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Transformations of phenolic compounds in cocoa shell during *in vitro* colonic fermentation

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ABSTRACT

Cocoa shell is a by-product generated by the cocoa processing industry, notable for its high content of phenolic compounds and methylxanthines, and recognized for their biological properties. The majority of cocoa phenolic compounds are not absorbed in the small intestine and reach the colon, where they can be catabolized by the gut microbiota, influencing their bioavailability and bioactivity. This research aimed to study the changes that phenolic compounds from cocoa shell flour (CSF) and extract (CSE) undergo during colonic fermentation after gastrointestinal digestion, using an *in vitro* model and a targeted metabolomics approach. A decrease in the concentration of most parental phenolic compounds was observed, with a simultaneous increase in phenyl-γ-valerolactones, phenylvaleric acids, and phenylpropanoic acids. Benzoic acids, phenylpropanoic acids, phenylacetic acids, and benzaldehydes were the compounds found in the highest concentrations. Additionally, phenolic compounds in CSF were metabolized more slowly than those in CSE. This may be due to the matrix effect that protects the compounds from degradation during colonic fermentation. These findings further support the potential of cocoa shells as a food ingredient rich in phenolic compounds and bioavailable metabolites, which may exert beneficial effects in the colon and at the systemic level.

1. Introduction

Cocoa is an important agricultural crop cultivated predominantly in tropical regions and is highly valued for its role in the chocolate manufacturing industry (Aprotosoaie et al., 2016). The processing of cocoa beans involves several steps, including the removal of the pod husk, which surrounds the seeds, and the generation of cocoa shells during the roasting process. This by-product, often perceived as waste, has traditionally been used as mulch in agricultural practices (Panak Balentić et al., 2018). Recent studies have confirmed the non-toxicity of cocoa shells, supporting their safe use as a food ingredient. Toxicological assessments, including acute and sub-chronic studies in animal models, have shown no lethality or adverse histopathological effects, with heavy metal levels well within safe limits. Considering these findings and their composition, cocoa shell presents a promising option for upcycling as a valuable ingredient in food products. The cocoa shell is comprised mainly of dietary fiber (59%), carbohydrates (69%), proteins (15%), and fats (8%). Cocoa shells also provide a balanced amino acid profile, making them a good source of plant-based protein with essential amino acids like lysine, leucine, and phenylalanine, as well as minerals, especially potassium (Gil-Ramírez et al., 2024). Furthermore, cocoa shells are rich in bioactive compounds such as methylxanthines, including theobromine and caffeine, and phenolic compounds, highlighting protocatechuic and vanillic acids and mono- and polymeric flavan-3-ols (Rebollo-Hernanz et al., 2022). Phenolic compounds are plant secondary metabolites widely studied for their biological properties and

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potential health benefits such as reduced risk of chronic diseases (Rahman et al., 2021). Specifically, flavonoids found in cocoa are recognized for their potential cardiovascular health benefits. They aid in preventing the oxidation of LDL-cholesterol and promote vasodilation, thereby enhancing blood flow and reducing blood pressure (Martin and Ramos, 2021).

While plant matrices, including cocoa shell, are rich in phenolic compounds, not all of these are effectively absorbed in the gastrointestinal tract. This highlights the importance of the release and absorption of phenolic compounds in the digestive tract, which are key to exerting their physiological effects on the organism. Thus, researching the bioaccessibility and bioavailability of these compounds during gastrointestinal digestion becomes essential. However, numerous factors can affect this process, such as interactions with other dietary components, pH, intestinal transit time, food matrix, and food processing (Shahidi and Pan, 2022). Our latest investigations on the bioaccessibility and bioavailability of phenolic compounds found in cocoa shell during *in vitro* digestion revealed that, although certain phenolic compounds were degraded during digestion, notably flavonols and flavones, the digestive process led to the release of other compounds such as hydroxybenzoic acids and flavan-3-ols. Furthermore, the food matrix, characterized by a high fiber content, exerted a protective effect on these phenolic compounds, allowing a substantial proportion of them to reach the colon (Cañas et al., 2022b). On the other hand, the bioaccessible fractions of phenolic compounds demonstrated antioxidant and free radical scavenging abilities and ameliorated oxidative stress in intestinal and hepatic cells (Cañas et al., 2023). Additionally, cocoa shell digested fractions effectively reduced lipid accumulation in hepatic cells by modulating cholesterol and fat metabolism pathways (Braojos et al., 2024). The cocoa shell also exhibited vasodilatory properties in aged rats, which could contribute to the prevention of diseases associated with endothelial dysfunction (Rodríguez-Rodríguez et al., 2022). Nevertheless, the majority of cocoa shell phenolic compounds, in particular complex and polymeric ones, are not absorbed in the small intestine and reach the large intestine, where intestinal bacteria convert them into low molecular weight catabolites. In this sense, phenolic compounds can act as prebiotic molecules stimulating the proliferation of protective bacterial strains, thus positively modulating the intestinal microbiota and preventing microbial imbalances (dysbiosis) linked to various gastrointestinal pathologies. Furthermore, these compounds are potentially capable of exerting anti-inflammatory, antioxidant, antiproliferative, and antidiabetic effects in the colon (Lippolis et al., 2023). So, the intake of natural sources rich in phenolic compounds, such as cocoa shells, emerges as a promising strategy to convey health benefits.

Phenolic compounds in cocoa shells are highly bound to fiber, which could be considered “antioxidant dietary fibers”, allowing them to resist digestion and reach the colon intact. There, they can be released during the fiber fermentation process carried out by the intestinal microbiota (Pérez-Jiménez and Saura-Calixto, 2017). While the interactions between cocoa-derived phenolic compounds and the gut microbiota have been investigated (Sorrenti et al., 2020), to date, the colonic fermentation of phenolic compounds present in cocoa shells has not been explored yet. Given this backdrop, our hypothesis is that phenolic compounds from cocoa shells could be released and transformed during colonic fermentation, potentially leading to the formation of bioactive metabolites with potentially protective properties. The aim of this investigation was to examine the impact of colonic fermentation on the phenolic compound transformation in cocoa shell flour (CSF) and extract (CSE) using an *in vitro* fecal fermentation model and a targeted metabolomics approach based on UHPLC-ESI-MS/MS.

2. Materials and methods

2.1. Chemicals

For *in vitro* gastrointestinal digestion, ammonium carbonate, calcium chloride dihydrate, magnesium chloride hexahydrate, potassium dihydrogen phosphate, porcine pepsin and porcine pancreatin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrochloric acid, potassium chloride, sodium bicarbonate, sodium chloride, and sodium hydroxide were supplied by Panreac Química SLU (Barcelona, Spain). For *in vitro* colonic fermentation, iron sulfate heptahydrate, L-cysteine hydrochloride monohydrate and PBS buffer were purchased from Panreac AppliChem (Barcelona, Spain). Di-potassium hydrogen phosphate and soluble starch were obtained from Carlo Erba Reagents (Milan, Italy). (+)-Arabinogalactan, guar gum, inulin, mucin from pig stomach, type III, pectin from citrus fruits, potassium chloride, sodium bicarbonate, sodium chloride, sodium salts of casein from cow's milk, tryptone, tween 80, xylans from birchwood and yeast extract were purchased from Sigma-Aldrich (St. Louis, MO, USA). Bile salts, buffered peptone water (ISO), calcium chloride, magnesium sulfate monohydrate, and monobasic potassium phosphate were acquired from Fluka Chemika (Neu-Ulm, Switzerland). Phenolic standards, including (−)-epicatechin, 2-hydroxy-3-(3'-hydroxyphenyl)acetic acid, 3-(3',4'-dihydroxyphenyl)propanoic acid, 3,4,5-trihydroxybenzoic acid, 3,4-dihydroxybenzaldehyde, 3,4-dihydroxybenzoic acid, 3',4'-dihydroxycinnamic acid, 3-caffeoylquinic acid, 3-hydroxybenzoic acid, 3'-hydroxycinnamic acid, 3-(3'-hydroxyphenyl)propanoic acid, 4'-hydroxy-3',5'-dimethoxycinnamic acid, 4-caffeoylquinic acid, 4-hydroxy-3,5-dimethoxybenzoic acid, 4'-hydroxy-3'-methoxycinnamic acid, 4-hydroxybenzaldehyde, 4-hydroxybenzoic acid, 4'-hydroxycinnamic acid, 3-(4'-hydroxyphenyl)propanoic acid, 5-caffeoylquinic acid, benzene-1,2,3-triol, benzoic acid, kaempferol, quercetin, and rutin were obtained from Sigma-Aldrich (St. Louis, MO, USA). 5-(3',4'-Dihydroxyphenyl)-γ-valerolactone and 5-(3'-hydroxyphenyl)-γ-valerolactone were synthesized in house and kindly supplied by Prof. Curti using the synthetic strategy previously reported (Curti et al., 2015). Chemical names of catabolites are reported according to Kay et al. (2020). For the UHPLC analysis, all reagents (UHPLC grade) were obtained from VWR International (Milan, Italy).

2.2. Sample preparation

The cocoa shell was supplied as dry material by Chocolates Santoclides (Castrocontrigo, León, Spain) and kept at 4 °C until required. The cocoa shell was milled with an IKA A10 laboratory grinder (IKA Werke, Staufen, Germany) to yield cocoa shell flour (CSF), which was then stored in sealed flasks at −20 °C until needed. An aqueous extract (CSE) was prepared from the CSF through hydrothermal extraction, in accordance with a method previously optimized (Rebollo-Hernanz et al., 2021). Specifically, the CSF, at a solid-to-solvent ratio of 0.02 g mL^{-1}, was added to boiling water (100 °C) and agitated for 90 min. The resulting extract was filtered, frozen at −20 °C, freeze-dried (Delta-2-24/LSC plus, Martin Christ, Osterode am Harz, Germany), and stored at −20 °C for future use.

2.3. In vitro simulated gastrointestinal digestion

Both CSF and CSE were subjected to *in vitro* gastrointestinal digestion following the harmonized INFOGEST protocol (Brodkorb et al., 2019), with slight modifications as previously optimized (Cañas et al., 2022a). Briefly, the oral phase was simulated by mixing 1 g of CSF or 100 mg of CSE with simulated salivary fluid (SSF), and the mixture was kept under agitation (2 min, 37 °C, pH 7), utilizing an overhead shaker Reax 2 (Heidolph Instruments, Schwabach, Germany). Amylase was not added due to the absence of starch in the samples. For the gastric phase, the oral phase was combined with simulated gastric fluid (SGF) and a porcine pepsin solution (2000 U mL^{-1} of digestion). The samples were then incubated under constant stirring (2 h, 37 °C, pH 3). The intestinal phase was simulated by mixing the gastric phase with simulated intestinal fluid (SIF), which contained pancreatin (100 U trypsin activity mL^{-1} of digest). The samples were incubated under agitation (2 h, 37 °C,

pH 7). Finally, the samples were freeze-dried and stored at −20 °C until further use. A digestion blank was prepared for each digestion phase, containing the corresponding simulated digestive fluids and enzymes.

2.4. Growth medium and fecal slurry

The growth medium and the fecal slurry were prepared according to a protocol previously described by Bresciani et al. (2018). Briefly, the growth medium was prepared by dissolving 2.5 g of soluble starch, 2.5 g of peptone, 2.5 g of tryptone, 2.25 g of yeast extract, 1.5 g of casein, 1 g of pectin, 1 g of mucin, 1 g of xylan, 1 g of guar gum, 0.5 g of inulin, 0.5 g of arabinogalactan, 0.4 g of cysteine-HCl, 2.25 g of NaCl, 2.25 g of KCl, 0.75 g of $NaHCO_3$, 0.35 g of $MgSO_4$, 0.25 g of K_2HPO_4, 0.25 g of KH_2PO_4, 0.4 g of $CaCl_2$, 0.0025 g of $FeSO_4{\cdot}7H_2O$, 0.2 g of bile salts, and 0.5 mL of Tween 80 in distilled water, adjusting to a final volume of 1 L. Subsequently, 1.8 mL of the growth medium was aliquoted into glass vessels. The containers and the remaining medium were sterilized by autoclaving at 121 °C for 15 min and stored at 4 °C until use.

Fresh fecal samples were collected from two healthy donors without intestinal diseases and who had not taken antibiotics during the 3 months before the study. Donors followed a controlled diet free of phenolic compounds for 2 days prior to feces collection. This diet excluded tea and coffee, both regular and decaffeinated, as well as alcohol, vegetables, fruits and fruit juices, chocolate and cocoa-based products, whole grain bread and cereals, spices like curry, herbs, and vegetable oils. The allowed foods included white bread, pasta, white rice, butter, meat, eggs, fish, potatoes, mushrooms, milk, plain yogurt, and cheese (Pereira-Caro et al., 2023). The feces were promptly stored in anaerobic conditions and processed within 2 h after collection. The fecal slurry was prepared by pooling the feces with sterilized PBS 1% (w/v) using a stomacher homogenizer (PBI International, Milan, Italy) to obtain a 10% (w/w) slurry suspension, used as a fermentation starter. The Ethics Committee of Area Vasta Emilia Nord (AVEN) approved the collection and use of the fecal slurries (protocol no. 796/2018/sper/unipr).

2.5. In vitro colonic fermentation

In vitro fermentation was performed according to the method of Dall'Asta et al. (2012) with some modifications. Sample aliquots of the fermentation substrates, namely CSF and CSE were dissolved in Dulbecco's PBS solution for 2 h under stirring at room temperature. For this, the total yield of the previously digested intestinal fraction was used to conduct colonic fermentation. For CSF, 4.0 g of the digested intestinal fraction were dissolved in 32 mL of PBS, and for CSE, 1.2 g of the digested intestinal fraction were dissolved in 16 mL of PBS. Blank samples containing growth medium and fecal slurry and abiotic controls containing the growth medium and the substrate were prepared. Digestion controls containing growth medium, fecal slurry, and the intestinal phase without CSF and CSE were also prepared. The fermentation was carried out by adding 400 μL of the substrate solutions in a sterilized glass vessel containing 1.8 mL of growth medium. Subsequently, 1.8 mL of fecal slurry was added, obtaining a final volume of 4 mL. To establish an anaerobic environment, vessels were sealed using rubber seals and purged of air using a double needle with nitrogen (N_2). After the fermentation started, the samples were incubated at 37 °C for 48 h in agitation at 200 strokes min^{-1} in a Dubnoff bath (ISCO, Milan, Italy). Samples were collected at 0, 2, 6, 24 and 48 h for further analysis. Some additional precautions were followed after 24 h fermentation for samples incubated for 48 h: batches were degassed with a sterile syringe to reduce the pressure caused by the microbial fermentation; then, vessels were flushed with N_2 to maintain an anaerobic environment, and sterilized fresh growth medium (1 mL) was added prior re-flushing N_2. Finally, acetonitrile was added to the fermentation solution (10% v/v) to stop the fermentation, and the fermented samples were frozen at −80 °C until further use.

2.6. Extraction of phenolic compounds and fecal metabolites

Phenolic compounds and the catabolites generated during the *in vitro* fermentation were extracted following a slightly modified version of a previously described method (Moreno-Ortega et al., 2022). Briefly, 300 μL of the fermented samples were mixed with 1.2 mL of acidified ethyl acetate (formic acid 0.1%, v/v). The samples were shaken using a vortex for 30 s and sonicated in an ultrasonic bath for 10 min. Samples were vortexed again for 30 s and sonicated for 5 min. Then, they were centrifuged at 14462×*g* (Centrisart® A–14C, Sartorius Lab Instruments GmbH and Co. KG, Goettingen, Alemania) for 10 min, collecting the supernatant. The resulting pellet was mixed with 0.5 mL of acidified ethyl acetate (formic acid 0.1%, v/v), vortexed for 30 s, sonicated for 10 min and again vortexed (30 s) and sonicated (5 min). The supernatant obtained was combined with the supernatant collected from the first extraction. Samples were evaporated using a centrifugal vacuum concentrator (SpeedVac Savant SPD121P, Thermo Fisher Scientific Inc., San Jose, CA, USA) and reconstituted in 200 μL of water: methanol 50:50 (v/v) with 0.1% formic acid. The samples were then vortexed for 30 s, sonicated for 5 min, and centrifuged at 14462×*g* for 5 min. The samples were filtered through a 0.22 μm pore size syringe nylon filter. Finally, they were stored at −80 °C until UHPLC-ESI-MS/MS analysis.

2.7. UHPLC-ESI-MS/MS analysis

Phenolic compounds were assessed through a UHPLC DIONEX Ultimate 3000 fitted with a TSQ Vantage triple quadrupole mass spectrometer (MS) (Thermo Fisher Scientific Inc., San Jose, CA, USA), equipped with a H-ESI-II heated-electrospray ionization source (Thermo Fisher Scientific Inc., San Jose, CA, USA). Chromatographic and ionization parameters were selected according to a previous work (Brindani et al., 2017), with slight modifications. For UHPLC, solvent A was 0.01% v/v formic acid in water and solvent B was acetonitrile containing 0.01% v/v formic acid. The gradient started with 5% of solvent B and was maintained for 0.5 min. The concentration of B was progressively increased to 40% over a 7-min interval. After that, the proportion of solvent B was further raised to 80% in 1 min, held constant for 2 min, and then the initial conditions were restored in 0.5 min and maintained for 3 min to re-equilibrate the column. This resulted in a total run time of 14 min. A flow rate of 0.4 mL min^{-1} was set. The H-ESI-II interface was adjusted to a capillary temperature of 275 °C, while the source heater was maintained at 250 °C. The flow rate for sheath gas (N_2) was established at 40 (arbitrary units), the auxiliary gas (N_2) flow rate at 5, and the sweep gas flow rate was adjusted to 15. The source voltage was 3 kV, the capillary voltage was −9 V, and the tube lens voltage was −53 V. The analysis of phenolic compounds and their catabolites in fermented CSF and CSE was conducted using a Kinetex Evo C18 column (100 × 2.1 mm; 2.6 μm particle size, Phenomenex, CA, USA) for separation, installed with a precolumn cartridge (Phenomenex). The column oven was set at a temperature of 40 °C and the injection volume was 5 μL. A total of 92 compounds (both parent compounds and gut microbiota catabolites) were monitored through selective reaction monitoring (SRM) mode (Table S1 in Supplementary Information). Quantification was performed with calibration curves of standards, when available, or using the most structurally similar compound. Chromatograms and mass spectral data were collected using Xcalibur software version 2.1 (Thermo Fisher Scientific Inc.).

2.8. Statistical analysis

Digestion controls containing gastrointestinal fluids, growth medium, and fecal slurry, without CSF and CSE, were used to subtract the values of the fermented samples. This ensured that the values measured in the fermented samples were not influenced by the residual presence of phenolic compounds from the fecal sample used. Results are expressed as mean ± standard deviation (SD) (n = 3). Data were analyzed by one-

way analysis of variance (ANOVA) and *post-hoc* Tukey's test to compare the same fermented sample at different incubation periods (0, 2, 6, 24, or 48 h), and to compare the contribution of the different phenolic compounds at the same incubation period. Differences were considered significant at $p < 0.05$. All statistical analyses were performed using IBM SPSS Statistics 28.0.1.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism 8.0 (San Diego, CA, US). XLSTAT2021 was employed to run multivariate analysis: principal component analysis (PCA) and agglomerative hierarchical clustering (AHC). The PCA was performed on a Pearson correlation matrix to neutralize scale effects among variables. The clustering analysis utilized Euclidean distance as the dissimilarity metric, with Ward's method employed as the aggregation criterion.

3. Results

3.1. Catabolism of phenolic compounds in CSF and CSE during in vitro colonic fermentation

CSF and CSE were incubated with fecal material for 48 h after *in vitro* gastrointestinal digestion. Throughout the *in vitro* colonic fermentation of CSF and CSE, up to 36 compounds were identified, as indicated in Table S1. Twelve families of compounds were detected: flavan-3-ols (n = 1), flavonols (n = 4), diphenylpropan-2-ol derivatives (n = 2), phenyl-γ-valerolactones (n = 2), phenylvaleric acids (n = 3), cinnamic acids (n = 3), cinnamoylquinic derivatives (n = 2), cinnamoylaminoacid derivatives (n = 6), phenylpropanoic acids (n = 4), phenylacetic acids (n = 1), benzoic acids (n = 6), and benzaldehydes (n = 2). The profile of phenolic compounds showed slight differences between CSF and CSE. In particular, CSF was associated with 35 different compounds, while CSE was associated with 32. Most compounds were identified at the beginning of the incubation (T0 and T2) and decreased during the fermentation process.

3.1.1. Changes in cocoa shell flour

The concentration of phenolic compounds during the colonic fermentation of CSF at different time points is summarized in Fig. 1A–B and detailed in Table 1. At the beginning of the fermentation, the total concentration of phenolic compounds was 345.4 μmol 100 g^{-1}. After 2 h of fermentation, there were no significant differences in the total concentration of phenolic compounds compared to the start of the fermentative process. After 6 h, a reduction in phenolic content was observed, which became more pronounced after 24 and 48 h. Phenylpropanoic acids emerged as the predominant class of phenolic compounds at the beginning of the fermentation process (T0). In particular, 3-(4'-hydroxyphenyl)propanoic acid was initially the most abundant compound, with a concentration of 136.3 μmol 100 g^{-1}. Although it exhibited a reduction of 41.3% by the end of the fermentation, it remained the predominant compound at all incubation times, reaching a final concentration of 80.0 μmol 100 g^{-1}. On the other hand, 3-(3'-hydroxyphenyl)propanoic acid, present in very low amounts at baseline, showed an increase of 99.2%, achieving a concentration of 67.2 μmol 100 g^{-1} at 48 h. Additionally, 3-(3',4'-dihydroxyphenyl)propanoic acid, not detected initially, was identified at the end of the fermentation process.

Benzoic acids were the second most abundant class, with 3,4-dihydroxybenzoic acid (86.1 μmol 100 g^{-1}) and 4-hydroxybenzoic acid (28.7 μmol 100 g^{-1}) notably abundant at T0. Both compounds remained relatively stable in the first 2 h of fermentation and decreased slightly until T6, and then they exhibited a relevant drop in the following times. In contrast, benzoic acid, which presented an initial concentration of 18.0 μmol 100 g^{-1}, did not show a significant decrease and was still detected at the end of the fermentation process. Another phenolic class present at high concentrations at T0 was the benzaldehydes, including 3,4-dihydroxybenzaldehyde (34.6 μmol 100 g^{-1}) and 4-hydroxybenzaldehyde (8.4 μmol 100 g^{-1}). These compounds experienced declines of 98.6% and 95.2%, respectively, at the end of the fermentation process. In the group of phenylacetic acids, only 2-hydroxy-2-(3'-hydroxyphenyl) acetic acid was present throughout the fermentation process, suffering a decrease of 74.5% at the end.

The most significant cinnamoyl-amino acid derivatives included *N-p*-coumaroyl-L-aspartate, *N-p*-coumaroyl-L-glutammate, isomer 1, and *N-p*-coumaroyl-L-tyrosine, which were not found after 48 h of fermentation. 4'-Hydroxycinnamic acid stood out as the main cinnamic acid at T0, with a concentration of 1.7 μmol 100 g^{-1}, but it was not detected in the following times. The same catabolism was observed for 4'-hydroxy-3'-methoxycinnamic acid. Conversely, 3',4'-dihydroxycinnamic acid remained quite stable during the fermentation process.

As regards flavonoids, flavonols were principally detected after 2 h of incubation, with quercetin-rutinoside (13.9 μmol 100 g^{-1}), quercetin (10.9 μmol 100 g^{-1}), and kaempferol (2.7 μmol 100 g^{-1}) as the main compounds, although they disappeared after 6 h of fermentation.

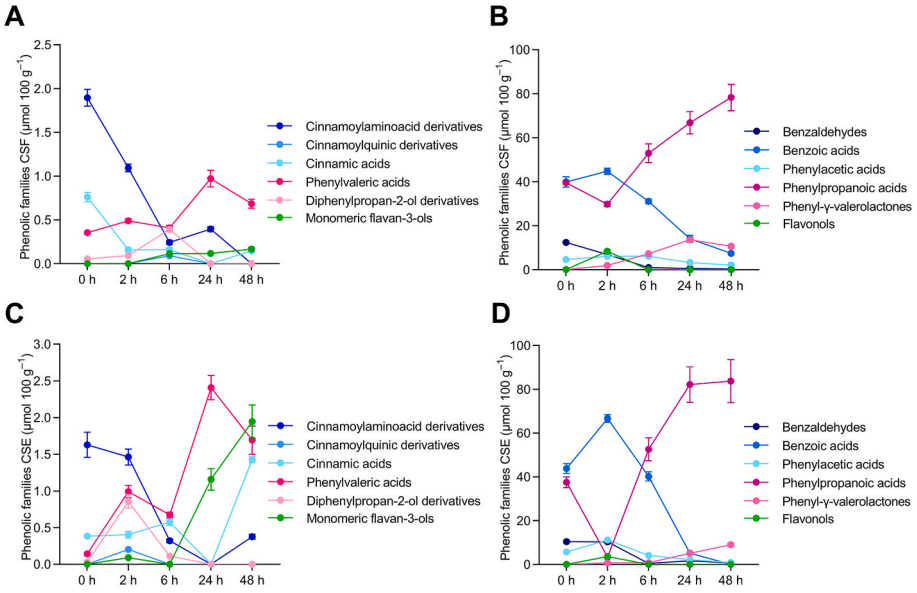

Fig. 1. Effects of *in vitro* colonic fermentation on families of phenolic compounds in CSF at low (**A**) and high concentrations (**B**), and in CSE at low (**C**) and high concentrations (**D**), during different incubation periods (0, 2, 6, 24, 48 h). The results are reported as mean ± SD ($n = 3$).

Table 1
Concentration of individual phenolic compounds (μmol 100 g^{-1}) in the cocoa shell flour (CSF) at baseline (T0) and after 2, 6, 24 and 48 h of *in vitro* colonic fermentation (T2, T6, T24 and T48). The nomenclature of catabolites is reported as proposed by Kay et al. (2020). Common names are indicated under brackets.

Compounds	T0	T2	T6	T24	T48
Cocoa shell flour					
Flavan-3-ols					
(−)-Epicatechin	n.d.	n.d.	0.3 ± 0.1^{a}	0.2 ± 0.0^{a}	0.3 ± 0.1^{a}
Flavonols					
Kaempferol	n.d.	2.7 ± 0.6	n.d.	n.d.	n.d.
Quercetin	n.d.	10.9 ± 3.3	n.d.	n.d.	n.d.
Quercetin-arabinoside	0.4 ± 0.1	0.3 ± 0.1	n.d.	n.d.	n.d.
Quercetin-rutinoside	n.d.	13.9 ± 0.0	n.d.	n.d.	n.d.
Diphenylpropan-2-ol derivatives					
1-(Hydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol	0.2 ± 0.1^{b}	0.2 ± 0.0^{b}	0.9 ± 0.1^{a}	n.d.	n.d.
1-(3',4'-Dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol	n.d.	0.1 ± 0.0	0.2 ± 0.0	n.d.	n.d.
Phenyl-γ-valerolactones					
5-(3'-Hydroxyphenyl)-γ-valerolactone	n.d.	n.d.	0.4 ± 0.0^{b}	0.2 ± 0.1^{b}	0.8 ± 0.2^{a}
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone	0.6 ± 0.1^{c}	6.6 ± 0.9^{b}	20.1 ± 0.8^{a}	23.4 ± 2.5^{a}	20.0 ± 2.4^{a}
Phenylvaleric acids					
4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.3 ± 0.0^{bc}	0.4 ± 0.1^{ab}	0.5 ± 0.1^{a}
4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid	0.2 ± 0.0^{c}	0.3 ± 0.0^{c}	0.3 ± 0.1^{c}	0.5 ± 0.1^{b}	0.8 ± 0.1^{a}
4-Hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid	0.8 ± 0.1^{b}	1.1 ± 0.1^{a}	0.5 ± 0.0^{c}	0.8 ± 0.1^{b}	n.d.
Cinnamic acids					
4'-Hydroxycinnamic acid (*p*-Coumaric acid)	1.7 ± 0.2	n.d.	n.d.	n.d.	n.d.
3',4'-Dihydroxycinnamic acid (Caffeic acid)	0.4 ± 0.1^{a}	0.5 ± 0.1^{a}	0.5 ± 0.1^{a}	n.d.	0.3 ± 0.1^{a}
4'-Hydroxy-3'-methoxycinnamic acid (Ferulic acid)	0.6 ± 0.1	n.d.	n.d.	n.d.	n.d.
Cinnamoylquinic derivatives					
5-*O*-*p*-Coumaroylquinic acid	n.d.	n.d.	0.3 ± 0.0	n.d.	n.d.
Cinnamoyl-amino acid derivatives					
N-*p*-Coumaroyl-L-aspartate	2.7 ± 0.3	1.4 ± 0.1	n.d.	n.d.	n.d.
N-*p*-Coumaroyl-L-glutammate isomer 1	1.8 ± 0.1	0.5 ± 0.0	n.d.	n.d.	n.d.
N-*p*-Coumaroyl-L-glutammate isomer 2	0.5 ± 0.1	n.d.	n.d.	n.d.	n.d.
N-*p*-Coumaroyl-L-tyrosine	1.3 ± 0.2^{a}	1.1 ± 0.1^{a}	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	n.d.
N-Caffeoyl-3-hydroxy-L-tyrosine	n.d.	0.4 ± 0.0^{a}	0.3 ± 0.1^{a}	0.3 ± 0.1^{a}	n.d.
N-Feruloyl-L-aspartate	0.2 ± 0.0^{c}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.4 ± 0.0^{a}	n.d.
Phenylpropanoic acids					
3-(4'-Hydroxyphenyl) propanoic acid	136.3 ± 4.2^{a}	92.1 ± 6.6^{b}	134.1 ± 22.1^{a}	60.0 ± 11.6^{c}	80.0 ± 14.4^{bc}
3-(3'-Hydroxyphenyl) propanoic acid	0.5 ± 0.1^{d}	6.0 ± 0.3^{cd}	14.4 ± 1.6^{c}	54.2 ± 5.9^{b}	67.2 ± 8.8^{a}
3-(3',4'-Dihydroxyphenyl) propanoic acid (Dihydrocaffeic acid)	n.d.	n.d.	n.d.	1.5 ± 0.0	5.6 ± 0.2
3-(4'-Hydroxy-3'-methoxyphenyl) propanoic acid (Dihydroferulic acid)	n.d.	0.6 ± 0.0	n.d.	n.d.	n.d.
Phenylacetic acids					
2-Hydroxy-2-(3'-hydroxyphenyl)acetic acid (3-Hydroxymandelic acid)	16.1 ± 2.7^{b}	20.4 ± 1.1^{a}	17.2 ± 0.8^{ab}	5.6 ± 1.2^{c}	4.1 ± 0.5^{c}
Benzoic acids					
Benzoic acid	18.0 ± 1.6^{ab}	24.5 ± 3.7^{a}	n.d.	20.3 ± 4.8^{a}	13.3 ± 0.4^{b}
4-Hydroxybenzoic acid	28.7 ± 2.7^{ab}	29.6 ± 1.3^{a}	24.2 ± 2.7^{b}	n.d.	n.d.
3-Hydroxybenzoic acid	0.5 ± 0.1^{d}	2.1 ± 0.2^{a}	1.1 ± 0.0^{c}	1.6 ± 0.0^{b}	n.d.
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	86.1 ± 11.0^{a}	86.5 ± 3.9^{a}	61.5 ± 3.1^{b}	2.5 ± 0.6^{c}	1.2 ± 0.2^{c}
3,4,5-Trihydroxybenzoic acid (Gallic acid)	0.4 ± 0.1^{b}	0.7 ± 0.1^{a}	0.3 ± 0.0^{b}	n.d.	n.d.
4-Hydroxy-3,5-dimethoxybenzoic acid (Syringic acid)	4.2 ± 0.8	4.9 ± 0.5	n.d.	n.d.	n.d.
Benzaldehydes					
4-Hydroxybenzaldehyde (4-Formylphenol)	8.4 ± 0.9^{a}	0.6 ± 0.2^{b}	0.2 ± 0.0^{b}	0.7 ± 0.1^{b}	0.4 ± 0.0^{b}
3,4-Dihydroxybenzaldehyde (Protocatechualdehyde)	34.6 ± 2.4^{a}	22.5 ± 2.1^{b}	2.7 ± 0.2^{c}	0.4 ± 0.1^{c}	0.5 ± 0.0^{c}
Total phenolic compounds	**345.4 ± 28.1^{a}**	**331.0 ± 25.3^{a}**	**280.2 ± 31.8^{b}**	**173.1 ± 27.3^{c}**	**195.0 ± 27.5^{c}**

Results are reported as mean ± SD (n = 3). Different lower-case letters (a, b, c, d) used within a row indicate significant differences among different incubation periods ($p < 0.05$). n.d.: not detected.

(−)-Epicatechin and its specific microbial catabolites, including diphenylpropan-2-ol derivatives, phenyl-γ-valerolactones and phenylvaleric acids, were mainly observed during the fermentation process and were absent or present at low concentrations at T0. Notably, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone appeared as the principal flavan-3-ol catabolite at all fermentation times, reaching a concentration of 20.1 μmol 100 g^{-1} after 6 h of fermentation and remaining stable until 48 h.

3.1.2. Changes in cocoa shell extract

Concerning the concentration of phenolic compounds in the CSE (Fig. 1C–D, Table 2), at the end of the intestinal phase, and before the fermentation process was 348.3 μmol 100 g^{-1}. After 2 h, a decrease in the phenolic compounds concentration was observed, which continued to decline towards the end of the fermentation. Benzoic acids were the most abundant group. Specifically, 3,4-dihydroxybenzoic acid was the predominant compound at T0, with a concentration of 83.0 μmol 100 g^{-1}, followed by 4-hydroxybenzoic acid (38.2 μmol 100 g^{-1}) and benzoic acid (25.3 μmol 100 g^{-1}). These compounds, along with other benzoic acids, decreased their concentration throughout the colonic fermentation process. As regards benzaldehydes, 3,4-dihydroxybenzaldehyde and 4-hydroxybenzaldehyde showed high concentrations at T0 (27.1 and 9.4 μmol 100 g^{-1}, respectively), but decreased rapidly and consistently during the fermentation process. In the case of phenylacetic acids, 2-hydroxy-2-(3'-hydroxyphenyl)acetic acid registered an initial concentration of 19.8 μmol 100 g^{-1}, and experienced a decrease of 98.0% at the end of fermentation.

In the case of phenylpropanoic acids, 3-(4'-hydroxyphenyl)propanoic acid stood out as the compound with the highest concentration at T0, reaching 130.6 μmol 100 g^{-1}. However, it was only detected until T6. Conversely, the concentrations of 3-(3',4'-dihydroxyphenyl)propanoic and 3-(3'-hydroxyphenyl)propanoic acids increased throughout the fermentation process. Additionally, after 48 h, 3-(3'-hydroxyphenyl)

Table 2
Concentration of individual phenolic compounds (μmol 100 g^{-1}) in the cocoa shell extract (CSE) at baseline (T0) and after 2, 6, 24 and 48 h of *in vitro* colonic fermentation (T2, T6, T24 and T48). The nomenclature of catabolites is reported as proposed by Kay et al. (2020). Common names are indicated under brackets.

Compounds	T0	T2	T6	T24	T48
Cocoa shell extract					
Flavan-3-ols					
(−)-Epicatechin	n.d.	0.2 ± 0.0[b]	n.d.	0.3 ± 0.1[b]	0.7 ± 0.2[a]
Flavonols					
Kaempferol	n.d.	7.0 ± 2.0	n.d.	n.d.	n.d.
Quercetin-arabinoside	0.3 ± 0.0	n.d.	n.d.	n.d.	n.d.
Diphenylpropan-2-ol derivatives					
1-(Hydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol	0.1 ± 0.0[b]	1.5 ± 0.3[a]	0.3 ± 0.0[b]	n.d.	n.d.
1-(3',4'-Dihydroxyphenyl)-3-(2",4",6"-trihydroxyphenyl)-propan-2-ol	n.d.	0.2 ± 0.0	n.d.	n.d.	n.d.
Phenyl-γ-valerolactones					
5-(3'-Hydroxyphenyl)-γ-valerolactone	n.d.	n.d.	n.d.	n.d.	1.0 ± 0.1
5-(3',4'-Dihydroxyphenyl)-γ-valerolactone	0.5 ± 0.0[c]	1.5 ± 0.2[b]	2.5 ± 0.4[a]	1.5 ± 0.4[b]	2.4 ± 0.5[a]
Phenylvaleric acids					
4-Hydroxy-5-(4'-hydroxyphenyl)valeric acid	n.d.	0.4 ± 0.0[b]	0.7 ± 0.1[a]	0.4 ± 0.0[b]	0.6 ± 0.1[a]
4-Hydroxy-5-(3'-hydroxyphenyl)valeric acid	n.d.	0.4 ± 0.1	0.4 ± 0.1	n.d.	n.d.
4-Hydroxy-5-(3',4'-dihydroxyphenyl)valeric acid	0.5 ± 0.1[b]	1.1 ± 0.2[a]	1.0 ± 0.0[a]	0.3 ± 0.1[b]	n.d.
Cinnamic acids					
3',4'-Dihydroxycinnamic acid (Caffeic acid)	0.8 ± 0.1[a]	0.8 ± 0.2[a]	0.6 ± 0.1[ab]	n.d.	0.5 ± 0.0[b]
4'-Hydroxy-3'-methoxycinnamic acid (Ferulic acid)	0.5 ± 0.0	n.d.	1.2 ± 0.1	n.d.	n.d.
Cinnamoylquinic derivatives					
5-*O*-Caffeoylquinic acid	n.d.	0.4 ± 0.0	n.d.	n.d.	n.d.
Cinnamoyl-amino acid derivatives					
N-*p*-Coumaroyl-L-aspartate	2.1 ± 0.6	0.9 ± 0.2	n.d.	n.d.	n.d.
N-*p*-Coumaroyl-L-glutammate isomer 1	1.5 ± 0.3	n.d.	n.d.	n.d.	n.d.
N-*p*-Coumaroyl-L-glutammate isomer 2	0.6 ± 0.1	n.d.	n.d.	n.d.	n.d.
N-*p*-Coumaroyl-L-tyrosine	1.3 ± 0.2[a]	0.9 ± 0.1[b]	0.2 ± 0.0[c]	n.d.	0.1 ± 0.0[c]
N-Caffeoyl-3-hydroxy-L-tyrosine	n.d.	0.4 ± 0.0	0.4 ± 0.0	n.d.	n.d.
N-Feruloyl-L-aspartate	0.2 ± 0.0[b]	0.5 ± 0.1[a]	0.5 ± 0.1[a]	n.d.	n.d.
Phenylpropanoic acids					
3-(4'-Hydroxyphenyl) propanoic acid	130.6 ± 16.9	n.d.	148.5 ± 31.7	n.d.	n.d.
3-(3'-Hydroxyphenyl) propanoic acid	0.3 ± 0.0[c]	4.9 ± 0.3[c]	13.8 ± 0.6[b]	24.0 ± 4.7[a]	28.8 ± 6.9[a]
3-(3',4'-Dihydroxyphenyl) propanoic acid (Dihydrocaffeic acid)	n.d.	0.6 ± 0.1[b]	0.6 ± 0.1[b]	n.d.	2.6 ± 0.5[a]
3-(4'-Hydroxy-3'-methoxyphenyl)propanoic acid (Dihydroferulic acid)	n.d.	0.9 ± 0.2	n.d.	n.d.	n.d.
Phenylacetic acids					
2-Hydroxy-2-(3'-hydroxyphenyl)acetic acid (3-Hydroxymandelic acid)	19.8 ± 0.5[a]	21.5 ± 1.6[a]	12.8 ± 0.6[b]	0.7 ± 0.2[c]	0.4 ± 0.1[c]
Benzoic acids					
Benzoic acid	25.3 ± 0.0	n.d.	21.3 ± 1.2	n.d.	n.d.
4-Hydroxybenzoic acid	38.2 ± 7.9[a]	39.6 ± 3.4[a]	33.8 ± 5.0[a]	n.d.	n.d.
3-Hydroxybenzoic acid	0.9 ± 0.1[b]	n.d.	0.4 ± 0.0[c]	1.4 ± 0.2[a]	n.d.
3,4-Dihydroxybenzoic acid (Protocatechuic acid)	83.0 ± 7.1[a]	83.1 ± 2.8[a]	68.6 ± 6.4[b]	0.1 ± 0.0[c]	n.d.
3,4,5-Trihydroxybenzoic acid (Gallic acid)	n.d.	0.5 ± 0.1	0.4 ± 0.1	n.d.	n.d.
4-Hydroxy-3,5-dimethoxybenzoic acid (Syringic acid)	5.3 ± 0.5	4.6 ± 0.3	n.d.	n.d.	n.d.
Benzaldehydes					
4-Hydroxybenzaldehyde (4-Formylphenol)	9.4 ± 2.4[a]	1.5 ± 0.4[b]	0.4 ± 0.0[b]	0.5 ± 0.1[b]	n.d.
3,4-Dihydroxybenzaldehyde (Protocatechualdehyde)	27.1 ± 1.3[a]	18.4 ± 1.3[b]	1.1 ± 0.1[c]	n.d.	0.3 ± 0.1[c]
Total phenolic compounds	**348.3 ± 38.1[a]**	**191.8 ± 13.9[b]**	**309.5 ± 46.7[a]**	**29.2 ± 5.8[c]**	**37.4 ± 8.5[c]**

Results are reported as mean ± SD (n = 3). Different lower-case letters (a, b, c) used within a row indicate significant differences among different incubation periods ($p < 0.05$). n.d.: not detected.

propanoic acid was the most abundant compound, with a concentration of 28.8 μmol 100 g^{-1}.

Among the cinnamoyl-amino acid derivatives, *N*-*p*-coumaroyl-L-aspartate, *N*-*p*-coumaroyl-L-glutammate, isomer 1, and *N*-*p*-coumaroyl-L-tyrosine were the predominant compounds at the beginning of fermentation. These compounds, along with the rest of the cinnamoyl-amino acid derivatives, were completely degraded. Two cinnamic acids were detected: 3',4'-dihydroxycinnamic acid remained relatively stable during the first 6 h of fermentation and dropped in the following times, while 4'-hydroxy-3'-methoxycinnamic acid was only detected until T6.

Regarding flavonols, kaempferol was the predominant compound after 2 h of fermentation. The fermentation process increased the concentration of (−)-epicatechin and its microbial catabolites (diphenylpropan-2-ol derivatives, phenyl-γ-valerolactones and phenylvaleric acids). In particular, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone was the predominant microbial catabolite, reaching 2.4 μmol 100 g^{-1} after 6 h of fermentation.

3.2. Temporal distribution of phenolic catabolites and classes in CSF and CSE during in vitro colonic fermentation

The proportions of phenolic catabolites throughout the colonic fermentation of CSF and CSE are detailed in Table S2, and Table S3, respectively. In this case, data are expressed as a percentage of the total content of phenolic compounds measured before colonic fermentation. Concerning CSF (Table S2), at the initial stage of incubation (T0), benzoic acids accounted for 30.3%, followed by phenylpropanoic acids (30.0%). Among the benzoic acids, 3,4-dihydroxybenzoic acid was the main compound (18.9%), followed by 4-hydroxybenzoic acid (6.3%), and benzoic acid (4.0%). In the group of phenylpropanoic acids, 3-(4'-hydroxyphenyl)propanoic acid stood out, comprising 29.9%. Benzaldehydes also contributed significantly (9.4%), with 3,4-dihydroxybenzaldehyde being the most notable compound (7.6%). Phenylacetic acids accounted for 3.5%, with 2-hydroxy-2-(3'-hydroxyphenyl)acetic acid being the only compound detected.

Following 2 h of fermentation, benzoic acids remained prominent, representing 32.6% of the total. This was mainly due to the compound 3,4-dihydroxybenzoic acid, which aligns with data at T0. Phenylpropanoic acids were also significant (21.6%), with 3-(4'-hydroxyphenyl)propanoic acid being the main contributor (20.2%), and 3-(3'-

hydroxyphenyl)propanoic acid increasing significantly its contribution (1.3%). Cinnamoyl-amino acid derivatives decreased and benzaldehydes were present at 5.0%, almost entirely attributed to 3,4-dihydroxybenzaldehyde (4.9%). Flavonols appeared and accounted for 6.1%, with quercetin-rutinoside (3.0%) and quercetin (2.4%) standing out. Also, 5-(3',4'-dihydroxyphenyl)-γ-valerolactone and 2-hydroxy-2-(3'-hydroxyphenyl)acetic acid increased and reached 1.4% and 4.5%, respectively.

After 6 h of incubation, phenylpropanoic acids emerged as the predominant compound family, reaching 32.6%, with 3-(4'-hydroxyphenyl)propanoic acid being the predominant compound (29.4%) and 3-(3'-hydroxyphenyl)propanoic acid reaching 3.2%. The relative contribution of benzoic acids decreased (19.1%). Phenyl-γ-valerolactones had a significant increase, representing 4.5%, mainly due to 5-(3',4'-dihydroxyphenyl)-γ-valerolactone (4.4%).

After 24 h of fermentation, phenylpropanoic acids remained the dominant phenolic group (25.4%). Within this class, 3-(4'-hydroxyphenyl)propanoic and 3-(3'-hydroxyphenyl)propanoic acids were the major contributors, with 13.2% and 11.9%, respectively. In contrast, benzoic acids reduced their contribution to 5.5%, with benzoic acid standing out (4.5%). Conversely, phenyl-γ-valerolactones increased their content to 5.2%, mainly due to 5-(3',4'-dihydroxyphenyl)-γ-valerolactone, which accounted for 5.1%. Finally, after 48 h of fermentation, a similar trend was observed. Phenylpropanoic acids remained dominant (33.5%), followed by phenyl-γ-valerolactones (4.6%), and benzoic acids (3.2%), being the representative compounds the same as those identified at 24 h.

Considering CSE (Table S3), benzoic acids (33.5%) were the main phenolic group at the beginning of colonic fermentation. Within this group, 3,4-dihydroxybenzoic acid was the most prevalent, reaching 18.2%, followed by 4-hydroxybenzoic acid (8.4%), and benzoic acid (5.5%). Phenylpropanoic acids accounted for 28.7%, largely due to the presence of 3-(4'-hydroxyphenyl)propanoic acid (28.6%). Benzaldehydes contributed 8.0%, with 3,4-dihydroxybenzaldehyde standing out (5.9%). Phenylacetic acids accounted for 4.3%, with 2-hydroxy-2-(3'-hydroxyphenyl)acetic acid being the only compound detected.

When the fermentation reached 2 h, benzoic acids still stood out as the most representative group, accounting for 28.0% of the total. Phenylacetic acids accounted for 4.7% and benzaldehydes for 4.3%. Cinnamoylquinic derivatives and 3-(4'-hydroxyphenyl)propanoic acid dropped, while 3-(3'-hydroxyphenyl)propanoic acid increased its proportion (1.1%). Moreover, the class of diphenylpropan-2-ol derivates and the compound 5-(3',4'-dihydroxyphenyl)-γ-valerolactone increased.

Conversely, after 6 h of fermentation, phenylpropanoic acids (35.7%) emerged as the predominant group of compounds, while benzoic acids accounted for 27.3%. In this case, the most notable compounds were 3-(4'-hydroxyphenyl)propanoic acid (32.6%) and 3,4-dihydroxybenzoic acid (15.0%), respectively. While the class of diphenylpropan-2-ols dropped, the compound 5-(3',4'-dihydroxyphenyl)-γ-valerolactone increased, reaching 0.6%. After 24 and 48 h, the majority of compounds were present in low amounts or not detected, with the exception of 3-(3'-hydroxyphenyl)propanoic acid, which accounted for 5.3% and 6.3%, respectively.

The changes in the distribution of phenolic classes in CSF during colonic fermentation are also shown in Fig. 2A and Table S4, considering each compound's relative contribution regardless of the initial amounts. A similar trend was observed as the CSF was primarily composed of benzoic acids (39.9%), phenylpropanoic acids (39.6%), and benzaldehydes (12.4%) at the beginning of the incubation. During the fermentation, all phenolic families exhibited a reduction in their proportion, except for phenylpropanoic acids (78.4%) and phenyl-γ-valerolactones (10.7%), which increased by 2.0 and 53.5-fold, respectively. Considering CSE, as illustrated in Fig. 2B and detailed in Table S4, benzoic acids initially accounted for 43.8%, followed by phenylpropanoic acids (37.6%) and benzaldehydes (10.5%). Within the first 2 h, the proportion of benzoic acids increased to 66.7%, although they disappeared at the end of the incubation. In contrast, the proportion of phenylpropanoic acids increased to 83.8% after 48 h. Other phenolic groups, notably phenyl-γ-valerolactones, also increased to 9.1% at the end of the colonic fermentation.

PCA and AHC analysis (Fig. 3) were performed to explain the dynamics of phenolic compounds in CSF and CSE throughout the colonic fermentation. Following the execution of PCA, a total of nine principal components (PCs) were identified, as shown in Fig. 3A. The cumulative variability explained by the first two PCs stood at 70.4%. PC1, accounting for 54.9% of the total variability, exhibited positive loadings for phenylpropanoic (15.0%), benzoic (14.5%), and phenylacetic acids (13.5%). PC2, responsible for 15.5% of the overall variability, had positive associations with the cinnamoylquinic derivatives (27.2%), diphenylpropan-2-ol derivatives (23.3%), and cinnamoyl-amino acid derivatives (12.3%). Both PC1 and PC2 allowed to discriminate samples based on the fermentation time, with later times (T24 and T48) mainly in the top right quadrant and early time points (T0, T2) mainly in the bottom left quadrant. The CSE fermented samples presented generally higher PC1 and PC2 scores than CSF-fermented samples.

Regarding hierarchical clustering, the dendrogram yielded two main clusters (Fig. 3B). The first principal cluster was mainly composed of initial and early-stage samples: CSF-0, CSF-2, CSE-0, and CSE-2. This suggested that these samples showed similar behavior during the first hours of fermentation. The second cluster mainly included samples from CSF at 6, 24, and 48 h, along with samples from CSE at the same time points (6, 24, and 48 h). This cluster formation implied significant similarity in the phenolic compound pattern among samples at T6, T24, and T48. These results are consistent with the findings from the PCA.

4. Discussion

The negative ecological impact of agricultural and industrial practices has increased the concern about sustainability and environmental preservation. In this sense, the revaluation of agrifood by-products obtained from these practices can be an effective strategy for transitioning to a sustainable and circular economic model (Jimenez-Lopez et al.,

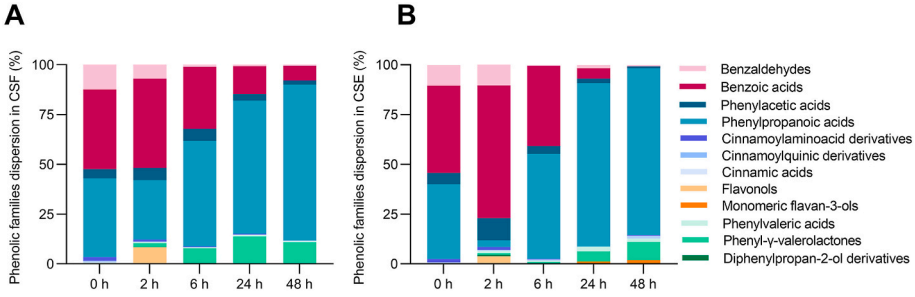

Fig. 2. Temporal distribution of phenolic compounds families during *in vitro* colonic fermentation. The data are shown for different incubation periods (0, 2, 6, 24, 48 h), for CSF (**A**) and CSE (**B**) and are expressed as a percentage (%) of initial total phenolic content specific to each time point.

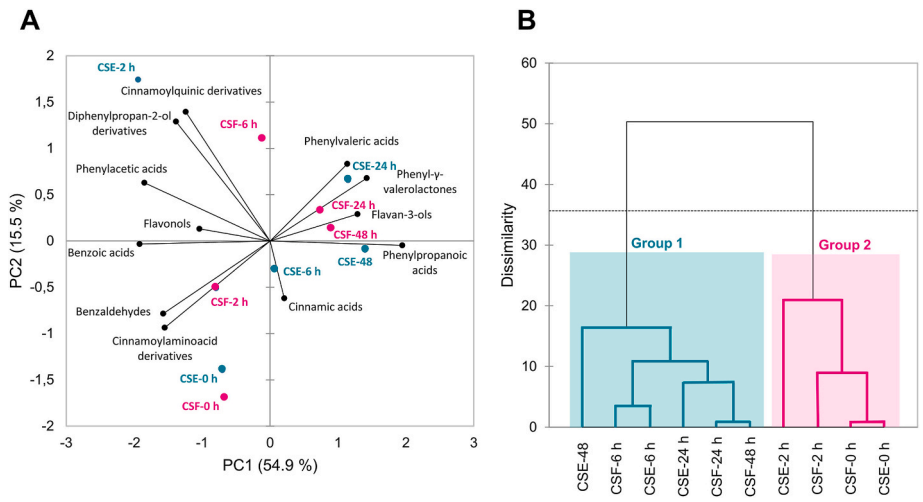

Fig. 3. Biplot illustrating sample scores and loadings for each variable in the principal component analysis (PCA) (**A**), and agglomerative hierarchical clustering (AHC) (**B**), depicting the behavior of phenolic compounds in CSF and CSE during *in vitro* colonic fermentation.

2020). The cocoa shell is distinguished by its high dietary fiber and phytochemical content. Given its composition, this byproduct should be considered a valuable resource for developing novel functional foods (Rojo-Poveda et al., 2020). In previous research following simulated gastrointestinal digestions, we demonstrated that the phenolic compounds from cocoa shells were bioaccessible and potentially bioavailable (Cañas et al., 2022b). Furthermore, both CSF and CSE exhibited antioxidant effects in mitigating cellular oxidative stress, as previously reported (Cañas et al., 2023). This suggested that cocoa shells could be incorporated into the diet as an ingredient rich in bioactive compounds, thereby offering potential health benefits. In the present investigation, we examined how *in vitro* colonic fermentation influences the phenolic profile of digested CSF and CSE, to further define the prospects of this by-product.

In the first comparative analysis of the two matrices, the number of phenolic compounds changed slightly between CSF and CSE (35 and 32 compounds, respectively), suggesting that both matrices possess a similar phenolic profile. This minor difference might be influenced by the matrix effect of CSF. Specifically, the presence of fiber in the CSF may hinder the release of certain compounds during the CSE extraction process, since some phenolic compounds are bound to cell wall components (Mosele et al., 2018). Furthermore, during the CSE preparation at 100 °C, it should be considered that high temperatures can also induce the transformation or degradation of specific compounds, despite heat facilitates the release of phenolic compounds from the matrix into the aqueous medium (Antony and Farid, 2022).

The phenolic compounds identified did not remain stable during the course of *in vitro* colonic fermentation, regardless of the matrix. Throughout the process, some compounds experienced a decrease, while others showed an increase. Additionally, a higher presence of compounds was recorded at the beginning of the incubation compared to the end. This variation could be attributed to the metabolic activity of the colonic microbiota, which may catabolize these compounds over time (Domínguez-Avila et al., 2021).

Benzoic acids were the most abundant family. Specifically, the compound 3,4-dihydroxybenzoic acid emerged as the predominant phenolic compound, which is consistent with findings from previous research on the phenolic profile of cocoa shells (Cañas et al., 2022b; Rebollo-Hernanz et al., 2021). During the first 2 h of fermentation, it was observed that the concentrations of 3,4-dihydroxybenzoic acid and 4-hydroxybenzoic acid, the predominant phenolic compounds, remained constant. However, this phenomenon does not imply that these compounds resist microbial degradation. They may be subject to degradation and simultaneous formation by the colonic microbiota, being both precursors and substrates. For example, 4-hydroxybenzoic acid could derive from the degradation of 3-(4′-hydroxyphenyl)propanoic acid, present at the beginning of the fermentation process, and could be catabolized into benzoic acid (Lee et al., 2022; Yang et al., 2020). Indeed, after 6 h of fermentation, an overall decrease in the concentration of benzoic acids was observed. For CSE, the reduction in the levels of 3,4-dihydroxybenzoic acid coincided with an increase in the presence of 3-hydroxybenzoic acid, suggesting biotransformation, in accordance with previous literature (Yang et al., 2020).

The disappearance of benzoic acids at the end of fermentation suggested that gut microbes can break benzoic acids into simpler compounds. For instance, 3,4,5-trihydroxybenzoic and 3,4-dihydroxybenzoic acids can be converted into benzene-1,2,3-triol and 1,2-dihydroxybenzene, respectively (Selma et al., 2009). However, these compounds were not detected in this study. Finally, the increases of benzoic acid after 6 and 24 h in CSE and CSF, respectively, could be attributed to its role as an intermediate and final metabolite in many degradation pathways (Bento-Silva et al., 2020).

Benzaldehydes were present at the beginning of the fermentation. In particular, the concentration of 3,4-dihydroxybenzaldehyde, which could be derived from the degradation of quercetin, experienced a decrease as the process continued (Justesen and Arrigoni, 2001). This reduction could be due to its conversion into 4-hydroxybenzaldehyde (De Ferrars et al., 2014), although this compound could also originate from the reduction of 4-hydroxybenzoic acid (Zhu et al., 2018). Finally, 4-hydroxybenzaldehyde also decreased, likely due to its transformation into other end products.

The levels of phenylpropanoic acids experienced an increase at the end of the fermentation process. In particular, the significant increment in the concentrations of 3-(3'-hydroxyphenyl)propanoic and 3-(3',4'-dihydroxyphenyl)propanoic acids has been previously reported. These compounds can be produced from the degradation of several precursors through multiple pathways, including cinnamate derivatives and flavonoids (Rechner, 2004).

The concentration of cinnamic acids was relatively low, and they were not detected at the end of the fermentation process. This could be due to their degradation into other metabolites, such as phenylpropanoic, phenylacetic and benzoic acids (Leonard et al., 2021). Previous research has highlighted that the main metabolites of 3', 4'-dihydroxycinnamic acid are 3-(3',4' -dihydroxyphenyl)propanoic acid, via reduction of its double bond, and 3-(3'-hydroxyphenyl)propanoic or 3-(4'-hydroxyphenyl)propanoic acids, resulting from dihydroxylation reactions (Vollmer et al., 2017). This is consistent with the *in vitro* fermentation conducted, as the concentration of some

phenylpropanoic acids increased while cinnamic acids decreased in both matrices. Cinnamoylquinic acids were also detected in very low quantities during the fermentation process. The compound 5-*O*-caffeoylquinic acid, the only cinnamoylquinic acid identified, disappeared probably due to degradation by microbial esterases. This degradation occurs through the hydrolysis of the ester bond between quinic and cinnamic acids (Sova and Saso, 2020; Tomas-Barberan et al., 2014). The cinnamoyl-amino acid derivatives identified at the beginning of the fermentation process were also catabolized by the end of the process. These compounds are commonly found in cocoa and its by-products, including cocoa shells (Lechtenberg et al., 2012).

Concerning flavonols, it was observed that quercetin-arabinoside, which appeared in low concentration at the beginning of fermentation, was not detected after 6 h. This was likely due to its hydrolysis by enzymes from the colonic microbiota. This process led to the production of the aglycone quercetin, resulting in an increase in its content. The aglycones found, in turn, were not detected after 6 h of incubation, which confirmed that they were metabolized into ring fission products, including phenylpropanoic, phenylacetic and benzoic acid derivatives, as reported in the literature (AL-Ishaq et al., 2021; Di Pede et al., 2020).

(−)-Epicatechin, the only flavan-3-ol detected, remained stable in CSF from 6 h, while it showed a significant increase in CSE at the end of fermentation. This suggests that it was possibly converted to other metabolites and simultaneously produced from other compounds, such as procyanidins. In detail, the increase in epicatechin levels could result from the hydrolysis of dimer B2, or from compounds like epigallocatechin-gallate and epicatechin-gallate when they lose their gallate residue (Serra et al., 2011). (−)-Epicatechin can be transformed into phenylvaleric acids and phenyl-γ-valerolactones, which can be metabolized into phenylpropanoic, benzoic, and cinnamic acids. These compounds originated through the successive loss of carbon atoms from the lateral chain via β-oxidation (Mena et al., 2019).

This study revealed differences in the metabolism of phenolic compounds between CSF and CSE during colonic fermentation. In particular, the degradation of cinnamoylquinic derivatives, cinnamoyl-amino acid derivatives, and benzoic acids was slower in the CSF compared to CSE. This behavior suggested that CSF may act as a barrier that limits the accessibility of these compounds to the colonic microbiota, thereby extending their presence during fermentation. In contrast, the rapid degradation of these compounds in CSE indicates a greater bioaccessibility, likely due to the absence of a food matrix. This difference highlights the importance of considering matrix effects on the bioaccessibility and bioavailability of phenolic compounds. Often, some phenolic compounds do not reach the colon because they are absorbed or degraded early in the gastrointestinal tract. Strategies such as encapsulation have been developed to protect these compounds from degradation during the early stages of digestion, allowing their controlled release in the colon (Grgić et al., 2020). CSF could play a similar protective role by slowing down the availability of phenolic compounds to the colonic microbiota, thereby lengthening their permanence and their potential bioactive activity during fermentation. Considering these results, it is evident that both matrices could have different applications in the food industry. While CSF could be useful in formulations that aim to prolong the release of phenolic compounds in the colon, CSE could be employed in products designed for a more immediate release of these compounds, like nutraceuticals or pharmaceutical products.

It is essential that phenolic compounds reach the colon, as they have the potential to modulate the colonic microbiota and lead to the production of metabolites with higher bioactivity (Kasprzak-Drozd et al., 2021). Several studies indicate that consuming a diet high in phenolic compounds can contribute to maintaining the balance of gut microbiota, increasing the levels of beneficial microbes such as *Bifidobacterium* and *Lactobacillus*, modulating the proportion between *Firmicutes* and *Bacteroides*, and inhibiting the growth of pathogenic bacteria (Wang et al., 2022). A diet rich in phenolic compounds may also contribute to reducing oxidative stress and inflammation, which is especially beneficial in intestinal diseases like colitis (Li et al., 2023; Wan et al., 2021). Additionally, some research suggests that the intake of phenolic compounds, including those found in cocoa, could potentially reduce the risk of certain types of cancer, such as colorectal cancer (Andújar et al., 2012; Zhao and Jiang, 2021).

To sum up, cocoa shell contains a large amount of phenolic compounds that may exert beneficial properties in the body. However, factors such as matrix processing and gastrointestinal digestion influence the bioaccessibility and bioavailability of these compounds. The transit to the colon is a crucial step since the intestinal microbiota can biotransform them into metabolites with higher biological activity. These findings suggest the possibility of incorporating cocoa shells as a nutraceutical ingredient, with the aim of reducing the risk of developing chronic diseases.

5. Conclusion

Cocoa shell phenolic compounds underwent deep transformations throughout the colonic fermentation process. Most phenolic compounds like benzoic acids and cinnamates decreased, except for certain phenylpropanoic acids, phenyl-γ-valerolactones, and phenylvaleric acids, which increased. This highlights the critical role played by the intestinal microbiota in the biotransformation of phenolic compounds in the colon. Additionally, differences between matrices were observed, with phenolic compounds from CSF being released and degraded more slowly than those from CSE. The fibrous matrix of CSF could offer protection against the degradation of phenolic compounds, thus facilitating a prolonged interaction between these compounds and the intestinal microbiota. These findings support our hypothesis that phenolic compounds from cocoa shells could be released and transformed during colonic fermentation, potentially leading to the formation of bioactive metabolites with potentially protective properties. Cocoa shells could be a functional food ingredient that could benefit human health, especially intestinal health.

CRediT authorship contribution statement

Silvia Cañas: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing. **Nicole Tosi:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Vanesa Núñez-Gómez:** Conceptualization, Investigation, Writing – review & editing. **Daniele Del Rio:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Pedro Mena:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Writing – review & editing. **Yolanda Aguilera:** Conceptualization, Project administration, Supervision, Writing – review & editing. **María A. Martín-Cabrejas:** Conceptualization, Project administration, Supervision, Writing – review & editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100930.

Data availability

Data will be made available on request.

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