Spinach consumption ameliorates the gut microbiota and dislipaemia in rats with diet-induced non-alcoholic fatty liver disease (NAFLD).

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of fat in liver cells, which causes serious health consequences. Animal and human studies suggest that the gut microbiota plays a role in the pathogenesis of NAFLD. Here, we investigated whether spinach consumption could ameliorate high-fat-diet-induced disturbances of certain intestinal bacterial groups and products derived from their metabolism, such as short-chain fatty acids (SCFAs) and microbial phenolic catabolites. Attention is also paid to blood lipids and glucose. For the study, a rat model of high-fat-diet-induced NAFLD was used. There were six experimental groups; NC (normal diet), NB (normal diet+2.5% spinach), NA (normal diet+5% spinach), HC (high-fat diet), HB (high-fat diet+2.5% spinach) and HA (high-fat diet+5% spinach). The rats consumed these diets for five weeks, and after that were sacrificed and plasma, urine, intestinal content, faeces and liver samples were taken. Biochemical parameters were analyzed in plasma, phenolic catabolites were quantified in faeces, urine, plasma and liver by UPLC-ESI-MS/MS, and the analysis of the microbiota and SCFAs in the intestinal content was performed by qPCR and GLC. Consumption of a high-fat diet caused NAFLD and dislipaemia and altered the gut microbiota and the pattern of SCFAs and phenolic gut microbial catabolites. Supplementation with spinach partially ameliorated some alterations induced by the high-fat diet, in particular by increasing *Lactobacillus* counts, reducing the fasting glucose and total and LDL-cholesterol and preventing excess liver cholesterol accumulation, thereby improving the values of the steatosis biomarkers.

Keywords: spinach; NAFLD; rat model; microbiota; cholesterol

Introduction.

Non-alcoholic fatty liver disease (NAFLD) is defined as the accumulation of fat in liver cells, which causes serious health consequences such as steatohepatitis, fibrosis and cirrhosis in late stages of the disease. This disease has aroused general interest during its widespread dissemination in developed and semi-developed countries due to its association with obesity, hyperlipidaemia, metabolic syndrome and diabetes mellitus.[1,2] The formation of NAFLD is caused by an imbalance between the high intake of lipids and carbohydrates in food and insufficient metabolism in the liver, as well as by the availability of lipids within the body through the transport of lipoproteins.[3] The main symptoms of NAFLD include the accumulation of triglycerides in hepatocytes and insulin resistance, which are evidenced by the presence of hyperinsulinemia, hyperglycaemia and increased production of VLDL and LDL.[4]

Animal and human studies suggest that the gut microbiota plays a role in the pathogenesis of NAFLD, as the liver is connected to the intestine through the portal vein and is therefore susceptible to changes in the microbiota and its metabolites.[5,6] The progression of NAFLD is also associated with the overproduction of intestinal bacteria and increased intestinal permeability - which causes a greater uptake of monosaccharides from the lumen, favours *de novo* fatty acids and triglycerides synthesis, and contributes to fat deposition in hepatic vacuoles, thereby increasing inflammation and oxidative hepatic stress due to the absorption of fermentation products such as ethanol and lipopolysaccharides.[5,7]

Changes in the patient's lifestyle have been proposed for the treatment of NAFLD: mainly dietary changes, weight loss and physical exercise. According to research, the best diet for improving this condition is the Mediterranean, due to its specific characteristics: low intake of saturated fat and processed sugars and high intake of fruits and vegetables - which provide a wide range of bioactive compounds, such as unsaturated fats and antioxidant compounds, including vitamins C and E, carotenoids and phenolic compounds.[8] Also, there is rising interest in the use of prebiotics, probiotics and their symbiosis as a means to diminish the effects of NAFLD, by reducing markers of inflammation, such as NF-kB and TNF-α[7], and reducing the synthesis of pro-inflammatory cytokines.[9] The use of prebiotics for NAFLD treatment has proven to be beneficial since body weight, food intake and insulin sensitivity decrease and glucose homeostasis improves.[10,11]

The gut microbial fermentation of prebiotic compounds like dietary fiber leads to the generation of short-chain fatty acids (SCFAs) that may have beneficial effects in the prevention and treatment of NAFLD and metabolic syndrome. Among the SCFAs, propionate and butyrate are linked to the regulation of the metabolism of hepatic lipids and glucose, whereas acetate is mainly used in the hepatic synthesis of lipids and cholesterol. Butyrate activates β-oxidation and cholesterol

transport *via* lipoproteins, resulting in decreased fatty deposits and insulin resistance, thereby lessening the triglyceride content. Propionate is involved in the inhibition of hepatic cholesterol synthesis and in the regulation of triglyceride and HDL levels.[12-14]

Furthermore, other bioactive compounds present in plant-based food are also considered as prebiotic, modulating the intestinal microbiota. Like indigestible carbohydrates, dietary polyphenols are not completely absorbed from the gastrointestinal tract and are metabolised by the gut microbiota. Recently, it has been proposed that at least some of the biological activities ascribed to food polyphenols are due to their microbial phenolic (colonic) catabolites. So, the phenolic compounds and their metabolites may accumulate to exert local and/or systemic physiological effects or even modulate the intestinal bacterial population by acting as potential prebiotics. There is evidence of the inhibition by derivatives of phenolic compounds of the growth of pathogenic bacteria such as *Clostridium perfringens*, *Clostridium difficile, Escherichia coli* and *Salmonella tiphymurium,* and of the development of *Bacteroides*, *Bifidobacterium, Lactobacillus, Prevotella* and butyrate-producing Firmicutes.[15-17]

Green leafy vegetables, such as spinach, are commonly consumed in the Mediterranean diet and they have been endowed with functional properties -antioxidant, anti-inflammatory, anti-proliferative, anti-obesity, hypoglycemic, hypolipidemic- due to their content of bioactive compounds. In particular, spinach is primarily composed of water (91.4%), and contains small amounts of protein (2.9%), carbohydrate (3.6%), and fat (0.4%). The lipid fraction is mainly composed of mono- and poly-unsaturated fatty acids (e.g., alpha-linolenic acid, linoleic acid, oleic acid) and trace levels of saturated fatty acids (e.g., capric acid, myristic acid, stearic acid. Spinach is also source of fiber –mainly insoluble– with an average content in the range 2-3 g/100 g, and provides important amounts of vitamins (K, A, folate, C) and minerals (Mg, K, Fe).[18,19]

Besides, spinach provides different classes of phenolic compounds, carotenoids and chlorophylls. The total carotenoid content is in the range of 7 to 17 mg/100 g, being lutein, β-carotene and zeaxanthin the most representative in its carotenoid profile.[20]. Among phenolic compounds, spinach has notable amounts of flavonoids (100-300 mg/100 g) such as patuletin, spinacetin, spinatoside and jaceidin. The predominant phenolic acids (40-125 mg/100 g) are *o*-coumaric, ferulic and *p*-coumaric, and the main lignans found in spinach are lariciresinol, secoisolariciresinol and pinoresinol.[19]

To the best of our knowledge, studies on the effect of spinach intake on the gut microbiota are still scarce. Several animal studies involving the intake of spinach thylakoids reported modulation of the gut microbiota, improving insulin response and the appetite regulation.[21-23]

On this basis, the aim of the present study was to evaluate the effect of spinach consumption on certain intestinal bacterial groups and the production of SCFAs and microbial phenolic catabolites in rats with high-fat-diet-induced NAFLD, and to explore relationships with changes in the plasma glucose and lipid profile, thereby elucidating the role of spinach intake in the dietary treatment of NAFLD and in the management of metabolic syndrome features.

Methods and materials.

Experimental design.

The experimental protocol was approved by the Ethical Committee of Animal Experimentation of the University of Murcia and by the General Directorate of Livestock and Fisheries of the C.A.R.M. (No. A1320140701). The sample size was determined using a method based on the law of diminishing return[24], which gave a sample size that was more than sufficient. So, 44 male adult Sprague-Dawley rats (8 weeks of age) were grouped in two groups (n=22) according to the diet: the normal diet (NC) (Teklad Global 14% Protein Rodent Maintenance Diet TD-2014; Harlan Laboratories) and a high-fat diet (HC) (Atherogenic rodent diet TD-02028; Harlan Laboratories). These diets were administered for a 2-week-acclimation period and, after this time, a 5-week intervention period with spinach started. For that, animals from each group were randomly allocated to 3 subgroups according to the type of diet (NC or HC) and the amount of spinach supplemented (0, 2.5 or 5%). So, there were six experimental groups; two control groups (n=6 rats/group) consuming the spinach-free diets NC and HC, and four experimental groups (n=8 rats/group): NA (NC+5% spinach), NB (NC+2.5% spinach), HA (HC+5% spinach) and HB (HC+2.5% spinach) (Fig. 1; Table 1). During the 5-week intervention period, rats were placed individually in metabolic cages under the same environmental conditions and were given free access to feed and drink. Food and water intake was measured daily and rats were weighed at weekly intervals. At the end of the experimental period, rats were sacrificed and the obtained biological samples (blood, intestinal content, urine and liver) were stored at -80 °C until analyzed.

Spinach and preparation of the experimental diets.

Spinach (*Spinacia oleracea*) was purchased from a local supermarket as a fresh-cut product (Florette SAS, Milagro, Navarra, Spain). The edible part was boiled for 10 minutes to remove oxalic acid[25], the water was discarded, and then the cooked spinach was lyophilized and ground. The powdered samples were stored at 4°C until their use. The spinach-enriched diets were prepared by mixing the standard diet or the high-fat diet (previously pulverized pellets) with 2.5% and 5% freeze-dried spinach powder, water was added to each of the mixtures until a non-sticky dough was formed. The pellets were then prepared using a pastry bag and dried in a tray dryer at 60 °C for 21 h. Dried pellets with spinach were packed in polythene bags and stored in the

refrigerator until they were used. The nutritional composition of the experimental diets is shown in Table 1.

Onset of NAFLD

In order to corroborate the presence of steatosis, hepatic tissue was examined by means of histopathological tests carried out by the Service of Pathological Anatomy of the Veterinary Hospital of the University of Murcia. As illustrated in Fig. 2, in the H groups (HC, HB, HA) grade 3 hepatic steatosis was observed, as characterised by the swelling of hepatocytes and fat accumulation in 50-75% of the hepatocytes. So, the liver fat content resulted 5-fold higher in the H groups, compared to the N groups. The high-fat diet altered the plasma lipid profile by increasing the relative contribution (%) of LDL to total plasma cholesterol. Furthermore, the high-fat diet increased body weight (1.7-fold) and the activity of the liver enzymes aspartate aminotransferase (AST) (1.5-fold) and alanine aminotransferase (ALT) (1.4-fold), compared to the N groups (data not shown). As shown in Table 7, the liver cholesterol content was 33-fold higher in the HC than in the NC group.

Analysis of the microbiota by quantitative polymerase chain reaction (qPCR).

To perform its quantification, the microbiota was extracted using the QIAmp DNA Stool Mini Kit (QUIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The qPCR amplification and detection were carried out using a BioRad CFX96 real time PCR system (BioRad Laboratories, Marnes la Coquette, France); the process consisted of an initial cycle of 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and 1 min at 60 °C as the annealing temperature. The reaction mixture was composed of SYBR Green PCR Master Mix (Applied Biosystems), 0.2 µM of each specific primer (Table 2) and 1 µL of template DNA in a final volume of 25 µL. Melt curve analysis was performed immediately after amplification, to distinguish the targeted PCR product, and was quantified by a NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). Calibration curves were created using serial 10-fold dilutions, corresponding to 10 to 10^8 gene copies/g. The bacterial concentration in each sample was calculated by comparing the threshold cycle (Ct) values obtained from standard curves, and the results were expressed as the number of gene copies/g of intestinal content. The samples were analyzed in duplicate with at least two independent PCR runs.

Analysis of Short-Chain Fatty Acids (SCFAs) by Gas Chromatography with Flame Ionisation Detector (GC-FID).

The method proposed by Mateo-Anson et al.[36], with modifications, was followed to determine the SCFAs in the intestinal content. For this, 100 mg of sample were homogenised with 650 µL of a mixture of formic acid (20%), methanol and 2-ethyl butyric acid as an internal standard (2

mg/mL in methanol), at a ratio of 1/4.5/1 (v/v), sonicated for 5 min at room temperature and then centrifuged at 16110 g for 15 min. The supernatant obtained was filtered through a polytetrafluoroethylene filter with a diameter of 13 mm and a pore size of 0.22 µm (VWR International, USA) and analyzed by GC-FID. The chromatographic analysis was performed using an Agilent 7890A GC-FID system equipped with an automatic injector (7683B) (Agilent Technologies, Santa Clara, USA). For the chromatographic separation, a Nukol ™ column (Supelco, USA) - 30 m long, internal diameter 0.25 mm, film thickness 0.25 µm - was used. Helium was used as the carrier gas at a flow rate of 25 mL/min. The oven temperature ramp was as follows: initially 80 °C and held constant for 5 min, then increased to 185 °C at a rate of 5 °C/min. The temperature of the detector and the injector was 220 °C, the air flow was 400 mL/min and the hydrogen flow was 30 mL/min. A commercial multi-standard (SUPELCO, USA) of certified quality and composition - containing SCFAs (acetic acid, propionic acid, isobutyric acid, butyric acid, isovaleric acid, valeric acid, isocaproic acid and heptanoic acid) at a concentration of 10 mM - was used to prepare multi-standards of 1, 5 and 10 mM.

Analysis of microbial phenolic catabolites by ultra-performance liquid chromatography tandem mass spectrometry with electrospray ionisation (UPLC-ESI-MS/MS).

The analysis of microbial phenolic catabolites in faeces, urine, plasma and liver from groups NC, NA, HC and HA was carried out by UPLC-ESI-MS/MS, as previously described.[37,38]

In brief, 1 g or 1 mL of sample was extracted with 9 mL of 95% methanol containing 0.1 µg/mL *trans*-cinnamic acid-*d5* as internal standard. The mixture was shaken at room temperature for 15 min in an orbital shaker and then centrifuged for 5 min at 4 °C (5000 g) in a SIGMA 3–30 K centrifuge (Sigma Laborzentrifugen, Osterode am Harz, Germany). The supernatant was transferred into a 10-mL calibrated flask and the volume was adjusted to 10 mL with 95% methanol. Later, an aliquot (5 mL) was evaporated, reconstituted with 10 mL of 0.01 N H_2SO_4 in water and submitted to solid phase extraction (SPE) using a Biotage Isolute ENV+ 1-g cartridge (Biotage, Uppsala, Sweden). The extract was filtered through a 0.22-µm filter before injection. The analyses were performed on a Waters Acquity UPLC system (Waters, Milford, MA, USA) consisting of a binary pump, an on-line vacuum degasser, an autosampler and a column compartment. Separation of the phenolic compounds was achieved on a Waters Acquity HSS T3 column (1.8 µm, 100 mm × 2.1 mm), kept at 40 °C. Mobile phases of 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B) were used. The flow was 0.4 mL/min, and the gradient profile was 0 min, 5% B; from 0 to 3 min, a linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 min, a linear gradient to 45% B; from 9 to 11 min, a linear gradient to 100% B; from 11 to 13 min, a wash at 100% B; from 13.01 to 15 min, back to the initial conditions of 5% B. The injection volume was 10 µL. All the metabolites analyzed eluted in 11 min, with a total run time and column equilibration of 17 min. The MS system used was a

Waters Xevo TQ (Milford, Massachusetts, USA) triple quadrupole mass spectrometer, coupled with an electrospray interface and polarity switching option during acquisition. To optimise detection, each metabolite was directly infused in the MS system in combined mode with 50/50 v/v of solvents A and B. The characteristic MS conditions were automatically optimised using a Waters Acquity IntelliStart (Milford, Massachusetts, USA), optimising the ionisation mode, cone volt energy and collision energy. The two most-abundant fragments were selected for each metabolite to establish an MRM (multiple reaction monitoring) quantitative method. The first transition, corresponding to the most-abundant fragment, was used as the quantifier ion, and the second as the qualifier ion.

Plasma biochemical parameters and indexes of insulin resistance [Log(HOMA-IR)] and insulin sensitivity [QUICKI].

Glucose, insulin, total cholesterol, HDL-cholesterol, LDL-cholesterol, VLDL-cholesterol and total triglycerides (TG) were analyzed in plasma samples using an automatic analyser (AU 600 Olympus Life, Germany) at the Veterinary Hospital of the University of Murcia. The logarithm of the homoeostasis model of insulin resistance [Log (HOMA-IR)] was calculated using the formula: log [(fasting insulin (μU/mL) x fasting glucose (mmol/L))/22.5]. The quantitative insulin sensitivity check index (QUICKI) was calculated using the formula: 1/[log(fasting insulin μU/mL) + log(fasting glucose mmol/L)].[39]

Statistical analyses.

The data were analyzed with the statistical package IBM SPSS version 24.0. A one-way analysis of variance (ANOVA) and Tukey's test for pairwise comparison were used to determine significant differences in the variables analyzed as a function of the diet consumed. The relationships between variables were examined using Pearson correlation coefficients. Values of $p < 0.05$ were considered statistically significant. The results are expressed as the mean ± standard deviation.

Results.

Daily nutrient and phenolic compound intake.

As can be seen from Table 3, the intakes of food, energy, protein and carbohydrates did not differ significantly among the experimental groups. Compared to rats fed the normal diet (NC, NB, NA), in rats fed the high-fat diet (HC, HB, HA) significantly-higher intake of fat (4.3-fold) and lower intakes of fiber (2.2-fold lower) and total phenolics (10.5-fold lower) were observed. However, within experimental groups NC, NB, NA and HC, HB, HA the daily intakes of fiber and phenolic compounds did not differ despite spinach supplementation. The type of diet –normal *vs* high-fat– had no effect on carotenoid intake, which was determined by the amount (%) of

spinach added to the diet. That is, carotenoid intake showed dose dependency; so, significantly higher intakes were observed for groups NA and HA, whose diets were supplemented with 5% spinach.

Effect on the microbiota and SCFAs production

As shown in Table 4, intake of the high-fat diet significantly reduced the counts of *Lactobacillus* (-18%), *Bifidobacterium* (-5%) and, to a lesser extent, *Clostridia* and *Bacteroides*. No statistically-significant changes were observed for *Enterococcaceae* or other groups. Spinach intake weakly increased the counts (+2%) of *Bifidobacterium* in rats fed either the normal or high-fat diet, whilst the increase in *Lactobacillus* was 9% in rats fed the high-fat diet supplemented with spinach. Overall, intake of the high-fat diet led to reduced production of SCFAs in comparison to the normal diet. So, when comparing the control groups (NC *vs* HC), the reductions observed were 66% for acetate, 45% for propionate, 46% for butyrate and 78% for other SCFAs (sum of isobutyrate, isovalerate, valerate and caproate). In rats fed the normal diet, spinach intake further reduced the acetate production, in a dose-dependent manner, achieving statistical significance in the NA group compared to NC (-33%), whereas the inverse behaviour was observed upon consumption of the high-fat diet enriched with spinach (acetate production increased by 44%). Consumption of either the normal or high-fat diet supplemented with spinach resulted in increased propionate production, although the changes were only statistically significant in the case of the normal diet (groups NB and NA). As illustrated in Fig. 3, the Ac:Pr ratio was 46% higher in HC rats compared to NC rats. In rats fed the normal diet, spinach intake significantly reduced the Ac:Pr ratio, by 44 and 54% in groups NB and NA, respectively. In contrast, in rats fed the high-fat diet the Ac:Pr ratio remained unchanged, regardless of the supplementation with spinach.

Microbial phenolic catabolites

In general, the production of faecal microbial phenolic catabolites (Table 5) was reduced in rats consuming the high-fat diet, although in rats fed the high-fat diet supplemented with 5% spinach (NA), a general tendency of the faecal concentrations of phenolic catabolites to increase was observed. Nevertheless, statistical significance was only achieved for *p*-coumaric acid. The major phenolic catabolites identified in faeces were 3-(3-hydroxyphenyl) propanoic acid, *trans*-ferulic acid and hydroferulic acid. The largest variations in mean concentration between the NC/NA and HC/HA groups were observed for sinapic acid (15-fold), hydroferulic acid (13-fold) and *trans*-ferulic acid (12-fold). Conversely, the faecal concentrations of 4-hydroxybenzoic acid and 3-(4-hydroxyphenyl) propionic acid were higher in the HC/HA groups than in NC/NA. All the faecal phenolic catabolites were also detected in urine (Table 6), where the highest concentrations were found for 3-(3-hydroxyphenyl) propanoic acid, 3-(4-hydroxyphenyl) propionic acid, *trans*-ferulic

acid and hydroferulic acid. Again, the largest variations in mean concentration between the NC/NA and HC/HA groups were observed for sinapic acid (6-fold), hydroferulic acid (6-fold) and *trans*-ferulic acid (3-fold). As observed for faeces, the concentrations of 4-hydroxybenzoic acid were higher in the HC/HA groups than in NC/NA. The only catabolite identified in plasma was 4-hydroxybenzoic acid, whereas 4-hydroxybenzoic acid, vanillic acid and caffeic acid were detected in liver (Table 7).

Biochemical parameters and indexes of insulin resistance/sensitivity

As illustrated in Fig. 3, intake of the high-fat diet worsened the plasma lipid profile, by increasing triglycerides, total cholesterol and LDL-cholesterol, and reducing HDL-cholesterol. Spinach intake (5%) ameliorated the lipid profile by significantly reducing total cholesterol, LDL and triglycerides (non-significantly). The VLDL levels were higher in rats fed the normal diet, but no significant changes were observed for VLDL throughout the study. Nevertheless, despite this general improvement of the lipid profile, HDL continued to drop.

As shown in Fig. 4, fasting glucose was higher in rats fed the high-fat diet, and dietary intervention with spinach significantly reduced its levels in a dose-dependent manner. A similar trend was observed in rats fed the normal diet, although the changes were not statistically significant. Neither the type of diet (normal *vs* high-fat) nor spinach intake had a statistically significant impact on the indexes of insulin resistance [Log(HOMA-IR)] or insulin sensitivity [QUICKI]. This indicates that insulin homeostasis was not greatly altered despite the onset of NAFLD.

Relationships between variables

Fiber intake showed a statistically-significant positive correlation with *Lactobacillus* ($R = 0.95$; $p = 0.015$) and acetate ($R=0.85$; $p = 0.030$). Phenolic compounds intake showed significant correlations with several parameters: among them should be highlighted the positive correlations with *Lactobacillus* ($R = 0.91$; $p = 0.011$), *Bifidobacterium* ($R = 0.86$; $p = 0.027$), acetate ($R = 0.92$; $p = 0.010$) and propionate ($R = 0.88$; $p = 0.022$), and the negative correlations with Enterobacteriaceae ($R = -0.82$; $p = 0.044$) and the Ac:Pr ratio ($R = -0.85$; $p = 0.031$). The Ac:Pr ratio was positively correlated with the plasma glucose ($r = 0.999$; $p = 0.030$) and triglycerides (non-significant); so, the lower the ratio, the lower the glucose and triglycerides levels. Negative correlations were found between plasma glucose and *Lactobacillus* ($R = -0.999$; $p = 0.032$) and *Bifidobacterium* (non-statistically significant). Moreover, *Lactobacillus* and *Bifidobacterium* exhibited inverse relationships with triglycerides, total cholesterol and LDL.

Discussion

Overall, consumption of the high-fat diet caused hepatic steatosis, altered the plasma lipid profile, increased fasting glucose and affected the microbiota, reducing the abundance of beneficial

bacterial groups such as *Lactobacillus* and *Bifidobacterium.* Alteration of the microbiota was coupled with lower production of microbial phenolic catabolites and SCFAs and an increased Ac:Pr ratio. Such alterations are in agreement with previous animal studies involving the consumption of high-fat diets and could be explained by a combination of higher intakes of saturated fat and sugars and the lower intakes of fibre and phenolic compounds.[10,41-44]
It is well stablished that high fat diets alter the composition of gut microbiota by increasing the ratio Firmicutes to Bacteroidetes and by inducing an overall decrease in bacterial diversity. Moreover, dietary fatty acids quality affects gut microbiota alterations as it has been reported in a rodent study in which diets rich in saturated fat –particularly those formulated with milkfat like that we used in the present study– had a negative impact on the ratio Firmicutes to Bacteroidetes.[43] Similarly, an *in vitro* study showed that fats rich in saturated fatty acids (butter oil) reduced the growth of *Lactobacillus delbrueckii* whilst those richer in unsaturated fatty acids (lineseed, olive, cod liver and turnip rape oil) promoted its growth.[45] However, the mechanism by which the different type of fatty acids modulate gut microbiota is yet poor elucidate. It is proposed that fatty acids may act in cell membrane, interfere with energy production, inhibit enzymatic activities, impair nutrient absorption and generate toxic compounds to cells, leading to growth inhibition or even bacterial death.[46]

In rats consuming the normal diet (NB/NA), spinach intake ameliorated the pattern of SCFAs, as the Ac:Pr ratio was significantly diminished through reduction of acetate and enhancement of propionate production. This is considered a positive effect since a lower Ac:Pr ratio is preferable, as it may reduce serum lipid levels and the risk of cardiovascular disease. The SCFAs elicit effects on glucose and lipid metabolism. In the liver, the fate of acetate is *de novo* lipogenesis and cholesterogenesis, whilst propionate has been reported to inhibit cholesterol synthesis, reduce visceral and liver fat, and to have beneficial effects on glucose metabolism. Butyrate has also been studied for its roles in nourishing the colonic mucosa and preventing colon cancer, as well as for its possible involvement in lipid metabolism, by regulating and slowing down fat transport from the intestine.[10,14,47]

The observed improvement in the SCFAs profile cannot be fully explained by the weak changes in the microbiota or by marked differences in the intakes of fiber and/or phenolic compounds. Neither can it be attributed to carotenoid intake, whose effect on gut bacteria is considered negligible.[48] In this context, *in vitro* studies have shown that changes in the SCFAs pattern are not always accompanied by deep modifications of the microbiota, even under experimental conditions involving the presence of fiber.[49] Similarly, varied results -either increases or decreases- regarding SCFAs production have been obtained in human studies on the impact of

fibre or prebiotic consumption on the composition and function of the human gastrointestinal microbiota.[50]

In rats fed the high-fat diet (HB/HA), spinach supplementation was unable to prevent the increase in liver fat accumulation but partially ameliorated some metabolic alterations induced by this diet. It is known that both *Bifidobacterium* and *Lactobacillus* could differentially attenuate obesity comorbidities induced by a high-fat diet (e.g. by lowering blood glucose, insulin or liver steatosis), partly due to strain-specific effects on metabolic-syndrome-related phylotypes of the gut microbiota.[51] In line with this, it was reported that supplementation with 5% spinach gave an improvement in the antioxidant status and a slight decrease in plasma triglycerides for rats consuming a high-fat and cholesterol diet.[52] Contrary to what was observed in rats consuming the normal diet, intake of the spinach-enriched high-fat diet increased acetate production but did not reduce the Ac:Pr ratio. However, in the context of NAFLD, this enhancement of acetate production could be interpreted as positive since acetate has been linked to suppression of adipocyte lipolysis, thus reducing the free fatty acid flux to the liver and mitigating fatty-liver-induced deterioration of glucose homeostasis.[14] This is consistent with the observed reduction in plasma cholesterol, LDL and triglycerides, and, more importantly, with the dose-dependent reduction of fasting glucose levels that denotes better glucose homeostasis in groups HB and HA.

A question therefore arises as which phytocotonstituents of spinach are responsible for the observed effects. As mentioned before, our reported intakes of fiber and phenolic compounds alone cannot explain changes in microbiota and SCFAs but plant cell compartments such as spinach thylakoids has been shown to modulate microbiota by increasing *Lactobacillus* counts and improving glucose tolerance. However, the mechanism behind the beneficial effect of thylakoids in relation to microbiota was regarded as unknown. So, the authors proposed two possible explanations. One was that the thylakoids themselves influence the growth of the bacteria in the intestine directly at the molecular level. The other explanation was an indirect effect of thylakoids, whereby a reduction in appetite and food intake may affect bacterial composition in the intestine.[21].

Therefore, we hypothesize that the presence of intact thylakoids in the experimental diets may have contributed to improvement of microbiota and, in turn, to the reductions we have observed in plasma glucose and lipids. Also, products of bacterial metabolization of fibre (SCFAs) and phenolic compounds may also play a role. In this regard, the microbial phenolic catabolite 4-hydroxybenzoic acid was increased in the plasma and liver of rats consuming the high-fat diet plus 5% spinach (Tables 6 and 7). This compound has been shown to have a hypoglycaemic effect

after oral administration to diabetic rats, possibly mediated by an increase in the peripheral glucose consumption.[53,54] Moreover, 4-hydroxybenzoic acid has been reported to lower the values of plasma total cholesterol, triglycerides and hepatic cholesterol in rats fed a cholesterol diet.[55] Interestingly, in our study we observed a marked reduction in liver cholesterol in rats consuming a high-fat diet plus 5% spinach, which was accompanied by liver accumulation of 4-hydroxybenzoic acid and carotenoids, mainly β-carotene. This remarkably-positive result might arise from the combined effects of the 4-hydroxybenzoic acid derived from gut bacterial metabolism, which, as mentioned above, is able to reduce liver cholesterol[55], and carotenoids, that may display hypocholesterolemic effects through a mechanism involving the inhibition of cholesterol synthesis in the liver.[56] Further studies in rodents involving carotenoids and carotenoid-rich foods supported their ability to decrease liver cholesterol as well as improve the plasma lipid profile.[57-60] Hence, both these antioxidant compounds, when accumulated in liver, could exert a synergistic action against NAFLD.

Last but not least, other natural antioxidants (NAO) present in spinach may have contributed to the effects reported herein. NAO is comprised primarily of aromatic polyphenols, including *p*-coumaric acid derivatives, flavonoids, and other hydrophilic molecules, and has been reported to improve glucose tolerance, blood lipids and antioxidant status[19,60] Also, chlorophylls have been linked to the hypolipidemic effects of spinach consumption[19] and other spinach compounds such as the oxyprenylated phenylpropanoid auraptene should be considered in the future for their possible role in glucose homeostasis and lipid metabolism.[61-63]

Conclusions.

Under our experimental conditions, consumption of a high-fat diet caused NAFLD and dislipaemia and altered the gut microbiota and the pattern of SCFAs and phenolic gut microbial catabolites. Supplementation with spinach partially ameliorated some of the alterations induced by the high-fat diet, in particular by increasing *Lactobacillus* counts, reducing fasting glucose and total and LDL-cholesterol and preventing excess cholesterol accumulation in the liver. The effects appear to arise from combined actions of the different components provided by the diets (carotenoids, fiber, phenolic compounds) as well as from products of microbial metabolism, such as phenolic catabolites (e.g. 4-hydroxybenzoic acid) and SCFAs.

Conflict of interest statement.

The authors declare no conflict of interests.

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FIGURES

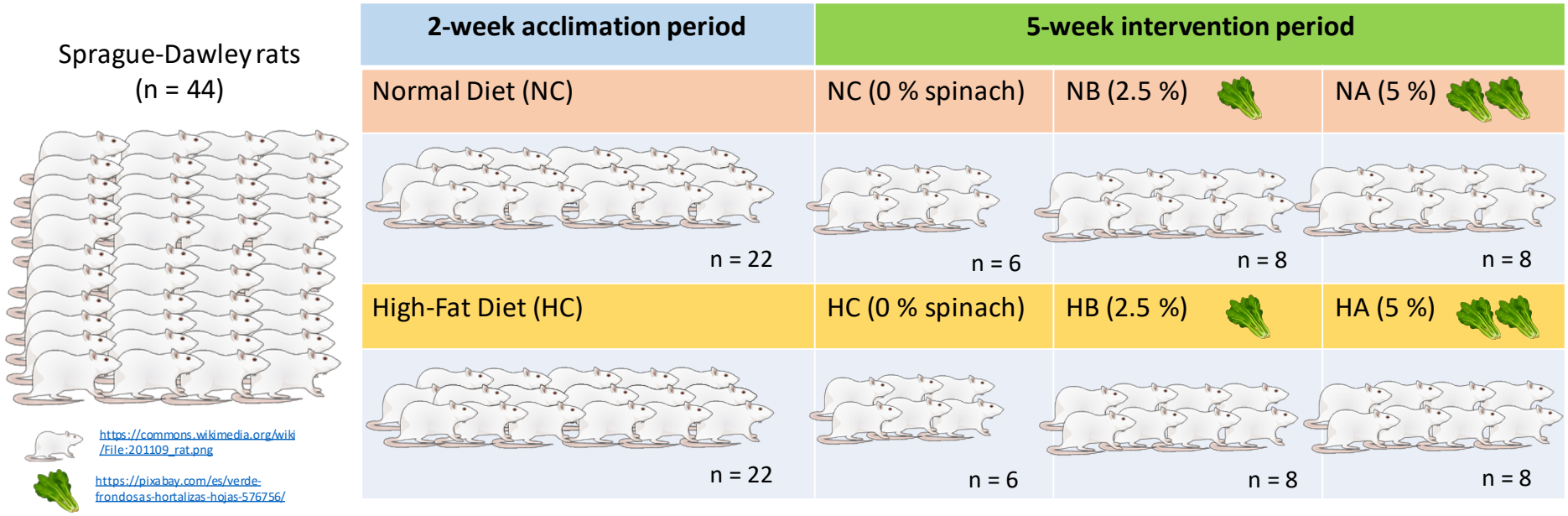

Fig. 1. Experimental design of the study.

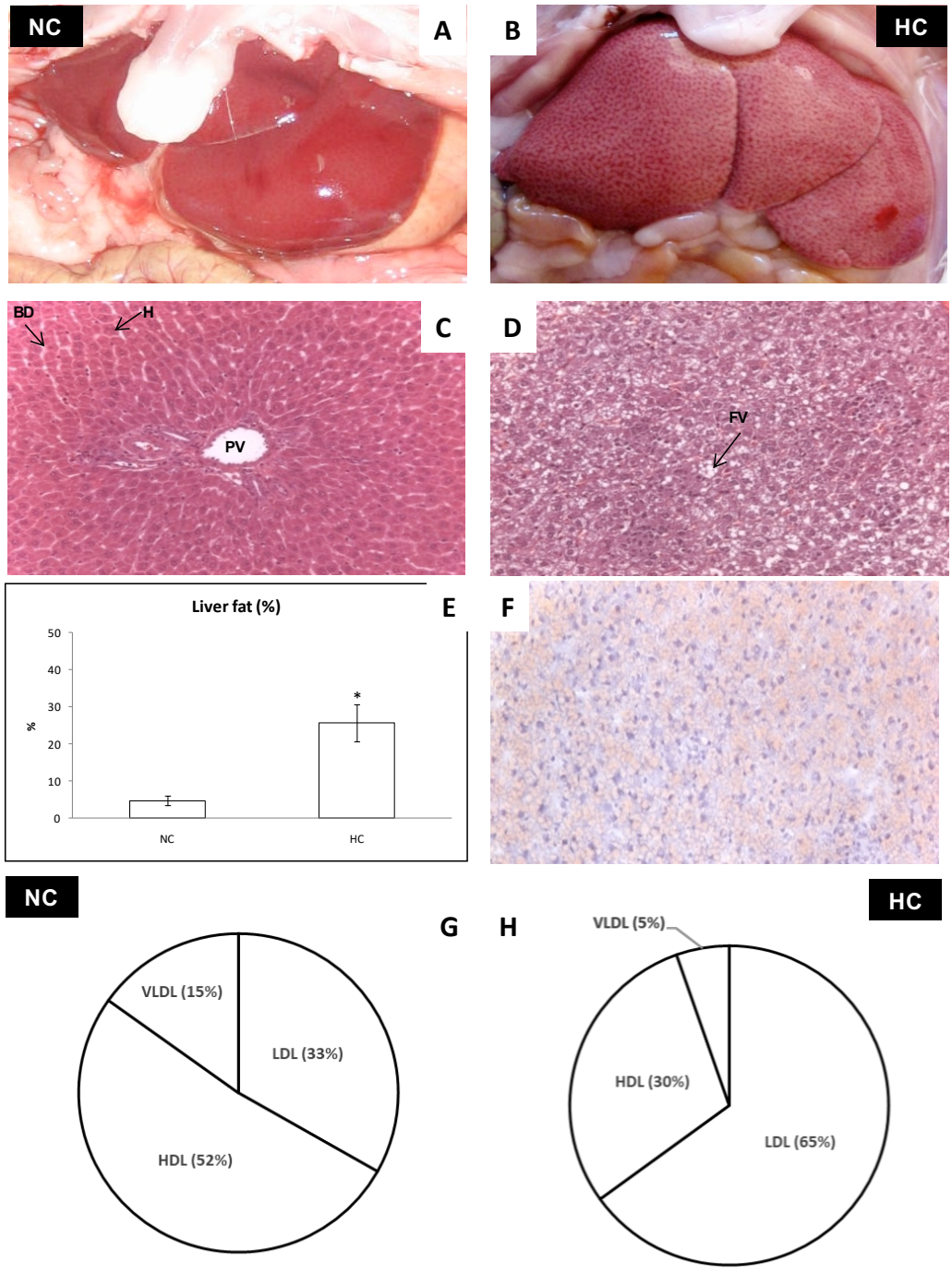

Fig. 2. Representative macro and light microscopic photographs (20x) of liver of groups NC (standard diet) and HC (high-fat diet). Panels A, B: visual aspect of rat liver during dissection to obtain liver samples. Panels C, D: microscopic images with H&E staining. Panel F: microscopic image with Sudan red staining. HC: high-fat diet, NC: standard diet. PV: portal vein, H: hepatocyte, BD: bile duct, FV: fat vacuole. Panel E: liver fat content at the end of the study. Panels G, H: relative contribution (%) of LDL, HDL and VLDL to total plasma cholesterol in groups NC and HC. *Indicates a significant statistical difference ($p<0.05$).

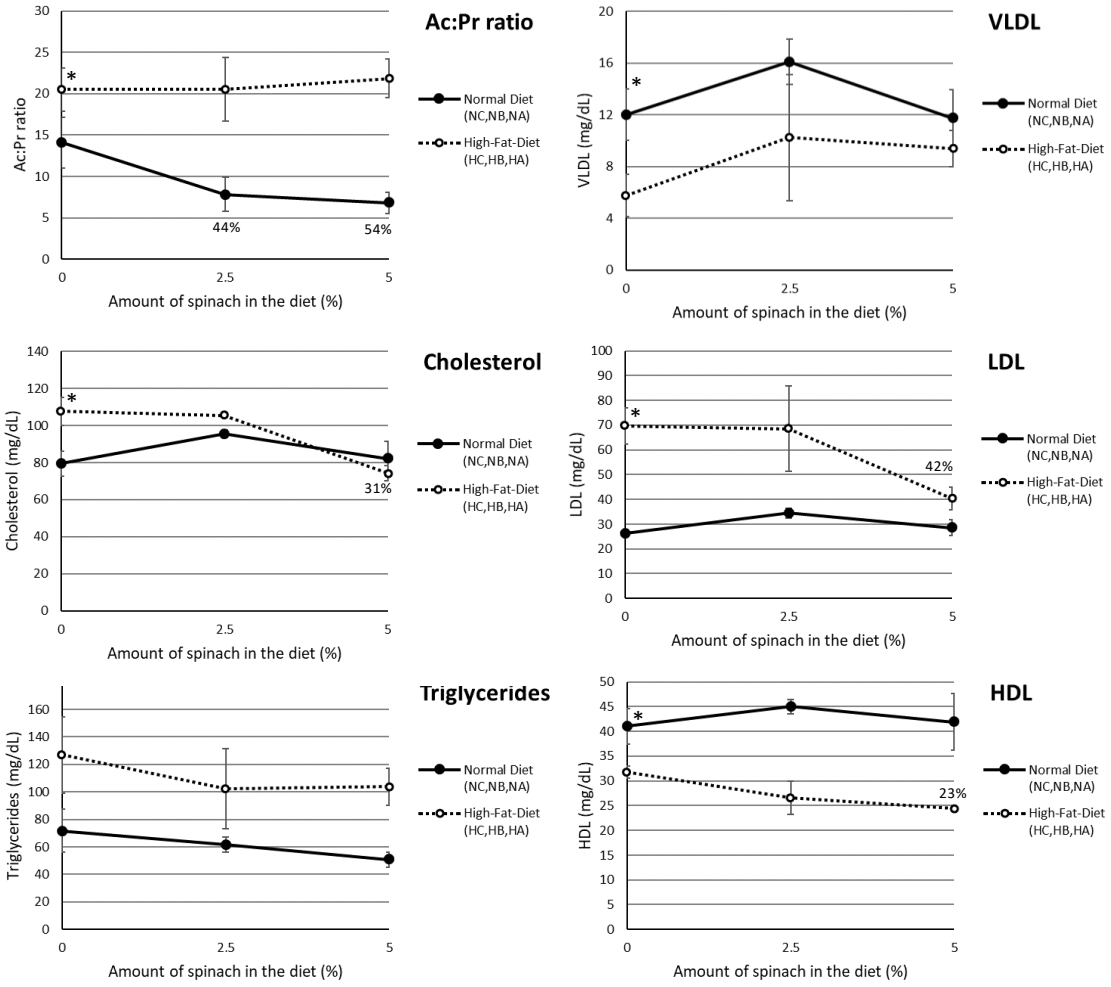

Fig. 3. Changes in the acetate to propionate (Ac:Pr) ratio and plasma lipids. Statistically significant changes (%) are indicated in the graphics ($p < 0.05$). *Indicates significant statistical differences ($p<0.05$) between NC and HC.

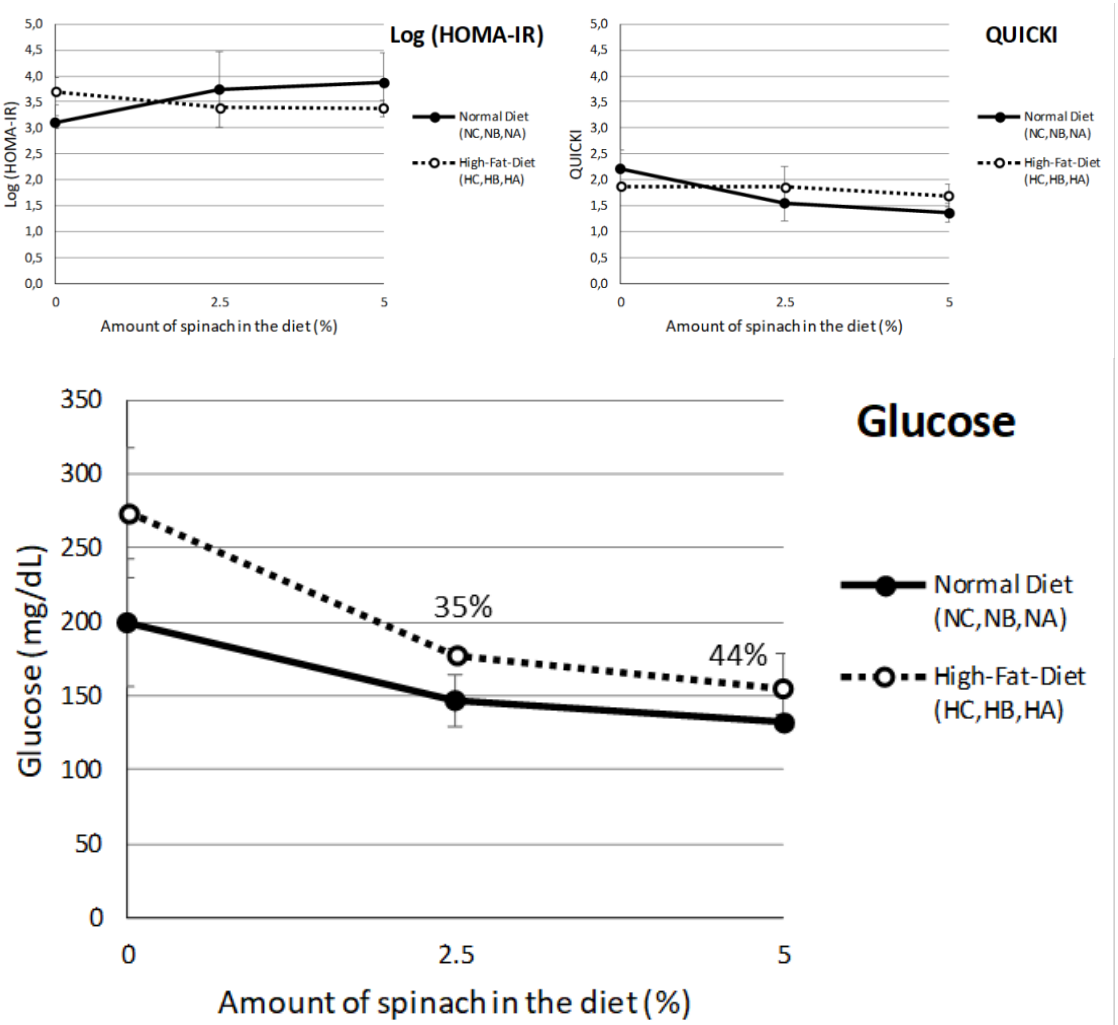

Fig. 4. Changes in fasting glucose and insulin resistance/sensitivity i

TABLES

Table 1. Nutritional characteristics of the diets used in the intervention study.

Nutrient amount (per 100 g)	NC	NB	NA	HC	HB	HA
Energy (kcal)	290	292.7	287.5	450	437.7	438
Protein (g)	14.3	14.98	15.46	17.3	17.71	18.12
Carbohydrates (g)	48.0	47.21	45.41	46.9	41.4	42
Fat (g)	4.0	4.01	4.03	21.20	20.78	20.37
Total saturated (g)	0.6	0.7	0.9	12.8	12.6	12.5
Total monounsaturated (g)	0.7	0.7	0.7	5.6	5.5	4.4
Total polyunsaturated (g)	2.1	2.5	2.8	1.0	1.4	1.8
Cholesterol (g)	--	--	--	1.25	1.22	1.19
Dietary fiber (g)[1]	21.2	21.3	22.6	6.9	12.4	11.8
Total phenolics (mg GAE)[2]	188	192	196	20	21	22
Total carotenoids (μg)[3]	--	305	909	--	305	909

NC: Normal diet (Teklad Global 14% Protein Rodent Maintenance Diet, 2014)
HC: High-fat diet (Atherogenic Rodent Diet, TD.02028, Harlan) containing casein (19.5%), sucrose (32.5%), anhydrous milkfat (21%). Major fatty acids (palmitic > oleic > stearic > myristic).
NB: normal diet+2.5% spinach, NA: normal diet+5% spinach, HB: high-fat diet+2.5% spinach, HA: high-fat diet+5% spinach
NB NA, HB and HA: Estimated data from diet data sheets and proximate composition of spinach
[1]Dietary fibre analysed using AOAC 985.29 method.[26]
[2]GAE; Gallic acid equivalents analysed using Folin Ciocalteu's Phenol reagent.[27]
[3]Total carotenoids -the sum of β-carotene, α-carotene and lutein- were analysed in lyophilised spinach by HPLC.[28] The amounts in the diets were calculated on the basis of the amount (%) added to the diets.

Table 2. Bacterial groups, standard cultures, primers and annealing temperatures used in this study.

Microbial target	Strain used for standard curve	Primer sequence 5'-3'	Mt *	Reference
Bacteroides group *Bacteroides-Prevotella-Porphiromonas*	*Bacteroides thetaiotaomicron* DSMZ2079	F: GGTGTCGGCTTAAGTGCCAT R: CGGA(C/T)GTAAGGGCCGTGC	64	29
Bifidobacterium	*Bifidobacterium longum* NCIMB8809	F: GATTCTGGCTCAGGATGAACGC R: CTGATAGGACGCGACCCCAT	60	30
Enterobacteriaceae	*Escherichia coli* LMG2092	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	63	31
Enterococcaceae	*Enterococcus faecalis* IPLAIF3/1	F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTGT	61	29
Lactobacillus	*Lactobacillus gasseri* IPLAIF1/6	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	60	32,33
Clostridia IV (*C. leptum-F. praustnitzi*)	*Clostridium leptum DSMZ735*	F: TTAACACAATAAGTWATCCACCTGG R: ACCTTCCTCCGTTTTGTCAAC	60	34
Total bacteria	*Escherichia coli* LMG2092	F: AGAGTTTGATCCTGGCTCAG R: GGCTGCTGGCACGTAGTTAG	50	35

*Mt: Melting temperature (ºC).

Table 3. Daily intake of food, energy, macronutrients, dietary fibre, total phenolics and total carotenoids.

Daily nutrient intake	NC	NB	NA	HC	HB	HA
Food (g)	13.5±3.8^{a}	8.7±3.7^{a}	10.5±2.9^{a}	9.4±4.3^{a}	10.1±4.6^{a}	7.4±4.1^{a}
Energy (kcal)	26.0±10.6^{a}	19.8±6.6^{a}	20.7±11.2^{a}	32.5±9.4^{a}	34.3±11.8^{a}	25.6±8.3^{a}
Protein (g)	1.3±0.5^{a}	1.0±0.3^{a}	1.1±0.6 ^{a}	1.3±0.4^{a}	1.4±0.5^{a}	1.1±0.3^{a}
Carbohydrates (g)	4.3±1.7^{a}	3.2±1.1^{a}	3.3±1.8^{a}	3.4±1.0^{a}	3.2±1.1^{a}	2.5±0.8^{a}
Fat (g)	0.4±0.1^{b}	0.3±0.1^{b}	0.3±0.2^{b}	1.5±0.4^{a}	1.6±0.6^{a}	1.2±0.4^{a}
Cholesterol (mg)	--	--	--	90.3±26.1^{a}	95.7±32.8^{a}	69.7±22.7^{a}
Dietary fiber (g)	1.9±0.8^{a}	1.4±0.5^{abc}	1.6±0.9^{ab}	0.5±0.1^{d}	1.0±0.3^{bcd}	0.7±0.2^{cd}
Total phenolics (mg GAE)	16.9±6.9^{a}	13.0±4.4^{a}	14.1±7.6^{a}	1.4±0.4^{b}	1.6±0.6^{b}	1.3±0.4^{b}
Total carotenoids (μg)	--	20.6±6.9^{b}	55.5±35.4^{a}	--	23.9±8.2^{b}	53.2±17.3^{a}

$^{a-c}$ Different superscript letters within the same row mean statistical significance ($p<0.05$); ANOVA.

Table 4. Composition of the microbiota (log_{10}cells/g) and concentrations of SCFAs (mmol/g) in the intestinal content.

Study group	NC	NB	NA	HC	HB	HA
Bacterial groups						
Bacteroides	11.3±0.1^{a}	11.3±0.1^{a}	11.4±0.1^{a}	11.0±0.3^{b}	11.2±0.2^{ab}	11.3±0.2^{ab}
Bifidobacterium	8.3±0.2^{ab}	8.5±0.1^{a}	8.5±0.1^{a}	7.9±0.3^{c}	8.0±0.2^{bc}	8.1±0.2^{bc}
Enterobacteriaceae	8.6±0.6^{ab}	8.5±0.9^{b}	8.5±0.7^{b}	8.9±0.4^{ab}	9.0±0.5^{ab}	9.5±0.5^{a}
Enterococcaceae	6.7±0.3^{a}	6.4±0.4^{a}	6.9±0.5^{a}	6.6±0.3^{a}	6.6±0.2^{a}	6.7±0.4^{a}
Lactobacillus	9.8±0.1^{a}	9.3±0.3^{ab}	9.6±0.3^{a}	8.0±1.1^{c}	8.6±0.6^{bc}	8.8±0.7^{abc}
Clostridia	10.6±0.1^{a}	10.6±0.1^{a}	10.6±0.1^{a}	10.0±0.2^{b}	10.1±0.1^{b}	10.1±0.4^{b}
Total bacteria	8.9±0.2^{ab}	8.9±0.2^{ab}	9.0±0.4^{a}	8.5±0.4^{b}	8.7±0.2^{ab}	8.8±0.4^{ab}
SCFAs						
Acetate	96.7±6.7^{a}	85.5±8.8^{a}	64.2±9.9^{b}	32.7±7.0^{d}	42.7±4.4^{cd}	47.2±7.0^{c}
Propionate	7.2±1.6^{b}	11.5±2.8^{a}	10.2±2.5^{a}	1.6±0.2^{c}	2.2±0.5^{c}	2.2±0.2^{c}
Butyrate	2.5±0.8^{abc}	5.5±4.4^{a}	4.8±3.2^{ab}	1.4±0.3^{abc}	0.6±0.1^{c}	0.8±0.2^{bc}
Other SCFAs	4.3±0.7^{b}	7.3±2.2^{a}	6.3±3.2^{ab}	1.0±0.2^{c}	1.2±0.3^{c}	1.0±0.3^{c}

$^{a-d}$ Different superscript letters within the same row indicate statistical significance ($p<0.05$); ANOVA.

Table 5. Concentrations of phenolic catabolites in faeces (μg/g).

Study group/metabolite	NC	NA	HC	HA
Pyrogallol	10.8±5.5[a]	9.2±3.9[a]	9.2±2.2[a]	11.3±3.6[a]
4-hydroxybenzoic acid	10.1±4.7[ab]	2.7±0.9[b]	13.8±4.3[ab]	22.4±9.8[a]
p-Coumaric acid	5.2±2.1[a]	1.8±0.5[bc]	0.2±0.1[c]	2.9±1.2[ab]
3-(4-hydroxyphenyl) propionic acid	16.0±8.5[ab]	3.1±1.3[b]	45.5±5.0[a]	52.3±3.7[a]
3-(3-hydroxyphenyl) propanoic acid	356.5±87.8 [a]	225.0±134.5[ab]	76.2±21.2[b]	251.5±63.1[ab]
Vanillic acid	13.6±5.4[a]	2.2±1.0[b]	0.4±0.1[b]	1.4±0.3[b]
Caffeic acid	1.0±0.2[a]	0.5±0.4[ab]	0.02±0.01[b]	0.2±0.09[b]
trans-Ferulic acid	186.3±38.4[a]	73.4±39.2[b]	0.60±0.4[c]	21.8±12.1[bc]
Hydroferulic acid	64.5±54.9 [a]	25.2±30.5[ab]	1.5±0.1[b]	5.4±2.6[ab]
Sinapic acid	10.3±5.0 [a]	4.8±1.8[b]	0.3±0.02[b]	0.7±0.5[b]
Protocatechuic acid	2.9±0.7[a]	1.3±0.7[a]	1.6±2.6[a]	3.0±0.9[a]
Total catabolites	677.2±213.2[a]	348.9±214.8[ab]	149.3±37.0[b]	372.6±98.3[ab]

[a-c] Different superscript letters within the same row indicate statistical significance ($p<0.05$); ANOVA.

Table 6. Concentrations of polyphenol metabolites in urine (μg/mg creatinine) and plasma (μg/mL).

Study group/metabolite	NC	NA	HC	HA
Urine				
Pyrogallol	1.2±0.8[a]	2.1±1.0 [a]	1.4±0.5[a]	1.6±0.79 [a]
4-hydroxybenzoic acid	2.0±1.2[b]	2.5±1.1[ab]	4.5±2.2[ab]	4.7±2.2[a]
p-Coumaric acid	1.3±0.9[a]	1.7±1.7[a]	0.5±0.3[a]	0.7±0.4[a]
3-(4-hydroxyphenyl) propionic acid	9.1±7.0[a]	8.9±2.3[a]	6.1±1.9[a]	6.4±3.2[a]
3-(3-hydroxyphenyl) propanoic acid	63.9±25.2[a]	60.2±20.3[a]	14.3±14.5[b]	21.6±14.8[b]
Vanillic acid	1.1±0.5[a]	1.1±0.4[a]	0.3±0.2[b]	0.23±0.2[b]
Caffeic acid	0.3±0.1[a]	0.3±0.2[a]	0.2±0.1[a]	0.2±0.1[a]
trans-Ferulic acid	6.3±4.2[ab]	12.1±6.1[a]	3.1±3.3[b]	2.8±2.4[b]
Hydroferulic acid	7.4±5.1[a]	7.0±3.8[a]	1.3±1.9[b]	0.9±0.8[b]
Sinapic acid	0.5±0.5[a]	0.5±0.2[a]	nd	0.2±0.1[a]
Protocatechuic acid	0.2±0.1[a]	0.3±0.3[a]	0.1±0.08[a]	0.11±0.10 [a]
Total catabolites	93.3±45.5[a]	96.3±37.4[a]	31.3±24.9[b]	39.7±25.0[b]
Plasma				
4-hydroxybenzoic acid	153.2±78.4 [ab]	165.5±72.1[a]	24.8±1.2[b]	47.5±21.7[ab]

[a-c]Different superscript letters within the same row mean statistical significance ($p<0.05$); ANOVA.
nd; not detected

Table 7. Concentrations of cholesterol (mg/g), carotenoids (μg/g) and polyphenol metabolites (μg/g) in liver.

Study group/metabolite	NC	NA	HC	HA
Cholesterol*	2.24±0.46[b]	1.93±0.45[b]	73.83±10.38[a]	2.33±0.50[b]
Lutein**	nd	nd	nd	0.03±0.06
α-carotene	nd	nd	nd	0.15±0.10
β-carotene	nd	0.20±0.09[b]	nd	1.28±0.47[a]
Total carotenoids	nd	0.20±0.09[b]	nd	1.45±0.51[a]
4-hydroxybenzoic acid	1.62±1.07[a]	0.28±0.26[a]	1.75±0.92[a]	4.44±4.67[a]
Vanillic acid	0.13±0.16[a]	0.32±0.34[a]	0.13±0.02[a]	0.05±0.06[a]
Caffeic acid	0.12±0.09[a]	0.36±0.29[a]	0.16±0.05[a]	0.27±0.14[a]

[a-c]Different superscript letters within the same row mean statistical significance ($p<0.05$); ANOVA.
nd; not detected
*Liver cholesterol was extracted using a Sigma Aldrich Cholesterol Extraction Kit (MAK175) and analysed by GC-FID.[40]
**Liver carotenoids (lutein, α-carotene and β-carotene) were analysed by HPLC.[28]