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ABSTRACT

The present work aimed at understanding gut microbiota bioconversion of phenolic compounds (PC) and organic acids in predigested *Hibiscus sabdariffa* (Hb) calyces and the mixture of Hb and Agave (*Agave tequilana* Weber) fructans (AF). With this purpose, dried Hb and Hb/AF were predigested with enzymatic treatment, and then fermented in a dynamic *in vitro* model of the human colon (TIM-2). After HPLC-ESI-QToF-MS analysis of samples taken at 0, 24, 48 and 72 h of fermentation, it was observed that hydroxycinnamic acids, flavanols, flavonols, and anthocyanins were mainly transformed into derivatives of hydroxyphenylpropionic, hydroxyphenylacetic and hydroxybenzoic acids. Moreover, organic acids, such as hydroxycitric and hibiscus acids, were formed along with unidentified lactones and reduced compounds. Interestingly, no differences were observed between microbial-derived metabolites formed after the fermentation of Hb and Hb/AF. In conclusion, colonic fermentation of polyphenol-rich Hb yields a wide range of microbial phenolic metabolites with potential effects on health.

1. Introduction

In the last decades, the understanding of the functional properties of plant-foods has been of remarkable interest due to the presence of bioactive molecules, such as phenolic compounds (PC). Consumption of PC has been associated with a lower incidence of certain chronic diseases, such as type 2 diabetes, cardiovascular disease or dyslipidemia (Cory, Passarelli, Szeto, Tamez, & Mattei, 2018; Serino & Salazar, 2019). Although isolated PC have been shown to be efficient from a health point of view, when combined with other bioactive compounds, such as dietary fiber (DF), synergistic effects can take place (Jacobs & Tapsell, 2013). In contrast, the presence of DF can interfere in the bioaccessibility and bioavailability of PC, limiting their uptake in the upper gastrointestinal tract. Non-bioaccessible PC bound to DF can reach the colon and be transformed by the colonic microbiota into metabolites, which are responsible for many of the health effects associated to dietary PC (Crozier, Jaganath, & Clifford, 2009). The main metabolites and/or catabolites generated by the bioconversion of PC are aromatic and phenolic acids with hydroxyl groups, or mono- or dimethoxy analogues with a sidechain of one to five carbons (Williamson & Clifford, 2010).

Therefore, the potential effects of PC on health depend not only on the initial amount in the food but also on the bioactivity of the metabolites formed during passage through the gastrointestinal tract (Bohn et al., 2015). PC have been considered as part in the concept of "three P's for gut health" which includes probiotics, prebiotics and (poly)phenols (Espín, González-Sarrías, & Tomás-Barberán, 2017). In some cases, they have even been considered as potential prebiotics, matching the currently accepted definition of prebiotics as "*a non-digestible compound that through its metabolization by microorganisms in the gut, modulated the composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host*" (Gibson et al., 2017). Therefore, it is relevant to further understand the non-bioaccesible fraction of a plant-food rich in PC and DF, because of the potential physiological relevance that the combined presence of the two bioactive compounds in the food matrix can provide to human health.

Agave (*Agave tequilana* Weber) fructans (AF) are considered as an important source of soluble DF. After fermentation by the intestinal microbiota DF in AF can produce a considerable amount of short chain

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fatty acids (SCFA), butyric acid specifically, and induce the growth of *Bifidobacterium* (Koenen, Rubio, Mueller, & Venema, 2016). On the other hand, *Hibiscus sabdariffa* (Hb) calyces are also rich in DF, with up to 40% DF depending of the variety (Sáyago-Ayerdi, Velázquez-López, Montalvo-González, & Goñi, 2014). It also contains organic acids such as hibiscus or hydroxycitric acids, and extractable PC as anthocyanins (cyanidin-3-glucoside, delphinin-3-glucoside, delphinidin-3-sambubioside, or cyanidin), phenolic acids, and flavonoids, together with non-extractable polyphenols such as condensed tannins and hydrolysable tannins (Borrás-Linares et al., 2015). In the present work, the combination of AF and Hb has been studied because it is considered a relevant source of antioxidant DF, which has previously shown to have positive effects on the lipid profile of hypercholesterolemic rats (Sáyago-Ayerdi, Mateos, et al., 2014) and metabolic hormones and inflammatory biomarkers in plasma and adipose tissue in obese mice (Moyano et al., 2016). In addition, Hb and Hb/AF have recently shown the capacity to modify gut microbiota abundance of *Bifidobacterium, Bacteroides* and *Catenibacterium* during *in vitro* fermentation (Sáyago-Ayerdi, Zamora-Gasga, & Venema, 2020). The aim of the present work was to evaluate the bioconversion of PC in predigested *Hibiscus sabdariffa* (Hb) calyces and the mixture of Hb and agave fructans (AF) assessed using a dynamic *in vitro* model of the human colon (TIM-2).

2. Materials and methods

2.1. Materials and reagents

AF were donated by the company Agaves de la Costa S. A. de C.V. Hb calyces from the 'Cruza Negra' variety were purchased on a market in Tepic (Nayarit, Mexico). All solvents and reagents were of analytical grade unless otherwise stated. Gallic acid, quercetin, 3,4-dihydroxyphenylpropionic acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxbenzoic acid, 3-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, catechin, epicatechin, garcinia acid and cyanidin chloride were acquired from Sigma-Aldrich (Madrid, Spain). Formic acid and acetonitrile (HPLC grade) were acquired from Panreac (Madrid, Spain). All media components used in TIM-2 system were purchased at Tritium Microbiology (Eindhoven, The Netherlands).

2.2. Predigestion of Hibiscus sabdariffa (Hb) calyces

Hb calyces were predigested mimicking physiological conditions according to a published standard protocol (Sáyago-Ayerdi, Velázquez-López, et al., 2014) to eliminate the bioaccessible PC, soluble sugars, and to digest the protein. Firstly, a double enzymatic hydrolysis was carried out with pepsin (pH 1.5, 37 °C, 1 h) and pancreatin (pH 6.9–7.5, 37 °C, 4 h). Afterwards, samples were centrifuged (20 min, 8000*g*) and the supernatant was diafiltrated to obtain the non-digestible fraction (Venema, 2015). The predigested Hb was used as sample and the mixture of Hb/AF was prepared in a proportion of 70:30.

2.3. Microbiota collection and standardization

Fecal samples were obtained from three donors from Maastricht University - Campus Venlo in The Netherlands. Volunteers were healthy, 28–47 years old, who declared not suffering any gastrointestinal disease, and not having taken antibiotics at least three months before the start of the study. Donors placed the fresh fecal samples in a plastic jar containing a gastight bag with an anaerocult strip (AnaeroGen™, Cambridge, UK) and samples were transported to the laboratory after no more than 2 h. To standardize the microbiota, a fecal pool was prepared (500 g) in an anaerobic cabinet (Sheldon Lab – Bactron IV, Cornelius, OR, USA) according to Venema, Nuenen, Smeets-Peeters, Minekus, and Havenaar (2000). The mixture was prepared with a dialysate solution (1:1, v/v) (content per liter: 2.5 g dipotassium hydrogen phosphate trihydrate, 4.5 g sodium chloride, 0.005 g ferrous sulphate heptahydrate, 0.5 g magnesium sulphate heptahydrate, 0.45 g calcium chloride dihydrate, 0.05 g ox-bile, 0.4 g *L*-cysteine hydrochloride; pH 5.8) and glycerol (15% w/w final concentration) as cryoprotective agent. The fecal material was aliquoted, frozen in liquid nitrogen, and stored at −80 °C until inoculation into the TIM-2.

2.4. In vitro colonic fermentation of predigested Hibiscus sabdariffa (Hb) calyces and the mixture of Hb and agave fructans (AF) in the dynamic TIM-2 system

TIM-2 system is a validated, dynamic and computer-controlled *in vitro* model that mimics human proximal large intestine using microbiota from human origin (Venema et al., 2000), that uses a simulated ileal effluent medium (SIEM) as a culture media for the samples and standardizes the fecal samples before fermentation (Maathuis, Hoffman, Evans, Sanders, & Venema, 2009). In the present study, TIM-2 system was inoculated with 70 mL of the standardized microbiota (described above). After creating an anaerobic environment by flushing with gaseous nitrogen, the microbiota was adapted to the model conditions with the simulated ileal effluent medium (SIEM) for 20 h, which mimics the indigestible fraction of a high fiber, polyphenol-free diet. The composition of SIEM is detailed in Maathuis et al. (2009). After a 4 h starvation, period which allowed the fermentation of the available carbohydrates by bacteria, the test sample (Hb or Hb/AF) was added. Aliquots of each sample were collected at time-point zero (t_0) along with a control sample, with SIEM medium but without sample. The predigested sample (Hb or Hb/AF) was continuously mixed with SIEM without indigestible carbohydrates (pectin, starch, xylan and arabinogalactan) in three different units (7.5 g/day, 2.5 mL/h), and samples were collected during fermentation at 24 h, 48 h and 72 h and stored at −80 °C until analysis.

2.5. Extraction of phenolic compounds (PC) from Hb calyces, predigested Hb and fermented samples (Hb and Hb/AF)

PC were extracted using the method developed by Pérez-Jiménez et al. (2008). Briefly, 1 g of sample (Hb calyces previously grounded and sieved through a 5 μm mess, or 1 g of predigested Hb) was weighed in duplicate and extracted with 2 N hydrochloric acid in aqueous methanol (50:50, v/v) for 1 h at room temperature with constant shaking. Then, samples were centrifuged for 15 min at 3000 *g* and supernatants were separated. The pellets were extracted again with acetone/water (70:30, v/v) for 1 h at room temperature with constant shaking and centrifuged for 15 min at 3000 *g*. After centrifugation, supernatants were combined and made up to 100 mL. An aliquot of 1 mL was concentrated under reduced pressure using a vacuum concentrator system (Speed-Vac, Thermo Fisher Scientific Inc., Waltham, MA, USA), and then resuspended in the same volume of 1% formic acid in deionized water (v/v), filtered (0.45 μM pore-size, cellulose-acetate membrane filters), dispensed in chromatographic vials and stored at −80 °C until analysis.

Regarding fermented samples, a 1 mL aliquot in an eppendorf was centrifuged for 30 min at 14,000*g* (4 °C), filtered (0.45 μm pore size cellulose-acetate membrane filter), dispensed in chromatographic vials and stored at −80 °C until analysis.

2.6. Characterization and quantification of the phenolic content and organic acids of Hb, predigested Hb and fermented samples by LC-ESI-QToF analysis

PC and organic acids from Hb and predigested Hb and metabolites from colonic fermentation were characterized using HPLC-ESI-QToF following the procedure described in Gómez-Juaristi, Martínez-López, Sarria, Bravo, Mateos (2018a, 2018b). Analyses were performed on an Agilent 1200 series LC system coupled to an Agilent 6530A Accurate-Mass Quadrupole Time-of-Flight (Q-ToF) with ESI-Jet Stream

Technology (Agilent Technologies). Compounds were separated on a reverse-phase Ascentis Express C18 (15 cm × 3 mm, 2.7 μm) column (Sigma-Aldrich Quimica, Madrid) preceded by a Supelco 55215-U guard column at 30 °C. Five μL of sample were injected and separated using a mobile phase consisting of Milli-Q water (phase A) and acetonitrile (phase B), both containing 0.1% formic acid, at a flow rate of 0.3 mL/min. The mobile phase was initially programmed with 90% of solvent A and 10% of B. The elution program increased to 30% of solvent B in 10 min, 40% solvent B in 5 min, and 50% of solvent B in 5 min. Then, the initial conditions (10% solvent B) were recovered in 2 min and maintained for 8 min. The QToF acquisition conditions were as follows: drying gas flow (nitrogen, purity > 99.9%) and temperature were 10 L/min and 325 °C, respectively; sheath gas flow and temperature were 6 L/min and 250 °C, respectively; nebulizer pressure was 25 psi; cap voltage was 3500 V and nozzle voltage was 500 V. The selected mass range was from 100 up to 970 *m/z* in negative or positive mode and fragmentor voltage was 150 V. Data were processed with Mass Hunter Workstation Software. External calibration curves were prepared with the following standards: gallic acid, quercetin, 3,4-dihydroxyphenylpropionic acid, 3-hydroxyphenylpropionic acid, 3,4-dihydroxyphenylacetic acid, 4-hydroxyphenylacetic acid, 3,4-dihydroxbenzoic acid, 3-hydroxybenzoic acid, *p*-coumaric acid, caffeic acid, catechin, epicatechin, garcinia acid and cyanidin chloride, all at six different concentration levels (from 0.001 to 20 μM). Limits of detection and quantification ranged from 0.003 to 0.007 μM and from 0.006 to 0.009 μM, respectively. The inter- and intra-day precision of the assay (as the coefficient of variation, was from 3.9 to 9.2%) were considered acceptable and allowed the quantification of phenolic compounds and their metabolites (quantified as equivalents of the respective parent molecule).

2.7. Statistical analysis

All analyses were performed in triplicate (n = 3) and results expressed as mean values and standard deviations. Hb and predigested Hb values were compared by *t*-Student's test, and results from Hb and Hb/AF fermentation were analyzed by a two-way ANOVA/Fisher's least significant differences test ($p < 0.05$). All analyses were performed using STATISTICA software, version 10.0 (StatSoft. Inc. 1984–2007, Tulsa, OK, USA).

3. Results and discussion

Hb is a relevant source of PC, which has already shown beneficial health properties (Borrás-Linares et al., 2015; Moyano et al., 2016; Sáyago-Ayerdi, Velázquez-López, et al., 2014). Evaluation of Hb digestive stability is essential to better estimate the amount of phytochemicals that might be available for absorption in the upper gastrointestinal tract and/or to undergo bioconversion in the colon by the intestinal microbiota. With this purpose, PC and organic acids of Hb were identified and quantified in the samples obtained after incubation with pepsin and pancreatin, involved in human gastric and intestinal digestion, respectively. Similarly, these compounds were quantified in the fractions obtained after *in vitro* digestion, specifically in the non-digestible fraction obtained by centrifugation and filtration by dialysis, containing the compounds non absorbed in the upper gastrointestinal tract. Finally, the non-digestible fraction of Hb, alone or in combination with AF, were submitted to *in vitro* colonic fermentation using a standardized human fecal inoculum.

3.1. Identification and quantification of the PC and organic acids content of Hb and predigested Hb

Changes in the phenolic constituents as well as specific organic acids (hydroxycitric and hibiscus acids and derivatives) in the Hb calyces and after the simulated gastrointestinal digestion were analyzed using HPLC coupled ESI-QToF-MS in both negative and positive mode. Compounds were identified on the basis of their relative retention time and accurate mass spectra, using commercial standards and/or information previously reported in the literature. Retention time (RT), molecular formula, molecular weight, accurate mass of the quasimolecular ion $[M-H]^-$ or $[M]^+$ after negative or positive ionization, MS^2 fragments and location (Hb, predigested Hb and/or fermented samples) of the main compounds identified in samples by HPLC-ESI-QToF-MS are provided in Table 1. The individual quantification of the identified PC and organic acids in Hb and predigested Hb are shown in Table 2, and that in fermented Hb and Hb/AF is shown in Table 3. Metabolite characterization was supported by commercial standards and/or previously published results (Gómez-Juaristi et al., 2018a, 2018b; Borrás-Linares et al., 2015 and/or Piovesana, Rodrigues, and Zapata Noreña, 2018). The characterization of PC in undigested Hb using solvent extraction yielded a different profile than that after gastrointestinal digestion. It is interesting to take into account the latter profile because some PC could be released during digestion and thus be bioaccessible for absorption in the small intestine.

Forty-nine and thirty-eight PC along with five and four organic acids were identified in Hb and predigested Hb, respectively (Table 1). Hibiscus acid, hibiscus acid hydroxyethylester and hibiscus acid dimethylester were characterized by their corresponding quasimolecular ions at *m/z* 189.0041, 235.0459 and 217.0354, respectively, and the common fragment ion at *m/z* 127, corresponding to the typical losses of water and carbon dioxide from hibiscus acid. Hydroxycitric acid was identified thanks to its $[M-H]^-$ at *m/z* 207.0146 and fragment ions at *m/z* 189 and 115, corresponding to the dehydrated molecule and the formation of trihydroxybutan-1,3-dien-1-one, respectively. Finally, 3-deoxy-*D*-lyxo-heptulosaric acid (DHA) was identified based on its $[M-H]^-$ at *m/z* 221.0.303 and previous published articles such as Borrás-Linares et al. (2015), among others. Hibiscus acid was the most abundant organic acid (78.1 and 63.6% of the total organic acids in Hb and predigested Hb, respectively) followed by hydroxycitric acid (19.9 and 35.2%) and other organic acids such as DHA and hibiscus acid hydroxyethylester, which were present in both samples (Table 2). Additionally, very low amounts of hibiscus acid dimethylester were detected in Hb, although not in the digested sample. Apparently, an average of 60% of hydroxycitric acid (garcinia acid) might be able to cross the intestinal barrier, which is relevant since this compound has been related with weight loss in rats (Carvajal-Zarrabal et al., 2009; Sáyago-Ayerdi, Mateos, et al., 2014) and mice (Villalpando-Arteaga et al., 2013).

Complete calyces of Hb are a rich source of hydroxycinnamic acids, primarily caffeoylquinic acids ($[M-H]^-$ at *m/z* 353.0878 and fragment ions at *m/z* 191, 179 and 135 corresponding to quinic acid, caffeic acid and molecule after losing carbon dioxide, respectively). Particularly 3-, 4- and 5-caffeoylquinic acids were identified based on previous studies (Gómez-Juaristi et al., 2018a, 2018b) and the stable elution order that follows these isomers with similar chromatographic conditions. Additionally, two caffeoylquinic acid isomers at 7.8 and 8.6 min were identified although the position of the esterifed hydroxyl moiety of quinic acid could not be specified. Other hydroxycinnamoylquinic acids characterized in Hb and predigested Hb, such as coumaroylquinic and feruloylquinic acids, showed similar behavior during MS analysis, with a deprotonated ion at *m/z* 337.0929 and 367.1035, respectively, along with fragment ions at *m/z* 191 corresponding to quinic acid, in addition to free coumaric acid (at *m/z* 163) and ferulic acid (at *m/z* 193), respectively. Three isomers of caffeoylshikimic acid were also identified based on its quasimolecular ion at *m/z* 335.0761 in agreement with Borrás-Linares et al. (2015). Other hydroxycinnamates identified were 2-*O*-trans-caffeoyl-hydroxycitric acid ($[M-H]^-$ at *m/z* 369.0463 and fragment ions at *m/z* 179 and 135) and methyl-chlorogenate ($[M-H]^-$ at *m/z* 367.1035 and fragment ions at *m/z* 353, 191 and 179), with fragment ions compatible with caffeoylquinic acid structure. Finally, free hydroxycinnamic acids such as caffeic, coumaric and ferulic acids were identified in both Hb and predigested Hb supported by their MS analysis and commercial standards.

Table 1
LC-QToF identification of phenolic compounds and organic acids in complete calyces of *H. sabdariffa L.* (Hb), predigested calyces (predigested Hb) and Hb fermented in a dynamic *in vitro* model of the human colon (TIM-2).

Name	RT (min)	Molecular formula	Molecular weight	*[M-H]$^-$ or [M]$^+$	MS^2 Fragments	Location		
						Hb	Predigested Hb	Fermented Hb
HIBISCUS ACID AND RELATED COMPOUNDS								
Hydroxycitric acid	2.0	$C_6H_8O_8$	208.0219	207.0146	189;115	Yes	Yes	No
Hibiscus acid	2.1	$C_6H_6O_7$	190.0114	189.0041	127	Yes	Yes	Yes
3-Deoxy-*D-lyxo*-heptulosaric acid (DHA)	2.3	$C_7H_{10}O_8$	222.0376	221.0303		Yes	Yes	No
Hibiscus acid hydroxyethylester	3.1	$C_8H_{12}O_8$	236.0532	235.0459	127	Yes	Yes	No
Hibiscus acid dimethylester	3.5	$C_8H_{10}O_7$	218.0427	217.0354	127	Yes	No	No
HYDROXYCINNAMIC ACIDS AND RELATED COMPOUNDS								
3-Caffeoylquinic acid	3.7	$C_{16}H_{18}O_9$	354.0951	353.0878	191;179;135	Yes	Yes	No
Coumaroylquinic acid	5.2	$C_{16}H_{18}O_8$	338.1002	337.0929	191;163	Yes	Yes	No
5-Caffeoylquinic acid	5.5	$C_{16}H_{18}O_9$	354.0951	353.0878	191;179	Yes	Yes	No
4-Caffeoylquinic acid	6.1	$C_{16}H_{18}O_9$	354.0951	353.0878	191;179;173; 135	Yes	Yes	No
Methyl-chlorogenate	6.5	$C_{17}H_{20}O_9$	368.1107	367.1035	353;191;179	Yes	Yes	No
2-*O-trans*-caffeoyl-hydroxycitric acid	6.6	$C_{15}H_{14}O_{11}$	370.0536	369.0463	179; 135	Yes	Yes	No
Caffeic acid	7.2	$C_9H_8O_4$	180.0423	179.0350	135	Yes	Yes	Yes
Caffeoylquinic acid isomer	7.8	$C_{16}H_{18}O_9$	354.0951	353.0878	191;179	Yes	Yes	No
Coumaroylquinic acid	8.0	$C_{16}H_{18}O_8$	338.1002	337.0929	191;163	Yes	Yes	No
Coumaroylquinic acid	8.2	$C_{16}H_{18}O_8$	338.1002	337.0929	191;163	Yes	Yes	No
Caffeoylquinic acid isomer	8.6	$C_{16}H_{18}O_9$	354.0951	353.0878	191	Yes	No	No
Caffeoylshikimic acid	8.5	$C_{16}H_{16}O_8$	336.0845	335.0761		Yes	Yes	No
Methyl-chlorogenate	9.2	$C_{17}H_{20}O_9$	368.1107	367.1035	179	Yes	Yes	No
Coumaric acid	9.7	$C_9H_8O_3$	164.0473	163.0401	119	Yes	Yes	Yes
Caffeoylshikimic acid	9.6	$C_{16}H_{16}O_8$	336.0845	335.0761	179	Yes	Yes	No
Caffeoylshikimic acid	10.0	$C_{16}H_{16}O_8$	336.0845	335.0761	179	Yes	Yes	No
Methyl-chlorogenate	10.2	$C_{17}H_{20}O_9$	368.1107	367.1035	353;191;179	Yes	Yes	No
Feruloylquinic acid	10.3	$C_{17}H_{20}O_9$	368.1107	367.1035	191;193	Yes	Yes	No
Ferulic acid	11.2	$C_{10}H_{10}O_4$	194.0579	193.0506	149;134;178	No	Yes	No
OTHER PHENOLIC ACIDS AND RELATED COMPOUNDS								
Gallic acid	2.6	$C_7H_6O_5$	170.0215	169.0142	125; 79; 69	Yes	Yes	Yes
Protocatechuic acid glucoside	3.0	$C_{13}H_{16}O_9$	316.0794	315.0722	153; 109	Yes	Yes	No
Pyrogallol	3.2	$C_6H_6O_3$	126.0317	125.0244	51	No	No	Yes
3,4-Dihydroxybenzoic acid (protocatechuic acid)	3.9	$C_7H_6O_4$	154.0266	153.0193	109	Yes	Yes	Yes
3,4-Dihydroxyphenylacetic acid	4.4	$C_8H_8O_4$	168.0423	167.0350	123; 108	No	No	Yes
3-Hydroxybenzoic acid	5.8	$C_7H_6O_3$	138.0317	137.0244	93	Yes	Yes	Yes
4-Hydroxybenzoic acid	6.0	$C_7H_6O_3$	138.0317	137.0244	93	Yes	Yes	Yes
Methylgallate	6.3	$C_8H_8O_5$	184.0372	183.0299	124;78	Yes	Yes	Yes
3,4-Dihydroxyphenylpropionic acid	6.6	$C_9H_{10}O_4$	182.0579	181.0506	137	No	No	Yes
4-Hydroxyphenylacetic acid	6.9	$C_8H_8O_3$	152.0473	151.0401	107	No	No	Yes
Syringic acid	7.9	$C_9H_{10}O_5$	198.0528	197.0455	182;153	Yes	Yes	Yes
3-Methoxy-4-hydroxyphenylacetic acid	8.8	$C_9H_{10}O_4$	182.0579	181.0506	137	No	No	Yes
5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone	9.2	$C_{11}H_{12}O_4$	208.0736	207.0663	163	No	No	Yes
4-Hydroxyphenylpropionic acid	9.4	$C_9H_{10}O_3$	166.0630	165.0557	121	No	No	Yes
3-Hydroxyphenylacetic acid	9.4	$C_8H_8O_3$	152.0473	151.0401	107; 121	No	No	Yes
3-Methoxy-4-hydroxyphenylpropionic acid	10.5	$C_{10}H_{12}O_4$	196.0736	195.0663	136	No	No	Yes
3-Hydroxyphenylpropionic acid	10.7	$C_9H_{10}O_3$	166.0630	165.0557	121	Yes	Yes	Yes
FLAVONOIDS								
Epigallocatechin	4.1	$C_{15}H_{14}O_7$	306.0740	305.0667	179	Yes	Yes	Yes
Kaempherol-3-sambubioside (Leucoside)	4.4	$C_{26}H_{28}O_{15}$	580.1428	579.1355	285	Yes	Yes	No
Myricetin 3-sambubioside	8.4	$C_{26}H_{28}O_{17}$	612.1326	611.1254	317	Yes	Yes	No
Quercetin-3-sambubioside	9.6	$C_{26}H_{28}O_{16}$	596.1377	595.1305	301	Yes	Yes	No
Quercetin-3-*O*-glucoside (*iso*quercitrin)	10.8	$C_{21}H_{20}O_{12}$	464.0955	463.0882	301; 300; 179; 151	Yes	No	No
Quercetin-3-*O*-galactoside	11.0	$C_{21}H_{20}O_{12}$	464.0955	463.0882	301; 300; 151	Yes	Yes	No
Kaempherol-3-*O*-rutinoside	11.7	$C_{27}H_{30}O_{15}$	594.1585	593.1512	285	Yes	Yes	No
Methyl-epigallocatechin	11.8	$C_{16}H_{16}O_7$	320.0896	319.0823	179	Yes	Yes	No
Kaempferol-3-glucoside	12.2	$C_{21}H_{20}O_{11}$	448.1006	447.0933	285	Yes	Yes	No
Kaempherol-3-glucoside	12.4	$C_{21}H_{20}O_{11}$	448.1006	447.0933	285	Yes	Yes	No
Kaempherol 3-*O*-(6″-*O*-acetyl) glucoside	12.7	$C_{23}H_{22}O_{12}$	490.1111	489.1038	285	Yes	No	No
Myricetin	13.4	$C_{15}H_{10}O_8$	318.0376	317.0303	179	Yes	No	No
Syringetin-3-*O*-glucoside	14.0	$C_{23}H_{24}O_{13}$	508.1217	507.1144	345	Yes	No	No
Kaempherol-3-*p*-coumaroylglucoside	15.9	$C_{30}H_{26}O_{13}$	594.1373	593.1301	285	Yes	No	No
Quercetin	16.1	$C_{15}H_{10}O_7$	302.0427	301.0354	151	Yes	No	Yes
Kaempherol	18.7	$C_{15}H_{10}O_6$	286.0477	285.0405	119	Yes	No	Yes
ANTHOCYANINS AND ANTHOCYANIDINS								
Delphinidin-3-sambubioside	3.1	$C_{26}H_{29}O^+_{16}$	597.1456	597.1453	303	Yes	Yes	No
Delphinidin	3.2	$C_{15}H_{11}O^+_7$	303.0556	303.0552		Yes	No	No
Delphinidin-3-glucoside	3.4	$C_{21}H_{21}O^+_{12}$	465.1033	465.1026	303	Yes	No	No
Cyanidin-3-sambubioside	4.6	$C_{26}H_{29}O^+_{15}$	581.1506	581.1501	287	Yes	Yes	No
Cyanidin-3-glucoside	4.7	$C_{21}H_{21}O^+_{11}$	449.1084	449.1074	287	Yes	No	No
Cyanidin	4.7	$C_{15}H_{11}O^+_6$	287.0556	287.0553		Yes	Yes	No

*$[M-H]^-$ or $[M]^+$: Hibiscus acid, hydroxycinnamic acids, phenolic acids and their related compounds along with flavonoids were characterized in negative ionization by looking for the quasimolecular ion $[M-H]^-$, while anthocyanidins were analysed in positive mode, $[M]^+$.

Caffeolquinic acids represented 70.9% and 81.3% of the total hydroxycinnamic acids in Hb and digested Hb, respectively (Table 2). Among the other hydroxycinnamates identified, 2-*O*-trans-caffeoyl-hydroxycitric acid (13.1% and 5.3%, respectively) was the most abundant, followed by coumaroylquinic acids (7.3% and 5.9%, respectively), caffeoylshikimic acids (4.7% and 4.8%, respectively), methyl-chlorogenate (3.6% and 2.0%, respectively) and feruoloylquinic acid (0.04% and 0.05%, respectively). Free hydroxycinnamic acids such as caffeic, coumaric and ferulic acids were present in very low concentrations compared to esterified hydroxycinnamic acids (adding up to 0.3% and 0.5% of total hydroxycinnamic acids in Hb and digested Hb, respectively). The amounts of these compounds did not increase significantly after digestion of Hb since hydroxycinnamates were poorly hydrolyzed. Hydroxycinnamic acids and related compounds represented 5.6% and 18.5% of the total polyphenols quantified in Hb and predigested Hb samples, respectively (Table 2).

Flavonoids were another important group of PC characterized in the samples headed by glycosidic derivatives of flavonols. Quercetin-3-sambubioside, quercetin-3-*O*-glucoside (*iso*quercitrin) and quercetin-3-*O*-galactoside showed a compatible $[M-H]^-$ at *m/z* 595.1305, 463.0882 and 463.0882 along with fragment ions at *m/z* 301 corresponding to quercetin after losing sugar moiety. A similar fragmentation pattern was observed for kaempherol-3-sambubioside ($[M-H]^-$ at *m/z* 579.1355), kaempherol-3-*O*-rutinoside ($[M-H]^-$ at *m/z* 593.1512), kaempherol-3-glucoside ($[M-H]^-$ at *m/z* 447.0933), kaempherol-3-*O*-(6″-O-acetyl)-glucoside ($[M-H]^-$ at *m/z* 489.1038) and kaempherol-3-*p*-coumaroyl-glucoside ($[M-H]^-$ at *m/z* 593.1301) and common fragment ion at *m/z* 285 corresponding to kaempherol. Additionally, myricetin-3-sambubioside and syringetin-3-*O*-glucoside were identified based on their respective quasimolecular ion at *m/z* 611.1254 and 507.1144 and fragment ions at *m/z* 317 and 345 corresponding to myricetin and syringetin, respectively. Free quercetin, myricetin and kaempherol were also identified supported by MS analysis and commercial standards. Along with the mentioned flavonols, flavanols such as epigallocatechin ($[M-H]^-$ at *m/z* 305.0667 and fragment ion at *m/z* 179) and methyl-epigallocatechin ($[M-H]^-$ at *m/z* 319.0823 and fragment ion at *m/z* 179) were also characterized in both samples. Flavonoids represented 19.4% and 9.6% of the total polyphenols in Hb and predigested Hb, respectively. Glycosidic derivatives (sambubioside, glucoside and galactoside) of quercetin along with the free form accounted for 46.4% and 42.5% of the total flavonoids identified in both Hb and predigested Hb, respectively. Myricetin-3-sambubioside along with myricetin represented 37.0% in Hb, while predigested Hb only had myricetin-3-sambubioside equivalent to 29.5% of the total polyphenols. Kaempherol was also characterized in Hb, mainly forming glycoside derivatives (sambubioside, rutinoside, glucoside, acetyl-glucoside or coumaroyl-glucoside); these, together with the aglycone kaempherol, amounted to 16.4% and 27.2% of the total flavonoids quantified in Hb and predigested Hb, respectively. To end, syringetin-3-glucoside was only quantified in Hb in very low amount (0.1% of the total flavonoids), as well as flavanols which were also in very low quantity (0.2% and 0.8% of the total flavonoids in Hb and predigested Hb, respectively).

Regarding simple phenolic acids, most compounds were characterized based on commercial standards and the literature (Gómez-Juaristi et al., 2018a, 2018b; Gómez-Juaristi, Sarria, Martínez-López, Bravo, & Mateos, 2019). Protocatechuic acid glucoside ($[M-H]^-$ at *m/z* 315.0722 and fragment ions at *m/z* 153 and 109, corresponding to protocatechuic acid and moiety after losing carbon dioxide, respectively) and methyl-gallate ($[M-H]^-$ at *m/z* 183.0299 and fragment ion at *m/z* 124 corresponding to the molecule after losing carbon dioxide) were identified based on MS analysis. Gallic acid was the most abundant compound, representing 40.1% and 48.3% of this group for Hb and predigested Hb, respectively. Minor amount of gallic acid was found as methyl-gallate, which levels decreased after *in vitro* digestion (2.5% and 0.9% of the total phenolic acids in Hb and predigested Hb, respectively). Protocatechuic acid-glucoside was very abundant in Hb (30.9% of the total phenolic acids), but it was partially hydrolyzed during *in vitro* digestion, decreasing down to 22.6% of the total phenolic acids in the predigested sample in contrast to the amount of free protocatechuic acid, which was 1.8% in Hb compared to 10.4% in predigested Hb. Syringic acid and 4-hydroxyphenylpropionic acid along with 3- and 4-hydroxybenzoic acids completed the group of phenolic acids, representing 24.7% and 17.7% of the total of this group in Hb and predigested Hb, respectively. This group of compounds was the least abundant of all the PC identified in Hb, representing 0.8 and 2.1% of the total phenolics in Hb and predigested Hb, respectively.

Anthocyanidins and anthocyanins were the most abundant group of polyphenols characterized in Hibiscus calyces in positive ionization. Particularly, delphinidin-3-sambubuioside ($[M]^+$ at *m/z* 597.1453) and delphinidin-3-glucoside ($[M]^+$ at *m/z* 465.1026) showed a common fragment ion at *m/z* 303 corresponding to their precursor delphinidin. Likewise, MS spectra allowed characterizing cyanidin-3-sambubuioside ($[M]^+$ at *m/z* 581.1501) and cyanidin-3-glucoside ($[M]^+$ at *m/z* 449.1084) with a common fragment ion corresponding to cyanidin at *m/z* 287. Delphinidin and cyanidin were also identified in both Hb and predigested Hb supported by standards. This group represented 74.3 and 69.7% of the total PC identified in Hb and predigested Hb, respectively. Delphinidin and cyanidin, mainly as glycosylated derivatives (sambubioside and glucoside), were the main anthocyanins identified in Hb along with minor amounts of free delphinidin and cyanidin (approx. 0.7% of the total anthocyanidins in both samples). In the predigested samples, delphinidin-3-sambubioside and cyanidin-3-sambubuioside (99.4% of the total anthocyanidins) along with a negligible amount of cyanidin (0.6% of the total anthocyanidins) were the only compounds quantified.

Hb contained 4588 mg of soluble PC in 100 g dry matter (Table 2). This result is in line with those reported by Sáyago-Ayerdi, Arranz, Serrano, and Goñi (2007), who found a content of soluble phenols of 3% (dry matter) in a beverage obtained from hibiscus calyces after PC quantification using the Folin-Ciocalteau assay. In contrast, the results here obtained were one order of magnitude higher than those described by Piovesana et al. (2018), who reported a phenolic content of 509 mg/100 g of fresh matter, although a limited identification of PC was done, as only thirteen PC were quantified along with six unidentified compounds. In part, the difference may be attributed to the different geographical origin (Porto Alegre, Brazil), as it is well known that soluble PC content of Hb can vary depending of the cultivar. Borrás-Linares et al. (2015) characterized 25 varieties of Hb grown in Mexico and described a great variation, between 2400 and 10000 mg gallic acid equivalent/100 g dry matter, although none was 'Cruza Negra', the variety used in the present study. However, in a previous study by Duarte-Valenzuela, Zamora-Gasga, Montalvo-González, and Sáyago-Ayerdi (2016), a content of 4152 mg gallic acid equivalents/100 g dry matter for 'Cruza Negra' was reported, which is close to the amount quantified in the present study. In addition, the individual characterization of PC here reported is in agreement with in Da-Costa-Rocha, Bonnlaender, Sievers, Pischel, and Heinrich (2014) and in Borrás-Linares et al. (2015). It is noteworthy that Hb is a photoperiod shrub that requires high sun exposure to increase the anthocyanin content, and that agronomic conditions play an important role in improving these compounds. 'Cruza Negra' variety characterized by possessing black calyces, rich in PC. The large differences between varieties highlights the need to specify always the genotype used in the research works, since many publications only mention that the studied Hb was acquired in a supermarket. In this sense, the Hb variety used in the present study contained a high amount of anthocyanidins and anthocyanins, 3407 mg/

Table 2
Content of individual phenolic compounds and organic acids present in complete calyces of *H. sabdariffa L.* (Hb) and predigested Hb. Results represent the mean ± standard deviation (n = 3). N.D.: not detected; dw: dry weight.

RT (min)	Proposed Compound	Hb (mg/100 g dw)	Predigested Hb (mg/100 g dw)
HIBISCUS ACID AND RELATED COMPOUNDS			
2.0	Hydroxycitric acid (garcinia acid)	90 ± 1[a]	62 ± 1[b]
2.1	Hibiscus acid	356 ± 2[a]	112.9 ± 0.5[b]
2.3	3-deoxy-*D-lyxo*-heptulosaric (DHA)	8.8 ± 0.3[a]	2.0 ± 0.2[b]
3.1	Hibiscus acid hydroxyethylester	0.19 ± 0.02[a]	0.022 ± 0.004[b]
3.5	Hibiscus acid dimethylester	0.05 ± 0.01	N.D.
TOTAL ORGANIC ACIDS (mg/100 g d.w.)		***455 ± 3[a]***	***177 ± 2[b]***
HYDROXYCINNAMIC ACIDS AND RELATED COMPOUNDS			
3.7	3-Caffeoylquinic acid	53 ± 2[a]	47.1 ± 0.7[a]
5.2	Coumaroylquinic acid	6.5 ± 0.7[a]	4.7 ± 0.1[a]
5.5	5-Caffeoylquinic acid	63 ± 2[a]	57.2 ± 0.5[a]
6.1	4-Caffeoylquinic acid	44 ± 1[a]	45 ± 4[a]
6.5	Methyl-chlorogenate	1.48 ± 0.04[a]	0.39 ± 0.07[b]
6.6	2-*O-trans*-caffeoyl-hydroxycitric acid	33.5 ± 0.7[a]	10.1 ± 0.9[b]
7.2	Caffeic acid	0.20 ± 0.05[a]	0.23 ± 0.04[a]
7.8	Caffeoylquinic acid isomer	18 ± 3[a]	4.7 ± 0.4[b]
8.0	Coumaroylquinic acid	4.6 ± 0.3[a]	2.6 ± 0.2[b]
8.2	Coumaroylquinic acid	7.6 ± 0.4[a]	3.9 ± 0.3[b]
8.5	Caffeoylshikimic acid	0.49 ± 0.01[a]	0.17 ± 0.01[b]
8.6	Caffeoylquinic acid isomer	2.2 ± 0.2	N.D.
9.2	Methyl-chlorogenate	5.0 ± 0.3[a]	2.58 ± 0.02[b]
9.6	Caffeoylshikimic acid	7.5 ± 0.1[a]	4.6 ± 1.3[b]
9.7	Coumaric acid	0.62 ± 0.04[a]	0.21 ± 0.03[b]
10.0	Caffeoylshikimic acid	4.05 ± 0.01[a]	4.3 ± 1.6[a]
10.2	Methyl-chlorogenate	2.72 ± 0.05[a]	0.90 ± 0.06[b]
10.3	Feruloylquinic acid	0.108 ± 0.002[a]	0.09 ± 0.02[a]
11.2	Ferulic acid	N.D.	0.6 ± 0.1
TOTAL (mg/100 g d.w.) (%)		***255 ± 12[a] (5.6%)***	***189 ± 9[b] (18.6%)***
FLAVONOIDS			
4.1	Epigallocatechin	1.6 ± 0.1[a]	0.55 ± 0.01[b]
4.4	Leucoside	37 ± 2[a]	4.8 ± 0.7[b]
8.4	Myricetin −3-sambubioside	82 ± 4[a]	29 ± 1[b]
9.6	Quercetin-3-sambubioside	42 ± 1[a]	29 ± 2[b]
10.8	Quercetin-3-*O*-glucoside (*iso*quercitrin)	5.1 ± 0.7	N.D.
11.0	Quercetin-3-*O*-galactoside	93 ± 4[a]	13 ± 1[b]
11.7	Kaempherol-3-*O*-rutinoside	31 ± 2[a]	10 ± 2[b]
11.8	Methyl-epigallocatechin	0.07 ± 0.01[b]	0.21 ± 0.02[a]
12.2	Kaempherol-3-glucoside	3.8 ± 0.2[b]	4.40 ± 0.07[a]
12.4	Kaempherol-3-glucoside	20.67 ± 0.06[a]	7.2 ± 0.10[b]
12.7	Kaempherol 3-*O*-(6″-*O*-acetyl) glucoside	2.6 ± 0.1	N.D.
13.4	Myricetin	248 ± 10	N.D.
14.0	Siringetin-3-*O*-glucoside	0.54 ± 0.02	N.D.
15.9	Kaempherol-3-*p*-coumaroylglucoside	17 ± 1	N.D.
16.1	Quercetin	273 ± 19	N.D.
18.7	Kaempherol	34 ± 3	N.D.
TOTAL (mg/100 g d.w.) (%)		***891 ± 46[a] (19.4%)***	***97 ± 7[b] (9.6%)***
PHENOLIC ACIDS DERIVATIVES			
2.6	Gallic acid	13.87 ± 0.09[a]	10.4 ± 0.5[b]
3.0	Protocatechuic acid glucoside	10.7 ± 0.5[a]	3 ± 1[b]
3.9	3,4-Dihydroxybenzoic acid (protocatechuic acid)	0.62 ± 0.04[b]	2.2 ± 0.2[a]
5.8	3-Hydroxybenzoic acid	1.74 ± 0.05[a]	0.8 ± 0.7[a]
6.0	4-Hydroxybenzoic acid	1.0 ± 0.2[a]	0.5 ± 0.3[a]
6.3	Methylgallate	0.86 ± 0.03[a]	0.2 ± 0.1[b]
7.9	Syringic acid	5.8 ± 0.2[a]	2.5 ± 0.2[b]
10.7	3-Hydroxyphenylpropionic acid	0.05 ± 0.01[a]	0.02 ± 0.01[b]
TOTAL (mg/100 g d.w.) (%)		***34.6 ± 1.1[a] (0.8%)***	***22 ± 2[b] (2.1%)***
ANTHOCYANIDINS AND ANTHOCYANINS			
3.1	Delphinidin-3-sambubioside	1764 ± 99[a]	283 ± 13[b]
3.2	Delphinidin	15 ± 1	N.D.
3.4	Delphinidin-3-glucoside	179 ± 15	N.D.
4.6	Cyanidin-3-sambubioside	1341 ± 83[a]	423 ± 19[b]
4.7	Cyanidin-3-glucoside	97 ± 7	N.D.
4.7	Cyanidin	9.9 ± 0.6[a]	4.3 ± 0.6[b]
TOTAL (mg/100 g d.w.) (%)		***3407 ± 206 (74.2%)***	***710 ± 33 (69.7%)***
TOTAL PHENOLIC COMPOUNDS (mg/100 g d.w.) (%)		***4588 ± 265 (100%)***	***1018 ± 52 (100%)***

100 g dry matter, which is an added value. In the study by Borrás-Linares et al. (2015), most Hb varieties showed an anthocyanin content ranging from 0 to 2967 mg/100 g dry matter and only 3 out of the 25 evaluated cultivars showed a higher content than than in 'Cruza Negra' used in the present study (4085–4408 mg /100 g dry matter). Additionally, Sindi, Marshall, and Morgan (2014) reported low values for total anthocyanins, ranging from 0 to 600 mg/100 g dry matter, highlighting the potential of 'Cruza Negra' variety.

Table 3
Phenolic compounds and organic acid content in fermented predigested Hibiscus (Hb) and the mix Hb and Agave Fructans (Hb/AF, 70/30) obtained using a dynamic *in vitro* model of the human colon.[a]

No	Name	RT (min)	0 h (μmoles)		24 h (μmoles)		48 h (μmoles)		72 h (μmoles)	
			Hb/AF	Hibiscus	Hb/AF	Hibiscus	Hb/AF	Hibiscus	Hb/AF	Hibiscus
HIBISCUS ACID AND RELATED COMPOUNDS										
1	Hibiscus acid	2.1	N.D.	N.D.	0.10 ± 0.02^{Aa}	0.02 ± 0.02^{Aa}	Traces	0.03 ± 0.02^{Aa}	Traces	0.04 ± 0.02^{Aa}
HYDROXYCINNAMIC ACIDS AND RELATED COMPOUNDS										
2	Caffeic acid	7.2	0.04 ± 0.02^{Aa}	0.01 ± 0.01^{Aa}	0.03 ± 0.03^{Aa}	0.08 ± 0.02^{Aa}	0.05 ± 0.05^{Aa}	0.09 ± 0.05^{Aa}	0.08 ± 0.07^{Aa}	0.2 ± 0.1^{Aa}
3	Coumaric acid	9.5	0.02 ± 0.02^{Aa}	0.018 ± 0.007^{Aa}	0.02 ± 0.01^{Aa}	0.03 ± 0.01^{Aa}	0.07 ± 0.03^{Aa}	0.075 ± 0.005^{Aa}	0.09 ± 0.05^{Aa}	0.106 ± 0.006^{Aa}
FLAVONOIDS										
4	Epigallocatechin	4.1	N.D.	N.D.	0.03 ± 0.01^{Aa}	0.052 ± 0.006^{Ba}	0.06 ± 0.02^{Ab}	0.117 ± 0.005^{Bb}	0.09 ± 0.02^{Ac}	0.18 ± 0.01^{Bc}
5	Myricetin	13.4	N.D	N.D.	0.07 ± 0.04^{Aa}	0.03 ± 0.05^{Aa}	0.12 ± 0.09^{Aa}	0.06 ± 0.05^{Aa}	0.16 ± 0.15^{Aa}	0.10 ± 0.09^{Aa}
6	Quercetin	16.1	N.D.	N.D.	0.016 ± 0.009^{Aa}	0.028 ± 0.008^{Aa}	0.024 ± 0.007^{Aa}	0.029 ± 0.009^{Aa}	0.04 ± 0.01^{Aa}	0.034 ± 0.004^{Aa}
PHENOLIC ACIDS AND RELATED COMPOUNDS										
7	Gallic acid	2.6	0.19 ± 0.02^{Aab}	0.18 ± 0.09^{A}	0.13 ± 0.06^{Aa}	0.41 ± 0.08^{B}	0.3 ± 0.1^{Ab}	0.80 ± 0.06^{B}	0.5 ± 0.1^{Ac}	1.37 ± 0.03^{B}
8	Pyrogallol	3.2	0.03 ± 0.03^{Aa}	N.D.	0.07 ± 0.04^{Aa}	0.08 ± 0.05^{Aa}	0.11 ± 0.05^{Aa}	0.102 ± 0.001^{Aa}	0.13 ± 0.05^{Aa}	0.19 ± 0.03^{Aa}
9	3,4-Dihydroxybenzoic acid (protocatechuic acid)	3.9	0.4 ± 0.2^{Aa}	0.3 ± 0.1 ^{Aa}	0.35 ± 0.02^{Aa}	0.38 ± 0.02 ^{Aa}	0.8 ± 0.4^{Aa}	0.6 ± 0.3 ^{Aa}	1.3 ± 0.6^{Aa}	1.0 ± 0.7^{Aa}
10	3,4-Dihydroxyphenylacetic acid	4.4	N.D.	N.D.	2.6 ± 0.1^{Aa}	2.97 ± 0.02^{Aa}	4 ± 1^{Aa}	4 ± 2^{Aa}	6 ± 3^{Aa}	6 ± 3^{Aa}
11	3-Hydroxybenzoic acid	5.8	0.52 ± 0.07^{Aa}	0.18 ± 0.06^{Aa}	0.16 ± 0.09^{Aa}	0.17 ± 0.02^{Aa}	0.4 ± 0.2^{Aa}	0.22 ± 0.09^{Aa}	0.6 ± 0.2^{Aa}	0.3 ± 0.2^{Aa}
12	4-Hydroxybenzoic acid	6.0	0.66 ± 0.03^{Aa}	0.38 ± 0.04^{Aa}	0.6 ± 0.4^{Aa}	0.23 ± 0.01^{Aa}	0.7 ± 0.5^{Aa}	0.4 ± 0.2^{Aa}	0.6 ± 0.2^{Aa}	0.5 ± 0.3^{Aa}
13	Methylgallate	6.3	N.D.	N.D.	0.05 ± 0.03^{Aa}	0.06 ± 0.01^{Aa}	0.11 ± 0.08^{Aa}	0.11 ± 0.06^{Aa}	0.2 ± 0.1^{Aa}	0.2 ± 0.1^{Aa}
14	3,4-Dihydroxyphenylpropionic acid	6.6	1.0 ± 0.4^{Aa}	1.6 ± 0.1^{Aa}	2.4 ± 0.6^{Aab}	4.3 ± 0.2^{Bc}	5 ± 2^{Abc}	6 ± 2^{Abc}	7 ± 3^{Abc}	7 ± 4^{Abc}
15	4-Hydroxyphenylacetic acid	6.9	1.1 ± 0.5^{Aa}	1 ± 1^{Aa}	1.3 ± 0.9^{Aa}	3 ± 1^{Aab}	4 ± 2^{Aabc}	8 ± 6^{Aabc}	8 ± 4^{Abc}	11 ± 5^{Ac}
16	Syringic acid	7.9	0.03 ± 0.01^{Aa}	0.08 ± 0.04^{Aab}	0.04 ± 0.01^{Aa}	0.10 ± 0.02^{Bb}	0.08 ± 0.01^{Ab}	0.14 ± 0.03^{Bb}	0.09 ± 0.02^{Ab}	0.16 ± 0.01^{Bc}
17	3-Methoxy-4-hydroxyphenylacetic acid	8.8	0.3 ± 0.1^{Aa}	0.22 ± 0.01^{Aa}	3 ± 1^{Ab}	5 ± 4^{Ab}	7 ± 5^{Ab}	7.9 ± 0.6^{Ab}	10 ± 7^{Ab}	10 ± 6^{Ab}
18	5-(3′,4′-Dihydroxyphenyl)-γ-valerolactone (DHPV)	9.2	N.D.	N.D.	0.430 ± 0.009^{Aa}	0.386 ± 0.001^{Aa}	0.7 ± 0.2^{Aa}	0.6 ± 0.3^{Aa}	0.9 ± 0.4^{Aa}	0.8 ± 0.6^{Aa}
19	3-Hydroxyphenylacetic acid	9.4	1.1 ± 0.7^{Aa}	0.8 ± 0.1^{Aa}	4.4 ± 0.9^{Aa}	8 ± 4^{Aa}	16 ± 9^{Aa}	13 ± 11^{Aa}	21 ± 14^{Aa}	17 ± 16.56^{Aa}
20	4-Hydroxyphenylpropionic acid	9.4	N.D.	N.D.	10 ± 6^{Aab}	8 ± 2^{Aa}	30 ± 6^{Ac}	20 ± 6^{Aabc}	53 ± 12^{Ad}	32 ± 10^{Acd}
21	3-Methoxy-4-hydroxyphenylpropionic acid	10.5	1.75 ± 0.09^{Aa}	1.96 ± 0.03^{Aa}	2.0 ± 0.1^{Aa}	2.14 ± 0.08^{Aa}	3 ± 1^{Aa}	3 ± 1^{Aab}	5 ± 2^{Aab}	4 ± 3^{Aab}
22	3-Hydroxyphenylpropionic acid	10.7	35 ± 10^{Aa}	37 ± 12b ^{Aa}	78 ± 29^{Abc}	91 ± 34 ^{Acd}	123 ± 38^{Acd}	146 ± 31 ^{Ad}	173 ± 27^{Ad}	193 ± 33 ^{Ad}
TOTAL (μmoles)			42 ± 12^{Aa}	44 ± 14 ^{Aa}	126 ± 46 ^{Abc}	106 ± 39 ^{Ab}	212 ± 62^{Acd}	196 ± 67^{Abcd}	285 ± 72^{Ad}	286 ± 75^{Ad}

[a] Results are represented as the mean ± standard deviation (n = 3). Different uppercase letters indicate significant differences between samples on each time. Different lowercase letters indicate significant differences between times for one sample. N.D.: not detected.

Regarding predigested Hb, quantification of the samples showed 1018 mg PC per 100 g dry matter, which implies that only about 22% of the total PC initially quantified in Hb would be available for the fermentation process, and the remaining (78%) could be considered potentially bioaccessible in the small intestine. However, it is important to bear in mind that Hb contains a relevant amount of condensed and hydrolysable tannins (Mercado-Mercado et al., 2015) that can be available for the gut microbiota in the residues of the calyces as part of the indigestible fraction.

3.2. Identification and quantification of the phenolic compounds and metabolites of the fermented predigested Hb and Hb/AF

It is well stablished that many of the health effects attributed to PC are related to the metabolites produced during the bioconversion by gut microbiota and, therefore, the colon is a key organ in the biotransformation of PC. Thus, the residues obtained after predigestion of Hb calyces, equivalent to the non-digestible fraction of Hb, submitted to *in vitro* colonic fermentation alone or mixed with AF (Hb/AF, 70:30). As mentioned above, we aimed at assessing if the dietary fiber in combination with the non-digestible fraction of Hb could generate some metabolites with beneficial health effects other than those in predigested Hb. In this sense, the validated dynamic *in vitro* model of the colon (TIM-2), allowed to evaluate the effect of human microbiota on the metabolism of PC present in Hb. TIM-2 presents the following advantages: it can simulate the kinetic conditions in the colonic tract and has a dialysis membrane that removes metabolites from the lumen, preventing the accumulation, which may lead to inhibition or even death of the microorganisms (Venema et al., 2000). In addition, it allows simultaneous screening of different metabolites, for instance short chain fatty acids, ammonia, polyphenols, minerals, etc. (Koenen et al., 2016). Table 3 summarizes the quantification of PC and organic acids derived

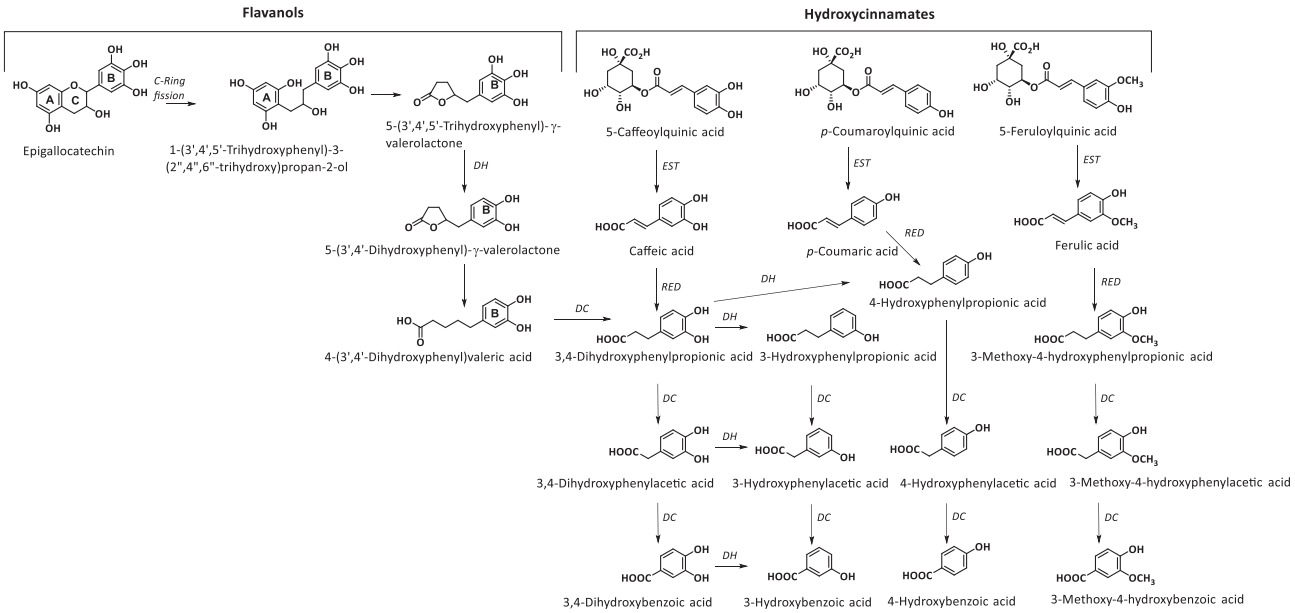

Fig. 1. Gut microbiota biotransformation pathways of hydroxycinnamates and flavanols contained in predigested complete calyces of hibiscus (*H. sabdariffa* L.). DC: decarboxylases; DH: dehydrogenases; EST: esterases; RED: reductases.

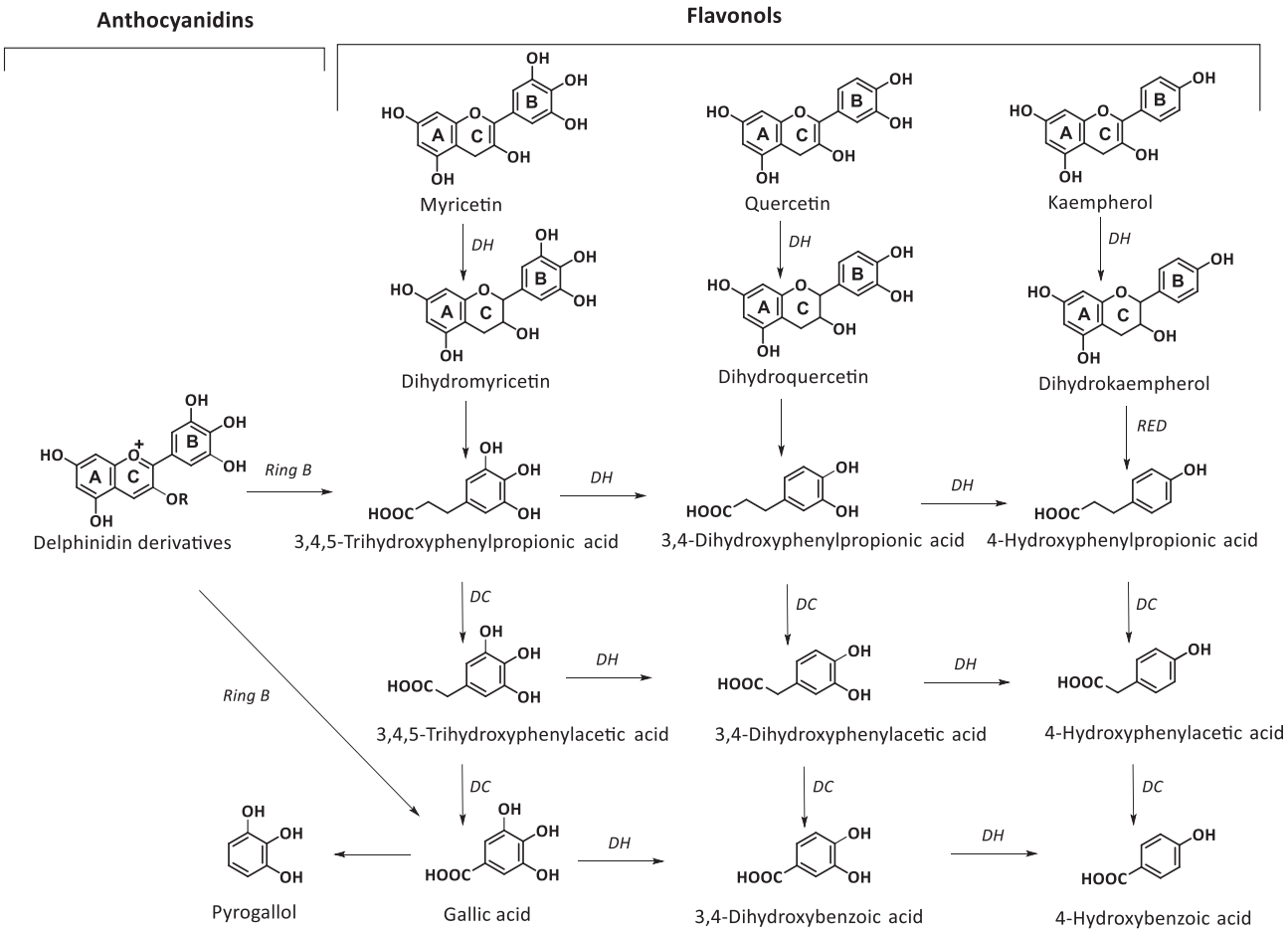

Fig. 2. Gut microbiota biotransformation pathways of flavonols and anthocyanidins contained in predigested complete calyces of hibiscus (*H. sabdariffa* L.). DC: decarboxylases; DH: dehydrogenases.

from the predigested Hb or Hb/AF after *in vitro* microbial colonic fermentation for 24, 48 and 72 h.

Firstly, regarding hibiscus acid and related compounds, only hibiscus acid was detected at a very low concentration or traces due to the lability of these compounds. Thus, hydroxycitric acid present in the digested samples was unstable and converted into its more stable lactone (Majeed, Badmaev, & Rajendran, 1998). Hibiscus acid was also susceptible to be reduced by the microbiota. Both the colonic metabolites along with the organic acids absorbed in the small intestine are responsible for the biological activity of these compounds. A recent review highlights the potential of organic acids in body weight management (Izquierdo-Vega et al., 2020). In particular, hydroxycitric acid promotes weight loss, suppresses fatty acid synthesis, and increases lipid oxidation, while hibiscus acid showed antimicrobial, antihypertensive, and enzyme inhibitory effects (alpha-amylase and alpha-glucosidase).

Hb is a rich source of hydroxycinnamates (18.6% of the total phenols in the digested Hb), but only caffeic and coumaric acids were detected in the fermented samples (in both Hb and Hb/AF). These compounds were also present at baseline but their content increased over fermentation time, with no difference ($p > 0.05$) between Hb and Hb/AF. This result indicates an extensive hydrolysis of hydroxycinnamates (caffeoylquinic, coumaroylquinic, caffeoylshikimic and 2-*O*-trans-caffeoyl-hydroxycitric acids) to form the abovementioned caffeic and coumaric acids. Likewise, absence of methyl-chlorogenate in the fermented samples suggests its complete hydrolysis to form chlorogenic acid and its subsequent hydrolysis into caffeic and quinic acids. Ferulic acid was not detected due to the low amounts of feruloylquinic acid and ferulic acid present in the predigested samples. Formation of reduced forms of hydroxycinnamic acids has been previously described in bioavailability studies carried out with coffee or yerba mate, which are rich sources of hydroxycinnamic acids (Gomez-Juaristi et al., 2018a, 2018b, 2019). Thus, dihydrohydroxycinnamic acids like dihydrocaffeic acid (3,4-dihydroxyphenylpropionic acid), dihydrocoumaric acid (hydroxyphenylpropionic acid) and dihydroferulic acid (4-hydroxy-3-methoxyphenylpropionic acid) were characterized in the fermented samples. Dihydrocaffeic acid might dehydroxylate, yielding 3- or 4-hydroxyphenylpropionic acid to contribute to the total amount of dihydrocoumaric acid (hydroxyphenylpropionic acid) (Fig. 1). Phenylpropionic acids might also evolve to form phenylacetic acid derivatives by the action of decarboxylases, yielding 3,4-dihydroxyphenylacetic, 3-hydroxy-4-methoxy-phenylacetic, 4-hydroxyphenylacetic and 3-hydroxyphenylacetic acids. Finally, decarboxylation of phenylacetic acid derivatives might result in the formation of benzoic acid derivatives, contributing to the total amount of 3,4-dihydroxybenzoic acid (protocatechuic acid) and 3- and 4-hydroxybenzoic acids quantified in the fermented samples.

Regarding the flavonoid group (9.6% of total PC quantified in the digested Hb), glycosylated derivatives of quercetin, myricetin, kaempherol and syringentin were absent in the samples fermented with human feces. Thus, hydrolysis of glycosylated derivatives to produce the corresponding aglycones, such as quercetin and myricetin detected in the samples, was the first biotransformation that took place by the intestinal microbiota. However, the low amount of these flavonols, up to 0.10–0.16 μmoles of mirycetin and 0.034–0.04 μmoles of quercetin in fermented Hb and Hb/AF, respectively, evidence the susceptibility to further biotransformation during fermentation. Consequently, colonic catabolism of kaempherol, quercetin, myricetin and syringetin results in their conversion into their respective dihydroderivatives, such as dihydroquercetin (taxifolin) for example, which can be further metabolized into mono-, di-, tri-hydroxyphenylpropionic acids or dimethylhydroxyphenylpropionic acid, respectively (Fig. 2). Subsequently, these compounds evolve to form phenylacetic acid derivatives, according to their chemical structure (Jaganath, Mullen, Lean, Edwards, & Crozier, 2009; Peng et al., 2014), and then into benzoic acids.

Within the flavonoid group, epigallocatechin was identified in the fermented samples although at low concentration (about 0.10 μmoles in both samples after 72 h), whereas no epicatechin derived from dehydroxylation of epicatechin gallate was detected. In turn, 5-(3′,4′-dihydroxypheynyl)-γ-valerolactone (DHPVL) was identified ($[M-H]^-$ at m/z 207.0663 and fragment ion at m/z 163), originated from C-ring fission of the flavan-3-ol molecule to produce the corresponding diphenylpropan-2-ol that is further converted into the diphenyl-γ-valerolactone (Gómez-Juaristi et al., 2019), which was quantified in the fermented samples and tended to accumulate over time (Table 3). Further biotransformation of DHPVL might evolve to dihydroxyphenylvaleric acid (not detected in this study), and 3,4-dihydroxyphenylpropionic acid and its monohydroxylated derivatives (3- and 4-dihydroxyphenylpropionic acids) which would subsequently form phenylacetic acid and benzoic acid derivatives, contributing to the formation of common phenolic acids (Fig. 1). It is remarkable that phenyl-γ-valerolactones and phenylvaleric acids have been described as exclusive microbial metabolites of flavan-3-ols (Gómez-Juaristi et al., 2019).

Hb contained a minor amount of phenolic acid derivatives, that after predigestion amounted up to 2.1% of the total PC in Hb. Protocatechuic acid glucoside was extensively hydrolyzed while methylgallate accumulated during colonic fermentation (from 0.05 μmoles up to 0.2 μmoles in both samples). The other phenolic acids observed, gallic, protocatechuic, 3-hydroxybenzoic, 4-hydroxybenzoic, and 4-hydroxyphenylpropionic acids) showed a tendency to accumulate over time as already mentioned, since most were common metabolites of the biotransformation of hydroxycinnamic acids and flavonoids.

Finally, Hb is known for its high content in anthocyanins and anthocyanidins. Particularly, the predigested Hb contained delphinidin-3-sambubioside and cyanidin-3-sambubioside, as well as free cyanidin (69.7% of the total PC quantified in predigested Hb). None of these compounds were detected in the fermented samples. Previous results have reported that after microbial deglycosylation, ring fission of the aglycone releases two parts, one derived from the A-ring and the second from the B-ring, which then undergo simultaneous catabolism (Aura et al., 2005; González-Barrio, Edwards, & Crozier, 2011). Regarding B-ring fission, both phenyl acids and benzoic acids have been reported as the microbial metabolites of anthocyanidins (Fig. 2). González-Barrio et al. (2011) proposed a complete colonic pathway for cyanidin including different alternative conversion pathways for the B-ring, and protocatechuic acid (benzoic acid) and 3,4-dihydroxyphenylpropionic acid (phenyl acid) were proposed as initial metabolites. The other most abundant anthocyanidin contained in Hb, delphinidin, might follow similar biotransformation pathways, which apart from trihydroxyphenylpropionic acid, that is dehydroxylated to produce dihydroxyphenylpropionic acid, might also produce gallic acid and pyrogallol, both compounds identified in the fermented samples. The fission of ring A of anthocyanidins could generate trihydroxybenzaldehyde, which could further be converted into phloroglucinol, but these were not detected in the present study (Forester & Waterhouse, 2008; González-Barrio et al., 2011) (Fig. 1).

Analysis of the biotransformation pathways of hydroxycinnamic acids, flavonoids, and anthocyanins and anthocyanidins by the microbiota revealed the formation of common metabolites such as phenylpropionic, phenylacetic and benzoic acids, as already discussed. Although some of these metabolites were present in predigested samples at baseline, their content increased over fermentation time. Regarding derivatives of phenylpropionic acid, 3,4-dihydroxyphenylpropionic and 3-methoxy-4-hydroxyphenylpropionic acids were less abundant than the monohydroxylated derivatives (3- and 4-hydroxyphenylpropionic acids). The content of the latter two increased up 193 and 32 μmoles in Hb, and up to 173 and 53 μmoles in Hb/AF, respectively, after 72 h of fermentation versus 7 and 4.5 μmoles of 3,4-dihydroxyphenylpropionic and 3-methoxy-4-hydroxyphenylpropionic acids in both samples, respectively. In spite of the high variability between replicates, a significant direct relationship was observed between their concentration and fermentation time ($p < 0.05$). Phenylacetic acids represented an important group of colonic metabolites, headed by 3-hydroxyphenylacetic acid (up to 17 and 21 μmoles in Hb and Hb/AF, respectively, at 72 h). Moreover, an average content of 10 μmoles of 4-hydroxyphenylacetic

and 3-methoxy-4-hydroxyphenylacetic acids was quantified in both Hb and Hb/AF after 72 h of fermentation, and finally 3,4-dihydroxyphenylacetic acid was also observed at minor levels (up to 6 μmoles for both samples at 72 h). The high variability between replicates may have blunted significant differences in the metabolite contents produced over fermentation time, although the accumulation of 4-hydroxyphenylacetic and 3-methoxy-4-hydroxyphenylacetic acids showed a significant ($p < 0.05$) relationship with time. Finally, 3,4-dihydroxybenzoic acid along with 3- and 4-hydroxybenzoic acids were characterized in the fermented samples in substantially lower amounts than phenylpropionic and phenylacetic acid derivatives, with protocatechuic acid (3,4-dihydroxybenzoic acid) being the most abundant compound of this group of metabolites (about 1 μmol after 72 h of incubation in Hb and Hb/AF). There were no significant differences between conditions although the was a tendency to accumulate over time (Table 3).

Another important aim of this study was to evaluate the influence that AF could have on this biotransformation of PC. In this sense, Table 3 shows no significant difference between metabolites characterized in both fermented samples (Hb and Hb/AF). Changes in gut microbiota upon feeding predigested Hb, AF, oligofructans (OF) and the mixture Hb/AF to the microbiota in TIM-2 have been previously studied (Sáyago-Ayerdi et al., 2020). About 45 bacterial genera were identified, of which 10 showed important changes after fermentation, mainly dependent on the fermentation time and type of substrate, primarily with a high relative abundance of *Bifidobacterium*, *Bacteroides* and *Catenibacterium*,. The genera *Bacteroides* was inhibited after 24 h fermentation of Hb, which might be related to the antimicrobial activity of Hb (Borrás-Linares et al., 2015). Similar results had been published in animal studies, mainly with flavonoids in apple juice or cocoa drink that are able to reduce *Bacteorides* and promote *Bifidobacterium* and *Lactobacillus* (Cardona, Andres-Lacueva, Tulipani, Tinahones, & Queipo-Ortuno, 2013; Etxeberria et al., 2013). The use of AF as a source of DF or prebiotics improved the relative abundance of *Bifidobacterium* (Sáyago-Ayerdi et al., 2020), but it did not affect the bioconversion of PC during the colonic fermentation.

It is important to mention that the quantified phenolic metabolites in the fermented samples showed a recovery of about 30% after 72 h, with a tendency to accumulate during fermentation. A large number of studies have demonstrated the presence of colonic metabolites in plasma and urine after consuming dietary sources of PC, increasing the residence of phenolic metabolites in the body, thus resulting in an extended bioactivity. A recent review provided an overview of studies that have detected and/or quantified microbial phenolic metabolites, covering *in vitro* experimental studies and human clinical trials (Marhuenda-Muñoz et al., 2019). Similarly, Mosele, Macià, and Motilva (2015) also reviewed the biological effects associated with these microbial phenolic metabolites. To further understand the promising activity of these microbial phenolic metabolites obtained by *in vitro* models, it is necessary to carry out human trials.

4. Conclusion

The present study revealed a wide spectrum of microbial-derived metabolites formed after the fermentation of Hb and the mixture of Hb/AF with human fecal microbiota in TIM-2, a validated colon model. Simple phenolic acid derivatives of phenylpropionic, phenylacetic and benzoic acids, common in the biotransformation of flavonoids, as well as hydroxycinnamic acids and anthocyanidins, were characterized upon fermentation of Hb and Hb/AF. Phenolic microbial metabolites are key to understand the bioactivity of Hb polyphenols, which were significantly more abundant than metabolites produced from organic acids, such as hydroxycitric and hibiscus acids, that are better absorbed in the small intestine. Further research is needed to understand the biological effects of these microbial phenolic metabolites as well as their potential health applications. This research sets the ground for future studies with the identification of Hb and Hb/AF microbial metabolites produced by the gut microbiota.

CRediT authorship contribution statement

S.G. Sáyago-Ayerdi: Resources, Investigation, Writing - review & editing. **K. Venema:** Methodology, Software, Validation, Resources, Writing - review & editing, Project administration. **M. Tabernero:** Methodology, Validation. **B. Sarriá:** Writing - review & editing. **L. Bravo:** Resources, Writing - review & editing. **R. Mateos:** Methodology, Validation, Resources, Writing - review & editing, Visualization, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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