Dietary intake of table olives exerts antihypertensive effects in association with changes in gut microbiota in spontaneously hypertensive rats

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Abstract

Arbequina table olive (AO) consumption lowers blood pressure (BP) in spontaneously hypertensive rats (SHR). This study evaluates whether dietary supplementation with AO induced changes in the gut microbiota that are consistent with the purported antihypertensive effects. Wistar-Kyoto rats (WKY-c) and SHR-c received water, while SHR-o were supplemented by gavage with AO (3.85 g/kg) for 7 weeks. Faecal microbiota was analysed by 16S rRNA gene sequencing. SHR-c showed increased Firmicutes and decreased Bacteroidetes compared to WKY-c. AO supplementation in SHR-o decreased BP by approximately 19 mmHg, and reduced plasmatic concentrations of malondialdehyde and angiotensin II. Moreover, reshaped faecal microbiota associated with antihypertensive activity by lowering *Peptoniphilus* and increasing *Akkermansia*, *Sutterella*, *Allobaculum*, *Ruminococcus*, and *Oscillospira*. Also promoted the growth of probiotic strains of *Lactobacillus* and *Bifidobacterium* and modified the relationship of *Lactobacillus* with other microorganisms, from competitive to symbiotic. In SHR, AO promotes a microbiota profile compatible with the antihypertensive effects of this food.

1. Introduction

Hypertension is the main preventable risk factor for cardiovascular disease and premature death worldwide.[1] Primary hypertension is induced by the interaction of non-modifiable genetic factors, which determine the risk of cardiovascular disease, with modifiable environmental factors, such as overweight and unhealthy lifestyles.[1]

An important element in the prevention of cardiovascular disease is the adherence to healthy dietary habits like those offered by the Mediterranean diet (MD). The MD has been widely studied, with strong evidence showing that it promotes cardiovascular health and prevents obesity and hypertension.[2] The consumption of the core elements of the MD (fruits, virgin olive oil, cereals, vegetables, nuts, legumes, and fish) is associated with a lower risk of cardiovascular disease and lower blood pressure (BP).[3]

Extra virgin olive oil (EVOO) has anti-inflammatory, antioxidant, and vasodilator properties, which reduce the atherosclerotic burden.[3,4] EVOO decreases BP in the spontaneously hypertensive rat (SHR) model[5] and in individuals at a high risk of developing cardiovascular disease.[6] In addition to oleic acid, olive oil contains polyphenols and pentacyclic triterpenes that have antioxidant, anti-inflammatory, and cardioprotective effects.[4] Phenolic compounds from EVOO contribute to the protection of blood lipids from oxidative stress, as established by the Commission Regulation (EU) No. 432/2012 document.[7]

It is well known that during olive oil milling, only a minimal portion of the bioactive compounds is extracted along with the oil. The rest remains in the mill by-products, like olive pomace or olive pulp, which are used to prepare dietary supplements or ingredients for animal feed.[8] These by-products have been studied

as potential sources of bioactive compounds. For example, olive pomace powders have been shown to have gastrointestinal health benefits as they stimulate the production of short-chain fatty acids (SCFA) by the gut microbiota, which has well-known beneficial effects.[9] The consumption of bread enriched with olive fibre has been observed to have beneficial effects on the host gut, increasing the abundance of probiotic bacteria such as *Bifidobacteriaceae* and *Lactobacillales*.[10] However, there are only a few studies on the protective effects of table olive consumption on cardiovascular variables.

In recent years, interest has focused on the relationship between gut microbiota and the host health status. Since the gut microbiota produces active metabolites that are involved in several physiological processes, an altered microbiota may be implicated in the development of cardiometabolic diseases.[11] Studies on animal models and humans have shown that the gut microbiota of hypertensive individuals has lower bacterial diversity and a different taxonomic composition compared to normotensive controls.[12] Some studies also suggest a possible causal role of gut dysbiosis in the pathogenesis of hypertension.[13]

Recently, our group reported that dietary supplementation with Arbequina table olives (AO) for 7 weeks lowered BP in SHR from the second week until the end of the intervention.[14] Therefore, in view of the role of the gut microbiota in the regulation of cardiovascular functions, the present study analysed differences in the faecal microbiota composition of hypertensive and normotensive rats and evaluated the hypothesis that dietary AO supplementation promotes the growth of bacteria involved in BP modulation in SHR.

2. Materials and methods

2.1. Table olives

Table olives of the Arbequina variety (AO), harvested in the 2016-2017 season and subjected to natural fermentation in brine, were obtained from Cooperativa del Camp (Maials, Lleida, Spain). The composition of AO (g/100 g of destoned olives) consisted of 21.0 lipids, 1.6 proteins, 7.2 fibre, and 4.3 salt, with metabolizable energy of 211 kcal (868 kJ). The AO content of pentacyclic triterpenes and phenolic compounds was 3308 ± 195 mg/kg and 1048 ± 85 mg/kg of destoned olive (n = 5). The detailed content of bioactive compounds in AO is shown in Table S1**†.** AO was prepared as a homogeneous suspension of the edible part of the olive, at a dose of 3.85 g/kg of animal weight which is equivalent to the intake of 30 AO by a person weighing 60 kg, as previously described.[14]

2.2. Animals

The study was approved by the Animal Experimentation Ethics Committee of the Universitat de Barcelona (Ref. 105/17) and by the Generalitat de Catalunya (Ref. 9468), complying with the European Community Guidelines for the care and management of laboratory animals. Male spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto (WKY) controls, all at the age of 11-week-old, were obtained from Envigo Laboratories (Huntingdon, United Kingdom). The animals were distributed into groups of two rats per cage and maintained under controlled conditions of temperature (22 ± 2 °C), humidity (50 ± 10%), and a 12-hour light-dark cycle. During the whole experiment, the rats were fed a standard diet (2014 Teklad Global 14%, Harlan, Barcelona, Spain) and water *ad libitum*.

2.3. Experimental design

At 14 weeks of age, the SHR group was randomly distributed into 2 groups, the untreated SHR (SHR-c n = 7) and the SHR supplemented with AO (SHR-o, n = 6). The third group was constituted of untreated WKY rats (WKY-c n = 8). During a period of 7 weeks, the control groups (WKY-c and SHR-c) received water by gavage at a volume of 10 mL/kg while the SHR-o had the corresponding dose of AO suspension.[14] The body weight of the rats, as well as food and water consumption were measured at 14 and 21 weeks of age.

2.4. Blood pressure

Systolic (SBP), diastolic blood pressure (DBP) and heart rate (HR) were determined in WKY-c, SHR-c, and SHR-o rats at 14 and 21 weeks of age. Measurements were performed using a non-invasive automatic BP analyser for rodents (LE5001 Harvard Apparatus, Panlab, Barcelona, Spain) as previously described.[14]

2.5. Analysis of malondialdehyde, angiotensin II, IL6 and TNF-α in plasma

At the end of the experiments, overnight fasted rats were anaesthetized by intramuscular injection of ketamine (90 mg/kg, Imalgene®, Merial, Lyon, France) and xylazine (10 mg/kg, Rompun®, Bayer Hispania SL, Sant Joan Despí, Barcelona, Spain). Blood was collected from WKY-c, SHR-c, and SHR-o rats and transferred to EDTA-K_3-coated tubes. Plasma samples were used to determine relevant biomarkers involved in the development of hypertension. Lipid peroxidation was assessed by measuring malondialdehyde (MDA) using the method described by Ohkawa *et al*.[15] Enzyme-linked immunosorbent assay (ELISA) kits from FineTest (Wuhan, Hubei, China) were used to determine angiotensin II (ANG II) (Ref. ER1637) and tumour necrosis factor α (TNF-α) (Ref. ER1393) while the kit

for interleukin 6 (IL6) (Ref. SEA079Ra) was provided by Cloud Clone Crop (Katy, TX, USA).

2.6. Collection of faecal samples

Stool samples were collected at 14 and 21 weeks of age in clean conditions. Faeces were collected directly into a sterile Eppendorf, frozen immediately in liquid N_2, and stored at -80 °C until use.

2.7. DNA extraction and purification

Microbial DNA was extracted from stool samples using the QIAamp PowerFecal DNA kit (Mo Bio Laboratories, Carlsbad, CA, USA). DNA quantification was performed using the NanoDrop ND-100 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), and the purity of the extraction was verified by agarose gel electrophoresis.

2.8. Analysis of the 16S rRNA gene

The V3 and V4 hypervariable regions of the bacterial 16S rRNA gene were amplified by PCR using the specific primers PCR1_Forward (50bp): 5'–TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG–3' and PCR1_Reverse (55bp): 5'–GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC–3'. Samples were sequenced using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) at the Genomics and Bioinformatics Service of the Universitat Autònoma de Barcelona (Bellaterra, Barcelona, Spain). The analysis of the 16S rRNA gene was performed using the BaseSpace app 16S Metagenomics and the MiSeq Reporter software v 2.6 provided by the Illumina MiSeq platform. The raw paired-end reads were trimmed considering a Phred quality score equal to or

greater than 30. The filtered sequences were analysed using the ClassifyReads algorithm, a high-performance implementation of the Ribosomal Database Project (RDP),[16] with further sequence homology analysis by the RDP SeqMatch tool using the Greengenes database (v. 13.5) as a reference.

2.9. Statistical analysis

Microbiota analysis only included taxa with a percentage of reads higher than 0.001%. Alpha diversity was analysed using the Chao1 and Shannon indices and the number of species observed. Beta diversity was estimated based on Bray-Curtis dissimilarities, using analysis of similarities (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) with Bonferroni post-hoc test to determine differences between microbial communities. Both tests were performed in RStudio (R v. 4.2.2; R Core Team, Vienna, Austria) using the vegan package v. 2.4-6. Beta diversity was visualised using principal coordinate analysis from the 16s Metagenomics app.

The normality of data was assessed using the Shapiro-Wilk test. The faecal microbiota composition analysis was performed by two-way ANOVA for the factors strain and age (14 and 21 weeks) in WKY-c and SHR-c groups. The Benjamini-Hochberg procedure was used to control the false discovery rate (FDR 5%) in multiple comparisons. The effect of AO supplementation on the SHR-c and SHR-o faecal microbiota at 21 weeks of age was analysed using an independent Student *t*-test or the Mann-Whitney *U* test, according to data distribution. Correlation between faecal microbiota with BP, MDA and ANG II was performed using the Spearman rank correlation, and the correlation between microbiota genera was visualized by a network plot using Cytoscape v 3.9.1.[17] The statistical analysis of

body weight, feed intake, water consumption, BP, MDA, ANG II, IL6 and TNF-α was performed by one-way ANOVA considering treatment (water or AO) as the independent variable following of Bonferroni post-hoc test. Statistical significance was considered when $p < 0.05$. The sample size of the WKY-c (n = 8), SHR-c (n = 7), and SHR-o (n = 6) were established, considering an 80% power to detect a difference greater than or equal to 0.26 units for the Shannon index and changes of 16 mmHg or more in SBP, assuming in both cases a 5% significance level in a two-sided test. Statistical analysis was performed with GraphPad Prism v 8.0.2 (La Jolla, CA, USA).

3. RESULTS

3.1. General characteristics

Table 1 shows the body weight as well as the food and water intake at the beginning and the end of the experiment, that is, at 14 and 21 weeks of age. No changes were found among groups in body weight gain and feed consumption. Conversely, the water consumption was higher in SHR-c and SHR-o than in the WKY-c rats ($p < 0.05$).

WKY-c rats gave measurements of SBP and DBP within the normotensive range, whereas SHR-c and SHR-o showed hypertensive values, both at 14 and 21 weeks of age (Table 1). The supplementation of AO for 7 weeks induced a decrease of 20 mmHg in SBP and 19 mmHg in DBP in the SHR-o compared to the SHR-c at the end of the experiment (Table 1). Moreover, the supplementation induced a non-significant decrease in heart rate (HR) of SHR-o with respect to SHR-c at the end of the 7 weeks of administration.

3.2. Malondialdehyde, angiotensin II, IL6 and TNF-α in plasma

Lipid peroxidation determined at 21 weeks of age was enhanced a 24% in plasma from SHR-c compared to WKY-c ($p > 0.05$). AO supplementation prevented the increase in the concentration of MDA in SHR-c yielding a decrease of 39% and a reduction of 20%, in WKY-c, as shown in Table 2 ($p < 0.05$). Plasma concentration of ANG II was higher in SHR-c compared with WKY-c ($p < 0.05$). However, ANG II concentration showed a reduction of 32% in rats administered with AO compared to SHR-c ($p < 0.05$) without reaching the values of WKY-c (Table 2). Quantification of IL6 and TNF-α showed similar plasma concentrations in control groups rats (WKY-c and SHR-c), and no differences were observed in rats supplemented with AO.

3.3. Differences in faecal microbiota composition and age-related changes in WKY rats and SHR controls

After bacterial DNA sequencing, a total of 4,182,795 reads that passed quality filtering (PF) were generated, and each faecal sample produced an average of 154,918 ± 9,041 PF reads. Similar alpha diversity ($p > 0.05$) was found for the WKY-c rats and SHR-c at 14 and 21 weeks of age (Fig. 1A). PCoA at the genus level also showed no differences, according to ANOSIM ($R = 0.07$; $p = 0.134$) and PERMANOVA ($F = 1.41$; $p = 0.139$), between the rat strains in beta diversity at both the ages examined (Fig. 1B).

Faecal microbiota composition was evaluated from the relative abundances of the different taxonomic levels (Fig. 1C). In both WKY-c rats and SHR-c, at 14 weeks of age, Firmicutes (WKY-14w: 78.9% and SHR-14w: 82.5%) was the main phylum, followed by Bacteroidetes (WKY-14w: 17.1% and SHR-14w: 12.3%), Actinobacteria (WKY-14w: 1.89% and SHR-14w: 3.60%), and Proteobacteria (WKY-14w: 1.42% and SHR-14w: 1.05%), with the remaining phyla accounting for less than 1% of the total faecal bacteria. No significant differences between strains were found at 14 weeks of age except for

Proteobacteria, which were significantly increased in WKY-c rats (p = 0.024) (Fig. 1C) and remained higher at 21 weeks of age (WKY-21w: 1.59% and SHR-21w: 1.26% p = 0.016). The comparison of the faecal microbiota between WKY-c rats and SHR-c at 21 weeks of age (Fig. 1C) showed an increase in the abundance of Firmicutes in SHR-c (WKY-21w: 72.5% and SHR-21w: 82.0% p = 0.014) and a higher abundance of Bacteroidetes in WKY-c rats (WKY-21w: 23.1% and SHR-21w: 12.2% p = 0.003). At the phylum level, there was an increase in the Firmicutes to Bacteroidetes (F/B) ratio in SHR-c, which was 2-fold higher than that of WKY-c rats at 21 weeks of age (p = 0.026) (Fig. 1D).

Lactobacillus, *Blautia*, *Ruminococcus,* and *Turicibacter* were the most abundant genera in both WKY rats and SHR throughout the study period, without differences between groups (Fig. 2). No differences in the relative abundances of the different genera were found between strains at 14 weeks of age, except for *Allobaculum* which was superior (p < 0.05) in SHR-c with respect to WKY-c but showed no differences at 21 weeks of age (Fig. 2). However, important differences were found at 21 weeks of age, where the relative abundances of *Phascolarctobacterium*, *Parabacteroides*, *Prevotella, Desulfovibrio, Sutterella,* and *Akkermansia* were higher (p < 0.05) in WKY-c rats with respect to SHR-c.

3.4. Key bacteria related to blood pressure

The association between the relative abundance of faecal microbiota and BP in WKY-c and SHR-c at 21 weeks of age was estimated by the analysis of Spearman rank correlation (Fig. 3). A direct association between the relative abundance of the phylum Firmicutes, including the *Sarcina* genus and the *Lactobacillus acidophilus* species were established with SBP and DBP (p < 0.05). Although this positive

association was also set up for the genera *Alkaliphilus* and *Peptoniphilus*, the correlation was only significant with DBP ($p < 0.05$).

On the other hand, an inverse significant association ($p < 0.05$) between the relative abundance of faecal microbiota was settled for the phylum Bacteroidetes, including *Parabacteroides, Bacteroides, Prevotella*, and *Flavobacterium* genera, the phylum Proteobacteria including the genera *Desulfovibrio* and *Sutterella* and SPB and DPB. Although the phylum Verrucomicrobia yielded a non-significant correlation with BP ($p > 0.05$), this inverse association was significant ($p < 0.05$) for the *Akkermansia* genus and *Akkermansia muciniphila* species (Fig. 3). Noteworthy that from the Firmicutes phylum, only the *Phascolarctobacterium* genus showed a negative relationship with BP ($p < 0.05$).

Spearman rank correlation analysis was also performed to estimate the association between gut microbiota and plasma concentrations of MDA and ANG II in hypertensive and normotensive rats. Fig. 3 shows that the phylum Firmicutes, along with the genera *Lactobacillus*, *Blautia*, *Alkaliphilus,* and *Sarcina* showed a direct association with MDA ($p < 0.05$); whereas only the phylum Firmicutes and *Sarcina* were correlated directly with ANG II ($p < 0.05$). Conversely, the genera *Phascolarctobacterium*, *Allobaculum*, *Parabacteroides*, *Sutterella,* and *Akkermansia*, along with the species *Bifidobacterium animalis*, *Bifidobacterium thermacidophilum,* and *Akkermansia muciniphila* were inversely correlated with lower values of lipid peroxidation ($p < 0.05$). While the phylum Bacteroidetes and the genera *Phascolarctobacterium*, *Bacteroides*, *Prevotella*, *Desulfovibrio* and *Akkermansia* showed an inverse association with ANG II ($p < 0.05$).

3.5. Effect of AO consumption on SHR faecal microbiota

The effect of AO on the diversity and abundance of SHR-c and SHR-o faecal microbiota was studied from a total of 1,950,997 PF reads with an average of 150,077 ± 15,909 PF reads per sample. Daily administration of AO for seven weeks did not affect the alpha diversity ($p > 0.05$) (Fig. 4A) and beta diversity of the microbial communities of SHR (ANOSIM: R = 0.17, $p = 0.083$ and PERMANOVA: F = 2.47, $p = 0.077$) (Fig. 4B). At 21 weeks of age, the main phyla, Firmicutes (SHR-c: 82.0% and SHR-o: 80.4%) and Bacteroidetes (SHR-c: 12.2% and SHR-o: 11.7%) showed similar abundances in both the untreated and treated groups, whereas only Actinobacteria (SHR-c: 3.09% and SHR-o: 5.99% $p = 0.047$) was significantly increased in the AO supplemented group (Fig. 4C). Moreover, AO intake did not modify the F/B ratio (Fig. 4D).

At the genus level, the most remarkable effects of AO consumption were the dramatic increase in the treated group of the growth of *Allobaculum* (SHR-c: 1.25% and SHR-o: 3.54% $p = 0.031$), *Sutterella* (SHR-c: 0.03% and SHR-o: 0.12% $p = 0.038$) and *Akkermansia* (SHR-c: 0.001% and SHR-o: 0.016% $p = 0.013$) (Fig. 5A). Moreover, AO elicited in the treated group a reduction in the growth of *Peptoniphilus* (SHR-c: 0.15% and SHR-o: 0.06% $p = 0.017$), *Blautia* ($p = 0.049$), *Oscillospira* ($p = 0.048$), and *Ruminococcus* ($p = 0.049$) (Fig. 5A).

Supplementation with AO also increased the abundance of *Akkermansia muciniphila* ($p = 0.019$), *Lactobacillus acidophilus* ($p = 0.003$), *Lactobacillus crispatus* ($p = 0.008$), *Bifidobacterium animalis* ($p = 0.014$), *Bifidobacterium thermacidophilum* ($p = 0.028$), and *Ruminococcus flavefaciens* ($p = 0.041$), but reduced the abundance of *Ruminococcus gnavus* ($p = 0.019$) (Fig. 5B).

3.6. Faecal microbiota co-occurrence networks

To evaluate interactions between bacterial taxa in the faecal microbiota of each group, we used Spearman rank correlation to create co-occurrence networks, where only significant correlations were considered (*rho* > 0.6 and $p < 0.05$). Positive correlations indicate cooperative or interdependent relationships between taxa, while negative correlations suggest a competitive relationship. The WKY-c group microbial network consists of 21 nodes (genera) and 53 edges (33 positive correlations and 20 negative correlations), where the mean number of relationships between bacterial taxa (degree) was 5.05, and the genera with the highest number of relationships were *Coprococcus* (degree 8), *Bifidobacterium*, and *Bacteroides* (degree 7). The SHR-c group network consisted of 23 nodes and 41 edges (16 positive and 25 negative correlations), where the mean number of relationships between taxa (3.56) was lower than that observed in WKY-c, and the most related genus was *Oscillospira* (degree 6), with a predominance of competitive relationships between bacterial genera. In the SHR-o group, after the supplementation with AO, the microbial network included 23 nodes and 52 edges (31 positive and 21 negative correlations), with an increase in the mean number of relationships between bacteria (4.52). Noteworthy that AO supplementation favoured the presence of *Akkermansia* within the network (degree 8) as well as the establishment of a higher number of positive correlations between taxa (Fig. 6).

4. Discussion

In a previous study, we found that the dietary supplementation with AO had anti-hypertensive effects in SHR but did not affect the BP in normotensive WKY rats.[14] Recently, the development of cardiometabolic pathologies, including hypertension has been associated with changes in the microbiota.[11,13] The mechanisms by which

gut dysbiosis affects cardiovascular homeostasis may involve an imbalance in the production of the microbiota-derived metabolites, alteration of the immune system, increased sympathetic nervous system activity, alterations in gut barrier integrity, and intestinal inflammation.[13] In addition, intestinal microbiota produces bioactive compounds that may have hypertensive or anti-hypertensive effects.[13] Therefore, we have compared the profile of the microbiota of WKY-c with that of SHR-c to identify the species, genera, or phyla that correlate with BP. Furthermore, we have analysed the changes in the microbiota composition induced by the supplementation with AO in SHR.

The microbiota of SHR-c and WKY-c rats showed similar alpha and beta diversities at 14 and 21 weeks of age in agreement with the findings of Abboud *et al*.[18] and Guo *et al*.[19] in the same rat strains. Differences in species richness and diversity in SHR take place from 25 weeks of age,[20,21] suggesting that the loss of diversity in the microbiota of hypertensive rats may occur in older animals than those used in the present study. At the phylum level, the faecal microbiota composition of WKY-c and SHR-c were similar at 14 weeks of age. However, at 21 weeks of age, the microbiota of SHR-c differs from that of age-matched WKY animals with a higher abundance of Firmicutes in SHR-c compared to WKY-c rats, a finding consistent with previous studies that described Firmicutes expansion as a characteristic of SHR gut dysbiosis.[12]

Given the differences in faecal microbiota composition observed between WKY-c and SHR-c at 21 weeks, a Spearman correlation analysis was conducted between the relative abundance of microorganisms and the measurements of BP. In the phylum Firmicutes, the genera *Sarcina*, *Alkaliphilus*, and *Peptoniphilus* as

well as the species *Lactobacillus acidophilus*, exhibited a direct association with BP since their relative abundance increased in hypertensive animals. Conversely, a lower abundance of *Phascolarctobacterium* in SHR-c than in WKY-c was inversely associated with SBP and DBP, which is consistent with results reported by Guo *et al*.[19] Similar results were found in hypertensive individuals and in patients with coronary artery disease, showing a lower abundance of *Phascolarctobacterium* than in healthy populations.[22]

Reduced abundance of the phylum Bacteroidetes has been described as a characteristic of the gut microbiota associated with hypertension.[12] Our results show that the lower relative abundance of four genera of this phylum, namely *Parabacteroides*, *Bacteroides*, *Prevotella*, and *Flavobacterium,* was correlated with hypertension. This inverse association between *Parabacteroides* and arterial hypertension confirms previous reports.[24] Moreover, the lower abundance of *Parabacteroides*, *Bacteroides,* and *Flavobacterium* was described in the gut microbiota of hypertensive rats.[12,23] Other taxa found in less abundance in SHR-c with respect to WKY-c, and inversely associated with BP, were the phylum Proteobacteria along with the genera *Desulfovibrio* and *Sutterella.* From the phylum Verrucomicrobia an inverse correlation was found for the genera *Akkermansia* and the specie *Akkermansia muciniphila.* Diverging results have been reported for *Desulfovibrio*, since its abundance was decreased in 9-month-old SHR compared to WKY controls,[23] and increased in rats transplanted with microbiota from spontaneously hypertensive stroke-prone rats[24] as well as in hypertensive individuals.[25] The inverse association of *Sutterella* with BP has also been established in SHR.[19] This genus was also found to be reduced in hypertensive

patients.[26] Furthermore, a high relative abundance of *Bifidobacterium animalis* in Wistar rats has been associated with normotension.[27] The drop in *Akkermansia* observed in SHR-c may also correlate with hypertension, as previously observed by Guo *et al*.[19] and Robles-Vera *et al.*[12] in the same strain.

Therefore, the analysis of faecal microbiota allowed the identification of taxa in SHR-c associated with high BP. Sixteen taxa were estimated to closely correlate with BP in rats, six of them showed a direct association with BP whereas ten exhibited an inverse relationship. Once the microbiota components associated with BP were identified, we conducted the second part of the study aimed at evaluating the effect of AO supplementation on SHR. We first confirmed the results of Franco-Ávila *et al.*[14] showing that AO supplementation lowers BP in SHR, with a reduction of 20 mmHg of the SBP and 19 mmHg of the DBP. The dose of AO chosen (3.85 g/kg) is equivalent to human consumption of 30-small-sized Arbequina olives which is about double the daily intake recommended by the MD pyramid.[28] The dose of AO used did not affect body weight, probably because AO has a low-calorie density (211 kcal in 100 g of edible portion) which represent approximately an additional 5% of the daily energy intake in SHR-o with respect to SHR-c.

Some components of table olives are known to affect the composition of the gut microbiota. For example, oleic acid promotes the biodiversity of intestinal bacteria[29] and stimulates the proliferation of species that produce SCFAs, which have anti-inflammatory activity and a role in the reduction of total cholesterol.[30] In addition to oleic acid, the AO that was administered in our study contained approximately 1 g of polyphenols and 3.3 g of pentacyclic triterpenes per kg of the edible part of the olive that could influence the gut microbiota composition of SHR-o. In this sense,

table olive polyphenols can act as prebiotics, inhibiting the growth of pathogenic bacteria such as *Escherichia coli*, and stimulating probiotic *Bifidobacteria*.[30] Most of the ingested polyphenols are not absorbed in the small intestine and enter the large intestine, where they promote the growth of beneficial bacteria.[31] Polyphenols can also be converted into active metabolites which may exert postbiotic effects.[31] Similar properties have been described for pentacyclic triterpenes when interacting with the intestinal microbiota.[32]

In our results, the AO supplementation reduced the abundance of *Peptoniphilus* and increased *Akkermansia (A. muciniphila)* and *Sutterella*, which are changes that have been suggested to ameliorate hypertension.[19,27,33] Concerning *Akkermansia*, this genus has been proposed as a biomarker of gut microbiota dysbiosis and some studies have reported an association between increased abundance and reduced prevalence of hypertension, obesity, and type-2 diabetes.[33] In hypertensive rat models, the supplementation with quinoa[19] or treatment with minocycline[34] lowered BP which was accompanied by a higher abundance of *Akkermansia* compared to the control animals. In addition, this genus has been demonstrated to be influenced by dietary components, such as the polyphenol quercetin that was able to increase *Akkermansia* in Wistar rats consuming a high-fat sucrose diet.[35] Sutterella was also incremented after the supplementation with wasabi thus preventing the development of hypertension in a model of obesity and metabolic syndrome in Wistar rat.[27] In accordance with our study, these authors also found an increase in the abundance of *Allobaculum*,[27] an SCFA-producing genus.[36] *Allobaculum* is also associated with a reduction of BP in SHR[19,20,27] and has been described to mediate the hypotensive effects of berberine.[37] Furthermore, *Allobaculum* was associated

with the increment of the expression of tight-junction proteins (which regulate epithelial permeability) in the large intestine[38] and negatively correlated with proinflammatory cytokines present in the blood.[39]

We also observed that AO supplementation reduced the faecal abundance of *Ruminococcus* and *Oscillospira*, both related to the production of trimethylamine, involved in the development of atherosclerosis.[40,41] The abundance of *Ruminococcus* is positively correlated with SBP[27] and atrial fibrillation.[42] On the other hand, the abundance of *Oscillospira* is positively correlated with hypertension, as shown in a Wistar rat model of obesity and metabolic syndrome[27] and in the spontaneously hypertensive heart failure rat model.[23] These results suggest that the effect of AO supplementation in reducing the abundances of *Ruminococcus* and *Oscillospira* may contribute to the prevention of cardiovascular disease.

AO promoted the growth of probiotic strains of *Lactobacillus* and *Bifidobacterium*. Its effects on increasing the abundance of *Lactobacillus acidophilus* may be relevant because, as shown in a clinical trial with elderly patients, this species was related to a reduction in BP and the restoration of plasma concentrations of total cholesterol, triglycerides, LDL, and HDL.[43] In SHR, Hidalgo *et al*.[29] showed that olive oil supplementation lowered systolic BP, an effect that correlated well with a higher abundance of *Lactobacillus* in the gut microbiota. Pentacyclic triterpenes from *Olea europaea* L. also promote the growth of *Lactobacillus*[44] therefore, an effect of these bioactive compounds cannot be excluded. In our study, the increased abundance of *Lactobacillus acidophilus* was paralleled by an increase in *Allobaculum*, as described by Mendes *et al*.[45]

AO supplementation increased the abundance of probiotic *Bifidobacterium*, an effect also observed in animals supplemented with olive oil.[46] The genus *Bifidobacterium* has also been associated with protective effects against hypertension in SHR.[27] AO increased the abundance of *Bifidobacterium animalis*, which may be relevant because it upregulates the release of anti-inflammatory cytokines in the human intestinal HT-29 cell line.[47] In addition, *Bifidobacterium animalis* can promote acetate production and regulate the Gpr43 receptor involved in BP regulation.[48]

The co-occurrence network has allowed us to study the interactions between microorganisms within the microbial community of each group, and to identify the number of relationships that a taxon establishes within the community, independently of its abundance.[49] Genera with similar abundance have been observed in WKY-c and SHR-c, although the interaction between taxons was different. In SHR-c, bacteria belonging to the phylum Firmicutes, namely, *Coprococcus, Oscillospira, Ruminococcus, Blautia, Lactobacillus*, *Allobaculum,* and *Alkaliphilus* have fewer connections with other bacteria and mainly establish competitive relationships. In addition, bacteria from the phylum Bacteroidetes associated with normal BP values, show a lower number of interactions within the SHR-c microbiota. Likewise, it was observed that *Sutterella*, also associated with a normotensive state, establishes cooperative relationships in WKY-c, while in SHR-c its role is mainly competitive. AO supplementation for 7 weeks has generated changes in the interactions of the SHR-o microbiota. An increase in the number of bacteria with significant participation within the community as well as the cooperative relationships between taxa was found. On the other hand, in addition

445 to changes in the relative abundance of some genera, AO supplementation has
446 modified the relationship of some bacteria. For example, in the SHR-o group,
447 *Lactobacillus* shows symbiotic relationships, while in SHR-c their interactions were
448 clearly competitive. In addition to increasing the abundance of *Akkermansia*, the
449 AO supplementation favoured a higher connection in the microbiota of the treated
450 animals. The co-occurrence study indicates that AO supplementation promotes
451 greater interaction between the genera associated with normal BP with the rest of
452 the microorganisms in the bacterial community.

453 Since the antihypertensive effect of olive components is associated with the
454 reduction of oxidative and inflammatory status,[50,52] as well as with the regulation of
455 the renin-angiotensin system (RAS) in SHR,[50,51] we have included in our study the
456 analysis of plasma concentrations of MDA, ANG II, IL6, and TNF-α.

457 Our results showed a higher concentration of MDA in SHR-c compared to WKY-c at
458 21 weeks of age, which is consistent with previous findings indicating that SHR develops
459 high BP concomitantly with an increase of oxidative stress markers.[50] We also observed
460 that plasma concentration of ANG II in SHR-c is 3-fold higher than that in WKY-c as
461 previously reported.[54] Moreover, the supplementation with AO elicited a decrease in the
462 plasmatic concentrations of MDA (39%) and ANG II (32%) in SHR-o compared to SHR-c.
463 These results are consistent with other studies indicating that an antihypertensive effect
464 in SHR rats is related to a decrease in oxidative stress biomarkers after the administration
465 of an extra-virgin olive oil enriched with polyphenols[51] and an oleuropein-enriched olive
466 leaf extract.[52]

467 Given the implication of the gut microbiota on blood pressure,[34] we performed a
468 correlation analysis between bacterial taxa and the concentrations of MDA and ANG II.

The relationship between bacterial taxa with MDA and ANG II showed a similar trend as that described for faecal microbiota and BP. Among the bacteria associated to MDA and ANG II, the AO supplementation reduced the relative abundance of *Blautia* and increased the relative abundance of the genera *Allobaculum* and *Sutterella*, as well as the species *Bifidobacterium animalis* and *Bifidobacterium thermacidophilum*, all inversely associated with MDA. In addition, the relative abundance of *Akkermansia* and the species *Akkermansia muciniphila*, inversely related to the concentrations of MDA and ANG II, increased in the supplemented group. It is noteworthy that an association between *Akkermansia* and the RAS system has been described previously.[53]

Among the effects of ANG II that are linked to hypertension, it is known that this hormone is able to activate the MAPK pathway through the ATR1 receptor, and initiate the signalling cascade leading to the production of proinflammatory cytokines.[52,54] However, in our study, despite observing a higher plasma ANG II concentration in SHR-c, no differences in plasmatic concentration of IL6 and TNF-α were observed in any group of rats. Similar results were reported for ANG II and inflammatory cytokines in plasma from WKY and SHR rats by Vazquez *et al*.[51]

Conclusions

In conclusion, our results show that AO supplementation has prebiotic effects, inducing a microbiota profile compatible with its reported antihypertensive activity, which is accompanied by a reduction in plasma MDA and ANG II. Dietary AO also stimulates the growth of probiotic species of the genera *Lactobacillus* and *Bifidobacterium* as well as taxa, such as *Akkermansia*, *Allobaculum,* and *Sutterella,* known for their antihypertensive and cardioprotective properties. These results

support the view that regular consumption of table olives may have beneficial health effects.

Author Contributions

Joana M. Planas: conceptualization, methodology, formal analysis, resources, writing-original draft, writing-review and editing, supervision, funding acquisition. Miquel Moretó: conceptualization, methodology, formal analysis, resources, writing-original draft, writing-review and editing. Aldo Gómez-Contreras: investigation, formal analysis, writing-original draft, writing-review and editing. M. Emília Juan: investigation, formal analysis, writing-review and editing. Lluïsa Miró: investigation, writing-review and editing. Talia Franco-Ávila: investigation, writing-review and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declared no conflict of interest.

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Data availability statement

Data for this paper, including body weight, food and water consumption, blood pressure, heart rate, malondialdehyde and 16S rRNA gene raw reads are available at the Science Data Bank at http://www.doi.org/10.57760/sciencedb.06495.

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Figure captions

Fig. 1. Differences between the faecal microbiota composition of Wistar-Kyoto (WKY-c) and spontaneously hypertensive rats (SHR-c) at 14 and 21 weeks of age. (A) Alpha diversity was evaluated with the Chao 1 Index, the Shannon Index, and the number of species. (B) Beta diversity at the genus level was assessed by ANOSIM and PERMANOVA tests adjusted by Bonferroni post-hoc analysis and visualized by principal coordinate analysis (PCoA) plot. (C) Relative abundance at the phylum level; and (D) the Firmicutes/Bacteroidetes (F/B) ratio. Results are expressed as the mean ± SEM of the relative abundance in WKY-c (n = 8) and SHR-c (n = 7). Two-way ANOVA with Bonferroni post-hoc test was used to analyse alpha diversity, relative abundance at phylum level and F/B ratio between groups. *$p < 0.05$ indicate statistically significant differences between the strains of the same age.

Fig. 2. Relative abundance at the genus level of faecal microbiota of SHR-c and WKY-c rats at 14 and 21 weeks of age. The graph depicts the phyla Firmicutes (Firm), Bacteroidetes (Bact), Actinobacteria (Actn), Proteobacteria (Prot), and Verrucomicrobia (Verr). Results are expressed as the mean ± SEM of the relative abundance in WKY-c (n = 8) and SHR-c (n = 7). Two-way ANOVA with Bonferroni post-hoc test was used for comparison between groups. *$p < 0.05$ and **$p < 0.01$ indicate statistically significant differences between the strains of the same age; #$p < 0.05$ indicate the differences due to age within the same rat strain.

Fig. 3. Heatmap of the Spearman rank correlation between the relative abundance of the bacterial faecal microbiota of SHR-c and WKY-c rats and systolic blood pressure (SBP), diastolic blood pressure (DBP), malondialdehyde (MDA) as well as angiotensin II (ANG II) at 21 weeks of age. The bacteria are grouped by phyla Firmicutes (Firm), Bacteroidetes

(Bact), Actinobacteria (Actn), Proteobacteria (Prot), and Verrucomicrobia (Verr). Colours range from red (negative correlation) to green (positive correlation). *$p < 0.05$ and **$p < 0.01$ indicate statistically significant correlations.

Fig. 4. Effect of the daily intake of Arbequina table olives (AO) during seven weeks in SHR. (A) Alpha diversity was evaluated with the Chao 1 Index, the Shannon Index, and the number of species. (B) Beta diversity at the genus level was assessed using ANOSIM and PERMANOVA tests and visualized using a principal coordinate analysis (PCoA) plot. (C) Relative abundance at the phylum level; and (D) the Firmicutes/Bacteroidetes (F/B) ratio. Data are expressed as mean ± SEM, and differences in alpha diversity, relative abundance and F/B ratio between SHR-c (n = 7) and SHR-o (n = 6) were analysed by *t*- Student or the Mann-Whitney *U* test.

Fig. 5. Effect of the daily consumption of Arbequina table olives for 49 days on (A) relative abundance at the genus level; and (B) species, in SHR faecal microbiota. Data are expressed as the mean ± SEM of the relative abundance and differences between SHR-c (n = 7) and SHR-o (n = 6) were analysed by *t*- Student or the Mann-Whitney *U* test. *$p < 0.05$ and **$p < 0.01$ indicate statistically significant differences.

Fig. 6. Co-occurrence network plots of the Spearman rank correlation among key genera. Faecal microbiota was analysed in WKY-c and SHR-c that were orally administered with water for 7 weeks as well as SHR-o supplemented by gavage with Arbequina table olives during the same experimental period. Genera are linked when the correlation is significant ($p < 0.05$). Node size indicates relative abundance.

Table 1. General characteristics of WKY and SHR animals after the supplementation of Arbequina table olives (AO) at a dose of 3.85 g/kg or water during the experiment.

	WKY-c	SHR-c	SHR-o
Body weight (g)			
14-wk-old	270 ± 7[a]	269 ± 4[a]	272 ± 6[a]
21-wk-old	347 ± 10[a]	325 ± 4[a]	323 ± 8[a]
Feed intake (g/day)			
14-wk-old	18.2 ± 0.5[a]	17.9 ± 0.2[a]	18.4 ± 0.2[a]
21-wk-old	18.0 ± 0.3[a]	17.4 ± 0.2[a]	16.8 ± 0.5[a]
Water intake (mL/day)			
14-wk-old	21.8 ± 0.7[a]	36.8 ± 2.3[b]	37.4 ± 2.3[b]
21-wk-old	21.1 ± 0.4[a]	30.3 ± 1.6[b]	29.9 ± 1.5[b]
SBP (mmHg)			
14-wk-old	147 ± 3[a]	209 ± 3[b]	209 ± 3[b]
21-wk-old	152 ± 2[a]	228 ± 4[b]	208 ± 7[c]
DBP (mmHg)			
14-wk-old	98 ± 3[a]	168 ± 4[b]	166 ± 3[b]
21-wk-old	109 ± 4[a]	177 ± 5[b]	158 ± 4[c]
HR (bpm)			
14-wk-old	395 ± 11[a]	458 ± 6[b]	458 ± 14[b]
21-wk-old	404 ± 13[a]	456 ± 8[b]	446 ± 6[b]

Results are presented as mean ± SEM in the WKY-c (n = 8), SHR-c (n = 7) and SHR-o (n = 6) groups. Data were analysed by one-way ANOVA followed by multiple comparison Bonferroni test. Means without a common letter differ, $p < 0.05$. SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate.

Table 2. Biomarkers of the development of hypertension in WKY and SHR animals after the supplementation of Arbequina table olives (AO) at a dose of 3.85 g/kg or water during the experiment.

	WKY-c	SHR-c	SHR-o
Malondialdehyde (μM)			
21-wk-old	14.7 ± 1.8[a]	19.3 ± 1.4[a]	11.8 ± 0.5[b]
Angiotensin II (pg/mL)			
21-wk-old	1277.5 ± 155.9[a]	3704.8 ± 380.4[b]	2517.0 ± 336.0[c]
IL6 (pg/mL)			
21-wk-old	3.6 ± 0.02	3.7 ± 0.02	3.8 ± 0.03
TNF-α (pg/mL)			
21-wk-old	2.1 ± 0.2	2.9 ± 0.4	2.7 ± 0.3

Results are presented as mean ± SEM in the WKY-c (n = 8), SHR-c (n = 7) and SHR-o (n = 6) groups. TNF-α, tumour necrosis factor α; IL6, interleukin 6. Data were analysed by one-way ANOVA followed by multiple comparison Bonferroni test. Means without a common letter differ, $p < 0.05$.

Figure 1

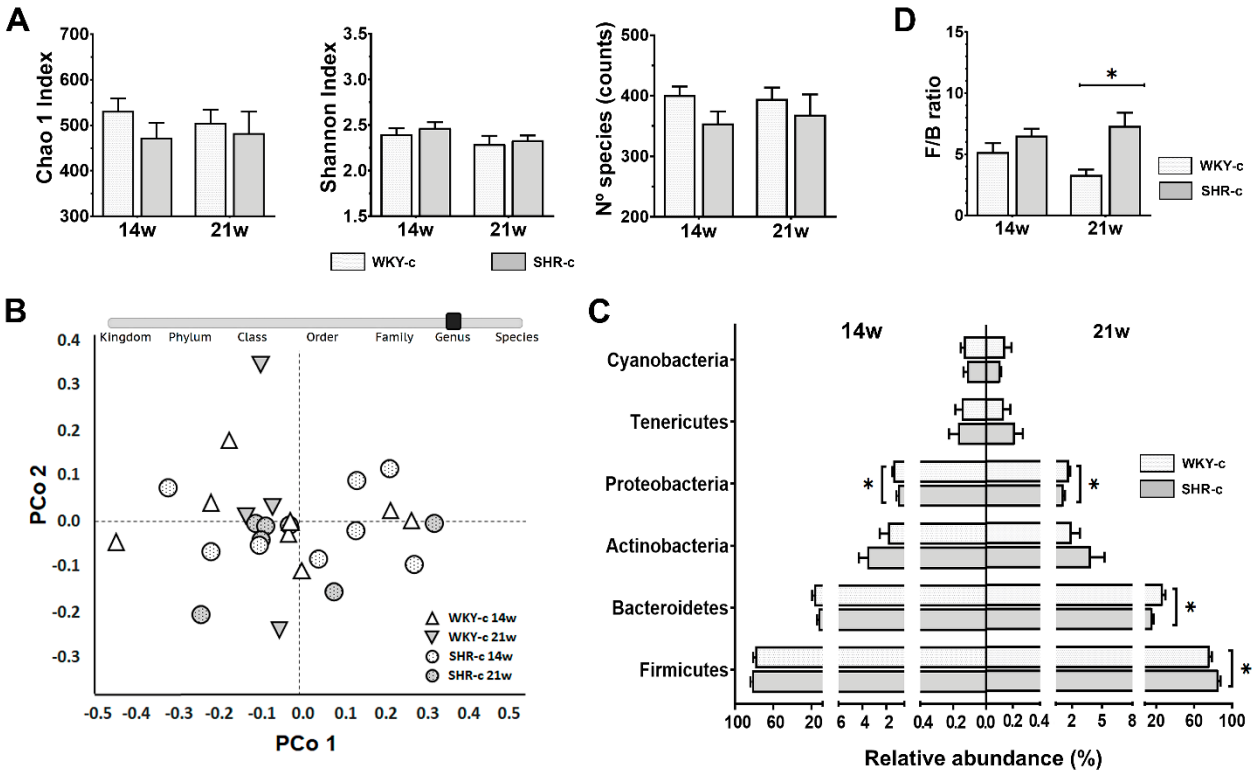

A
Chao 1 Index
Shannon Index
Nº species (counts)
14w
21w
WKY-c
SHR-c
D
F/B ratio
*
B
Kingdom
Phylum
Class
Order
Family
Genus
Species
PCo 2
PCo 1
WKY-c 14w
WKY-c 21w
SHR-c 14w
SHR-c 21w
C
Cyanobacteria
Tenericutes
Proteobacteria
Actinobacteria
Bacteroidetes
Firmicutes
Relative abundance (%)

Figure 2

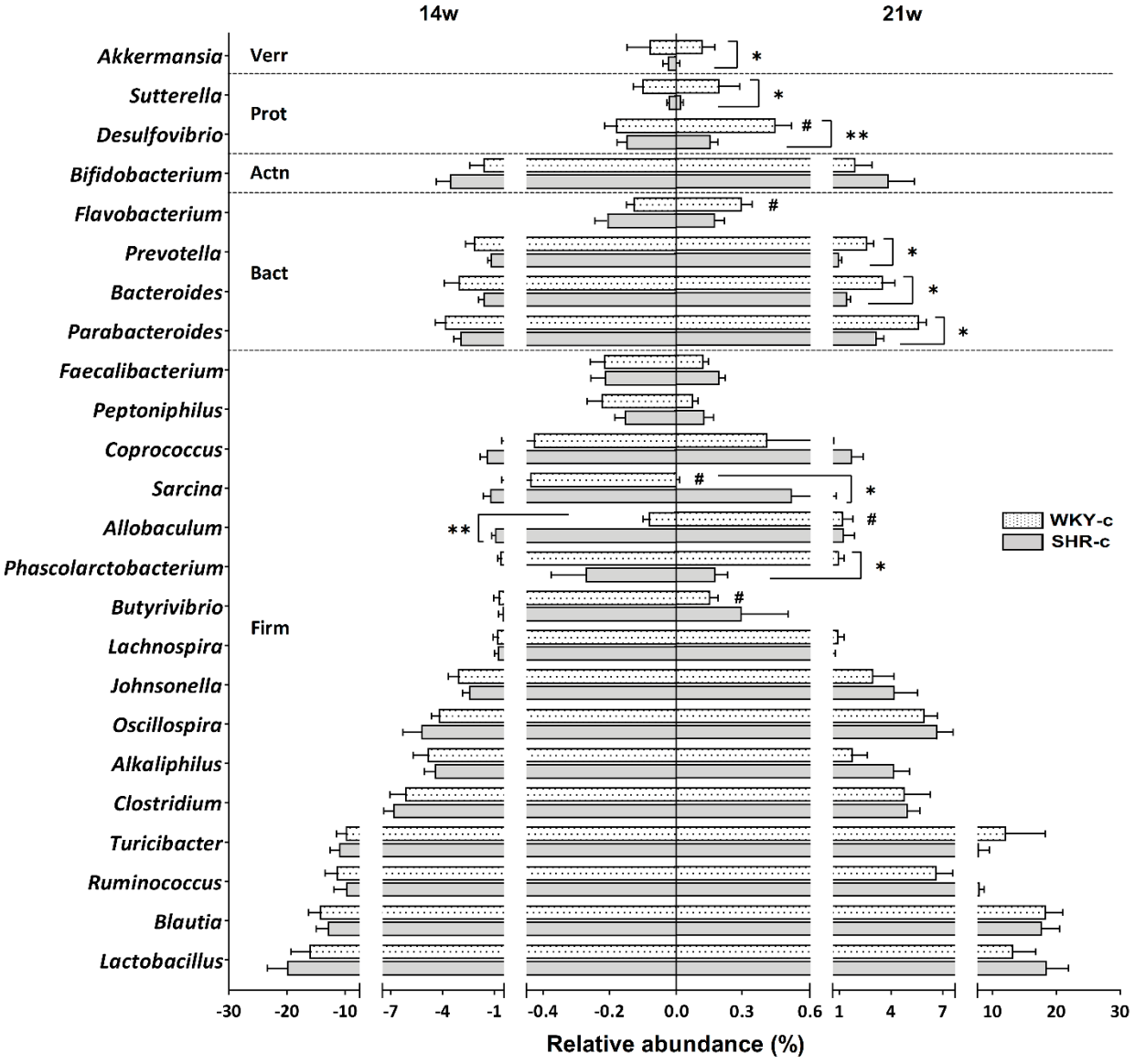

14w
21w
Akkermansia
Sutterella
Desulfovibrio
Bifidobacterium
Flavobacterium
Prevotella
Bacteroides
Parabacteroides
Faecalibacterium
Peptoniphilus
Coprococcus
Sarcina
Allobaculum
Phascolarctobacterium
Butyrivibrio
Lachnospira
Johnsonella
Oscillospira
Alkaliphilus
Clostridium
Turicibacter
Ruminococcus
Blautia
Lactobacillus
Verr
Prot
Actn
Bact
Firm
WKY-c
SHR-c
-30 -20 -10 -7 -4 -1 -0.4 -0.2 0.0 0.3 0.6 1 4 7 10 20 30
Relative abundance (%)

Figure 3

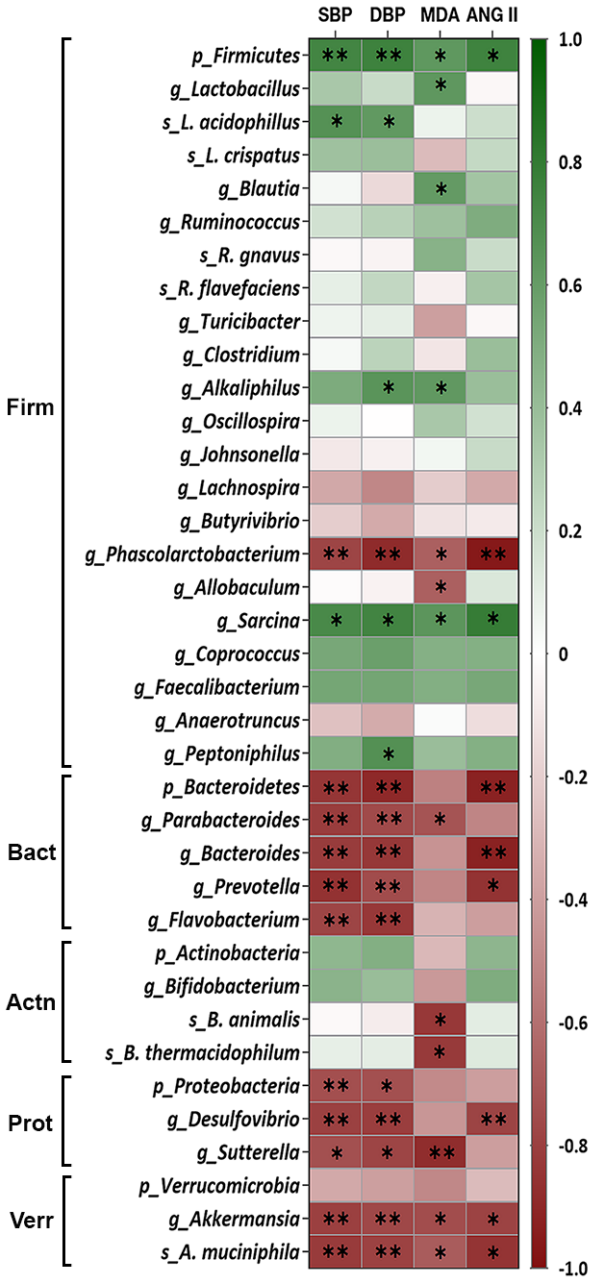

SBP
DBP
MDA
ANG II
1.0
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
-1.0
Firm
Bact
Actn
Prot
Verr
p_Firmicutes
g_Lactobacillus
s_L. acidophillus
s_L. crispatus
g_Blautia
g_Ruminococcus
s_R. gnavus
s_R. flavefaciens
g_Turicibacter
g_Clostridium
g_Alkaliphilus
g_Oscillospira
g_Johnsonella
g_Lachnospira
g_Butyrivibrio
g_Phascolarctobacterium
g_Allobaculum
g_Sarcina
g_Coprococcus
g_Faecalibacterium
g_Anaerotruncus
g_Peptoniphilus
p_Bacteroidetes
g_Parabacteroides
g_Bacteroides
g_Prevotella
g_Flavobacterium
p_Actinobacteria
g_Bifidobacterium
s_B. animalis
s_B. thermacidophilum
p_Proteobacteria
g_Desulfovibrio
g_Sutterella
p_Verrucomicrobia
g_Akkermansia
s_A. muciniphila

Figure 4

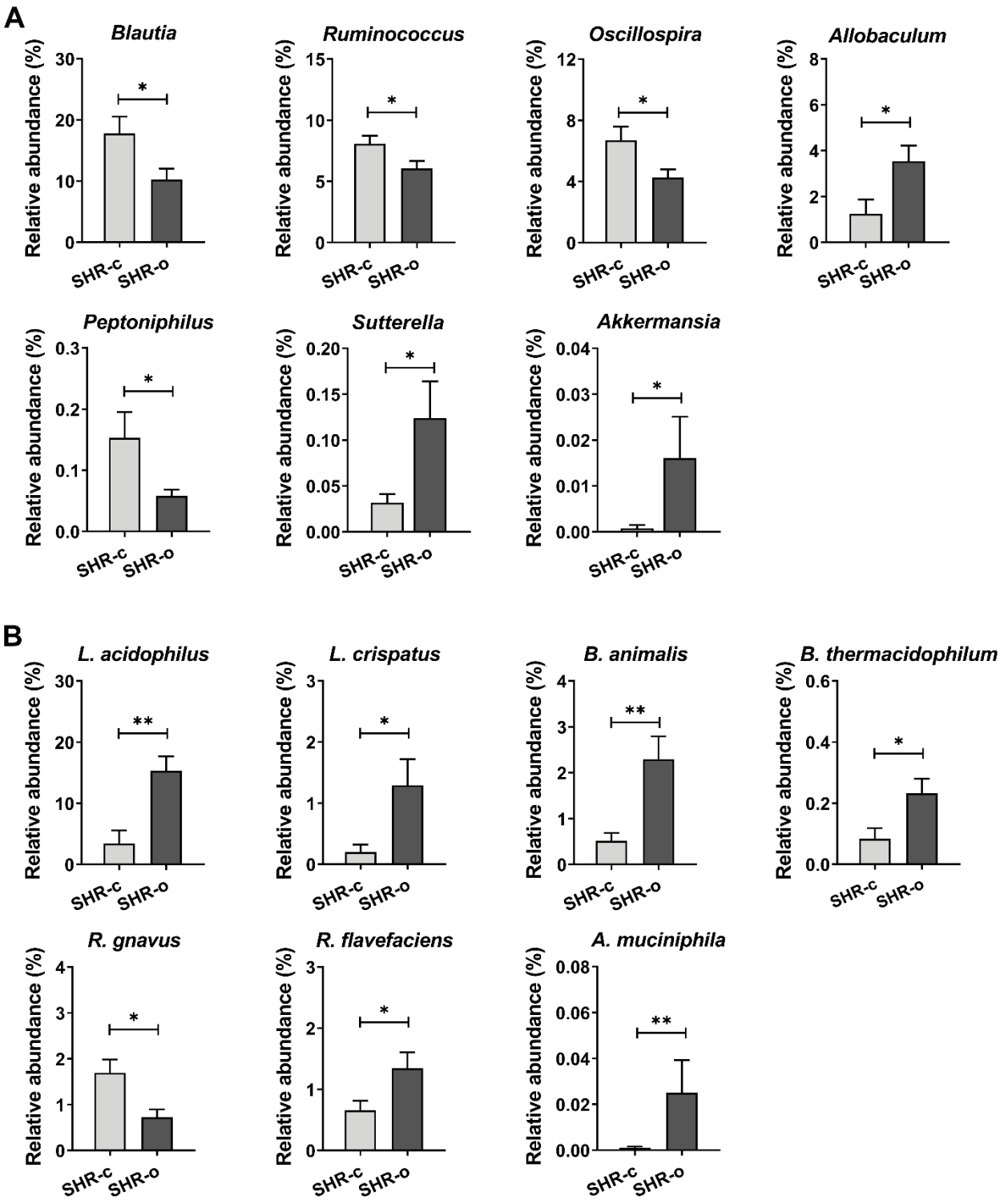
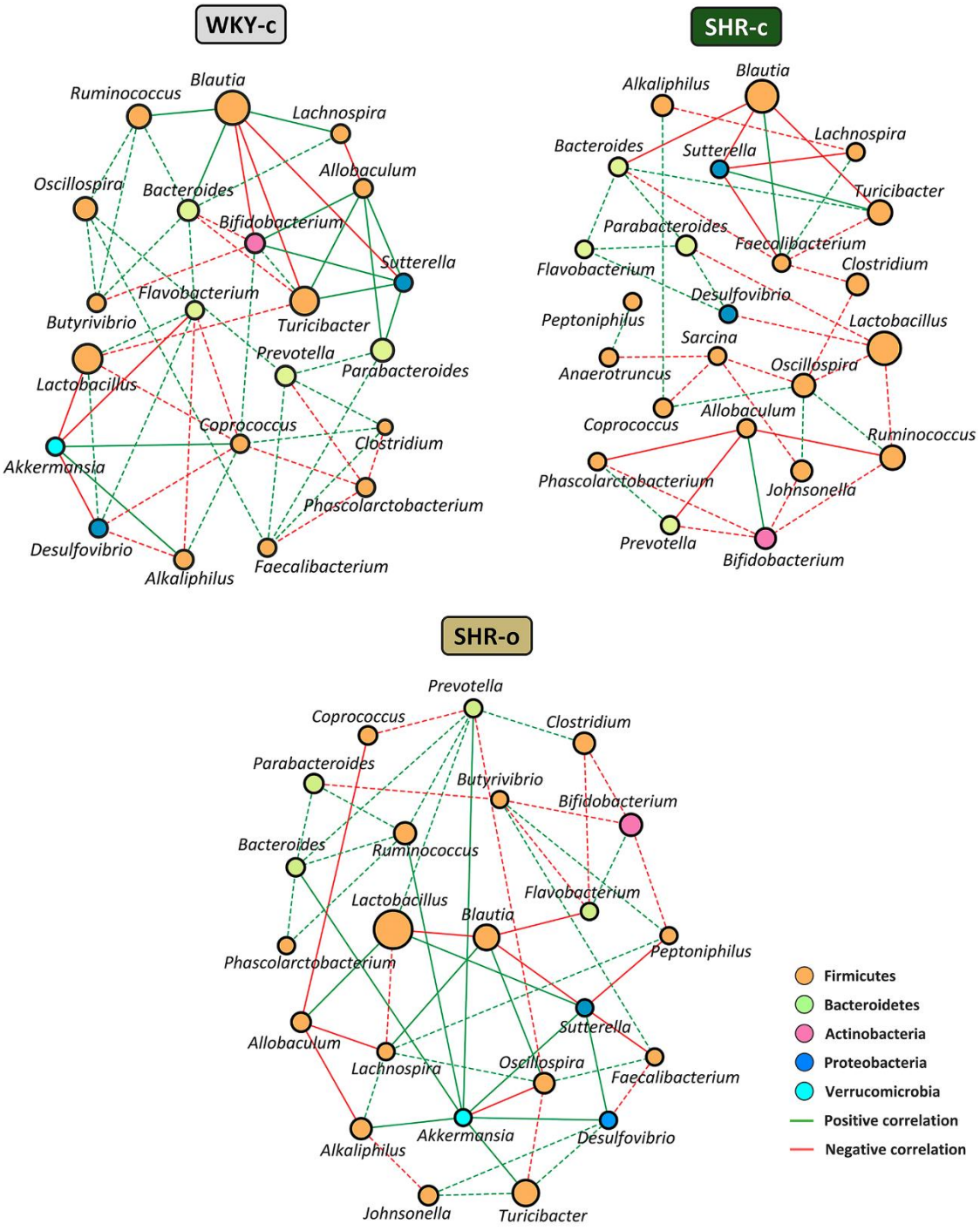

A
Blautia
Ruminococcus
Oscillospira
Allobaculum
Peptoniphilus
Sutterella
Akkermansia
Relative abundance (%)
SHR-c
SHR-o
B
L. acidophilus
L. crispatus
B. animalis
B. thermacidophilum
R. gnavus
R. flavefaciens
A. muciniphila

Figure 5


WKY-c
Ruminococcus
Blautia
Lachnospira
Oscillospira
Bacteroides
Allobaculum
Bifidobacterium
Sutterella
Flavobacterium
Butyrivibrio
Turicibacter
Prevotella
Parabacteroides
Lactobacillus
Coprococcus
Clostridium
Akkermansia
Phascolarctobacterium
Desulfovibrio
Faecalibacterium
Alkaliphilus
SHR-c
Alkaliphilus
Blautia
Lachnospira
Bacteroides
Sutterella
Turicibacter
Parabacteroides
Faecalibacterium
Clostridium
Flavobacterium
Desulfovibrio
Peptoniphilus
Lactobacillus
Sarcina
Anaerotruncus
Oscillospira
Coprococcus
Allobaculum
Ruminococcus
Phascolarctobacterium
Johnsonella
Prevotella
Bifidobacterium
SHR-o
Prevotella
Coprococcus
Clostridium
Parabacteroides
Butyrivibrio
Bifidobacterium
Bacteroides
Ruminococcus
Lactobacillus
Flavobacterium
Blautia
Peptoniphilus
Phascolarctobacterium
Sutterella
Allobaculum
Lachnospira
Oscillospira
Faecalibacterium
Akkermansia
Desulfovibrio
Alkaliphilus
Johnsonella
Turicibacter
Firmicutes
Bacteroidetes
Actinobacteria
Proteobacteria
Verrucomicrobia
Positive correlation
Negative correlation