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Study of antioxidant capacity and metabolization of quebracho and chestnut tannins through *in vitro* gastrointestinal digestion-fermentation

Silvia Molino[a], Mariano Fernández-Miyakawa[b], Samuele Giovando[d], José Ángel Rufián-Henares[a,c,*]

[a] Departamento de Nutrición y Bromatología, Instituto de Nutrición y Tecnología de los Alimentos, Centro de Investigación Biomédica, Universidad de Granada, Spain
[b] Instituto de Patobiología, Instituto Nacional de Tecnología Agropecuaria/Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina
[c] Instituto de Investigación Biosanitaria ibs.GRANADA, Universidad de Granada, Spain
[d] Centro Ricerche per la Chimica Fine Srl, San Michele Mondovì (CN), Italy

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ABSTRACT

Quebracho (QUE) and chestnut (CHE) are natural sources of tannins, but there are no connection between QUE, CHE and human health. The study investigated the antioxidant response and metabolization of tannin extracts trough *in vitro* digestion-fermentation. The FRAP assay pointed a higher reducing capacity of CHE than QUE (6.90 vs. 5.07 mmol Trolox/g), in contrast to a stronger scavenging activity of QUE (8.16 mmol Trolox/g vs 6.70 mmol Trolox/g). The results obtained showed a decrease of the antioxidant capacity of tannins after microbial fermentation, but a high prebiotic activity through release of short-chain fatty acids of both CHE (11.14 mmol/g) and QUE (4.79 mmol/g) was observed. The UPLC-MS investigations on digested and fermented tannins gave an identification and semi-quantification of 18 compounds, including hydrolysable and condensed tannins and their metabolites. The results represent a valid basis for further studies on the potential use of these wood extracts in human diet.

1. Introduction

Tannins are secondary metabolites widely distributed in the plant kingdom, extracted from many types of trees and plants and can be present in barks, leaves, wood and also in fruits and roots. These plants generally used for tannin production may contain up to 40% tannin by weight (van Diepeningen et al., 2004). Most raw materials used for industrial production are Chestnut wood (18% of tannin in wood on dry matter), Quebracho hardwood and Mimosa barks (24% in both), Tara pods and Chinese or Turkish gallnut (50% in both). In particular, *Schinopsis lorentzii* Engl. and *Schinopsis balansae* Engl., known as red quebracho, are evergreen tree species widespread in the dense subtropical forests of Gran Chaco (Argentina), Bolivia and Paraguay, while *Castanea sativa* Mill. trees, commonly named chestnut, is found across the Mediterranean region.

The quebracho wood extract (QUE) is among the most industrially produced source of tannins, predominantly composed of oligomers of profisetinidins. It is composed of oligomers in which flavonoid units are condensed together to obtain molecular weight (MW) ranging from 1.000 to 20.000 Da. The composition of QUE was described by Pasch, Pizzi, and Rode (2001) using MALDI-TOF mass spectrometry and Venter, Senekal, et al. (2012), Venter, Sisa, et al. (2012) reported a more detailed description of the linear structures of the polyanthocyanidins oligomers. CHE is characterized by the presence of hydrolysable tannins, composed of a carbohydrate core whose hydroxyl groups are esterified with phenolic acids with a MW ranging from 300 to 5.000 Da (Mueller-Harvey & McAllan, 1992). The chemical composition of CHE has been clarified by using MALDI-TOF mass spectrometry which states that it is an ellagic-type hydrolysable tannin (Pasch & Pizzi, 2002). Castalagin, with the isomer vescalagin, represents around 30% of the product. It has been shown that these substances and their higher oligomers are present in this tannin and are quite stable since they come from the rearrangement of polypentagalloyl glucose, naturally occurring in chestnut wood (Pasch & Pizzi, 2002). The higher oligomers contain repeating units of polygalloyl glucose chain, where galloyl groups can be linked differently to each other (Radebe, Rode,

Abbreviations: QUE, quebracho wood extracts; CHE, chestnut wood extract; SCFAs, short-chain fatty acids; GAR+, global antioxidant response+; MW, molecular weight; FRAP, ferric reducing ability of plasma; ABTS, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)

* Corresponding author at: Departamento de Nutrición y Bromatología, Facultad de Farmacia, Universidad de Granada, Campus Universitatio de Cartuja, 18071 Granada, Spain.
E-mail address: jahrufian@ugr.es (J.Á. Rufián-Henares).

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Pizzi, Giovando, & Pasch, 2013).

QUE and CHE are already commercialized in animal feeding, especially for cattle and poultry (Buccioni et al., 2017; Carrasco et al., 2018). Several studies have reported that addition of QUE and CHE to animal feed improved the nutrition and the animal health in both ruminants and monogastric animals (Buccioni et al., 2017; Diaz Carrasco et al., 2016; Henke et al., 2017; López-Andrés et al., 2013; Redondo, Chacana, Dominguez, & Fernandez Miyakawa, 2014) Due to their chemical composition, these compounds exert antiviral, antimicrobial, scavenging and antimutagenic effects locally in the intestine as unabsorbable complex structures (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009). However, since a little amount of tannins or their metabolites might be absorbed from the gastrointestinal tract, systemic effects associated to improvement of endogenous antioxidant activity in different organs are observed (Cires, Wong, Carrasco-Pozo, & Gotteland, 2017; Serrano et al., 2009). Tannins may interfere with the digestion of nutrients, binding proteins or delaying the absorption of sugar and lipids (Cires et al., 2017). In particular, complexes with proteins are given by the tannins numerous hydroxyl groups and depend on proline content and size of the proteins (Hagerman & Butler, 1981). Given the antinutrient effect, these compounds could be studied as functional substances. There are studies that reported the possible use of tannins in nutrition for celiac disease, by their potential cross-linking activity of wheat gluten (Girard, Bean, Tilley, Adrianos, & Awika, 2018), or in diabetes mellitus thanks to their anthyperglycemic activity (Cires et al., 2017; Williamson, 2013; Yin et al., 2011).

Even though tannins have shown important biological properties, little is known about the bioavailabilty and biological effects of QUE and CHE extracts in humans. In particular, in order to understand how these extracts determine health benefits, it is crucial to study the metabolization of tannins during digestion and fermentation processes. Several authors have tried to understand the metabolisation of tannins contained in fruits (i.e. apples or grapes) or directly of molecules like proanthocyanidins (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009; Aura et al., 2013; Bazzocco, Mattila, Guyot, Renard, & Aura, 2008; Stoupi, Williamson, Drynan, Barron, & Clifford, 2010), evidencing the production of phenolic acids such as 2-(3,4-dihydroxyphenyl) acetic acid, 2-(3-hydroxyphenyl) acetic acid, 2-(4-hydroxyphenyl) acetic acid and 3-(3-hydroxyphenyl) propionic acid.

Taking all this information into account, the overall aim of the present report was to unravel the possible use of QUE and CHE as potential ingredients for the development of functional foods. In order to reach this goal, three different sub-objectives were defined, after *in vitro* digestion and fermentation of the samples: (i) To study the global antioxidant response of QUE and CHE. (ii) To determine their bioactivity on the gut microbiota, reflected by the production of short chain fatty acids (SCFAs). (iii) To investigate the evolution of the polyphenolic profile of tannins extracts.

2. Material and methods

2.1. Plant material, chemicals and reagents

Tannin extracts (QUE and CHE), obtained by hot water extraction, are commercialized by Silvateam Spa (San Michele di Mondoví, Italia) as powder. QUE, a profisetinidin condensed tannin of 6,25 of degree of polymerization, was previously characterized by Pasch et al. (2001). The composition of CHE, a hydrolysable ellagitannin with 30% of isomers castalagin and vescalagin as representative substances, was described by Pasch & Pizzi (2002). Reagents and inulin were from Sigma-Aldrich (Germany) and Alpha-Aesar (United Kingdom). All chemical reagents used for all the assays, digestion, fermentation, as well as HPLC and UPLC-ESI-MS analysis were of analytical grade.

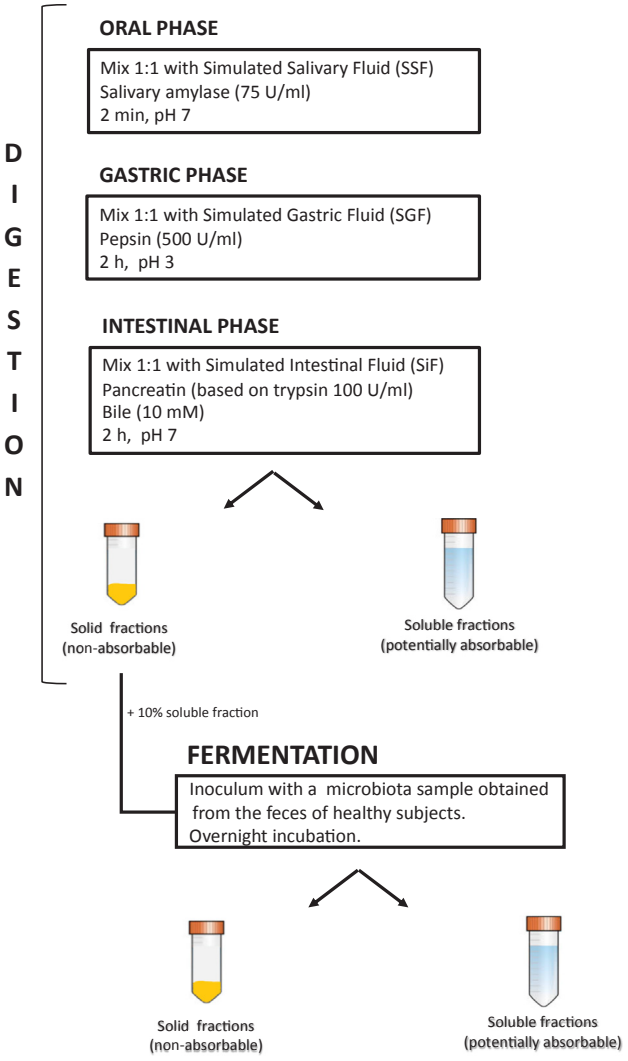


Fig. 1. Schematic description of the *in vitro* digestion and fermentation processes.

2.2. In vitro digestion-fermentation

The *in vitro* digestion-fermentation (Fig. 1) was performed as described by the GAR + method (Pérez-burillo, Rufián-henares, & Pastoriza, 2018a). The samples were subjected to oral, gastric and intestinal digestion and then to a further step of fermentation with the microbiota present in faeces, obtained from healthy donors (Mean Body Index = 21.2). In the oral phase, 5 mL of simulated salivary fluid with α-amylase and 25 μL of $CaCl_2$ were added to 5 g of tannin extract, following incubation at 37 °C for 2 min. Then, 10 mL of simulated gastric fluid with pepsin and 5 μL of $CaCl_2$ were added and the pH was lowered to 3.0 by adding 1 N HCl; the mixture was then incubated at 37 °C for 2 h. Finally, 20 mL of simulated intestinal fluid with bile salts, pancreatin and 40 μL of $CaCl_2$ were added and the pH was raised to 7.0 with 1 N NaOH. The mixture was incubated at 37 °C for 2 h. After that, the tubes were immersed in iced water to stop the enzymatic reactions. A centrifugation of the mixture at 6000 rpm for 10 min at 4 °C enabled the separation of the solid fractions from the supernatants (potentially absorbable solution). Then, 10% of the supernatant was added to the solid residue in order to mimic the fraction that is not readily absorbed after digestion, while the remaining part was stored at −80 °C until further analysis.

The digested wet-solid residues (500 mg) derived from the digestion process were subjected to fermentation (Pérez-burillo, Rufián-Henares,

& Pastoriza, 2018b), by adding 7.5 mL of fermentation final solution (peptone water + resazurine) and 2 mL of inoculum (32% faeces in phosphate buffer 100 mM, pH 0 7.0). Anaerobic atmosphere was produced by bubbling nitrogen through the mix; then the mix was incubated at 37 °C for 20 h under oscillation. Immediately afterwards, the samples were immersed in ice, to stop microbial activity, and centrifuged at 6000 rpm for 10 min. The supernatant was collected as a soluble fraction potentially absorbed after fermentation and stored at -80 °C. The solid residue, representing the non-absorbed fraction after fermentation, was also stored in order to measure the direct antioxidant capacity.

2.3. Antioxidant capacity

The antioxidant capacity was assessed on three fractions (the supernatants derived from digestion and fermentation and the solid residue remaining after fermentation), in order to evaluate the global antioxidant response (GAR+) of the extracts (Pérez-burillo et al., 2018a, 2018b). The antioxidant capacity was determined with two different methods, presented below [FRAP (ferric reducing ability of plasma) and ABTS (2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) methods]. All the results were corrected considering their respective blanks (enzymes, chemicals and inoculum).

2.3.1. Antioxidant activity

$TEAC_{FRAP}$ and *$TEAC_{ABTS}$ assays* were carried on the supernatants from digestion and fermentation, using 20 µL of sample and 280 µL of reagent solution (FRAP or ABTS). The solid residues were analysed following the QUENCHER procedure described by Gökmen, Vural Serpena, and Fogliano (2009). This procedure was applied to the lyophilized insoluble fractions resultant after fermentation, using 3 mg of sample and 6 mL of reagent solution (FRAP or ABTS).

$TEAC_{FRAP}$ assay. The ferric reducing antioxidant power was estimated as described by Benzie and Strain (1996), adapted to a microplate reader. Trolox stock solutions ranging from 0.01 to 1.00 mg/mL were used to perform the calibration curves. The results obtained are expressed as mmol Trolox equivalents per g of fresh sample.

$TEAC_{ABTS}$ assay. The radical scavenging activity was evaluated following the procedure by Re et al. (1999). The antioxidant capacity was estimated in terms of radical scavenging activity. Calibration was performed with a Trolox stock solution, used as reference standard (0.01–1.00 mg/mL). The results obtained were expressed as mmol Trolox equivalents per g of fresh sample.

2.3.2. Global antioxidant response+ (GAR+)

GAR + reflect the overall complete antioxidant capacity of the analysed products. In this way, it is calculated as the resultant from the sum of the antioxidant capacity of the supernatant obtained after digestion (potentially bioaccessible), the supernatant obtained after fermentation (potentially bioaccessible) and the solid residue remaining after fermentation (non bioaccessible). In the supplemental information of Perez-Burillo, Rufian-Henares, and Pastoriza (2018) are indicated as all the factors to take into account in order to estimate the correct antioxidant capacity through the digestion and fermentation processes.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2018.07.056.

2.4. SCFAs analysis

Three different SCFAs were determined by HPLC: acetic, propionic, and butyric acids. Standard solutions were quantified with concentrations ranging from 10,000 to 5 ppm. Inulin, a commercial prebiotic fibre, was also analysed for comparison. The sample did not require any pre-treatment before injection. Briefly, the SCFA standards were prepared in the mobile phase at concentrations ranging from 5 to 10,000 ppm. After the fermentation process, 1 mL of supernatant from the fermentation was centrifuged to remove solid particles, filtered through a 0.22 µm nylon filter, and finally transferred to a vial for HPLC analysis. The results were expressed as mmol of SCFA per g of dry food.

2.5. Extraction procedure for mass spectroscopy analysis

Mass spectroscopy (MS) analysis was conducted on the supernatants derived from digestion and the supernatants obtained after the fermentation process. The extraction was achieved following the discontinuous extraction method, adapted to micro-extraction, described by Esteban-Muñoz Barea-Álvarez, Oliveras-López, Giménez-Martínez, Rufián-Henares and Olalla-Herrera (2018). Briefly, 1 mL of each sample was mixed with diethyl ether (50:50, v/v) and left at 4 °C for 18 h under darkness. The supernatants were collected and the pellets were mixed with diethyl ether (50:50, v/v). After 5 min the supernatants were collected, and the step was repeated once. Then, the supernatants were combined, desiccated with anhydrous sodium sulphate and evaporated to dryness. The resulting residue was re-collected from the flask with 1 mL of a 50:50 water/methanol solution and the mixture was well shaken. The extracts were filtered with 20 µm Waters Millipore membrane and stored at 4 °C until further use.

2.6. UPLC-DAD-ESI-QTOF-MS analysis

Chromatographic separation was performed with an Acquity Ultra Performance Liquid Chromatograph (UPLC) system (Waters, Milford), UPLC column—ACQUITY UPLC BEH C18 2.1 mm × 100 mm, 1.7 µm (Waters) with precolumn in-line filter (Waters, Milford). The mobile phase consisted of solvents A (water containing 0.5% acetic acid) and B (100% acetonitrile). The column temperature was set at 40 °C, and the mobile phase flow rate at 0.4 mL/min. Analysis started with 95% A and 5% B; then the concentration of A was decreased to 5% (15 min). Finally, initial gradients were recovered over 0.10 min to re-equilibrate the column for 2.9 min.

Mass data, obtained on a Sinapt G2 TOF in negative ion electrospray ionization (ESI), were recorded in the range of 50–1200 *m/z*. Source operating parameters are defined hereafter: capillary and cone voltages were set at 2.5 kV and 35 V respectively, the desolvation temperature at

Table 1
Antioxidant capacity obtained from FRAP and ABTS assays.

	$TEAC_{FRAP}$		$TEAC_{ABTS}$	
	QUE	*CHE*	*QUE*	*CHE*
Supernatant from digestion	$2.33 \pm 0.02^{a,b,A}$	$4.40 \pm 0.05^{a,A}$	$3.29 \pm 0.16^{a,A}$	$5.70 \pm 0.16^{a,A}$
Supernatant from fermentation	$2.52 \pm 0.01^{a,B}$	$2.43 \pm 0.05^{b,B}$	$2.07 \pm 0.078^{b,B}$	$2.30 \pm 0.15^{b,B}$
Solid from fermentation	$0.22 \pm 0.001^{b,C}$	$0.056 \pm 0.001^{b,C}$	$1.35 \pm 0.22^{c,C}$	$0.16 \pm 0.02^{c,C}$
Total – GAR+	5.07	6.90	6.70	8.16

Results expressed as mmol Trolox equivalents per g of fresh sample. The data represent means ± standard deviation (n = 3).
Different lower-case letters within the same column and upper-case letters for the same antioxidant assay indicate statistically significant differences ($p < 0.05$). In the table, QUE:Quebracho wood extract; CHE: Chestnut wood extract; GAR+: Global Antioxidant Capacity.

500 °C and the source temperature at 100 °C. UPLC system and Mass spectrometer were controlled by MassLynx® v4.1 software (Waters, Milford, USA). In particular, the analysis of MS spectra was performed with MarkerLynx, a suite included within the MassLynx software. Identification of the peaks and quantification was carried out with standard solutions (for gallic acid, syringic acid, vanillic acid, p-coumaric acid, ellagic acid and quercetin). The determination of the compounds without standard was performed with the mass spectral data obtained on Q-TOF (Table 1). The molecules separated by UPLC were analysed by MassLynx in their $[M-H]^-$ deprotonated molecular formulas together with the interpretation of MS fragmentations. The selected compounds were then searched against the Kyoto Encyclopaedia of Genes and Genomes (KEGG) and Chemspider. The peak area of the identified compounds was used to provide semi-quantitative information for comparison purposes (Table 3).

2.7. Statistical analysis

All measurements were performed at least in triplicate and expressed as means ± standard deviations. Statistical analysis of the data was carried out with Statgraphics Centurion XVI by using analysis of the unpaired Student's *t*-test in order to determine the statistical significance ($p < 0.05$) between different groups.

3. Results and discussion

3.1. Antioxidant capacity as a result of in vitro gastrointestinal digestion

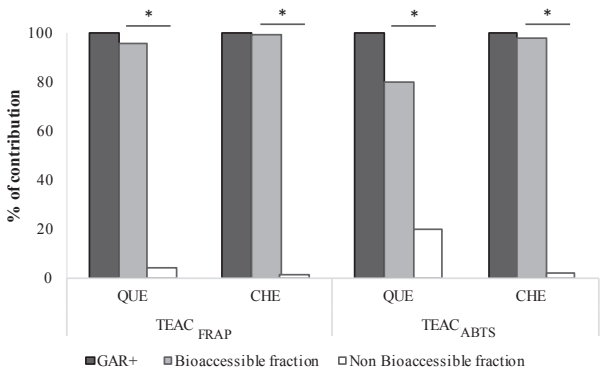
Global antioxidant capacity (GAR+) has been established as a suitable method to evaluate total antioxidant capacity of fresh and processed foods (Pastoriza, Delgado-Andrade, Haro, & Rufián-Henares, 2011). The GAR+ was determined in the soluble fraction obtained after enzymatic digestion, as well as in the soluble and insoluble fractions of fermented QUE and CHE. As GAR+ is the total antioxidant capacity of the three fractions, it is expressed as a single value. Table 1 and Fig. 2 present the results obtained by $TEAC_{FRAP}$ and $TEAC_{ABTS}$ assays. CHE had a higher reducing capacity (6.90 mmol Trolox/g) than QUE (5.07 mmol Trolox/g) with the $TEAC_{FRAP}$ assay (Table 1). Comparing these values with those reported by Pérez-burillo et al. (2018a, 2018b), both QUE and CHE have an antioxidant capacity 70–820 fold higher than regular foods (i.e. yoghurt, whole grain bread, lentils and peanuts). This potent reducing activity could be explained by the composition of the extracts, which are polyphenols concentrates. In particular, in the case of QUE, it was calculated by Venter, Senekal, et al. (2012), Venter, Sisa, et al. (2012) that the 95% consisted of proanthocyanidins, while only the remaining 5% were soluble sugars.

Subsequently, the contribution of the bioaccessible fractions (supernatants from digestion and fermentation) vs. the solid residue to the global reducing capacity were compared (Fig. 2). The antioxidant capacity of supernatants of both QUE and CHE was significantly higher ($p < 0.05$) than the solid residues. This means that in general, tannins extracts could exert their reducing activity not only in the intestinal tract, but also at systemic level, possibly reaching several organs. The high contribution of QUE and CHE supernatants (95.7 and 99.2%, respectively) suggest that most of the reducing capacity is exerted by the soluble fractions, with a small reducing capacity released in the solid residue. The liquid fraction obtained after digestion of QUE was statistically ($p < 0.05$) less antioxidant than that obtained after fermentation (Table 1), reinforcing the idea of the release of bioactive compounds (mainly polyphenols and their metabolites) due to the activity of microbiota enzymes. On the contrary, the 1.8-fold decrease of the reducing capacity of CHE after fermentation, indicate that this extract is better metabolized through the intestinal enzymatic action.

Regarding the $TEAC_{ABTS}$ method, the global antioxidant capacity ranged from 6.7 (CHE) and 8.16 (QUE) mmol Trolox per g of sample (Table 1). The results are 39–146 times higher than those of fresh and processed foods reported by Pérez-burillo et al. (2018a, 2018b). Similar to the $TEAC_{FRAP}$, the discrepancy with foods could be explained by the high content in phenolic compounds of the studied extracts. The contribution of the bioaccessible fractions of CHE was statistically higher ($p < 0.05$) than those of QUE, which accounted 98.1 and 79.9% of the total, respectively (Table 1). The outstanding data is the high level of scavenging activity against ABTS radical recorded for the solid residue of QUE, contrary to the reducing activity measured with the FRAP method. Thus, despite QUE presented the highest GAR+, less than half could be absorbed. Finally, the bioaccessible fractions obtained after digestion were 1.6 and 2.5 times more antioxidant ($p < 0.05$) than those released after microbial fermentation for QUE and CHE, respectively (Table 1). Thus, the metabolites released after digestion not only displayed an important reducing capacity but also and even stronger antiradical activity.

3.2. Potential prebiotic activity

The analysis of SCFAs is a good indicator of the effect of a foodstuff on the gut microbiota (Pérez-burillo et al., 2018a). The high content of SCFAs evidences the fermentative activity, as by-products of the colonic microbiota metabolism. Some recent studies suggest that polyphenols and their metabolites could selectively stimulate some microorganisms metabolic pathways, like SCFAs production (Bolca, Van de Wiele, & Possemiers, 2013; Tzounis et al., 2011). Fig. 3 depicts the levels of total SCFAs, acetate, propionate and butyrate released after fermentation of QUE and CHE extracts. CHE gave rise to a higher generation of total



* Indicates statistically significant differences ($p < 0.05$).

Fig. 2. Contribution of bioaccessible fractions (supernatants from digestion and fermentation) and solid residue to the antioxidant capacity of foods.

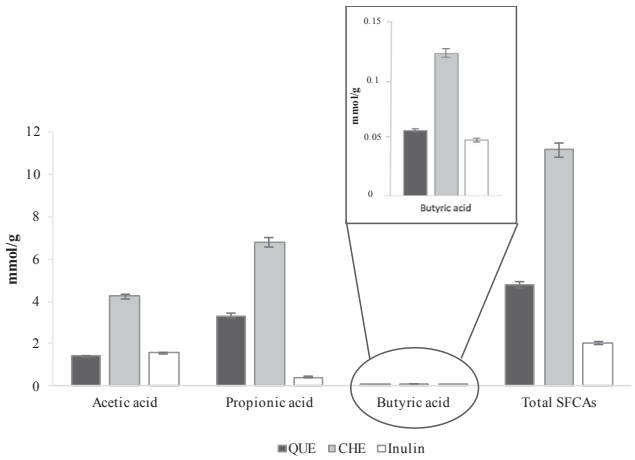


Fig. 3. Release of SCFAs (mmol per g of fresh food) of QUE and CHE after fermentation.

SCFAs in comparison to QUE (11.14 and 4.79 mmol/g, respectively).

When the production of individual SCFAs was analysed, statistically significant differences ($p < 0.05$) were observed between the two tannin extracts after the fermentation for acetic, propionic and butyric acids, being higher for CHE (Fig. 3). This difference might be explained by the different composition of these products; while QUE principally consists of a mixture of condensed tannins, CHE possesses a high concentration of hydrolysable tannins, which probably are easily available for microbial fermentation. Comparing results with those of inulin fermentation, lower levels of only acetic acid were obtained in QUE treatment. Propionic acid values were higher for both extracts (7.8 and 16-times higher for QUE and CHE, respectively). While the amount of butyrate obtained for QUE was almost the same as inulin, CHE were 2.6 times higher. It should be taken into account that inulin is a fructose polymer and the most common energy sources of microbiota associated with SCFAs production are carbohydrates that escape absorption in the small intestine. Therefore, given the composition of the analysed extracts (mainly polyphenols), the generation of SCFAs by the gut microbiota was surprisingly high. Aura et al. (2013) reported similar values for the production of total SCFAs by proanthocyanidin, while grape and wine was reported as a higher releaser, probably due to the presence in the food matrixes of polysaccharides such as pectins. On the other hand, another study conducted on apple and ciders reported low levels of production of total SCFAs, despite the content of polysaccharides (Bazzocco et al., 2008).

According to Gibson et al. (2017), tannins could be suggested as important prebiotic substrates, since they are fermented by gut microbiota, producing SCFAs. It is noteworthy to mention that the relationship between gut microbiota and tannins is bi-directional, since tannins are metabolized by the gut microbiota but also tannins act on different colonic pathways and processes due to their bacteriostatic-bactericidal activity (Duda-Chodak, Tarko, Satora, & Sroka, 2015). The relative proportions of SCFAs in the gastrointestinal tract can vary depending on (i) the fermented substrate, (ii) individuals, (iii) different life stages. The predominant SCFAs in the human gut is acetic acid, followed by propionic and butyric acids (Verbeke et al., 2015). In this study, the main SCFA released after fermentation was propionic acid, for both QUE and CHE, followed by acetic and butyric acids. In support of these findings, Pereira-Caro, Borges, et al. (2015), Pereira-Caro, Oliver, et al. (2015) reported that orange juice polyphenols determined the generation of acetate and propionate by the gut microbiota. More specifically, the bioavailability of orange juice polyphenols depends on the production of hydroxy- and methoxyphenyl propionic acids by the gut microbiota, metabolites that are readily absorbed.

Table 2
Tannins and metabolites identified in supernatants derived from digestion and fermentation of QUE and CHE.

N.	Compound	Molecular formula	m/z $[M-H]^-$	MS fragments	RT (min)
1.	Gallic acid	$C_7H_6O_5$	167.0137	125.0244	1.59
2.	Catechin	$C_{15}O_6H_{14}$	289.0712	271.0611; 151.04; 137.0244	2.91
3.	Procyanidin B1	$C_{30}O_{12}H_{26}$	577.135	289.0717; 141.04	3.31
4.	Tannin dimer	$C_{30}O_{11}H_{26}$	561.14	289.0712; 271.0611; 151.04	3.53
5.	Syringic acid	$C_9H_{10}O_5$	197.0450	169.0506; 153.0557	4.03
6.	Epicatechin gallate	$C_{22}O_{10}H_{18}$	441.08	303.05; 169.014	4.05
7.	Pentagally glucose	$C_{41}O_{26}H_{32}$	939.1104	787.09; 169.0172	4.18
8.	Tannin trimer	$C_{45}O_{16}H_{38}$	833.2082	681.16; 561.14; 151.04	4.44
9.	Vanillic acid	$C_8O_4H_8$	167.034	123.00	4.52
10.	Gallocatechin	$C_{15}O_7H_{14}$	305.66	167.0414; 137.0211	4.53
11.	Tannin tetramer	$C_{60}O_{21}H_{50}$	1105.2766	833.2082; 681.16; 561.14	4.67
12.	*p*-Coumaric acid	$C_9O_3H_8$	163.039	–	5.06
13.	Sinapinic acid	$C_{11}O_5H_{12}$	223.0606	–	5.45
14.	Urolithin A	$C_{13}O_4H_8$	227.034	199.04; 183.04	5.64
15.	Urolithin B	$C_{13}O_3H_8$	211.039	183.04	5.98
16.	Hydroxyphenyl acetic acid	$C_8O_3H_8$	151.04	107.05	6.19
17.	Ellagic acid	$C_{14}O_8H_6$	300.998	257,009	6.78
18.	Quercetin	$C_{15}O_7H_{10}$	301.0353	283.0248; 273.04	6.79

Table 3
Semi-quantitative analysis of the soluble tannins and their metabolites in supernatants derived from digestion and fermentation of QUE and CHE.

N.	Compound	BPQ digested		BPQ fermented		CHE digested		CHE fermented	
		mDa	Area/g	mDa	Area/g	mDa	Area/g	mDa	Area/g
1.	Gallic acid	1.5	1.72×10^3	0.3	6.56×10^5	–	–	−0.4	2.16×10^4
2.	Catechin	2.8	3.47×10^5	−0.3	2.12×10^5	−1.5	4.52×10^3	0.7	2.91×10^4
3.	Procyanidin B1	4.6	1.48×10^4	–	–	–	–	–	–
4.	Tannin dimer	−0.4	1.01×10^6	4.1	1.04×10^6	4.6	1.69×10^5	2.7	1.60×10^5
5.	Syringic acid	2	3.95×10^4	0.7	1.57×10^6	0.2	2.58×10^3	1.2	8.05×10^4
6.	Epicatechin gallate	2.9	1.54×10^4	−2.5	1.35×10^4	4.9	8.98×10^3	–	–
7.	Pentagally glucose	–	–	–	–	4.9	1.81×10^4	–	–
8.	Tannin trimer	0.3	1.87×10^6	−1.2	1.17×10^6	0.2	1.03×10^5	1.6	1.52×10^5
9.	Vanillic acid	0.1	1.14×10^5	−0.3	3.00×10^5	1.2	4.00×10^4	0.8	3.50×10^4
10.	Gallocatechin	0.5	4.91×10^4	5.1	4.38×10^4	5	1.17×10^4	–	–
11.	Tannin tetramer	−0.5	3.84×10^4	0.5	4.85×10^4	4.7	1.47×10^4	−1.5	6.97×10^3
12.	*p*-Coumaric acid	0.1	2.85×10^4	−0.3	1.65×10^5	0.2	2.15×10^4	0.3	8.82×10^4
13.	Sinapinic acid	–	–	2.1	2.34×10^5	–	–	0.5	7.31×10^4
14.	Urolithin A	–	–	1.3	1.02×10^5	0.5	2.62×10^4	0.7	5.24×10^5
15.	Urolithin B	–	–	–	–	1.5	3.24×10^4	0.3	8.70×10^4
16.	Hydroxyphenyl acetic acid	0.7	2.20×10^5	1	6.55×10^5	0.2	7.59×10^4	0.9	1.07×10^5
17.	Ellagic acid	0.7	6.03×10^3	0.2	2.72×10^4	−0.4	4.87×10^3	0.5	1.21×10^4
18.	Quercetin	–	–	1.3	6.10×10^4	–	–	−1.3	9.83×10^3

3.3 Mass spectrometry

Table 2 shows the 18 compounds identified and Table 3 provides a semi-quantitative information of the analysis of the supernatant obtained from digestion-fermentation of both QUE and CHE. Quantification by standard solutions of gallic acid, syringic acid, vanillic acid, and p-coumaric acid are shown in Table 4, while ellagic acid and quercetin were not quantifiable. The samples were a highly complex variety of hydrolizable tannins, condensed tannins and phenolic acids (Fig. 4). As regard QUE, it is well known that its composition is characterized by the presence of condensed tannins (Venter, Senekal, et al. (2012), Venter, Sisa, et al. (2012)). After digestion, some tannin complexes (in abundance order: tannin trimer > tannin dimer > tannin tetramer > catechins) were still found, while the pentamer, hexamer and heptamer were no longer present, probably due to the activity of digestive enzymes. As described by Venter, Senekal, et al. (2012), Venter, Sisa, et al. (2012), the oligomers are homologous series characterized by a catechin molecule and an increasing number of *ent*-fisetinidiol-4α-ol. Besides these compounds, proanthocyanidin B1, gallocatechin, and epicatechin-gallate were identified. During digestion, other small metabolites such as vanillic acid and hydroxyphenyl acetic acid were also produced. Due to the intestinal microbiota metabolism, a different profile of the bioaccessible fractions derived from fermentation was observed. As already mentioned, the predominant compounds were tannins oligomers, since they could not be metabolised. The absence of procyanidin B1 could explain the metabolization to hydroxyphenyl acetic acid, as reported also by Rios et al. (2003). The increase of the presence of small molecules, such as vanillic, syringic and p-coumaric acids derive from catechins (Cires et al., 2017) were identified. Some compounds not present before (such as the tannin monomer sinapinic acid and quercetin) were also identified.

The profile of CHE tannins has been previously described by Pasch & Pizzi (2002). Compounds, in abundance order: tannin dimer > tannin trimer > tannin tetramer > catechin, were obtained from the supernatant derived from the digestion of CHE. Nevertheless, it should be noted that the amount of tetramer was higher in the bioaccessible fraction from digestion than that obtained after fermentation. Some of the characteristic molecules of CHE, such as pentagallyglucose, gallocatechin and epicatechin, were identified only in the digested bioaccessible fraction. After digestion, large compounds such as castalagin and vescalagin described by Pasch and Pizzi (2002) were not found, probably because of their hydrolysis during the acidic gastric phase. After fermentation, the amount of dimer, trimer and catechin increased, probably due to the metabolization of the former compounds. As previously mentioned, the microbial metabolism was responsible of the production of different compounds such as sinapinic acid and quercetin, or the increase of other metabolites like hydroxyphenyl acetic, p-coumaric, vanillic acid and ellagic acids. Ellagic acid produced urolithin A and urolithin B after microbial metabolization. The production of urolithins by the gut microbiota was deeply described by Tomás-Barberán et al. (2017), that also put a focus on the different metabotype able to generate these metabolites.

The results obtained support the hypothesis about transformation of tannins during the gastrointestinal digestion and subsequent metabolization by gut microbiota. Thus, new bioactive compounds are released, which in turn could determine health effects in human beings. For example, the production from ellagitannins and ellagic acid from urolithins (A and B) is interesting, since they are absorbed in the intestine and exert anti-inflammatory and anticancer effects, as well as antimicrobial activity (Espín, Larrosa, García-Conesa, & Tomás-Barberán, 2013). Furthermore, Verbeke et al. (2015) reported specific health effects for some of the detected microbial metabolites, i.e. antimicrobial activity of hydroxyphenyl acetic acid and no oestrogenic activity for urolithin A and urolithin B. However, the physiological

Table 4
Quantification of single polyphenols (mg/kg of fresh tannin extract) insupernatants derived from digestion and fermentation of QUE and CHE.

	QUE		CHE	
	Digested fraction	Fermented fraction	Digested fraction	Fermented fraction
Gallic acid	8.92 ± 0.02	975.9 ± 2.53	N.Q.	91.6 ± 0.23
Syringic acid	180.1 ± 0.46	7581.9 ± 20.4	1.27 ± 0.01	160.14 ± 0.45
Vanillic acid	497.2 ± 1.49	1126.3 ± 2.82	96.8 ± 0.25	15.9 ± 0.05
p-Coumaric acid	162.2 ± 0.41	808.5 ± 2.18	71.4 ± 0.19	360.2 ± 0.83

N.Q. – not quantifiable.

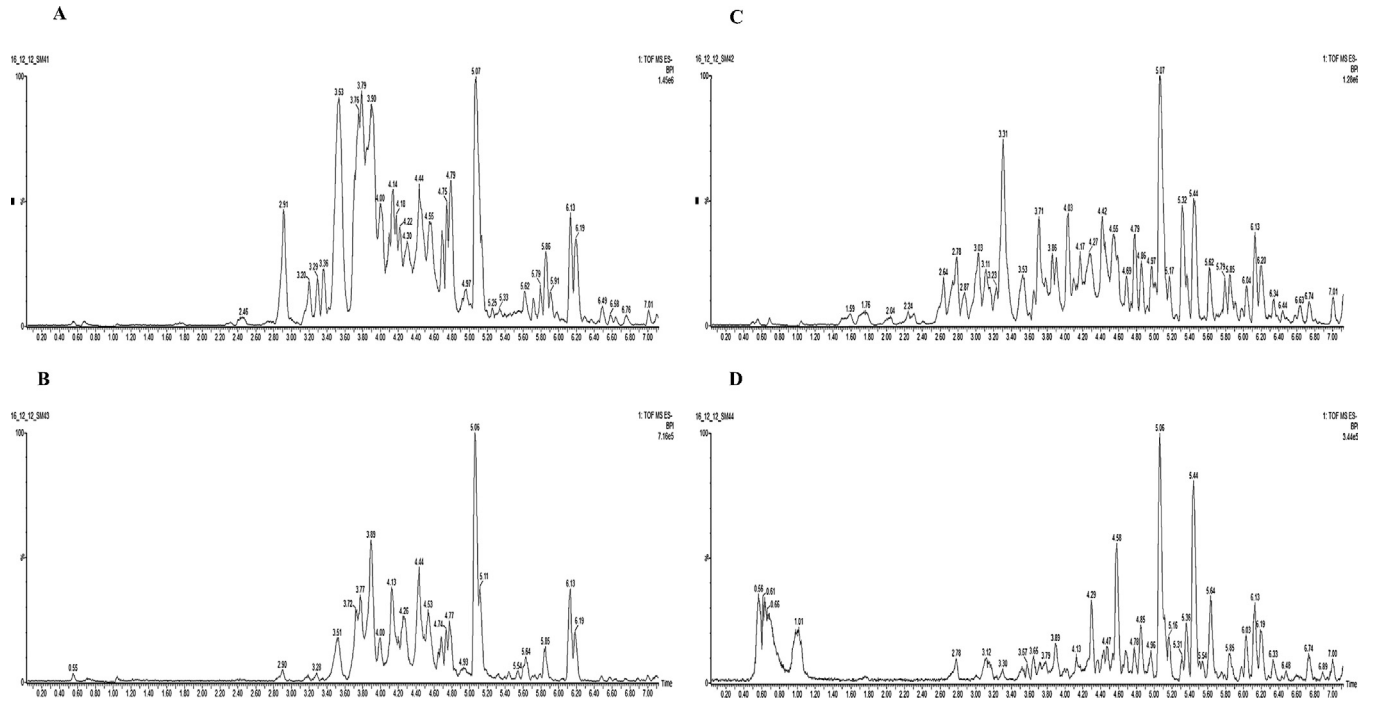


Fig. 4. UPLC-MS total ion current (TIC) chromatograms of supernatants derived from digestion and supernatants derived from fermentation of QUE (3A and 3B) and CHE (3C and 3D).

relevance of other compounds is still unknown and deserves further studies.

4. Conclusion

This is the first study that reports the effect of *in vitro* gastrointestinal digestion and fermentation of tannin wood extracts on the production of SCFAs, antioxidant capacity and evolution of the polyphenols profile. The global antioxidant response obtained from two assays indicated a higher reducing capacity of the CHE, while QUE showed higher antiradical activity. Tannins have been proven to be important substrates for microbial fermentation and production of SCFAs. In particular, the supernatant derived from the fermentation of CHE had the highest concentration of SCFAs. The UPLC-MS analysis represents the first tentative identification and a semi-quantitative analysis of the polyphenolic content of QUE and CHE, that allows for carrying out a preliminary investigation of their metabolization during digestion and colonic fermentation. These results constitute the basis for further studies on the interaction of these wood extracts with complex food matrices. Subsequently, a nutritional intervention with healthy adults could allow for the investigation of how QUE and CHE affect the colon microbial composition and function, being therefore used as potential ingredients for functional foods formulation.

Ethics statement

No ethics approval was needed in order to perform the research activities related with this paper since all of them were done *in vitro*.

Conflict of interest

The authors declare that there are no conflicts of interest.

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