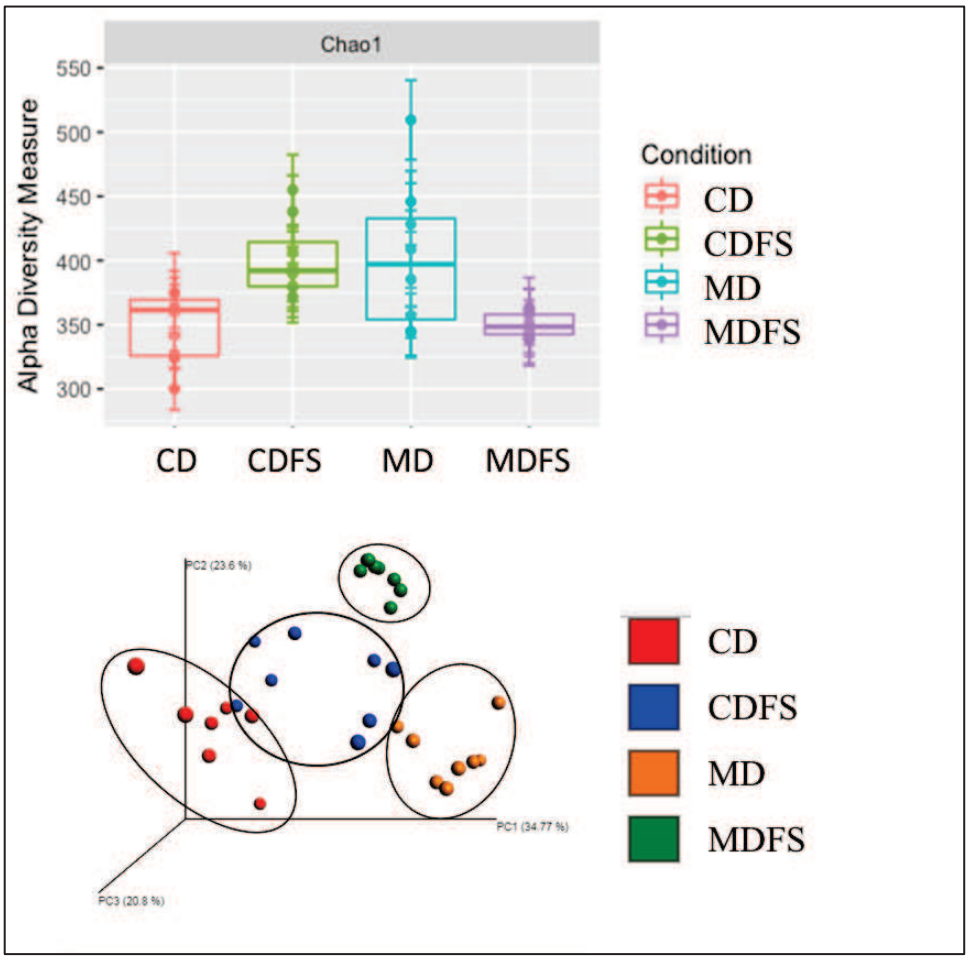


Figure. 3.7. α-diversity and PCoA scores plot of the 4 intervention groups: control diet (CD), control diet with flaxseed (CDFS), malnourished diet (MD) and malnourished diet with flaxseed (MDFS). **a)** α-diversity plot showed higher microbial diversity of CDFS vs CD (although no significance), and a significant less diversity in MDFS vs MD. **b)** (The scores plot obtained from PCA of control diet +8%flaxseed, malnourished diet mice and malnourished +8%flaxseed diet mice demonstrates differentiation between interventions based on microbiome profiles. PC 1 captured 34.8% of the variance, PC 2 captured 23.6% of the variance and PC3 captured 20.8% of the variance.

3.3.5.2 Bacterial shifts in the gut microbiota among interventions

To further identify the significantly changed bacteria among the four different intervention groups, LEfSe (Linear discriminant analysis Effect Size) analyses for different taxa were conducted.

Effect of Flaxseed on Microbiota Independently of Diet

Three taxa were consistently different in mice given flaxseed according to LEfSe analysis, regardless of their status as control or malnourished (**Figure 3.8**). The genus *Turicibacter* was enriched in flaxseed-fed mice in both the CDFS and MDFS groups, while *Ruminococcaceae* and *Coprococcus* were depleted in both CDFS and MDFS groups.

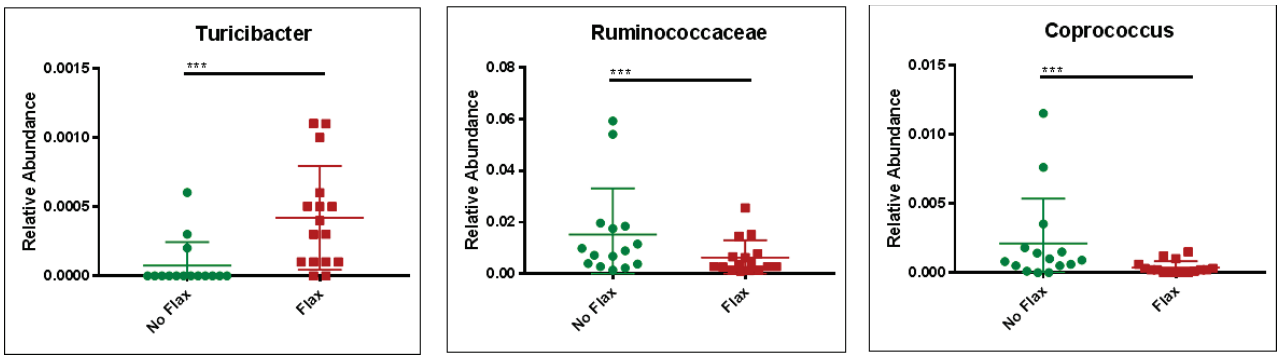


Figure 3.8. Differences in relative abundance of fecal microbial bacteria depending on the presence or absence of flaxseed lignans on diet.

Impact of the flaxseed on gut microbiota in a standard diet

Main differences in fecal microbial community in the control group diet interventions observed came from *Firmicutes* phylum. Mainly, CDFS intervention decreased *Lactobacillus, Coprococcus* and *Erysipelotrichaceae* (**Figure 3.9**).

Impact of flaxseed on gut microbiota in malnutrition diet

The LEfSe analysis in fecal microbial community from malnourished mice +/- flaxseed showed that flaxseed caused more extensive restructuring of the microbiota in malnourished mice. Malnourished mice given flaxseed had decreased *Coriobacteraceae*, *Erysipelotrichaceae, Lactobacillus*, *Sutterella*, *Akkermansia*, and *Lactococcus*, and increased *Turicibacter*, *Oscillospira*, *Ruminococcus*, and *S24-7* (**Figure 3.9**).

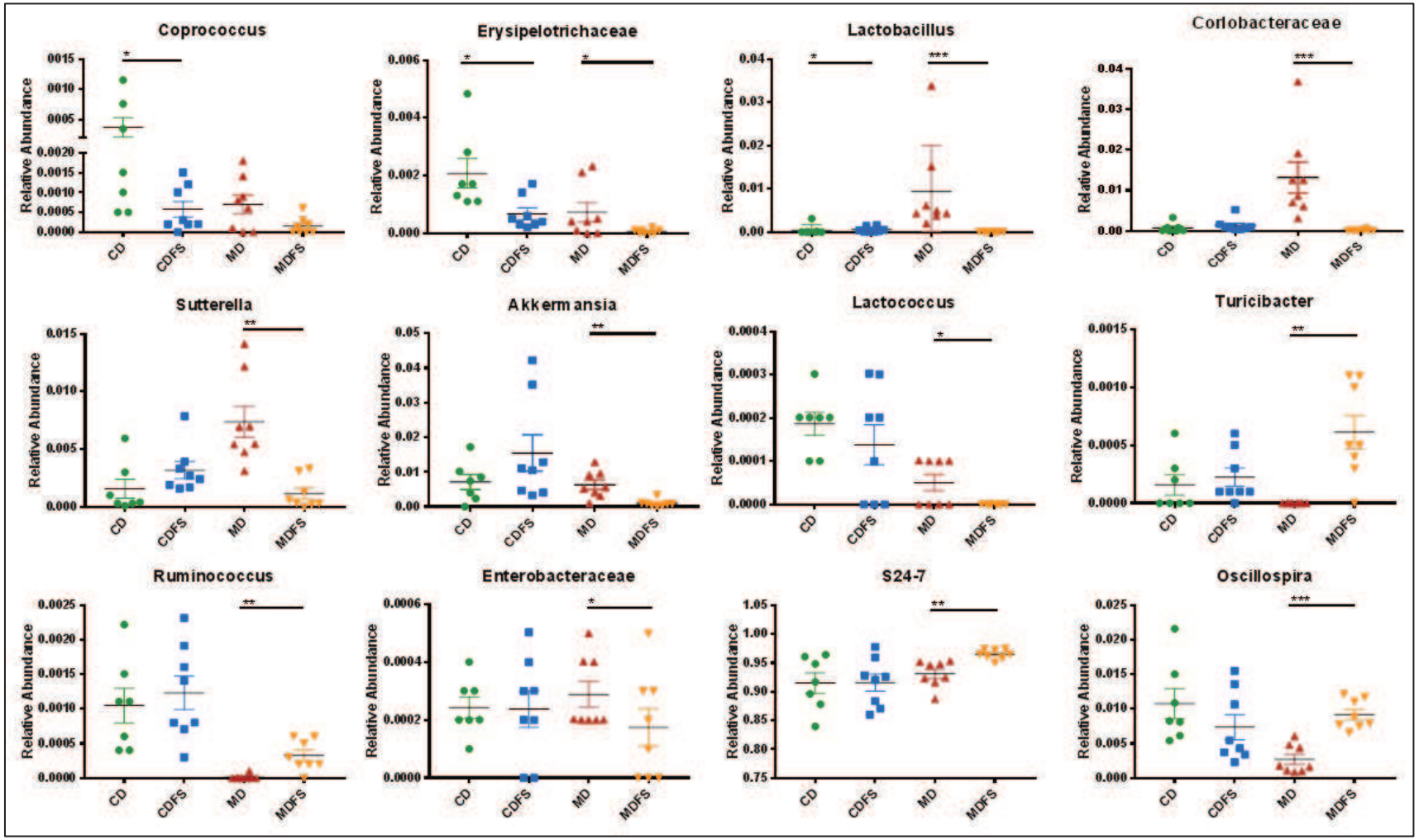


Fig 3.9. Relative abundance of fecal microbial bacteria that showed some differences between at least two of the 4 intervention groups: control diet (CD), control diet with flaxseed (CDFS), malnourished diet (MD) and malnourished diet with flaxseed (MDFS).

3.4 Discussion

In the present study we investigated the early-life impact of lignans on microbial metabolism during healthy development and malnutrition in a mouse model. This is the first time the impact the lignans on malnourished mice has been studied. Results indicate that diet can shape the microbiota and impact the animal. The presence of flaxseed in the mouse standard diet had impact in different ways. In CDFS mice, there was a trend to increase the gut microbiota diversity, increased significantly the weight gain, although there were no differences in the amount of food consumed and finally the anti-inflammatory cytokine IL-10 was also increased. On the other hand, the presence of flaxseed in the malnourished diet had an opposite impact: the mice showed decreased weight gain and their tail length was significantly smaller, although they ingested the same amount of food and calories. The microbiome analysis also revealed significantly less taxonomic diversity in this group.

This is consistent with a previous study of Brown et al. [12] which compared CD group with MD; similar to this study, we also found that MD had lower weight and tail length despite the calorie intake was the same.

Regarding the physiology, goblet cells have a protective role in the intestine through the production of protective bioactive compounds including mucins [183–185]. These molecules are sequestered and concentrated within the mucus layer acting as a barrier, and provide defense against pathogens as well as prevent bacterial penetration of the intestinal epithelium, and they play a key role in the maintenance of healthy intestinal homeostasis [185–187]. We found differences in the mucosal defenses when comparing CDFS with MDFS. In this case MDFS had a thinner inner mucus layer. It has been shown before [188] that the thinning of the inner mucus layer can be caused after

antibiotic treatment accompanied by a dramatic change in the microbial community structure. Although dietary flaxseed has exacerbated acute colonic mucosal injury and inflammation in a colitis model[189] it also has shown beneficial effects like increasing the number of goblet cells and therefore the thickness of the mucus layer in healthy black mice[190] and all these changes have been linked to the shift of the microbiota community. In this experiment the number of goblet cells didn't change between interventions, but the mucus layer thickness did as described before.

Prebiotics are known for their ability to increase beneficial species like *Bifidobacterium* but also other beneficial species like *Ruminococcus, Roseburia/Enterococcus rectale group, Faecalibacterium prausnitzii* among others which are related to health benefits on the host[188]. In this study, we demonstrated that the flaxseed induced change in the colonic microbial community structure. In both situations, CDFS and MDFS, there were differences when compared to CD and MD, respectively. In both cases flaxseed caused an increase of *Turicibacter,* and a *decrease* of *Coprococcus* and *Erysipelotrichaceae. Akkermansia* and *Sutterella* increased although were not significant in CDFS compared to CD while they significantly decreased in MDFS in comparison with MD group. Akkermansia has been identified as an important mucosa-associated Gram-negative species necessary for maintaining intestinal immune homeostasis, increasing gut barrier integrity. And its abundance has been proposed to be enhanced in polyphenol-rich diets in the past[165,172]. In this study Akkermansia is richer in CDFS than CD but lower in MDFS than MD showing the possibility that Akkermansia richness also depends in the total of the diet and not just of the content in polyphenol. Increases in *Sutterella* have been associated with Metabolic Syndrome, while Akkermansia has been inversely correlated with Metabolic Syndrome[191]. Another interesting difference found was that *Oscillospira* was lower in

the CDFS and higher in the MDFS. Oscillospira has been associated with leanness, or less BMI which would agree with our results, but it also has been correlated to less inflammation. Surprisingly, *Oscillospira* has never been cultured, although it is constantly detected when analyzing the human microbiome[191]. Moreover, since there are multiple strains of *Oscillospira* in the human gut, we should study all of them and determine which physiological aspects are shared among all, which would allow us to full understand the clinical significance of *Oscillospira*.

Another interesting bacterium which has been highly localized to the gastrointestinal tracts of homeothermic animals from the Bacteroidales order but that has not been cultured yet is the S24-7 family. Little is known but they are increasingly being recognized as a predominant member of the gut microbiota[191]. In our study there was an increase in S24-7 in the MDFS versus MD. S24-7 belongs to Bacteroidetes phylum, it seems that S24-7 might also be able to metabolize flaxseed lignans and take advantage from it, although its culture with SDG would be required to confirm this assumption.

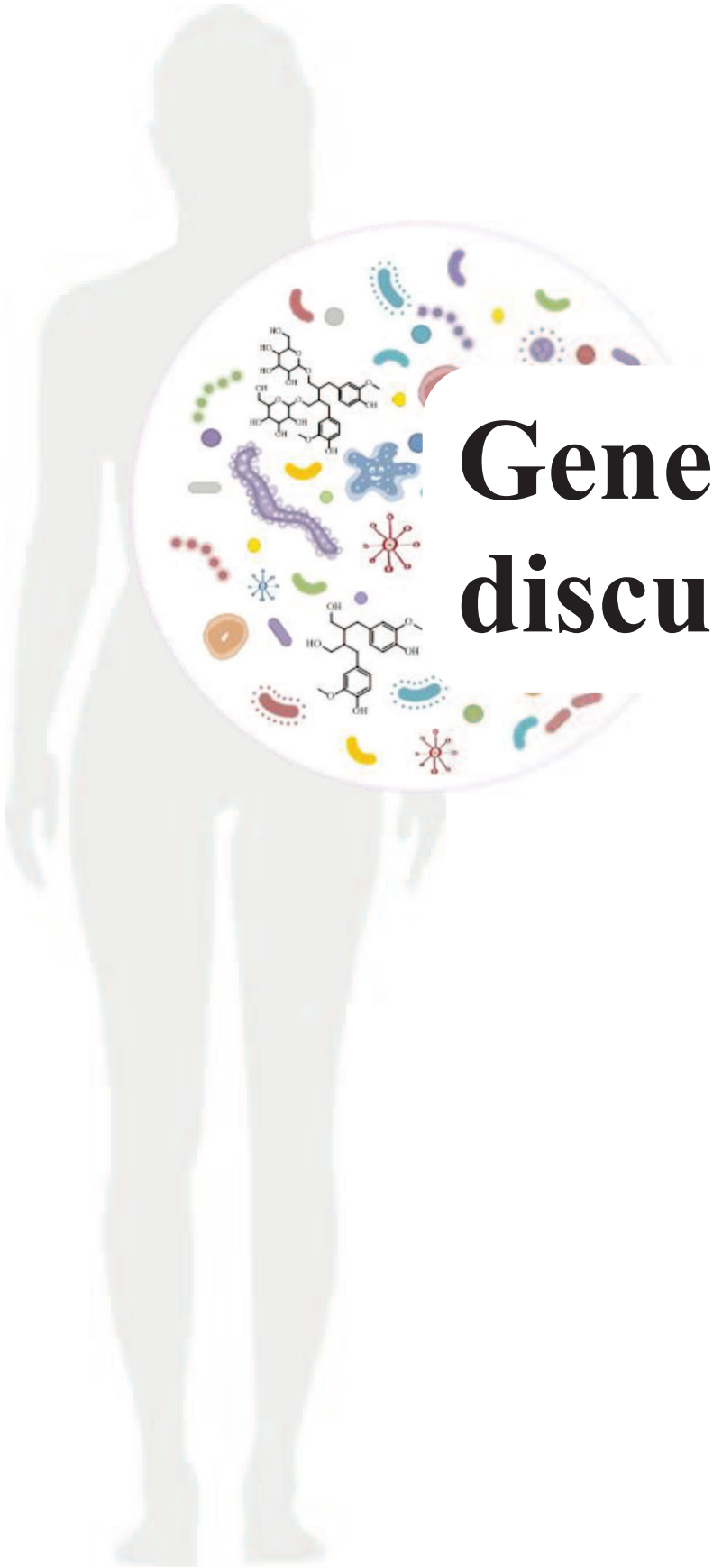
In the first week of diet the malnourished group had a higher metabolite concentration, and this could be explained, as the reaction because to a more stressful and unhealthy diet. Although in a chronic consumption of the different diets, after 3 weeks, the ones with a standard diet could take more advantage of the flaxseed metabolites, so EL and ED resulted being in higher concentrations and MDFS mice couldn't metabolize lignans as much as CDFS mice, having lower levels of ED and EL. *Akkermansia, Ruminococcus* and *Sutterella* resulted to be more abundant when higher lignan metabolites (ED, EL) were found and *Oscillospira* resulted to be less abundant when higher ED and EL were found. Further studies are needed to fully understand if

there is a swap in the gut community over the time which correlates with lignan metabolites formation.

3.5 Summary

In conclusion, we were able to see four different microbiomes after each intervention. Diet altered the microbiome community and we could see a different behavior depending on the standard or malnourished diet adding or not flaxseed. The study of the lignan impact on gut microbiota and its effects in health doesn't seem to be an easy equation to solve. Also after these findings we still cannot distinguish if lignans can be considered as prebiotic compounds because of the complexity of the microbiota, and it is difficult to distinguish a healthy from an unhealthy microbiome.

It seems reasonable to think after seeing the differences in the behavior of the growth in mice and its different concentration in the healthy metabolites after the three weeks intervention that flaxseed can be a good prebiotic in standards diets, but also can be harmful in malnourishment. Further studies need to be done with longest treatments to fully understand how they act and how they impact in the health. It would be very interesting to explore the possible intervention with lignans plus probiotics in both situations, normal and malnutrition diets.



General discussion

General discussion

It is well-recognized that flaxseed is the main contributor in diet[3]. Although in Spain, specifically in the PREDIMED population, which is discussed in this work, the main contributor for the dietary lignan intake was olive oil. After olive oil, wheat products were the following contributor to the overall lignan intake, then tomatoes and their byproducts, red wine, kiwis and other fruits and vegetables. Pinoresinol is the main lignan compound of olive oil, and this is the reason why pinoresinol was the lignan most consumed in this study, followed by 1-acetoxypinoresinol and lariciresinol. Secoisolariciresinol, which is the main lignan compound found in flaxseed was the fifth lignan polyphenol consumed by the PREDIMED population. The metabolism of all different lignans converges to the enterolignans ED and EL formation. And the health effects of them are linked to the production and absorption of them into the body through the colonocyte after the microbial conversion.

The results from this work showed that yogurt consumption and high lignan intake were linked to a healthier cardiovascular parameters profile. Previous studies on yogurt and lignan consumption have shown beneficial effects on human health, but as shown in the present study joint consumption of these foods had a stronger impact on CVR-P and was associated with lower cholesterol and LDL-c levels and a trend to lower TGs levels. Some of the benefits associated to yogurt intake are cardiovascular and gastrointestinal health, weight management, and type 2 diabetes, among others [141,142]. It is the first time that these associations between them both are shown. Although, other studies in the past have correlated lignan metabolites to better cardiovascular risk parameters. Yogurt can have a probiotic role when ingested and lignans could act as

prebiotic, enhancing the health on the host. Despite probiotic properties from yogurt have been demonstrated in the past[75,138–140], it is the first time that lignans are considered as potential prebiotics, thus more studies are needed to confirm this assumption. . Full-fat yogurt was correlated with higher LDL-c and lower TG levels, while low-fat yogurt was correlated with lower total cholesterol, HDL-c and LDL-c, but with no impact on TGs. It has been suggested that the potential underlying mechanisms for weight loss or the prevention of weight gain could be stimulatory effects on the growth of beneficial intestinal bacteria [144]. Manipulation of the intestinal microbiota can be beneficial for maintaining health in adults as it has been pointed before by Zapata et al. [147]. In this work, higher lignan and yogurt consumption was associated with lower levels of total cholesterol, LDL-c and TGs, while HDL-c values did not decrease, indicating an improved lipid profile. Yogurt consumption did not affect serum glucose levels. Microbiota associated with yogurt intake seems to metabolize lignans more efficiently but also lignans intake may help to modulate gut microbiota by increasing the beneficial strains [90,135]. Although the associations were clear and statistically significant there are some limitations in this study, the first is that since the data obtained was from an elderly population at high cardiovascular risk we may limit the generalization of these results. The second one, discussed also in chapter 2 is that the calculation of lignan intake has been done with FFQs and Phenol-Explorer. FFQs sometimes can under or overestimate the intake of some foods. And some foods may have different levels or concentrations of lignans, depending on the cooking, storage, or variety ingested [108,151]. Although the database is very extensive, possible confounding variables such as stress, or mental health among others, were not recorded. Finally, since this was an observational study, a cause-effect relationship cannot be established from the results.

The study in vitro allowed the discovery of ten new bacterial species as responsible of the SDG metabolism for the first time. SDG is the main lignan compound in flaxseed, and since flaxseed is the most common source of lignans worldwide it makes important to study its metabolism to the enterolignans ED and EL. From Bacteroidetes phyla *B. thetaiotaomicron* was the first time found to be responsible to metabolize SDG into SECO. Four species from Firmicutes phyla were found to be responsible for the metabolism of SDG into SECO for the first time, Fa*ecalibacterium prausnitzii, Bacillus licheniformis, Peptostreptococcus russellii* and *Sarcina ventriculi*. Also, *L. johnsonii* and *gasseri* to be also part of this group and *Lachnospira multipara* and *Veillonella* were also able to demethylate and dehydroxylate SECO into ED. Other species were found responsible of the metabolism of SDG such as *Bacteroides fragilis, B. ovatus, B. dorei* and P. *distasonis* and have been described before with this ability[24,66–68]. Actinobacteria, such as *B. adolescentis* and *B. longum*, have been previously studied by Gaya et al.[35], and according to them exhibited the ability to produce SECO, in this work same results were found. *Akkermansia muciniphila* was able convert SECO into EL; this is very interesting since *Akkermansia* has been inversely associated with cardiovascular diseases, diabetes, obesity and low-grade inflammation[168]*F. prausnitzii* has gained interest as a potential probiotic strain, since it has been negatively correlated in diseases like Parkinson's disease, Crohn's disease, ulcerative colitis and obesity[166,167]. Recently, has been linked to healthy human microbiota, and changes in its abundance have been linked to dysbiosis[167]. Evidence suggests that some strains of *A. muciniphila* and *F. prausnitzii* are good candidates as next-generation probiotics [168,169].

The experiment *in vitro* recreating an artificial gut microbiota under anaerobic conditions, allowed the study of the metabolism of lignans by a community. In 48

hours, any SDG was detected, and in the short period of 24 hours the community had already produced the EL and ED. Meaning that the community was able to metabolize SDG lignan and convert it into EL and ED. The simulation of the gut conditions *in vitro* is challenging. The main factors affecting the gut microbiota *in vivo* are the diet, life style, stress, which impacts in the pH, food transit time, etc. When simulating the microbiota in vitro, the broth of culture is not the same as in the gut, and the pH can be smoothly different. In this experiment it was not controlled, but in future experiments it should be controlled all the time, since it can be crucial for the survival of some bacteria.

Shifts in the community with the presence of SDG were also studied. Bacteroidetes increased significantly their abundance with the presence of SDG. These findings correlate with other studies of *in vitro* incubations with tea and wine polyphenols[171,172]. A higher ratio Bacteroidetes:Firmicutes has been linked to a healthy diet and lifestyle and consequently a better health on the host. Another interesting difference between the communities was that the presence of SDG had a negative impact in *Escherichia coli* relative abundance. Most *E. coli* strains do not cause disease, but virulent strains can cause gastroenteritis, urinary tract infections, neonatal meningitis, hemorrhagic colitis, and Crohn's disease[173–175]. Since Akkermansia muciniphila was found to capable to metabolize SDG and in the past some studies have shown that the presence of polyphenols caused an increase of the beneficial *Akkermansia muciniphila*[165], its increase in the community with SDG was expected, although any increase was observed in this experiment. Since the presence of lignans gave differences in the bacterial community, in principle they could act as prebiotic. Although the possible beneficial effect on the host is still necessary to be studied to conclude that lignans can be considered prebiotics.

The last study that comprises this thesis was to investigate the early-life impact of lignans on microbial metabolism during healthy development and malnutrition in a mouse model. This study also wants to put the shed light on the fact that lignans can be considered prebiotics. Up to our knowledge this is the first time that the impact of lignans is studied in a malnourished model. The impact of lignans in the healthy and malnourished mice was very different. Comparing between healthy mice and healthy mice supplemented with flaxseed, there was a trend to increase the gut microbiota diversity in the last one. CDFS also increased significantly their weight gain, although there were no differences in the calories consumed. And IL-10, an anti-inflammatory cytokine was also increased. Regarding the malnourished model, flaxseed had an opposite impact in the growth of the mice. MDFS mice had less weight gain and a tail significantly smaller. And surprisingly the microbiome analysis revealed significantly less taxonomic diversity in this group. The microbiome diversity has been linked to health in the past, as more diversity, as more healthy a living being is[192]. The physiology of the mice gut was also studied. The mucus layer thickness and goblet cell counts were assessed. Goblet cells have a protective role in the intestine, which includes the production of protective bioactive compounds such as mucins [183–185]. Mucins can act as a barrier, provide defense against pathogens and have a key role in the maintenance of healthy intestinal homeostasis[185–187]. When comparing CDFS with MDFS a completely different behavior was shown. MDFS had a thinner inner mucus layer. Dietary flaxseed has exacerbated acute colonic mucosal injury and inflammation in a colitis model[189] it also has shown beneficial effects like increasing the number of goblet cells and therefore the thickness of the mucus layer in healthy black mice[190] and all these changes have been linked to the shift of the microbiota community. In this experiment the number of goblet cells did not change between interventions, but the mucus layer

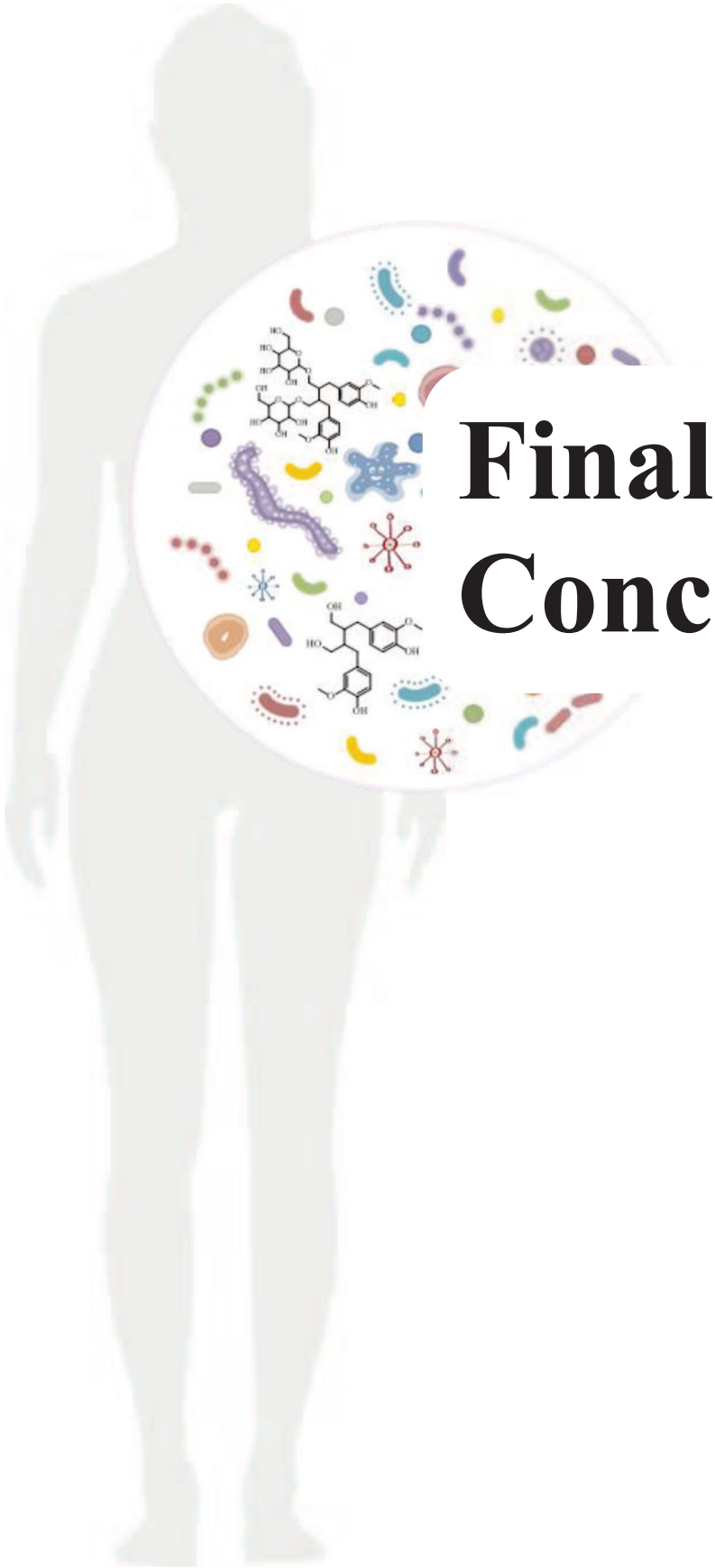
thickness did. So the flaxseed had the opposite impact in the physiology of the gut depending on the mice model.

Prebiotics are known for their ability to increase beneficial species which are related to health benefits on the host[188]. In this study, we demonstrated that the flaxseed induced change in the colonic microbial community structure. The presence of flaxseed vs no flaxseed in the diets caused an increase of *Turicibacter,* and a *decrease* of *Coprococcus* and *Erysipelotrichaceae.* However *Akkermansia* and *Sutterella* had an opposite behavior when comparing CDFS vs CD and MDFS vs MD. *Akkermansia* and *Sutterella* increased although were not significant in CDFS compared to CD while they significantly decreased in MDFS in comparison with MD group. As I mentioned before Akkermansia is a very interesting one since is being proposed as a new probiotic. The microbial difference between flaxseed interventions shows the possibility that is important the whole diet for the health and not just the content in a polyphenol. And it brings the question to the point that the administration of probiotics together with the lignan should be studied. Akkermansia abundance has been proposed to be enhanced in polyphenol-rich diets in the past[165,172].

Comparing the results from the study *in vitro* and *in vivo* of the impact of lignans on microbiota, both agrees in an increase of the phylum Bacteroidetes. Although, just in the study with mice there were an increase in *Akkermansia muciniphila*, in the control group supplemented with flaxseed. It is very difficult to simulate the gut microbiome, since it is an open system very complex. *In vitro* studies allow us to control all the variables, but *in vivo* studies are more representative of the reality and the real consequences. The study with mice was all done with females, it should be repeated with male mice to ensure that have the same behavior, since some of the results can be gender dependent.

The new open concept of prebiotics let the door open to introduce in this category new food ingredients. The results from this work show us very different behavior of lignans in health and disease. So when describing prebiotics and probiotics we have to be very cautious in which situation it is recommended to use and how it has been proved. And probably we are closer to more specific person to person dietary recommendations than to general recommendations for most of the people.

Future investigations to elucidate the role of lignans as prebiotics on gut microbiota are needed. Study the impact of probiotics on healthy and malnourished mice together with lignans supplementation would be a very interesting way to observe if they can act together improving the health host. Specially, in the malnourished situation since their microbiota could lack of the ability of metabolizing lignans, and therefore have a negative impact on them. And finally, if it is proved that they can act synergistically; a study in humans would be needed to confirm the results. The specificity and safety of the strain used for such a purpose will be indispensable to ensure its success. The importance of microbiota on health host is a relative new concept that is gaining importance among scientists but also everyday more people are aware about it. Therefore, all the efforts invested to clarify and understand how it works and its potential health impacts appear to be few when you look at the potential of what is considered nowadays as the new organ.



Final Conclusions

Final conclusions

To sum up, from the results obtained in these studies performed during the thesis derived the following conclusions:

1. The joint consumption of yogurt and high lignan content foods had a stronger impact on CVR-P and was associated with lower cholesterol and LDL-c levels and a trend to lower TGs levels.

2. Total and low-fat yogurt intake were correlated with higher weight, but yogurt intake together with a high lignan diet did not produce any increase in weight.

3. These findings suggest that an associative effect of lignans and yogurt may ameliorate CVR-P in humans. Therefore, daily low-fat yogurt consumption in a healthy, well-balanced diet with a high content of lignan-rich foods, such as flaxseed or extra virgin olive oil, may be recommended to enhance the beneficial effects of these two foods when ingested separately, at least in elderly populations.

4. In this work 10 new strains have been described for the first time as responsible for the metabolism of SDG, among them, *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* which are already potential probiotics.

5. The study of an artificial community with SDG increased Bacteroides and decreased *Escherichia coli.*

6. Diet and lignan content of the diet altered the microbiome community and we could differentiate clusters for each intervention: control diet, control diet with 8% flaxseed, malnourished diet and malnourished diet with 8% flaxseed.

7. However lignans cannot be considered as prebiotic compounds because of the complexity of the microbiota, and the differences showed between standard and malnourished mice. But they can be considered potential prebiotics in standard mice.

Bibliography

Bibliography

1. WHO | Cardiovascular diseases (CVDs). *WHO*. 2017.

2. Landete JM, Arqués J, Medina M, Gaya P, de Las Rivas B, Muñoz R. Bioactivation of Phytoestrogens: Intestinal Bacteria and Health. *Crit Rev Food Sci Nutr*. 2016;56(11):1826-43.

3. Adlercreutz H. Lignans and Human Health. *Crit Rev Clin Lab Sci*. October 2008.

4. Gaya P, Medina M, Sánchez-Jiménez A, Landete J. Phytoestrogen Metabolism by Adult Human Gut Microbiota. *Molecules*. 2016;21(8):1034.

5. Creus-Cuadros A, Tresserra-Rimbau A, Quifer-Rada P, et al. Associations between Both Lignan and Yogurt Consumption and Cardiovascular Risk Parameters in an Elderly Population: Observations from a Cross-Sectional Approach in the PREDIMED Study. *J Acad Nutr Diet*. 2017;117(4):609-622.e1.

6. Gibson GR, Hutkins R, Sanders ME, et al. Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nat Rev Gastroenterol Hepatol*. 2017;14(8):491.

7. WHO | Joint child malnutrition estimates - Levels and trends (2016 edition). *WHO*. 2017.

8. Smith MI, Yatsunenko T, Manary MJ, et al. Gut Microbiomes of Malawian Twin Pairs Discordant for Kwashiorkor. *Science (80-)*. 2013;339(6119):548-554.

9. Subramanian S, Huq S, Yatsunenko T, et al. Persistent gut microbiota immaturity in malnourished Bangladeshi children. *Nature*. 2014;510(7505):417-421.

10. Blanton L V, Charbonneau MR, Salih T, et al. Gut bacteria that prevent growth

impairments transmitted by microbiota from malnourished children. *Science (80-)*. 2016;351(6275).

11. Schwarzer M, Makki K, Storelli G, et al. Lactobacillus plantarum strain maintains growth of infant mice during chronic undernutrition. *Science (80-)*. 2016;351(6275):854-857.

12. Brown EM, Wlodarska M, Willing BP, et al. Diet and specific microbial exposure trigger features of environmental enteropathy in a novel murine model. *Nat Commun*. 2015;6.

13. Quideau S, Deffieux D, Douat-Casassus C, Pouységu L. Plant Polyphenols: Chemical Properties, Biological Activities, and Synthesis. *Angew Chemie Int Ed*. 2011;50(3):586-621.

14. Del Rio D, Costa LG, Lean MEJ, Crozier A. Polyphenols and health: what compounds are involved? *Nutr Metab Cardiovasc Dis*. 2010;20(1):1-6.

15. Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: chemistry, bioavailability and effects on health. *Nat Prod Rep*. 2009;26(8):1001-1043.

16. Del Rio D, Rodriguez-Mateos A, Spencer JPE, Tognolini M, Borges G, Crozier A. Dietary (poly)phenolics in human health: structures, bioavailability, and evidence of protective effects against chronic diseases. *Antioxid Redox Signal*. 2013;18(14):1818-92.

17. Scalbert A, Morand C, Manach C, Rémésy C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother*. 2002;56(6):276-282.

18. Bohn T. Dietary factors affecting polyphenol bioavailability. *Nutr Rev*. 2014;72(7):429-452.

19. Scalbert A, Williamson G. Dietary intake and bioavailability of polyphenols. *J Nutr*.

2000;130(8S Suppl):2073S-85S.

20. Marín L, Miguélez EM, Villar CJ, Lombó F. Bioavailability of Dietary Polyphenols and Gut Microbiota Metabolism: Antimicrobial Properties. *Biomed Res Int*. 2015;2015:1-18.

21. Pandareesh MD, Mythri RB, Srinivas Bharath MM. Bioavailability of dietary polyphenols: Factors contributing to their clinical application in CNS diseases. *Neurochem Int*. 2015;89:198-208.

22. Stitch SR, Toumba JK, Groen MB, et al. Excretion, isolation and structure of a new phenolic constituent of female urine. *Nature*. 1980;287(5784):738-740.

23. Setchell KDR, Lawson AM, Mitchell FL, Adlercreutz H, Kirk DN, Axelson M. Lignans in man and in animal species. *Nature*. 1980;287(5784):740-742.

24. Clavel T, Doré J, Blaut M. Bioavailability of lignans in human subjects. *Nutr Res Rev*. 2006;19(2):187-196.

25. Landete JMM. Plant and mammalian lignans: A review of source, intake, metabolism, intestinal bacteria and health. *Food Res Int*. 2012;46(1):410-424.

26. Kajla P, Sharma A, Sood DR. Flaxseed—a potential functional food source. *J Food Sci Technol*. 2015;52(4):1857-1871.

27. Mazur WM, Uehara M, Wähälä K, Adlercreutz H. Phyto-oestrogen content of berries, and plasma concentrations and urinary excretion of enterolactone after a single strawberry-meal in human subjects. 2017.

28. Johnsson P, Kamal-Eldin A, Lundgren LN, Aman P. HPLC method for analysis of secoisolariciresinol diglucoside in flaxseeds. *J Agric Food Chem*. 2000;48(11):5216-9.

29. Milder IEJ, Arts ICW, van de Putte B, Venema DP, Hollman PCH. Lignan contents of Dutch plant foods: a database including lariciresinol, pinoresinol, secoisolariciresinol and matairesinol. *Br J Nutr*. 2005;93(3):393-402.

30. López-Biedma A, Sánchez-Quesada C, Delgado-Rodríguez M, Gaforio JJ. The biological activities of natural lignans from olives and virgin olive oils: A review. *J Funct Foods*. 2016;26:36-47.

31. Owen RW, Giacosa A, Hull WE, Haubner R, Spiegelhalder B, Bartsch H. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil. *Eur J Cancer*. 2000;36(10):1235-47.

32. Owen RW, Mier W, Giacosa A, Hull WE, Spiegelhalder B, Bartsch H. Identification of lignans as major components in the phenolic fraction of olive oil. *Clin Chem*. 2000;46(7):976-88.

33. Brenes M, Hidalgo FJ, García A, et al. Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *J Am Oil Chem Soc*. 2000;77(7):715-720.

34. Tetens I, Turrini A, Tapanainen H, et al. Dietary intake and main sources of plant lignans in five European countries. *Food Nutr Res*. 2013;57.

35. Gaya P, Peirotén Á, Medina M, Landete JM. Bifidobacterium adolescentis INIA P784: The first probiotic bacterium capable of producing enterodiol from lignan extracts. *J Funct Foods*. 2017;29:269-274.

36. Xie L-H, Akao T, Hamasaki K, et al. Biotransformation of Pinoresinol Diglucoside to Mammalian Lignans by Human Intestinal Microflora, and Isolation of Enterococcus faecalis Strain PDG-1 Responsible for the Transformation of (+)-Pinoresinol to (+)-Lariciresinol. *Chem Pharm Bull (Tokyo)*. 2003;51(5):508-515.

37. Possemiers S, Bolca S, Eeckhaut E, Depypere H, Verstraete W. Metabolism of isoflavones, lignans and prenylflavonoids by intestinal bacteria: producer phenotyping and relation with intestinal community. *FEMS Microbiol Ecol*. 2007;61(2):372-383.

38. Liu Z, Saarinen NM, Thompson LU. Sesamin is one of the major precursors of mammalian lignans in sesame seed (Sesamum indicum) as observed in vitro and in rats.

J Nutr. 2006;136(4):906-12.

39. Thompson LU, Boucher BA, Liu Z, Cotterchio M, Kreiger N. Phytoestrogen Content of Foods Consumed in Canada, Including Isoflavones, Lignans, and Coumestan. *Nutr Cancer*. 2006;54(2):184-201.

40. Hu C, Yuan Y V., Kitts DD. Antioxidant activities of the flaxseed lignan secoisolariciresinol diglucoside, its aglycone secoisolariciresinol and the mammalian lignans enterodiol and enterolactone in vitro. *Food Chem Toxicol*. 2007;45(11):2219-2227.

41. Prasad K. Hydroxyl radical-scavenging property of secoisolariciresinol diglucoside (SDG) isolated from flax-seed. *Mol Cell Biochem*. 1997;168(1-2):117-123.

42. Prasad K. Antioxidant activity of secoisolariciresinol diglucoside-derived metabolites, secoisolariciresinol, Enterodiol, and enterolactone. *Int J Angiol*. 2000;9(4):220-225.

43. Kitts DD, Yuan YV, Wijewickreme AN, Thompson LU. Antioxidant activity of the flaxseed lignan secoisolariciresinol diglycoside and its mammalian lignan metabolites enterodiol and enterolactone. *Mol Cell Biochem*. 1999;202(1/2):91-100.

44. Hutchins AM, Martini MC, Olson BA, Thomas W, Slavin JL. Flaxseed Consumption Influences Endogenous Hormone Concentrations in Postmenopausal Women. *Nutr Cancer*. 2001;39(1):58-65.

45. Pianjing P, Thiantanawat A, Rangkadilok N, Watcharasit P, Mahidol C, Satayavivad J. Estrogenic Activities of Sesame Lignans and Their Metabolites on Human Breast Cancer Cells. *J Agric Food Chem*. 2011;59(1):212-221.

46. Adlercreutz H, Bannwart C, Wähälä K, et al. Inhibition of human aromatase by mammalian lignans and isoflavonoid phytoestrogens. *J Steroid Biochem Mol Biol*. 1993;44(2):147-53.

47. Prasad K. Suppression of phosphoenolpyruvate carboxykinase gene expression by secoisolariciresinol diglucoside (SDG), a new antidiabetic agent. *Int J Angiol*. 2002;11(2):107-109.

48. Wang W, Liu LQ, Higuchi CM, Chen H. Induction of NADPH:quinone reductase by dietary phytoestrogens in colonic Colo205 cells. *Biochem Pharmacol*. 1998;56(2):189-95.

49. Hemmings SJ, Barker L. The effects of dietary flaxseed on the Fischer 344 rat: I. Development, behaviour, toxicity and the activity of liverγ-glutamyltranspeptidase. *Cell Biochem Funct*. 2004;22(2):113-121.

50. Imran M, Ahmad N, Anjum FM, et al. Potential protective properties of flax lignan secoisolariciresinol diglucoside. *Nutr J*. 2015;14:71.

51. Prasad K. A Study on Regression of Hypercholesterolemic Atherosclerosis in Rabbits by Flax Lignan Complex. *J Cardiovasc Pharmacol Ther*. 2007;12(4):304-313.

52. Prasad K. Serum biochemical changes in rabbits on a regular diet with and without flax lignan complex following a high-cholesterol diet. *Int J Angiol*. 2008;17(1):27-32.

53. Prasad K. Flax Lignan Complex Slows Down the Progression of Atherosclerosis in Hyperlipidemic Rabbits. *J Cardiovasc Pharmacol Ther*. 2009;14(1):38-48.

54. Penumathsa S V., Koneru S, Thirunavukkarasu M, Zhan L, Prasad K, Maulik N. Secoisolariciresinol Diglucoside: Relevance to Angiogenesis and Cardioprotection against Ischemia-Reperfusion Injury. *J Pharmacol Exp Ther*. 2006;320(2):951-959.

55. Penumathsa SV, Koneru S, Zhan L, et al. Secoisolariciresinol diglucoside induces neovascularization-mediated cardioprotection against ischemia–reperfusion injury in hypercholesterolemic myocardium. *J Mol Cell Cardiol*. 2008;44(1):170-179.

56. Felmlee MA, Woo G, Simko E, Krol ES, Muir AD, Alcorn J. Effects of the flaxseed

lignans secoisolariciresinol diglucoside and its aglycone on serum and hepatic lipids in hyperlipidaemic rats. *Br J Nutr*. 2009;102(3):361.

57. Sano T, Oda E, Yamashita T, et al. Antithrombic and anti-atherogenic effects of partially defatted flaxseed meal using a laser-induced thrombosis test in apolipoprotein E and low-density lipoprotein receptor deficient mice. *Blood Coagul Fibrinolysis*. 2003;14(8):707-12.

58. Vanharanta M, Voutilainen S, Rissanen TH, Adlercreutz H, Salonen JT. Risk of Cardiovascular Disease–Related and All-Cause Death According to Serum Concentrations of Enterolactone. *Arch Intern Med*. 2003;163(9):1099.

59. Adlercreutz H, Mousavi Y, Clark J, et al. Dietary phytoestrogens and cancer: in vitro and in vivo studies. *J Steroid Biochem Mol Biol*. 1992;41(3-8):331-337.

60. Erkkilä AT, Herrington DM, Mozaffarian D, Lichtenstein AH. Cereal fiber and whole-grain intake are associated with reduced progression of coronary-artery atherosclerosis in postmenopausal women with coronary artery disease. *Am Heart J*. 2005;150(1):94-101.

61. van der Schouw YT, Sampson L, Willett WC, Rimm EB. The usual intake of lignans but not that of isoflavones may be related to cardiovascular risk factors in U.S. men. *J Nutr*. 2005;135(2):260-6.

62. Bloedon LT, Szapary PO. Flaxseed and Cardiovascular Risk. *Nutr Rev*. 2004;62(1):18-27.

63. Hallund J, Tetens I, Bügel S, et al. Daily consumption for six weeks of a lignan complex isolated from flaxseed does not affect endothelial function in healthy postmenopausal women. *J Nutr*. 2006;136(9):2314-8.

64. Hallund J, Ravn-Haren G, Bügel S, Tholstrup T, Tetens I. A lignan complex isolated from flaxseed does not affect plasma lipid concentrations or antioxidant capacity in healthy postmenopausal women. *J Nutr*. 2006;136(1):112-6.

65. Clavel T, Henderson G, Alpert C-A, et al. Intestinal Bacterial Communities That Produce Active Estrogen-Like Compounds Enterodiol and Enterolactone in Humans. *Appl Environ Microbiol*. 2005;71(10):6077-6085.

66. Clavel T, Henderson G, Engst W, et al. Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside. *FEMS Microbiol Ecol*. 2006;55(3).

67. Clavel T, Borrmann D, Braune A, Doré J, Blaut M. Occurrence and activity of human intestinal bacteria involved in the conversion of dietary lignans. *Anaerobe*. 2006;12(3):140-7.

68. Roncaglia L, Amaretti A, Raimondi S, Leonardi A, Rossi M. Role of bifidobacteria in the activation of the lignan secoisolariciresinol diglucoside. *Appl Microbiol Biotechnol*. 2011;92(1):159-168.

69. Woting A, Clavel T, Loh G, Blaut M. Bacterial transformation of dietary lignans in gnotobiotic rats. *FEMS Microbiol Ecol*. 2010;72(3):507-514.

70. Jin J-S, Hattori M. Further Studies on a Human Intestinal Bacterium *Ruminococcus* sp. END-1 for Transformation of Plant Lignans to Mammalian Lignans. *J Agric Food Chem*. 2009;57(16):7537-7542.

71. Jin J-S, Hattori M. Human intestinal bacterium, strain END-2 is responsible for demethylation as well as lactonization during plant lignan metabolism. *Biol Pharm Bull*. 2010;33(8):1443-7.

72. Clavel T, Lippman R, Gavini F, Doré J, Blaut M. Clostridium saccharogumia sp. nov. and Lactonifactor longoviformis gen. nov., sp. nov., two novel human faecal bacteria involved in the conversion of the dietary phytoestrogen secoisolariciresinol diglucoside. *Syst Appl Microbiol*. 2007;30(1):16-26.

73. Jin J-S, Zhao Y-F, Nakamura N, Akao T, Kakiuchi N, Hattori M. Isolation and

characterization of a human intestinal bacterium, Eubacterium sp. ARC-2, capable of demethylating arctigenin, in the essential metabolic process to enterolactone. *Biol Pharm Bull*. 2007;30(5):904-11.

74. Xie L-H, Akao T, Hamasaki K, Deyama T, Hattori M. Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and isolation of Enterococcus faecalis strain PDG-1 responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol. *Chem Pharm Bull (Tokyo)*. 2003;51(5):508-15.

75. Guarner F, Perdigon G, Corthier G, Salminen S, Koletzko B, Morelli L. Should yoghurt cultures be considered probiotic? *Br J Nutr*. 2005;93(6):783.

76. Marchesi JR, Adams DH, Fava F, et al. The gut microbiota and host health: a new clinical frontier. *Gut*. 2016;65(2):330-339.

77. Williamson G, Clifford MN. Role of the small intestine, colon and microbiota in determining the metabolic fate of polyphenols. *Biochem Pharmacol*. March 2017.

78. Bäckhed F, Ley RE, Sonnenburg JL, Peterson DA, Gordon JI. Host-Bacterial Mutualism in the Human Intestine. *Science (80-)*. 2005;307(5717).

79. Bäckhed F, Fraser CM, Ringel Y, et al. Defining a Healthy Human Gut Microbiome: Current Concepts, Future Directions, and Clinical Applications. *Cell Host Microbe*. 2012;12(5):611-622.

80. Claesson MJ, Jeffery IB, Conde S, et al. Gut microbiota composition correlates with diet and health in the elderly. 2012.

81. Karlsson F, Tremaroli V, Nielsen J, Backhed F. Assessing the Human Gut Microbiota in Metabolic Diseases. *Diabetes*. 2013;62(10):3341-3349.

82. Sartor RB. Genetics and Environmental Interactions Shape the Intestinal Microbiome to Promote Inflammatory Bowel Disease Versus Mucosal Homeostasis. *Gastroenterology*.

2010;139(6):1816-1819.

83. Sartor RB. Microbial Influences in Inflammatory Bowel Diseases. *Gastroenterology*. 2008;134(2):577-594.

84. Honda K, Littman DR. The Microbiome in Infectious Disease and Inflammation. *Annu Rev Immunol*. 2012;30(1):759-795.

85. Li DY, Tang WHW. Gut Microbiota and Atherosclerosis. *Curr Atheroscler Rep*. 2017;19(10):39.

86. Wallace BD, Wang H, Lane KT, et al. Alleviating cancer drug toxicity by inhibiting a bacterial enzyme. *Science*. 2010;330(6005):831-5.

87. FAO/WHO. Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food; Ontario, Canada. April 30, May 1, 2002.

88. Roberfroid M, Gibson GR, Hoyles L, et al. Prebiotic effects: metabolic and health benefits. *Br J Nutr*. 2010;104(S2):S1-S63.

89. Gibson GR, Roberfroid MB, Kamm M, et al. Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr*. 1995;125(6):1401-12.

90. Steinert RE, Sadabad MS, Harmsen HJM, Weber P. The prebiotic concept and human health: a changing landscape with riboflavin as a novel prebiotic candidate? *Eur J Clin Nutr*. 2016;(April):1-6.

91. Parkar SG, Trower TM, Stevenson DE. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe*. 2013;23:12-19.

92. Peters U, Poole C, Arab L. Does tea affect cardiovascular disease? A meta-analysis. *Am J Epidemiol*. 2001;154(6):495-503.

93. Peterson J, Dwyer J, Adlercreutz H, Scalbert A, Jacques P, McCullough ML. Dietary lignans: physiology and potential for cardiovascular disease risk reduction. *Nutr Rev*. 2010;68(10):571-603.

94. Krauss RM, Eckel RH, Howard B, et al. AHA Dietary Guidelines: revision 2000: A statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation*. 2000;102(18):2284-99.

95. de la Fuente-Arrillaga C, Vázquez Ruiz Z, Bes-Rastrollo M, Sampson L, Martinez-González MA. Reproducibility of an FFQ validated in Spain. *Public Health Nutr*. 2010;13(9):1364-1372.

96. Martínez-González MA, Fernández-Jarne E, Serrano-Martínez M, Wright M, Gomez-Gracia E. Development of a short dietary intake questionnaire for the quantitative estimation of adherence to a cardioprotective Mediterranean diet. *Eur J Clin Nutr*. 2004;58(11):1550-1552.

97. Elosua R, Marrugat J, Molina L, Pons S, Pujol E. Validation of the Minnesota Leisure Time Physical Activity Questionnaire in Spanish men. The MARATHOM Investigators. *Am J Epidemiol*. 1994;139(12):1197-209.

98. Fitó M, Guxens M, Corella D, et al. Effect of a traditional Mediterranean diet on lipoprotein oxidation: a randomized controlled trial. *Arch Intern Med*. 2007;167(11):1195-203.

99. Salas-Salvadó J, Fernández-Ballart J, Ros E, et al. Effect of a Mediterranean diet supplemented with nuts on metabolic syndrome status: one-year results of the PREDIMED randomized trial. *Arch Intern Med*. 2008;168(22):2449-58.

100. Martínez-González MA, García-Arellano A, Toledo E, et al. A 14-item Mediterranean diet assessment tool and obesity indexes among high-risk subjects: the PREDIMED trial. Peiró C, ed. *PLoS One*. 2012;7(8):e43134.

101. García-Calzón S, Gea A, Razquin C, et al. Longitudinal association of telomere length and obesity indices in an intervention study with a Mediterranean diet: the PREDIMED-NAVARRA trial. *Int J Obes (Lond)*. 2014;38(2):177-82.

102. Salas-Salvadó J, Bulló M, Babio N, et al. Reduction in the incidence of type 2 diabetes with the Mediterranean diet: results of the PREDIMED-Reus nutrition intervention randomized trial. *Diabetes Care*. 2011;34(1):14-9.

103. Delisle H, Batal M. The double burden of malnutrition associated with poverty. *Lancet*. 2016;387(10037):2504-2505.

104. Grijalva-Eternod CS, Wells JCK, Cortina-Borja M, et al. The Double Burden of Obesity and Malnutrition in a Protracted Emergency Setting: A Cross-Sectional Study of Western Sahara Refugees. Grais RF, ed. *PLoS Med*. 2012;9(10):e1001320.

105. Bourke CD, Berkley JA, Prendergast AJ. Immune Dysfunction as a Cause and Consequence of Malnutrition. *Trends Immunol*. 2016;37(6):386-398.

106. Gordon JI, Dewey KG, Mills DA, Medzhitov RM. The Human Gut Microbiota and Undernutrition. *Sci Transl Med*. 2012;4(137):137ps12-137ps12.

107. Yatsunenko T, Rey FE, Manary MJ, et al. Human gut microbiome viewed across age and geography. *Nature*. 2012;486(7402):222-7.

108. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: food sources and bioavailability. *Am J Clin Nutr*. 2004;79(5):727-747.

109. Boto-Ordóñez M, Urpi-Sarda M, Queipo-Ortuño MI, Tulipani S, Tinahones FJ, Andres-Lacueva C. High levels of Bifidobacteria are associated with increased levels of anthocyanin microbial metabolites: a randomized clinical trial. *Food Funct*. 2014;5(8):1932-8.

110. Kechagia M, Basoulis D, Konstantopoulou S, et al. Health benefits of probiotics: a

review. *ISRN Nutr*. 2013;2013:481651.

111. Fung TT, Rimm EB, Spiegelman D, et al. Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. *Am J Clin Nutr*. 2001;73(1):61-67.

112. Jacobs DRJ, Steffen LM. Nutrients, foods, and dietary patterns as exposures in research: a framework for food synergy. *Am J Clin Nutr*. 2003;78(3):508S-513.

113. Babio N, Becerra-Tomás N, Martínez-González MÁ, et al. Consumption of Yogurt, Low-Fat Milk, and Other Low-Fat Dairy Products Is Associated with Lower Risk of Metabolic Syndrome Incidence in an Elderly Mediterranean Population. *J Nutr*. 2015;145(10):2308-16.

114. Díaz-López A, Bulló M, Martínez-González MA, et al. Dairy product consumption and risk of type 2 diabetes in an elderly Spanish Mediterranean population at high cardiovascular risk. *Eur J Nutr*. 2015;55(1):349-360.

115. Martínez-González MÁ, Corella D, Salas-Salvadó J, et al. Cohort profile: design and methods of the PREDIMED study. *Int J Epidemiol*. 2012;41(2):377-85.

116. Predimed.es - Home. 2003.

117. Estruch R, Ros E, Salas-Salvadó J, et al. Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med*. 2013;368(14):1279-90.

118. Fernández-Ballart JD, Piñol JL, Zazpe I, et al. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *Br J Nutr*. 2010;103(12):1808-16.

119. Martínez-González MA, Fernández-Jarne E, Serrano-Martínez M, Wright M, Gomez-Gracia E. Development of a short dietary intake questionnaire for the quantitative estimation of adherence to a cardioprotective Mediterranean diet. *Eur J Clin Nutr*.

2004;58(11):1550-2.

120. Elosua R, Garcia M, Aguilar A, Molina L, Covas MI, Marrugat J. Validation of the Minnesota Leisure Time Physical Activity Questionnaire In Spanish Women. Investigators of the MARATDON Group. *Med Sci Sports Exerc*. 2000;32(8):1431-7.

121. O'Brien E, Waeber B, Parati G, Staessen J, Myers MG. Blood pressure measuring devices: recommendations of the European Society of Hypertension. *BMJ*. 2001;322(7285):531-6.

122. Warnick GR, Knopp RH, Fitzpatrick V, Branson L. Estimating low-density lipoprotein cholesterol by the Friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints. *Clin Chem*. 1990;36(1):15-9.

123. Mataix J. *Tablas de Composición de Alimentos [Food Composition Tables]*. 4th ed. Granada: Universidad de Granada; 2003.

124. Scalbert A. Phenol-Explorer.

125. SAS version 9.3 [computer program]. SAS Institute Inc, Cary NC; 2011.

126. de Pascual-Teresa S, Moreno D a., García-Viguera C. Flavanols and Anthocyanins in Cardiovascular Health: A Review of Current Evidence. *Int J Mol Sci*. 2010;11(4):1679-1703.

127. Zhang W, Wang X, Liu Y, et al. Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects. *Br J Nutr*. 2008;99(6):1301-9.

128. Adolphe JL, Whiting SJ, Juurlink BHJ, Thorpe LU, Alcorn J. Health effects with consumption of the flax lignan secoisolariciresinol diglucoside. *Br J Nutr*. 2010;103(7):929-938.

129. During A, Debouche C, Raas T, Larondelle Y. Among plant lignans, pinoresinol has the

strongest antiinflammatory properties in human intestinal Caco-2 cells. *J Nutr*. 2012;142(10):1798-805.

130. Pellegrini N, Valtueña S, Ardigò D, et al. Intake of the plant lignans matairesinol, secoisolariciresinol, pinoresinol, and lariciresinol in relation to vascular inflammation and endothelial dysfunction in middle age-elderly men and post-menopausal women living in Northern Italy. *Nutr Metab Cardiovasc Dis*. 2010;20(1):64-71.

131. Milder IE, Feskens EJ, Arts IC, Bueno-de-Mesquita HB, Hollman PC, Kromhout D. Intakes of 4 dietary lignans and cause-specific and all-cause mortality in the Zutphen Elderly Study. *Am J Clin Nutr*. 2006;84(2):400-405.

132. Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. *Pharmacol Res*. 2013;69(1):52-60.

133. Lee HC, Jenner AM, Low CS, Lee YK. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Res Microbiol*. 2006;157(9):876-84.

134. Rastmanesh R. High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction. *Chem Biol Interact*. 2011;189(1-2):1-8.

135. Parkar SG, Trower TM, Stevenson DE. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe*. 2013;23:12-9.

136. Kemperman RA, Bolca S, Roger LC, Vaughan EE. Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities. *Microbiology*. 2010;156(Pt 11):3224-31.

137. Etxeberria U, Fernández-Quintela A, Milagro FI, Aguirre L, Martínez JA, Portillo MP. Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. *J Agric Food Chem*. 2013;61(40):9517-33.

138. Brigidi P, Swennen E, Vitali B, Rossi M, Matteuzzi D. PCR detection of

Bifidobacterium strains and Streptococcus thermophilus in feces of human subjects after oral bacteriotherapy and yogurt consumption. *Int J Food Microbiol*. 2003;81(3):203-209.

139. Mater DDG, Bretigny L, Firmesse O, et al. Streptococcus thermophilus and Lactobacillus delbrueckii subsp. bulgaricus survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiol Lett*. 2005;250(2):185-7.

140. Elli M, Callegari ML, Ferrari S, et al. Survival of Yogurt Bacteria in the Human Gut. *Appl Environ Microbiol*. 2006;72(7):5113-5117.

141. Pei R, Martin DA, DiMarco DM, Bolling BW. Evidence for the Effects of Yogurt on Gut Health and Obesity. *Crit Rev Food Sci Nutr*. April 2015.

142. Glanville JM, Brown S, Shamir R, Szajewska H, Eales JF. The scale of the evidence base on the health effects of conventional yogurt consumption: findings of a scoping review. *Front Pharmacol*. 2015;6(October):1-12.

143. Cormier H, Thifault É, Garneau V, et al. Association between yogurt consumption, dietary patterns, and cardio-metabolic risk factors. *Eur J Nutr*. 2015.

144. Jacques PF, Wang H. Yogurt and weight management. *Am J Clin Nutr*. 2014;99(5 Suppl):1229S-34S.

145. Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain–gut–enteric microbiota axis. *Nat Rev Gastroenterol & Hepatol*. 2009;6(5):306-314.

146. Alcock J, Maley CC, Aktipis CA. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. *Bioessays*. 2014;36(10):940-949.

147. Zapata HJ, Quagliarello VJ. The microbiota and microbiome in aging: potential implications in health and age-related diseases. *J Am Geriatr Soc*. 2015;63(4):776-81.

148. Jacques PF, Tucker KL. Are dietary patterns useful for understanding the role of diet in

chronic disease? *Am J Clin Nutr*. 2001;73(1):1-2.

149. Jacobs DR. What comes first: the food or the nutrient? Executive summary of a symposium. *J Nutr*. 2014;144(4 Suppl):543S-546S.

150. Jacobs DR, Tapsell LC, Temple NJ. Food Synergy : The Key to Balancing the Nutrition Research Effort. 33(2):507-529.

151. Vallverdú-Queralt A, Arranz S, Medina-Remón A, Casals-Ribes I, Lamuela-Raventós RM. Changes in phenolic content of tomato products during storage. *J Agric Food Chem*. 2011;59(17):9358-65.

152. Oomah D. Flaxseed as a functional food source.

153. Bressa C, Bailén-Andrino M, Pérez-Santiago J, et al. Differences in gut microbiota profile between women with active lifestyle and sedentary women. Dasgupta S, ed. *PLoS One*. 2017;12(2):e0171352.

154. Harris K, Kassis A, Major G, Chou CJ. Is the Gut Microbiota a New Factor Contributing to Obesity and Its Metabolic Disorders? *J Obes*. 2012;2012:1-14.

155. Singh RK, Chang H-W, Yan D, et al. Influence of diet on the gut microbiome and implications for human health. *J Transl Med*. 2017;15(1):73.

156. Kemperman RA, Bolca S, Roger LC, Vaughan EE. Novel approaches for analysing gut microbes and dietary polyphenols: challenges and opportunities. *Microbiology*. 2010;156(11):3224-3231.

157. Letunic I, Bork P. Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res*. 2016;44(W1):W242-W245.

158. Eliasson C, Kamal-Eldin A, Andersson R, Åman P. High-performance liquid chromatographic analysis of secoisolariciresinol diglucoside and hydroxycinnamic acid

glucosides in flaxseed by alkaline extraction. *J Chromatogr A*. 2003;1012(2):151-159.

159. Juri Rappsilber †, Yasushi Ishihama †,‡ and, Mann* M. Stop and Go Extraction Tips for Matrix-Assisted Laser Desorption/Ionization, Nanoelectrospray, and LC/MS Sample Pretreatment in Proteomics. 2002.

160. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem*. 1987;162(1):156-159.

161. Chomczynski P, Sacchi N. The single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction: twenty-something years on. *Nat Protoc*. 2006;1(2):581-585.

162. Comeau AM, Douglas GM, Langille MGI. Microbiome Helper: a Custom and Streamlined Workflow for Microbiome Research. Eisen J, ed. *mSystems*. 2017;2(1):e00127-16.

163. LangilleLab. 16S Bacteria and Archaea Standard Operating Procedure.

164. Clemente JC, Ursell LK, Parfrey LW, Knight R. The Impact of the Gut Microbiota on Human Health: An Integrative View. *Cell*. 2012;148(6):1258-1270.

165. Roopchand DE, Carmody RN, Kuhn P, et al. Dietary Polyphenols Promote Growth of the Gut Bacterium Akkermansia muciniphila and Attenuate High-Fat Diet-Induced Metabolic Syndrome. *Diabetes*. 2015;64(8):2847-58.

166. Feng J, Tang H, Li M, et al. The abundance of fecal Faecalibacterium prausnitzii in relation to obesity and gender in Chinese adults. *Arch Microbiol*. 2014;196(1):73-77.

167. Miquel S, Martín R, Rossi O, et al. Faecalibacterium prausnitzii and human intestinal health. *Curr Opin Microbiol*. 2013;16(3):255-261.

168. Cani PD, de Vos WM. Next-Generation Beneficial Microbes: The Case of Akkermansia muciniphila. *Front Microbiol*. 2017;8:1765.

169. Brodmann T, Endo A, Gueimonde M, et al. Safety of Novel Microbes for Human Consumption: Practical Examples of Assessment in the European Union. *Front Microbiol*. 2017;8:1725.

170. Muñoz-González C, Moreno-Arribas MV, Rodríguez-Bencomo JJ, et al. Feasibility and application of liquid–liquid extraction combined with gas chromatography–mass spectrometry for the analysis of phenolic acids from grape polyphenols degraded by human faecal microbiota. *Food Chem*. 2012;133(2):526-535.

171. Lee HC, Jenner AM, Low CS, Lee YK. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Res Microbiol*. 2006;157(9):876-884.

172. Kemperman RA, Gross G, Mondot S, et al. Impact of polyphenols from black tea and red wine/grape juice on a gut model microbiome. *Food Res Int*. 2013;53(2):659-669.

173. Terlizzi ME, Gribaudo G, Maffei ME. UroPathogenic Escherichia coli (UPEC) Infections: Virulence Factors, Bladder Responses, Antibiotic, and Non-antibiotic Antimicrobial Strategies. *Front Microbiol*. 2017;8:1566.

174. Amézquita-López BA, Soto-Beltrán M, Lee BG, Yambao JC, Quiñones B. Isolation, genotyping and antimicrobial resistance of Shiga toxin-producing Escherichia coli. *J Microbiol Immunol Infect*. July 2017.

175. Sabouri S, Sepehrizadeh Z, Amirpour-Rostami S, Skurnik M. A minireview on the in vitro and in vivo experiments with anti-Escherichia coli O157:H7 phages as potential biocontrol and phage therapy agents. *Int J Food Microbiol*. 2017;243:52-57.

176. DeBoer MD, Lima AAM, Oría RB, et al. Early childhood growth failure and the developmental origins of adult disease: do enteric infections and malnutrition increase risk for the metabolic syndrome? *Nutr Rev*. 2012;70(11):642-653.

177. Kau AL, Ahern PP, Griffin NW, Goodman AL, Gordon JI. Human nutrition, the gut microbiome and the immune system. *Nature*. 2011;474(7351):327-36.

178. Goyal A, Sharma V, Upadhyay N, Gill S, Sihag M. Flax and flaxseed oil: an ancient medicine & modern functional food. *J Food Sci Technol*. 2014;51(9):1633-53.

179. Teodósio NR, Lago ES, Romani SA, Guedes RC. A regional basic diet from northeast Brazil as a dietary model of experimental malnutrition. *Arch Latinoam Nutr*. 1990;40(4):533-47.

180. Hutlab. Galaxy.

181. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. *Genome Biol*. 2011;12(6):R60.

182. BD Cytometric Bead Array (CBA) Mouse Inflammation Kit Instruction Manual.

183. Van Klinken BJ, Dekker J, Büller HA, Einerhand AW. Mucin gene structure and expression: protection vs. adhesion. *Am J Physiol*. 1995;269(5 Pt 1):G613-27.

184. Thim L. Trefoil peptides: from structure to function. *Cell Mol Life Sci*. 1997;53(11-12):888-903.

185. Wlodarska M, Willing B, Keeney KM, et al. Antibiotic Treatment Alters the Colonic Mucus Layer and Predisposes the Host to Exacerbated Citrobacter rodentium-Induced Colitis. *Infect Immun*. 2011;79(4):1536-1545.

186. Hollingsworth MA, Swanson BJ. Mucins in cancer: protection and control of the cell surface. *Nat Rev Cancer*. 2004;4(1):45-60.

187. Wlodarska M, Willing BP, Bravo DM, Finlay BB. Phytonutrient diet supplementation promotes beneficial Clostridia species and intestinal mucus secretion resulting in protection against enteric infection. *Sci Rep*. 2015;5:9253.

188. Scott KP, Antoine J-M, Midtvedt T, van Hemert S. Manipulating the gut microbiota to maintain health and treat disease. *Microb Ecol Health Dis*. 2015;26:25877.

189. Zarepoor L, Lu JT, Zhang C, et al. Dietary flaxseed intake exacerbates acute colonic mucosal injury and inflammation induced by dextran sodium sulfate. *Am J Physiol Gastrointest Liver Physiol*. 2014;306(12):G1042-55.

190. Power KA, Lepp D, Zarepoor L, et al. Dietary flaxseed modulates the colonic microenvironment in healthy C57Bl/6 male mice which may alter susceptibility to gut-associated diseases. *J Nutr Biochem*. 2016;28:61-69.

191. Lim MY, You HJ, Yoon HS, et al. The effect of heritability and host genetics on the gut microbiota and metabolic syndrome. *Gut*. 2017;66(6):1031-1038.

192. Eckburg PB, Bik EM, Bernstein CN, et al. Diversity of the Human Intestinal Microbial Flora.

Annex

RESEARCH
Original Research: Brief

Associations between Both Lignan and Yogurt Consumption and Cardiovascular Risk Parameters in an Elderly Population: Observations from a Cross-Sectional Approach in the PREDIMED Study

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ABSTRACT

Background The study of dietary patterns is gaining interest. Although the health benefits of yogurt and lignans have been investigated separately, to our knowledge there are no studies on their associative effects.

Objective The aim of this study was to evaluate a possible association between yogurt and lignans using biomarkers of cardiovascular disease risk in an elderly population.

Design We conducted a cross-sectional analysis of the association between baseline dietary information and cardiovascular risk parameters using food frequency questionnaires.

Participants We enrolled 7,169 Spanish participants of the PREDIMED (Prevención con Dieta Mediterránea) study (elderly men and women at high cardiovascular risk) from June 2003 to June 2009.

Main outcome measures Cardiovascular risk parameters, including cholesterol, triglycerides, glucose, body mass index, weight, waist circumference, and blood pressure were measured.

Statistical analysis General linear models were used to assess the relationship between categorical variables (yogurt, total dairy intake, lignans, and yogurt plus lignans) and cardiovascular risk parameters.

Results The consumption of either yogurt or lignans seems to have beneficial effects on human health, but the consumption of both showed greater improvement in some cardiovascular health parameters. Indeed, participants with a higher consumption of both yogurt and lignans showed lower total cholesterol (estimated β-coefficients= −6.18; P=0.001) and low-density lipoprotein cholesterol levels (β=−4.92; P=0.005). In contrast, participants with lower yogurt and lignan consumption had a higher body mass index (β=0.28; P=0.007) and weight (β=1.20; P=0.008).

Conclusions High lignan and yogurt consumption is associated with a better cardiovascular risk parameters profile in an elderly Mediterranean population. Further research is warranted to determine the mechanisms and consequences of this potential effect.

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POLYPHENOLS, SUCH AS LIGNANS,[1] ARE METABOlized by microbiota in the colon, and their metabolites have been shown to have health benefits. Some polyphenols have even been considered as prebiotics because of their ability to alter the microbiota profile and levels.[2] The main lignan polyphenols are pinoresinol, matairesinol, secoisolariciresinol, 1-acetoxypinoresinol, lariciresinol, syringaresinol, and isolariciresinol (Figure). Lignan intake has been related to beneficial health effects, including prevention of cancer and cardiovascular diseases.[3] Flaxseed and other seeds have high lignan concentrations, as do some fruits and vegetables, as well as beverages, such as wine, coffee, and tea.[4]

Probiotics are live microorganisms that confer health benefits on the host when administered in adequate amounts.[5]

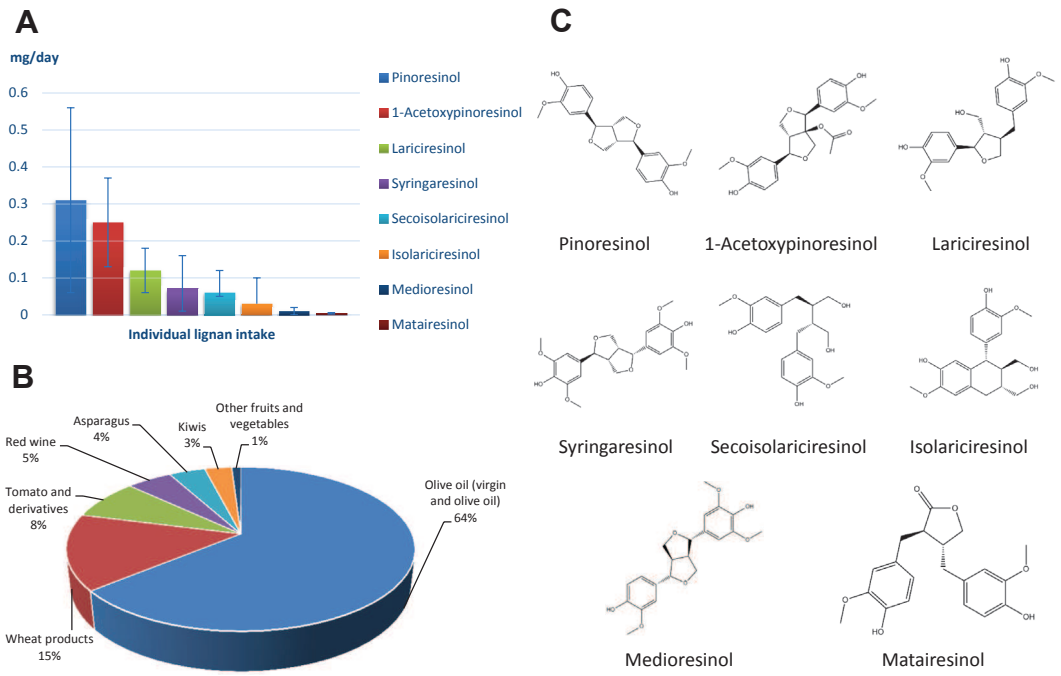

Figure. Lignan intake of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED cohort at baseline. (A) Mean intake of each individual lignan: pinoresinol, 1-acetoxypinoresinol, lariciresinol, syringaresinol, secoisolariciresinol, isolariciresinol, medioresinol and matairesinol. (B) Percentage of the lignan food sources. (C) Chemical structures of lignan compounds studied.

Most of the probiotics currently consumed by humans come from fermented dairy products, such as yogurt produced using cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.[6] The benefits attributed to probiotics include the prevention and management of diarrhea, enhancement of immune response, and improved lactose digestion and absorption.[7]

Because nutrients and foods are consumed in combination, nutritional epidemiology recognizes the importance of studying the effect of dietary patterns on health.[8] Food synergy is defined as additive or more than additive influences of foods and food constituents on health, linking dietary patterns and foods with disease prevention.[9]

Previous studies carried out within the PREDIMED (Prevención con Dieta Mediterránea) framework have shown an association between yogurt consumption and a decrease in the incidence of the metabolic syndrome and type 2 diabetes.[10,11] However, the associative effects of yogurt and lignan consumption have not been studied to date. The aim of this work was to assess the health benefits of lignans and yogurt consumption on cardiovascular risk parameters, such as the lipid profile, glycemic profile, body mass index, and blood pressure in a well-characterized elderly population.

METHODS

Study Design

A cross-sectional study was performed using baseline data from the PREDIMED cohort. A detailed description of the study has been published elsewhere.[12,13] Baseline data collection was carried out in Spain from June 2003 to June 2009. Briefly, the PREDIMED study was a large prospective, multicenter, randomized, controlled trial aimed to assess the effect of the traditional Mediterranean diet on the primary prevention of clinical cardiovascular events in elderly participants at high risk and was undertaken from October 2003 to December 2010. The 7,447 eligible participants were randomized to one of the following intervention groups: Mediterranean diet supplemented with extra virgin olive oil, Mediterranean diet supplemented with nuts, or a control diet (low-fat diet) group. The trial was stopped after a median follow-up of 4.8 years after determining the benefits of the Mediterranean diets in the prevention of major cardiovascular events (myocardial infarction, stroke, or cardiovascular death) compared to the low-fat group.[14]

This study was conducted according to the guidelines of the Declaration of Helsinki and all procedures involving human participants and patients were approved by the Institutional Review Boards of the participating centers (clinical trial registration: ISRCTN of London, England: 35739639). Written informed consent was obtained from all the participants.

Population Characteristics, Cardiovascular Risk Parameters, Anthropometric Measures, and Diet

Of the 7,447 participants, 278 were excluded: 275 because they had not completed the food frequency questionnaire (FFQ) at baseline, and in 3 participants the high-density lipoprotein (HDL) cholesterol values were missing. The final number of participants included was 7,169.

To assess the diet and lifestyle characteristics of the study population, the participants filled out the following validated questionnaires: a 137-item semi-quantitative FFQ,[15] a

14-point questionnaire on adherence to the traditional Mediterranean diet,[16] and the Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire.[17] The participants also completed a general questionnaire to provide information about lifestyle habits, concurrent diseases, and medication use.

Body weight and height were measured with minimum clothing and no shoes using calibrated scales and wall-mounted stadiometers, respectively. Blood pressure was measured in triplicate in a sitting position using a semi-automatic sphygmomanometer (Omron HEM-705CP), with a 5-minute interval between each measurement, and recording the mean of the three values according to the procedure recommended by the European Hypertension Society.[18] Biochemical analyses were performed in local laboratories. Glucose was measured by the glucose–oxidase method, cholesterol by esterase–oxidase–peroxidase, triglycerides by glycerol–phosphate oxidase–peroxidase, and HDL cholesterol by direct measurement. All the local laboratories fulfilled the external quality-control requirements. When triglycerides were <300 mg/dL (3.39 mmol/L), low-density lipoprotein (LDL) cholesterol was calculated with the Friedewald formula.[19] A concordance study of nine laboratories was conducted. A mean of 200 samples from each laboratory was analyzed for total cholesterol, HDL cholesterol, and triglycerides. The Medical Research Institute of the Mar Laboratory, which uses ABX-Horiba commercial kits in a PENTRA-400 autoanalyzer (ABX-Horiba), was used as the reference. One center was unable to provide samples for the concordance study. The concordance analysis of lipid measurements showed, respectively, an r^2 between 0.85 (95% CI 0.77 to 0.90) and 0.97 (95% CI 0.95 to 0.98) for total cholesterol; between 0.82 (95% CI 0.78 to 0.83) and 0.92 (95% CI 0.89 to 0.95) for HDL cholesterol; between 0.81 (95% CI 0.73 to 0.87) and 0.99 (95% CI 0.99 to 0.99) for triglycerides; and between 0.82 (95% CI 0.74 to 0.88) and 0.96 (95% CI 0.99 to 0.99) for glucose.

Categories of Lignan and Yogurt Consumption

Total energy and nutrient intake were calculated using Spanish food composition tables.[20] Lignan intake was calculated by multiplying the content of lignans in a particular food item (mg/g) by the daily consumption of this food (g/day). Data regarding the lignan content in foods were obtained from the Phenol-Explorer database.[21] Values of lignan intake were divided into low or high with the median being the cut point, or into tertiles, depending on the analysis.

The FFQ included questions concerning the consumption of dairy products. In the validation study, the intra-class correlation coefficient between dairy product consumption from the FFQ and repeated food records was 0.84.[15] Responses to individual dairy items of the FFQ were converted to mean daily consumption (g/day) and categorized into total yogurt (including full-fat and low-fat) and total dairy intake without yogurt (including all types of milk, cheeses, custard, whipped cream, and ice cream). The consumptions were then divided into the following categories: 0 yogurt/day, from 0 to <1 yogurt/day, and ≥1 yogurt/day, or tertiles, depending on the analysis. The total dairy consumption was divided in tertiles.

Lignan, dairy, and other nutrient intake were adjusted for total energy intake because it is associated with disease risk and is usually proportional to nutrient intake.

Statistical Analysis

Descriptive analyses were conducted to compare the baseline characteristics across categories of yogurt consumption at baseline. Values are presented as mean±standard deviation for continuous variables and frequencies (and percentages) for categorical variables. For continuous variables, the differences between groups were analyzed using an analysis of variance test. The χ^2 test was used for categorical data.

General linear models were used to assess the relationship between categorical exposure variables (lignans, yogurt, total dairy products, and yogurt plus lignans) and cholesterol, triglycerides, blood pressure, glucose, and weight. Multivariate models were adjusted for recruitment center, sex, age, smoking, soft drinks, carbohydrates, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, n-3 fatty acids, family history of heart disease, diabetes, hypertension, and dairy, fiber, and energy intake. A test for linear trend was performed with the use of the resulting variable as a continuous variable.

Given the prebiotic nature of lignans, it is plausible that yogurt consumption may have differential effects on cardiovascular risk parameters, depending on the intake of these polyphenolic compounds. Therefore, to test for statistical interactions between lignans and yogurt in different cardiovascular risk parameters, stratified analyses were performed and interaction *P* values were calculated.

All statistical analyses were conducted using SAS software.[22] All *t* tests were two-sided and *P* values <0.05 were considered significant.

RESULTS

The baseline characteristics of the study participants are summarized in Table 1. Around 23% of the population did not consume any yogurt (1,631 participants), 54% consumed <1 yogurt per day (3,840 participants), and 24% consumed ≥1 yogurts per day (1,698 participants).

Around 23% of the population did not consume any yogurt (1,631 participants), 54% consumed <1 yogurt per day (3,840 participants) and 24% consumed ≥1 yogurts per day (1,698 participants). The distribution of sex, smoking, level of education, energy expenditure during leisure time, age, and participants with hypertension and cholesterol was significantly different between groups. In contrast, participants with diabetes were equally distributed among the three yogurt groups. Participants with the highest yogurt intake also had the highest intake of carbohydrates, protein, and fiber, but the lowest cholesterol intake. Nonconsumers had higher blood pressure, glucose, and triglycerides, but lower body mass index (BMI; calculated as kg/m^2) and HDL cholesterol levels. There were no significant differences between yogurt consumption and Mediterranean diet adherence (Mediterranean diet score).

Lignans and Food Sources

The Figure shows the individual lignan intake and the chemical structures of the different lignans, as well as the main lignan food sources ingested by the PREDIMED cohort. The lignan most frequently consumed was pinoresinol (0.31±0.25 mg/day), followed by 1-acetoxypinoresinol (0.25±0.12 mg/day), lariciresinol (0.12±0.06 mg/day), syringaresinol (0.07±0.09 mg/day), secoisolariciresinol (0.06±0.06 mg/day),

Table 1. Baseline characteristics of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) cohort according to categories of yogurt consumption assessed by food frequency questionnaire adjusted for energy

Characteristic	Nonconsumers	<1 yogurt/day	≥1 yogurt/day	*P* value
	←——— *n (%)* ———→			
No. of participants (n=7,169)	1,631 (22.7)	3,840 (53.6)	1,698 (23.7)	
Sex, women	663 (40.6)	2,201 (57.3)	1,216 (71.6)	<0.001[a]
Smoking				<0.001[a]
Never	784 (48.0)	2,382 (62.0)	1,217 (71.7)	
Current	393 (24.1)	622 (16.2)	187 (11.0)	
Former	454 (27.8)	836 (21.8)	294 (17.3)	
Education				0.01[a]
University	68 (4.2)	148 (3.8)	55 (3.2)	
Secondary	309 (18.9)	754 (19.6)	271 (15.9)	
Elementary	1,254 (76.9)	2,938 (76.5)	1,372 (80.8)	
Arterial hypertension[b]	1,310 (80.3)	3,197 (83.3)	1,420 (83.6)	0.01[a]
Diabetes[c]	813 (49.8)	1,808 (47.08)	848 (49.9)	0.06[a]
Hypercholesterolemia[d]	1,134 (69.5)	2,804 (73.0)	1,247 (73.4)	0.01[a]
	←——— *mean±standard deviation* ———→			
Age (y)	67.3±6.2	66.8±6.1	67.5±6.0	<0.001[e]
Energy expenditure[f] **(MET**[g]**-h/day)**	4.0±4.2	3.9±4.0	3.7±3.7	0.04[e]
Dietary pattern (g/day)				
Mediterranean diet adherence score	8.5±1.9	8.7±1.9	8.7±1.9	0.61[e]
Total dairy	279.0±207.1	378.9±204.1	494.9±228.2	<0.001[e]
Yogurt, total	0.0±0.0	65.3±38.6	196.6±94.6	<0.001[e]
Low-fat yogurt	0.0± 0.0	44.5±44.2	142.3±116.1	<0.001[e]
Milk, total	235.5±194.1	266.7±185.8	271.6±184.0	0.37[e]
Low-fat milk	174.8±194.9	221.1±198.5	234.9±196.1	0.02[e]
Cream and whipped cream	0.39±3.39	0.65±5.79	0.30±2.0	0.01[e]
Cheese	15.1±17.1	14.6±15.6	12.6±15.7	<0.001[e]
Low-fat cheese	11.2±21.5	13.7±18.9	17.2±23.0	<0.001[e]
Dairy desserts	13.7±43.3	10.5±26.1	9.1±28.5	0.07[e]
Other dairy[h]	1.75±6.7	1.76±5.3	1.97±6.7	0.23[e]
Soft drinks	21.7±72.6	18.7±63.8	14.1±47.5	0.008[e]
Nutrient intake[i]				
Total energy (kcal/day)	2,300±600	2,351.6±581.6	2,046.6±483.5	<0.001[e]
Carbohydrates (g/day)	234.7±46.3	238.0±42.9	242.6±36.0	<0.001[e]
Protein (g/day)	87.8±14.4	92.3±13.9	96.6±13.4	<0.001[e]
SFA[j] (g/day)	25.8±6.4	25.3±6.0	24.4±5.1	<0.001[e]
MUFA[k] (g/day)	50.1±11.6	48.8±11.3	47.2±10.4	<0.001[e]
PUFA[l] (g/day)	16.2±5.5	15.8±5.3	15.2±4.7	<0.001[e]
Fiber (g/day)	24.6±7.6	25.5±7.8	26.3±7.0	<0.001[e]
Total cholesterol (mg/day)	368.9±111.1	368.9±116.5	357.5±91.9	<0.001[e]
n-3 fatty acids (g/day)	2.2±0.79	2.2±0.80	2.2±0.73	0.07[e]
Lignans (mg/day)	0.59±0.2	0.60±0.2	0.61±0.2	0.04[e]

(*continued on next page*)

Table 1. Baseline characteristics of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) cohort according to categories of yogurt consumption assessed by food frequency questionnaire adjusted for energy (*continued*)

Characteristic	Nonconsumers	<1 yogurt/day	≥1 yogurt/day	*P* value
	←——————*mean±standard deviation*——————→			
Cardiovascular risk parameters				
Body mass index	29.6±3.5	29.9±3.7	30.0±3.7	0.50[e]
Systolic blood pressure (mm Hg)	149.7±19.1	148.6±19.0	148.2±19.1	0.40[e]
Diastolic blood pressure (mm Hg)	83.2±10.2	82.9±10.0	82.2±10.5	0.01[e]
Glucose (mg/dL)[m]	123.5±39.8	121.1±42.0	122.1±41.3	0.44[e]
Lipid profile (mg/dL)				
Total cholesterol[n]	210.7±38.3	210.7±38.1	212.2±38.2	0.22[e]
HDL cholesterol[n]	52.8±13.0	53.8±14.3	55.6±13.9	<0.001[e]
LDL cholesterol[n]	130.3±33.5	130.2±33.4	130.2±34.4	0.99[e]
Triglycerides[o]	142.1±79.1	136.8±83.7	132.5±67.3	0.08[e]

[a] χ^2 tests.
[b] Arterial hypertension was defined as systolic blood pressure ≥140 mm Hg, diastolic blood pressure ≥90 mm Hg, or taking antihypertensive medication.
[c] Diabetes was diagnosed when fasting plasma glucose concentrations of ≥126.1 mg/dL (≥7.0 mmol/L), 2-hour plasma glucose concentrations of ≥200.0 mg/dL (≥11.1 mmol/L) after an oral dose of 75 g glucose, or insulin treatment.
[d] Hypercholesterolemia was defined as LDL-cholesterol ≥160 mg/dL (4.16 mmol/L), HDL-cholesterol ≤40 mg/dL (1.04 mmol/L), or antihyperlipidemic medication.
[e] One-way analysis of variance tests.
[f] In physical activity at leisure time.
[g] MET=metabolic equivalent.
[h] Cream cheese and condensed milk.
[i] Food frequency questionnaire was used to estimate the dietary pattern by multiplying the frequency of consumption of all food items by the mean portion size using Spanish food composition tables and was carried out by trained dietitians.
[j] SFA=saturated fatty acids.
[k] MUFA=monounsaturated fatty acids.
[l] PUFA=polyunsaturated fatty acids.
[m] To convert mg/dL glucose to mmol/L, multiply by 0.0555. To convert mmol/L glucose to mg/dL, multiply mmol/L by 18.0. Glucose of 100 mg/dL=5.55 mmol/L.
[n] To convert mmol/L cholesterol to mg/dL, multiply by 38.7. To convert mg/dL cholesterol to mmol/L, multiply mg/dL by 0.0259. Cholesterol of 193 mg/dL=5.00 mmol/L.
[o] To convert mg/dL triglycerides to mmol/L, multiply mg/dL by 0.0113. To convert mmol/L triglycerides to mg/dL, multiply mmol/L by 88.6. Triglyceride of 159 mg/dL=1.80 mmol/L.

isolariciresinol (0.03±0.07 mg/day), medioresinol (0.01±0.01 mg/day), and matairesinol (0.004±0.002 mg/day). The main lignan food sources were olive oil (>60%), wheat products (about 15%), tomatoes and derivatives (8%), red wine (5%), asparagus (4%), kiwis (3%), and other fruits and vegetables. Table 2 (available online at www.andjrnl.org) shows the main food sources of each lignan.

Lignan Intake and Cardiovascular Risk Parameters

Table 3 shows the relationship between lignan intake and cardiovascular risk parameters. Participants with the highest (>0.67 mg/day) and medium (0.46 to 0.67 mg/day) lignan intakes had significantly lower plasma glucose levels (estimated β-coefficients=−6.08; $P<0.001$ and $\beta=-4.16$; $P=0.002$, respectively) compared to those with the lowest lignan intake (P trend=0.02). No significant associations were observed for other cardiovascular risk parameters across the lignan groups.

Total Yogurt, Full-Fat Yogurt, Low-Fat Yogurt, or Dairy Intake and Cardiovascular Risk Parameters

The associations between the intake of yogurt, full-fat yogurt, low-fat yogurt, or other dairy products and Cardiovascular Risk Parameters are presented in Table 3. Participants consuming any kind of yogurt had significantly lower total cholesterol levels ($\beta=-2.92$, $P=0.02$ for <1 yogurt/day, and $\beta=-3.33$; $P=0.03$ for ≥1 yogurt/day; P trend=0.03) compared with nonconsumers. Those with the highest intake (≥1 yogurt/day) also had lower triglyceride levels ($\beta=-6.94$; $P=0.02$) compared to nonconsumers. In addition, in both groups, yogurt consumption was associated with higher weight ($\beta=0.90$; $P=0.004$ and $\beta=0.88$; $P=0.02$ for 1 yogurt/day and ≥1 yogurt/day, respectively; P trend=0.007).

Low intake of full-fat yogurt was associated with higher weight and higher diastolic blood pressure ($\beta=0.78$; $P=0.01$ and $\beta=0.81$; $P=0.02$, respectively). An intake of ≥1 full-fat yogurt/day was correlated with a decrease in triglyceride levels ($\beta=-9.33$; $P=0.03$). However, there were no significant differences in the other cardiovascular risk parameters. Regarding low-fat yogurt, consumers of 1 yogurt/day had lower total cholesterol values ($\beta=-4.40$; $P<0.001$), HDL cholesterol ($\beta=-1.05$; $P=0.01$), LDL cholesterol ($\beta=-3.80$; $P<0.001$), and diastolic blood pressure ($\beta=-0.076$; $P=0.02$), but a higher weight ($\beta=0.64$; $P=0.03$) compared with nonconsumers.

Finally, association between total dairy intake and cardiovascular risk parameters was examined. A total dairy intake of >500 g/day was associated with lower total cholesterol ($\beta=-4.36$; $P=0.002$) and diastolic blood pressure ($\beta=-0.78$; $P=0.04$),

Table 3. Association between yogurt, dairy, or lignans consumption and cardiovascular risk parameters of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) cohort

General linear models	Model[a]	β (95% CI)	*P* value	β (95% CI)	*P* value	*P* trend
Lignans intake		←——*Group 1 vs Group 0*[b]——→		←——*Group 2 vs Group 0*[b]——→		
Total cholesterol (mg/dL)[c]	1	1.08 (−1.26 to 3.42)	0.37	1.50 (−0.83 to 3.84)	0.21	
	2	1.19 (−1.20 to −3.60)	0.73	2.42 (−0.20 to 5.05)	0.07	0.60
HDL[d] cholesterol (mg/dL)[c]	1	0.34 (−0.49 to 1.19)	0.42	1.04 (0.20 to 1.88)	0.01	
	2	−0.15 (−1.01 to 0.71)	0.20	0.05 (−0.89 to 0.99)	0.92	0.81
LDL[e] cholesterol (mg/dL)[c]	1	−2.46 (−7.42 to 2.49)	0.33	−5.51 (−10.45 to −0.57)	0.03	
	2	0.96 (−1.25 to 3.18)	0.20	2.38 (−0.03 to 4.80)	0.05	0.29
Triglycerides (mg/dL)[f]	1	−2.46 (−7.42 to 2.49)	0.33	−5.51 (−10.45 to −0.57)	0.03	
	2	−1.68 (−6.77 to 3.40)	0.51	−2.57 (−8.14 to 2.98)	0.36	0.03
Glucose (mg/dL)[g]	1	−2.93 (−5.51 to −0.34)	0.03	−2.46 (−5.04 to 0.12)	0.62	
	2	−4.16 (−6.78 to 1.54)	0.002	−6.08 (−8.95 to −3.21)	<0.001	0.02
BMI[h]	1	−0.10 (−0.31 to 0.10)	0.32	−0.17 (−0.37 to 0.037)	0.11	
	2	0.01 (−0.20 to 0.22)	0.92	0.11 (−0.12 to 0.34)	0.36	0.29
Weight (kg)	1	−0.12 (0.72 to 0.47)	0.68	−0.086 (−0.68 to 0.51)	0.78	
	2	0.45 (−0.17 to 1.08)	0.53	0.65 (−0.03 to 1.32)	0.06	0.57
Waist circumference (cm)	1	−0.49 (−1.06 to 0.09)	0.10	−0.99 (−1.57 to −0.41)	0.001	
	2	−0.22 (−0.82 to 0.37)	0.46	−0.26 (−0.92 to 0.40)	0.44	0.005
SBP[i] (mm Hg)	1	0.94 (−0.25 to 2.14)	0.12	0.67 (−0.53 to 1.88)	0.27	
	2	0.97 (−0.26 to 2.19)	0.12	1.31 (−2.06 to 2.67)	0.06	0.90
DBP[j] (mm Hg)	1	0.06 (−0.58 to 0.71)	0.35	−0.26 (−0.91 to 0.38)	0.42	
	2	0.18 (−0.48 to 0.84)	0.59	0.27 (−0.46 to 1.01)	0.46	0.09

	Model[ak]	β (95% CI)	*P* value	β (95% CI)	*P* value	*P* trend
Total yogurt		←*<1 yogurt/day vs nonconsumers*→		←*≥1 yogurt/day vs nonconsumers*→		
Total cholesterol (mg/dL)	1	−3.00 (−5.40 to 0.62)	0.01	−3.34 (−6.17 to −0.51)	0.02	
	2	−2.92 (−5.30 to −0.53)	0.02	−3.33 (−6.20 to −0.48)	0.02	0.03
HDL cholesterol (mg/dL)	1	−0.31 (−1.17 to 0.54)	0.48	0.08 (−0.93 to 1.10)	0.87	
	2	−0.41 (−1.27 to 0.44)	0.34	−0.14 (−1.16 to 0.88)	0.78	0.81
LDL cholesterol (mg/dL)	1	−1.74 (−3.94 to 0.46)	0.12	−2.45 (−5.05 to 0.15)	0.06	
	2	−1.65 (−3.87 to 0.55)	0.14	−2.39 (−5.04 to 0.23)	0.07	0.06
Triglycerides (mg/dL)	1	−5.10 (−10.16 to −0.06)	0.05	−8.47 (−14.46 to −2.48)	0.005	
	2	−4.14 (−9.19 to 0.91)	0.11	−6.94 (−12.97 to −0.91)	0.02	0.07
Glucose (mg/dL)	1	−0.93 (−3.56 to 1.71)	0.49	1.33 (−1.79 to 4.45)	0.40	
	2	−0.87 (−3.51 to 1.72)	0.50	1.82 (−1.30 to 4.94)	0.25	0.50
BMI	1	0.14 (−0.06 to 0.35)	0.18	0.11 (−0.14 to 0.36)	0.39	
	2	0.14 (−0.06 to 0.35)	0.18	0.13 (−0.12 to 0.38)	0.32	0.11
Weight (kg)	1	0.88 (0.26 to 1.49)	0.005	0.75 (0.02 to 1.48)	0.04	
	2	0.90 (0.29 to 1.52)	0.004	0.88 (0.15 to 1.69)	0.02	0.007
Waist circumference (cm)	1	0.51 (−0.088 to 1.11)	0.09	−0.17 (−0.88 to 0.54)	0.63	
	2	0.59 (−0.01 to 1.19)	0.055	0.04 (−0.67 to 0.76)	0.90	0.42
SBP (mm Hg)	1	−0.33 (1.57 to 0.91)	0.60	−0.42 (−1.89 to 1.06)	0.58	
	2	−0.37 (−1.61 to 0.87)	0.55	−0.48 (−1.96 to 0.99)	0.52	0.62
DBP (mm Hg)	1	−0.04 (−0.71 to 0.62)	0.89	−0.25 (−1.04 to 0.54)	0.53	
	2	−0.04 (−0.71 to 0.62)	0.90	−0.22 (−1.01 to 0.58)	0.59	0.65

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Table 3. Association between yogurt, dairy, or lignans consumption and cardiovascular risk parameters of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) cohort (*continued*)

	Model[ak]	β (95% CI)	*P* value	β (95% CI)	*P* value	*P* trend
Full-fat yogurt		←<1 yogurt/day vs nonconsumers→		←≥1 yogurt/day vs nonconsumers→		
Total cholesterol (mg/dL)	1	1.70 (−0.63 to 4.04)	0.15	−1.72 (−5.65 to 2.22)	0.39	
	2	1.19 (−1.28 to 3.66)	0.34	−2.43 (−6.47 to 1.61)	0.24	0.047
HDL cholesterol (mg/dL)	1	0.36 (−0.47 to 1.20)	0.39	0.95 (−0.46 to 2.36)	0.19	
	2	0.56 (−0.32 to 1.44)	0.21	0.99 (−0.45 to 2.44)	0.18	0.12
LDL cholesterol (mg/dL)	1	2.11 (−0.03 to 4.25)	0.05	−1.95 (−5.58 to 1.67)	0.29	
	2	1.81 (−0.45 to 4.08)	0.12	−2.38 (−6.10 to 1.34)	0.21	0.047
Triglycerides (mg/dL)	1	0.03 (−4.91 to 4.97)	0.99	−7.60 (−15.94 to 0.73)	0.07	
	2	−1.90 (−7.12 to 3.31)	0.47	−9.33 (−17.87 to −0.79)	0.03	0.02
Glucose (mg/dL)	1	−2.27 (−4.84 to 0.30)	0.08	−0.81 (−5.14 to 3.51)	0.71	
	2	−1.85 (−4.55 to 0.84)	0.18	0.63 (−3.77 to 5.03)	0.78	0.98
BMI	1	0.23 (0.03 to 0.44)	0.03	0.24 (−0.11 to 0.58)	0.18	
	2	0.19 (−0.03 to 0.40)	0.08	0.22 (−0.13 to 0.58)	0.22	0.06
Weight (kg)	1	0.72 (0.13 to 1.32)	0.02	0.51 (−0.50 to 1.52)	0.32	
	2	0.78 (0.15 to 1.41)	0.01	0.70 (−0.33 to 1.74)	0.18	0.037
Waist circumference (cm)	1	0.70 (0.12 to 1.28)	0.02	0.25 (−0.74 to 1.24)	0.61	
	2	0.48 (−0.14 to 1.09)	0.13	0.16 (−0.85 to 1.17)	0.75	0.30
SBP (mm Hg)	1	0.82 (−0.38 to 2.02)	0.18	1.84 (−0.22 to 3.91)	0.08	
	2	0.43 (−0.83 to 1.70)	0.50	1.33 (−0.77 to 3.44)	0.21	0.42
DBP (mm Hg)	1	1.06 (0.41 to 1.70)	0.001	0.63 (−0.48 to 1.76)	0.27	
	2	0.81 (0.13 to 1.49)	0.02	0.36 (−0.76 to 1.50)	0.52	0.31
Low-fat yogurt						
Total cholesterol (mg/dL)	1	−3.51 (−5.62 to −1.40)	0.001	−1.79 (−4.51 to 0.93)	0.20	
	2	−4.40 (−6.65 to −2.15)	<0.001	−2.87 (−5.75 to 0.01)	0.05	0.08
HDL cholesterol (mg/dL)	1	−0.89 (−1.64 to −0.13)	0.02	−0.38 (−1.35 to 0.59)	0.44	
	2	−1.05 (−1.85 to −0.24)	0.01	−0.63 (−1.65 to 0.40)	0.23	0.57
LDL cholesterol (mg/dL)	1	−3.00 (−4.93 to −1.06)	0.002	−1.17 (−3.65 to 1.31)	0.36	
	2	−3.80 (−5.87 to −1.72)	<0.001	−2.11 (−4.75 to 0.52)	0.11	0.20
Triglycerides (mg/dL)	1	−0.43 (−4.89 to 4.03)	0.85	−4.36 (−10.13 to 1.40)	0.14	
	2	−0.82 (−5.59 to 3.95)	0.73	−4.53 (−10.60 to 1.56)	0.14	0.34
Glucose (mg/dL)	1	1.56 (−0.76 to 3.88)	0.19	2.69 (−0.33 to 5.70)	0.08	
	2	1.29 (−1.18 to 3.76)	0.31	2.80 (−0.36 to 5.97)	0.08	0.39
BMI	1	0.0007 (−0.18 to 0.18)	0.99	−0.104 (−0.35 to 0.14)	0.40	
	2	0.12 (−0.08 to 0.32)	0.23	0.04 (−0.21 to 0.30)	0.72	0.35
Weight (kg)	1	0.24 (−0.29 to 0.78)	0.38	0.058 (−0.64 to 0.76)	0.87	
	2	0.64 (0.06 to 1.21)	0.03	0.55 (−0.18 to 1.30)	0.14	0.025
Waist circumference (cm)	1	0.18 (−0.35 to 0.71)	0.51	−0.80 (−1.49 to −0.11)	0.02	
	2	0.55 (−0.02 to 1.12)	0.06	−0.30 (−1.02 to 0.42)	0.42	0.67
SBP (mm Hg)	1	−0.87 (−1.96 to 0.22)	0.12	−1.37 (−2.79 to 0.04)	0.06	
	2	−0.62 (−1.79 to 0.55)	0.30	−1.12 (−2.62 to 0.37)	0.14	0.28
DBP (mm Hg)	1	−0.94 (−1.52 to −0.35)	0.001	−0.96 (−1.72 to −0.20)	0.01	
	2	−0.076 (−1.38 to −0.13)	0.02	−0.76 (−1.56 to 0.04)	0.06	0.25

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Table 3. Association between yogurt, dairy, or lignans consumption and cardiovascular risk parameters of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) cohort (*continued*)

	Model[ak]	β (95% CI)	*P* value	β (95% CI)	*P* value	*P* trend
Total dairy[l]		←——*Group 1 vs Group 0*[m]——→		←——*Group 2 vs Group 0*[m]——→		
Total cholesterol (mg/dL)	1	−4.34 (−6.70 to −2.17)	<0.001	−4.52 (−7.18 to −1.86)	<.001	
	2	−4.30 (−6.60 to −2.01)	<0.001	−4.36 (−7.09 to −1.62)	0.002	<0.001
HDL cholesterol (mg/dL)	1	−1.06 (−1.87 to −0.25)	0.01	−1.07 (−2.02 to −0.11)	0.03	
	2	−0.87 (−1.69 to −0.005)	0.04	−0.65 (−1.63 to 0.33)	0.19	0.005
LDL cholesterol (mg/dL)	1	−2.41 (−4.50 to −0.32)	0.02	−2.23 (−4.68 to 0.22)	0.07	
	2	−2.34 (−4.45 to −0.22)	0.03	−2.18 (−4.70 to 0.33)	0.09	0.048
Triglycerides (mg/dL)	1	−4.35 (−9.15 to 0.45)	0.07	−2.93 (−8.58 to 2.71)	0.31	
	2	−4.74 (−9.58 to 0.11)	0.06	−4.13 (−9.93 to 1.65)	0.16	0.37
Glucose (mg/dL)	1	3.35 (0.85 to 5.85)	0.008	8.93 (5.99 to 11.87)	<0.001	
	2	2.64 (0.14 to 5.14)	0.04	7.89 (4.89 to 10.88)	<0.001	<0.001
BMI	1	0.15 (−0.05 to 0.35)	0.15	0.11 (−0.12 to 0.35)	0.34	
	2	0.075 (−0.13 to 0.28)	0.46	−0.011 (−0.25 to 0.23)	0.93	0.68
Weight (kg)	1	0.12 (−0.46 to 0.71)	0.67	−0.14 (−0.83 to 0.56)	0.70	
	2	−0.08 (−0.67 to 0.50)	0.78	−0.53 (−1.24 to 0.18)	0.15	0.82
Waist circumference (cm)	1	0.18 (−0.38 to 0.75)	0.52	−0.22 (−0.90 to 0.46)	0.52	
	2	−0.05 (−0.62 to 0.52)	0.86	0.70 (−1.39 to −0.003)	0.05	0.16
SBP (mm Hg)	1	−0.25 (−1.43 to 0.92)	0.67	−0.82 (−2.22 to 0.59)	0.25	
	2	−0.28 (−1.46 to 0.90)	0.64	−0.77 (−2.21 to 0.67)	0.29	0.025
DBP (mm Hg)	1	−0.14 (−0.77 to 0.48)	0.65	−0.57 (−1.32 to 0.19)	0.14	
	2	−0.22 (−0.85 to 0.41)	0.50	−0.78 (−1.53 to −0.019)	0.04	0.02

[a]Model 1: adjusted for recruitment center, sex, and age; Model 2: adjusted for recruitment center, sex, age, smoking, soft drinks, carbohydrates, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, n−3 fatty acids, and dairies.
[b]Lignans groups were formed according to tertiles, group 0: <0.46 mg/day, group 1: 0.46 to 0.67 mg/day, and group 2: >0.67 mg/day.
[c]To convert mmol/L cholesterol to mg/dL, multiply by 38.7. To convert mg/dL cholesterol to mmol/L, multiply mg/dL by 0.0259. Cholesterol of 193 mg/dL=5.00 mmol/L.
[d]HDL=high-density lipoprotein.
[e]LDL=low-density lipoprotein.
[f]To convert mg/dL triglycerides to mmol/L, multiply mg/dL by 0.0113. To convert mmol/L triglycerides to mg/dL, multiply mmol/L by 88.6. Triglyceride of 159 mg/dL=1.80 mmol/L.
[g]To convert mg/dL glucose to mmol/L, multiply by 0.0555. To convert mmol/L glucose to mg/dL, multiply mmol/L by 18.0. Glucose of 100 mg/dL=5.55 mmol/L.
[h]BMI=body mass index.
[i]SBP=systolic blood pressure.
[j]DBP=diastolic blood pressure.
[k]Model 2 replacing dairies by fiber.
[l]Total dairy groups were formed according to tertiles, group 0: <200 g/day, group 1: 200 to 500 g/day, and group 2: >500 g/day.
[m]Total dairy comprises whole/low-fat/nonfat milk, condensed milk, ice cream, custard, and all types of cheeses (ricotta, cured cheeses).

Table 4. General linear models for the association between cardiovascular risk parameters and the joint intake of yogurt and lignans of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) cohort

		Low Lignan Intake (<0.6 mg/day) (n=3,525)		High Lignan Intake (>0.6 mg/day) (n=3,644)		
General linear models	**Model[a]**	**β (95% CI)**	***P* value**	**β (95% CI)**	***P* value**	***P* interaction**
Total cholesterol (mg/dL)	Model 1					0.05
	<1 yogurt/day vs nonconsumers	−2.22 (−5.74 to 1.29)	0.21	−3.71 (−6.96 to −0.46)	0.02	
	≥1 yogurt/day vs nonconsumers	0.63 (−3.61 to 4.88)	0.77	−6.48 (−10.28 to −2.67)	<0.001	
	Model 2					0.01
	<1 yogurt/day vs nonconsumers	−2.57 (−6.03 to 0.89)	0.14	−3.83 (−7.05 to −0.62)	0.02	
	≥1 yogurt/day vs nonconsumers	−0.59 (−3.63 to 4.81)	0.78	−6.18 (−9.97 to −2.40)	0.001	
HDL cholesterol (mg/dL)	Model 1					0.27
	<1 yogurt/day vs nonconsumers	0.59 (−0.65 to 1.83)	0.35	−1.17 (−2.36 to 0.01)	0.05	
	≥1 yogurt/day vs nonconsumers	0.62 (−0.87 to 2.12)	0.41	−0.49 (−1.87 to 0.89)	0.49	
	Model 2					0.79
	<1 yogurt/day vs nonconsumers	0.07 (−1.18 to 1.32)	0.91	−1.14 (−2.32 to 0.04)	0.06	
	≥1 yogurt/day vs nonconsumers	−0.21 (−1.74 to 1.32)	0.79	−0.48 (−1.87 to 0.91)	0.50	
LDL cholesterol (mg/dL)	Model 1					0.16
	<1 yogurt/day vs nonconsumers	−0.96 (−4.18 to 2.25)	0.55	−2.45 (−5.48 to 0.57)	0.11	
	≥1 yogurt/day vs nonconsumers	1.39 (−2.49 to 5.28)	0.48	−5.54 (−9.06 to −2.03)	0.002	
	Model 2					0.05
	<1 yogurt/day vs nonconsumers	−1.09 (−4.25 to 2.08)	0.50	−2.74 (−5.72 to 0.24)	0.07	
	≥1 yogurt/day vs nonconsumers	1.91 (−1.95 to 5.76)	0.33	−4.92 (−8.41 to −1.43)	0.005	
Triglycerides (mg/dL)	Model 1					0.05
	<1 yogurt/day vs nonconsumers	−9.41 (−16.97 to −1.86)	0.01	−1.07 (−7.86 to 5.72)	0.76	
	≥1 yogurt/day vs nonconsumers	−8.76 (−1.79 to 0.38)	0.06	−7.36 (−15.30 to 0.58)	0.07	
	Model 2					0.21
	<1 yogurt/day vs nonconsumers	−7.53 (−15.18 to 0.11)	0.05	−1.31 (−8.15 to 5.53)	0.71	
	≥1 yogurt/day vs nonconsumers	−5.93 (−15.19 to 3.33)	0.21	−7.98 (−15.94 to −0.015)	0.049	
Glucose (mg/dL)	Model 1					0.08
	<1 yogurt/day vs nonconsumers	−0.55 (−4.40 to 3.30)	0.78	−1.19 (−4.80 to 2.43)	0.52	
	≥1 yogurt/day vs nonconsumers	1.91 (−2.73 to 6.56)	0.42	0.98 (−3.25 to 5.22)	0.65	
	Model 2					0.07
	<1 yogurt/day vs nonconsumers	−0.47 (−3.66 to 2.71)	0.77	−0.89 (−4.76 to 2.99)	0.65	
	≥1 yogurt/day vs nonconsumers	−2.20 (−5.15 to 0.75)	0.14	−1.92 (−5.41 to 1.57)	0.28	

(*continued on next page*)

Table 4. General linear models for the association between cardiovascular risk parameters and the joint intake of yogurt and lignans of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) cohort (*continued*)

		Low Lignan Intake (<0.6 mg/day) (n = 3,525)		High Lignan Intake (>0.6 mg/day) (n = 3,644)		
General linear models	Model[a]	β (95% CI)	*P* value	β (95% CI)	*P* value	*P* interaction
BMI[b]	Model 1					0.11
	<1 yogurt/day vs nonconsumers	0.22 (−0.08 to 0.52)	0.14	0.07 (−0.22 to 0.37)	0.63	
	≥1 yogurt/day vs nonconsumers	0.25 (−0.11 to 0.61)	0.18	−0.003 (−0.35 to 0.34)	0.98	
	Model 2					0.44
	<1 yogurt/day vs nonconsumers	0.28 (−0.02 to 0.58)	0.007	0.06 (−0.23 to 0.36)	0.65	
	≥1 yogurt/day vs nonconsumers	0.51 (0.15 to 0.88)	0.006	−0.04 (−0.39 to 0.31)	0.81	
Weight (kg)	Model 1					0.94
	<1 yogurt/day vs nonconsumers	1.21 (0.33 to 2.09)	0.006	0.56 (−0.30 to 1.42)	0.20	
	≥1 yogurt/day vs nonconsumers	1.05 (−0.01 to 2.10)	0.05	0.47 (−0.53 to 1.48)	0.35	
	Model 2					0.42
	<1 yogurt/day vs nonconsumers	1.20 (0.32 to 2.09)	0.008	0.57 (−0.29 to 1.43)	0.20	
	≥1 yogurt/day vs nonconsumers	1.35 (0.27 to 2.43)	0.01	0.39 (−0.62 to 1.41)	0.45	
Waist circumference (cm)	Model 1					0.05
	<1 yogurt/day vs nonconsumers	0.70 (−0.14 to 1.54)	0.10	0.36 (−0.49 to 1.22)	0.40	
	≥1 yogurt/day vs nonconsumers	0.25 (−0.76 to 1.26)	0.63	−0.42 (−1.42 to 0.58)	0.41	
	Model 2					0.37
	<1 yogurt/day vs nonconsumers	0.70 (−0.15 to 1.55)	0.11	0.35 (−0.51 to 1.21)	0.42	
	≥1 yogurt/day vs nonconsumers	0.42 (−0.61 to 1.46)	0.42	−0.54 (−1.55 to 0.47)	0.29	
SBP[c] (mm Hg)	Model 1					0.96
	<1 yogurt/day vs nonconsumers	0.26 (−1.50 to 2.02)	0.77	−0.88 (−2.63 to 0.87)	0.32	
	≥1 yogurt/day vs nonconsumers	−0.05 (−2.18 to 2.08)	0.96	−0.72 (−2.77 to 1.34)	0.49	
	Model 2					0.69
	<1 yogurt/day vs nonconsumers	−0.26 (−2.03 to 1.50)	0.77	−1.14 (−2.86 to 0.59)	0.20	
	≥1 yogurt/day vs nonconsumers	−0.23 (−2.38 to 1.93)	0.84	−1.09 (−3.13 to 0.95)	0.30	
DBP[d] (mm Hg)	Model 1					0.08
	<1 yogurt/day vs nonconsumers	0.09 (−0.85 to 1.04)	0.85	−0.15 (−1.09 to 0.79)	0.76	
	≥1 yogurt/day vs nonconsumers	−0.02 (−1.16 to 1.12)	0.97	−0.34 (−1.45 to 0.76)	0.54	
	Model 2					0.08
	<1 yogurt/day vs nonconsumers	−0.08 (−1.03 to 0.86)	0.86	−0.12 (−1.05 to 0.82)	0.80	
	≥1 yogurt/day vs nonconsumers	0.16 (−0.99 to 1.32)	0.78	−0.12 (−1.23 to 0.99)	0.83	

[a]Model 1: adjusted for recruitment center, sex, and age; Model 2: additionally adjusted for smoking, soft drinks, carbohydrates, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, family history of heart disease, diabetes, and hypertension.
[b]BMI=body mass index.
[c]SBP=systolic blood pressure.
[d]DBP=diastolic blood pressure.

and higher plasma glucose levels (β=7.89; P<0.001). Total dairy intake of 200 to 500 g/day was associated with lower total cholesterol (β=−4.30; P<0.001), HDL cholesterol (β=−0.87; P=0.04), and LDL cholesterol (β=−2.34; P=0.03), and higher glucose levels (β=2.64; P=0.04). Significant linear associations were found for total dairy intake and total cholesterol (P trend<0.001), HDL cholesterol (P trend=0.005), LDL cholesterol (P trend=0.048), glucose (P trend<0.001), systolic blood pressure (P trend=0.025), and diastolic blood pressure (P trend=0.02).

Joint Analysis of Lignans and Yogurt Consumption

Table 4 shows the results of the general linear models used to assess the association between yogurt consumption and different Cardiovascular Risk Parameters stratified by lignan intake. The participants with the highest consumption of lignans (>0.6 mg/day) and total yogurt had significantly lower levels of total cholesterol (β=−6.18; P=0.001; P interaction=0.01) and LDL cholesterol (β=−4.92; P=0.005; P interaction=0.05), and triglycerides (β=−7.98; P=0.049; P interaction=0.21), although the P for interaction was not significant in the latter. Participants with a higher consumption of yogurt but a lower intake of lignans (<0.6 mg/day) had a significantly higher BMI (β=0.51; P=0.006) and weight (β=1.35; P=0.01), while those with high lignan intake showed no differences in BMI and weight (β=−0.04; P=0.81; P interaction=0.41 and β=0.391; P=0.45; P interaction=0.42, respectively).

DISCUSSION

The present cross-sectional study, evaluated the ameliorative effects of lignans, yogurts, and the joint consumption of lignans and probiotics on cardiovascular risk parameters in humans. Previous studies on yogurt and lignan consumption have shown beneficial effects on human health; but, as shown in the present study, joint consumption of these foods had a stronger impact on cardiovascular risk parameters and was associated with lower cholesterol and LDL cholesterol levels and a trend to lower triglyceride levels. To our knowledge, this is the first study to suggest that polyphenol and yogurt intake can improve cardiovascular risk parameters and particularly the lipid profile.

Some polyphenols can be metabolized and absorbed through the gut barrier, but they usually reach the colon, where they are metabolized by the microbiota and absorbed.[23] Lignans are metabolized by the intestinal microbiota to enterodiol and enterolactone.[1,24] There is some evidence indicating that lignan-rich foods are protective against cardiovascular disease and some cancers, including breast, colon, and prostate cancer.[3,25,26] In this study, a higher lignan intake was associated with a decrease in glucose levels. In addition, stratified analyses related to sex showed lower glucose levels in both men and women (data not shown). Pinoresinol was the lignan most frequently ingested, mainly (96%) from olive oil. In a study of plant lignans by During and colleagues,[27] pinoresinol showed the strongest anti-inflammatory effect in the human intestine. In a cross-sectional study including 242 males and females in northern Italy, matairesinol was associated with lower vascular inflammation and endothelial dysfunction.[28] In a prospective cohort study including 570 men,[29] the evaluation of four lignans (lariciresinol, pinoresinol, secoisolariciresinol, and matairesinol) found that the intake of matairesinol was inversely associated with mortality due to a reduction in cardiovascular disease and cancer. In this population, matairesinol was the lignan least consumed, and the main food sources of lignans were olive oil, wheat, tomato, red wine, asparagus, and kiwis (Table 2, available at www.andjrnl.org).

The gut microbiota can also be influenced by the diet, which has a direct impact on the gut environment, including transit time and pH.[30] The prebiotic effect of polyphenols has been studied previously,[2] and it has been suggested that polyphenols may affect the relative viability of beneficial bacterial groups, such as *Firmicutes* and *Bacteroides*.[31-33] The polyphenol–microbiota interaction is evident,[34,35] but more holistic approaches involving the use of high-throughput "omics" tools are needed to shed light on the physiological relevance of this interaction in humans.

As a probiotic, yogurt has benefits for consumer health. Its functional properties have been confirmed by studies on the metabolic activity of yogurt bacteria in the human intestine.[6,36-38] As a functional food, yogurt has been associated with benefits for cardiovascular and gastrointestinal health, weight management, and type 2 diabetes, among others.[39,40] In the present study, total and low-fat yogurt intake were correlated with higher weight, but yogurt intake together with a high lignan diet did not produce any increase in weight. Obesity is a cardiovascular risk parameter and is related to increased levels of triglycerides, LDL cholesterol, and cholesterol, and decreased HDL cholesterol levels. In accordance with Cormier and colleagues,[41] yogurt consumption was associated with lower levels of cholesterol and triglycerides. Stratified analyses on sex showed some differences between men and women, with >1 yogurt/day being associated with lower cholesterol and triglyceride levels in men but a higher BMI and weight in women. Full-fat yogurt was correlated with higher LDL cholesterol and lower triglyceride levels, while low-fat yogurt was correlated with lower total cholesterol, HDL cholesterol, and LDL cholesterol, but with no impact on triglycerides. It has been suggested that the potential underlying mechanisms for weight loss or the prevention of weight gain could be stimulatory effects on the growth of beneficial intestinal bacteria.[42] An alternative mechanism of action is that yogurt consumption induces higher satiety, and therefore, a reduction in appetite.[39] This latter effect could also involve microbiota, with microbial manipulation of eating behavior via the nervous system and the gut–brain axis.[43,44] A study by Zapata and colleagues[45] concluded that manipulation of the intestinal microbiota may be beneficial for maintaining health in older adults.

High lignan and yogurt consumption was associated with lower levels of total cholesterol, LDL cholesterol, and triglycerides, while HDL cholesterol values did not decrease, indicating an improved lipid profile. Yogurt consumption did not affect serum glucose levels, but these levels significantly increased when the total dairy intake was considered. On the one hand, it seems that microbiota associated with yogurt intake could metabolize lignans more efficiently and, on the other hand, lignans may help to modulate gut microbiota by increasing the beneficial strains.[33,46]

Studying the role of diet nutrients in chronic conditions such as cardiovascular diseases is complex because "we don't

eat nutrients, we eat foods."[47] In addition, limiting analysis to individual nutrients might fail to take into account many potential interactions between dietary components and requires a large sample size and adjustment for other nutrients. Therefore, study of the synergy between foods and bioactive compounds could be a useful approach in the prevention of disease.[48,49]

This study has taken a novel approach by focusing on the potential health benefits of lignans, yogurt, and their joint consumption; nevertheless, some limitations should be noted. Firstly, the data obtained were from an elderly population at high cardiovascular risk, which can limit the generalization of the results. Secondly, lignan intake was calculated with FFQs and Phenol-Explorer, which is the most comprehensive polyphenol database available, although information about some foods is still limited. It should also be considered that polyphenol content in foods can differ according to the preparation method, maturity at harvesting, environmental factors, or storage conditions.[1,50] It is important to be aware of the fact that some confounding variables, such as lifestyle or stress, among others, could be ignored because they were not recorded in the questionnaires. Finally, because this was an observational study, a cause–effect relationship cannot be established from the results, and therefore, the hypothesis should be confirmed in future clinical trials.

CONCLUSIONS

These findings suggest that an associative effect of lignans and yogurt may ameliorate cardiovascular risk parameters in humans. Therefore, daily low-fat yogurt consumption in a healthy, well-balanced diet with a high content of lignan-rich foods, such as flaxseed or extra virgin olive oil, may be recommended to enhance the beneficial effects of these two foods when ingested separately, at least in elderly populations. Additional clinical trials focusing on the differences in lignan metabolites in yogurt consumers and nonconsumers are needed. In addition, the development of modifications in microbiota communities after yogurt and lignan intake should be studied, and how these modification affect human health should be evaluated.

References

1. Manach C, Scalbert A, Morand C, Remesy C, Jimenez L. Polyphenols: Food sources and bioavailability. *Am J Clin Nutr*. 2004;79(5):727-747.
2. Boto-Ordóñez M, Urpi-Sarda M, Queipo-Ortuño MI, Tulipani S, Tinahones FJ, Andres-Lacueva C. High levels of Bifidobacteria are associated with increased levels of anthocyanin microbial metabolites: A randomized clinical trial. *Food Funct*. 2014;5(8):1932-1938.
3. Adlercreutz H. Lignans and human health. *Crit Rev Clin Lab Sci*. 2007;44(5-6):483-525. Review.
4. Landete JM. Plant and mammalian lignans: A review of source, intake, metabolism, intestinal bacteria and health. *Food Res Int*. 2012;46(1):410-424.
5. Food and Agriculture Organization/World Health Organization. *Guidelines for the evaluation of probiotics in food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food*. Ontario, Canada: April 30-May 1, 2002.
6. Guarner F, Perdigon G, Corthier G, Salminen S, Koletzko B, Morelli L. Should yoghurt cultures be considered probiotic? *Br J Nutr*. 2005;93(6):783.
7. Kechagia M, Basoulis D, Konstantopoulou S, et al. Health benefits of probiotics: A review. *ISRN Nutr*. 2013;2013:481651.
8. Fung TT, Rimm EB, Spiegelman D, et al. Association between dietary patterns and plasma biomarkers of obesity and cardiovascular disease risk. *Am J Clin Nutr*. 2001;73(1):61-67.
9. Jacobs DRJ, Steffen LM. Nutrients, foods, and dietary patterns as exposures in research: A framework for food synergy. *Am J Clin Nutr*. 2003;78(3 suppl):508S-513S.
10. Babio N, Becerra-Tomás N, Martínez-González MÁ, et al. Consumption of yogurt, low-fat milk, and other low-fat dairy products is associated with lower risk of metabolic syndrome incidence in an elderly Mediterranean population. *J Nutr*. 2015;145(10):2308-2316.
11. Díaz-López A, Bulló M, Martínez-González MA, et al. Dairy product consumption and risk of type 2 diabetes in an elderly Spanish Mediterranean population at high cardiovascular risk. *Eur J Nutr*. 2015;55(1):349-360.
12. Martínez-González MÁ, Corella D, Salas-Salvadó J, et al. Cohort profile: Design and methods of the PREDIMED study. *Int J Epidemiol*. 2012;41(2):377-385.
13. Predimed (Prevención con Dieta Mediterránea). www.predimed.es. Published 2003. Accessed February 8, 2016.
14. Estruch R, Ros E, Salas-Salvadó J, et al. Primary prevention of cardiovascular disease with a Mediterranean diet. *N Engl J Med*. 2013;368(14):1279-1290.
15. Fernández-Ballart JD, Piñol JL, Zazpe I, et al. Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. *Br J Nutr*. 2010;103(12):1808-1816.
16. Martínez-González MA, Fernández-Jarne E, Serrano-Martínez M, Wright M, Gomez-Gracia E. Development of a short dietary intake questionnaire for the quantitative estimation of adherence to a cardioprotective Mediterranean diet. *Eur J Clin Nutr*. 2004;58(11):1550-1552.
17. Elosua R, Garcia M, Aguilar A, Molina L, Covas MI, Marrugat J. Validation of the Minnesota Leisure Time Physical Activity Questionnaire In Spanish Women. Investigators of the MARATDON Group. *Med Sci Sports Exerc*. 2000;32(8):1431-1437.
18. O'Brien E, Waeber B, Parati G, Staessen J, Myers MG. Blood pressure measuring devices: Recommendations of the European Society of Hypertension. *BMJ*. 2001;322(7285):531-536.
19. Warnick GR, Knopp RH, Fitzpatrick V, Branson L. Estimating low-density lipoprotein cholesterol by the Friedewald equation is adequate for classifying patients on the basis of nationally recommended cutpoints. *Clin Chem*. 1990;36(1):15-19.
20. Mataix J. *Tablas de Composición de Alimentos [Food Composition Tables]*, 4th ed. Granada: Universidad de Granada; 2003.
21. Neveu V, Perez-Jiménez J, Vos F, Crespy V, du Chaffaut L, Mennen L, Knox C, Eisner R, Cruz J, Wishart D, Scalbert A. Phenol-Explorer: An online comprehensive database on polyphenol contents in foods. 2010. http://dx.doi.org/10.1093/database/bap024.
22. SAS [computer program]. Version 9.3. Cary, NC: SAS Institute Inc; 2011.
23. Scalbert A, Morand C, Manach C, Rémésy C. Absorption and metabolism of polyphenols in the gut and impact on health. *Biomed Pharmacother*. 2002;56(6):276-282.
24. de Pascual-Teresa S, Moreno DA, García-Viguera C. Flavanols and anthocyanins in cardiovascular health: A review of current evidence. *Int J Mol Sci*. 2010;11(4):1679-1703.
25. Zhang W, Wang X, Liu Y, et al. Dietary flaxseed lignan extract lowers plasma cholesterol and glucose concentrations in hypercholesterolaemic subjects. *Br J Nutr*. 2008;99(6):1301-1309.
26. Adolphe JL, Whiting SJ, Juurlink BHJ, Thorpe LU, Alcorn J. Health effects with consumption of the flax lignan secoisolariciresinol diglucoside. *Br J Nutr*. 2010;103(7):929-938.
27. During A, Debouche C, Raas T, Larondelle Y. Among plant lignans, pinoresinol has the strongest antiinflammatory properties in human intestinal Caco-2 cells. *J Nutr*. 2012;142(10):1798-1805.
28. Pellegrini N, Valtueña S, Ardigò D, et al. Intake of the plant lignans matairesinol, secoisolariciresinol, pinoresinol, and lariciresinol in relation to vascular inflammation and endothelial dysfunction in middle age-elderly men and post-menopausal women living in Northern Italy. *Nutr Metab Cardiovasc Dis*. 2010;20(1):64-71.

29. Milder IE, Feskens EJ, Arts IC, Bueno-de-Mesquita HB, Hollman PC, Kromhout D. Intakes of 4 dietary lignans and cause-specific and all-cause mortality in the Zutphen Elderly Study. *Am J Clin Nutr*. 2006;84(2):400-405.
30. Scott KP, Gratz SW, Sheridan PO, Flint HJ, Duncan SH. The influence of diet on the gut microbiota. *Pharmacol Res*. 2013;69(1):52-60.
31. Lee HC, Jenner AM, Low CS, Lee YK. Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Res Microbiol*. 2006;157(9):876-884.
32. Rastmanesh R. High polyphenol, low probiotic diet for weight loss because of intestinal microbiota interaction. *Chem Biol Interact*. 2011;189(1-2):1-8.
33. Parkar SG, Trower TM, Stevenson DE. Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe*. 2013;23:12-19.
34. Kemperman RA, Bolca S, Roger LC, Vaughan EE. Novel approaches for analysing gut microbes and dietary polyphenols: Challenges and opportunities. *Microbiology*. 2010;156(Pt 11):3224-3231.
35. Etxeberria U, Fernández-Quintela A, Milagro FI, Aguirre L, Martínez JA, Portillo MP. Impact of polyphenols and polyphenol-rich dietary sources on gut microbiota composition. *J Agric Food Chem*. 2013;61(40):9517-9533.
36. Brigidi P, Swennen E, Vitali B, Rossi M, Matteuzzi D. PCR detection of *Bifidobacterium* strains and *Streptococcus thermophilus* in feces of human subjects after oral bacteriotherapy and yogurt consumption. *Int J Food Microbiol*. 2003;81(3):203-209.
37. Mater DDG, Bretigny L, Firmesse O, et al. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* survive gastrointestinal transit of healthy volunteers consuming yogurt. *FEMS Microbiol Lett*. 2005;250(2):185-187.
38. Elli M, Callegari ML, Ferrari S, et al. Survival of yogurt bacteria in the human gut. *Appl Environ Microbiol*. 2006;72(7):5113-5117.
39. Pei R, Martin DA, DiMarco DM, Bolling BW. Evidence for the effects of yogurt on gut health and obesity [published online ahead of print April 15, 2015]. *Crit Rev Food Sci Nutr*. http://dx.doi.org/10.1080/10408398.2014.883356.
40. Glanville JM, Brown S, Shamir R, Szajewska H, Eales JF. The scale of the evidence base on the health effects of conventional yogurt consumption: Findings of a scoping review. *Front Pharmacol*. 2015;6(October):1-12.
41. Cormier H, Thifault É, Garneau V, et al. Association between yogurt consumption, dietary patterns, and cardio-metabolic risk factors. *Eur J Nutr*. 2016;55(2):577-587.
42. Jacques PF, Wang H. Yogurt and weight management. *Am J Clin Nutr*. 2014;99(5 suppl):1229S-1234S.
43. Rhee SH, Pothoulakis C, Mayer EA. Principles and clinical implications of the brain–gut–enteric microbiota axis. *Nat Rev Gastroenterol Hepatol*. 2009;6:306-314.
44. Alcock J, Maley CC, Aktipis CA. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. *Bioessays*. 2014;36(10):940-949.
45. Zapata HJ, Quagliarello VJ. The microbiota and microbiome in aging: Potential implications in health and age-related diseases. *J Am Geriatr Soc*. 2015;63(4):776-781.
46. Steinert RE, Sadabad MS, Harmsen HJM, Weber P. The prebiotic concept and human health: A changing landscape with riboflavin as a novel prebiotic candidate? *Eur J Clin Nutr*. 2016;(April):1-6.
47. Jacques PF, Tucker KL. Are dietary patterns useful for understanding the role of diet in chronic disease? *Am J Clin Nutr*. 2001;73(1):1-2.
48. Jacobs DR. What comes first: The food or the nutrient? Executive summary of a symposium. *J Nutr*. 2014;144(4 suppl):543S-546S.
49. Jacobs DR, Tapsell LC, Temple NJ. Food synergy: The key to balancing the nutrition research effort. *Public Health Rev*. 2012;33(2):507-529.
50. Vallverdú-Queralt A, Arranz S, Medina-Remón A, Casals-Ribes I, Lamuela-Raventós RM. Changes in phenolic content of tomato products during storage. *J Agric Food Chem*. 2011;59(17):9358-9365.

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STATEMENT OF POTENTIAL CONFLICT OF INTEREST

R. M. Lamuela-Raventos reports serving on the board of and receiving lecture fees from Research Foundation on Wine and Nutrition (FIVIN), receiving lecture fees from Cerveceros de España, and receiving lecture fees and travel support from PepsiCo. J Salas-Salvadó reports serving on the board of and receiving grant support through his institution from the International Nut and Dried Fruit Council, receiving consulting fees from Danone, and receiving grant support through his institution from Eroski and Nestlé. R. Estruch reports serving on the board of and receiving lecture fees from the FIVIN, serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research, receiving lecture fees from Cerveceros de España and Sanofi-Aventis, and receiving grant support through his institution from Novartis. F. Arós reports receiving payment for the development of educational presentations from Menarini and AstraZeneca. X. Pintó reports serving on the board of and receiving grant support through his institution from the Residual Risk Reduction Initiative (R3i) Foundation; serving on the board of Omegafort; serving on the board of and receiving payment for the development of educational presentations, as well as grant support through his institution, from Ferrer; receiving consulting fees from Abbott Laboratories; receiving lecture fees, as well as grant support through his institution, from Merck and Roche; receiving lecture fees from Danone and Esteve; receiving payment for the development of educational presentations from Menarini; and receiving grant support through his institution from Sanofi-Aventis, Kowa, Unilever, Boehringer Ingelheim, and Karo Bio. Sponsor's role: The funding sources played no role in study design; collection, analysis, or interpretation of data; writing of the manuscript; or the decision to submit for publication. No potential conflict of interest was reported by the other authors.

FUNDING/SUPPORT

The authors would like to express their gratitude for financial support from Instituto Danone, CICYT (AGL2013-49083-C3-1-R) from the Spanish Ministry of Economy and Competitively, Generalitat de Catalunya (GC) 2014 SGR 773, and CIBEROBN from the Instituto de Salud Carlos III. The funding sources played no role in the study design, the collection, analysis, or interpretation of data, the writing of the manuscript; or the decision to submit for publication.

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The authors would like to thank all the volunteers involved in the PREDIMED study for their valuable cooperation. The authors would also like to thank Marta Iguacen for her assistance.

Table 2. Mean intake of lignan compounds and their food sources of 7,169 elderly Spanish participants at high cardiovascular risk from the PREDIMED (Prevención con Dieta Mediterránea) study

Lignan	Intake (mg/day)	SD[a]	Food sources
Pinoresinol	0.31	0.25	Olive oil (96%), asparagus (0.7%), refined wheat (0.6%), whole-grain wheat (0.6%)
1-Acetoxypinoresinol	0.25	0.12	Olive oil (100%)
Lariciresinol	0.12	0.06	Wheat (67%), whole-grain wheat (11%), tomato (6.5%), asparagus (4%)
Secoisolariciresinol	0.06	0.06	Kiwi (37%), asparagus (31%), red wine (19%), whole-grain wheat (6%)
Syringaresinol	0.07	0.09	Whole-grain wheat (81%), asparagus (10%), kiwis (3%), red wine (3%)
Isolariciresinol	0.03	0.07	Red wine (100%)
Medioresinol	0.01	0.01	Whole-grain wheat (53%), tomato (21%), kiwi (15%), asparagus (8%)
Matairesinol	0.004	0.002	Red wine (74%), asparagus (8%), tea (6%), whole-grain wheat (6%)

[a]SD=standard deviation.

Conference communications

Yoghurts and lignans work synergistically to improve cardiovascular risk parameters in an elderly population

A. Creus-Cuadros[1], A. Tresserra-Rimbau[1,2], P. Quifer-Rada[1,2], M.A. Martínez-González[2,3], D. Corella[2,4], J. Salas-Salvadó[2,5], M. Fitó [2,6], R. Estruch[2,7] , E. Gómez-Gracia[2,8], J. Lapetra[2,9], F. Arós[2,10], M. Fiol[2,11], E. Ros[2,12], Ll. Serra-Majem[2,13], X.Pintó[2,14], M. Ruiz-Canela[2,3], J. V. Sorli[2,4], J. Basora[2,5], H. Schoeder [2,6] and R.M. Lamuela-Raventós[1,2*], on behalf of the PREDIMED Study Investigators.

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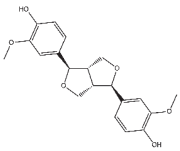
Introduction

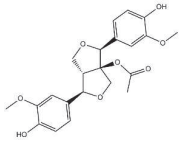
Lignans are of increasing interest due to their potential anticarcinogenic, estrogenic and antioxidant activities. After ingestion, plant lignans are deglycosylated and converted to the enterolignans by colonic bacteria. Several studies have shown a potential protective effect of these metabolites against cardiovascular diseases and some cancers like colon and breast cancer.
Probiotics are live microorganisms that confer a health benefit on the host when they administered in adequate amounts. Nowadays, most of the probiotics consumed come from fermented dairy products such as yogurt (produced using a culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*).

Objective

We hypothesized that yogurts may improve lignan bioavailability, increasing absorption by colonic mucosa and/or facilitating the bioactivation of polyphenols, lowering cardiovascular risk factors.

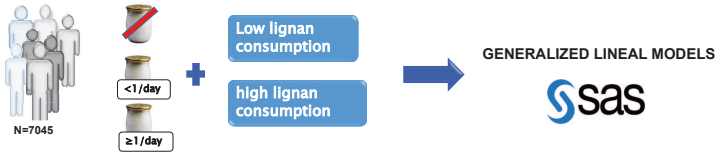


Pinoresinol



1–Acetoxypinoresinol

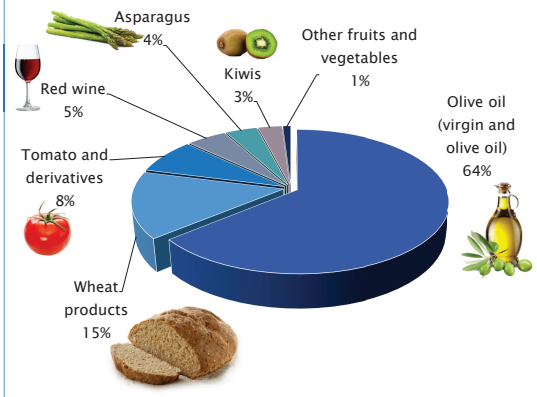
Study design and method



In the present study, we used data from the PREDIMED study, a cohort that includes 7447 elderly participants at high cardiovascular risk. Intake of lignans was calculated by matching food consumption data from baseline food frequency questionnaires with the Phenol explorer database on the polyphenol content of each reported food. General lineal models were used to assess the association between lignans and yogurt and different cardiovascular risk parameters.

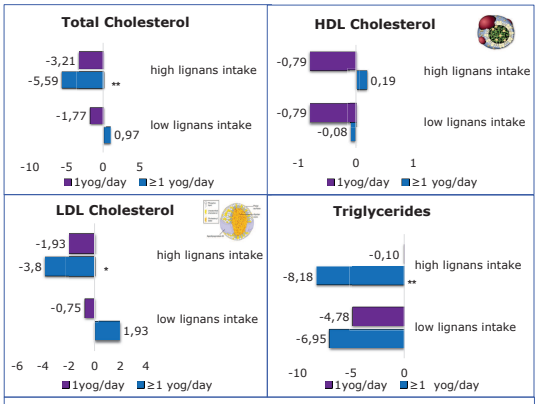
Results and Conclusions

DISTRIBUTION OF THE LIGNANS FOOD SOURCES IN THE PREDIMED COHORT



In this figure, the main food sources of lignans are shown. Olive oil was the main sources, representing more than 60% of the intake. Wheat products and tomato were the the 3rd and the 4th source of lignans intake by the Predimed. The remaining 17% came from other sources like red wine (5%), asparagus (4%), kiwis (3%) and other fruits and vegetables.

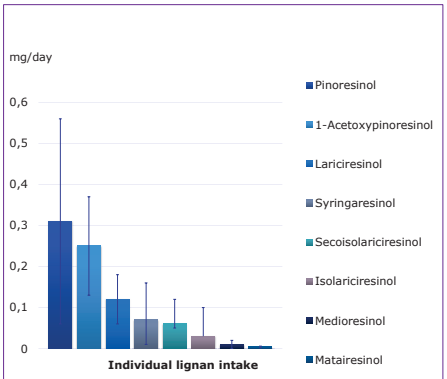
INFLUENCE OF SIMULTANEOUSLY INTAKE OF YOGHURT AND LIGNANS ON BIOCHEMICAL PARAMETERS



Plots represents the GLM coefficients. All of them are adjusted by sex, age, smoking, intake of soft drinks, fiber, energy, carbohydrates and saturated fatty acids.
*p<0,05; **p<0,005 ;***p<0,0005

Participants with higher consumption of lignans and yoghurt had significantly lower Total cholesterol, LDL cholesterol and Triglyceride levels. No significant differences were observed in HDL cholesterol and Glucose. Volunteers with higher consumption of yoghurts but lower intake of lignans had significantly higher weight. But there were no significant differences when people had higher consumption of yoghurt and lignans together.

AVERAGE INTAKE OF EACH INDIVIDUAL LIGNAN



The most consumed lignan was pinoresinol (0.31±0.25mg/d), followed by 1-acetoxypinoresinol (0.25±0.12 mg/d), lariciresinol (0.12±0.06mg/d), syringaresinol (0.07±0.09mg/d), secoisolariciresinol (0.06±0.06mg/d), isolariciresinol (0.03±0.07mg/d), medioresinol (0.01±0.01mg/d) and matairesinol (0.004±0.002mg/d).

Our findings suggest that a synergistic effect of lignans and yogurt ameliorates cardiovascular risk parameters (lower total cholesterol, LDL cholesterol and triglycerides) in humans. Therefore, daily yogurt consumption in a healthy, well-balanced diet with lignan-rich foods could be recommended to enhance the beneficial effects of these two foods obtained when ingested separately, at least in elderly populations.

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Clinical Trial Registration

International Standard Randomized Controlled Trial Number (ISRCTN of London, England) 35739639.

Acknowledgments

We would like to thank to the Instituto Danone, and to the CICYT (AGL2013-49083-C3-1-R) from the Spanish Ministry of Economy and Competitively the Generalitat de Catalunya (GC) 2014 SGR 773, and to the CIBEROBN from the Instituto de Salud Carlos III, ISCIII (CIBEROBN).

References

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Yoghurts and lignans work synergistically decreasing LDL cholesterol

A. Creus-Cuadros[1], A. Tresserra-Rimbau[1,2], P. Quifer-Rada[1,2], J. Salas-Salvadó[2,3], M.A. Martínez-González[2,4], D. Corella[2,5], R. Estruch[2,6], M. Fitó[2,7] and R.M. Lamuela-Raventós[1,2*], on behalf of the PREDIMED Study Investigators

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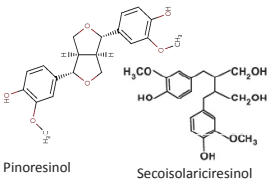
Introduction

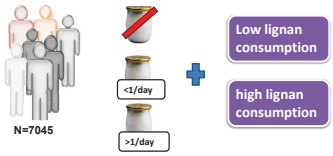
Lignans are interesting from a public health point of view as they constitute, along with isoflavonoids, the major phytoestrogen intake in western population and they play a role in the prevention of cardiovascular and other chronic diseases. Probiotics are live microorganisms that confer a health benefit on the host when they are administered in adequate amounts. Nowadays, most of the probiotics consumed by humans come from fermented dairy products as yoghurt (produced using a culture of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*).

Objective

We hypothesized that yoghurts may improve lignans bioavailability, increasing absorption by colonic mucosa and/or facilitating the bioactivation of polyphenols, lowering cardiovascular risk factors.



Study design and method



We used data from the PREDIMED study, a cohort that included 7447 elderly participants at high cardiovascular risk. Intake of lignans was calculated by matching food consumption data from baseline food frequency questionnaires with the Phenol explorer database on the polyphenol content of each reported food. General lineal models were used to assess the association between lignans and yoghurt and different cardiovascular risk parameters.

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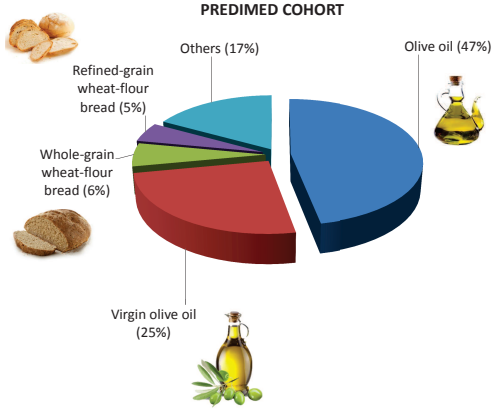
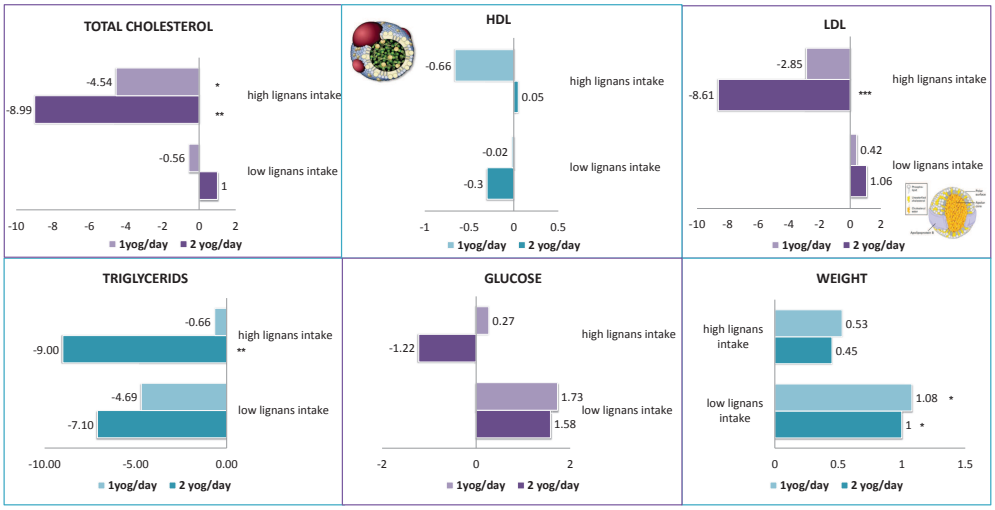


In this figure, the main food sources of lignans are shown. Olive oil and virgin olive oil were the main sources, representing more than 70% of the intake. Whole-grain and refined-grain wheat flour bread were the 3rd and the 4th source of lignans intake by the Predimed. The remaining 17% came from other sources like broccoli, cabbagge, apricots, garlics and pears, among others.

INFLUENCE OF SIMULTANEOUSLY INTAKE OF YOGHURT AND LIGNANS ON BIOCHEMICAL PARAMETERS



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*p<0,05; **p<0,005 ;***p<0,0005

Participants with higher consumption of lignans and yoghurt had significantly lower Total cholesterol and LDL cholesterol. However, no differences were observed when separated analyses of lignans and yoghurt were performed. In addition, participants with higher consumption of lignans and higher consumption of yoghurts had lower Triglycerid levels. No significant differences were observed in HDL cholesterol and Glucose.

Volunteers with higher consumption of yoghurts but lower intake of lignans had significantly higher weight. But there were no significant differences when people had higher consumption of yoghurt and lignans together.

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« Yoghurts and lignans work synergistically decreasing cholesterol LDL »

ICPH 2015

TOURS, FRANCE

USE OF HIGH RESOLUTION MASS SPECTROMETRY TOOLS FOR THE SCREENING OF THE POLYPHENOLIC METABOLIC DIFFERENCES BETWEEN WINE AND DEALCOHOLIZED WINE

Anna Creus-Cuadros[1], Paola Quifer-Rada[1,2], Xiaohui Guo[1], Mariel Colmán-Martínez[1], Mercè Mercader Martí[3], Rosa M. Lamuela-Raventós[1,2]

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[2]*CIBER Fisiopatología de la obesidad y nutrición (ciberobn) and RETICS RD06/0045/0003. Institute of Health Carlos III, Spain*
[3]*Miguel Torres, Vilafranca del Penedès, Spain*

Introduction

Red wine is a rich source of polyphenols. Its moderate intake is associated with beneficial effects on health, such as the prevention of cardiovascular disease. Up to now, only a few studies have focused on the bioavailability of polyphenols in a wine matrix based on the presence or absence of alcohol. Nowadays, there is no consensus as to whether alcohol increases polyphenol bioavailability by improving polyphenol solubility or by increasing their elimination as a result of the diuretic effect. The aim of this study was to analyze the phenolic profile and their plasma metabolites after an acute intervention of wine and dealcoholized wine (DW) in order to test if the alcohol present in the wine matrix affected phenolic absorption and metabolism.

Objective

To analyze the phenolic profile and their metabolites in plasma after an acute intervention of wine and dealcoholized wine (DW) in order to test if the alcohol present in the wine matrix affects the phenolic absorption and metabolism.

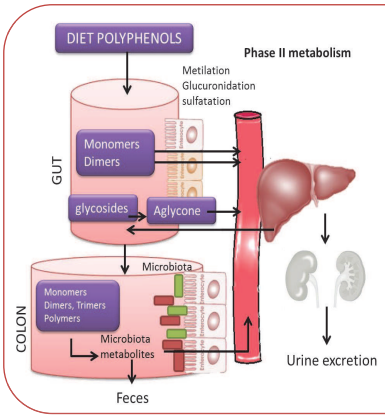
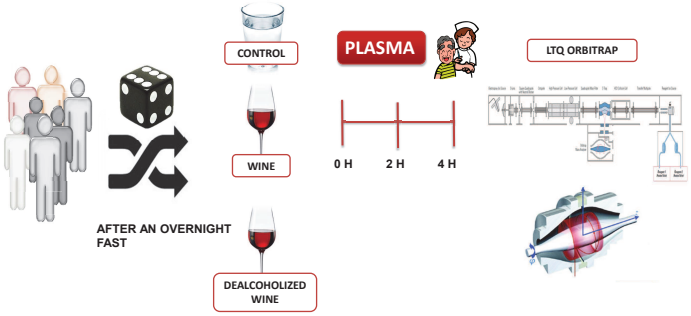


Figure 1. Routes for dietary polyphenols and their metabolism. Polyphenols could undergo metilation, glucuronidatation and sulfatation in the enterocyte as well as in the liver cells. Conjugated metabolites could be eliminated by urine excretion but they also could be released back to the lumen. Thus, the non absorbed polyphenols and the released polyphenols could be metabolized by the colon microbiota or excreted by feces.

Study design and method

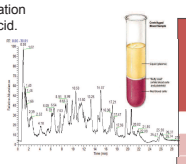


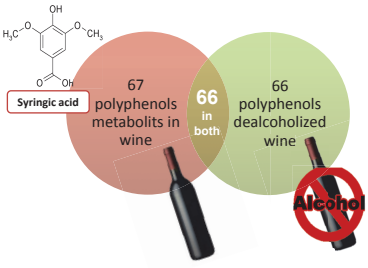
A randomized crossover intervention study was carried out. After an overnight fast the volunteers consumed wine or DW and samples of plasma were collected at 0h, 2h and 4h.
Liquid chromatography coupled to high resolution mass spectrometry was used for an accurate identification of polyphenols and polyphenolic metabolites in plasma

Results

Table1. Table shows the Total Polyphenol concentration in wine and DW expressed in equivalents of Gallic acid.

Total polyphenols	[Mean Conc.] meq GA/L	SD	CV
WINE	2686.2	138.0	5.1
DW	2179.9	114.5	5.3





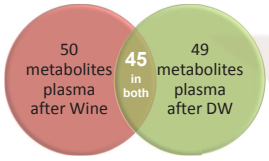


Table2. Table only shows phenolic metabolites that were different between RW and DRW interventions.

POLYPHENOLIC METABOLIC DIFFERENCES	PLASMA AFTER INTERVENTION			
	WINE		DEALCOHOLISED WINE	
	2H	4H	2H	4H
HYDROXYBENZOIC ACIDS				
Protocatechuic acid glucoside		✓	✓	✓
4-hydroxyhippuric acid *		✓	✓	✓
Syringic acid	✓	✓		
Vanillic acid sulfate 1		✓	✓	✓
Vanillic acid sulfate 2		✓	✓	✓
Gallic acid ethyl ester*	✓		✓	✓
PHENYLACETIC ACIDS				
Homovanillic acid sulfate		✓	✓	✓
Homoprotocatechuic acid *		✓	✓	✓
Homovanillic acid*				✓
CYNNAMIC ACIDS				
Dihydrocaffeic acid sulfate		✓	✓	✓
Caffeic acid*				✓
Ferulic acid 4-*O*-glucuronide				✓
p-coumaric acid *		✓	✓	✓
o-coumaric acid*		✓		
STILBENES				
trans-resveratrol-4'-*O*-glucuronide*		✓		
trans-resveratrol*		✓	✓	✓
FLAVONOLS				
kaempferol-3-glucoside*	✓		✓	✓
kaempferol*	✓			
FLAVANOLS				
Epicatechin*				✓
(Epi)catechin sulfate		✓	✓	✓
taxifolin*	✓			

Metabolites of benzoic acids, hydroxycinnamic acid, stilbenes, flavones, flavonols and their derivatives were identified; including metabolites derived from the microbiota metabolism such as propionic acids, phenylacetic acids, hydroxyphenylpentanoics acids and valerolactones. Also, several conjugated metabolites of the phase II metabolism were identified such as glucuronidated and sulfated metabolites. For example protocatechuic acid was observed in wine and its conjugated form protocatechuic-*O*-glucoside acid was identified in plasma. In addition, methylated, methyl-glucuronidated, diglucuronidated, di- and trisulfated and sulfated-glucuronidated conjugated forms occurred during the phase II metabolism were also identified.

Conclusions

- Dealcoholized wine had lower concentration of polyphenols than wine.
- 67 and 66 phenolic compounds were identified in wine and dealcoholized wine, respectively. Syringic acid was only identified in the wine sample.
- 50 and 49 polyphenolic metabolites were identified in plasma after wine and DW consumption, respectively.
- Plasma polyphenols remained detectable longer after the DW intervention (at least 2h), possibly due to the diuretic effect of the ethanol.

References

1. Tresserra-Rimbau, A. *et al.* 2013.
2. Tresserra-Rimbau, A. *et al.* 2014.
3. Chiva-Blanch, G. *et al.* 2012.
4. Chiou, Y.-S. *et al.* 2014.
5. Queipo-Ortuño, M. I. *et al.* 2012.
6. Ortuño, J. *et al.* 2010.
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22nd Young Research Fellow Meeting 2015

SCREENING THE POLYPHENOLIC METABOLIC DIFFERENCES BETWEEN WINE AND DEALCOHOLIZED WINE IN PLASMA BY HIGH RESOLUTION MASS SPECTROMETRY

Anna Creus-Cuadros[1], Paola Quifer-Rada[1,2], Guo Xiaohui[1], Mariel Colmán-Martínez[1], Mercè Mercader Martí[3], Rosa M. Lamuela-Raventós[1,2]

[1]*Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain*
[2]*CIBER Fisiopatología de la obesidad y nutrición (ciberobn) and RETICS RD06/0045/0003. Institute of Health Carlos III, Spain*
[3]*Miguel Torres, Vilafranca del Penedès, Spain*

Introduction

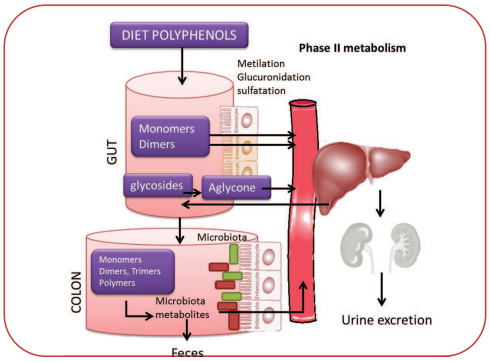
Red wine is a rich source of polyphenols. Its moderate intake is associated with beneficial effects on health, such as cardiovascular disease prevention. Up to now, only a few studies have focused on the bioavailability of polyphenols considering the matrix of wine, such as alcohol. Nowadays, there is no consensus if alcohol increases polyphenols bioavailability by improving polyphenol's solubility or increases the elimination as a result of the diuretic effect. The aim of this study was to analyze the phenolic profile and their metabolites in plasma after an acute intervention of wine and dealcoholized wine (DW) in order to test if the alcohol present in the wine matrix affected phenolic absorption and metabolism.

Objective

To analyze the phenolic profile and their metabolites in plasma after an acute intervention of wine and dealcoholized wine (DW) in order to test if the alcohol present in the wine matrix affects the phenolic absorption and metabolism.

Method

Liquid chromatography coupled to high resolution mass spectrometry was used for an accurate identification of polyphenols and polyphenolic metabolites in plasma at 0h, 2h and 4h after an acute consumption of wine and dealcoholized wine.

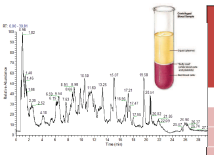


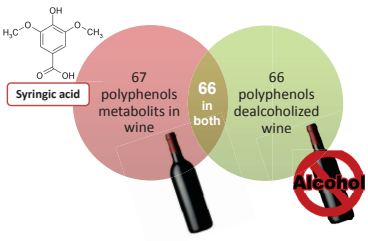
Study design

Results

Table1. Table shows the Total Polyphenol concentration in wine and DW expressed in equivalents of Gallic acid.

Total polyphenols	[Mean Conc.] meq GA/L	SD	CV
WINE	2686.2	138.0	5.1
DW	2179.9	114.5	5.3





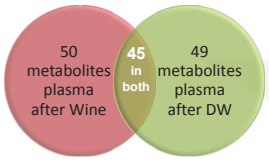


Table2. Table only shows phenolic metabolites that were different between RD and DRW interventions.

POLYPHENOLIC METABOLIC DIFFERENCES	PLASMA AFTER INTERVENTION			
	WINE		DEALCOHOLISED WINE	
	2H	4H	2H	4H
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PHENYLACETIC ACIDS				
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Homoprotocatechuic acid *		✓	✓	✓
Homovanillic acid*				✓
CYNNAMIC ACIDS				
Dihydrocaffeic acid sulfate		✓	✓	✓
Caffeic acid*				✓
Ferulic acid 4-*O*-glucuronide				✓
p-coumaric acid *		✓	✓	✓
o-coumaric acid*		✓		
STILBENES				
trans-resveratrol-4'-*O*-glucuronide*		✓		
trans-resveratrol*		✓	✓	✓
FLAVONOLS				
kaempferol-3-glucoside*	✓		✓	✓
kaempferol*	✓			
FLAVANOLS				
Epicatechin*				✓
(Epi)catechin sulfate		✓	✓	✓
taxifolin*	✓			

Metabolites of benzoic acids, hydroxycinnamic acid, stilbenes, flavones, flavonols and their derivatives were identified; including metabolites derived from the microbiota metabolism such as propionic acids, phenylacetic acids, hydroxyphenylpentanoics acids and valerolactones. Also, several conjugated metabolites of the phase II metabolism were identified such as glucuronidated and sulfated metabolites. For example protocatechuic acid was observed in wine and its conjugated form protocatechuic-*O*-glucoside acid was identified in plasma. In addition, methylated, methyl-glucuronidated, diglucuronidated, di- and trisulfated and sulfated-glucuronidated conjugated forms occurred during the phase II metabolism were also identified.

Conclusions

- Dealcoholized wine had lower concentration of polyphenols than Wine.
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7. Manach, et al. 2004.

Effects of bioactive compounds from dealcoholized red wine with two different polyphenol contents, on endothelial function in subjects with metabolic syndrome and high cardiovascular risk

G. Sasot[1], A. Creus-Cuadros[1], M. Mercader-Martí[4], RM. Lamuela-Raventós[1,2] , R. Estruch[2,3]

[1]Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain
[2]CIBER Physiopathology of obesity and nutrition (CIBEROBN), Institute of Health Carlos III, Spain
[3]Department of Internal Medicine, Hospital Clínic, Institute of Biomedical Investigation August Pi i Sunyer (IDIBAPS), University of Barcelona, Spain
[4]Miguel Torres, Vilafranca del Penedés, Spain

Introduction

Metabolic syndrome (MetS) is generally considered to be a cluster of conditions including impaired glucose metabolism, central obesity, elevated triglycerides with reduced HDL-cholesterol and elevated blood pressure. Several studies revealed that mortality and risk from cardiovascular disease (CVD) and coronary heart disease (CHD) are higher in subjects with MetS. Epidemiological studies and intervention clinical trials have shown that dealcoholized red wine (DRW) and moderate consumption of red wine (RW) are inversely associated with cardiovascular risk factors. Both, RW and DRW, reduce the expression of cell adhesion molecules (CAM) indicating a possible additional protective effect due to the non-alcoholic fraction of red wine.

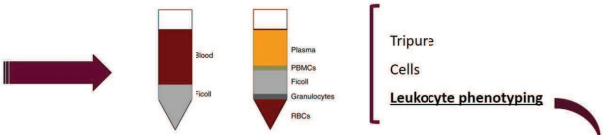
Study design and Methods

A **randomized**, **open**, **prospective**, and **controlled** clinical trial, running in **parallel** was performed in 36 subjects with MetS to evaluate the effects of wine polyphenols

n=54
Age (y): 70 ±3,8
BMI (kg/m²): 26,1 ±8,7

375 mL/day of **DRW**, dealcoholized red wine with grape extract (**DRWEx**) or **water** were administered during **three months**

Anthropometric measurements and **blood biochemical analysis** were carried out at **baseline** and **after each intervention**



LFA-1a, MAC-1a, VLA-4 (CD49d), CD15, CD40, CD31, CD36, hCCR2 → ✓ **T-Lymphocytes** (CD2), ✓ **Monocytes** (CD14)

CD34, CD133, KDR → ✓ Endothelial progenitor cells (**EPC**)

CD34, CD45, CD146 → ✓ Circulating endothelial cells (**CEC**)

Levels of **EPC**, **CEC** and **leukocyte cell membrane receptors** were analysed by **flow cytometry**

Results

After DRW and DRWEx interventions, we observed a decrease on body weight, body mass index, waist circumference, total cholesterol concentration and an improvement on blood pressure. Moreover, there was an increase in the number of EPCs and a significant decrease in levels of CECs and, T-lymphocyte and monocyte expression.

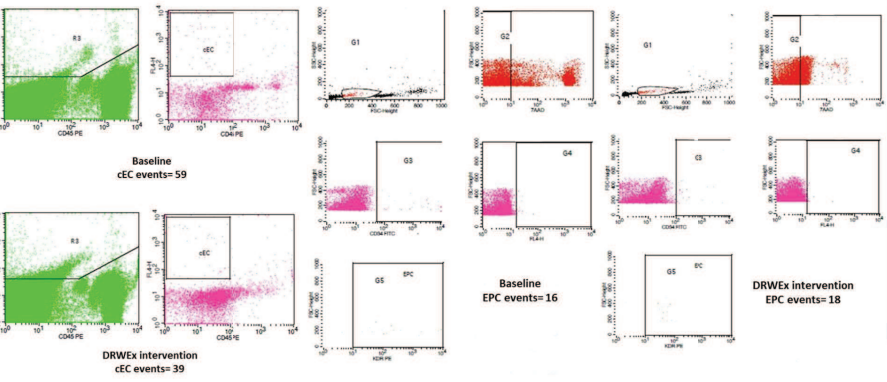


Figure 1. Levels of CEC and EPC in one subject at baseline and after DRWEx intervention

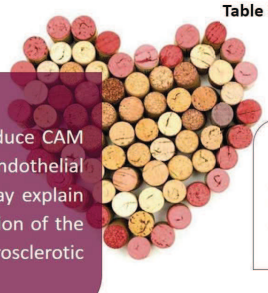
	EPC	CEC
Control		
Before	14.5 ±6.0	42.6 ±16.3
After	12.6 ± 9.7	42.4 ± 17.2
Difference (95% CI)	-1.88 (-6.49, 2.74)	-0.25 (-19.86, 19.36)
DRW		
Before	24.1 ± 15.5	91.3 ±117.8
After	21.4 ± 9.0	40.1 ± 29.0
Difference (95% CI)	-2.75 (-18.11, 12.61)	-51.13 (-128.51, 26.26)*
DRWEx		
Before	18.9 ±9.5	82.8 ± 58.2
After	30.0 ±27.5	36.9 ± 20.9
Difference (95% CI)	11.10 (-11.88, 34.08)	-45.90 (-86.03, -5.77)*

95% CI: 95% confidence intervals of the differences after the intervention.
*$P \leq 0,05$, compared to before each intervention

Table 1. Results of analysis of endothelial cells (EPC and CEC). Expressed in number of c

Conclusions

We conclude that the non-alcoholic fraction of wine, rich in polyphenols, may reduce CAM and CEC, known markers of severity of CVD, and increase EPC, a marker of endothelial regeneration, in a population at high risk of CVD due to MetS. These features may explain why DRW and moderate RW consumption suggests an improvement in the condition of the vascular endothelium and possibly contributes to delay the development of atherosclerotic plaques.



Financial support

Authors would like to express their gratitude for financial support from INCOMES proje supported by Ministerio de Economía y Competitividad through the INNPRONTA progra And CICYT (AGL2013-49033-C3-1-R), Instituto de Salud Carlos III (CIBERObn) and th Generalitat de Catalunya (GC) 2014 SGR 773.

Diferencias nutricionales entre una dieta rica en antioxidantes y una dieta baja en antioxidantes

Sara Hurtado-Barroso[1,2], Jose Fernando Rinaldi Alvarenga[1], Paola Quifer-Rada[1,2], Anna Creus-Cuadros[1], Rosa María Lamuela-Raventós[1,2]

[1]Department ofNutrition, Food Science, and Gastronomy, School of Pharmacy, INSA-University of Barcelona, Barcelona, Spain

[2]CIBER Physiopathology of obesity and nutrition (CIBERobn), Instituto de Salud Carlos III, Madrid, Spain

INTRODUCCIÓN

La dieta mediterránea, rica en hortalizas y frutas, previene enfermedades crónicas tales como las enfermedades cardiovasculares debido en parte al alto contenido en compuestos con propiedades antioxidantes para la salud.

OBJETIVO

Valorar si con el seguimiento de una dieta típicamente mediterránea, rica en antioxidantes, respecto a una dieta con un consumo de 2 piezas de fruta u hortalizas (pobre en antioxidantes), mejoraba el aporte de antioxidantes.

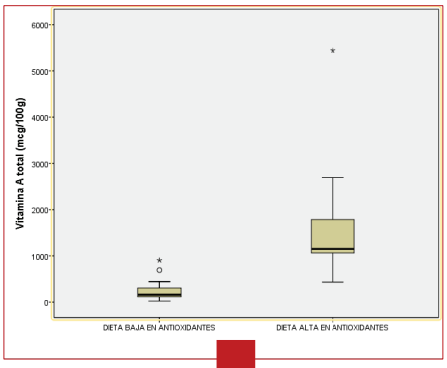
DISEÑO DEL ESTUDIO

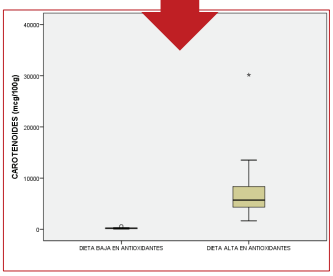
- Randomizado
- Cruzado
- Aleatorizado

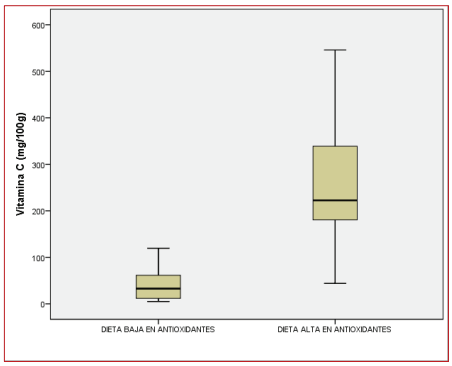
18 varones
18-32 años
No fumadores
Sanos

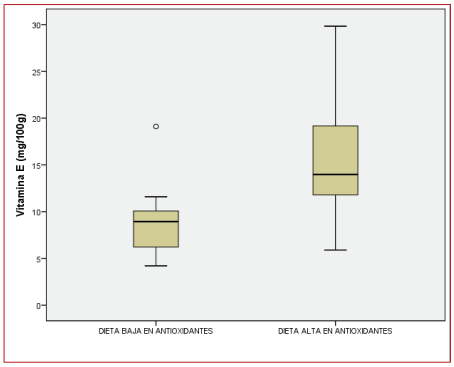
REGISTROS DIETÉTICOS DE LOS 3 ULTIMOS DÍAS

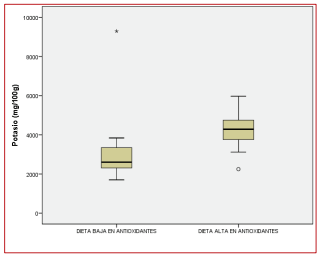
RESULTADOS

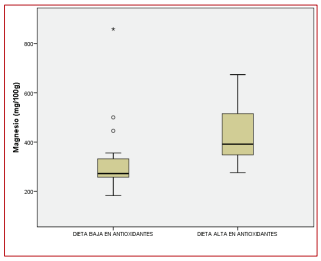












CONCLUSIÓN

Las recomendaciones nutricionales van dirigidas a aumentar el consumo de alimentos ricos en antioxidantes (frutas y hortalizas), incrementar la ingesta de las vitaminas antioxidantes (A, E y C), de los carotenoides; así como de algunos minerales tales como el potasio y magnesio.

Assessment of different doses of polyphenols from dealcoholized red wine on endothelial function in subjects with Metabolic Syndrome

G. Sasot[1], A. Creus-Cuadros[1], R. Estruch[2,3], M. Mercader-Martí[4], RM. Lamuela-Raventós[1,2]

[1]Nutrition and Food Science Department, XaRTA, INSA. Pharmacy School, University of Barcelona, Av. Joan XXIII s/n Barcelona, Spain
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n=36
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BMI (kg/m²): 26,1 ±8,7

375 mL/day of **DRW**, dealcoholized red wine with grape extract (**DRWEx**) or **water** were administered during **three months**

Anthropometric measurements and **blood biochemical analysis** were carried out at **baseline** and **after each intervention**

Tripure
Cells
Leukocyte phenotyping

LFA-1a, MAC-1a, VLA-4 (CD49d), CD15, CD40, CD31, CD36, hCCR2 → ✓ **T-Lymphocytes** (CD2)
✓ **Monocytes** (CD14)
CD34, CD133, KDR → ✓ Endothelial progenitor cells (**EPC**)
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Levels of **EPC**, **CEC** and **leukocyte cell membrane receptors** were analysed by **flow cytometry**

Results

After DRW and DRWEx interventions, we observed a decrease on body weight, body mass index, waist circumference, total cholesterol concentration and an improvement on blood pressure. Moreover, there was an increase in the number of EPCs and a significant decrease in levels of CECs and, T-lymphocyte and monocyte expression.

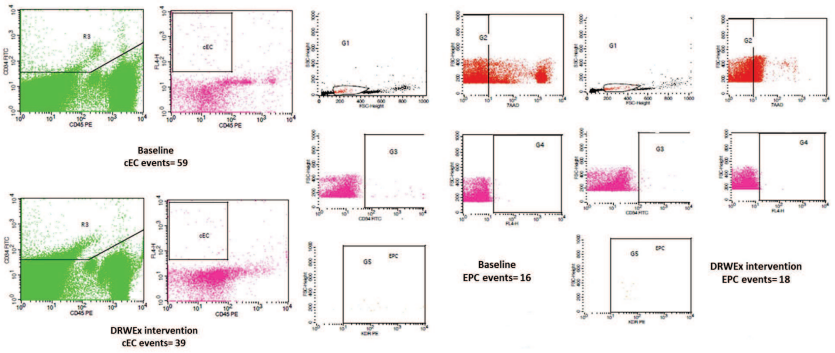

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Table 1. Results of analysis of endothelial cells (EPC and CEC). Expressed in number of cells

Conclusions

We conclude that the non-alcoholic fraction of wine, rich in polyphenols, may reduce CAM and CEC, known markers of severity of CVD, in a population at high risk of CVD due to MetS. This reduction suggests an improvement in the condition of the vascular endothelium and possibly contributes to delay the development of atherosclerotic plaques. An increased number of EPC is inversely associated with risk factors of CVD.

Financial support

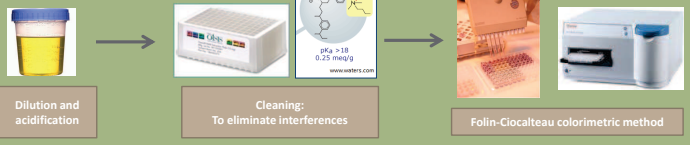
Authors would like to express their gratitude for financial support from INCOMES project supported by Ministerio de Economía y Competitividad through the INNPRONTA program. And CICYT (AGL2013-49083-C3-1-R), Instituto de Salud Carlos III (CIBERObn) and the Generalitat de Catalunya (GC) 2014 SGR 773.

DEVELOPMENT OF NEW BIOMARKERS FOR NUTRITIONAL EPIDEMIOLOGY

Anna Tresserra-Rimbau, Paola Quifer-Rada, Miriam Martínez-Huélamo, Anna Creus-Cuadros, Gemma Sasot, Mariel Colmán-Martínez, Xiaohui Guo, Rosa M. Lamuela-Raventós.

[1]*Department of Nutrition and Food Science-XARTA-INSA, School of Pharmacy, University of Barcelona, Barcelona, Spain.*
[2]*Centro de Investigación Biomédica en Red de la Fisiopatología de la Obesidad y Nutrición (ciberobn). Instituto de Salud Carlos III, Madrid, Spain.*

Total polyphenols in urine → As a biomarker of → **Consumption of fruits and vegetables**



Dilution and acidification | Cleaning: To eliminate interferences | Folin-Ciocalteau colorimetric method

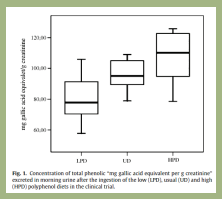


Fig. 1. Concentration of total phenolic "mg gallic acid equivalent per g creatinine" excreted in morning urine after the ingestion of the low (LPD), usual (UD) and high (HPD) polyphenol diets in the clinical trial.

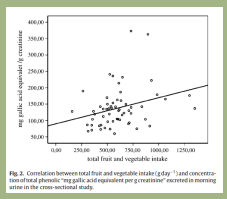


Fig. 2. Correlation between total fruit and vegetable intake (g·day⁻¹) and concentration of total phenolic "mg gallic acid equivalent per g creatinine" excreted in morning urine in the cross-sectional study.

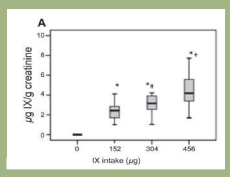
Results from a clinical trial | Results from a cross-sectional study within the PREDIMED trial

Medina-Remón A. Bioanalysis. 4(22):2705-13, 2012
Medina-Remón A. Anal. Chim. Acta. 639: 54-60, 2009

Isoxanthohumol in urine → As a biomarker of → **Consumption of beer**

Cleaning: To eliminate interferences | LC-ESI-QqQ-MS/MS

Box plot of urinary excretion of IX in males after different doses of IX from beer.



Results from a clinical trial

Quifer-Rada P. J. Nutr. 144: 484-488, 2014
Quifer-Rada P. Anal. Chem. 85:5547-54, 2013

To assess the effect of diet on health it is necessary to accurately determine nutrient and food intakes. Traditional dietary assessment methods, such as food frequency questionnaires or 24-h recalls are subjective and they do not consider bioavailability and metabolism. On the other hand, nutritional biomarkers have become a good alternative to estimate dietary intake because they are objective, accurate, consider bioavailability and they are useful to monitor dietary intervention accomplishment.

Martínez-Huélamo M. Foof Chem. 168: 203-210, 2015

Results from a clinical trial

Intervention with → Tomato juice / Tomato juice + olive oil

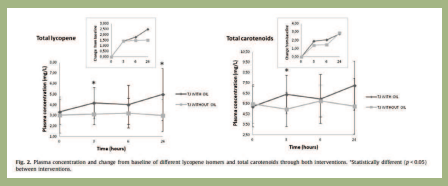
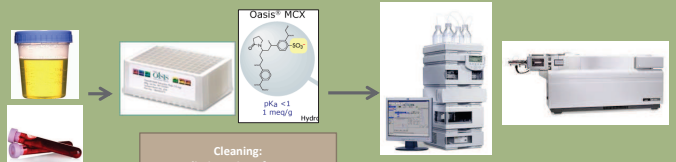


Fig. 2. Plasma concentration and change from baseline of different lycopene isomers and total carotenoids through both interventions. *Statistically different ($p < 0.05$) between interventions.



Cleaning: To eliminate interferences | LC-ESI-QqQ-MS/MS

Lycopene and β-carotene in plasma → As a biomarker of → **Consumption of tomatoes**

Regueiro J. Br. J. Nutr. 111: 1680-85, 2014

Results from a clinical trial

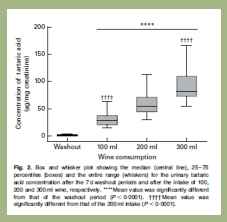
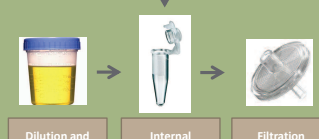


Fig. 2. Box and whisker plot showing the median (central line), 25–75 percentiles (boxes) and the entire range (whiskers) for the urinary tartaric acid concentration after the 7-d washout periods and after the intake of 100, 200 and 300 ml wine, respectively. ****Mean value was significantly different from that of the washout period ($P < 0.001$). ††††Mean value was significantly different from that of the 200 ml intake ($P < 0.001$).

^{2}H-labelled isotope standard solution



Dilution and acidification | Internal standard | Filtration (0.20 μm) | LC-ESI-QqQ-MS/MS

Tartaric acid in urine → As a biomarker of → **Consumption of wine**

Conclusions

Total polyphenols in urine are a good biomarker of fruits and vegetables (and their derivatives) consumption and it is very well correlated with polyphenol intake, while isoxanthohumol, a new biomarker developed by our group, is used to accurately measure beer consumption. Other examples are tartaric acid quantification in urine for wine consumption and lycopene and beta-carotene in plasma for tomato products. It is necessary, however, to further investigate new biomarkers for other key foods.

Acknowledgements

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