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Levels of lipid-derived gut microbial metabolites differ among plant matrices in an *in vitro* model of colon fermentation

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ABSTRACT

This study explored differences in microbial lipid metabolites among sunflower seeds, soybeans, and walnuts. The matrices were subjected to *in vitro* digestion and colonic fermentation. Defatted digested materials and fiber/phenolics extracted therefrom were added to sunflower oil (SO) and also fermented. Targeted and untargeted lipidomics were employed to monitor and tentatively identify linoleic acid (LA) metabolites. Walnut fermentation produced the highest free fatty acids (FFAs), LA, and conjugated LAs (CLAs). Defatted digested walnuts added to SO boosted FFAs and CLAs production; the addition of fibre boosted CLAs, whereas the addition of phenolics only increased 9e,11z-CLA and 10e,12z-CLA. Several di-/tri-hydroxy-C18-FAs, reported as microbial LA metabolites for the first time, were annotated. Permutational multivariate analysis of variance indicated significant impacts of food matrix presence and type on lipidomics and C18-FAs. Our findings highlight how the food matrices affect CLA production from dietary lipids, emphasizing the role of food context in microbial lipid metabolism.

1. Introduction

The human gut microbiota has been described as a symbiotic ecosystem, playing a crucial role in physiological human activities, including metabolism and immune system modulation (Makki et al., 2018). This ecosystem is a complex community containing 100 trillion microorganisms including fungi, viruses, archaea and bacteria (Tremaroli & Bäckhed, 2012). These microorganisms are thriving on dietary components that escape digestion and absorption in the small intestine, preferably dietary fiber and proteins (Zeng et al., 2022). This may due to the amounts of ingested food surpassing the digestion potential of the host, or due to nutrients being resistant to digestive enzymes because of the structural complexity of the food matrix or to specific dietary compounds (Kan et al., 2020; Wong & Jenkins, 2007). For example, cell wall integrity would impair macronutrient utilization from plant tissues (Capuano et al., 2018).

Dietary lipids are important macronutrients since they contain essential fatty acids (FAs) and allow the absorption of lipophilic vitamins. Polyunsaturated fatty acids (PUFAs) that escape absorption in the small intestine can be metabolized by certain members of human gut microbiota, such as *Lactobacillus*, *Roseburia* and *Bifidobacteri*um, mostly as a detoxification pathway, also known as biohydrogenation (Gorissen et al., 2015; Kishino et al., 2013). Linoleic acid (LA), one of the most abundant PUFAs in plants, is converted into several FA metabolites, such as conjugated LAs (CLAs), oxo- or hydroxy-FAs, and various C18:1 FAs (Gorissen et al., 2015; Salsinha et al., 2018; Yang et al., 2017). Many of these metabolites have been shown to benefit gut health, from ameliorating chemical-induced acute colitis in animal models to inhibiting the *in vitro* growth of human colon cancer cells (Beppu et al., 2006; Borniquel et al., 2012; Brandão & Ribeiro, 2018; Devillard et al., 2007; Hontecillas et al., 2002; Ogawa et al., 2005; Salsinha et al., 2018). Whether the metabolic pathways for the generation of some metabolites are known, the modulation of lipid metabolism by food-related or dietary factors is far less understood.

Lipidomics is emerging as a powerful technique for the detection and identification of a large number of lipophilic molecules with diverse

Abbreviations: LA, linoleic acid; CLA, conjugated linoleic acid; FA, fatty acid; QC, quality control; Permutational multivariate analysis of variance, PERMANOVA; oxoOME, oxo-octadecenoic acid; DiHOME, dihydroxy-octadecenoic acid; DiHODE, dihydroxy-octadecadienoic acid; TriHOME, trihydroxy-octadecanoic acid.

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structures (Wang et al., 2022; Wu et al., 2020). High resolution mass spectrometry (MS) enables the investigation of different classes of molecules based on the charge, size, masses, shapes and MS/MS characteristic fragmentation pathways. Targeted lipidomics focuses on a limited number of defined lipophilic compounds in order to accurately quantify them. In contrast, untargeted lipidomics can provide more comprehensive information on the overall lipid composition without predefined screening conditions. Lipidomics has been used to fill the knowledge gap in those lipids with biological effects, from the identification and quantification to metabolic pathways (Chen et al., 2022; Wang et al., 2022).

In a previous work, we have shown that the level of cell integrity may affect the level of LA metabolite in an *in vitro* model of colonic fermentation of soybeans (Huyan et al., 2022). Next to structural features, the composition of the diet can also affect microbial metabolism of specific substrates. For instance, it has been showed that the fiber types could modulate the microbial catabolism of tryptophan, inducing different concentrations and profile of tryptophan catabolites (Huang et al., 2022). The present study was designed to further investigate the effect of the food matrix on microbial metabolism of dietary lipids, particularly linoleic acid. We did this by comparing three lipid-rich food matrices, namely sunflower seeds, soybeans and walnuts differing in the amount and type of oil (i.e. fatty acid composition) as well as in their proximate composition. Lipid metabolism by the gut microbiota was monitored by a combination of targeted and untargeted lipidomics approaches. By means of a targeted approach, several known LA metabolites and precursor FAs were quantified whereas several other lipid metabolites were identified or putatively annotated by using the lipidomics untargeted approach.

2. Materials and methods

2.1. Materials

Sunflower seeds, soybeans and walnuts were purchased from a local supermarket (Wageningen, the Netherlands) and stored at room temperature in a dry place. All the samples were used within five weeks of their purchase. High purity CLAs, *cis*-9, *trans*-11-CLA (9z,11e-CLA), *trans*-9, *trans*-11-CLA (9e,11e-CLA), *trans*-10, *cis*-12-CLA (10e,12z-CLA), 11e-octadecenoic acid (vaccenic acid) and 12-hydroxy-9(z)-octadecenoic acid (12-hydroxy-9z-C18:1) were purchased from Larodan (Solna, Sweden). Free fatty acids (FFAs), including palmitic acid (hexadecanoic acid, C16:0), *n*-heptadecanoic acid (C17:0), stearic acid (octadecanoic acid, C18:0), oleic acid (9z-octadecenoic acid, C18:1), LA (9z,12z-octadecadienoic acid, C18:2), α-linolenic acid (9z,12z,15z-octadecatrienoic acid, C18:3), and their methyl esters, (+)catechin, 2,5-dihydroxybenzoic acid, benzoic acid, caffeic acid, ellagic acid, epicatechin gallate, ferulic acid, gallic acid, and all other chemicals of analytical grade used in this study were purchased from Sigma-Aldrich (St. Louis MO, USA). Heat-stable α-amylase, protease and amyloglucosidase were from the commercial kit Total Dietary Fiber purchased from Megazyme International (Wicklow, Ireland).

2.2. Preparation of samples

In this study, we have followed a sequential approach where we first investigated the lipid metabolism in the three matrixes, then we investigated the potential sources of the observed differences by first fermenting the defatted material with the same oil (so to exclude differences induced by the specific type of oil) and then by fermenting fibre and phenolic compounds extracted from the matrixes with the same oil (to investigate which component of the defatted matrix may explain the differences). An overview of the sample preparation and experimental design is presented in Fig. 1.

2.2.1. Preparation of sunflower seeds, soybeans and walnuts

Raw sunflower seeds and walnuts were mashed by a 6875D Freezer/Mill machine (Retsch Technology GmbH, Haan, Germany). The soybeans were cooked at 121 °C for 10 min with distilled water (1:3, w/v) and gently ground. All materials were then loaded on a stack of sieves with 4 aperture sizes: 1000–2000, 425–1000, 250–425, and 125–250 μm, and the particles of sizes ranging from 250 to 425 μm were collected. This particle size range was selected because previous work has demonstrated that this size of walnut can be representative of what is generated in the bolus upon mastication and because soybean particles of smaller size contain broken cells (Huyan et al., 2022; McArthur & Mattes, 2020). The obtained materials were dried and then stored at 4 °C and pre-digested within 48 h. Sunflower oil was extracted from the residues of sunflower seed samples by using Soxhlet extraction and stored at 4 °C before use.

2.2.2. In vitro digestion of sunflower seeds, soybeans and walnuts

The materials prepared as described in 2.2.1 were subjected to a simulated *in vitro* gastrointestinal digestion according to the INFOGEST protocol (Brodkorb et al., 2019) slightly modified as described in our previous study (Huyan et al., 2022). Briefly, 0.7 g of each food was mixed with Milli Q water to produce a paste of 5 g. The paste was then subjected to oral, gastric and intestinal phase sequentially. After the intestinal phase of the simulated digestion, the entire tube was centrifuged at 4500*g* for 20 min to obtain the residual pellets, which will be

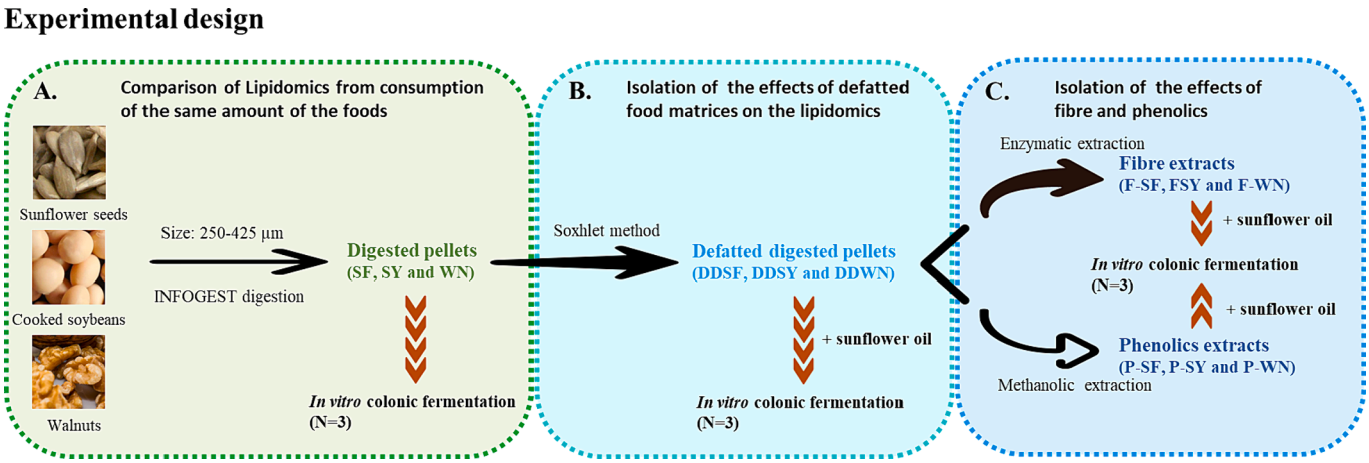


Fig. 1. Flow chart of the sample preparation and experimental design. Notes: SF, sunflower seeds; SY, soybeans; WN, walnuts; DDSF, defatted digested sunflower seeds; DDSY, defatted digested soybeans; DDWN, defatted digested walnuts; F-SF, sunflower seed fiber extracts; F-SY, soybean fiber extracts; F-WN, walnut fiber extracts; negative control, sample with only media; P-SF; sunflower seed phenolics extracts; P-SY, soybean phenolics extracts; P-WN, walnut phenolics extracts.

from now on referred to as digested pellets. The digested pellets were collected, dried, weighed and then stored at −20 °C and characterized in terms of fiber, protein and lipid content. Afterwards, one part of the digested pellets was directly subjected to simulated *in vitro* colonic fermentation and another part was used for the extraction of phenolics and fiber.

2.2.3. Determination and collection of lipids, fiber, protein and polyphenols

2.2.3.1. Defatting of the digested pellets and determination of fatty acid composition. Soxhlet extraction was applied to raw samples and digested pellets. The defatted sample were defined as defatted digested pellets, namely, defatted digested sunflower seeds (defatted digested sunflower seed), defatted digested soybeans (defatted digested soybean) and defatted digested walnuts (defatted digested walnut). The extracted lipids were collected, concentrated and methylated to fatty acid methyl esters (FAMEs) by mixing with NaOH/MeOH (Huyan et al., 2022). The resulting FAMEs were analyzed by gas-chromatography equipped with a flame ionization detector fitted with a capillary FA-free Stabil wax-DA column (30 m × 0.25 μm × 0.25 μm, Restek, Bellefonte, PA, USA). One μL of samples was injected and the oven temperature was set at 150 °C for 0.035 min and subsequently increased to 250 °C at a rate of 10 °C/min and then maintained at that temperature for 25 min. The injector and detector were operated at 250 °C. Nitrogen was used as a carrier gas. The FAME peaks were identified by comparing retention times to known standards and quantified by using external calibration curves prepared for each FAME.

2.2.3.2. Determination of protein content. DUMAS Flash EA 1112 Protein analyzer (Thermo Fisher Scientific, Massachusetts, USA) was utilized to measure the protein content of raw samples and defatted digested pellets. The obtained values were normalized by a control sample to exclude the amount of digestive enzymes added. A Conversion factor of 6.39 for sunflower seeds; 5.7 for soybeans and 6.25 for walnuts was used for the calculation of protein content.

2.2.3.3. Preparation and determination of fiber and phenolics from digested pellets. Determination and collection of fiber was performed using a Total Dietary Fiber assay kit from Megazyme International (K-TDFR-100A, Bray, Ireland) following a modified AOAC enzymatic–gravimetric method (991.42, AACC, 2000). Briefly, defatted digested sunflower seed, defatted digested soybean and defatted digested walnut (the amount obtained after digestion of 0.7 g of each food and defatting of the corresponding amount of digested pellet from 2.2.2), and their raw materials, were first digested by heat-stable α-amylase at 98–100 °C for 1 h and then digested with protease at 60 °C for 30 min, followed by amyloglucosidase treatment at 60 °C for 30 min to remove protein and starch. Four volumes of 96 % ethanol, warmed to 60 °C before use, were added and the residue was then collected and washed with ethanol and acetone. The residue was oven-dried (105 °C) overnight in an air oven, weighed and stored in a dark place at room temperature until use (within 2 weeks).

Phenolics were extracted as described in previous publications with some modifications (Kostić et al., 2019; Le et al., 2019; Ni et al., 2021). Briefly, defatted digested sunflower seed, defatted digested soybean and defatted digested walnut (the amount obtained after digestion of 0.7 g of each food and defatting of the corresponding amount of digested pellet from 2.2.2) were treated with methanol (1:5, v/v) and sonicated. This process was repeated five times, and the resulting phenolic fractions were pooled together and concentrated by rotational evaporation with reduced pressure. The phenolic fractions also passed a filter (15 mm Ø, 0.2 μm regenerated cellulose filter, Phenomenex, Torrance, USA) before being added to the fermentation.

The composition of phenolics was analyzed by an UHPLC (LC-40, Shimadzu, Kyoto, Japan) coupled with QTOF-MS 9030 (Shimadzu, Kyoto, Japan). Chromatography was performed using a BEH C18 column (2.1 × 100 mm, 1.7 μm). The column temperature was set at 40 °C. The mobile phases consisted of (A) water with 5 mM ammonium formate and 0.1 % formic acid, and (B) acetonitrile with 5 mM ammonium formate and 0.1 % formic acid. A multistep elution dual-mode gradient was developed as follows: at 0.0 min (5 % B; 0.40 mL min^{-1}) a gradient begun up to 0.5 min (15 % B; 0.4 mL min^{-1}), and a second step was set to 3.0 min (15 % B; 0.4 mL min^{-1}), then the third step 8.5 min reached 95 % B and slightly increased the flow (95 % B; 0.5 mL min^{-1}), subsequently an isocratic step was executed during three minutes and twenty seconds, 11.9 min (95 % B; 0.50 mL min^{-1}), 12.0 min (5 % B; 0.40 mL min^{-1}) a reconditioning period up to 14.0 min (5 % B; 0.40 mL min^{-1}). The sample injection volume was 5 μL.

The samples, including the prepared standards ((+)catechin, 2,5-dihydroxybenzoic acid, benzoic acid, caffeic acid, ellagic acid, epicatechin gallate, ferulic acid, gallic acid), were recorded by full scan acquisition in both positive and negative ionization modes. QTOF system was equipped with an electrospray ionization interface (ESI). Conditions of the negative ionization mode are as follows: nebulizing gas flow 230 L min^{-1}; heating gas pressure 10.0 L min^{-1}; temperature 300 °C and capillary voltage –3500 V. The capillary voltage in positive ESI was + 4500 V, other source settings were the same as for ESI(–). Both methods consisted of a full scan MS ranging from *m/z* 50–1200, followed by the acquisition of product ion spectra, ranging from *m/z* 50–1000, for the twenty most intensive ions of the survey spectra throughout the chromatographic run (MS/MS) with a collision energy of 35 eV and with a collision energy spread of 20 eV. The total cycle time of MS and MS/MS methods took 0.1 and 0.05 s. The phenolics were quantified by using a six-point calibration curve from prepared external standards ranged from 25 μg/L-1.5 mg/L.

2.3. In vitro colonic fermentation

In vitro colonic fermentation was conducted as described elsewhere (Huang et al., 2021) with some modifications. Three healthy adults (23–29 years old) who declared that they did not smoke and had not consumed antibiotics for 6 months, donated fresh faecal samples. Healthy volunteers gave written consent for a single faecal donation and their anonymity was granted at all times. According to the guidelines of the Medical Ethical Advisory Committee of Wageningen University (METC-WU), this research did not need ethical approval.

The faecal samples were prepared with anaerobic sterilized phosphate buffer (1:20, w/v), which consisted of 8.8 g/L K_2HPO_4, 6.8 g/L KH_2PO_4 and 0.1 g sodium thioglycolate in demi-water as well as the addition of 15 mg/L sodium dithionite before use, in a Stomacher 400 circulator (Seward, UK). The basal medium, containing 5.22 g/L K_2HPO_4, 16.32 g/L KH_2PO_4, 2 g/L $NaHCO_3$, 2 g/L yeast extract, 2 g/L peptone, 1 g/L mucin, and 0.5 g/L L-cysteine HCl, was used. Each treatment was prepared by filling sterilized penicillin bottles with 10 % faecal inoculum, 60 % basal medium and 30 % sterile water with one material from one group of the following samples: digested pellets (2.2.2), defatted digested pellets (2.2.3.1), fiber or phenolics extracts from the digested pellets (2.2.3.3). In the experiment with digested pellets (see also panel A in Fig. 1), the whole of each of the pellets obtained after digestion of the three matrixes was used; the amount of digested pellet was therefore different for the three matrixes, reflecting the *in vitro* digestibility of the corresponding matrix; in the experiments with the defatted digested pellets (see panel B in Fig. 1). To each of the digested, defatted pellet the same amount of extracted sunflower oil (0.20 ± 0.01 g) was added as LA in source because LA is the only PUFA in sunflower oil. Finally, in the experiments with fiber/phenolics (see panel C in Fig. 1), the extracts obtained from the whole of each of the digested and defatted pellet was used. In these experiments, the same amount of extracted sunflower oil (0.20 ± 0.01 g) was added as LA in source. The final volume of each sample was 70 mL and nitrogen was flushed to create an anaerobic condition. Each fermentation was carried

out in triplicate. In addition, one extra sample containing sunflower oil (0.20 ± 0.01 g) was fermented as a positive control. A negative control containing only the standard media with no extra addition was also included. The bottles were shaken at 100 rpm at 37 °C.

The sampling time was set at 8, 24, and 48 h, and the samples were collected with sterile syringes and needles. The collected samples were stored in −20 °C for further analysis. Following collection, pressure measurement of fermentation samples was taken to ensure that no gas exchange occurred with the external environment.

2.4. Extraction and determination of fatty acid from in vitro colonic fermentation

Before the FA extraction, an internal standard (*n*-heptadecanoic acid) was added to the collected samples without any other treatments, and FAs were extracted with a solution (1:2, v/v) of chloroform/methanol (1:1, v/v), according to the procedure of Bligh-Dyer (Bligh & Dyer, 1959). The resulting samples were divided into two parts. The solvent of one part was evaporated and the residue was reconstituted to a final volume of 1 mL 2-propanol/methanol/water (65:30:5) before analysis of lipidomics. Another part was directly injected into the GC-FID for the measurement of selected FAs, including five LA metabolites, that is, 9e,11e-CLA, 9z,11e-CLA, 10e,12z-CLA, 12-hydroxy,9z-C18:1 and vaccenic acid, and five long-chain FFAs, palmitic acid, stearic acid, oleic acid, LA and linolenic acid, with the same column, program and conditions described in 2.2.3.1.

2.5. Untargeted lipidomics analysis

2.5.1. UHPLC-QTOF-HRMS untargeted lipidomics method

The untargeted lipidomics determination followed the method described elsewhere with some modifications (Rubert et al., 2017). Briefly, an UHPLC (LC-40, Shimadzu, Kyoto, Japan) system equipped with BEH C18 (2.1 × 100 mm, 1.7 μm) analytical column and maintained at 60 °C was optimized. The mobile phases consisted of (A) water/methanol (95/5, v/v) with 5 mM ammonium formate and 0.1 % formic acid, and (B) 2-propanol/methanol/water (65/30/5, v/v/v) with 5 mM ammonium formate and 0.1 % formic acid. A multistep elution dual-mode gradient was developed as follows: at 0.0 min (10 % B; 0.30 mL min^{-1}) a gradient began up to 1.0 min (50 % B; 0.4 mL min^{-1}), and a second step was set to 5.0 min (80 % B; 0.3 mL min^{-1}), then the third step 11.0 min reached 100 % B and slightly increased the flow (100 % B; 0.4 mL min^{-1}), subsequently, an isocratic step was executed during four min and a half, 15.5 min (100 % B; 0.40 mL min^{-1}), 15.1 min (10 % B; 0.30 mL min^{-1}) a reconditioning period up to 17.5 min (10 % B; 0.30 mL min^{-1}). The sample injection volume was 5 μL. In order to avoid systematic bias due to analytical variation, all samples were injected under a randomized sequence and six quality control (QC) samples from samples of digested pellets and/or defatted digested pellets were prepared and analyzed at regular intervals through the analysis.

QTOF-MS 9030 (Shimadzu, Kyoto, Japan) was used for microbial metabolic lipid fingerprint. An Electrospray ionization interface (ESI) ion source was applied. The negative ionization mode was used because it can identify more FFAs (Rubert et al., 2017), with the following source ESI (-) settings: nebulizing gas flow 2 L min^{-1}; heating gas pressure 10 L min^{-1}; temperature 300 °C; and capillary voltage −3500 V. The method consisted of a full scan MS ranging from *m/z* 100–1200, followed by the acquisition of product ion spectra, ranging from *m/z* 100–1000, for the twenty most intensive ions of the survey spectra throughout the chromatographic run (MS/MS) with a collision energy of 35 eV with a collision energy spread of 20 eV. The total cycle time of MS and MS/MS methods took 0.1 and 0.05 s.

2.5.2. Lipidomics data mining strategy and analysis

For data analysis of untargeted lipidomics, MS-Dial (version 4.90), MS-Finder (version 3.52) software and MetaBoAnalyst (version 5.0, https://www.metaboanalyst.ca) were employed. Data mining was performed in MS-Dial using an automated algorithm with a retention time range (RT) (0.4–16 min), and peak finding (*m/z* range was 100–1000). Subsequently, RT and *m/z* alignment of the respective peaks was executed using RT and *m/z* tolerances of 0.2 min and 0.01 Da, 0.05 Da for MS1 tolerance and 0.02 Da for MS2 tolerance, respectively. The spots in the network were exported into MS-Finder for structure elucidation. MS1 and MS2 tolerances were set to 0.001 Da and 0.01 Da. The formula finder was exclusively processed with C, H, and O atoms. The potential candidates for each metabolite were obtained by consulting all databases in MS-Finder and then ranked according to their similarity scores, which were calculated based on the comparison between the experimental MS/MS spectra and those in databases. The internal database, that is HMDB (Human), Urine (Human), Saliva (Human), Feces (Human), Serum (Human), CSF (Human), SMPDB (Human), LipidMAPS (lipids), YMDB (Yeast), ECMDB (*E. coli*), BMDB (Bovine), FooDB (Food), PlantCyc (Plant), ChEBI (Biomolecules), T3DB (toxin), STOFF (Enviroment), DrugBank (Drug), STOFF (Environment), BLEXP (blood exposome), NPA (Natural products Atlas), NANPDB (Natural product), COCONUT (Natural products), KNApSAcK (Natural product), PubChem (Biomolecules), and UNPD (Natural products) were checked.

The top 2 ranked candidates of C18-related metabolites were selected and reported as 'Putatively annotated' features and those that matched the retention time, *m/z* and MS spectra of the pure standards were reported as 'Identified' features. The LC-MS data were exported and divided into two matrices, 'identified molecule features' and 'LA related molecule features'. The data were then analyzed by MetaBoAnalyst. Molecular features in at least 50 % of the QCs, with coefficients of variation less than 30 % across the QCs, were selected.

2.6. Statistical analysis

Mean values ± standard error of the mean (SEM) or standard deviation (SD) were used to express the results. Differences in proximate composition of the three matrixes and digested matrixes were evaluated using a one-way ANOVA. Differences in the concentration of fatty acids at each fermentation time were evaluated by two-way repeated-measures analysis of variance (ANOVA) followed by a Tukey post-hoc test with a correction for multiple comparisons using statistical hypothesis testing performed by GraphPad Prism 9.1.0 (GraphPad Software, La Jolla, CA). Lipidomics of samples was analyzed with a Bray–Curtis dissimilarity matrix using the vegan package for R (version 4.3.1). The dissimilarity matrix was then projected with non-metric multidimensional scaling to visualize similarities among samples. Permutational multivariate analysis of variance (PERMANOVA) followed by a Benjamini-Hochberg *p* value adjustment was furthermore conducted to compare differences among lipidomics obtained from each food. A value of $p < 0.05$ was considered statistically significant, unless stated elsewhere.

3. Results

3.1. Composition of the selected matrices before and after in vitro digestion

The content of protein, fiber and lipid in the raw sunflower seeds, soybeans and walnuts samples and their digested pellets are shown in Fig. 2.

After digestion, more walnuts were collected, followed by sunflower seeds and then soybeans (Fig. 2A). The lipid content of the three matrices followed the order of walnuts > sunflower seeds > soybeans but the lipid content of digested walnuts was very close to digested sunflower seeds (Fig. 2B). The total amount of PUFAs (C18:2 and C18:3, in this case) was higher in digested walnuts than in digested sunflower seeds, and the PUFA content in digested walnuts and digested sunflower seeds was much higher than digested soybeans. However, the fiber

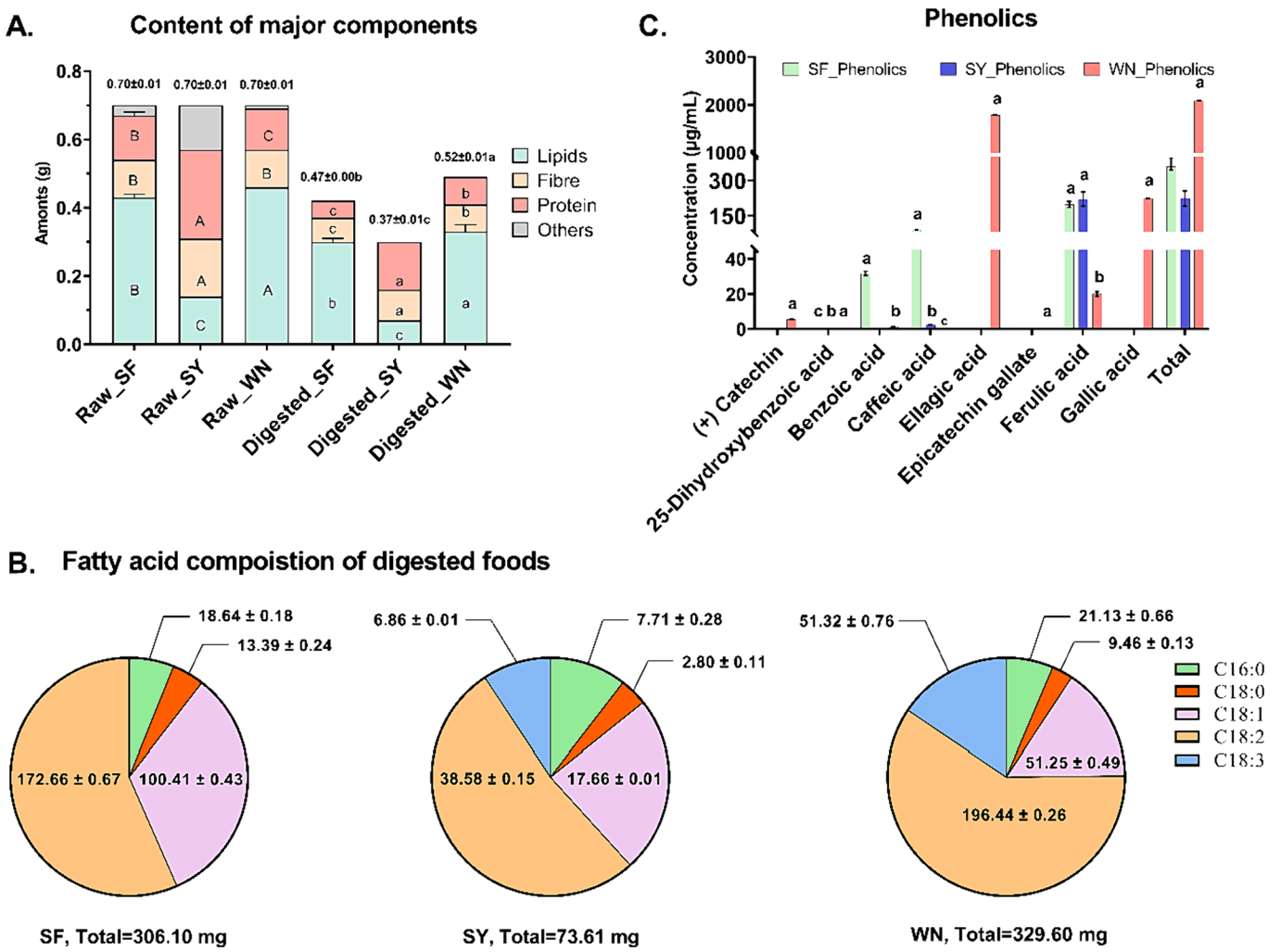


Fig. 2. Characterization of the chemical composition of sunflower seeds, soybeans and walnuts before and after *in vitro* simulated gastrointestinal digestion. (A) content of fibre, protein and lipids of raw and digested foods; (B) content and composition of fatty acid from digested foods (mg); and (C) content and composition of phenolics from digested foods (μg/g of food). Notes: the results are expressed as mean ± SD (n = 3); In panel A, capital letters indicate a significant difference in the content of each component among the food matrixes before digestion, while the lower case letters indicate a significant difference among digested foods ($p < 0.05$, *t*-test or one-way ANOVA followed by a Tukey post-hoc test); In panel C, the lower case letters indicate a significant differences in the content of each phenolic among different food matrices. Abbreviations: SF, sunflower seeds; SY, soybeans; WN, walnuts. The quantity of digested samples was what remained after digestion of 0.70 ± 0.01 g of raw corresponding sample.

content in soybeans was higher than in sunflower seeds and walnuts before and after digestion (Fig. 2A). The absolute fiber content of the digested pellets was lower than the original material. This was ascribed to the soluble fiber solubilized in the digested fluids and removed after centrifugation. Soybeans also contained twice as much proteins than sunflower seeds and walnuts. After digestion, soybeans lost around 50 % of the lipids, fiber and protein compared to walnuts which lost 30 % of those components. Furthermore, walnuts also contained the highest levels of most of the detected phenolics while the content of caffeic and ferulic acids was higher in sunflower seeds and soybeans (Fig. 2C).

3.2. Production of lipid metabolites after in vitro digestion and fermentation of the three matrixes

To compare the level of LA metabolites after *in vitro* digestion and fermentation the three food matrices, the whole of the digested pellets was *in vitro* fermented and ten FFAs, namely palmitic acid, stearic acid, oleic acid, LA, linolenic acid, three CLAs (10e,12z-CLA, 9z,11e-CLA, 9e,11e-CLA), vaccenic acid and 12-hydroxy,9z-C18:1, were monitored and showed in Fig. 3.

The levels of total FFAs and free LA were the highest during the colonic fermentation of digested walnuts, as shown in Fig. S. A1 and A2. *In vitro* colonic fermentation of walnuts also produced the highest levels of the three measured CLAs but produced almost the same amount of 9z,11e-CLA as sunflower seed and soybeans at 24 h when its levels were decreasing. *In vitro* colonic fermentation of sunflower seeds and soybeans produced similar levels of CLAs but the levels of total FFA and free LA were higher in sunflower seeds. The levels of 12-hydroxy,9z-C18:1, the precursor of some CLAs, were the same in the three samples during the fermentation except at 24 h when the amount was the highest during fermentation of soybeans. Vaccenic acid, one of the later products of LA microbial metabolism was found in higher amounts in the fermentation of walnuts and sunflower seeds, and its concentration was not significantly different between walnuts and sunflower seeds.

3.3. Production of lipid metabolites after in vitro fermentation of sunflower oil in the presence of the defatted pellets

To isolate the effect of the food matrix on the production of lipid metabolites (so to remove the effect of different amounts and type of oils in the three matrixes), an experiment was devised where the digested pellets were defatted and added to the same amount of the same oil, i.e.,

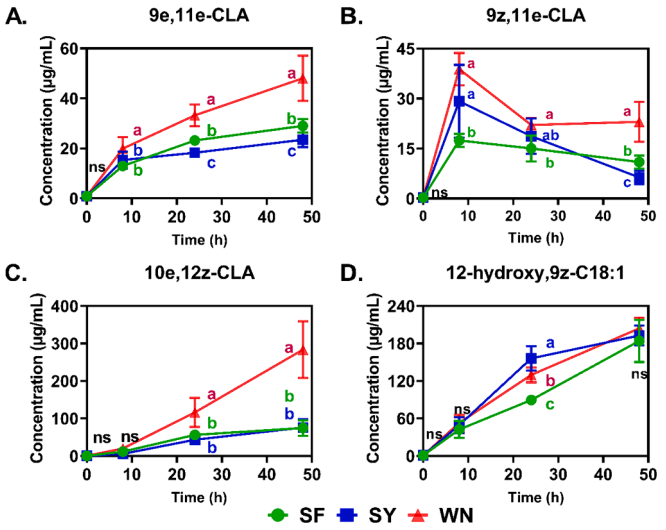


Fig. 3. Concentrations of (A) 9e,11e-CLA, (B) 9z,11e-CLA, (C) 10e,12z-CLA, and (D) 12-hydroxy,9z-C18:1 during fermentation of digested sunflower seeds, soybeans, and walnuts. Different lowercase letters with the corresponding colors to the treatments indicate significant differences in the level of LA metabolites. Notes: CLA, conjugated linoleic acid; SF, sunflower seeds; SY, soybeans; WN, walnuts.

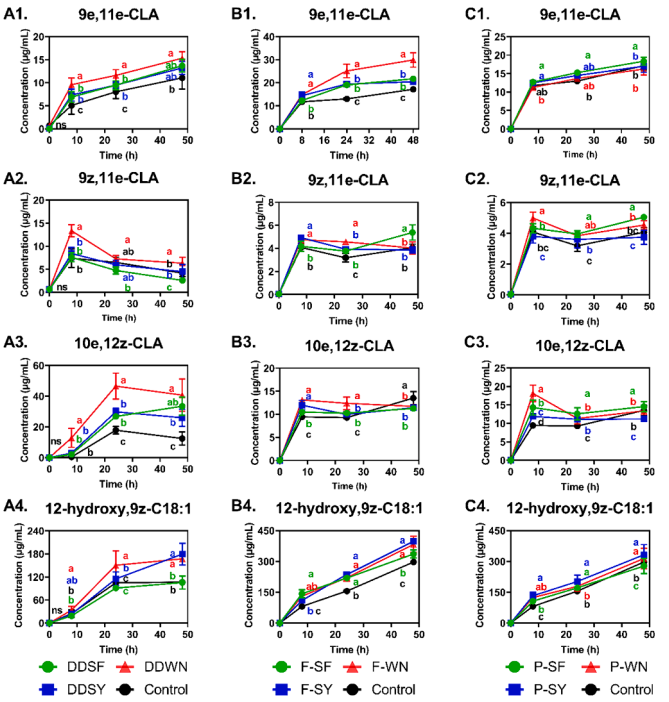


Fig. 4. Concentrations of (1) 9e,11e-CLA, (2) 9z,11e-CLA, (3) 10e,12z-CLA, and (4) 12-hydroxy,9z-C18:1 during fermentation of (A) defatted digested foods (i.e. sunflower seeds, soybeans, and walnuts), (B) fibre extracts and (C) phenolic acid extracts from defatted digested foods (i.e. sunflower seeds, soybeans, and walnuts) defatted digested sunflower seeds, soybeans, and walnuts added to the same amount of sunflower oil. Different lowercase letters with the corresponding colors to the treatments indicate significant differences in the level of LA metabolites. Notes: CLA, conjugated linoleic acid; control, sample with only media and sunflower oil; DDSF, defatted digested sunflower seeds; DDSY, defatted digested soybeans; DDWN, defatted digested walnuts; ns, no significance; F-SF, sunflower seed fiber extracts; F-SY, soybean fiber extracts; F-WN, walnut fiber extracts; negative control, sample with only media; P-SF; sunflower seed phenolics extracts; P-SY, soybean phenolics extracts; P-WN, walnut phenolics extracts.

sunflower oil previously extracted from sunflower seeds. The amount of digested pellets used was not the same but reflected the amounts obtained after *in vitro* digestion of the corresponding matrix (see panel b in Fig. 1 and Fig. 2). By this, the effects of three food matrices on microbial metabolism of lipids were isolated and compared.

As shown in Fig. S1 B1-2, the addition of defatted digested pellets (defatted digested sunflower seed, defatted digested soybean and defatted digested walnut) increased the release of FFA and the production of FA metabolites from sunflower oil compared to the control (where only the sunflower oil was fermented) regardless of the type of defatted digested matrices at most of the fermentation times. Still, the addition of defatted digested walnut to sunflower oil produced the highest levels of total FFAs and LA at most fermentation times, as well as the highest level of CLAs and hydroxy fatty acid (Fig. 4 A1-4). Although the addition of defatted digested soybean produced the same level of 9e,11e-CLA as defatted digested sunflower seed, the level of its precursor, i.e., 12-hydroxy,9z-C18:1, was higher in defatted digested soybean than in defatted digested sunflower seed at 24 and 48 h. Still, the vaccenic acid was produced the higher in sunflower seeds and walnut matrices (Fig. S1 B3).

3.4. Production of lipid metabolites after fermentation of sunflower oil with fibres or phenolics extracted from defatted food matrices

To further understand the differences in LA metabolism observed upon addition of the three defatted digested pellets, fiber or phenolics were isolated from the defatted digested pellets and co-fermented with sunflower oil. The amount of fiber and phenolics added was not the same but reflected the amount of fiber or phenolics obtained from the corresponding amount of each defatted digested matrix from which they were extracted (see panel C in Fig. 1 and Fig. 2). Therefore, according to Fig. 2, digested pellets obtained after digestion of 0.70 g of soybeans and walnuts contained the highest amount of fiber and polyphenols, respectively.

The addition of fiber to sunflower oil increased the levels of total FFAs and LA metabolites at most of the fermentation times, as Fig. S1 C1-2 show. Addition of walnut fiber produced the highest concentration of 9e,11e-CLA after 24 h and the other two CLAs at 24 h (Fig. 4 B1-3), although the amount of fiber extracted by walnuts was lower than from soybeans.

Compared to the addition of fiber, phenolics were less effective in increasing the total FFAs and LA (Fig. S1 D1 and D2), but the levels of 9z,11e-CLA and 10e,12z-CLA (Fig. 4 C2-3) were increased by the addition of the phenolics at 8 h when the levels of these two CLAs started to decline. At such time, the addition of walnut phenolics to sunflower oil produced the highest amounts of the metabolites.

3.5. Identification of LA metabolites by untargeted lipidomics and lipid profiles from the fermentations

Based on the untargeted lipidomics analysis by LC-MS, we identified and putatively annotated some C18-FAs that may be products of microbial LA metabolism from the fermentation of the three digested and defatted digested foods after 48 h, as shown in Table 1. Many FAs with one or several hydroxy functional group(s), such as ricinoleic acid (hydroxy-octadecenoic acid), 9,10-DiHOME (dihydroxy-octadecenoic acid), and 9,12,13-TriHOME (trihydroxy-octadecenoic acid) were identified or putatively annotated. No donor- or matrix-specific metabolites were observed.

NMDS plots were generated to visualize the variations in microbial lipidomics and C18-FA compositions, resulting from the *in vitro* colonic fermentation of the digested pellets or defatted digested pellets (Fig. 5). Despite the same profile of LA microbial metabolites from the three foods, the composition of these microbial metabolites differed among the foods. For example, soybeans produced the highest level of a di-

Table 1
Identified and putatively annotated C18-metabolites related to the microbial linoleic acid metabolism.

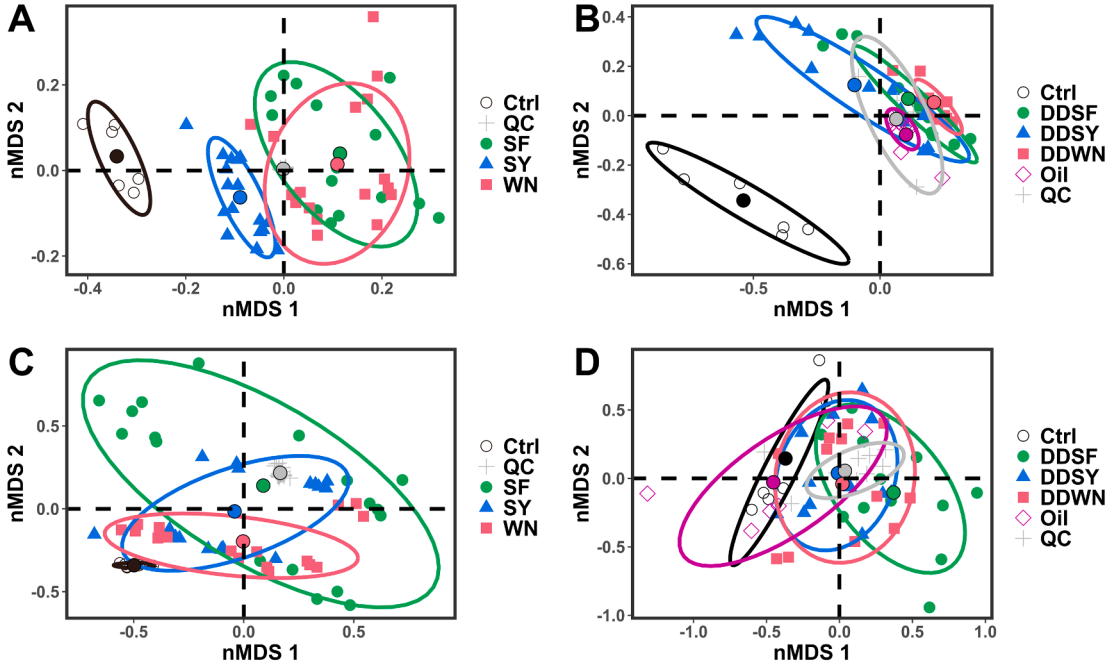
RT/ min	*m/z*	Formula	Adduct type	Common name(s) *
Identified				
7.11	295.23	C18H32O3	[M-H]-	12-OxoOME(9Z)
7.06	297.24	C18H34O3	[M-H]-	Ricinoleic acid
Putatively annotated				
7.30	299.26	C18H36O3	[M-H]-	9 / 10 / 11 / 12-Hydroxy stearic acid**
6.29	311.22	C18H32O4	[M-H]-	(12,13) / (9,10)-DiHODE
6.28	313.24	C18H34O4	[M-H]-	(9,10) / (12,13)-DiHOME
6.55	313.24	C18H34O4	[M-H]-	(9,10) / (12,13)-DiHOME
6.80	315.25	C18H36O4	[M-H]-	(9,10) / (9,12)-dihydroxy stearic acid
5.78	329.23	C18H34O5	[M-H]-	(9,10,13) / (9,12,13)-TriHOME
5.98	331.25	C18H36O5	[M-H]-	(9,10,13) / (9,10,12)-trihydroxy-octadecanoic acid

Note: * top 2 ranked from MS-Finder and Common names are from Human Metabolome Database (hmdb.ca) and LIPIDMAPS (lipidmaps.org); ** the same FAs that achieve the same score;

hydroxy FA (i.e., (9,10)/(9,12)-dihydroxy stearic acid) compared to sunflower seeds and walnuts (Fig. S2). The cluster of soybean samples was differently positioned in the plot compared to those of walnuts and sunflower seeds in the NMDS plots and closer to the cluster of control samples, while the clusters of sunflower seeds and walnuts partly overlapped. Despite this, the PREMANOVA analysis demonstrated significant lipidomics from these two foods. Additionally, the inclusion of defatted digested matrices induced significant alterations in both lipidomics and C18-FA compositions ($p < 0.1$). Specifically, the lipidomics of defatted digested sunflower seed and defatted digested walnut samples displayed significant difference compared to defatted digested soybean, despite the cluster of the three defatted digested pellets are largely overlapped. Although the addition of defatted digested walnut to sunflower oil produced the higher levels of CLAs compared to the other two matrices, defatted digested walnut did not result in different C18-FA composition. Additionally, significant differences in lipidomics were observed among the donors.

4. Discussion

A substantial amount of dietary components escapes digestion and absorption in the small intestine and enters the colon, and these components can be metabolized by the microbial communities residing in the colon resulting in the synthesis of a variety of microbial metabolites. This also includes dietary lipids, despite microbial metabolism of lipids is far less studied than fiber or proteins. In a previous study, we found that the lipid chemical forms (i.e., FFAs versus triglycerides) or the presence of an intact soybean cell wall can modulate the level of LA metabolites produced by the gut microbiota (Huyan et al., 2022). To further investigate how differences in the food matrix properties may affect lipid microbial metabolism in the large intestine, sunflower seeds,



Comparison	SF vs SY	SF vs WN	SY vs WN	SF vs Ctrl	SY vs Ctrl	WN vs Ctrl
Lipidomics	<0.001	0.0019	<0.001	<0.001	<0.001	<0.001
C18_FA	0.0888	0.0248	0.0372	<0.001	0.0011	0.0044

Comparison	DDSF vs DDSY	DDSF vs DDWN	DDSY vs DDWN	DDSF vs Ctrl	DDSY vs Ctrl	DDWN vs Ctrl	DDSF vs Oil	DDSY vs Oil	DDWN vs Oil	Ctrl vs Oil
Lipidomics	0.0192	0.0602	0.0058	<0.001	0.0109	<0.001	<0.001	0.0884	<0.001	<0.001
C18_FA	0.0367	0.2259	0.4268	0.0108	0.0106	0.0887	0.0301	0.0889	0.2338	0.8365

Fig. 5. Non-metric Multi-dimensional Scaling (NMDS) plots based on the level of microbial metabolites after 48 h *in vitro* fermentation: (A) NMDS based on lipidomics profiles from fermentation of digested pellets; (B) NMDS based on the lipidomics profiles from fermentation of defatted digested pellets; (C) NMDS based on level of C18-FAs found in untargeted lipidomics method after fermentation of digested pellets; and (D) NMDS based on level of C18-FAs after fermentation of defatted digested pellets. The tables embedded present the *p* values of each comparison between two treatments using permutational multivariate analysis of variance (PERMANOVA) followed by a Benjamini-Hochberg *p* value adjustment (upper table for digested foods; lower table for defatted digested foods and oils). Abbreviation of sample names: Ctrl, control (sample with only addition of media and faecal sample); SF, digested sunflower seeds; SY, digested soybeans; WN, digested walnuts; DDSF, defatted digested sunflower seeds; DDSY, defatted digested soybeans; DDWN, defatted digested walnuts; Oil, sunflower oil; QC, quality control.

soybeans and walnuts were selected as lipid-rich foods with different nutritional composition, such as the content of proteins, polyphenols, fiber, lipids as well as different lipid unsaturation levels.

The first goal of this study was to determine the impact of the degree of dietary lipid digestion on the level of microbial lipid metabolites in the colon. To reach this goal, we have first subjected the same amount of the three plant-based lipid-rich matrices to an *in vitro* simulated gastrointestinal digestion. Subsequently, we have fermented the residual material (digested pellets) using an *in vitro* model of colonic fermentation with inoculums from three independents donors. As expected, the levels of FFAs, free LA and CLAs after *in vitro* fermentation were highly affected by the amount of lipids remaining in the digested pellets, which followed the order walnuts > sunflower seeds > soybeans. However, the amount of LA released or CLA produced was not always proportional to the amount of lipids present in the digested pellets. Indeed, whereas the soybeans produced the lowest levels of metabolites given the lowest lipid content in the digested pellets, it was also expected the sunflower seeds and walnuts would produce comparable amounts of the metabolites according to the lipid content of their residual pellets. However, walnut samples produced the highest levels of CLAs. This suggests that the amount of undigested lipids has a large effect on the level of LA metabolites in the large intestine but that other factors such as the accessibility and composition of lipids (e.g. FA composition), as well as the content in fiber and other compounds (e.g., metal ions) in the pellets may play a role.

To better understand these factors, we then excluded the effect of lipid accessibility and composition by defatting the digested pellets and fermenting the defatted digested pellets with the same amount of sunflower oil in which LA is the main FA. We observed that the addition of the defatted digested pellets to sunflower oil increased the production of LA metabolites (e.g., 10e,12z-CLA at 48 h) indicating that LA microbial metabolism can be affected by the presence of dietary components other than lipids. Despite the same amount of the same oil was used, walnut defatted digested pellets produced the highest levels of CLAs at most of the fermentation times but the differences in the level of CLAs among the three defatted digested pellets was smaller than the difference produced by the fermentation of the digested pellets. To understand the differences observed in the lipid metabolism in the presence of the three defatted digested matrices, we isolated fiber and phenolics to separately test their effects on lipid microbial metabolism. It is known that fiber sustains the growth of microorganisms while phenolic compounds have a duplibiotic effect, i.e., both antimicrobial and prebiotic effects, on the microorganisms (Rodríguez-Daza et al., 2021; Tomás-Barberán & Espín, 2019). In this case, soybeans had a slightly higher fiber content while the contents of detected phenolics followed the order of walnuts > sunflower seeds > soybeans, and ferulic acid is the main phenolic acid in sunflower seeds and soybeans while ellagic acid and gallic acid are the main phenolic acids in walnut sample. Whereas fiber addition stimulated the production of higher levels of FFAs, addition of those phenolics showed a modest effect on the generation of FFAs. Although the levels of 9e,11e-CLA and 12-hydroxy,9z-C18:1 were much higher upon addition of fiber compared to phenolics, this did not result in higher levels of all measured CLAs. For example, the level of 10e,12z-CLA and 9z,11e-CLA in the sample of walnut and sunflower seed phenolics was higher than that of fiber at 8 h. Hence, the production of some lipid microbial metabolites (e.g., 10e,12z-CLA and 9e,11e-CLA) can be promoted by dietary components, such as fiber and phenolics. The results of this set of experiment may help understanding the differences observed when comparing the production of LA metabolites upon the addition of the three defatted digested matrices to oil. Despite this, this observation of the effects of fiber and phenolics may not be fully physiologically realistic because of the loss of soluble fiber in the preparation of our digested pellet sample.

Further, 12-hydroxy,9z-C18:1 is an early metabolite and the precursor of 9e,11e-CLA (Salsinha et al., 2018), and both sunflower seeds and walnuts showed higher levels of 9e,11e-CLA but lower level of 12-hydroxy,9z-C18:1 than soybeans. From the results, it can be suggested that this pathway, i.e., from the production of 12-hydroxy,9z-C18:1 to its conversion to 9e,11e-CLA, can be promoted by sunflower seed and walnut fiber. The addition of digested sunflower seeds to sunflower oil produced a higher level of vaccenic acid, which is the later product of 9z,11e-CLA (Gorissen et al., 2015; Song & Kennelly, 2003), than soybeans. This suggests a more rapid formation and conversion of the CLA in the presence of sunflower seeds and walnuts compared to soybeans, thereby demonstrating a more active microbial metabolism. Given the data from Fig. 4B and 3C, fiber and phenolics were responsible for this. Moreover, phenolics in three matrices were also responsible for the conversion of 10e,12z-CLA despite differences in phenolic profile and content. Furthermore, the higher levels of total FFAs in the fermentation using defatted digested walnut matrix may also indicate an intense microbial activity, because the differences in the level of FFA release can only depend on the lipases expressed by bacteria and no longer on differences in the content of lipids or LA.

Other than the five monitored LA metabolites, microbial metabolism of lipid can also produce a much larger number of lipid metabolites. In the present study, we report the formation of several di- and tri- hydroxy fatty acids from the lipid microbial metabolism of C18 PUFA(s) by human gut microbiota using an untargeted lipidomics approach. Some of those hydroxy FAs, for instance, 12,13-DiHOME and 9,12,13-TriHOME are identified as oxylipins and can be found as metabolites in human or animals formed from the activity of enzymes, such as cyclooxygenase, lipooxygenase, and cytochrome P450 (Buckner et al., 2021; King et al., 2021). Unlike conjugated fatty acids (i.e., CLAs), the biological effect of those hydroxy fatty acids produced by the metabolism of lipids by gut microbiota is limitedly reported (Devillard et al., 2009). Many of those lipids are important biomarkers in the study of inflammation and are related to human health such as stimulating brown adipose tissue activity which constitutes an appealing target for the treatment of metabolic disorders and increases skeletal muscle fatty acid uptake (Lynes et al., 2017; Stanford et al., 2018). Some lipid bacterial metabolites were also detected in stools of neonates, and 12,13-DiHOME, for example, could lead to PPARγ activation in excess of 50 μM and is likely to associate with impeding immune tolerance (Levan et al., 2019). It is known that CLAs produced in the gut from LA microbial metabolism can be absorbed by epithelial cells but not distributed systemically (Druart et al., 2014; Levan et al., 2019). However, whether those FAs with hydroxy or other function groups would pass the epithelial barrier and then play a systemic effect is still unknown.

Additionally, we employed NMDS plots to visualize differences among the three matrices and the control sample (only lipid and food matrices added) in terms of lipid profiles. The inclusion of each food matrices resulted in significant different C18-FA ($p < 0.1$) and lipidomics ($p < 0.001$) compositions. To further investigate the effects from food matrices on both lipid profiles, we analyzed the lipidomics and C18-FA data from defatted digested matrices fermented with SO. Again, the addition of the defatted digested matrices to the oil resulted in significant change in the lipidomics and C18-FAs ($p < 0.1$) compared to oil alone. Interestingly, the introduction of oil did not lead to significant difference in C18-FA compositions when compared to the control sample. This suggests that the production of FA metabolites from microbial metabolism is strongly associated with the amount/quality of non-lipid components in food matrices. Moreover, although defatted digested walnut exhibited the higher levels of CLAs, it did not change the C18-FA composition compared to defatted digested sunflower seed, defatted digested walnut and sample with only oil added. This further confirms the specific improving effects of dietary fibre and phenolics in defatted digested walnut on the microbial CLA production. Moreover, the lipid profiles of the samples clustered by the donors showed larger differences (Adonis $p < 0.001$, data not shown) compared to the same samples clustered by matrices (Adonis $p < 0.1$). This suggest that diet is a major contributor of variability in lipid profile but interindividual differences might contribute even more.

5. Conclusion

Taken together, our results showed that a higher lipid content in a food matrix and a reduced lipid digestibility would result in higher levels of LA metabolites, such as CLAs and hydroxy FAs. However, our data also suggest that other components of the food matrix, like fiber and phenolics, will also play an important role in formation of LA metabolites by modulating microbial community or activity. Specifically, in relation to the three food matrices studied here we showed that different lipid profiles are produced when the same amount of each matrix is digested and fermented by the gut microbiota and walnuts produced the higher levels of CLAs. Furthermore, we reported, for the first time, various lipid metabolites with multiple hydroxy groups, including mono-, di- and tri-hydroxy FAs as potential microbial metabolites of lipids. Attention to the biological effects of those compounds should be paid because their formation in the gut may result in potential local effects. In the present study, no donor- or matrix-specific LA metabolites were observed. This may be one limitation of current study. The future research should consider more biological replication and diverse dietary compounds in modulating the microbial lipid metabolism by human gut microbiota.

CRediT authorship contribution statement

Zongyao Huyan: Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nicoletta Pellegrini:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Josep Rubert:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Wilma T. Steegenga:** Writing – review & editing, Supervision, Formal analysis, Conceptualization. **Edoardo Capuano:** Writing – review & editing, Supervision, Resources, Project administration, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2024.114230.

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