TITLE: Red wine consumption is associated with fecal microbiota and malondialdehyde in a human population.

ABSTRACT

Objectives: Red wine intake has been associated with a lower risk of cardiovascular disease, its polyphenol content being the primary cause of antioxidant and anti-inflammatory properties attributed to this beverage. However, the way in which these activities are exerted is not yet clear, although some authors have proposed that intestinal microbiota could be implicated. **Methods:** The association between red wine intake, inflammation and oxidative stress parameters and fecal microbial populations has been explored in 38 adult volunteers. Food intake was recorded by means of an annual food frequency questionnaire (FFQ). Energy, cholesterol and ethanol intake were analyzed using the nutrient Food Composition Tables developed by CESNID and polyphenol intake was obtained from the Phenol-Explorer Database. Fecal levels of *Akkermansia, Bacteroides, Bifidobacterium, Blautia coccoides* group, *Clostridium leptum* group, *Lactobacillus* group *and Faecalibacterium prausnitzii* were determined by quantitative PCR. Serum concentrations of C-reactive protein (CRP), malondialdehyde (MDA), total antioxidant capacity (TAC), cholesterol, triglycerides and glucose were analyzed by standard methods. **Results:** Subjects with regular consumption of red wine (mean = 100 ml/day) had lower serum concentrations of MDA and lower fecal levels of *B. coccoides, C. leptum, Bifidobacterium* and *Lactobacillus.* A positive association between MDA levels and *B. coccoides* and *Lactobacillus* was also found. **Conclusion:** Regular consumption of red wine appears to be associated with a reduced serum lipoperoxidation in which the intestinal microbiota may be involved.

INTRODUCTION

In the last few decades, several studies have suggested that a moderate consumption of red wine, which is characteristic of some dietary patterns such as the Mediterranean one, is an important factor in the prevention of several pathologies related to oxidative stress [1]. Besides its alcohol content, the moderate intake of which has been related to a positive effect on health [2], red wine provides other components with additional benefits beyond those of alcohol alone [3,4]. Red wine is a natural food source of antioxidants, among which, are phenolic compounds, especially flavonoids, lignans and stilbenes, contained in the skins and seeds of red grapes [5]. Apart from the effects that these phenolic compounds exert on the organoleptic properties of this alcoholic beverage, some authors have proposed their antioxidant capacity as the main reason for the beneficial health effects ascribed to the moderate consumption of red wine [6,7]. In this way, Estruch *et al.* found lower levels of plasma biomarkers of oxidative stress in healthy men after the consumption of red wine, compared with those consuming gin [8]. Other authors did not only find lower levels of oxidative stress with the intake of this beverage, but also higher levels in antioxidant defense [8,9]. Nevertheless, the way in which red wine exerts its antioxidant actions is not clear. Evidence from animal and human studies suggests that supplementation with polyphenol-rich foods, such as red wine could also differentially influence the intestinal bacterial populations, which have been reported to be responsible of the transformation of these compounds into other with a higher bioavailability and bioactivity [10]. In this context, the aim of this work is to bear out the association between the regular consumption of red wine and some biomarkers of antioxidant status, lipid peroxidation and inflammation, as well as to analyze the existence of differences in the intestinal microbiota according to the consumption of this beverage.

MATERIALS AND METHODS

Participants

The study sample involved 38 healthy adults (27 females, 11 males; aged from 55 to 67 years old). Exclusion criteria were: previous diagnosis of cancer, autoimmune or digestive diseases and consumption of vitamin or mineral supplements, probiotics/prebiotics or antibiotics, during the previous month. Ethical approval was obtained from the Regional Ethical Committee of Asturias and an informed written consent was obtained from each volunteer.

Nutritional Assessment

Dietary intake was assessed by means of an annual semi-quantitative food frequency questionnaire (FFQ), detailing 160 items. Trained dieticians asked about cooking practices, number and quantity of ingredients used in each recipe (e.g., type of oil or milk used) and other information relevant to the study, such as the consumption of skin in fruits. During an interview, subjects were asked item-by-item whether they usually ate each food and, if so, how much they usually ate. For this purpose, 3 different serving sizes of each cooked food were presented in pictures to the participants so that they could choose from up to 7 serving sizes (from “less than the small one” to “more than the large one”). For some of the foods consumed, amounts were recorded in household units, by volume, or by measuring with a ruler. To record the consumption of alcoholic beverages, each participant was asked if they consumed them regularly, and if so, they were asked about the type and amount, for which household measures, such as a glass, a bottle, etc., were used. Methodological issues concerning dietary assessment have been detailed elsewhere [11]. Food intake was analyzed for energy, cholesterol and ethanol

content by using the nutrient Food Composition Tables developed by CESNID [12]. Polyphenol content was obtained from Phenol-Explorer Database [13].

Height was registered by using a stadiometer with an accuracy of ±1 mm (Año-Sayol, Barcelona, Spain). Subjects were barefoot, in an upright position and with the head positioned in the Frankfort horizontal plane. Weight was measured on a scale with an accuracy of ± 100 g (Seca, Hamburg, Germany). Body mass index (BMI) was calculated from the formula weight (kg) /height (m)^2.

Microbiological and Biochemical Analyses

Each volunteer was asked to provide a fecal sample and a blood sample directly after the nutritional assessment period. Blood samples were drawn after a 12-hour fast and subsequently centrifuged and divided in aliquots. These biological samples were immediately frozen at -80 °C and stored until further analyses.

One gram of fecal sample was used for DNA extraction with the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) and the DNA obtained was used for quantification of the different bacterial populations (*Akkermansia*, *Bacteroides*, *Bifidobacterium*, *Blautia coccoides* group, *Clostridium leptum* group, *Lactobacillus* group, and *Faecalibacterium prausnitzii*) by quantitative polymerase chain reaction as previously described [14].

Serum levels of C-reactive protein (CRP) were determined by CRP Human Instant ELISA (eBioscience, San Diego, C.A).

Malondialdehyde (MDA) concentrations in serum were determined with the spectrophotometric method of lipid peroxidation LPO-586 (Byoxytech, Oxis

International, Portland, OR). This kit uses the reaction of a chromogenic reagent with MDA, without interference from 4-hydroxyalkenals (hydrochloric acid solvent procedure), in aqueous samples at 45ºC. One molecule of MDA reacts with 2 molecules of reagent to yield a stable chromophore with maximal absorbance at 586 nm [15]. The within-run coefficient of variation ranged from 1.2 to 3.4%, depending on the concentration of MDA.

Total antioxidant capacity (TAC) in serum was determined with the colorimetric assay P40117 (Innoprot, Innovative Technologies in Biological Systems, Derio, Vizcaya, Spain). In this method, Cu^{2+} is converted to Cu^{+} by both small molecules and protein. The reduced ion is chelated with a colorimetric probe giving a broad absorbance peak around 450 nm, proportional to the TAC [16].

Serum cholesterol was measured using cholesterol oxidase, esterase and peroxidase; triglycerides by the lipase method; and glucose by the hexoquinase method. All were analyzed with a DimensionXpand plus (Siemens, Erlangen, Germany) and in an independent laboratory (Análisis Clínicos Blanco, Gijón, Asturias, Spain).

Statistical Analysis

Statistical analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL). Data are presented as means ± SD for continuous variables and as percentages for categorical ones. Goodness of fit to normal distribution was investigated by Kolmogorov-Smirnov test. Given that the intake of anthocyanins, dihydrochalcones, dihydroflavonols, flavones, total phenolic acids, hidroxibenzoic and hidroxicinnamic acids, and stilbenes intake and fecal levels of *Clostridium leptum* showed a skewed distribution, they were logarithmically transformed for statistical analysis. All the

variables included in this work were analyzed according to the intake of red wine. For this purpose, the sample was divided between those who had consumed red wine during the previous year and those had not. Thus, a dichotomous variable was created between non-consumers (n=16) and consumers (n=22) of this beverage. Significant differences between means were calculated by multivariate-analysis of variance to allow for covariate adjusting. Gender, body mass index and energy intake were included as covariates in the model. For descriptive purposes, mean and SD were presented on untransformed variables. Pearson bivariate correlations between fecal microbial groups and serum MDA, CRP and TAC were also carried out. The conventional probability value (0.05) for significance was used in the interpretation of results.

RESULTS

General characteristics of the sample: energy, cholesterol, and ethanol intake; and serum levels of cholesterol, triglycerides and glucose, together with body mass index and smoking habit, distributed by red wine consumption, are shown in Table 1. Values of all the variables evaluated were similar in both samples with the exception of ethanol intake, which was higher in the red wine consumers.

Daily mean intake of polyphenol classes and subclasses, according to red wine consumption, is presented in Table 2. The intake of flavonoids and stilbenes was higher in the consumers. Anthocyanins, dihydroflavonols, and flavanols were the flavonoids slightly increased in this group of subjects, together with hydroxybenzoic and hydroxyphenylacetic acids within phenolic acids.

The differences in the fecal levels of intestinal microbial groups as well as in the serum biochemical parameters between red wine consumers and non-consumers were also

investigated (Table 3). With regard to the fecal microbial populations, subjects who consumed red wine regularly had lower levels of *Bifidobacterium*, *B. coccoides*, *C. leptum*, and *Lactobacillus*. This group also presented lower serum concentrations of MDA. No significant differences were found for serum levels of CRP and TAC.

To examine whether fecal microbiota may be associated with the levels of oxidative stress and inflammation, Pearson bivariate correlations between bacterial groups and serum parameters were performed (Table 4). A positive association was found between MDA and *B. coccoides* and *Lactobacillus*, whereas no statistically significant differences were found for MDA and the rest of the microbial groups analyzed or between these groups and CRP or TAC.

DISCUSSION

In literature, a wide range of beneficial health effects have been attributed to the moderate consumption of red wine [17]. Our data highlighted that the consumption of this beverage in the context of a regular diet could be associated with reduced levels of MDA and hence with lower lipid peroxidation in our human sample. This effect has been traditionally attributed to the antioxidant capacity of the compounds present in red wine. However, although red wine consumption contributed to a higher flavonoid, phenolic acid, and stilbene intake in the sample, this was too low to observe any differences in the pool of total polyphenol intake between consumers and non-consumers. Accordingly, we did not find differences in serum antioxidant status between both groups. This finding differs from previous studies reporting a positive association between red wine intake and total antioxidant capacity in serum [18-20]. In this regard, some factors should be considered: first, the mean consumption of red wine in our study (100.07 ml/day) is much lower than the 375-400 ml/day reported by others

[9,21]; second, it is likely that the effect from supplementation studies with high doses of red wine during short periods of time (2-4 weeks) has a different effect with respect to that of regular consumption. It is also likely that the variability in the consumption of this beverage could exert different effects between subjects. Moreover, it is of special interest to consider the potential role of the rest of the polyphenols or antioxidants ingested in the diet because, as we observed in this study, the amount of antioxidants provided by other food groups, such as fruits or vegetables, could counteract the differences in the total antioxidant pool between consumers and non-consumers. It should also be taken into account that the grape variety, cultivation, processing and ageing of wine can determine the final polyphenol content of red wines [22].

The linkage between red-wine consumption and health is under investigation. MDA is one of the most abundant products of lipid peroxidation cytotoxins formed in foods or endogenously and it is probably the most widely used marker of lipid peroxidation in humans [23,24]. There is no consensus in literature about the usefulness of MDA in predicting risk of mortality, but comparison with previous studies in other human populations of serum concentrations of this compound reveal that red wine consumers in our sample had concentrations of MDA similar to those found in subjects with a lower risk of mortality [25]. In addition to the impact of red wine on oxidative stress, light to moderate red wine consumption has also been associated with reduced inflammation [19]. We have not found significant differences in CRP concentrations between red wine consumers and non-consumers. Although CRP has been identified by several authors as significant predictor of cardiovascular events, the concentrations found in the total of the sample are within the low risk range (0.11-0.55 mg/dl) previously described [26].

Recent studies have indicated that the intestinal microbiota may be responsible, in part, for the beneficial effects described for red wine polyphenols as the bacterial modification of these compounds, resulting in metabolites with greater intestinal absorption than the original phenolic compounds and improved antioxidant activity at systemic level [27-32]. Some authors have proposed that all individuals have their own unique signature of intestinal microbiota, the composition of which could be modulated by long-term changes in diet [31,33,34]. We found that the dominant microbiota in the feces of the regular red wine consumer and the non-consumer groups was slightly different. Red wine consumers had lower levels of *Blautia coccoides, Clostridium leptum, Bifidobacterium,* and *Lactobacillus,* which is in accordance with the antibacterial activity of polyphenols reported in other studies [35-37]. It is probably that the cell-wall structure of the different bacterial groups could determine their susceptibility to the antimicrobial effect of phenolic compounds, where gram-positive bacteria are more sensitive than gram-negative bacteria [38-40]. The levels of *Akkermansia, Bacteroides,* and *F. prausnitzii* were no different between consumers and non-consumers, suggesting that these microorganisms may be less sensitive to the "antimicrobial" effect of phenolic compounds or that they may benefit from the antioxidant activity. Other authors have found increased levels of microbial groups from *Proteobacteria* and *Fusobacteria* after supplementation with this beverage [41]. Although epidemiological analyses did not establish causality, together with the limited sample size, the positive correlations observed between MDA and *B. coccoides* and *Lactobacillus* are in agreement with the hypothesis we have put forward regarding the implication of changes in the microbiota in the beneficial effects attributable to this beverage. In a future, it will be desirable to extend the sample size in order to increase the statistical power of the study and to stratify the consumption of red wine in low-

214 medium-high consumers to deepen in the association between this beverage with the
215 intestinal microbiota and oxidative stress and to determine whether it is dose dependent.

216 **CONCLUSION**

217 Regular consumption of red wine appears to be associated with a reduced serum
218 lipoperoxidation in which the intestinal microbiota may be involved.

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Table 1. General characteristics of the study sample according to the consumption of red wine.

	Non-consumption (N = 16)	**Consumption (N = 22)** [a]
Age (y)	61.44 ± 2.58	60.73 ± 3.74
Male sex (%)	25.0	31.8
Energy intake (kcal/d)	1798.81 ± 368.74	2022.29 ± 650.16
Cholesterol intake (mg/d)	282.71 ± 88.23	323.12 ± 141.15
Ethanol intake (g/d)	2.24 ± 5.00	12.01 ± 9.12 **
Serum cholesterol (mg/dl)	237.19 ± 26.12	228.05 ± 44.94
Serum triglycerides (mg/dl)	113.31 ± 30.82	119.55 ± 64.85
Serum glucose (mg/dl)	98.63 ± 16.66	97.09 ± 10.61
BMI (kg/m^2)	26.26 ± 3.70	25.51 ± 3.18
Smoking habit (%)	24.4	30.0

[a] Mean intake of red wine = 100.07 ml/d.

Results are presented as mean ± SD and percentage (%).

BMI = Body Mass Index

** $p \leq 0.001$

Table 2. Mean intake of polyphenol classes and subclasses according to the consumption of red wine.

	Non-consumption (N = 16)	Consumption (N = 22) [a]
Total polyphenols (mg/d)	2310.07 ± 1081.53	2364.06 ± 1051.47
Flavonoids (mg/d)	368.70 ± 196.10	562.61 ± 292.11 *
Anthocyanins	4.68 ± 11.30	45.72 ± 48.60 *
Dihydrochalcones	3.39 ± 2.27	2.64 ± 3.11
Dihydroflavonols	0.83 ± 2.72	5.46 ± 4.34 *
Flavanols	153.24 ± 133.46	290.26 ± 201.86 *
Flavanones	148.30 ± 96.77	163.45 ± 206.98
Flavones	4.40 ± 3.18	4.29 ± 6.27
Flavonols	53.86 ± 55.24	50.81 ± 32.27
Phenolic acids (mg/d)	119.37 ± 67.47	197.04 ± 201.48
Hydroxybenzoic acids	5.53 ± 3.92	25.40 ± 31.80 *
Hydroxycinnamic acids	113.76 ± 66.04	171.43 ± 190.27
Hydroxyphenylacetic acids	0.08 ± 0.12	0.21 ± 0.16 *
Stilbenes (mg/d)	0.56 ± 1.71	3.57 ± 2.76 **
Lignans (mg/d)	0.97 ± 0.46	1.02 ± 0.32
Other polyphenols (mg/d)	10.93 ± 5.16	17.46 ± 13.02

[a] Mean intake of red wine = 100.07 ml/d.

Analysis adjusted by gender, body mass index and energy intake.

Results are presented as estimated marginal means ± SD on untransformed variables.

* $p \leq 0.05$ ** $p \leq 0.001$

Table 3. Mean values of fecal microbial groups and biochemical parameters according to the consumption of red wine.

	Non-consumption (N = 16)	**Consumption (N = 22)** [a]
Microbial groups (log no. cells/g):		
Akkermansia	6.64 ± 1.93	6.51 ± 1.82
Bacteroides	9.50 ± 0.64	9.31 ± 0.65
Bifidobacterium	8.32 ± 0.78	7.89 ± 0.58 *
Blautia coccoides	8.57 ± 0.86	6.89 ± 1.59 **
Costridium leptum	10.02 ± 0.38	9.12 ± 0.82 **
Lactobacillus	6.35 ± 1.23	5.49 ± 1.2 *
Faecalibacterium prausnitzii	6.90 ± 0.77	6.49 ± 0.75
Biochemical parameters:		
Serum MDA (µM)	2.18 ± 0.44	1.84 ± 0.52 *
Serum CRP (pg/ml)	1641.53 ± 1293.94	1122.05 ± 1079.37
Serum TAC (mM)	0.35 ± 0.08	0.33 ± 0.09

[a] Mean intake of red wine = 100.07 ml/d.

MDA = Malondialdehyde; CRP = C-Reactive Protein; TAC = Total Antioxidant Capacity

Analysis adjusted by gender, body mass index and energy intake.

Results are presented as estimated marginal means ± SD on untransformed variables.

* $p \leq 0.05$ ** $p \leq 0.001$

Table 4. Pearson bivariate correlations between serum biochemical parameters and fecal microbial groups (log no. cells/g) (N = 38)

	Serum MDA (µM)	**Serum CRP (pg/ml)**	**Serum TAC (mM)**
Akkermansia	-0.125	0.203	-0.168
Bacteroides	-0.067	0.124	-0.126
Bifidobacterium	0.043	0.125	0.008
Blautia coccoides	0.443*	0.203	0.170
Clostridium leptum	0.183	0.192	0.127
Lactobacillus	0.331*	0.168	0.201
Faecalibacterium prausnitzii	-0.195	0.085	0.069

MDA = Malondialdehyde; CRP = C-Reactive Protein; TAC = Total Antioxidant Capacity

* $p \leq 0.05$