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In vitro gastrointestinal bioaccessibility and colonic fermentation of lignans from fresh, fermented, and germinated flaxseed†

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This research assessed the influence of fermentation and germination as well as of particle size on lignan bioaccessibility from flaxseed by simulated *in vitro* gastrointestinal digestion. *In vitro* simulated colonic fermentation was used to study lignan release and its conversion into enterolignans. In addition, tea was included as a representative sample to investigate the stability of lignans in the gastrointestinal tract. Only secoisolariciresinol (SECO) was detected in flaxseed samples. SECO bioaccessibility in fermented flaxseed was highest among all matrices but limited to ≈1% ($P < 0.001$). Lignan bioaccessibility was significantly influenced by particle size too ($P < 0.001$ for both). In the colon, fermented flaxseed produced the highest SECO release among all flaxseed samples (≈65%), and the highest conversion of enterolignan (≈1.0%), whereas the conversion of lignans in tea brew was relatively high (≈15%). Lignan conversion varies greatly among donors due to inter-individual differences in microbiota activity. Food fermentation could be a viable strategy for increasing lignan release and conversion to enterolignan.

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Introduction

Lignans are phenolic compounds that belong to the class of non-flavonoid polyphenols that can exert both oestrogenic and anti-oestrogenic effects.[1] Some lignans can be metabolized into enterolignans, *i.e.* enterodiol and enterolactone, by the human intestinal microbiota[2] through deglycosylation, oxidation, and demethylation.[3] These metabolites have been reported to have beneficial health effects. Epidemiologic studies have shown that the intake of lignan-rich foods aids in lowering the risk of several cancers[4] and cardiovascular diseases,[5] demonstrating their antioxidant properties as well as estrogenic activity.[6] Differences in microbiota composition may lead to differences in the absorption and excretion patterns of enterolignans.[2]

It is widely known that not all of the constituents in a food matrix are fully bioavailable. The fraction of a nutrient or non-nutrient available to the human body for physiological functions and/or storage is known as bioavailability.[7] As a result, investigating the bioavailability of a food component is essential to predict its biological activity.[8]

Food processing affects the lignan content of foods[9] and the levels and bioavailability of bioactive compounds.[10] Reducing the particle size of plant tissue, for instance, increases the fraction of broken cells thus increasing the fraction of potentially bioavailable intracellular compounds.[11] However, most lignans, similar to phenolic compounds in general, are covalently or physically bound to plant cell walls and thus have low bioavailbility.[12] As a result, enzymatic treatments and fermentation processes targeting bran structure have been investigated to improve the bioavailability of lignans.[13] In addition, germination and fermentation are also reported to affect lignan content,[14,15] but how these processing methods affect the bioavailability of lignans is unknown.

Since animal and human studies are time-consuming and expensive and may raise ethical concerns, *invitro* approaches that predict the behaviour of phytochemicals during GI digestion have been developed. This approach enables the screening of a relatively large number of samples and/or conditions to study the separate and combined effects of each phase of digestion on the release and availability of phytochemicals, which would be impossible with *in vivo* approaches.[8] Bioaccessibility is the fraction of a potentially available compound released from its matrix in the gastrointestinal (GI)

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tract and thus becomes available for intestinal absoption.[16] Furthermore, it has been demonstrated that bioaccessibility as determined using *in vitro* models can be well associated with bioavailability determined in human studies and animal models.[17]

To date, there are relatively few reports on the effect of various processing techniques on the bioaccessibility of lignans from food. Thus, the present study investigated lignan bioaccessibility from fresh, fermented, and germinated flaxseed, and the effect of particle size using simulated *in vitro* digestion models. In addition, tea was used to study the stability of lignans under *in vitro* simulated gastrointestinal conditions. Moreover, the release of lignans and their conversion into enterolignans was investigated through *in vitro* colonic fermentation with human fecal inoculums.

Materials and methods

Chemicals and reagents

Porcine pepsin (P6887), porcine pancreatin (P1750, 4× USP), and porcine bile salt preparation (B8631) were purchased from Sigma-Aldrich (Merck KGaA, Germany). KCl, KH_2PO_4, NaCl, $MgCl_2{\cdot}(H_2O)_6$, $CaCl_2{\cdot}(H_2O)_2$, and pure ethanol were purchased from VWR International B.V. (Netherlands). KH_2PO_4, NaCl, $(NH_4)_2CO_3$, $NaHCO_3$, NaOH, HCl, and Tween 80 were purchased from Sigma-Aldrich Chemie B.V. (Netherlands), as well as yeast extract, peptone, mucine, and L-cysteine HCl.

Acetonitrile, methanol, sodium acetate, sodium hydroxide, glacial acetic acid, formic acid, pure standards of pinoresinol (PINO), matairesinol (MATA), secoisolariciresinol (SECO), enterolactone (EL), enterodiol (END) and glycocholic acid (glycine-1 ^{13}C, 13C-GA) were purchased from Sigma-Aldrich Chemie NV (Zwijndrecht, Netherlands). For enzymatic hydrolysis, β-glucuronidase with an activity of ≥300 000 units per g of solid and a sulfatase activity of ≥10 000 units per g of solid type H-1 from *Helix pomatia* were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). The *Rhizopus oryzae* fungus for the fermentation of flaxseed, mung beans, and soybeans was obtained from Startercultures.eu/starterculturen.nl. All of the chemicals used were of analytical and chromatographic grade.

Preparation of samples

Preparation of fermented flaxseed. Flaxseed was fermented following a procedure previously described with some modifications.[18] Flaxseed produces mucilage, a gel-like layer formed when seeds are soaked in water. Water causes the polysaccharide in the epidermal cell layer of the seed coat to become viscous.[19] The mucilage halts the formation of a compact white mycelium. Therefore, mung beans were used as a supplementary ingredient with a ratio of 1 : 1 to form a white layer of compact mycelium around the beans and bind them into a dense, firm cake. Mung beans were rinsed and soaked in water for 12 h, then were dehulled, split in half, and boiled in demineralized water (1 : 2, w/v) for 30 minutes. Subsequently, the flaxseed was rinsed, drained, and boiled for 3 minutes. Then, flaxseed and mung beans were mixed (1 : 1, w/w) to surround the beans with a white layer of compact mycelium. The sample was allowed to cool to below 36 °C and transferred to a container with vinegar (20 mL). Next, the samples were inoculated with the fungi *Rhizopus oryzea* at the level of 0.1% (w/w) of boiled samples. Finally, the samples were placed in perforated polyethylene bags and incubated for 24–48 hours at 25–30 °C, or until a compact white mycelium formed.

Preparation of germinated flaxseed. Flaxseed was soaked in water for 1 h. The seeds were placed in a container (60 × 150 mm), 20 g per cup, and kept in the dark and at room temperature (RT) for germination. The sprouts were rinsed with water every 12 hours to prevent microbial growth. The germination process took 6 days.

Preparation of tea brews. To make the tea brew, one tea bag was placed in boiling tap water (100 mL per g of tea leaves). After infusing the tea for 10 minutes, the various samples were allowed to cool before 50 mL of each sample was filtered through a 1.2 m Acrodisc filter (Gelman Sciences, Ann Arbor, USA). The samples were kept at −20 °C until analysis.

Sample milling. The collected fresh, fermented, and germinated flaxseed were milled to obtain coarse (>2.5 mm mesh per sieve) and fine (<0.5 mm mesh per sieve) fractions, freeze-dried and stored at −20 °C until further analysis.

Simulated *in vitro* gastrointestinal digestion

All samples were digested using a static *in vitro* digestion system consisting of a simulated oral, gastric, and intestinal phase, with modifications.[20,21] The compositions (%, w/w) of the simulated salivary fluid (SSF), simulated gastric fluid (SGF, pH 3.0 ± 0.05), and simulated intestinal fluid (SIF, pH 7.0 ± 0.05) were as reported.[20]

For the oral phase, flaxseed samples (5 g) or tea brew (5 mL) were mixed with the SSF electrolyte stock solution (3.5 mL, 37 °C), $CaCl_2$ (0.025 mL, 0.3 M), and Milli-Q water (1.475 mL) (Veolia water, Veolia Water Solutions, and Technologies Netherlands B.V.). The final volume was 10 mL.

Subsequently, an oral bolus (10 mL) was mixed with SGF (7.5 mL, 37 °C) to start the gastric phase. The pH was adjusted to 3 with HCl (0.2 mL, 1 M). Then, $CaCl_2$ (0.005 mL, 0.3 M) was added. Finally, Milli-Q water was added to obtain the final 20 mL (approximately 0.698 mL). Porcine pepsin stock solutions (1.6 mL, 25 000 units per mL, 37 °C) (2000 units per mL in final chyme) was added the SGF. Then, the chyme was shaken for 2 h (37 °C). At the end of the gastric digestion step, NaOH (0.15 mL, 1 M) was added to adjust the SGF to pH 7.0, inhibiting the gastric enzyme activity.

Thereafter, NaOH (0.15 mL, 1 M) was added to 20 mL of gastric chyme to reach pH 7.0. Subsequently, SIF (11 mL, 37 °C), fresh bile stock solution (2.5 mL, to reach a final con-

centration of 10 mM in the intestinal chyme), and $CaCl_2$ (0.04 mL, 0.3 M) were added. Then, Milli-Q water (1.31 mL) was added. Finally, pancreatin solution (5 mL, 1600 U mL^{-1} amylase activity) was added to reach a final volume of 40 mL. At the end of the intestinal step, samples were incubated for 2 hours (37 °C) on a rotating device.

In parallel, control samples with no digestive enzymes added were tested to see if these enzymes affect lignan bioaccessibility. Milli-Q water was added to replace the enzyme solutions. All gastric and intestinal digests were centrifuged (4 °C, 20 000*g*, 10 minutes) to stop the enzymatic reaction. Supernatants (5 mL) were collected and filtered through a 0.22 μm membrane filter (Phenomenex, Netherlands) for lignan analysis. Then, the remaining pre-digested samples were freeze-dried for further use in *in vitro* colonic fermentation.

$$\text{Release of SECO}(\%) = \frac{\text{SECO in the supernatant after colonic fermentation, } \mu g}{\text{SECO in pre-digested supernatantafter gastrointestinal digestion, } \mu g} \times 100$$

$$\text{Conversion yield}(\%) = \frac{\text{Enterolignans in the supernatant after colonic fermentation, } \mu g}{\text{Lignans in pre-digested supernatantafter gastrointestinal digestion, } \mu g} \times 100$$

The bioaccessibility of lignans was defined as the content of lignans that was detected in the supernatants after digestion, divided by the total lignans initially present in the samples before digestion:

$$\text{Bioaccessibility}(\%) = \frac{\text{Lignans in the supernatant after digestion, } \mu g}{\text{Lignans in the samples before digestion, } \mu g} \times 100$$

In vitro colonic fermentation

Fresh fecal samples were donated by three healthy adults (one Malay and two Dutch, aged 25–40 years old, with a body mass index (BMI) of 18.5–23.9), who declared no smoking and no antibiotic consumption for 6 months before the beginning of the study. The fecal samples were prepared according to protocols previously reported, with modifications.[22,23] Briefly, feces (20.0 g) were dissolved in anaerobic phosphate buffer (100 mL) before homogenization in a stomacher bag. The phosphate buffer contained 8.8 g L^{-1} K_2HPO_4, 6.8 g L^{-1} of KH_2PO_4, and 0.1 g L^{-1} of sodium thioglucolate. The resulting fecal suspension was filtered and considered to be the fecal microbiota supernatant. The colon medium consisted of 5.22 g L^{-1} K_2HPO_4, 16.32 g L^{-1} KH_2PO_4, 2.0 g L^{-1} $NaHCO_3$, 2.0 g L^{-1} yeast extract, 2.0 g L^{-1} peptone, 1.0 g L^{-1} mucine, 0.5 g L^{-1} L-cysteine HCl, and 2.0 mL L^{-1} Tween 80. Both phosphate buffer and colon medium were flushed with nitrogen (30 minutes) before autoclaving.

Fecal supernatant (7 mL) was added to a penicillin bottle containing a buffered colon medium (43 mL). Then, pre-digested fresh, fermented, or germinated flaxseed (1 g) or tea sample (10 mL) was added, and sterile water was added to make up the mixture of fecal supernatant and the colon medium to 70 mL. The fermentation started with continuous shaking (60 rpm, 37 °C) under anaerobic conditions. In parallel, one control without the fecal supernatant was included to determine the release of lignans from the food matrix. All incubations were duplicated, and supernatants were collected at time intervals (2, 8, and 24 h). All supernatants were centrifuged immediately when sampled and stored at −20 °C until further use. For the *in vitro* colonic fermentation, only the pre-digested samples with a fine particle size were used. Two replicates were performed using two independent samples.

The release of SECO was defined as the content of SECO detected in the supernatants after fermentation, divided by the total SECO present in the pre-digested samples after *in vitro* gastrointestinal digestion. Then, the conversion yield was defined as the content of enterolignans that was detected in the supernatants after colonic fermentation, divided by the total lignans initially present in the pre-digested samples after *in vitro* gastrointestinal digestion:

Lignan extraction

Lignans were extracted by alkaline hydrolysis as suggested by Peñalvo *et al.*, Milder *et al.*, and Nørskov *et al.* with slight modifications.[24–26] In brief, 20 mg of coarse and fine fresh, fermented, and germinated flaxseed were extracted under alkaline conditions (1 mL, 0.3 M NaOH in methanol/water, 8/2, v/v) at 60 °C for 1 h. Samples were centrifuged, and the supernatants were transferred to plastic tubes and evaporated under N_2 flow at 60 °C. The extracts from pre-digestion and *in vitro* fermentation treatments were subjected to hydrolysis by adding to them 0.6 mL of β-glucuronidase/sulfatase (2 mg mL^{-1} in 50 mM sodium acetate buffer, pH 5) and also to brew tea (1 mL, without alkaline extraction) using a vortex incubator (37 °C, 16 h). The hydrolysis was stopped by adding formic acid (0.5 mL, 0.4%), and the extracts were centrifuged. Subsequently, 300 μL of acetonitrile was added, and the samples were transferred to a HPLC vial for LC-MS/MS analysis.

LC-MS/MS analysis of lignans

A Nexera UPLC system (Shimadzu Corporation, Kyoto, Japan) was used, coupled with a LCMS-8050 triple quadrupole mass spectrometer (Shimadzu Corporation, Kyoto, Japan). 5 μL of sample was injected into an Acquity Premier BEH C18 column, 1.7 μm, 2.1 × 100 mm BEH (Waters Chromatography B.V., 4879 AH Etten-Leur, the Netherlands). The flow rate was set at 0.3 mL min^{-1} and the column temperature at 40 °C. The mobile phases consisted of 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) with the following elution profile (*t* in [min]/[%B]): (0.0/15), (9.0/75), (11.0/75), (13.0/15), (18.0/15). MS data were collected for 18 minutes.

All analytes were analyzed in positive ionisation mode. The turbo ion-spray ionization voltage was 4.0 kV. The electrospray ionization probe, desolvation line, and heat block were all set to 300, 250, and 400 degrees Celsius, respectively. The collision-induced dissociation gas had a pressure of 4 kPa, and the flow rates of the drying gas, nebulizer gas, and heating gas were set at 10 mL min^{-1}, 3 mL min^{-1}, and 10 mL min^{-1}, respectively. The electrode voltage of Q1 pre-bias (collision cell energy entrance potential), collision cell Q2 (collision energy), Q3 pre-bias (collision cell energy exit potential), and parent and fragment ion *m*/*z* of the multiple reaction monitoring transitions were optimized using support software (Shimadzu Corporation, Kyoto, Japan). The dwell time ranged from 11 ms to 16 ms for the analytes for single reaction monitoring (SRM). The most abundant fragment ions for SECO, 13C-GA, PINO, MATA, EL, and ENT were 363.2 → 137.05 (collision energy: −24 V), 467.20 → 413.3 (collision energy: −18 V), 359.2 → 341.15 (collision energy: −9 V), 359.2 → 137.10 (collision energy: −27 V), 299.2 → 133.0 (collision energy: −16 V), and 303.0 → 107.0 (collision energy: −25 V), respectively, and therefore were selected for quantitation. The representative chromatograms of fresh flaxseed, the intestinal phase of simulated *in vitro* digestion, and 2 h and 24 h of *in vitro* simulated colonic fermentation of fresh flaxseed are provided in Fig. S1.†

Method validation

Calibration curves. All standards were dissolved in 100% acetonitrile and maintained at −20 °C. A working solution (50 μg mL^{-1}) consisting of all the lignans and ^{13}C-GA in water/acetonitrile (1 : 1, v/v) was prepared on the day of the analysis and used for the preparation of the calibration curves (12.5–2500 ng mL^{-1}).

Matrix effect. The matrix effect (ME) was calculated as an indicator of ionization suppression or enhancement. The known amounts of lignan standard solution (10 ul, 2.5 mg L^{-1}) were spiked with tea and digested supernatants (method without extraction), pre-digested tea, and fresh, germinated, and fermented flaxseed (total method). For pre-digested fresh, germinated, and fermented flaxseed, the standards were added immediately after the extraction solvent and for tea and digested supernatants, immediately before the addition of β-glucuronidase/sulfatase was added. SECO, PINO, MATA, and enterolactone recoveries were relatively high (111–134%). The results showed that the extraction method effectively extracts the analytes from all samples.

Statistical analysis

Data of bioaccessibility are presented as mean ± standard deviation (SD) from duplicate analyses of two independent digestions. Data for release of SECO during *in vitro* colonic fermentation, and conversion into enterolignans, are averages from 3 donors. Each *in vitro* fermentation was performed in duplicate. One-way analysis of variance (ANOVA) was used to assess the significant differences in the bioaccessibility of fresh, fermented, and germinated flaxseed and tea brew among different digestion phases. An additional ANOVA was used to test the differences in bioaccessibility values obtained with or without the inclusion of digestive enzymes in the simulated *in vitro* digestion. Two-way ANOVA followed by Tukey's HSD multiple comparison tests were used to test the effect of the type of processing (fresh, fermented and germinated flaxseed), particle size (fine and coarse) and their interaction on lignan bioaccessibility. Pearson's correlation was used to identify the correlation between the amount of SECO released and its conversion to EL by correlating data for each donor at all fermentation times. Then, a repeated ANOVA measurement followed by Tukey's HSD multiple comparison *post-hoc* tests were applied to test the difference in SECO release and conversion yield into enterolignans during *in vitro* colonic fermentation of fresh, germinated and fermented flaxseed and tea brew among the 3 donors. An additional repeated ANOVA measurement followed by Tukey's HSD multiple comparison *post-hoc* tests were used to test the differences in SECO release and conversion yield into enterolignans of fresh, germinated, and fermented flaxseed and tea brew within three time points. *P*-values <0.05 were regarded as statistically significant. Statistical analyses were performed using the IBM SPSS Statistics version 25 software (IBM Corp).

Results and discussion

Effect of processing and particle size on lignan bioaccessibility during simulated *in vitro* gastro-intestinal digestion of flaxseed matrixes

In the present research, the effects of fermentation and germination as well as particle size reduction on lignan bioaccessibility were assessed using simulated *in vitro* gastrointestinal digestion. In addition to the flaxseed matrixes, tea brew was included as a representative sample to study the stability of lignans in the gastrointestinal environment and their interactions with digestive fluids. SECO was detected in fresh, germinated, and fermented flaxseed. Only PINO and MATA were found in tea brew (Table 1). The SECO content was the highest in germinated flaxseed, with values of 15.6 ± 1.1 mg per g dry weight (DW) and 14.6 ± 0.6 mg per g DW in fine and coarse particles, respectively. Fermented flaxseed showed the second highest content of SECO with values of 11.1 ± 2.1 mg per g DW and 9.8 ± 0.6 mg per g DW in fine and coarse particles, respectively. Fresh flaxseed has the lowest SECO content with values of 3.0 ± 0.5 mg per g DW and 2.1 ± 0.4 mg per g DW in fine and coarse particles, respectively.

As a general observation, bioaccessibility of lignans from the flaxseed food matrixes was very low (1% and less). Our findings are in accordance with a study on flaxseed that showed no lignans were released during *in vitro* simulated gastrointestinal digestion.[27]

Both the type of processing and particle size significantly affected the bioaccessibility of SECO ($P < 0.001$ for both). No interaction between particle size and samples was observed ($P = 0.142$). The bioaccessibility was highest in fermented flaxseed, reaching around 1.03%, while it was 0.87% and 0.67% in

Table 1 Bioaccessibility (%) of lignans during simulated *in vitro* gastrointestinal digestion of fine and coarse particles of fresh, germinated, and fermented flaxseed and tea brew (mean ± SD, n = 2)

Fine*			Coarse		
Oral	Gastric	Intestinal	Oral	Gastric	Intestinal
Flaxseed (SECO)[a]					
0.67 ± 0.07^{A}	0.72 ± 0.05^{A}	0.87 ± 0.09^{A}	0.61 ± 0.12^{A}	0.69 ± 0.08^{A}	0.67 ± 0.02^{A}
Germinated flaxseed (SECO)[b]					
0.47 ± 0.10^{A}	0.52 ± 0.05^{A}	0.55 ± 0.04^{A}	0.37 ± 0.05^{A}	0.50 ± 0.04^{A}	0.53 ± 0.04^{A}
Fermented flaxseed (SECO)[ac]					
0.70 ± 0.06^{A}	0.72 ± 0.01^{A}	1.03 ± 0.04^{B}	0.63 ± 0.06^{A}	0.69 ± 0.08^{A}	1.00 ± 0.22^{B}

Oral	Gastric	Intestinal
Tea brew (PINO and MATA)		
88.5 ± 8.42^{A}	67.0 ± 3.51^{B}	24.4 ± 4.15^{C}

Different lower case letters indicate within samples (fresh, germinated, and fermented flaxseed); upper case letters indicate within the digestion phase. * Within this particle size a significant difference in bioaccessibility (P < 0.05) is indicated. SECO = secoisolariciresinol; PINO = pinoresinol; MATA = matairesinol.

fine and coarse particles of fresh flaxseed, respectively. Germinated flaxseed presented the lowest bioaccessibility of SECO (0.5%) for both particle sizes.

Fresh flaxseed showed a significant difference in SECO bioaccessibility between the digestion phases in fine and coarse particles, P = 0.047 and P = 0.002, respectively. Similarly, fermented flaxseed showed a significant difference in SECO bioaccessibility among digestion phases in fine and coarse particles, P = 0.019 and P = 0.024, respectively. However, in germinated flaxseed, coarse size showed a significant difference in SECO bioaccessibility between the digestion phases (P = 0.017) but not for fine size (P = 0.066).

In tea brew, the lignan content was 330.5 ± 29.7 ng mL^{-1}. In tea brew, the bioaccessibility of lignans was about 88% after the oral phase and decreased to approximately 70% after the gastric phase (P = 0.04), and further decreased to around 25% after the intestinal phase.

Furthermore, the addition of digestion enzymes did not significantly affect the bioaccessibility of SECO in flaxseed samples for fine (P = 0.640) and coarse sizes (P = 0.261). Similar to flaxseed samples, digestion enzymes did not significantly affect the bioaccessibility of MATA and PINO in tea brew (P = 0.062).

Our finding is in line with previous research that showed that milling and crushing of flaxseed, *i.e.* decreasing the particle size, will enhance bioavailability.[28] To the best of our knowledge, no data are available on the bioaccessibility of lignans during fermentation using *Rhizopus oyrzae*. Fungi of the genus *Rhizopus* have been traditionally used in food fermentation, specifically of tempeh in South-East Asia including Malaysia, for centuries. *Oryzae* is considered to be GRAS by the FDA but not by the EFSA, which mostly raises an issue related to mycotoxin presence.[29] The chance that the fungus may survive the passage through the gastrointestinal tract, as well as colonize and thrive in the ecology of the large intestine is negligible. Nevertheless, a study on fermented raw pumpkin oil cake proved an effective release of bound phenolics did occur, which may indicate an increase in bioaccessibility.[30] Likewise, a study on fermented rice bran with *Rhizopus oryzae* reported an increased phenolic content compared to the unfermented samples[31] by releasing insoluble bound phenolic compounds *via* the activity of exogenous enzymes.[32]

To the best of our knowledge, there are no published data on the bioaccessibility of lignans in germinated food. However, one study has shown that the content and bioavailability of phenolic compounds increased in germinated compared to non-germinated grains.[33] This would be due to the activity of endogenous enzymes releasing bound phenolic compounds, which increased bioaccessibility.[34]

The particle size of foods affects the bioaccessibility of phytochemicals. A study showed that the bioaccessibility of the antioxidant and total phenolic content of carob flour improved with a reduction of the particle size.[35] Similarly, another study proved that the reduction in particle size in wheat bran increased ferulic acid bioaccessibility.[36] Grinding breaks down plant cells, increasing the yield and bioaccessibility.[37]

All in all, our findings indicate that the SECO bioaccessibility from the food matrix may be altered according to the way foods are processed.

The results show here for the first time that lignans are unstable under simulated gastrointestinal conditions by using tea brew. Other studies have reported that polyphenols may be unstable under simulated *in vitro* gastrointestinal digestion, with only 20% remaining intact at the end of the intestinal phase.[38] Degradation, epimerization, hydrolysis, and oxidation of polyphenols are significant transformations during digestion, particularly after the transition from the gastric to the intestinal environment.[39] Furthermore, a study speculated that the presence of bile salt in the intestinal phase would reduce campesterol, β-sitosterol, and sitosterol in beverages due to reduced solubility of sterols,[40] which may have impacted the bioaccessibility of lignans as well. However, the observation that lignans could be degraded during gastric and intestinal digestion is relevant when designing strategies to optimize the health effect of lignans.

We also calculated the net amount of parent lignans that would be available to our body when the simultaneous effect of fermentation and germination on content and bioaccessibility is considered. The amount of SECO available for absorption was highest in fine and coarse fermented flaxseed, with values of 113 μg per g DW and 98 μg per g DW, respectively. Germinated flaxseed showed the second highest SECO content in the intestinal phase with values of 85.8 μg per g DW and 77.4 μg per g DW in fine and coarse sizes, respectively. Then, fresh flaxseed has the lowest SECO content in the intestinal phase, with values of 26.1 μg per g DW and 14.1 μg per g DW in fine and coarse sizes, respectively.

From the findings mentioned above, it was concluded that fermentation and germination could increase the amount of circulating lignans. During fermentation, the endogenous and bacterial enzymes could modify grain constituents, thus affecting the absorption and bioavailability of bioactive compounds.[41] Meanwhile, during germination, the soaking process activated the enzymes to break down the active compounds.[42] Fermentation and germination are relatively simple technologies yet they are widely used to increase the nutrient density in bioactive compounds by releasing the bound fraction in the food matrix, thereby increasing the bioaccessibility.[43]

Lignans released from the food matrix during *in vitro* colonic fermentation

Next, the release of lignans in the *in vitro* colonic fermentation phase was further investigated. Only pre-digested fine particles were used since this particle size showed a higher bioaccessibility than the coarse size. The release of SECO from fine particles of fresh, germinated, and fermented flaxseed during *in vitro* colonic fermentation is shown in Fig. 1. A control was added, where the flaxseed samples were dissolved in the fermentation medium without fecal inoculum.

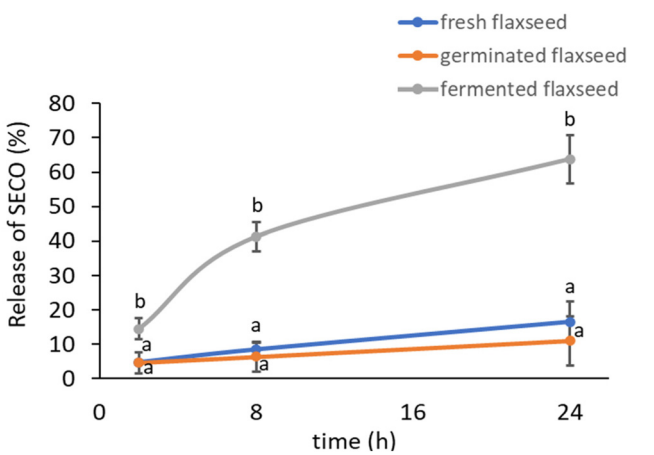

Fig. 1 Release of SECO (% of the pre-digested samples) in the supernatant during *in vitro* colon fermentation of fine particles of fresh, germinated, and fermented flaxseed. Data are from 3 donors (mean ± SD, n = 3). Different lower case letters within samples indicate a significant difference in the % SECO released during three time points (2, 8, 24 h) in the supernatant ($P < 0.05$). SECO = secoisolariciresinol.

Notably, the SECO can be already detected in the supernatant after 2 hours of fermentation. A significant increase was found in SECO released in fermented flaxseed from 2 to 24 hours of fermentation. The SECO released was significantly increased from 8 to 24 h in flaxseed and germinated flaxseed. After 24 hours of *in vitro* fermentation, fermented flaxseed produced the highest SECO release among all samples, approximately 65%, showing that the metabolic activity of the fungus *Rhizopus oryzae* may help the release of lignans in the colon. The SECO released was comparable between fresh and germinated flaxseed (approximately 10%). However, a significantly lower percentage of SECO was released in the control (approximately 2.5%). This clearly demonstrates that microbiota activity in the large intestine aids in releasing SECO from the plant matrix.

Lignan conversion to enterolignan during *in vitro* colonic fermentation

In the present study, the conversion of lignans to enterolignans was also investigated. Only enterolactone (EL) was detected in the samples after colonic fermentation, probably because of the rapid dehydrogenation of END into EL.[44] SECO conversion to EL during *in vitro* colon fermentation of fresh, germinated, and fermented flaxseed is shown in Fig. 2. The conversion of EL was significantly higher in fermented flaxseed at all times, ranging from 0.5% to 1.0%. *In vitro* colonic fermentation of fresh and germinated flaxseed produced a lower conversion to EL of approximately 0.1% after 24 hours of fermentation, with no significant difference between the samples.

The fermentation time did not significantly affect the conversion of SECO in EL in fresh ($P = 0.881$) and germinated flaxseed ($P = 0.801$), while the production of EL was significantly increased from 8 to 24 hours of fermentation in the fermented flaxseed ($P = 0.024$). As expected, no EL was detected in the control samples because microbiota are necessary for the conversion of SECO to EL.

Furthermore, the correlation between SECO release and conversion to EL in fresh, germinated and fermented flaxseed at 24 h of fermentation was evaluated. SECO release in all flaxseed samples was significantly positively correlated with EL conversion at 24 h of fermentation ($r = 0.999$, $P < 0.029$). It was concluded that the more lignans released, the higher the conversion to EL because more substrate is available.

The net amount of EL available to our body when the simultaneous effect of fermentation and germination on content and conversion of SECO is considered was calculated. The EL content produced after colonic fermentation was by far at its highest in fermented flaxseed, with a value of 116.6 μg per g DW, followed by germinated flaxseed with a value of 10.9 μg per g DW. The EL production in fresh flaxseed had its lowest value of 4.0 μg per g DW. Fermented flaxseed had an approximately 29-fold difference compared to fresh flaxseed. In addition, the germination of flaxseed would substantially

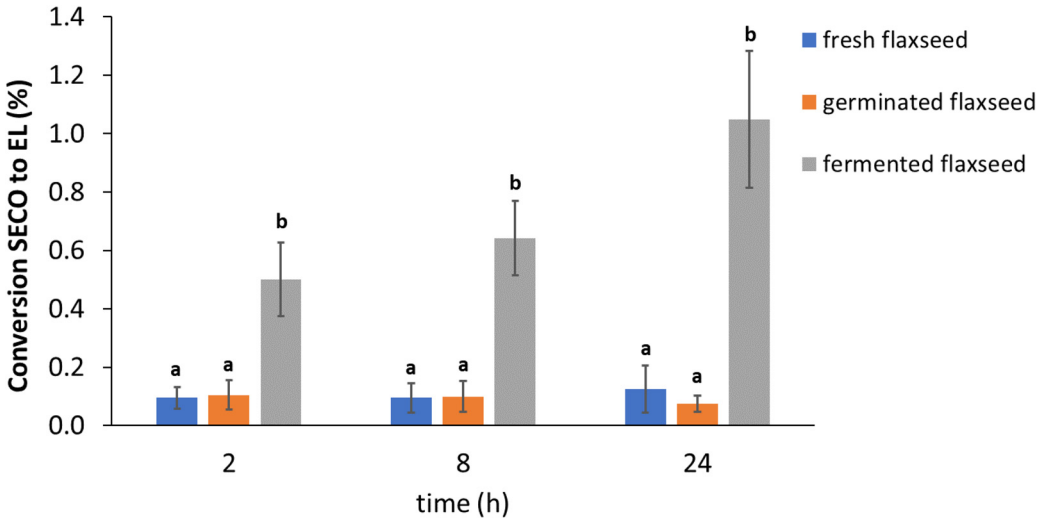

Fig. 2 Lignan (SECO) conversion to EL during *in vitro* colon fermentation of fresh, germinated, and fermented flaxseed. Data are from 3 donors (mean ± SD, n = 3). Different lower case letters within samples indicate a significant difference in the SECO conversion during three time points (2, 8, 24 h) in the supernatant (P < 0.05). SECO = secoisolariciresinol; EL = enterolactone.

increase EL production with a nearly 3-fold difference compared to fresh flaxseed. According to the aforementioned results, it was concluded that food fermentation and germination could significantly enhance EL production from flaxseed.

The inter-individual differences were looked into further and reported data for the single donors are shown in Fig. 3. Donor 3 had the highest EL conversion rate of all the samples, around 1.5% in fermented flaxseed. Nearly a 5-fold difference in EL production was observed in fresh and fermented flaxseed between the highest and lowest producers.

The gut microbiota pathway for the formation of enterolignans begins with demethylation and dehydroxylation of SECO, followed by oxidation of ED to EL.[45] Factors such as diet, genetics,[46] gender,[28] and age[47] affect the conversion of lignans to EL by modulating microbiota.[48] In the present research, the variability observed in lignan conversion among donors is great, clearly due to differences in the individual microbiota. It was suggested that microbiota dehydrogenation of lignans to generate EL is a crucial step in the colon that could explain major variations in EL production.[49] The ability

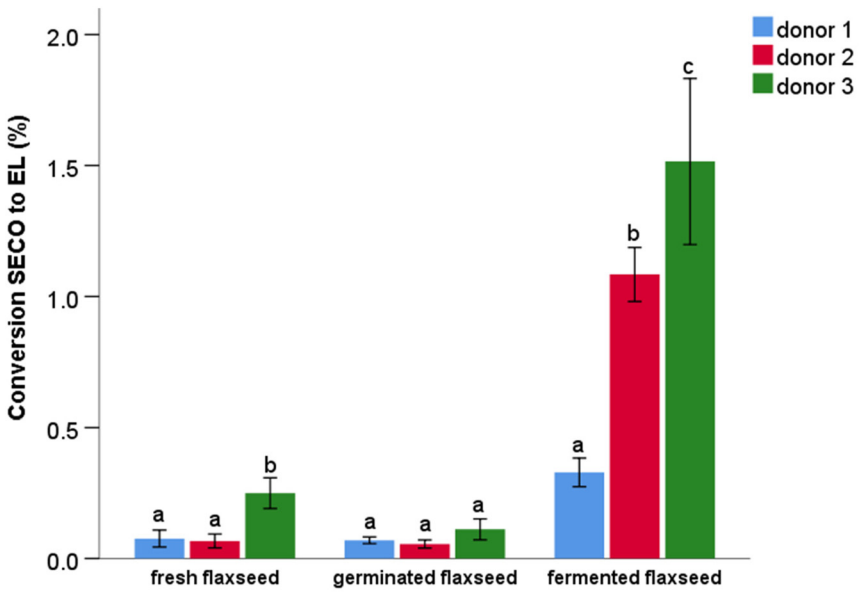

Fig. 3 Lignan (SECO) conversion to EL at 24 hours in *in vitro* colon fermentation of fresh, germinated, and fermented flaxseed. Data are from two replications (mean ± SD, n = 2). Different lower case letters indicate a significant difference in SECO conversion to EL among the three donors in each sample. SECO = secoisolariciresinol; EL = enterolactone.

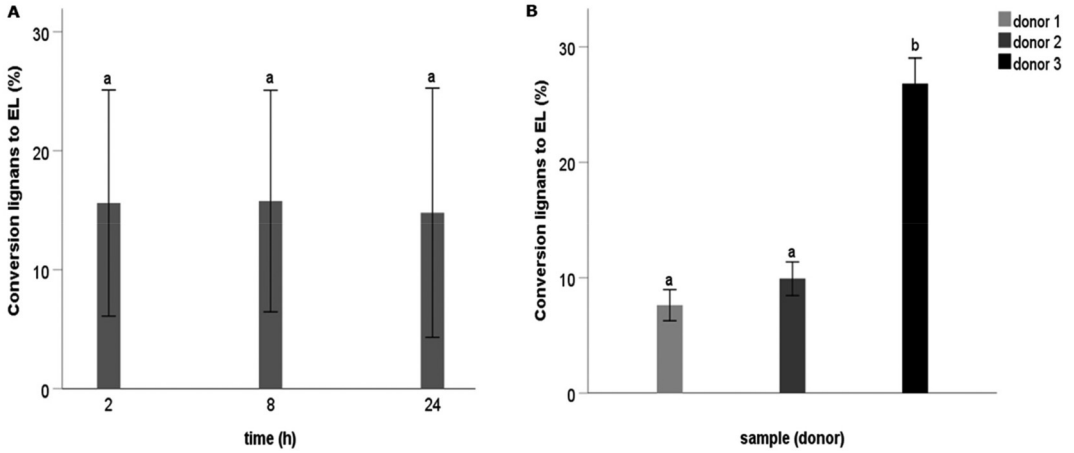

Fig. 4 Lignan (PINO and MATA) conversion to EL during *in vitro* colon fermentation of tea brew. (A) Data are from 3 donors (mean ± SD, n = 3) at different fermentation times; (B) data are from two replications of tea brew (mean ± SD, n = 2) of 3 donors. Different lower case letters indicate a significant difference ($P < 0.05$) in lignan conversion to EL. EL = enterolactone.

of gut microbiota to metabolize lignans to EL is determined by the presence of a metabolite production gradient that gives rise to "high producers" and "low producers" of metabolites.[50] To date, only a few microbiota species have been identified as enabling the production of EL. In particular, EL production has been related to the abundance of *Ruminococcus*,[51] *Bacteroides*, and *Eggerthella* species.[52] Thus, microbiota differences are a factor that influences the outcome of the health effects of lignans.[44] Furthermore, a study hypothesized that if high lignan products are consumed for an extended period of time, the microbiota will most likely adapt to metabolizing lignans, increasing the plasma concentration of the resulting enterolignans.[27]

Only PINO and MATA were detected in the tea brew in colonic fermentation. The conversion of lignans (PINO and MATA) in tea brew was relatively high, approximately 15% (Fig. 4A), which was much higher than the conversion rate observed from flaxseed matrixes. The most likely explanation for this difference in conversion yield may be the different level of lignans to which microbiota have been exposed during the fermentation experiments. This amount would be highest when germinated flaxseed was fermented with a value of 15.1 mg of lignans (in 1 g of fermented material), followed by fermented flaxseed, with a value of 11.1 mg. The lowest lignan content after the colonic fermentation was in fresh flaxseed, with a value of 3.0 mg. Compared to flaxseed matrixes, the amount of lignans in 10 mL of tea brew used in the fermentation was several orders of magnitude lower, *i.e.* 0.003 mg. We can speculate that the high lignan concentration may have saturated the capacity of the microbiota for lignan conversion into enterolignans. Furthermore, the presence of fibre and its fermentation in the flaxseed may have delayed microbial metabolism of lignan as suggested elsewhere.[27] Another possible explanation for the higher conversion of lignans in tea is that MATA can be directly converted to EL, whereas SECO needs to be converted to MATA or END prior to EL production.[53]

The influence of inter-individual differences on the conversion of lignans to EL was further examined (Fig. 4B). Donor 3 significantly showed again the highest production of EL (approximately 25%) compared to others.

Conclusion

In the current study, for the first time, a systematic investigation of lignan bioaccessibility during gastrointestinal digestion, its release, and conversion to EL in colonic fermentation from differently processed flaxseed is reported. Our findings highlight that only a limited fraction of lignans are released in the small intestine and that processing and particle size reduction have a limited effect on the bioaccessibility of lignans. Fermentation, however, might represent a potential strategy for enhancing lignan release and conversion to EL in the large intestine. Further research into particle size reduction can be conducted to assess the impact on lignan bioaccessibility and conversion to EL.

Author contributions

Umi Kalsum Hussain Zaki: conceptualization, investigation, formal analysis, writing – original draft. Christos Fryganas: conceptualization, investigation, formal analysis, writing – review and editing. Laura Trijsburg: writing – review and editing. Edith Feskens: writing – review and editing. Edoardo Capuano: supervision, conceptualization, investigation, writing – review and editing.

Conflicts of 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.

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