

# Effects of the combination of $\omega$ -3 PUFAs and proanthocyanidins on the gut microbiota of healthy rats

Sara Ramos-Romero<sup>a,\*</sup>, Mercè Hereu<sup>a</sup>, Eunice Molinar-Toribio<sup>a,1</sup>, María Pilar Almajano<sup>b</sup>, Lucía Méndez<sup>c</sup>, Isabel Medina<sup>c</sup>, Núria Taltavull<sup>d</sup>, Marta Romeu<sup>d</sup>, Maria Rosa Nogués<sup>d</sup> and Josep Lluís Torres<sup>a</sup>

<sup>a</sup> Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain;  
sara.ramos@iqac.csic.es (S.R.-R.); merce.hereu@iqac.csic.es (M.H.);  
eice7@hotmail.com (E.M.-T.); josepluis.torres@iqac.csic.es (J.L.T.)

<sup>b</sup> Chemical Engineering Department, Technical University of Catalonia, Barcelona, Spain; m.pilar.almajano@upc.edu (M.P.A)

<sup>c</sup> Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain;  
luciamendez@iim.csic.es (L.M.); medina@iim.csic.es (I.M.)

<sup>d</sup> Faculty of Medicine and Health Science, Universitat Rovira i Virgili, Reus, Spain ;  
nuria.taltavull@urv.cat (N.T.); marta.romeu@urv.cat (M.R.);  
mariarosa.nogues@urv.cat (M.R.N.)

## \* Corresponding Author

Dr. Sara Ramos-Romero

Institute of Advanced Chemistry of Catalonia (IQAC-CSIC), Jordi Girona, 18-26,  
08034 Barcelona, Spain

Phone: (+34) 934006100; Fax: (+34) 932045904

E-mail: sara.ramos@iqac.csic.es

---

<sup>1</sup> Present address: Department of Biochemistry, Faculty of Natural Sciences, Exact and Technology, University of Panama, Panama City, Panama

## ABSTRACT

$\omega$ -3 Polyunsaturated fatty acids (PUFAs) reduce risk factors for cardiovascular diseases (CVD) and other pathologies that involve low-grade inflammation. They have recently been shown to exert complementary functional effects with proanthocyanidins. As the reduction of health-promoting gut bacteria such as lactobacillus and bifidobacteria has been linked to a number of alterations in the host, the aim of this study was to determine whether PUFAs and proanthocyanidins also cooperate in maintaining well-balanced microbiota. To this end, rats were supplemented for 6 months with eicosapentaenoic acid (EPA)/docosahexaenoic acid (DHA) 1:1 (16.6 g/kg feed); proanthocyanidin-rich grape seed extract (GSE, 0.8 g/kg feed); or both. Plasma adiponectin, cholesterol, and urine nitrites were measured. Gut bacterial subgroups were evaluated in fecal DNA by qRT-PCR. Short-chain fatty acids (SCFAs) were determined in feces by gas chromatography. Body and adipose tissue weights were found to be higher in the animals given  $\omega$ -3 PUFAs, while their energy intake was lower. Plasma cholesterol was lower in  $\omega$ -3 PUFA supplemented groups, while adiponectin and urine nitrites were higher.  $\omega$ -3 PUFAs reduced the population of Lactobacillales and *L. acidophilus* after 6 months of supplementation. GSE significantly reduced *L. plantarum* and *B. longum*. The combination of  $\omega$ -3 PUFAs and GSE maintained the health-promoting bacteria at levels similar to those of the control group. Acetic acid was increased by the  $\omega$ -3 PUFA individual supplementation, while the combination with GSE kept this value similar to the control value. In conclusion, while individual supplementations with  $\omega$ -3 PUFAs or GSE modify the populations of Lactobacillus, Bifidobacterium and microbial products (SCFAs), their combination maintains the standard proportions of these bacterial subgroups and their function while also providing the cardiovascular benefits of  $\omega$ -3 PUFAs.

**KEYWORDS:** microbiota, proanthocyanidins; EPA/DHA; Lactobacillus; Bifidobacterium

## HIGHLIGHTS

- $\omega$ -3 PUFAs reduce the populations of gut Lactobacilliales and *L. acidophilus*
- $\omega$ -3 PUFAs reduce the concentration of the microbial product acetic acid in feces
- GSE reduces the populations of gut *Lactobacillus plantarum* and *Bifidobacterium longum*
- $\omega$ -3 PUFAs promote weight gain and fat accumulation in healthy rats
- Combined,  $\omega$ -3 PUFAs and GSE maintain balanced gut bacteria and short-chain fatty acids

## 1. INTRODUCTION

$\omega$ -3 Polyunsaturated fatty acids ( $\omega$ -3 PUFAs) and polyphenols are bioactive compounds present in common foodstuffs (Quideau, Deffieux, Douat-Casassus, & Pouysegue, 2011; Tapiero, Ba, Couvreur, & Tew, 2002). Eicosapentaenoic acid (EPA, 20:5, n-3) and docosahexaenoic acid (DHA, 22:6, n-3) are the major  $\omega$ -3 PUFAs of marine origin. Different  $\omega$ -3 PUFAs may have different effects, many of them related to the prevention of cardiovascular diseases (CVD). We recently showed that mixtures of EPA and DHA at different proportions have different effects on CVD risk factors in rats: EPA/DHA 1:1 and 2:1 decrease inflammation, while oxidative stress is more effectively reduced by a 1:2 mixture in obese hypertense rats (Molinar-Toribio et al., 2015). The 1:1 mixture was the most effective at reducing protein carbonylation (Méndez et al., 2013) and CVD risk markers (Lluís et al., 2013) in rats fed a standard diet. The molecular mechanism through which  $\omega$ -3 PUFAs exert their preventive effects include shift of lipids from the  $\omega$ -6 to the  $\omega$ -3 metabolic pathway and the modulation of genes associated with both lipid catabolism and anabolism (Poudyal, Panchal, Diwan, & Brown, 2011). Consequently,  $\omega$ -3 PUFAs help maintain the normal levels of systemic biomarkers of CVD, such as plasma cholesterol and triglycerides, oxidative stress, and blood pressure (Poudyal et al., 2011). Despite hypertension and other CVD risk factors having been linked to gut dysbiosis (Yang et al., 2015) and the possibility that they may be averted by probiotics (Miremadi, Sherkat, & Stojanouska, 2016), the effect of  $\omega$ -3 PUFAs on gut microbiota has hardly been studied. Studies of the effects of  $\omega$ -3 PUFAs on microbiota have mainly focused on the major bacterial phyla Bacteroidetes and Firmicutes.  $\omega$ -3 PUFAs from flaxseed seem to decrease the proportion of Bacteroidetes (Liu, Hougen, Vollmer, & Hiebert, 2012), and those from fish oil lower the population of Firmicutes (Yu et al., 2014). A reduction in the Bacteroidetes/Firmicutes ratio has been linked to weight gain and other metabolic conditions, such as insulin resistance, in part by the synthesis of short-chain fatty acids (SCFAs) (Canfora, Jocken, & Blaak, 2015): end products of the fermentation of indigestible dietary components of the host diet.

Polyphenols are a family of phytochemicals and are widespread in plant foods; they have a common structure including at least two phenol groups (Bravo, 1998; Quideau et al., 2011). Dietary polyphenols and their metabolites exert a beneficial effect through a combination of mechanisms that may include the reduction of inflammation and

oxidative stress (Pan, Lai, & Ho, 2010; Salvadó, Casanova, Fernández-Iglesias, Arola, & Bladé, 2015), as well as inhibition of intestinal glycosidases and of glucose transporters that reduce postprandial glycaemia (Williamson, 2013). Moreover, polyphenols can modulate the intestinal microbial composition and modify the metabolic activity of gut bacteria in humans (Duda-Chodak, 2012). Proanthocyanidins are oligomeric and polymeric flavan-3-ols composed mainly of (epi)catechin and its gallic acid esters:-( -Epicatechin and (+)-catechin from tea and cocoa protect commensal anaerobes and probiotics such as *Bifidobacterium* spp. and *Lactobacillus* spp., and they also inhibit potential pathogenic bacteria in humans (Lee, Jenner, Low, & Lee, 2006). Similarly, flavan-3-ols from grape products reduce the growth of *Clostridium* spp., while increasing the populations of other subgroups such as *Lactobacillus*, in rats (Dolara et al., 2005). This effect on *Lactobacillus* as well as on other groups such as *Bifidobacterium* has also been reported in humans after daily intake of red wine for one month (Queipo-Ortuño et al., 2012).

It has recently been shown that  $\omega$ -3 PUFAs and proanthocyanidins exert collaborative functional effects such as a reduction of plasma insulin, leptin, and perigonadal fat accumulation in obese rats (Ramos-Romero et al., 2016), and decrease C-reactive protein concentration in rats with hypercholesterolemia (Sekhon-Loodu et al., 2014). As alterations in these variables have been associated with a reduction in health-promoting gut bacteria such as *Lactobacillus* and *Bifidobacteria* (Arbolea, Watkins, Stanton, & Ross, 2016; Cani et al., 2007; Guardamagna et al., 2014), we decided to study whether  $\omega$ -3 PUFAs and proanthocyanidins also cooperate in maintaining the levels of putatively beneficial gut bacteria. Thus, the aim of this study is to explore the possible complementary function of a combination of dietary  $\omega$ -3 PUFAs (EPA/DHA 1:1) and proanthocyanidins in a grape seed extract (GSE) on the proportions of health-promoting bacteria in rats.

## 2. MATERIALS AND METHODS

### 2.1 Animals

A total of twenty-eight female Wistar-Kyoto rats from Charles River Laboratories (Wilmington, MA, USA), aged 8-10 weeks, were used. Female rats were chosen for consistency with our previous studies of  $\omega$ -3 PUFAs (Lluís et al., 2013; Molinar-Toribio et al., 2015; Ramos-Romero et al., 2016; Taltavull et al., 2014). All animal handling was carried out in the morning to minimize the effects of circadian rhythms. All the procedures strictly adhered to the European Union guidelines (EU Directive 2010/63/EU) for the care and management of laboratory animals, and were approved by the CSIC Subcommittee for Bioethical Issues (reference no. CEEA-12-007).

### 2.2 Experimental design

The rats were housed two to three per cage under controlled conditions of humidity (60%), and temperature ( $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ ) with a 12 h light-12 h dark cycle. The rats were randomly divided into 4 dietary groups ( $n=7/\text{group}$ ): STD, the control group;  $\omega$ -3, a group supplemented with EPA/DHA 1:1 (16.6 g/kg feed); GSE, a group supplemented with 0.84 g GSE/kg feed; and  $\omega$ -3 + GSE, a group supplemented with both EPA/DHA 1:1 and GSE. The experimental diets were pelletized in-house by lyophilization from frozen emulsions. To prevent oxidation and contamination by fungi, the dry pellets were vacuum-packed and stored at  $4^{\circ}\text{C}$  until use. To ensure that all the diets were isocaloric, appropriate amounts of soybean oil was added to the feed preparations that were not supplemented with  $\omega$ -3 PUFAs. To guarantee a proper mixture of the different components and an adequate consistency of the final pellet, soybean lecithin and porcine gelatin were added. The feed compositions are shown in Table 1. Following the reported conversion of animal doses into human equivalent doses (Reagan-Shaw, Nihal, & Ahmad, 2008), the  $\omega$ -3 PUFA supplementation was equivalent to 0.15 mL  $\omega$ -3 PUFAs/kg human and day; and GSE supplementation was equivalent to 6.7-9.5 mg GSE/kg human and day. The standard diet Global 2014 was from Harlan Teklad Inc. (Indianapolis, IN, USA). Porcine gelatin type A 240/60 was from Juncà (Girona, Spain). Soybean lecithin Topcithin 50<sup>TM</sup> was from Cargill (Barcelona, Spain). EPA/DHA 1:1 was obtained by mixing appropriate quantities of the commercial fish oils AFAMPES 121 EPA (A.F.A.M.S.A., Vigo, Spain), EnerZona Omega 3 RX (Milan, Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98, S.L., Barcelona, Spain). The EPA/DHA

1:1 ratio was chosen on the basis of previous results (Méndez et al., 2013; Molinar-Toribio et al., 2015). Soybean oil, obtained from unrefined organic soya beans (first cold pressing), was from Clearspring Ltd (London, UK). The GSE Grajfnol® was from JF-Natural Products (Tianjin, China). The GSE consisting of fine Grajfnol® powder contains 98% grape seed (poly)phenols with the following composition: total proanthocyanidins (UV) ≥95%; oligomeric proanthocyanidins, ≥60%; procyanidin dimer B2 (HPLC) ≥1.8%; ash, -1.5%; weight loss on drying, -5.0%. All the groups had free access to water and feed.

### 2.3 Data and sample collection

Body weight and feed intake were measured after 0, 6, 12, 18 and 24 weeks of the experiment. Feed intake per kg of body weight and day was estimated by dividing the total intake per cage by the total weight of the animals in that cage and the number of days. Energy intake was calculated as estimates of metabolizable energy based on the Atwater factors, assigning 4 kcal/g protein, 9 kcal/g fat, and 4 kcal/g available carbohydrate.

For urine collection, during weeks 14-16 of the experiment, the rats were randomized and placed in metabolic cages and deprived of food for 18 h and all the urine produced during that period was collected. Fecal samples were collected by abdominal massage at week 23 at the same time in the morning.

At the end of the experiment, the rats were fasted overnight and anesthetized intraperitoneally with ketamine and xylazine (80 and 10 mg/kg body weight, respectively). Blood was collected by cardiac puncture and stored at 80°C until analysis. Perigonadal adipose tissue samples were washed with 0.9% NaCl solution, dried and weighed.

### 2.4 CVD-related markers in plasma and urine

Systolic and diastolic blood pressures were measured after week 24 of the experiment. The rats were restrained in a rat pocket and maintained at 32 °C. Systolic and diastolic blood pressure were measured by the tail-cuff method, using a non-invasive automatic blood pressure analyzer (Panlab; Barcelona, Spain) as previously described (Bunag, 1973). To obtain stable responses and to reduce variability associated with circadian rhythms, the operations were performed in a quiet place and always at the same time in the morning. Data are presented as the mean of four measurements.

Plasma total cholesterol, HDL-cholesterol (HDLc), LDL-cholesterol (LDLc), and triglycerides were measured using a spectrophotometric method and the corresponding kits from Spinreact (Girona, Spain) as described by Bucolo *et al.* (Bucolo & David, 1973; Méndez *et al.*, 2013). Plasma adiponectin was measured using the ELISA kit from Millipore (Billerica, MA, USA).

The stable end product of NO, NO<sub>2</sub><sup>-</sup>, was quantified in urine by a modification of the Griess reaction. Briefly, the urine was lyophilized from frozen samples and concentrated 5-fold. The concentrates (50 µL) were mixed with sulfanilamide 1% in 1.2 N HCl (60 µL) and 0.3% aqueous N-(1-naphthyl)ethylene-diamine dihydrochloride 0.3% (60 µL) for 10 min at room temperature. Absorbance was measured spectrophotometrically at 550 nm. The concentration of NO<sub>2</sub><sup>-</sup> was calculated using a calibration curve made using NaNO<sub>2</sub>.

## 2.5 Fecal Microbial Subgroups

The levels of total bacteria, the Bacteroidetes and Firmicutes phyla, the Lactobacillales and Bifidobacteriales orders, and the *Lactobacillus plantarum*, *Lactobacillus acidophilus* and *Bifidobacterium longum* species were determined in fecal DNA by quantitative real-time polymerase chain reaction (qRT-PCR).

Total DNA was extracted from feces using the QIAamp® DNA Stool Mini Kit from QIAGEN (Hilden, Germany). The DNA concentration was quantified using a Nanodrop 8000 Spectrophotometer (ThermoScientific, Waltham, MA, USA). All DNA samples were diluted to 20 ng/µL.

qRT-PCR cycling conditions were as follows: 5 s at 95°C; then 45 cycles of: 5 s at 95°C, 30 s at the primer-specific annealing temperature (Table 2), and 30 s at 72°C (extension).

Following amplification, to determine the specificity of the qRT-PCR reactions, melting curve analysis was carried out by treatment for 2 s at 95°C and for 15 s at 65°C, followed by a temperature gradient up to 95°C at a rate of 0.11°C/s, with five fluorescence recordings per °C. The relative DNA abundances for the different genes were calculated from the second derivative maximum of their respective amplification curves (*C<sub>p</sub>*, calculated in triplicate), considering *C<sub>p</sub>* values to be proportional to the dual logarithm of the inverse of the specific DNA concentration, following the equation:  $[DNA_a]/[DNA_b] = 2^{C_{pb}-C_{pa}}$  (Pfaffl, 2001). Total bacteria were normalized as 16S rDNA gene copies per mg of wet feces (copies/mg).



## 2.6 Short-Chain Fatty Acids in Feces

SCFAs (acetic acid, propionic acid, butyric acid and pentanoic acid) were analyzed by gas chromatography in feces after week 23 using the method proposed by Schwiertz (Schwiertz et al., 2010) with some modifications. Briefly, fecal samples were freeze-dried and weighed (~50 mg dry matter with  $10^{-4}$  g precision). A solution of oxalic acid (0.1 M) and the internal standard, caproic acid (0.1 mM), in acetonitrile/water 3:7, was added to each sample (0.025 mL/mg dry feces). Then, SCFAs were extracted using a horizontal shaker (30 min, 4°C) and the suspension was centrifuged (15 min, 16000 g, 4°C). The supernatant was passed through a nylon filter (0.45 µm) into a GC vial. SCFAs were analyzed using a Shimadzu (Kyoto, Japan) gas chromatograph (GC2025) with an automatic injector (AOC20i) at 240°C, with a flame ionization detector (Shimadzu 2025) at 240°C, equipped with an HP-Innowax capillary column (Agilent, Santa Clara, CA, USA) (30 m x 0.25 mm i.d. x 0.25 µm f.d.). The injection volume was 1 µL, the carrier gas was helium at a flow of 1 mL/min and the mode of injection was splitless. The oven temperature program was 50°C (3 min) then slope 8°C/min to 180°C (0 min) and slope 50°C/min to 200°C (5 min). Other conditions were: gas helium flow, 30 mL/min; hydrogen flow, 40 mL/min; and airflow 400 mL/min.

## 2.7 Statistical analysis

The results are expressed as mean values with their standard errors (SEM). After verifying the variance equality and the normal distribution, statistical significance was determined by ANOVA for repeated measures of body weight or one-way ANOVA with each group as variable, and Tukey's multiple comparison test was used for mean comparisons. Differences were considered significant when  $P < 0.05$ . All data manipulation and statistical analysis was performed using Graph Pad Prism 5 (Graph Pad Software, Inc., San Diego, CA, USA).

### 3. RESULTS

#### 3.1 Feed/energy intakes, body weight and perigonadal adipose tissue

The rats fed the  $\omega$ -3 PUFAs enriched diets ( $\omega$ -3 and  $\omega$ -3+GSE groups) had consumed significantly ( $P<0.05$ ) less feed and energy than the STD group after 24 weeks (Supplementary Material Table S1).

Body weight was similar for all groups at the beginning of the experiment (144.0 g, SEM 2.6). After 24 weeks of the diets, the animals supplemented with  $\omega$ -3 PUFAs had significantly higher ( $P<0.05$ ) body weights ( $\omega$ -3 275.7 g, SEM 9.0 g;  $\omega$ -3+GSE 275.6 g, SEM 8.1) than the other groups (STD 254.4 g, SEM 5.4; and GSE 262.1 g, SEM 4.5) (Figure 1a). Similarly, perigonadal fat was significantly higher in animals supplemented with  $\omega$ -3 PUFAs ( $P<0.01$ ) than in non-supplemented and GSE-supplemented rats (Figure 1b).

#### 3.2 Risk Factors of CVD

Blood pressure and plasma total cholesterol, HDLc, LDLc, triglycerides and adiponectin were measured after 24 weeks of supplementation (Table 3). Systolic and diastolic blood pressures were similar between the groups at the end of the study. Plasma cholesterol was significantly ( $P<0.01$ ) lower in animals supplemented with  $\omega$ -3 PUFAs ( $\omega$ -3 and  $\omega$ -3+GSE groups) than in the other two groups (STD and GSE). All the groups presented similar concentrations of plasma HDLc and triglycerides. Plasma LDLc levels presented some differences (Table 3), but they were all below 1.28 mmol/L, which may be considered a reference value (Ihedioha, Noel-Uneke, & Ihedioha, 2013). Plasma adiponectin was higher in the groups supplemented with  $\omega$ -3 PUFAs than in the STD group; while this difference was significant only for animals supplemented with both  $\omega$ -3 PUFAs and GSE.

Urine nitrites were similar between the groups and slightly higher ( $P=0.123$ ) in animals fed  $\omega$ -3 PUFAs compared to those in the STD group (Table 3).

#### 3.3 Proportions of Fecal Bacterial Populations

The proportions of the major bacterial phyla (Figure 2), and selected probiotics (Figure 3) were determined in fecal DNA. The percentages of Bacteroidetes and Firmicutes were similar in the groups supplemented with  $\omega$ -3 PUFAs and/or GSE (Figure 2a, b).

294 The population of Lactobacillales was significantly ( $P<0.01$ ) lower in the  $\omega$ -3 group  
295 than in the STD group; while supplementation with proanthocyanidins (GSE and  $\omega$ -  
296 3+GSE groups) did not modify the proportion of Lactobacilli (Figure 3a). The  
297 percentage of Bifidobacteriales was similar in all the groups (Figure 3d). The proportion  
298 of *L. acidophilus* was lower ( $P<0.001$  vs STD) in animals supplemented with  $\omega$ -3  
299 PUFAs, independently of the presence of proanthocyanidins (Figure 3b). The  
300 proportions of *L. plantarum* (Figure 3c) and *B. longum* (Figure 3e) were lower ( $P<0.05$   
301 vs STD) in animals supplemented with GSE; while the combination of GSE with  $\omega$ -3  
302 PUFAs did not affect the levels of these populations.

#### 304 3.4 Short-chain fatty acids

305 SCFAs were determined in feces from rats supplemented for 23 weeks (Table 4).  
306 The animals supplemented only with  $\omega$ -3 PUFAs showed increased SCFA content with  
307 respect to the other groups; with the concentration of acetic acid being significantly  
308 higher ( $P<0.05$ ). The valeric acid concentration was significantly ( $P<0.05$ ) lower in the  
309 groups supplemented with GSE.

#### 4. DISCUSSION

The present study focuses on the effect of the combination of  $\omega$ -3 PUFAs (EPA/DHA 1:1) and proanthocyanidins (mostly oligomers consisting of 2 to 4 epicatechin units in GSE) on gut health-promoting commensal bacteria, and the possible influence of their combination on the functional effects of  $\omega$ -3 PUFAs on CVD risk factors. The 1:1 proportion of  $\omega$ -3 PUFAs was chosen on the basis of previous results with healthy and rats affected by CVD (Lluís et al., 2013; Méndez et al., 2013; Molinar-Toribio et al., 2015). In the present study, we show that GSE may enhance the effects of  $\omega$ -3 PUFAs on CVD risk factors. The reduction in plasma cholesterol by  $\omega$ -3 PUFAs agrees with previous studies where fish oil supplementation decreased total plasmatic fatty acids in healthy rats (Méndez et al., 2013). Plasma cholesterol is inversely related to adiponectin levels, as reverse cholesterol transport is accelerated by adiponectin through increasing high-density lipoprotein assembly in the liver (Matsuura et al., 2007). Here, we show that plasma adiponectin increased in animals fed  $\omega$ -3 PUFAs, and increased even further when GSE was added to the supplementation (Table 3). Plasma adiponectin levels are related to the endothelial vasodilation response (Ouchi et al., 2003), probably because adiponectin stimulates the production of NO (Z. V. Wang & Scherer, 2008), which partially agrees with our results from urine nitrites ( $\omega$ -3 groups show nonsignificant higher values than the STD group).

Besides CVD risk factors, the first observable effects of  $\omega$ -3 PUFAs were changes in feed intake and body weight, independently of GSE supplementation. Our results for feed intake corroborate the satiety effect induced by  $\omega$ -3 PUFAs, previously reported by other authors (Parra et al., 2008). Despite the fact that  $\omega$ -3 PUFAs reduced feed intake, they increased body weight gain in our animals (Figure 1), an effect also detected in normoweight women who consume  $\omega$ -3 PUFAs (Iso et al., 2001). The body weight increase induced by  $\omega$ -3 PUFA intake in normoweight individuals could be related to a post-prandial increase of the chylomicron response (Griffo et al., 2014) and to the lower response of cholecystokinin and GLP-1, associated with faster gastric emptying of fat (Robertson et al., 2002). Moreover,  $\omega$ -3 PUFAs may promote fat accumulation through the stimulation of adipocyte growth and differentiation via expression of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) (Chambrier et al., 2002). The weight gain observed in lean animals supplemented with  $\omega$ -3 PUFAs is in apparent contradiction with the observations of other authors who report the anti-obesogenic effect of  $\omega$ -3

PUFAs in obese individuals (Buckley & Howe, 2010; Lorente-Cebrián et al., 2013). This anti-obesogenic effect of  $\omega$ -3 PUFAs seems to be related to a reduction in obesity-associated low-grade inflammation (Calder, 2013; Flock, Rogers, Prabhu, & Kris-Etherton, 2013). This state has been linked to metabolic endotoxemia, commonly derived from gut dysbiosis, in turn typically induced by an energy-dense diet (Kaliannan, Wang, Li, Kim, & Kang, 2015). The anti-obesogenic effect of  $\omega$ -3 PUFAs may only be evident within a pro-inflammatory environment and not in normoweight individuals with well-balanced microbiota. Fat gain induced by  $\omega$ -3 PUFAs in lean rats may be viewed as a beneficial contribution to the physiological role of adipose tissue as a fuel reservoir that keeps fat from being deposited in organs.

A reduction in the population of probiotic bacteria is a risk factor for the development of many intestinal conditions, including diarrhea, obesity, irritable bowel syndrome, inflammatory bowel disease, and even tumors (Gareau, Sherman, & Walker, 2010; Guarner & Malagelada, 2003). Our results show that  $\omega$ -3 PUFAs and proanthocyanidins induced changes in the proportions of several subgroups and species of probiotics (Figure 3). The reduction of Lactobacillales induced by  $\omega$ -3 PUFAs is in agreement with the fact that polyunsaturated fatty acids reduce the adherence of most probiotic lactobacilli (Kankaanpää, Yang, Kallio, Isolauri, & Salminen, 2004). Specifically, the incorporation of a given PUFA into bacterial fatty acids is clearly observed when lactobacilli are cultured in broth supplemented with that particular fatty acid, and these changes seem to influence microbial adhesion to intestinal surfaces (Kankaanpää et al., 2004). Proanthocyanidins in GSE counteracted this reduction in Lactobacillales, possibly by promoting the growth of lactobacilli as observed in rats given tea and wine phenolics (Dolara et al., 2005; Lee et al., 2006). The effects of polyphenols on the adhesion and viability of probiotics are complex, and depend on the chemical structure of the phenolic compound and on the membrane composition (proteins, enzymes and lipids) of any particular species (Bustos et al., 2012; Parkar, Stevenson, & Skinner, 2008; Parkar, Trower, & Stevenson, 2013). These pleiotropic effects can modify cell permeability and finally result in the loss of protons, other ions, and macromolecules (Bustos et al., 2012). Specifically, epicatechin and other proanthocyanidin monomers inhibit the growth of *Lactobacillus rhamnosus* (Parkar et al., 2008) and the adhesion of both *Lactobacillus plantarum* and *Lactobacillus acidophilus* to enterocytes (Bustos et al., 2012) *in vitro*. This inhibitory effect on some *Lactobacillus* species is in agreement with our results, which show a reduction in the

populations of *Lactobacillus plantarum* and *Bifidobacterium longum* as result of GSE supplementation. Combined supplementation with  $\omega$ -3 PUFAs and GSE also counteracted the effect of  $\omega$ -3 PUFAs on the excreted concentration of acetic acid, an SCFA product of bacterial metabolism. Acetic acid contributes to lipogenesis via acetyl-CoA and regulates levels of plasma cholesterol (Fushimi et al., 2006), in agreement with the present results on body weight gain (Figure 1) and cholesterolemia (Table 3). Higher concentrations of SCFAs in feces have been observed in overweight and obese rats and humans (Byrne, Chambers, Morrison, & Frost, 2015; Canfora et al., 2015; Schwietz et al., 2010).

## 5. CONCLUSIONS

In conclusion, supplementation of rats with  $\omega$ -3 PUFAs increases their weight gain and perigonadal fat, reduces the populations of several probiotics and increases the levels of acetic acid, a product of bacterial metabolism. Combined supplementation of  $\omega$ -3 PUFAs and proanthocyanidins from grape seed counteracts the effects of the  $\omega$ -3 PUFAs on health-promoting lactobacillus and bifidobacterium, and on acetic acid, while maintaining their beneficial effects on cholesterolemia.

**Acknowledgments:** Language revision by Christopher Evans is appreciated.

**Funding:** This research was supported by the Spanish Ministry of Science and Innovation (through grants AGL2009-12374-C03-01, -02 and -03; and AGL2013-49079-C2-1,2-R). The Spanish Ministry of Economics and the Panamanian Government awarded graduate fellowships to M.H. (BES2014-068592) and E.M.-T. (SENACYT/IFRHU), respectively.

**Author Contributions:** S.R.-R. and E. M.-T. performed the *in vivo* experiments; S.R.-R. and M.H. performed the qRT-PCR experiments; M.P.A. performed the SCFA experiments; N.T. and M.R. evaluated the CVD risk factors; S.R.-R. and L.M. analyzed the data; S.R.-R. and J.L.T. wrote the paper; S.R.-R., M.R.N., I.M. and J.L.T. conceived and designed the experiments. All authors have approved the final article

**Conflicts of Interest:** The authors declare no conflict of interest.

# **TABLES**

**Table 1.** Composition of experimental diets.

<b>Ingredients (g)</b>	<b>STD</b>	<b>ω-3</b>	<b>GSE</b>	<b>ω-3 + GSE</b>
Flour <sup>1</sup>	1000.0	1000.0	1000.0	1000.0
tert-butylhydroquinone	0.08	0.08	0.08	0.08
Porcine gelatin	25.0	25.0	25.0	25.0
Soybean lecithin	6.0	6.0	6.0	6.0
Soybean oil	17.4	-	17.4	-
ω-3 PUFAs <sup>2</sup>	-	17.4	-	17.4
Grajfnol®	-	-	0.88	0.88
Protein (% by weight)	16.4	16.4	16.4	16.4
Carbohydrate (% by weight)	46.6	46.6	46.5	46.5
Fat (% by weight)	6.2	6.2	6.2	6.2
Energy from protein (%)	21.3	21.3	21.3	21.3
Energy from carbohydrate (%)	60.5	60.5	60.5	60.5
Energy from fat (%)	18.2	18.2	18.2	18.2
Total energy density (kcal/g) <sup>3</sup>	3.1	3.1	3.1	3.1

<sup>1</sup> Standard flour (Teklad Global 2014). <sup>2</sup> The amount of EPA/DHA was 25 mg/kg body weight. <sup>3</sup> Energy density is estimated as *metabolizable energy* based on the Atwater factors, assigning 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrate.

416 **Table 2.** qRT-PCR primers and conditions.

Target bacteria	Annealing temp. (°C)	Sequence (5'-3')	Positive control	Ref.
Total Bacteria	65	F: ACT CCT ACG GGA GGC AGC AGT R: ATT ACC GCG GCT GCT GGC	<sup>1</sup>	(Hartman et al., 2009)
Bacteroidetes	62	F: ACG CTA GCT ACA GGC TTA A R: ACG CTA CTT GGC TGG TTC A	<i>Bacteroides fragilis</i>	(Ismail et al., 2011)
Firmicutes	52	F: CTG ATG GAG CAA CGC CGC GT R: ACA CYT AGY ACT CAT CGT TT	<i>Lactobacillus brevis</i>	(Haakensen, Dobson, Deneer, & Ziola, 2008) (Muhling, Woolven-Allen, Murrell, & Joint, 2008)
Lactobacillales	60	F: AGC AGT AGG GAA TCT TCC A R: CAC CGC TAC ACA TGG AG	<i>Lactobacillus acidophilus</i>	(Walter et al., 2001)
<i>L. acidophilus</i>	64	F: AGC TGA ACC AAC AGA TTC AC R: ACT ACC AGG GTA TCT AAT CC	<i>Lactobacillus acidophilus</i>	(Walter et al., 2001)
<i>L. plantarum</i>	55	F: GCC GCC TAA GGT GGG ACA GAT R: TTA CCT AAC GGT AAA TGC GA	<i>Lactobacillus plantarum</i>	(Walter et al., 2001)
Bifidobacteriales	55	F: CTC CTG GAA ACG GGT GG R: GGT GTT CTT CCC GAT ATC TAC A	<i>Bifidobacterium longum</i>	(Queipo-Ortuno et al., 2013)
<i>B. longum</i>	50	F: GTT CCC GAC GGT CGT AGA G R: GTG AGT TCC CGG CAT AAT CC	<i>Bifidobacterium longum</i>	(R. F. Wang, Cao, & Cerniglia, 1996)

417 <sup>1</sup> Positive control of Total Bacteria was the same as those the result was rated with





**Table 3** CDV risk factors in rats supplemented with  $\omega$ -3 PUFAs and/or GSE for 24 weeks.

	STD		$\omega$ -3		GSE		$\omega$ -3 + GSE	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Systolic pressure (mmHg)	123.5	4.0	123.2	2.4	114.8	3.6	117.2	3.1
Diastolic pressure (mmHg)	84.0	4.0	96.2	7.0	94.6	7.4	91.3	2.1
Plasma cholesterol (mmol/L)	4.7 <sup>a</sup>	0.2	3.8 <sup>bc</sup>	0.1	4.4 <sup>ab</sup>	0.1	3.5 <sup>c</sup>	0.2
Plasma HDLc (mmol/L)	1.5	0.1	1.6	0.1	1.5	0.1	1.5	0.1
Plasma LDLc (mmol/L)	0.4 <sup>a</sup>	0.1	0.4 <sup>a</sup>	0.0	0.7 <sup>b</sup>	0.0	0.6 <sup>c</sup>	0.0
Plasma triglycerides (mmol/L)	1.7	0.1	1.7	0.1	1.9	0.1	1.6	0.1
Plasma adiponectin ( $\mu$ g/mL)	21.7 <sup>a</sup>	2.9	30.0 <sup>ab</sup>	2.1	22.0 <sup>a</sup>	2.8	32.1 <sup>bc</sup>	1.5
Urine nitrites (ng/mL)	1.3	0.3	2.5	0.9	1.5	0.1	2.1	0.2

Means with different letters differ,  $P < 0.05$ . Comparisons were performed using one-way ANOVA and Tukey's post-hoc tests.

**Table 4.** Short-chain fatty acids determined in feces from rats supplemented with  $\omega$ -3 PUFAs and/or GSE for 23 weeks.

	STD		$\omega$ -3		GSE		$\omega$ -3 + GSE	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Acetic acid	5.54 <sup>a</sup>	1.26	9.10 <sup>b</sup>	1.36	6.03 <sup>a</sup>	1.62	4.48 <sup>a</sup>	1.89
Propionic acid	1.18	0.43	1.47	0.54	1.11	0.51	1.24	0.63
Butyric acid	0.26	0.07	0.31	0.11	0.30	0.14	0.24	0.14
Valeric acid	0.15 <sup>ab</sup>	0.07	0.21 <sup>a</sup>	0.08	0.09 <sup>b</sup>	0.05	0.10 <sup>b</sup>	0.02

SCFA content is expressed as mmol of caproic acid equivalents/g dry feces. Means with different letters differ,  $P < 0.05$ . Comparisons were performed using one-way ANOVA and Tukey's post-hoc tests.

## 433 BIBLIOGRAPHY

- 434 Arboleya, S., Watkins, C., Stanton, C., & Ross, R. P. (2016). Gut Bifidobacteria Populations in  
435 Human Health and Aging. *Frontiers in Microbiology*, 7. doi: 10.3389/fmicb.2016.01204
- 436 Bravo, L. (1998). Polyphenols: Chemistry, dietary sources, metabolism, and nutritional  
437 significance. *Nutrition Reviews*, 56(11), 317-333.
- 438 Buckley, J. D., & Howe, P. R. C. (2010). Long-Chain Omega-3 Polyunsaturated Fatty Acids May  
439 Be Beneficial for Reducing Obesity-A Review. *Nutrients*, 2(12), 1212-1230. doi:  
440 10.3390/nu2121212
- 441 Bucolo, G., & David, H. (1973). Quantitative determination of serum triglycerides by the use of  
442 enzymes. *Clin Chem*, 19(5), 476-482.
- 443 Bunag, R. D. (1973). Validation in awake rats of a tail-cuff method for measuring systolic  
444 pressure. *Journal of Applied Physiology*, 34(2), 279-282.
- 445 Bustos, I., Garcia-Cayuela, T., Hernandez-Ledesma, B., Pelaez, C., Requena, T., & Carmen  
446 Martinez-Cuesta, M. (2012). Effect of Flavan-3-ols on the Adhesion of Potential  
447 Probiotic Lactobacilli to Intestinal Cells. *Journal of Agricultural and Food Chemistry*,  
448 60(36), 9082-9088. doi: 10.1021/jf301133g
- 449 Byrne, C. S., Chambers, E. S., Morrison, D. J., & Frost, G. (2015). The role of short chain fatty  
450 acids in appetite regulation and energy homeostasis. *International Journal of Obesity*,  
451 39(9), 1331-1338. doi: 10.1038/ijo.2015.84
- 452 Calder, P. C. (2013). Omega-3 polyunsaturated fatty acids and inflammatory processes:  
453 nutrition or pharmacology? *British Journal of Clinical Pharmacology*, 75(3), 645-662.  
454 doi: 10.1111/j.1365-2125.2012.04374.x
- 455 Canfora, E. E., Jocken, J. W., & Blaak, E. E. (2015). Short-chain fatty acids in control of body  
456 weight and insulin sensitivity. *Nat Rev Endocrinol*, 11(10), 577-591. doi:  
457 10.1038/nrendo.2015.128
- 458 Cani, P. D., Neyrinck, A. M., Fava, F., Knauf, C., Burcelin, R. G., Tuohy, K. M., . . . Delzenne, N. M.  
459 (2007). Selective increases of bifidobacteria in gut microflora improve high-fat-diet-  
460 induced diabetes in mice through a mechanism associated with endotoxaemia.  
461 *Diabetologia*, 50(11), 2374-2383. doi: 10.1007/s00125-007-0791-0
- 462 Chambrier, C., Bastard, J. P., Rieusset, J., Chevillotte, E., Bonnefont-Rousselot, D., Therond, P., .  
463 . . Vidal, H. (2002). Eicosapentaenoic acid induces mRNA expression of peroxisome  
464 proliferator-activated receptor gamma. *Obesity Research*, 10(6), 518-525. doi:  
465 10.1038/oby.2002.70
- 466 Dolara, P., Luceri, C., De Filippo, C., Femia, A. P., Giovannelli, L., Caderni, G., . . . Cresci, A.  
467 (2005). Red wine polyphenols influence carcinogenesis, intestinal microflora, oxidative  
468 damage and gene expression profiles of colonic mucosa in F344 rats. *Mutation*  
469 *Research-Fundamental and Molecular Mechanisms of Mutagenesis*, 591(1-2), 237-246.  
470 doi: 10.1016/j.mrfmmm.2005.04.022
- 471 Duda-Chodak, A. (2012). The inhibitory effect of polyphenols on human gut microbiota. *Journal*  
472 *of Physiology and Pharmacology*, 63(5), 497-503.
- 473 Flock, M. R., Rogers, C. J., Prabhu, K. S., & Kris-Etherton, P. M. (2013). Immunometabolic role of  
474 long-chain omega-3 fatty acids in obesity-induced inflammation. *Diabetes-Metabolism*  
475 *Research and Reviews*, 29(6), 431-445. doi: 10.1002/dmrr.2414
- 476 Fushimi, T., Suruga, K., Oshima, Y., Fukiharu, M., Tsukamoto, Y., & Goda, T. (2006). Dietary  
477 acetic acid reduces serum cholesterol and triacylglycerols in rats fed a cholesterol-rich  
478 diet. *British Journal of Nutrition*, 95(5), 916-924. doi: 10.1079/bjn20061740
- 479 Gareau, M. G., Sherman, P. M., & Walker, W. A. (2010). Probiotics and the gut microbiota in  
480 intestinal health and disease. *Nature Reviews Gastroenterology & Hepatology*, 7(9),  
481 503-514. doi: 10.1038/nrgastro.2010.117

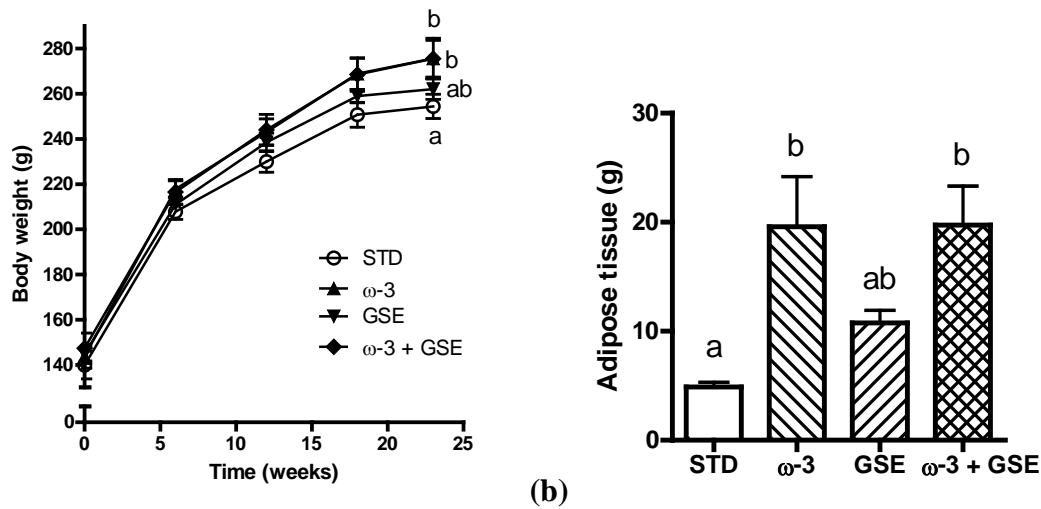
- Griffo, E., Di Marino, L., Patti, L., Bozzetto, L., Annuzzi, G., Cipriano, P., . . . Rivellese, A. A. (2014). Test meals rich in marine long-chain n-3 polyunsaturated fatty acids increase postprandial chylomicron response. *Nutrition Research*, 34(8), 661-666. doi: 10.1016/j.nutres.2014.07.005
- Guardamagna, O., Amaretti, A., Puddu, P. E., Raimondi, S., Abello, F., Cagliero, P., & Rossi, M. (2014). Bifidobacteria supplementation: Effects on plasma lipid profiles in dyslipidemic children. *Nutrition*, 30(7-8), 831-836. doi: 10.1016/j.nut.2014.01.014
- Guarner, F., & Malagelada, J. R. (2003). Gut flora in health and disease. *Lancet*, 361(9356), 512-519. doi: 10.1016/s0140-6736(03)12489-0
- Haakensen, M., Dobson, C. M., Deneer, H., & Ziola, B. (2008). Real-time PCR detection of bacteria belonging to the Firmicutes Phylum. *Int J Food Microbiol*, 125(3), 236-241. doi: 10.1016/j.ijfoodmicro.2008.04.002
- Hartman, A. L., Lough, D. M., Barupal, D. K., Fiehn, O., Fishbein, T., Zasloff, M., & Eisen, J. A. (2009). Human gut microbiome adopts an alternative state following small bowel transplantation. *Proc Natl Acad Sci U S A*, 106(40), 17187-17192.
- Ihedioha, J. I., Noel-Uneke, O. A., & Ihedioha, T. E. (2013). Reference values for the serum lipid profile of albino rats (*Rattus norvegicus*) of varied ages and sexes. *Comparative Clinical Pathology*, 22(1), 93-99.
- Ismail, N. A., Ragab, S. H., Elbaky, A. A., Shoeib, A. R., Alhosary, Y., & Fekry, D. (2011). Frequency of Firmicutes and Bacteroidetes in gut microbiota in obese and normal weight Egyptian children and adults. *Arch Med Sci*, 7(3), 501-507. doi: 10.5114/aoms.2011.23418
- Iso, H., Rexrode, K. M., Stampfer, M. J., Manson, J. E., Colditz, G. A., Speizer, F. E., . . . Willett, W. C. (2001). Intake of fish and omega-3 fatty acids and risk of stroke in women. *Jama-Journal of the American Medical Association*, 285(3), 304-312. doi: 10.1001/jama.285.3.304
- Kaliannan, K., Wang, B., Li, X. Y., Kim, K. J., & Kang, J. X. (2015). A host-microbiome interaction mediates the opposing effects of omega-6 and omega-3 fatty acids on metabolic endotoxemia. *Sci Rep*, 5, 11276. doi: 10.1038/srep11276
- Kankaanpää, P., Yang, B., Kallio, H., Isolauri, E., & Salminen, S. (2004). Effects of polyunsaturated fatty acids in growth medium on lipid composition and on physicochemical surface properties of lactobacilli. *Applied and Environmental Microbiology*, 70(1), 129-136. doi: 10.1128/aem.70.1.129-136.2004
- Lee, H. C., Jenner, A. M., Low, C. S., & Lee, Y. K. (2006). Effect of tea phenolics and their aromatic fecal bacterial metabolites on intestinal microbiota. *Research in Microbiology*, 157(9), 876-884. doi: 10.1016/j.resmic.2006.07.004
- Liu, T., Hougen, H., Vollmer, A. C., & Hiebert, S. M. (2012). Gut bacteria profiles of *Mus musculus* at the phylum and family levels are influenced by saturation of dietary fatty acids. *Anaerobe*, 18(3), 331-337. doi: 10.1016/j.anaerobe.2012.02.004
- Lluís, L., Taltavull, N., Muñoz-Cortés, M., Sánchez-Martos, V., Romeu, M., Giralt, M., . . . Nogués, M. R. (2013). Protective effect of the omega-3 polyunsaturated fatty acids: Eicosapentaenoic acid/Docosahexaenoic acid 1:1 ratio on cardiovascular disease risk markers in rats. *Lipids in Health and Disease*, 12, 140-140. doi: 10.1186/1476-511x-12-140
- Lorente-Cebrián, S., Costa, A. G. V., Navas-Carretero, S., Zabala, M., Martínez, J. A., & Moreno-Aliaga, M. J. (2013). Role of omega-3 fatty acids in obesity, metabolic syndrome, and cardiovascular diseases: a review of the evidence. *Journal of Physiology and Biochemistry*, 69(3), 633-651. doi: 10.1007/s13105-013-0265-4
- Matsuura, F., Oku, H., Koseki, M., Sandoval, J. C., Yuasa-Kawase, M., Tsubakio-Yamamoto, K., . . . Yamashita, S. (2007). Adiponectin accelerates reverse cholesterol transport by increasing high density lipoprotein assembly in the liver. *Biochemical and Biophysical Research Communications*, 358(4), 1091-1095. doi: 10.1016/j.bbrc.2007.05.040

- Méndez, L., Pazos, M., Gallardo, J. M., Torres, J. L., Pérez-Jiménez, J., Nogués, R., . . . Medina, I. (2013). Reduced protein oxidation in Wistar rats supplemented with marine omega 3 PUFAs. *Free Radical Biology and Medicine*, 55, 8-20. doi: 10.1016/j.freeradbiomed.2012.11.004
- Miremadi, F., Sherkat, F., & Stojanouska, L. (2016). Hypocholesterolaemic effect and anti-hypertensive properties of probiotics and prebiotics: A review. *Journal of Functional Foods*, 25, 497-510. doi: 10.1016/j.jff.2016.06.016
- Molinar-Toribio, E., Pérez-Jiménez, J., Ramos-Romero, S., Romeu, M., Giralt, M., Taltavull, N., . . . Torres, J. L. (2015). Effect of n-3 PUFA supplementation at different EPA:DHA ratios on the spontaneously hypertensive obese rat model of the metabolic syndrome. *British Journal of Nutrition*, 113(6), 878-887. doi: 10.1017/S0007114514004437
- Muhling, M., Woolven-Allen, J., Murrell, J. C., & Joint, I. (2008). Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. *Isme Journal*, 2(4), 379-392. doi: 10.1038/ismej.2007.97
- Ouchi, N., Ohishi, M., Kihara, S., Funahashi, T., Nakamura, T., Nagaretani, H., . . . Matsuzawa, Y. (2003). Association of hypoadiponectinemia with impaired vasoreactivity. *Hypertension*, 42(3), 231-234. doi: 10.1161/01.hyp.0000083488.67550.b8
- Pan, M.-H., Lai, C.-S., & Ho, C.-T. (2010). Anti-inflammatory activity of natural dietary flavonoids. *Food & Function*, 1(1), 15-31. doi: 10.1039/c0fo00103a
- Parkar, S. G., Stevenson, D. E., & Skinner, M. A. (2008). The potential influence of fruit polyphenols on colonic microflora and human gut health. *International Journal of Food Microbiology*, 124(3), 295-298. doi: 10.1016/j.ijfoodmicro.2008.03.017
- Parkar, S. G., Trower, T. M., & Stevenson, D. E. (2013). Fecal microbial metabolism of polyphenols and its effects on human gut microbiota. *Anaerobe*, 23, 12-19. doi: 10.1016/j.anaerobe.2013.07.009
- Parra, D., Ramel, A., Bandarra, N., Kiely, M., Martínez, J. A., & Thorsdottir, I. (2008). A diet rich in long chain omega-3 fatty acids modulates satiety in overweight and obese volunteers during weight loss. *Appetite*, 51(3), 676-680. doi: 10.1016/j.appet.2008.06.003
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res*, 29(9), e45.
- Poudyal, H., Panchal, S. K., Diwan, V., & Brown, L. (2011). Omega-3 fatty acids and metabolic syndrome: Effects and emerging mechanisms of action. *Progress in Lipid Research*, 50(4), 372-387. doi: 10.1016/j.plipres.2011.06.003
- Queipo-Ortuño, M. I., Boto-Ordoñez, M., Murri, M., Gómez-Zumaquero, J. M., Clemente-Postigo, M., Estruch, R., . . . Tinahones, F. J. (2012). Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers. *American Journal of Clinical Nutrition*, 95(6), 1323-1334. doi: 10.3945/ajcn.111.027847
- Queipo-Ortuno, M. I., Seoane, L. M., Murri, M., Pardo, M., Gomez-Zumaquero, J. M., Cardona, F., . . . Tinahones, F. J. (2013). Gut microbiota composition in male rat models under different nutritional status and physical activity and its association with serum leptin and ghrelin levels. *PLoS One*, 8(5), e65465. doi: 10.1371/journal.pone.0065465
- Quideau, S., Deffieux, D., Douat-Casassus, C., & Pouysegur, L. (2011). Plant Polyphenols: Chemical Properties, Biological Activities, and Synthesis. *Angewandte Chemie-International Edition*, 50(3), 586-621. doi: 10.1002/anie.201000044
- Ramos-Romero, S., Molinar-Toribio, E., Pérez-Jiménez, J., Taltavull, N., Dasilva, G., Romeu, M., . . . Torres, J. L. (2016). The combined action of omega-3 polyunsaturated fatty acids and grape proanthocyanidins on a rat model of diet-induced metabolic alterations. *Food & Function*, 7(8), 3516-3523. doi: 10.1039/c6fo00679e
- Reagan-Shaw, S., Nihal, M., & Ahmad, N. (2008). Dose translation from animal to human studies revisited. *Faseb Journal*, 22(3), 659-661. doi: 10.1096/fj.07-9574LSF

- Robertson, M. D., Jackson, K. G., Fielding, B. A., Morgan, L. M., Williams, C. M., & Frayn, K. N. (2002). Acute ingestion of a meal rich in n-3 polyunsaturated fatty acids results in rapid gastric emptying in humans. *American Journal of Clinical Nutrition*, 76(1), 232-238.
- Salvadó, M. J., Casanova, E., Fernández-Iglesias, A., Arola, L., & Bladé, C. (2015). Roles of proanthocyanidin rich extracts in obesity. *Food & Function*, 6(4), 1053-1071. doi: 10.1039/c4fo01035c
- Schwiertz, A., Taras, D., Schafer, K., Beijer, S., Bos, N. A., Donus, C., & Hardt, P. D. (2010). Microbiota and SCFA in lean and overweight healthy subjects. *Obesity (Silver Spring)*, 18(1), 190-195.
- Sekhon-Loodu, S., Catalli, A., Kulka, M., Wang, Y., Shahidi, F., & Rupasinghe, H. P. V. (2014). Apple flavonols and n-3 polyunsaturated fatty acid-rich fish oil lowers blood C-reactive protein in rats with hypercholesterolemia and acute inflammation. *Nutrition Research*, 34(6), 535-543. doi: 10.1016/j.nutres.2014.05.002
- Taltavull, N., Muñoz-Cortés, M., Lluís, L., Jové, M., Fortuño, À., Molinar-Toribio, E., . . . Nogués, M. R. (2014). Eicosapentaenoic acid/docosahexaenoic acid 1:1 ratio improves histological alterations in obese rats with metabolic syndrome. *Lipids in Health and Disease*, 13, 31. doi: 10.1186/1476-511x-13-31
- Tapiero, H., Ba, G. N., Couvreur, P., & Tew, K. D. (2002). Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. *Biomedicine & Pharmacotherapy*, 56(5), 215-222. doi: 10.1016/s0753-3322(02)00193-2
- Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., & Hammes, W. P. (2001). Detection of Lactobacillus, Pediococcus, Leuconostoc, and Weissella species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl Environ Microbiol*, 67(6), 2578-2585. doi: 10.1128/aem.67.6.2578-2585.2001
- Wang, R. F., Cao, W. W., & Cerniglia, C. E. (1996). PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Applied and Environmental Microbiology*, 62(4), 1242-1247.
- Wang, Z. V., & Scherer, P. E. (2008). Adiponectin, cardiovascular function, and hypertension. *Hypertension*, 51(1), 8-14. doi: 10.1161/hypertensionaha.107.099424
- Williamson, G. (2013). Possible effects of dietary polyphenols on sugar absorption and digestion. *Molecular Nutrition & Food Research*, 57(1), 48-57. doi: 10.1002/mnfr.201200511
- Yang, T., Santisteban, M. M., Rodriguez, V., Li, E., Ahmari, N., Carvajal, J. M., . . . Mohamadzadeh, M. (2015). Gut dysbiosis is linked to hypertension. *Hypertension*, 65(6), 1331-1340. doi: 10.1161/hypertensionaha.115.05315
- Yu, H.-N., Zhu, J., Pan, W.-s., Shen, S.-R., Shan, W.-G., & Das, U. N. (2014). Effects of Fish Oil with a High Content of n-3 Polyunsaturated Fatty Acids on Mouse Gut Microbiota. *Archives of Medical Research*, 45(3), 195-202. doi: 10.1016/j.arcmed.2014.03.008

FIGURES

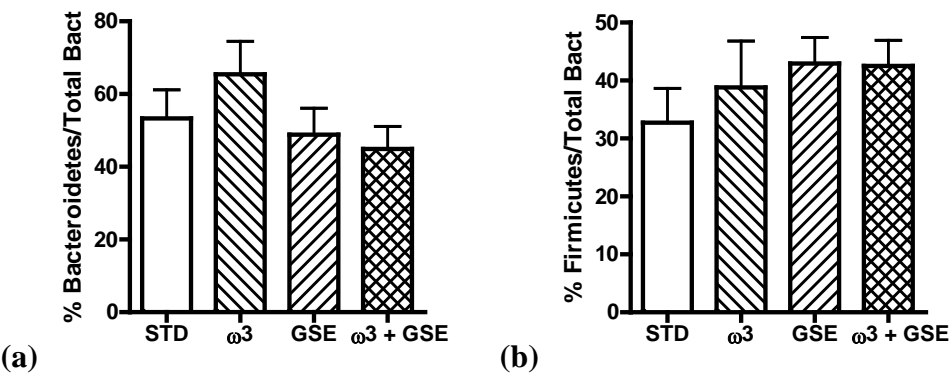
Figure 1.-



**Figure 1.** Body weight (a) and perigonadal fat (b) of rats fed the different diets for 24 weeks: STD, ○; ω-3, ▲; GSE, ▼; ω-3 + GSE, ◆. The curves corresponding to the ω-3 and ω-3+GSE groups are superimposed. The data represent means with their standard errors. Comparisons were performed using two-way ANOVA for repeated measures (a) or one-way ANOVA followed by Tukey's post-hoc test (b). Means with different letters differ, P<0.05.

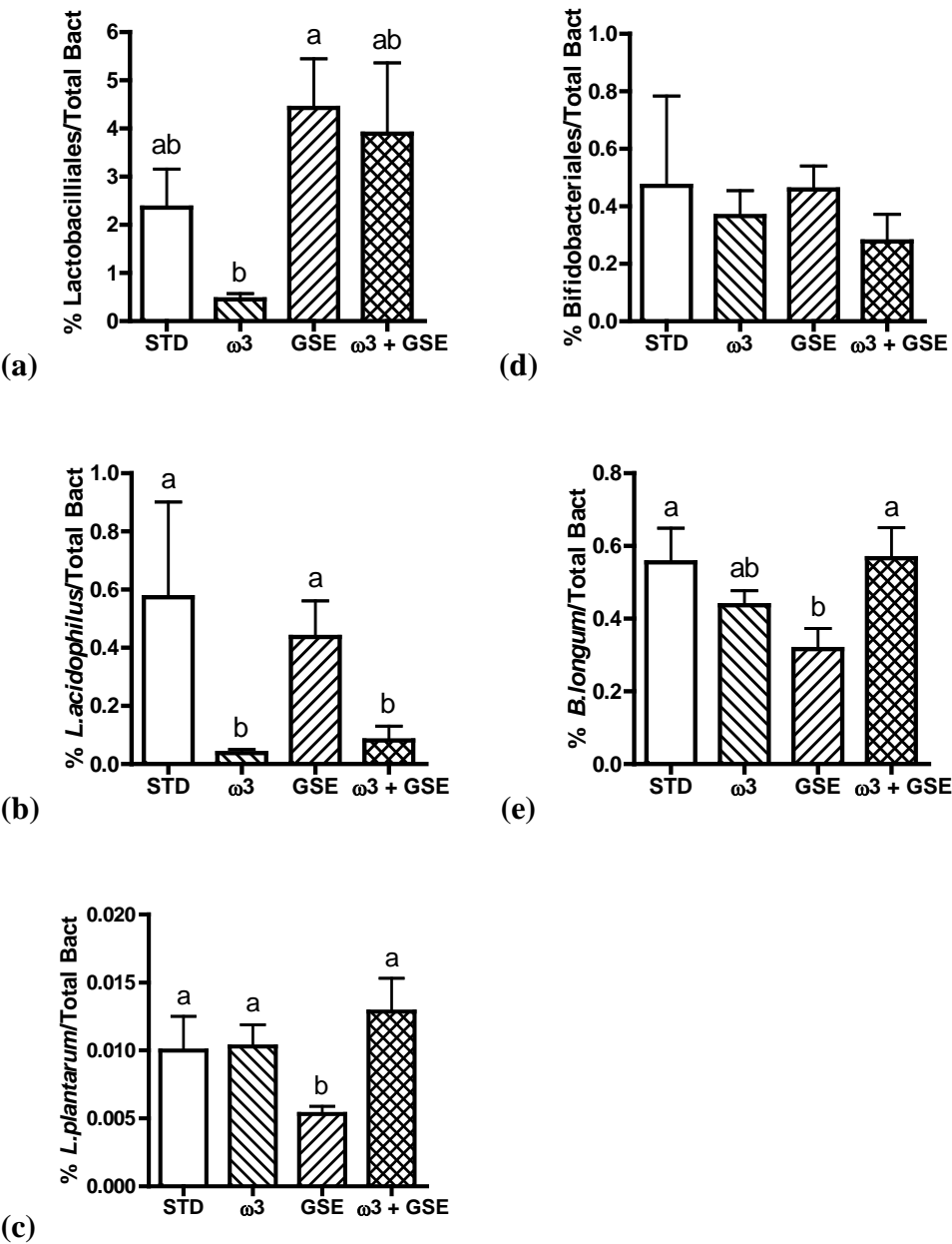


**Figure 2.-**



**Figure 2.** Bacteroidetes (a) and Firmicutes (b) in fecal samples from rats fed the different diets (STD,  $\omega$ -3, GSE, or  $\omega$ -3 + GSE) for 23 weeks. The data represent means with their standard errors. Comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test.

**Figure 3.-**



**Figure 3.** Lactobacillales (a), *Lactobacillus acidophilus* (b), *Lactobacillus plantarum* (c), Bifidobacteriales (d), and *Bifidobacterium longum* (e) in fecal samples from rats fed the different diets (STD, ω-3, GSE or ω-3 + GSE) for 23 weeks. The data represent means with their standard errors. Comparisons were performed using one-way ANOVA followed by Tukey's post-hoc test. Means with different letters differ, P<0.05.

Supplementary material

**Table S1.** Feed and energy intakes, and CDV risk factors in rats supplemented with  $\omega$ -3 PUFAs and/or GSE for 24 weeks.

	STD		$\omega$ -3		GSE		$\omega$ -3 + GSE	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Feed intake (g/kg bw/day)	59.4 <sup>a</sup>	2.6	41.4 <sup>b</sup>	4.5	56.4 <sup>a</sup>	4.2	40.0 <sup>b</sup>	3.6
Energy intake <sup>1</sup> (kcal/kg bw/day)	184.0 <sup>a</sup>	8.1	128.4 <sup>b</sup>	14.1	174.8 <sup>a</sup>	13.0	123.9 <sup>b</sup>	11.1

<sup>1</sup> Energy intake is estimated as metabolizable energy based on Atwater factors, which assign 4 kcal/g to protein, 9 kcal/g to fat, and 4 kcal/g to available carbohydrates.

Means with different letters differ,  $P < 0.05$ . Comparisons were performed using one-way ANOVA and Tukey's post-hoc test.