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ABSTRACT

Scope: This work aimed at investigating absorption, metabolism and bioavailability of phenolic compounds after consumption of wholegrain bread or bread enriched with an aleurone fraction.

Methods and results: Two commercially available breads were consumed by 15 participants on three occasions and matched for either the amount of ferulic acid in the bread or the amount of bread consumed. Urine was collected for 48 h from all the volunteers for phenolic metabolite quantification. Blood samples were collected for 24 h following bread consumption in 5 participants. A total of 12 and 4 phenolic metabolites were quantified in urine and plasma samples, respectively. Metabolites were sulfate and glucuronic acid conjugates of phenolic acids, and high concentrations of ferulic acid-4'-*O*-sulfate, dihydroferulic acid-4'-*O*-sulfate and dihydroferulic acid-*O*-glucuronide were observed. The bioavailability of ferulic acid was significantly higher from the aleurone-enriched bread when all ferulic acid metabolites were accounted for.

Conclusions: The study shows that low amounts of aleurone-enriched bread resulted in equivalent plasma levels of ferulic acid as wholegrain bread. This could suggest that, if the absorbed phenolic metabolites after wholegrain product intake exert health benefits, equal

levels could be reached through the consumption of lower doses of refined products enriched in aleurone fraction.

The study shows that low amounts of bread enriched in aleurone, the metabolically active innermost part of the bran, resulted in equivalent plasma levels of ferulic acid as wholegrain bread. This could suggest that, if the absorbed phenolic metabolites after wholegrain product intake exert health benefits, equal levels could be reached through the consumption of lower doses of refined products enriched in aleurone fraction.

1 INTRODUCTION

Increasing epidemiological, mainly observational, evidence suggests that wholegrain cereal consumption is linked to a reduction in the risk of chronic and degenerative diseases such as type 2 diabetes, cardiovascular disease, obesity, and cancer [1-4]. Although the mechanisms proposed for potential preventative effects have not been elucidated and remain speculative, the amounts of dietary fiber, vitamins, minerals and phytochemicals in wholegrain cereal appear to have key roles [5]. Inflammation might mediate the process, as suggested by the effect of wholegrain cereals on inflammatory markers such as C-reactive protein (CRP), TNF-α, plasminogen activator inhibitor-1 and interleukin (IL)-6 [6-9]. Despite the evidence and the high levels of bioactive nutrients and non-nutrients, wholemeal cereal consumption remains lower than recommended in guidelines. In the United States, the consistent promotion of wholegrain products led to a 23.4% rise in consumption from 2008 to 2010. However, the present average intake of 14 g/day is still considerably lower than the recommended intake of at least 48 g/day [10].

Whole-wheat is the main wholegrain cereal consumed in Western countries and represents an important source of dietary fiber [11]; minerals such as potassium, phosphorus, magnesium; vitamins, mainly niacin and folate; and phenolic compounds [12]. Phenolic

compounds in wheat bran include primarily hydroxycinnamic acids among which ferulic acid is the most abundant representing the 70 to 90%, caffeic acid, *p*-coumaric acid, sinapic acid, and benzoic acid derivatives, including protocatechuic acid, *p*-hydroxybenzoic acid, salicylic acid, vanillic acid; and syringic acid [12, 13]. The whole-wheat kernel is usually processed to obtain the bran fraction which contains 45–50% of the aleurone layer. The aleurone layer is a metabolically active monolayer that is the innermost part of the bran and represents 5–8% of the wheat kernel [12, 14]. The aleurone layer is usually removed during milling for its firm attachment to the external integuments [15]. However, the aleurone has a high content of fiber, protein, minerals, phytates, B vitamins (niacin, riboflavin and folate), lipidic compounds, plant sterols, and phenolic compounds [12]. Moreover, being protected from the environment, its content of pollutants such as pesticide residues, aflatoxins and heavy metals is reduced compared to the pericarp [16]. Due to its high content in nutrients and low level of contaminants, the development of new techniques to separate the aleurone from other bran layers has been actively sought by the food industry [16].

Most phenolic acids exist in the aleurone layer and in the wholegrain in three forms; soluble free acids; soluble conjugated moieties esterified to sugars or other low molecular weight compounds; and insoluble bound moieties that are esterified to the fiber fraction, mainly arabinoxylans. Ferulic acid, in particular, occurs partly in the free glycosylated form, but principally (~92%) covalently esterified with plant cell wall polysaccharides by *O*-5 position linkage [11, 17]. Moreover, ferulic acid undergoes dimerization *in planta* to form oligomers, namely dihydrodiferulic acids (ferulic acid dimers) and ferulic trimers [18, 19]. The linkage of hydroxycinnamates with the fiber fraction reduces the bioaccessibility [13] and bioavailability [20] of the phenolic fraction. Although, recently proposed technological

bioprocessing demonstrated the capacity to improve ferulic bioaccessibility [13, 21], it is necessary to consider the complex set of transformations that cereal phenolics are subjected to within the human body following ingestion.

Several studies have demonstrated that phenolic compounds are extensively metabolized and heavily modified after ingestion. After polyphenol-rich food intake, circulating phase-II and microbiota-derived catabolites are predominant, whereas native compounds rarely appear in circulation. Minor chemical modifications in the small intestine include the formation of aglycones that undergo conjugation by phase II enzymes at the enterocyte, in the liver and at the tissue level after absorption [22, 23]. In contrast, a substantial amount of unmodified native compounds reach the colon where they are subjected to microbial catabolism that results in structurally modified catabolites (23). These catabolites are then absorbed through the portal circulation and undergo phase II metabolism [24]. Generally, the phenolic-fiber linkage in wholegrain products prevents almost all the phenolics from upper intestine absorption. The vast majority of these molecules reach the colon where the gut microbiota β-glucosidase and esterase activity permits a slow and continuous release of bound phenolics and ferulic acid [25]. The continuous release causes prolonged metabolite circulation in the bloodstream, which could explain the health benefits correlated with sustained wholegrain consumption [11].

Building on previous *in vitro* [26, 27] and animal studies [28] performed by our group the aim of this investigation was to assess the human absorption, metabolism and bioavailability of phenolic compounds following the consumption of whole-wheat. We also aim to measure and compare the concentration of metabolites in urine and plasma following the consumption of wholegrain bread or bread enriched with aleurone fraction.

2 MATERIALS AND METHODS

2.1 Participants

Fifteen participants were recruited to take part in the study. Selected participants were 8 males and 7 females, 26 ± 4 years old, 62.4 ± 13.5 kg, 1.7 ± 0.1 m and 21 ± 3 kg/m^2 (data expressed as mean ± SD). Exclusion criteria included: not being pregnant or nursing; not being diagnosed with gluten intolerance or metabolic disorders such as hypertension, dyslipidemia, glucose intolerance or diabetes; non users of vitamin/mineral/micronutrient supplementation, and not taking any medication/drugs or antibiotic therapy. All participants were informed about the purpose of the study and provided written informed consent before their inclusion in the trial. Relevant principles of *Good Clinical Practice* were followed throughout the study. The study was approved by the Ethics Committee for Parma Hospital and University and registered on ClinicalTrials.gov, identification number: NCT02353234. A flow diagram of the progress through the phases of the trial is reported in supporting information figure 1.

2.2 Study design

In a single-blind, randomized, crossover study design two commercially available breads (Barilla S.P.A.) were consumed by each participant on three separate occasions. The phenolic composition and concentrations of the two breads were first confirmed [27] prior to meal portion calculation. The nutritional values and the ferulic acid content per portion are reported in Table 1.

Each participant was tested under three acute treatments that differed for the amount of ferulic acid or the bread quantity consumed. Each acute feeding experiment was separated by a minimum 1-week washout period. The bread products consumed consisted of: WGB - 94 g of wholegrain bread containing approximately 87 mg of total ferulic acid; AB-94 - 94 g of a commercial wheat bread enriched in aleurone fraction (6% w/w of the inner part of aleurone [26]) containing approximately 43 mg of total ferulic acid, which matches WGB for total bread consumed; AB-190 - 190 g of a commercial bread enriched in aleurone fraction (6% w/w of the inner part of aleurone [26]), containing approximately 87 mg of total ferulic acid, which matches WGB for the total amount of ingested ferulic acid.

Participants were asked to consume a polyphenol free diet two days prior to and two days after laboratory visits. A list of permitted and forbidden foods was supplied to facilitate participant adherence to the dietary restrictions. Additionally, a food diary was provided to record all meals and to check for compliance to the recommended diet. During the laboratory visits participants collected their urine following fasting (t_0), and after product consumption at: 0-3, 3-6, 6-10, 10-14, 14-24, 24-28, 28-34 and 34-48 h. The urine volumes were recorded and two aliquots of 2 mL each were stored at −80 °C until analysis. Five randomly selected participants provided 3 mL blood samples taken at fasting (t_0) and at 30 min, 1 h, 2 h, 4 h, 7 h, and 24 h following bread consumption. Blood was collected in K_2EDTA tubes, centrifuged for 15 min at 4,000 rpm and ≈ 1.2 mL of plasma was separated and stored at −80 °C until analysis. The study design is shown in Fig. 1.

2.3 Chemicals

All chemicals and solvents were of analytical grade. All solvents and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Vanillic acid (3-methoxy-4-hydroxybenzoic acid), 3-hydroxybenzoic, 4- hydroxybenzoic, protocatechuic acid (3,4-dihydroxybenzoic acid), 3',4'-dihydroxyphenylacetic acid, 3-(3'-hydroxyphenyl)propionic acid, 3-(4'-hydroxyphenyl)propionic acid, hippuric acid, 4-hydroxyhippuric acid, and ferouloylglycine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ferulic acid-4'-*O*-sulphate disodium salt, isoferulic acid-3'-*O*-*β*-D-glucuronide, dihydroferulic acid 4'-*O*-sulfate sodium salt, dihydroisoferulic acid 3-*O*-*β*-D-Glucuronide, and dihydrocaffeic acid-3'-*O*-sulphate sodium salt were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). Ultrapure water from MilliQsystem (Millipore, Bedford, MA, USA) was used throughout the experiment.

2.4 Urine and plasma extraction

Urine samples were centrifuged at 14,000 rpm for 10 min and 700 µL of supernatant was diluted 1:2 (v/v) adding acidified water (0.1% formic acid v/v). Samples were then filtered through a 0.45 µm nylon filter before analysis.

Plasma samples were extracted for analysis following the method previously reported by Renouf [29]. Briefly, 400 µL of plasma was added to 1.2 mL of ethanol. Samples were vortexed for 5 min, centrifuged at 14,000 rpm for 10 min and 1.3 mL of supernatant was dried under a rotary-vacuum evaporation. The pellet was re-suspended with 65 µL of acidified water (1% formic acid)/methanol (50:50, v/v) and samples were vortexed again to dissolve the pellet and finally centrifuged at 14,000 rpm for 5 min prior to analysis.

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156 ***2.5 uHPLC/MS^n Analysis***

157 Urine and plasma samples were analyzed by ultra-high performance liquid chromatography
158 coupled with mass spectrometry (uHPLC/MS) to identify and quantify the phenolic
159 metabolites after bread consumption. An Accela ultra-high performance liquid
160 chromatography (uHPLC) 1250 apparatus equipped with a linear ion trap mass spectrometer
161 (LIT-MS) (LTQ XL, Thermo Fisher Scientific Inc., San Jose, CA, USA) fitted with a heated-ESI
162 (H-ESI-II) probe (Thermo Fisher Scientific Inc.) was used. Compound separations were
163 performed with a XSELECT^{TM} HSS PFP column (50 x 2.1 mm), 2.5 µm particle size (Waters,
164 USA). A preliminary analysis was carried out in full scan, data-dependent MS^3 mode,
165 scanning from a mass to charge (m/z) of 100 to 800 to perform a preliminary assessment of
166 the main phenolic metabolites present in the samples. On the basis of the obtained
167 information, a second preliminary analysis was carried out in full MS^2 and MS^3 mode to
168 confirm the identification of the detected metabolites and to record the retention time.
169 Finally, quantification of all metabolites in biological samples was achieved using the single
170 reaction monitoring (SRM) mode. The analyses were carried out in negative ionization
171 mode, with a capillary temperature of 275°C, while the source was set at 250°C. The sheath
172 gas flow was 40 units, while auxiliary and sweep gases were set to 5 units. The source
173 voltage was 3 kV. The capillary voltage and tube lens were −9 and −53 V, respectively. For
174 chromatographic analysis, the mobile phase, pumped at a flow rate of 0.3 mL/min, was
175 composed of acetonitrile containing 0.1% formic acid (solvent A) and 0.1% formic acid in
176 water (solvent B). A linear gradient bringing A from 5 to 50% over 6.5 min was carried out
177 for separation. All metabolites were fragmented using a collision-induced dissociation (CID)

of 30, using helium gas for the collision. Where available, phenolic acid catabolites were quantified using a calibration curve prepared with the standard compound. When not available, the conjugated metabolites were quantified with the most structurally similar compound (Table 2).

2.6 Statistical analysis and absorption kinetic parameters

Based on available published data [30], using an F test and an ANOVA test with repeated measures, it was estimated that 12 participants in each treatment group would give sufficient power (α error of 0.05, 80% power) to detect a significant difference of 5.0 μmol of excreted ferulic acid.

Urine and plasma data were analyzed with a statistical software (SPSS, Chicago, IL, USA, version 21). All data were expressed as mean values ± SEM. A general linear model repeated measures (ANOVA, LSD post-hoc test) analysis was performed to compare each metabolite among the three treatments. Statistical significance was set at $p < 0.05$. Kinetic parameters of the plasmatic metabolites detected after bread consumption, including area under curve (AUC), plasma maximum concentration (C_{max}), time to reach the maximum plasma concentration (T_{max}), and, when available, apparent half-life ($T_{1/2}$) were calculated by means of pharmacokinetic functions for Microsoft Excel. Data on pharmacokinetic parameters were presented as mean values ± SEM.

3 RESULTS

All participants consumed the control bread (wholegrain bread) once and the breads enriched in aleurone fraction twice for a total of three experimental trials. The portion of

each meal was calculated to ensure that the same dose of ferulic acid (WGB vs AB-190) or the same quantity of bread (WGB vs AB-94) was administered. This approach allowed us to determine the bioavailability of ferulic acid in wholegrain products compared to aleurone-rich bread, and to test the possible influence of the consumed dose on the phenolic acid absorption and metabolism. All participants consumed the entirety of provided meals, which were well tolerated.

Using LC-MS analysis, 12 metabolites were identified, or tentatively identified, and quantified in urine samples, whereas only 4 metabolites were quantifiable in plasma samples. The spectrometric characteristics of the detected catabolites are reported in Table 3. The phenolic composition of the tested products were mostly free or bound phenolic acids [27] with no parent compound detected in the analyzed urine and plasma samples

3.1 Identification and Quantification of Urinary Metabolite

Among the 12 quantified urinary metabolites, 7 compounds were detected as sulfate conjugates and one as a glucuronide conjugate, highlighting the importance of the interaction of native phenolic compounds with intestinal and hepatic sulfotransferases (SULTs) and uridine-5'-diphosphate glucuronosyltransferases (UGTs). The presence of only one glucuronidated metabolite could suggest a minor interaction of phenolic acids of wholegrain and aleurone fractions with UGTs, compared with SULTs [28]. Besides the sulfate and glucuronidated forms, one metabolite was detected in its free, aglyconic form, and three compounds were found as nitrogen-containing conjugates, a reaction strictly linked to microbial metabolism [31].

The most abundant metabolites in terms of cumulative excretion appeared to be hippuric acid and hydroxyhippuric acid. Two isomers of hydroxyhippuric acid were detected in all samples, but they were quantified together using the available standard. These nitrogen-containing metabolites were monitored due to their possible origin from phenolic compounds. However, the initial amount and the trend of the excretion curves suggests several potential origins as previously reported [23] (Supporting information Fig. 2). Since ferulic acid is the main phenolic compound, both in wholegrain and in aleurone-rich bread, high concentrations of ferulic acid metabolites (ferulic acid-4'-*O*-sulfate, dihydroferulic acid-4'-*O*-sulfate and dihydroferulic acid-*O*-glucuronide) were recovered in urine samples (Fig. 2). If the ferulic acid-4'-*O*-sulfate (Fig. 2a) indicates an interaction of the native compound with the SULTs, the dihydroferulic metabolites (Fig. 2b and 2c) provides evidence of a microbial action on the double C-C bond prior to hepatic sulfation or glucuronidation. Colonic catabolism also resulted in the production of feruloylglycine [32] (Fig. 2d). Fig. 3 shows the cumulative excretion of dihydrocaffeic acid sulfate (Fig. 3a) and sinapic acid sulfate (Fig. 3b), with the latter tentatively identified due to the lack of a commercial standard. As previously reported for ferulic acid colonic metabolites, the importance of the microbiota on the biotransformation of native phenolic compounds was demonstrated by the presence of dihydrocaffeic acid sulfate and other low molecular weight metabolites such as vanillic acid-4-*O*-sulfate (Fig. 3c) and hydroxybenzoic acid sulfate (Fig. 3d), and of a negligible amount of 3-(phenyl)propionic acid-*O*-sulfate (Supporting information Fig. 2c). These metabolites could originate from all the native phenolic acids in the diet, not specifically to ferulic acid. For this reason, these specific metabolites cannot be taken into account when ferulic acid bioavailability is evaluated. Finally, 3',4'-

dihydroxyphenylacetic acid was the only identified unconjugated compound (Supporting information Fig. 2d). The concentration in urine appeared to increase throughout the 48 h sampling period with no noticeable difference across the products ingested.

By observing the slopes of the 48-h cumulative curves, the metabolic origin of each metabolite could be interpreted. Specifically, a steep slope recorded within the 0-3 h time period is suggestive of early absorption in the small intestine, and of a subsequent conjugation at the enterocyte or hepatic level. Hence, ferulic acid-4'-*O*-sulfate was the only metabolite derived from this metabolic path, indicating that the native compound could be partially absorbed in the small intestine (Fig. 2a). An increase in the slope at 3-6 h indicates a release of the most accessible fiber-linked-phenolic compounds by microbiota enzymatic activity. The release of native compounds in the colon led to increased absorption and further conjugation. For example, ferulic acid-4'-*O*-sulfate (Fig. 2a), sinapic acid sulfate (Fig. 3b) and hydroxybenzoic acid sulfate (Fig. 3b) curves exhibited a increased rate of appearance within 3 and 6 h. Finally, increasing slopes detected from 6 to 48 h explain a deeper microbiota catabolism. In particular, metabolites excreted during these excretion periods could be associated with i) a long transient time in the large intestine, according to normal metabolism of dietary fiber-rich products [33], which caused a slower release of less accessible fiber-linked phenolics; ii) the microbial enzymatic activity needed to convert the released phenolic compounds into lower molecular weight molecules (as vanillic acid, hydroxybenzoic acid (Fig. 3c and d), 3-(phenyl)propionic acid (Supporting information Fig. 2c)), or into nitrogen-containing conjugates (hippuric acid, hydroxyhippuric acid (Supporting information Fig. 2a and 2b) and feruloylglycine (Fig. 2d)), or into reduced forms

(dihydrocaffeic acid (Fig. 3a) and dihydroferulic acid (Fig. 2b and 2c)); or iii) the hepatic SULTs and UGTs activity on phenolics absorbed from the colon.

Observation of the 48-h urinary excretion (Fig. 2, 3 and Supporting information Fig. 2) suggests that the consumption of AB-190 (bread enriched with aleurone fraction that contained the same dose of ferulic acid of the wholegrain bread) is generally linked to higher excretion of almost all the detected metabolites, with peculiar differences for feruloylglycine, hippuric acid, dihydrocaffeic acid sulfate, and sinapic acid sulfate.

3.2 Bioavailability

To calculate the bioavailability of specific wheat phenolics, we have computed the ratio between the total metabolite 48h urinary excretion and the total intake of parent compounds through the three fed bread portions.

Although the 48-h cumulative excretion of dihydrocaffeic acid sulfate and sinapic acid sulfate appeared significantly higher after the consumption of the aleurone enriched bread, the bioaccessibility and bioavailability of caffeic or sinapic acid cannot be quantified as the portion meal was not standardized for their respective contents.

On the contrary, the bioavailability of ferulic acid can be evaluated by focusing specifically on its direct metabolites, such as ferulic acid-4'-*O*-sulfate, dihydroferulic acid-4'-*O*-sulfate and dihydroferulic acid-*O*-glucuronide, and by correlating their total amount to the amount of native free and bound ferulic acid provided through each test meal. Fig. 4 shows that the bioavailability of ferulic acid in aleurone enriched bread containing the same dose of ferulic acid (AB-190) was slightly higher than after the control wholegrain product, but this difference did not reach a statistical significance. On the contrary, the bioavailability of

ferulic acid in bread (AB-94) enriched in aleurone fraction consumed in same quantity as wholegrain bread was significantly higher.

3.3 Plasma kinetics of phenolic compounds

The second target of the study focused on plasma pharmacokinetics of phenolic compounds in bread. Contrary to urinary data, only 4 metabolites were detected in quantifiable concentrations in plasma samples. Hippuric acid and hydroxyhippuric acid (where the concentration was calculated considering both of the isomers) had the highest concentration among all circulating metabolites (Supporting information Fig. 3a and b). Although hippuric acid exhibited a peak plasma concentration at 7 h, indicating a possible colonic degradation of native phenolic compounds, the absorption curves appeared unclear and these metabolites could not be directly associated with phenolic acid metabolism. On the contrary, the presence of ferulic acid-4'-*O*-sulfate and dihydroferulic acid-4'-*O*-sulfate can be strictly linked to bread consumption (Fig. 5). The curve reported for ferulic acid-4'-*O*-sulfate (Fig. 5a) clearly demonstrates the absorption and the rapid conjugation of the native ferulic acid in the first part of the gastrointestinal tract, confirming what was observed in urines. The evidence was supported by the calculated T_{max} value close to 1 h. The circulating concentrations rapidly decreased within 3 h and progressively until 24 h. Although the curve derived from the consumption of the product enriched in aleurone fraction containing less ferulic acid (AB-94) showed a lower C_{max} and AUCs value, no significant differences were observed in the kinetic parameters among the three products. The dihydroferulic acid-4'-*O*-sulfate absorption curve (Fig. 5b) distinctly outlined a maximum peak at 7 h, which was higher after the consumption of AB-190, in accordance with its colonic formation [34], and

its concentration decreased before the last collection time point. However, because of the lack of intermediate time points between 7 h and 24 h it was impossible to exclude the presence of additional peak concentrations within this period.

4 DISCUSSION

The present study compared, for the first time, the *in vivo* human absorption, metabolism and bioavailability of phenolic compounds present in two types of bread: wholegrain bread and wheat bread enriched in aleurone fraction. We standardized bread intake based on the dose of the main phenolic acid, ferulic acid, and accounted for free and bound phenolics, as well as ferulic acid oligomers [27].

Previous research has compared the *in vitro* early digestion of wholegrain bran to the aleurone fraction. Zaupa and colleagues reported the *in vitro* bioaccessibility of the main phenolic compounds were higher in the aleurone flour compared to unfractionated bran [26]. These results were corroborated when the same approach was used to measure the *in vitro* bioaccessibility of phenolic compounds in wholegrain flour bread or aleurone-rich flour bread. All the main phenolic acids were significantly more bioaccessible in the bread enriched in aleurone fraction [27]. Despite small intestine bioaccessibility, it is important to assess colonic bioaccessibility, as phenolic compounds are poorly absorbed in the small intestine [23]. *In vitro* colonic biotransformation has revealed the capacity of colonic microbiota enzymes to catabolize ferulic oligomers, to release fiber-linked compounds, and to convert them into simple metabolites, such as ferulic and dihydroferulic acid and other aromatic metabolites such as phenylpropionic, phenylacetic and benzoic acid derivatives

[18, 35, 36], microbial metabolites which have been reported to be efficiently absorbed at large intestinal level [37].

Existing animal studies that fed a whole-wheat meal or aleurone-rich meal led to the identification of both human and microbial metabolites that are mainly conjugated with sulfate or glucuronic acid residues [28, 38, 39]. Calani and colleagues (2014) fed rats a control pellet or a wheat aleurone-rich pellet. Fifteen metabolites were quantified and were mostly sulfate conjugated. Of these fifteen, six metabolites were detected in the present human *in vivo* study including: ferulic acid-4'-*O*-sulfate, dihydroferulic acid-4'-*O*-sulfate, vanillic acid-4-*O*-sulfate, 3-(phenyl)propionic acid-*O*-sulfate, 3',4'-dihydroxyphenylacetic acid and hippuric acid. In the same rat feeding study the 24-h urinary recovery for the majority of metabolites was higher when the aleurone-rich pellet was consumed, demonstrating a higher absorption of aleurone phenolic compounds [28]. A better absorption of the phenolic acid of aleurone fraction rather than wholegrain was also established by Nørskov and colleagues (2013). Pigs that consumed aleurone-rich wheat flour for 72 h had a higher phenolic metabolite plasma concentration in comparison to a whole-wheat diet. However, the phenolic compound intake was not standardized between the two diets [39].

No human studies are available on aleurone phenolic acid metabolism and absorption. However, some human intervention studies that investigated wholegrain cereal consumption reported urinary excretion of ferulic acid conjugates, feruloylglycine and sinapic acid conjugates [40]. Additionally, plasma circulating concentrations of ferulic acid conjugates, vanillic acid, 3-(hydroxyphenyl)propionic acid conjugates, and sinapic acid conjugates [21, 40], have been reported as ranging from nanomolar to low micromolar. The absence of some plasma microbial catabolites following wholegrain bread intake in the

present study could be explained by the lower dose of ferulic acid (~3-3.5 fold lower respectively), than previously utilized in other studies.

In the present study we standardized ferulic intake between the wholegrain bread and the aleurone bread trials and found a total bioavailability of 4-8%. The bioavailability of ferulic acid in humans was higher than in rats fed aleurone-rich pellets where it was approximately 0.7% [28]. In contrast, ferulic acid bioavailability was similar, at 4-5.5%, when rats were fed a cereal-based meal [20]. Additionally, other human studies that examined whole-wheat bread or high-bran cereals report a 3-10% bioavailability of ferulic acid [21, 40]. Other studies that report wholegrain ferulic acid bioavailability, with the exception of Calani et al (2014), do not consider the amount of ferulic acid dimers or trimers contained in the test meal.

As bioavailability could have been influenced both by the type of ferulic acid source (wholegrain flour or aleurone fraction) and by the amount of bread (with a possible bulk effect and/or absorption saturation), the chosen study design allowed us to discriminate between these two potential co-factors. When the same amount of ferulic acid was fed (87mg, but the two bread amounts were different), the bioavailability did not result significantly different between aleurone enriched and wholemeal breads (Fig.4), unless feruloyglycine was accounted for in the calculation (Supporting information Fig. 4). When, however, the same amount of bread was fed (94g, but the total ferulic acid intake was different), ferulic acid from aleurone-enriched bread was significantly better absorbed and excreted. Nevertheless, it must be pointed out that the excretion of feruloylglycine in the bioavailability calculation could be misleading, since the concentration of this metabolite was detectable already at the beginning (T_0) of each treatment, as also previously reported

[40], suggesting that its 48 h-cumulative excretion could result from the catabolism of other ingested or biotransformed compounds.

Plasma levels of ferulic acid metabolites were not significantly different after the three feedings, indicating that even with half dose of ferulic acid (introduced with AB-94), consumers could reach plasma levels of ferulic acid metabolites comparable to those obtained from a higher dose introduced through wholemeal bread or higher volumed of aleurone bread.

The higher ferulic bioavailability in bread enriched with the aleurone fraction compared to wholegrain bread could be explained by the different bran structure. Increased bioaccessibility, and hence bioavailability, of wheat aleurone phenolics has been demonstrated in the literature by applying different approaches, like enzymatic treatments targeting specific linkages in wheat bran [35, 41], fermentation systems, used as sources of specific enzymes [21, 41], or high energy grinding, increasing the specific surface of the bran fractions [16, 35]. Conversely, the higher bioavailability we observed following the lower ferulic consumption suggests a potential negative dose-metabolic response explained by a reduction in the capacity to metabolize and absorb phenolic acids caused by a higher level of intake. Future studies should focus on establishing the mechanisms involved in the absorption of phenolic acids from aleurone-rich products.

In recent years, the consumption of (poly)phenol-rich foods has been largely connected to beneficial health effects [42, 43]. As such, evaluating the metabolism and bioavailability of phenolic acids (particularly ferulic acid) in widely consumed staple products, such as bread, becomes of particular interest. Additionally, more studies are appearing in the literature trying to unravel the mechanisms behind the protective effects exerted by wholegrain

foods, with some of these highlighting the possible involvement of phenolic acids. For example, the circulating levels of specific phenolic metabolites detected after wholegrain consumption in humans, such as ferulic acid conjugates, vanillic acid and hydroxyphenylpropionic acid, have been specifically related to a reduction in pro / anti-inflammatory cytokine ratios. These metabolites result in an immune-modulatory effect [21] and a reduction in TNF-α and an enhancement in IL-10 correlated to the improvement of the inflammation status [44]. Furthermore, two of the main phenolic acid metabolites (3,4-dihydroxyphenylacetic acid and 3-hydroxyphenylpropionic acid) have been described *in vitro* to potentiate glucose-stimulated insulin secretion in a beta cell line INS-1E and in rat pancreatic islets [45]. Also, these metabolites are reported to protect against beta cell dysfunction and death induced by tert-butyl hydroperoxide, through the activation of protein kinase C and the extracellular regulated kinase pathways [45]. Moreover, the dihydroxylated microbial compound 3,4-dihydroxyphenylacetic acid, as well as 3,4-dihydroxyphenylpropionic, exhibited anti-inflammatory properties, modulating the production of the main pro-inflammatory cytokines, as TNF-α, IL-1β and IL-6, in LPS-stimulated peripheral blood mononuclear cells. Contrarily, 4-hydroxyhippuric acid exerted its anti-inflammatory property only acting on TNF-α secretion [46]. Finally, very recently, Malunga and colleagues described ferulic acid and feruloylated arabinoxylan mono-/oligosaccharides as possible inhibitors of sucrase and maltase functions of α-glucosidase in Caco-2 and rat intestinal cells; moreover glucose uptake in Caco-2 cells was partially inhibited by their presence [47].

Limited evidence is available on potential human health benefits due to aleurone. Rats fed with wheat aleurone did not experience improvement of any parameter associated with

obesity, such as body weight gain, adiposity, fasting blood glucose, plasma insulin and leptin, anti-inflammatory markers and oxidative stress markers [48]. Ounnas and colleagues (2014) demonstrated that plasma eicosapentaenoic acid concentration increased in rats fed with wheat aleurone, while docosahexaenoic acid and omega-6 fatty acids were not affected [49]. In humans, the consumption of wheat aleurone-rich foods significantly lowered the plasma concentration of the inflammatory marker, C-reactive protein, which is an independent risk factor for CVD. No changes were observed in other markers of inflammation, antioxidant status or endothelial function [50]. However, it remains to be established whether the potential health benefits reported for wholegrain consumption are dependent on a specific component or a combination of components present in wholegrains and possibly in aleurone fractions.

In conclusion, this is the first human intervention study in which phenolic compound metabolism, absorption and bioavailability in wholegrain bread have been compared to those introduced through bread enriched with aleurone layer. The results of this study, besides showing a general increased bioavailability of ferulic acid in aleurone-rich bread, demonstrate that high levels of ferulic acid metabolites can be achieved in humans with the consumption of relatively low amounts (4 slices) of white, aleurone-enriched bread. We are only able to speculate as if the potential mechanisms behind the health effects linked to wholegrains are maintained with aleurone-rich food consumption. However, if the circulating phenolic metabolites after intake of wholegrain products are involved in physiological benefits [44], this study demonstrates that the same levels could be reached through consumption of refined products enriched in aleurone fractions. This study paves the way for future research focused on measuring the modulation of health related

biomarkers by aleurone and its phenolics in both health individuals as well as those with disease risk factors.

Supporting material

mnfr201600238-sup-0001-supporting_information_rev1_OK

We thank all the volunteers who participated in the study.

LB, FS and DDR designed the research; LB conducted the experiments, collected samples and data, analyzed samples, drafted the manuscript; RL and EDA were medical study supervisors and responsible for hematic sample collection; CM provided the bread products and organized the portioning; FS, MN, MD, SR and FB critically read and improved the protocol and the manuscript; DDR secured the funding and has the primary responsibility for the final content; all authors: read and approved the final manuscript. CM is an employee of Barilla SpA. LB, FS, RL, EDA, MN, MD, SR, FB, and DDR had no competing financial interests.

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TABLE 1

Nutritional values and ferulic acid content per portion of testing meals.

	WGB Wholegrain Bread (94 g)	**AB-94 Aleurone-Enriched Bread (94 g)**	**AB-190 Aleurone-Enriched Bread (190 g)**
Energy (kcal)	246	245	496
Energy (kJ)	1035	1035	2092
Fats (g)	5.5	3.9	8.0
Carbohydrates (g)	35.3	40.7	82.3
Fiber (g)	7.5	5.6	11.4
Proteins (g)	10.2	8.9	18.0
Ferulic Acid (mg)	87	43	87

TABLE 2

Standard compounds used for metabolite quantification.

Metabolites	**Standard compounds used for quantification**

3',4'-Dihydroxyphenylacetic acid	3',4'-Dihydroxyphenylacetic acid
Hippuric acid	Hippuric acid
4-Hydroxyhippuric acid **Hydroxyhippuric acid**	4-Hydroxyhippuric acid
Hydroxybenzoic acid sulfate	Dihydrocaffeic acid-3-*O*-sulfate
3-(Phenyl)propionic acid-*O*-sulfate	Dihydrocaffeic acid-3-*O*-sulfate
Vanillic acid-4-*O*-sulfate	Dihydroferulic acid-4'-*O*-sulfate
Feruloylglycine	Feruloylglycine
Dihydrocaffeic acid-3-*O*-sulfate	Dihydrocaffeic acid-3-*O*-sulfate
Ferulic acid-4'-*O*-sulfate	Ferulic acid-4'-*O*-sulfate
Dihydroferulic acid-4'-*O*-sulfate	Dihydroferulic acid-4'-*O*-sulfate
Sinapic acid-*O*-sulfate	Ferulic acid-4'-*O*-sulfate
Dihydroferulic acid-*O*-glucuronide	Dihydro(iso)ferulic acid-3-*O*-glucuronide

TABLE 3

Spectrometric characteristics used for metabolite quantification.

Metabolites	RT	$[M-H]^-$ (m/z)	MS^2	MS^3	Sample Detection
3',4'-Dihydroxyphenylacetic acid	2.06	167	123		Urine
Hippuric acid	2.75	178	134		Urine, Plasma
4'- Hydroxyhippuric acid	1.93	194	100		Urine, Plasma
Hydroxyhippuric acid	2.17	194	150		
Hydroxybenzoic acid sulfate	2.52	217	137	93, 137	Urine
3-(Phenyl)propionic acid-*O*-sulfate	2.88	245	165	147, 121	Urine
Vanillic acid-4-*O*-sulfate	2.78	247	167	123, 152, 108	Urine
Feruloylglycine	3.69	250	206, 100, 149, 175, 191		Urine
Dihydrocaffeic acid-3-*O*-sulfate	2.55	261	181	137, 119	Urine
Ferulic acid-4'-*O*-sulfate	3.53	273	193	149, 178, 134	Urine, Plasma
Dihydroferulic acid-4'-*O*-sulfate	2.94	275	195	151, 136, 180, 177	Urine, Plasma
Sinapic acid-O-sulfate	3.10	303	223	208, 179, 191	Urine
Dihydroferulic acid-*O*-glucuronide	3.14	371	195	119, 151, 149, 136	Urine

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Legends for figures

FIGURE 1

Graphical representation of the study design.

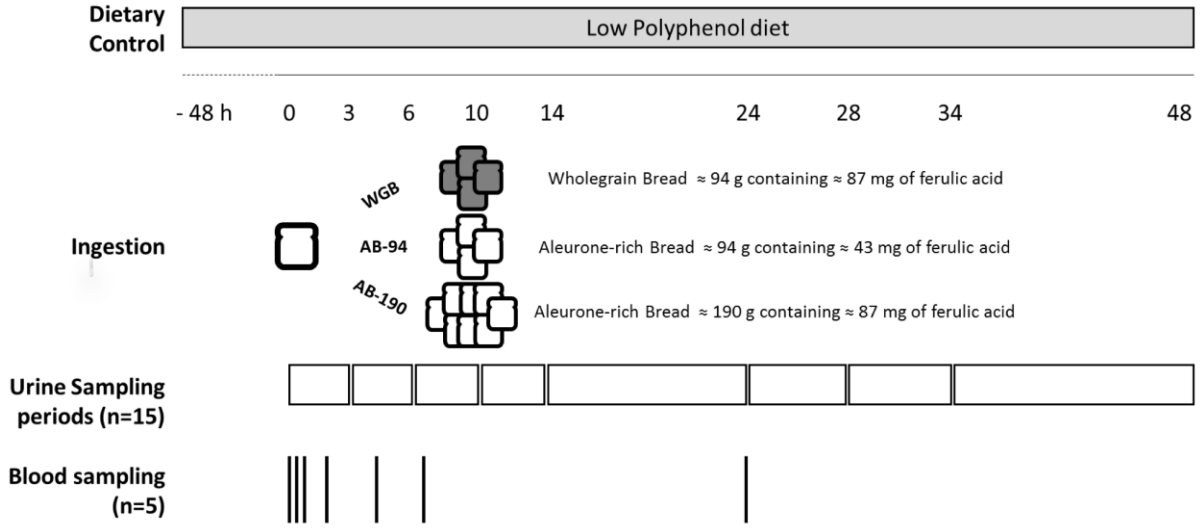

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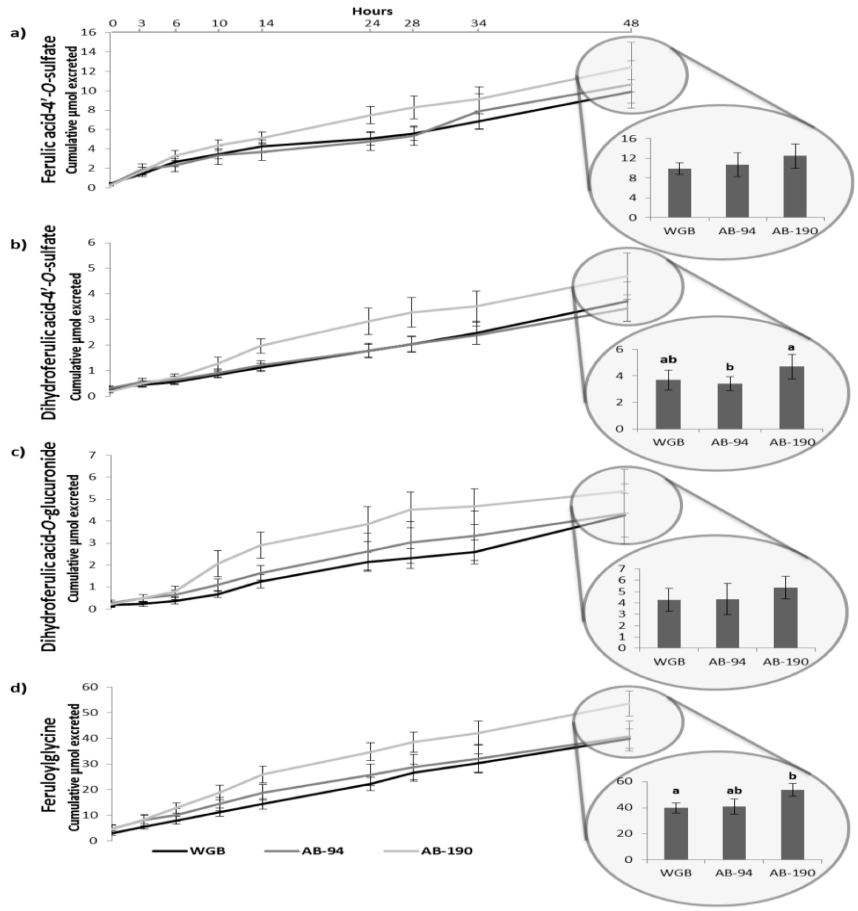
FIGURE 2

Cumulative urinary excretion of ferulic acid metabolites: a) Ferulic acid-4'-*O*-sulfate, b) Dihydroferulic acid-4'-*O*-sulfate, c) Dihydroferulic acid-*O*-glucuronide, and d) Feruloylglycine. Values are expressed as cumulative µmol excreted ± SEM (n = 15). Different letters mean significant differences comparing the three consumed product. A general linear model for repeated measures (ANOVA, LSD post-hoc test) was used to compare each metabolite among the three treatments. Statistical significance was set at $p < 0.05$. WGB consists of 94 g of wholegrain bread, containing approximately 87 mg of total ferulic acid; AB-94 consists of 94 g of a commercial bread enriched in aleurone fraction, containing approximately 43 mg of total ferulic acid; AB-190 consists of 190 g of a commercial bread enriched in aleurone fraction, containing approximately 87 mg of total ferulic acid.



600 **FIGURE 3**

Cumulative excretion of phenolic acid metabolites: a) Dihydrocaffeic acid-3-*O*-sulfate, b) Sinapic acid sulfate, c) Vanillic acid-4-*O*-sulfate, and d) Hydroxybenzoic acid sulfate. Values are expressed as cumulative µmol excreted ± SEM (n = 15). Different letters mean significant differences comparing the three consumed product. A general linear model for repeated measures (ANOVA, LSD post-hoc test) was used to compare each metabolite among the three treatments. Statistical significance was set at $p < 0.05$. WGB consists of 94 g of wholegrain bread, containing approximately 87 mg of total ferulic acid; AB-94 consists of 94 g of a commercial bread enriched in aleurone fraction, containing approximately 43 mg of total ferulic acid; AB-190 consists of 190 g of a commercial bread enriched in aleurone fraction, containing approximately 87 mg of total ferulic acid.

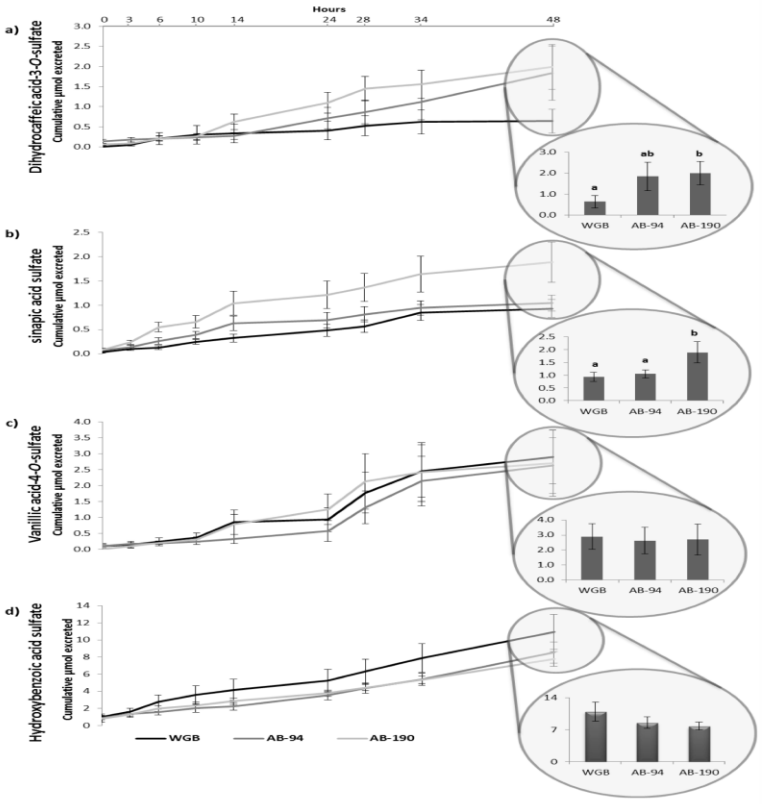


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FIGURE 4

Ferulic acid bioavailability calculated comparing the total amount of ferulic acid consumed by the 15 volunteers and the quantity excreted through its main metabolites, namely ferulic acid-4'-*O*-sulfate, dihydroferulic acid-4'-*O*-sulfate and dihydroferulic acid-*O*-glucuronide. Different letters mean significant differences comparing the three consumed product. A general linear model for repeated measures (ANOVA, LSD post-hoc test) was used to compare the bioavailability ($p < 0.05$). WGB consists of 94 g of wholegrain bread, containing approximately 87 mg of total ferulic acid; AB-94 consists of 94 g of a commercial bread enriched in aleurone fraction, containing approximately 43 mg of total ferulic acid; AB-190 consists of 190 g of a commercial bread enriched in aleurone fraction, containing approximately 87 mg of total ferulic acid.

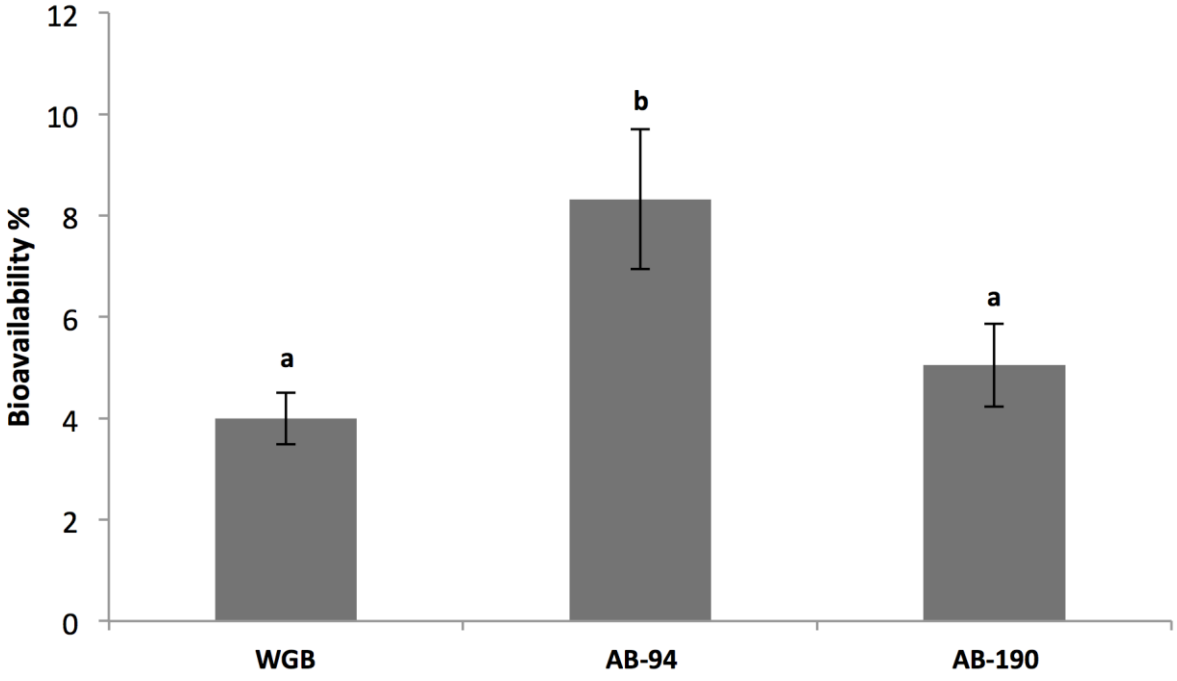

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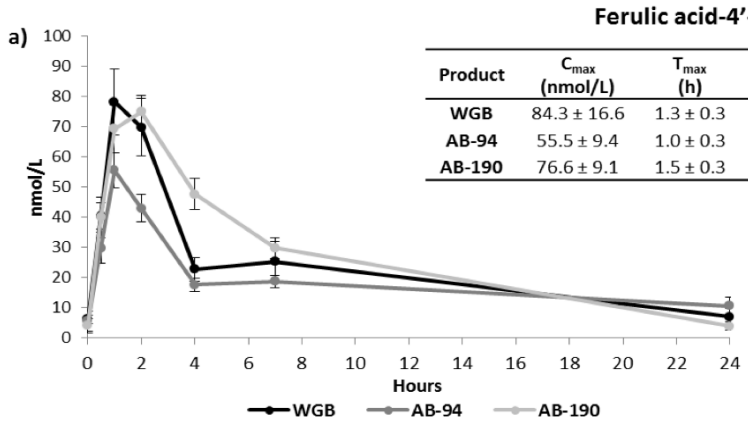
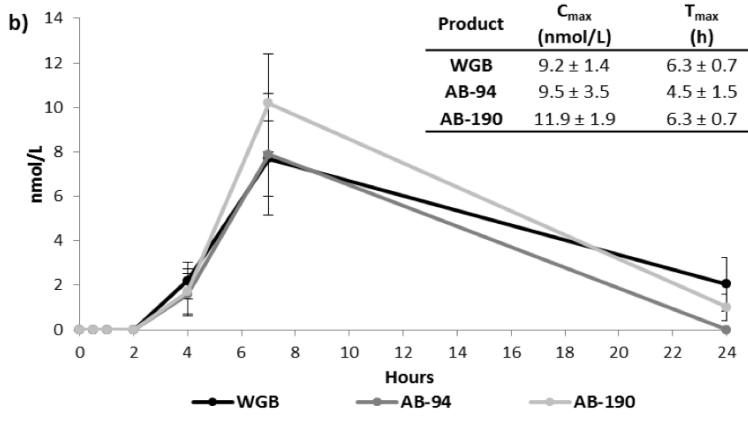
FIGURE 5

Absorption kinetics of the phenolic metabolites detected in plasma. a) Ferulic acid-4'-*O*-sulfate, and b) Dihydroferulic acid-4'-*O*-sulfate. Values are expressed as nmol/L ± SEM (n = 5). A general linear model for repeated measures (ANOVA, LSD post-hoc test) was used to compare pharmacokinetic parameters among the three treatments. Statistical significance was set at $p < 0.05$. No statistically significant difference was observed. WGB consists of 94 g of wholegrain bread, containing approximately 87 mg of total ferulic acid; AB-94 consists of 94 g of a commercial bread enriched in aleurone fraction, containing approximately 43 mg of total ferulic acid; AB-190 consists of 190 g of a commercial bread enriched in aleurone fraction, containing approximately 87 mg of total ferulic acid.



Ferulic acid-4'-*O*-sulfate

Product	C_{max} (nmol/L)	T_{max} (h)	$T_{1/2}$ (h)	AUC (nmol/L*h)
WGB	84.3 ± 16.6	1.3 ± 0.3	8.4 ± 3.3	554.3 ± 149.4
AB-94	55.5 ± 9.4	1.0 ± 0.3	16.9 ± 6.4	443.6 ± 66.4
AB-190	76.6 ± 9.1	1.5 ± 0.3	7.4 ± 0.9	634.7 ± 48.3



Dihydroferulic acid-4'-*O*-sulfate

Product	C_{max} (nmol/L)	T_{max} (h)	$T_{1/2}$ (h)	AUC (nmol/L*h)
WGB	9.2 ± 1.4	6.3 ± 0.7	NA	99.8 ± 31.4
AB-94	9.5 ± 3.5	4.5 ± 1.5	NA	82.8 ± 39.3
AB-190	11.9 ± 1.9	6.3 ± 0.7	NA	114.8 ± 32.3