RESEARCH ARTICLE

Gut metabolites and bacterial community networks during a pilot intervention study with flaxseeds in healthy adult men

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Scope: Flaxseeds contain the phytoestrogens lignans that must be activated to enterolignans by intestinal bacteria. We investigated the impact of flaxseeds on fecal bacterial communities and their associations with fecal and blood metabolites.

Methods and results: Nine healthy male adult subjects ingested 0.3 g/kg/day flaxseeds during 1 week. Gut bacteria as well as blood and fecal metabolites were analyzed. Ingestion of flaxseeds triggered a significant increase in the blood concentration of enterolignans, accompanied by fecal excretion of propionate and glycerol. Overall diversity and composition of dominant fecal bacteria remained individual specific throughout the study. Enterolactone production was linked to the abundance of two molecular species identified as *Ruminococcus bromii* and *Ruminococcus lactaris*. Most dominant species of the order *Bacteroidales* were positively associated with fecal concentrations of either acetic, isovaleric, or isobutyric acid, the latter being negatively correlated with blood levels of triglycerides. The relative sequence abundance of one *Gemmiger* species (*Ruminococcaceae*) and of *Coprococcus comes* (*Lachnospiraceae*) correlated positively with blood levels of LDL cholesterol and triglycerides, respectively.

Conclusion: Flaxseeds increase enterolignan production but do not markedly alter fecal metabolome and dominant bacterial communities. The data underline the possible role of members of the family *Ruminococcaceae* in the regulation of enterolignan production and blood lipids.

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1 Introduction

The human intestine is home to trillions of microbial cells referred to as the intestinal microbiota. The use of antibiotics, germfree mice, and fecal transplantations has demonstrated that gut bacterial communities take over important functions for their host such as the exclusion of pathogens or the regulation of host energy metabolism and immune responses [1, 2]. However, in genetically susceptible individuals and under the influence of detrimental environmental factors, endogenous

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Abbreviations: **ED**, enterodiol; **EL**, enterolactone; **FID**, flame ionization detector; **TG**, triglycerides

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bacteria can also favor the development of chronic disorders such as diabetes, allergies, and inflammatory bowel diseases [3, 4]. Strict anaerobes of the phyla *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, and *Verrucomicrobia* are dominant members of the human intestinal microbiota. They carry out important metabolic functions such as the conversion of indigestible food components (e.g. polysaccharides, polyphenols) and host-derived substrates (e.g. mucin and bile salts), thereby producing short-chain fatty acids and bioactive metabolites [5–8].

A paradigm example of bioactive metabolites of bacterial origin in the gut is the production of enterolignans [9]. Enterodiol (ED) and enterolactone (EL) were first detected in primates in 1980 and were thought to be new steroids [10], underlining that lignans share structural features with endogenous hormones and are thus considered as phytoestrogens. Two years later, the same authors reported that urinary lignans originate from plant substrates [11]. The conversion of plant lignans by gut bacteria was studied more in detail shortly thereafter [12]. Secoisolariciresinol, matairesinol, pinoresinol, lariciresinol, and glycosylated derivatives thereof are the main lignans in plants. They occur in numerous food items illustrating their relevance with respect to nutritional habits in Westernized countries [13]. Flaxseed (*Linum usitatissimum*) is by far the main source of secoisolariciresinol diglucoside (SDG) in food (300 mg/100 g) and is thus one of the major contributors of enterolignan production in human subjects [14, 15]. In addition to containing lignans, flaxseeds are also rich in omega-3 fatty acids and indigestible carbohydrates (each 15 to 20% w/w) [16].

Due to their structural similarity with estrogens, and to possible antioxidant properties and modulation of host enzyme activities, enterolignans have been extensively studied for health-promoting effects [17]. Flaxseeds and the lignans they contain are proposed to be of benefit for the treatment or prevention of metabolic and cardiovascular diseases as well as breast and prostate cancer [18–22]. However, although evidence in culture systems and experimental models is good, there is urgent need for state-of-the-art randomized clinical trials. No matter what health effects lignans have, intestinal bacteria are essential for their activation from plant substrates. Hence, in order to investigate interindividual differences and molecular mechanisms underlying the health effects of lignans, it is crucial to study gut bacterial communities.

Despite the discovery of the bacterial activation of lignans as early as the 1980's [11, 12], lignan-converting strains were first isolated 15 years later [23]. The production of enterolignans from SDG results from the activity of phylogenetically and metabolically diverse bacteria that catalyze sequential activation via deglycosylation, demethylation, dehydroxylation, and dehydrogenation [24]. Deglycosylation and demethylation seem not to be limiting steps thanks to functional redundancy between different species and the ability of one strain to metabolize different substrates [25]. Moreover, the main species catalyzing dehydroxylation, i.e. *Eggerthella lenta*, belongs to the core gut microbiome in humans and is thus dominant and prevalent in the intestine [26]. In contrast, we showed that enterolactone-producing communities belong to subdominant populations and only one enterolactone-producing species has been isolated and characterized to date [27, 28]. Interestingly, ED and EL have been described as being more biologically active than plant lignans. Thus, it is sound to hypothesize that gut microbial profiles influence enterolignan production phenotypes with possible ensuing consequences on physiological effects. Besides numerous in vitro work based on fermentation experiments using human fecal slurries [29, 30], there are only two studies that investigated serum enterolactone concentrations and fecal bacteria in human subjects [31, 32]. The study by Holma *et al.* [31] focused solely on the comparison between two groups of individuals (low or high serum enterolactone), fecal bacteria were analyzed in a targeted manner, and the intervention included a synbiotic mixture but no lignan substrates [31]. Very recently, Hullar *et al.* [32] conducted a sequencing-based study of fecal microbiota diversity and composition in relation to enterolignan production phenotypes in 115 premenopausal women consuming their usual diet. To the best of our knowledge, there are still no data available in humans on the interaction between gut bacterial profiles and enterolignan production phenotypes in response to targeted dietary intake of plant lignans.

In the present work, we assessed the effect of a dietary intervention with flaxseeds in healthy male adult subjects. Primary outcomes were diet-induced changes in serum enterolignan concentrations and fecal bacterial communities as analyzed by high-throughput 16S rRNA gene sequencing. Additional outcomes included nontargeted metabolite analysis via NMR spectroscopy and targeted measurement of SCFA in feces as well as blood lipids, since flaxseeds contain substantial amounts of fibers and unsaturated fatty acids.

2 Materials and methods

2.1 Subjects

Ten healthy male adults between 20 and 40 years of age were recruited at the university campus in Weihenstephan (Freising, Germany). Exclusion criteria were allergy to flaxseeds, underweight or obesity, chronic diseases (e.g. inflammatory bowel diseases, allergies, diabetes), antibiotic, pre- and probiotic treatment, and participation in another clinical trial in the last 2 months preceding the study. Participants were informed about the aims and risks of the study and gave their written informed consent. The study protocol was approved by the ethics committee of the medical faculty of the Technische Universität München (approval number 5346/12). All clinical aspects of the study were supervised by a physician. A code (P-01 to P-10) was assigned to each participant by the study coordinator at inclusion to ensure blinded handling of samples by the technical staff. The study protocol is

available at the German Clinical Trials Register under accession number DRKS00003609.

2.2 Experimental design and sample collection

This longitudinal pilot study comprised three main periods of 1 week each (Fig. 1): (i) a run-in period, where participants consumed their usual diet but were asked not to consume the lignan-rich products flaxseeds, sesame seeds and rye, as well as pro- and prebiotics; (ii) the flaxseed intervention using a dose of 0.3 g per kg bodyweight per day. Individual daily portions of ground flaxseeds (Bauck, Rosche, Germany) were kept in 50-mL Falcon tubes and stored at room temperature. The participants were instructed to consume the entire portion every day, splitting it into several servings and adding it raw to beverages or prepared food; (iii) a wash-out period (similar to run-in).

At study start and on three occasions thereafter (time point T1–T3), i.e. at the end of each aforementioned study period, the participants' medical status was monitored with the help of questionnaires and samples were collected as follows: a total volume of 17.7 mL venous blood was drawn using the S-Monovette® K2-EDTA (2.7 mL) and serum gel (2 × 7.5 mL) (Sarstedt, Nümbrecht, Germany). One EDTA and one serum sample were analyzed by Synlab (München, Germany) for measurement of erythrocytes, leucocytes, thrombocytes, hemoglobin, hematocrit, triglycerides (TG), HDL- and LDL cholesterol in order to determine basal participants' characteristics and to follow possible effects of the flaxseed intervention on blood lipids. The third blood sample was used to obtain serum by centrifugation (10 min, 2000 × *g*) after 20–30 min coagulation for quantification of lignans. At sampling point T1-3, participants were instructed to defecate directly into 1 L sterile PP snap cap containers (Plastiques Gosselin, Borre, France) to prevent contaminations by toilet water and materials. Fresh fecal samples were kept at 4°C under aerobic conditions for a maximum of 4.5 h, aliquoted into screw-cap tubes, and stored at −80°C until further processed.

2.3 High-throughput 16S ribosomal RNA (rRNA) gene sequence analysis

Volumes of 600 μL DNA stabilization solution (STRATEC biomedical), 400 μL Phenol:chloroform:isoamyl alcohol (25:24:1; Sigma-Aldrich) and an amount of 500 mg autoclaved 0.1 mm glass beads (Roth) were added to frozen fecal aliquots (150–800 mg). Microbial cells were disrupted by mechanical lysis (3 × 30 s at maximum speed) using a FastPrep®-24 (MP Biomedicals) fitted with a 24 × 2 mL cooling adaptor. After heat treatment (95°C, 5 min) and centrifugation (15 000 × *g*, 5 min, 4°C), supernatants were treated with RNase (0.1 μg/μL) for 30 min at 37°C. Metagenomic DNA was purified using gDNA columns (Macherey-Nagel) following the manufacturer's instructions. Concentrations and purity were controlled using the NanoDrop® system (Thermo Scientific) and samples were stored at 4°C during library preparation and at −20°C thereafter for longer storage.

The V3/V4 region of 16S rRNA genes was amplified (25 cycles) from 24 ng of metagenomic DNA using the bacteria-specific primers 341F and 785R [33] following a two-step procedure to limit amplification bias [34]. Amplicons were purified using the AMPure XP system (Beckmann), pooled in an equimolar amount, and sequenced in paired-end modus (PE275) using a MiSeq system (Illumina, Inc.) following the manufacturer's instructions and a final DNA concentration of 10 pM and 15% (v/v) PhiX standard library.

2.4 Sequence analysis

Raw read files were demultiplexed (allowing a maximum of two errors in barcodes) and each sample was processed using usearch [35] following the UPARSE approach [36]. First, all reads were trimmed to the position of the first base with quality score <3 and then paired. The resulted sequences were size filtered excluding those with assembled size <380 and >440 nucleotides. Paired reads with expected error >3 were further filtered out and the remaining sequences were trimmed by ten nucleotides on each side to avoid GC bias and nonrandom base composition. For each sample, sequences were de-replicated and checked for chimeras with UCHIME [37]. Sequences from all samples were merged, sorted by abundance, and operational taxonomic units (OTUs) were picked at a threshold of 97% similarity. Finally, all sequences were mapped back to the representative sequences resulting in one OTU table for all samples.

Only those OTUs with a relative abundance above 0.5% total sequences in at least one sample were kept. RDP classifier [38] was used to assign taxonomic classification to the OTUs representative sequences (80% confidence). A phylogenetic tree was constructed using fasttree [39]. The OTU table was rarefied to the minimum count of sequences observed and distances between microbial profiles were calculated using the generalized Unifrac procedure [40]. For estimation of diversity within samples (*alpha*-diversity), the Shannon index was calculated and transformed to the corresponding effective number of species as described by Jost [41]. Effective species counts reflect better the true diversity within samples when compared with metrics like species richness as these are less affected by the number of rare species.

2.4 Enterolignan quantification in serum

Samples were homogenized by vortexing for 1 min at room temperature and a volume of 400 μL was added to 1 mL 0.15 M sodium acetate buffer (pH 4) supplemented with 15 μL enzymatic mix (*Helix pomatia*, Sigma-Aldrich). Samples were

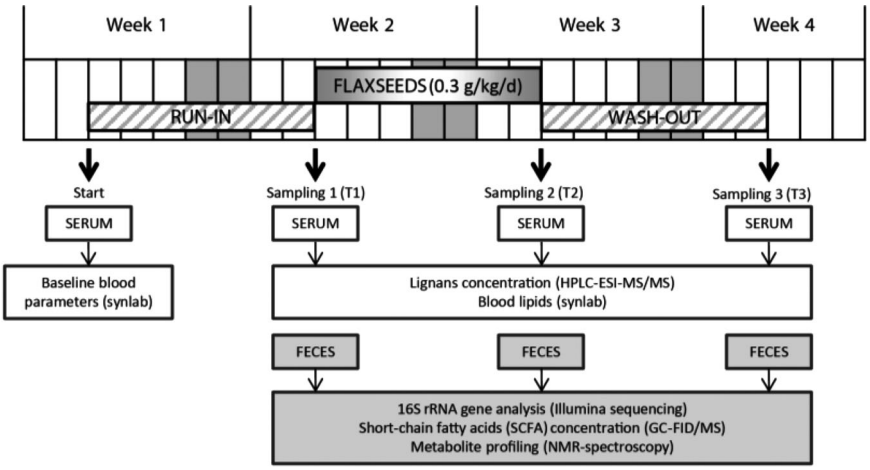

Figure 1. Schematic view of the study design. This pilot study included nine participants and was divided in three periods of 1 week each, as detailed in the Materials and methods section. Samples were collected in the middle of the week so as to minimize weekend-related confounding factors with respect to dietary behavior. FID, flame ionization detector.

hydrolyzed overnight at 37°C. Supernatants (14 000 rpm, 5 min, 20°C) were used for extraction using SPE columns (Sigma-Aldrich). Columns were washed with 3 mL methanol and equilibrated with 3 mL 0.15 M sodium acetate buffer prior to sample loading. After washing with 3 mL acetate buffer, a volume of 3 mL methanol was used for elution. Eluates were vacuum-dried under nitrogen flow, re-suspended with 100 μL methanol in 0.1% acetic acid (20:80, v/v), and used for measurement by LC combined with tandem MS using multiple reaction monitoring.

LC-MS/MS analysis was carried out on an API 4000 triple quadrupole MS/MS system (Applied Biosystems) connected to an Agilent Technologies 1200 LC system. Metabolites were separated using a Luna C18 column (150 mm × 3.0 mm, 100 Å, 5 μm) (Phenomenex) combined with a C18 guard column (4.0 mm × 2.0 mm) (Phenomenex). The flow rate was 300 μL/min and the elution gradient as follows: The percentage of solution B (methanol/0.1% acetic acid/isopropanol, 90/10/0.01, v/v) in A (0.1% acetic acid/isopropanol, 99/1, v/v) was raised from 15 to 95% within 10 min and held at 96% for 4 min. The column was equilibrated for 5 min with 15% B. The Analyst 1.6 software was used for data processing. Calibration curves were determined by measuring standard dilution series (four replicates): 1, 2, 4, 6, 8, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800, 1000 2000, and 4000 μM. Detection limits were determined based on S/N obtained from blank biological matrices. Limits were 2.53, 3.27, 0.62, and 1.12 nM for matairesinol, secoisolariciresinol, ED, and EL, respectively.

2.5 SCFA quantification in feces

The procedure for extraction and measurement was adapted from a previously published procedure [42]. Briefly, 100 ± 5 mg feces was resuspended by vortexing for 2 min in 1 mL 0.5% H_3PO_4 (85%, Merck) containing 25 μL internal standard, i.e. 4-methylvaleric acid (Sigma-Aldrich) in ethyl acetate (>99.5%, Sigma-Aldrich). SCFA in aqueous supernatants (10 min, 18 000 × *g*) were extracted with 1 mL ethyl acetate and the upper organic phase was used for measurement by GC with flame ionization detection (GC/FID) (Agilent Technologies instrument 6890N).

Data acquisition was performed using the ChemStation software. The GC was fitted with a DB-WAX ETR (30 m × 250 μm id × 0.5 μm film thickness) (J&W Scientific) and hydrogen was used as the carrier gas in the constant flow mode (1 mL/min). Injection was made in the splitless mode with a volume of 1 μL at 250°C. The column was initially maintained at 90°C, then increased to 150°C within 4 min, to 170°C within 4 min, and finally to 250°C within 4 min and hold for 2 min. The FID temperature was 200°C and nitrogen was used as make-up gas at 25 mL/min. Template chromatograms are shown in Supporting Information Fig. 1. Seven-point calibration functions were generated using authentic reference compounds (each point in triplicate). Linear regression was confirmed by ratios (SCFA/internal standard) of both areas under the curve and amounts. Recovery rates (from 98.1 ± 7.9 for propionic acid to 109.6 ± 1.8 for valeric acid) were determined in triplicate by spiking one fecal sample with known amounts of the internal standard and SCFA. Final SCFA concentrations were calculated using the linear standard equations and were expressed as microgram per gram dry weight.

2.6 NMR fecal metabolite profiling

Aqueous fecal extracts were obtained as follows: about 50 mg feces was dissolved in 1 mL H_2O:ACN (1:1) and homogenized with ceramic beads and a steal ball (NucleoSpin, Macherey-Nagel Dueren, Germany) for 3 min in TissueLyser II (Qiagen, Hilden, Germany). After centrifugation (10 min, 14 000 rpm), supernatants were dried down in a SpeedVac Concentrator (Thermo Scientific, Waltham, MA, USA), reconstituted in

200 uL NMR buffer (90% D_2O, 0.1% TSP, 500 mmol PO4, pH 7.4), and immediately analyzed via NMR spectroscopy.

For an overview of all molecules present in the samples, a standard 1D pulse sequence [recycle delay (RD)-90°-t1-90°-tm-90°-acquire FID] was acquired, with water suppression irradiation during RD of 2 s, mixing time (tm) set on 200 ms and a 90° pulse set to 9 μs, collecting 512 scans into 64 K data points with a spectral width of 12 ppm. Processing of the spectra was carried out in TopSpin 3.2 (Bruker BioSpin, Rheinstetten, Germany). FIDs were multiplied by an exponential decaying function corresponding to a line broadening of 0.3 Hz before Fourier transformation. All spectra were manually phased, baseline corrected, and calibrated to TSP (δ 0.00). Data were imported to Matlab (Mathworks, MA, USA) and further processed, i.e. water region removed (δ 4.75–5.10) and normalized using probabilistic quotient normalization [43]. PCA and OPLS-DA score plots were calculated in Matlab. To illustrate metabolites driving the separation of samples in the scores plot, the covariance and correlation coefficients of the respective groups were calculated and shown in a covariance-correlations-plot [44].

2.7 Statistics

All datasets, including OTU counts and abundances as well as concentrations of SCFA, blood lipids, and lignans are provided in Supporting Information Table 1. Unless otherwise stated, data are presented as mean ± SD. Statistical analyses were performed in the R programming environment. For all tests, *p*-values below 0.05 were considered to be indicative of significant effects. The Benjamini-Hochberg method was used for adjustment after multiple testing. However, because the present study is explorative and our intention was to generate hypotheses on possible associations between fecal bacterial populations and metabolites production, and because *p*-values adjustment is still a matter of debate in clinical science [45], interpretations were based on *p*-values prior to adjustment. Nevertheless, for the sake of clarity and transparency, adjusted *p*-values are provided in Supporting Information Table 1.

The effect of intervention on OTU and taxonomic counts as well as metabolite concentrations was tested using one-way repeated measures ANOVA followed by paired t-test for pairwise comparisons. Pearson correlation coefficients across all prevalent OTUs (detected in at least ten samples) against selected fecal and blood metabolites were calculated and visualized using the Hmisc and corrgram packages. OTUs abundances of zero were treated as missing values and thus did not contribute to the correlations. For the network analysis, only strong (absolute coefficient >0.5) and significant ($p < 0.01$) correlations were considered. For *beta*-diversity analysis, generalized Unifrac distances were calculated using the package GUniFrac [40]. For visualization of the relationships between bacterial profiles, two methods, a hierarchical clustering (Ward's method) dendrogram and a nonparametric multiple dimensional scaling (NMDS) plot, were computed using the packages vegan and ade4.

Table 1. Baseline characteristics of the nine male adult participants

Parameter (unit)	mean ± SD
Age (y)	31.1 ± 3.1
BMI (kg/m²)	23.5 ± 3.0
Hemoglobin (g/dL)	16.0 ± 0.8
Hematocrit (%)	46.0 ± 2.2
Thrombocytes (x 10^3/μL)	206 ± 40
Leucocytes (/nL)	5.2 ± 0.7
Triglycerides (mg/dL)	94 ± 33
HDL-cholesterol (mg/dL)	60 ± 14
LDL-cholesterol (mg/dL)	131 ± 39
LDL/HDL ratio	2.4 ± 1.1

BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

3 Results

3.1 Ingestion of flaxseeds increases serum enterolignan concentrations

Nine participants completed the study. Their characteristics at baseline are shown in Table 1. One participant (P-06) was excluded from the study during the run-in period due to antibiotics prescription. After the 7-day-long intervention with flaxseeds (0.3 g/kg/day), two participants reported slight and sporadic abdominal pain, one other participant reported increased flatulence, and one last participant reported slight and sporadic abdominal pain, increased flatulence and constipation.

Ingestion of flaxseeds triggered a significant, approximately ten-fold increase in serum concentrations of both ED and EL, with median values of approximately 20 and 60 nM after intervention, respectively (Fig. 2). Two individuals were characterized by unchanged to low increased levels of EL after intervention (P-03 and P-10). The serum concentrations of matairesinol and secoisolariciresinol were not affected by treatment when calculated on all participants.

Because approximately one-fifth of the dry weight of flaxseeds consists of indigestible carbohydrates, and in order to assess the effects of the dietary intervention not only on bacterial diversity and composition but also functions, we determined concentrations of SCFA, the main bacterial products of indigestible carbohydrate fermentation, in feces. The intervention had minor effects on fecal SCFA (Supporting Information Table 1). We detected the following mean concentrations of SCFA in feces (μg/g dry weight) (n = 27): 1790 ± 605 (acetate), 996 ± 438 (propionate), 1237 ± 534 (butyrate), 105 ± 67 (isobutyrate), 187 ± 92 (valerate), 178 ± 126 (isovalerate). Concentrations of propionate were consistently higher after flaxseed intervention, and decreased again after

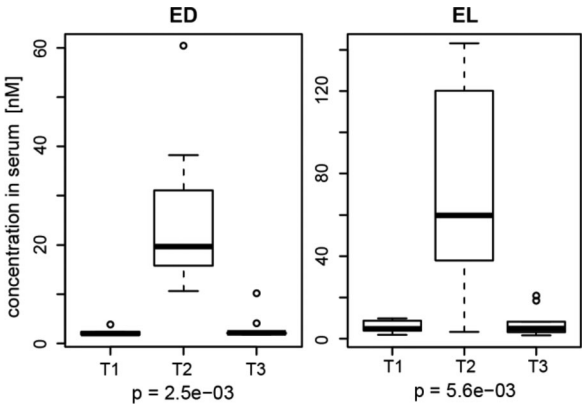

Figure 2. Ingestion of flaxseeds increases serum enterolignan concentrations. Enterodiol (ED) and enterolactone (EL) were measured in the serum of participants (n = 9) at start (T1), immediately after intervention (T2) and 1 week post-intervention (T3) using LC coupled with MS, as described in details in the Methods section. Data are shown as boxplots indicating median values, first and third quartile, and the most extreme data point located ≤1.5 × inter-quartiles range from the box (whiskers). Outliers are shown as individual points.

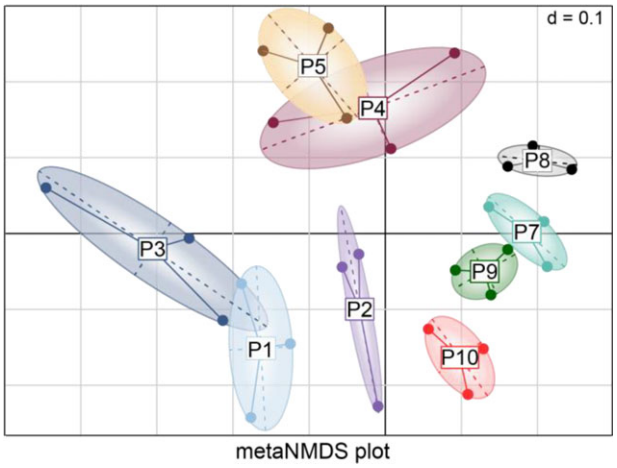

Figure 3. Dominant fecal bacterial communities are not altered by flaxseed intervention. Similarities between individual fecal bacterial profiles before, immediately after, and 1 week after the short-term dietary intervention with flaxseeds were investigated by means of high-throughput 16S sequencing. Interprofile relationships based on generalized UniFrac distances are depicted as a nonparametric multidimensional scaling (NMDS) plot made of two dimensions. Abbreviations: P, participant number.

the 1-week wash-out (Supporting Information Fig. 2). The intervention also had minor effects on blood lipids. Levels of HDL-cholesterol decreased in most individuals throughout the 2-week study period, whereas LDL-cholesterol levels did not change significantly (Supporting Information Fig. 2).

3.2 Dominant fecal bacterial communities are not altered by flaxseed intervention

On a total of 27 fecal samples sequenced (nine participants across three time points), we analyzed a set of 693 307 sequences after quality- and chimera-check as well as OTUs filtering. The number of sequences per sample was 25 678 ± 5140.

Multidimensional analyses of phylogenetic distances between samples revealed that fecal bacterial profiles clustered significantly according to individuals, independent of measurement time points (before or after intervention) (Fig. 3). This speaks in favor of marked interindividual differences in dominant fecal bacterial communities and indicates that the 7-day-long intervention with 0.3 g/kg/day flaxseeds did not induce consistent shifts in the overall phylogenetic makeup of bacterial communities. The dataset contained a total of 126 OTUs, ranging between 47 and 100 per sample. Shannon effective species counts per sample ranged from 10 to 40 and were not affected in a consistent manner by the dietary intervention (Supporting Information Fig. 2).

As expected, fecal bacterial communities were dominated by members of the phyla *Bacteroidetes* and *Firmicutes*, followed by smaller relative abundance of *Actinobacteria* and *Proteobacteria* (Supporting Information Table 1). *Verrucomicrobia* were also detected in five of nine participants. This composition of dominant fecal bacterial communities was not significantly affected by flaxseed ingestion at nearly all taxonomic levels analyzed (phyla to genera) (Supporting Information Fig. 3). The occurrence of only one genus, *Coprococcus*, with relative abundances >1% increased throughout the study period, but responses were highly dependent upon individuals (Supporting Information Fig. 2). At the phylotype level, there was a transient increase in the relative abundance of one OTU (S387) associated with flaxseed ingestion (Supporting Information Fig. 2). This OTU occurred at relatively low abundances and could be assigned unambiguously to the species *Ruminococcus lactaris* on basis of the 382 nt sequenced.

3.3 Specific bacterial groups are associated with target metabolite concentrations

To go beyond overtime changes in concentrations of enterolignans, SCFA and lipids due to the intervention (when considering all nine individuals grouped together), we looked at individual-specific associations between fecal bacterial composition and target metabolites. Therefore, we established a network of correlations between the relative abundance of OTUs and the fecal or blood concentration of metabolites analyzed by targeted approaches, considering only those OTUs occurring in more than 9 of the 27 samples measured (Fig. 4). We obtained a total of 38 OTUs that showed significant positive or negative correlations ($p < 0.01$). The taxonomic identification, prevalence, and mean abundances of these OTUs are shown in Table 2. In particular, 29 of the 70 correlations observed between OTUs and metabolites concerned a total of 15 most prevalent and

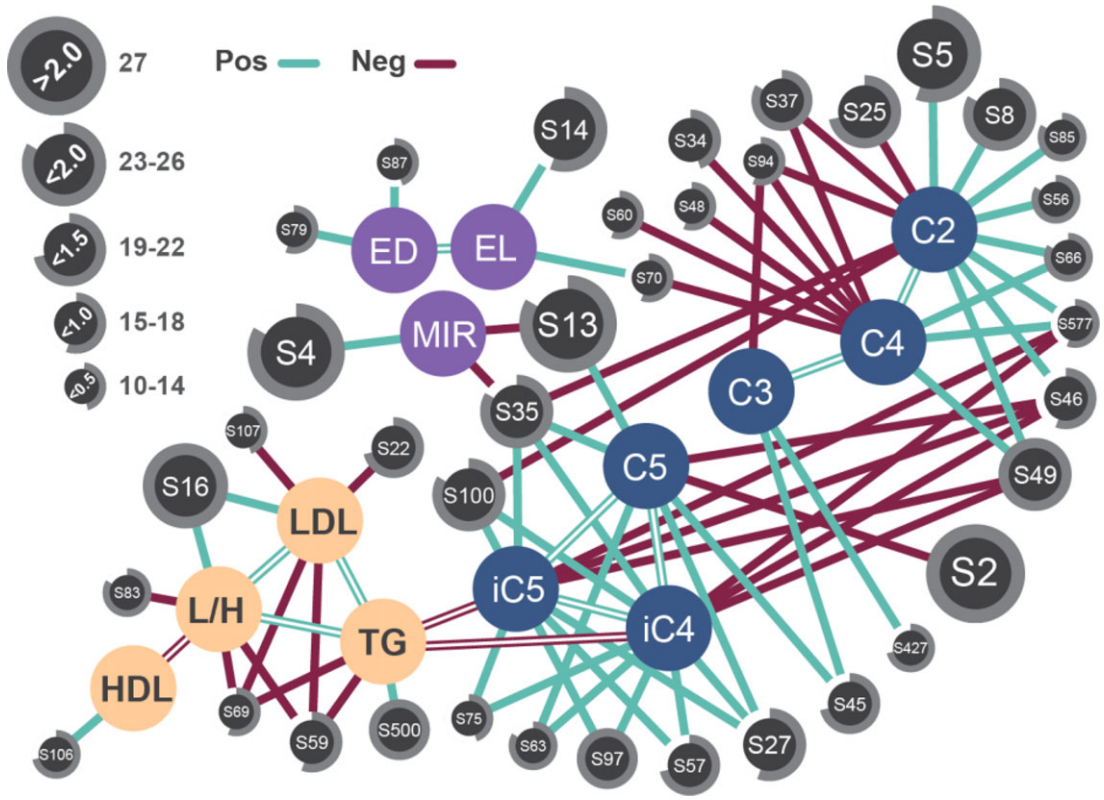

Figure 4. Specific bacterial groups are associated with blood and fecal metabolite concentrations. The correlation network across molecular species and selected metabolites was constructed in R and visualized in Cytoscape 3 [88]. The identification of molecular species shown in the correlation network is given in Table 2. OTUs and metabolites characterized by significant and strong correlations ($p < 0.01$; abs. $r > 0.5$) are shown. Red and green edges indicate negative and positive correlations, respectively. White lines over connecting edges indicate correlations in between metabolites. Variables are color-coded as follows: OTUs, black; enterolignans, purple; SCFA, blue; blood lipids, beige. The size of OTU nodes corresponds to the average relative abundance of the given OTU (as percentage of total sequences) across all samples in which it was detected. The outer ring indicates the number of samples that were positive for the particular OTU (maximum of 27 samples). C2, acetic acid; C3, propionic acid; C4 butyric acid; iC4, isobutyric acid; C5, valeric acid; iC5, isovaleric acid; ED, enterodiol; EL, enterolactone; HDL, HDL-cholesterol (high-density lipoprotein); LDL, LDL cholesterol (low-density lipoprotein); L/H, LDL to HDL ratio; MIR, matairesinol; Neg, negative correlations; Pos, positive correlations; TG, triglycerides.

dominant OTUs that occurred in more than half of the samples at abundances around or above 1% total sequences (S2, S4, S5, S8, S13, S14, S16, S22, S25, S27, S35, S49, S97, S100, and S500). Dot plots depicting key correlations between some of these OTUs (their abundance) and the concentration of respective metabolites, as detailed below, are shown in Supporting Information Fig. 4.

We found one OTU (S14) identified as *Ruminococcus bromii* with a mean abundance of 1.9% in five of nine individuals, which was positively associated with blood concentrations of EL. Interestingly, this OTU was not detected in the two individuals characterized by no to low increase in blood levels of EL after dietary intervention (P-03 and P-10). There were also two unknown members of the order *Clostridiales* (S13, S35) that correlated negatively with blood concentrations of matairesinol, whereas one dominant OTU representative of the newly described species *Fusicatenibacter saccharivorans* (S4) within the family *Lachnospiraceae* (phylum *Firmicutes*) correlated positively with matairesinol.

Many of the observed significant correlations were related to fecal levels of SCFA, with acetic (C2) and isobutyric (iC4) acids being most densely connected (≥10 links to molecular species). Main OTUs positively associated with isovaleric acid (iC5) and iC4 belonged to the order *Bacteroidales* (S27, S97), the family *Ruminococcaceae* (S57, S75, S35), or the genus *Subdoligranilum* (S100). The latter two OTUs (S35, S100) were also negatively associated with concentrations of C2 together with S25 (*Dorea longicatena*), whereas *Hungatella* (S8) and *Bacteroides* (S5) spp. correlated positively with C2. Also, one OTU closely related to *Anaerostipes hadrus* (S49) and found in all individuals correlated positively with both C2 and

Table 2. Features of molecular species significantly associated with metabolite concentrations (see Fig. 4)

OTU id	Species[a)]	Similarity	prev	tot-%[b)]	pos-%[b)]
S2	*Bacteroides dorei*	100.0%	27	6.0	6.0
S4	*Fusicatenibacter saccharivorans**	100.0%	25	2.3	2.5
S5	*Bacteroides stercoris*	99.8%	16	1.3	2.2
S8	*Clostridium sphenoides*	95.8%	24	1.1	1.3
S13	*Oscillospira guilliermondii*	95.5%	24	1.9	2.1
S14	*Ruminococcus bromii**	99.7%	15	1.1	1.9
S16	*Gemmiger formicilis*	100.0%	27	1.7	1.7
S22	*Lactobacillus rogosae*	100.0%	21	0.8	1.0
S25	*Dorea longicatena**	100.0%	21	0.9	1.2
S27	*Parabacteroides merdae*	100.0%	18	0.7	1.1
S34	*Alistipes timonensis*	90.1%	11	0.3	0.8
S35	*Oscillibacter ruminantium*	93.5%	24	1.2	1.4
S37	*Barnesiella intestinihominis*	99.8%	24	0.6	0.7
S45	*Alistipes onderdonkii*	100.0%	21	0.4	0.5
S46	*Bacteroides fragilis**	100.0%	18	0.4	0.6
S48	*Collinsella aerofaciens**	100.0%	15	0.2	0.3
S49	*Anaerostipes hadrus*	100.0%	27	1.1	1.1
S56	*Clostridium amygdalinum*	96.9%	24	0.4	0.4
S57	*Oscillibacter valericigenes*	94.3%	23	0.5	0.6
S59	*Eubacterium siraeum**	100.0%	15	0.3	0.6
S60	*Ruminococcus callidus**	99.2%	18	0.3	0.5
S63	*Oscillibacter ruminantium*	95.6%	24	0.2	0.2
S66	*Romboutsia ilealis*	99.0%	26	0.2	0.3
S69	*Eubacterium xylanophilum*	98.4%	16	0.2	0.4
S70	*Moorella humiferrea*	87.3%	18	0.2	0.3
S75	*Oscillospira guilliermondii*	93.5%	12	0.1	0.3
S79	*Escherichia coli*	100.0%	16	0.1	0.2
S83	*Fusicatenibacter saccharivorans*	96.3%	24	0.3	0.4
S85	*Oribacterium sinus*	95.3%	25	0.4	0.4
S87	*Turicibacter sanguinis**	99.0%	13	0.02	0.1
S94	*Alistipes indistinctus*	94.8%	16	0.1	0.1
S97	*Alistipes shahii*	99.5%	27	0.9	0.9
S100	*Gemmiger formicilis*	96.6%	24	1.1	1.2
S106	*Alistipes obesi**	100.0%	23	0.2	0.2
S107	*Sporobacter termitidis*	91.1%	10	0.1	0.2
S427	*Clostridium lavalense*	95.6%	22	0.1	0.2
S500	*Coprococcus comes**	99.2%	27	1.0	1.0
S577	*Ruminococcus gnavus**	99.5%	16	0.3	0.5

a) OTU sequences (381–407 nt) were searched for closest related species with a standing name in nomenclature using EzTaxon [87]. OTUs that could be identified down to the species level, i.e. only one hit with >97% sequence similarity was identified, are marked with asterisks.
b) Refers to the mean relative sequence abundance of the corresponding OTU when considering either all 27 samples (tot-%) or only those samples that were positive for the given OTU (pos-%).
id, identity; prev, prevalence (number of samples positive for the given OTU).

butyric acid (C4), and negatively with iC5 and iC4. The most prevalent and dominant OTU in the network (S2) was related to *Bacteroides dorei* and *Bacteroides vulgatus* (≥99% sequence similarity) and was negatively associated with fecal concentrations of valeric acid (C5).

Finally, with respect to blood lipids, levels of TG correlated negatively with iC4 and positively with the abundance of one OTU identified as *Coprococcus comes* (S500) and characterized by a prevalence of 100% and a mean abundance of 1%. Concentrations of LDL cholesterol were overall negatively associated with members of the *Firmicutes* (S59, S69, S83, S107), in particular with one species of the genus *Lactobacillus* (S22), whereas *Gemmiger* sp. (S16) within the *Ruminococcaceae* was positively associated with LDL cholesterol.

3.4 Nontargeted fecal metabolome is linked to specific microbiota profiles and is marginally affected by flaxseeds

To gain a nontargeted overview of flaxseed-induced fluctuations in dominant gut metabolites, including diet-, host-, and microbiome-derived metabolites [46], we analyzed fecal extracts using NMR spectroscopy. When compared with bacterial phylogenetic makeups that were most similar for one given subject overtime, hierarchical clustering of NMR signals revealed metabolite profiles with intermingled subject-time effects (Fig. 5A). A consistent deep branching was evident for two participants (P1 and P3) characterized by very specific metabotypes, including high excretion of

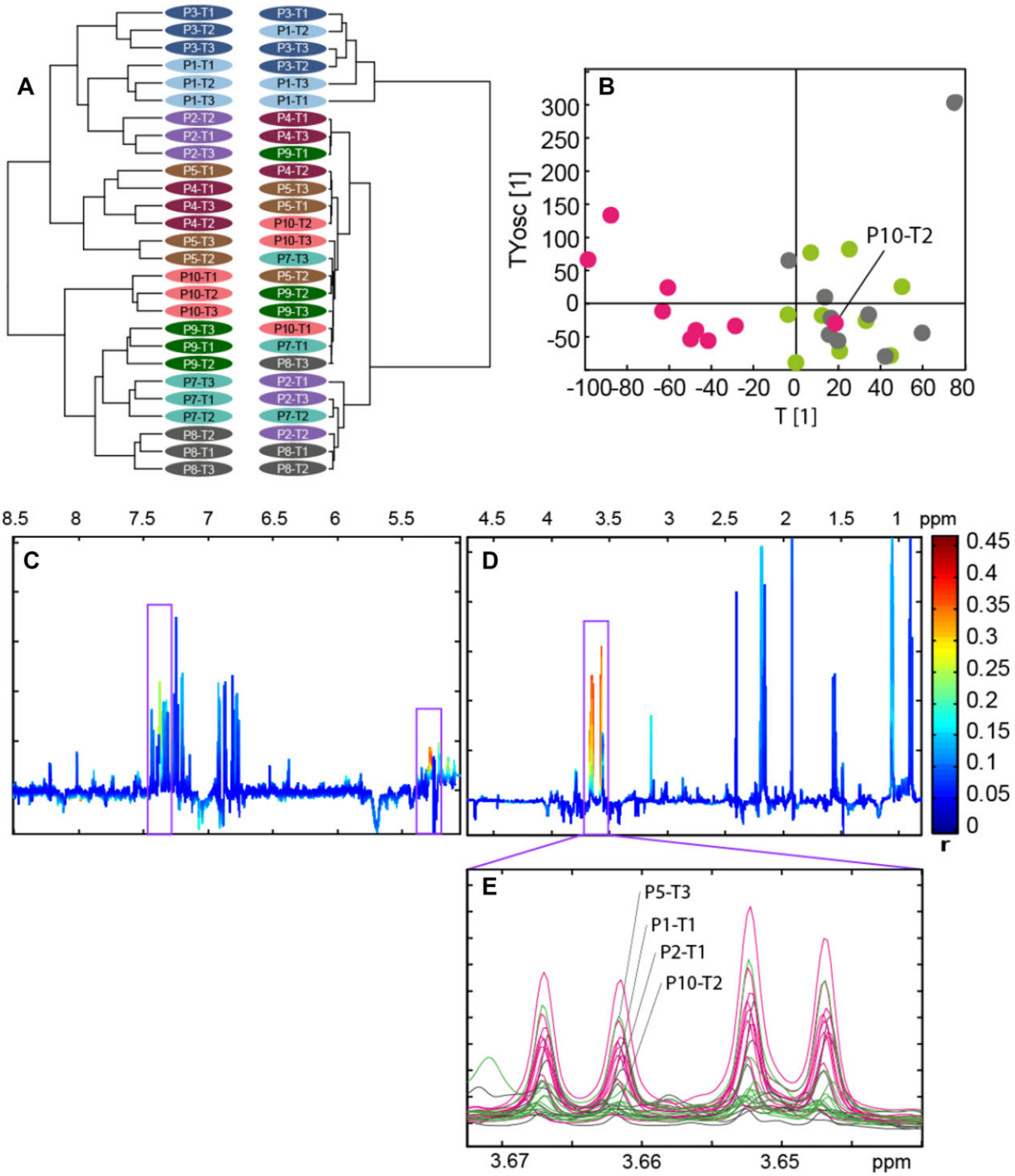

Figure 5. Flaxseeds trigger subtle changes in fecal metabolomes. (A) Hierarchical clustering showing interprofile relationships based on 16S rRNA gene sequencing (left dendrogram) and NMR-derived signal intensities (right dendrogram). (B) OPLS-DA supervised analysis of aqueous extracts from feces analyzed with NMR spectroscopy. The plot illustrates differences between fecal samples at T2 (after intervention; magenta dots) and T1 and 3 (gray and green, respectively). Biomarkers of intervention in the aromatic area (C) and the aliphatic and carbohydrate area (D) are shown. Zoom-in panel (E) shows individual NMR signals corresponding to glycerol, which were color-coded according to time as in panel B. Outlier samples are labeled with participant number and time point of measurement: Participants 1 and 2 (both glucose excreters) showed high levels of glycerol at T1 (baseline). Participant 5 kept high levels of glycerol throughout T3 (after wash-out phase). P, participant number; T, time point of measurement.

succinate and 5-aminovalerate, as well as higher signals for short-chain fatty acids, alanine, and formate (Supporting Information Fig. 5). In feces of these two participants at all three time points, we found three dominant OTUs with higher proportions and prevalence (S46, S55, and S577), which could be identified down to the species level as *Bacteroides fragilis*, *Clostridium nexile*, and *Ruminococcus gnavus*, respectively (Supporting Information Fig. 6). Additional individual-specific patterns included unusual high signals for glucose in feces of two samples (P1 and P2) at baseline, possibly due to remnant levels originating from complex dietary carbohydrates (Supporting Information Fig. 5). OPLS-DA supervised analysis across all subjects and time points indicated a separation according to intervention, although goodness of the model was very low (R2Y = 0.42, Q2Y = 0.01), wherefore both loadings plot and raw spectra were consulted for putative biomarker identification (Fig. 5B). Biomarkers of intervention in the aromatic area included a signal with chemical shift at δ 7.376 (t), putatively assigned to the enterolignans, and one unassigned metabolite (δ 5.27 (t)), which was however present at T2 in only two participants (Fig. 5C). In the aliphatic and carbohydrate area, excretion of glycerol was specific postintervention in all but one participant (P10) (Fig. 5D/E).

4 Discussion

Despite the importance of the gut microbiota in regulating phytoestrogens bioavailability, and the knowledge accumulated on single bacterial strains involved in lignan activation, links between enterolignan production capacities and intestinal bacterial communities as measured by culture-independent techniques have not yet been investigated in human subjects consuming plant lignans. In the present study, we performed a pilot dietary intervention in healthy adult men and thereby showed the impact of flaxseeds, as primary source of enterolignans precursors, on the gut ecosystem and depicted interrelationships between gut bacterial composition and metabolites production.

The blood concentrations of enterolignans that we detected at baseline (2–10 nM) are comparable to reference values from the literature [5]. Flaxseeds ingestion triggered a sharp increase in the concentration of both ED and EL (up to 143 nM after intervention), similar to what has been observed using identical amounts of flaxseeds (0.3 g/kg/day) [47]. Interestingly, whereas all nine individuals in our study were characterized by a marked increase in blood levels of ED, two participants seemed not to respond to flaxseeds intervention regarding EL production. The bacterial phylogenetic diversity in feces was quite different in these two participants, implying that EL-producer or nonproducer profiles can be variable, and most likely also that less dominant communities not captured by our method are associated with EL production. In a previous study including 24 male and female adult subjects, we found that EL-producing bacteria occurred at mean cell counts of 3×10^5 CFU/g in feces and were not detectable in one male subject [27]. Based on batch culture of fecal samples from 100 female donors, Possemiers *et al*. reported production of EL in only 39% of samples, but the detection limit of the method used in this paper was not specified [48]. Hence, additional studies are needed to reach consensus on the prevalence of EL producers in human populations, but it is already clear that dehydrogenation is a limiting step in the activation of dietary lignans and that more effort is required for the identification of additional lignan-dehydrogenating strains [28, 49].

Taxonomic composition of the fecal bacterial communities in our study is representative of healthy gut microbiomes as reported by others. However, richness (≤100 species per sample) is lower than values obtained in recent metagenomic (ca. 150) [26] or amplicon studies (>300) [50–52]. This is most likely due to the stringent parameters that we used for picking [36] and filtering molecular species (0.5% abundance threshold on top of chimera check [37]) in order to prevent analysis of spurious OTUs. A very recent study based on an innovative low-error amplicon sequencing approach reported a total of 101 ± 27 species per individual after analysis of fecal samples from 37 healthy adult subjects from the United States [53].

Based on the analysis of 27 samples overtime from 9 participants, we observed no major shifts in diversity and composition of dominant fecal bacterial communities following intervention with flaxseeds. This tends to challenge the recent consensus that the gut microbiome is highly dependent on diet [54], but agrees with the former notion that dominant bacterial populations in distal parts of the intestine are relatively stable over a timespan of months without drastic changes in diet or medication [55]. Other recent studies have also underlined the stability of bacterial diversity overtime, i.e. individuals harbor specific dominant bacterial species that persist even over decades [50, 56]. Altogether, it is safe to say that most dominant and prevalent bacteria detected in feces are individual-specific and remain in the ecosystem for long periods of time, but they can be subjected to quantitative fluctuations that are dependent on environmental factors. These factors include substantial changes in dietary intake, but ingestion of ca. 30 g flaxseeds per day for a week seems not to fall into this category. It is also possible that flaxseed intervention triggers more subtle changes in diversity and composition (e.g. affecting slow growing or dormant bacteria) that may require analysis over a longer timespan than the 1-week period analyzed in the present study to become detectable.

In contrast to the very clear individual-specific patterns observed for dominant bacterial communities, NMR-derived metabolite profiles in feces were dependent on both individuals and the time point of sampling, without clear relation to flaxseed supplementation. This supports the concept that the fecal metabolome is influenced by varying daily dietary intake to a higher extent than bacterial populations [57]. We previously observed this effect also in human urine, where both diet and interindividual differences were found to contribute to urinary metabolite profiles [58]. However, we also acknowl-

edge the limitation of our pilot design that precludes discrimination of a low number of subjects according to main fecal metabotypes. Two subjects stood out of the study population due to high excretion of succinate and 5-aminovalerate, which was linked to higher abundance of the molecular species *R. gnavus*, *B. fragilis*, and *C. nexile*. Succinate is a common fermentation product of cellulolytic bacteria such as ruminococci [59, 60] and is also produced by *B. fragilis* [61, 62], and *Clostridium* spp. were shown to be involved in 5-aminovalerate metabolism [63]. Besides the enterolignans, glycerol was the only metabolite showing an increase after intervention. In previous studies, glycerol was found to be specific for the fecal metabolome in humans compared with rats and mice [64], and increase abundance was reported in Crohn's disease patients [65]. In the present intervention trial, it is likely that glycerol is a surrogate marker for the fatty acids contained in ground flaxseeds that reach the colon and are hydrolyzed by bacteria [66, 67].

Our sequencing data point at one single molecular species, for which the occurrence was increased after ingestion of flaxseeds and returned to basal sequence abundance 1 week after the end of intervention. However, this OTU accounted for a minor proportion of sequence counts across the 27 samples (≤1%). The fact that it was identified as a member of *R. lactaris*, and that another dominant species of this genus, *R. bromii*, correlated positively with blood concentrations of EL, suggest that ruminococci species should be investigated in more detail for their involvement in enterolignan production. Jin *et al.* [49] previously reported the isolation of two strains capable of catalyzing enantioselective dehydrogenation of enterodiol. Strain END-1 was tentatively named *Ruminococcus* sp., but is in fact most closely related to *Blautia producta* and *Blautia coccoides* according to newest taxonomic classification [68]. These findings reflect the likelihood that conversion of lignan by bacteria in the gut is strain-specific, since other strains of *B. producta* were not able to catalyze dehydrogenation of ED [27]. On the other hand, one other most dominant and prevalent member of the family *Ruminococcaceae*, namely *Oscillospira guilliermondii*, correlated negatively with blood concentrations of matairesinol. The fact that different members of one family may be associated with different steps of activation of dietary lignans could explain the discrepancy between previous findings on correlations between enterolignan production and bacterial composition as measured using 16S probes that target entire phylogenetic groups [27, 48]. The impact of the newly described species *Fusicatenibacter saccharivorans* on lignan metabolism in the gut may also be worth investigating, since one corresponding OTU was found in most of the samples at a mean abundance >2% and correlated positively with blood concentrations of matairesinol [69]. Despite the reported ability of bifidobacteria to deglycosylate SDG and the fact that flaxseeds contain a substantial amount of indigestible carbohydrates, we did not observe a bifidogenic effect associated with flaxseed ingestion [70], most likely because many other bacteria in the gut can compete for these substrates [25, 52]. A recent study stated that low density of *Lactobacillales* is associated with low serum enterlactone concentrations [31], yet we did not detect members of this order in our study and thus cannot draw any conclusion.

A very recently published study by Hullar *et al.* [32] assessed intestinal bacterial communities via 16S rRNA gene sequencing in relation to enterolignan production phenotypes, using fecal samples collected from 115 premenopausal women in the United States. Importantly, the subjects included in the latter study consumed their usual diet, whereas our study focuses on changes linked to dietary supplementation of enterolignan substrates. One of the main findings reported by Hullar *et al.* was that enterolignan production is positively associated with taxa richness, although the amplitude of changes was not clearly defined. In the present study, effective counts of molecular species were not affected by dietary intervention and were not associated with blood levels of enterolignans. In our hands, molecular species occurring at relative abundances <0.5% total sequence counts have a high probability to correspond to taxa not present in the original gut sample of interest. Hence, our analysis is based on stringent parameters for filtering spurious OTUs, which, besides the low number of participants included in our study, may explain the discrepancies observed. Also, although our analysis pointed at the possible involvement of ruminococci in mechanisms underlying enterolignan production, Hullar *et al.* [32] reported that relative abundances of the genera *Moryella*, *Acetanaerobacterium*, *Fastidiosipila*, and *Streptobacillus* was <0.7% and was significantly increased in high EL excreters. Additional studies will be required to test the causal role of these bacterial taxa in enterolignan production.

The concentrations of SCFA that we measured in fecal samples of nine healthy male adult subjects are in the range of values reported in the literature [42]. Our data suggest that ingestion of flaxseeds is associated with increased propionic acid production. Others found similar results using ground flaxseeds in a continuous culture system inoculated with a cow gut microbiota [71]. Concentrations of both propionic and acetic acid correlated positively with those of butyric acid, yet all three fatty acids were disconnected from valeric as well as iso-butyric and -valeric acid in the network analysis. Molecular species identified as *Anaerostipes* sp., *B. fragilis*, and *R. gnavus* for instance correlated positively with the former but negatively with the latter SCFA, in agreement with the known SCFA production by these bacteria [72, 73]. Overall, SCFA were connected to many molecular species, underlining the complex trophic chains responsible for SCFA production in the gut [74, 75]. The most dominant and abundant OTU in the network was closely related to *B. dorei* and correlated negatively with concentration of valeric acid, which may reflect growth requirements as observed for other members of this genus [76].

Fecal concentrations of both iso-butyric and -valeric acid correlated negatively with blood levels of TG in our population, which is in line with two dietary intervention studies from the literature, one in rats using lipids from porpoise

adipose tissue that contains substantial amounts of isovaleric acid [77] and one in steers fed chromium [78]. Both studies reported opposite associations between serum TG and isovaleric acid. We also observed that one OTU occurring in all 27 samples and identified as *C. comes* correlated negatively with blood TG. A recent study in obese type-2 diabetes patients described a decrease in sequence abundance of this species following bariatric surgery, but did not report correlation with TG [79]. With respect to cholesterol levels, one molecular species within the lactobacilli detected in 78% of the samples correlated positively with LDL cholesterol. Lactobacilli were reported to be characteristic of individuals with type-2 diabetes [80], yet probiotic intervention has been associated with cholesterol-lowering effects [81]. These studies emphasize the need to refine analysis down to the strain level. Finally, flaxseeds are associated with potent beneficial effects with respect to various metabolic conditions, including for instance hypertension [82] or glucose homeostasis [83]. We noted a decrease in HDL-cholesterol levels following the intervention, although values remained in the normal range (>40 mg/dL) for all samples but one participant after the wash-out phase. Interestingly, an increase in HDL-cholesterol was also observed in children and adolescents with hypercholesterolemia after 4 weeks of dietary flaxseed supplementation [84]. Moreover, circulating levels of cholesterol may not reflect the situation in metabolically relevant organs (e.g. liver, fat tissue) [85], and effects are most likely gender-specific [86].

In conclusion, based on snap-shot analysis of fecal and systemic blood samples, we found that flaxseeds trigger enterolignan production and may be associated with fecal propionate and glycerol levels, but they have only a faint impact on dominant gut bacterial community structure. The work sheds light on single bacterial groups that must be investigated further for their role in enterolignan production and host lipid metabolism. This is the first intervention trial linking enterolignan production capability and intestinal bacterial profiles in human. Despite the advantage of analyzing intraindividual changes overtime where each participant acts as its own control, we acknowledge the pilot and explorative aspect of the work and the low number of participants included in the study. Moreover, considering rapid dynamics of metabolites absorption in the gut, repeated measures over several consecutive days in feces and collection of 24-h urine samples may be worth considering in future investigations. Nonetheless, the dominant bacteria that we identified can now be used as basis for hypothesis-driven assessment of the structure and functions of gut bacterial populations in a higher number of participants, which is essential for understanding interindividual differences that underlie the activation of dietary phytoestrogens and thus potential health effects.

S.R., T.S., and T.C.: designed the research; K.K., S.H., S.P., B.S., T.S., and T.C.: conducted the research; K.H.E., D.H., P.S.K., and S.R.: provided essential reagents and materials; I.L., S.H., S.P., B.S., and T.C.: analyzed data and performed statistical analysis; I.L., K.K., S.H., and T.C.: wrote paper; T.C. had primary responsibility for final content.

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