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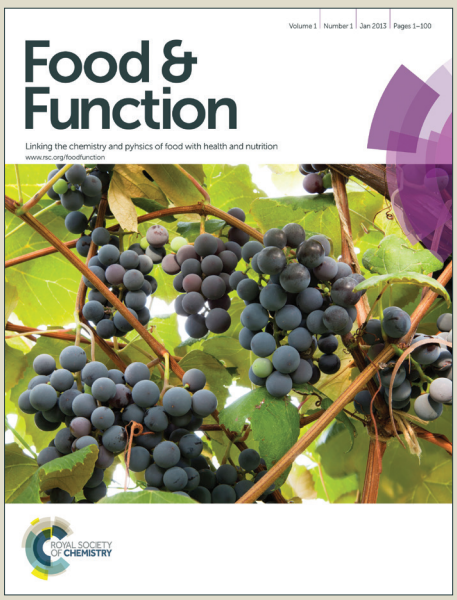

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TITTLE: Phenolic compounds from red wine and coffee are associated with specific intestinal microorganisms in allergic subjects

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ABSTRACT

Dietary modulation of gut microbiota, suggested to be involved in allergy processes, has recently attracted much interest. While several studies have addressed the use of fibres to modify intestinal microbial populations, information about other components, such as phenolic compounds, is scarce. The aim of this work was to identify the dietary components able to influence the microbiota in 23 subjects suffering from rhinitis and allergic asthma, and 22 age and sex-matched controls. Food intake was recorded by means of an annual food frequency questionnaire. Dietary fibres were obtained from Marlett *et al.* tables and Phenol-Explorer Database was used for phenolic compounds intake. Quantification of microbial groups was performed by Ion Torrent 16S rRNA gene-based analysis. Results showed a direct association between the intake of red wine, source of stilbenes, and the relative abundance of *Bacteroides*, and between coffee, rich in phenolic acids, and the abundance of *Clostridium, Lactococcus* and *Lactobacillus* genera. Despite epidemiological analyses not establishing causality, these results support the association between polyphenol rich beverages and faecal microbiota in allergic patients.

KEYWORDS: allergy; phenolic compounds; microbiota; red wine; coffee

INTRODUCTION

Large amounts of data have discussed the involvement of intestinal bacteria in the initiation and amplification of inflammatory processes, allergies and autoimmune diseases [1]. During the last few years, there has been an increasing interest in the study of gut microbiota, using high throughput techniques, in order to establish associations between the gut microbes and these pathologies [2,3]. Allergy is a disorder of the immune system characterized by a hypersensitive reaction induced by certain types of antigens referred to as allergens. Lifestyle changes in western countries may be interfering in the mutualistic relationship between bacteria and host, leading to an increase in the incidences of this disease [4]. Although it has been proposed that some food components, such as probiotics, prebiotics and antioxidants, are critical players in the correct maintenance of the immune system, their association with the microbiota in immunological disorders has not yet been adequately described [5]. Apart from probiotics and prebiotics, other bioactive compounds from diet, such as phenolic compounds, are able to modulate the intestinal microbiota [6]. Evidence from animal and human studies has shown that supplementing diet with polyphenol-rich food, such as red wine [7], tea [8], cocoa [9] or blueberry [10,11], produces modifications in the intestinal bacterial populations. Despite the unclear impact of these microbial changes on health, polyphenols have shown promising results in different trials with animal models of allergy [12] and autoimmunity [13]. It could be considered that some of the potentially health effects of polyphenols on these pathologies may be due to their impact on the gut microbiota composition due to the microbial bio-conversion of polyphenolic compounds into other bioactive compounds with more potent anti-oxidant and/or anti-inflammatory activity [14].

Giving these evidences, the aim of our work was the identification of dietary components associated with the faecal microbiota of a sample of allergic patients. The data resulting from this work could be useful for generating hypotheses that can be used, in the future, for the design of intervention studies aimed to test the effect of specific diets on the symptoms or the course of this disease or for the design of new functional foods targeted at this group.

SUBJECTS AND METHODS

Participants

Twenty three subjects suffering from rhinitis and allergic asthma were randomly selected according to the clinical criteria recommended by the European Community Respiratory Health Survey [15], functional criteria (spirometry and bronchial challenge test with methacholine) and immunological criteria (determination of specific IgE to some key antigens and positive cutaneous tests for those key antigens). Subjects diagnosed as having autoimmune diseases, inflammatory bowel disease (IBD) or other diseases known to affect the intestinal function, as well as subjects who had undergone medical treatment with oral corticoids, immunosuppressive agents, monoclonal antibodies, antibiotics or immunotherapy during the previous 6 months were not considered for this study. Twenty two age and sex matched subjects from the same population were recruited as controls.

Ethics approval for this study (reference code AGL2010-14952; grant title "Towards a better understanding of gut microbiota functionality in some immune disorders") was obtained from the Bioethics Committee of CSIC (Consejo Superior de Investigaciones Científicas) and from the Regional Ethics Committee for Clinical Research (Servicio de

Salud del Principado de Asturias) in compliance with the Declaration of Helsinki. All determinations were performed with fully informed written consent from all participants involved in the study.

Nutritional assessment

Dietary intake of the previous year was assessed by means of a semi-quantitative FFQ referring to 160 items. During a personal 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. Special attention was paid to cooking practices, number and amount of ingredients used in each recipe, as well as questions concerning menu preparation (e.g., type of oil, type of milk used) and other relevant information for the study, such as the consumption of skin in fruit. Food intake was analysed for energy using the nutrient Food Composition Tables developed by CESNID [16], dietary fibre (total and subtypes) from Marlett food composition tables [17], and the phenolic compounds content in foods was completed using the Phenol Explorer Database [18].

Anthropometric measures

Body mass index (BMI) was calculated from the formula: weight (kg) / height $(m)^2$. Height was registered using a stadiometer with an accuracy of ±1 mm (Año-Sayol, Barcelona, Spain). Subjects stood 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).

Microbiological analyses

Faeces were collected in an interval of 7 days after nutritional interviews. Fresh faecal material (between 10 and 50 g per person) was collected in a sterile container and immediately manipulated and homogenized within a maximum of 3 h from defecation. During the waiting period, from defecation to homogenization, samples were kept at 4°C. Thirty millilitres of RNAlater solution (Applied Biosystems, Foster City, CA) was added to 10 g of sample, and the mixture was homogenized in a sterile bag, using a stomacher apparatus (IUL Instruments, Barcelona, Spain) with three cycles at high speed, 1 min per cycle. Homogenized samples were then stored at -80°C until use.

Faecal DNA extraction, 16S rRNA amplification sequencing of 16S rRNA gene-based amplicons and the sequence-based microbiota analysis were performed according to Hevia *et al*. [19]. The raw sequences reported in this article have been deposited in the NCBI Short Read Archive (SRA) (study accession number: SRP028162).

Statistical analysis

Statistical analysis was performed using IBM-SPSS version 19.0 (SPSS-Inc., Chicago). For descriptive purposes, mean values were presented on untransformed variables. Linear regression analysis was used to investigate the association between the intake of dietary fibre (total and subtypes) and classes of phenolic compounds with faecal microbial genera. We also introduced sex, energy intake and age as covariates. The main food sources of the dietary components previously related to microbiota were selected and placed in a multiple stepwise regression analysis to explore whether their

association with microbial groups remained with independence of covariates and other related variables included in the model. The statistical parameters employed were β (standardized regression coefficient) and R^2 (coefficient of multiple determinations). The conventional probability value for significance (0.05) was used in the interpretation of results.

RESULTS

General characteristics of the sample, mean intake of energy, dietary fibre (total and subtypes) and polyphenol classes in allergy patients and controls are compared in Table 1. No significant differences were found for any of the variables under study, with the exception of lignan intake, which was higher in the control group.

Results from linear regression analysis between the intake of dietary components and microbial genera, in patients and controls are presented in Tables 2 and 3. Positive associations were identified between the intake of total and insoluble fibre with the relative abundance of *Clostridium* in allergic subjects (Table 2). Also, Clostridium, *Lactococcus* and *Lactobacillus,* were directly associated with phenolic acids, and *Bacteroides* with stilbenes (Table 3). Given the high correlation between phenolic compounds and fibres from foods, an additional stepwise regression analysis was conducted to explore the relative importance of total and insoluble fibre and phenolic acids intake on *Clostridium*. Phenolic acid intake was found to be an independent contributor to this microbial group ($R^2 = 0.338$; $\beta = 0.581$; $p = 0.004$) data not shown).

With the aim of exploring the associations observed in allergy subjects, the main food sources of phenolic acids and stilbenes were calculated (Figure 1). Coffee, identified as one of the top contributors of phenolic acids, was found to be an independent contributor to *Lactococcus, Lactobacillus* and *Clostridium* variation. Also, red wine, accounting for 95% of the intake of stilbenes, was positively associated with the relative abundance of *Bacteroides* in faeces (Table 4).

DISCUSSION

The importance of a well-balanced colonic microbiota as a key factor in the modulation of human immunity is more and more recognized in the last years. Our results represent a first step in broadening the knowledge of the association between diet and microbiota in allergic patients, supporting the interaction between phenolic compounds and microbiota, and pointing to a specificity between them, to the extent that only certain microbial groups have been associated with the intake of these compounds, and because the observed associations in allergic were not extrapolated to the controls. Though a possible explanation could be the existence of differences in the intake of these compounds, we have not found any, except for lignans which represented a low proportion of total polyphenol intake and were not associated with any microbial genera. Thus, it seems more probable that intra-group variability in microbiota composition may involve the different diet-microbiota associations observed in allergic subjects with respect to those of the control [20,21].

From all the evaluated dietary components previously associated with microbiota [22,23], phenolic acids and stilbenes were independently associated with some bacterial genera in the allergic patients. Despite the fact that the benefits of increasing the levels of *Lactococcus, Lactobacillus*, *Clostridium and Bifidobacterium* in allergic patients are not well documented, studies using animal models have proposed that the administration of some of these bacteria is able to modulate the allergic response, by means of T cell response regulation [24]. In relation to this, it has been shown that oral administration of a mix of several *Clostridium* strains attenuated disease in a mice model of allergic diarrhoea through the activation of T regulatory cells [25].

Although a positive association between the intake of fibre and *Clostridium* was also detected, in accordance with scientific evidence about the interaction of this component on microbiota modulation [26], this appeared to be linked to phenolic acid consumption, since its association disappeared when the model was controlled by these phenolic compounds. In this regard, the nutritional assessment of the whole diet, carried out in this work, may have some advantages with respect to intervention studies, since the mixture of phenolic compounds provided by diet, together with other dietary components contained in the phenol-containing foods, such as fibres, may improve the faecal environment, interacting with the behaviour of some bacterial groups [27].

In spite of the low coffee intake in our sample, in comparison with other European countries (mean 60.7 ml/d *vs.* 270 ml/d) [28], our results pointed to a positive association between this beverage and *Lactococcus, Lactobacillus* and *Clostridium*. The effect of coffee on intestinal microbiota is not yet clear. Results from an animal model indicate that this drink could limit the growth of some bacterial groups, such as *Clostridium* and *Escherichia coli* and, at the same time, encourage others as *Bifidobacterium* [29]. This bifidogenic effect of coffee has also been found in intervention studies with humans [30], in accordance with our results. However, given the nature of this study, we are not able to analyse factors such as the variety of coffee, its degree of roasting or processing, that could have an impact on its polyphenol content [31].

In relation to red wine, it has been suggested that the intake of one of its major stilbene, resveratrol, could prevent the development of some allergies [32]. Therefore, apart from the antioxidant, anti-inflammatory and anti-allergic properties widely described for red wine phenolics [33-35], our results support a potential role for this beverage in microbiota modulation, by means of its association with *Bacteroides*, as has previously been

suggested [7]. At this point, it should be taken into account that the statistical power of our study may be limited by the relatively small sample size, and that the intake of phenolic compounds in the sample could be insufficient, when compared with that of intervention studies, to have an impact on other members of the intestinal microbiota. Longitudinal studies considering the changes in the microbiota structure from the first ages of allergic individuals could be interesting to complement this work.

Despite epidemiological analyses not establishing causality, these results support the association between polyphenol rich beverages, such as coffee and red wine, on faecal microbiota in allergic patients. These descriptive results will be useful for future research focused on the relationship between diet and microbiota, although more investigation is needed in order to corroborate these data before making dietary recommendations.

ACKNOWLEDGMENTS

This study was financed by European Union FEDER funds and the Spanish Plan Nacional de I+D (grant AGL2010-14952). Adriana Cuervo is in receipt of a grant from Alimerka Foundation. We show our greatest gratitude to all the volunteers participating in the study.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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338 **Table 1.** General description of the studied variables in allergy patients and controls.

	Allergic (N=23)	Control (N= 22)
Age (y)	39.39 ± 11.28	39.18 ± 9.50
Male sex (%)	43.5	31.8
BMI (kg/m^2)	26.27 ± 3.94	25.00 ± 3.63
Energy (kcal /d) [a]	1995.80 ± 429.14	2187.52 ± 565.21
Total fibre (g/d) [a,b]	15.85 ± 6.88	17.53 ± 8.05
Soluble fibre (g/d) [a,b]	2.65 ± 1.24	2.62 ± 0.96
Insoluble fibre (g/d) [a,b]	13.21 ± 5.72	14.91 ± 7.14
Phenolic compounds:		
Flavonoids (mg/d) [a,b]	428.32 ± 259.88	383.39 ± 350.66
Phenolic acids (mg/d) [a,b]	333.21 ± 210.46	307.37 ± 262.16
Lignans (mg/d) [a,b]	0.78 ± 0.23	1.04 ± 0.50 *
Stilbenes (mg/d) [a,b]	1.59 ± 2.79	0.63 ± 0.77

339 Multivariate analysis adjusted by [a] age, gender and [b] energy intake. Results are presented as
340 estimated marginal mean ± SD and percentage (%). * $p \leq 0.05$

341 **Table 2.** Linear regression analysis between dietary intake of fibre (total and subtypes)
342 and dominant microbial genera, in patients with allergy and controls.

		Total fibre [a]	Soluble fibre [a]	Insoluble fibre [a]
Bacteroides	**A**	-0.238	-0.228	-0.229
	C	0.135	0.262	0.117
Bifidobacterium	**A**	-0.022	0.006	-0.028
	C	-0.178	-0.220	-0.171
Blautia	**A**	-0.272	-0.116	-0.301
	C	-0.023	-0.055	-0.018
Lactococcus	**A**	0.099	-0.107	0.149
	C	-0.067	-0.091	-0.064
Lactobacillus	**A**	0.564	0.252	0.549
	C	-0.029	-0.039	-0.028
Clostridium	**A**	0.777*	0.524	0.809*
	C	-0.290	-0.287	-0.289
Faecalibacterium	**A**	-0.488	-0.516	-0.459
	C	-0.334	-0.377	-0.327
Streptococcus	**A**	-0.178	-0.294	-0.139
	C	-0.021	-0.063	-0.016

343 A = Allergy (N = 23); C = Control (N = 22). [a] Derived from a linear regression analysis
344 including age, sex and energy intake as covariates. Results are expressed as β (standardized
345 regression coefficient). Units: microbial genera (%), dietary components (g/d).* $p \leq 0.05$.

346 **Table 3.** Linear regression analysis between dietary intake of phenolic compounds and
347 microbial genera in patients with allergy and controls.

		Flavonoids [a]	Phenolic acids [a]	Lignans [a]	Stilbenes [a]
Bacteroides	**A**	-0.037	-0.333	0.073	0.631*
	C	0.093	0.047	-0.314	0.184
Bifidobacterium	**A**	-0.443	0.146	0.053	-0.023
	C	-0.300	-0.265	-0.240	-0.038
Blautia	**A**	-0.141	-0.136	-0.024	0.317
	C	0.106	-0.006	-0.166	0.202
Lactococcus	**A**	-0.031	0.635*	-0.015	-0.193
	C	-0.155	-0.159	-0.173	-0.240
Lactobacillus	**A**	0.162	0.567*	-0.250	-0.349
	C	-0.115	-0.005	-0.521	-0.098
Clostridium	**A**	0.125	0.630*	-0.150	0.024
	C	-0.067	-0.090	0.109	-0.005
Faecalibacterium	**A**	0.229	0.096	0.094	0.294
	C	-0.111	-0.139	0.570	-0.082
Streptococcus	**A**	-0.211	0.289	-0.151	-0.272
	C	-0.173	0.203	-0.105	0.011

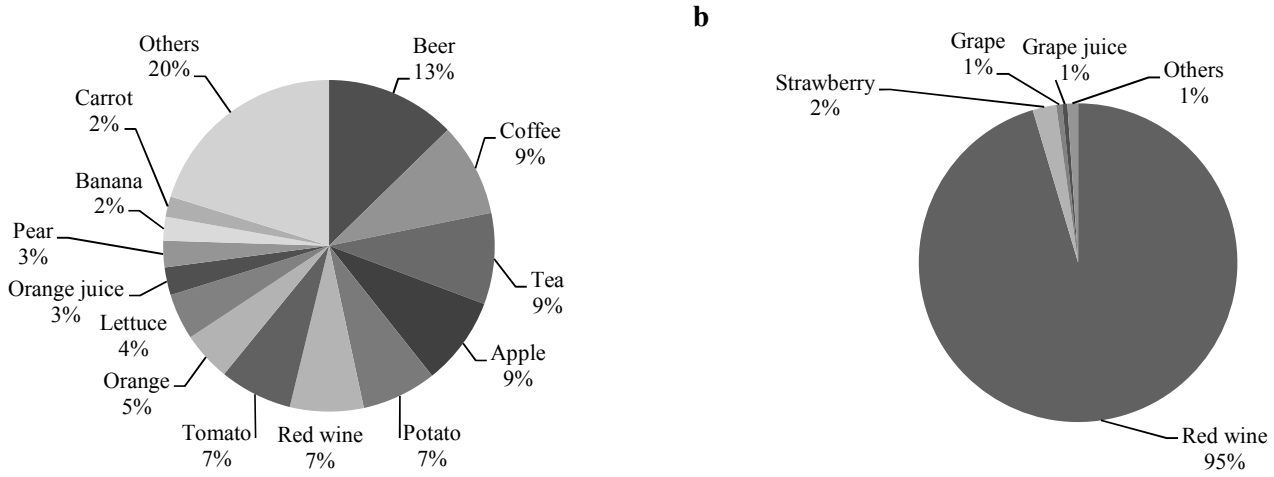
348 A = Allergy (N = 23); C = Control (N = 22). [a] Derived from a linear regression analysis
349 including age, sex and energy intake as covariates. Results are expressed as β (standardized
350 regression coefficient). Units: microbial genera (%), dietary components (mg/d).* $p \leq 0.05$, ** p
351 ≤ 0.001.

352 **Table 4.** Multiple stepwise regression analysis for prediction of bacterial genera relative
353 abundance by the intake of the main food sources of phenolic acids and stilbenes in
354 allergic patients.

	Predictors	Intake (g/d)	R^2	β	p
Bacteroides [a]	Red wine	45.34 ± 79.64	0.325	0.570	0.004
Lactococcus [b]	Coffee	60.65 ± 56.08	0.434	0.659	0.001
Lactobacillus [c]	Coffee		0.221	0.470	0.024
Clostridium [d]	Coffee		0.336	0.579	0.004

355 (N = 23) *β:* standardized regression coefficient; R^2: coefficient of multiple determinations.
356 Variables included in the model: [a] age, gender, energy, red wine, strawberry, grape and grape
357 juice intake; [b, c, d] age, gender, energy, beer, coffee, tea, apple, potato, red wine, tomato, orange,
358 lettuce, orange juice, pear, banana and carrot intake. Only significant results are presented.

359 **Figure 1.** (**a**) Main food sources of phenolic acids in allergic subjects. (**b**) Main food sources of stilbenes in allergic subjects.



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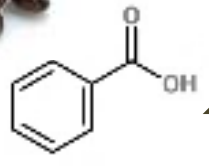
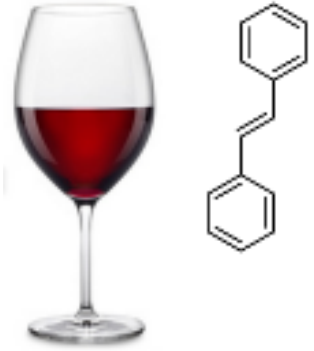


Phenolic acids



Stilbenes

Lactobacillus

Lactococcus

Clostridium

Bacteroides

?

T reg cells

Intestinal mucosa