Sex-Dependent Colonic Microbiota Modulation by Hazelnut (*Corylus avellana* L.) Dietary Fibers

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SUPPLEMENTARY TEXT

Materials & methods

Determining the monosaccharide compositions and linkage profiles of the extracted IDFs and SDFs

Monosaccharide compositions and linkage characterizations of extracted IDFs and SDFs were analyzed using a gas chromatography coupled with mass spectrometry (GC/MS), as previously described by Pettolino *et al.*[1]. Briefly, samples were firstly subjected to carboxyl reduction. For monosaccharide composition, pre-reduced samples were exposed to alditol acetate derivatization protocol, where hydrolysis of SDFs and IDFs were achieved using trifluoroacetic acid (TFA) and sulfuric acid, respectively.[1] The monosaccharides were quantified as their alditol acetate derivatives on a capillary column (RTX-2330, Restek Corp., Bellefonte, PA, USA) by GC/MS (Shimadzu QP-2010 Ultra, Shimadzu Corp. Kyoto, Japan). For linkage characterizations, pre-reduced samples were subjected to a partial-methylation process, followed by exposure to alditol acetate derivatization protocol.[1] Partially methylated alditol acetates were run on GC/MS coupled with RTX-2330 column and quantified as previously specified.[1] The GC/MS setup conditions were as follows; injection temperature: 240 °C; injection volume 1 µL (split ratio: 1:15); helium was used as a carrier gas at 1.14 mL/min; oven temperature was set to 160 °C. This temperature was held for 7.15 min, then ramped at 4 °C/min to 220 °C, held for 4.10 min, and then ramped at 2.90 °C/min to 240 °C, held for 5.15 min and finally ramped at 10.8 °C/min to 260 °C, with a final hold of 5.10 min. The mass selective detector (MSD) transfer line was maintained at 260 °C.

Results and Discussion

Monosaccharide and glycosidic linkage compositions of DFs extracted from natural hazelnut, roasted hazelnut, and hazelnut skin

Natural hazelnut, roasted hazelnut, and hazelnut skin possess structurally different IDFs

IDFs were composed mainly of glucose, xylose, arabinose, galacturonic acid, and mannose (**Table S1**), indicating that hazelnut DFs are mainly composed of cellulose, pectic polysaccharides, and xyloglucans. Glycosidic linkage analyses also confirmed these predictions (**Table S1**). This is in an agreement with our previous research[2] and others who worked on the DFs of dicotyledonous plants (to which hazelnut belongs), which are composed mainly of cellulose, pectic polysaccharides, and xyloglucans.[3-5] However, monosaccharide compositions of extracted DFs differed among groups, suggesting that their structural features vary. For example, IDFs of hazelnut skin had a significantly (p<0.05) higher amount of galacturonic acid (11.24%), compared to those from roasted (4.41%) and natural (6.61%) hazelnuts. To quantify and provide detailed insights into the structural features of IDFs, we examined their glycosidic linkage profiles in detail.

Hemicelluloses: The main hemicelluloses found in dicotyledonous plants are heteromannans and xyloglucans.[5] 4-Man and 4,6-Man linkages are components of the heteromannan chain.[1,6] In addition to possessing t-Gal units (indicative of the number of branches), heteromannans could also contain 4-Glc equivalent to 4-Man; and 4,6-Glc equivalent to 4,6-Man linkages (Pettolino et al., 2012). Thus, by summing up the 4-Man, 4-Glc (equivalent to 4-Man), 4,6-Man, 4,6-Glc (equivalent to 4,6-Man) and t-Gal (equivalent to branches), heteromannans present in the insoluble fractions of natural hazelnut, roasted hazelnut, and hazelnut skin were calculated to be 8.07%, 8.59%, and 2.84%, respectively. The remaining 4,6-Glc was assumed to be part of xyloglucan. Moreover, xyloglucans also contain the 4-Glc linkage (equivalent to 4,6-Glc) and 2-Xyl (equivalent to or less than branching points), in addition to having t-Fuc and t-Xyl in their branching points.[1,6] By summing up the 4,6-Glc, 4-Glc, 2-Xyl, t-Fuc, and t-Xyl, xyloglucans were found to contain 19.22%, 22.98%, and 17.20% of the insoluble fractions of natural hazelnut, roasted hazelnut, and hazelnut skin, respectively. The presence of 2-Xyl, t-Fuc, and t-Xyl glycosidic linkages suggest that xyloglucans found in the insoluble fractions of natural hazelnut, roasted hazelnut, and hazelnut skin possess X and F side chains as branching units. In addition, by calculating the ratio of xylose-related linkages to glucose-related linkages, branching densities of these xyloglucans were estimated to be 0.61, 0.53, and 0.70 for the xyloglucans of natural hazelnut, roasted hazelnut, and hazelnut skin, respectively. These suggest that xyloglucans in the insoluble fraction of hazelnut skin were more densely branched, compared to that of natural hazelnut, whereas that of roasted hazelnut was the least densely branched.

Cellulose: 4-Glc is a common glycosidic linkage present in starch, cellulose, and hemicelluloses of plants. Since hazelnut has been known to contain only limited amount of starch (< 2%)[7] and samples used in this study were extensively treated with pancreatin (that contains amylases), all the detected 4-Glc linkages in these samples very likely belong to cellulose and hemicelluloses. By subtracting 4-Glc linkages assigned to hemicelluloses from total 4-Glc linkages, we estimated the cellulose contents in the insoluble fraction of the samples to be 39.95%, 46.56%, and 49.77% for natural hazelnut, roasted hazelnut, and hazelnut skin, respectively.

Pectic polysaccharides: The presences of high number of 1,4-GalA linkages (a typical bond found in the linear chain of pectic polysaccharides [homogalacturonan, HG]), and 2-Rha and 2,4-Rha linkages (typical linkages found in the hairy region of pectic polysaccharides [rhamnogalacturonan I, RGI]) indicate that the insoluble fractions of the extracted DFs contain a significant amount of pectic polysaccharides. RGI/HG ratios of the pectic polysaccharides were estimated by calculating the rhamnose to galacturonic acid residues ratio, which were found to be 0.59, 0.37, and 0.25 for natural hazelnut, roasted hazelnut, and hazelnut skin, respectively. These data suggest that pectic polysaccharides in the insoluble fractions of natural hazelnut possess more hairy regions than that of roasted hazelnut, and pectic polysaccharides of hazelnut skin are the least branched. The rhamnose/galacturonic acid ratio obtained in this study is lower than the ones obtained in our previous study[2] in which the rhamnose/galacturonic acid ratio for IDFs of natural hazelnut, roasted hazelnut, and hazelnut skin were calculated to be 0.49, 0.64, and 0.49, respectively. These differences between studies could be attributed to the different harvesting years because harvesting time has been known to impact the structural features of structural polysaccharides of plants.[8]

Branching densities of the RGI regions of hazelnut pectic polysaccharides were also estimated by calculating the ratio of 2,4-Rha linkage (branching units) to total rhamnose-related linkages, which were found to be 0.47, 0.49, and 0.55 for natural hazelnut, roasted hazelnut, and hazelnut skin, respectively, suggesting that RGI region of hazelnut pectic polysaccharides are heavily branched. The presence of arabinose linkages [5-Ara(*f*), 3,5-Ara(*f*), 2,3,5-Ara(*f*) and t-Ara(*f*)] as well as galactose related linkages [t-Gal and 4-Gal] indicate that the branching points of RGI regions are made mainly of arabinan and galactan molecules.

The total pectic polysaccharide amount present in the insoluble fractions of samples were estimated by summing up the 2-Rha, 2,4-Rha, t-Ara(*f*), 5-Ara(*f*), 3,5-Ara(*f*), 2,3,5-Ara(*f*), t-Gal, 4-Gal, t-GalA, and 4-GalA. These values were found to be 25.46%, 16.76%, and 22.71% for natural hazelnut, roasted hazelnut, and hazelnut skin, respectively.

Natural hazelnut, roasted hazelnut, and hazelnut skin possess structurally different SDFs

Arabinose, galacturonic acid, galactose, xylose, glucose, rhamnose, and mannose were mainly detected in SDFs of the samples (**Table S2**), indicating that the SDFs of hazelnut samples are mainly composed of pectic polysaccharides and hemicelluloses (mainly xyloglucans and [hetero]mannans). Glycosidic linkage analyses also confirmed the presence of such polysaccharide molecules (**Table S2**), which is in an agreement with our previous research.[2] Like the IDFs, monosaccharide compositions of SDFs differ among groups, suggesting that their structural features vary.

Pectic polysaccharides: Total pectic polysaccharide contents of hazelnut DFs were estimated by summing up the pectic polysaccharide related linkages [2-Rha, 2,4-Rha, t-Ara(*f*), 5-Ara(*f*), 3,5-Ara(*f*), 2,3,5-Ara(*f*), t-Gal, 4-Gal, t-GalA, and 4-GalA]. These values were calculated to be 67.8%, 63.9%, and 57.7% for natural hazelnut, roasted hazelnut, and hazelnut skin samples, respectively. In addition, rhamnose/galacturonic acid ratios for natural hazelnut, roasted hazelnut, and hazelnut skin samples were calculated to be 0.65, 0.35, and 0.18, respectively, suggesting that pectic polysaccharides in the soluble fractions of hazelnut skin possess longer linear (smooth) regions and less branched (hairy) regions, compared to those from natural and roasted hazelnuts. Moreover, like the ones in insoluble fractions of the samples, the ratio of the 2,4-Rha linkage to total rhamnose-related linkages (calculated to be 48%, 63% and 51% for natural hazelnut, roasted hazelnut, and hazelnut skin samples, respectively) suggests that RGI of the pectic polysaccharides in soluble fractions of the samples are densely branched. Arabinose/galactose ratio of the samples (1.64, 1.84, and 1.78 for natural hazelnut, roasted hazelnut, and hazelnut skin samples, respectively) suggests that the RGI of the pectic polysaccharides in the soluble fractions of the samples contains more arabinan as a branching point than galactan molecules.

Hemicelluloses: The presences of significant amounts of mannoses, glucose and xylose suggest that soluble fractions of the samples are composed mainly of mannan and xyloglucan. Detection of significant amounts of 4-Man, 4-Glc, and 4,6-Glc also confirms this, as they are the typical linkages of mannan and xyloglucans. Mannan polymers could possess branching points at the O-6 positions of mannose residues; however, no 4,6-Man linkages were detected, suggesting that mannans detected in the soluble fractions have a linear structure. Such linear mannans are also detected in many other plants.[9,10] Therefore, the relative abundances of mannan polymers in the samples were assumed to be equal to the that of mannose units, which are 4.1%, 7.7%, and 8.2% for natural hazelnut, roasted hazelnut, and hazelnut skin samples, respectively.

Xyloglucan contents of the samples were estimated to be 13.4% and 18.4% for natural and roasted hazelnut samples, respectively by summing up the 4,6-Glc, 4-Glc and t-Xyl (equal to the 4,6-Glc). Detection of only t-Xyl suggests that xyloglucans of soluble fractions of natural and roasted hazelnut samples have X-type side chains.[5,11] On the other hand, no evidence of the 4,6-Glc linkage was detected in the soluble fraction of the hazelnut skin sample, suggesting that, unlike natural and roasted hazelnut samples, xyloglucans found in the soluble fraction of hazelnut skin have a linear structure.

SUPPLEMENTARY TABLES

Table S1. Monosaccharide and glycosidic linkage compositions of IDFs of roasted hazelnut, natural (raw) hazelnut, and hazelnut skin (mole basis, %)*.

Monosaccharide / Glycosidic linkage	Amount (%, mole)		
	Roasted hazelnut	Natural hazelnut	Hazelnut skin
Rhamnose	1.63 ± 0.41 ^{B}	3.89 ± 0.39 ^{A}	2.84 ± 0.78 ^{AB}
Fucose	1.43 ± 0.12	2.16 ± 1.38	1.05 ± 0.20
Arabinose	7.03 ± 0.49 ^{B}	11.66 ± 1.32 ^{A}	4.93 ± 0.16 ^{C}
Xylose	11.25 ± 0.27 ^{B}	12.58 ± 0.61 ^{A}	12.54 ± 0.52 ^{A}
Mannose	3.92 ± 0.27 ^{A}	3.10 ± 0.64 ^{AB}	2.30 ± 0.34 ^{B}
Galactose	5.36 ± 0.26 ^{A}	5.14± 0.39 ^{A}	3.70 ± 0.24 ^{B}
Galacturonic acid	4.41 ± 0.21 ^{B}	6.61 ± 0.50 ^{B}	11.24 ± 0.71 ^{A}
Glucose	64.65 ± 1.18 ^{A}	53.83 ± 1.86 ^{B}	61.30 ± 1.20 ^{A}
Glucuronic acid	0.32 ± 0.01 ^{B}	1.02 ± 0.04 ^{A}	0.09 ± 0.00 ^{C}
t-Fuc(*p*)	1.43 ± 0.12	2.16 ± 1.38	1.05 ± 0.20
t-Ara(*f*)	1.87 ± 0.13 ^{B}	2.67 ± 0.30 ^{A}	0.53 ± 0.02 ^{C}
5-Ara(*f*)	2.97 ± 0.21 ^{B}	4.38 ± 0.49 ^{A}	3.21 ± 0.10 ^{B}
3,5-Ara(*f*)	1.28 ± 0.09 ^{B}	2.52 ± 0.29 ^{A}	0.16 ± 0.01 ^{C}
2,3,5-Ara(*f*)	0.91 ± 0.06 ^{B}	2.09 ± 0.24 ^{A}	1.03 ± 0.03 ^{B}
2-Rha(*p*)	0.83 ± 0.21 ^{B}	2.08 ± 0.21 ^{A}	1.28 ± 0.35 ^{B}
2,4-Rha(*p*)	0.80 ± 0.20 ^{B}	1.82 ± 0.18 ^{A}	1.56 ± 0.43 ^{A}
t-Xly(*p*)	6.02 ± 0.14 ^{B}	9.26 ± 0.45 ^{A}	2.81 ± 0.12 ^{C}
2-Xyl(*p*)	1.83 ± 0.04 ^{C}	3.32 ± 0.16 ^{B}	3.82 ± 0.16 ^{A}
2,4-Xyl(*p*)	1.63 ± 0.04 ^{A}	–	1.41 ± 0.06 ^{B}
2,3,4-Xyl(*p*)	1.77 ± 0.04 ^{B}	–	4.49 ± 0.19 ^{A}
t-Man(*p*)	0.45 ± 0.03 ^{B}	–	0.88 ± 0.13 ^{A}
4-Man(*p*)	2.45 ± 0.17 ^{A}	2.42 ± 0.50 ^{A}	0.94 ± 0.14 ^{B}
4,6-Man(*p*)	1.02 ± 0.07 ^{A}	0.69 ± 0.14 ^{B}	0.48 ± 0.07 ^{B}
t-Gal(*p*)	1.67 ± 0.08	1.85 ± 0.14	–
4-Gal(*p*)	3.69 ± 0.18	3.29 ± 0.25	3.69 ± 0.23
t-GalA(*p*)	1.30 ± 0.06 ^{C}	1.90 ± 0.14 ^{B}	3.30 ± 0.21 ^{A}
4-GalA(*p*)	3.11 ± 0.15 ^{C}	4.71 ± 0.35 ^{B}	7.95 ± 0.50 ^{A}
t-Glc(*p*)	0.52 ± 0.01 ^{B}	0.17 ± 0.01 ^{C}	0.59 ± 0.01 ^{A}
4-Glc(*p*)	56.06 ± 1.02 ^{A}	47.67 ± 1.64 ^{B}	55.47 ± 1.08 ^{A}
4,6-Glc(*p*)	8.07 ± 0.15 ^{A}	5.99 ± 0.21 ^{B}	5.24 ± 0.10 ^{C}
t-GlcA(*p*)	0.32 ± 0.01 ^{B}	1.02 ± 0.04 ^{A}	0.10 ± 0.00 ^{C}

* Values are represented as mean ± standard error. Statistical analyses were done between fiber groups for each constituent, and values possessing different capital letters are statistically different (Tukey's test, $p<0.05$).

Table S2. Monosaccharide and glycosidic linkage compositions of soluble DFs of roasted hazelnut, natural (raw) hazelnut, and hazelnut skin (mole basis, %)*.

Monosaccharide	Amount (%, mole)		
	Roasted hazelnut	Natural hazelnut	Hazelnut skin
Rhamnose	6.53 ± 0.48 ^{B}	8.23 ± 0.00 ^{A}	6.56 ± 0.55 ^{B}
Fucose	1.35 ± 0.12	1.93 ± 0.36	1.10 ± 0.68
Arabinose	24.98 ± 1.85 ^{A}	24.83 ± 1.13 ^{A}	9.72 ± 0.96 ^{B}
Xylose	8.86 ± 0.96 ^{B}	13.20 ± 0.64 ^{A}	8.81 ± 1.93 ^{B}
Mannose	7.73 ± 1.38 ^{A}	4.05 ± 1.04 ^{B}	8.19 ± 0.51 ^{A}
Galactose	13.57 ± 2.20 ^{A}	15.18 ± 0.41 ^{A}	5.46 ± 0.04 ^{B}
Galacturonic acid	18.84 ± 3.05 ^{B}	19.53 ± 0.53 ^{B}	35.97 ± 0.23 ^{A}
Glucose	14.97 ± 5.14 ^{AB}	12.74 ± 0.16 ^{B}	23.50 ± 0.81 ^{A}
Glucuronic acid	0.71 ± 0.24	0.31 ± 0.00	0.69 ± 0.02
t-Ara(*f*)	4.92 ± 0.36 ^{A}	4.46 ± 0.20 ^{A}	2.11 ± 0.21 ^{B}
5-Ara(*f*)	8.20 ± 0.61 ^{A}	5.75 ± 0.26 ^{B}	5.22 ± 0.52 ^{B}
3,5-Ara(*f*)	4.31 ± 0.32 ^{B}	7.49 ± 0.34 ^{A}	–
2,3,5-Ara(*f*)	7.55 ± 0.56 ^{A}	7.12 ± 0.33 ^{A}	2.39 ± 0.24 ^{B}
2-Rha(*p*)	2.39 ± 0.18 ^{C}	4.25 ± 0.32 ^{A}	3.23 ± 0.27 ^{B}
2,4-Rha(*p*)	4.13 ± 0.31 ^{A}	3.98 ± 0.18 ^{AB}	3.33 ± 0.28 ^{B}
t-Xly(*p*)	8.86 ± 0.96 ^{B}	13.20 ± 0.64 ^{A}	8.81 ± 1.93 ^{B}
t-Man(*p*)	3.90 ± 0.70 ^{A}	1.71 ± 0.44 ^{B}	4.52 ± 0.28 ^{A}
4-Man(*p*)	3.83 ± 0.68	2.33 ± 0.60	3.67 ± 0.23
4-Gal(*p*)	13.57 ± 2.20 ^{A}	15.18 ± 0.41 ^{A}	5.46 ± 0.04 ^{B}
t-GalA(*p*)	7.34 ± 1.19 ^{B}	8.03 ± 0.22 ^{AB}	9.69 ± 0.06 ^{A}
4-GalA(*p*)	11.50 ± 1.86 ^{B}	11.50 ± 0.31 ^{B}	26.28 ± 0.17 ^{A}
t-Glc(*p*)	0.93 ± 0.32 ^{A}	0.53 ± 0.01 ^{AB}	0.29 ± 0.01 ^{A}
4-Glc(*p*)	9.95 ± 4.42 ^{B}	11.11 ± 0.14 ^{B}	23.22 ± 0.80 ^{A}
4,6-Glc(*p*)	4.24 ± 1.46 ^{A}	1.14 ± 0.01 ^{A}	-
t-GlcA(*p*)	0.71 ± 0.24	0.31 ± 0.04	0.69 ± 0.02

* Values are represented as mean ± standard error. Statistical analyses were done between fiber groups for each constituent, and values possessing different capital letters are statistically different (Tukey's test, $p<0.05$).

Table S3. Composition of commercial chow that was used to prepare the modified chow and used to feed mice during the acclimatization period.*

Constituent	Amount (%)	Vitamin	Amount (minimum)
Dry matter	88	Vitamin A (IU/kg)	24000
Crude protein	20	Vitamin D_3 (IU/kg)	3000
Crude cellulose	5	Vitamin E (mg/kg)	300
Ash	5.2	Vitamin K_3 (mg/kg)	30
Oil	2.7	Vitamin B_1 (mg/kg)	20
Calcium	0.9	Vitamin B_2 (mg/kg)	20
Phosphorous	0.6	Vitamin B_6 (mg/kg)	12
Lycine	0.96	Vitamin B_{12} (μg/kg)	100
Methionin	0.40	Nicotinic acid (mg/kg)	100
Meth+Cys	0.66	Pantotenic acid (mg/kg)	41
Sodium	0.14	Biotin (mg/kg)	6,3
Metabolic energy (Kcal/Kg)	2600	Cholin (mg/kg)	1000

***** Ingredient table was provided by the manufacturer (MBD Deney Hayvanları Yemi, Gebze, Kocaeli, Türkiye).

SUPPLEMENTARY FIGURES

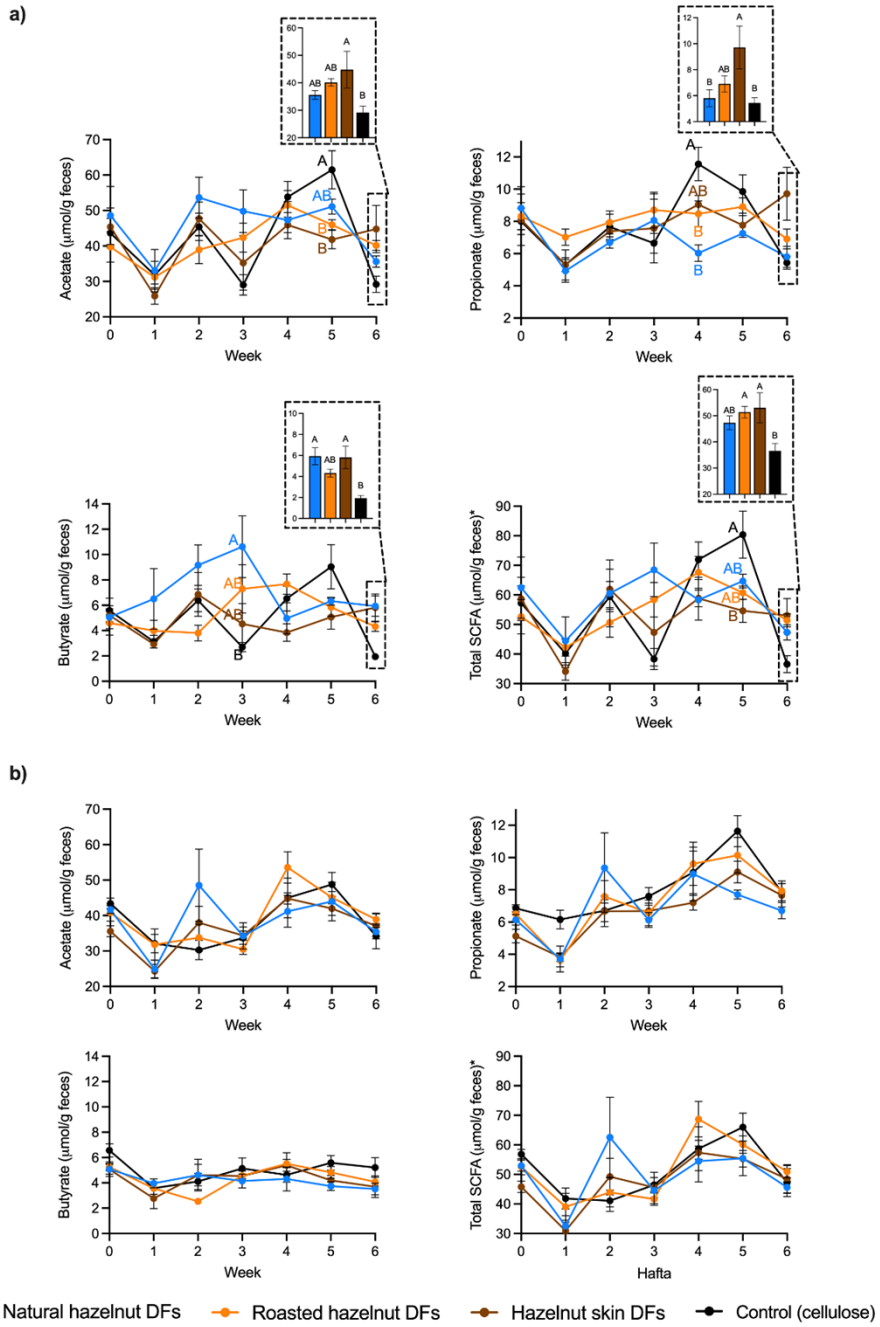

Figure S1. Weekly changes in the absolute amounts of acetate, propionate, butyrate and total SCFA in the feces of **a)** female and **b)** male mouse during the feeding period. Error bars represent the standard error of means (n=6). In each weeks' samples, statistical analyses were done between groups for each week, and values possessing different capital letters are statistically different (Tukey's test, P<0.05). *Total SCFA = acetate + propionate + butyrate.

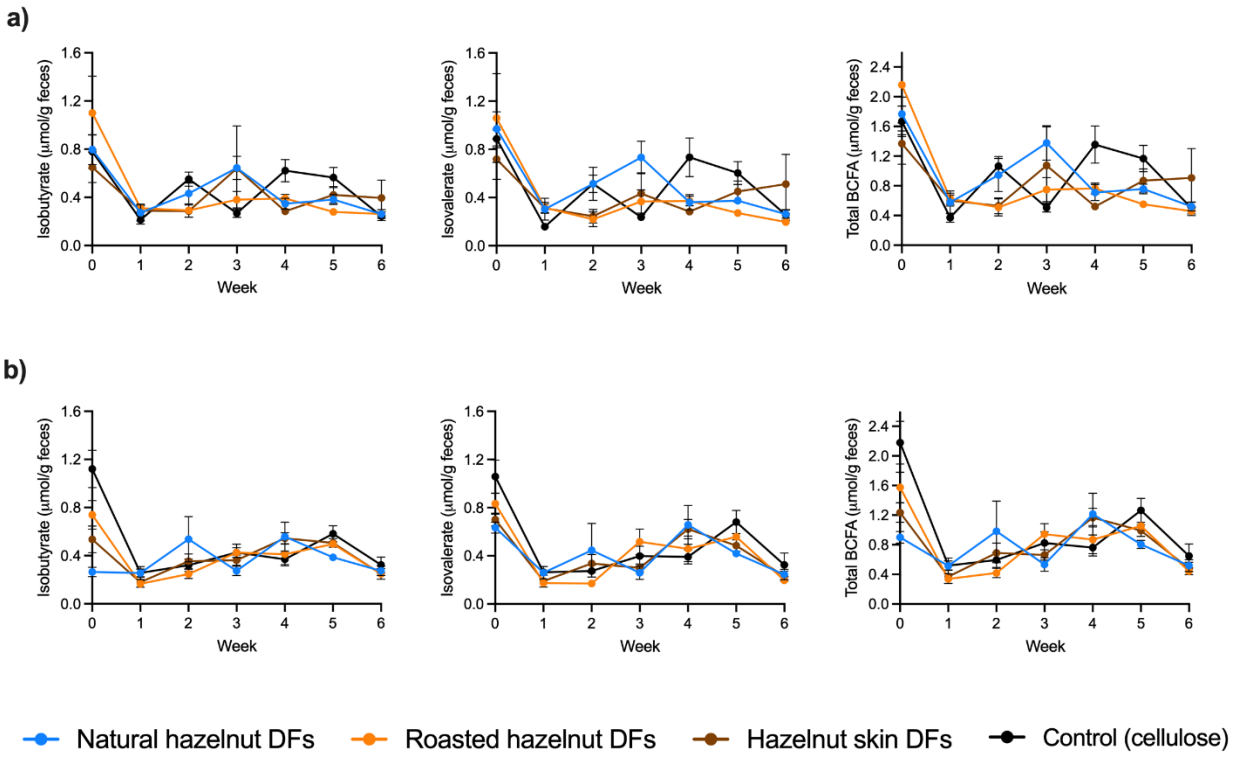

Figure S2. Weekly changes in the absolute amounts of isobutyrate, isovalerate, and total BCFA in the feces of **a)** female and **b)** male mouse during the feeding period. Error bars represent the standard error of means (n=6). No statistical differences were found between groups within each week (Tukey's test, P>0.05). *Total BCFA = isobutyrate + isovalerate.

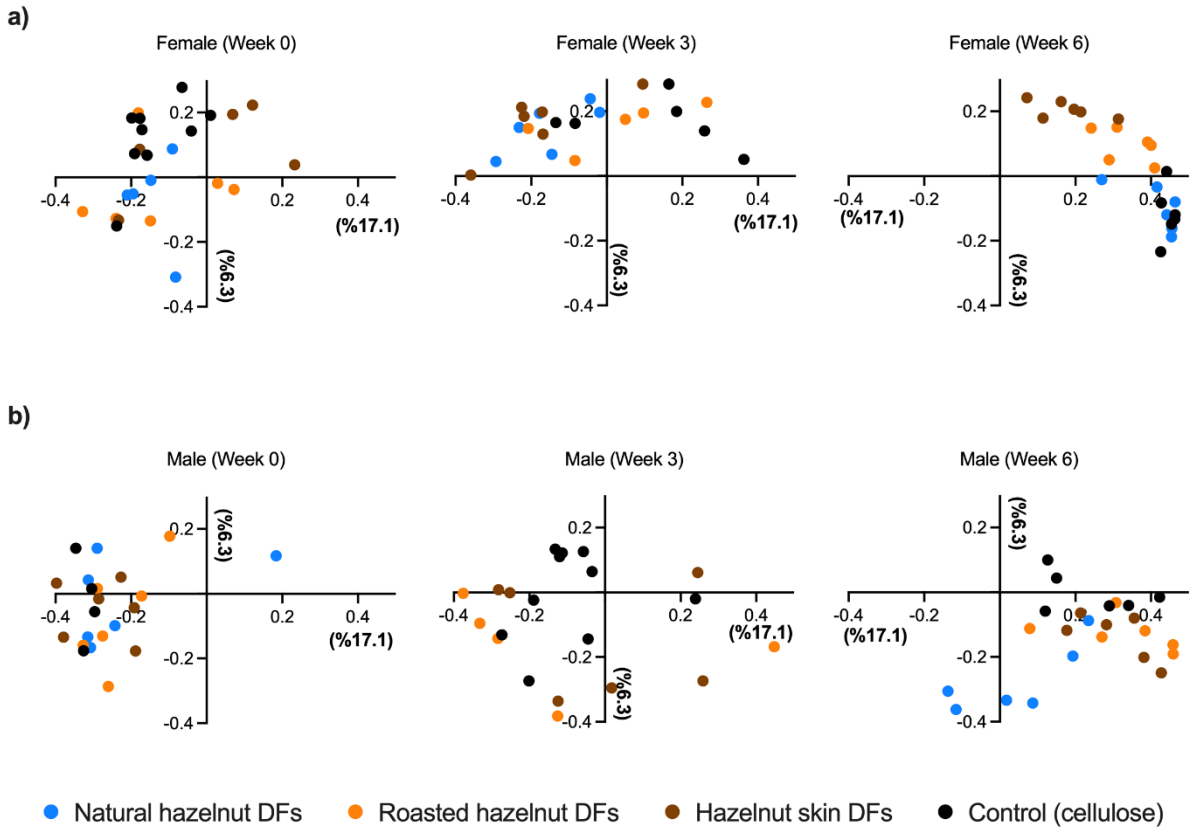

Figure S3. ThetaYC dissimilarity of fecal microbial communities of **a)** female and **b)** male mouse at weeks 0, 3, and 6 of feeding period based on the relative abundances of OTUs at 97% similarity level.

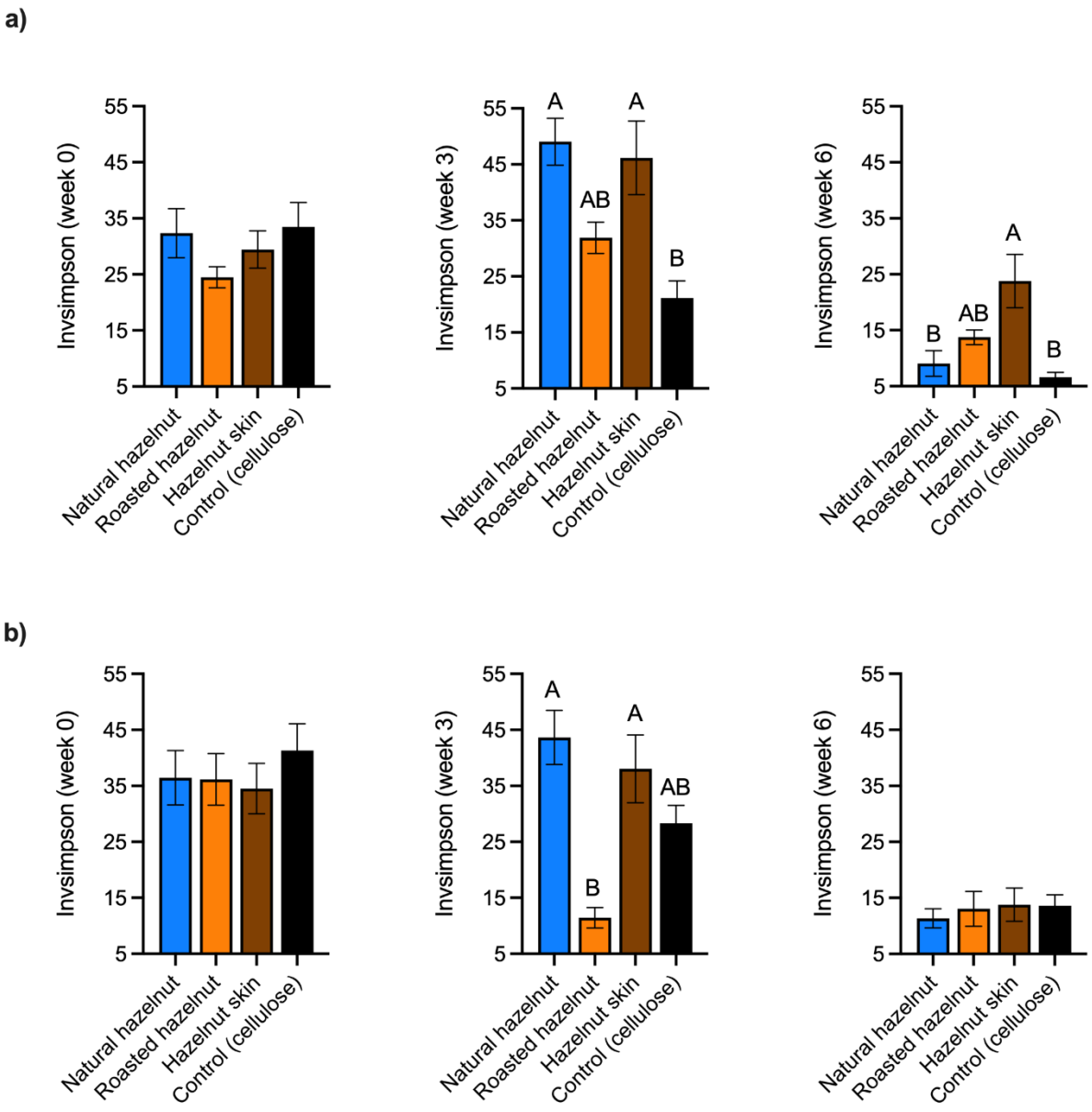

Figure S4. Changes in α-diversity of the fecal microbiota communities of a) female and b) male mouse, as measured by Invsimpson index calculator. Error bars represent the standard error of means (n=6). Different letters represent significant differences between samples ($P<0.05$).

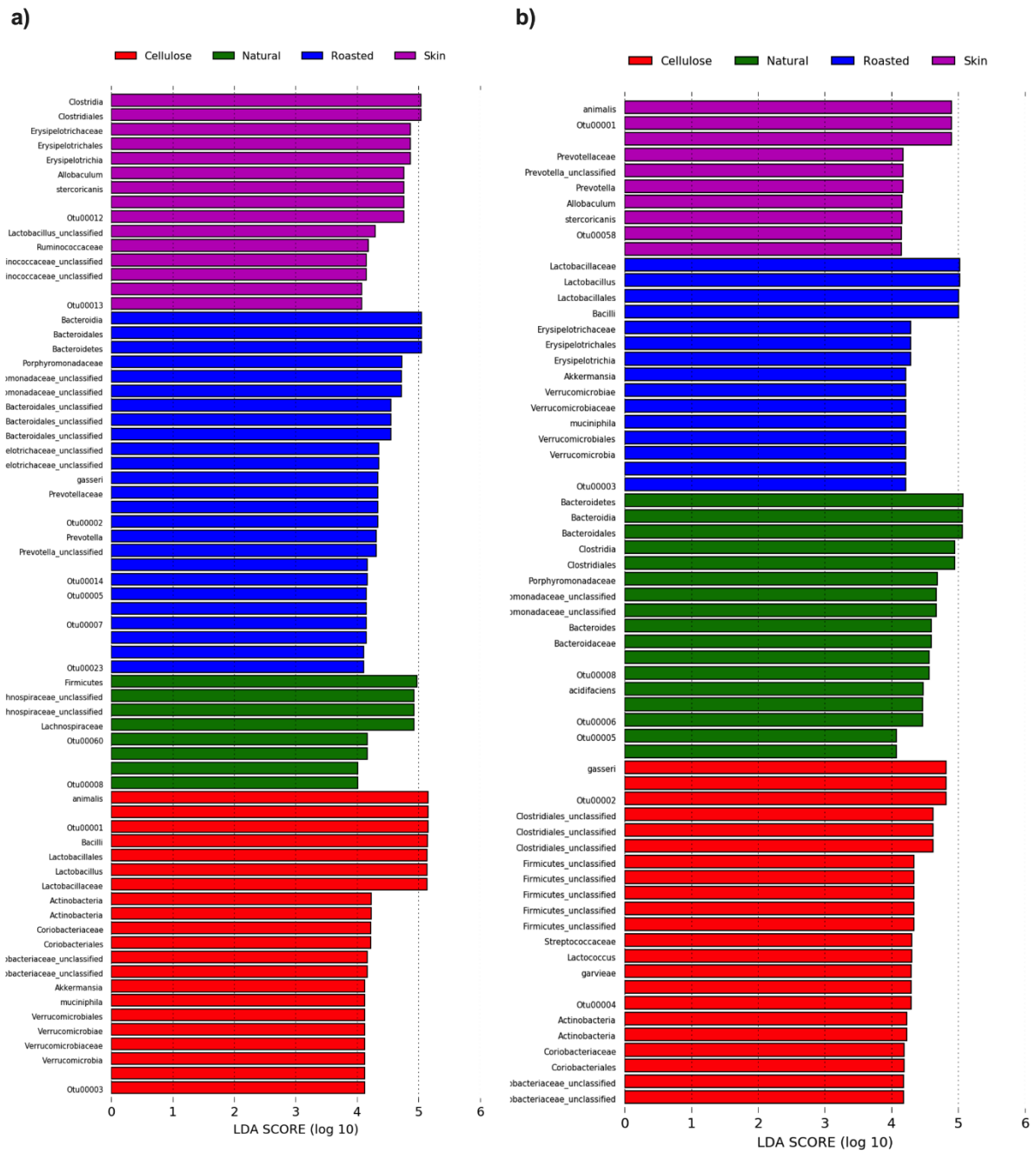

Figure S5. LDA results for a) female and b) male obtained as a result of LEfSe in the **a)** female and **b)** male mouse feces at the end of the feeding period (week 6) (LDA>4.0).

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