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Metabolite Production during in Vitro Colonic Fermentation of Dietary Fiber: Analysis and Comparison of Two European Diets

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ABSTRACT: Metabolite production and antioxidant released during colonic fermentation of naturally occurring dietary fiber (DF) from two European diets (Mediterranean and Scandinavian) were determined. With this aim, DF and associated components were isolated from both whole diets, as well as from cereals and fruits and vegetables comprising the diets. DF was used as substrate for colonic fermentation in a dynamic in vitro model of the colon, samples were collected, and fermentation metabolites were analyzed. Statistical differences between samples were observed in the concentrations of short-chain fatty acids and ammonia and in the ratio acetate/propionate/butyrate. Whole grain cereal DF generated a larger amount of propionate than refined flour cereal DF. Fruit and vegetable DF generated higher amounts of butyrate than cereal DF. Most antioxidant compounds were released from DF during in vitro colonic fermentation. It is concluded that different sources of DF may play a specific role in health maintenance mediated by metabolites produced during colonic fermentation.

KEYWORDS: short-chain fatty acids, ammonia, dietary fiber, colonic fermentation, Mediterranean diet, Scandinavian diet

INTRODUCTION

The health benefits of dietary fiber (DF) have been widely studied, starting with the description of its bulking effect[1] to novel proposals in glycemic response regulation,[2] satiety capacity,[3] and prebiotic activity[4] or as a carrier of antioxidant compounds.[5] Metabolomic studies have demonstrated that some DF health benefits are mediated by metabolites produced during gut bacterial fermentation.[6]

Among the metabolites generated during colonic fermentation with potential health effects, short-chain fatty acids (SCFA) have been the focus of a large number of studies. Protective properties for the colonic epithelium have being attributed to butyrate,[7] which is used as the main energy source by colonocytes[8] and plays a role as an inflammatory and pain perception modulator[8,9] and in counteracting oxidative stress at the epithelium level.[10] On the other hand, propionate has been shown to have a promising effect on hepatic lipogenesis regulation in animal models[11] and a modulatory effect of hormones implicated in appetite and energy intake regulation produced in the intestine[12] and adipose tissue.[13] However, not all metabolites produced during colonic fermentation may be associated with health benefits, and some of them may have deleterious health effects. Ammonia in low concentrations alters intestinal mucosa, promotes tumor cell growth, and facilitates virus infection.[14,15]

The analysis of microbiota metabolites derived from DF colonic fermentation is a challenge for nutritional science. Several intervention studies had used fecal samples, as a noninvasive procedure, as an approximation to evaluate colonic metabolite production. However, due to the difficulty of sampling the content of the human colon in vivo (especially the proximal colon), human studies addressing in situ metabolite production during colonic fermentation are scarce. Urine biomarkers produced exclusively by the microbiota were proposed as a noninvasive methodology to have a partial view on changes in microbiota metabolism.[16] An accurate alternative was proposed to profile metabolite production by using stable isotope labeled substrates such as [U-^{13}C]-glucose[17] and starch.[18] Interestingly, this methodology allows the prediction of the conversion of substrates into metabolites, analyzing the ^{13}C incorporation.[19] Combining this technology with 16S rRNA-based stable isotope probing, it is possible to identify the bacterial groups involved in the fermentation.[18,19] This accurate methodology has a wide applicability in studies of physiological benefit associated with synthetic or extracted polymers. Most (cited) studies in the literature analyze the fermentative properties of single dietary fiber sources, whereas the existing knowledge concerning the fermentative properties of naturally occurring DF present in whole diets remains scarce.

DF daily intake is relatively low in most developed countries, and fiber enrichment of processed food is a strategy to reach the daily recommendations. To facilitate the labeling procedure, the Codex Alimentarius commission, comprising FAO and WHO experts, had proposed a guideline for nutritional claims (Alinorm 09/32/26) in which a new official definition of DF was included, as follows: *DF means carbohydrate polymers with ten or more monomeric units, which are not hydrolyzed by the endogenous enzymes in the small intestine of humans.*[20]

This definition acknowledges the difference between naturally occurring fiber and synthetic or extracted polymers. For the two latter a physiological benefit should be demonstrated first, to be

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considered as DF. Naturally occurring fiber has associated physiological benefits, as demonstrated by epidemiological evidence. However, naturally occurring DF differs in amount, composition, structure, and associated compounds in different foodstuffs, such as fruits, vegetables, and cereals, and therefore the physicochemical, fermentative, and bulking properties of these foodstuffs would be different. It would thus be necessary to analyze these different DF sources according to their health-related effects, for example, on colonic metabolite spectra.

Therefore, in this study we aimed to investigate the fermentative properties of the DF from two whole European diets, from which DF and associated compounds [present in these diets from Murcia (Spain) and Copenhagen (Denmark)] were analyzed in a previous study.[21] In the present work we used a combination of in vitro methodologies as an approach to evaluate the health-related colonic metabolite spectra and concentrations produced by the human colonic microbiota during the fermentation of naturally occurring DF in these diets.

MATERIALS AND METHODS

Chemicals. *Dietary Fiber by Indigestible Faction Methodology.* Pancreatin from porcine pancreas (P3292), lipase (EC 3.1.1.3; L-3126), bile acids from porcine bile extract (B-8631), and α-amylase (EC 3.2.1.1) were purchased from Sigma-Aldrich (Steinheim, Germany); pepsin (EC 3.4.23.1) from porcine stomach mucosa (≥2000 units/mg protein) was purchased from Merck (Darmstadt, Germany); and amyloglucosidase from *Aspergilllus niger* (EC 3.2.1.3) was purchased from Roche Diagnostics (Barcelona, Spain).

In Vitro Colonic Fermentation and Microbial Metabolite Analysis. Pectin, xylan, arabinogalactan, amylopectin, casein, and vitamin mix were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands); starch and cysteine·HCl were obtained from BDH (Amsterdam, The Netherlands); Tween, $K_2HPO_4 \cdot 3H_2O$, NaCl, $FeSO_4 \cdot 7H_2O$, $MgSO_4 \cdot 7H_2O$, and $CaCl_2 \cdot 2H_2O$ were obtained from Merck (Amsterdam, The Netherlands); and bactopentone and ox bile were obtained from Oxoid (Haarlem, The Netherlands). All of the chemicals were of the highest chemical purity. Antioxidant associated compounds were extracted with acetone, 1-butanol, ethanol, and methanol that were purchased from Panreac Química S.A. (Madrid, Spain). These reagents were of analytical grade. Gallic acid was obtained from Sigma-Aldrich Química (Madrid, Spain).

Dietary Fiber Samples. Estimates of plant food consumption in the selected diets from Murcia (Spain) and Copenhagen (Denmark) were based on the European Prospective into Cancer and Nutrition (EPIC) study publications.[22,23] Table 1 summarizes the plant-derived daily intake estimated for each diet. Foods were locally acquired in Danish and Spanish markets, and the edible portion of each plant food as eaten (raw or cooked) was freeze-dried and milled. In the fruits and cereals groups, portions were kept as described by the intake data[22,23] using in the diet of Copenhagen brown bread ("Schwarzbrot"; Bolderslev, Denmark/Kohberg Brod A/S) and refined wheat flour bread ("Pan rustico"; S.L. Madrid) in the diet from Murcia. Diet nutritional characteristics were described previously.[21] DF was determined according to the method described by Saura Calixto[24] with some modifications to increase the amount of DF isolated per run. Briefly, 9 g of freeze-dried samples (whole diets or their starchy food or nonstarchy food components) was digested in subsequent steps simulating physiological conditions: (i) pepsin (0.6 mL of a 300 mg/mL solution in 0.2 M HCl−KCl buffer, pH 1.5, 40 °C, 1 h); (ii) pancreatin (3 mL of a 5 mg/mL solution in 0.1 M phosphate buffer, pH 7.5, 37 °C, 6 h); (iii) lipase (3 mL of a 7 mg/mL solution in 0.1 M phosphate buffer, pH 7.5, 37 °C, 6 h); (iv) porcine bile extract (3 mL of a 17.5 mg/mL solution in 0.1 M phosphate buffer, pH 7.5, 37 °C, 6 h); (v) α-amylase (3 mL of a 120 mg/mL solution in 0.1 M Tris−maleate buffer, pH 6.9, 37 °C, 16 h); and (vi) 300 μL of amyloglucosidase (45 min, 60 °C). Samples were transferred to dialysis tubes (12000−14000 molecular weight cutoff, Visking dialysis tubing; Medicell International Ltd., London, U.K.) and dialyzed against water for 48 h at 25 °C to remove digested compounds. Dialysis retentate was collected and freeze-dried to be used as fermentation substrate. DF intake estimation is summarized in Table 1. A detailed description of DF constituent analysis was given previously.[21]

Table 1. Origin of Consumption and Dietary Fiber Intake in the Diets from Murcia and Copenhagen

	Murcia (g/day/person)	Copenhagen (g/day/person)
Diet-Derived Food Daily Consuption[a]		
starchy foods	**318.28**	**373.84**
cereal-derived products	215.88	289.67
potatoes	75.15	83.03
legumes	27.25	1.14
nonstarchy foods	**651.66**	**291.28**
vegetables	247.57	119.55
fruits	401.74	164.65
cocoa products	2.35	7.14
Dietary Fiber Intake Estimation[b]		
insoluble DF	**42.83**	**32.03**
NSP	12.20	8.47
resistant starch	3.74	4.04
insoluble resistant protein	16.12	12.91
Klason lignin and minerals	10.77	6.61
soluble DF	**15.72**	**13.10**
NSP	13.52	11.33
soluble resistant protein	2.20	1.77
associated antioxidant compounds	**3.75**	**2.62**

[a] Data calculated on the basis of the EPIC study.[22,23] [b] Data from Tabernero et al.[21]

In Vitro Colonic Fermentation. In vitro colonic fermentation of DF was performed by simulating human large intestinal conditions in the TIM-2 model. Details of this model are summarized elsewhere.[25−27] Each unit was filled with simulated ileal efflux media (SIEM) containing (g/L) 4.7 pectin, 4.7 xylan, 4.7 arabinogalactan, 4.7 amylopectin, 23.5 casein, 39.2 starch, 17 Tween 80, 23.5 bactopeptone, 2.5 $K_2HPO_4 \cdot 3H_2O$, 4.5 NaCl, 0.005 $FeSO_4 \cdot 7H_2O$, 0.5 $MgSO_4 \cdot 7H_2O$, 0.45 $CaCl_2 \cdot 2H_2O$, 0.4 bile, and 0.4 cysteine·HCl, plus 1 mL of a vitamin mixture containing (per L) 1 mg of menadione, 2 mg of D-biotin, 0.5 mg of vitamin B12, 10 mg of pantothenate, 5 mg of nicotinamide, 5 mg of *p*-aminobenzoic acid, and 4 mg of thiamin.[25] The system was inoculated with a standardized fecal microbiota derived from eight healthy volunteers as described previously.[25] In brief, feces from eight volunteers were mixed in an anaerobic cabinet, and 200 g was used to inoculate a fermentor. Semicontinuously SIEM was added to the microbiota for 40 h, after which samples were aliquoted and snap-frozen in liquid nitrogen as described before.[25] In this way a standardized microbiota was obtained that contained all of the microbial species present in fecal samples [similarity with fecal material 82.5% based on a phyolgenetic microarray analysis, 85% based on DGGE; Simpson's index of diversity 175 and 148 for feces and standardized microbiota, respectively[28]] and was of a quantity sufficient to do multiple experiments with the same starting microbiota. After 16 h of stabilization in TIM-2, during which SIEM was fed,[28] the system was starved for approximately 2 h to allow

full fermentation of all fermentable substrates from the SIEM, which would otherwise shield production of SCFA and other microbial metabolites from the two tested diets. Samples taken after stabilization, but just prior to DF addition, were measured for microbial metabolites, but production of metabolites at this time point was artificially set to zero to reflect only production from the DF. After stabilization, DF from the whole diets or from the starchy or nonstarchy foods of these diets was dissolved in dialysate and was added to the modules at a rate of 1 mL/h, thus accomplishing the daily DF intake from both populations.[22,23] The incubations were run for 72 h under strict anaerobiosis, at a pH of 5.8 and a temperature of 37 °C, with continuous removal of fermentation products by an internal dialysis system and simulation of peristaltic movements, as described in detail before.[25] Luminal and dialysate samples were collected each 24 h and immediately frozen for further analysis.

Microbial Metabolite Analysis in Lumen and Dialysate Samples. Each 24 h, 10 mL of luminal content was anaerobically extracted from the TIM-2 system and divided into aliquots, which were immediately frozen in liquid nitrogen and stored for subsequent analysis. At the same time, dialysate samples were obtained, frozen, and stored for low molecular weight metabolite analysis. Briefly, branched-chain fatty acids (BCFA) and SCFA were analyzed gas chromatographically in the supernatant of samples (12000 rpm, 5 min) combined with formic acid (20%), methanol, and 2-ethyl butyrate as internal standard (2 mg/mL) as described before.[25] Samples were injected (0.5 μL) on a GC column (Stabilwax-DA, 15 m, 0.53 internal diameter, and 0.1 μm stationary phase; Variam Chrompack, Bergen op Zoom, The Netherlands) installed in a GC system (Chrompack CP9001) as described before.[25] Concentrations were calculated on the basis of a standard calibration curve of each of the different SCFA or BCFA in the linear concentration range of 0.01−10 mmol/L.

Ammonia and L/D-lactate were analyzed enzymatically, with a Cobas Mira Plus autoanalyzer (Roché Almere, The Netherlands) as was described previously.[25] Concentrations in the samples were determined via comparison with a series of standard solutions with known concentrations in the range of 0.01−5 mmol/L.

Antioxidant Associated Compounds Analysis. Lumen samples obtained after 72 h of in vitro colonic fermentation were centrifuged (15 min, 3000*g*), and supernatant and residue were separated before being freeze-dried. Dried soluble and insoluble powder was collected for each sample, and 0.5 g was placed in a capped centrifuge tube; 20 mL of acidic methanol/water (50:50, v/v; pH 2) were added, and the tube was thoroughly shaken at room temperature for 1 h. The tube was centrifuged at 2500*g* for 10 min and the supernatant recovered. 20 mL of acetone/water (70:30, v/v) were added to the residue, and shaking and centrifugation were repeated. Methanolic and acetonic extracts were combined and used to determine the antioxidant capacity associated with extractable antioxidants. The residues were treated with HCl/buthanol/$FeCl_3$ (5:95, v/v) at 100 °C for 3 h. Samples were then centrifuged (2500*g* for 10 min) and supernatants recovered. After two washings with HCl/buthanol/$FeCl_3$ (5:95, v/v), the final volume was adjusted to 25 mL by obtaining nonextractable polyphenols (NEPP), mainly condensed tannins (proanthocyanidins) not extracted by the previous aqueous−organic procedure.[29]

Total polyphenols (PP) in extracts were determined according to the Folin−Ciocalteu method in supernatants.[30] Briefly, test sample (0.5 mL) was mixed with 1 mL of Folin−Ciocalteu reagent and swirled. After 3 min, 10 mL of sodium carbonate solution (75 g/L) was added and mixed. Additional distilled water was mixed thoroughly by inverting the tubes several times. After 1 h, the absorbance at 750 nm was recorded. The results were expressed as milligram gallic acid equivalents per gram of dry matter. Absorbances at 555 nm of NEPP extractions were compared with carob pod (*Ceratonia siliqua*) proanthocyanidin standard (Nestle, Ltd., Vers-Chez-les Blanes, Switzerland), and results were expressed as milligram condensed tannin equivalents per gram of original dry matter.

Statistics and Calculations. Cumulative concentrations of SCFA, BCFA, lactate, and ammonia were calculated by taking measurements in dialysate and luminal content and the volume of each sample into account, as described elsewhere.[25] Results were plotted versus time, and after fitting the data, a linear equation was used to estimate the metabolite production per 24 h. Mean values were compared by one-way ANOVA using SPSS.09. Fermentations were run in duplicate, and all analyses performed on DF and fermentation metabolites and antioxidants were conducted in triplicate.

RESULTS

DF Composition, Fermentation, and Metabolite Production. In a previous study we compared two different European diets, Mediterranean style (diet from Murcia, Spain) and a typically Scandinavian dietary pattern also known as the Atlantic diet (diet from Copenhagen, Denmark), giving special attention to the composition and main sources of DF.[21] Summarizing, Table 1 shows the main dietary characteristics of both diets and the results from DF analysis. For the current study nondigestible fractions of these complete diets, mainly DF and associated compounds, were isolated. The same process was used to obtain the nondigestible fraction of the cereal products (starchy foods) and fruit and vegetable (nonstarchy foods) content of each diet. Table 2 summarizes the composition of purified nondigestible fractions that were obtained and used as substrate during in vitro colonic fermentations in TIM-2.

Lumen and dialysate samples were collected during 72 h, each 24 h, and metabolite concentrations were analyzed. SCFA and lactate concentrations derived from carbohydrate fermentation are summarized in Table 3. Results are presented in millimoles of metabolite per gram of DF. Acetate was the SCFA most abundant in all fermentations. In both diets analyzed,

Table 2. Fermentable Substrates Daily Added to the Fermentation Media

	Mediterranean diet			Scandinavian diet		
	whole diet	cereals	fruit and vegetable	whole diet	cereals	fruit and vegetable
total carbohydrates (g)	1.40	1.14	1.66	1.15	1.69	0.54
protein (g)	0.97	1.53	0.45	0.78	1.29	0.24
ratios[a]						
sCHO/iCHO	0.85	1.38	0.61	0.90	1.25	0.30
tCHO/protein	1.44	0.74	3.69	1.47	1.31	2.25

[a] sCHO/iCHO, ratio soluble carbohydrates/insoluble carbohydrates; tCHO/protein, ratio total carbohydrates/total protein.

Table 3. Production of Short-Chain Fatty Acids (SCFA) and Lactate[a]

		whole diet DF	cereal DF	fruit and vegetable DF
total SCFA	Mediterranean	5.59 ± 0.03 a1	4.58 ± 0.67 b1	5.15 ± 0.77 ab1
	Scandinavian	5.48 ± 0.54 a1	4.18 ± 0.50 b1	7.98 ± 0.52 c2
acetate (A)	Mediterranean	2.73 ± 0.02 a1	2.31 ± 0.12 b1	2.76 ± 0.15 a1
	Scandinavian	2.75 ± 0.14 a1	1.82 ± 0.10 b2	3.74 ± 0.43 c2
propionate (P)	Mediterranean	1.59 ± 0.22 a1	1.70 ± 0.24 a1	1.41 ± 0.15 a1
	Scandinavian	2.01 ± 0.22 a2	1.39 ± 0.13 b2	2.25 ± 0.26 a2
butyrate (B)	Mediterranean	1.27 ± 0.20 a1	0.57 ± 0.56 a1	0.97 ± 0.54 a1
	Scandinavian	0.71 ± 0.17 a2	0.97 ± 0.26 a1	1.99 ± 0.17 b2
ratio A:P:B	Mediterranean	49:28:23	50:37:12	54:27:19
	Scandinavian	50:37:13	44:33:23	47:28:25
lactate	Mediterranean	0.27 ± 0.07 a1	0.03 ± 0.03 b1	0.18 ± 0.11 a1
	Scandinavian	0.06 ± 0.03 a2	0.03 ± 0.01 a1	0.34 ± 0.01 b2

[a] Results are expressed as average mmol of metabolite per gram of dietary fiber followed by the standard deviation. Different letters (a−c) indicate statistical difference between results in the same row ($p < 0.05$). Different numbers (1 or 2) indicate statistical difference between Mediterranean and Scandinavian results (in the same column) ($p < 0.05$).

Table 4. Production of Branched-Chain Fatty Acids (BCFA) and Ammonia[a]

		whole diet DF	cereal DF	fruit and vegetable DF
total BCFA	Mediterranean	0.04 ± 0.02 a1	0.04 ± 0.04 a1	0.05 ± 0.02 a1
	Scandinavian	0.05 ± 0.04 a1	0.07 ± 0.07 a1	0.03 ± 0.01 a1
isobutyrate	Mediterranean	0.01 ± 0.01 a1	0.02 ± 0.02 a1	0.02 ± 0.02 a1
	Scandinavian	0.02 ± 0.01 a1	0.04 ± 0.04 a1	0.01 ± 0.002 a1
isovalerate	Mediterranean	0.03 ± 0.02 a1	0.02 ± 0.01 a1	0.03 ± 0.02 a1
	Scandinavian	0.03 ± 0.03 a1	0.03 ± 0.03 a1	0.02 ± 0.01 a1
ammonia	Mediterranean	0.96 ± 0.02 a1	1.41 ± 0.32 b1	1.18 ± 0.23 ab1
	Scandinavian	1.08 ± 0.16 a1	1.12 ± 0.19 a1	2.03 ± 0.37 b2

[a] Results are expressed as average mmol of metabolite per gram of dietary fiber followed by the standard deviation. Different letters (a−c) indicate statistical difference between results in the same row ($p < 0.05$). Different numbers (1 or 2) indicate statistical difference between Mediterranean and Scandinavian results (in the same column) ($p < 0.05$).

concentrations of total SCFA were significantly lower during cereal DF fermentation than during fermentation of whole diet DF, and particularly, acetate concentrations were lower than in the rest of the DF samples tested ($p < 0.05$). During colonic fermentation of DF of fruits and vegetables consumed in Copenhagen, the concentrations of total SCFA, acetate, and butyrate were significantly higher than determined during the fermentation of DF of the whole diet and cereal DF sample of this diet.

Compared with the data obtained from the fermentation of the fruit and vegetable DF from the Mediterranean diet, metabolite concentrations (SCFA, acetate, butyrate, and lactate) were significantly lower than from the Scandinavian fruit and vegetable sample ($p < 0.05$). The production of acetate and propionate was significantly higher during the fermentation of the cereal DF from the Mediterranean diet than from the Scandinavian diet ($p < 0.05$).

The SCFA profile was similar in terms of proportion of acetate in both complete diets, but the Scandinavian diet showed a higher propionate percentage than the Mediterranean, which had a higher butyrate percentage. During cereal DF fermentation, samples obtained from both diets generated relatively high amounts of propionate, around 35% of tSCFA. During fruit and vegetable DF fermentation, both samples generated relatively high amounts of butyrate, around 20% of tSCFA. Lactate concentration was low in all of the analyses performed (<0.4 mmol/g DF), and no statistical differences were observed between samples.

BCFA and ammonia were generated during in vitro fermentation and results, expressed as millimoles per gram of substrate added to the system, are summarized in Table 4. Concentrations of total BCFA, isobutyrate, and isovalerate were very low in all samples analyzed (<0.1 mmol/g of fiber), and no statistical differences were observed between samples. Ammonia concentrations were lower during the fermentation of whole diets than

Table 5. Antioxidant Associated Compounds Released during in Vitro Colonic Fermentation[a]

		whole diet DF	cereal DF	fruit and vegetable DF
released PP	Mediterranean	1.52 ± 0.54 a1	0.34 ± 0.10 b1	1.30 ± 0.26 a1
	Scandinavian	1.17 ± 0.09 a1	0.65 ± 0.25 b2	2.58 ± 0.54 c2
released NEPP	Mediterranean	1.25 ± 0.23 a1	0.73 ± 0.12 b	1.92 ± 0.46 c1
	Scandinavian	1.17 ± 0.10 a1	ND[b]	2.62 ± 0.39 b2
residual PP	Mediterranean	0.41 ± 0.14 a1	0.08 ± 0.03 b1	1.01 ± 0.08 c1
	Scandinavian	0.28 ± 0.02 a1	0.14 ± 0.04 b2	0.50 ± 0.15 c2
residual NEPP	Mediterranean	0.56 ± 0.09 a1	0.13 ± 0.03 b	1.19 ± 0.01 c1
	Scandinavian	0.48 ± 0.22 a1	ND	1.06 ± 0.67 a1

[a] Results are expressed as average mg gallic acid or condensed tannins per gram of dry matter (for PP and NEPA, respectively) followed by the standard deviation. Different letters (a−c) indicate statistical difference between results in the same row ($p < 0.05$). Different numbers (1 or 2) indicate statistical difference between Mediterranean and Scandinavian results (in the same column) ($p < 0.05$). [b] ND = not detected.

in the cereal DF (significant in samples from the Mediterranean diet) and fruit and vegetable DF (significant in samples from the Scandinavian diet).

Antioxidant Compounds Released during in Vitro Colonic Fermentation. After in vitro colonic fermentation, luminal content was collected and centrifuged, and antioxidant compounds were analyzed in both the soluble (released antioxidants) and insoluble (residual antioxidants) fractions. Antioxidants were analyzed as total PP and NEPP (Table 5). No significant differences were observed when the in vitro colonic fermentation was compared with the two whole diets in any of the antioxidant fractions defined in this study. However, PP released was higher during the fermentation of the cereal and fruit and vegetable samples from the diet from Copenhagen than from the diet from Murcia. This fact was also observed in the data obtained from the released condensed tannins of the sample of fruit and vegetable from the diet from Copenhagen, which was higher than those analyzed in the corresponding sample from the diet of Murcia. The content in extractable PP of the residual fraction was higher in the samples of cereals from the diet from Copenhagen than in the diet from Murcia, whereas the opposite was observed in the fruit and vegetable sample. The concentrations of NEPP from the cereal sample from Copenhagen, both released and residual, were below the limits of detection of the methodology used in this study. In most cases the highest concentrations of antioxidants, both PP and NEPP, were observed in the fruit and vegetable samples from both diets.

Estimation of Daily Production of Colonic Fermentation Metabolites. Estimation of the daily production of major fermentation metabolites (SCFA and ammonia) was done on the basis of the results obtained in this work (Tables 3 and 4) and data on food intake published specifically for the regions studied.[22,23] Results, expressed as millimoles of SCFA or ammonia per day per person, are summarized in Table 6. In addition, the contributions from cereal DF or fruit and vegetable DF fermentation were calculated as a percentage of the total value (given in boldface), assuming the whole diet as 100% of the production.

Daily total SCFA, acetate, and butyrate production was higher in the Mediterranean diet than in Scandinavian diet, whereas no significant differences were observed in the daily production of propionate and ammonia. When the ratio of acetate/propionate/butyrate was compared between diets, the most relevant difference was seen in the proportions of propionate (higher in the Scandinavian diet) and butyrate (higher in the Mediterranean diet). Ammonia production was lower than SCFA and was similar in both diets.

Table 6. Estimation of Daily Production of SCFA and Ammonia in Mediterranean and Scandinavian Diets Based on Fermentation Results (This Study) and Dietary Intake[22,23] and the Contribution of the Cereal DF and Fruit and Vegetable DF to the Total

		Mediterranean		Scandinavian	
	DF	mmol per capita	%	mmol per capita	%
tSCFA		348		261	
	cereal	142	**47**	144	**58**
	fruit and vegetable	162	**53**	104	**42**
acetate (A)		170		131	
	cereal	71	**45**	63	**56**
	fruit and vegetable	87	**55**	49	**44**
propionate (P)		99		96	
	cereal	53	**55**	48	**62**
	fruit and vegetable	44	**45**	29	**38**
butyrate (B)		79		34	
	cereal	18	**38**	34	**57**
	fruit and vegetable	30	**63**	26	**43**
ratio (A:P:B)		49:28:23		50:37:13	
ammonia		60		51	
	cereal	44	**54**	39	**60**
	fruit and vegetable	37	**46**	26	**40**

In the Scandinavian diet, cereal DF fermentation contributes more than fruit and vegetable DF fermentation to the total SCFA, propionate, and ammonia concentrations. In the

Mediterranean diet fruit and vegetable dietary fibers were the main contributors to butyrate production (Table 6).

DISCUSSION

SCFA and ammonia were the most abundant metabolites derived from fermentation of the nondigestible components of the two European diets by colonic microbiota. Cereal DF samples from both diets differed in the production of acetate and propionate and in its contribution to the total production of the diet. These differences could be related to the fact that cereal samples used in the Mediterranean diet were composed of refined product, whereas in the Scandinavian diet they were composed of whole grain derived product. Cereals are the main contributor to DF in the diet from Copenhagen (58%). The ratio of SCFA in the Scandinavian diet was particularly high in propionate, and cereal DF was an important contributor to the total propionate production. Cereal DF is particularly high in β-glucan and arabinoxylans, which have been described as propionogenic DF.[31,32] The DF in cereal grain is located primarily in the outer layers, which are removed during the refinement process. This fact could explain the differences observed in the metabolites produced during the fermentation of cereal DF samples from the two different whole diets. Rat hepatocyte primary culture experiments suggest an important role of propionate in the regulation of hepatic lipogenesis[11] with a potential effect in cholesterol homeostasis. In addition, propionate has been recently related to increase plasmatic GLP-1 concentrations[12] and to regulate leptin and resistin expression in adipose tissue,[13] suggesting a role in energy intake and insulin resistance.

In the Mediterranean diet selected for this study the DF content was derived almost equally from the intake of cereals and fruits and vegetables. The production of SCFA derived from the fermentation of the DF from the whole diet was particularly high in butyrate. There is a scientific consensus in the protective role of butyrate for the large intestine epithelium.[7] Butyrate is taken up by colonocytes and is their main energy source.[8] Protective properties for colonic epithelium have been associated with this metabolite due to its ability to promote cell differentiation, cell cycle control, and induction of apoptosis in damaged cells, and it may modulate the inflammatory process by interaction with lamina propia macrophages.[7] In addition, a role of butyrate in epithelial oxidative stress protection has been proposed,[10] as well as a role in reduction of pain perception.[9] The main contribution to the total butyrate production in the Mediterranean diet is associated with the fermentation of fruits and vegetables (63%), and it could be due to the high ratio of total carbohydrate/protein in this DF, which may be associated with a promotion of growth of butyrogenic bacteria. Ongoing analysis in microbiota composition during fermentation may elucidate this.

Correlation of the whole set of data obtained in the analysis of this study revealed a positive relationship between total SCFA and propionate produced and certain DF compounds as resistant starch (R^2 = 0.77 for total SCFA and R^2 = 0.90 for propionate, respectively), the ratio total carbohydrate (tCHO)/protein present in DF (R^2 = 0.77 and 0.84, respectively), and the amount of resistant protein (R^2 = 0.79, correlated only with propionate concentration). A negative correlation was observed between total SCFA and propionate produced and the amount of antioxidant associated compounds (R^2 = 0.81 and 0.87, respectively) and the concentration of insoluble NSPs (R^2 = 0.84, correlated only with propionate concentration). No correlation was found between acetate and butyrate production and the DF composition. Modulation of total SCFA, and specifically propionate, may be related to the composition of dietary fiber and associated compounds reaching the colon. Due to the important role that propionic acid may play in health,[11−13] it would be relevant to confirm these results in further analysis.

Ammonia is the main metabolite derived from protein fermentation. The high toxicity of ammonia is avoided by a rapid absorption and metabolism in the liver, where it is transformed into urea to be eliminated in the urine. Low concentrations of ammonia (5−10 mM) alter nucleic acid synthesis, modify intestinal mucosa, increase the growth of tumor cells, and promote virus infection.[14] Increased levels of plasma ammonia are responsible for hepatic coma.[15] Ammonia concentrations may be reduced with prebiotic intake, as observed in cirrhotic patients.[15] Fecal ammonia content varies between 12 and 30 mmol/day and is dependent on the intake of protein-rich food, such as fish and meat.[33] These values are considerably lower than the estimations described in the present study (60 and 51 mmol/day for the whole diets from Murcia and Copenhagen, respectively). However, post-mortem analysis revealed that ammonia concentrations are higher in proximal colon (from 10 to 60 mmol/kg) and distal colon (from 20 to 60 mmol/kg) than in feces (from 12 to 40 mmol/kg).[34] The ammonia concentrations estimated in this work are in the range of colonic measurements, although it should be considered that in vivo ammonia production could be higher due to internal (mucus, enzymes, epithelial exudates) and external (protein-rich foods as meat and fish) protein sources that were not included in the present study. Also, fecal concentrations do not reflect production, as ammonia is also taken up by the epithelium as eluded to above.

Comparison of ammonia concentration and nondigestible substrate composition gave a negative relationship between ammonia concentration and the ratio tCHO/total protein found in the fermented samples (R^2 = 0.72). This inverse relationship could be explained by the bacteria using carbohydrates as energy source and protein for biomass incorporation, reducing nitrogen sources for proteolytic fermentation and, consequently, ammonia production. Moreover, a negative correlation was observed between ammonia formation and DF content of total carbohydrates (R^2 = 0.72), insoluble NSPs (R^2 = 0.85), and the amount of antioxidant associated compounds (R^2 = 0.77). Therefore, reducing the ammonia formation may be associated with the composition of DF, and they are potential modulators that could play an important role in intestinal health maintenance.

The role of DF as carrier of dietary antioxidants has being recently reviewed,[5] and the impact of colonic fermentation on the release of dietary antioxidants from DF matrix has been analyzed in this work. By comparison of the rate of DF associated PP released or remaining in the residual fraction after fermentation, the largest fraction of these compounds was released to the luminal soluble fraction (solubilized rates vary from 56% during the fermentation of fruit and vegetable sample from Murcia diet to 84% during the fermentation of fruit and vegetable sample from Copenhagen; not shown). The same was observed for the release of DF associated NEPP (solubilized rates vary from 62% during the fermentation of fruit and vegetable sample from Murcia diet to 85% during the fermentation of cereal sample from Murcia; data not shown). Comparable portions of solubilized PP were observed during in vitro colonic cocoa fermentation.[35] In vivo studies have described health-related properties of antioxidant compounds associated with DF,

suggesting positive effects on cardiovascular disease prevention and also in gastrointestinal health, including prevention of colon cancer risk.[5] Further research will be needed to evaluate the heath-related implications that the luminal release of PP and NEPP may have.

To summarize, we conclude, on the basis of the discriminatory results from the two different European diets, that the source and composition of naturally occurring DF have an effect on the metabolite production during colonic fermentation and, hence, on the physiological benefits reported for the metabolites produced from DF. As not all DF are equal, this should be taken into account during dietetic recommendations. Special focus on the intake of nonrefined cereals may be recommended in cardiovascular disease prevention, whereas fruits and vegetables may play a relevant role in intestinal health maintenance. Moreover, the ratio tCHO/protein of DF could be considered as an indicator of the ammonia formation during colonic fermentation.

Encouraging the general population to increase the intake of naturally occurring DF is a pertinent nutritional policy, especially in Western countries. However, to improve nutritional strategy in specific risk populations, recommendations should emphasize specific target food intake, as we show here that different DF sources lead to production of different metabolites, with specific reported health effects. Thus, specific sources of certain DF can be efficient in specific disease prevention.

This study has laid the groundwork to be able to make these specific recommendations by deciphering the contribution of diet (components) to health benefit through the production of beneficial microbial metabolites.

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ABBREVIATIONS USED

SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids; DF, dietary fiber; EPIC, European Prospective into Cancer and Nutrition; TIM-2, TNO's gastrointestinal model of the colon; ratio A:P:B, ratio acetate/propionate/butyrate; sCHO/iCHO, ratio soluble carbohydrates/insoluble carbohydrates; tCHO/protein, ratio total carbohydrates/total protein; NSP, nonstarch polysaccharide; PP, polyphenols; NEPP, nonextractable polyphenol.

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