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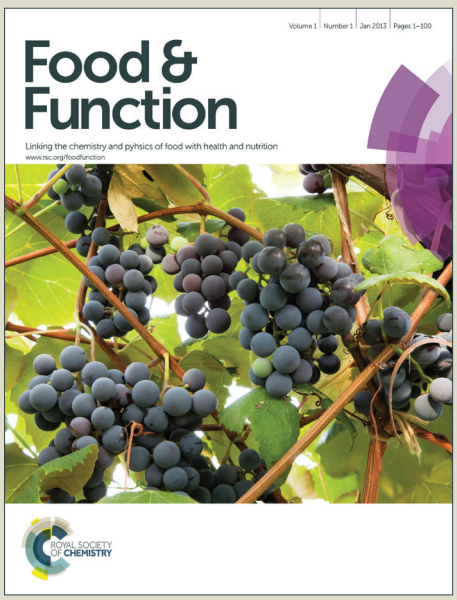

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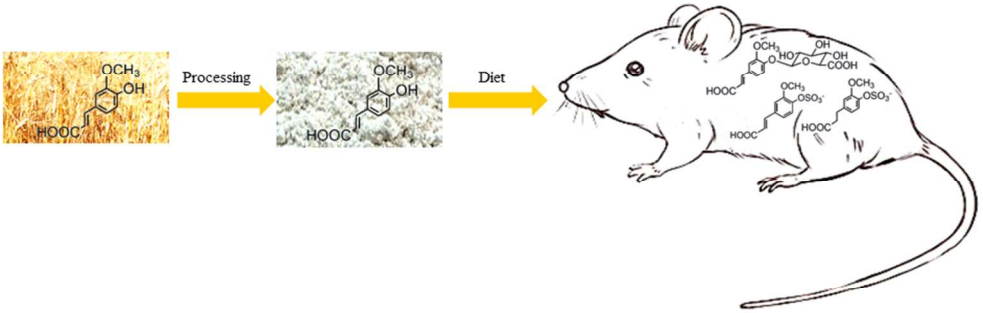

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Bioavailability and metabolism of hydroxycinnamates in rats fed durum wheat aleurone fractions.

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Running title: Bioavailability of aleurone hydroxycinnamates in rats.

Abbreviations: WA-A (wheat aleurone A), WA-B (wheat aleurone B), UHPLC-MS^n (ultra-high performance liquid chromatography coupled to multistage mass spectrometry), CT (control pellet), CID (collision induced dissociation), SIM (selective ion monitoring), SULTs (sulfotransferases), UGTs (uridine-5'-diphosphate glucuronosyltransferases), $[M-H]^-$ (negatively charged molecular ion), GI (gastrointestinal).

Abstract

The consumption of whole meal cereals has been associated with reduced risk of several chronic diseases, and the mechanisms behind these protective effects may be linked, besides dietary fiber and micronutrients, to an increased intake of hydroxycinnamates contained in the bran. Among bran fractions, aleurone usually contains the highest concentration of ferulic acid and diferulic acid esters linked to arabinoxylans representing the most relevant subclasses. The aim of the present study was to evaluate the absorption of hydroxycinnamates by measuring the urinary metabolite profiles of rats fed with the two different aleurone fractions (the inner part of the aleurone, named wheat aleurone A, WA-A, and the outer part, named wheat aleurone B, WA-B). An acute feeding experiment with two rat groups consuming equivalent amounts of total ferulic acid from the different aleurone fractions was carried out to evaluate ferulic acid bioavailability as affected by different sources. A chronic feeding experiment was also conducted with two rat groups consuming the same amount of the two different aleurone fractions, carried out to investigate the short-term metabolism and absorption of aleurone phenolics. The results revealed higher increases in the 24 h-excretion of phenolic metabolites/catabolites in aleurone fed rats compared to rats fed with a regular diet. Specifically, in the chronic feeding, ferulic acid was more bioavailable when WA-A was ingested. Based on previous observations, demonstrating various positive physiological responses to ferulic acid and ~~to~~ aleurone fractions characterized by higher phenolic bioavailability, our results ~~clearly~~ indicate that the WA-A fraction has potentially interesting nutritional characteristics, that might be used for the formulation of new wheat based products.

Keywords: durum wheat aleurone, ferulic acid, phenolic metabolites, urinary excretion, bioavailability.

1 Introduction

A growing body of evidence indicates that whole meal cereals may contribute to the prevention of chronic-degenerative diseases. Their consumption has been associated with reduced risk of cardiovascular disease,[1] type 2 diabetes,[2] and some types of cancer.[3,4] Although the mechanisms behind these potential protective effects remain to be elucidated, a higher intake of dietary fiber, micronutrients and phytochemicals may be implicated. These bioactive components are located mainly in the bran, which accounts for 10-14% of wheat kernel[5] and can be further separated into aleurone layer, hyaline layer, testa and the inner and outer pericarp.[6,7] Among these bran fractions, aleurone usually contains the highest concentration of phenolic compounds, mainly hydroxycinnamates, the most abundant of which are ferulic acid and diferulic acid esters linked to arabinoxylans.[6]

The esterification of phenolic compounds to fiber is known to limit their bioaccesibility,[8] and, as a consequence, adversely impact on bioavailability.[9] Several studies have investigated the bioavailability of whole grain-derived phenolics in animals and humans[10-12] and the issue of poor bioaccesibility has been approached by applying bioprocessing methods aimed at increasing the concentration of free ferulic acid.[13-14] However, most studies did not consider the complex set of transformations undergone by cereal phenolics within the body where, following ingestion, phenolic compounds are extensively metabolized throughout the gastrointestinal (GI) tract, with phase II metabolites and microbiota-derived catabolites largely exceeding the parent compounds in the circulatory system. Chemical modifications initially occur in the lumen of the small intestine with, when present, cleavage of sugar moieties and aglycones undergoing conjugation by phase II enzymes at the enterocyte level.[15] Further phase II metabolism may also occur in the liver and other organs.[16] However, a substantial amount of phenolics are not absorbed in the upper GI tract and reach the colon, where they are catabolised by the local microbiota resulting in further modified in their chemical structure.[17,18]

The resulting microbial catabolites can then undergo phase II metabolism locally and/or be absorbed and reach the liver where they can be subject to enzymatic metabolism before re-entering the systemic blood circulation and finally being excreted in urine. In a recent study, the effect of wheat

aleurone structure on hydroxycinnamate absorption and metabolism was investigated in a diet-induced obese mice model.[19] The investigators applied a non-targeted LC-MS metabolomic approach and were able to distinguish different aleurones on the base of the excreted metabolites. Enzymatic aleurone pre-processing led to an increased excretion of ferulic acid as sulphate and glycine conjugates, reflecting the increase in the free, more bioavailable ferulic acid in the animal diet. In contrast, mice fed with native or cryo-grounded aleurone showed a urinary phenolic profile richer in microbial catabolites, including 3-(hydroxyphenyl)propionic acids and hippuric acid.[19]

We have recently reported the *in vitro* high bioaccessibility of various nutritionally relevant components in a specially obtained wheat aleurone fraction,[20] suggesting the need for further studies to evaluate if this increased bioaccessibility results in an increased bioavailability *in vivo*. Thus, the aim of the present study was to evaluate the *in vivo* absorption of hydroxycinnamates by measuring the urinary phenolic profile of rats fed with the two different aleurone fractions.[20] The study was divided in two sub-studies, set up to account both for total ferulic acid and aleurone intake: i) an acute feeding experiment with two rat groups consuming equivalent amounts of total ferulic acid from the different aleurone fractions, designed to evaluate ferulic acid bioavailability as affected by the different source matrix, and ii) a chronic feeding experiment with two rat groups consuming the same amount of the two different aleurone fractions, performed to investigate the short-term metabolism and absorption of aleurone phenolic compounds.

2 Results

2.1 Phenolic composition of rat feeds

The phenolic content of each rat feed is summarized in Table 1, whereas the mass spectral characteristics of the identified phenolic compounds are presented in Supplemental Table 1. It can be seen that amounts ranging from 97 to 99% of the total quantified phenolics were recovered bound to the polysaccharidic fraction of the cell wall. Ferulic acid and related hydroxycinnamates were by far the major components, with a total content equal to 12.83, 26.90 and 8.54 mg/g for WA-A, WA-B and CT, respectively.

2.2 Identification of urinary phenolic compounds

Using LC-MS^n, 21 urinary phenolic constituents were identified, of which 15 have also been quantified in both feeding studies and in all three groups. Identification was obtained by comparison with reference compounds, when available. However, most glucuronide and sulphate phenolic conjugates, for which reference compounds were not available, were partially identified by comparing their MS^3 spectra with MS^2 spectra of corresponding aglycone standards, as reported in Supplemental Figure 1, 2, 3 and 4. The complete list of all the identified/partially identified urinary compounds, with their mass spectral characteristics, is presented in Table 2.

None of the parent phenolic compounds present in the three feeding pellets were recovered in rat urine, indicating substantial metabolic and/or catabolic transformations of all the ingested compounds. Among the identified phenolics, 12 compounds were recovered as sulphate and glucuronide conjugates, indicating interaction with sulphotransferases (SULTs) and uridine-5′-diphosphate glucuronosyltransferases (UGTs) in the rat GI tract or post-absorption in the liver. However, only four of the 12 metabolites were recovered as glucuronides, indicating that the aleurone-associated phenolics interact principally with rat SULTs rather than UGTs.

Ferulic acid was recovered both as sulphate or glucuronide conjugates, while dihydroferulic acid formed only a sulphate derivative. One peak was identified as a sulphate derivative of coumaric acid by comparing its MS^3 ion spectra with those obtained in MS^2 experiments with a coumaric acid standard.

Three peaks had a negatively charged molecular ion ($[M-H]^-$) at *m/z* 165. Two of three had a very similar fragmentation pattern, with the most abundant ion at *m/z* 121, suggesting that they were 3-(hydroxyphenyl)propionic acids, and one was identified as 3-(3′-hydroxyphenyl)propionic acid on the basis of co-chromatography with the authentic standard. The third peak with $[M-H]^-$ at *m/z* 165 had a weak MS^2 ion at *m/z* 121, and a major fragment at *m/z* 147, indicating a hydroxyphenylpropionic-like structure, considering also its elution close to the 3′-isomer.

Two peaks with a $[M-H]^-$ at *m/z* 261 and 357, yielded an *m/z* 181 MS^2 ion which upon MS^3 yielded an *m/z* 137 fragment and a minor ion at *m/z* 163. This indicates that the peak at *m/z* 261 was a 3-(hydroxyphenyl)propionic acid-*O*-sulphate, while *m/z* 357 peak is a 3-(hydroxyphenyl)propionic acid-*O*-glucuronide. The MS^3 spectra of both the glucuronide and the sulphate did not match with the MS^2 spectrum of dihydrocaffeic acid, which has hydroxyl groups at the 3′ and 4′ positions. Moreover, the sulphate form did not co-chromatograph with a dihydrocaffeic-3′-*O*-sulphate standard and yielded different MS^3 fragment ions. On the other hand, both conjugating forms of dihydroxyphenylpropionic acid shared their MS^3 spectra with MS^2 fragment ions of a 3-(3′,5′-dihydroxyphenyl)propionic acid standard. Therefore, based on these findings, the peaks at *m/z* 261 and 357 could tentatively identified as the sulphate and glucuronide conjugates of 3-(3′,5′-dihydroxyphenyl)propionic acid, identified previously as biomarkers of intake of whole grain-derived alkylresorcinols.[21] A sulphated derivative of a hydroxymethoxybenzoic acid was also detected in rat urine. It had a $[M-H]^-$ at *m/z* 247 which fragmented to form the corresponding aglycone (*m/z* 167) through the 80 *m/z* loss of a sulphate group. The MS^3 produced main ions at *m/z* 123 and 152 and a minor ion at m/z 108. This compound shared the MS^3 fragmentation pattern with those obtained in MS^2 with a 3-methoxy-4-hydroxybenzoic acid (vanillic acid) standard. This indicates that the compound is a vanillic acid-*O*-sulphate, previously detected in rat urine after consumption of different processed aleurones.[19] The probable vanillic acid metabolite is the 4-*O*-sulphate. Interestingly, in the study performed by Pekkinen et al.,[19] urinary excretion of vanillic acid-*O*-sulphate was higher in mice urine fed with free ferulic acid than in mice groups fed with processed aleurones. These findings led to the hypothesis that vanillic acid conjugate was mainly derived from ferulic acid via *β*-oxidation and sulphation in the liver. Finally, enterolactone and enterodiol, two mammalian lignans produced by the colon microbiota, were detected both in free and conjugated form, possibly derived from the lignans known to be located in the aleurone layer.[22]

2.3 Acute feeding (ferulic acid bioavailability)

An equal amount of total ferulic acid was consumed by both rat groups in the acute feeding experiment (approximately 35 mg, equivalent to 180 μmoles). This approach allowed a careful evaluation of the bioavailability of ferulic acid derived from the two aleurones.

The intake of both aleurones, compared with the control pellet, significantly increased urinary excretion of several phenolic compounds. Figure 1 and Supplemental Figure 5 show the cumulative urinary excretion of the most relevant phenolic metabolites. The 24 h-urinary excretion of hippuric acid and 3-(3′-hydroxyphenyl)propionic acid, as well sulphated and glucuronidated forms of ferulic acid, increased significantly after ingestion of both aleurone groups compared to rodents fed with the control pellet. Despite a slight increase in ferulic acid-*O*-sulphate, the difference between the two aleurone groups was not statistically significant. The WA-A fed rats showed a statistically significant increase in the excretion of several other phenolic metabolites when compared to WA-B fed or control animals. Coumaric acid-*O*-sulphate, 3-(phenyl)propionic acid-*O*-sulphate, vanillic acid-*O*-sulphate, 3-(hydroxyphenyl)propionic-*O*-sulphate, dihydroferulic-*O*-sulphate, besides enterolactone and its glucuronide were all excreted in significantly greater amounts by the WA-A fed animals. Hippuric acid was the most abundant phenolic to be excreted in urine in all three groups, and reached an overall 24 h-excretion up to 23.6 μmol in the WA-A group. The elevated urinary excretion of hippuric acid occurring after aleurone consumption is only partially associated the ingestion of the aleurones, because hippuric acid is derived from other degradation pathways, including those of aromatic amino acid catabolism.[16] Two dihydroxyphenylacetic acids, one of which was identified as the 3′,4′-isomer, increased only in the WA-B fed group. Its excretion peaked from 6 to 24 h, implying the colon as a site of formation. This phenolic acid had previously been identified as one of the main human microbial metabolite formed after *in vitro* fermentation of 8-*O*-4-dehydrodiferulic acid, one of the ferulate dimers present in wheat.[23] It must be noted that the 24 h-excretion of dihydroferulic acid-*O*-sulphate was significantly more abundant in the WA-A fed rats, and that the most of its urinary excretion occurred at last time points. Enterolactone was previously recovered in a colon fermentation model with WA, wherein its concentration increased

until 24 h fermentation,[24] in keeping with the present study where most urinary excretion of enterolactone and its glucuronide occurred 12 -24 h after WA intake.

Sulphated and glucuronidated ferulic acid metabolites were excreted mainly 2-3 h after ingestion, pointing out the fact that WA-derived ferulic acid are probably absorbed primarily in the small intestine. In contrast, as noted previously, dihydroferulic acid-*O*-sulphate was mainly excreted at later collection periods, especially for WA-A rodents, indicating the colon as its likely site of absorption and an effective release and reduction of bound ferulic acid, which went unabsorbed in the upper GI tract. However, the formation of the sulphate metabolite from diferulic acids in the colon is also a possibility, as demonstrated previously in an *in vitro* study with human fecal fermentations.[23]

As a general way to interpret figure 1, these cumulative curves could provide interesting information about the site of production of the excreted metabolites. For example, if the curve slope is steep during later time points, this means the metabolite has been produced later in time, indicating colonic origin (i.e. dihydroferulic acid and enterolactone). If, on the other hand the cumulative excretion curves are steeper in earlier collection time points, the metabolite should have been produced at intestinal or hepatic level (i.e. ferulic acid conjugates).

Focusing on total ferulic acid intake, an estimation of its bioavailability can be obtained by measuring the 24 h-excretion of ferulic acid-*O*-sulphate, ferulic acid-*O*-glucuronide and dihydroferulic acid-*O*-sulphate, the three metabolites likely to be derived from the ferulic acid skeleton, and comparing this figure with the actual intake of ferulic acid monomers and small oligomers. The complete excretion of these metabolites was equal to 1.25 µmol in WA-A fed rats, corresponding to 0.69 ± 0.24% (mean ± SD) of the total ferulic acid ingested with WA-A, and 0.88 µmol in WA-B rodents, accounting for the 0.49 ± 0.27% of total ferulic acid intake, but this difference did not reach statistical significance.

2.4 Chronic feeding (short term metabolism and absorption of phenolic compounds)

In chronic feeds, as was observed after acute supplementation, several phenolic metabolites were significantly higher amounts in 24 h-urine after WA-A and WA-B rats compared with the control pellet

fed animals (Figure 2 and Supplemental Figure 6). In this case, since WA-A and WA-B groups consumed a same amount of aleurone, their intake of phenolics was different. As shown in Table 1, the total ferulic acid content in WA-B was more than twice that of WA-A. In general, chronic administration of both aleurones led to an increase of excreted phenolic compounds. Most were excreted in proportion to the actual phenolic intake, resulting significantly higher levels in urine of WA-B fed rats. However, some compounds did not follow this trend, highlighting a higher bioavailability of WA-A over WA-B phenolics. This observations applies to ferulic acid-*O*-glucuronide, dihydroferulic acid-*O*-sulphate, vanillic acid-*O*-sulphate (Figure 2), and a 3-(hydroxyphenyl)propionic acid and a dihydroxyphenylacetic acid (Supplemental Fig. 6).

3 Discussion

The present study evaluated the modification of the urinary phenolic profile of rats treated with durum wheat-derived aleurone fractions, investigating specifically the bioavailability of ferulic acid.

As expected, in the acute feeding experiment, several urinary phenolic acids increased in rodents fed with both WAs compared with a control group. However, some were significantly more abundant in the 24 h urine of WA-A fed rodents compared to their WA-B counterparts. In particular, a total ferulic acid bioavailability of ~1% was obtained by monitoring the 24 h-urinary excretion of catabolites formed directly by modification of the ferulic acid side chain. This figure contrasts with data previously reported in the literature, where a urinary recovery of ferulic acid reaching a 4-5.5% was observed in rats fed with cereal-based meals.[9] In another study, 2.3% of ferulic acid was excreted in rat urine after consumption of wheat bran.[11] In a human intervention carried out with bioprocessed and control bread, both with a similar bran content, a 24 h-urinary recovery of ferulic acid equal to 10% and 4% for bioprocessed and control breads was reported, respectively.[14] In better agreement with what was observed in the current investigation is a human study performed with breads fortified with rye bran, where 24 h urinary ferulic acid excretion ranged from 0.4 to 1% of the ingested dose.[25] One of the reasons of these inconsistencies is, however, linked to the fact that these reported studies did not take into account the amount of diferulic

and triferulic acids present in cereal bran, whereas these molecules were fully characterised and accounted for, in the present investigation. Only Kern *et al.* who performed a human feeding study with high-bran breakfast cereals, have included *cis*- ferulic acid and diferulic acids in the estimate of total hydroxycinnamate consumption.[12] However, they considered only the intake of *trans*-ferulic acid when estimating a 3.1% 24 h-excretion in urine. If other ferulic acid-linked compounds had been included this figure would have been somewhat lower.

In the present study, since the WA-A and B supplements contained substantial amounts of ferulic acid oligomers (dimers and trimers) (Table 1), these compounds were included in the total ferulic acid intake. Andreasen *et al.* have reported that diferulic acids are absorbable in the upper GI tract of rats, appearing in plasma after 1 h of ingestion.[26] They also demonstrated that human and rat colonic microbiota express esterase activity towards three isomers of diferulic acids from model compounds and dietary cereal bran,[26] so providing a mechanism for the release of ferulic acid from dietary diferulates prior to absorption. It has also been reported that human fecal incubations hydrolyse 8-*O*-4-dehydrodiferulic acid result in the formation of products, including ferulic and dihydroferulic acid.[23] Based on these findings, diferulic and triferulic acids should be included in the overall amount of ferulate intake in studies on ferulic acid bioavailability.

The chronic feeding experiment confirmed the observations made in the acute study that with the exception of 3-(3'-hydroxyphenyl)propionic acid, the spectrum of excreted phenolic compounds was not in line with the differences in the total amount of phenolics consumed by the two groups of rats. Specifically, with a total daily ferulic acid intake of 25.7 mg and 53.8 mg with WA-A and WA-B, respectively, at least a doubling of the excretion of ferulic metabolites would be anticipated after the WA-B feeding. In practice this is not what occurred as the ratio between WA-B and WA-A excreted ferulic acid-*O*-sulphate and ferulic acid-*O*-glucuronide was only 1.4. When it comes to the main microbial metabolite of ferulic acid, dihydroferulic acid-*O*-sulphate, its urinary excretion was higher, but not significantly higher, in urine collected from the WA-A fed rats. These observations confirm what was

observed after the acute study, and point to the higher bioavailability of ferulic acid from WA-A, at least under the experimental conditions that were employed.

Among the possible explanations for this higher bioavailability, the different particle size of the two aleurone fractions could have played a role, as WA-A had particle size <100 μm, whereas WA-B ranged between 300−425 μm.[20] However, this would contrast with previous *in vitro* and *in vivo* studies with wheat aleurone, which did not report any difference in bioavailability between aleurone fractions with different particle sizes.[19,24] It must be noted though that the difference in particle sizes of the aleurones used in these two studies (191 vs 65 μm) was less pronounced than in the current investigation.

Finally, it must be pointed out that these results will have to be confirmed in humans, as many obvious differences occur between the two models, in particular considering the diversity of the respective colonic microbiota.

4 Experimental

4.1 Chemicals

Sodium hydroxide, citric acid, *trans*-ferulic acid, *trans*-isoferulic acid, sinapic acid, caffeic acid, 4-hydroxybenzoic, 2-, 3-, 4-coumaric acid, vanillic acid (3-methoxy-4-hydroxybenzoic acid), protocatechuic acid (3,4-dihydroxybenzoic acid), 3',4'-dihydroxyphenylacetic acid, 3-(3′-hydroxyphenyl)propionic acid, 3-(4'-hydroxyphenyl)propionic acid, 3-(3',4',-dihydroxyphenyl)propionic acid (aka dihydrocaffeic acid), 3-(3',5'-dihydroxyphenyl)propionic acid, 3-(3'-methoxy-4'-hydroxyphenyl)propionic acid (aka dihydroferulic acid), and hippuric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) as well enterolactone and enterodiol. Homovanillic acid (3'-methoxy-4'-hydroxyphenylacetic acid) was purchased from Extrasynthese (Genay Cedex, France). Ferulic acid-4′-*O*-sulphate disodium salt, isoferulic acid-3'-*O*-β-D-glucuronide, dihydrocaffeic acid-3'-*O*-sulphate sodium salt from Toronto Research Chemical, (Toronto, Canada).

4.2 Animals and Treatment

The rats were cared for in accordance with the European Council Directive 86/609/EEC on the care and use of laboratory animals (OJ L 358). The protocols were performed under license from the French Ministry of Agriculture (license No. A380727) and approved by the local animal ethics committee (license No. 113_ LBFA-FO-01). The rats were housed under conditions of constant temperature at 25°C, humidity 50%, and a standard light-dark cycle (12h/12h). Standard diet (A04, SAFE, France) and tap water were provided ad libitum.

4.3 Aleurone fractions

The aleurone fractions were provided by Barilla G. e R. F.lli, Parma (Italy) and obtained as follow: durum wheat (*Triticum turgidum* L. subsp. *durum*) kernels went through three debranning steps. The material obtained was micronized and separated by a turbo separator into the inner and outer parts of the aleurone layer. The inner part of the aleurone was named wheat aleurone A (WA-A) and the outer part was named wheat aleurone B (WA-B), as reported by Zaupa and colleagues.[20]

4.4 (Poly)phenol analysis

The phenolic components of the regular feeding pellet and of two aleurones types have been extracted and quantified by means UHPLC-MS^n. Briefly, 50 mg of sample were extracted with 6 mL of water. The sample was vortexed for 2 min, left under agitation for 30 min at room temperature and centrifuged at 9200 x g for 10 min. Finally, the supernatant was taken and stored at -20°C prior to UHPLC-MS^n analysis. For the extraction of the bound phenolic compounds, the residue was further digested with 1.5 mL of 2.0 mol L^{-1} sodium hydroxide at room temperature for 1 h. After alkaline hydrolysis the mixture was adjusted to pH 3.0 by adding 1.35 mL of 3.0 mol L^{-1} citric acid. The acidic aqueous extract was partitioned against 6 mL of ethyl acetate after which the ethyl acetate reduced to dryness *in vacuo* and the residue dissolved in methanol. Bound extract were kept at -20°C in the dark prior to UHPLC-MS^n analysis. The quantification of phenolic compounds was performed by calibration with commercial standards. Dimeric and trimeric ferulic acids with respective $[M-H]^-$ values of *m/z* 385 and 577, were analyzed in full scan MS^2 mode and quantified as ferulic acid equivalents. Further MS^3 experiment was

carried out to obtain a better identification of these compounds because of unavailability of equivalent commercial standards.

4.5 Acute feeding

Twelve male Wistar rats (Charles River Laboratories, body weight 375-400 g) were divided randomly into two groups. The rats were housed individually in metabolic cages for collection of urine and feces. All rats had been fasted overnight before being orally fed a single pellet of either WA-A or WA-B. Based on reported analyses, the dose of total ferulic acids (ferulic, diferulic and triferulic acids) was 35 mg/rat. Thus, 2.8 g of WA-A (ca. 35 mg of total ferulic acid) and 1.3 g of WA-B (ca. 35 mg of total ferulic acid) were fed. Each pellet was mixed with 2.5 mL of water and 12.5 mg of commercial white sugar.

Total urine samples were collected at 0 h, then over 30 min periods after the ingestion of the pellet during the first 7 h, and then at 340 min intervals over the next 17 h. The volume of urine was recorded and the samples were immediately frozen at -80°C prior to analysis.

4.6 Chronic feeding

Thirty male Wistar rats (Charles River Laboratories, baseline body weight 75-100 g) were housed individually and acclimated before being divided into three experimental groups (n = 10). The first group was provided with control pellets, while the second and third groups with a WA-A and WA-B pellets, respectively, a period for 12 weeks. Body weights and food intake were recorded weekly. The WA pellets were prepared by mixing 2 g of aleurone layer with 2.5 mL of water and 12.5 mg of commercial white sugar. The control pellets (CT) were prepared by mixing 1 g of A04 standard carbohydrate diet (A04, Safe, Augy, France) with 2.5 mL of water and 12.5 mg of commercial white sugar. The mass density of 1 g of A04 standard diet was the equivalent of 2 g of the WA layer flour. The WA and control pellets were prepared daily. The daily intake of total ferulic acid was 8.5 mg in the control group, while in the WA-A and B groups was equal to 25.7 mg and 53.8 mg, respectively.

At the end of experiment, blood was collected from fasted rats and plasma was stored at -80°C. Finally, the excretion of phenolic compounds and their metabolites was estimated in urine samples collected overnight during the final day before sacrifice.

4.7 UHPLC-MS^n analysis of urinary phenolic metabolites

Urine samples of rats were diluted with 0.1% aqueous formic acid and filtered through a 0.45 μm nylon filter before UHPLC-MS^n analysis, using an Accela UHPLC 1250 equipped with linear ion-trap-mass spectrometer (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-electrospray ionization probe (H-ESI-II; Thermo Fisher Scientific Inc.).

A preliminary analysis was carried out in full scan, data-dependent MS^3 scanning from *m/z* 100 to 800 to perform a preliminary investigation on main urinary metabolites. On the basis of the obtained information, the main urinary metabolites were monitored in MS^2 or MS^3 mode. The MS functioned in negative ionization mode, with capillary temperature at 275 °C, while the source at 250 °C. The sheath gas flow was 40 units, while auxiliary and sweep gases were set to 5 units. The source voltage was 3 kV. The capillary voltage and tube lens were -5 and -68 V, respectively. For UHPLC, mobile phase A was 0.1% formic acid in water and phase mobile B was acetonitrile containing 0.1% formic acid. Separations were performed with a Kinetex PFP column (50 × 2.1 mm), 2.6 μm particle size (Phenomenex, Torrance, CA, USA). The mobile phase, pumped at a flow rate of 0.2 mL/min, comprised a programme of 0-1 min - 2% B, 1-12 min - 2% to 35% of B. All metabolites were fragmented in MS^2 or MS^3 using a CID of 30, with the sole exception of enterodiol and enterolactone, for which a CID of 40 in MS^2 (for free forms) and in MS^3 (for conjugated forms) was used. Helium gas was used for CID. Free forms of phenolic acids were quantified in MS^2 mode, while phenolic acid conjugates were quantified in MS^3 mode, except for enterodiol and enterolactone metabolites, which were quantified using selective ion monitoring (SIM).

Where possible, phenolic acid catabolites were quantified using a calibration curve prepared with a reference compound. When such standards were not available, catabolites were quantified using a structurally related compound. Specifically, coumaric acid-*O*-sulphate, 3-(phenyl)propionic acid-*O*-

sulphate and 3-(hydroxyl)phenylpropionic acid-*O*-sulphate were quantified in 3-(4'-hydroxyphenyl)propionic acid-3'-*O*-sulphate (aka dihydrocaffeic acid-3'-*O*-sulphate) equivalents. Enterolactone-*O*-glucuronide was quantified in SIM using its aglycone equivalent. Vanillic acid-4-*O*-sulphate and dihydroferulic-4′-*O*-sulphate were quantified using ferulic acid-4'-*O*-sulphate. Ferulic acid-4'-*O*-glucuronide was quantified in isoferulic acid-3′-*O*-glucuronide equivalents.

4.8 Statistical analysis

The phenolic acid content of the diets is reported as mean ± SD, while urinary excretion was reported as mean ± SEM. Urinary excretion of phenolic metabolites distributions were tested for normality and compared by ANOVA using LSD as a post hoc test. The bioavailability of ferulic acid in WA-A and WA-B groups was expressed as mean % values ± SD, and compared between the two groups using a *t* test for independent samples. Statistical analyses were performed with SPSS Version 20.0 (SPSS Inc., Chicago, IL, USA).

5 Conclusions

In summary, this study assessed the urinary phenolic profile of rats in two feeding experiments with different durum wheat aleurone fractions and revealed increases in the 24 h-excretion of phenolic metabolites compared to rats fed with a regular diet. More specifically, only marginally in the acute study, but consistently after the chronic feeding, ferulic acid, the main phenolic acid in cereals, was more bioavailable when ingested in the form of WA-A, which represents the inner part of the aleurone layer and has a characteristic <100 μm particle size. These results confirm what had already been shown *in vitro* in a previous study by our group.[20] Despite only a few works have demonstrated *in vivo* beneficial effects of ferulic acid, and only in rodents,[27-29] but based on the observations of Pekkinen and collegaues[19], reporting various positive physiological responses to aleurone fractions characterized by higher phenolic bioavailability, our results suggest that the WA-A fraction has potentially interesting nutritional characteristics, that might be used for the formulation of new wheat based products.

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Table 1. Phenolic acid content in the wheat aleurones (WA-A and WA-B) and control pellet (CT).

	WA-A		WA-B		CT	
	Free	Bound	Free	Bound	Free	Bound
4-Hydroxybenzoic acid	0.06 ± 0.01	0.03 ± 0.00	0.12 ± 0.02	nd	nd	nd
4'-Coumaric acid	0.03 ± 0.00	0.11 ± 0.01	0.29 ± 0.02	0.70 ± 0.07	0.07 ± 0.01	1.15 ± 0.06
Caffeic acid	nd	nq	nd	0.04 ± 0.00	nd	0.07 ± 0.00
trans-Ferulic acid	0.30 ± 0.05	5.38 ± 0.25	0.31 ± 0.06	8.95 ± 0.63	0.06 ± 0.01	3.33 ± 0.34
Ferulic acid isomer	nd	0.79 ± 0.01	nd	2.86 ± 0.44	nd	1.17 ± 0.25
Sinapic acid	0.04 ± 0.02	0.24 ± 0.00	nd	0.17 ± 0.02	nd	0.11 ± 0.03
Sinapic acid isomer	nd	nd	nd	0.04 ± 0.01	nd	0.04 ± 0.00
Dimeric ferulic acids	nd	5.91 ± 0.51	nd	13.50 ± 2.23	nd	3.66 ± 0.23
Trimeric ferulic acids	nd	0.44 ± 0.05	nd	1.26 ± 0.00	nd	0.33 ± 0.03
Total ferulic acid*	12.83 ± 0.77		26.90 ± 3.86		8.54 ± 0.25	

Data expressed as mg/g ± SD (n = 3). nd: not detected; nq: not quantifiable; * free + bound compounds

Table 2. UHPLC-MS^n identification of urinary phenolic metabolites.

RT	Compound	$[M-H]^-$ (*m/z*)	MS^2 ions (*m/z*)	MS^3 ions (*m/z*)
8.55	3′-Coumaric acid	163	119	
6.76	Hydroxyphenylpropionic acid-like	165	147 (100), 121 (2)	
7.59	3-(3′-Hydroxyphenyl)propionic acid	165	121 (100), 119 (2), 147 (1)	
8.20	3-(Hydroxyphenyl)propionic acid	165	121 (100), 147 (11)	
4.26	Dihydroxyphenylacetic acid	167	123 (100), 149 (10)	
4.82	3′,4′-Dihydroxyphenylacetic acid	167	123 (100), 95 (5)	
5.36	Hippuric acid	178	134	
12.80	Enterolactone	297	253 (100), 189 (14), 121 (9), 251 (8), 165 (6)	
10.80	Enterodiol	301	253 (100), 283 (23), 271 (16), 241 (14), 251 (3)	
7.88	Coumaric acid-*O*-sulphate	243		243→163: 119
7.08	3-(Phenyl)propionic acid-*O*-sulphate	245		245→165: 121 (100), 119 (2)
6.51	Vanillic acid-4-*O*-sulphate	247		247→167: 123 (100), 152 (95), 108 (12)
5.96	3-(Hydroxyphenyl)propionic acid-*O*-sulphate	261		261→181: 137 (100), 163 (2)
8.03	Ferulic acid-4′-*O*-sulphate	273		273→193: 149 (100), 178 (68), 134 (9)
7.07	Dihydroferulic acid-4′-*O*-sulphate	275		275→195: 136 (100), 151 (99), 177 (39), 123 (17), 149 (7), 180 (3), 119 (3), 59 (3)
3.76	3-(Hydroxyphenyl)propionic acid-*O*-glucuronide	357		357→181: 137 (100), 163 (8)
6.41	Ferulic acid-4'-*O*-glucuronide	369		369→193: 149 (100), 178 (43), 134 (7)
11.40	Enterolactone-*O*-sulphate	377		377→297: 253 (100), 189 (25), 165 (12), 121 (9), 251 (7)
9.70	Enterodiol-*O*-sulphate	381		381→301: 253 (100), 283 (23), 271 (16), 241 (14), 251 (3)
10.30	Enterolactone-*O*-glucuronide	473		473→297: 253 (100), 189 (21), 121 (8), 251 (8), 165 (7)
8.97	Enterodiol-*O*-glucuronide	477		477→301: 253 (100), 283 (36), 241 (15), 175 (12), 271 (11)

The relative abundance for each ion is reported in brackets.

Figure legends

Figure 1. Cumulative urinary excretion curves of most phenolic metabolites during the acute feeding. CT: control group. WA-A: wheat aleurone A group. WA-B: wheat aleurone B group. Each curve point is expressed as mean values (n=4) of µmol excreted. A different letter indicates significantly different cumulative excretions ($p<0.05$).

Figure 2. Excretion of most phenolic metabolites in the 24 h urine, in the chronic feeding experiment. CT: control group. WA-A: wheat aleurone A group. WA-B: wheat aleurone B group. Data are expressed as mean values (µmol ± SEM, with n=6 for each group). A different letter indicates significantly different cumulative excretions ($p<0.05$).

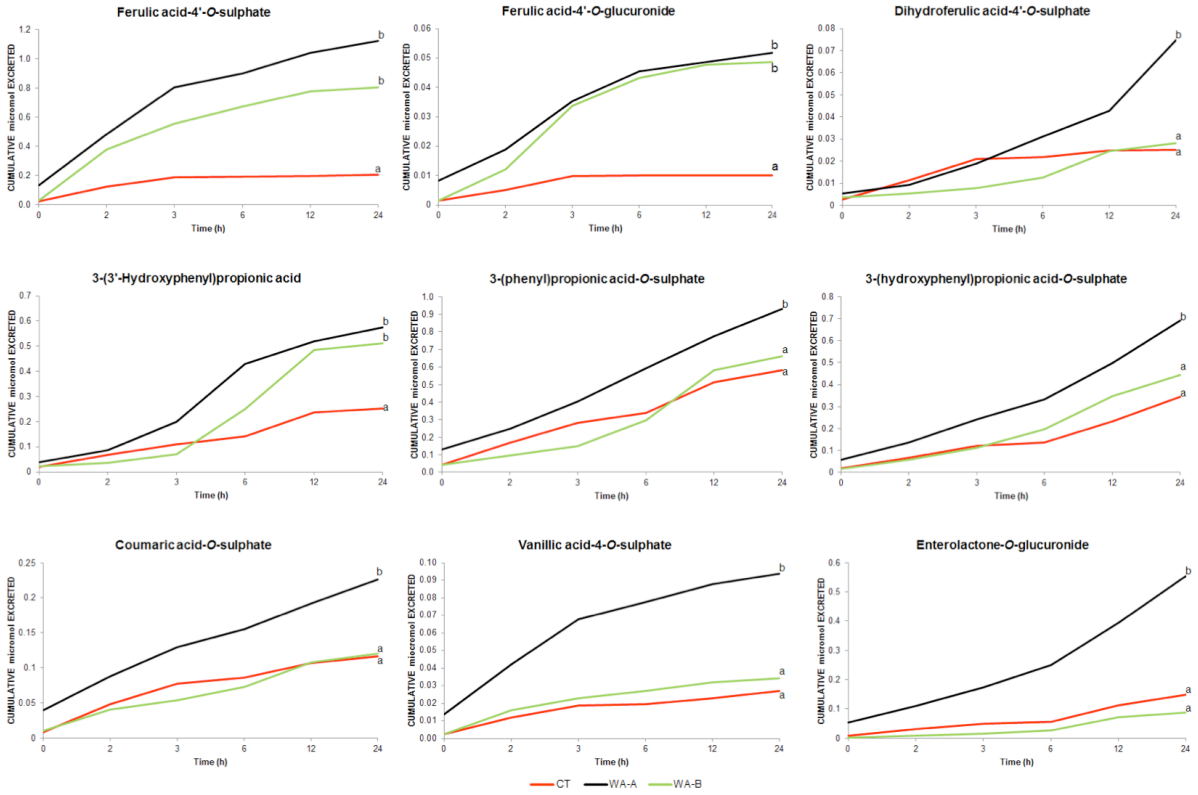

Figure 1

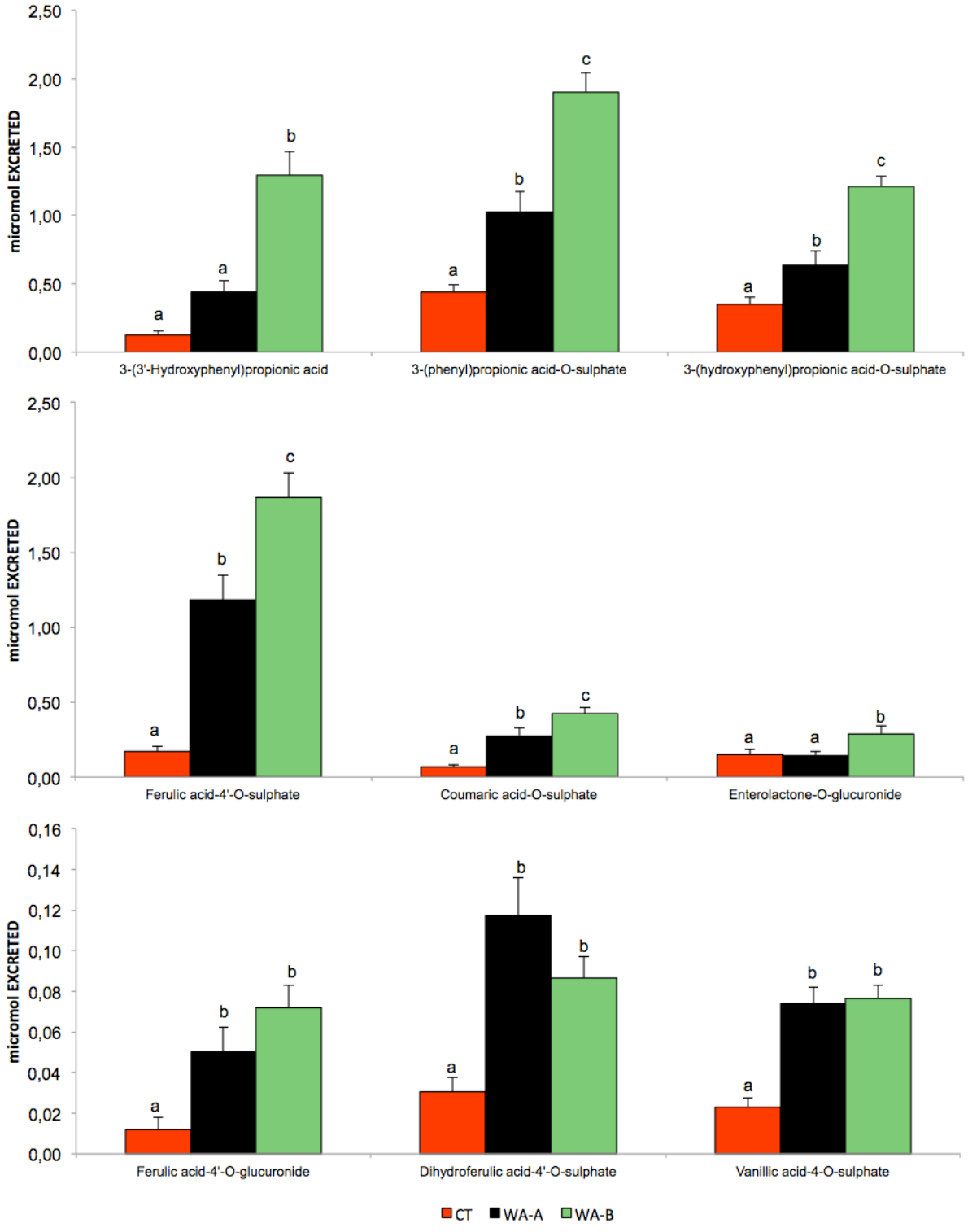

Figure 2