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Digestion and gut-microbiota fermentation of cocoa melanoidins: An *in vitro* study

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ABSTRACT

This research aimed to compare the *in vitro* digestibility and fermentability of melanoidins extracted from cocoa nibs fast-roasted with a fluidized bed roaster (FR-melanoidins), melanoidins from cocoa nibs slow-roasted in a convective oven (SR-melanoidins), and native high molecular weight compounds extracted from unroasted cocoa nibs (UR-HMWC). Data showed that both types of melanoidin extracts, especially FR-melanoidins, better release polyphenols during digestion and earlier produce short-chain fatty acids during *in vitro* fermentation than UR-HMWC. The delayed catabolism of UR-HMWC resembled that of dense fibers, while FR- and SR-melanoidins resembled oligosaccharides. The production of SCFA was positively correlated with the increase in *Bacteroides* sp. The profile of the genera identified in the proximal colon showed the differences in fermentability between FR- and SR-melanoidins, with FR-melanoidins having the higher relative growth of nonpathogenic bacteria. This study demonstrated the *in vitro* digestibility of bound phenolic compounds and the potential prebiotic activity of cocoa melanoidins.

1. Introduction

Roasting leads to many changes in cocoa (*Theobroma cacao* L.) composition due to chemical reactions. Native high molecular weight compounds (HMWC) of cocoa (12 to 150 kDa) such as polysaccharides, proteins, and condensed polyphenols, may undergo depolymerization reactions, producing compounds with lower molecular weight (Oracz & Nebesny, 2019). Simultaneously, water-soluble cocoa melanoidins, which are heterogeneous polymers (30 to 70 kDa), are produced via the Maillard reaction (MR) involving polyphenols in their structure (Oracz & Nebesny, 2019). Therefore, the composition of the soluble fraction of cocoa above 12 kDa changes upon roasting.

As reviewed by Echavarría, Pagán, and Ibarz (2012); Tagliazucchi and Bellesia (2015); and Wang, Qian, and Yao (2011), food melanoidins are poorly-digestible compounds with low ability to release low molecular mass compounds, thus passing by the gastrointestinal tract to be mainly recovered in feces. So far, this simulation has not been performed with cocoa melanoidins. According to Pérez-Burillo, Rajakarunab, Pastoriza, Paliy, and Rufián-Henares (2020), the content of released polyphenols from melanoidins extracted from dark chocolate upon *in vitro* gut microbiota fermentation is exceptionally higher than that from other dietary melanoidins. Therefore, it is possible that the enzymes of the upper digestive system release a significant amount of this kind of low molecular weight compounds from cocoa melanoidins.

The molecular structure of cocoa melanoidins is not fully defined. It is well known that they are heterogenous polymers mainly formed by aldol condensations of highly reactive α-dicarbonyl compounds (a carbohydrate-based skeleton) and partially branched by amino

Abbreviations: AH, Alkaline hydrolysis; BC, Blank control; BCFAs, Branched-chain fatty acids; d.b., dry basis; DC, Distal colon; FR, Fast roasting / roasted, or fluidized-bed roasting/roasted; HMWC, High molecular weight compounds; LOD, Limit of detection; LOQ, Limit of quantification; NC, Negative control; PC, Proximal colon; PD, Phylogenetic distances; RT, Room temperature; SCFAs, Short-chain fatty acids; SR, Slow roasting/roasted, or oven roasting/roasted; UR, Unroasted, or native.

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compounds (Echavarría et al., 2012). Melanoidins also contain covalently bound polyphenols (Oracz, Nebesny, & Zyzelewicz, 2019; Oracz & Zyzelewicz, 2019) that certainly confer antioxidant activity to this advanced MR product (Fogliano, 2014; Oracz & Zyzelewicz, 2019; Quiroz-Reyes & Fogliano, 2018; and Zhang, Zhang, Troise, & Fogliano, 2019). The water-soluble cocoa extracts containing melanoidins are mainly composed of proteins (15 to 20 % w/w), carbohydrates (8 to 16 % w/w), and water (≈5 %) (Oracz & Nebesny, 2018). The exact amount of polyphenols is difficult to assess. Upon chemical hydrolysis, a moiety of 200 to 500 mg of total polyphenols is released from 100 g of cocoa extracts d.b. (cut-off 12.5 kDa,). They are mainly represented by epicatechin and catechin, as reported by Oracz et al. (2019).

The microbiota's capacity to use food melanoidins as a source of carbon is well established (Pérez-Burillo et al., 2020; and van Boekel et al., 2010). Pérez-Burillo et al. (2020) demonstrated via *in vitro* experiments that the growth of microorganisms of the genera *Bifidobacterium,* the formation of short-chain fatty acids (SCFAs), and the release of polyphenols were favored with the presence of melanoidins extracted from chocolate with respect to the control; however, many details of the utilization of cocoa melanoidins are still missing.

The kinetics of fermentation of cocoa melanoidins along the gut is still unknown. It is well known that the microbiota profile of the ascendent, transversal, and descendent parts of the colon strongly differ, as the pH gradually increases from about 5.5 in the cecum to about 6.9 in the rectum. The change in pH along the colon reflects the bacterial fermentative production of acids upon the breakdown of carbohydrates by bacteria under anaerobic conditions. Thus, the greatest fermentative activity is associated with the highest availability of carbohydrate substrates in the cecum and proximal colon (Tannock, 2017), while the fermentation of proteins has been reported to be the highest in the distal large intestine (van der Wielen, Moughan, & Mensink, 2017). In this framework, it is worth to investigate how a heterogeneous polymer like the melanoidins is utilized across the colon.

In our previous study (Peña-Correa, Ataç Mogol, Van Boekel, & Fogliano, 2022), we demonstrated that a roasting technique based on almost 100 % forced convective heat transfer (represented by a fluidized bed roaster) favored the production of pyrazines in cocoa nibs, compared to roasting cocoa over aluminum trays inside an oven. Pyrazines are intermediate MR-volatile organic compounds capable of producing advanced MR products like melanoidins (Echavarría et al., 2012). As the formation of pyrazines is boosted by the fluidized bed technique, the formation of melanoidins could also be favored, as reported in another of our studies (Peña-Correa, Ataç Mogol, Fryganas, & Fogliano, 2023) in which we found that the brown color of HMWC extracted from fluidized bed roasted cocoa is more intense than that from convective oven roasted cocoa. Therefore, the ratio of melanoidins / native-HMWC in water-soluble melanoidins extracts may be determined by the roasting technique, affecting their interaction with digestive enzymes and gut microbiota.

The objective of this study is to compare the native HMWC extracted from unroasted cocoa nibs and the extracts containing melanoidins obtained from fluidized-bed roasted cocoa, and oven-roasted cocoa, on: (i) their potential to store and release phenolic compounds via alkaline hydrolysis and simulated *in vitro* digestion; (ii) the release of polyphenols and the production of SCFAs in *in vitro* simulated proximal colon (PC) and distal colon (DC), via batch fermentation experiments; and (iii) the modification of the bacteria communities of the PC and DC due to the use of melanoidins as carbon source.

2. Materials and methods

2.1. Materials and consumables

Fermented and dried Forastero cocoa beans (*T. cacao* L.) from Ivory Coast, with a water content of 6.14 ± 0.02 % w/w, were supplied by Olam International (Koog aan de Zaan, The Netherlands). Pepsin, bile salt, pancreatin, K_2HPO_4, KH_2PO_4, $NaHCO_3$, yeast extract, peptone, mucin, L-Cysteine HCL, and Tween-80 were purchased from Sigma Aldrich (St. Louis, USA). Polytetrafluoroethylene (PTFE) filters were purchased from Phenomenex (Niederlassung, Germany). The following standards were obtained in analytical standard quality (purity ≥ 97 %) from Sigma Aldrich (St. Louis, USA): (-)-epicatechin, (+)-catechin, procyanidin B2, chlorogenic acid, gallic acid, caffeic acid, ferulic acid, acetic acid, propionic acid, isobutyric, butyric acid, isovaleric acid. The system of PureLab Ultra (ELGA LabWater, Lane End, UK) prepared the MilliQ water.

2.2. Roasting process of cocoa nibs

Fermented and dried cocoa beans (180 g) were heated in a preheated pan using an induction stove (ATAG BV, The Netherlands) with constant stirring (4 min) and then cooled (40 °C). The shells were manually removed and winnowed with an air gun. The cotyledons (i.e., the cocoa nibs) were softly cracked with a mortar and pestle and then sieved to retain the cocoa particles between 4.0 and 7.0 mm. The sieved cocoa nibs (water content of 4.31 ± 0.11 % w/w) were kept in vacuum bags at −20 °C until the roasting experiments.

The roasting process of sieved cocoa nibs in a fluidized bed roaster and a convective oven was performed as described in our previous study (Peña-Correa, Mogol, & Fogliano, 2023). Briefly, samples of cocoa nibs (70 g) were roasted in a preheated electric fluidized bed coffee roaster (Toper Optical Roaster, Izmir, Turkey) at 140 °C for 3 min and 43 s. This study refers to this technique as fast roasting (FR). For the oven roasting procedure, batches of 70 g of cocoa nibs were dispersed on aluminum trays and then placed in a pre-heated electric convective oven (VWR International B.V., Breda, The Netherlands) at 140 °C for 45 min and 16 s. This procedure was referred to as slow roasting (SR) in this study. The roasting times were determined by a water content of 1.0 ± 0.1 % w/w in roasted cocoa nibs. Unroasted (UR) cocoa nibs (200 g) were separated as a control. All the samples were vacuum-packed and stored at −20 °C until usage.

2.3. Preparation of cocoa powder and extraction of water-soluble cocoa extracts

A defatting procedure with petroleum ether was performed to obtain cocoa powder (defatted ground cocoa nibs), as described in our previous study (Peña-Correa et al., 2023). Briefly, cocoa nibs were twice-ground using a screw juicer (Vital Max Oscar 900, Hurom, Korea). Ground cocoa (100 g) was defatted with petroleum ether (4 × 200 mL). After each defatting step, the mix was stirred (15 min, RT) and then allowed to precipitate for 1 h. The upper layers containing the solvent with the cocoa butter were pipetted off, pooled, and centrifuged (2950 g,10 min, RT). The solvent containing cocoa butter was discarded, and the pellets and the sediments (cocoa powder) were air-dried (48 h, RT) in a fume hood while they were gently stirred with a spatula every 12 h. The fat-free and solvent-free cocoa samples, namely cocoa powder (CP), were put into airtight plastic containers and stored at −20 °C until thew were used for chemical analysis.

To obtain native high molecular-weight compounds and high molecular-weight melanoidins, an extractive procedure was performed based on our previous study (Peña-Correa et al., 2023), with some modifications. Briefly, 10 g of cocoa powder was thoroughly mixed with 80 mL of Milli-Q water using a homogenizer (Ultra-Turrax®, T25 digital, Probe T4, IKA, Staufen, Germany) for 4 min at 9000 rpm, RT. The mixture was capped and placed in a shaking hot water bath (70 °C, 20 min, 80 rpm) and centrifuged (22680*g* for 15 min) (Avanti ultra-centrifuge, Rotor ID 16250, Beckman Coulter, USA). The pellet was discarded, and the supernatant was successively vacuum-filtered through Whatman filter papers Nr 4, 44, and 602.

The last filtrate was dialyzed with a dialysis tubing cellulose membrane (14 kDa cut-off, 76 mm flat-width, Sigma Aldrich, Darmstadt,

Germany) for 24 h at 4 °C against 4 L of MilliQ water. The water was changed 3 times. The dialyzed part (the permeate) was discarded, and the retentate, which corresponded to cocoa compounds > 14 kDa, was freeze-dried (Alpha 1–2 LDplus, Christ, Germany) at −82 °C and 1.0 mbar. The workflow of the obtention of cocoa extracts > 14 kDa and their usage throughout this study are represented in Fig. 1.

2.4. Alkaline hydrolysis of cocoa melanoidins

The alkaline hydrolysis method described by (Oracz et al., 2019) was adapted to estimate the content of covalently-bound phenolic compounds in native cocoa HMWC extracted from unroasted cocoa (UR) and cocoa melanoidins obtained from FR and SR cocoa nibs. Briefly, 20 mg of freeze-dried cocoa extracts were hydrolyzed (90 min, 30 °C) with 2 mL of a solution containing 2 M NaOH, 2 % (w/w) ascorbic acid, and 20 mM EDTA. Then, the mixture was adjusted to pH 1.0 with 5 M HCl and filled up to 10 mL with MilliQ water. Finally, the mixture was centrifuged (3220 g, 10 min, RT), and the supernatant was stored at −20 °C for further analysis. The pellet was discarded.

2.5. In vitro digestion of cocoa melanoidins

To evaluate the content of polyphenols that can be released from UR-HMWC, FR-melanoidins, and SR-melanoidins in the upper gastrointestinal tract, the INFOGEST® static *in vitro* method described by Brodkorb et al. (2019) was performed with some modifications. Briefly, the simulated digestion fluids of the oral (SSF), gastric (SGF), and intestinal (SIF) phases were prepared. All the solutions involved in this method were kept in a water bath at 37 °C. To simulate the formation of a bolus, a sample of 1.2 g of HMWC was added with 6.8 mL SSF, 120 μL 0.3 M $CaCl_2$, and 31.2 mL of Milli-Q water. The mixture was vortexed (20 s). Then, 36 mL of SGF, and the pH was adjusted to 3.0 by 1 M HCl. Then 24 μL of 0.3 M $CaCl_2$, and 7.68 mL of pepsin solution (25000 U/mL) were added. Milli-Q water was added to reach a final volume of 100 mL. The mixture was placed in an incubator (VWR International B.V., Breda, The Netherlands) provided with a rotating unit (37 °C, 2 h, 120 rpm). The enzymatic hydrolysis of the gastric phase was terminated by adjusting the pH to 7.0 by 1 M NaOH, thus producing a simulated chyme.

To mimic the intestinal phase (the formation of a chyle), 52.8 mL SIF, 12 mL of bile salt solution (28.8 mg/ml), 192 μL 0.3 M $CaCl_2$, and 24 mL pancreatin solution (amylase activity 1600 U/mL) were immediately added. Milli-Q water was added to fill up to 190 mL approximately. The samples were incubated (37 °C, 120 rpm, 2 h). Finally, the enzymatic activity was halted by immersing the bottles in an ice bath for 30 min. To remove the precipitated material of the chyle, the samples were centrifuged (3220g, 10 min).

The supernatant was ultrafiltered in an ultrafiltration stirring cell unit (Amicon, model 8400, 400 mL capacity, Millipore, Billerica, MA USA) provided with a 10 kDa membrane (Mycrodyn Nadir, Nadir FM UP020Pes, Sterlitech, Auburn, WA USA) under a positive pressure of 4.5 bar generated by nitrogen supply. Three washing steps with 30 mL of Milli-Q water were done on the retentate when its volume was about 20 mL. The retentate containing non-digested water-soluble cocoa compounds > 10 kDa was filled up to 50 mL with Milli-Q water, and the permeate containing digested low molecular weight compounds (Fig. 1) was filled up to 250 mL with Milli-Q water. Both were stored at −20 °C

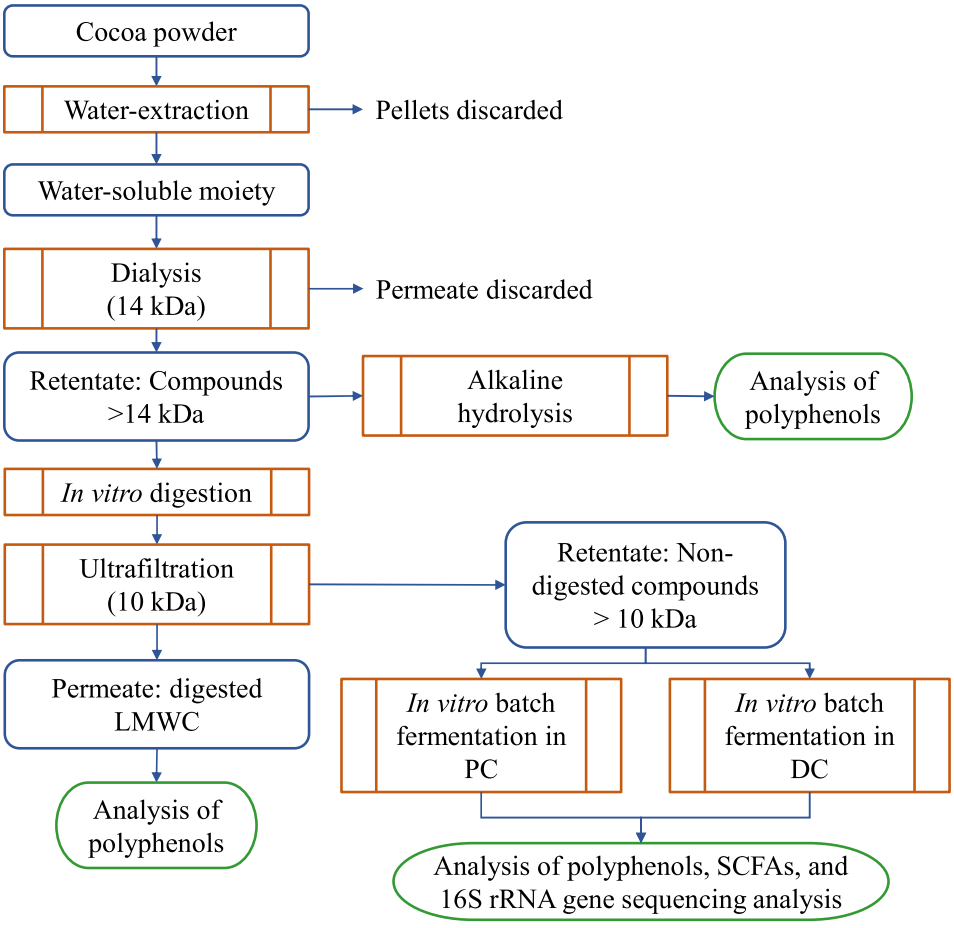

Fig. 1. Workflow of the experiments performed in this study from the obtention of water-soluble cocoa extracts > 14 kDa, followed by their alkaline hydrolysis and *in vitro* digestion, and finishing with the *in vitro* batch fermentation experiments. LMWC stands for low molecular weight compounds, PC for proximal colon, DC for distal colon, and SCFAs for short-chain fatty acids.

for further usage. The method was performed in duplicate for each type of cocoa extract.

2.6. In vitro batch fermentation of non-digested cocoa HMWC

2.6.1. Preparation of fecal inoculum

The SHIME® system (PRODIGEST, Belgium) is a continuous bioreactor capable of mimicking the fermentation along the human large intestine. By following the method described by Rovalino-Córdova, Fogliano, and Capuano (2021), we used this system for separately and simultaneously conditioning the fecal material of two donors (healthy volunteers of Caucasian ethnicity, non-smokers, aged between 25 and 35 years old with no history of antibiotic treatment for at least 6 months before stool collection) in bioreactors mimicking the conditions of the proximal and distal colon.

2.6.2. In vitro batch fermentation

The *in vitro* batch fermentation of the non-digested compounds > 10 kDa was performed as previously described by Rovalino-Córdova et al. (2021), with some modifications. Briefly, buffered colon mediums were carefully prepared to simulate the pH conditions of the proximal (pH = 5.8 ± 0.1) and distal colon (pH = 6.5 ± 0.1) (See composition in Table S1). Then, sterile penicillin bottles were added with 7.7 mL of sterilized buffered colon medium and 14.8 mL of solution transporting the non-digested compounds > 10 kDa (8.4 mg/mL). The bottles were closed with rubber and aluminum caps. To create anaerobic conditions, the headspace of the bottles was replaced by flushing with nitrogen. Then, 2.5 mL of the fecal biomass prepared in the SHIME® system was inoculated. Negative control (NC) and blank control (BC) were prepared by respectively replacing the solution transporting non-digested cocoa extracts and the SHIME®-stabilized fecal biomass, with sterile MilliQ water. Immediately after the inoculation, the fermentative bottles were vortexed for 5 s; after that, 1.5 mL of biomass was taken (corresponding to time zero sampling) and immersed in an ice bath. Right after, the bottles, namely batch bioreactors, were incubated over a rotating shaker (37 °C, 120 rpm). Samples of 1.5 mL were taken again after 2, 5, and 24 h of fermentation. This experiment was performed in duplicate for each biological donor.

The samples of biomasses were centrifuged (4 °C, 20.000*g*, 10 min). The supernatants were reserved for further chemical analysis, and the pellets for DNA extraction and sequencing. Both were kept at −20 °C.

2.7. Analysis of polyphenols

To determine the release of epicatechin, catechin, procyanidin B2 (P-B2), chlorogenic acid, gallic acid, caffeic acid, and ferulic acid, the supernatants obtained in Sections 2.4 and 2.6.2, and the permeates of Section 2.5 were thawed and then filtrated with PTFE filters. By duplicate, 80 μL of the filtrated sample and 80 μL of methanol were transferred into amber vials, and then vortexed for 30 s. The extracts were analyzed by using a Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan), according to the method described in our previous investigation (Peña-Correa et al., 2023).

2.8. Analysis of short-chain fatty acids (SCFAs)

The supernatants obtained in Section 2.6.2 were thawed and then filtrated with PTFE filters. By duplicate, 100 μL of the samples were transferred into GC-amber vials. The analysis was performed by using a Shimadzu GC-2014 gas chromatograph (Kyoto, Japan) equipped with a flame-ionization detector (FID), a capillary fatty acid-free Stabilwax-DA column (1 μm × 0.32 mm × 30 m) (Restek, Bellefonte, PA, USA), and a split injector, according to the setting conditions described by Guo et al. (2020). Nitrogen was used as the carrier gas, and standard solutions of acetic, propionic, butyric, isovaleric, and isobutyric acids in concentrations of 5 to 500 mg/L were prepared and used for identification and quantification. The limits of detection and quantification are presented in Table S2.

2.9. DNA extraction, quantification, and sequencing of barcoded amplicons

The cell pellets of bacterial cultures of fermentative times 0 h and 24 h of donor 1 obtained in section 2.6.2 were used for DNA isolation using QIAamp PowerFecal Pro DNA Kit (Qiagen). DNA yield was quantified using Qubit HS Fluorescence (Invitrogen). Of each DNA sample, 2.5 μL was used for bacterial 16S rRNA gene amplification by PCR targeting the variable V3 and V4 region using the primers Pro341F CCTACGGGNBGCASCAG and Pro805R GACTACNVGGGTATCTAATCC, both with universal extension as recommended by 16S Metagenomic Sequencing Library (Takahashi, Tomita, Nishioka, Hisada, & Nishijima, 2014). After initial amplification of the 16S target region, amplicons were purified and analyzed on a Bioanalyzer using DNA1000 chips. After that, indexing PCR was done using Nextera UD indexes adapters (Illumina). Barcoded amplicons were quantified using Qubit HS Fluorescence and equimolar pooled. Sequencing was performed on the Illumina MiSeq instrument using v2 flowcell and chemistry with 4 pM library loading concentration. Paired-end reads sequencing was obtained by 2 × 251 cycles. Base-calling and subsequent data demultiplexing were performed using bcl2fastq v2.20.0.422.

2.10. Data processing

The bioinformatics analysis of sequences was performed in the QIIME2 program (Quantitative Insights Into Microbial Ecology) version 2022.2.0 (Bolyen et al., 2019).

The samples were demultiplexed by the sequencing facility allowing the elimination of barcodes. Illumina short-reads were processed with the DADA2 pipeline. The DADA2 includes merging of reads, the inspection of read quality, quality filtering and trimming of reads, dereplication and error rate learning, sample inference for the determination of true sequence variants, construction of sequence table and removal of chimeric reads. Primers were removed with the DADA2 *trimLeft* function. The sequences were grouped in amplicon sequence variants (ASV) with 99 % similarity. To classify the sequences according to their taxonomic information, the *q2-feature-classifier* plugin was used based on the Vsearch alignment method (Rognes, Flouri, Nichols, Quince, & Mahé, 2016) with the SILVA v132 database (Quast, Pruesse, Yilmaz, Gerken, Schweer, Yarza, & Glöckner, 2013).

2.11. Analysis of microbiota data

The 16S rRNA gene sequencing analyses were conducted in RStudio, version 3.2.4, utilizing the Phyloseq (McMurdie & Holmes, 2013) and Microbiome packages (Lahti & Shetty, 2019) to import sample data and calculate alpha and beta diversity metrics. Alpha diversity was estimated with four indexes: Shannon, Chao 1, Inverted Simpson, and Phylogenetic distance (PD). The significance of categorical variables was determined using the nonparametric Wilcoxon test for two category comparisons or the Kruskal-Wallis test when comparing three or more categories. For beta diversity, principal coordinate analysis (PCoA) was performed to identify a clustering pattern of microbial composition as a function of the variables of interest using permutation-based methods (PERMANOVA, permuted multivariate analysis of variance, using the Adonis2 library) for Weighted UniFrac and Unweighted UniFrac distances (Fierer et al., 2010).

The correlation analysis between microbial genera abundance and the SCFA and BCFA data was performed using Kendall correlation test using the cor.mtest function from the corrplot stats package. The p values were adjusted with Bonferroni coorelation test.

2.12. Statistical analysis of parametric data

Polyphenols and SCFAs data were statistically analyzed via ANOVA. The Least Significant Differences (LSD) method with 95 % significance was applied using the statistic software StatGraphics Centurion XVIII (StatGraphics Technologies Inc., USA). Principal Component Analysis (PCA) was performed using R commander 3.6.1 (R Foundation, Austria) and R studio (RStudio Team, USA).

3. Results and discussion

3.1. Phenolic compounds bound to cocoa melanoidins, and their bioavailability upon in vitro digestion

Phenolic compounds present in plants mainly include free polyphenols, which can be extracted by organic solvents, and polyphenols bound to native HMWC like cellulose, hemicellulose, pectin, protein, and arabinoxylans by ester and C–C covalent linkages (Zhu, Li, Deng, Li, & Zhang, 2020). Certain thermally processed foods like cocoa contain another source of bound polyphenols, i.e., melanoidins (Oracz et al., 2019). Chemical alkaline hydrolysis (AH) and enzymatic hydrolysis (like in the simulated *in vitro* digestion) can cleave covalent bonds and release low molecular weight compounds. The content of released polyphenols upon AH and *in vitro* digestion of native cocoa HMWC and cocoa melanoidins are presented in Table 1.

The most predominant phenolic compound integrated to UR-HMWC and cocoa melanoidins was epicatechin, followed by catechin and procyanidin B2 (P-B2). Ferulic, chlorogenic, caffeic, and gallic acids were found below the LOQ. These results are aligned with the content of free polyphenols present in cocoa, as we reported our previous study (Peña-Correa et al., 2023), which used the same cocoa samples of this investigation.

As presented in Table 1, the AH treatment demonstrated that UR-HMWC holds a significantly higher content of epicatechin, catechin, and P-B2 than FR- and SR-extracts containing melanoidins. The total content of released polyphenols from FR- and SR-melanoidins is about 40 and 60 % of the total polyphenols of UR-HMWC, respectively. The depolymerization of native HMWC, and the formation of melanoidins determined the polyphenols content in FR- and SR-extracts; however, the final ratio of native HMWC and melanoidins (both carrying polyphenols) is unknown. The absolute values of AH-released polyphenols from cocoa melanoidins and unroasted cocoa extracts are aligned with those reported by Oracz et al. (2019); however, they detected more types of bound phenolic acids and found a higher content of bound polyphenols in roasted cocoa extracts than in UR-HMWC. The conditions of the roasting experiments could mainly explain these divergences (e.g., they roasted whole beans).

The content of total polyphenols released upon *in vitro* digestion showed a similar trend to the AH treatment: The total amount released from UR-HMWC was higher than that from FR- and SR-melanoidins (Table 1). However, there were differences in the individual phenolic compounds, especially in catechin, which was higher in FR-melanoidins, followed by SR-melanoidins. The native form of catechin in cocoa is (+)-catechin. During roasting, free (-)-epicatechin epimerizes to (-)-catechin (De Taeye, Kankolongo Cibaka, Jerkovic, & Collin, 2014). Although both catechin enantiomers have different features, they produce the same fragment ions under the LC-MS/MS analysis. Consequently, the sum of both enantiomers is measured as one compound and is generally referred to as catechin. This explains the increase in free catechin content during the roasting process of cocoa reported in various studies (De Taeye, Bodart, Caullet, & Collin, 2017; Żyżelewicz a et al., 2016), including our previous study, which used the same cocoa samples (Peña-Correa et al., 2023). As free catechin content increases in cocoa during roasting, bound catechin to melanoidins may increase too. This hypothesis suggests that (i) the ratio of generated melanoidins / native HMWC is higher in FR cocoa extracts than in SR extracts, and (ii) catechin may be a key compound to further unraveling the gray area between native HMWC and melanoidins in roasted cocoa extracts.

The differences in digestibility of UR-HMWC *vs.* cocoa extracts containing melanoidins can be appreciated by considering the AH-released polyphenols data as 100 % (Table 1). FR- and SR-melanoidins released the highest percentage of epicatechin, catechin, and P-B2 than UR-HMWC, especially epicatechin, which was fully digested from FR-melanoidins. These results suggest that the compactness of SR- and FR-melanoidins and/or the strength of the covalent bonds of polyphenols within cocoa melanoidins are lower than in UR-HMWC. The higher digestibility of FR-melanoidins compared with SR-melanoidins supports the hypothesis abovementioned: there could be more generated melanoidins and less native HMWC in FR cocoa extracts than in SR cocoa extracts. As reported in one of our previous studies (Peña-Correa et al., 2023), the oven roasting process probably led to lower heat penetration of the cores of cocoa nibs than the fluidized bed technique, thus preserving more native HMWC.

This investigation showed for the first time the potential of cocoa melanoidins to release bound polyphenols before entering the gut, and the modulation of the bioavailability of bound polyphenols via the formation of cocoa melanoidins. In line with our results, the *in vitro* digestion of coffee extracts containing melanoidins (Cut-off: 10 kDa) led to an increase in antioxidant activity in contrast to NaCl-treated and untreated coffee extracts (Rufián-Henares & Morales, 2007). The authors suggested that digestive enzymes modify water-soluble high molecular-weight molecules (including melanoidins), and increase the bioavailability of low molecular-weight structures (Rufián-Henares & Morales, 2007). In contrast, the *in vitro* digestion of melanoidins elaborated in a model system resulted in no formation of degradation products with nominal molecular masses below 3000 Da (Ames, Wynne, Hofmann, Plos, & Gibson, 1999). These differences may be ascribed to the limited types of reactants of the model systems (lysine and glucose), in contrast to the large variety of amino acids, peptides, proteins, carbonyl compounds (e.g., reducing sugars and lipid oxidation products), and polyphenols present in unroasted cocoa and green coffee.

Table 1
Phenolic compounds content (mg/100 g cocoa extract d.b.) released from native cocoa HMWC and cocoa melanoidins upon alkaline hydrolysis and *in vitro* digestion.

Treatment	Sample	Epicatechin	Catechin	P- B2	Sum
Alkaline hydrolysis	UR-HMWC	286.1 ± 27.3^{c}	232.2 ± 31.3^{c}	99.1 ± 13.9^{b}	626.8 ± 52.3^{c}
	FR-melanoidins	109.4 ± 10.9^{a}	97.8 ± 10.2^{a}	42.3 ± 5.4^{a}	268.2 ± 28.5^{a}
	SR-melanoidins	179.2 ± 68.7^{b}	155.4 ± 59.8^{b}	56.7 ± 18.2^{a}	400.7 ± 151.9^{b}
In vitro digestion	UR-HMWC	195 ± 6.0^{C} (68 %)	21.8 ± 1.1^{A} (9 %)	47.8 ± 4^{B} (48 %)	264.7 ± 6.1^{B} (42 %)
	FR-melanoidins	113.5 ± 11.9^{A} (104 %)	53.3 ± 10.7^{C} (54 %)	37.2 ± 8.4^{AB} (88 %)	204.1 ± 14.9^{A} (76 %)
	SR-melanoidins	133.3 ± 5.9^{B} (74 %)	35.4 ± 3.4^{B} (23 %)	30.6 ± 6.9^{A} (54 %)	210.5 ± 9.6^{A} (53 %)

The results correspond to mean ± standard deviation (percentage of digestion with respect to alkaline hydrolysis). Lowercase and uppercase superscript letters in the same column represent significant differences ($p < 0.05$) for alkaline hydrolysis and *in vitro* digestion data, respectively.

3.2. Production of short-chain fatty acids (SCFAs) in in vitro batch fermentation experiments

The catabolism of cocoa melanoidins by gut microbiota via *in vitro* batch fermentation may be reflected in the formation of the SCFAs acetic, propionic, butyric, and the branched-chain SCFAs (BCFAs) isobutyric and isovaleric acids. The data of the blank control (BC) (absence

of bacteria inoculum) was below the LOD, thus confirming the clear performance of the experiment and the absence of SCFAs in our cocoa extracts. The means of concentration of SCFAs in biomasses containing UR-HMWC, FR-melanoidins, SR-melanoidins, and in negative controls (absence of test product) (Table S3) were normalized to plot the PCAs (81.8 % variability) shown in Fig. 2 and Fig. S1. The formation of SCFAs was strongly correlated with the presence of cocoa extracts in the fermentative mediums (Fig. 2A) and with the DC (Fig. 2B). It means that gut microorganisms used UR-HMWC and cocoa melanoidins as a carbon source, and that the distal section of the colon is likely the most active site for their catabolism.

The PCA of Fig. S1 showed that there was not much effect of the

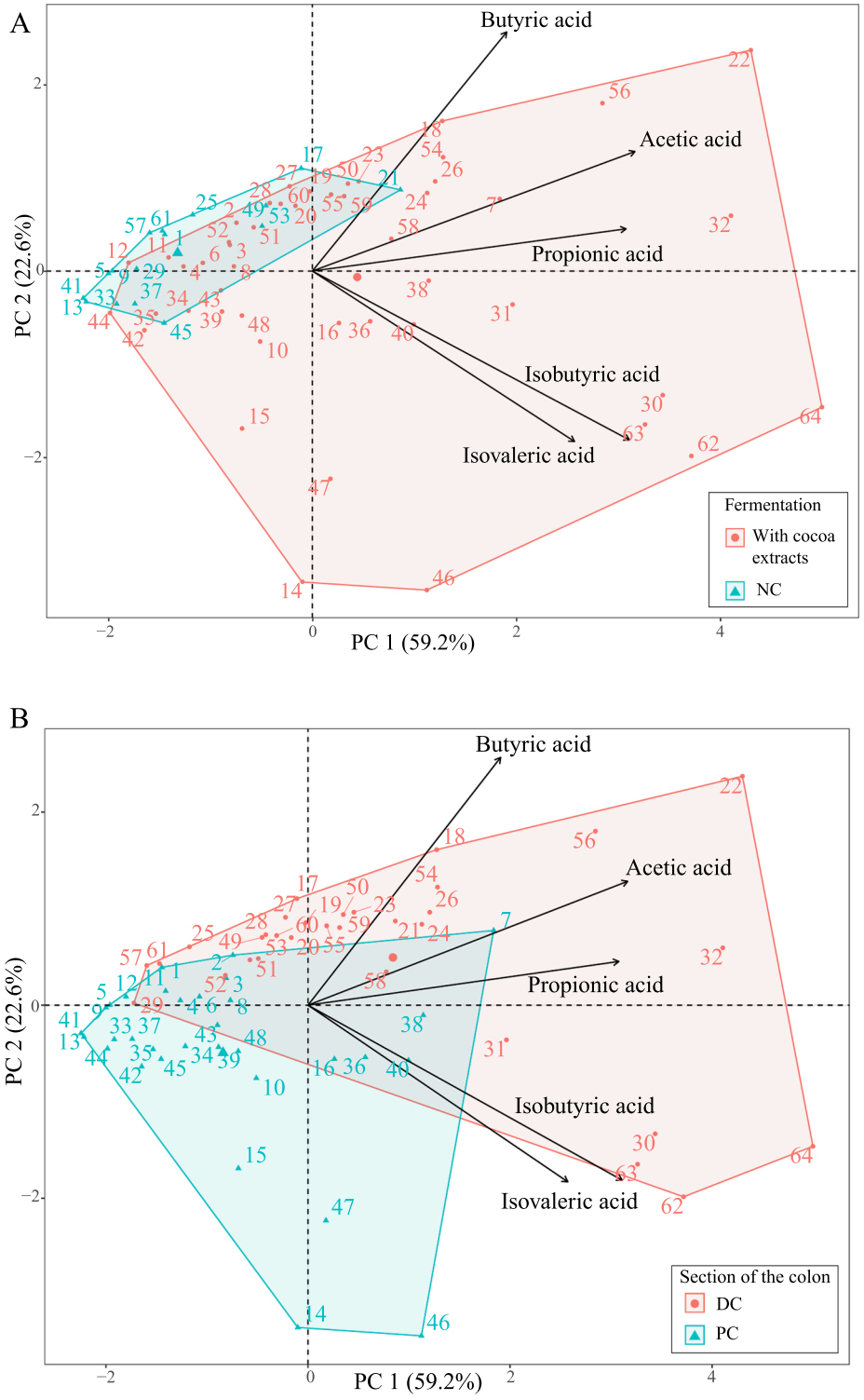

Fig. 2. PCA-cluster analysis of the means of SCFAs data of *in vitro* batch fermentation experiments containing microbiota of donors 1 and 2 by clustering: (A) the presence of cocoa extracts and the negative control (NC); and (B) the section of the colon. Scores 1 to 16 correspond to proximal colon (PC), and 17 to 32 to distal colon (DC) batch fermentations with biological material of donor 1; scores 33 to 48 correspond to PC, and 49 to 64 to DC batch fermentations with biological material of donor 2. Detailed information of the scores can be found in Table S3.

different biological donors in the production of SCFAs. For that reason, only the data of the duplicates of fermentation of one donor (donor 1) was used for further detailed analysis of the factors type of cocoa extract, fermentation time, and section of the colon. The authors acknowledge there could be limitations of using the data of one individual. However, every microbiota is an unique and organized system. For that reason, we did not considered appropriate to average data of different individuals. Instead, we focused the analysis of the above mentioned factors on the data of donor 1, and provided the statistical analysis of the SCFA and BCFA of the second donor in the supplements (Table S4). This in turn may provide sharper insights on the fermentability of cocoa melanoidins by gut microbiota.

The dynamics of generation of SCFAs during *in vitro* batch fermentation of native cocoa HMWC and cocoa melanoidins in PC- and DC-simulated bioreactors inoculated with pre-stabilized gut microbiota of donor 1 are presented in Fig. 3. Data of isobutyric and isovaleric acids were summed and represented as BCFAs. At first glance, it is evident that DC-bacteria produced higher acetic, propionic, and butyric acid content than PC-bacteria. This trend was reflected in the PCA (Fig. 2B). Fig. 3 also demonstrates that the most abundant metabolite was acetic acid, as regularly reported in other studies (Cher & Yassour, 2021; Li, Faden, & Zhu, 2020; and Pérez-Burillo et al., 2020).

The inoculums taken from the SHIME® continuous system carried SCFAs and BCFAs, thus explaining the load of these compounds in batch

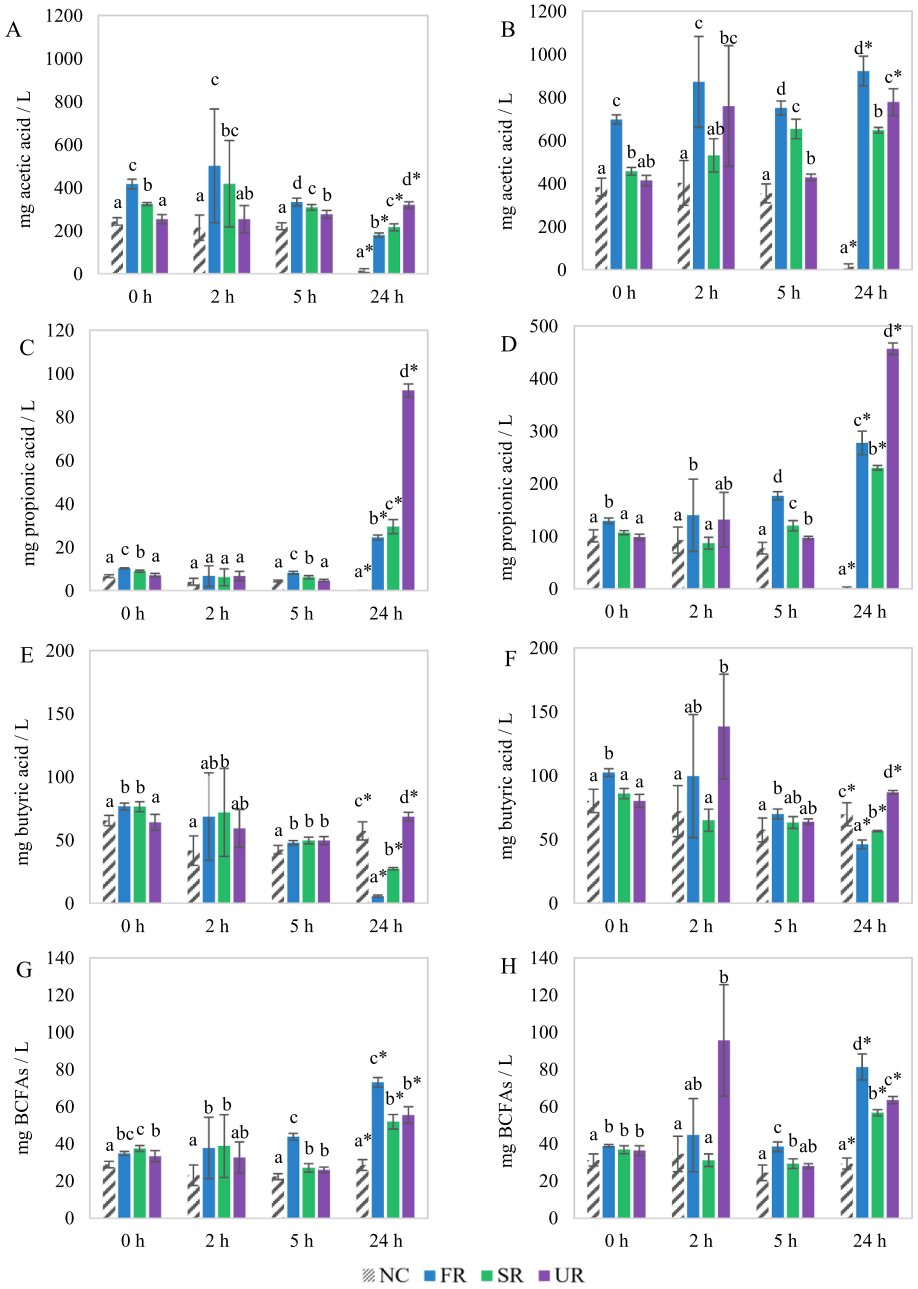

Fig. 3. Formation of acetic acid, propionic acid, butyric acid, and branched short-chain fatty acids (BCFAs) (sum of isobutyric and isovaleric acids) during *in vitro* the batch fermentation of non-digested cocoa compounds > 10 kDa with the gut microbiota of donor 1. The cocoa extracts were obtained from fast roasted (FR), slow roasted (SR), and unroasted (UR) cocoa. NC stands for negative control. Panels A, C, E, and G correspond to bioreactors mimicking the proximal colon, and panels B, D, F, H, to the distal colon. The error bars show the standard deviation. Different lowercase letters identify significant differences ($p < 0.05$) among the source of cocoa HMWC within each fermentation time. Asterisks * at time 24 represent statistical differences ($p < 0.05$) with respect to time 5 h for the same type of cocoa extract.

fermentation biomasses at time 0 h. However, the abundance of these metabolites was not even within the treatments and control. The concentration of SCFAs was higher in biomasses containing FR and SR melanoidins, and the content of BCFAs was higher with the presence of the three cocoa extracts than in the NC-biomasses. As discussed before, the blank controls proved that cocoa extracts were SCFA and BCFA free; therefore, these differences might be due to the fast production of these metabolites from the moment of the inoculation of the SHIME®-stabilized bacteria in the batch bioreactors until the cooling process of the extracted sample.

The data of SCFAs corresponding to 2 h of fermentation showed a high standard deviation. Likely, the adaptation of bacteria to the new fermentative environments was not yet even within the replicates; therefore, we do not consider time 2 h a good time-point to discuss the formation of SCFAs.

The formation of acetic acid after 5 and 24 h of fermentation of UR-HMWC, FR-, and SR-cocoa melanoidins significantly increased compared with the NCs (Fig. 3, panels A and B). In both PC and DC systems, the biomasses containing FR-melanoidins showed the highest concentration of acetic acid after 5 h of incubation, followed by SR-melanoidins ($p < 0.05$). After 24 h of fermentation in PC-systems, the acetic acid content in biomasses containing FR- and SR-melanoidins was significantly reduced. In contrast, the DC fermentative medium with FR-melanoidins showed a significant increase of acetic acid, while it was steady in that of SR-melanoidins. In both PC- and DC-biomasses containing UR-HMWC, the content of acetic acid increased significantly from 5 to 24 h of incubation ($p < 0.05$). The delayed usage of UR-HMWC by gut bacteria to produce acetic acid may reflect the complexity of native polymeric compounds.

Bacteria responsible for the transformation of dietary macronutrients into propionic acid were very active in the DC (Fig. 3D). The formation of this metabolite was about 5 folds of that in the PC (Fig. 3C). In both kinds of colon representing systems, the formation of propionic acid after 5 h of fermentation followed this order: FR-melanoidins > SR-melanoidins > UR-HMWC > NC ($p < 0.05$), meaning that bacteria showed a higher preference for melanoidins. After 24 h of incubation, the fermentation of UR-HMWC showed a considerable increase in propionic acid, higher than that of FR- and SR-melanoidins ($p < 0.05$), thus demonstrating again the delayed usage of this substrate. Maldonado-Mateus et al. (2021) found aligned results when fermenting the unsoluble fraction of unroasted cocoa powder with gut microbiota via *in vitro* experiments.

The formation of butyric acid in PC and DC bioreactors (Fig. 3, panels E and F) displayed similar trends. After 5 h of fermentation, the generation of butyric acid was significantly higher with the presence of the three cocoa extracts, with no statistical differences among them. After 24 h, the production of butyric acid decreased in biomasses containing FR- and SR-melanoidins and increased in those containing UR-HMWC and in the NC ($p < 0.05$). The similarity between UR-HMWC and NC after 24 h of incubation suggests that such a rise is not caused by a delayed use of the cocoa substrate to generate butyric acid. It could be a cross-feeding effect. Butyrate, as a crucial energy source for diverse metabolic functions in the gut (Mirzaei et al., 2022), could have been used by specific microorganisms present in biomasses containing FR- and SR-melanoidins, while it was not significantly consumed in NC– and UR-HMWC-biomasses.

Branched short-chain fatty acids (BCFAs) are considerably produced when proteins are present (Rios-Covian, González, Nogacka, Arboleya, Salazar, & Gueimonde, 2020). A considerable moiety of cocoa extracts containing melanoidins (15 to 20 % w/w) corresponds to proteins (Oracz & Nebesny, 2018). According to the literature, the fermentation of proteins better occurs in the distal colon (van der Wielen et al., 2017). However, our batch *in vitro* experiment showed similar trends in formation of BCFAs in both types of *in vitro* colon bioreactors (Fig. 3, panels G and H). Continuous *in vitro* systems can better mimic the saccharolytic and proteolytic activity across the colon.

BCFAs content in PC and DC systems ranged from 20 to 40 mg/L after 5 h of fermentation and from 20 to 80 mg/L after 24 h. In both time-points, biomasses containing FR-melanoidins showed the highest BCFAs production ($p < 0.05$), while those containing SR-melanoidins and UR-HMWC showed similar BCFAs content. These results strongly suggest that FR-melanoidins contain a higher protein moiety than SR-melanoidins and UR-HMWC, thus supporting the hypothesis above-mentioned that the ratio of melanoidins/native-HMWC in FR-extracts is higher than that in SR-extracts. The formation of BCFAs was boosted from 5 to 24 h of fermentation for the three kinds of cocoa extracts ($p < 0.05$), probably due to the complexity of the protein moiety of these compounds.

In general, SCFAs data showed that FR- and SR-melanoidins were well accepted by gut microorganisms of donor 1 from 5 h onwards, while UR-HMWC took more time to be catabolized. Although the concentration of SCFA in biomasses of donor 2 tended to be lower than that of donor 1 (Table S4), the dynamics of fermentation were similar. Our study demonstrated that cocoa melanoidins are more usable by gut bacteria than native HMWC, and provided relevant insights to understand the gut-fermentability of these compounds. The delayed usage of UR-HMWC resembles the fermentation of slowly breakdown fiber like cellulose (Bai et al., 2021) and lemon pectin (van Trijp et al., 2020), while the advanced fermentation of FR- and SR-melanoidins by gut microbiota resembles the degradation of galactooligosaccharides and fructooligosaccharides (van Trijp et al., 2020).

Unlike our results, the *in vitro* fermentation of high molecular weight material extracted from roasted coffee showed significantly higher total SCFAs generation after 24 h of incubation than at previous fermentation times (Reichardt, Gniechwitz, Steinhart, Bunzel, & Blaut, 2009). The proximal composition of coffee extracts showed a higher content of carbohydrates (25–50 % w/w) than that of cocoa extracts (8 to 16 % w/w in > 12.4 kDa fraction) (Oracz & Nebesny, 2018). Complex water-soluble carbohydrates present in coffee beans with a molecular weight up to 200 kDa (e.g., arabinogalactan type II and galactomannans) could have determined the fermentability of coffee extracts (Oosterveld, Harmsen, Voragen, & Schols, 2003).

3.3. Release of phenolic compounds in the colon

The catabolism of food melanoidins by gut microbiota can generate free polyphenols, among other low molecular weight compounds (Pérez-Burillo et al., 2020). During the *in vitro* fermentation of UR-HMWC, FR-, and SR-melanoidins, we found a release of phenolic compounds; however, they did not show significant differences compared to the control without bacteria (data shown in Table S5). In principle, this indicates the absence of a specific fermentation capacity by the gut bacteria. In contrast, Pérez-Burillo et al. (2020) found a significant release of polyphenols from pre-digested chocolate melanoidins by gut microorganisms via *in vitro* batch fermentation experiments. Besides the difference in the source of melanoidins, two other relevant differences between our experiments and theirs could explain this divergence: (i) After centrifuging the *in vitro*-produced chyle, Pérez-Burillo & colleagues used the pellet and 10 % of the supernatant for further *in vitro* batch fermentations. In our case, we discarded the pellet and used the retentate of ultrafiltration for gut microbiota fermentation experiments, as described in Fig. 1. (ii) Pérez-Burillo & colleagues used a ten-times higher concentration of digested fraction in their bioreactors (500 mg /10 mL) than that used in our study.

3.4. Changes in gut microbiota diversity and profile

A total of 6,031,020 sequences were obtained after filtering and checking chimera sequences of the 16S rRNA region V3-V4 from the samples. The sequencing depth of the data processed by DADA2 ranged from 125,562 to 125,671 sequences per sample. A total of 82 taxa within the phylum *Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes,*

Fusobacteria, Latescibacteria, Lentisphaerae, Patescibacteria, Planctomycetes, Proteobacteria, and *Verrucomicrobia* were identified in biomasses containing microbiota of Donor 1.

Alpha diversity analysis was performed considering the factors: fermentation time, section of the colon, and kind of cocoa extract. Shannon index is presented in Fig. 4, while Chao 1, Inv. Simpson, and PD are shown in Fig. S2. The fermentation time did not affect the richness and diversity of the microbial community (Shannon, $p = 0.164$ (Fig. 4A); Chao 1, $p = 0.781$; Inv. Simpson, $p = 0.220$; and PD, $p = 0.146$ (Fig. S2)). The alpha diversity analysis of the factors section of the colon and kind of cocoa extract was performed only with the data of time 24 h. According to Shannon ($p = 3.7$ e-5) (Fig. 4B), Chao 1 ($p = 3.6$ e-5), Inv. Simpson ($p = 3.7$ e-5), and PD ($p = 3.7$ e-5) indexes (Fig. S2), the richness of the taxa of DC-biomasses was significantly higher than that of PC, thus explaining the huge formation of SCFAs in these bioreactors. The alpha diversity was not affected by the presence of the different kinds of cocoa extracts (Shannon, $p = 0.470$ (Fig. 4C); Chao 1, $p = 0.370$; Inv. Simpson, $p = 0.275$; and PD, $p = 0.928$ (Fig. S2)). These results demonstrate that native cocoa HMWC and cocoa extracts containing melanoidins (FR and SR) did not compromise gut microbial diversity of donor 1.

The beta diversity analysis based on Weighted and Unweighted UniFrac distances of the factors section of the colon and kind of cocoa extract was performed only with the data of time 24 h, as observed in Fig. 5. The PCA of Weighted UniFrac and Unweighted UniFrac were explained by 91.4 % and 60.2 % of the variability, respectively. The beta diversity analysis of the factor section of the colon revealed notorious differences ($p < 0.001$) between PC and DC (Fig. 5 panels A and B). Both alpha and beta diversity differences in the sections of the colon were expected as buffered pH conditions strongly determine the kinds and the number of microorganisms (Mailhe et al., 2018). Aligned results along the *in vitro* simulated colon sections were reported by Faria Duque et al. (2021).

The human gut microbiome is shaped by factors such as diet, environment, and genetic background. Different diets may affect beta diversity (the degree of change in composition between treatments) (H. Li et al., 2016). The incorporation of native cocoa HMWC and cocoa melanoidins did not significantly change the beta diversity of the bacterial community (Weighted, $p = 0.123$; and Unweighted, $p = 0.423$) (Fig. 5 panels C and D). However, the clusters of Fig. 5C were not fully overlapping: the NC is almost separated, and UR-HMWC shifted slightly from FR- and SR-melanoidins. It means that microbial community of donor 1 tends to change when they are fed with cocoa extracts, and some particular differences occur in bacteria fed with cocoa melanoidins. These divergences can be appreciated in detail in the microbiota profile in Fig. 6.

The taxonomic classification at the genus level revealed that the microbiota profile of donor 1 was affected by fermentation time, kind of cocoa extract, and section of the colon factors (Data in Fig. 6 and Table S6). The most abundant microorganisms in PC-biomasses at time zero belonged to the genus *Megasphaera* (33 to 60 %) (Fig. 6A). *Megasphaera* sp. have demonstrated a preference for acidic environments (Firrman et al., 2022). The relative abundance of *Megasphaera* sp. in both colon-representing systems notoriously decreased in NC-biomasses after 24 h of fermentation. The incorporation of UR-HMWC in PC and DC bioreactors, and SR-melanoidins in DC bioreactors, did not change this trend. In contrast, when feeding with FR-melanoidins in both colon-representing systems and with SR-melanoidins in PC, the relative abundance of *Megasphaera* sp. increased. These results demonstrate that microorganisms of the genera *Megasphaera* preferred cocoa melanoidins over native HMWC.

Species belonging to the genera *Bacteroides* are equipped with a wide range of carbohydrate-depolymerizing enzymes, which confer them the ability to degrade complex carbohydrates (Reichardt et al., 2009). *Bacteroides*-related bacteria may dominate the more distal parts of the large intestine, as the luminal pH increases to 6.5 (den Besten et al., 2013). It explains their almost exclusivity to DC systems in our study. They were the most abundant microorganisms in DC-biomasses by covering 40 to 50 % of the relative abundance of the detected genus at time zero (Fig. 6B). The relative abundance of *Bacteroides* sp. in NC-DC-biomasses did not change notoriously after 24 h of fermentation. In contrast, the presence of cocoa extracts increased it, with no notorious difference among the three test products. The growth in *Bacteroides* sp., and the significant increase in SCFAs and BCFAs (especially acetic and propionic acids) in DC bioreactors containing cocoa extracts (Fig. 3 panels B and D) were positively corelated, as presented in the correlation analysis in Fig. 7. This correlation was expected as butyrate and acetate are typical fermentation products of the microorganisms of the genus *Bacteroides* (Macy & Probst, 1979). In line with these results, the *in vitro* batch fermentation of melanoidins elaborated in a model system (Ames et al., 1999) and with coffee melanoidins (Reichardt et al., 2009) showed a significant increase in *Bacteroides* after 24 h of incubation.

Bacteroides sp. were almost absent in PC compartments at time zero (Fig. 6A). Interestingly, the PC-fermentative systems led to differences among cocoa extracts after 24 h of fermentation. The presence of FR-melanoidins showed a remarkable relative increase of species belonging to genera *Bacteroides*. At that point, it turned the second most abundant genus, thus changing notoriously the bacteria community profile. The PC-biomasses containing UR-HMWC and SR-melanoidins showed a slight increase in *Bacteroides* sp., but did not lead to drastic changes in bacteria profiles. The advanced formation of acetic and propionic acid in PC bioreactors containing FR-melanoidins (Fig. 3, panels A and C) may explain such a proliferation. Because the systems were buffered and closed, *Bacteroides* sp. that grew after 5 h did not die during the following fermentation time. Considering that FR-melanoidin extracts contain more melanoidins than SR-melanoidin extracts, we conclude that cocoa melanoidins have a potential prebiotic effect along the whole large intestine, while native HMWC are more selectively used in the distal region.

Species of the genera *Bifidobacterium* were the second most abundant in the PC systems at time zero, ranging from 17 to 26 % (Fig. 6A). *Bifidobacterium* sp. are acid-tolerant, and their proliferation is affected when fecal pH increases (Henrick et al., 2018). However, they competed with *Megasphaera* sp. for the second place in relative genera abundance in DC systems (about 10 %). The relative abundance of microorganisms of the genera *Bifidobacterium* in the NC-PC systems was not affected after 24 h of fermentation. In contrast, it was reduced in the DC, thus demonstrating its low resistance to pH close to neutrality. The reduction was more profound when adding cocoa extracts in both PC and DC bioreactors, especially in those containing FR- and SR-melanoidins. These results, and the negative correlation between the relative abundance of *Bifidobacterium* sp. and the formation of SCFA and BCFA (Fig. 7) may initially suggest that cocoa extracts inhibit the relative growth of microorganisms of the genera *Bifidobacterium*. However, *Bacteroides*- and *Bifidobacterium*-related bacteria metabolize dietary fibers as carbon sources; therefore, they compete for similar substrates, especially short-chain fructooligosaccharides (Biavati, Vescovo, Torriani, & Bottazzi, 2000). As a consequence, the growth of *Bacteroides* and the decrease of *Bifidobacterium*, or vice versa, may simultaneously occur (Cher & Yassour, 2021). This rationale explains the differences with previous studies that demonstrated that chocolate-obtained melanoidins (Pérez-Burillo et al., 2020) and bread-crust-extracted melanoidins (Borrelli & Fogliano, 2005) favored the growth of *Bifidobacterium* sp, and reduced the proliferation of microorganisms of the genera *Bacteroides*. In other words, the microbiota profile of the donor determines the results of *in vitro* studies.

The slight separation of the cluster UR from FR and SR in the Weighted UniFrac analysis (Fig. 5C) was driven by the exceptional growth of species belonging to the genus *Bacteroides* and *Megasphaera* when fed with FR- and SR-melanoidins. *Bacteroides* species are beneficial as they can establish stable and long-term contact with the host and can degrade dietary fiber into SCFAs, thus providing energy for cells,

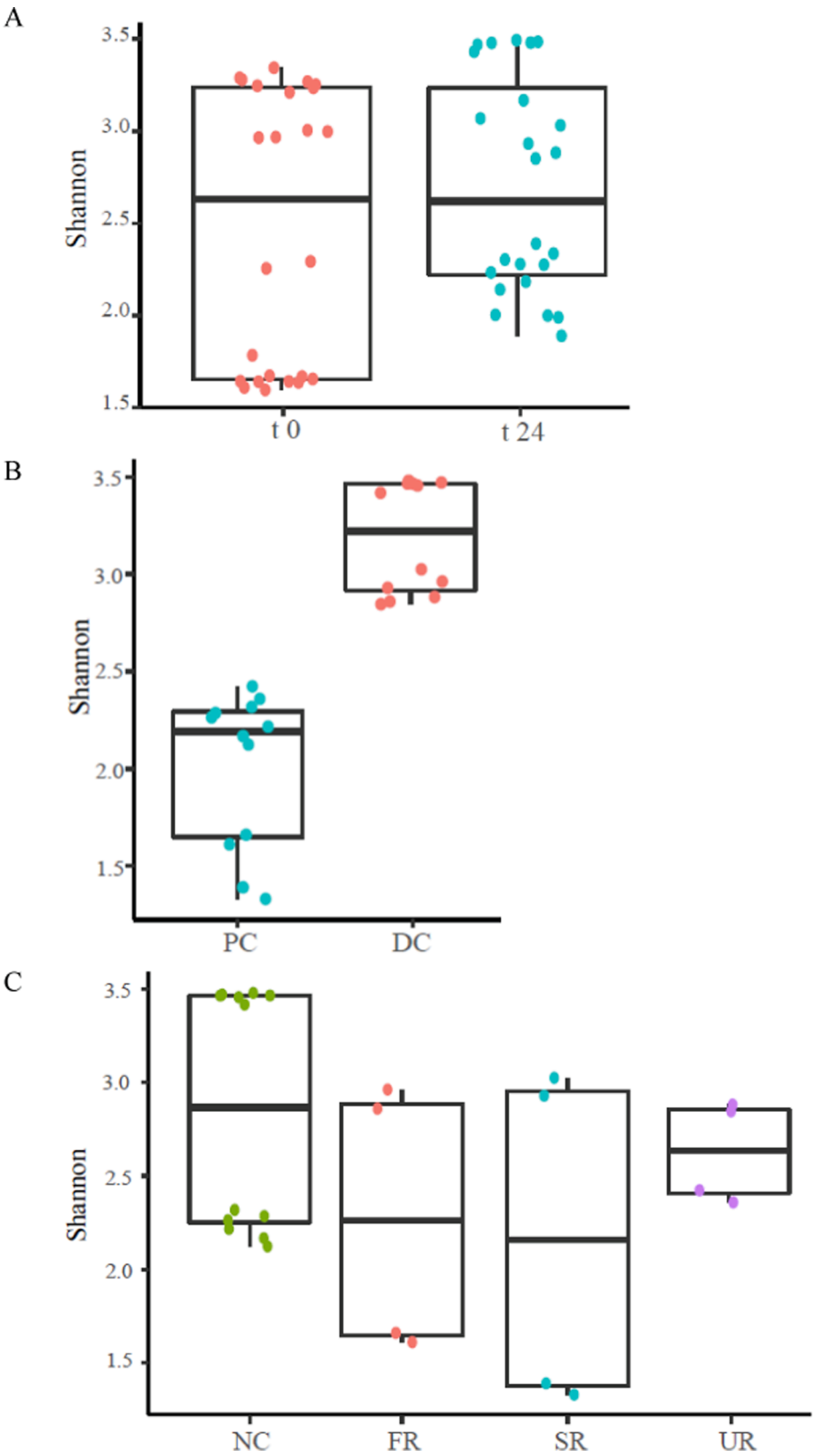

Fig. 4. Alpha diversity using Shannon Index for gut microbiota community of donor 1 from different perspectives. Panel A shows the fermentation time, representing t0 and t24 the time zero and 24 h of fermentation, respectively. Panel B discriminates the sections of the colon, corresponding PC and DC to proximal and distal colon, respectively. Panel C shows the kinds of cocoa extract, where NC, UR, FR, and SR stand for negative control, unroasted, fast roasted, and slow roasted cocoa extracts, respectively.

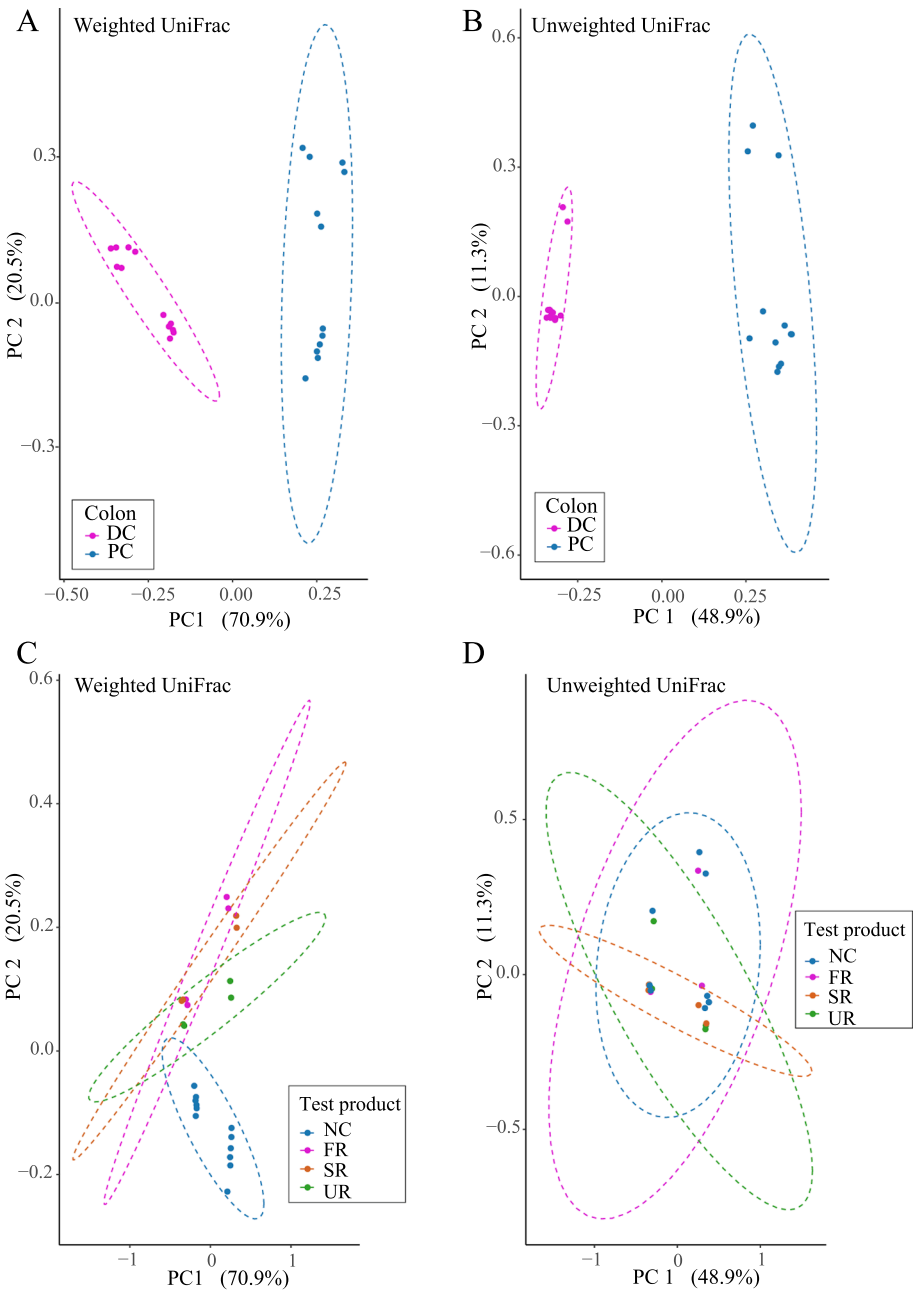

Fig. 5. Beta diversity of gut microbiota community of donor 1 explained by Weighted (A and C) and Unweighted (B and D) UniFrac distances from different perspectives: Section of the colon (A and B), corresponding PC and DC to proximal and distal colon, respectively; and kind of cocoa extract (C and D), where NC, UR, FR, and SR stand for negative control, unroasted, fast roasted, and slow roasted cocoa, respectively.

promoting barrier function, and reducing the occurrence of inflammatory reactions (Deng et al., 2020). *Megasphaera* species are also nonpathogenic; they have been negatively associated with diarrhea disease instead (Carey et al., 2021). Though Fig. 5C did not separate FR and SR, Fig. 6A evidenced the more profound effect of FR-melanoidins in increasing the relative abundance of *Bacteroides* sp. in the PC.

The tendency of the NC to form a separated cluster in the Weighted UniFrac analysis (Fig. 5C) could be explained by the abundance of microorganisms of the genus *Enterococcus*, *Escherichia-Shigella*, and *Clostridium sensu stricto 1* after 24 h of fermentation. The relative abundance of species of these genera was significantly reduced or even eliminated when incorporating cocoa extracts in the batch *in vitro* fermentation of both colon-representing systems. The relative abundance of microorganisms of the abovementioned genera was negatively correlated with the formation of acetic, propionic, isobutyric, and isovaleric acids (Fig. 7). According to the literature, species of the genus *Enterococcus*, *Escherichia-Shigella*, and *Clostridium sensu stricto 1* cover several known pathogenic bacteria for humans (Gorbach, 1996), and their population decrease with the presence of SCFA (Mirzaei et al., 2022). So, our results suggest an indirect antibacterial effect of cocoa extracts, especially cocoa FR-melanoidins, by stimulating the proliferation of SCFAs-producers.

The positive correlation between butyric acid and the growth of *Escherichia-Shigella* sp. displayed in Fig. 7 was determined by the high concentration of this compound in the NCs (Fig. 3E-F). As discussed in Section 3.2, this important source of energy likely accumulated in those bioreactors because of the minimal presence of bacteria capable of catabolize it.

Microorganisms of the genus *Lachnoclostridium*, *Akkermansia*, *Sutterella*, and *Parabacteroides* were exclusive of the DC fermentative

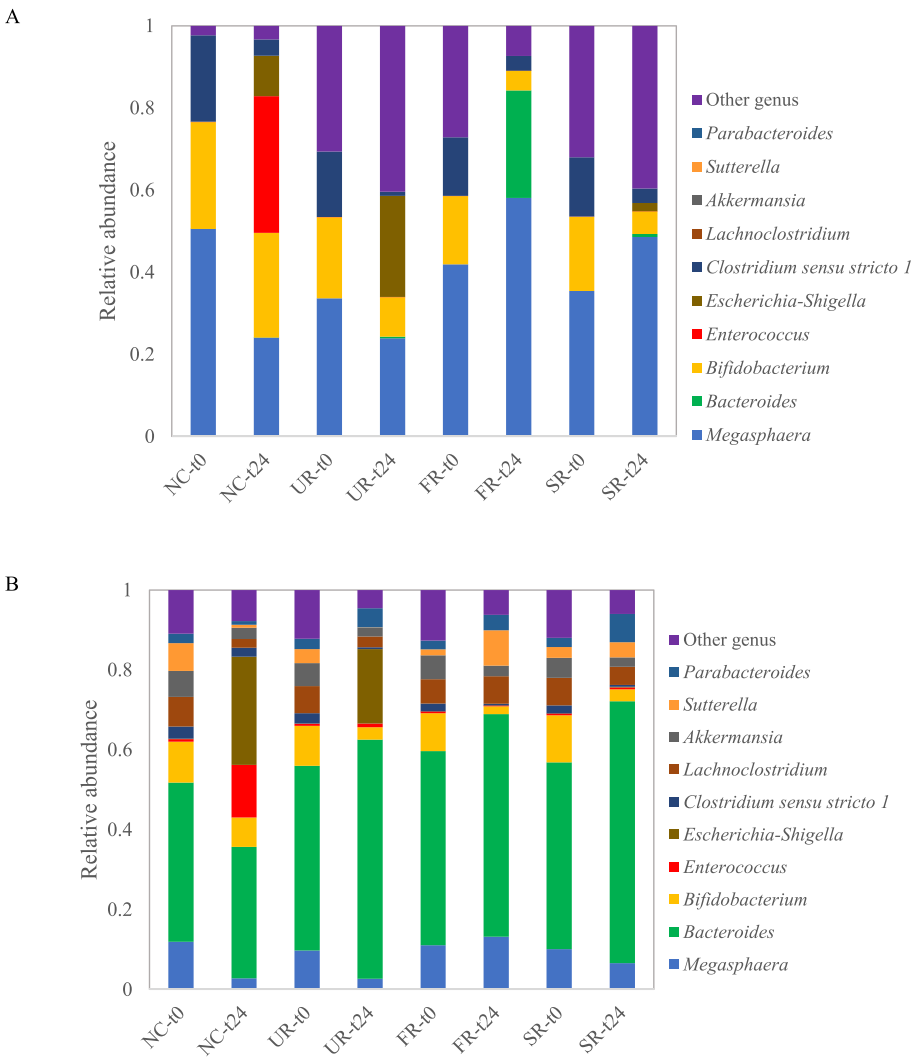

Fig. 6. Relative abundance of different genus in the microbiota of donor 1 stabilized in the SHIME® continuous system, followed by *in vitro* batch fermentations experiments mimicking the conditions of the proximal colon (Panel A) and the distal colon (Panel B) at the initial time of fermentation (0) and after 24 h (t24). The first two bars correspond to the negative control (NC), and the rest to the fermentations containing different kinds of cocoa extracts obtained from unroasted (UR), fast roasted (FR), and slow roasted (SR) cocoa nibs.

systems (Fig. 6B). Other microorganisms belonging to different genera out of the top ten were summed and presented in Fig. 6 (purple bar). The list is shown in Table S7.

The effect of roasting on the interaction of the insoluble fraction of cocoa powder with gut microbiota via *in vitro* experiments also revealed differences in microbiota profile (Maldonado-Mateus et al., 2021). Unroasted cocoa powder favored the increase in the relative abundance of *Veillonella* genera, while roasted cocoa favored *Faecalibacterium* genera. The genera *Veillonella* was found in the microbiota community of donor 1 of this study (Table S7); however, its abundance was not significant.

4. Conclusions

We proved via *in vitro* digestion experiments that the roasting process of cocoa improves the digestibility of polyphenols bound to water-soluble compounds > 14 kDa via the formation of melanoidins. A higher relative content of polyphenols was digested from melanoidins extracted from fluidized-bed-roasted cocoa nibs (FR-melanoidins), followed by melanoidins extracted from convective-oven-roasted (or slow-roasted) cocoa nibs (SR-melanoidins). The lowest relative digestion of polyphenols was found in native high molecular weight compounds obtained from unroasted cocoa (UR-HMWC). The molecular structure of native HMWC would be more compact than that of cocoa melanoidins. The higher digestibility of FR-melanoidins over SR-melanoidins suggests this extract contains more melanoidins and less native HMWC.

The response of gut microbiota fed with cocoa extracts revealed that just after 5 h of fermentation, FR- and SR-melanoidins were highly accepted by gut bacteria, as demonstrated with the formation of SCFAs and BCFAs, while UR-HMWC was delayed up to 24 h. The content of these metabolites was higher when fermenting FR-melanoidins compared to SR-melanoidins. Native HMWC resembled the fermentation of a dense fiber like cellulose, whereas both extracts containing cocoa melanoidins were more rapidly metabolized as if they were oligosaccharides, especially FR-melanoidins.

This study demonstrated that cocoa melanoidins are capable of improving the balance of pathogenic and nonpathogenic genera of donor 1 by increasing the relative abundance of the latter, especially the *Bacteroides* sp. The proliferation of the nonpathogenic microorganisms was correlated with the production of SCFAs and BCFAs, and most of the species belonging to the pathogenic genus are sensitive to those metabolites, thus explaining the indirect antibacterial effect of cocoa extracts, especially melanoidins.

The results of this investigation strongly suggest that roasting

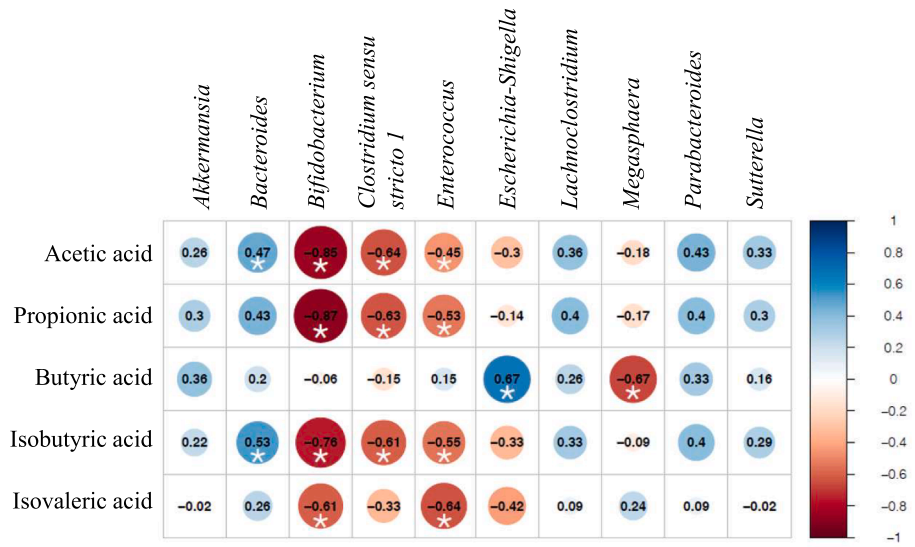

Fig. 7. Correlation analysis between the abundance of the top-ten bacterial genera and the formation of acetic, propionic, butyric, isobutyric, and isovaleric acids at the end of the fermentation (24 h). The color and the size of the circles indicates the magnitude of correlation. Asterisks (*) indicate significant correlations ($p < 0.05$) according to Kendall's correlation.

changes the structure of water-soluble compounds of cocoa > 14 kDa by degrading more native HMWC and producing cocoa melanoidins. This in turn improves the accessibility of digestive enzymes and gut microbiota to catabolize polymeric structures. Fluidized bed roasting, a fast and low-carbon footprint technique, outstood over traditional oven roasting by showing more profound effects on the Maillardization of native HMWC. The modulation of the formation of MR products like melanoidins by roasting process could allow the formulation of healthier cocoa-based products, more friendly to the human digestive system and health.

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Ethical statement

This research falls outside of human or animal studies, so institutional ethical approval was not required.

CRediT authorship contribution statement

Ruth Fabiola Peña-Correa: Conceptualization, Methodology, Data curation, Formal analysis, Writing – original draft, Investigation, Project administration, Funding acquisition. **Zixuan Wang:** Methodology, Formal analysis, Data curation. **Victoria Mesa:** Data curation, Statistical analysis, Writing – review & editing, Validation. **Burçe Ataç Mogol:** Methodology, Validation, Investigation, Supervision. **Julian Paul Martínez-Galán:** Data curation, Validation. **Vincenzo Fogliano:** Writing – review & editing, Supervision, Methodology.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2023.105814.

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