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RESEARCH ARTICLE

In vitro bioaccessibility and gut biotransformation of polyphenols present in the water-insoluble cocoa fraction

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Scope: Cocoa, especially the water-insoluble cocoa fraction (WICF), is a rich source of polyphenols. In this study, sequential in vitro digestion of the WICF with gastrointestinal enzymes as well as its bacterial fermentation in a human colonic model system were carried out to investigate bioaccessibility and biotransformation of WICF polyphenols, respectively.
Methods and results: The yield of each enzymatic digestion step and the total antioxidant capacity (TAC) were measured and solubilized phenols were characterized by MS/MS. Fermentation of WICF and the effect on the gut microbiota, SCFA production and metabolism of polyphenols was analyzed. In vitro digestion solubilized 38.6% of WICF with pronase and Viscozyme L treatments releasing 51% of the total phenols from the insoluble material. This release of phenols does not determine a reduction in the total antioxidant capacity of the digestion-resistant material. In the colonic model WICF significantly increased of bifidobacteria and lactobacilli as well as butyrate production. Flavanols were converted into phenolic acids by the microbiota following a concentration gradient resulting in high concentrations of 3-hydroxyphenylpropionic acid (3-HPP) in the last gut compartment.
Conclusion: Data showed that WICF may exert antioxidant action through the gastrointestinal tract despite its polyphenols being still bound to macromolecules and having prebiotic activity.

Keywords:
Digestion / Flavanols / Phloroglucinolysis / Prebiotic / SCFA

1 Introduction

The dietary-insoluble fraction (DIF) includes, apart from the conventional dietary fiber (carbohydrate polymers that are not hydrolyzed by endogenous enzymes in small intestine of human beings), other indigestible compounds such as fractions of resistant starch, proteins, polyphenols and other associated compounds [1]. The DIF concept was proposed as a more realistic and physiological tool to study the nutritional value of a food or even a diet [2].

In this context, the concept of antioxidant dietary fiber (ADF) was coined to indicate the insoluble moiety of some foods having antioxidant activity [3]. ADF is of relevance for technological, physiological and nutritional implications of antioxidant compounds associated with the indigestible fraction of many foods. Most of the phenolic compounds in cereals are covalently bound to cell wall polysaccharides [4] and in some fruits the content of non-extractable polyphenols (mainly hydrolyzable tannins and proanthocyanidins associated with dietary fiber and proteins) is about

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Abbreviations: 3,4-DHBA, 3,4-dihydroxybenzoic acid; **3-HPA,** 3-hydroxyphenylacetic acid; **3-HPP,** 3-hydroxyphenylpropionic acid; **ABTS,** 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid); **ADF,** antioxidant dietary fiber; **DIF,** dietary insoluble fraction; **FISH,** fluorescence in situ hybridization; **mDP,** mean degree of polymerization; **MRM,** multiple reaction monitoring; **TAC,** total antioxidant capacity; **WICF,** water-insoluble cocoa fraction

five-folds (112–126 mg/100 g of fresh fruit) that of free polyphenols (19–28 mg/100 g of fresh fruit). Also melanoidins, the brown polymers formed by proteins and carbohydrates through the Maillard reaction in many processed foods, are potent antioxidants. They escape digestion and small intestinal absorption thus potentially behaving as ADF in vivo [5, 6].

Antioxidant compounds present in DIF may have a physiological relevance in maintaining a reducing environment in the intestinal lumen. They can exert their action through a surface reaction along the gastrointestinal tract [7] thus preventing the damage caused by radicals on the intestinal cells [8]. During the transit time, DIF antioxidant compounds may be released by the digestive enzymes of small intestine thus becoming bioaccessible. Once they reach the colon, microbial enzymes may release them from macromolecules thus permitting the absorption through the colon in their original chemical forms or after further microbial metabolism. This process would explain the delayed absorption of polyphenol metabolites that was often recorded after cereal or grape dietary fiber consumption [9, 10] and it is the basis of the wide range of biological activities that dietary polyphenols showed at low nanomolar concentration [4, 11–14].

Among the dietary constituents, cocoa highly contributes to the intake of water-insoluble polyphenols. Cocoa has a high total phenol content (up to 224 ± 66.4 μmol catechins/g) [15] and the benefits of its consumption on human cardiovascular health have been associated mainly with the polyphenols moiety [16, 17]. The beneficial properties of cocoa DIF were investigated by Lecumberri et al. [18] in hypercholesterolemic rats. Starting from cocoa husks, they isolated a material consisting of 60% dietary fiber on dry matter basis, whose main part (83%) was DIF [19]. They showed that a 21-day intervention with the cocoa fiber-enriched diet reduced blood lipid concentration (cholesterol and triglycerides) and lipid peroxidation without any effects on total antioxidant capacity (TAC), on the activity of antioxidant enzymes and on the hepatic levels of glutathione [18]. To explain their results the authors suggested that the potential absorption of polyphenols from cocoa-insoluble material might have played a role in the observed effects probably through systemic circulation and/or through gut microbiota action.

The objective of this study was to assess changes in the polyphenol fraction of water-insoluble cocoa fraction (WICF) by in vitro digestion simulating the human gastrointestinal process. The therewith linked changes in the antioxidant capacity of the insoluble materials obtained at each digestion step were measured by the QUENCHER method [20]. Additionally, the potential of the WICF as a prebiotic material, a source of short chain fatty acid (SCFA) and of polyphenol metabolites was investigated by a three-stage continuous culture system (gut model), validated to mimic the human colonic microbial environment [21, 22].

2 Materials and methods

2.1 Materials

HPLC water and methanol were purchased from Merck (Darmstadt, Germany). Ethanol, *n*-hexan and sodium hydroxide were from Carlo Erba (Milan, Italy). Cellulose, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), diammonium salt and pronase were obtained from Fluka (Steinheim, Germany). Hydrochloric acid and Folin reagent were from Riedel de-Haën (Seelze, Germany). Pepsin, pancreatin from porcine pancreas and Viscozyme L were purchased from Sigma (St. Louis, MO, USA). Dialysis tubes were from Spectrum Laboratories (Rancho Dominguez, CA, USA), mod. Spectra/Por (3500 Da cutting off). For fluorescence in situ hybridization (FISH) analysis, paraformaldehyde and 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) were purchased from Sigma. PBS Tablets were from Oxoid (UK). Phloroglucinol dihydrate (98%, HPLC grade) was obtained from Fluka. Pure procyanidin B1 and B2 and all phenolic acids were purchased from Sigma.

2.2 Production of WICF

Insoluble cocoa fraction was obtained from an alkali-treated commercial cocoa powder (Perugina, Perugia, Italy) by a series of successive water washes and centrifugation. Briefly cocoa was suspended in water (45 g cocoa: 270 mL water) aliquoted in 50 mL tubes, vortexed for 1 min (Super Mixer, Continental Equipment) and centrifuged at $3220 \times g$ for 15 min (IEC CL3OR centrifuge, Thermo Scientific, France). This procedure was repeated for 15 times. Supernatants were discarded and the pellet was freeze-dried (Flexi-Dry MP freeze-drier, FTS Systems, New York, USA).

2.3 In vitro digestion

Enzymatic hydrolyses of the isolated WICF were sequentially performed as described by Kedia et al. [23]. Briefly, 1 g of water-insoluble cocoa powder was dissolved in 60 mL water and treated with 3 mL pepsin solution (800–2500 U/mg; 0.5 mg/mL, 0.9% NaCl; pH 2, 37°C for 1 h). After digestion the tube was centrifuged at $3220 \times g$ for 15 min, pepsin digested material was taken for further analysis while the pellet was dissolved with 6 mL pancreatin solution ($4 \times$ United States Pharmacopoeia specifications; 0.5 mg/mL in 20 mM $NaPO_4$ buffer – 10 mM NaCl; pH 8, 37°C for 1 h). The same procedure was applied on pancreatin-digested sample before treating the pellet with 2 mL pronase solution (4.7 U/mg; 1 mg/mL; pH 8, 37°C for 1 h). After centrifugation and separation of the supernatant, the pronase digested sample was finally treated with 100 μL Viscozyme L, which is a multi-enzyme complex containing a wide range of carbohydrases, including arabanase, cellulase,

β-glucanase, hemicellulase and xylanase (pH 4, 37°C for 1 h).

The in vitro digestion of three samples of WICF was carried out and results of each analysis were reported as mean ± SD. Three control samples without the addition of the enzymes were also run in parallel.

2.4 TAC

TAC of soluble material obtained after each digestion step (supernatants collected after centrifugation) was measured by the ABTS method [24].

The measurement on the insoluble fraction was performed by the QUENCHER method as previously described [20]. Briefly, 6 mL of ABTS work solution, prepared through dilution of the stock with ethanol–water (50:50), were added to 10 mg of freeze-dried sample. The mixture was shaken for 30 min and then centrifuged for 2 min at 3220 × *g* to obtain a clean solution whose absorbance at 734 nm was measured. Each measure was performed in triplicate and results were expressed as mean ± SD (mmol Trolox equivalents [TE]/kg).

2.5 Analysis of total phenol content

Phenol content of soluble material obtained by each digestion step was measured by Folin-Ciocalteau method [25]. Each measure was performed in triplicate and results were expressed as mean ± SD (mg gallic acid/100 g of cocoa).

Procyanidins and phenolic acids from previously acidified (to a pH <2) soluble materials were also extracted by ethyl acetate [26] and the extracts analyzed by LC/MS/MS as reported below.

2.6 Acid-catalyzed degradation of oligomeric procyanidins in the presence of phloroglucinol and HPLC-DAD analysis

Acid-catalyzed degradation in the presence of phloroglucinol was performed under the conditions previously described [27] with minor modifications. Briefly, a solution of 0.1 N HCl in methanol, containing 50 g/L phloroglucinol and 10 g/L ascorbic acid was prepared. Ten milligrams of lyophilized sample was dissolved in 1 mL of the reagent solution and the reaction was performed at 50°C for 25 min. Degradation products were analyzed by RP-HPLC-DAD immediately after the acid-catalyzed reaction.

A Shimadzu LC-20A Prominence chromatographic system (Kyoto, Japan) equipped with a diode array detector (DAD detector SPD-M20A) was used. Separation was performed on a Supelcosil LC-318 (250 × 4.6 mm, with particle size of 5 μm, Supelco, Bellefonte, PA, USA) at 35°C. Eluent A was water, eluent B was water/formic acid 0.1% v/v and eluent C was acetonitrile. The flow rate was kept constant throughout the analysis at 1 mL/min. The elution program used was as follows: 1% B isocratic during all the analysis; 3% C isocratic from 0 to 5 min, 3–8% C linear from 5 to 9 min, 8% C isocratic from 9 to 15 min, 8–9% C linear from 15 to 16 min, 9% C isocratic from 16 to 22 min, 9–14% C linear from 22 to 25 min, 14–20% C linear from 25 to 35 min, 20–40% C linear from 35 to 46 min, 40–99% C linear from 46 to 47 min, 99% C isocratic from 47 to 50 min, 99–3% C linear from 50 to 51 min and re-equilibration of the column from 51 to 56 min under initial gradient conditions. DAD detection was performed at 254, 280 and 330 nm. The injection volume was 1 μL. The mean degree of polymerization (mDP) was measured by calculating the molar ratio of all the flavan-3-ol units (phloroglucinol adducts plus terminal units) to epicatechin and catechin corresponding to terminal units, as described [28].

2.7 In vitro fermentation in a three-stage continuous culture colonic model system – gut model

The used three-stage culture system comprised three glass fermenters of increasing working volume, simulating the proximal (vessel 1 [V1], 280 mL), transverse (vessel 2 [V2], 300 mL) and distal colon (vessel 3 [V3], 320 mL). V1 was fed by means of a peristaltic pump with complex colonic model growth medium (CMGM) [22]. The three fermenters were connected in series, with V1 feeding V2, which sequentially fed V3 finally overflowing into the waste. Culture pH was maintained at 5.5 (V1), 6.2 (V2) and 6.8 (V3) respectively. All vessels were kept at 37°C by means of a circulating water-bath and the system was kept anaerobic by continuously sparging with O_2-free N_2.

Fecal samples from one healthy donor (one male, 30 years of age, omnivore, free of any known metabolic and gastrointestinal diseases, not taking probiotic-, prebiotic- supplements and antibiotics for the 6 months prior fecal sample donation) were collected on site, they were kept in an anaerobic cabinet (10% H_2, 10% CO_2, 80% N_2), they were diluted 1:5 w/w in anaerobic PBS (0.1 mol/L PBS (pH 7.4), 150 mM NaCl) and finally they were homogenized in a stomacher (Seward, Worthing, West Sussex, UK) for 2 min. The vessels of the colonic model were then inoculated with 100 mL of this fecal slurry and they were kept separated for a 24-h period in order to stabilize bacterial populations. After 24 h (Ti) the colonic model growth medium flow between vessels was initiated and the system was run for eight full volume turnovers to allow for steady state to be achieved (SS1). At SS1, samples were obtained on three consecutive days to confirm steady state status through SCFA profiles and FISH analyses. Taking into account the operating volume (900 mL) and the retention time (36 h) of the colonic model system, the WICF prepared as described below, was added daily for a further eight volume turnovers upon which steady state 2 (SS2) was achieved. Samples on three consecutive days were obtained to establish SS2 as described for SS1.

The cocoa material to feed the gut model was prepared from WICF by the procedure introduced by Saura-Calixto et al. [2] with some modifications. Briefly, 20 g cocoa were dissolved in 120 mL water containing α-amylase, 1 mL (3000 Ceralpha units/mL; pH 7, 100°C for 35 min) and protease, 2.5 mL (350 tyrosine units/mL; pH 7, 60°C for 30 min). Both enzyme preparations were provided by Megazyme in the Total Dietary Fibre Kit.

After performing the enzyme treatments, the samples were transferred into dialysis tubes (3500 Da cutting off) and dialyzed against water for 6 days at room temperature. Dialysis retentates were freeze-dried and they were used to feed V1 at 1% w/v.

The current experimental design was limited to a single run. The results of intervention at SS2 can be directly compared to that of SS1; in this way the use of a single system can provide significant results [29].

2.8 Bacterial analysis by FISH

Enumeration of bacterial populations of the gut model's samples, obtained at SS1 and SS2, was performed by FISH analysis, as described by Martìn-Pelàez et al. [30]. The hybridization was carried out using genus- and group-specific 16S rRNA gene-targeted oligonucleotide probes labelled with Cy3 (Sigma-Aldrich, Poole, UK). The probes, as reported in Table 1, were: Eub338 I-II-III for total bacteria [31], Bac303 for *Bacteroides–Prevotella* spp. [32]; Bif164 for *Bifidobacterium* genus [33]; Lab158, for the *Lactobacillus–Enterococcus* group [34]; Enter1432 for enteric bacteria group [35] and Chis150 for *Clostridium histolyticum* group [36].

2.9 SCFAs analysis

SCFA content of samples was evaluated by the method developed by Zhao et al. [37], using 2-ethylbutyric acid as the internal standard. The analysis was carried out using a GC Hewlett Packard (Agilent) 5890 Series II (HP, Crawley, West Sussex, UK), equipped with a column FFAP (30 m × 0.53 mm, diameter = 0.50 μm, J&W Scientific, Agilent Technologies, South Queensferry, West Lothian, UK) and a flame ionization detector (FID). Helium was supplied as the carrier gas at a flow rate of 14 mL/min. The temperature of the flame ionization detector and the injection port was 300 and 280°C, respectively. The initial oven temperature was 100°C, maintained for 0.5 min, raised to 150°C at 8°C/min, then increased to 250°C at 50°C/min, and finally held at 250°C for 2 min. The run time for each analysis was 10.75 min. Data handling was carried out with Atlas Lab software (thermo Lab Systems, Mainz, Germany).

2.10 Polyphenol metabolites

Polyphenol metabolite concentrations in the three vessels before and after feeding the gut model with cocoa-insoluble dietary fiber were measured by performing ethyl acetate extraction of HCl acidified samples [26] and by analysis of the extracts by LC/MS/MS as reported in the following section.

2.11 Characterization of phenolic acids and procyanidins by LC/MS/MS analysis

The LC/MS/MS analyses were carried out using a mass spectrometer model Sciex API 3000 triple-quadrupole by Applied Biosystem (Toronto, Canada) with interface TurboIonSpray (TIS), coupled with an HPLC binary micropumps (Perkin Elmer, USA, mod. Series 200) as previously described [9].

For procyanidins, an Inertsil ODS-3V 5 μm 4.6 × 250 mm (GLScience, Torrance, CA, USA) column was used, with water 0.1% formic acid (solvent A) and CH_3CN (solvent B) as the mobile phases. Applied gradient profile was as follows: 0–12 min 95% A–5% B, 12–16 min 60% A–40% B, 16–26 min 50% A–50% B and 26–30 min 95% A–5% B.

For phenolic acids, a Prodigy C18 particle size 5 μm 150 mm × 4.60 mm column (Phenomenex, Torrance, CA, USA) and the following mobile phases were used: water 0.1% formic acid (solvent A) and methanol (solvent B). The following gradient elution was used: 0–10 min 95% A–5% B,

Table 1. Oligonucleotide probes used in this study for FISH analysis

Target genus or group	Probe	Sequence (5′ to 3′)	Hybridization-washing temperature
Most bacteria	EUB338I[a)]	GCTGCCTCCCGTAGGAGT	46–48
Most bacteria	EUB338II[a)]	GCAGCCACCCGTAGGTGT	46–48
Most bacteria	EUB338III[a)]	GCTGCCACCCGTAGGTGT	46–48
Bacteroides spp.	Bac303	CCAATGTGGGGGACCTT	46–48
Bifidobacterium spp.	Bif164	CATCCGGCATTACCACCC	50–50
Enterobacteriaceae	Enter1432	CTTTTGCAACCCACT	46–48
Clostridium cluster I and II	Chis150	TTATGCGGTATTAATCTYCCTTT	50–50
Lactobacillus–Enterococcus spp.	Lab158	GTATTAGCAYCTGTTTCCA	50–50

a) These probes are used together in equimolar concentrations.

10–12 min 55% A–45% B, 12–15 min 45% A–55% B, 15–22 min 100% and 22–24 min 95% A–5% B.

Nebulization temperature was 400°C. Flow rate was 0.8 mL/min. Data acquisition was performed in MRM (multiple reaction monitoring) in negative ions mode. The capillary voltage was 4500 V and ions derived from de-protonation of cocoa procyanidins were selected. The declustering potential and the collision energy were optimized for each compound. Typical fragmentation patterns of monitored compounds in the insoluble cocoa fraction and in the sample from gut vessels are shown in Table 2.

2.12 Statistical analysis

FISH and SCFA data as well as the concentration of metabolites were analyzed by one-way Anova method, using the Tukey post-hoc test analysis when significance of overall difference was below the set limit ($p < 0.05$).

Analyses were performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

3 Results and discussion

3.1 Enzymatic in vitro digestion of WICF

3.1.1 Effect of the enzyme treatments on the solubilization of WICF

The steps of the in vitro sequential enzymatic treatment on WICF adopted in this study is schematized in Fig. 1. The figure reports the yields and the TAC measured for both, insoluble and soluble materials, at each digestion step. A negligible amount of phenols was solubilized and no modification of TAC was recorded in the sample run without enzymes addition (control), thus confirming that the washing procedure exhaustively solubilized all the potentially soluble material.

Data showed that 38.1% of cocoa powder analyzed in this study were water-soluble while 61.9% were insoluble. The latter moiety constitutes the WICF which have a content of polysaccharide of 61.4%. In total, 38.6% of WICF (23.9 g of 61.9 g) was solubilized considering the action of all digestive enzymes. Regarding the action of individual enzymes, both pepsin and pancreatin solubilized 11.0% of the treated material (6.8 g from 61.9 g was solubilized by pepsin and 6.1 g from 55.1 g was solubilized by pancreatin) while the combined action of pronase and Viscozyme L (mimicking the lower gut hydrolysis) solubilized a further 18% of the material obtained by previous enzymatic treatment (11.0 g from 49.0 g insoluble material obtained by pepsin+pancreatin digestion). Interestingly, the digestion with Viscozyme L showed the highest yield of extraction (19.1% solubilized material) compared to the previously applied enzymes (4.1% yield by pronase and 11.0% by pancreatin and by pepsin).

The overall figure indicates that WICF represents approximately 60% of dietary cocoa. Following digestion in the small intestine, as mimicked here by sequential in vitro digestion, about 75% (47.0 g from 61.9 g) of this insoluble fraction may reach the colon where bacteria play a major role in fermenting it. Microflora action leads to the solubilization of a further 17.8% (11.0 g from 61.9 g) that is potentially absorbable through the colon.

3.1.2 TAC

TACs of insoluble cocoa fractions were measured for the first time using the Quencher method, which allows a direct

Table 2. LC/MS/MS fragmentation parameters for the detection in MRM mode of phenolic compounds and their metabolites

	Parent ion (*m/z*)	Fragments (*m/z*)
Procyanidin dimers	577	289, 407, 425, 125
Procyanidin trimers	865	287
Procyanidin tetramers	1153	577, 289
−(Epi)catechin	289	245
+(Epi)catechin gallate	441	289
5-(3,4-Dihydroxyphenyl)-γ-valerolactone	207	163, 122
5-(3-Methoxy-4-hydroxyphenyl)-γ-valerolactone	221	206, 162
3-HPP	165	121, 106, 77
3-HPA	151	107, 65
3,4-DHBA, Protocatechuic acid	153	109
2-(3,4-Dihydroxyphenil)acetic acid	167	123, 95
t-3-(4-Hydroxy-3-methoxy-phenyl)prop-2-enoic acid (Ferulic acid)	193	134, 178
3-(3,4-Dihydroxyphenyl)-2-propenoic acid (Caffeic acid)	179	135
4-Hydroxy-3-methoxybenzoic acid (Vanillic acid)	167	123, 95
Benzoylaminoacetic acid (Hippuric acid)	178	134
3,4,5-Trihydroxybenzoic acid (Gallic acid)	169	125
3-(3,4-Dihydroxycinnamoyl)quinate (Chlorogenic acid)	353	191
3-(4-Hydroxyphenyl)prop-2-enoic acid (Coumaric acid)	163	119

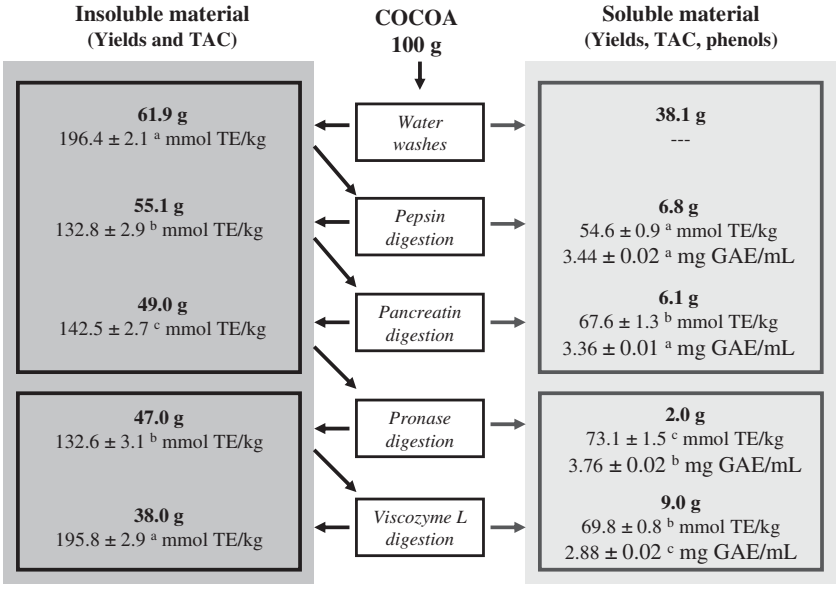

Figure 1. Yields, total phenols and TAC measured by the QUENCHER method of insoluble and soluble materials produced during in vitro digestion of WICF. Different letters next to the values of TAC and of phenol concentration indicate a significant difference ($p > 0.05$).

measure of radical scavenging capacity of the solid material by avoiding any extraction procedure [20].

Data reported in Fig. 1 showed a slight increase in the TAC of the extracts up to the pronase treatment, the TAC of material solubilized by pronase is 33.9% higher than that obtained by pepsin (73.1 ± 1.5 versus 54.6 ± 0.9 mmol TE/kg). The TAC of Viscozyme extract was similar to that of pronase extract (69.8 ± 0.8 versus 73.1 ± 1.5 mmol TE/kg). Considering that the extracts of Viscozyme digestion were obtained from material already treated with three proteolytic enzymes, this result indicates that a significant part of antioxidant compounds was linked to the polysaccharide moiety of WICF.

From the TAC results measured from the insoluble material, it can be highlighted that the sequential action of digestive enzymes modified the reducing capacity of the insoluble fraction. The action of the proteases significantly reduced the TAC of the insoluble material (196.4 ± 2.1 mmol TE/kg in the untreated cocoa water-insoluble material versus 132.6 ± 3.1 mmol TE/kg in the insoluble material after pronase digestion). Interestingly, the TAC of the insoluble material increased again after Viscozyme treatment (195.8 ± 2.9 mmol TE/kg) thus suggesting that the hydrolysis of polysaccharides made bioaccessible some antioxidants that were previously buried in the structure of the insoluble material. Digestive enzymes were able to modify the chemical structure of the insoluble materials allowing the bound polyphenols to be exposed on the surface of the matrix thus exerting their reducing properties against the $ABTS^{+}$ radical during the measure by the QUENCHER method.

From a physiological point of view, these data highlighted the in vivo potential role of WICF along the gastrointestinal tract to act as a scavenger of free radicals present in the upper and lower gastrointestinal tract [8].

The antioxidant action of WICF appear of particular relevance considering the absolute value of its TAC which is twofold higher than that of many whole cereals and comparable to that of buckwheat [20].

3.1.3 Phenol concentration of soluble materials

Phenol concentration of soluble materials obtained by each digestion step and measured by Folin Ciocaulteau method is reported in Fig. 1. In agreement with the TAC data, phenol concentration of extracts obtained by pronase was higher than the phenol concentration of the soluble materials obtained from the other enzyme treatments.

Total amount of phenols in soluble materials was 925 mg gallic acid equivalent (GAE)/100 g cocoa. A significantly higher amount of polyphenols was released upon pronase treatment compared to the other enzyme treatments (271 versus 217, 235, 202 mg for pepsin, pancreatin and Viscozyme L treatment, respectively).

In all, these data confirmed that half of WICF polyphenols became bioaccessible and, therefore, potentially absorbable in the colon upon the action of microbial proteases and polysaccharidases which here was mimicked by pronase and Viscozyme L, respectively.

Summarizing, the modification of chemical structure of cocoa-insoluble materials by sequential enzyme digestions had a double positive effect: it caused a high TAC of insoluble residues passing through the gastrointestinal tract thus being potentially beneficial for colorectal cancer chemoprevention [38]; and it increased the bioaccessibility

of polyphenols, as shown by the phenol content in the soluble extracts.

3.1.4 Characterization of phenolic and procyanidinic fraction of soluble materials

A qualitative MS/MS analysis of the supernatants derived from the enzyme hydrolysis confirmed the release of different phenolic acids, procyanidin monomers and dimers from the insoluble fraction. In particular, three different dimer isomers were found, originating from fragmentation of molecular ion 577→289. Previous studies on cocoa proanthocyanidins showed similar results, with hydrolysis of proanthocyanidins into epicatechin dimers and monomers [39]. Some data suggested that monomers released from insoluble cocoa fraction after gastric and intestinal digestion are partially available for absorption [40, 41].

A more detailed chemical characterization of all soluble extracts was obtained by a specific quantitative analysis of catechins and procyanidins composition before and after phloroglucinolysis, using HPLC-DAD. Phloroglucinolysis was performed in order to obtain the complete de-polymerization of the procyanidins present in the solubilized material allowing the release of the free terminal of the chain (catechin or epicatechin) and their quantification as catechin and epicatechin adducts [28]. This approach was useful to assess the prevalence of catechin and epicatechin in procyanidins. Results are summarized in Table 3: all samples obtained after proteases digestion (pepsin, pancreatin and pronase) showed the presence of catechin, epicatechin and procyanidin B2, while B1 dimer was released only by pepsin hydrolysis. After the action of Viscozyme L, epicatechin (but no catechin or procyanidins) was found. These data suggest that the catechin and dimeric procyanidins, particularly B1, were mainly linked to the protein moiety of the insoluble material, while a minor part of epicatechin was associated with the polysaccharide moiety.

The prevalence of the epicatechin in cocoa procyanidins has been supported by previous papers [42, 43], highlighting the prevalence of procyanidinic dimers B2 (epicatechin-4B-8-epicatechin), B5 (epicatechin-4B-6-epicatechin), trimer C1 (epicatechin-4B-8-epicatechin-4B-8-epicatechin) and tetramer D (epicatechin-4B-8- epicatechin-4B-8-epicatechin-4B-8-epicatechin) in processed chocolate. In Table 3, also the data obtained from the same soluble materials, analyzed after acid-catalyzed phloroglucinolysis and the mDP obtained by phloroglucinolysis [28] are reported. The absolute amount of catechins and procyanidins was probably underestimated due to the significant tendency of flavonoids monomers to oxidize to quinonic form, and then to polymerize in vitro, decreasing their solubility. This may account for the discrepancy between the very high antioxidant capacity of the extract and the relatively low amount of phenols measured by HPLC-DAD. It is likely that more complex procyanidins are present in the soluble materials and they might have influenced the TAC values. This suggestion has been confirmed by the presence of a significant quantity of catechin and epicatechin (either as terminal monomers and as adducts), measured after acid-catalyzed phloroglucinolysis. Concerning the mDP, we highlighted values ranging from 1.83 (calculated for procyanidins present in soluble fraction obtained by pancreatin) and 2.88 (calculated form procyanidins present in soluble fraction obtained after the pronase digestion of the insoluble pellet). These data are well correlated with the mDP recently reported in cocoa powder (3.09) by Hellstrom et al. [42] The data reported in this study confirm our results: about 50% of the total quantity of extractable procyanidins consist of high-degree polymerized procyanidins (>10 polymerization degree) and a significant percentage of unextractable procyanidins was found.

Table 3. Concentration of catechin, epicatechin, procyanidins B1 and B2 in soluble cocoa fractions (upper part of the table) and monomeric composition of polymeric procyanidins (lower part of the table)

Cocoa sample	Catechin	Epicatechin	Procyanidin B1	Procyanidin B2	
Pepsin	6.62±0.14 b	8.61±0.21 b	10.49±0.35 a	5.17±0.31 b	
Pancreatin	2.03±0.19 c	3.74±0.20 c	n.d.	1.48±0.30 c	
Pronase	0.76±0.04 d	1.53±0.14 d	n.d.	0.68±0.08 c	
Viscozyme L	n.d.	1.38±0.23 d	n.d.	n.d.	
Total	9.41	15.26	10.49	7.33	
	Terminal catechin[a)]	Terminal epicatechin[a)]	Catechin adduct[a)]	Epicatechin adduct[a)]	mDP
Pepsin	9.57±0.17 b	12.26±0.91 b	4.40±0.31 b	7.37±0.71 b,c	2.78
Pancreatin	5.14±0.21 c	11.90±0.30 b	1.44±0.20 c,d	12.75±1.68 b	1.83
Pronase	1.75±0.04 d	4.89±0.12 b	0.75±0.04 d	7.43±0.29 b,c	2.88
Viscozyme L	2.55±0.34 d	4.20±0.19 b	2.31±0.36 c	5.82±0.41 c	2.51
Total	19.02	33.25	8.91	33.37	

Results are expressed as milligrams per 100 g of cocoa powder. Mean±SD ($n = 3$) followed by the same letter (a, b, c, d), within a column, are not significantly different ($p > 0.05$). n.d., not detected; mDP, mean degree of polymerization.
a) Determined after phloroglucinolysis procedure.

In all, phloroglucinolysis analysis confirmed that both monomers (with prevalence of epicatechin), dimers (with prevalence of B2) and the putative quantity of high molecular weight procyanidins (>3 polymerization degree), all prevalently containing epicatechin monomer [43], were linked to protein and to insoluble fiber of cocoa, and can be partially released after enzyme digestion. These analytical data are in good agreements with the antiradical properties of the cocoa insoluble fiber measured by the QUENCHER method.

3.2 Gut model experiment on WICF

To investigate the effect of dietary cocoa fiber on gut microbiota, WICF was pre-digested by gastric and duodenal enzymes, and then dialyzed to retain the high molecular weight and the insoluble material. The digestion-resistant material was finally used to feed a gut model using a procedure validated in many previous studies [44–47]. Prebiotic activity, production of SCFA and metabolism of phenolic compounds were investigated as detailed below.

3.2.1 Prebiotic in vitro activity

Changes in bacteria populations in the gut model fed with cocoa dietary fiber, as measured by the FISH analysis, are depicted in Fig. 2. Results indicated that cocoa dietary fiber was a good substrate for human gut microbiota in vitro. A significant increase in lactobacilli numbers in vessel 1 ($p<0.01$) and in vessel 3 ($p<0.05$) as well as an increase in bifidobacteria in all vessels (V1, $p<0.01$; V2, $p<0.05$ and V3, $p<0.01$) was recorded at SS2 compared to SS1. The increase in lactobacilli and bifidobacteria by cocoa dietary fiber suggested its potential prebiotic activity in vivo [48], which can be related not only to the polysaccharide moiety, but also to the flavanol compounds. In fact, the ability of catechin in a human fecal batch culture (at a concentration of 150 mg/L but not at 1000 mg/L) to increase the growth of bifidobacteria was recently reported by Tzounis et al. [49].

Contrary to coffee dietary fiber and coffee brews which were shown to be fermented by the *Bacteroides–Prevotella* group with propionate production [50, 51], cocoa dietary fiber was not a preferred substrate for this bacterial group (see Fig. 2). The different carbohydrate compositions of coffee and cocoa, namely galactomannans and arabinogalactans in the high molecular weight coffee fraction [51] versus cellulose as well as minor amounts of hemicellulose and pectic substances [18] in cocoa dietary fiber might account for this difference. The coexistence of fermentable polysaccharides and free flavanol monomers in cocoa which are both able to modify the gut microbiota can open new possibilities for the prebiotic action of dietary components.

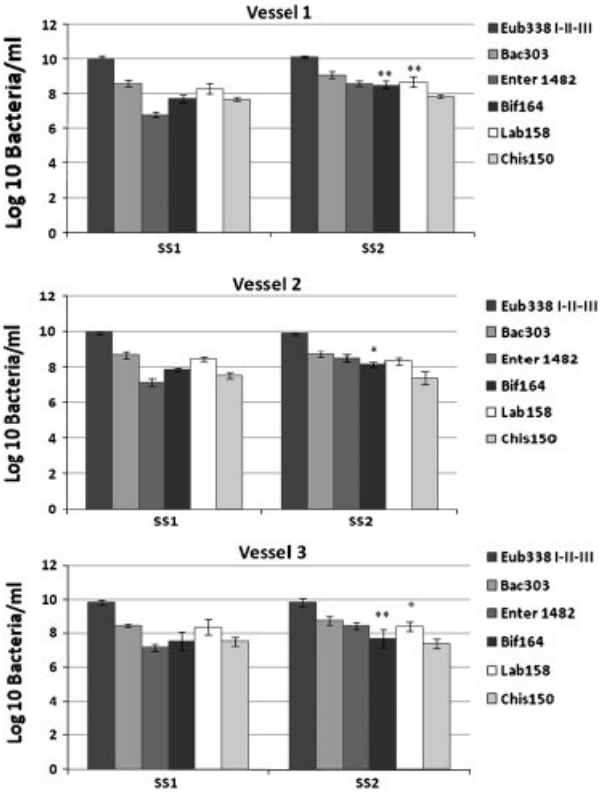

Figure 2. Bacterial populations (Log10 cells/mL) recovered from the three different vessels (V1, V2 and V3) of the colonic model system as measured by the FISH analysis. Steady state 1 (SS1) and 2 (SS2) represent the stationary point reached by the gut model before and after the addition of the cocoa-insoluble material. Significant differences between SS1 and SS2 in bacteria populations within the same vessel and relative to each bacteria are indicated as follows: $^{*}p<0.05$; $^{**}p<0.01$; Tukey's test abbreviations of the legends are reported in Table 1.

3.2.2 Microbial metabolites

3.2.2.1 SCFA

The relative concentrations of SCFA in the three vessels at SS1 and SS2 are reported in Fig. 3. Interestingly, a significant increase in butyric acid in all three vessels (in vessel 1, 16.48 ± 1.714 mM versus 19.48 ± 0.221, a 1.18-fold increase, $p<0.05$; in vessel 2, 13.48 ± 1.412 mM versus 19.42 ± 0.912, a 1.44-fold increase, $p<0.01$; in vessel 3, 14.31 ± 0.912; 17.18 ± 0.594 mM, a 1.20-fold increase, $p<0.05$, respectively) at SS2 compared to SS1 was found.

Butyrate production is of great importance as it has trophic effect on gut epithelium, it modulates proliferation and apoptosis and effects gene expression in colonic epithelial cells [52, 53].

In a previous work, performed by Mäkivuokko et al. [54] the increase in concentrations of SCFA, in particular butyrate concentrations by fermentation of pre-digested

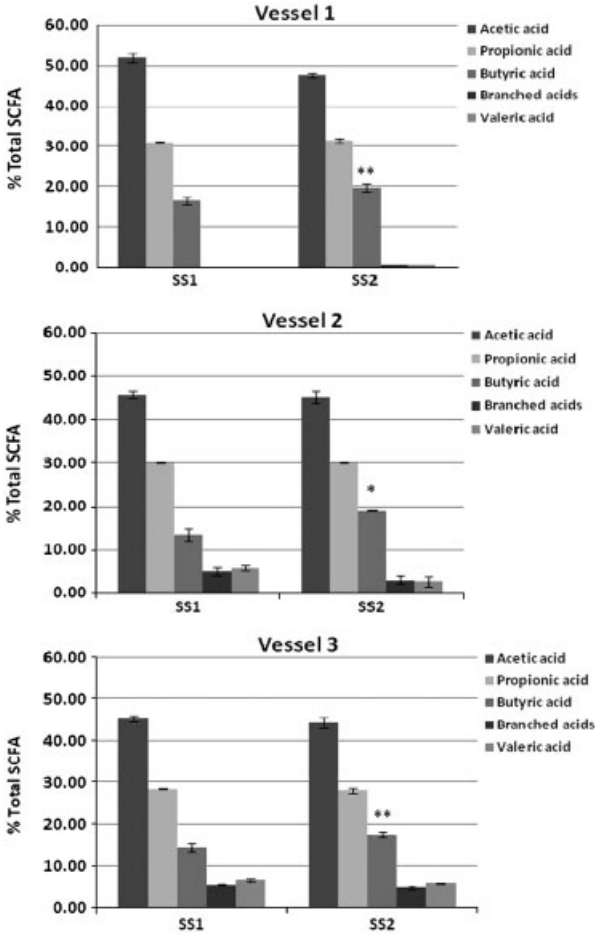

Figure 3. SCFAs in samples recovered from the three different modules (V1, V2 and V3) of the colonic model system steady state 1 (SS1) and 2 (SS2) represent the stationary point reached by the gut model before and after the addition of the cocoa-insoluble material. Significant differences between SS1 and SS2 in SCFA concentrations within the same vessel and relative to each SCFA are indicated as follows: $^{*}p<0.05$; $^{**}p<0.01$; Tukey's test.

cocoa mass was only found when 2% polydextrose was added to cocoa mass. As the concentrations of SCFA for fermentation of pre-digested cocoa mass in combination with polydextrose were similar to the concentrations measured previously for polydextrose alone and as the concentrations after cocoa mass fermentation did not differ from baseline the authors concluded that, unlike in our study, cocoa mass alone did not give rise to SCFA production.

The different concentration and type of pre-digested cocoa mass added to the system, the different gut model used and especially the longer running time in our study (10 versus 2 days) may account for the different results between the two studies.

Unfortunately, in the Mäkivuokko study [54] the bacterial population was not investigated by FISH and only total bacterial count was estimated; thus, it was not possible to compare the two studies for microbiota composition.

3.2.2.2 Microbial metabolites of cocoa polyphenols

The gut metabolism of different flavonoids was elucidated using the pig cecal microflora [55, 56]. However, the products of cocoa dietary fiber metabolism by human gut microflora in a three-stage culture system mimicking the human colon were investigated in this study for the first time.

3-Hydroxyphenylpropionic acid (3-HPP), 3-hydroxyphenylacetic acid (3-HPA) and 3,4-dihydroxybenzoic acid (3,4-DHBA) were the phenolic acids detected in the three vessels. A fourth unknown compound having an MRM fragmentation peak of m/z 289→245 (characteristic of (epi)catechin) was also found. As it was not possible to univocally identify this compound having a retention time longer than (epi)catechin, it was named (epi)catechin derivative.

Cocoa polyphenol metabolite concentrations in the three vessels are shown in Fig. 4. Significant increases in 3-HPP concentration ($p<0.01$) in the three vessels, of 3-HPA concentration ($p<0.05$) in vessel 1 and vessel 3 and of

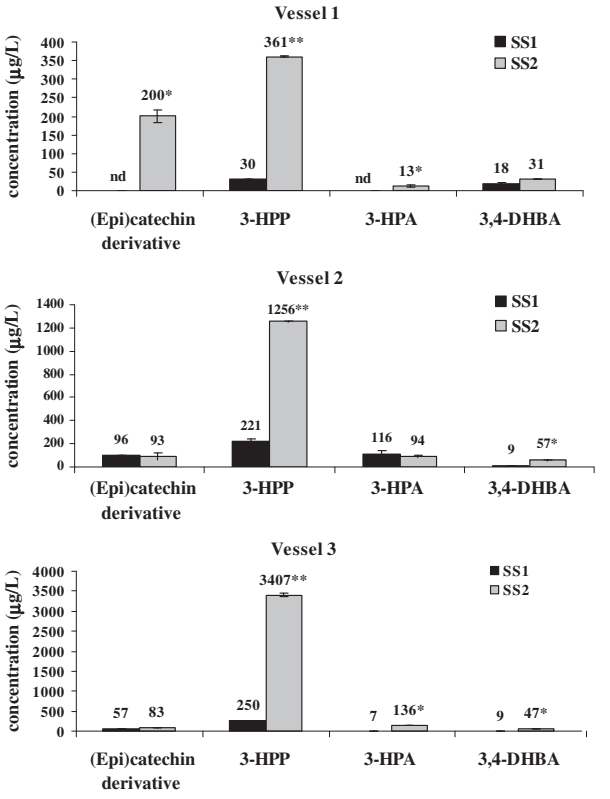

Figure 4. Concentration of cocoa polyphenols' metabolites in the three vessels of gut model fed with cocoa-insoluble material. Relatively to each metabolite: * indicates $p<0.05$ for SS2 versus SS1; ** indicates $p<0.01$ for SS2 versus SS1. Steady state 1 (SS1) and 2 (SS2) represent the stationary point reached by the gut model before and after the addition of the cocoa-insoluble material.

3,4-DHBA concentration ($p<0.05$) in vessel 2 and vessel 3, were found after feeding with the digestion-resistant cocoa fraction.

Four recent studies investigated procyanidins dimer and/or (epi)catechin metabolism by microflora [26, 49, 57, 58]. In three of them [26, 49, 57] human microflora and in one pig ceca content [58] were used in a single, stirred batch-culture, to follow the fermentation of pure compounds or a procyanidin dimer fraction (extracted from grape-seed) over a time interval ranging from 8 to 48 h.

Our data are in agreement with these previous studies that showed that phenolic acids are the major metabolites of cocoa polyphenols. Looking at specific metabolites, our finding of a high concentration of 3-HPP in all the vessels, as well as the absence of 5-phenyl-γ-valerolactone and of 5-(4′-hydroxyphenyl)-γ-valerolactone were in accordance with data reported by Stoupi et al. [57] for procyanidin B2 fermentation taking the samples after 48 h. According to Stoupi et al. [57] the longer fermentation time than in the work by Appeldoorn and co-workers [26] or that by Tzounis et al. [49] (48 h versus 24 or 8 h respectively) determined the consumption of γ-valerolactones and of 3,4-dihydroxyphenyl acetic acid and the increase in 3-HPP in the fermentation vessel. Phenyl-γ-valerolactones may form variously hydroxylated phenyl valeric acids and those in turn, by β-oxidation, may form 3-HPP. On the other hand, 3-HPA might be formed through α-oxidation of 3-HPP [57] and 3,4-DHBA was hypothesized to derive from rapid degradation of the intermediate 3,4-dihydroxyphenyl acetic acid.

The absence of valerolactones and of 3,4-dihydroxyphenyl acetic acid in this study might be explained in a similar manner. In the three-stage gut model, which was continuously fed with 1% w/v cocoa dietary fiber, an equilibrium condition between material fed and its degradation products is reached, determining an increase in the final metabolites and the absence of the intermediate ones. The differences to the previous studies especially regarding the type and relative amount of metabolites, as well as different hydroxylation patterns, might be due to different compositions of fermented matrix (cocoa dietary fiber in this study versus purified procyanidins extracts or pure compounds alone or in combination in the previous studies), to the different compositions of microbiota of to the respective donors or the different experimental intestinal models used.

However, the use of the gut model had some advantages compared to single batch cultures as it allowed us to distinguish the metabolism in three different vessels mimicking the three different regions of the colon. Data showed that the concentration of each metabolite was very different in the three vessels ranging from 13 up to 361 μg/L in the first vessel (representing the ascendant colon), from 94 up to 1256 μg/L in the second vessel (representing the transversal colon) and from 47 up to 3407 μg/L in the third vessel (representing the descendent colon). This is the first study showing this gradient of concentration for the production of flavonoid metabolites along the lower gut.

The significance of this study is limited by the fact that the gut model cannot reflect the human variability; therefore the physiological relevance of this finding should be further investigated also by means of human studies.

4 Concluding remarks

In recent years, there has been growing interest by food scientists in the physiological relevance of antioxidant material reaching the lower gut. Perez-Jimenez et al. [59] demonstrated that procyanidin content in food is largely underestimated and they suggested that the measurement of non-extractable polyphenols may be crucial in assessing reliable dietary intakes of polyphenols [10, 60]. This is of particular importance when the health benefits exerted by polyphenols bound to dietary fiber throughout the gastrointestinal tract are investigated.

(i) In this context the results of our study on WICF provided new insight about the potential physiological relevance of DIF in the gastrointestinal tract, showing that: insoluble polyphenols are able to exert antioxidant action through the whole gastrointestinal tract, despite being still bound to other macromolecules;
(ii) human digestive process solubilizes a significant part of the bound polyphenols and it increases their bioaccessibility;
(iii) WICF has prebiotic activity and it determines an increase in butyrate production. The association between fermentable polysaccharides and some flavonoids, such as the catechins, may be very effective in the modification of microflora;
(iv) the concentration of phenol metabolite is very different in the various tracts of the colon reaching a maximum value in the terminal tract.

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