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Characterization and Fermentability of an Ethanol Soluble High Molecular Weight Coffee Fraction

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Brews from differently roasted Arabica coffees were shown to contain 8−12% ethanol soluble substances with molecular masses greater than 2 kDa, possibly contributing to their dietary fiber contents. About 13% of these substances were nondigestible carbohydrates, mainly arabinogalactans. The nondigestible high molecular weight ethanol soluble fraction (HESF) of the medium roasted coffee brew was further characterized and subjected to in vitro fermentation with human fecal bacteria. In addition to carbohydrates, HESF contained proteins/peptides (~20%), but the main fraction was composed of structurally unknown Maillard reaction products. From NMR spectroscopy, we conclude that intact caffeic and ferulic acid derivatives were not incorporated into the melanoidins to a significant extent. Stepwise ultrafiltration and gel filtration indicated a large variation in the molecular weights of HESF constituents. Coffee HESF was shown to be less fermentable by fecal bacteria than soluble coffee fiber isolated by the enzymatic-gravimetric methodology, and because of its lower carbohydrate content, less short-chain fatty acids were produced during the fermentation. Total cell counts, destructive chemical analysis, and NMR spectroscopy indicated that coffee carbohydrates are the preferred substrates for colonic microbiota. However, NMR spectra, absorbances at 405 nm, and nonprotein nitrogen contents showed that noncarbohydrate and nonprotein compounds were also utilized to some extent but the bacterial species involved in this degradation remain to be identified.

KEYWORDS: Coffee dietary fiber; polysaccharides; melanoidins; fermentability; gut microbiota; enzymatic-gravimetric methodology; NMR; FISH

INTRODUCTION

Recent studies showed that coffee beverages contain substantial amounts of soluble dietary fiber (*1, 2*), which is well utilized by human fecal microbiota (*2*). Major components of the dietary fiber fraction isolated from coffee brews are complex type II arabinogalactans and galactomannans with a low degree of substitution (*2*).

We recently analyzed the dietary fiber contents of various coffee brews by applying the widely accepted enzymatic-gravimetric methodology (*3*) and showed that the contents and structural characteristics of coffee dietary fiber are influenced by various parameters such as the coffee type, the degree of roast, and the brewing procedure (*4*). However, it is well-known that some fiber compounds such as ethanol soluble nondigestible oligosaccharides are not determined by using the enzymatic-gravimetric methodology (*5*). An alternative approach has therefore been used by Díaz-Rubio and Saura-Calixto to determine dietary fiber in coffee brews (*1*): Following enzymatic treatments, a high molecular weight fraction was isolated by using dialysis (molecular weight cutoff (MWCO) = 12−14 kDa). After acid hydrolysis, the fiber monosaccharides were determined semispecifically by applying a colorimetric method (dinitrosalicylic acid). Although avoiding an ethanol precipitation step, this method may also lead to underestimation of the dietary fiber content because galactomannans and arabinogalactans with molecular weights <12 kDa (*6*) are excluded.

Although the high molecular weight fraction of coffee brews also contains considerable amounts of structurally unknown compounds derived from the Maillard reaction (melanoidins), a clear distinction between melanoidins and poly- or oligosaccharides is not possible because carbohydrates are thought to

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be integral components of coffee melanoidin complexes (*7, 8*). Model melanoidins were shown to have some physiological properties similar to dietary fiber. They are mostly indigestible (*9, 10*), show high water holding capacities (*11*), and are capable of adsorbing organic molecules, such as bile acids (*12*). Model melanoidins were also shown to be fermented by gut microbiota, possibly affecting the microbial composition (*13–15*). Coffee dietary fiber isolated by the enzymatic-gravimetric procedure was previously reported to contain unknown, brown colored components (*2, 4*). However, the relevance of food borne melanoidins as dietary fiber still needs to be addressed.

We expected coffee brews to contain considerable amounts of high molecular weight components (melanoidins, ethanol soluble polysaccharides) that are not detected as dietary fiber using the enzymatic-gravimetric procedure but either belong to the dietary fiber complex, by definition (*16*), or possess fiberlike properties. Therefore, the aim of our study was to analyze the content and to determine the chemical characteristics and fermentability of this high molecular weight ethanol soluble coffee fraction.

MATERIALS AND METHODS

General. Reagents for fluorescence in situ hybridization (FISH) were of molecular biological grade. All other chemicals and solvents were of analytical grade or the highest purity available. Chemical characterization and fermentation experiments were carried out in duplicate and triplicate, respectively.

Materials. Light, medium, and dark roasted Arabica coffee samples (origin Colombia) were kindly provided by Tchibo Manufacturing GmbH Co. KG (Hamburg, Germany). Coffee beans were ground to an average particle size of 450 μm.

Coffee Preparation. Drip coffee beverages were prepared using a standard coffee maker, No. 4 sized cone paper filters, ground coffee (50 g), and medium hard water (1 L; 69 mg/L Ca^{2+}, 3.9 mg/L Mg^{2+}, 136 mg/L HCO_3^-) resulting in about 900 mL of coffee beverage. Dry weights of coffee brews were determined gravimetrically following freeze drying.

Determination and Preparation of the Ethanol Soluble High Molecular Weight Fraction. The contents of the ethanol soluble high molecular weight material from light, medium, and dark roasted Colombian coffee drip brews were analyzed gravimetrically by using the freeze-dried coffee brew extracts (1.0 g) as starting material. Coffee dietary fiber was removed according to the enzymatic-gravimetric methodology (*3*), as recently applied to coffee brews (*2, 4*) using a commercially available enzyme kit (BIOQUANT, Merck, Darmstadt, Germany). After treatments with heat-stable α-amylase, protease, and amyloglucosidase, the total dietary fiber fraction was precipitated in 76% ethanol and separated by filtration (glass filter crucibles, porosity 2, pore size 40−90 μm, partially filled with Celite 545). After removing ethanol by rotary evaporation at 40 °C and reduced pressure, the remaining solution was dialyzed (benzoylated cellulose, MWCO 2 kDa, Sigma-Aldrich Chemie GmbH, Steinheim, Germany) against water. The ethanol soluble high molecular weight fraction obtained after dialysis ($HESF_D$) was freeze-dried, weighed, and subjected to total carbohydrate analysis as detailed below.

For further characterization, chemical analysis, and fermentation experiments, the ethanol soluble high molecular weight fraction was isolated on a larger scale from the medium roasted coffee brew (freeze-dried powder, 10 g) according to the described procedure but using ultrafiltration instead of dialysis. Ultrafiltration was carried out using two tangential flow devices (polyethersulfone membrane, MWCO 3 kDa, Vivaflow 50, Sartorius AG, Goettingen, Germany) connected in series and a flexible-tube pump (Masterflex L/S pump drive 7554-85 and easy-load pump head 7518-00, Cole-Parmer Instrument Co., Vernon Hills, IL). The preparation of the high molecular weight ethanol soluble fraction was performed in triplicate. The freeze-dried retentates ($HESF_{UF}$, >3 kDa) were homogenized in a mortar and used as detailed below.

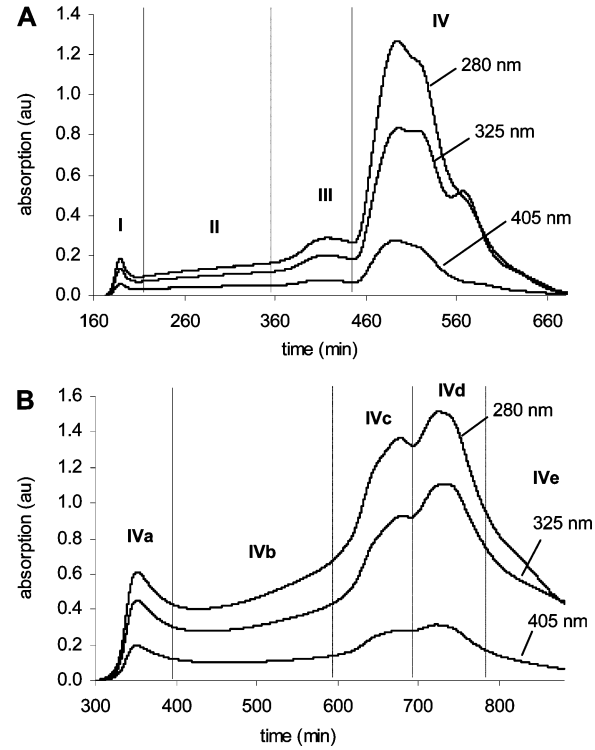

Figure 1. Fractionation of a high molecular weight ethanol soluble coffee fraction ($HESF_{UF}$) using Sephadex G-50 (**A**) and Sephadex G-25 (**B**) chromatography and photo diode array detection with recording wavelengths at 405, 325, and 280 nm.

Further Fractionation of the Ethanol Soluble High Molecular Weight Fraction. $HESF_{UF}$ (600 mg) was further fractionated using gel filtration chromatography [L-7100 pump, L-7455 photodiode array detector equipped with a preparative flow cell (Merck/Hitachi, Darmstadt, Germany), Serva Linear II fraction collector (Serva-Technik GmbH & Co KG, Heidelberg, Germany)]. A glass column with flow adaptors (Kronlab, Sinsheim, Germany; 2.5 cm × 100 cm) was filled with Sephadex G-50 medium (Amersham Biosciences, Freiburg, Germany) swollen in water, which was also used as an eluent. $HESF_{UF}$ (~300 mg) dissolved in water (4.5 mL) was applied using a six-way valve and a 5 mL sample loop. The flow rate was 1.0 mL/min, and fractions were collected every 20 min and combined according to the chromatogram (**Figure 1A**). Fractions from two runs were pooled, freeze-dried, and subjected to chemical characterization or, in case of fraction IV, further fractionated using Sephadex G-25 medium gel filtration chromatography. Sephadex G-25 chromatography was carried out in one run using the instrumentation and column as detailed for Sephadex G-50 chromatography, water as an eluent and a flow rate of 0.5 mL/min. Fractions were collected every 10 min and combined according to the chromatogram (**Figure 1B**), freeze-dried, and subjected to chemical analysis as described below.

Additional fractionation of $HESF_{UF}$ was performed by an ultrafiltration procedure. Ultrafiltration was carried out with centrifugal ultrafiltration devices (Vivaspin 20, polyethersulfone membrane, Sartorius AG, Goettingen, Germany) with MWCO of 100, 50, 10, and 3 kDa. After dissolving 200 mg of $HESF_{UF}$ in 20 mL of water, fractions >100 kDa, 50−100 kDa, 10−50 kDa, and 3−10 kDa were obtained by stepwise ultrafiltration, freeze-dried, and weighed.

Determination of Absorbance at 405 and 280 nm. Aqueous solutions (0.1 mg/mL) of $HESF_{UF}$ or its subfractions were measured spectrophotometrically at 280 and 405 nm (1 cm path length) using water as a blank.

Determination of Total Carbohydrates and Carbohydrate Characterization. The total carbohydrate content, calculated as the sum of anhydosugars, and the monosaccharide compositions were determined using the alditol acetate methodology: Neutral sugars were released by Saeman hydrolysis as modified by Englyst et al. (prehydrolysis with 12 M H_2SO_4 for 5 min at room temperature, hydrolysis using 2 M

Table 1. 16S rRNA Targeting Oligonucleotide Probes Used for FISH

probe	sequence (5′−3′)	OPD code	target organism	reference
EUB mix			total bacteria	(*35*)
EUB 338	GCTGCCTCCCGTAGGAGT	S-D-Bact-0338-a-A-18		(*36*)
EUB 785	CTACCAGGGTATCTAATCC	S-D-Bact-0785-a-A-19		(*37*)
EUB 927	ACCGCTTGTGCGGGCCC	S-D-Bact-0927-a-A-17		(*38*)
EUB 1055	CACGAGCTGACGACAGCCAT	S-D-Bact-1055-a-A-20		(*37*)
EUB 1088	GCTCGTTGCGGGACTTAACC	S-D-Bact-1088-a-A-20		(*37*)
Erec 482	GCTTCTTAGTCARGTACCG	S-*-Erec-0482-a-A-19	*Clostridium coccoides-Eubacterium rectale* group	(*39*)
Bac 303	CCAATGTGGGGGACCTT	S-*-Bacto-0303-a-A-17	*Bacteroides-Prevotella* group	(*40*)
Bif 164	CATCCGGCATTACCACCC	S-G-Bif-0164-a-A-18	bifidobacteria	(*41*)

H_2SO_4 for 60 min at 100 °C) (*17*) and analyzed as their alditol acetates (following $NaBH_4$ reduction and acetylation) by GC-FID (*18*). GC conditions used are detailed in Gniechwitz et al. (*2*).

Carbohydrate structures were also characterized by methylation analysis, which was carried out according to Nunes and Coimbra (*19*) with minor modifications, as previously described (*2*). Identification and quantification of partially methylated alditol acetates was carried out by GC-MS and GC-FID, as detailed previously (*2*). Molar response factors according to Sweet et al. (*20*) were used for quantification.

Determination of the Total Nitrogen Content. The total nitrogen content was analyzed after Kjeldahl digestion using the spectrophotometric procedure described by Willis et al. (*21*).

Amino Acid Analysis. Dried samples (1 mg) were hydrolyzed with 6 M HCl (1 mL) for 24 h at 110 °C. The acid was removed by rotary evaporation at reduced pressure and 40 °C, and the amino acids were extracted in 4% (w/v) sulfosalicylic acid (50 mL). The extracts were adjusted to 100 mL, centrifuged, and filtered. The amino acids were analyzed by HPLC and fluorescence detection after derivatization with *o*-phthaldialdehyde as described by Bytof et al. (*22*).

Fecal Samples and Culture Conditions. Fecal samples were collected from one healthy volunteer who had no previous history of gastrointestinal disorders and had not undergone antibiotic therapy within 6 months prior to the study. Fermentations were carried out using an up-scaled version (batch culture volume = 100 mL) of the procedure previously described (*2*). The final concentration of the $HESF_{UF}$ was 3.0 mg/mL. The control contained no substrate. Aliquots (30 mL) were taken after 0 h, 24 h, and 48 h of incubation and analyzed, as described below.

Determination of Optical Density, pH and Short-Chain Fatty Acids. Spectrophotometric determination of optical density (OD), determination of pH, and gas chromatographic determination of short-chain fatty acids (SCFA) were performed, as described previously (*2*).

Preparation of Fermented Samples for Chemical Analysis. Whereas unfermented coffee fractions were analyzed as prepared, an additional cleanup was necessary for the chemical analysis of fermented samples: Cells were removed from batch culture aliquots by centrifugation (6 min, 4 °C, 10 000*g*). Low molecular weight material (<3 kDa) was removed from the cell-free fermented samples using the same ultrafiltration procedure, as described for the preparation of $HESF_{UF}$. The retentates (>3 kDa) were freeze-dried and chemically analyzed, as described before (total carbohydrate content, monosaccharide composition, total nitrogen content, total amino acid content, absorbance at 405 and 280 nm). In addition, the freeze-dried retentates were analyzed by NMR spectroscopy, as detailed below.

NMR Spectroscopy. Structural changes of $HESF_{UF}$ were monitored by NMR over the course of fermentation using two-dimensional (2D) $^{13}C-^{1}H$ correlation spectroscopy, via the HSQC (heteronuclear single quantum coherence) experiment. Aliquots of fermented samples were taken after 0, 24, and 48 h of fermentation and prepared as described above. Samples (50 mg for 0 h-, 24 h-, and 48 h-samples, respectively) were dissolved in deuterium oxide (0.7 mL). HSQC experiments of the fermented samples were performed on a Bruker DMX-750 with a cryogenic probe whereas both the Bruker DMX-750 but also a Bruker DRX500 instrument (Rheinstetten, Germany) were used to analyze model compounds. Experiments were performed at 300 K, and chemical shifts (δ) were referenced to the signals of acetone, which was used as internal standard (δ_H 2.22 ppm; δ_C 30.89 ppm). To assign specific signals, the following model compounds were measured under the same conditions: arabinogalactan (from larch wood), carob galactomannan (from *Ceratonia siliqua*), maltose, cellobiose, rutinose, albumin (from bovine serum), 5-*O*-caffeoylquinic acid, and L-phenylalanine.

Table 2. Dry Matter, Soluble Dietary Fiber (SDF), and High Molecular Weight Ethanol Soluble Coffee Fraction ($HESF_D$) Contents of Light, Medium, and Dark Roasted Coffee Brews and Content and Composition of Total Carbohydrates in $HESF_D$

	light roasted	medium roasted	dark roasted
dry matter (g/100 mL)	1.47 ± 0.01	1.58 ± 0.01	1.60 ± 0.00
SDF (mg/100 mL)[a]	289 ± 2[b]	350 ± 4	349 ± 9
$HESF_D$ (mg/100 mL)	112 ± 6[b]	187 ± 11	194 ± 3
total carbohydrates in fraction $HESF_D$ (g/100 g)	12.94 ± 0.46	12.58 ± 1.33	12.89 ± 1.38
monosaccharide composition[c]			
rhamnose (mol%)	7.9	8.2	7.8
glucose (mol%)	7.7	6.6	7.9
mannose (mol%)	6.2	5.0	6.7
arabinose (mol%)	32.6	35.3	31.7
galactose (mol%)	45.6	45.0	45.8

[a] Results previously published (*4*). [b] Light roasted coffee showed significantly ($p < 0.05$) lower SDF and $HESF_D$ contents than medium or dark roasted coffee. [c] Monosaccharide composition of total carbohydrates in fraction $HESF_D$, anhydro sugars.

Fluorescence in Situ Hybridization and Enumeration of Bacterial Cells. The oligonucleotide probes used in this study are listed in **Table 1**. Batch culture aliquots (1.0 mL) were centrifuged (5 min, 4 °C, 12000*g*). The pellet was resuspended in PBS (0.5 mL), added to paraformaldehyde (1.5 mL; 4% aqueous solution), and fixed for 4 h at 4 °C. Fixed samples were stored at −80 °C until further use. For microscopic sample preparation, Teflon coated 8-well-slides (Roth, Karlsruhe, Germany) with a well diameter of 6 mm were used. The fixed samples were homogenized for 1 min at full speed in a Gyroprep (UniEquip Laborgerätebau and -vertriebs GmbH, Martinsried, Germany) and diluted depending on the probe used. Tween solution (10 μL; 0.01%) was applied to each well, and the fixed and diluted fecal samples (10 μL) were added. The air-dried suspension was dehydrated for 3 min each in 60, 80, and 96% ethanol. In case of using probes targeting gram-positive groups of bacteria, samples were treated with lysozyme buffer pH 7.2 (14 μL; 1 mg/mL lysozyme, 100 mM Tris-HCl, 50 mM EDTA) for 10 min at 4 °C. Subsequently, the lysozyme was removed with bidistilled H_2O, and the slides were air-dried and dehydrated, as described. Each well was covered with a mixture of hybridization buffer (10 μL; 0.9 M NaCl, 10 mM Tris HCl pH 7.4, 0.01% SDS) and probe solution (1 μL; 10 pmol/μL in hybridization buffer), except for Bac303, which was used at 4-fold concentration in hybridization buffer with 30% (v/v) formamide. The slides were kept in a moist chamber for overnight hybridization and washed in hybridization buffer for 30 min at a temperature 2 °C higher than the one used for hybridization. The slides were air-dried and mounted with Vectashield (Vector Laboratories, Peterborough, U.K.). Samples were counted with an Axioplan2 imaging microscope (Carl Zeiss, Oberkochen, Germany), equipped with a servo-controlled microscope stage (EM14MOT, Merzhäuser, Wetzlar, Germany), a Plan-Neofluar 40× dry and 100× oil immersion objective (Carl Zeiss, Oberkochen, Germany), and the fluorescence filter set 15 (excitation: 546 nm; emission: 590 nm) for Cy3 excitation.

Table 3. Chemical Characteristics of the Ethanol Soluble High Molecular Weight Fraction ($HESF_{UF}$) from a Medium Roasted Colombian Coffee Brew and of its Subfractions HESF I - IVe (Obtained by GPC)

			HESF Subfraction							
		$HESF_{UF}$	I	II	III	IVa	IVb	IVc	IVd	IVe
yield	(g/100 g)		10.7	11.4	13.0	4.6	20.3	16.9	13.9	9.2
total carbohydrates[a]	(g/100 g)	11.46 ± 0.43	33.40 ± 0.47	34.37 ± 1.79	9.16 ± 0.50	3.81 ± 0.18	10.98 ± 0.59	6.93 ± 0.13	4.07 ± 0.12	3.05 ± 0.14
monosaccharide composition[b]										
rhamnose	(mol%)	9.5	11.6	7.6	19.4	16.1	5.9	8.2	12.1	14.4
glucose	(mol%)	4.6	5.4	3.3	7.1	17.2	12.9	20.1	22.4	27.2
mannose	(mol%)	5.1	3.3	3.7	8.6	6.4	7.6	7.0	6.6	7.5
arabinose	(mol%)	27.7	32.9	26.1	32.2	15.1	19.6	22.3	22.0	17.9
galactose	(mol%)	53.2	46.8	59.3	32.7	45.2	54.1	42.5	36.9	33.0
total nitrogen	(g/100 g)	4.88 ± 0.25	3.76 ± 0.42	4.25 ± 0.34	1.62 ± 0.07	6.76 ± 0.33	6.98 ± 0.08	4.38 ± 0.04	3.56 ± 0.39	2.10 ± 0.12
nitrogen from amino acids	(g/100 g)	2.94 ± 0.05	2.23 ± 0.07	1.62 ± 0.04	0.69 ± 0.03	2.28 ± 0.03	2.42 ± 0.04	0.69 ± 0.03	1.43 ± 0.03	0.10 ± 0.01
"unknown" nitrogen[c]	(g/100 g)	1.94	1.53	2.63	0.93	4.48	4.56	3.69	2.13	2.00
total amino acids[d]	(g/100 g)	20.02 ± 0.37	15.58 ± 0.50	11.10 ± 0.24	4.55 ± 0.12	15.86 ± 0.29	17.59 ± 0.21	4.75 ± 0.20	9.88 ± 0.22	0.68 ± 0.07
amino acid composition[b]										-[e]
Asx	(mol%)	14.2	14.2	14.7	15.6	13.7	16.4	16.4	18.3	
Glx	(mol%)	25.2	27.1	25.8	25.5	24.5	31.0	26.0	25.6	
Ser	(mol%)	4.3	6.1	4.1	4.5	3.1	3.5	4.3	4.5	
His	(mol%)	1.8	1.3	2.1	1.7	1.7	1.1	1.9	2.6	
Gly	(mol%)	21.3	22.5	21.0	22.2	24.3	16.5	20.0	16.8	
Thr	(mol%)	3.8	3.7	3.6	4.1	3.9	4.6	3.6	5.2	
Arg	(mol%)	1.0	0.4	0.6	1.7	0.5	0.5	0.3	0.8	
Ala	(mol%)	6.3	6.6	7.3	6.9	5.9	7.3	6.2	6.5	
Tyr	(mol%)	1.6	1.7	1.0	0.3	2.8	1.0	0.8	0.5	
Val/Met	(mol%)	3.4	3.0	3.5	3.3	2.9	3.2	2.8	3.5	
Phe	(mol%)	3.3	3.4	2.4	2.5	4.7	2.7	3.3	2.5	
Ile	(mol%)	3.8	2.9	4.1	3.0	4.0	3.7	3.8	4.0	
Leu	(mol%)	6.7	5.8	7.3	6.2	6.7	7.2	7.1	6.9	
Lys	(mol%)	3.3	1.3	2.4	2.3	1.3	1.5	3.6	2.5	
absorbance at 405 nm[f]	(au)	0.261	0.312	0.224	0.073	0.273	0.172	0.138	0.277	0.300
absorbance at 280 nm[f]	(au)	0.893	0.697	0.643	0.202	0.732	0.631	0.553	0.959	1.107

[a] Anhydro sugars. [b] Molar percentages of total carbohydrate or total amino acid contents, respectively. [c] Nitrogen deriving from structurally unknown compounds (calculated). [d] Anhydro amino acids. [e] Not listed (due to low total amino acid content only three amino acids were measurable). [f] Aqueous solutions (0.1 mg/mL) $d = 1$ cm.

Table 4. Comparison of Selected Structural Characteristics of Arabinogalactans Present in the Ethanol Soluble High Molecular Weight Fraction ($HESF_{UF}$) and the Soluble Dietary Fiber Fraction (SDF) from a Medium Roasted Colombian Coffee Brew

glycosidic-linkage ratio	$HESF_{UF}$	SDF[a]
3-Gal*p*/6-Gal*p*	1.9	2.8
(3-Gal*p* + 6-Gal*p*)/3,6-Gal*p*	2.7	1.9
T-Ara*f*/3,6-Gal*p*	3.0	1.1
T-Ara*f*/5-Ara*f*	1.5	3.6

[a] Results previously published (*4*).

RESULTS AND DISCUSSION

Contents and Characteristics of the Ethanol Soluble High Molecular Weight Coffee Fraction ($HESF_{D/UF}$). Using dialysis (MWCO 2 kDa), drip brews from light, medium, and dark roasted Colombian coffees were shown to contain 112−194 mg/100 mL of high molecular weight compounds that are not detected as dietary fiber ($HESF_D$) using the widely accepted enzymatic-gravimetric procedure (*3*) (**Table 2**). These fractions contributed to 8−12% of the coffee brew dry matter. There was a significant increase in the $HESF_D$ contents from light to medium roasted coffee but no further increase for the dark roasted brew. Similar effects of the roasting degree were observed upon analyzing the soluble dietary fiber (SDF) contents of coffee beverages (*4*) (**Table 2**). However, only about 13% of this high molecular weight fraction were carbohydrates and therefore dietary fiber by definition (*16*). The monosaccharide composition of $HESF_D$ was comparable for all degrees of roast (**Table 2**) and revealed that ethanol soluble carbohydrates were mainly arabinogalactans. This is in accordance with results reported by Bekedam et al. (*23*): arabinose and galactose made up about 80 mol% of carbohydrates present in a coffee fraction that was soluble in 80% ethanol. Compared to SDF arabinogalactans (*4*), $HESF_D$ carbohydrates were characterized by lower galactose/arabinose ratios (1.3−1.4 vs 2.1−3.2).

The high molecular weight ethanol soluble fraction was isolated on a larger scale from the medium roasted coffee brew using ultrafiltration instead of dialysis ($HESF_{UF}$). The average yield of $HESF_{UF}$ (∼11% of the coffee brew dry matter) was similar to the yield of $HESF_D$ (∼12%).

Further analysis and fermentation experiments were carried out with $HESF_{UF}$. Its monosaccharide composition was slightly different from $HESF_D$. However, the main monosaccharide residues were also arabinose and galactose, accounting for ∼80% of the total carbohydrates (**Table 3**). Apart from the already mentioned galactose/arabinose ratio, arabinogalactans from $HESF_{UF}$ and SDF varied also in other structural characteristics as analyzed by methylation analysis (**Table 4**): the $HESF_{UF}$ arabinogalactans were less branched as indicated by a higher (3-Gal*p* + 6-Gal*p*)/3,6-Gal*p* ratio (2.7 vs 1.9). In addition, $HESF_{UF}$ arabinogalactans were characterized by lower 3-Gal*p*/6-Gal*p* (1.9 vs 2.8) and T-Ara*f*/5-Ara*f* ratios (1.5 vs 3.6). Furthermore, $HESF_{UF}$ showed a much higher ratio of T-Ara*f*/3,6-Gal*p* than SDF (3.0 vs 1.1). Nunes and Coimbra also

Table 5. Production of SCFA after 24 and 48 h of Fermentation of a High Molecular Weight Ethanol Soluble Coffee Fraction ($HESF_{UF}$) Compared to the Control

	acetate (mM)		propionate (mM)		butyrate (mM)		total (mM)	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
$HESF_{UF}$	3.70 ± 0.70	4.63 ± 0.01	1.17 ± 0.99	1.89 ± 0.03	0.59 ± 0.09	0.85 ± 0.02	5.47 ± 0.97	7.36 ± 0.05
control	1.12	1.55	0.06	0.05	0.04	0.03	1.22	1.63

Table 6. Total Carbohydrate, Total Nitrogen, and Amino Acid Nitrogen Contents as Analyzed after 0, 24, and 48 h of Fermentation of a High Molecular Weight Ethanol Soluble Coffee Fraction ($HESF_{UF}$) by Human Fecal Microbiota

		0 h	24 h	48 h
yield	(g/100 g)	72.9	55.8	61.9
total nitrogen	(g/100 g)	3.55 ± 0.06	2.55 ± 0.01	1.96 ± 0.02
total amino acids[a]	(g/100 g)	12.41 ± 0.67	10.39 ± 0.58	7.91 ± 0.86
nitrogen from amino acids	(g/100 g)	1.85 ± 0.09	1.53 ± 0.07	1.17 ± 0.11
"unknown" nitrogen[b]	(g/100 g)	1.70	1.02	0.79
total carbohydrates[c]	(g/100 g)	14.44 ± 0.28	6.57 ± 0.13	4.88 ± 0.10
rhamnose[c]	(g/100 g)	1.21 ± 0.02	0.57 ± 0.03	0.19 ± 0.02
glucose[c]	(g/100 g)	0.36 ± 0.03	0.23 ± 0.02	1.28 ± 0.00
mannose[c]	(g/100 g)	0.51 ± 0.04	0.27 ± 0.01	0.21 ± 0.00
arabinose[c]	(g/100 g)	4.34 ± 0.18	2.50 ± 0.12	1.10 ± 0.09
galactose[c]	(g/100 g)	8.02 ± 0.21	3.00 ± 0.02	2.10 ± 0.04

[a] Anhydro amino acids. [b] Nitrogen deriving from structurally unknown compounds (calculated). [c] Anhydro sugars.

observed high T-Ara*f*/3,6-Gal*p*-ratios (2.1−2.6) in melanoidins they isolated from the 75% ethanol soluble fractions of roasted Brazilian coffees (*8*).

The differences in arabinogalactan structures in $HESF_{UF}$ and SDF may partially explain their different ethanol solubilities. However, ethanol solubility may also be affected by linkages to structurally unknown compounds (e.g., Maillard reaction products (MRP)). The presence of high amounts of MRPs in $HESF_{UF}$ was indicated by a strong brown color and UV-activity (aqueous solution, 0.1 mg/mL, absorbances at 405 and 280 nm; **Table 3**), which was 3−4-fold higher than observed for the SDF fraction (aqueous solution, 0.25 mg/mL, 0.206 au and 0.509 au for 405 and 280 nm, respectively) (*4*). In addition, $HESF_{UF}$ contained 4.88 g/100 g nitrogen, only about 60% of which derived from amino acids. Carbohydrates and amino acids only accounted for 31.5% of the HESF, again indicating the importance of structurally unknown MRPs for this ethanol soluble high molecular weight material.

Stepwise ultrafiltration of $HESF_{UF}$ showed that its constituents substantially differed in their molecular weights, most of the compounds being found in fractions >100 kDa (40%) or 3−10 kDa (53%). Fractions 10−50 kDa and 50−100 kDa accounted only for 5 and 2%, respectively. The broad molecular weight range of compounds present in $HESF_{UF}$ is also reflected in the chromatograms obtained by gel filtration using Sephadex G-50 (**Figure 1A**). The majority of compounds eluted after ∼440 min, as indicated by the elution profile measured at 405 nm, 325, or 280 nm and by the yields of the fractions (**Table 3**). Because the structures and therefore the conformations of compounds in $HESF_{UF}$ are essentially unknown, a calibration of the gel-filtration chromatography with comparable standard substances is not possible. However, a 1 kDa dextran standard eluted after 442 min, indicating a rather low molecular weight of compounds of fraction IV. The yield of fraction IV was roughly comparable to the proportion of the 3−10 kDa fraction obtained by ultrafiltration (65% vs 53%). However, it is possible that secondary interactions between coffee melanoidins and the gel matrix occur, leading to an underestimation of molecular weights. Further fractionation of fraction IV using Sephadex G-25 gel chromatography indicated the complexity of the material in the lower high molecular weight range (**Figure 1B**).

Chemical analysis of the fractions obtained by gel-filtration chromatography revealed that the early eluted components (subfractions I and II) had the highest carbohydrate contents (33 and 34%, respectively) (**Table 3**). It may be concluded that longer polysaccharide chains are involved in melanoidin structures present in fractions I and II, compared to fractions III to IVe, leading to higher molecular weights. However, the gel-filtration fractions rather are complex mixtures than pure compounds. Therefore, it cannot be ruled out that they also contain ethanol-soluble carbohydrates, which are not part of melanoidin structures. Arabinose and galactose proportions (**Table 3**) showed that carbohydrate components of all $HESF_{UF}$ subfractions are mainly arabinogalactans. Arabinose/galactose ratios ranged from 0.3 (subfraction IVa) to 1.0 (subfraction III) and follow no trend regarding the assumed molecular weights. Surprisingly high glucose proportions were measured for subfractions IVa to IVe. However, we cannot fully rule out that compounds other than glucose, which may have been released or formed from MRP during acid hydrolysis coeluted with glucitolperacetate, thus leading to an overestimation of the glucose content.

$HESF_{UF}$ subfractions largely differed in their total nitrogen (1.6−7.0%) and total amino acid contents (0.7−17.6%), but no correlations to molecular weight or absorbances at 405 and 280 nm were distinct.

To sum up, the data show that the high molecular weight coffee fraction, which is not determined as dietary fiber, is composed of a broad spectrum of compounds that differ in their molecular weights and chemical compositions although some coarse similarities (e.g., in the carbohydrate composition) exist. Because of the high content of chemically unknown substances, as well as the distinct brown color and UV-activity, it may be concluded that this coffee fraction is mainly composed of melanoidins with low carbohydrate and protein contents and this seems especially true for those subfractions with assumed lower molecular weights. The structures of coffee melanoidins, like those of food melanoidins in general, are still largely unknown. However, oligo- or polysaccharides and/or peptides/proteins as well as phenolic compounds are proposed as components of these complex structures in coffee deriving from the Maillard reaction (*7, 8, 23*).

Fermentability of the Ethanol Soluble High Molecular Weight Coffee Fraction. Although HESF is not determined as dietary fiber (enzymatic-gravimetric method), it is partially composed of nondigestible oligo- or polysaccharides that are however most likely incorporated into melanoidin structures. The fermentability of $HESF_{UF}$ was investigated by in vitro fermentation experiments with human fecal bacteria. Increasing OD over the course of fermentation indicated that $HESF_{UF}$ was utilized by fecal microbiota in vitro. However, this increase was less distinct (ca. 0.3 units) when compared to fermentation of SDF (ca. 0.45 units) (*2*) despite fermenting twice the amount

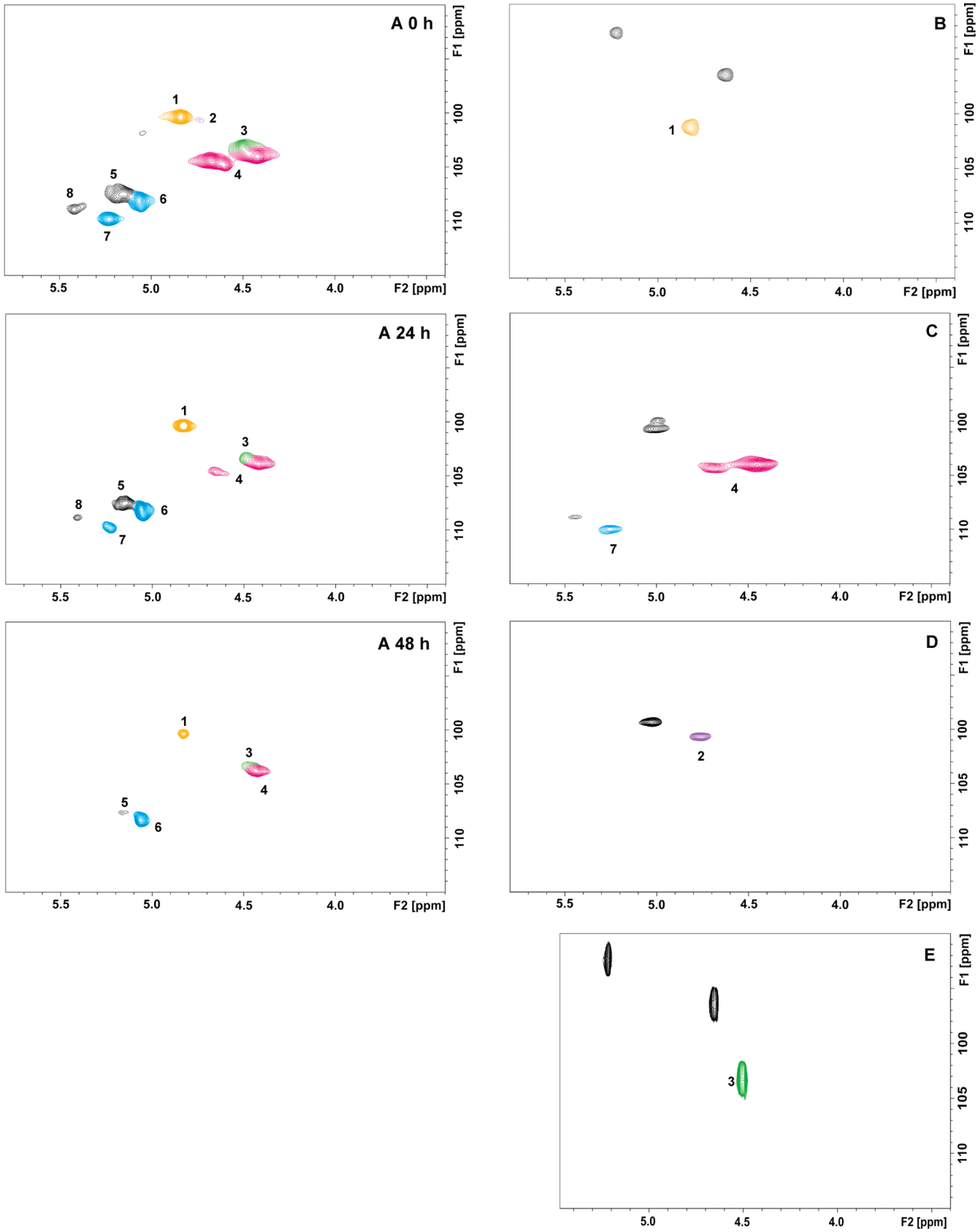

Figure 2. Anomeric region of HSQC spectra for a high molecular weight ethanol soluble coffee fraction ($HESF_{UF}$) (**A**) after 0, 24 and 48 h of fermentation, rutinose (**B**), larch arabinogalactan (**C**), carob galactomannan (**D**), and cellobiose (**E**). The color code is as follows: yellow, rhamnopyranosyl residues; purple, mannopyranosyl residues; magenta, galactopyranosyl residues; green, glucopyranosyl residues; blue, arabinofuranosyl residues. For signal numbers refer to text and **Table 7**.

of $HESF_{UF}$. Because $HESF_{UF}$ contained considerably less carbohydrates than SDF, these results indicate that noncarbohydrate components of $HESF_{UF}$ are less fermentable than coffee carbohydrates.

Table 7. Assignment of Characteristic Carbohydrate Correlations Present in HSQC Spectra of $HESF_{UF}$ (Presented in **Figure 2**)

correlation ($\delta H^1/\delta C^{13}$)	No. (**Figure 2**)	model compound for comparison	assignment	reference
4.84/100.39 ppm	**1**	rutinose	α-linked T-Rha*p*	-
4.84/100.39 ppm	**2**	carob galactomannan	β-linked 4-Man*p*	(*25*)
4.50/103.27 ppm	**3**	cellobiose	β-linked nonreducing Glc*p*	-
4.77−4.34/103.2−104.8 ppm	**4**	larch arabinogalactan	β-linked Gal*p*[a]	(*26*)
5.15/107.5 ppm	**5**	-	not assigned	-
5.07/108.08 ppm	**6**	-	α-linked 5-Ara*f*[b]	-
5.23/109.82 ppm	**7**	larch arabinogalactan	α-linked T-Ara*f*[b]	(*27, 28*)
5.42/108.9 ppm	**8**	-	not assigned	-

[a] A differentiation between correlations from T-Gal*p*, 3-Gal*p*, 6-Gal*p* and 3,6-Gal*p* was not attempted in this study. [b] Tentatively assigned (see text).

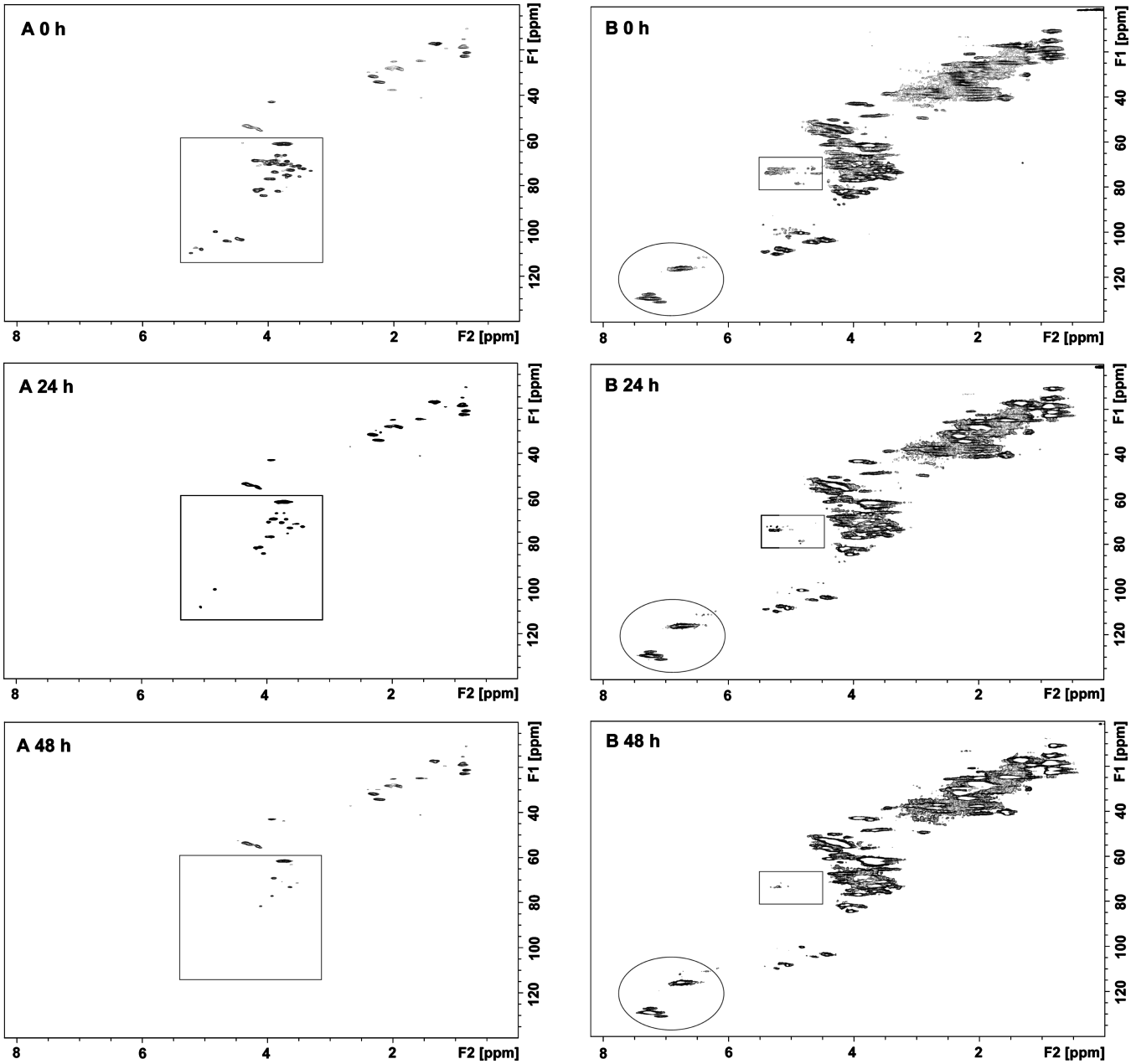

Figure 3. HSQC spectra of a high molecular weight ethanol soluble coffee fraction ($HESF_{UF}$) after 0, 24, and 48 h of fermentation displayed at high (**A**) and low (**B**) contour levels.

After 24 h of fermentation, acetate, propionate, and butyrate were produced in low concentrations in a molar ratio of ∼68:21:11 (**Table 5**), demonstrating higher proportions of propionate and butyrate than recently reported for the fermentation of coffee SDF (81:15:4) (*2*). Fermentation studies using larch arabinogalactan or guar as substrates showed that the microbial degradation of galactomannans leads to the production of higher molar proportions of acetate than the degradation of arabinogalactans (*24*). Since SDF polysaccharides were characterized by higher proportions of galactomannans than $HESF_{UF}$ carbohydrates (∼50 mol% vs ∼5 mol% mannose), this is a possible explanation for the differences in molar ratios of SCFA (although arabinogalactans from larch and galactomannans from guar are not completely comparable to those from coffee).

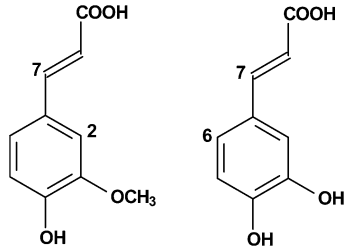

Figure 4. Ferulic acid (left) and caffeic acid (right); carbons and protons that were thought to show characteristic correlation signals in the HSQC spectra are numbered.

Chemical analysis showed that $HESF_{UF}$ polysaccharides were well degraded: after 24 h of fermentation, the remaining high molecular weight material contained 55% and after 48 h 66% less carbohydrates than the 0 h-sample (**Table 6**). Still, compared to the fermentation of coffee SDF, where about 91% of SDF carbohydrates were degraded after 24 h of fermentation (*2*), the carbohydrate degradation was less extensive. However, we also showed that coffee arabinogalactans were less utilized than galactomannans (*2, 6*). In addition, $HESF_{UF}$ arabinogalactans are possibly less degradable than SDF arabinogalactans because they show lower T-Ara*f*/5-Ara*f* and 3-Gal*p*/6-Gal*p* ratios. Also, it can not be ruled out that the high proportion of MRPs in coffee $HESF_{UF}$ negatively affect carbohydrate degradation. Finally, the lower bacterial growth, which was assumed to be due to limited substrate availability, may have resulted in a slower utilization of carbohydrates.

It was also shown that the total content of amino acids decreased over the course of fermentation (**Table 6**). However, no significant amounts of branched SCFA, known as microbial products from protein utilization, were measured after 24 or 48 h of fermentation (data not shown). In addition to polysaccharides and amino acids, MRPs were also utilized by fecal microbiota, as indicated by the decreasing contents of "unknown", non-amino acid nitrogen (**Table 6**). Despite the highly complex nature of this high molecular weight coffee fraction, $HESF_{UF}$ fermentation was successfully monitored by using 2D-NMR $^{13}C-^{1}H$ correlation experiments. To ensure comparability, the same sample amounts (residual high molecular weight material after 0, 24, and 48 h; 50 mg of each) were analyzed using the same conditions (NMR instrument, number of scans, temperature, solvent), and spectra were compared at similar contour levels. The relative decrease in the signals observed in the carbohydrate anomeric region from 0 to 24 h and 48 h of fermentation is shown in **Figure 2A**. By using carbohydrate model compounds and data available from the literature (*25–28*), most signals in the anomeric region could be assigned to $^{1}H/^{13}C$ correlations of specific monosaccharide residues (summarized in **Table 7**;). Correlations **6** (5.07 ppm/108.08 ppm) and **7** (5.23 ppm/109.82 ppm) were only tentatively assigned to α-linked 5-Ara*f* and to T-Ara*f*, respectively. The C-1 chemical shift of T-Ara*f* from larch arabinogalactans was reported to be around 110 ppm (*27, 28*), whereas 5-Ara*f* is not known as larch arabinogalactan constituent (*27–29*). This is in accordance with our larch arabinogalactan spectrum (**Figure 2C**). However, for the anomeric carbons of 5-Ara*f* and T-Ara*f* from arabinans, chemical shifts of ∼108 ppm were reported (*30–32*). Thus, signal **6** may also include the anomeric T-Ara*f* correlation signal.

NMR spectroscopy confirmed that the carbohydrates in $HESF_{UF}$ were readily utilized by fecal microbiota. **Figure 3** shows the HSQC spectra of 0 h-, 24 h-, and 48 h-samples at high (**A**, left) and low (**B**, right) contour levels. Considering the spectra at higher contour levels, the intensities of the correlations marked by a rectangle decrease over time. All carbohydrate correlations (with the exception of the methyl correlation from rhamnose) are located in this part of the spectrum. At low contour levels (**Figure 3B**), the spectra of the fermented samples revealed no obvious decrease in the intensity of correlations, which are located in the region of phenolic, aromatic, or olefinic proton/carbon correlations (denoted in a circle). Phenolic compounds are thought to be incorporated into coffee melanoidins (*7, 8, 23, 33*), and chlorogenic acids are often proposed to play a role in coffee melanoidin formation. However, characteristic correlations from intact caffeic or ferulic acid moieties (**Figure 4**) of chlorogenic

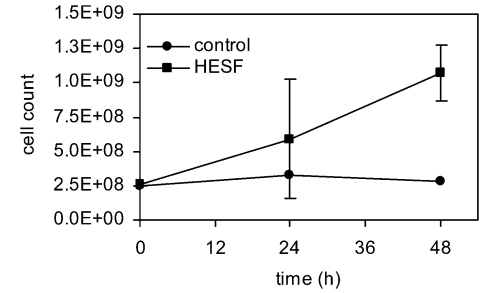

Figure 5. Total cell counts during fermentation of a high molecular weight ethanol soluble coffee fraction ($HESF_{UF}$) (after 0, 24, and 48 h) as analyzed by fluorescence in situ hybridization. Control = medium and bacteria without substrate.

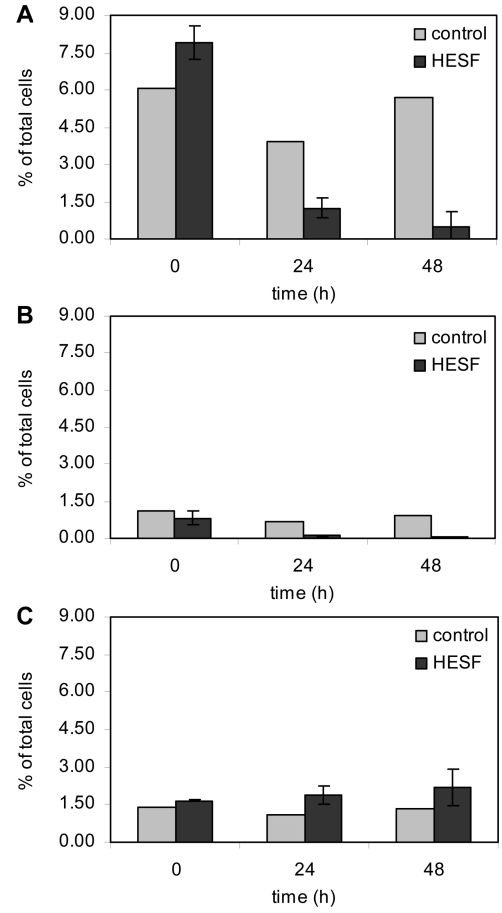

Figure 6. Cell counts of different bacterial groups during fermentation of a high molecular weight ethanol soluble coffee fraction ($HESF_{UF}$) (after 0, 24, and 48 h) as analyzed by fluorescence in situ hybridization: (**A**) *Eubacterium rectale-Clostridium coccoides* group; (**B**) bifidobacteria; (**C**) *Bacteroides-Prevotella* group. Control = medium and bacteria without substrate.

acids were absent in the $HSQC_{UF}$ (0 h-sample): no correlations assigned to H-7/C-7 (7.49 ppm/146.86 ppm) or H-6/C-6 (7.01 ppm/123.40 ppm) of caffeic acid (spectrum of 5-*O*-caffeoylchinic acid not shown) were present even at contour levels close to the noise. The characteristic correlations deriving from H-7/C-7 (~7.6−7.7 ppm/147 ppm) and H-2/C-2 (~7.1−7.2 ppm/112 ppm) of ferulic acid (*34*) were also not present in the HSQC spectrum of $HESF_{UF}$. Absence of these correlations implies that intact caffeic or ferulic acid moieties are not (or insignificantly) incorporated into the melanoidins of the ethanol soluble high molecular weight coffee fraction. Hence, the circled correlations (**Figure 3A**) are suggested to derive from other compounds. The spectra at low contour levels (**Figure 3B**) also revealed that, in addition to polysaccharides, other yet unknown compounds (correlations delineated by a rectangle) are utilized by fecal bacteria. In this region, no correlations of proteins or carbohydrates are expected, as confirmed by the HSQC spectra of carbohydrates (larch arabinogalactan, carob galactomannan, and cellobiose) and bovine serum albumin (spectrum not shown) and comparison with data from the literature. This finding indicates a degradation or modification of unknown noncarbohydrate and nonprotein compounds deriving from the Maillard reaction. In the marked region (rectangle), correlations of noncarbohydrate and nonprotein carbon atoms bound to a proton and a heteroatom are expected (e.g., HC−O or HC−N), but no specific suggestions are possible at this point. To sum up, the results of our in vitro study indicate that noncarbohydrate and nonprotein moieties of coffee melanoidin complexes are fermented in the colon as well. However, whether or not the high molecular weight coffee fraction, which is not measured as dietary fiber using the enzymatic gravimetric procedure, adds to the dietary fiber content of coffee beverages beyond its carbohydrate content remains to be addressed.

Effects on the Composition of the Fecal Microbiota. FISH analysis was carried out to monitor changes in the proportion of dominant bacterial groups during degradation of $HESF_{UF}$. The proportions of three dominant bacterial groups relative to the total cell counts at 0, 24, and 48 h (**Figure 5**) were compared. Whereas the proportions of members of the *Eubacterium rectale-Clostridium coccoides* group and of bifidobacteria decreased (**Figure 6A** and **6B**, respectively), the proportion of members of the *Bacteroides-Prevotella* group was fairly stable over 48 h of incubation (**Figure 6C**). Thus, members of the *Bacteroides-Prevotella* group were possibly capable of partly utilizing $HESF_{UF}$ as a substrate. Because bacteroides are well-known for their ability to degrade various polysaccharides including polysaccharides from coffee brews (*2*), we assume that members of this group are responsible for $HESF_{UF}$ polysaccharide degradation. The small increase in the total cell count was most likely due to substrate limitations, owing to the low carbohydrate content of $HESF_{UF}$. However, the polysaccharide composition with arabinogalactans as dominating carbohydrates might also have some impact.

A large proportion of the microbiota remained unidentified and further investigations are needed to reveal which bacterial groups are involved in the degradation of these high molecular noncarbohydrate and nonprotein coffee compounds.

ABBREVIATIONS USED

5-Ara*f*, (1→5)-linked arabinofuranosyl residues; 3-Gal*p*, (1→3)-linked galactopyranosyl residues; 6-Gal*p*, (1→6)-linked galactopyranosyl residues; 3,6-Gal*p*, (1→3,6)-linked galactopyranosyl residues; FISH, fluorescence in situ hybridization; $HESF_D$ and $HESF_{UF}$, high molecular weight ethanol soluble coffee fraction, obtained by dialysis and ultrafiltration, respectively; HSQC, heteronuclear single quantum coherence; MRP, Maillard reaction products; MWCO, molecular weight cutoff; OD, optical density; PBS, phosphate buffered saline; SDF, soluble dietary fiber; T-Ara*f*, terminally linked arabinofuranosyl residues; T-Gal*p*, terminally linked galactopyranosyl residues; T-Rha*p*, terminally linked rhamnopyranosyl residues; TRIS, tris(hydroxymethyl)aminomethane.

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