Impact of lignans in oilseed mix on gut microbiome composition and enterolignan production in younger healthy and premenopausal women: an *in vitro* study

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Research

Keywords: Flaxseed, enterolignans, enterolactone, enterodiol, gut microbiome, metabolism

Posted Date: December 13th, 2019

DOI: https://doi.org/10.21203/rs.2.18756/v1

Version of Record: A version of this preprint was published at Microbial Cell Factories on April 3rd, 2020.
See the published version at https://doi.org/10.1186/s12934-020-01341-0.

Abstract

Background

Dietary lignans belong to the group of phytoestrogens together with coumestans, stilbenes and isoflavones, and themselves do not exhibit oestrogen-like properties. Nonetheless, the gut microbiota converts them into enterolignans, which show chemical similarity to the human oestrogen molecule. One of the richest dietary sources of lignans are oilseeds, including flaxseed. The aim of this study was to determine the concentration of the main dietary lignans in an oilseed mix, and evaluate the gut microbiota-dependent production of enterolignans for oestrogen substitution in young and premenopausal women. The oilseed mix was fermented in a pH-controlled batch culture system inoculated with women's faecal samples. The lignan content and enterolignan production were measured by ultra-high-performance liquid chromatography – tandem mass spectrometry (UHPLC-MS/MS), and the gut-derived microbial communities were profiled by 16S rRNA gene-based next-generation sequencing.

Results

In vitro batch culture fermentation of faecal samples inoculated with oilseed mix for 24 h resulted in a substantial increase in enterolactone production in younger women and an increase in enterodiol in the premenopausal group. As for the gut microbiota, different baseline profiles were observed as well as different temporal dynamics, mainly related to *Clostridiaceae*, and *Klebsiella* and *Collinsella* spp.

Conclusions

Despite the small sample size, our results revealed that lignan-rich oilseeds have a strong influence on the faecal microbiota of both younger and premenopausal females, leading to a different enterolignan profile being produced. More studies are needed to evaluate the long-term effects of lignan-rich diets on the gut microbiota and find out how enterolactone-producing bacterial species could be increased. Diets rich in lignans could potentially serve as a safe supplement of oestrogen analogues to satisfy cellular needs for endogenous oestrogen and deliver numerous health benefits, provided that the premenopausal woman microbiota is capable of converting dietary precursors to enterolignans.

Background

Dietary lignans are phytoestrogens present in plants as aglycones or glycosides [1]. Around 70 different plant species are rich in various lignans in their roots, rhizomes, stems, leaves, seeds, and fruits [2]. In particular, grains with bran and oilseeds, especially flaxseeds, are extremely rich sources [3, 4]. The most studied aglycone forms of dietary lignans are secoisolariciresinol (Seco), lariciresinol (Lari), pinoresinol (Pino) and matairesinol (Mat) (Fig. **1**), whose range of concentration varies from 88.06 mg/100 g of fresh weight (FW) to 436.99 mg/100 g FW [3]. Secoisolariciresinol diglucoside (SDG), i.e. the glycosylated form

of Seco, represents around 1% of the dry weight of flaxseeds [3], and its concentration ranges from 11.9 to 25.9 mg/g [5]. This has led to flaxseed becoming the most common source of enterolignan precursors for research purposes.

Dietary lignans do not exhibit oestrogen-like properties themselves but are known to be metabolised by the gut microbiota into enterolignans (or mammalian lignans), i.e. enterodiol (ED) and enterolactone (EL). Given that EL, more than ED, is able to ligate the oestrogen receptor ER-α [6-9] and modulate endogenous oestrogen levels [10], it would be interesting to investigate the production of enterolignans by premenopausal-related gut microbiota layouts, following a lignan-rich diet. The amplitude of the modulating effects of enterolignans is expected to be determined by the biological level of endogenous oestrogen: enterolignans would exert antagonistic activity with normal levels of oestrogen, while acting as weak oestrogens when their levels are low [11]. Additionally, it has been suggested that circulating enterolignans may stimulate the synthesis of sex hormone-binding globulin, which binds sex hormones, reducing their systemic levels and biological activity and, consequently, the risk of hormone-dependent cancer [12]. EL may also inhibit aromatase, an enzyme that converts androgens to oestrogens in fat, muscle and other tissues [13]. Numerous human clinical trials and studies in animal models have demonstrated the association between high concentrations of ED and EL in blood and urine and various health benefits, such as normal blood lipids [14], reduced risk of breast cancer [15] and osteoporosis [9, 16] , as well as improved signs of metabolic syndrome [17]. In particular, a number of studies have revealed that the anti-cancerous effect of enterolignans is higher than that of their precursors (i.e. dietary lignans) and that the decreased risk of cancer correlates with blood and urinary levels of enterolignans but not with lignan-rich diets [18-20], thus emphasizing the relevance of the gut microbiota as a key mediator of their effect.

According to in vitro studies from Peterson et al. [21], the faecal microbiota can metabolise 100% of Lari, producing 46% of EL and 54% of ED, while the microbial conversion of other lignans has been reported to be incomplete (in 24 hours 72% of SDG, 55% of Pino and 62% of Mat are metabolised into enterolignans, and only 21% of SDG will get converted to EL). Furthermore, it has been shown that one-third of adults' microbiotas could directly produce EL, skipping the stage of ED [22], that some intestinal microbes convert most of ED to EL [23], and that the females' microbiota is able to produce more ED and EL than the male's one [24]. Even age has been reported to affect the production of enterolignans, with the child microbiota being less capable of converting dietary lignans into ED and EL [25] .

Although the lignan transformation by the gut microbiota is recognized to be essential in the protection against menopausal symptoms as well as certain hormone-dependent chronic diseases (e.g. cancer, cardiovascular disease and osteoporosis) [26-28], to date only a few bacterial species (often subdominant) have been identified as enabling such a transformation [28, 29]. In particular, EL production has been related to the abundance of *Ruminococcus* species, i.e. *R. bromii* and *R. lactaris* [30], as well as to those of *Lactobacillus-Enterococcus* [31] and *Methanobrevibacter*, an archaeon central to the syntrophic hydrogen metabolism that may be important for EL production [32]. On the other hand,

low-EL excreter phenotypes have been associated with the pathobiont, sulphite-reducing bacterium, *Bilophila*, as well as microbial signatures of epithelial dysfunction and inflammation [33].

In an attempt to bridge this gap, providing further insights into bacterial taxa capable of converting lignans into EL/ED, here we investigated the lignan profile of a commercially prepared blend of oilseeds as a functional dietary supplement for women, by ultra-high-performance liquid chromatography - tandem mass spectrometry (UHPLC-MS/MS). We then used this as a substrate for inoculating stool samples of young and pre-menopausal women in *in vitro* anaerobic batch culture fermentation experiments. The aim of these experiments was to determine the production of oilseed mix-derived enterolignans and to explore differences in bacterial taxa that could be involved in lignan transformation. We investigated the dynamics of individual lignans (Seco, Lari, Pino and Mat) as well as enterolignans (ED and EL), gut microbial profiles and metabolic end products of microbial fermentation. We used anaerobic stirred batch cultures i*n vitro* systems, inoculated with faecal samples of young and premenopausal women, which simulated the physicochemical characteristics of the distal colon. The faecal-derived microbial communities were profiled at 0, 5 and 24 hours by 16S rRNA-gene next-generation sequencing whereas the dietary lignan content was measured by UHPLC-MS/MS.

Result

Sample hydrolysis, extraction and quantification

The methodology for the extraction of lignans from food samples as well as of enterolignans from fermentation faecal samples was adapted from the work of Nørskov and Knudsen [34] as well as the work of Milder et al. [35] with minor modifications. The combination of solvent-based extraction and alkaline hydrolysis was used to release lignans from food or faecal matrix. On the other side, enzymatic extraction with β-glucuronidase/sulfatase was performed in order to degrade glycosidic bonds and to release lignan aglycones. Solid Phase Extraction method was used to prepare samples for UHPLC-MS/MS analysis.

A UHPLC-MS/MS method was developed for accomplishing high resolution and accuracy of the signals. Deuterated standard Secoisolariciresinol-d^6 (Seco-d6) was used as reference standard. A multiple reaction monitoring (MRM) method was set up in negative ion mode for every analyte and quantification was established using the most intense MRM signal transition with 8-16 points calibration curves of pure analytical standards. The obtained MRM detection parameters are given in Table **1**.

Determination of the lignan content in the oilseed mix

Obtained concentrations (mg/100 g of dry matter) of free and total (free plus bound) Seco, Lari, Pino and Mat in the oilseed mix are listed below in Table **2**.

Analysis of enterolignans in fermented samples

The results of batch culture fermentation experiments revealed differences in the concentration of individual lignans and enterolignans at different time points in samples with added oilseed mix between young healthy (YD) and premenopausal (PD) donors (Table **3**).

The average sum of total individual lignans in the YD samples falls about 10 times during the first 5 h of fermentation, with a slight increase at 24 h. The concentration of Mat and Pino in this group decreased significantly, whereas Seco only slightly decreased and Lari increased. At the same time, amounts of total enterolignans in YD samples increased more than 10 times in 24 h, with EL expanding nearly 20 times but ED being slightly reduced.

In contrast, the analysis of fermentation samples from PDs showed different dynamics of individual lignans: total lignans increased almost 4 times in 24 h with only Pino falling about 10 times during 24 h, while levels of Seco, Mat and Lari expanded. Additionally, PD samples showed that the amount of total enterolignans halved in 24 h, with ED levels expanding nearly 60 times, but EL dropping almost 100 times from 0 h to 24 h, suggesting the inability of faecal bacteria from PDs to convert ED to EL. It is also worth noting that all premenopausal participants entered the experiment with very different initial levels of enterolignans (particularly, ED), resulting in a high value of standard deviation.

Fig. **2** shows the dynamics of Seco, Lari, Pino and Mat in the samples from YDs (a) and PDs (b) with added oilseed mix in 24-h fermentation experiments. While very different concentrations of lignans between YDs and PDs can be seen at T0.2, at T5 their levels are almost identical as well as at T24, except for Mat, whose level increased almost 500 times in the PD group.

Fig. **3** illustrates the dynamics of ED and EL in YD and PD fermentation samples during the 24-h batch culture experiment. As for ED, it can be seen that the levels are equally low in YD and PD samples at T0.2 and T5 while at T24, the value in PD samples is almost 37 times higher than in YD samples.

On the other hand, the average EL concentration is extensively higher in PD samples at T0.2, falling slightly at T5 and experiencing a tremendous fall at T24. Conversely, EL levels in YD samples are insignificant at the beginning, rise slightly at T5 and are boosted at T24. Taken together, our results clearly demonstrate different dynamics of lignan transformation in different age groups of women: adding oilseed mix to YD samples leads to the overall falling of levels of dietary lignans and to the production of enterolignans. Contrary to that, in PDs, Mat levels increase after 24 h, with increased level of ED but decreased levels of EL. These observations suggest that PDs could have unbalanced, i.e. dysbiotic intestinal microbial communities, with very poor conversion of dietary lignans and inability to efficiently convert ED or Mat to EL.

Impact of oilseed mix on gut-derived microbial communities from younger healthy and premenopausal women

The faecal microbial communities from PDs were profiled over time and compared with those of YDs to assess whether the different dynamics of lignan transformation were reflected in different layouts and

trajectories of the gut microbiota. The 16S rRNA gene-based next-generation sequencing yielded a total of 1,809,764 high-quality reads, with an average of 33,514 ± 7,194 sequences per sample, binned into 2,623 amplicon sequence variants (ASVs) at 99% similarity.

No significant differences were observed in alpha diversity across the entire dataset, regardless of the sample origin (PD vs. YD), experimental condition (oilseed mix vs. inulin vs. negative control) and time point (T0.2 vs. T5 vs. T24) (p value > 0.05, Wilcoxon test). However, the biodiversity of both PD and YD gut microbiota tended to decrease over time (Additional file 1: Fig. **S1**). This trend was more noticeable in PD samples in the presence of the oilseed mix while, in the same condition, the biodiversity of YD samples was generally maintained.

The Principal Coordinates Analysis (PCoA) of inter-sample variation, based on weighted (Additional file 2: Fig. **S2**) and unweighted (Fig. **4**) UniFrac distances, showed a significant separation between microbial communities from PDs and YDs (p value < 1×10^{-4}, permutation test with pseudo-*F* ratio). Within each group (PD and YD), the samples still segregated significantly according to both the experimental condition and the time point (p value < 0.001), suggesting a differential impact of supplements over time, likely related to the baseline microbial community.

At T0.2, a number of significant taxonomic differences were indeed observed between PDs and YDs. In particular, compared to YDs, the gut-derived microbial ecosystem of PDs showed increased relative abundance of some Bacteroidetes members, especially *Bacteroides* and *Rikenellaceae*, as well as of *Enterobacteriaceae* and *Ruminococcaceae* genera (p value ≤ 0.05, Wilcoxon test). On the other hand, *Coriobacteriaceae*, especially *Collinsella*, and *Streptococcus* were far more represented in the baseline microbiota of YDs vs. PDs (p value ≤ 0.05) (Additional file 3: Fig. **S3**).

With specific regard to the impact of oilseed mix, both common and unique microbial signatures were observed in response to treatment. Among the shared features, it is worth noting that 24 h of fermentation with the supplement resulted in decreased proportions of *Ruminococcaceae* and various *Lachnospiraceae* members, and increased amounts of *Enterobacteriaceae* (p value ≤ 0.1). However, the latter increase was markedly different between groups (ranging from 0.2 to 78.5% in YDs and from 0.1 to 27.7% in PDs) and largely attributable to the different representation of *Klebsiella*, whose 24-h relative abundance was 51.3 vs. 1.7% in the YD and PD group, respectively. At species level, *Klebsiella* ASVs were found to be variously assigned to *K. pneumoniae* and *K. aerogenes*, with the latter being overall more prevalent, regardless of the origin of the stool samples. In addition to this, it is interesting to point out that the family *Clostridiaceae* only increased in PD-derived microbial communities, as well as the genus *Collinsella*, whose trend was exactly opposite (i.e. decreased) in YD-related samples (p value ≤ 0.2) (Fig. **5**).

Discussion

In this work, we determined the concentration of four main dietary lignans in an oilseed mix commercially prepared from the following ingredients: pumpkin, sunflower seeds, buckwheat and millet flakes, milled flaxseed, hemp and chia. Compared to flaxseed, the oilseed mix had 4 times less free lignans with Seco being 8.2 times less and Mat almost 6 times less than in flaxseed. Comparable volumes of Lari in both flaxseed and oilseed mix were found: 44.2 mg/100 g and 44.9 mg/100 g, respectively. Seco and Mat are the main substrate for enterolignans, with Seco being converted to EL through ED, and Mat being directly converted to EL. Differences in lignan concentration were discussed previously [5, 36] and are thought to be related to variance in the crop, climate, storage conditions and other factors. Thus, it was expected to obtain concentrations that would diverge from previously reported data.

We then used the oilseed mix in batch culture fermentation experiments to evaluate the dynamics of enterolignan production by gut-derived microbial communities from healthy younger (YD) vs. premenopausal (PD) female donors. Adding 0.5 g of oilseed mix to stool samples of YDs resulted in 10 times increased concentration of EL and a decrease in ED in 24 h, as compared to the negative control. Contrary to that, the same amount of supplement in the presence of PD samples resulted in a significant ED boost, whose 24-h concentration increased by 110 times compared to the negative control and by 80 times compared to the YD group under the same experimental conditions. However, despite such a high volume of ED, EL levels continued to decline, probably indicating the failure of bacterial communities from PDs to efficiently convert ED to EL. Consistent with this, i.e. with the inability of PD-related microbiotas to produce EL, high levels of Mat were found at the end of fermentation.

The biotransformation of dietary lignans to EL and ED is a complex process believed to involve microbial consortia capable of performing four major catalytic reactions – O-deglycosylation, O-demethylation, dihydroxylation, and dehydrogenation – with each reaction being catalyzed by a specialized group of bacteria that often constitute a minor component of the overall microbiome [29-31]. Though evidence of association between specific enterolignans and certain human gut bacteria has been provided, such as that between high urinary excretion of EL and increased proportions of Ruminococcus spp. while decreased amounts of Bilophila [31, 37], the complexity and diversity of the gut microbiota are considered essential to maximise lignan conversion and thus influencing human exposure to enterolignans. In this regard, here we demonstrated that YDs and PDs have a different intestinal microbiota profile and that these profiles undergo different temporal dynamics in the presence of oilseed mix, potentially contributing to the different production pattern of EL/ED, as observed in our in vitro batch culture fermentations. In particular, PDs were found to be significantly depleted in Collinsella, while being enriched in the pro-inflammatory Enterobacteriaceae family, as well as in a number of Bacteroidetes members, including Bacteroides and Rikenellaceae, that typically thrive on high-fat diets and are enriched in obese gut microbiomes [38]. As for the impact of the oilseed mix, both YD and PD gut-derived microbial communities showed an enrichment over time in Enterobacteriaceae, specifically Klebsiella, but with a 24-h abundance far greater in the former. As previously observed [39, 40], several Klebsiella spp. – closely related to K. pneumoniae - are capable of biotransforming SDG to Seco, with the latter representing a substrate for EL production through microbial metabolism. The differential representation of Klebsiella could therefore help to explain the greater levels of EL in YD- vs. PD-related samples. On the other hand, it

is worth noting the differential trend of Collinsella, whose relative abundance increased in PD-related microbial communities while it decreased in YD-related ones, which suggests a possible role of this taxon in EL/ED conversion. Interestingly, Collinsella has recently been described as a bacterial genus involved in the biotransformation of dietary phytoestrogens in equol, possibly contributing to the desirable health benefits of isoflavones in postmenopausal women [41, 42]. Finally, the PD gut-derived microbial ecosystem appeared to be enriched in the Clostridiaceae family, whose members are already known to be variously involved in lignan metabolism [43, 44] .

Conclusion

Despite the small sample size and the obvious limitations of an *in vitro* study, our findings revealed that the healthy younger women's microbiota is more efficient in converting dietary lignans into enterolignans, especially enterolactone, than the older premenopausal women's microbiota. In addition, our study points to *Klebsiella* and *Collinsella* spp. as previously neglected actors of the human gut microbiota with a strong potential to be directly involved in EL/ED production. Future studies in larger cohorts are needed to confirm our findings and to understand how we can modulate the microbiota composition, favouring the microbial species capable of producing enterolactone more effectively. If a woman's microbiota will effectively convert dietary lignans into EL, a consistent consumption of lignan-rich food will potentially preserve a woman's health in the long term, preventing or at least delaying the onset of degenerative conditions typically associated with menopause.

Methods

Oil seed samples

Eurocan Ltd (UK) provided five different organic raw oils seeds (flaxseed, chia, hulled sunflower, hulled pumpkin, hulled hemp), organic buckwheat and millet flakes, as well as ready-made oilseed mix "MightyMix". Oilseed mix was milled using Hinari Genie MB280 electric grinder. Duplicates 25 mg of the mix were stored in separate 2-ml Eppendorf tubes at -20°C. The present oilseed mix nutritional profile was characterised by Campden BRI (Chipping Campden) Ltd, accredited to ISO17025:2005 by UKAS by official reference methods (fat: Weibull Stoldt; sugars: HPLC; total dietary fibre: AOAC; protein: Kehjdahl) as reported in Table S1.

Collection and stool sample preparation

Faecal samples were donated by three healthy younger (aged 25-30 years) and three older premenopausal female donors (aged 40-55 years). All donors confirmed to be healthy of metabolic and gastrointestinal conditions, were not taking prebiotic or probiotic supplements, did not have antibiotic treatments in the previous six months before the study. The information on the donors' health status, lifestyle habits, clinical anamnesis, and medicine use was collected with pre-informative questionnaire. All faecal samples were collected on site, kept at – 20°C and used within a maximum of 15 min after

collection. Samples were diluted 1/10 w/v in anaerobic PBS (0.1 mol/l phosphate buffer solution, pH 7.4) and homogenized (Stomacher 400, Seward, West Sussex, UK) for 2 min at 460 paddle-beats.

In vitro batch culture fermentation experiments

Batch culture fermentation method was carried out as previously described by Costabile et al. [45]. Each vessel was inoculated with 5 ml of fresh faecal slurry (1/10 w/v) for both healthy and premenopausal subjects. A known prebiotic compound inulin (Raftilose P95, 95% oligosaccharide, β(2-1)-fructan, of which 60% w/w glucose-fructose, 40% w/w fructose, degree of polymerization, 3–10) serving as a positive control was added to a separate batch-culture vessel. A further vessel was prepared under the same conditions but without the addition of any compound (negative control, ctr) whereas another vessel was used to add the seed mix. Batch

culture fermenters were ran under anaerobic conditions for a period of 24 h during which samples (5 ml) were collected at time 0.2, 5 and 24 h. Samples were stored at −80 °C until required for 16S rRNA gene-based next-generation sequencing analysis and ultra-high-performance liquid chromatography (UHPLC) – tandem mass spectrometry (MS/MS) quantification.

Lignan extraction methods

The methodology for the extraction of lignans from single food samples and enterolignans from faecal samples was adapted from the work of Nørskov and Knudsen [34] as well as work of Milder et al. [35] and optimised in regards to the weight and the character of the samples. To each oilseed sample (25 mg), 1 ml of n-hexane, was added. The samples were vortexed and left at room temperature with gentle agitation for 20 min. Samples were centrifuged at 13200 rpm, at 4°C for 10 min. The supernatant was discarded, and pellets were kept for the next steps.

Extraction of free lignans from oilseed samples and fermentation samples

Oilseed free lignan samples

Pellets were extracted with 0.5 ml of 100% methanol, vortexed and left at room temperature with gentle agitation for 10 min. After centrifugation at 13200 rpm, at 4°C for 10 min, the supernatant was collected into clean 2-ml Eppendorf tubes and left at nitrogen stream to evaporate for dryness.

Fermentation enterolignans samples

One milliliter of fermentation sample was extracted with 0.5 ml of 100% methanol, sonicated for 10 min, and then kept with gentle agitation for 10 min. Next, samples were centrifuged at 13200 rpm, for 10 min at 4°C, and the supernatant was collected into clean 2-ml tubes and evaporated for dryness.

Afterwards, dried fermentation and oilseed samples were incubated for 16 h at 37°C with added ß-glucuronidase/sulfatase from Helix pomatia (freshly dissolved in 0.05 M sodium acetate buffer with concentration of 2 mg/ml), cooled and added with 0.5 ml of acidified water (0.4% of formic acid) to stop the hydrolysis, vortexed and then centrifuged at 13200 rpm for 10 min at 4°C. Samples were ready for SPE.

Extraction of total lignans from oilseed samples

Following defatting, pellets were extracted with 0.5 ml of 0.3 M NaOH in 70% methanol, vortexed, and then incubated for 1 h with gentle agitation under 60°C. After cooling down, pH was adjusted by adding 20 µl of glacial acetic acid (pH=5). Next, samples were centrifuged for 10 min at 13200 rpm at 4°C; the supernatant was collected into 2-ml clean Eppendorf tubes and evaporated for dryness under a nitrogen stream. Enzymatic hydrolysis was performed by adding 0.6 ml of ß-glucuronidase/sulfatase from Helix pomatia (same as above) to each dried sample and setting samples for overnight incubation under 37°C coupled with gentle agitation. Afterwards, samples were cooled down, added with 0.5 ml of acidified water (0.4% of formic acid), vortexed and then centrifuged at 13200 rpm, at 4°C for 10 min. Supernatants were collected into clean Eppendorf tubes ready for solid phase extraction (SPE).

Solid Phase Extraction (SPE) of lignans and enterolignans

SPE was performed using 1-ml cartridges Strata® C-18 (55 µm, 70Å) from Phenomenex UK and SPE Vacuum Manifold. Waste was collected into 15-ml Falcon tubes, and final samples were collected into clean 2-ml Eppendorf tubes. After assembling manifold, cartridges were prepared as follows: with locked taps, 0.5 ml of acetonitrile was added to each cartridge and left for 10 min, then drained out. Next, with locked taps, 0.5 ml of LC-MS water was added, then drained after 10 min. After this, samples were added for slow elution through C18 material, followed by 0.5 ml of methanol, added twice to each cartridge and let to elute slowly until dry, afterwards, the vacuum was applied to dry the sorbent. Each cartridge was eluted with 0.4 ml of acetonitrile, and after draining, the vacuum was applied to facilitate full elution. Samples were then evaporated under a nitrogen stream and stored at -20°C. Aliquots of 0.5 ml of LC-MS water containing the internal standard (seco-d^6) at the final concentration of 20 ng/ml and fermentation samples were added with 100 µl of LC-MS water. Samples were vortexed and 200 µl was dispersed into well plates ready for LC-MS analysis.

Microbial DNA extraction

Total microbial DNA was extracted from around 250 mg of *in vitro* fermentation samples using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. DNA concentration and quality were evaluated using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

16S rRNA gene-based next-generation sequencing, bioinformatics and statistics

The V3-V4 hypervariable region of the 16S rRNA gene was PCR-amplified using the primer set 341F/805R, as previously reported [46]. PCR products of about 460 bp were purified using a magnetic bead-based system (Agencourt AMPure XP; Beckman Coulter, Brea, CA) and indexed by limited-cycle PCR using Nextera technology (Illumina, San Diego, CA). Indexed libraries, further cleaned up as described above, were pooled at equimolar concentration, denatured and diluted to 6 pmol/l. Sequencing was performed on an Illumina MiSeq platform using the 2×250 bp protocol, according to the manufacturer's instructions. Sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA; BioProject ID PRJNA592433).

The obtained paired-end reads were processed using a pipeline combining PANDAseq [47] and QIIME2 [48, 49]. High-quality reads were filtered and clustered into amplicon sequence variants (ASVs) at 99% similarity through an open-reference strategy performed with DADA2 [50]. Taxonomy was assigned using the vsearch classifier [51] against Greengenes database as a reference (release May 2013). Alpha diversity was measured using the number of observed ASVs and the Faith's Phylogenetic Diversity (PD whole tree). Beta diversity was computed based on weighted and unweighted UniFrac distances and visualized on a Principal Coordinates Analysis (PCoA) plot. For the identification of *Klebsiella* species, ASVs assigned to the genus *Klebsiella* were subjected to BLAST analysis [52]. Statistics was performed using R Studio 1.0.44 on R software version 3.3.2 [53] implemented with the packages stats and vegan [54]. The significance of data separation in the PCoA plot was tested by a permutation test with pseudo-*F* ratio using the function adonis in vegan. Bar plots were built using the R packages made4 [55] and vegan. Non-parametric tests (Kruskal-Wallis test or Wilcoxon test, paired or unpaired as needed) were achieved using the stats package. A p value ≤ 0.05 was considered statistically significant; a p value between 0.05 and 0.2 was considered a tendency.

Ultra-high-performance liquid chromatography (UHPLC)–MS/MS

Acquity H class UPL chromatography equipment was used and separations were performed on an Aquity UPLC® HSS PFP 1.8 µm 2.1 x 100 mm C18 (Waters, UK) class column with a column protection of Acquity UPLC® HSS T3 1.8 µm Van Guard™ pre-column 3/Pk 2.1 x 5 mm column (Waters, UK) at a flow rate of 0.65 ml/min at 30°C. The mobile phases A and B consisted of 100% LC-MS water and 100% acetonitrile, respectively. The gradient started at 95% phase A and 5% phase B, was held constant for 6 min, then phase B increased to 75% during 0.9 min, with the subsequent increase to 95% for 0.1 min, followed by 99% increase of phase A during the last 2 min. The total run for each sample was 10 min. Sample injection volume was 2 µl. The negative mode was used for ionisation. Detection was performed using Xevo TQ-micro (Waters, UK) quadrupole mass spectrometer, which facilitates the detection of low concentrated analytes. Parent and daughter ions (m/z) are described for each compound in Table 1 together with cone voltage and collision energy. The analysis data were collected and analysed using MassLynx software.

Abbreviations

SDG – secoisolariciresinol diglucoside

Seco - secoisolariciresinol

Lari - lariciresinol

Pino - pinoresinol

Mat- matairesinol

ED-enterodiol

EL-enterolactone

ER- oestrogen receptor

SPE-solid phase extraction

LC-MS-Liquid Chromatography – Mass Spectrometry

MRM-Multiple reaction monitoring

UHPLC-Ultra-High-Performance Liquid Chromatography

YD-younger donor

PD-premenopausal donor

Declarations

Acknowledgements

We thank the student Oghenerukevwe Obayiuwana for the technical assistance during the *in vitro* fermentation experiments and lignan extraction.

Availability of data and materials

The datasets used in the current study are available from the corresponding author. 16S rRNA gene sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive (NCBI SRA; BioProject ID PRJNA592433).

Supplementary information

Additional files. Additional table (Table **1S**) and figures (**FigS1, S2, S3**).

Authors' contributions

GC and AC designed the experiments and wrote the manuscript. AK performed the *in vitro* fermentation experiments and UHPLC-MS/MS analysis. EK prepared the samples for DNA analysis. MB, ST and PB carried out all 16S rRNA-gene next-generation sequencing and analysis, respectively. All authors read and approved the final manuscript.

Funding

This research received no external funding.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Tables

Due to technical limitations, all Tables available in the Supplementary Files section below.

Figures

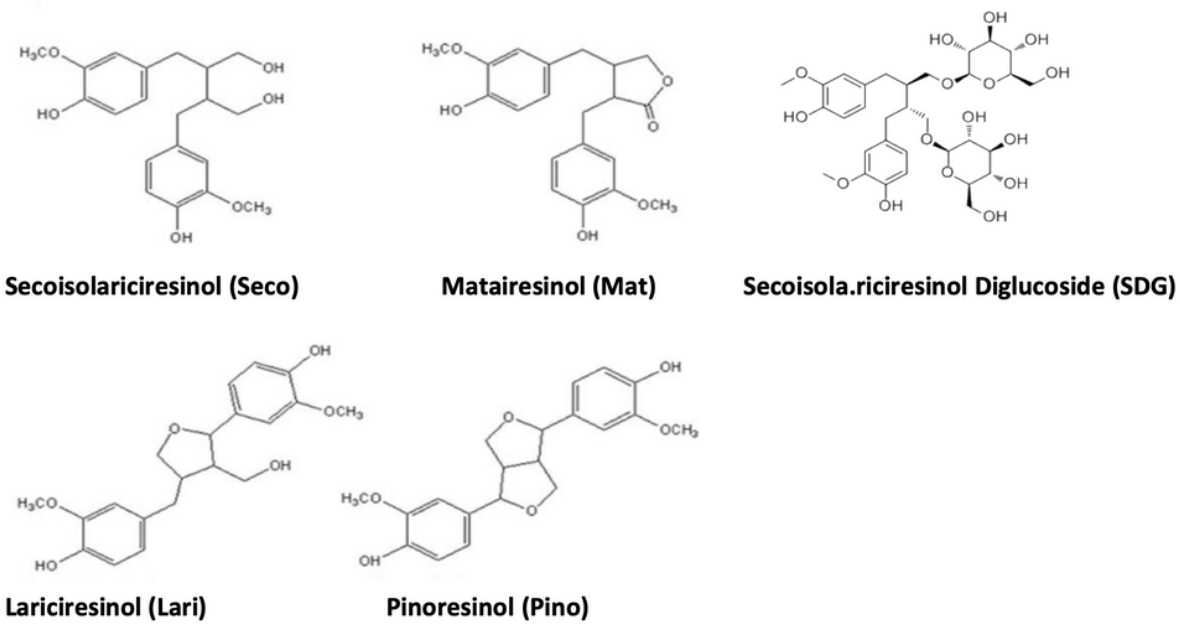

Figure 1

Dietary lignans. Secoisolariciresinol (Seco); Matairesinol (Mat); Secoisolariciresinol Diglucoside (SDG); Lariciresinol (Lari); Pinoresinol (Pino)

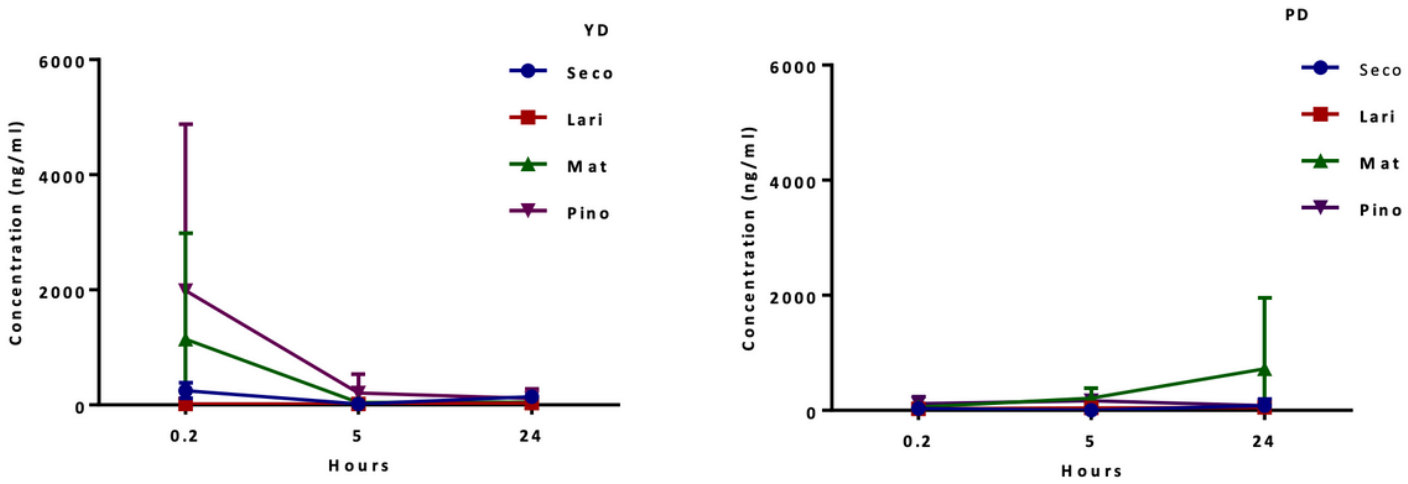

Figure 2

Dynamics of individual dietary lignans over 24 h of fermentation in faecal samples from younger healthy donors (YD, a) and premenopausal donors (PD, b) with added oilseed mix

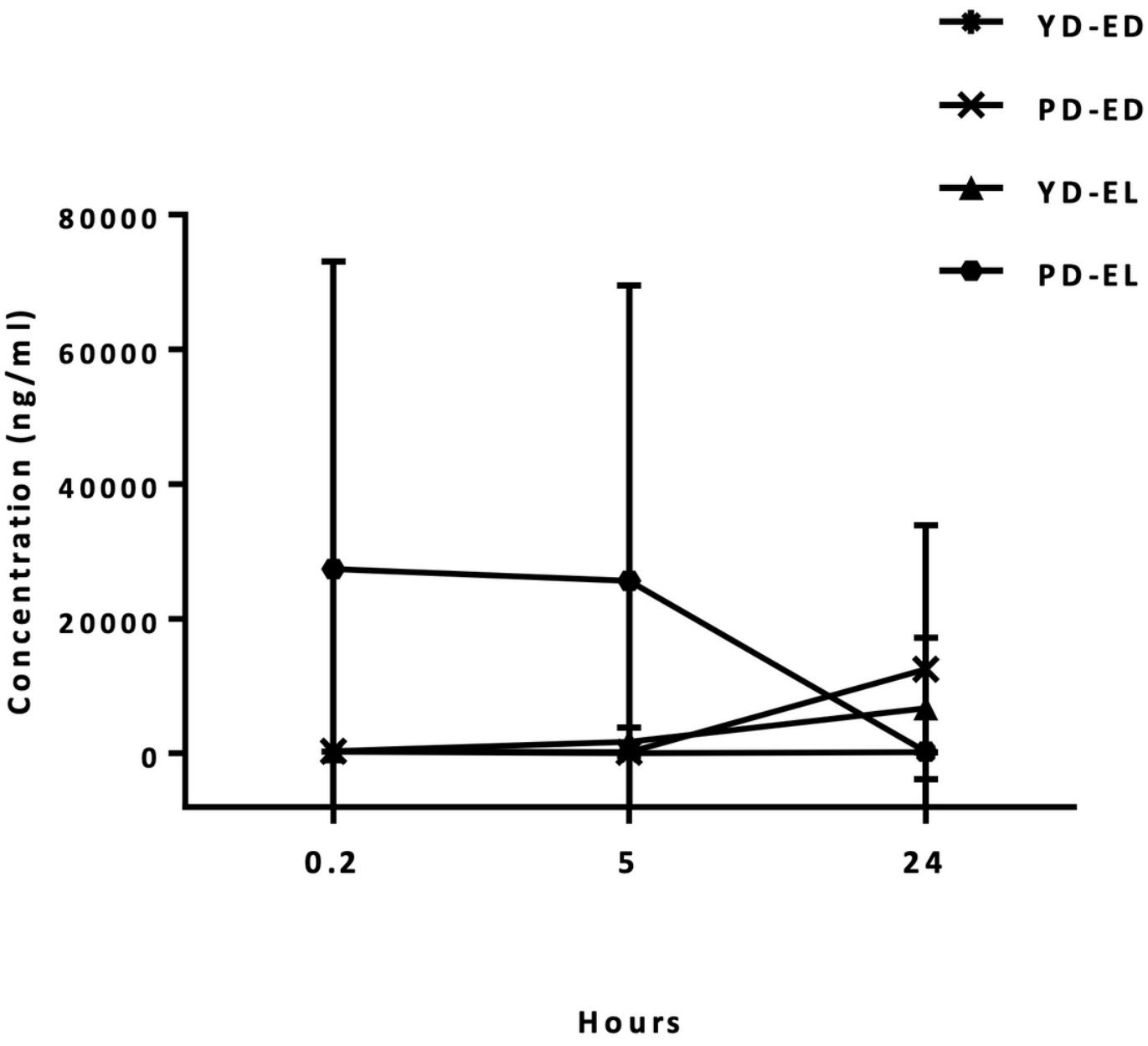

Figure 3

Concentration of enterodiol (ED) and enterolactone (EL) in fermentation samples from younger healthy donors (YD) and premenopausal donors (PD) at 0.2 (T0.2), 5 (T5) and 24 hours (T24)

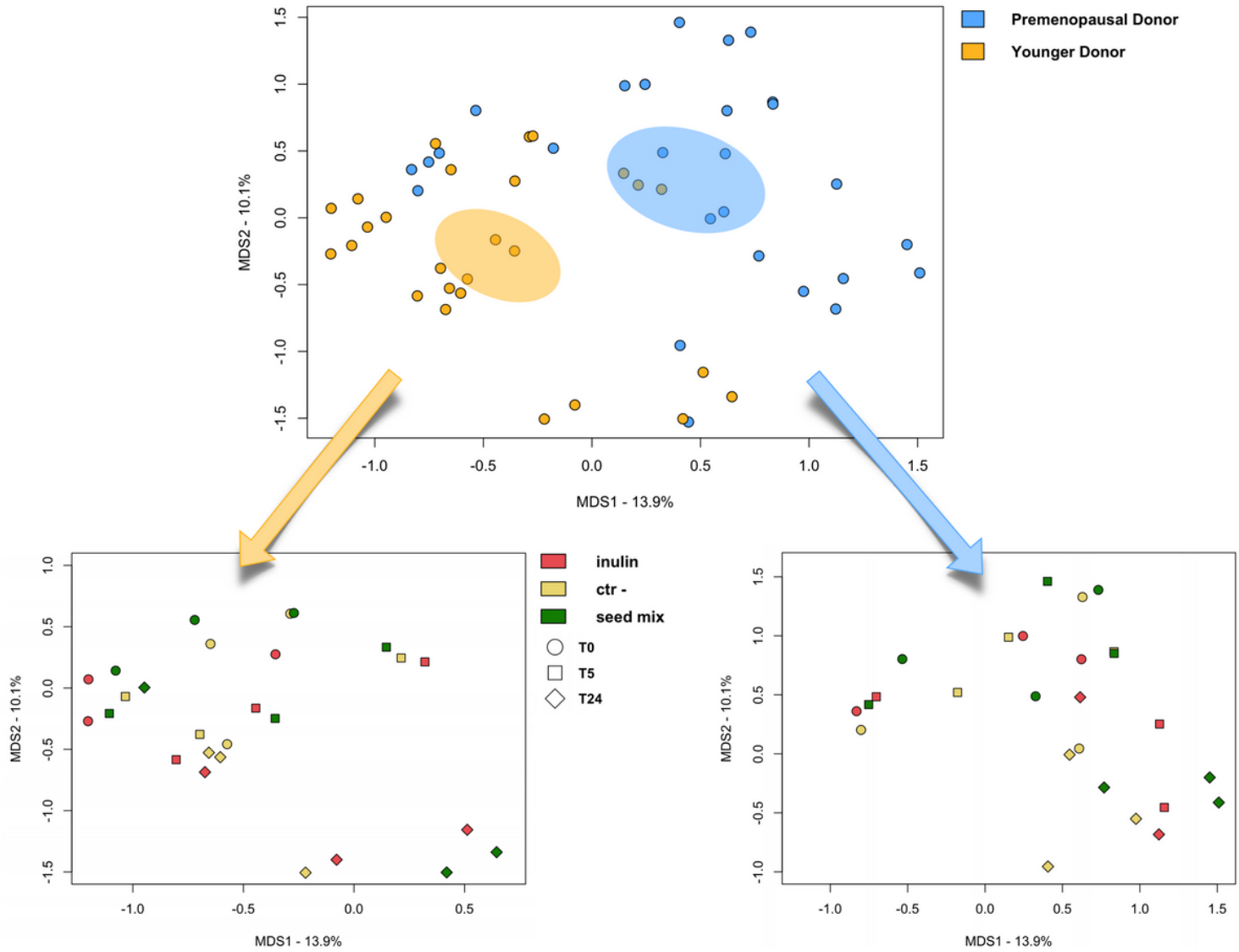

Figure 4

Gut-derived microbial communities of premenopausal women separate from those of younger healthy women and are affected by oilseed mix. Principal Coordinates Analysis (PCoA) of the gut-derived microbial communities, based on unweighted UniFrac distances. A significant separation between premenopausal and younger healthy women was found (upper panel), as well as between different experimental conditions and time points (lower panels) (p value < 1 × 10-4, permutation test with pseudo-F ratio).

Supplementary Files

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