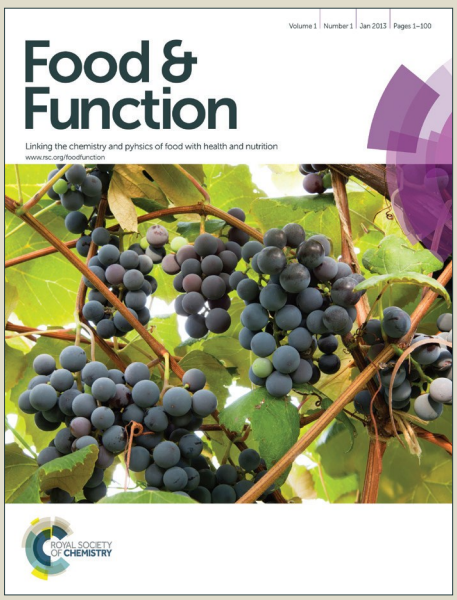
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Title

Red wine polyphenols modulate fecal microbiota and reduce markers of the metabolic syndrome in obese patients.

Authors

Moreno-Indias Isabel[1,2§], Sánchez-Alcoholado Lidia[1§], Perez-Martinez P[2,3], Andrés-Lacueva C[4,5], Cardona Fernando[1,2*], Tinahones Francisco[1,2*], Queipo-Ortuño María Isabel[1,2]

[1]Clinical Management Unit of Endocrinology and Nutrition of the Virgen de la Victoria Hospital, Biomedical Research Institute of Malaga (IBIMA), Malaga, Spain

[2]Biomedical Research Networking Center for Pathophysiology of Obesity and Nutrition, CIBERobn, Madrid, Spain

[3]Lipid and Atherosclerosis Unit, IMIBIC/Reina Sofia University Hospital/University of Cordoba, Spain.

[4]Biomarkers and Nutrimetabolomic Lab. Department of Nutrition and Food Science, XaRTA, INSA, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain

[5] INGENIO-CONSOLIDER Program, Fun-c-food CSD2007-06, Barcelona, Spain

[§] These authors contributed equally to this work.

Corresponding Author: Francisco Tinahones, Clinical Management Unit of Endocrinology and Nutrition, Virgen de la Victoria Hospital, Malaga, Spain. e-mail: fjtinahones@hotmail.com and Fernando Cardona, Biomedical Research Institute of Malaga (IBIMA), Malaga, Spain e-mail: fernandocardonadiaz@gmail.com

Abstract

This study evaluated the possible prebiotic effect of a moderate intake of red wine polyphenols on modulating the gut microbiota composition and the improvement of the risk factors for the metabolic syndrome in obese patients. Ten metabolic syndrome patients and ten healthy subjects were included in a randomized, crossover, controlled intervention study. After a washout period, the subjects consumed red wine and de-alcoholized red wine over a 30-day period for each. The dominant bacterial composition did not differ significantly between the study groups after the two red wine intake periods. In the metabolic syndrome patients, red wine polyphenols significantly increased the number of fecal bifidobacteria and *Lactobacillus* (intestinal barrier protectors) and butyrate-producing bacteria (*Faecalibacterium prausnitzii* and *Roseburia*) at the expense of less desirable groups of bacteria such as LPS producers (*Escherichia coli* and *Enterobacter cloacae*). The changes in gut microbiota in these patients could be responsible for the improvement in the metabolic syndrome markers. Modulation of the gut microbiota by red wine could be an effective strategy for managing metabolic diseases associated with obesity.

Key words: gut microbiota, obesity, metabolic syndrome, red wine polyphenols, LPS and biochemical biomarkers.

Introduction

The metabolic syndrome (MetS) is a cluster of medical conditions, including obesity and insulin resistance, that increase the risk of developing type 2 diabetes and cardiovascular diseases, which have become an important epidemic worldwide.[1] Recent studies have demonstrated that obesity and the MetS may be associated with substantial changes in the composition and metabolic function of the gut microbiota [2]. Several years ago, Vijay-Kumar *et al.,*[3] using transgenic mice, showed evidence of the direct relationship between the development of the MetS, a malfunction of the innate immune system, and changes in the composition of the gut microbiota.

The beneficial effects of prebiotic food products on energy homoeostasis, satiety regulation, body weight gain and change in the composition of the gut microbiota have recently been analyzed in studies using both animal and human models.[4] Together with data from obese animals and patients, these studies support the hypothesis that the gut microbiota composition (especially the number of bifidobacteria) may contribute to the modulation of metabolic processes associated with the MetS, specifically obesity and type 2 diabetes.[5, 6]

Several intervention studies in humans and animals have provided further evidence for the protective effects of polyphenols in the direction of modulation of vascular and platelet function, blood pressure, and an improved plasma lipid profile.[7-9] Plant polyphenols, organic compounds found in numerous plant species and their fruits, are being actively studied as potential treatments for components of the MetS.[10-12] The use of red wine polyphenols may be a potential mechanism for prevention of cardiovascular and metabolic alterations associated with obesity. Agouni et al provided strong evidence of an improvement in obesity-associated alterations, including glucose and lipid metabolism, as well as endothelial and cardiac functions due to the beneficial

effects of red wine polyphenols on both vascular and cardiac functions in a Zucker fatty rat model.[13]

The phenolic components in wine also have an effect on the microbiota. Queipo-Ortuño *et al.*[14] have recently shown that red wine consumption can significantly modulate the growth of select gut microbiota in healthy humans. The consumption of red wine polyphenols significantly increased the number of *Enterococcus*, *Prevotella*, *Bacteroides*, *Bifidobacterium*, *Bacteroides uniformis*, *Eggerthella lenta*, and the *Blautia coccoides-Eubacterium rectale* group, while the quantity of *Lactobacillus* spp. was unaltered. Resveratrol has been identified as responsible for changes in the intestinal microbiota in rats, with an increase in *Bifidobacterium* and *Lactobacillus* levels.[15] Anthocyanins have *in vitro* bacteriostatic activity against *Staphylococcus spp., Salmonella spp., Helicobacter pylori,* and *Bacillus cereus* among others.[16, 17] Catechins and epicatechins affect the growth of selected microflora, resulting in an increase in the growth of the *Blautia coccoides–Eubacterium rectale* group, *Bifidobacterium spp.* and *Escherichia coli*, as well as having an inhibitory effect on the *Clostridium histolyticum* group.[18]

A dietary modulation of the gut microbiota and its metabolic output could positively influence host metabolism and thus constitute a potential coadjuvant approach in the management of obesity and associated metabolic disorders. Thus, our aim was to examine the possible prebiotic effect of a moderate intake of red wine polyphenols on the modulation of the gut microbiota composition and the improvement in the MetS risk factors in obese patients.

Materials and Methods

Study subjects and design

The study involved 20 Caucasian adult men aged 48±2 years (range 45-50 years). Ten obese participants met the criteria for the MetS and 10 were healthy subjects (control group). The MetS patients were recruited if they fulfilled at least three of the updated criteria for the diagnosis of the MetS according to the National Cholesterol Education Program's Adult Treatment Panel III (ATP III): Waist circumference > 102 cm; HDL cholesterol < 40 mg/dL in men; serum triglycerides ≥ 150 mg/dL; fasting blood glucose 110 to 126 mg/dL and blood pressure ≥ 130/85 mmHg.

Exclusion criteria were established type 2 diabetes; body mass index (BMI) >40 kg/m2; acute or chronic infection, inflammatory disease or endocrine disorders; history of cancer; leukocytosis ($>10\times10^9$ cells/L); anti-inflammatory, corticosteroid, hormone, or antibiotic drug treatment; a history of alcohol abuse or drug dependence; and a restrictive diet or a weight change ≥5 kg during the 3 months prior to the study. None of the 20 volunteers received antibiotic therapy, prebiotics, probiotics, synbiotics, vitamin supplements or any other medical treatment influencing intestinal microbiota during the 3 months before the start of the study or during the study. A randomized, crossover controlled intervention study was performed. The study was divided into 4 periods: an initial washout period of two weeks (baseline) during which the participants did not consume any red wine, followed by two intervention periods of 30 days each during which participants drank only red wine (272 mL/day) or de-alcoholized red wine (272 mL/day) separated by a washout period of 15 days. Each participant provided 3 different fecal samples: a baseline sample after the washout period and a sample at the end of each 30-day period. Fasting blood samples and 24-h urine were also collected at baseline and after each intake period. The participants did not smoke or drink other alcoholic beverages and they were only advised to follow the same qualitative dietary recommendations according to the American Heart Association dietary guidelines [19], in

the absence of any low-in-polyphenols washout diet to mimic their normal free-living conditions as much as possible. Participants were also asked to maintain the same level of physical activity throughout the study. At baseline and after each intervention period, a medical examination and structured nutrient intake and physical activity questionnaires were completed. This information was converted into dietary data using the Professional Diet Balancer software (Cardinal Health Systems Inc, Edina, MN). The Ethics Committee of the Virgen de la Victoria Hospital approved the clinical protocol. All the participants gave written informed consent.

Anthropometric measures

Body weight, height, waist and hip circumference were measured according to standardized procedures.[20]

Laboratory measurements

Blood samples were collected after an overnight fast. The serum was separated in aliquots and immediately frozen at −80ºC. Serum biochemical parameters were measured in duplicate. Serum albumin, glucose, cholesterol, HDL cholesterol, triglycerides (Randox Laboratories Ltd., Antrium, UK), bilirubin (Dimension Vista System, Siemens, Tarrytown, NY), uric acid, C-reactive protein **(**Dimension autoanalyzer from Dade Behring Inc. Deerfield, IL, USA), gamma-glutamyl transpeptidase, glutamate-oxaloacetate transaminase, and glutamic pyruvic transaminase (Wako Bioproducts, Richmond, VA, USA) were all measured using standard enzymatic methods. Low-density lipoprotein cholesterol was calculated using the Friedewald formula. The insulin was analyzed using an immunoradiometric assay (BioSource International, Camarillo, CA, USA), showing a 0·3% cross-reaction with proinsulin. The intra- and inter-assay CV were 1·9% and 6·3%, respectively.

Resveratrol and dihydroresveratrol metabolites were analyzed in 24-h urine samples as biomarkers of red wine intake, by using the technique described by Urpi-Sarda et al. [21-23] The resveratrol metabolites were quantified by using the commercial and available standards. Dihydroresveratrol was provided by Biopharmalab SL and the concentrations of dihydroresveratrol metabolites were quantified by using a dihydroresveratrol calibration curve.[24] Similarly, ethylglucuronide was measured in 24-h urine samples by liquid chromatography (LC Agilent series 1200 coupled with a hybrid quadrupole time-offlight QSTAR Elite; Applied Biosystems/MDS Sciex).

Limulus amebocyte lysate assays

Serum concentrations of LPS were measured by endotoxin assay, based on a limulus amebocyte extract with a chromogenic limulus amebocyte lysate (LAL) assay (QCL-1000; Lonza Group Ltd). Samples were diluted in pyrogen-free water and heated to 70ºC for 10 min to inactivate endotoxin neutralizing agents that inhibit the activity of endotoxin in the LAL assay. Pyrosperse reagent (Lonza Group Ltd), which is a metallo-modified polyanionic dispersant, was added at a ratio of 1:200 (vol:vol) to test samples before LAL testing to minimize interference in the reaction. All samples were tested in duplicate, and results were accepted when the intra-assay CV was 10%. The endotoxin content was expressed as endotoxin units (EU) per milliliter. Exhaustive care was taken to avoid environmental endotoxin contamination, and all material used for both sample preparation and the test was pyrogen-free.

DNA extraction from fecal samples

Fecal samples were collected and immediately stored at −80°C until analysis. DNA extraction from 200 mg of stools was done using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA

concentration was determined by absorbance at 260 nm, and the purity was estimated by determining the A260/A280 ratio with a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA).

2.5. Analysis of fecal microbiota by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE)

Fecal samples from each subject were examined by determining PCR-DGGE profiles. The V2-V3 region of the 16S rRNA genes (positions 339 to 539 in the *Escherichia coli* gene) of bacteria in the fecal samples was amplified with the primers HDA1-GC (5′-**CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G**CC TAC GGG AGG CAG CAG T-3′; (the GC clamp is in boldface) and HDA2 (5′-GTA TTA CCG CGG CTG CTG GCA C-3′). Aliquots (2 μL) of DNA were amplified by RT-PCR (20 μL final volume) in a 7500 Fast RT-PCR Systems instrument using Fast SYBR Green Master Mix and 200 nM of each of the universal primers HDA1-GC/HDA2 with the following amplification program: initial denaturation at 95° for 20 s, amplification using 45 cycles including denaturation at 95°C for 3 s, annealing at 55°C for 30 s and extension at 72°C for 1 min.

After RT-PCR, 15 μL of products were mixed with 6 μL loading dye before loading. Electrophoresis was performed with a DCode™ Universal Mutation Detection System instrument (Bio-Rad). 6% polyacrylamide gels were prepared and electrophoresed with 1 × TAE buffer prepared from 50 × TAE buffer (2 M Tris base, 1 M glacial acetic acid, 50 mM EDTA). The denaturing gradient was formed by using two 6% acrylamide (acrylamide/bisacrylamide ratio, 37.5:1) stock solutions (Bio-Rad). The gels contained a 20-80% gradient of urea and formamide, increasing in the direction of electrophoresis. Electrophoretic runs were in a Tris-acetate-EDTA buffer (TAE 1x) (40 mmol/L Tris, 20 mmol/L acetic acid, and 1 mmol/L EDTA, pH 7.4) at

130 V and 60°C for 4.5 h. Electrophoresis was stopped when a xylene cyanol dye marker reached the bottom of the gel. Gels were stained with ethidium bromide (0.5 mg/L) for 5 min, rinsed with deionized water, viewed by UV transillumination and photographed with Gelcapture image acquisition software (DNR Bio-Imaging Systems Ltd). Similarities between banding patterns in the DGGE profile were calculated based on the presence and absence of bands and expressed as a similarity coefficient (Cs). Gels were analyzed using BioNumerics software (Applied Maths, Sint-Martens-Latem, Belgium). Normalized banding patterns were used for cluster analysis. The Dice similarity coefficient was used to calculate pairwise comparisons of the DGGE fingerprint profiles obtained. A Cs value of 100% indicates that DGGE profiles are identical while completely different profiles result in a Cs value of 0%. The UPGMA (unweighted pair group method with arithmetic mean) algorithm was used for construction of dendrograms.

Sequencing of bands from DGGE gels

Bands were excised from DGGE gels with a sterile razor, placed in 40 µL sterile water and incubated at 4°C for diffusion of DNA into the water. DNA was used in a second PCR with HDA1/2 primers without a GC clamp (initial denaturation 95° for 20 s, followed by 45 cycles including denaturation at 95°C for 3 s, annealing at 55°C for 15 s and extension at 72°C for 10 s). PCR products were diluted until 20 ng/µL, purified with ExoSAP-IT (USB corporation, Miles Road, Cleveland, Ohio, USA) and sequenced in an ABI 3130 (Applied Biosystems) using the BigDie-Kit-Standard. Nucleotide sequence data obtained were analyzed using MicroSeqID v2.1.1 software (Applied Biosystems).

Microbial quantification by real-time quantitative PCR

Specific primers targeting different bacterial genera were used to characterize the fecal microbiota by real-time quantitative PCR (Table 1).[25-38] Briefly, real-time quantitative PCR experiments were performed with a LightCycler 2.0 PCR sequence detection system using the FastStart DNA Master SYBR Green kit (Roche Diagnostics, Indianapolis, IN, USA). All PCR tests were carried out in duplicate with a final volume of 20 μL, containing 1 μL of each fecal DNA preparation and 200 nM of each primer (Table 1). The thermal cycling conditions used were as follows: an initial DNA denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, primer annealing at optimal temperature (Table 1) for 20 s, and extension at 72°C for 15 s. Finally, melt curve analysis was performed by slowly cooling the PCRs from 95 to 60°C (0.05°C per cycle) with simultaneous measurement of the SYBR Green I signal intensity. Melting-point-determination analysis allowed the confirmation of the specificity of the amplification products.

The bacterial concentration from each sample was calculated by comparing the Ct values obtained from the standard curves with the LightCycler 4.0 software. Standard curves were created using serial tenfold dilution of pure cultures of DNA, corresponding to 10^1–10^{10} copies/gram of feces. The different strains used were obtained from the Spanish Collection of Type Cultures (CECT) (*Bacteroides vulgatus* NCTC 11154, *Fusobacterium varium* NCTC 10560, *Enterococcus faecalis* CECT 184, *Enterobacter cloacae* CECT 194, *Clostridium perfringens* CECT 376) and the American Collection of Type Cultures (ACTC) (*Bifidobacterium bifidum* ATCC 15696, *Lactobacillus casei* ATCC 334D-5, *Prevotella intermedia* ATCC 25611D-5, *Clostridium histolyticum*, ATCC 19401, *Eggerthella lenta*, ATCC 25559, *Bacteroides uniformis* ATCC8492, *Ruminococus productus*, ATCC 27340D-5, *Faecalibacterium*

prausnitzii (ATCC 27768) and Leibniz-Institut DSMZ (*Roseburia intestinalis* L1-82). The data presented are the mean values of duplicate real-time PCR analyses.

Red wine composition

The red wine and de-alcoholized red wine used in this study were elaborated with the Merlot grape variety, from the Penedes appellation. The de-alcoholized red wine had the same composition and polyphenolic compounds as the red wine, except for the ethanol.[14] The phenolic profile of the red wine was determined by HPLC with diode-array detection as described previously[39] and the resveratrol and piceid contents were determined by HPLC with diode-array detection as described by Romero-Pérez *et al.*[40] The description of the daily alcohol and polyphenol consumption from the 272 mL of red wine and de-alcoholized red wine used in this study is shown in Table 2.

Statistical analysis

Results are expressed as mean values and standard deviations. The statistical analysis was performed with SPSS 15.0 software (SPSS Inc., Chicago, IL, USA). The 16S rRNA gene copy values were converted into logarithmic values before the statistical analysis. A Wilcoxon signed-rank test with a Bonferroni post hoc test was used to compare the treatments (changes in biochemical and anthropometric variables and in bacterial number) in each study group. The Mann-Whitney U test was used to compare the MetS patients with the healthy subjects at baseline and after red wine and de-alcoholized red wine intake periods. One-way analysis of variance (ANOVA) for repeated measures with the Bonferroni post hoc test was used to compare changes in the dietary analysis in response to the intervention treatments in each study group. Student's *t*-test for independent samples was used to analyze changes in dietary analysis between the MetS and the healthy subjects at baseline and after the two red wine intake periods. The Spearman correlation coefficient was calculated to estimate the linear correlations

between variables. A multivariate regression analysis was performed to identify individual bacteria as independent predictors for blood pressure, plasma lipid profile and inflammation markers in both study groups after the polyphenol interventions (red wine and de-alcoholized red wine). Statistical significance was set at a *P* value of <0.05.

Results

Anthropometric and biochemical measurements

The biochemical and anthropometric characteristics of the patients and controls are shown in Table 3. As expected, subjects in the MetS group had a significantly higher weight, waist and hip circumferences, BMI, DBP, SBP, glucose, GGT, triglycerides, total cholesterol, CRP and LPS and significantly lower HDL cholesterol than the healthy subjects at baseline. On the other hand, in the MetS group, after the red wine and de-alcoholized red wine intake periods, we observed a statistically significant decrease in the SBP, DBP, glucose, triglycerides, total cholesterol, CRP and LPS and a significant increase in the serum level of HDL cholesterol with respect to baseline. Nevertheless, the healthy subjects exhibited a significant decrease in the levels of GPT and plasma cholesterol after the red wine and de-alcoholized red wine intake periods with respect to baseline. No significant differences in the anthropometric and biochemical variables were found when comparing the red wine and de-alcoholized red wine intake periods within the MetS and the healthy groups. Finally, after the red wine and de-alcoholized red wine intake periods we only found significant differences in weight, waist and hip circumferences, BMI, GGT and triglycerides between the two study groups (Table 3).

Diet and intervention compliance

The red wine and de-alcoholized red wine were well tolerated by all the volunteers, who all completed the study, and no intolerance or adverse events were reported. No significant differences in dietary intake data were found during the study in the MetS and healthy subjects or between the two study groups at basal level and in the different intake periods (Table 4).

Resveratrol metabolites derived from phase II metabolism and dihydroresveratrol produced by intestinal microbiota were significantly increased in urine after both red wines intakes compared to basal level.

After red wine and de-alcoholized red wine intake resveratrol metabolites were significantly increased compared to baseline [4.49 µmol (95% CI: 1.36, 7.63)] and [5.03 µmol (95% CI: 2.25, 7.80)] ($P<0.001$) respectively. Total dihydroresveratrol after de-alcoholized red wine and red wine were also significantly increased compared with baseline 3.58 µmol (95% CI: 1.18, 6.17) and 4.57 µmol (95% CI: 0.42, 8.79), respectively, ($P<0.001$).

Alcoholic intake was monitored after the two treatments by urinary ethylglucuronide output. After red wine intake the urinary ethylglucuronide concentration was significantly increased compared with baseline [358% (95% CI: 146, 570%) ($P<0.05$)]. Nevertheless, no significant differences in urinary ethylglucuronide concentration were observed between de-alcoholized red wine and baseline [36% (95% CI: 25, 47%) ($P=0.638$)]. Compliance with the red wine intervention was ensured by empty bottles returned and analyzing participants' reports.

PCR-DGGE fingerprint analysis and bacterial band identification in the fecal samples.

Variations were found in the presence or absence (qualitative) and intensity (quantitative) of the bands at baseline with respect to red wine period in both the MetS

patients and the healthy subjects in the host-specific fingerprints. DGGE band profiles showed differences in band richness between the two groups. Analyzing the diversity of microbiota, we found that at baseline there was a significant difference in the mean DGGE bands between the MetS patients and the healthy subjects (11.4 ±1.3 vs. 15.9±1.4; P<0.001). However, after the red wine and de-alcoholized red wine periods the differences in band richness were not significantly different between the MetS patients and the healthy subjects (17.4±1.8 vs. 17.9±1.6, P=0.520 and 18.2±1.5 vs. 18.6±1.4, P=0.545, respectively). On the other hand, some bands were observed in fingerprints from all the periods (in a different lane but at the same position), indicating that specific species of the predominant microbiota were common to all groups.

The Dice similarity coefficient was used to calculate the similarity index of the DGGE band profiles for the two participant groups after the red wine and de-alcoholized red wine intake periods. The mean similarity index in the MetS patients was 29.6± 7.13% and. 30.1±7.42% respectively and in healthy subjects it was 27.08 ± 8.07% and 28.1±7.75% respectively, with no significant differences between the study groups during these two intervention periods (P=0.469 and P=0.563).

All the bands from all subject profiles at baseline and after the red wine and de-alcoholized red wine periods in the two study groups were cloned and sequenced to identify the dominant microbiota and the sequence similarity matches for bands were analyzed by MicroSeqID v2.1.1 software. Bacterial identification showed that the majority of the bacteria represented in our fingerprints corresponded to four phyla (Table 4). Most of the sequences belonged to *Firmicutes* and *Bacteroidetes,* with the rest distributed among *Actinobacteria* and *Proteobacteria*. Nevertheless, we also observed important differences between the MetS patients and the healthy subjects in the frequencies of different genera within these phyla at baseline. In the MetS patients at

baseline, we found an increase in the frequencies of *Bacteroides, Clostridium* and *Escherichia* accompanied by a decrease in the frequencies of *Prevotella* and the absence of *Lactobacillus* and *Bifidobacterium* with respect to healthy subjects. Finally, after red wine and de-alcoholized red wine intake we found no differences in the frequency of appearance at different taxa levels between both study groups (Table 5).

Comparative analysis of gut microbiota communities between the MetS patients and healthy subjects at baseline and after the red wine and de-alcoholized red wine intake periods.

Changes in the bacterial population abundance at the phylum and genus levels were assessed in the fecal samples of the two study groups at baseline and after each intervention period (Table 6). At baseline, a significant increase in the number of *Proteobacteria* and *Firmicutes* was found in the MetS patients with respect to the healthy subjects, while after the red wine and de-alcoholized red wine intake periods no significant differences at the phylum level were found between the study groups. Nevertheless, in the MetS subjects we observed a significant increase in the number of *Fusobacteria* and *Bacteroidetes* and a significant decrease in *Firmicutes* after the red wine and de-alcoholized red wine periods with respect to baseline. Also, in the healthy group we observed a significant increase in *Bacteroidetes* when compared baseline with both the red wine and the de-alcoholized red wine intake periods.

Within *Firmicutes*, in the MetS patients we found a significant decrease in the number of the *Clostridium* and the *Clostridium histolyticum* group accompanied by a significant increase in the quantities of the *Blautia Coccoides-Eubacterium rectale* group, *Faecalibacterium prausnitzii, Roseburia* and *Lactobacillus* after the red wine and de-alcoholized red wine intake periods compared to baseline. In the healthy group we have only observed a significant increase in the number of *Faecalibacterium prausnitzii*

and *Roseburia* through the intervention study. Moreover, the significant differences in the bacteria number of *Blautia coccoides- Eubacterium rectale group, Clostridium, Clostridium histolyticum group* and *Lactobacillus* observed at the baseline level between the study groups disappeared after the two red wine intervention periods.

Within *Bacteroidetes*, a significant decrease in the number of *Bacteroides* and a significant increase in *Prevotella* were observed in the MetS group after red wine and de-alcoholized red wine intake compared to baseline. Similarly, in the healthy group, a significant decrease in the number of *Bacteroides uniformis* was found only when compared to baseline with the red wine and de-alcoholized red wine periods. In addition, at baseline we found a significantly higher quantity of *Bacteroides* and *Parabacteroides distasonis* and a less amounts in the number of *Prevotella* in the MetS group with respect to the healthy group. Nevertheless any difference was observed after the two red wine intake period when compared the study groups.

Within *Actinobacteria* significant increases in the number of *Bifidobacterium* and *Eghertella lenta* were observed in the MetS patients and healthy subjects after the red wine and de-alcoholized red wine periods with respect to baseline. The significant difference found at basal level in this two genera when compared Mets and healthy volunteers was not found after the red wine and de-alcoholized red wine periods.

Finally, at baseline, within *Proteobacteria* we observed a significant increase in the number of *Escherichia coli* and *Enterobacter cloacae* in the MetS group compared to healthy subjects, but these significant differences disappeared after the red wine intake periods. Moreover, in the MetS group, a significant decrease in the number of *Escherichia coli* and *Enterobacter cloacae* was observed after the red wine and de-alcoholized red wine intake with respect to the baseline period. No significant

differences were found in the healthy subjects in the quantity of *Escherichia coli* and *Enterobacter cloacae* when compare baseline with the red wine intake periods.

Relationship between gut microbiota composition and blood pressure, plasma glucose level, plasma lipid profile and inflammation markers in both study groups. After the polyphenol interventions (red wine and de-alcoholized red wine), in the MetS patients we found a significant univariate correlation between changes in the amount of specific bacteria at different taxa level and plasma triglycerides, cholesterol, HDL-cholesterol, glucose and CRP (Table 7). In the healthy subjects, however, we only observed significant univariate correlations between changes in the amount of specific bacteria and HDL-cholesterol, glucose and SBP (Table 8).

In the MetS group, using a multivariate regression analysis that included all the bacterial groups analyzed, only the increase in *Actinobacteria* (P=0.005, β= 1.11, R^2=0.99) and *Lactobacillus* (P<0.001, β=0.224, R^2=0.99) and the decrease in *Clostridium histolyticum* (P=0.029, β= -0.194, R^2=0.99) and *Escherichia coli* (P=0.029, β= -0.194, R^2=0.99) predicted the triglyceride reduction. Moreover, the increase in the number of *Bifidobacterium* (P=0.001, β= 1.004, R^2=0.99) and *Faecalibacterium prausnitzii* (P=0.001, β= 1.10, R^2=0.99) were associated with the reductions in plasma cholesterol and glucose levels respectively. On the other hand, the decrease in CRP was predicted by the decrease in *Clostridium* (P=0.040, β=-0.762 R^2=0.97), and the reduction in plasma LPS levels was associated with *Bifidobacterium* growth (P=0.015, β= 0.342, R^2=0.750) and the decrease in the number of *Enterobacter cloacae* (P=0.032, β=-0.564, R^2=0.98). In the healthy group, after a multivariate regression analysis, only the decrease in *Clostridium* was associated with the decrease in SBP (P=0.001, β=-1.019, R^2=0.99).

Discussion

In the current study we have shown that the differences in the composition of fecal microbiota found between the MetS patients and the healthy subjects disappeared after a regular intake of red wine and de-alcoholized red wine polyphenols during one month. Specifically, there was a significant increase in the abundance of intestinal barrier protectors and butyrate-producing bacteria and a significant decrease in LPS producers in the MetS group after polyphenol interventions (red wine and de-alcoholized red wine). In order to analyze the fecal microbiota characteristics in MetS conditions, as well as to observe the effect of red wine polyphenols on this microbiota, we had to exclude the influence of confounding factors such as age, gender, diet and race from this study.

PCR-DGGE was used to analyze the predominant fecal bacterial populations in order to compare the bacterial diversity and similarity between the two study groups after the red wine and de-alcoholized red wine intake periods. The results revealed that the diversity and similarity of the dominant bacterial composition in the MetS patients were not significantly different from that of the healthy subjects after the two red wine interventions.

Sequence analysis of all DGGE bands obtained allowed the association of specific bacterial genotypes with the MetS or a healthy status. Previous studies have shown a dominance of *Bacteroidetes*, *Firmicutes* and *Proteobacterias* in the feces of both groups of subjects. The main differences found were at the genus-division of bacteria within these phyla at baseline between the MetS patients and the healthy subjects. We found that within *Bacteroidetes,* the *Prevotella* genus was associated with healthy subjects, while the *Bacteroides* genus was prevalent in the MetS group. Given

the concept of "enterotypes", i.e., the assignment of an individual microbiome into a given enterotype based upon the relative enrichment of that microbiome in one of three genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2) or *Ruminococcus* (enterotype 3)[41], the MetS gut microbiomes could be classified into enterotype 1 and the healthy microbiomes could be classified into enterotype 2. In addition, the apparent baseline association between the *Escherichia* genus and the MetS group, as well as the lack of *Bifidobacterium* and *Lactobacillus* genera in the same group, is noteworthy. However, after the intake of red wine and de-alcoholized red wine, the dominant microbiota genera were not significantly different in the MetS patients compared to healthy subjects, demonstrating the prebiotic capacity of the red wine polyphenols, able to change the dominant microbiota community associated with the MetS condition.

As DGGE is considered a semi-quantitative tool for monitoring the dynamics of the predominant bacterial species of fecal microbiota, an additional analysis with real-time quantitative PCR was performed to obtain a quantitative estimation of the changes found in the gut microbiota between the MetS patients and healthy subjects at baseline and after the red wine and de-alcoholized red wine intake periods. We found that at baseline the MetS patients had a significantly lower number of *Bifidobacterium, Egghertella lenta, Prevotella, Blautia Coccoides-Eubacterium rectale* group, *Lactobacillus, Faecalibacterium prausnitzii* and *Roseburia* and significantly more *Proteobacteria, Firmicutes, Escherichia coli, Enterobacter cloacae, Bacteroides, Parabacteroides distasonis, Clostridium* spp. and *Clostridium histolitycum* in their gut microbiota compared to healthy subjects. After the polyphenol interventions (red wine and de-alcoholized red wine), however, we found no significant differences in the microbiota between the two study groups.

After the red wine and de-alcoholized red wine intake periods, in the MetS patients we observed a significant decrease in *Bacteroides* and a significant increase in protectors of the gut mucosal barrier, such as *Bifidobacterium* spp and *Lactobacillus* spp, possibly due to their capacity to degrade phenolic compounds such as anthocyanin metabolites.[42] Other studies by Vendram *et al.* [43] and Hidalgo *et al.*[44] also indicated that anthocyanins seemed to increase the number of *Bifidobacterium* spp. and *Lactobacillus* spp. in the human gut microbiota of healthy subjects. Moreover, previous studies have also suggested that dietary polyphenols may help to improve the growth of certain *Lactobacillus* strains, that may mitigate the inflammation by promoting the normalization of intestinal microflora and exclusion of pathogens, decreasing intestinal permeability, improving the intestine´s immunological barrier functions and alleviating the intestinal inflammatory response.[45]

In addition, Hidalgo *et al.*[44] investigated the effect of gallic acid, which is a structural component of ellagitannins, on human gut microflora, showing a clear inhibition of the growth of potentially harmful gut bacteria of the *Clostridium histolyticum* group, which includes important pathogens closely related to the progression of colon cancer and the onset of inflammatory bowel disease. Other intervention study with cocoa flavan-3-ols in healthy volunteers has shown that they enhance the growth of *Lactobacillus* spp. and *Bifidobacterium* spp. and limit the growth of the *Clostridium histolyticum* group. [46] Similar results have been reported by us in the number of *Clostridium histolyticum* group after red wine and de-alcoholized red wine intake periods in MetS patients, suggesting that red wine polyphenols have an inhibitory effect on the growth of these bacteria. Differences in the cell surface structures could explain why Gram-positive clostridial type bacteria are more sensitive to the bactericidal effects of this compounds that are Gram-negative species. [47]

At baseline, the MetS patients weighed significantly more than their healthy controls and after red wine and de-alcoholized red wine consumption, the MetS patients showed a greater reduction in body weight than healthy controls, although this reduction was not statistically significant. The weight decrease observed in this study is probably related to the alteration in the balance between the *Bacteroidetes* and *Firmicutes* groups in favor of *Bacteroidetes*, in part possibly due to the polyphenol content of the wine. This prevalence of *Bacteroidetes* found following regular wine ingestion (red wine and de-alcoholized red wine) could be due to *Firmicutes* possessing a disproportionately smaller number of glycan-degrading enzymes than *Bacteroidetes*, this being the possible mechanism by which polyphenols may exert their effect on lowering weight.[48] Moreover, our results clearly showed that red wine and de-alcoholized red wine significantly decreased SBP and DBP in the MetS patients and these results agree with those of other studies, which have reported that polyphenols reduce elevated blood pressure.[49]

Interestingly, polyphenols and polyphenols plus ethanol significantly reduced blood glucose levels in the MetS patients compared to baseline, possible due to the shift in the gut microbiota to a higher proportion of Gram-positive relative to Gram-negative bacteria produced by the presence of polyphenols. It has been found that type 2 diabetes mellitus might be associated with the dominance of Gram-negative bacteria in the gut.[50] This study have shown that polyphenol and polyphenol plus ethanol intake positively affected the growth of the *Blautia coccoides-Eubacterium rectale* group, *Faecalibacterium prausnitzii* and *Roseburia* the most abundant intestinal butyrate producing bacteria. Previous studies have shown that butyrate induces mucin synthesis [51], decreases bacterial transport across the epithelium [52], and improves gut integrity by increasing tight junction assembly [53]. Moreover, we have found that the increase in the

abundance of *Faecalibacterium prausnitzii* was associated to the decrease in blood glucose levels in MetS patients. This association may be related to the role of the short-chain fatty acids such as butyrate formed by this gut microbiota on the regulation of the levels of gut hormones such as glucose-dependent insulinotropic polypeptide and glucagon-like peptide 1, which have important effects on carbohydrate metabolism. [54]

In addition to this regulation in insulin sensitivity, we found that the regular intake of red wine, with or without ethanol, generated significant decreases in the plasma levels of triglycerides and total cholesterol and an increase in plasma levels of HDL-cholesterol in the MetS patients during the study. In this study, the decrease observed in the plasma cholesterol concentration could be related to the significant increase in *Bifidobacterium* induced by red wine polyphenols, a bacterial genus that has the capacity to produce beneficial organic acids (lactate and acetate) and the ability to inhibit the growth of pathogenic bacteria[55], and that has been previously associated with the reduction of plasma cholesterol levels.[56, 57] Moreover, we observed a negative association between the number of *Bifidobacterium* and the plasma cholesterol levels in the MetS patients in our study. Additionally, gut microbiota can increase energy metabolism and have a systemic effect on host lipid metabolism, especially increasing triglyceride clearance.[58] On the other hand, the significant increased in the numbers of *Eggerthella lenta* (bacteria able to degrade resveratrol into dihydroresveratrol) found in both study group was related with the significant enhance of dihydroresveratrol found after de-alcoholized red wine and red wine intake with respect to baseline. Claus *et al.* showed an association between the genus Eggerthella and host metabolism and especially hepatic triglyceride levels in mice. [59]

Low grade inflammatory signaling has been suggested to be one of the mechanisms linking gut microbiota to the MetS.[60] In our study we observed that LPS

plasma concentrations were significantly increased two–fold in the MetS patients with respect to healthy subjects at baseline. This plasma endotoxin increase may derive from enhanced LPS production by gut microbiota (metabolic endotoxemia) or from increased intestinal LPS absorption.[61] But after the red wine and de-alcoholized red wine intake periods the plasma endotoxin load (LPS) was significantly reduced, accompanied by a concomitant decrease in CRP (a well-defined biomarker for low-grade inflammation), resulting in an important alleviation of the inflammatory condition. This situation may be explained by the observed decrease in the number of lipopolysaccharide producers (*Escherichia coli* and *Enterobacter cloacae*)[62] and the increase in intestinal barrier protectors such as *Bifidobacterium* spp after the polyphenol and polyphenol plus ethanol intake periods in the MetS patients compared to the baseline level. Moreover, we found a significant negative and a significant positive association between *Bifidobacterium* spp and *Enterobacter cloacae*, respectively, and the plasma level of LPS in the MetS patients. Similarly, Cani *et al.*[58] using animal models, observed an inverse correlation between the number of *Bifidobacterium* and the LPS levels after a high-fat diet. These data show that changes in gut microbiota produced by red wine and de-alcoholized red wine may cause a decrease in the release of LPS in the bloodstream of the host due to an enhancement of the intestinal barrier integrity produced by changes in the gut microbiota, improving insulin sensitivity and obesity in the MetS patients.

These study findings indicate that polyphenols or small ethanol doses plus polyphenol intake for a short time can generate an important change in the gut microbiota, which may influence the host metabolism. Moreover, we also observed that small ethanol doses did not block the polyphenols from doing their protective work.

Conclusion

The moderate intake of red wine by obese adults with the MetS resulted in positive effects on the composition of the gut microbiota and a reduction in the metabolic syndrome risk markers. Due to the dominating role of diet in shaping the composition of the gut microbiota, modulation of the gut microbiota by nutrients with prebiotic properties such as red wine could be an effective strategy for managing metabolic diseases associated with obesity.

Acknowledgements : The research group belongs to the "Centros de Investigación en Red" [CIBER, CB06/03/0018] of the "Instituto de Salud Carlos III". Isabel Moreno Indias was supported by a "Sara Borrell" postdoctoral contract (CD12/00530), María Isabel Queipo-Ortuño acknowledges support from the "Miguel Servet Type I" program (CP13/00065) and Fernando Cardona acknowledges support from the "Miguel Servet Type II" program (CP13/ 00023) from the Instituto de Salud Carlos III, Madrid, Spain, and co-founded by Fondo Europeo de Desarrollo Regional - FEDER. We are grateful to Torres S.A. for providing the red wine used in this study.

List of abbreviations: MetS (metabolic syndrome); PCR-DGGE (polymerase chain reaction-denaturing gradient gel electrophoresis); CECT (Spanish Collection of Type Cultures); ACTC (American Collection of Type Cultures), DBP (Diastolic blood pressure); SBP (Systolic blood pressure); GGT (Gamma-glutamyl transferase); GOT (Glutamic oxaloacetic transaminase); GPT (Glutamic pyruvic transaminase); CRP (C reactive protein).

Authors' contributions to the manuscript: FC, FJT and MIQO: conception and design of the research; IMI, LSA, PPM, CAL, FC and MIQO: research work; IMI, FC and MIQO: statistical analysis and data interpretation; IMI, FC, FJT and MIQO wrote the paper, and PPM, CAL, FJT and MIQO provided critical revision.

Conflict of interest statement: All authors declare the absence of any conflict of interest.

References

1. R. H. Eckel, S. M. Grundy and P. Z. Zimmet, *Lancet*, 2005, **365**, 1415-1428.

2. H. Tilg and A. Kaser, *J. Clin. Invest.*, 2011, **121**, 2126-2132.

3. M. Vijay-Kumar, J. D. Aitken, F. A. Carvalho, T. C. Cullender, S. Mwangi, S. Srinivasan, S. V. Sitaraman, R. Knight, R. E. Ley and A. T. Gewirtz, *Science*, 2010, **328**, 228-231.

4. N. M. Delzenne, A. M. Neyrinck and P. D. Cani, *Microb. Cell Fact.*, 2011, **10**, S10.

5. M. Roberfroid, G. R. Gibson, L. Hoyles, A. L. McCartney, R. Rastall, I. Rowland, D. Wolvers, B. Watzl, H. Szajewska, B. Stahl, F. Guarner, F. Respondek, K. Whelan, V. Coxam, M. J. Davicco, L. Leotoing, Y. Wittrant, N. M. Delzenne, P. D. Cani, A. M. Neyrinck and A. Meheust, *Br. J. Nutr.*, 2010, **104**, S1-63.

6. N. M. Delzenne, A. M. Neyrinck, F. Backhed and P. D. Cani, *Nat. Rev. Endocrinol.*, 2011, **7**, 639-646.

7. J. M. C. Hodgson, K. D., *J. Sci. Food Agric.*, 2006, **86**, 2492–2498.

8. F. Cardona, C. Andres-Lacueva, S. Tulipani, F. J. Tinahones and M. I. Queipo-Ortuno, *J. Nutr. Biochem.*, 2013, **24**, 1415-1422.

9. R. Puupponen-Pimia, T. Seppanen-Laakso, M. Kankainen, J. Maukonen, R. Torronen, M. Kolehmainen, T. Leppanen, E. Moilanen, L. Nohynek, A. M. Aura, K. Poutanen, F. A. Tomas-Barberan, J. C. Espin and K. M. Oksman-Caldentey, *Mol. Nutr. Food Res.*, 2013, **57**, 2258-2263.

10. E. P. Cherniack, *Nutrition*, 2011, **27**, 617-623.

11. I. Perez-Torres, A. Ruiz-Ramirez, G. Banos and M. El-Hafidi, *Cardiovasc. Hematol. Agents Med. Chem.*, 2013, **11**, 25-37.

12. N. Osakabe, *J. Clin. Biochem. Nutr.*, 2013, **52**, 186-192.

13. A. Agouni, A. H. Lagrue-Lak-Hal, H. A. Mostefai, A. Tesse, P. Mulder, P. Rouet, F. Desmoulin, C. Heymes, M. C. Martinez and R. Andriantsitohaina, *Plos One*, 2009, **4**, e5557.

14. M. I. Queipo-Ortuno, M. Boto-Ordonez, M. Murri, J. M. Gomez-Zumaquero, M. Clemente-Postigo, R. Estruch, F. Cardona Diaz, C. Andres-Lacueva and F. J. Tinahones, *Am. J. Clin. Nutr.*, 2012, **95**, 1323-1334.

15. M. Larrosa, M. J. Yanez-Gascon, M. V. Selma, A. Gonzalez-Sarrias, S. Toti, J. J. Ceron, F. Tomas-Barberan, P. Dolara and J. C. Espin, *J. Agric. Food Chem.*, 2009, **57**, 2211-2220.

16. R. Puupponen-Pimia, L. Nohynek, S. Hartmann-Schmidlin, M. Kahkonen, M. Heinonen, K. Maatta-Riihinen and K. M. Oksman-Caldentey, *J. Appl. Microbiol.*, 2005, **98**, 991-1000.

17. L. J. Nohynek, H. L. Alakomi, M. P. Kahkonen, M. Heinonen, I. M. Helander, K. M. Oksman-Caldentey and R. H. Puupponen-Pimia, *Nutr. Cancer*, 2006, **54**, 18-32.

18. X. Tzounis, J. Vulevic, G. G. Kuhnle, T. George, J. Leonczak, G. R. Gibson, C. Kwik-Uribe and J. P. Spencer, *Br. J. Nutr.*, 2008, **99**, 782-792.

19. R.H. Eckel, J.M. Jakicic, J.D. Ard, de Jesus JM, Houston Miller N, Hubbard VS, Lee IM, Lichtenstein AH, Loria CM, Millen BE, Nonas CA, Sacks FM, Smith SC Jr, Svetkey LP, Wadden TA, Yanovski SZ, Kendall KA, Morgan LC, Trisolini MG, Velasco G, Wnek J, Anderson JL, Halperin JL, Albert NM, Bozkurt B, Brindis RG, Curtis LH, DeMets D, Hochman JS, Kovacs RJ, Ohman EM, Pressler SJ, Sellke FW, Shen WK, Smith SC Jr, Tomaselli GF and American College of Cardiology/American Heart Association Task Force on Practice Guidelines, *Circulation*, 2014, **129**, S76-99

20. C.W. Callaway, The Airlie (VA) Consensus Conference-Standardization of anthropometric measurements, In: T. Loham, A. Roche, R. Martorel, ed, Champaign, IL: Human Kinetics, USA, 1988, 20-37.

21. R. Zamora-Ros, M. Urpi-Sarda, R. M. Lamuela-Raventos, R. Estruch, M. Vazquez-Agell, M. Serrano-Martinez, W. Jaeger and C. Andres-Lacueva, *Clin. Chem*., 2006, **52**, 1373-1380.

22. U.S. M. Zamora-Ros R, Lamuela-Raventós RM, Estruch R, Martínez-González MA, Bulló M, Arós F, Cherubini A, Andres-Lacueva C., *Free Radic. Biol. Med.*, 2009, **46**, 1562-1566.

23. M. Urpi-Sarda, R. Zamora-Ros, R. Lamuela-Raventos, A. Cherubini, O. Jauregui, R. de la Torre, M. I. Covas, R. Estruch, W. Jaeger and C. Andres-Lacueva, *Clin. Chem*., 2007, **53**, 292-299.

24. M.E. Juan, I. Alfaras, J.M. Planas, *J. Agric. Food. Chem.*, 2010, **58**,7472–7475.

25. X. Guo, X. Xia, R. Tang, J. Zhou, H. Zhao and K. Wang, *Lett. Appl. Microbiol.*, 2008, **47**, 367-373.

26. J. M. Delroisse, A. L. Boulvin, I. Parmentier, R. D. Dauphin, M. Vandenbol and D. Portetelle, *Microbiol. Res.*, 2008, **163**, 663-670.

27. M. K. Friswell, H. Gika, I. J. Stratford, G. Theodoridis, B. Telfer, I. D. Wilson and A. J. McBain, *PLoS One*, 2010, **5**, e8584.

28. J. E. Stach, L. A. Maldonado, A. C. Ward, M. Goodfellow and A. T. Bull, *Environ. Microbiol.*, 2003, **5**, 828-841.

29. T. Matsuki, K. Watanabe, J. Fujimoto, T. Takada and R. Tanaka, *Appl. Environ. Microbiol.*, 2004, **70**, 7220-7228.

30. A. Z. Bekele, S. Koike and Y. Kobayashi, *FEMS Microbiol. Lett.*, 2010, **305**, 49-57.

31. T. Rinttila, A. Kassinen, E. Malinen, L. Krogius and A. Palva, *J. Appl. Microbiol.*, 2004, **97**, 1166-1177.

32. S. K. Lau, P. C. Woo, G. K. Woo, A. M. Fung, M. K. Wong, K. M. Chan, D. M. Tam and K. Y. Yuen, *Diagn. Microbiol. Infect. Dis.*, 2004, **49**, 255-263.

33. C. Liu, Y. Song, M. McTeague, A. W. Vu, H. Wexler and S. M. Finegold, *FEMS Microbiol. Lett.*, 2003, **222**, 9-16.

34. J. Tong, C. Liu, P. Summanen, H. Xu and S. M. Finegold, *Anaerobe*, 2011, **17**, 64-68.

35. V. K. Sharma, E. A. Dean-Nystrom and T. A. Casey, *Mol. Cell. Probes.*, 1999, **13**, 291-302.

36. D. K. Anbazhagan, G.G.; Mansor, M.; Yan, G.O.S.; Yusof, M.Y.; Sekaran, S.D., *Afr. J. Microbiol. Res.*, **4**, 1186-1191.

37. A.N. Payne, C. Chassard, M. Zimmermann, P. Müller, S. Stinca and C. Lacroix, *Nutr. Diabetes*., 2011, **1**, e12.

38. N. Larsen, F.K. Vogensen, F.W. van den Berg, D.S. Nielsen, A.S. Andreasen, B.K. Pedersen, W.A. Al-Soud, S.J. Sørensen, L.H. Hansen, M. Jakobsen, *PLoS One*, 2010, **5**, e9085.

39. M. Ibern-Gomez, C. Andres-Lacueva, R.M. Lamuela-Raventos and A.L. Waterhouse American Journal of Enology and Viticulture *Am. J. Enol. Vitic.*, 2002, **53**, 218-221.

40. A. I. Romero-Perez, M. Ibern-Gomez, R. M. Lamuela-Raventos and M. C. de La Torre-Boronat, *J. Agric. Food Chem.*, 1999, **47**, 1533-1536.

41. M. Arumugam, J. Raes, E. Pelletier, D. Le Paslier, T. Yamada, D. R. Mende, G. R. Fernandes, J. Tap, T. Bruls, J. M. Batto, M. Bertalan, N. Borruel, F. Casellas, L. Fernandez, L. Gautier, T. Hansen, M. Hattori, T. Hayashi, M. Kleerebezem, K.

Kurokawa, M. Leclerc, F. Levenez, C. Manichanh, H. B. Nielsen, T. Nielsen, N. Pons, J. Poulain, J. Qin, T. Sicheritz-Ponten, S. Tims, D. Torrents, E. Ugarte, E. G. Zoetendal, J. Wang, F. Guarner, O. Pedersen, W. M. de Vos, S. Brunak, J. Dore, H. I. T. C. Meta, M. Antolin, F. Artiguenave, H. M. Blottiere, M. Almeida, C. Brechot, C. Cara, C. Chervaux, A. Cultrone, C. Delorme, G. Denariaz, R. Dervyn, K. U. Foerstner, C. Friss, M. van de Guchte, E. Guedon, F. Haimet, W. Huber, J. van Hylckama-Vlieg, A. Jamet, C. Juste, G. Kaci, J. Knol, O. Lakhdari, S. Layec, K. Le Roux, E. Maguin, A. Merieux, R. Melo Minardi, C. M'Rini, J. Muller, R. Oozeer, J. Parkhill, P. Renault, M. Rescigno, N. Sanchez, S. Sunagawa, A. Torrejon, K. Turner, G. Vandemeulebrouck, E. Varela, Y. Winogradsky, G. Zeller, J. Weissenbach, S. D. Ehrlich and P. Bork, *Nature*, 2011, **473**, 174-180.

42. M. Boto-Ordonez, M. Urpi-Sarda, M. I. Queipo-Ortuno, S. Tulipani, F. J. Tinahones and C. Andres-Lacueva, *Food Funct.*, 2014, **5**, 1932-1938.

43. S. Vendrame, S. Guglielmetti, P. Riso, S. Arioli, D. Klimis-Zacas and M. Porrini, *J. Agric. Food Chem.*, 2011, **59**, 12815-12820.

44. M. Hidalgo, Oruna-Concha, J., Kolida, S.,Walton, G.E., Kallithraka, S., Spencer, J.P., de Pascual-Teresa, S., *J. Agric. Food Chem.*, 2007, **60**, 3882–3890.

45. D. Hervert-Hernandez, C. Pintado, R. Rotger and I. Goni, *Int. J. Food Microbiol.*, 2009, **136**, 119-122.

46. X. Tzounis, A. Rodriguez-Mateos, J. Vulevic, G.R. Gibson, C. Kwik-Uribe and J.P. Spencer, *Am. J. Clin. Nutr*. 2011, **93**, 62–72.

47. G.R. Gibson, E.R. Beatty, X. Wang and J.H. Cummings, *Gastroenterology*, 1995, **108**, 975–82

48. R. Rastmanesh, *Chem. Biol. Interact.*, 2011, **189**, 1-8.

49. R. Lopez-Sepulveda, R. Jimenez, M. Romero, M. J. Zarzuelo, M. Sanchez, M. Gomez-Guzman, F. Vargas, F. O'Valle, A. Zarzuelo, F. Perez-Vizcaino and J. Duarte, *Hypertension*, 2008, **51**, 1088-1095.

50. P. D. Cani, Neyrinck, A. M., Fava, F., Knauf, C., Burcelin, R.G., Tuohy, K.M., Gibson, G.R., Delzenne, N.M. , *Diabetologia* 2007, **50**, 2374-2383.

51. N. Burger-van Paassen, A. Vincent, P.J. Puiman, M. van der Sluis, J. Bouma, G. Boehm, J.B. van Goudoever, I. van Seuningen and I.B. Renes, *Biochem. J.* 2009, **420**, 211–219.

52. K. Lewis, F. Lutgendorff, V. Phan, J.D. Soderholm, P.M. Sherman and D.M. McKay, *Inflamm. Bowel. Dis.* 2010, **16**, 1138–1148.

53. L.Y. Peng, Z. Li, R.S. Green, I.R. Holzman and J. Lin, *J. Nutr.* 2009, **139**, 1619–1625.

54. H.V. Lin, A. Frassetto, E.J. Jr Kowalik, A.R. Nawrocki, M.M. Lu, J.R. Kosinski, J.A. Hubert, D. Szeto, X. Yao, G. Forrest, D.J. Marsh, *PLoS One,* 2012, **7**, e35240.

55. D. M. Saulnier, S. Kolida and G. R. Gibson, *Curr. Pharm. Des.*, 2009, **15**, 1403-1414.

56. K. Tahri, J. Crociani, J. Ballongue and F. Schneider, *Lett. Appl. Microbiol.*, 1995, **21**, 149-151.

57. S. Rabot, M. Membrez, A. Bruneau, P. Gerard, T. Harach, M. Moser, F. Raymond, R. Mansourian and C. J. Chou, *FASEB J.*, 2010, **24**, 4948-4959.

58. V. R. Velagapudi, R. Hezaveh, C. S. Reigstad, P. Gopalacharyulu, L. Yetukuri, S. Islam, J. Felin, R. Perkins, J. Boren, M. Oresic and F. Backhed, *J. Lipid Res*., 2010, **51**, 1101-1112.

59. I. Martínez, D.J. Perdicaro, A.W. Brown, S. Hammons, T.J. Carden, T.P. Carr, K.M. Eskridge and J. Walter, *Appl. Environ. Microbiol.*. 2013, 79, 516-524.

60. P. D. Cani, J. Amar, M. A. Iglesias, M. Poggi, C. Knauf, D. Bastelica, A. M. Neyrinck, F. Fava, K. M. Tuohy, C. Chabo, A. Waget, E. Delmee, B. Cousin, T. Sulpice, B. Chamontin, J. Ferrieres, J. F. Tanti, G. R. Gibson, L. Casteilla, N. M. Delzenne, M. C. Alessi and R. Burcelin, *Diabetes*, 2007, **56**, 1761-1772.

61. G. Musso, R. Gambino and M. Cassader, *Diabetes Care*, 2010, **33**, 2277-2284.

62. N. Fei and L. Zhao, *ISME J.*, 2013, **7**, 880-884.

Table 1. Primers used for real-time PCR

Target group	Oligonucleotide sequence (5′-3′)	Reference
Bacteroidetes	CATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	*Guo et al., 2008* [25]
Bacteroides	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG	*Guo et al., 2008* [25]
Lactobacillus	GAGGCAGCAGTAGGGAATCTTC GGCCAGTTACTACCTCTATCCTTCTTC	*Delroisse et al., 2008* [26]
Fusobacteium	CCCTTCAGTGCCGCAGT GTCGCAGGATGTCAAGAC	*Friswell et al., 2010* [27]
Firmicutes	ATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	*Guo et al., 2008* [25]
Actinobacteria	CGCGGCCTATCAGCTTGTTG CCGTACTCCCCAGGCGGGG	*Stach et al., 2003* [28]
Bifidobacterium	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	*Matsuki et al., 2002* [29]
Prevotella	GGTTCTGAGAGGAAGGTCCCC TCCTGCACGCTACTTGGCTG	*Bekele et al., 2010* [30]
Enterococcus	CCCTTATTGTTAGTTGCCATCATT ACTCGTTCTTCCCATGT	*Rinttila et al., 2004* [31]
Proteobacteria	CATGACGTTACCCGCAGAAGAAG CTCTACGAGACTCAAGCTTGC	*Friswell et al., 2010* [27]
Clostridium Cluster IV	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	*Matsuki et al., 2004* [29]
Eghertella lenta	TGGCGAACGGGTGAGTAA AGGCCCGGGAA CGTATTCAC	*Lau et al., 2004* [32]
Blautia coccoides–Eubacterium rectale group	CGGTACCTGACTAAGAAGC AGTTTCATTCTTGCGAACG	*Rinttila et al., 2004* [31]
Clostridium histolyticum	ATGCAAGTCGAGCGA(G/T)G	*Rinttila et al., 2004* [31]

group	TATGCGGTATTAATCT(C/T)CCTTT	
Bacteroides uniformis	TCCGTTTTCCACTTATAAGA GGGTTBCCCCATTCGG	*Liu et al.*, *2003* [33]
Parabacteroides distasonis	TGCCTATCAGAGGGGGATAAC GCAAATATTCCCATGCGGGAT	*Tong et al., 2011* [34]
Escherichia coli	GACTGCAAAGACGTATGTAGATTCG ATCTATCCCTCTGACATCAACTGC	*Sharma et al., 1999* [35]
Enterobacter cloacae	CGAGAGCCTGUTGCTG GAT TGGCTGACCCAAT	*Anbazhagan et al., 2010* [36]
Faecalibacterium prausnitzii	GGAGGAAGAAGGTCTTCGG AATTCCGCCTACCTCTGCACT	*Payne et al., 2011* [37]
Roseburia	TACTGCATTGGAAACTGTCG CGGCACCGAAGAGCAAT	*Larsen et al., 2010* [38]

Table 2. Daily polyphenol and alcohol consumption from 272 mL of red wine and 272 mL of de-alcoholized red wine used in this study.

	De-alcoholized red wine	Red wine	*P**
Total phenols (meq GA)[1]	733.02 ± 23.61	797.86± 102.63	0.426
Phenolic compounds (mg)[2]			
Flavan-3-ols			
Catechin	34.39 ± 3.63	33.60 ± 3.07	0.786
Epicatechin	19.20 ± 2.24	18.46 ± 2.11	0.699
Procyanidin B1	17.50 ± 2.10	17.52 ± 1.52	0.712
Procyanidin B2	12.92 ± 1.44	12.41 ± 0.74	0.502
Procyanidin B3	7.48 ± 0.08	6.85 ± 0.08	0.526
Procyanidin B4	13.19 ± 1.35	13.33 ± 1.54	0.934
Anthocyanins			
Delphinidin-3-glucoside	4.00 ± 0.44	4.15 ± 0.24	0.589
Petunidin-3-glucoside	3.27 ± 0.31	3.34 ± 0.29	0.755
Peonidin-3-glucoside	1.82 ± 0.16	1.84 ± 0.17	0.797
Malvidin-3-glucoside	13.56 ± 1.16	13.28 ± 1.21	0.787
Malvidin-(6-acetyl)-3-glucoside	2.83 ± 0.33	2.98 ± 0.26	0.563
Malvidin-(6-coumaroyl)-3-glucoside	0.96 ± 0.09	1.13 ± 0.07	0.066
Flavonols			
Quercetin-3-glucuronide	3.06 ± 0.39	3.23 ± 0.38	0.770
Quercetin	6.48 ± 0.64	7.25 ± 0.21	0.161
Isorhamnetin	0.80 ± 0.04	0.91 ± 0.07	0.114
Hydroxycinnamic acids			
2-S-Glutathionylcaftaric	2.93 ± 0.34	2.80 ± 0.27	0.956
trans-Caftaric	5.23 ± 0.44	5.06 ± 0.39	0.595
trans-Caffeic	3.31 ± 0.25	3.13 ± 0.22	0.246
trans-Coutaric	1.53 ± 0.14	1.42 ± 0.12	0.182
Stilbenes			
trans-resveratrol	0.74 ± 0.06	0.79 ± 0.10	0.352
cis-resveratrol	0.75 ± 0.04	0.76 ± 0.04	0.761
trans-piceid	2.86 ± 0.26	2.56 ± 0.31	0.160
cis-piceid	1.93 ± 0.24	2.10 ± 0.09	0.226
Hydroxybenzoic acids			
Gallic acid	19.90 ± 1.91	18.63 ± 1.74	0.306
Protocatechuic acid	1.59 ± 0.14	1.42 ± 0.17	0.246
Tyrosols			
Tyrosol	13.01 ± 1.06	11.86 ± 1.29	0.298
Alcoholic content (g)	<1	30	

*Comparison between red wine and de-alcoholized red wine polyphenols (Student's *t* test for independent samples)

[1] Total polyphenols: expressed as mean±SD (*n*=2) mequivalents of gallic acid (meq GA).[2] Results are expressed as mean±SD (*n*=2) mg/dose except for alcoholic content.

Table 3. Anthropometric and biochemical variables during the study of MetS patients and healthy subjects.

	MetS patients			Healthy subjects		
	Baseline (washout period)	**Red wine period**	**De-alcoholized red wine period**	**Baseline (washout period)**	**Red wine period**	**De-alcoholized red wine period**
Weight (kg)	113.30 ± 16.54$^{a, *}$	110.86 ± 16.13$^{a, §}$	110.02 ± 14.86$^{a, ¥}$	82.38 ±12.37^{a}	81.88 ± 13.10^{a}	81.68 ± 11.34^{a}
Waist (cm)	116.30 ± 10.90^{a,*}	116.00 ± 9.82$^{a, §}$	115.26 ± 10.82$^{a, ¥}$	96.2 ± 4.70^{a}	95.6 ± 5.75^{a}	95.01 ± 5.32^{a}
Hip (cm)	117.70 ± 8.45$^{a, *}$	117.0 ± 9.24$^{a, §}$	116.80 ± 8.98$^{a, ¥}$	104.4 ± 7.70^{a}	102.60 ± 6.84^{a}	101.9 ± 6.98^{a}
DBP (mmHg)	94.20 ± 9.83$^{a, *}$	85.20 ± 9.15^{b}	84.0 ± 8.52^{b}	82.60 ± 8.76^{a}	81.80 ± 8.12^{a}	81.12 ± 7.98^{a}
SBP (mmHg)	134.13 ± 10.52$^{a, *}$	123.6 ± 9.23^{b}	121.06 ± 8.45^{b}	118.45 ± 9.12^{a}	116.23 ± 9.91^{a}	115.93 ± 8.58^{a}
BMI (kg/m^2)	35.24 ± 4.21$^{a, *}$	34.49 ± 4.17$^{a, §}$	34.53 ± 4.23$^{a, ¥}$	27.52 ± 2.10^{a}	27.34 ± 2.31^{a}	27.27 ± 2.19^{a}
Glucose (mg/dL)	137.00 ± 16.78^{a,*}	107.30 ± 15.12^{b}	102.8 ± 12.7^{b}	100.6 ± 8.26^{a}	99.60 ± 8.90^{a}	97.2 ± 8.14^{a}
Uric acid (mg/dL)	5.02 ± 1.26^{a}	5.01 ± 1.03^{a}	4.97 ± 1.15^{a}	5.48 ± 0.99^{a}	5.01 ± 0.53^{a}	5.10 ± 0.67^{a}
GOT (mg/dL)	21.80 ± 8.16^{a}	20.5 ± 6.45^{a}	18.27 ± 4.67^{a}	22.20 ± 7.19^{a}	18.40 ± 3.25^{a}	17.76 ± 3.84^{a}
GPT (mg/dL)	47.0 ± 10.83^{a}	42.0 ± 9.30^{a}	43.4 ± 7.40^{a,}	46.80 ± 5.28^{a}	41.5 ± 3.95^{b}	39.09 ± 3.39^{a}
GGT (mg/dL)	43.20 ± 7.29$^{a, *}$	38.20 ± 7.22$^{a, §}$	39.0 ± 7.40$^{a, ¥}$	30.60 ± 5.81^{a}	27.00 ± 4.26^{a}	26.89 ± 5.01^{a}
Triglycerides (mg/dL)	365.80 ± 24.18$^{a, *}$	257.6 ± 22.55$^{b, §}$	248.92 ± 26.35$^{b, ¥}$	125.00 ± 18.09^{a}	111.2 ± 19.33^{a}	119.6 ± 19.62^{a}
Cholesterol (mg/dL)	289.40 ± 18.03$^{a, *}$	184.66 ±13.08^{b}	179.96 ± 17.98^{b}	191.40 ±10.15^{a}	180.40 ± 9.21^{b}	178.90 ± 8.57^{b}
LDL cholesterol (mg/dL)	138.60 ± 22.64^{a}	133.80 ± 26.73^{a}	131.6 ± 25.6^{a}	120.60 ± 23.88^{a}	117.60 ± 34.51^{a}	115.0 ± 31.24^{a}
HDLcholesterol (mg/dL)	41.60 ± 9.60^{a,*}	51.0 ± 9.84^{b}	52.80 ± 8.84^{b}	66.0 ±10.8^{a}	57.80 ± 11.20^{a}	58.66 ± 8.96^{a}
CRP (mg/L)	8.20 ± 2.57$^{a, *}$	5.37 ± 2.23^{b}	5.01 ± 2.06^{b}	4.46 ± 1.84^{a}	3.80 ± 1.56^{a}	3.59 ± 1.10^{a}
LPS (EU/ml)	0.28 ± 0.05$^{a, *}$	0.11 ± 0.03^{b}	0.11 ± 0.02^{b}	0.14 ± 0.07^{a}	0.12 ± 0.03^{a}	0.11 ± 0.01^{a}

Values are presented as means ± SD. N=10 subjects per group. DBP, Diastolic blood pressure, SBP, Systolic blood pressure; GGT, Gamma-glutamyl transferase; GOT, Glutamic oxaloacetic transaminase; GPT, Glutamic pyruvic transaminase, CRP, C reactive protein. Values in a row with different superscript letters are significantly different P<0.05

***** Differences between MetS patients and healthy subjects at baseline ***P<0.05***

§Differences between MetS patients and healthy subjects after red wine intake ***P<0.05***

$^{¥}$ Differences between MetS patients and healthy subjects after de-alcoholized red wine intake ***P<0.05***

Table 4. Results of energy and dietary intakes in MetS patients and healthy subjects at baseline and after the red wine and de-alcoholized red wine intake periods.

	MetS patients				Healthy subjects			
	Baseline (washout period)	**De-alcoholized red wine intervention**	**Red wine intervention**	***P**	**Baseline (washout period)**	**De-alcoholized red wine intervention**	**Red wine intervention**	***P**
Energy (kcal/d)	2007.7 ± 340.5 [a]	1919.8 ± 376.2 [a]	1915.3 ± 332.0 [a]	0.392	1978.5± 368.0 [a]	1930.7 ± 313.1 [a]	1985.3 ± 360.2 [a]	0.772
Total protein (g/d)	71.2 ± 22.2 [a]	70.8 ± 28.4 [a]	69.5 ± 30.0 [a]	0.126	70.7 ± 27.2 [a]	69.7 ± 20.8 [a]	68.6 ±28.8 [a]	0.888
Carbohydrates (g/d)	170.8 ± 66.0 [a]	165.2 ± 64.5 [a]	163.9 ± 57.1 [a]	0.345	167.0 ± 63.2 [a]	165.2 ± 67.7 [a]	166.8 ± 70.1 [a]	0.633
Dietary fiber (g/d)	17.0 ± 7.1 [a]	17.3 ± 5.6 [a]	16.9 ± 6.5 [a]	0.786	16.7 ± 5.5 [a]	17.1 ± 6.5 [a]	16.9 ± 5.8 [a]	0.176
Sugars (g/d)	68.5 ± 26.4 [a]	67.5 ± 30.7 [a]	68.1 ± 33.0 [a]	0.567	66.5 ± 30.3 [a]	66.1 ± 34.7 [a]	65.3 ± 37.9 [a]	0.165
Total lipids (g/d)	81.5 ± 33.6 [a]	80.7± 32.1 [a]	79.9 ± 36.2 [a]	0.661	77.8 ± 37.6 [a]	76.7 ± 40.3 [a]	77.6 ± 42.8 [a]	0.942
Total polyphenols (mg/d)	409.0±97.3 [a]	390.5±89.3 [a]	381.9±92.8 [a]	0.749	391.5±86.9 [a]	393.6±98.6 [a]	387.7±77.4 [a]	0.921

Values are presented as mean±SD. N=10 subjects per group. Energy, nutrient and total polyphenol contributions from interventions were excluded.

*Changes in outcome variables in response to the intervention treatment were determined by repeated-measures 1-factor ANOVA. $P<0.05$ (Bonferroni post hoc test)

A Student's *t*-test for independent samples was used to look for the differences between groups. Different superscript letters are significantly different, ($P<0.05$).

Table 5. Bacterial identification after the sequencing of the bands from the DGGE analysis of fecal samples at baseline and after the red wine and de-alcoholized red wine intake periods in both study groups.

	MetS patients			Healthy subjects			
Bacteria genus (sequencing results of the bands)	**Baseline (washout period[a] n=77**	**Red wine period[a] n=84**	**De-alcoholized red wine period[a] n=86**	**Baseline (washout period)[a] n=80**	**Red wine period[a] n=84**	**De-alcoholized red wine period[a] n=86**	**Sequence similarity (%)**
Phylum *Bacteroidetes*							
Genus *Bacteroides*	27 (35.06%)	20 (23.80%)	20 (23.25%)	15 (18.75%)	19 (22.61%)	20 (23.25%)	99.86
Genus *Prevotella*	9 (11.68%)	19 (22.61%)	20 (23.25%)	21 (26.25%)	19 (22.61%)	19 (22.09%)	99.95
Phylum *Firmicutes*							
Genus *Clostridium*	24 (31.16%)	14 (16.66%)	14 (16.27%)	17 (21.25%)	14 (16.66%)	13 (15.11%)	99.76
Genus *Lactobacillus*	0	13 (15.47%)	14 (16.27%)	9 (11.25%)	13 (15.47%)	15 (17.44%)	97.69
Phylum *Actinobacteria*							
Genus *Bifidobacterium*	0	12(14.28%)	13 (15.11%)	10 (12.50%)	13 (15.66%)	13 (15.11%)	99.99
Phylum *Proteobacteria*							
Genus *Campylobacter*	5 (6.49%)	2 (2.38%)	1 (1.16%)	3 (3.75%)	2 (2.38%)	2 (2.32%)	99.70
Genus *Acinetobacter*	3 (3.89%)	2 (2.38%)	2 (2.32%)	2 (2.50%)	2 (2.38%)	2 (2.32%)	99.68
Genus *Escherichia*	9 (11.68%)	2 (2.38%)	2 (2.32%)	3 (3.75%)	2 (2.38%)	2 (2.32%)	99.88

[a] Refers to the frequency (and percent) of each unique bacteria genus in the baseline or red wine or de-alcoholized red wine intake periods.

"n" refers to the number of bands cloned, sequenced and identified in each study group.

N= 10 subjects per group.

Table 6. Real-time quantitative PCR of microbiota phyla, genera, groups and species in both study groups.

	MetS patients			Healthy subjects		
	Baseline (washout period)	**Red wine period**	**De-alcoholized red wine period**	**Baseline (washout period)**	**Red wine period**	**De-alcoholized red wine period**
Proteobacteria	8.84 ± 1.78 [a, *]	7.58 ± 1.08 [a]	7.64±0.75 [a]	6.83 ± 1.89 [a]	6.53 ± 2.17 [a]	6.48±2.15 [a]
Escherichia coli	9.29 ± 2.69 [a, *]	7.41 ± 1.35 [b]	7.27±1.99 [b]	7.31 ± 1.41 [a]	7.32 ±2.97 [a]	7.28±1.78 [a]
Enterobacter cloacae	8.99± 1.84 [a, *]	7.01± 1.43 [b]	6.89±1.39 [b]	6.89± 1.32 [a]	6.78±1.28 [a]	6.63±1.32 [a]
Fusobacteria	6.56 ± 1.19 [a]	7.85 ± 0.96 [b]	7.63±0.99 [b]	6.39 ± 2.08 [a]	7.82 ± 1.63 [a]	7.75±1.23 [a]
Actinobacteria	7.87 ± 3.04 [a]	8.57 ± 2.49 [a]	8.69±2.18 [a]	8.76 ± 2.77 [a]	9.53 ± 2.24 [a]	9.67±1.97 [a]
Bifidobacterium	6.37 ± 1.54 [a, *]	10.03 ± 0.77 [b]	9.73±2.07 [b]	8.54 ± 1.95 [a]	10.65 ± 2.08 [b]	10.33±1.74 [b]
Egghertella lenta	8.00 ± 0.38 [a, *]	9.92 ± 0.95 [b]	9.74±0.84 [b]	9.05± 0.86 [a]	10.02 ± 1.03 [b]	9.94±0.84 [b]
Bacteroidetes	8.95 ± 0.5 [a]	9.78 ± 0.65 [b]	9.85±0.89 [b]	8.98 ± 0.63 [a]	10.18 ± 0.49 [b]	10.33±0.54 [b]
Bacteroides	9.28 ± 0.81 [a, *]	7.64 ± 2.59 [b]	7.47±1.25 [b]	8.34 ± 0.92 [a]	7.58 ± 2.14 [a]	7.48±1.68 [a]
Bacteroides uniformis	9.71 ± 0.69 [a]	8.74±1.41 [a]	9.46±0.94 [a]	10.25 ± 0.95 [a]	8.30±1.00 [b]	9.19±1.17 [b]
Parabacteroides distasonis	9.26 ± 0.73 [a, *]	9.62 ± 0.40 [a]	10.09±1.12 [a]	7.20 ± 2.40 [a]	8.92 ± 1.40 [a]	9.32±1.98 [a]
Prevotella	6.92 ± 0.69 [a,*]	8.74 ± 0.77 [b]	8.93±0.99 [b]	8.93 ± 0.72 [a]	9.40 ± 0.81 [a]	9.36±0.78 [a]
Firmicutes	9.92 ± 0.35 [a,*]	8.42 ± 0.63 [b]	8.31±0.75 [b]	8.38 ± 0.52 [a]	8.09 ± 0.91 [a]	7.97±0.42 [a]

Blautia coccoides-Eubacterium rectale group	4. 09 ± 0.60 [a, *]	6.69 ± 0.89 [b]	6.79±0.62 [b]	6.82 ± 0.68 [a]	7.27 ± 0.65 [a]	6.99±0.34 [a]
Enteroccocus	5.71 ± 1.42 [a]	5.90 ± 0.76 [a]	5.74±1.08 [a]	4.66 ± 0.81 [a]	4.71 ± 1.15 [a]	4.75±1.38 [a]
Clostridium	5.43 ± 1.69 [a, *]	3.13 ± 0.90 [b]	3.09±0.92 [b]	3.97 ± 1.42 [a]	3.56 ± 1.52 [a]	3.47±1.03 [a]
Clostridium histolyticum group	4.08±1.07 [a, *]	2.88 ± 0.55 [b]	3.10±0.50 [b]	3.16 ± 0.92 [a]	2.50± 0.96 [a]	2.59±0.77 [a]
Lactobacillus	4.30 ±1.61 [a, *]	6.83 ± 0.56 [b]	6.63±0.87 [b]	5.78 ± 1.43 [a]	6.34 ± 1.14 [a]	6.46±1.21 [a]
Faecalibacterium prausnitzii	6.90±0.96 [a, *]	9.45±1.12 [b]	9.32±1.09 [b]	8.23±1.21 [a]	9.57±1.32 [b]	9.49±0.98 [b]
Roseburia	8.42±1.12 [a, *]	10.85±1.43 [b]	10.78±1.34 [b]	9.80±1.17 [a]	11.21±1.37 [b]	11.07±1.10 [b]

Values are presented as means ± SD and expressed as $\log_{10}$ copies per gram of feces. N = 10 participants per group. Values in a row with different superscript letters are significantly different $P<0.05$

* Differences between MetS patients and Healthy subjects at baseline $P<0.05$
§Differences between MetS patients and Healthy subjects after red wine intake $P<0.05$
¥ Differences between MetS patients and Healthy subjects after de-alcoholized red wine intake $P<0.05$

Table 7. Correlations betweens gut microbiota composition and blood pressure, glucose level, plasma lipid profile and inflammation markers in MetS patients.

	Triglycerides	Cholesterol	HDL-cholesterol	LPS	CRP	Glucose
Actinobacterias	-0.989 (0.001)					
Clostridium	0.882 (0.048)				0.882 (0.048)	
Escherichia coli	0.972 (0.006)	0.942 (0.005)		0.915 (0.029)		
Lactobacillus	-0.915 (0.030)	-0.992 (0.007)				
Bacteroidetes	-0.916 (0.029)					
Bacteroides uniformis		-0.956 (0.011)				
Bifidobacterium		-0.908 (0.033)	0.917 (0.028)	-0.906 (0.034)		
Egghertella lenta			0.901 (0.037)			
Enterobacter cloacae				0.971 (0.029)		
Faecalibacterium prausnitzii						-0.997 (0.001)
Roseburia						-0.937 (0.030)

Correlations are reported by Spearman's Rho (r) and P-values are given in parentheses.

Statistical significance was set at a *P* value of <0.05

Table 8. Correlations betweens gut microbiota composition and blood pressure, glucose level and plasma lipid profile in healthy subjects.

	HDL-cholesterol	SBP	Glucose
Proteobacteria	-0.945 (0.015)	0.912 (0.031)	
Blautia Coccoides-Eubacterium rectale group	0.946 (0.015)		
Clostridium	-0.904 (0.035)	0.982 (0.003)	
Lactobacillus		-0.908 (0.003)	
Faecalibacterium prausnitzii			-0.907 (0.032)

Correlations are reported by Spearman's Rho (r) and P-values are given in parentheses. Statistical significance was set at a *P* value of <0.05

The moderate intake of red wine by obese adults with the MetS resulted in positive effects on the composition of the gut microbiota and a reduction in the metabolic syndrome risk markers.