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Beta-Glucan and Phenolic Compounds: Their Concentration and Behavior during in Vitro Gastrointestinal Digestion and Colonic Fermentation of Different Barley-Based Food Products

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Supporting Information

ABSTRACT: Among cereals, barley (*Hordeum vulgare* L.) is notable for its high content of bioactives such as β-glucan and phenolic compounds, but it is not used as widely in human nutrition as wheat. To compare the impact of food formulation and processing on barley bioactives, crackers, cookies, and fresh pasta were prepared combining wheat and barley flour. After quantification of β-glucan and PCs in the barley flour and barley-based products, their behavior during in vitro gastrointestinal digestion and colonic fermentation was studied. The β-glucan and PCs were not drastically affected by processing. The amount of bioaccessible compounds after gastrointestinal digestion was lower than the amount retained in the undigested fraction. After in vitro colonic fermentation, β-glucan was mainly metabolized to acetic and propionic acids and PCs to phenylpropionic and phenylacetic acids. Based on the results of the study, the daily ingestion of barley-based foods may contribute to the intake of beneficial bioactive compounds.

KEYWORDS: *barley (Hordeum vulgare L.), phenolic compounds, β-glucan, gastrointestinal digestion, colonic fermentation*

INTRODUCTION

Wheat is probably the most popular cereal, at least in many Western societies. Its use is widespread in the diet as the main ingredient of baked products (such as bread, cookies, cakes, crackers) and in a broad variety of other foods (e.g., fresh and dry pasta). However, while other types of cereals possess good nutritional properties, they are not used as widely for human nutrition.

Barley is normally linked with the elaboration of alcoholic beverages, especially beer but is not a common ingredient of other cereal-based foods, probably because of its limited technological and/or sensorial properties.[1] Nevertheless, it is possible to elaborate a wide variety of food products substituting a proportion of wheat flour with barley flour and thus offering the consumer similar products but with additional nutritional properties.[1,2]

Barley is rich in β-glucan, although the level depends greatly on the variety.[1] β-glucan belongs to the group of the soluble fiber and has an important physiological relevance since its intake has been associated with a lowering in plasmatic cholesterol,[2,3] an improvement in the glucemic index and insulin sensitivity,[4,5] and the production of beneficial short-chain fatty acids (SCFA).[6,7] In addition, barley is a rich source of phenolic compounds (PCs), such as phenolic acids and flavan-3-ols.[8] Therefore, the incorporation of barley into food formulas may contribute to enriching the final products with β-glucan and PCs that are negligible in wheat flour.[8,9] This could be an interesting proposal for opening new production lines for functional foods.

Major and mainly minor components present in cereal grains and flour can undergo modifications during processing or subsequent cooking before consumption.[1,8,10] In the same line, the bioavailability of bioactive food compounds in the body depends not only on their nature but also on the characteristics of the food matrix.[3,6,10,11]

Food processing has been shown to affect β-glucan of oat and barley containing foods by reducing the molecular weight[12−14] which in turn increases the solubility of β-glucan during in vitro digestion and may also change the metabolites profile during colonic fermentation.[14] β-glucan has been described reaching the colon in variable proportions, where it is catabolized to SCFA by colonic microbiota, mainly propionic, butyric, and acetic acids.[6] Increase of propionic and butyric acid levels have been linked to the health effects attributed to β-glucan such as reduction in plasma cholesterol levels and e modulation of altered thrombotic and inflammatory markers.[15,16]

On the other hand, it is well-known that gastrointestinal digestion impacts on the PCs present in food. Part of the PCs are released from the food matrix in the small intestine, and thus becoming susceptible to further absorption (bioaccessible fraction). Further, some phenolic compounds, such as the flavan-3-ols present in barley, have been reported to suffer losses

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due to the pH changes during gastrointestinal digestion. Results are, however, contradictory with some authors reporting no losses[17] while and others report losses of up to 100%.[18−20]

The nondigested PC fraction continues its transformation in the large intestine.[10,21,22] In the case of barley, the major part of the PCs are phenolic acids covalently bound to grain components, which limits their release during gastrointestinal digestion due to the lack of specific enzymes.[8,10,23] These bound PCs can be gradually released and metabolized by the colonic microbiota, so increasing their local bioaccesibility.[10,11,22,23] In this sense Anson et al.[24] conducted a colonic fermentation study of bread prepared with wheat bran rich in bound ferulic acid and observed the release of ferulic acid and the appearance of phenylpropionic and phenylacetic and benzoic acids derivatives as result of the microbial catabolic activity.

To understand the possible health effects associated with the intake of any bioactive compound, it is necessary to know the different events that occur along the human gut such as their bioaccessibility in the upper gastrointestinal tract and their possible transformations by colonic microbiota. Numerous authors have studied the phenolic content and antioxidant properties of barley grains, flour and barley-based products, but only one recent study has described the β-glucan and PC composition and their fate during digestion.[8] The aim of the present study includes (a) the description of β-glucan and the free and bound PC content in barley flour and in three different barley flour based-products (crackers, cookies, and fresh pasta), (b) the release from the food to the digesta (bioaccessible fraction) of β-glucan and PCs during an in vitro gastrointestinal digestion, and (c) the modification of the nonbioaccessible fraction by human microbiota performing an in vitro colonic fermentation.

MATERIALS AND METHODS

Chemicals and Reagents. Ultrapure water was obtained from a Milli-Q water purification system (Millipore Corp., Bedford, MA). Alpha-amylase, pepsin, pancreatin, bile salts, KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, (both HPLC-grade), and HCl (37%) were from Scharlau S.L. (Barcelona, Spain). $NaHCO_3$, KCl, $CaCl_2$, $MgCl_2 \cdot 6H_2O$, NaOH, $(NH_4)_2CO_3$, $FeSO_4 \cdot 7H_2O$, $MnSO_4 \cdot H_2O$, $ZnSO_4 \cdot 7H_2O$, $CoCl_2 \cdot 6H_2O$, $Mo(NH_4)_6O_{24} \cdot 4H_2O$, and $CuSO_4 \cdot 5H_2O$, NaCl, $Na_2SO_4 \cdot 10H_2O$ were purchased from Pancreac Quimica S.A. (Barcelona, Spain).

3-(3′,4′-Dihydroxyphenyl)propionic acid (also known aa dihydrocaffeic acid) was purchased from Fluka (Buchs, Switzerland). Ferulic acid, sinapic acid, 4-hydroxybenzoic acid, vanillic acid, catechin, 4-hydroxyphenylacetic acid, 3-(4′-hydroxyphenyl)propionic acid, acetic acid, propionic acid, and butyric acid were from Sigma-Aldrich (St. Louis, MO) while *p*-coumaric acid was from Extrasynthese (Genay, France).

Barley Grains and Flour. The barley (*Hordeum vulgare* L.) cultivar Oregon 14 waxy was selected for the elaboration of barley-based crackers, cookies and fresh pasta. The grain was kindly provided by Semillas Batlle, S.A. (Bell-lloc d'Urgell, Lleida, Catalonia, Spain). After collection and dehulling, the whole grain was ground in a Foss Cyclotec 1093TM (FOSS, Barcelona, Spain) mill equipped with a 0.5 mm screen. To avoid possible oxidation of the PCs, the flour was immediately used to elaborate the crackers, cookies, and fresh pasta.

Elaboration of Crackers, Cookies, and Fresh Pasta. The formulation of crackers, cookies, and fresh pasta was designed by our group after several tests. The amount of barley was selected in order to elaborate the three products without losing pasting properties. The amount and type of ingredients is detailed in Table 1, and all ingredients were assumed to be dry. Refined wheat flour was used for the elaboration of all products. All the products were prepared in the pilot plant of the Department of Food Technology of the University of Lleida (June 2016).

Table 1. Description of the Type and the Percentage of Ingredients (Dry Based) Used to Elaborate the Three Barley-Based Products: Crackers, Cookies, and Fresh Pasta

ingredient (%)	crackers	cookies	fresh pasta
barley flour	27	29	37
wheat flour	66	21	54
eggs		1.4	5
butter		20	
sugar		7	
black sugar		20	
salt	0.4	0.8	3
baking powder		0.8	
fresh yeast	0.6		
olive oil	6		
yolk			1

Elaboration of Crackers. All the ingredients were mixed, and the dough was left to stand for 30 min. After this time, squares (5 cm × 5 cm) were cut and left to stand for a further 30 min before the crackers were baked for 15 min at 200 °C in a convection oven (PE 46 SVR, Eurofred, Italy).

Elaboration of Cookies. Butter and sugar were manually homogenized in a bowl. After adding eggs and mixing for 5 min, bicarbonate (food grade) and wheat and barley flour were added, and all the ingredients were homogenized. The cookies (4 cm × 4 cm) were baked for 10 min at 200 °C in a convection oven (PE 46 SVR, Eurofred, Italy).

Elaboration of Fresh Pasta. Wheat and barley flour were kneaded with eggs and salt until homogenized. Strips (5 mm wide and 1.5 mm thickness) were cut with a pasta machine and left to stand for 10 min. After this time, the pasta was boiled in distilled water for 5 min.

A fraction of each product was freeze-dried and stored at −80 °C until the β-glucan and PC determination. The nonlyophilized fraction was immediately stored at −20 °C in sealed bags until the in vitro gastrointestinal digestion procedure.

In Vitro Gastrointestinal Digestion. The in vitro gastrointestinal digestion procedure involved oral, gastric, and intestinal steps. The method was adapted from the standardized static protocol proposed by Minekus et al.[25] Briefly, 5 g (wet base) of ground crackers, cookies, and fresh cooked pasta were weighed and mixed with simulated salivary fluid to start the oral digestion (pH 7 and 75 U/mL of α-amylase). In vitro oral digestion was performed for 5 min with continuous stirring in a water bath (37 °C) in the dark. Afterward, the simulated gastric solution and porcine pepsin (2000 U/mL) were added and the pH corrected to 3 with HCl. The samples were maintained at 37 °C for 1 h in an orbital shaker (200 rpm). For the duodenal digestion, samples obtained after the gastric step were mixed with simulated intestinal solution (pH 7, porcine bile salts 10 mmol/L, 100 U/mL pancreatin for crackers and fresh pasta digestion based on trypsin activity and 2000 U/mL for cookies digestion, based on lipase activity) and incubated at 37 °C for 2 h in an orbital shaker (200 rpm).

The samples obtained after gastric and intestinal digestion were centrifuged (10 min at 9000 rpm at 4 °C) to separate the bioaccessible fraction (supernatant) and the nonbioaccessible fraction (residue) (Figure 1). Both fractions were freeze-dried and stored at −80 °C for β-glucan and PC analysis. The intestinal residue was considered the portion of the food that reaches the colon and was subsequently fermented with human feces.

In Vitro Colonic Fermentation. Fecal samples were donated by three adults (28−36 years, BMI 18.5−24.9 kg/m^2) who declared no gastrointestinal alterations and reported no antibiotic use over the last 4 months before the collection day. The fresh feces was collected in anaerobic chambers containing anaerobic sacks (BD GasPack) to maintain anoxygenic conditions and used within 2 h after defecation.

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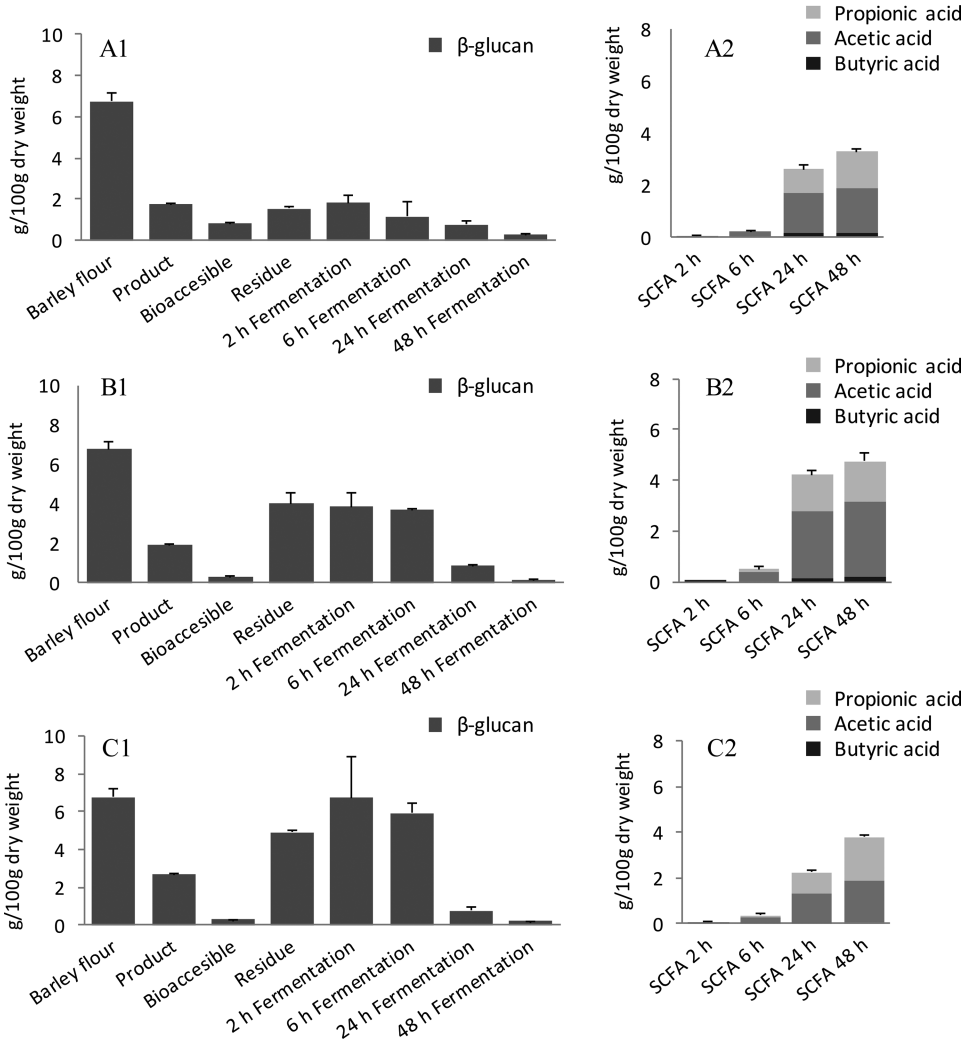

Figure 1. β-glucan content and its recovery after in vitro intestinal digestion and in vitro colonic fermentation (2 h, 6 h, 24 h, and 48 h) of cookies (A1), crackers (B1) and fresh pasta (C1). Generation of SCFA (acetic, propionic, and butyric acids) after 2 h, 6 h, 24 h, and 48 h of in vitro colonic fermentation of cookies (A2), crackers (B2), and fresh pasta (C2).

A fecal pool was used as microbial inoculum for the in vitro colonic fermentation. Fecal slurry (5% feces) was prepared by mixing the fecal pool with a prereduced carbonate-phosphate buffer prepared according to Mosele et al.[11] Fecal slurry (10 mL) was aliquoted in tubes containing 0.1 g of the freeze-dried intestinal residues of crackers, cookies, and fresh pasta. These samples were prepared in parallel with two controls: control 1, fecal slurry without the intestinal residue, and control 2, carbonate−phosphate buffer incubated with the intestinal residues.

The anaerobic chambers containing the tubes (samples and controls) were incubated in an orbital shaker (60 rpm) at 37 °C for 2, 6, 24, and 48 h. All incubations were performed in triplicate. Samples obtained from each time point were freeze-dried and stored at −80 °C until the colorimetric (β-glucan determination) and chromatographic analyses (PC and SCFA determination).

β-Glucan Determination. β-glucan determination was performed following the method described by Motilva et al.[26] Briefly, aliquots of the whole grain flour, barley-based products, in vitro digested samples, and fecal fermentation samples were mixed with 0.2 mL of aqueous ethanol (50% v/v), 4 mL of sodium phosphate buffer (20 mmol/L, pH 6.5), and 5 mL of water. The tubes were incubated at 100 °C for 3 min. After cooling at 50 °C, lichenase (0.2 mL, 10 U) was added and the tubes were incubated at 50 °C for 60 min. After incubation, 2 mL of sodium acetate buffer (200 mmol/L, pH 4.0) was added and the samples were filtered through Whatman no. 41 filters. A volume of 20 μL of each filtrate was transferred to a microplate and 20 μL of β-glucosidase (0.2 U) was added to each well. Blanks were prepared with 20 μL of each sample and 20 μL of sodium acetate buffer (50 mmol/L, pH 4.0). Samples were incubated at 45 °C for 10 min, and 210 μL of glucose oxidase/peroxidase (GOPOD) reagent was added and further incubated at 45 °C for 20 min. After cooling at 25 °C, the absorbance was read at 510 nm using a Multiscan GO spectrophotometer (Thermo Scientific, Vantaa, Finland). All the samples were quantified by reference to a calibration curve of D-glucose ranging from 0.05 mg/mL to 1 mg/mL in the well.

Phenolic Characterization by UPLC−MS/MS of Barley Flour, Barley-Based Products, and the Samples Obtained after in Vitro Gastrointestinal and Colonic Incubations. Aliquots of barley wholemeal, barley-based products, duodenal residue samples, and fecal fermentation samples were extracted with methanol/water/formic acid (79.5/19.5/1, v/v). The samples were sonicated for 30 s and centrifuged at 9000*g* for 10 min. The extraction process was repeated twice more, and the supernatants from the three extractions were pooled and filtered through 0.22 μm PVDF filter discs into amber glass vials and 2.5 μL was directly analyzed by liquid chromatography.

To extract the bound phenols, the residue obtained after the extraction of the free phenols was subjected to alkaline hydrolysis by adding 6 mL of 2 mol/L NaOH. The samples were left to stand for 12 h at room temperature for complete hydrolysis, sonicated for 1 min, and centrifuged at 9000*g* for 10 min. The supernatant was acidified with HCl 37% (w/w) to pH 2 and centrifuged, and the supernatant was mixed 1:1 with phosphoric acid, centrifuged (10 min at 9000*g*), and

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subjected to μSPE cleanup according to the method described by Serra et al.[27] A volume of 2.5 μL of the eluate was directly analyzed by liquid chromatography.

The phenolic analysis was performed by ultraperformance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) as the detector system (Waters, Milford, MA). The chromatographic column was an Acquity BEH C18 (100 mm × 2.1 mm i.d.) with a 1.7 μm particle size (Waters). The UPLC system was equipped with a binary pump system, and a gradient elution was used to separate the phenolic compounds. Eluent A was Milli-Q water/acetic acid (99.8:0.2, v/v), and eluent B was acetonitrile. The gradient was performed as follows: 0−5 min, 5−10% B; 5−10 min, 10−12.4% B; 10−18 min, 12.4−28% B; 18−23 min, 28−100% B; 23−25.5 min, 100% B; 25.5−27 min, 100−5% B; and 27−30 min, 5% B. For both methods, the flow-rate was 0.4 mL/min.

Tandem MS (MS/MS) analyses were carried out on a triple quadrupole detector (TQD) mass spectrometer (Waters) equipped with a Z-spray electrospray interface. The analyses were done in negative ion mode. The data were acquired with selected reaction monitoring mode (SRM). Two transitions were studied for each compound: the most abundant was used for quantification and the second one for confirmation purposes. Data acquisition was carried out with the MassLynx v 4.1 software.

Ferulic acid, sinapic acid, p-coumaric acid, catechin, dihydrocaffeic acid, 3-(4′-hydroxyphenyl)propionic acid, 4′-hydroxyphenylacetic acid, vanillic acid, and 4-hydroxybenzoic acid were quantified using their own calibration curves. Catechin-glucoside, catechin-diglucoside, gallocatechin, procyanidin B3, prodelphinidin B3 and B4, and procyanidin C2 were quantified as catechin. Diferulic and triferulic acids were quantified as ferulic acid.

Determination of SCFA in Fermentation Samples. Acetic, propionic, and butyric acids were determined in freeze-dried samples of colonic fermentation following the methodology described by Mosele et al.[21] Briefly, freeze-dried fecal fermentation samples (0.1 g) were mixed with 1 mL of acidified aqueous solution (1% phosphoric acid) containing 4-methyl valeric acid as the internal standard (IS). Samples were shaken for 15 min and centrifuged (10 min, 1800*g*, 4 °C). Supernatants were centrifuged (4 min, 8784*g*, 4 °C) and filtered (0.22 mm pore size filter) before chromatographic analysis.

The analysis of acetic, propionic, and butyric acid was performed by GC (Agilent 7890A series) using a capillary BP-21 column (30 m, 0.25 mm, 0.25 μm; SGE, Cromlab SL, Barcelona, Spain), coupled to a flame ionization detector (FID). The column temperature was programmed at 90 °C, rising by 15 °C/min until it reached 150 °C, then 5 °C/min to 170 °C, and then 20 °C/min to 240 °C, and maintained 3 min (total run time 14.5 min). Helium was the carrier gas (1 mL/min). Injection was carried out with a split injector (1:100) at 220 °C, detector temperature was 250 °C, and 1 μL of the solution was injected into the GC/FID system. Identification of the SCFAs was carried out according to the retention time of standard compounds, and their contents were determined with reference to the peak side of the IS. All samples were analyzed in triplicate.

RESULTS AND DISCUSSION

Many authors have been interested in the study of barley due to its high content of β-glucan, which is associated with beneficial physiological properties.[1−3,28] However, barley also contains other biomolecules, such as PCs, that should not be ignored since they also improve some health biomarkers.[29] Indeed, Behall et al.[2] and Gao et al.[29] suggested that the promising results observed after administration of barley-rich diets may also be due to the presence of PCs in barley.

Through a combination of sequential in vitro digestive steps (gastric, intestinal, and colonic), it is possible to estimate how β-glucan and PCs behave in different barley-based food products during digestion. The results presented in this work contribute with valuable information on how certain biomolecules could be transformed and metabolized during the digestion process to be bioaccessible to the body.

β-Glucan Content in Barley-Based Products and Transformations during in Vitro Gastrointestinal Digestion and Colonic Fermentation. Figure 1 describes graphically the initial amounts of β-glucan in the barley-based-products (g/100 g of dry weight), how they were released from the food matrix during the in vitro gastrointestinal digestion (bioaccessible fraction) and metabolized to SCFA during the in vitro colonic fermentation. The β-glucan content of the barley flour determined by the enzymatic hydrolysis was 6.75 g/100 g dry weight. Based on the dry weight, crackers, cookies and fresh pasta were elaborated with 27, 29, and 37% of barley flour, respectively (Table 1). Cooked fresh pasta was the barley-based product that contained the highest amount of β-glucan (2.67 g/100 g dry weight), followed by the crackers and cookies (1.90 and 1.77 g/100 g dry weight, respectively) (Figure 1). These values are close to the theoretically expected value based on the percentage of β-glucan added through the barley flour used in each of the three formulas which indicates that the processing did not degrade β-glucan in the barley-based products. Vasanthan et al.[30] and Fares et al.[9] observed, respectively, in pasta and bread that processing and heat treatment did not negatively affect the β-glucan content in the final products.

After in vitro intestinal digestion, β-glucan was more abundant in the residue (nonbioaccessible fraction) (Figure 1A1−C1) than in the supernatant. The amount of β-glucan detected in the residue (nonbioaccessible fraction) was even higher than that detected in the original product, especially in the crackers and fresh pasta with 4.00 and 4.89 g/100 g dry weight, respectively (Figure 1B1,C1). However, this apparent increase in β-glucan contents might be explained by a relative increase of this compound in the residue cause by a loss of material that was digested and passed to the supernatant. When calculating the portion of digested product assuming that the dry matter retained in the residue is not-digested product while the dry matter of the supernatant would correspond to digested and solubilized product, the β-glucan values match with the contents found in the products (detailed calculations can be found in Table S2 of the Supporting Information).

The fact that β-glucan levels were higher in the residue could be related with the solubility of β-glucan. At the pH values normally observed during intestinal digestion, β-glucan tends to form aggregates[28] decreasing its solubility. It was noted that the inclusion of β-glucan in dairy foods favored the depletion of LDL and total cholesterol in hypercholesterolemic adults[2,3] and improved glucose control and insulin sensitivity.[4,5] These beneficial effects have been linked to the capability of β-glucan to increase the viscosity of the intestinal content and thus retain food components and interfere with their enzymatic hydrolysis and/or absorption.[1,3,5] Although this physicochemical property seems to be coherent with explaining the biological function of β-glucans, the mode of action of β-glucan is still under discussion.[31]

A discrete metabolism of β-glucan was observed during the first 6 h of colonic fermentation when the nonbioaccessible fraction of the crackers, cookies, and fresh pasta was submitted to in vitro colonic fermentation (Figure 1A1−C1). After this time, the amount of β-glucan decreased abruptly to less than 0.30 g/100 g of dry weight after 48 h of colonic fermentation. As the β-glucan levels decreased during fermentation, a concomitant generation of SCFAs was observed (Figure 1A2−C2). They were detected at low amounts at initial fermentation times

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(0−6 h) but acetic and propionic acids increased considerable after 48 h, whereas butyric acid concentrations were considerably lower and its percentage did not exceed 6.5% of the total SCFs after 48 h of fermentation (Figure 1A2−C2). Acetic acid was the main SCFA detected after the colonic fermentation of the digestion residue of the crackers and cookies (2.96 and 1.69 g/100 g dry weight, respectively) while in the fresh pasta samples, propionic and acetic acids were produced in similar amounts at the end of the incubation period (48 h) (numeric data for SCF concentrations can be consulted in Table S1 of the Supporting Information). SCFAs are end products of fermentation of dietary fibers by the anaerobic intestinal microbiota, and data available in the literature sustain that the normal molar relation among acetic, propionic, and butyric acids is approximately 60:20:20.[16] Nevertheless, in the present study, the fermentation of barley-based products displaced this relation toward a major production of propionic acid. Some authors have published that food containing β-glucan promotes the production of propionic acid[6] whereas others have observed that β-glucan favors the generation of butyric acid.[16] Further, the SCFA profiles of the different products varied slightly among each other with rations between acetic, propionic, and butyric acid of 62:34:4, 51:43:6, and 48:52:0 for crackers, cookies, and fresh pasta, respectively, showing a trend toward a higher percentage of propionic acid in the following order: crackers < cookies < fresh pasta. Immerstrand et al.[16] analyzed the influence of molecular weight of β-glucan on SCFAs production and profile and found that the ratio of (propionic acid + butyric acid)/acetic acid increased with increasing molecular weight. Since different food processing treatments have been shown to affect the solubility, viscosity and molecular weight of β-glucan,[32,33] the higher percentage of propionic acid might indicate that more severe heat treatments such as baking might cause a degradation of high molecular β-glucan while milder conditions such as cooking preserves better the molecular structure of β-glucan. Propionic acid has been proposed to lower cholesterol levels and have beneficial effect on glucose and lipid metabolism but the mechanisms are not fully elucidated.[34,15] The acetate produced after fermentation of dietary fiber by intestinal microbiota is readily absorbed and transported to the liver where it can act as a substrate for acetyl-CoA formation, the precursor for endogenous cholesterol synthesis. Propionate has been suggested to act by impairing the acetate utilization, and thereby also cholesterol biosynthesis.[35−37]

In general, SCFAs are considered to be important for the energy metabolism, necessary to maintain the normal requirements for the biochemical routes for the synthesis and degradation of dietary compounds at the local level (colon) and in peripheral tissues and to maintain beneficial microbiota activity.[7] The enrichment of cereal-based food products with barley flour may, thus, be a good strategy to develop new food products with enhanced nutritional value.

Phenolic Compound (PC) Content in Barley-Based Products and Transformations during in Vitro Gastrointestinal Digestion and Colonic Fermentation. The free PC and bound PC contents of the flour, barley-based products and the supernatant obtained after gastric and intestinal digestion are listed in Tables 2−4. Regarding the amounts of PCs, and in accordance with other authors,[1,8−10] ferulic acid and flavan-3-ols (monomeric and dimeric forms) were the most abundant in barley flour and barely based-products. Ferulic acid was detected as the main component of the bound PC fraction, representing approximately 95% of these fraction (Table 2 and 4). Flavan-3-ols were the main components of the free PC fraction, with monomers (gallocatechin, catechin, and catechin-glucoside and -diglucoside) accounting for approximately 35%, dimers (procyanidin B3, prodelphinidin B3, and prodelphinidin B4) for 60% and trimers (procyanidin C2) for 1% of the free PCs.

Table 2. Content of Free and Bound Phenolic Compounds (PC) Detected in Barley Flour[a]

μmol/100 g dry weight	barley flour
Free Phenolics	
ferulic	2.72 ± 0.23
catechin	41.91 ± 2.35
catechin-glucoside	16.18 ± 0.39
catechin-diglucoside	2.90 ± 0.37
gallocatechin	4.43 ± 0.45
procyanidin B3	43.21 ± 1.27
prodelphinidin B3	53.21 ± 1.26
prodelphinidin B4	5.05 ± 0.21
procyanidin C2	2.16 ± 0.17
Total	**171.77 ± 10.22**
Bound Phenolics	
ferulic acid	444.06 ± 13.27
diferulic acid (decarboxylated)	3.78 ± 0.09
diferulic acid	9.60 ± 0.26
triferulic acid	0.80 ± 0.01
p-coumaric acid	12.12 ± 0.48
vanillic acid	2.54 ± 0.17
sinapic acid	2.54 ± 0.17
sinapoyl-hexose	8.61 ± 0.20
Total	**470.21 ± 13.33**

[a]Data are expressed as μmol/100 g dry weight (mean ± SD, n = 3).

Barley flour contained a total 171.77 μmol/100 g dry weight (100%) of free PCs among which prodelphinidin B3, procyanidin B3, and catechin were the main compounds (Table 2). The content of free PCs was similar in the crackers (59.3 μmol/100 g dry weight) and cookies (53.42 μmol/100 g dry weight) while fresh pasta (35.53 μmol/100 g dry weight) showed a lower value. The crackers and cookies had a similar phenolic profile when comparing to the barley flour used in their formulation, prodelphinidin B3 (31−32%), procyanidin B3 (27−29%), and catechin (27−33%) being the most important compounds in both products. On the other hand, procyanidin B3 (42%) was the major free PC in fresh pasta, followed by catechin (34%) and prodelphinidin B3 (14%). When taking into account that the crackers, cookies, and pasta were prepared with 27%, 29%, and 37% of barley flour, respectively, only the fresh pasta showed a decrease in the free PC content (35.53 μmol/100 g compared to the expected 63.55 μmol/100 g). Fresh cooked pasta was the product that underwent major modification in the amount and profile of the free PCs, probably due to leaching losses during the boiling process which was also observed by other authors.[9] Leaching not only resulted in a general loss of free PCs but also in a change in the PCs profile with considerable greater losses of prodelphinidin B3 compared to procyanidin B3 and catechin and may be related to the higher solubility of this compound in water due to the presence of additional hydroxyl groups in both B-rings of the dimer. Baking seems to affect the loss of free PCs to a lesser extent since the amounts and profiles of free PCs in the crackers and cookies (Table 3) were consistent with the amount of barley flour used (Table 2). On the other hand, bound PCs (concentration and

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Table 3. Content of Free Phenolic Compounds (PC) in Barley Flour-Based Products and in the Gastric and Intestinal Supernatant after in Vitro Digestion[a]

	cracker (27%)[b]			cookies (29%)[b]			fresh pasta (37%)[b]		
μmol/100 g dry weight	product	gastric	intestinal	product	gastric	intestinal	product	gastric	intestinal
ferulic	3.92 ± 0.12	1.39 ± 0.10	0.97 ± 0.11	1.70 ± 0.16	0.75 ± 0.08	0.49 ± 0.06	2.21 ± 0.21	0.50 ± 0.11	0.37 ± 0.01
catechin	15.99 ± 0.71	2.55 ± 0.38	0.95 ± 0.18	17.46 ± 2.79	n.d.	1.17 ± 0.16	12.08 ± 0.74	1.08 ± 0.13	n.d.
catechin-glucoside	n.d.	n.d.	n.d.	n.d.	n.d.	1.64 ± 0.14	n.d.	n.d.	n.d.
catechin-diglucoside	0.67 ± 0.36	0.34 ± 0.11	0.26 ± 0.04	1.01 ± 0.14	0.42 ± 0.05	0.36 ± 0.02	0.75 ± 0.13	0.24 ± 0.04	0.17 ± 0.00
gallocatechin	2.32 ± 0.22	1.19 ± 0.14	0.38 ± 0.08	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
procyanidin B3	16.16 ± 0.67	3.14 ± 0.43	0.46 ± 0.09	15.43 ± 1.37	3.03 ± 0.07	0.71 ± 0.11	14.96 ± 0.73	1.67 ± 0.14	0.51 ± 0.03
prodelphinidin B3	18.73 ± 2.33	0.97 ± 0.40	n.d.	16.62 ± 3.10	2.13 ± 0.54	n.d	5.15 ± 0.32	0.19 ± 0.02	n.d.
prodelphinidin B4	1.02 ± 0.13	n.d	n.d.	0.66 ± 0.13	n.d.	n.d.	n.d.	n.d.	n.d.
procyanidin C2	0.50 ± 0.01	0.12 ± 0.01	n.d.	0.54 ± 0.06	n.d.	n.d.	0.38 ± 0.06	n.d.	n.d
Total	**59.30 ± 3.97**	**9.69 ± 1.03**	**2.89 ± 0.34**	**53.42 ± 6.96**	**6.34 ± 0.47**	**4.37 ± 0.39**	**35.53 ± 1.97**	**3.54 ± 0.38**	**0.93 ± 0.12**

[a]Data are expressed as μmol/100 g dry weight (mean ± SD, $n = 3$). [b]Percentage of barley flour used to elaborate the barley-based products.

profile) were not negatively affected by either baking or boiling (Table 4), and concentrations were even higher than expected from the percentage of barley flour added. This is in line with Fares et al.[9] who observed a similar trend; the bound PCs in pasta were not affected by boiling and the concentration of ferulic acid increased after cooking.

To evaluate how PCs behave during the in vitro digestion process, we considered two fractions: (1) the bioaccessible fraction containing those PCs released during digestion (supernatant) and (2) the intestinal residue which was further incubated with human feces to elucidate the colonic metabolism of the nonbioaccessible PC fraction. After gastric digestion, the total free PCs recovered in the supernatant was 9.69 μmol, 6.34 μmol, and 3.54 μmol/100 g of dry weight for the crackers, cookies, and fresh pasta, respectively. These amounts correspond to 16.3%, 11.9%, and 10.0% of the initial content determined in the crackers, cookies, and fresh pasta submitted to digestion. (Table 3).

The recovery of free PC in the supernatants after intestinal in vitro digestion decreased further to 8.2%, 5.1%, and 2.6% in the cookies, crackers, and fresh pasta, respectively (Table 3). Besides the clear decrease in all free PCs of more than 90% of the initial amounts after in vitro digestion, it should also be noted that the dimer and trimer (procyanidin B3, prodelphinidin B3, prodelphinidin B4, and procyanidin C2) contents decrease more than that of the monomers which suggests that monomeric flavan-3-ols may be more stable under acid/alkaline conditions during in vitro digestion than their di- and trimeric forms.

The potential bioaccessibility of free PCs represented by those compounds that were recovered in the supernatants after gastric and intestinal digestions (Table 3) was low. The decrease of the free PCs may have different causes: The presence of β-glucans may have contributed to form a gel network that limits the solubilization of PCs during digestion, similarly to the ability to reduce the absorption of dietary cholesterol. Further, the low recovery of flavan-3-ols may be attributed to their ability to bind to proteins during the in vitro digestion.[38] An increasing degree of polymerization (DP) of proanthocyanidins also increases their ability to interact with other food components[39,40] which could explain why di- and trimeric flavan-3-ols were detected in lower amounts compared to the monomers (free PCs). However, the most probable explanation for the low concentration of flavan-3-ols detected in the intestinal supernatant is the instability of these compounds under GI digestion conditions. Similar losses were described in other studies on the stability of flavan-3-ol rich foods during in vitro digestion with reported losses of 64−100%[18−20,41] and were attributed to the degradation of flavan-3-ols at mild alkaline conditions during in vitro duodenal digestion. In the present study losses were detected already after gastric digestion. Other authors also observed losses during gastric digestion,[20] indicating that flavan-3-ols are prone to suffering degradation during their whole passage through the GI tract.

Although in all three barley-based products more than 90% of the initially present free PCs were lost and end concentrations ranged from 0.93 to 4.37, slight differences were observed between the different barley-based products, with the lowest values found in pasta and the highest in cookies indicating that processing and matrix have an impact on digestibility of free PCs. To what extent the differences in composition and processing between cookies, cracker, and fresh pasta may have influenced the differences observed is difficult to assess, and

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Table 4. Content of Bound Phenolic Compounds (PC) Detected in Barley Flour-Based Products and the Residues Collected after the in Vitro Gastrointestinal Digestion[a]

	cookie (27%)[b]		cracker (29%)[b]		fresh pasta (37%)[b]	
μmol/100 g dry weight	product	intestinal residue	product	intestinal residue	product	intestinal residue
ferulic acid	187.05 ± 13.93	339.50 ± 8.26	247.91 ± 14.56	446.47 ± 40.79	203.53 ± 20.33	336.81 ± 10.98
diferulic acid (decarboxylated)	1.07 ± 0.00	2.97 ± 0.10	0.76 ± 0.05	3.05 ± 0.17	0.74 ± 0.06	1.52 ± 0.18
diferulic acid	4.00 ± 0.45	9.61 ± 0.63	3.98 ± 0.29	7.56 ± 0.92	3.12 ± 0.15	3.06 ± 0.21
triferulic acid	0.20 ± 0.01	0.76 ± 0.05	0.16 ± 0.03	0.60 ± 0.02	0.10 ± 0.01	0.53 ± 0.02
p-coumaric acid	5.17 ± 0.36	10.67 ± 0.39	4.97 ± 0.06	11.68 ± 1.42	4.20 ± 0.36	13.91 ± 2.20
vanillic acid	0.95 ± 0.04	1.48 ± 0.16	1.32 ± 0.09	1.44 ± 0.17	0.70 ± 0.06	0.81 ± 0.11
sinapic acid	1.52 ± 0.20	2.32 ± 0.19	2.20 ± 0.33	1.50 ± 0.22	n.d.	n.d.
sinapoyl-hexose	2.52 ± 0.24	4.68 ± 0.43	3.56 ± 0.45	5.33 ± 0.82	2.91 ± 0.24	3.70 ± 0.48
Total	**196.15 ± 13.34**	**359.12 ± 9.36**	**258.66 ± 13.80**	**464.56 ± 42.83**	**211.19 ± 20.64**	**346.41 ± 11.08**

[a]Data are expressed as μmol/100 g dry weight (mean ± SD, *n* = 3). [b]Percentage of barley flour used to elaborate the barley-based products.

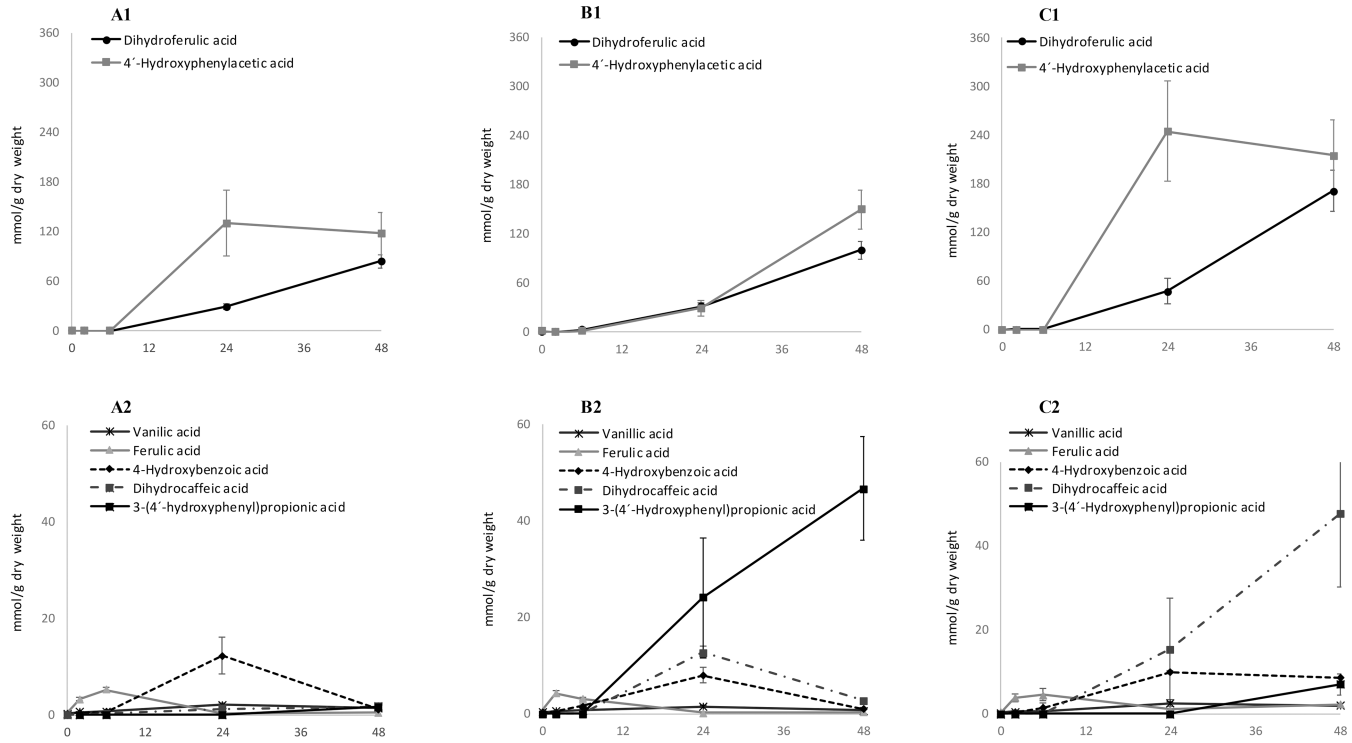

Figure 2

Figure 2. Main colonic phenol catabolites quantified in in vitro fermented cookies (A1), crackers (B1), and fresh pasta (C1); minor phenol catabolites quantified in fermented cookies (A2), crackers (B2), and fresh pasta (C2).

further research focused on the matrix and heat processing should be carried out.

Table 1 and Table 4 show the concentration of bound PCs in the barley flour, barley-based products, and intestinal residue (nonbioaccessible fraction), with ferulic acid being the main compound of this phenolic fraction. The results obtained after intestinal digestion showed that the concentration of some bound PCs (ferulic acid, *p*-coumaric acid, diferulic acid, and sinapoyl-hexose) in the residue from the crackers, cookies, and fresh pasta was higher than in the products before digestion. This apparent increase may be explained by the loss of material during the digestion process, similar to that observed for β-glucan contents. When taking into account the material loss during digestion, bound PCs actually decrease 87.6%, 70.0%, and 61.4%, respectively, in cookies, crackers, and fresh pasta (Table S3). The observed decrease corresponds mainly to a loss of ferulic acid, the main bound phenolic compound. As observed for free PCs, fresh pasta was the barley-based product with the highest losses and cookies the one with the lowest, which points to a better preservation of bound PCs in this latter product while food matrix of fresh pasta seems to preserve them to a lower degree. Differences in food composition and processing, which determine the matrix of the final product may influence the preservation of phenolic compounds during the digestion process. However, as already mentioned for free PCs, it is difficult to determine which of the mentioned factor is responsible for or has a major effect on the observed outcome.

The general picture after in vitro gastrointestinal digestion revealed that, for the three barley products, bound PCs (mainly ferulic acid) are present at considerably higher amounts than free PCs (flavan-3-ols) and after passing in vivo to the colon would form the main barley phenolics exposed to the degradation processes catalyzed by the colonic microbiota.

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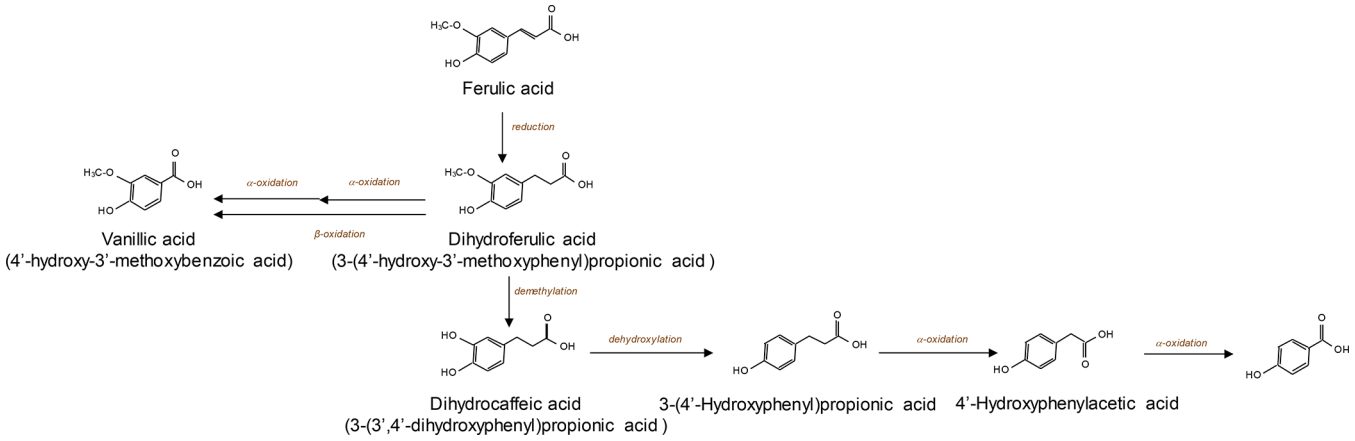

Figure 3. Proposed catabolic pathways for ferulic acid colonic degradation.

Results obtained from the colonic fermentation experiment showed an intense metabolism of PCs (Figure 3) in the three barley-based products. A total of seven catabolites were identified and quantified and include derivatives of cinnamic, phenylpropionic, phenylacetic and benzoic acids. Typical microbial flavan-3-ol catabolites, such as trihydroxy-, dihydroxy, and monohydroxyvalerolactones,[42] however, were not detected probably due to the low concentration of their precursors in the intestinal residue (<1 μmol/100 g dry weight residue; data not shown). Consequently, the microbial catabolites detected in this study are most probably arising from the hydrolysis of bound PCs, mainly ferulic acid, and their further degradation by the intestinal micriobiota. Seven phenolic metabolites were produced during the in vitro colonic fermentation of the intestinal residues, and fermentation profiles of these metabolites are shown in Figure 2. The proposed catabolic degradation pathways of ferulic acid are shown in Figure 3, and the involved degradation steps are in accordance with previous studies analyzing ferulic acid transformations in the colon.[42−45] The first catabolic event was the appearance of ferulic acid peaking at 2−6 h (Figure 2A2−C2) released from the food matrix, probably by the action of microbial cinnamoyl esterases. Ferulic acid, however, appeared as a transient peak and its concentration declined toward the later fermentation time-points (36−48 h). Dihydroferulic acid (3-(3′-methoxy-4′-hydroxyphenyl)-propionic acid) resulting from the reduction of the double bond of the ferulic acid structure increased and was one of the major catabolites detected at the end of the fermentation period (48 h) in all three barley-based products. Similarly, 4′-hydroxyphenylacetic acid, resulting from further demethylation, dehydroxylation, and α-oxidation of dihydroferulic acid, increased after 6 h of fermentation and was the main end catabolite in all three products (Figure 2). Other catabolites detected were dihydrocaffeic acid (3-(3′,4′-dihydroxyphenyl)-propionic acid) and 3-(4′-hydroxyphenyl)propionic acid, intermediate catabolites in the production of 4′-hydroxyphenyl-acetic acid (Figure 2). The 4-hydroxybenzoic acid resulted from an α-oxidation of 4′-hydroxyphenylacetic acid, while vanillic acid (3-methoxy-4-hydroxybenzoic acid) is the result of either one β-oxidation or two consecutive α-oxidations of dihydro-ferulic acid.

From the amounts detected, dihydroferulic acid and 4′-hydroxyphenylacetic acid can be classified as major catabolites while the remaining five compounds (vanillic acid, ferulic acid, dihydrocaffeic acid, 3-(4′-hydroxyphenyl)propionic acid, and 4-hydroxybenzoic acid) were classified as minor catabolites. With regard to the two major catabolites (Figure 2A1−C1) the amount of 4′-hydroxyphenylacetic acid increased during fermentation in all three products studied reaching a plateau at 24 h with, respectively, end concentrations of 117.70 ± 25.64 μmol, 149.39 ± 24.10 μmol, and 215.16 ± 43.22 μmol/100 g dry weight for the cookies, crackers, and fresh pasta. Dihydroferulic acid (3-(3′-methoxy-4′-hydroxyphenyl)propionic acid) was also detected in all three in vitro fermented barley-products in increasing amounts as the fermentation time progressed reaching concentrations of 83.97 ± 7.77, 99.77 ± 11.18, and 171.21 ± 25.90 μmol/100 g in the cookies, crackers, and fresh pasta, respectively. While crackers and cookies produced similar amounts of these two catabolites, higher levels were produced after fermentation of fresh pasta. This could be a consequence of the cooking process that might have caused disruption of the cell structure facilitating thus the liberation of bound PCs[46,47] and further degradation by the colonic microbiota.

Ferulic acid (3-(3′-methoxy-4′-hydroxyphenyl)cinnamic acid), vanillic acid (3-methoxy-4-hydroxybenzoic acid), and 4-hydroxybenzoic acid were detected in less abundant amounts in all products throughout the fermentation process (Figure 2A2−C2). Ferulic acid peaked at early time points (2−6 h), with lower amounts detected toward the end of the fermentation period. 4-Hydroxybenzoic acid showed a similar profile but peak concentrations were reached after 24 h of fermentation. Interestingly, in vitro colonic fermentation of fresh pasta led to the formation of considerable amounts of dihydrocaffeic acid (3-(3′,4′-dihydroxyphenyl)propionic acid), which was produced in increasing amounts and reached 47.62 μmol/100 g dry weight at 48 h while samples from the crackers produced considerable concentrations of 3-(4′-hydroxyphenyl)propionic acid during fermentation and reached an end concentration of 46.66 μmol/100 g. These latter two compounds were also detected in the other samples but at concentrations below 10 μmol/100 g of dry weight (Figure 2A2−C2). According to these results, the microbiota demonstrated an important role in the release and transformation of the bound PC retained in the undigested gastrointestinal residue to simple phenolic acids and in the generation of SCFA as a consequence of the β-glucan metabolism.

In conclusion, the use of barley flour as an ingredient in the formulation of crackers, cookies, and fresh pasta permitted the presence of bioactive compounds (β-glucan and PC) in the final product, because these fractions were not drastically affected by

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processing and cooking. After in vitro digestion of the three products, the results showed that the β-glucan and free PC were not completely released to the bioaccessible fraction. This can limit their bioavailability in the upper gastrointestinal tract as they are retained in the undigested residue. However, colonic catabolism plays an important role in generating SCFA and simple phenolic compounds from the β-glucan and PC which could exert a protective effect at the local level (colon) or be absorbed, thus improving the bioavailability of these fractions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b02240.

Digestion losses of intestinal residue during in vitro gastrointestinal digestion (Table S1), as well as short chain fatty acid (SCFA) (Table S2) and phenolic compounds (PCs) (Table S3) composition of in vitro fermented cookies, crackers, and fresh pasta (PDF)

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