Moderate intake of red wine promotes a significant increase of phenolic metabolites in human faeces

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Abstract. With the final aim of ascertaining consistent data about the changes of phenolic metabolites in faeces after a regular wine consumption, the present paper compiles the data from two previous human intervention studies: *a)* a pilot study ($n = 8$), that compared the ingestion of de-alcoholised red wine (272 mL/day), red wine (272 mL/day), or gin (100 mL/day) during 20 days, and *b)* a large trial study ($n = 41$, 33 cases and 8 controls), that assessed variability among individuals after the intake of red wine (250 mL/day, 28 days). Great coincidence was observed in the main phenolic metabolites identified in the faecal samples from both studies that included benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones and cinnamic acids. However, significant differences ($P < 0.05$) in the total phenolic content between faecal samples before and after the wine intervention was only observed for the large trial study (358 ± 270 and 625 ± 380 µg/g faeces, corresponding to the mean values before and after the intervention, respectively), emphasizing the large variability in the phenolic-metabolizing gut-microbial capacity among individuals. The overall results confirm that consumption of wine enhances phenolic metabolism, which might have physiological relevancy at the gut level.

1. Introduction

Recent scientific evidence suggests that dietary polyphenols, including red wine polyphenols, might exert biological effects at the gut level, including antinflamatory activity, modulatory effects on the gut microbiota composition, and interaction with cells, among others. However, these health benefits derived from moderate consumption of wine and other foods rich in polyphenols seem to be due to the metabolites formed during its passage among the gastrointestinal tract, rather than molecular forms originally present in food [1]. It is assumed that the major transforma-
tions of polyphenols in the human body are due to the colonic microbiota whose growth and metabolism could be also modulated by the phenolic metabolites, constituting what is named as a 'two-way interaction' between wine polyphenols and human microbiota [2]. Several authors have used *in vitro* models that simulate the conditions of the gastrointestinal tract in order to evaluate the metabolism of polyphenols by gut bacteria and also to assess the impact of the polyphenols and/or their metabolites on the growth and metabolic activity of gut microbiota [3–6]. However, human intervention studies are needed to get a deeper understanding on the effects of a moderate wine consumption, and also to take into account the variability of the human microbiota among individuals.

With the final aim of evaluating the potential biological effects of a moderate wine consumption on human microbiota and the phenolic metabolism involved, two

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intervention studies involving healthy volunteers have been carried out. Firstly, a pilot study ($n = 8$) was performed in order to evaluate the effect of alcohol and polyphenols content on the colonic microbiota modulation, and to determine the phenolic metabolites profile in faeces [7]. Secondly, a large trial study was realized considering a more relevant number of volunteers ($n = 41$) in order to assess differences in the phenolic-metabolizing activity among individuals [8]. In the present paper, we have accomplished results from both studies and have drawn final conclusions about the changes in the phenolic metabolite profile of faeces after the consumption of red wine.

2. Material and methods

2.1. Red wines

A young Merlot red wine (Penedês appellation, Spain) with a total polyphenol content of 798 mg of gallic acid /L was used for the pilot study [7]. For the large trial study, a young Pinot Noir red wine (Penedês appellation, Spain) selected for its high phenolic content (total polyphenol content = 1758 mg of gallic acid/L) was used. Both wines were kindly provided by Bodegas Miguel Torres SA (Catalonia, Spain).

2.2. Design of the intervention studies

The pilot study ($n = 8$) was a randomized, crossover, controlled intervention study that was divided into 4 consecutive periods: an initial washout period of 15 days (baseline) during which the volunteers did not consume any alcohol or red wine, followed by 3 consecutive periods of 20 days during which the participants drank only de-alcoholised red wine (272 mL/day), red wine (272 mL/day), or gin (100 mL/day) (Fig. 1). The large trial study was a randomized and controlled intervention study that only consisted in the moderate consumption of a young red wine by healthy volunteers ($n = 41$, 33 cases and 8 controls). The study was divided into 2 consecutive periods: a washout period (14 days) followed by a 28 days period during which the volunteers drank 250 mL/day of red wine, divided into 2 doses (Fig. 1). During both studies, participants maintained the restriction of drinking any other alcoholic beverages and followed a low-

polyphenols diet. These studies were carried out according to the rules and approval of the Bioethics committee.

2.3. Preparation of faecal solutions

Each volunteer provided a first baseline faecal sample after the washout period and a sample at the end of each intake period. These samples were stored at $-80°C$ awaiting analysis. For preparation of faecal solutions, samples were thawed at room temperature and weighted (1.0 g) in 15 mL sterile conical tubes. 10 mL of sterile saline solution (NaCl 0.9%, Fresenius Kabi, Spain) was added, vorterex and centrifuged (10 min, 10000 rpm, 4°C) two times. The supernatant (faecal solution) was filtered (0.22 μm) and diluted with acetonitrile (1:4, v/v, acetonitrile/faecal solution). 2.0 μL of sample were injected into the chromatographic system.

2.4. Analysis of phenolic metabolites

Faecal solutions were analysed by an UPLC-ESI-MS/MS method validated for the screening of more than 60 microbial derived phenolic metabolites, including phenols, and mandelic, benzoic, hippuric, phenylacetic, phenylpropionic, valeric and cinnamic acids [7]. Some improvements in detectability and quantification of phenolic metabolites by UPLC-ESI-MS/MS were further achieved by using an internal standard (4-hydroxybenzoic 2,3,5,6-d$_4$ acid) [8].

3. Results and discussion

Table 1 ensembles the phenolic metabolites determined in the faeces before and after the wine intake from both, pilot and large trial, studies. In the pilot study, 22 phenolic metabolites were detected, comprising benzoic acids, phenols, hippuric acids, phenylacetic acids, phenylpropionic acids, valeric acids, valerolactones and cinnamic acids. These same metabolites plus other 13 compounds (making a total of 35 metabolites) were found in the faecal samples from the large trial study. However, alcohol seemed not to influence the formation of microbial phenolic metabolites [7] or the composition of the colonic microbiota [9]. Differences in the number of the metabolites determined were attributed either to the improvements made
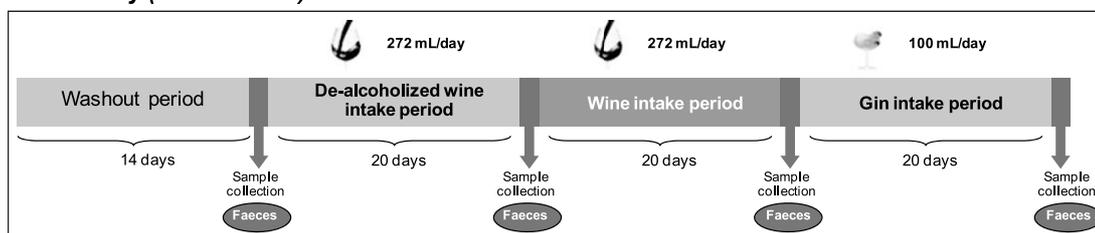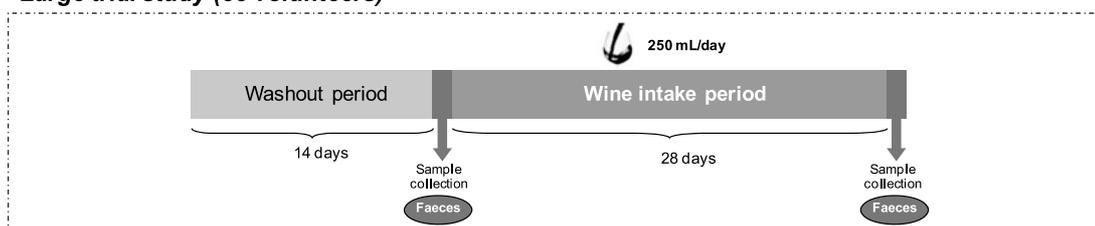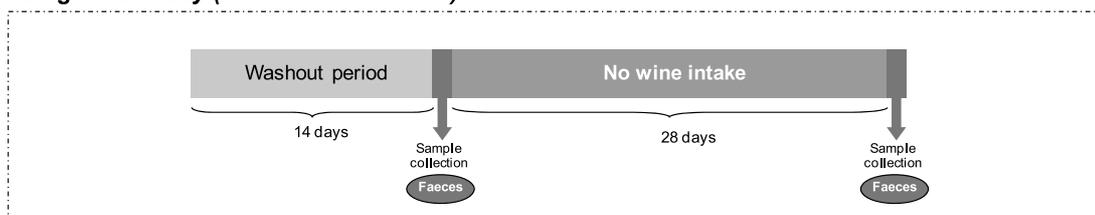
Fig. 1. Schemes of the wine intervention studies.

on the method to enhance its sensitivity, and/or to the higher phenolic content of the wine used (798 and 1758 mg of gallic acid/L in the pilot and large studies, respectively). No detectable amounts of the phenolic compounds presented in the wine (anthocyanins, flavan-3-ols, flavonols or stilbenes) were found in the faecal samples in none of both studies, indicating that they were completely metabolized during its passage through the gastrointestinal tract.

Among the microbial metabolites, benzoic, phenylacetic, 3-phenylpropionic and 4-hydroxy-5-phenyl-valeric acids were the most abundant phenolic metabolites at baseline (before wine intake) found in both intervention studies (data not shown). The content of phenolic compounds at baseline obtained in our studies were included within the concentration ranges previously reported in literature [10–15], in spite of the differences in methodology, number of volunteers, and sample preparation among studies. Nevertheless, it is necessary to point out the high inter-individual

variability observed in the concentrations of faecal phenolic metabolites within each study, which evidences the difficulty in establishing reference values and the importance of carrying out these studies with a large group of volunteers, under controlled diets, in order to obtain reliable and consistent conclusions.

The t-test for dependent samples and its corresponding non-parametric Wilcoxon matched-pairs test were realized to evaluate differences in the content of phenolic acid metabolites in faeces before and after the wine intervention period for both studies (Table 1). In the pilot study (8 volunteers), out of the 22 phenolic metabolites identified in the faecal samples, 8 compounds (mainly benzoic acids) showed statistically significant increases ($P < 0.05$) after the wine intake period, whereas no significant differences ($P > 0.05$) were found in the total phenolic metabolites content (119 ± 34 μg/g faeces and 192 ± 126 μg/g faeces before and after wine intervention). On the other hand, in the large trial study

Table 1

Phenolic metabolites quantified in faeces before and after the wine intervention period in a pilot study (8 volunteers) and in a large trial study (33 volunteers)

Compound	Pilot study		Large trial study	
	Before	After	Before	After
Mandelic acids				
3-Hydroxymandelic acid			+	+
Benzoic acids				
Gallic acid		+	+	+
3,5-Dihydroxybenzoic acid	+	+*	+	+*
Protocatechuic acid	+	+*	+	+*
3-O-Methylgallic acid		+*	+	+*
4-Hydroxybenzoic acid	+	+	+	+
4-O-Methylgallic acid			+	+
3-Hydroxybenzoic acid	+	+	+	+
Vanillic acid	+	+*	+	+*
Syringic acid		+*	+	+*
Benzoic acid	+	+	+	+
Salicylic acid	+	+	+	+
Phenols				
Catechol/Pyrocatechol			+	+
4-Methylcatechol			+	+
Hippuric acids				
4-Hydroxyhippuric acid			+	+
Phenylacetic acids				
3,4-Dihydroxyphenylacetic acid			+	+
4-Hydroxyphenylacetic acid	+	+	+	+
3-Hydroxyphenylacetic acid	+	+	+	+
4-Hydroxy-3-methoxyphenylacetic acid			+	+
Phenylacetic acid	+	+	+	+
Phenylpropionic acids				
3-(3′,4′-Dihydroxyphenyl)-propionic acid	+	+	+	+
3-(4′-Hydroxyphenyl)-propionic acid			+	+
3-(3′-Hydroxyphenyl)-propionic acid	+	+	+	+
3-Phenylpropionic acid	+	+*	+	+*
Valeric acids				
4-Hydroxy-5-(3′,4′-dihydroxyphenyl)-valeric acid		+	+	+*
4-Hydroxy-5-(3′-hydroxyphenyl)-valeric acid	+	+	+	+
4-Hydroxy-5-(phenyl)-valeric acid	+	+*	+	+*
Valerolactones				
5-(3′,4′,-dihydroxyphenyl)-γ-valerolactone			+	+
5-(3′-hydroxyphenyl)-γ-valerolactone				+*
5-(4′-hydroxyphenyl)-γ-valerolactone			+	+
Cinnamic acids				
Caffeic acid	+	+	+	+
p-Coumaric acid	+	+*	+	+
m-Coumaric acid			+	+
Ferulic acid	+	+	+	+
Isoferulic acid			+	+

*Mean values significantly different from baseline concentration ($P < 0.05$).

(33 volunteers), out of the 35 phenolic metabolites identified, 9 phenolic metabolites (mainly benzoic and 4-hydroxyvaleric acids) showed statistically significant increases after the wine intake period. Metabolites significantly changing after the red wine interventions mainly come from the catabolism of both flavan-3-ols and anthocyanins, the major flavonoids in red wine. Moreover, the total phenolic metabolites content was significantly higher ($P < 0.05$) in the samples after the wine intake ($625 \pm 380\ \mu g/g$ faeces) in comparison to
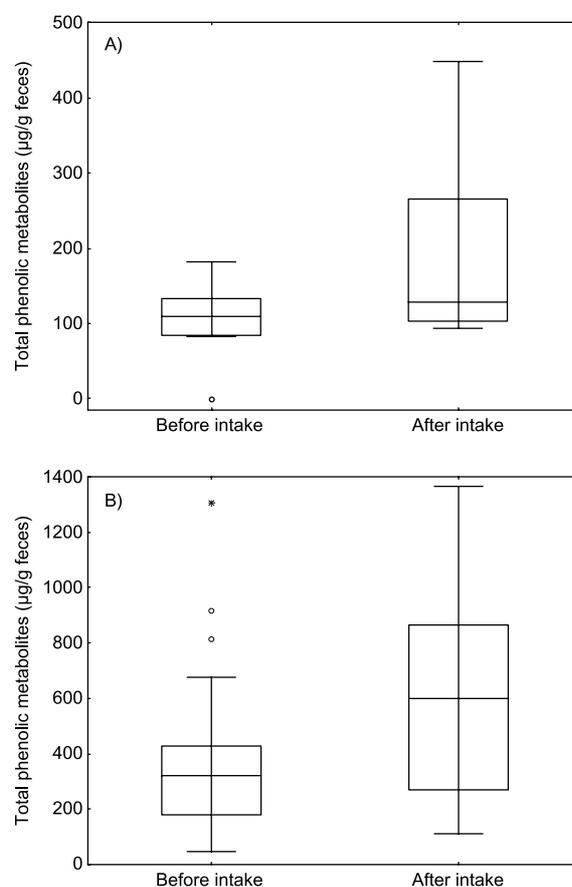
Fig. 2. Box and whiskers plots (-median, ▦ 25th–75th percentiles, ⊥ non-outlier range, O outliers and ✳ extremes) of the total phenolic metabolites in faeces before and after wine intake obtained in: A) the pilot study (8 volunteers); and B) the large trial study (33 volunteers).

the samples before (358 ± 270 µg/g faeces). To summarize data, Fig. 2 displays the box and whiskers plots (median, 25th and 75th percentiles, non-outlier range, outliers and extremes) of the total phenolic metabolites before and after the red wine intervention for both studies. In general, the 25th–75th percentiles were greater for the samples after the wine intake than samples before the wine intake, indicating that wine polyphenols metabolism enhances inter-individual variability further still.

As other authors have proposed, the human population can be classified into high, moderate and low metabolizers, taking into consideration the levels of a specific phenolic metabolite after an intervention with a source rich in the corresponding precursors [16, 17]. Thanks to the high number of volunteers considered under the large trial study, an attempt to distribute the volunteers by means of the total phenolic metabo-

lite content in faecal solutions was carried out. Thus, three groups could be established according to the total phenolic metabolites content in faeces after wine intervention: <500 µg/g faeces, 500–1000 µg/g faeces, and >1000 µg/g faeces [8]. These results suggest a different gut microbial capacity to metabolize wine polyphenols exists among the human population, as observed for polyphenols from other sources.

4. Conclusions

In conclusion, these studies reports complementary and valuable data on the basal levels of phenolic metabolites in faeces, which are in accordance to those reported in the literature. Results from both studies indicate that the microbial metabolic profile of faeces is significantly modified after moderate and reg-

ular intake of red wine polyphenols, which may be related to positive biological effects. Due to the large variability in gut microbiota among individuals, only intervention studies with a large number of volunteers could lead to conclusive results. Our findings support the relevance of the phenolic metabolites in the colon environment, and because of this, the possible gut biological effects associated with moderate wine consumption. From our point to view, more efforts are needed in the identification of wine-polyphenols metabolizing bacteria and in the determination of the bioactive properties of the main metabolites, especially in relation to the microbiota composition.

Acknowledgements

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