Influence of red wine polyphenols and ethanol on the gut microbiota ecology and biochemical biomarkers[1–4]

María Isabel Queipo-Ortuño, María Boto-Ordóñez, Mora Murri, Juan Miguel Gomez-Zumaquero, Mercedes Clemente-Postigo, Ramon Estruch, Fernando Cardona Diaz, Cristina Andrés-Lacueva, and Francisco J Tinahones

ABSTRACT

Background: Few studies have investigated the effect of dietary polyphenols on the complex human gut microbiota, and they focused mainly on single polyphenol molecules and select bacterial populations.

Objective: The objective was to evaluate the effect of a moderate intake of red wine polyphenols on select gut microbial groups implicated in host health benefits.

Design: Ten healthy male volunteers underwent a randomized, crossover, controlled intervention study. After a washout period, all of the subjects received red wine, the equivalent amount of dealcoholized red wine, or gin for 20 d each. Total fecal DNA was submitted to polymerase chain reaction (PCR)–denaturing gradient gel electrophoresis and real-time quantitative PCR to monitor and quantify changes in fecal microbiota. Several biochemical markers were measured.

Results: The dominant bacterial composition did not remain constant over the different intake periods. Compared with baseline, the daily consumption of red wine polyphenol for 4 wk significantly increased the number of *Enterococcus, Prevotella, Bacteroides*, *Bifidobacterium, Bacteroides uniformis, Eggerthella lenta, and Blautia coccoides–Eubacterium rectale* groups ($P < 0.05$). In parallel, systolic and diastolic blood pressures and triglyceride, total cholesterol, HDL cholesterol, and C-reactive protein concentrations decreased significantly ($P < 0.05$). Moreover, changes in cholesterol and C-reactive protein concentrations were linked to changes in the bifidobacteria number.

Conclusion: This study showed that red wine consumption can significantly modulate the growth of select gut microbiota in humans, which suggests possible prebiotic benefits associated with the inclusion of red wine polyphenols in the diet. This trial was registered at controlled-trials.com as ISRCTN88720134. *Am J Clin Nutr* 2012;95:1323–34.

INTRODUCTION

The human large intestine is an extremely active fermentation site and is inhabited by different bacterial species, reaching their highest concentrations in the colon (up to 10^{12} cells per gram of feces) (1). Not only does the composition of this bacterial ecosystem vary substantially among individuals, it is also dynamic and susceptible to change driven by dietary factors and diverse disease conditions. A balanced gut microbiota composition confers benefits to the host, whereas microbial imbalances are associated with metabolically mediated disorders (2). The intake of phytochemicals and their derived products exerts significant effects on the intestinal environment, modulating the gut microbiota composition and probably their functional effects in mammalian tissues (3). Polyphenols are phytochemicals abundantly present in our diet in diverse products, including tea, coffee, wine, fruit, vegetables, and chocolate (4). Polyphenol bioavailability and absorption may be influenced by its chemical structure, food matrix, and enterohepatic circulation, and a high percentage is not absorbed in the small intestine, arriving intact at the colon, where polyphenols may exert their regulatory function (5, 6). In an in vitro study using a gastrointestinal model, dietary polyphenol absorption was tested, and it was estimated that ~42% of dietary polyphenols become bioaccessible in the large intestine, where they may interact with microflora, and ~10% remained in the food matrix and were inaccessible after the whole digestion process (7). During wine consumption, polyphenolic oligomers such as procyanidins, conjugated polyphenols, esters, and phase II metabolites arrive in the colon (6, 8). Resveratrol metabolism has been extensively studied, suggesting a low bio-

[1] From the Laboratorio de Investigaciones Biomédicas del Hospital Virgen de la Victoria (Fundación IMABIS), Málaga, Spain (MIQ-O, MM, MC-P, and FCD); CIBER Fisiopatología de la Obesidad y la Nutrición (MIQ-O, MM, FCD, FJT, RE, and MB-O); the Department of Nutrition and Food Science, XaRTA, INSA, Faculty of Pharmacy, University of Barcelona, Barcelona, Spain (MB-O and CA-L); Laboratorio de Biología Molecular del Hospital Civil (Fundación IMABIS), Málaga, Spain (JMG-Z); the Department of Internal Medicine, Hospital Clínic, Institut d'Investigació Biomèdica August Pi i Sunyer, University of Barcelona, Barcelona, Spain (RE and MB-O); INGENIO-CONSOLIDER Program, Barcelona, Spain (CA-L); and Servicio Endocrinología y Nutrición del Hospital Universitario Virgen de la Victoria, Málaga, Spain (FJT).

[2] MB-O and MM contributed equally to this work.

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[4] Address reprint requests and correspondence to F Cardona Diaz, Laboratorio Investigación Biomédica 1ª Planta, Hospital Clínico Universitario Virgen de la Victoria, Campus de Teatinos s/n 29010 Málaga, Spain. E-mail: fernandocardonadiaz@gmail.com.

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availability after intestinal and liver metabolism and a fast excretion. At least 70% of the resveratrol ingested is absorbed and readily metabolized to resveratrol glucuronides and sulfates and found later in urine and plasma, where it is available for enterohepatic circulation (9–11). The polyphenols in red wine may modify the gut microbial composition by their antimicrobial properties and this, in turn, may affect their functional relations with the host (12, 13). Indeed, consumption of polyphenols has been suggested to have diverse benefits, such as improved gut health and a reduced risk of coronary artery disease (14).

Although a few intervention studies have investigated the effects of dietary polyphenols on the modulation of the human intestinal microbiota, the current study is the first to be conducted after wine consumption. Most studies addressing this topic have focused mainly on single-polyphenol molecules and selected bacterial groups (14, 15). The purpose of the current study was to evaluate the possible prebiotic effect of red wine polyphenols on the human gut microbiota by designing a randomized, crossover, controlled intervention. This aim was based on the hypothesis that the moderate intake of red wine polyphenols may modulate the gut microbiota, influencing the growth of specific bacterial groups capable of producing host health benefits.

SUBJECTS AND METHODS

Study subjects and design

The study involved 10 healthy adult men aged 48 ± 2 y (range: 45–50 y). The participants were not receiving treatment for diabetes, hypertension, or dyslipidemia, nor did they have any acute or chronic inflammatory diseases, infectious diseases, viral infections, cancer, or a previous cardiovascular event at study entry. They had not received any antibiotic therapy, prebiotics, probiotics, symbiotics, or vitamin supplements or any other medical treatment influencing intestinal microbiota during the 3 mo before the start of the study or during the study (including the washout period). A randomized, crossover, controlled intervention study was performed. The study was divided into 4 consecutive periods: an initial washout period of 15 d (baseline) during which the participants didnot consume any alcohol or red wine, followed by 3 consecutive periods of 20 d during which the participants drank only de-alcoholized red wine (272 mL/d), red wine (272 mL/d), or gin (100 mL/d). Each participant provided 4 different fecal samples: a first baseline sample after the washout period and a sample at the end of each 20-d period. Fasting blood samples and 24-h urine were also collected at baseline and after each period. The participants were asked not to change their dietary pattern and lifestyle habits during the study. The subjects were asked to avoid alcoholic beverages during the study. At baseline and after each intervention period, a medical examination and a nutrient intake and physical activity–structured questionnaires were made. This information was converted into dietary data by using the Professional Diet Balancer software (Cardinal Health Systems Inc). 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, and waist and hip circumferences were measured according to standardized procedures (16).

Laboratory measurements

Blood samples were collected after an overnight fast. The serum was separated in aliquots that were immediately frozen at −80°C. Serum biochemical measures were measured in duplicate. Serum albumin, glucose, cholesterol, HDL cholesterol, triglycerides (Randox Laboratories Ltd), bilirubin (Dimension Vista System), uric acid, C-reactive protein (Dimension autoanalyzer; Dade Behring Inc), γ-glutamyl transpeptidase, glutamate-oxaloacetate transaminase, and glutamic pyruvic transaminase (Wako Bioproducts) were all measured by using standard enzymatic methods. LDL cholesterol was calculated by using the Friedewald formula. Insulin was analyzed by using an immunoradiometric assay (BioSource International), showing a 0.3% cross-reaction with proinsulin. The intra- and interassay CVs were 1.9% and 6.3%, respectively.

In the 24-h urine samples, resveratrol metabolites were measured as a biomarker of consumption of de-alcoholized red wine and red wine intervention compliance (17, 18), jointly with dihydroresveratrol metabolites (9, 11), by using the technique described by Urpi-Sarda et al (19). The resveratrol metabolites were quantified by using the commercial and available standards; *trans*- and *cis*-resveratrol-3-*O*-glucuronide (98% purity each), *cis*-resveratrol-4′-*O*-glucuronide (96% purity), and *trans*-resveratrol-3-*O*-sulfate (98% purity) were purchased from Toronto Research Chemicals Inc. The *trans*-resveratrol-3-*O*-sulfate calibration curve was used to quantify *trans*-resveratrol-4′-*O*-sulfate, *cis*-resveratrol-4′-*O*-sulfate, and *cis*-resveratrol-3-*O*-sulfate. Dihydroresveratrol was provided by Biopharmalab SL. The concentrations of dihydroresveratrol metabolites were quantified by using the dihydroresveratrol calibration curve (11, 20).

Similarly, ethylglucuronide was measured in 24-h urine samples, as a biomarker of alcohol intake, by liquid chromatography (LC Agilent series 1200 coupled with a hybrid quadrupole time-of-flight QSTAR Elite; Applied Biosystems/MDS Sciex).

DNA extraction from fecal samples

Fecal samples were collected and immediately stored at −80°C until analyzed. DNA was extracted from 200 mg stool by using the QIAamp DNA stool Mini kit (Qiagen) 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).

Analysis of fecal microbiota by polymerase chain reaction–denaturing gradient gel electrophoresis

Fecal samples from each subject were examined by determining polymerase chain reaction (PCR)[5]–denaturing gradient gel electrophoresis (DGGE) profiles. The V2-V3 region of the 16S rRNA genes (positions 339–539 in the *Escherichia coli* gene) of bacteria in the fecal samples was amplified with the

[5]Abbreviations used: ATCC, American Type Culture Collection; BP, blood pressure; CECT, Spanish Type Culture Collection; CRP, C-reactive protein; Cs, similarity coefficient; DBP, diastolic blood pressure; DGGE, denaturing gradient gel electrophoresis; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; SBP, systolic blood pressure.

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′) generating a 200-bp product. Aliquots (2 μL) of DNA were amplified by real-time quantitative PCR (qPCR) (20 μL final volume) in a 7500 Fast RT-PCR Systems instrument by using Fast SYBR Green Master Mix and 200 nmol/L of each of the universal primers HDA1-GC/HDA2 with the following amplification program: initial denaturation at 95° for 20 s, amplification by 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 real-time qPCR, 15 μL of the products was 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 mol Tris base/L, 1 mol glacial acetic acid/L, and 50 mmol EDTA/L). 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 1×) (40 mmol Tris/L, 20 mmol acetic acid/L, and 1 mmol EDTA /L; 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 ultraviolet transillumination, and photographed with Gelcapture image acquisition software (DNR Bio-Imaging Systems Ltd). Samples were obtained from each subject after each period and analyzed on the same DGGE gel to avoid the possible influence of variations in electrophoretic conditions between different gels. Similarities between banding patterns in the DGGE profile were calculated based on the presence and absence of bands and expressed as a Cs (similarity coefficient). Gels were analyzed by using BioNumerics software (Applied Maths). 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 the DGGE profiles are identical, whereas completely different profiles result in a Cs value of 0%. The unweighted pair group method with arithmetic mean algorithm was used for construction of dendrograms.

Sequencing of selected bands from DGGE gels

Bands of specific interest 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 gas chromatograph 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), and sequenced in an ABI 3130 (Applied Biosystems) by using the BigDie-Kit-Standard. The nucleotide sequence data obtained were analyzed by using MicroSeqID v2.1.1 software (Applied Biosystems).

Microbial quantification by real-time qPCR

Specific primers targeting different bacterial genera were used to characterize the fecal microbiota by real-time qPCR (21–29) (**Table 1**). Briefly, quantitative PCR experiments were performed with a LightCycler 2.0 PCR sequence detection system by using the FastStart DNA Master SYBR Green kit (Roche Diagnostics). All PCR tests were carried out in duplicate with a final volume of 20 μL, containing 100 ng of each fecal DNA preparation and 200 nmol/L 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°C 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 by using a serial 10-fold dilution of DNA from pure cultures, corresponding to 10^1–10^{10} copies/g feces. The different strains used were obtained from the Spanish Type Culture Collection (CECT) (*Bacteroides vulgatus* National Collection of Type Cultures 11154, *Fusobacterium varium* National Collection of Type Cultures 10560, *Enterococcus faecalis* CECT 184, *Enterobacter cloacae* CECT 194, and *Clostridium perfringens* CECT 376) and the American Type Culture Collection (ATCC) (*Bifidobacterium bifidum* ATCC 15696, *Lactobacillus casei* ATCC 334D-5, *Prevotella intermedia* ATCC 25611D-5, *Clostridium histolyticum* ATCC 19401, *Eggerthella lenta* ATCC 25559, *Bacteroides uniformis* ATCC 8492, and *Ruminococus productus* ATCC 27340D-5). The data presented are the mean values of duplicate real-time qPCR analyses.

De-alcoholized red wine, red wine, and gin composition

De-alcoholized red wine and red wine used in this study were produced with the Merlot grape variety, from the Penedès appellation. The de-alcoholized red wine had the same composition and polyphenolic compounds as the red wine, except for ethanol (only 0.42%). The phenolic profile of de-alcoholized red wine and red wine was determined by HPLC with diode-array detection as described previously (30), and the resveratrol and piceid contents were determined by HPLC with diode-array detection as described by Romero-Pérez et al (31). The total phenolic content of several distilled alcoholic beverages was determined with the Folin-Ciocalteu method (32), and the alcoholic beverage selected was gin (38% alcohol) because the amount of phenols was not detectable. A description of the daily phenolic and alcoholic doses during treatment periods from both the wines and gin is included in **Table 2**. No significant differences in the phenolic content were identified between de-alcoholized red wine and regular red wine.

Statistical analysis

The results are expressed as means ± SDs. The statistical analysis was performed with SPSS 15.0 software (SPSS Inc).

TABLE 1
Primers used for real-time polymerase chain reaction

Target group	Oligonucleotide sequence (5′-3′)	Reference	Amplicon size
			bp
Bacteroidetes	CATGTGGTTTAATTCGATGAT AGCTGACGACAACCATGCAG	Guo et al, 2008 (21)	126
Bacteroides	GAGAGGAAGGTCCCCCAC CGCTACTTGGCTGGTTCAG	Guo et al, 2008 (21)	106
Lactobacillus	GAGGCAGCAGTAGGGAATCTTC GGCCAGTTACTACCTCTATCCTTCTTC	Delroisse et al, 2008 (22)	126
Fusobacterium	CCCTTCAGTGCCGCAGT GTCGCAGGATGTCAAGAC	Friswell et al, 2010 (23)	273
Firmicutes	ATGTGGTTTAATTCGAAGCA AGCTGACGACAACCATGCAC	Guo et al, 2008 (21)	126
Actinobacteria	CGCGGCCTATCAGCTTGTTG CCGTACTCCCCAGGCGGGG	Stach et al, 2003 (24)	600
Bifidobacterium	CTCCTGGAAACGGGTGG GGTGTTCTTCCCGATATCTACA	Matsuki et al, 2002 (25)	550
Prevotella	GGTTCTGAGAGGAAGGTCCCC TCCTGCACGCTACTTGGCTG	Bekele et al, 2010 (26)	121
Enterococcus	CCCTTATTGTTAGTTGCCATCATT ACTCGTTCTTCCCATGT	Rinttilä et al 2004 (27)	144
Proteobacteria	CATGACGTTACCCGCAGAAGAAG CTCTACGAGACTCAAGCTTGC	Friswell et al, 2010 (23)	195
Clostridium cluster IV	GCACAAGCAGTGGAGT CTTCCTCCGTTTTGTCAA	Matsuki et al, 2004 (25)	239
Eggerthella lenta	TGGCGAACGGGTGAGTAA AGGCCCGGGAA CGTATTCAC	Lau et al, 2004 (28)	1221
Blautia coccoides–Eubacterium rectale group	CGGTACCTGACTAAGAAGC AGTTTCATTCTTGCGAACG	Rinttilä et al, 2004 (27)	429
Clostridium histolyticum group	ATGCAAGTCGAGCGA(G/T)G TATGCGGTATTAATCT(C/T)CCTTT	Rinttilä et al, 2004 (27)	120
Bacteroides uniformis	TCCGTTTTCCACTTATAAGA GGGTTBCCCCATTCGG	Liu et al, 2003 (29)	350

The bacterial copy numbers were converted into logarithm values before the statistical analysis. The Friedman test was used to check changes in bacterial number, biochemical variables, and compliance between the intervention treatment. Wilcoxon's signed-rank tests with a Bonferroni post hoc test was used to compare the treatments. One-factor 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. Within and between the intervention periods, the differences are expressed as means and 95% CIs. Univariate correlations were calculated by using Pearson's *r*. A multivariate regression analysis was performed to identify individual bacteria as independent predictors for blood pressure (BP), lipid markers, and C-reactive protein (CRP). Statistical significance was set at a P value <0.05. All data are presented as means ± SDs.

RESULTS

Diet and intervention compliance

The drinks were well tolerated by all of the volunteers, all of whom completed the study, and no intolerance or adverse events were reported. Throughout the study, the participants maintained their usual diet and physical activity, taking care that both remained as stable as possible over the 4 periods. No significant differences in dietary intake data were observed during the study (**Table 3**).

Alcoholic intake was monitored after the 3 treatment periods by urinary ethylglucuronide output. After the red wine and gin intakes, urinary ethylglucuronide concentrations were significantly increased compared with baseline [358% (95% CI: 146, 570%) and 342% (95% CI: 159, 525%), respectively ($P < 0.05$)] and the de-alcoholized red wine period [606% (95% CI: 328, 885%) and 625% (95% CI: 319, 931), respectively ($P < 0.05$)]. No significant differences were observed between the de-alcoholized red wine and washout periods [36% (95% CI: 25, 47%; $P = 0.638$)] or between the red wine and gin periods [24% (95% CI: 2, 46%; $P = 1.000$)]. Compliance with the 3 interventions was ensured by counting the empty bottles returned and by analyzing the participants' reports. No carryover effect was observed in the study.

Resveratrol absorption and metabolism

Resveratrol metabolites were analyzed in 24-h urine samples as biomarkers of red wine intake (17–19). Resveratrol metabolites derived from phase II metabolism were significantly increased in urine after both red wines as compared with baseline and after gin intake. Dihydroresveratrol, produced by intestinal microbiota, also had a significantly higher concentration (9, 12).

The resveratrol metabolite concentrations were significantly higher after the de-alcoholized red wine and the red wine periods

TABLE 2
Daily polyphenol and alcohol consumption from 272 mL red wine, 272 mL de-alcoholized red wine, and 100 mL gin used in this study[1]

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

[1] GA, gallic acid; ND, not detected.
[2] Reflects the comparison between red wine and de-alcoholized red wine polyphenols (Student's *t* test for independent samples).
[3] Mean ± SD (all such values; $n = 2$).

[5.03 μmol (95% CI: 2.25, 7.80) and 4.49 μmol (95% CI: 1.36, 7.63), respectively] than at baseline [0.21 μmol (95% CI: 0.08, 0.33)] and after gin consumption [0.17 μmol (95% CI: −0.01, 0.35)] ($P < 0.001$). The resveratrol metabolite concentrations did not change significantly after the gin period compared with baseline ($P > 0.05$). Total dihydroresveratrol concentrations after de-alcoholized red wine and red wine were 3.70 μmol (95% CI: 1.21, 6.20) and 4.64 μmol (95% CI: 0.38, 8.91), respectively, which were significantly increased compared with baseline (0.13 μmol; 95% CI: 0.05, 0.20) and gin (0.17 μmol; 95% CI: −0.02, 0.35) ($P < 0.001$). The results are expressed in **Figure 1**.

Anthropometric and biochemical measurements

The anthropometric and biochemical variables of the participants during the study are shown in **Table 4**. A significant decrease was observed in diastolic BP (DBP; mm Hg) after the red wine period with respect to the washout period, whereas the systolic BP (SBP; mm Hg) decreased significantly after both the red wine and the de-alcoholized red wine periods. The highest decrease in DBP and SBP was nevertheless observed after the red wine period (97.40 ± 15.21 compared with 86.50 ± 11.60 and 145.40 ± 23.86 compared with 129.50 ± 17.60; $P < 0.05$). The effect of the 3 interventions on BMI and plasma concentrations of glucose, glutamic pyruvic transaminase, and LDL cholesterol did not differ significantly. However, a significant decrease was seen in the concentrations of glutamate-oxaloacetate transaminase, γ-glutamyl transpeptidase, triglycerides, HDL cholesterol, and CRP after both the de-alcoholized red wine and the red wine periods compared with the washout period. Finally, the uric acid and total cholesterol concentrations decreased significantly from baseline only after the red wine period.

PCR-DGGE and bacterial band identification

The generated host-specific fingerprints showed interindividual variation in the composition of the fecal microbiota, showing

TABLE 3
Result of energy and dietary intakes in the 10 subjects studied at baseline and after the 3 treatments[1]

	Baseline (washout period)	De-alcoholized red wine intervention	Red wine intervention	Gin intervention	*P*
Energy (kcal/d)	1721.3 (1421.0, 2294.0)	1813.7 (1515.6, 2165.6)	1792.3 (1535.0, 2111.8)	1849.1 (1602.6, 2173.4)	0.052
Total protein (g/d)	75.7 (64.8, 94.2)	85.7 (70.8, 103.6)	83.5 (69.8, 100.0)	91.9 (77.2, 111.8)	0.228
Carbohydrates (g/d)	167.0 (136.0,227.0)	151.2 (124.5, 182.7)	151.8 (126.1, 180.7)	155.8 (129.1, 187.1)	0.892
Dietary fiber (g/d)	16 (14.1, 17.9)	17.1 (14.6, 19.7)	16.9 (14.5, 19.5)	16.9 (13.8, 19.9)	0.834
Sugars (g/d)	69.5 (56.4, 82.7)	66.1 (30.7, 101.5)	70.1 (37.9, 102.3)	78.4 (37.9, 118.9)	0.527
Total lipids (g)	79.8 (63.6, 108)	91.7 (72.7, 111.1)	90.6 (78.8, 103.6)	91.5 (76.6, 105.7)	0.084
SFA (g/d)	24.0 (19.4, 32.1)	25.8 (20.4, 31.65)	26.0 (22.7, 29.2)	29.6 (23.5, 35.6)	0.164
MUFA (g/d)	40.8 (30.9, 57.0)	47.2 (36.5, 58.0)	46.9 (39.3, 54.7)	43.9 (35.3, 53.1)	0.176
PUFA (g/d)	11.6 (10.1, 13.0)	12.2 (9.8, 14.7)	11.8 (9.8, 13.9)	12.9 (8.4, 17.5)	0.598
Cholesterol (mg/d)	329.6 (307.4, 351.9)	351.0 (275.9, 426.1)	313.8 (261.9, 365.7)	350.3 (280.1, 420.5)	0.567
Vitamin C (mg/d)	82.3 (67.1, 97.5)	70.9 (49.7, 92.3)	72.2 (32.9, 111.4)	67.5 (50.6, 84.4)	0.564
Vitamin A (μg RE/d)	690.3 (574.3, 806.3)	798.6 (456.8, 1140.4)	679.3 (550.9, 807.6)	805.1 (449.5, 1160.7)	0.661
Vitamin E (mg/d)	5.5 (4.6, 6.6)	5.5 (3.9, 7.4)	6.2 (4.9, 7.7)	5.6 (3.9, 7.5)	0.816
Folic acid (μg/d)	237.9 (204.7, 271.3)	229.1 (181.1, 277.1)	209.3 (175.0, 243.6)	222.3 (159.3, 285.3)	0.633
Total polyphenols (mg/d)	425.0 (250.0, 651.0)	372.6 (189.4, 592.0)	369.5 (203.1, 584.2)	379.6 (225.1, 538.2)	0.942

[1] All values are means; 95% CIs in parentheses. $n = 10$ subjects. Energy, nutrient, and total polyphenol contributions from interventions were excluded. No changes were observed between baseline and interventions determined, $P > 0.05$ (repeated-measures 1-factor ANOVA with post hoc Bonferroni test). RE, retinol equivalent.

a high heterogeneity in electrophoretic patterns between the different subjects and between the different intake periods. Variations were found in the presence or absence (qualitative) and intensity (quantitative) of the bands.

DGGE band profiles (mean of bands: 16.8) were stable for each subject at baseline. Nevertheless, differences in band richness were found between the de-alcoholized red wine, the red wine, and the gin periods (de-alcoholized red wine, mean of bands: 17.4; red wine, mean of bands: 18.2; gin, mean of bands: 14.6). On the other hand, some bands were seen in fingerprints from all the participants, indicating that specific species of the predominant microbiota were common to the participants.

The Dice similarity coefficient was used to calculate the similarity coefficient between DGGE band profiles in the 4 different sampling periods for each participant. The median similarity coefficient values were 34.68%, 26.42%, and 28.33% for de-alcoholized red wine, red wine, and gin, respectively. These data showed that the dominant bacterial composition did not remain constant over the different intervention periods with respect to baseline. Cluster analysis of DGGE profiling confirmed that banding patterns were not closely related for the participants, revealing grouping according to the intake period. The DGGE gel and the results of the cluster analysis are shown in **Figure 2**.

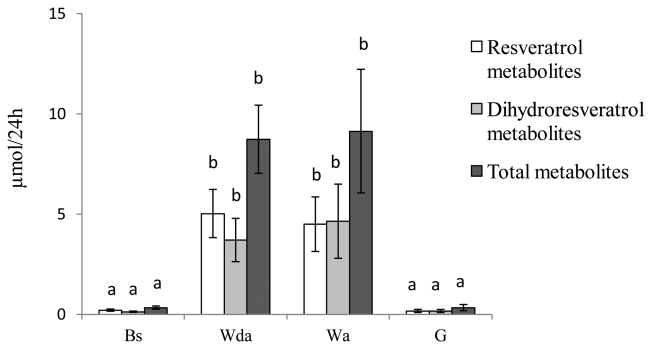

FIGURE 1. Mean (±SEM) resveratrol metabolites, dihydroresveratrol metabolites, and total metabolites (resveratrol and dihydroresveratrol) quantified in 24-h urine samples from 10 volunteers at Bs, Wda, Wa, and G. The changes between the intervention treatments were analyzed by using the Friedman test. Wilcoxon's signed-rank test was used to compare the treatments with one another. Values in a box of the same shade of gray with different lowercase letters are significantly different, $P < 0.05$ (Bonferroni post hoc test). Bs, baseline sample; G, sample after gin consumption; Wa, sample after red wine consumption; Wda, sample after de-alcoholized red wine consumption.

Selected bands from all volunteer profiles at baseline and in the different intake periods were sequenced and the sequence homology matches for bands were analyzed by MicroSeqID v2.1.1 software. Bacterial identification showed that most of the bacteria represented in our fingerprints corresponded to 5 phyla. Most of the sequences belonged to *Firmicutes* and *Bacteroidetes*, with the rest distributed among *Proteobacteria, Actinobacteria*, and *Fusobacteria* (**Table 5**). Nevertheless, we also observed important differences between baseline and the different intake periods and between polyphenol intake periods (de-alcoholized red wine and red wine) and the gin period in the distribution ratio of different genera within the *Bacteroidetes* and *Firmicutes* phyla. At baseline, the *Bacteroides* and *Prevotellaceae* frequencies were lower than those after polyphenol intake periods, whereas the *Clostridium* frequency was similar. After the de-alcoholized red wine and the red wine periods, the *Bacteroides, Clostridium*, and *Prevotellaceae* frequencies were similar; however, an increase in the *Bacteroides* and *Clostridium* frequencies and a disappearance of *Prevotellaceae* after the gin period were observed (Table 5).

Effect of wine polyphenol intake and ethanol on human fecal microbiota

Changes in the bacterial population content were assessed in the fecal samples of all volunteers at baseline and after each intervention period. The results obtained in the real-time qPCR experiments with the different primers are shown in **Tables 6** and **7.** Relevant differences were found in the bacteria number of 4 phyla after the different intake periods. *Proteobacteria, Fusobacteria, Firmicutes*, and *Bacteroidetes* changed significantly over the study. Only the *Actinobacteria* phyla did not change

TABLE 4
Anthropometric and biochemical variables during the study[1]

	Baseline (washout period)	De-alcoholized red wine period	Red wine period	Gin period	P^2
Weight (kg)	97.8 ± 21.3	97.8 ± 19.4	96.4 ± 20.6	97.2 ± 19.6	0.306
Waist (cm)	106.7 ± 14.3	106.5 ± 14.4	105.1 ± 14.5	105.7 ± 13.5	0.392
Hip (cm)	111.0 ± 10.4	109.0 ± 12.8	110.2 ± 11.1	110.8 ± 10.3	0.908
DBP (mm Hg)	97.4 ± 15.2[a]	91.0 ± 12.9[a]	86.5 ± 11.6[b]	98.4 ± 14.3[a]	0.026
SBP (mm Hg)	145.4 ± 23.9[a]	135.1 ± 24.6[b]	129.5 ± 17.6[b]	142.7 ± 22.3[a]	0.026
BMI (kg/m^2)	27.6 ± 3.2	27.6 ± 3.1	27.5 ± 2.9	27.6 ± 2.8	0.241
Glucose (mg/dL)	111.3 ± 23.1	104.5 ± 24.2	108.5 ± 16.4	108.8 ± 17.2	0.772
Uric acid (mg/dL)	5.7 ± 1.1[a]	5.3 ± 1.0[a]	5.0 ± 0.8[b]	5.4 ± 1.5[a]	0.018
GOT (mg/dL)	22.0 ± 7.3[a]	14.3 ± 4.0[b]	17.6 ± 13.4[b]	19.1 ± 8.0[a]	0.021
GPT (mg/dL)	46.4 ± 12.6	41.2 ± 7.7	42.0 ± 9.3	43.1 ± 6.9	0.888
GGT (mg/dL)	36.9 ± 25.6[a]	30.1 ± 13.5[b]	36.1 ± 16.3[b]	38.0 ± 27.7[a]	0.012
Triglycerides (mg/dL)	245.4 ± 231.7[a]	171.7 ± 206.7[b]	179.4 ± 177.1[b]	190.1 ± 222.5[b]	0.001
Cholesterol (mg/dL)	257.5 ± 88.6[a]	241.2 ± 94.9[a]	188.6 ± 61.6[b]	235.3 ± 91.4[a]	0.008
LDL cholesterol (mg/dL)	129.6 ± 41.9	123.5 ± 28.1	125.7 ± 30.3	130.6 ± 22.0	0.266
HDL cholesterol (mg/dL)	58.5 ± 16.7[a]	48.8 ± 17.1[b]	49.7 ± 14.3[b]	52.3 ± 16.5[a]	0.001
CRP (mg/L)	6.9 ± 2.6[a]	4.3 ± 2.3[b]	4.6 ± 2.5[b]	6.8 ± 3.7[a]	0.001

[1] All values are means ± SDs; $n = 10$ subjects. Means in a row with different superscript letters are significantly different, $P < 0.05$ (Wilcoxon's signed-rank test with post hoc Bonferroni test). CRP, C-reactive protein; DBP, diastolic blood pressure, GGT, γ-glutamyl transferase; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; SBP, systolic blood pressure.
[2] Derived by using the Friedman test.

significantly after any of the periods. After the red wine period, the bacterial concentrations of *Proteobacteria, Fusobacteria, Firmicutes* and *Bacteroidetes* were significantly increased compared with the washout period. On the other hand, the de-alcoholized red wine significantly increased the *Fusobacteria* concentration with respect to baseline. The concentrations of *Bacteroidetes* and *Firmicutes* decreased significantly after the de-alcoholized red wine period compared with the red wine period (Table 6).

Moreover, a significant change in the number of *Enterococcus*, *Clostridium*, *Clostridium histolyticum*, *Blautia coccoides–Eubacterium rectale*, *Bacteroides*, *Bacteroides uniformis*, *Prevotella*, Bifidobacterium, and *Eggerthella lenta* were found during the study. Within Firmicutes, the genus *Enterococcus* and the *Blautia coccoides–Eubacterium rectale* group increased significantly after consumption of de-alcoholized red wine and red wine compared with baseline. *Lactobacillus* concentrations remained unchanged throughout the study ($P > 0.05$), whereas *Clostridium* and the *Clostridium histolyticum* group increased significantly after gin consumption. Within Bacteroidetes, the genus *Bacteroides* and the *B. uniformis* species increased significantly after red wine intake, whereas the number of *Prevotella* decreased significantly after gin intake and increased significantly after red wine intake, both with respect to baseline. Finally, within the Actinobacteria phyla, we found that interventions with red wine and de-alcoholized red wine led to a significant increase in the number of Bifidobacterium and *Eggerthella lenta* compared with baseline (Table 7).

Bacterial changes predicted modifications in lipid markers, CRP, and BP

In addition to the reduction after polyphenol interventions (de-alcoholized red wine and red wine) in BP and concentrations of triglycerides, cholesterol, HDL cholesterol, and CRP with respect to baseline (Table 4), we also found after the same periods a significant univariate correlation between changes in the amount of specific bacteria and DBP (*Bacteroides*: $r = -0.406$, $P = 0.026$), SBP (*Enterococcus*: $r = -0.362$, $P = 0.049$; *Bacteroides*: $r = -0.362$, $P = 0.049$), plasma triglycerides (*Bacteroides*: $r = -0.364$, $P = 0.048$), cholesterol (*Bifidobacterium*: $r = -0.401$, $P = 0.028$; *Bacteroides*: $r = -0.363$, $P = 0.049$), HDL cholesterol (*Lactobacillus:* $r = -0.447$, $P = 0.013$; *Bacteroides*: $r = -0.469$, $P = 0.009$), and CRP (*Bifidobacteria:* $r = -0.430$, $P = 0.018$; *Lactobacillus*: $r = -0.405$, $P = 0.027$). With the use of a multivariate regression analysis that included all the bacterial groups analyzed, only the increment of *Bacteroides* predicted the DBP ($P = 0.48$, $R^2 = 0.364$) and SBP ($P = 0.03$, $R^2 = 0.369$) reductions and triglyceride ($P = 0.048$, $R^2 = 0.364$) and HDL cholesterol ($P = 0.001$, $R^2 = 0.732$) reductions. On the other hand, *Bifidobacterium* growth predicted the decrease in cholesterol ($P = 0.012$, $R^2 = 0.583$) and CRP ($P = 0.018$, $R^2 = 0.430$). This suggested that these bacterial groups could be partly implicated in the observed reduction in BP, plasma lipid markers, and CRP.

DISCUSSION

In this study we observed that red wine, de-alcoholized red wine, and gin produced significant changes in the fecal microbiota of all the participants. The DGGE analysis of the predominant fecal microbiota generated complex and relatively stable and unique profiles for each study subject. DGGE analysis showed low similarity index values between the different intake periods, with clustering of banding patterns characteristic for each intake period. These results indicate that changes in the gut microbiota composition of healthy subjects occur after a dietary intervention, which disagrees with previous studies regarding the subject specificity of the predominant fecal communities and their stability over time and resistance to perturbations (33, 34). Because DGGE is considered a semiquantitative tool for monitoring the dynamics

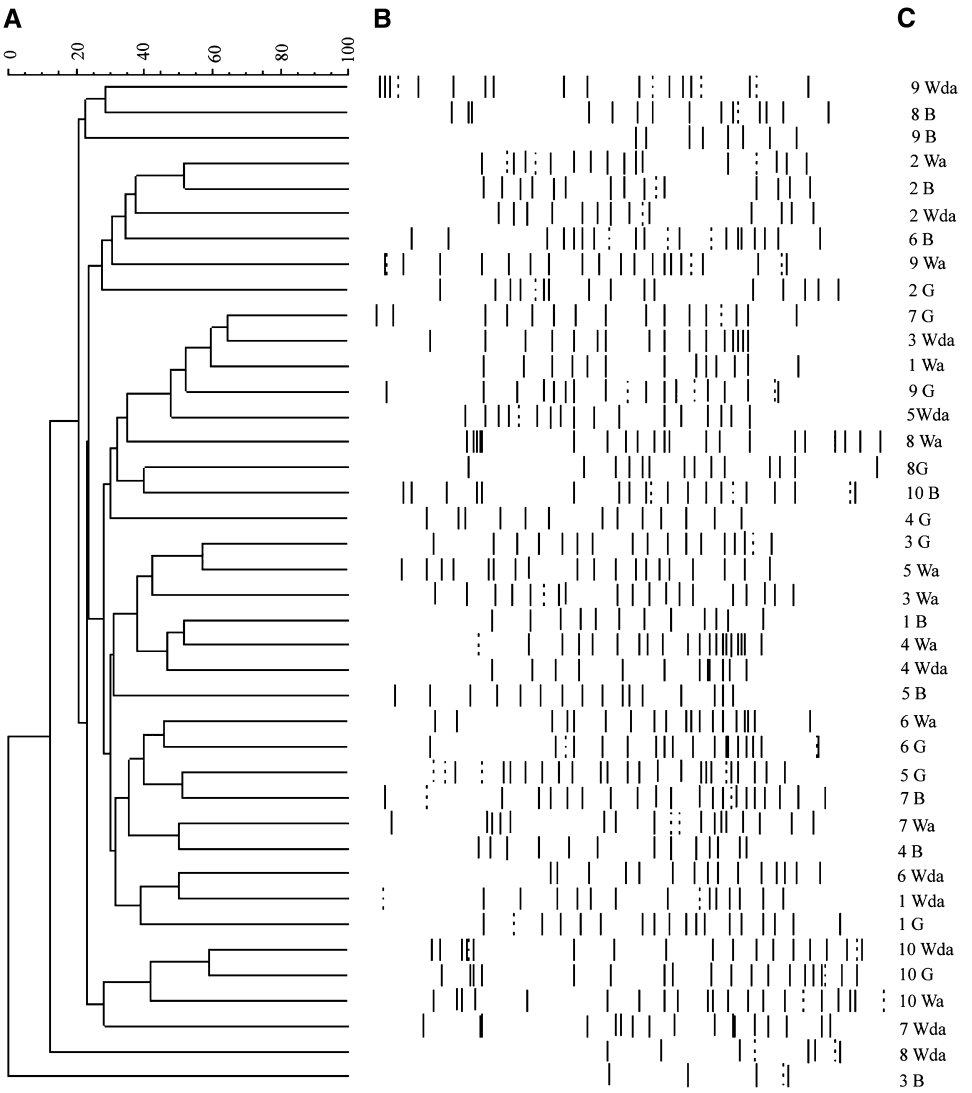

FIGURE 2. Dendrograms of electrophoretic band patterns obtained in the denaturing gel-gradient electrophoresis experiment with universal primers in the 4 different fecal samples collected from the 10 healthy subjects. A: cluster analysis; B: DGGE profiles of fecal samples; C: line graph. B, baseline sample; G, sample after gin consumption; Wa, sample after red wine consumption; Wda, sample after de-alcoholized red wine consumption.

of the predominant bacterial species of fecal microbiota, additional analysis with real-time qPCR was performed to obtain a quantitative estimation of the effect of the ethanol and polyphenol intakes on fecal bacteria populations. We found significant changes in the relative amounts of this microbiota, depending on the kind of beverage consumed. Sequencing results also showed differences in the microbiota composition during the study. After ethanol intake (gin period), there was an increase in the *Bacteroides* and *Clostridium* frequencies and a disappearance of *Prevotellaceae* as compared with polyphenol (de-alcoholized red wine period) or polyphenol plus ethanol intake (red wine period) or baseline (no polyphenol, no alcohol). These results suggest that the dominant genera in the intestinal microbiota of the volunteers was different at baseline and after polyphenol intake than after only ethanol intake. Moreover, the diversity of the fecal microbiota, according to the number of bands in DGGE profiles, was higher after the red wine period. We also found that phenolic compounds altered fecal microbiota, and consequently the *Bacteroides/Firmicutes* balance. After the red wine period, which was rich in polyphenols, there was a significant increase in the concentration of *Firmicutes* and *Bacteroidetes* in stool samples. However, no significant differences were detected in the number of *Lactobacillus* species after any intake period, which indicates that polyphenol or ethanol consumption had no effect on the growth of these bacteria. Similar results were reported by Tzounis et al (35), who found that after catechin intake the growth of *Lactobacillus* spp. remained relatively unaffected in an in vitro study, and by Yamakoshi et al (36), who observed no change in that genera after ingestion of procyanidin-rich extracts in healthy adults. Nevertheless, Tzounis et al recently found a significant increase in the growth of *Lactobacillus* spp. after a regular intake of a high-cocoa flavonol drink in both in vivo and in vitro studies (37). Although the *Actinobacteria* phyla did not change after any intake period, we found a significant increase in the *Proteobacteria, Firmicutes*, and *Bacteroidetes* phyla after the red wine period but not after the gin or de-alcoholized red wine periods, as compared with the initial washout period. These findings indicate that small ethanol doses plus polyphenol intake for a short time can generate an important

TABLE 5
Bacterial identification after the sequencing of the bands from the denaturing gradient gel electrophoresis analysis of fecal samples at baseline and in the 3 intake periods

Type bacteria genus (sequencing results of the bands)	Baseline (washout period)[1] (n = 35)	De-alcoholized red wine periods[1] (n = 38)	Red wine period[1] (n = 41)	Gin period[1] (n = 37)	Sequence similarity
	n (%)	*n* (%)	*n* (%)	*n* (%)	%
Phylum *Bacteroidetes*					
Genus *Bacteroides*	10 (28.57)	15 (39.47)	14 (34.14)	18 (48.64)	99.86
Unclassified *Prevotellaceae*	7 (20)	11 (28.94)	11 (26.82)	0	94.71
Phylum *Firmicutes*					
Genus *Clostridium*	5 (25.71)	4 (10.52)	5 (12.19)	13 (35.13)	98.73
Genus *Veillonella*	3 (8.57)	3 (7.89)	3 (7.31)	1 (2.70)	97.49
Phylum *Actinobacteria*					
Unclassified *Actinobacteria*	1 (2.85)	2 (5.26)	2 (4.87)	1 (2.70)	94.52
Phylum *Proteobacteria*					
Genus *Pseudomona*	1 (2.85)	1 (2.63)	1 (2.43)	1 (2.70)	97.70
Genus *Acinetobacter*	1 (2.85)	1 (2.63)	1 (2.43)	1 (2.70)	97.37
Unclassified *Enterobacteriales*	1 (2.85)	2 (5.26)	1 (2.43)	1 (2.70)	93.39
Unclassified *Campylobacterales*	1 (2.85)	1 (2.63)	2 (4.87)	0	94.27
Phylum *Fusobacterium*					
Unclassified *Fusobacteriaceae*	1 (2.85)	1 (2.63)	1 (2.43)	1 (2.70)	94.28

[1] Refers to the frequency (and percentage) of each unique bacteria genus in the baseline or de-alcoholized red wine or red wine or gin intake periods; *n* is the number of bands sequenced and identified in each intake period.

change in the gut microbiota, which may have influenced the host metabolism. Moreover, after the red wine period, there was a significant increase in the number of *Enterococcus, Bacteroides*, and *Prevotella* genera and an important decrease in the *Clostridium* genera and *Clostridium histolyticum* group. Similar results were found by others in rat models. Smith et al (38) found that the *Bacteroides* group increased significantly when a tannin-rich diet was given to rats, and Dolara et al (39) reported that when rats were treated with red-wine polyphenols they had significantly lower levels of *Clostridium* spp., and no change was observed in *Actynomices* genera within *Actinobacterias*, as in the current study. Predominant red wine polyphenols, such as flavan-3-ol monomers and procyanidins, are associated with antimicrobial activity, exerting their inhibitory effect on certain bacterial groups by binding to bacterial membranes (14, 40). Differences in cell surface structures could explain why Gram-positive clostridial-type bacteria are more sensitive to the bactericidal effects of these compounds than are Gram-negative *Prevotella* and *Bacteroides* species (41). The significant decrease found by us in the *Clostridium histolyticum group* suggests that red wine polyphenols have an inhibitory effect on the growth of these bacteria, which includes *Clostridium perfringens*—an important pathogen closely related with the progression of colonic cancer and the onset of inflammatory bowel disease (42). The same result was reported by Tzounis et al (37) in an intervention study using cocoa-derived flavonols. On the other hand, this study showed that polyphenol and polyphenol plus ethanol intake positively affected the growth of the *Blautia coccoides–Eubacterium rectale* group, *Bifidobacterium, Eggerthella lenta*, and *Bacteroides uniformis.* The last 2 microorganisms are able to degrade resveratrol into dihydroresveratrol, which has antiproliferative effects in human prostate cancer cells and has less antioxidant activity and less ability to inhibit DNA synthesis than resveratrol (13, 20, 43). We found that the significant increase in dihydroresveratrol found in urine in our study after polyphenol intake was related with the significant increase in numbers of *Eggerthella lenta* and *Bacteroides uniformis*.

A study by Barcenilla et al (44) showed that most of the butyrate-producing isolates from human fecal samples are related to the *Blautia coccoides–Eubacterium rectale* group. The presence of butyrate in the colon may be important for the prevention of colon cancer (45) or ulcerative colitis (46). In addition, the increased growth of *Bifidobacteria* has been associated with positive effects in the large intestine (47). This bacterial group has the capacity to produce beneficial organic acids (lactate and acetate) and the ability to inhibit the growth of pathogenic bacteria (48). Other previous in vitro studies using human feces inoculated with (+)-catechin have reported an increase in the growth of *Blautia coccoides–Eubacterium rectale* group and *Bifidobacterium* spp., which suggests a moderate prebiotic effect of monomeric flavan-3-ol on the intestinal microbiota (35). Vendrame et al (49) found a significant increase in the amount of *Bifidobacterium* after 6 wk of consumption of a wild blueberry drink, which suggests an important role of the polyphenol present in wild blueberries on the intestinal microbiota composition modulation. The similar data found in our study could suggest a possible prebiotic effect of the red wine polyphenol on the microbiota.

Bacterial population shifts caused by wine polyphenols may have an effect on host health. Our results clearly showed that de-alcoholized red wine and red wine significantly decrease SBP and transaminase concentrations. These results agree with those of other studies, which have reported that polyphenols suppress serum transaminase elevations and reduce elevated BP (50, 51). Uric acid concentrations decreased significantly during the red wine period. This can be explained by the significant increase of *Proteobacteria* observed in this stage, which has previously been reported to degrade uric acid (52). During the study we found that the regular intake of red wine polyphenols generated significant decreases in the plasma concentrations of triglycerides,

TABLE 6
Real-time polymerase chain reaction quantification of microbiota phyla during the study[1]

	Baseline (washout period)	De-alcoholized red wine period	Red wine period	Gin period	P^2
	log₁₀ copies/g feces	*log₁₀ copies/g feces*	*log₁₀ copies/g feces*	*log₁₀ copies/g feces*	
Proteobacteria	7.21 ± 1.50[a]	7.37 ± 1.92[a,b]	8.68 ± 2.43[b]	6.74 ± 2.19[a]	0.017
Actinobacteria	8.32 ± 2.78	8.53 ± 2.56	9.05 ± 2.48	7.66 ± 2.94	0.084
Fusobacteria	5.97 ± 1.71[a]	7.04 ± 1.09[b,c]	7.83 ± 0.99[b]	5.81 ± 1.72[a,c]	0.015
Firmicutes	8.62 ± 0.50[a]	8.54 ± 0.52[a]	9.41 ± 0.68[b]	7.88 ± 0.86[a]	0.006
Bacteroidetes	8.96 ± 0.87[a]	9.44 ± 0.82[a]	9.98 ± 0.64[b]	8.46 ± 1.38[a]	0.001

[1] All values are means ± SDs; $n = 10$ subjects. Wilcoxon's signed-rank test was used to compare the treatments with each other. Means in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).
[2] Derived by using the Friedman test.

total cholesterol, and HDL cholesterol. A previous study using a rat model showed that red wine polyphenols reduced circulating triglycerides and total cholesterol (53). Additionally, gut microbiota increase energy metabolism and have systemic effects on host lipid metabolism, especially increasing triglyceride clearance (54). We found that these significant reductions observed in BP, triglycerides, and HDL cholesterol may have been due in part to the polyphenol-induced increase in the growth of *Bacteroides* genera. The decrease observed in the cholesterol concentration could be related to the significant increase in *Bifidobacterium*—a bacteria genera previously associated with the reduction of plasma cholesterol concentrations (55). Finally, we noted a significant reduction in the concentration of CRP after de-alcholized red wine and red wine treatment. This could have been due to the increase seen in *Bifidobacterium* number. CRP is a blood marker of inflammation, and its concentration is a specific predictor of cardiovascular event risk in healthy subjects. Its reduction in our study links polyphenol intake to cardiovascular benefits in the host (56, 57).

One of the main limitations of our study was the lack of washout periods between interventions. The inclusion of washout periods between interventions would extend the study a further 6 wk, which would make it difficult to ensure compliance, and the subjects would be more inclined to withdraw from the study. Nonetheless, no carryover effect was observed in the study, and the absence of a washout period was therefore unlikely to affect the results obtained.

Finally, a limitation of the 16S rRNA gene–based method is that the function of the identified bacteria is unknown. Future studies using a microbial metagenomic sequencing analysis will be done to obtain information about the functional diversity of the bacterial community analyzed here and their effects on polyphenol metabolism and health.

In conclusion, this was the first in vivo study to show that regular moderate consumption of red wine could have a noteworthy effect on the growth of select gut microbiota. We found that red wine polyphenols can inhibit nonbeneficial bacteria from the human microbiota and potentiate the growth of probiotic bacteria such as bifidobacteria, which could be implicated in the reduction of CRP and cholesterol observed in our study, promoting health benefits in the host. Although further research is required, the results of this study suggest the possible prebiotic benefits associated with the inclusion of red wine polyphenols in the diet.

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TABLE 7
Genera, species, and groups within the phyla *Bacteroidetes, Firmicutes*, and *Actinobacteria* amplified by real-time polymerase chain reaction[1]

	Baseline (washout period)	De-alcoholized red wine period	Red wine period	Gin period	P^2
	log₁₀ copies/g feces	*log₁₀ copies/g feces*	*log₁₀ copies/g feces*	*log₁₀ copies/g feces*	
Enterococcus	5.18 ± 1.2[a]	6.94 ± 1.5[b]	7.10 ± 1.1[b]	4.84 ± 1.75[a]	0.001
Lactobacillus	5.24 ± 1.81	5.66 ± 1.71	5.59 ± 2.02	4.91 ± 2.12	0.204
Clostridium	4.70 ± 2.17[a]	3.98 ± 2.08[a]	3.34 ± 1.78[a]	6.48 ± 1.64[b]	0.013
Clostridium histolyticum group	3.62 ± 2.09[a]	3.75 ± 2.12[a]	3.19 ± 2.19[a]	6.62 ± 1.52[b]	0.001
Blautia coccoides–Eubacterium rectale group	4.95 ± 1.33[a]	6.64 ± 1.26[b,c]	6.86 ± 1.43[b]	5.13 ± 1.17[a,c]	0.001
Bacteroides	7.61 ± 1.71[a]	8.37 ± 0.95[a,b,c]	9.42 ± 0.85[b]	6.46 ± 1.81[a,c]	0.046
Bacteroides uniformis	8.52 ± 0.97[a]	9.37 ± 1.12[a,b]	9.98 ± 1.02[b]	7.96 ± 1.53[a]	0.001
Prevotella	6.87 ± 0.67[a]	7.19 ± 0.64[a,b]	7.85 ± 0.81[b]	4.88 ± 0.57[c]	0.013
Bifidobacterium	7.12 ± 2.28[a]	9.93 ± 1.85[b]	9.88 ± 1.78[b]	6.87 ± 2.74[a]	0.031
Eggerthella lenta	7.68 ± 1.41[a]	9.84 ± 1.65[b]	9.97 ± 1.77[b]	7.26 ± 1.87[a]	0.001

[1] Values are means ± SD; $n = 10$ subjects. Wilcoxon's signed-rank test was used to compare the treatments with each other. Means in a row with different superscript letters are significantly different, $P < 0.05$ (Bonferroni post hoc test).
[2] Derived by using the Friedman test.

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The authors' responsibilities were as follows—MIQ-O, CA-L, and FJT: conceived and designed the research; MIQ-O, MM, MB-O, JMG-Z, MC-P, and FCD: conducted the research; MIQ-O, MM, and FCD: performed the statistical analysis and interpreted the data; MIQ-O, MB-O, MM, FCD, and FJT: wrote the manuscript; and MIQ-O, CA-L, FJT, and RE: critically revised the manuscript. All authors have read and approved the final manuscript. None of the authors had any financial or other contractual agreements that might have caused conflicts of interest or that could be perceived as causing conflicts of interest.

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