Effects of Disintegration on *in Vitro* Fermentation and Conversion Patterns of Wheat Aleurone in a Metabolical Colon Model

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S *Supporting Information*

ABSTRACT: This work aimed to elucidate the effect of wheat aleurone integrity on its fermentability, i.e., the formation of short-chain fatty acids (SCFA) and microbial phenolic metabolites, in an *in vitro* model using human faecal microbiota as an inoculum. The structure of aleurone was modified by mechanical (dry grinding) or enzymatic (xylanase with or without feruloyl esterase) treatments in order to increase its physical accessibility and degrade its complex cell-wall network. The ground aleurone (smaller particle size) produced slightly more SCFA than the native aleurone during the first 8 h but a similar amount at 24 h (102.5 and 101 mmol/L, respectively). Similar colonic metabolism of ferulic acid (FA) was observed for native and ground aleurone. The enzymatic treatments of aleurone allowed a high solubilization of arabinoxylan (up to 82%) and a high release of FA in its conjugated and free forms (up to 87%). The enzymatic disintegration of aleurone's structure led to a higher concentration and formation rate of the colonic metabolites of FA (especially phenylpropionic acids) but did not change significantly the formation of SCFA (81 mmol/L for enzyme treated versus 101 mmol/L for the native aleurone).

KEYWORDS: *aleurone, ferulic acid, feruloyl esterase, particle size, short-chain fatty acids, xylanase treatment*

INTRODUCTION

The consumption of whole grain foods has been associated with health benefits, such as protection against obesity, diabetes, cardiovascular diseases, and cancers.[1] The presence of physiologically beneficial dietary fibers and numerous bioactive compounds, as well as the cereal structure, have a potential role in the health properties of whole grain foods.[2] In wheat grain, the nutritionally interesting compounds are mainly concentrated in the aleurone, making it the most nutritionally interesting fraction of wheat grain.[3]

Aleurone is a unicellular layer located between endosperm and the outer layers of wheat grain, which represents around 7% of the wheat grain dry mass.[4] It is made of hexagonal cells with an average size of 37−65 μm × 25−75 μm.[5] Its cell walls contain mainly insoluble arabinoxylans (AX) highly esterified with phenolic acids, β-glucans, cellulose, and proteins.[6,7] The most abundant phenolic acid is monomeric ferulic acid (FA), followed by minor amounts of dimeric FA, sinapic acid, and *p*-coumaric acid.[8,9] These phenolic compounds were shown to be mainly responsible for the antioxidant properties of wheat aleurone.[10] Besides its antioxidant properties, the wheat aleurone has been shown to be more susceptible than wheat bran to degradation by the colonic microflora *in vitro*[11] and *in vivo*.[12] Aleurone fermentation presented beneficial effects in the gut, and the metabolites of aleurone were also able to reduce the level of tumor promotion in human HT29 colon cells.[13]

The physical and physicochemical characteristics of dietary fiber, such as particle size, hydration properties, viscosity, solubility, molecular weight, and degree of branching, were shown to be involved in their effects on colonic function.[14−16] The adhesion of microbiota on the fiber (physical accessibility) is the first limiting parameter of fermentation besides the chemical structure of fiber components.[16] The solubility of AX from aleurone also influences their fermentation behavior, the soluble one being generally more rapidly fermented than the insoluble one. The highly branched AX with high arabinose/xylose ratio is also fermented more slowly.[11] The *in vitro* fermentation of water-extractable AX (WE-AX) from wheat flour increased the production of total short-chain fatty acids (SCFA), but this production was not affected by the molecular mass of the AX.[17] *In vivo*, water unextractable AX (WU-AX) from wheat was only partially fermented in the ceco-colon of rats and increased the level of butyrate, whereas WE-AX had a higher production of acetate.[14] However, most of these studies have been performed with isolated AX fiber polymers, which did not take into account the effects of the cereal matrix in which they were embedded.

Dietary fibers contain many co-passengers such as vitamins and antioxidants. In wheat aleurone, the most abundant co-passengers are phenolic compounds that are potentially strong antioxidants carried by AX. The health benefits of aleurone may

not be due only to the fermentation of its dietary fiber but also to the action of its co-passengers and their metabolites and/or even to their synergistic effects. Depending on the structure of fibers and how they are inserted in the cereal matrix, the phenolic co-passengers can be released throughout the gut, affecting its physiological activity.[18] For example, in wheat bran, most of the FA bound to AX is released in the colon.[19] However, changes of the wheat bran structure by ultrafine grinding or enzymatic processing was shown to increase the bioaccessibility of phenolic compounds in an *in vitro* model of the upper gastrointestinal tract.[20,21]

In this paper we have modified the structure of the wheat aleurone layer by dry and wet processes and studied the effects of the disintegration of aleurone structure on its fiber fermentability, i.e., the formation of SCFA and the microbial metabolites derived from its co-passenger FA, in an *in vitro* model using human faecal microbiota as an inoculum. Our previous work showed that the structure of wheat aleurone impacts its *in vitro* antioxidant capacity in gastric conditions.[22] The present work aimed to elucidate if the modification of the structure of the aleurone layer influences its fermentation pattern in a gut model.

■ MATERIALS AND METHODS

Wheat Aleurone Treatments. The starting aleurone layer material, named as "native" aleurone, was provided by Bühler AG (Uzwil, Switzerland). Its purity was estimated around 85%. It was obtained from wheat grains (90% of Swiss varieties, Arina and Runal medium hard wheat) by using a dry process described in the Bühler A.G. patent applications. Its chemical composition was 39.7 g fiber,[23] 22.2 g protein,[24] 13.3 g ash,[24] 9.8 g fat,[25] and 5.8 g starch[24] in 100 g wheat aleurone per dry matter.

The native aleurone was ground (fraction named as "ground") by using an impact mill (Hosokawa-Alpine, type 100 UPZ, Augsburg, Germany) with 0.3 mm sieve, 18 000 rpm rotor speed, and 2 kg/h feed speed. The mill was operated in cryogenic conditions by combining a cryogenic screw feeder with a liquid nitrogen supply (Micronis, Agen, France) to the impact mill. The grinding temperature was −90 °C. The temperature of the ground product was monitored and adjusted by varying the flow of liquid nitrogen.

The native aleurone was treated with two enzyme preparations: the xylanase enzyme preparation Grindamyl Powerbake 950 (Danisco, Denmark) and the A-type feruloyl esterase (FAE) from *Aspergillus niger* (activity on methyl ferulate 1.08 U/mg powder at 25 °C pH 5.0). These two enzymes were kindly provided by Jens Frisbak Sorensen (Danisco, Denmark) and Craig Faulds (IFR, Norwich, UK), respectively. In the first treatment, aleurone was hydrolyzed with 7 U of xylanase per gram of aleurone (fraction named as "Xyn"). The treatment was carried out in distilled water (ratio 0.15 g of aleurone/mL) at 20 °C for 16 h under stirring. In the second treatment (fraction named as "Xyn+FAE"), aleurone was first treated with 70 U of xylanase per gram of aleurone (ratio 0.15 g of aleurone/mL, distilled water) at 20 °C for 16 h, and then a feruloyl esterase was added to the solution (concentration 1.08 U of FAE/g aleurone). The reaction was allowed to continue at 20 °C for an additional 8 h under stirring. For both treatments, the enzymes were then inactivated by incubating the samples in a boiling water bath for 10 min. The treated aleurone fractions were freeze-dried and stored in plastic bags under vacuum and protected from light at 4 °C.

Microscopy of Aleurone Fractions. The aleurone fractions were first embedded in 2% agar and then fixed in 3.0% (w/v) paraformaldehyde and 1.0% (v/v) glutaraldehyde in 0.1 M Na-K phosphate buffer (pH 7.0), dehydrated in graded ethanol series, and embedded in hydroxyethyl methylacrylate resin (Leica Historesin embedding kit, Heidelberg, Germany). Polymerized samples were cut in 2 μm sections by a rotary microtome HM 355 (Microm Laborgeräte GmbH, Walldorf, Germany) using a tungsten carbon knife. The sections were transferred onto glass slides. Protein was stained with aqueous 0.1% (w/v) Acid Fuchsin (BDH Chemicals Ltd., Poole, Dorset, U.K.) in 1.0% acetic acid for 1 min. β-Glucan was stained with aqueous 0.01% (w/v) Calcofluor White (Fluorescent brightener 28, Aldrich, Germany) for 1 min. In exciting light (400−410 nm, emission >455 nm) intact cell walls stained with Calcofluor appeared blue and proteins stained with Acid Fuchsin appeared red. Starch was unstained and appeared black. In addition, the autofluorescence of the samples was detected by imaging the sections without staining using the same epifluorescence and emission wavelengths as mentioned above. The samples were examined with an Olympus BX-50 microscope (Olympus Corp., Tokyo, Japan). Micrographs were obtained using a PCO SensiCam CCD color camera (PCO AG, Kelheim, Germany) and the Cell^P imaging software (Olympus).

Particle Size of Aleurone Fractions. The particle size distribution of the wheat aleurone fractions was measured (from 0.02 to 2000 μm) by using a laser diffraction particle size analyzer Mastersizer 2000 (Malvern Instruments Ltd., U.K.). Aleurone fractions were suspended in ethanol directly in the measurement cell and analyzed when the obscuration was 10−20%. At least three parallel analyses were carried out for each sample. The total specific surface of aleurone fractions was calculated from the volume distribution given by the laser diffraction measurement using the following equation:

$$\text{sp surf} = \frac{6}{\rho D_{3,2}} \qquad (1)$$

where ρ is the density of the material (1.53 ± 0.02 g/cm^3 measured for each aleurone fraction with a pycnometer using nitrogen as the carrying gas) and $D_{3,2}$ is the surface weighted mean diameter.

Water Holding Capacity (WHC) of Aleurone Fractions. The water holding capacity was analyzed in triplicate by using a Bauman apparatus, which consists of a small water tank connected to a horizontal graduated capillary tube filled with deionized water. A sintered glass was placed on the top of the water tank with a Whatman grade 5 filter paper (Whatman, Little Chalfont, U.K.). The zero value was recorded from the graduated capillary tube after the stabilization of water (around 5 min). Aleurone sample (75 mg) was sprinkled on the wetted filter paper, and the water uptake was recorded every 2 min up to its stabilization (20 min at room temperature). A glass lid was set on the sintered glass during the measurement to minimize evaporative losses. The results were expressed as gram of water uptake per 1 g of aleurone sample dry matter.

Water Binding Capacity (WBC) of Aleurone Fractions. The water binding capacity was determined in duplicate by a simple centrifugation method modified by Santala et al.[26] A 100-mg portion of aleurone fractions was weighed in a Eppendorf tube, and 1 mL of water was added and mixed by vortex. The aleurone suspension was shaken at room temperature (20 °C) for 30 min and centrifuged (12 100g, 15 min). The supernatant was removed carefully by draining for 10 min at a 45° angle. The gained weight per gram of aleurone dry matter was determined as the WBC.

Porosity of Aleurone Fractions. The porosity and the mean pore size of aleurone fractions was determined at the Institut Européen des Membranes (UMR 5635, IEM, Montpellier) by using an Autopore IV 9500 mercury intrusion porosimeter (Micromeritics Instrument Corporation, Norcross, USA). Mercury was forced to enter into the pores of 1.0 g of aleurone fraction by applying a controlled increased pressure (from 0.003 to 413 MPa). The total porosity was calculated as the total volume of intruded mercury at the maximum pressure divided by the bulk volume of the sample. The mean pore size was obtained from the curve of the cumulative intrusion volume versus pore size.

Analysis of (Insoluble and Soluble) Arabinoxylans and β-Glucans of Aleurone Fractions. Neutral sugars were quantified as anhydro-sugars determined by gas−liquid chromatography (DB 225 capillary column) of their alditol acetates obtained after sulphuric acid hydrolysis (1 M, 2 h, 100 °C) of the aleurone fractions.[27] For soluble compounds, a prior extraction in water was carried out, and the extracts were hydrolyzed in similar conditions (1 M, 2 h, 100 °C). All

Table 1. Physical Characterization of Native and Treated Aleurone Fractions[a]

	aleurone			
	native	ground	Xyn	Xyn+FAE
particle size (d_{50} μm)	191 ± 4c[b]	65 ± 1d	236 ± 4b[b]	423 ± 25a[b]
specific surface (m²/g)	0.02 ± 0.00bc[b]	0.20 ± 0.00a	0.03 ± 0.00b[b]	0.01 ± 0.00c[b]
water holding capacity (mL/g)	2.99 ± 0.07a	2.29 ± 0.01b	3.06 ± 0.1a	2.23 ± 0.1b
water binding capacity (g/g)	2.96 ± 0.04	1.20 ± 0.01	2.68 ± 0.03	1.13 ± 0.00
porosity (%)	62.5	62.0	80.7	59.5
Median pore diameter (μm)	53.1	15.0	92.0	104.2
total AX (g/100 g)	24.3 ± 0.1a	25.4 ± 0.8a	22.7 ± 0.4ab	21.4 ± 0.2b
ratio arabinose/xylose	0.37 ± 0.01b	0.37 ± 0.01b	0.38 ± 0.01b	0.40 ± 0.00a
insoluble AX (g/100 g)[c]	23.4 ± 0.1	24.2 ± 0.8	12.8 ± 0.5	3.8 ± 0.4
soluble AX (g/100 g)	0.89 ± 0.04c	1.21 ± 0.02c	9.9 ± 0.3b	17.7 ± 0.3a
ratio arabinose/xylose	0.80 ± 0.02a	0.82 ± 0.03a	0.42 ± 0.01c	0.35 ± 0.01b
AX intrinsic viscosity (mL/g)[d]	204 ± 5	207 ± 5	34 ± 2	17 ± 2
total β-glucans (g/100 g)	3.91 ± 0.05a	3.35 ± 0.03b	3.82 ± 0.13a	3.01 ± 0.13c
soluble β-glucans (g/100 g)	0.93 ± 0.14	0.98 ± 0.11	2.32 ± 0.12	2.84 ± 0.02
total FA (mg/g)	6.6 ± 0.4a[b]	6.2 ± 0.2b	6.36 ± 0.05ab[b]	5.04 ± 0.04a[b]
bound FA (mg/g)	6.48 ± 0.4[b]	6.02 ± 0.2	5.29 ± 0.06[b]	0.67 ± 0.2[b]
conjugated FA (mg/g)	0.09 ± 0.01[b]	0.13 ± 0.01	0.45 ± 0.02[b]	0.68 ± 0.2[b]
free FA (mg/g)	0.01 ± 0.00c[b]	0.01 ± 0.00c	0.62 ± 0.01b[b]	3.70 ± 0.03a[b]

[a]Characterization represented by mean particle size (d_{50} in μm), specific surface (m²/g), water holding capacity (mL/g), and water binding capacity (g water/g aleurone). The degradation of the aleurone cell wall is represented by the amounts of insoluble and soluble arabinoxylans (AX) (g/100 g) and β-glucans (g/100 g), the molecular size of arabinoxylans, and the proportion of monomer ferulic acid (FA) present in bound, conjugated, and free forms. The data are presented as mean ± standard deviation. A letter after a numerical value (in a line) indicates significant differences ($p < 0.05$) between the samples. Lines without letters mean that no statistical analysis was performed due to either analysis in duplicates or data obtained by calculating. [b]Data from our previous work.[22] [c]Calculated as total AX minus soluble AX. [d]Calculated for peaks eluting between 11.8−18.9 mL for native and ground aleurone or 15−21 mL for Xyn and Xyn+FAE treated aleurone.

analyses were performed in triplicate. The concentration of β-glucan was measured by using the Megazyme mixed-linkage β-glucan kit (Megazyme, Ireland). For the soluble β-glucan quantification, the aleurone fractions were first treated in NaOH 200 mM (50 mg/mL) at 20 °C for 2 h and then centrifuged 10 min at 10 000g (25 °C). The β-glucan concentration of the solubilized extract was measured by the same Megazyme kit as previously. All analyses were performed at least in duplicate.

Molecular Size of Water-Soluble Polymers. The size of WE-AX in the different wheat aleurone fractions was estimated using a high-performance size-exclusion chromatography (HPSEC) system with online molar mass and viscosity determinations. Wheat aleurone fractions (400 mg) were first suspended in water (2 mL) and stirred for 30 min at room temperature on a rotating wheel. After centrifugation (4 °C) supernatant (1 mL) was placed for 10 min in boiling water bath, then centrifuged, and filtered over a 0.45 μm membrane prior to injection. The samples were injected (50 μL) at room temperature on the HPSEC system comprising two Shodex OH-pack columns in series, first a SB HQ 805 and second a SB HQ 804, eluted at 0.7 mL/min with 50 mM NaNO₃ containing 0.02% NaN₃. Online molar mass and intrinsic viscosity determinations were performed at room temperature using a multiangle laser-light scattering (MALLS) detector (mini-Dawn, Wyatt, USA; operating at three angles: 41°, 90°, and 138°), a differential refractometer (ERC 7517 A) (dn/dc = 0.146 mL/g), and a differential viscometer (T-50A, Viscotek, USA). In addition the system included an UV detector (UV 2000, ThermoSeparationProducts, USA) working at 280 nm for the detection of proteins and phenolic rich fractions. ASTRA 1.4 (Wyatt, USA) and TRISEC softwares were used to determine the weight average molar mass (M_w) and the intrinsic viscosity [η], respectively.

Phenolic Acids (Total, Conjugated, and Free Forms) of Aleurone Fractions. The phenolics acids (ferulic (Z)-FA + (E)-FA, sinapic, p-coumaric, and FA dimers, including 8,5′-diFA, 8-O-4′-diFA, 8,5′benzodiFA, and 5,5′-diFA, and trimers) were determined in their total ester-linked, conjugated, and free forms. The extraction of total ester-linked phenolic acids from the aleurone fractions, as well as the solvent and parameters of the RP-HPLC system have been described

by Antoine et al.[8] The extraction of conjugated and free phenolics acids from aleurone fractions was measured with a methodology adapted from Hemery et al.[20] and described by Rosa et al.[28]

Metabolical in Vitro Colon Model. Metabolical in vitro colon model experiments were performed with the aleurone fractions according to Barry et al.[29] with the following modifications. For measurement of SCFA and phenolic acid metabolites, 100 mg (on dry weight basis) of wheat aleurone fractions was weighed in the fermentation bottles (50 mL), and hydrated with 2 mL of 0.11 M carbonate−0.02 M phosphate buffer at initial pH 6.9 with minerals as described in Barry et al.,[29] 1 day before inoculation. Human feces were collected from 4 healthy volunteers, who had not received antibiotics for at least 6 months and had given a written consent. The collection of fecal samples was performed with an approval and according to the guidelines given by the Ethical Committee of Technical Research Centre of Finland. Freshly passed feces were immediately taken in an anerobic chamber or closed in a container with an oxygen consuming pillow (Anaerocult Mini; Merck, Darmstadt, Germany) and a strip testing the anaerobiosis (Anaerotest; Merck, Darmstadt, Germany). Faecal suspension was prepared under strict anaerobic conditities. Equal amounts of faecal samples were pooled and diluted to a 12.5% (w/v) suspension, 8 mL of which was dosed to the fermentation bottles to obtain a 10% (w/v) final faecal concentration described previously by Aura et al.[30] The fermentation experiments were performed in triplicate, and a time course of 0, 2, 4, 6, 8, and 24 h was followed using the same inoculum for all aleurone fractions. Incubation was performed at 37 °C in tightly closed bottles with magnetic stirring (250 rpm). Faecal background was also incubated in the same conditions without addition of aleurone samples.

Analysis of Metabolites. Gas formation during fermentation was measured as described in Nordlund et al.[31] Briefly, 100 mg (on dry weight basis) of aleurone fractions was weighed in headspace bottles, hydrated with 2 mL of 0.11 M carbonate−0.02 M phosphate buffer at initial pH 6.9 with minerals as described in Barry et al.,[29] 1 day before inoculation, and inoculated with 8 mL of faecal suspension. Bottles were sealed with rubber stoppers in constant rotation of 150 rpm. The stoppers were punctured with an infusion needle connected to a
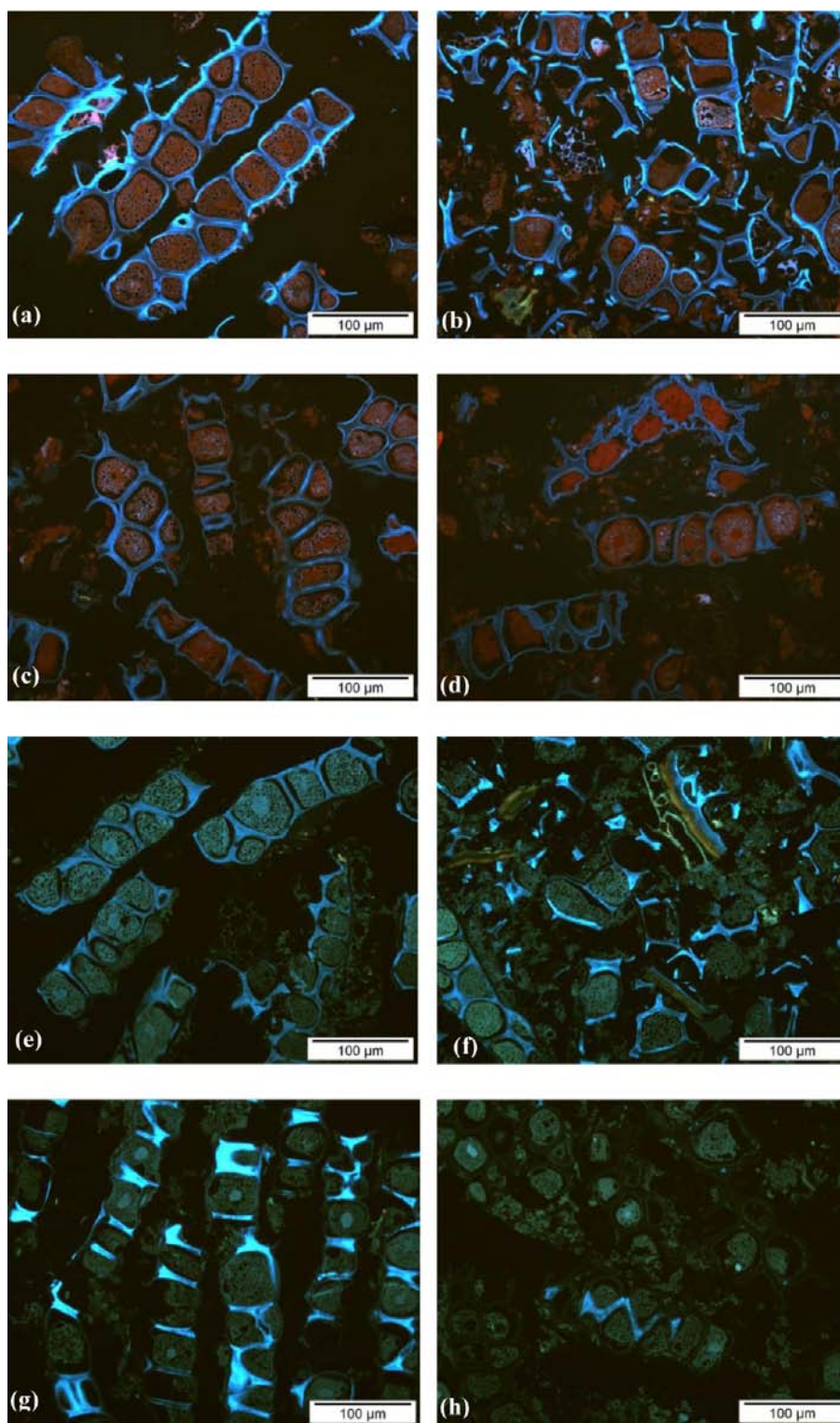
Figure 1. Microstructure of wheat aleurone fractions. (a−d) β-Glucans and aleurone cell walls were stained in blue and proteins in red (native (a), ground (b), Xyn (c), and Xyn+FAE (d) aleurone fractions). (e−h) Autofluorescence in aleurone cell walls mainly due to the aromatic structures of ferulic and p-coumaric acids (native (e), ground (f), Xyn (g), and Xyn+FAE (h) aleurone fractions).

pressure sensor and the devise recorded the gas pressure (Pa) in 20 min intervals. The measurement was continued for 26 h from the start of the incubation. SCFAs were analyzed by GC after diethyl ether extraction according to Aura et al.[30] The formation of SCFAs was expressed as a sum of acetic, propionic, and butyric acids. The relative proportions of the individual SCFAs were calculated from the averages of three replicates at time point 24 h. Phenolic acids were analyzed by

GC−MS instrument as described in Aura et al.[32] The phenolic acids were quantified with authentic standards. The standards benzoic acid (BA), 3-hydroxybenzoic acid (3-OHBA), 3-(4′-hydroxyphenyl)-propionic acid (4-OHPPA), and 3-(3′,4′-dihydroxyphenyl) propionic acid (3,4-diOHPPA) were products from Aldrich, (Steinheim, Germany). 4-Hydroxybenzoic acid (4-OHBA), 2-(3′-hydroxyphenyl)-acetic acid (3-OHPAc), and 2-(3′,4′-dihydroxyphenyl)acetic (3,4-
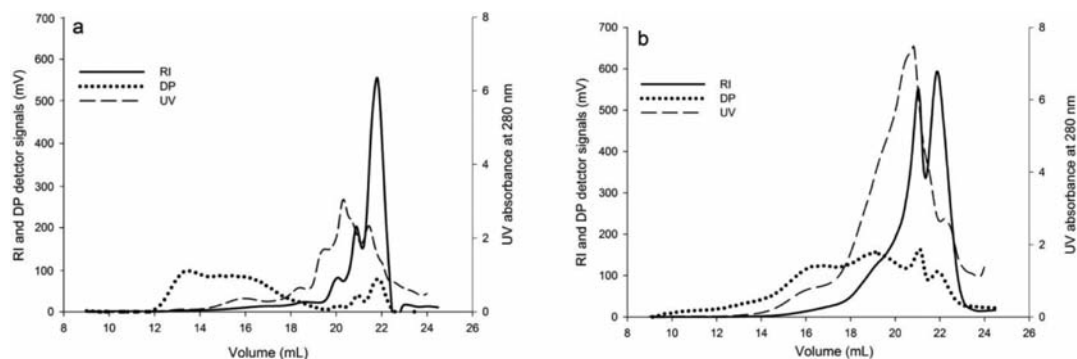
Figure 2. Elution profile of water-soluble material from (a) native and (b) xylanase-treated (Xyn) aleurone fractions analyzed by HPSEC. RI = refractometer index, DP = differential pressure.

diOHPAc) were purchased from Sigma (St. Louis, USA). 3-Phenylpropionic acid (3-PPA) and 3,4-dihydroxybenzoic acid (3,4-diOHBA), were from Fluka (Buchs, Switzerland), and 3-(3′-hydroxyphenyl)propionic acid (3OHPPA) was purchased from Alfa Aesar (Karlsruhe, Germany). 4-Methylcatechol (Aldrich, Steinheim, Germany), vanillic acid (3-methoxy-4-hydroxybenzoic acid; Fluka, Buchs, Switzerland)), 4-coumaric acid (Sigma, St.Louis, USA), gallic acid (Extrasynthése, Genay, France), and ferulic acid (Sigma-Aldrich, St. Louis, USA) were obtained as shown. The internal standard was 2-hydroxycinnamic acid (mainly trans; Aldrich Inc. H2, 280-9; 97%; St. Louis, USA). N-Methyl-N-trimethylsilyl-trifluoracetamide (MSTFA) from Pierce (Rockford, USA) and methoxyamine 2% hydrochloride in pyridine (MOX; Pierce, Rockford, USA) were used as the silylation reagents.

Statistical Analyses. The responses were measured in triplicates, and two-way ANOVA with repeated measures was used. Bonferroni adjustment was used to test the significances ($p < 0.05$) between the samples. The statistics were performed with MatLab Version R2008b. Significantly different response levels ($p < 0.05$) between aleurone fractions within a time point are indicated with different letters (a, b, c).

■ RESULTS

Structure of Native and Treated Aleurone Fractions. The native aleurone had 192 μm mean particle size (d_{50}) and 0.02 m^2/g specific surface. The mechanical treatment applied to the native aleurone reduced 3-fold its d_{50} and increased 10-fold its specific surface (Table 1). On the contrary, the enzyme (xylanase alone or associated with FAE)-treated aleurone fractions presented higher mean particles size (Table 1), especially when xylanase was associated with FAE ($d_{50} = 423$ μm). The physical structure of the aleurone fractions was visualized by microscopy (Figure 1). The native aleurone (Figure 1a) consisted mainly of the intact rows of aleurone cells (around 7−8 rows linked). The grinding clearly broke the aleurone cells, reducing the particle size (Figure 1b), but rows of aleurone cells remained after grinding. The physical structure of the enzyme-treated aleurone fractions (Figure 1c and 1d) was not pronouncedly modified and still presented many rows of cells linked. In these fractions, a release of protein from the aleurone cells (colored in red) was observed. The water holding capacity (WHC) of aleurone fractions was also affected by the treatments. The ground and the Xyn+FAE aleurone fractions presented similar WHC, which was lower than the WHC of the native aleurone and the Xyn aleurone (Table 1). A similar trend was observed for the water binding capacity (WBC) of the aleurone fractions (Table 1). The porosity of the ground and Xyn+FAE aleurone was similar to that of the native aleurone (about 60%), but the Xyn aleurone had higher porosity (80%).

The median pore diameter was higher in the Xyn and Xyn +FAE aleurone fractions (Table 1).

AX was present mainly in insoluble form, 96.3% and 95.2% in the native and ground aleurone fractions, respectively (Table 1). Both enzymatic treatments efficiently solubilized AX, as Xyn and Xyn+FAE aleurone fractions presented 43% and 82% of their AX in soluble form, respectively (Table 1). The degree of branching (ratio Arabinose/Xylose) was similar for all of the aleurone fractions (0.37), except for Xyn+FAE aleurone, which presented slightly higher value (0.40). Similar results were observed for β-glucans. Native and ground aleurone had similar amounts of total and soluble β-glucans (3.9 and 3.4 g β-glucan/100 g, being 24% and 29% in the soluble form, respectively). Both enzymatic treatments solubilized β-glucans (60 and 94% in the Xyn and Xyn+FAE aleurone fractions, respectively). The solubilization of AX and β-glucans in both enzymatically treated aleurone fractions was also noticed from the microscopic pictures (Figure 1c and d). The binding of Calcofluor reagent was reduced, indicating the degradation of β-glucans. It was also observed that cell walls became thinner and wrinkled, especially in the Xyn+FAE aleurone, which made it seem that the enzymatic treatment plasticized aleurone cell walls.

The water-soluble material of the different aleurone fractions was analyzed by HPSEC, and typical chromatograms are shown in Figure 2. Native and ground aleurone gave identical profiles, with a population eluting in the 12−18 mL elution range and associated with a strong viscosimetric signal (Figure 2a). These peaks were ascribed to high molecular weight AX and mixed linked β-glucans that exhibited an intrinsic viscosity of about 200 mL/g. Light scattering detector (not shown) indicated the presence of aggregates, and it was not possible to calculate a reliable molecular weight for these fractions. The integration of refractive index signal of these peaks indicated that they accounted for about 2% and 2.4% of the native and ground aleurone, respectively, which is in good agreement with chemical analysis for soluble AX and soluble β-glucans. Major peaks associated with strong UV and weak viscosimetric signals were observed in the 19−23 mL elution range and mostly corresponded to soluble proteins and low molecular weight compounds. The elution profile of enzyme-treated aleurone fractions (Figure 2b) were very different from the native one. The increase of the refractometric signal in the 15−21 mL elution range (and especially the 18−21 mL area, Figure 2b) demonstrated that AX were strongly solubilized by the xylanase treatment. Although the overall elution profile was close to the Xyn-treated aleurone fraction, a slightly higher increase in the intensity of the peaks in the 18−21 mL area was observed for

the Xyn+FAE-treated sample, indicating a higher solubilization extent for AX. A viscosimetric signal was associated with the refractive signal, indicating that the enzyme action ended more probably with polymers rather than oligomers. The strong 280 nm UV signal (Figure 2b) associated with these peaks indicated the presence of phenolic compounds (likely FA) and/or proteins. The calculation of the intrinsic viscosity of the polymers eluting in the 15−21 mL range (Table 1) indicated that low molecular weight polymers were obtained and clearly indicated that Xyn+FAE treatment produced smaller polymers than xylanase alone.

The native and ground aleurone fractions had FA mainly esterified to polysaccharides (98.5% and 97.7%, Table 1). The enzymatic treatments efficiently released FA from the polysaccharides in conjugated and free forms. The Xyn and Xyn+FAE aleurone fractions had, respectively, 17% and 87% of their FA in conjugated plus free forms (Table 1). The autofluorescence observed in Figure 1e−h was caused by the ferulic acid that was bound to arabinoxylan in the aleurone cell walls. A loss of autofluorescence can be observed when FA is released in the medium. This was partly observed in the Xyn (Figure 1g) and evident in the Xyn+FAE (Figure 1h) aleurone fractions, where the autofluorescence was detected only in the inner parts of the cell walls between aleurone cells, confirming the higher solubilization of FA in these two fractions.

Production of Colonic Metabolites during *in Vitro* Fermentation. The behavior of wheat aleurone fractions were compared in the metabolical batch *in vitro* colon model. This model used a pooled microbial population as a source of microbial enzymes for degradation of fiber complex and conversion of microbial metabolites.

The gas evolution in all of the four fermented aleurone fractions showed significant difference from the faecal control already at the 2 h time point (1.5×10^5 Pa) and remained higher (2.2×10^5 Pa) until 26 h (Figure 3). From 2 to 8 h, all of the aleurone fractions presented similar gas evolution patterns, and no significant differences were observed between the fractions. Native aleurone presented similar gas production trend as the other fractions until 8 h, but from 8 to 22 h the increase in gas production was higher than in the others. In
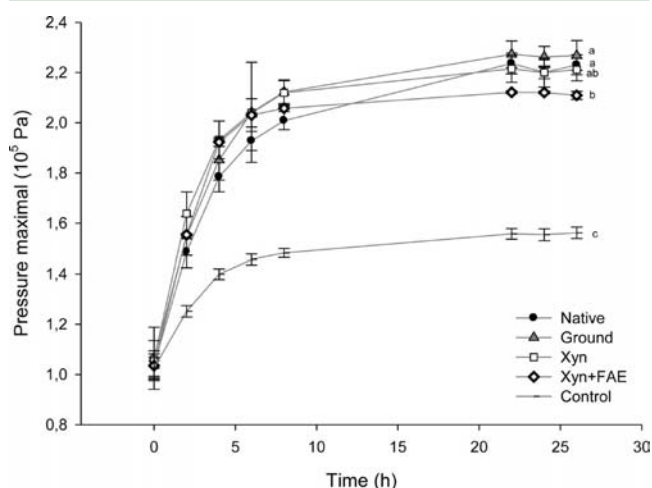
contrast, the production of gases in the Xyn+FAE remained constant from 8 to 26 h. The gas production rate of the ground and Xyn increased until 22 h, and after that the rate was stable. Thus, lower gas formation by Xyn+FAE aleurone was observed especially from 22 to 26 h, but it was still significantly higher than in the faecal control.

The total SCFA produced during the fermentation of aleurone fractions is presented in Figure 4a. At 2 h, the SCFA production by all of the aleurone fractions was already significantly higher than in the control. At 4 h, the ground aleurone produced a significantly ($p < 0.05$) higher amount of SCFA in comparison to the other fractions. From 6 to 24 h, both enzymatically treated fractions, especially the Xyn aleurone, produced lower SCFA compared with the native and ground aleurone. The ratio butyric/total SCFA was calculated. At 24 h, it was similar for the native (20.6 ± 0.1%), ground (19.7 ± 0.4%), and Xyn (19.7 ± 0.4%) aleurone fractions. However, the Xyn+FAE aleurone presented lower ratio butyric/total SCFA (17.6 ± 0.2%, $p < 0.05$). The production of each SCFA presented the same behavior as their sum (data not shown). The production of branched chains (2-Me-propionic and 3-Me-butyric acids) by all of the fractions was similar at 0 and 2 h (Figure 4b), but from 4 to 24 h the native and ground aleurone had greater production of branched chains. The two enzymatically treated aleurone fractions did not differ from the control until 8 h, presenting slightly higher production of branched chain only at 24 h.

In both enzyme-treated aleurone fractions, FA was rapidly metabolized from the beginning of the fermentation, as shown by its rapid decrease after 2 h (Figure 5a). In contrast, the FA from native and ground aleurone fractions, mainly esterified to AX, was slowly released from the matrix (Figure 5a). Its amounts only decreased from 8 h of fermentation (Figure 5a). A similar trend was observed for *p*-coumaric acid (data not shown). The formation of 3,4-dihydroxyphenylpropionic acid (3,4-diOHPPA) (Figure 5b) in all of the aleurone fractions was fast and transitory, as it was detected at 2 h. The 3,4-diOHPPA was probably further dehydroxylated to 3-hydroxyphenylpro-pionic acid (3OHPPA) (Figure 5c).[21,33] The formation of 3OHPPA was intensive in the Xyn and Xyn+FAE fractions at 2 and 4 h in comparison to native and ground aleurone fractions. The next metabolite, 3-phenylpropionic acid (3PPA), was formed at similar concentration in the native and ground aleurones and in the control, but both enzymatic aleurone treated fractions had significantly greater levels from 4 to 6 h (Figure 5d). The metabolite 3PPA increased concomittantly to the disappearance of 3OHPPA after 4 h even though 3PPA is not a specific metabolite.[31] Furthermore higher amounts of 3PPA were found only in the enzymatic aleurone treated fractions. 3,4-Dihydroxyphenylacetic acid, formed from left units of diferulates,[34] disappeared after 2 h (Figure 5e) probably because it was converted to 3-hydroxyphenylacetic acid (Figure 5f), which has been shown to occur for quercetin metabolites[35] in the same model. None of the treatments (mechanical or enzymatic) applied on aleurone enhanced significantly the conversion of 3,4-dihydroxyphenylacetic and 3-hydroxyphenylacetic acids, which were minor metabolites compared with the corresponding phenylpropionic acids.

Benzoic acid was also produced during fermentation in all aleurone fractions, but it was higher than control only at 0 and 2 h. The metabolite of benzoic acid, 4-hydroxybenzoic acid, had a transient appearance, as its concentration increased until 4 h and then decreased to reach the control baseline. Xyn+FAE-
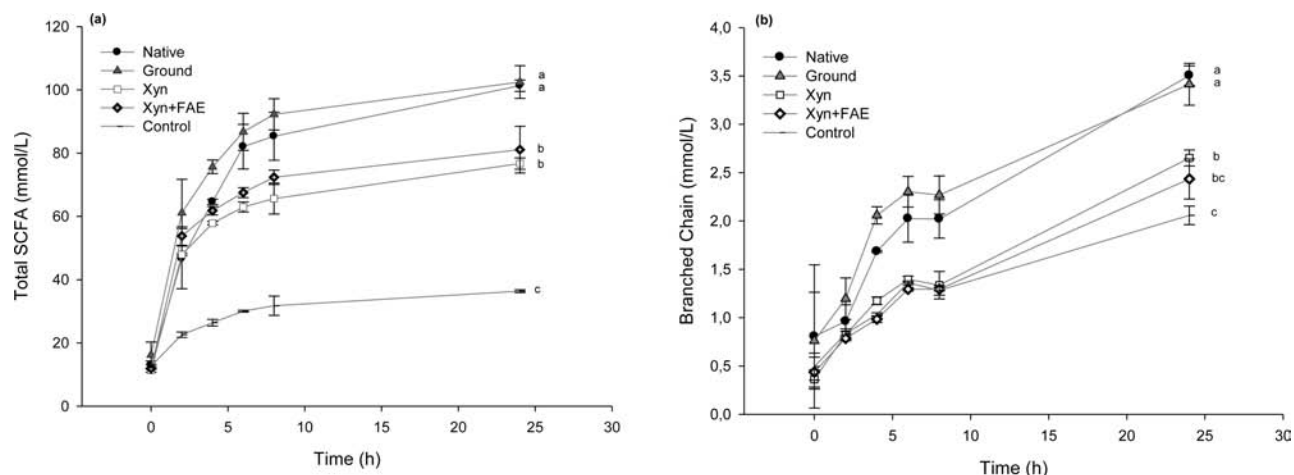

Figure 3. Time course of gas pressure (measured as maximal pressure, Pa) developed by human faecal microbiota from the native and processed wheat aleurone fractions. Different letters (a, b, c) indicate significant differences between samples at 26 h ($p < 0.05$).

Figure 4. Production of short-chain fatty acids (mmol/L) developed by *in vitro* fermentation (0−24 h) of native and processed wheat aleurone fractions. Total SCFA comprises the sum of butyric, propionic, acetic, and valeric acids (a). The branched chain comprises the sum of 2-Me-propionic and 3-Me-butyric acids (b). Different letters (a, b, c) indicate significant differences between samples at 24 h ($p < 0.05$).

treated aleurone had a tendency to produce less 4-hydroxybenzoic acid than the other aleurone fractions, being significantly lower at 4 and 8 h. The 3,4-dihydroxybenzoic acid was formed in the beginning of fermentation (significantly higher concentration in all aleurone fractions than control at 0 and 4 h), but its concentration decreased over time most probably due to its dehydroxylation to 3- or 4-hydroxybenzoic acids or less likely due to its decarboxylation to 3,4-dihydroxytoluene which increased, from 2 to 24 h. No major differences were observed in the formation of benzoic acid and its derivatives by applying mechanical or enzymatic treatments in the aleurone. The formation of these minor metabolites (<10 μM) is displayed in the Supporting Information.

Enterodiol is an intermediary mammalian lignan metabolite. Independently of the aleurone fractions considered, it appeared transiently at 2 h, after which its concentration decreased to be totally metabolized after 8 h (Table 2). No differences in enterodiol were observed between the aleurone fractions, except for the higher concentration in the Xyn aleurone fraction at 4 h. The enterolactone (metabolized from enterodiol) was formed already at the 0 h time point, and its concentration increased until 24 h in all of the aleurone fractions (Table 2). The Xyn+FAE aleurone had enterolactone accumulation lower than that of the other aleurone fractions.

In the beginning of the fermentation period, the pH of the faecal suspensions containing the aleurone fractions was 7.3, except in the Xyn+FAE aleurone, which had pH 7.0 at t = 0 h. In all of the aleurone fractions pH decreased rapidly up to 2 h and then slowly up to 24 h. After 2 h, both enzymatically treated aleurone fractions had significantly lower pH (6.7 and 6.6 in Xyn and Xyn+FAE, respectively) compared to that of native and ground fractions (pH = 6.9). All of the fractions reached similar pH (6.4) at the end (24h) of the fermentation trial, except the native aleurone (pH = 6.3). The pH evolution is displayed in the Supporting Information.

■ DISCUSSION

Effect of Disintegration of Aleurone by Dry Processing on Its Fermentation Pattern. In the present work, dry processing (grinding) applied on aleurone was not conclusively related to enhanced SCFA production during wheat aleurone fermentation. SCFAs are important end-products of microbial

fermentation. Among SCFA, butyrate has been reported to be the compound that mostly contributes to the colonic health.[36] The ground aleurone presented a slight tendency to produce more gases and SCFA than the native aleurone, but the total production volume and also the ratio of butyrate/total SCFA were statistically similar. The mechanical treatment applied to the native aleurone efficiently reduced 1/3 of its particle size and increased its specific surface (10-fold), enhancing the exposition of aleurone cell walls.[22,37] Stewart and Slavin[38] and Jenkins et al.[39] noticed that fine wheat bran produced higher SCFA and butyrate concentrations than large particles. However, Connolly et al.[40] observed an opposite effect for oat flakes. The higher amount of small particles in the ground aleurone decreased its water holding and binding capacities. This phenomenon was already observed by Auffret et al.[41] when grinding wheat bran. The ground aleurone also presented smaller pore size than native aleurone, which can affect its fermentability.[42] Thus, even if the ground aleurone had higher specific surface, which should increase the accessibility of the microbiota to the substrate, its lower hydratation properties (WHC and WBC) have probably decreased its fermentability.

The ground aleurone also had a tendency to produce more branched SCFA (formed from proteolytic fermentation), but this was not significantly different from the native aleurone. In the native and ground aleurone fractions, the highest amount (3.5 mmol/L at 24 h) of branched SCFA represented only 3.5% of the total straight SCFA. A similar amount was reported by Mateo Anson et al.,[43] and this value was within the range of those found in the human proximal and distal colons (3.4% and 7.5% of the total SCFA, respectively).[44]

During the fermentation of native and ground aleurone fractions, the amount of FA slightly increased in the inoculum preparation, probably due to the release of FA from the matrix by some bacterial esterases present in the human microbiota.[45] The dry processing (grinding) applied on aleurone reduced its particle size (to 1/3) but did not affect the release or the metabolism of FA during the fermentation period. This result was expected as the amount of insoluble fibers and bound FA in the ground aleurone was predominant and very similar to the value measured on the native aleurone. A slow release of FA from polysacharides could be however very interesting as it allows a continuous presence of a low concentration of
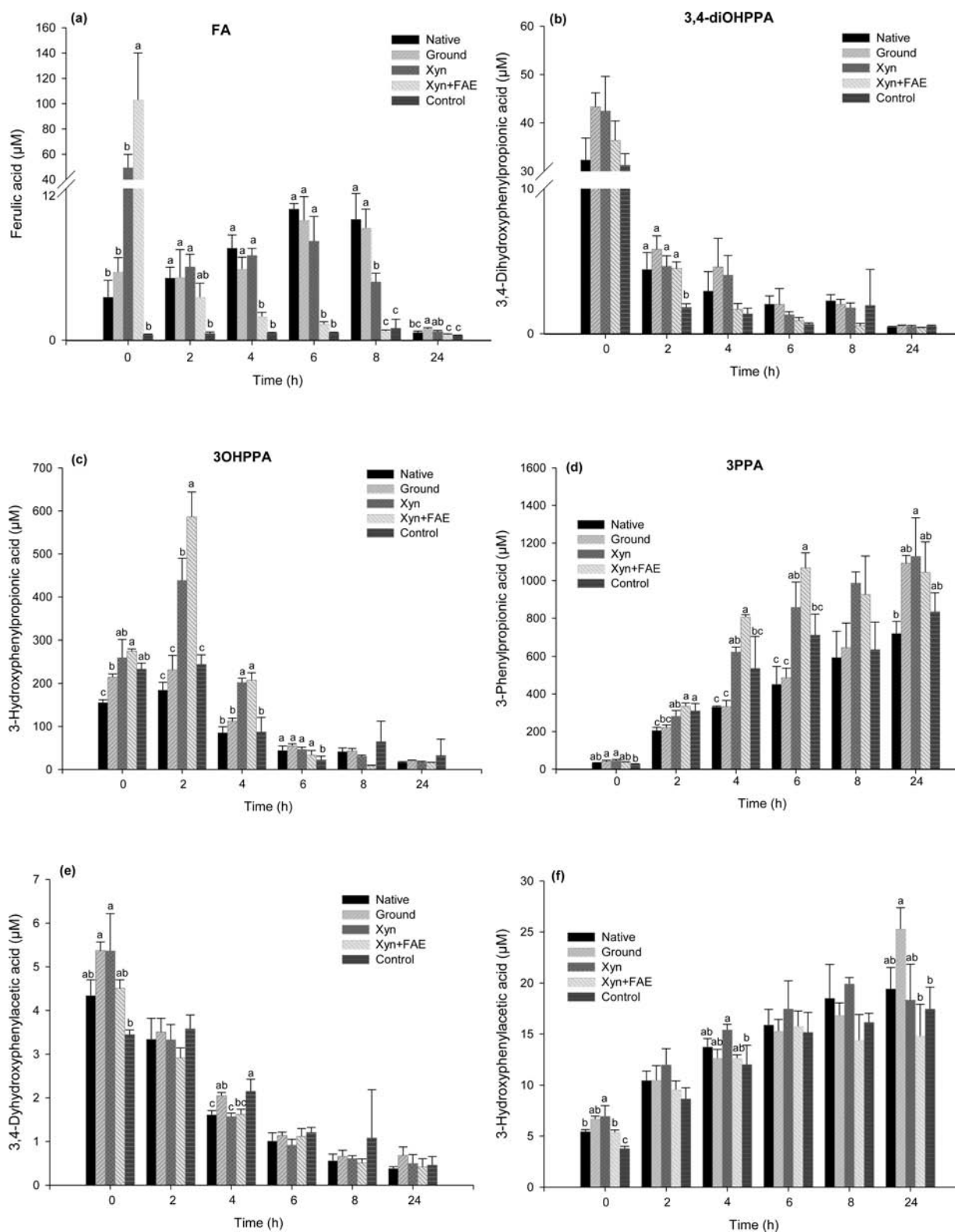
Figure 5. Formation of phenolic microbial metabolites (μM) developed by human faecal microbiota from native and processed wheat aleurone fractions. (a) Ferulic acid (FA). (b) 3,4-Dihydroxyphenylpropionic acid (3,4-diOHPPA). (c) 3-Hydroxyphenylpropionic acid (3OHPPA). (d) 3-Phenylpropionic acid (3PPA). (e) 3,4-Dihydroxyphenylacetic acid. (f) 3-Hydroxyphenilacetic acid. Different letters (a, b, c) indicate significant differences between samples within a time point ($p < 0.05$).

phenolic compounds in the colonic tract, which may have a high potential benefit.[46,47]

Effect of Enzymatic Aleurone Disintegration on Its Fermentation Pattern. Xylanase has already been used as a

pretreatment to solubilize AX of wheat bran.[48] Excepting our previous work,[22] there was until now no data available on the enzymatic treatment of wheat aleurone. In the present study, the effect of Xyn alone or associated with FAE on aleurone

Table 2. Formation of Enterodiol and Enterolactone (μM) during in Vitro Fermentation (0–24 h)[a]

time (h)	enterodiol (μM)					enterolactone (μM)				
	native	ground	Xyn	Xyn+FAE	control	native	ground	Xyn	Xyn+FAE	control
0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.51 ± 0.02a	0.57 ± 0.01a	0.53 ± 0.09a	0.52 ± 0.05a	0.27 ± 0.03b
2	0.36 ± 0.03ab	0.43 ± 0.03ab	0.42 ± 0.04ab	0.47 ± 0.07a	0.29 ± 0.04b	0.82 ± 0.13	0.77 ± 0.10	0.81 ± 0.08	0.72 ± 0.08	0.62 ± 0.11
4	0.22 ± 0.05bc	0.24 ± 0.01b	0.38 ± 0.03a	0.25 ± 0.03b	0.14 ± 0.04c	1.03a ± 0.11b	0.99 ± 0.09ab	1.17 ± 0.08ab	0.73 ± 0.11b	0.81 ± 0.11b
6	0.08 ± 0.02ab	0.16 ± 0.03a	0.13 ± 0.05ab	0.15 ± 0.04ab	0.06 ± 0.01b	1.32 ± 0.27	1.35 ± 0.03	1.41 ± 0.30	0.94 ± 0.04	0.97 ± 0.09
8	0.00 ± 0.00b	0.09 ± 0.02a	0.00 ± 0.00b	0.00 ± 0.00b	0.03 ± 0.05ab	1.40 ± 0.31ab	1.45 ± 0.13ab	1.80 ± 0.12a	0.97 ± 0.23b	1.05 ± 0.09b
24	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	1.50 ± 0.69	2.36 ± 0.34	2.11 ± 0.37	1.25 ± 0.22	1.37 ± 0.15

[a] A letter after the numerical value indicates significant differences ($p < 0.05$) between the samples within a time point.

fermentation was presented. The Xyn+FAE aleurone presented the lowest production of gases. As Xyn+FAE aleurone had a higher amount of soluble AX (82%) and β-glucans (94%), it was expected to be more easily degraded than the other aleurone fractions.[49] However, the Xyn+FAE aleurone presented a physical structure that could make its degradation more difficult. Its mean particle size was indeed very high (423 μm). This was probably due to an agglomeration of the particles occurring during the freezing/freeze-drying treatment applied after enzymatic treatment. Furthermore, the Xyn+FAE aleurone had low WHC and WBC, which can be explained by the intensive solubilization of dietary fibers, as WU-AX is known to bind more water than WE-AX.[50] The crystallinity of the Xyn+FAE fraction might also have been affected by the higher solubilization of fibers. This structural parameter could reduce its degradability, as already observed by Guillon et al.[42] when studying the fermentability of a sugar beet fiber. Furthermore, the high fiber solubilization could have modified its behavior during the freeze-drying step. Generally, freeze-drying increases the porosity of samples (as observed with Xyn aleurone having 80% porosity). However, this was not observed for Xyn+FAE aleurone, which had similar porosity as the native aleurone (around 60%). Thus, we can therefore reasonably assume that the physical structure of the Xyn+FAE aleurone fraction impeded the access of microbiota to their substrate and delayed the fermentation of this fraction.

The production of SCFA during the *in vitro* fermentation of aleurone was not improved by any of the enzymatic treatments. The characteristics of a cereal matrix (chemical composition and physical/structural characteristics) that can affect the production of SCFA are still not fully elucidated. For example, soluble rye bran extract showed faster fermentation and greater SCFA production than the insoluble residue of rye bran.[51] However, some of the studies on cereal matrices did not observe any differences between the SCFA production from insoluble and soluble fibers. The SCFA production during *in vitro* fermentation was therefore not affected by the higher fiber solubilization level of enzymatically treated wheat bran,[52] the differences in the molecular weight of AX and β-glucans,[17,53] or the soluble pentosan contents of bioprocessed wheat bran.[43] Besides the difference in solubility, the higher degree of branching of AX fiber can decrease its fermentation.[11] In the present work, the soluble AX from Xyn and Xyn+FAE aleurone and insoluble AX from native aleurone presented a similar degree of branching illustrated by the ratio A/X (being even higher for Xyn aleurone). Thus, even if the degree of solubilization and polymerization of AX was appropriate in the enzymatically treated fractions of aleurone to enhance its susceptibility for degradation by the intestinal microbiota, the physical state of the fraction impeded the access of the microbiota and retarded the fermentability of the fibers.

The enzymatic treatment used to degrade the aleurone structure was efficient to release FA in its conjugated and free forms (up to 87%). The release of FA in conjugated and free forms influenced its colonic metabolism. The fast decrease of FA (between 0 and 2 h) on Xyn and even faster on Xyn+FAE aleurone fractions indicates its fast metabolism by colonic microbiota. Enzyme-treated aleurone fractions also presented greater amounts of 3OHPPA and 3PPA. A recent study showed that the colonic metabolites derived from FA, such as 3OHPPA, could reduce prostanoid production in cells, indicating possible anti-inflammatory properties.[54] Even if FA was metabolized at a higher rate in the enzyme-treated aleurone

fractions than in the native one, the time course of FA metabolite formation was similar for all of the aleurone fractions in the current study, which is in agreement with Braune et al.[35] and Mateo Anson et al.[21] Moreover, large qualitative changes were not expected as the aleurone fractions presented similar composition.

In all aleurone fractions, benzoic acid, 3,4-dihydroxybenzoic, and 3,4-dihydroxytoluene were detected in statistically identical and much lower concentration than others FA metabolites. These metabolites can be derived from many compounds: directly from vanillic acid but also by β-oxidation of phenylpropionic acids.[55] Enterolactone can be formed either from enterodiol or directly from matairesinol.[56] The lower concentration of enterolactone in Xyn+FAE aleurone fraction may be due to the inhibition of its production by the low pH caused by the high amount of soluble phenolics in this fraction, as already observed for rye bran fractions in the same metabolical *in vitro* colon model.[57]

In conclusion, this *in vitro* gut fermentation study demonstrated that the structure of wheat aleurone strongly affected its microbial metabolism, i.e., the formation of SCFA, gases, and phenolic metabolites. Decrease of the particle size and concomitant increase of its specific surface area had a tendency to increase the production of total gas and SCFA by enhancing the microbial accessibility. The higher solubilization of cell-wall fibers, notably AX, accompanied or not by the release of its phenolic co-passenger FA did not increase the formation of SCFA in our study. Physical parameters (particle size, WHC, WBC, and porosity) are more suggested to affect the fermentation behavior of these enzymatically degraded fractions. The benefits of enzymatic aleurone disintegration were the high production of conjugated and free FA, which promoted FA colonic metabolites (notably phenylpropionic acids). Production of bioavailable FA from aleurone was also previously shown to improve the inhibition of lipid peroxidation in the early phase of digestion.[22] Therefore, processing aleurone in order to release bioactive compounds can promote beneficial effects in both the upper and lower part of the digestive tract. Enzymatically treated aleurone fractions could also potentially act as prebiotics, as AX with lower molecular size can stimulate the growth of bifidobacteria and lactobacilli in colon.[17,52] The present study related the colonic fermentability of structurally modified aleurone, contributing to elucidate the role of cereal processing on gastrointestinal events.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Evolution of pH and formation of benzoic acid derivatives (μM) during the *in vitro* fermentation (0–24 h) of aleurone fractions. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ACKNOWLEDGMENTS

■ ABBREVIATIONS

AX, arabinoxylans; FA, ferulic acid; FAE, feruloyl esterase; SCFA, short-chain fatty acids; WHC, water holding capacity; WBC, water binding capacity; WE-AX, water extractable arabinoxylans; WU-AX, water unextractable arabinoxylans; 3OHPPA, 3-hydroxyphenylpropionic acid; 3,4-diOHPP, 3,4-dihydroxyphenylpropionic acid; 3PPA, 3-phenylpropionic acid

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