New Players in the Relationship between Diet and Microbiota: The Role of Macromolecular Antioxidant Polyphenols

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Abstract

Purpose: Solid evidence has emerged supporting the role of polyphenols and fibers as gut microbiota modulators. These studies have been limited to the data available in food composition databases, which did not include the food content of non-extractable polyphenols (NEPP). The main objective of this work is to quantify the intake of the different types of dietary polyphenols including NEPP and to evaluate their impact on the composition and activity of the intestinal microbiota.

Methods: Cross-sectional descriptive study conducted on a sample of 147 adults with no declared pathologies. Dietary intake has been registered by a semi quantitative Food Frequency Questionnaire (FFQ) and transformed into extractable (EPP) and NEPP, and dietary fibers based on available databases. Major phylogenetic types of the intestinal microbiota were determined by qPCR and fecal SCFA quantification was performed by gas chromatography.

Results: NEPP account for two thirds of the total polyphenols intake. A combined analysis by stepwise regression model including all dietary fiber and (poly)phenols has identified hydrolysable (poly)phenol (HPP) intake, as the best predictor of *Bacteroides-Prevotella-Porphyromonas* group

and *Bifidobacterium* levels in feces. Also, HPP were positively associated with butyric acid, while insoluble fiber was identified as a predictor of propionic acid in feces.

Conclusion: The intake of macromolecular (poly)phenols could contribute to modulate the gut microbiota by increasing the levels of certain intestinal microorganisms with proven health benefits.

Keywords: Dietary polyphenols; macromolecular antioxidants; microbiota; non-extratable polyphenols

Introduction

Polyphenols, the most abundant antioxidants in the human diet, comprise a very complex group of molecules present in vegetable foods and beverages. Dietary polyphenols received considerable deal of attention over the last few decades, due to their potential role in preventing the onset and development of some highly prevalent pathologies, such as cardiovascular diseases [1–4], neurological disorders [5–8], diabetes [9–12] or inflammatory processes [13–16]. The evidence of these health-promoting effects are mostly supported by numerous epidemiological and interventional studies on the impact of the different classes and subclasses of (poly)phenols, either from regular diet or from polyphenol-enriched foods, on different biomarkers of oxidative stress or inflammation, among others. Recent data open new approaches to this field, showing the need of considering not only the amount of polyphenols consumed with the diet but also their intestinal absorption and the interaction with the intestinal microbiota [17–20]. At this point, the study of the dietary intake of the recently described macromolecular antioxidants [21–23] could be particularly relevant. Most epidemiological studies on phenolic compounds and health, addressed almost exclusively the intake of extractable polyhenols (EPP) [22], i.e., those phenolic compounds that may be extracted from food matrix by aqueous-organic solvents, considered as all food polyphenols for a long time. Indeed, most information available in composition databases is referred to them [24,25]. Nevertheless, a high poyphenol amount remains in the residue of these extractions, the non-extractable polyphenols (NEPP) [22]. They are consumed when foods are ingested, are partially bioavailable by the action of gut microbiota and have been related to several health benefits [23,26]. For these reasons, there is an increasing interest in this fraction of dietary antioxidants, also called macromolecular antioxidants, because they exhibit either polymeric nature (such as the high molecular weight non-extractable proanthocyanidins, NEPA) or are small phenolic compounds associated with macromolecules, e.g., dietary fiber (such as hydrolysable polyphenols, HPP). Due to their association with dietary fiber, NEPP require a partial degradation of dietary fiber to be released, what originates a mutual interaction between both food constituents, leading to the release of both short chain fatty acids (SCFA) and phenolic metabolites. Thus, this modifies the intestinal micro-ecology and contributes to the maintenance of gut homeostasis and physiology

[27,28]. Hence, the study of the associations between NEPP and gut microbiota in a particular population, not yet performed, could be of great utility to improve the current knowledge about the potential health effects of dietary phenolic compounds [22]. In this study we aimed at assessing the intake of dietary NEPP and its relationship with the gut microbiota in healthy adults.

Subjects and Methods

Participants

The sample of the study included 147 subjects from Asturias Region, in the North coast of Spain recruited between 2010 and 2018. Inclusion criteria were neither having been diagnosed with cancer, autoimmune or gastrointestinal diseases, nor having consumed antibiotics or probiotics/prebiotics one month prior to the study. All subjects were mentally and physically capable to participate in the study and gave informed written consent. Ethical approval was obtained from the Regional Ethics Committee for Clinical Research (Servicio de Salud del Principado de Asturias), ref. no. 17/2010 and the Ethical Committee of CSIC, in compliance with the Declaration of Helsinki.

Nutritional assessment

Dietary intake has been registered by a personal interview using an annual, semi quantitative Food Frequency Questionnaire (FFQ), which has been designed *ad hoc* for the purpose of this study and validated for dietary fibers and polyphenols by means of a 24 h recall method. During a personalized interview, expert dieticians asked volunteers, item by item, whether they usually ate each food and, if so, how much they ate. Methodological issues concerning dietary assessment have been described elsewhere [17]. Food intake was analyzed for energy, macronutrients, and total dietary fiber content by using the nutrient Food Composition Tables developed by the Centro de Enseñanza Superior de Nutrición Humana y Dietética (CESNID) [29]. Detailed information about fiber content was ascertained using the data published by Marlett *et* al. [30]. The content of extractable (EPP) and non-extractable polyphenols (NEPP), and its subclasess of hydrolysable polyphenols (HPP) and

non-extractable proanthocyanidins (NEPA), from fruit, vegetables, cereals, nuts and legumes was obtained from Arranz *et al*. [22].

Anthropometric measures

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

Blood biochemical analyses

A blood sample was drawn by venepuncture after approximately 12-hour fast and collected in separate tubes for serum and plasma. Samples were kept on ice and centrifuged (1000 × g, 15 minutes) within 2–4 hours after collection. Plasma and serum aliquots were storage at -20 °C until analyses were performed. Serum glucose, serum total cholesterol, serum HDL-cholesterol, serum LDL-cholesterol and serum triglycerides were determined by using an automated biochemical auto-analyser.

Total antioxidant capacity (TAC) in serum was determined by the colorimetric assay P40117 (Innoprot, Innovative Technologies in Biological Systems, Vizcaya, Spain). This method determines the conversion of, Cu^{2+} to Cu^{+} by serum small molecules and proteins. The reduced ion is chelated with a colorimetric probe, giving a broad absorbance peak around 450 nm, which is proportional to the TAC [31]. Serum malondialdehyde (MDA) concentrations were determined by the spectrophotometric method of lipid peroxidation LPO-586 (Byoxytech, Oxis International, Portland, OR) [32]. Serum levels of C-reactive protein (CRP) were determined by CRP Human Instant ELISA kit (eBioscience, San Diego, CA).

Fecal samples collection and processing

Feces were collected in an interval of 7 days after the nutritional interviews. Donors were provided with sterile plastic containers and were instructed about fecal collection. Feces were immediately transported to the laboratory (maximum 2–3 h from deposition) and frozen until analyses [33]. Prior to analyses fecal samples were melted, one gram of sample was weighed, diluted 1:10 in sterile phosphate-buffered saline solution (PBS) and homogenized in a Lab-Blender 400 stomacher (Seward Medical, London, UK) at full speed for 4 min. One mL of the homogenized samples was centrifuged (10,000g, 30 min, 4 °C). The pellet obtained was then used for fecal microbiota DNA extraction whereas the supernatant was filtered through 0.2-μm filters, mixed with 1/10 of ethyl butyric acid (1 mg/mL) as an internal standard and stored at −20 °C until gas chromatography (GC) analyses were performed. Fecal DNA was obtained by using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) as previously described [34]. Quantitative PCR (qPCR) targeting the 16S rRNA gene for the quantification of the most relevant intestinal bacterial groups (*Akkermansia, Bacteroides–Prevotella–Porphyromonas* group, *Bifidobacterium, Clostridium* cluster XVIa, *Lactobacillus* group and *Faecalibacterium*) was performed in a 7,500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using the SYBR Green PCR Master Mix (Applied Biosystems) as described before [34]. Samples were analysed in duplicate in two independent PCR runs.

Analysis of SCFA (acetic acid, propionic acid, isobutyric acid, butyric acid, and isovaleric acid) was performed in a gas chromatograph 6890N (Agilent Technologies Inc, Palo Alto, CA, USA) connected to a mass spectrometry (MS) 5973N detector (Agilent Technologies) and to a flame ionization detector (FID) as described previously [35].

Statistical analyses

With the software IBM-SPSS 24.0 (SPSS-210 Inc., Chicago), statistical analyses were performed. The normality of the variables analyzed was checked with Kolmogorov−Smirnov test. Those variables with a skewed distribution were logarithmically transformed for the analyses. A Student's t test and ANOVA analyses were used to assess the differences between quantitative variables

143 defined as dependent variables and categorical variables used as independent variables. To deepen
144 into the associations between phenolic compounds and dietary fibers and blood biomarkers Pearson
145 correlation analyses were conducted. A heatmap was generated under R version 3.3.3 package
146 heatmap.2. Also, the linear trend between these variables and fecal microbial was explored by
147 means of an stepwise regression model including gender, age, body mass index, alcohol
148 consumption and physical activity as covariates. A p-value of 0.05 was used in the interpretation of
149 results.

150 **Results**

151 A general description of the studied variables according to gender, age, BMI and some lifestyle
152 factors is presented in Table 1. The intake of total polyphenols (estimated as the sum of EPP and
153 NEPP) was significantly higher in the group of age of 50-65 years in comparison with subjects under
154 50 years and those above 65 years old ($p<0.001$). Intake of NEPA has been significantly increased in
155 subjects with BMI <25 Kg/m^2 respect to those in the BMI range of 25-30 Kg/m^2 ($p=0.007$) and ≥ 30
156 Kg/m^2 ($p=0.031$). Also, the intake of all phenolic compounds evaluated were significantly higher in
157 the alcohol-consuming group as well as in those who performed physical activity. No relevant
158 differences were identified in the number of depositions per week according to NEPA and HPP
159 intake.

160 The average intake of polyphenols, classified according to the type of phenolic extraction, is
161 presented in Table 2. The combined consumption of the set of EPP and NEPP (or macromolecular
162 antioxidants) in the sample was slightly higher than 1g/d. While fruits were the major contributors
163 to the intake of EPP in the sample, NEPP were mainly provided by fruits and cereals, both being the
164 best food sources of NEPA and HPP, respectively (Table 2).

165 The relative contribution of each of the groups of macromolecular antioxidants to the total intake of
166 (poly)phenols is represented graphically in Fig. 1. As shown, NEPP account for two thirds of the total
167 polyphenols intake. This contribution is provided by fruits (42.84%) followed by cereals (34.67%)
168 and, in a lesser percentage, legumes (10.65%), vegetables (9.93%) and dry fruits (1.92%). In the case
169 of EPP, fruits and cereals explain 75% of the intake. In order to confirm the high correlation expected
170 between the intake of these compounds and the different types of dietary fiber, a Pearson correlation

analysis has been conducted (Fig. 2). A significant high correlation (between *r*= 0.354 and 0.911: p<0.001) has been observed between dietary fiber and the different groups of polyphenols studied, which should be taken into account in the interpretation of the data.

To assess the impact that the intake of EPP and NEPP could exert on the composition and metabolic activity of the gut microbiota, sample subjects were categorized into tertiles according to the daily intake for each one of the compounds evaluated (EPP, NEPP, soluble and insoluble fibers) (Tables 3 and 4). Tertile 1 corresponding to the lowest intake and tertile 3 to the highest one. The results obtained showed that the subjects belonging to tertile 3 of EPP had higher fecal levels of *Bacteroides-Prevotella-Porphyromonas* group (9.2 *vs*. 8.1 Log n^{o}. cells per gram feces in tertile 1; *p. value*= 0.009), the genus *Bifidobacterium* (8.4 in comparison with 7.4 and 7.7 Log n^{o}. cells per gram feces in T1; *p.value*=0.008 and T2; *p. value*=0.041, respectively) and the species *Faecalibacterium prausnitzii* (7.7 *vs*. 6.9 Log n^{o}. cells per gram feces in T1; *p.value*=0.005). In addition to the mentioned microbial groups, subjects with a higher intake of NEPP displayed higher levels of the *Clostridium* cluster XIVa than subjects within the lowest one (8 *vs*. 6.6 Log n^{o}. cells per gram feces; *p.value*= 0.001). Moreover, those individuals with a higher intake of both types of phenolic compounds (EPP and NEPP) and those having the higher intake of soluble and insoluble fibers (tertile 3), also presented higher fecal concentration of butyric acid. Furthermore, a higher intake of insoluble fiber was also associated with a higher fecal excretion of propionic acid (15,3 vs. 11.6 and 12 mM in T1; *p.value*= 0.018 and T2; *p.value*= 0.030). Nevertheless, it is not possible to rule out that some of the observed relationships could be due to the high degree of correlation between the different types of dietary fiber and polyphenols. Therefore, trying to separately identify the best dietary fiber and/or phenolic predictors of the intestinal microbial groups and fecal SCFA, a stepwise linear regression model was carried out (adjusted for age, gender, BMI, physical activity and alcohol consumption since these anthropometric and lifestyle variables were related in our sample with the intake of the different types of phenolic compounds) (Table 5). The combined analysis of all dietary fibers and phenolic compounds included in the model (Table 5) has served to identify HPP intake as the best predictor of *Bacteroides* group and *Bifidobacterium* genus fecal levels whereas counts of *Clostridium*

198 cluster XIVa and *Faecalibacterium prausnitzii* in feces appeared to be age-dependent. Regarding
199 fecal SCFA, the main metabolic end-product of the bacterial metabolic fermentative activity, HPP
200 were positively associated with levels of butyric acid (β= 0.441, *p.value* <0.001), while insoluble
201 fiber persisted in the model as a predictor of propionic acid level in feces (β= 0.258, p.value =0.007)
202 (Table 5).

203 Finally, the possible correlation between the intake of phenolic compounds and some blood
204 biomarkers as serum glucose concentration, lipid profile and parameters related to the inflammatory
205 state and antioxidant capacity, has been explored (Fig. 3). A positive association between the intake
206 of (poly)phenols and levels of total and LDL cholesterol, has been detected, whereas the association
207 with malondialdehyde was inverse. No association with serum inflammatory or antioxidant capacity
208 has been evidenced.

209 **Discussion**

210 Epidemiological and experimental studies have documented the role of diet in health promotion.
211 Fruits and vegetables have been pointed as food groups with a protective effect against a plethora of
212 pathologies, based on their content in bioactive compounds, mainly fiber and antioxidants, where
213 phenolic compounds have shown to be particularly relevant [36]. Although mounting scientific
214 evidence in this field has suggested a possible synergistic effect of these bioactives in microbiota
215 modulation [37–39], most of these effects are referred to (poly)phenols that can be obtained by
216 aqueous-organic extractions from foods, the EPP [40]. Thus, the contribution of the fraction of
217 (poly)phenols remaining in food residues, called NEPP or macromolecular antioxidants, to gut
218 microbiota profile in healthy subjects has not been explored until now. Therefore, as far as we know,
219 this is the first observational study to identify the possible relationship existing among the intake of
220 macromolecular antioxidants, the intestinal microbiota and some serum biomarkers and
221 anthropometric factors.

222 Intake data for EPP and NEPP fractions obtained here are similar to previously reported data in the
223 Spanish population, based exclusively on solid foods. Moreover, it was also reported that NEPP

contributed over 70% of the total polyphenols intake [22], which represents approximately a 90% of the daily dietary intake of antioxidants. Data obtained here for NEPP intake are relevant because information on this aspect in different population is rather scarce [21,22,41,42]. Indeed, composition data for NEPP are quite limited as compared to those available for EPP, what hampers, for instance, the evaluation of NEPP intake from individual foods, as it has been done for EPP [23]. These NEPP are polymeric molecules linked to vegetable undigestible compounds such as complex polysaccharides that entrap polyphenols, facilitating their arrival to the large intestine where they can be accessible to the gut microbiota [23,43]. Thus, both physiologically and quantitatively, NEPP are an important group of bioactive compounds when studying diet-microbiota interactions. In this regard, data obtained in the present work revealed a differential effect of dietary (poly)phenols according to their structure. Thus, while subjects with the highest intake of both EPP and NEPP presented higher fecal levels of the *Bacteroides-Prevotella-Porphyromonas* group, the *Bifidobacterium* genus and the species *Faecalibacterium praunitzii*, NEPP exhibited an additional positive association with increased fecal levels of *Clostridium* cluster XIVa. Differences were detected as well for some microbial groups and SCFA among tertiles for soluble and insoluble dietary fibers. With this type of cross-sectional and observational study, it is not possible to establish a directionality or causality in the observed associations. However, given the very high degree of correlation existing between the phenolic compounds analyzed and soluble and insoluble fiber, a stepwise regression model has been carried out to analyze more in depth the dietary fiber and/or (poly)phenols predictors of intestinal microbial groups and SCFA, introducing as covariates in the model those anthropometric and lifestyle variables that were related in our sample with the intake of fiber and (poly)phenols (Table 1). From all the variables introduced, HPPs have been identified as the best predictor of fecal *Bifidobacterium* and *Bacteroides-Prevotella-Porphyromonas* groups. These results were in consonance with previous studies reporting a `prebiotic-like´ effect of macromolecular antioxidants by modulating populations and metabolic activity of certain microorganisms such as bifidobacteria or lactobacilli [44]. An enrichment in *Prevotella* was described in vegan populations and in subjects with high consumption of plant-based foods [45–47]. Also, an intervention with

whole-grain cereals -rich in HPP- in overweight/obese subjects led to sustained increases in the relative abundance of Firmicutes and Bacteroidetes [48].

Since NEPP reach the colon linked to the matrix of fermentable polysaccharides, our findings of higher levels of fecal butyrate in the higher consumption tertiles of polyphenols and dietary fibers were expectable. Despite the complexity of microbial interactions with these compounds, it seems reasonable to assume that the fermentation of fibers may selectively enhance the activity of certain butyrate producer's bacteria [49]. For example, *Bifidobacterium* has been shown to be indirectly involved in the intestinal production of butyrate through cross-feeding interactions with other intestinal microbial groups such as *Faecalibacterium praunitzii* [50,51]; interestingly, this specie was increased here in the subjects with the highest EPP or NEPP intake, although its main predictor was age. Moreover, this is a two-way interaction, where the presence of dietary fiber also contributes to the microbial transformation of polyphenols; for instance, it was observed in an *in vitro* model that the colonic transformation of NEPA was higher in the presence of a combination of soluble (highly fermentable) and insoluble dietary fiber than in the presence of only insoluble dietary fiber [52].

Finally, the positive relationship between the intake of NEPP and the higher blood lipid concentrations (total cholesterol and LDL cholesterol) could be *a priori* surprising. However, given the descriptive nature of the present study, we cannot discard that some of the participants had minor differences in their dietary habits as a consequence of a moderate increase in cholesterol levels, since a 36.8% of the sample presented a moderate increase in serum cholesterol levels, up to 240 mg/dL, thus above reference values (200 mg/dL), or that age might be influencing this association.

Despite (poly)phenols are quantitatively the main dietary antioxidants, the intake of NEPP does not seem to contribute to the total serum antioxidant capacity. As foods contain a wide variety of antioxidants, it is possible that we did not have enough variability in the sample as to observe differences in the total antioxidant capacity at the systemic level due to the intake of NEPP. It would be interesting in future studies to evaluate the impact of NEPP on the antioxidant capacity at

the colonic level; in this way, a preclinical study where rats were supplemented with a NEPP-rich product found a five-fold increase in cecum antioxidant capacity [53].

In summary, this study shows for the first time that the intake of macromolecular phenolic compounds could be an effective way to modulate gut microbiota, enhancing levels of relevant groups of intestinal bacteria. The different bacterial populations assessed in this study (*Akkermansia*, *Bacteroides* group, *Bifidobacterium*, *Clostridium* cluster *XIVa*, *Lactobacillus* group and *Faecalibacterium prausnitzii*) represent more than 95% of the overall phylogenetic types of the human intestinal microbiota [54]. However, further research in humans is needed to elucidate the specific effects of these (poly)phenols on different chronic diseases. To this end, it would be of great importance to develop specific composition tables that include detailed information on the amount of EPP and NEPP in a wide variety of foods and that allow the development of harmonized methods for the quantification of the intake of these phytochemicals.

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Table 1 Mean intake of phenolic compounds (g/day) in the study sample according to gender, age, BMI and life-style related variables

	TOTAL (EPP + NEPP)	EPP	NEPP (NEPA + HPP)	NEPA	HPP
Gender					
Male (n=43)	$1.33 \pm 0.60_a$	$0.32 \pm 0.16_a$	$1.01 \pm 0.45_a$	$0.33 \pm 0.21_a$	$0.68 \pm 0.29_a$
Female (n=104)	$1.22 \pm 0.62_a$	$0.31 \pm 0.17_a$	$0.92 \pm 0.46_a$	$0.32 \pm 0.23_a$	$0.60 \pm 0.26_a$
Age (y)					
<50 (n=46)	$1.10 \pm 0.42_a$	$0.27 \pm 0.11_a$	$0.83 \pm 0.31_a$	$0.26 \pm 0.14_a$	$0.57 \pm 0.19_a$
50-65 (n=60)	$1.58 \pm 0.95_b$	$0.39 \pm 0.18_b$	$1.19 \pm 0.50_b$	$0.43 \pm 0.25_b$	$0.76 \pm 0.30_b$
>65 (n=41)	$0.95 \pm 0.47_a$	$0.23 \pm 0.12_a$	$0.72 \pm 0.35_a$	$0.25 \pm 0.18_a$	$0.47 \pm 0.19_c$
BMI (kg/m^2)					
<25 (n=42)	$1.42 \pm 0.80_a$	$0.36 \pm 0.21_a$	$1.06 \pm 0.59_a$	$0.40 \pm 0.29_a$	$0.66 \pm 0.32_a$
25-30 (n=70)	$1.16 \pm 0.49_a$	$0.28 \pm 0.13_b$	$0.87 \pm 0.36_b$	$0.29 \pm 0.18_b$	$0.59 \pm 0.21_a$
>30 (n=65)	$1.26 \pm 0.57_a$	$0.31 \pm 0.15_a$	$0.95 \pm 0.43_{a,b}$	$0.30 \pm 0.18_b$	$0.65 \pm 0.30_a$
Alcohol consumption[α]					
Non-consumers (n=113)	$1.19 \pm 0.61_a$	$0.29 \pm 0.16_a$	$0.90 \pm 0.46_a$	$0.31 \pm 0.22_a$	$0.59 \pm 0.26_a$
Alcohol consumers (n=34)	$1.49 \pm 0.57_b$	$0.37 \pm 0.15_b$	$1.11 \pm 0.43_b$	$0.38 \pm 0.21_b$	$0.73 \pm 0.28_b$
Physical activity[β]					
Sedentary (n=51)	$1.02 \pm 0.48_a$	$0.25 \pm 0.13_a$	$0.77 \pm 0.35_a$	$0.25 \pm 0.17_a$	$0.52 \pm 0.21_a$
Active (n=58)	$1.37 \pm 0.73_b$	$0.33 \pm 0.19_b$	$1.03 \pm 0.54_b$	$0.36 \pm 0.27_b$	$0.67 \pm 0.31_b$
Smoking status					
Non-smoker (n=81)	$1.16 \pm 0.66_a$	$0.28 \pm 0.17_a$	$0.87 \pm 0.48_a$	$0.30 \pm 0.24_a$	$0.57 \pm 0.26_a$
Current smoker (n=22)	$1.34 \pm 0.64_b$	$0.32 \pm 0.16_a$	$1.02 \pm 0.49_a$	$0.34 \pm 0.23_a$	$0.68 \pm 0.33_a$
Deposition (times/week)					
<3 (n=9)	$0.90 \pm 0.49_a$	$0.21 \pm 0.12_a$	$0.70 \pm 0.37_a$	$0.22 \pm 0.17_a$	$0.48 \pm 0.21_a$
3-7 (n=23)	$1.42 \pm 0.58_a$	$0.35 \pm 0.16_b$	$1.07 \pm 0.42_b$	$0.39 \pm 0.22_a$	$0.68 \pm 0.25_a$
≥7 (n=77)	$1.18 \pm 0.67_a$	$0.29 \pm 0.17_{a,b}$	$0.89 \pm 0.50_{a,b}$	$0.30 \pm 0.24_a$	$0.59 \pm 0.29_a$

Results from T-student and ANOVA analyses are presented as mean ± standard deviation. Different subscript letters indicate significant statistical differences. EPP, extractable polyphenols; NEPP, non – extractable polyphenols; NEPA, non – extractable proanthocyanidins; HPP, hydrolyzable polyphenols; BMI, body mass index. [α]Alcohol consumers: men ≥ 10g/day; women ≥ 5 g/day. [β]Sedentary: walking less than 30 min/day.

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Table 2 Daily intake of macromolecular antioxidant polyphenols depending on their dietary origin.

	EPP (g/day)	NEPP (g/day)	NEPA (g/day)	HPP (g/day)
Cereals	0.08 ± 0.04	0.30 ± 0.16	-	0.29 ± 0.16
Dry fruits	0.01 ± 0.02	0.02 ± 0.04	0.01 ± 0.03	0.01 ± 0.02
Fruits	0.15 ± 0.13	0.45 ± 0.36	0.26 ± 0.21	0.19 ± 0.15
Legumes	0.01 ± 0.01	0.09 ± 0.09	0.05 ± 0.05	0.04 ± 0.04
Vegetables	0.06 ± 0.05	0.09 ± 0.08	-	0.09 ± 0.08
Total	0.31 ± 0.16	0.95 ± 0.46	0.32 ± 0.22	0.62 ± 0.27

Results presented as mean ± standard deviation. EPP, extractable polyphenols; NEPP, non – extractable polyphenols; NEPA, non – extractable proanthocyanidins; HPP, hydrolysable polyphenols. Cereals included rice, bread, pasta, biscuits, breakfast cereals and pastries. Dry fruits included chestnut, hazelnut, walnut, almond, peanut and pistachio. Fruits included: apple, apricot, avocado, banana, black table olive, blackberry, cherry, fig, grape, green table olive, kiwi, lemon, melon, mango, mandarin, orange, peach, pear, pineapple, plum, strawberry and watermelon. Legumes included chickpeas, beans and lentils. Vegetables contained artichoke, asparagus, aubergine, cabbage, chard, carrot, cauliflower, celery, cucumber, endive, garlic, green bean, kale, leek, lettuce, mushroom, onion, pepper, potato, pumpkin, spinach and tomato.

Table 3 Levels of the major fecal microbial groups and fecal short chain fatty acids (SCFA) in the study sample according to extractable polyphenols (EPP) and non-extractable polyphenols (NEPP) daily intake categorized into tertiles.

	EPP (g/day)			NEPP (g/day)		
	Tertile 1$^{\alpha}$	Tertile 2$^{\beta}$	Tertile 3$^{\gamma}$	Tertile 1$^{\delta}$	Tertile 2$^{\varepsilon}$	Tertile 3$^{\zeta}$
Microbiota (Log no. cells per gram feces) (n=135)						
Akkermansia	$5.68 \pm 2.47_{a}$	$5.97 \pm 2.24_{a}$	$5.95 \pm 1.82_{a}$	$5.47 \pm 2.69_{a}$	$6.14 \pm 1.92_{a}$	$5.98 \pm 1.84_{a}$
Bacteroides-Prevotella-Porphyromonas	$8.15 \pm 2.13_{a}$	$8.54 \pm 2.15_{a,b}$	$9.16 \pm 0.96_{b**}$	$7.83 \pm 2.76_{a}$	$8.81 \pm 1.12_{b*}$	$9.19 \pm 0.95_{b**}$
Bifidobacterium	$7.42 \pm 2.27_{a}$	$7.66 \pm 1.88_{a}$	$8.40 \pm 0.76_{b**}$	$7.07 \pm 2.76_{a}$	$8.05 \pm 0.89_{b**}$	$8.35 \pm 0.80_{b**}$
Clostridium cluster XIVa	$7.10 \pm 2.23_{a}$	$7.39 \pm 2.28_{a}$	$7.91 \pm 1.49_{a}$	$6.58 \pm 2.73_{a}$	$7.77 \pm 1.51_{b**}$	$7.99 \pm 1.49_{b**}$
Lactobacillus group	$6.28 \pm 2.12_{a}$	$5.80 \pm 1.80_{a}$	$6.14 \pm 1.25_{a}$	$5.97 \pm 2.52_{a}$	$6.08 \pm 1.28_{a}$	$6.14 \pm 1.25_{a}$
Faecalibacterium prausnitzii	$6.87 \pm 1.54_{a}$	$7.35 \pm 1.48_{a}$	$7.66 \pm 0.80_{b**}$	$6.91 \pm 1.51_{a}$	$7.31 \pm 1.52_{a,b}$	$7.65 \pm 0.81_{b**}$
SCFA (mM) (n=128)						
Acetic acid	$31.14 \pm 19.72_{a}$	$38.66 \pm 17.76_{a}$	$37.29 \pm 18.65_{a}$	$31.61 \pm 20.88_{a}$	$38.77 \pm 16.64_{a}$	$36.49 \pm 18.81_{a}$
Propionic acid	$11.16 \pm 7.35_{a}$	$14.01 \pm 6.82_{a}$	$13.80 \pm 7.75_{a}$	$11.22 \pm 7.66_{a}$	$13.46 \pm 6.06_{a}$	$14.15 \pm 8.14_{a}$
Isobutyric acid	$1.43 \pm 0.78_{a}$	$1.79 \pm 0.91_{a}$	$1.89 \pm 1.78_{a}$	$1.49 \pm 0.81_{a}$	$1.64 \pm 0.77_{a}$	$1.97 \pm 1.83_{a}$
Butyric acid	$7.94 \pm 5.39_{a}$	$10.37 \pm 6.11_{a}$	$12.52 \pm 9.59_{b**}$	$8.49 \pm 6.23_{a}$	$9.56 \pm 4.91_{a}$	$12.73 \pm 9.83_{b**}$
Isovaleric acid	$2.00 \pm 1.27_{a}$	$2.47 \pm 1.32_{a}$	$2.64 \pm 2.53_{a}$	$2.04 \pm 1.29_{a}$	$2.33 \pm 1.26_{a}$	$2.72 \pm 2.54_{a}$

Results derived from ANOVA analyses are presented as estimated marginal mean ± standard deviation. Range of EPP intake according to tertiles: $^{\alpha}$Tertile 1 (0.06-0.228 g/day), $^{\beta}$Tertile 2 (0.229-0.37 g/day), $^{\gamma}$Tertile 3 (0.371-1.33 g/day). Range of NEPP intake according to tertiles: $^{\delta}$Tertile 1 (0.21-0.704 g/day), $^{\varepsilon}$Tertile 2 (0.705-1.07 g/day), $^{\zeta}$Tertile 3 (1.08-3.84 g/day). Different subscript letters indicate significant statistical differences. *p≤0.05; **p≤0.01.

Table 4 Levels of the major fecal microbial groups and fecal short chain fatty acids (SCFA) in the study sample according to the intake of soluble and insoluble fibers categorized into tertiles.

	Soluble fiber (g/day)			Insoluble fiber (g/day)		
	Tertile 1^{α}	Tertile 2^{β}	Tertile 3^{γ}	Tertile 1^{δ}	Tertile 2^{ε}	Tertile 3^{ζ}
Microbiota (Log n°. cells per gram feces) (n=135)						
Akkermansia	$5.76 \pm 2.45_{a}$	$5.73 \pm 2.23_{a}$	$6.10 \pm 1.83_{a}$	$5.74 \pm 2.49_{a}$	$6.07 \pm 2.18_{a}$	$5.81 \pm 1.85_{a}$
Bacteroides-Prevotella-Porphyromonas	$8.27 \pm 2.14_{a}$	$8.46 \pm 2.11_{a,b}$	$9.14 \pm 1.07_{b*}$	$8.36 \pm 2.20_{a}$	$8.40 \pm 2.04_{a}$	$9.12 \pm 1.08_{a}$
Bifidobacterium	$7.41 \pm 2.25_{a}$	$7.73 \pm 1.93_{a,b}$	$8.36 \pm 0.69_{b**}$	$7.50 \pm 2.29_{a}$	$7.77 \pm 1.89_{a,b}$	$8.24 \pm 0.81_{b*}$
Clostridium cluster XIVa	$7.07 \pm 2.38_{a}$	$7.44 \pm 2.23_{a}$	$7.90 \pm 1.38_{a}$	$6.88 \pm 2.40_{a}$	$7.71 \pm 2.14_{a,b}$	$7.82 \pm 1.40_{b*}$
Lactobacillus group	$6.01 \pm 2.00_{a}$	$5.85 \pm 1.95_{a}$	$6.32 \pm 1.22_{a}$	$5.93 \pm 2.03_{a}$	$6.08 \pm 1.89_{a}$	$6.19 \pm 1.27_{a}$
Faecalibacterium prausnitzii	$6.96 \pm 1.52_{a}$	$7.37 \pm 1.51_{a,b}$	$7.57 \pm 0.86_{b*}$	$7.01 \pm 1.58_{a}$	$7.33 \pm 1.47_{a}$	$7.55 \pm 0.84_{a}$
SCFA (mM) (n=128)						
Acetic acid	$33.97 \pm 21.22_{a}$	$35.71 \pm 16.63_{a}$	$37.60 \pm 18.57_{a}$	$31.66 \pm 19.26_{a}$	$37.13 \pm 18.38_{a}$	$38.24 \pm 18.69_{a}$
Propionic acid	$12.72 \pm 8.35_{a}$	$11.68 \pm 6.01_{a}$	$14.51 \pm 7.43_{a}$	$11.57 \pm 7.28_{a}$	$11.97 \pm 6.41_{a}$	$15.34 \pm 7.91_{b*}$
Isobutyric acid	$1.61 \pm 1.01_{a}$	$1.53 \pm 0.68_{a}$	$1.97 \pm 1.77_{a}$	$1.55 \pm 0.90_{a}$	$1.55 \pm 0.67_{a}$	$2.02 \pm 1.84_{a}$
Butyric acid	$8.89 \pm 6.41_{a}$	$9.44 \pm 5.66_{a,b}$	$12.57 \pm 9.39_{b*}$	$8.18 \pm 5.79_{a}$	$9.16 \pm 4.96_{a}$	$13.48 \pm 9.78_{b**}$
Isovaleric acid	$2.20 \pm 1.50_{a}$	$2.17 \pm 0.98_{a}$	$2.73 \pm 2.55_{a}$	$2.15 \pm 1.46_{a}$	$2.21 \pm 0.99_{a}$	$2.77 \pm 2.59_{a}$

Results derived from ANOVA analyses are presented as estimated marginal mean ± standard deviation. Range of soluble fiber intake according to tertiles: $^{\alpha}$Tertile 1 (0.62-1.91 g/day), $^{\beta}$Tertile 2 (1.92-2.70 g/day), $^{\gamma}$Tertile 3 (2.71-6.88 g/day). Range of insoluble fiber intake according to tertiles: $^{\delta}$Tertile 1 (1.22-9.63 g/day), $^{\varepsilon}$Tertile 2 (9.64-14.01 g/day), $^{\zeta}$Tertile 3 (14.02-30.70 g/day). Different subscript letters indicate significant statistical differences. *$p \leq 0.05$; **$p \leq 0.01$.

Table 5 Results obtained from a stepwise multiple regression analysis to identify dietary predictors of fecal microbiota levels (log n^{o}. cells per gram feces) and fecal short chain fatty acids (SCFA) (mM).

	Predictors	**R^2**	**β**	**ρ valor**
Microbiota				
Bacteroides-Prevotella-Porphyromonas[a]	HPP (g/day)	0.080	0.284	0.004
Bifidobacterium[b]	HPP (g/day)	0.312	0.312	0.002
Clostridium cluster XIVa[c]	Age (y)	0.228	-0.477	< 0.001
Faecalibacterium prausnitzii[d]	Age (y)	0.311	-0.557	< 0.001
SCFA				
Propionic acid[e]	Age (y)	0.238	-0.368	< 0.001
	Insoluble fiber (g/day)		0.258	0.007
Butyric acid[f]	HPP (g/day)	0.194	0.441	< 0.001

Results derived from stepwise regression analysis; R^2: coefficient of multiple determinations; β: standardized regression coefficient. Variables included[a]: extractable polyphenols EPP), non-extractable polyphenols (NEPP) , non-extractable proanthocyanidins (NEPA) , hydrolyzable polyphenols (HPP) and soluble fiber. Variables included[b]: EPP, NEPP, NEPA, HPP, soluble and insoluble fiber. Variables included[c]: NEPP, NEPA, HPP and insoluble fiber. Variables included[d]: EPP, NEPP, NEPA, HPP and soluble fiber. Variables included[e]: insoluble fiber. Variables included[f]: EPP, NEPP, NEPA, HPP, soluble and insoluble fiber. Age, gender, body mass index, alcohol consumption and physical activity were introduced as covariates. Only significant results are presented.

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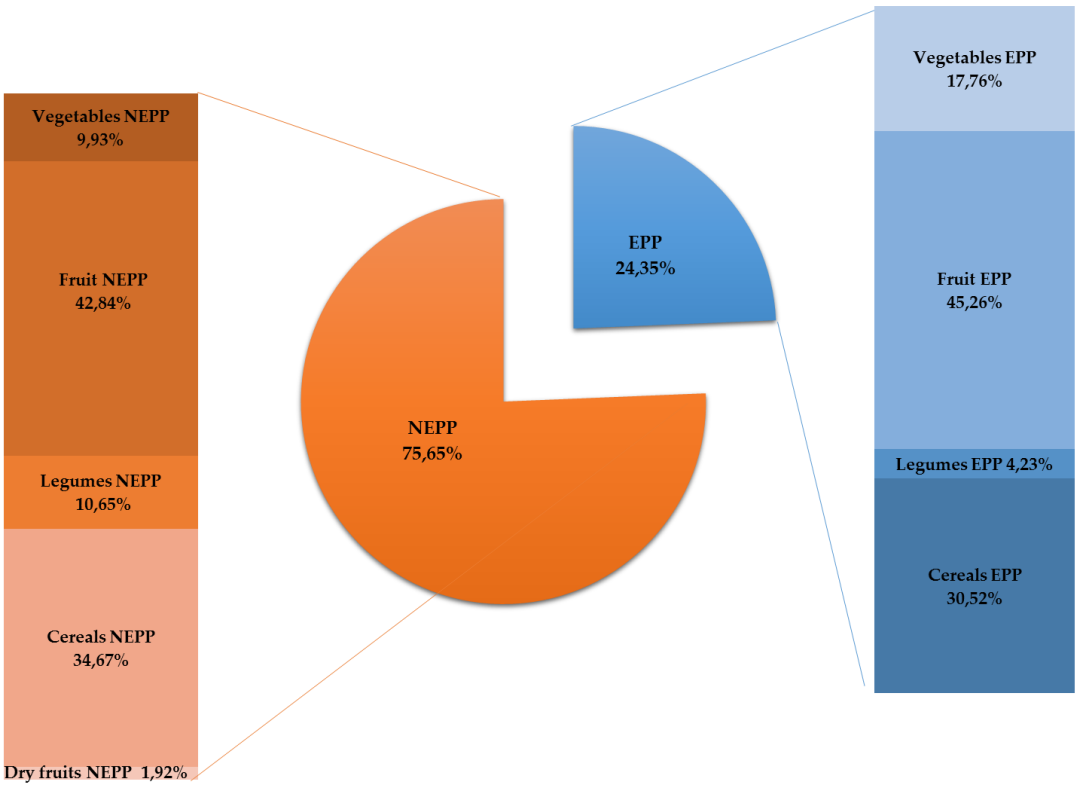

Fig. 1 Contribution (%) of the different types of polyphenols according to their solubility and foodstuff to the total polyphenol intake. EPP, extractable polyphenols; NEPP, non – extractable polyphenols; NEPA, non – extractable proanthocyanidins; HPP, hydrolyzable polyphenols.

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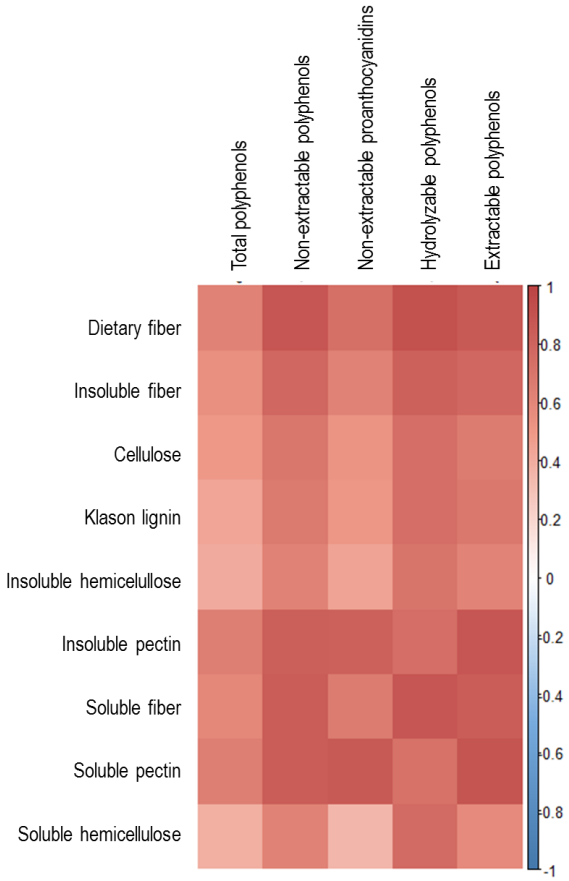

Fig. 2 Pearson correlation between phenolic intake (g/day) and dietary fiber (g/day). Columns correspond to extractable and non-extractable polyphenols; rows correspond to dietary fiber. Blue and red colours denote negative and positive association, respectively. The intensity of the colours represents the degree of association between these dietary polyphenols and the fiber matrix. All correlations were significant ($p \leq 0.01$).

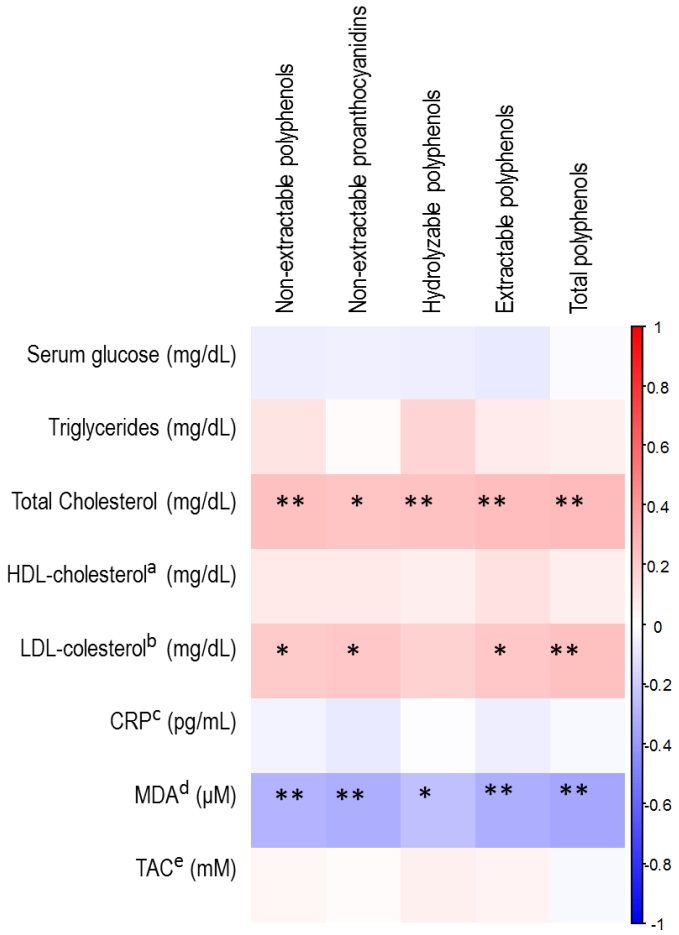

Fig. 3 Pearson correlation between phenolic intake (g/day) and blood biomarkers. Columns correspond to extractable and non-extractable polyphenols; rows correspond to biomarkers. Blue and red colours denote negative and positive association, respectively. The intensity of the colours represents the degree of association between these dietary polyphenols and the fiber matrix and asterisks indicate significant associations: *p≤0.05; **p≤0.01. [a]HDL, high-density lipoprotein. [b]LDL, low-density lipoprotein. [c]CRP, C-reactive protein. [d]MDA, malondialdehyde. [e]TAC, total antioxidant capacity.

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