Research Article

Received: 2 January 2013 Revised: 11 September 2013 Accepted article published: 14 November 2013 Published online in Wiley Online Library: 7 January 2014

(wileyonlinelibrary.com) DOI 10.1002/jsfa.6482

Bioaccessibility of lignans from flaxseed (*Linum usitatissimum* L.) determined by single-batch *in vitro* simulation of the digestive process

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Abstract

BACKGROUND: Flaxseed is an important source of lignan secoisolariciresinol diglucoside (SDG) and its aglycone, secoisolariciresinol (SECO). These phenolic compounds can be metabolized to the mammalian lignans enterodiol (ED) and enterolactone (EL) by human intestinal microflora. Flaxseed lignans are known for their potential health benefits, which are attributed to their antioxidant and phytoestrogenic properties. The focus of this study was to determine the bioaccessibility of plant and mammalian lignans in whole flaxseed (WF) and flaxseed flour (FF) throughout the entire digestive process. Moreover, the metabolic activity of intestinal microflora was evaluated.

RESULTS: A single-batch *in vitro* simulation of the digestive process was performed, including fermentation by the intestinal microflora in the colon. Bioaccessibility was calculated as (free lignan)/(total lignan). In digested WF, the bioaccessibility values of SECO, ED and EL were 0.75%, 1.56% and 1.23%, respectively. Conversely, in digested FF, the bioaccessibility values of SDG, ED and EL were 2.06%, 2.72% and 1.04%, respectively. The anaerobic count and short-chain fatty acids indicate that bacteria survival and carbohydrate fermentation occurred.

CONCLUSION: The contents of both SDG and ED were significantly higher in digested FF than in digested WF. FF facilitated the action of intestinal bacteria to release SDG and metabolize ED.

Keywords: bioaccessibility; flaxseed; lignans; *in vitro* digestion

INTRODUCTION

Flaxseed (*Linum usitatissimum* L) is known for its high content of lignans relative to other grains and legumes, of which secoisolariciresinol diglucoside (SDG) is the most important.[1,2] SDG can be metabolized to mammalian lignans by the gut microflora through a series of reactions: first, SDG undergoes hydrolysis to generate the aglycone secoisolariciresinol (SECO), which is then dehydrated and demethylated to produce enterodiol (ED), and, finally, ED can be oxidized to enterolactone (EL).[3] Flaxseed lignans and their mammalian metabolites are known to have a number of potential health benefits, including reducing the risk of breast, prostate and colon cancers, which has been attributed to its (anti)-estrogenic and antioxidant properties.[4,5] Furthermore, they can lower the total cholesterol, low-density lipoprotein (LDL) cholesterol and glucose concentrations in the blood, which could prevent cardiovascular diseases.[6] Depending on the rate of SDG metabolism by the intestinal microflora, intestinal epithelial cells can be exposed to relatively high concentrations of SECO, ED and EL.[4]

Several *in vitro* gastrointestinal models have been designed to study the reactions that occur during digestion. The simulator of the human intestinal microbial ecosystem (SHIME) involves five or six bioreactors with controlled pH conditions that simulate the stomach, the small intestine and the ascending, transverse and descending colon.[7,8] The TNO (Netherlands Organization for Applied Scientific Research) gastrointestinal model (TIM) has four computer-controlled chambers simulating the conditions in the stomach, duodenum, jejunum and ileum, which involve simulation of peristaltic movements by controlled squeezing and simulation of the absorption of nutrients and water.[9,10] Neither model incorporates mastication into the simulation, which is needed to obtain a complete digestive process. Recently, a new system was designed that uses a single bioreactor to study the passage of food through the stomach and small intestine. This system simulates the upper gastrointestinal tract and can be used to determine the survival of probiotics in different food matrixes. The simulation of the stomach and small intestine is performed in a flask with stirring to combine acid and gastric

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enzymes, specifically pepsin, to simulate the stomach and the pancreatic and bile salts in the neutral pH of the small intestine. This model is a more realistic representation of the conditions of the upper gastrointestinal tract.[11,12] To simulate the digestive process in the colon, the EnteroMix® simulator uses four reactors that create the conditions in the ascending, transverse, descending and sigmoid colon using a fecal inoculum obtained from one or more donors.[13,14]

Several studies on the metabolism of lignans have been conducted for complex SDG and free SDG.[3,15,16] Eeckhaut *et al.*[15] estimated the SDG bioavailability in the upper gastrointestinal tract through artificial stomach and intestinal digestion and investigated microbial fermentation in the colon to determine SDG metabolism in the large intestine. SDG was released in the large intestine and SECO was released by microbial action in the ascending colon. The SECO was then transformed to ED and EL in the transverse colon.[15] However, this study was conducted with a flax lignan concentrate (40%) as the food matrix in the gastric stimulation, a situation that is far from the actual conditions because it omits the effect of other flaxseed nutritional components. Usually, flaxseed is consumed as whole flaxseed or flaxseed flour. Therefore, it is important to know the bioaccessibility of flaxseed lignans by evaluating the contents of SDG and its metabolites that are generated through the entire digestive process (mastication – colon fermentation).

Bioaccessibility is the quantitatively determined amount of a substance that will potentially be available for absorption by the epithelial cells.[17,18] In contrast, bioavailability refers to the amount of metabolites absorbed by the body.[19,20] Recently, the bioaccessibility of micronutrients such as selenium,[21] iron, zinc,[22] arsenic[23] and lycopene,[24] among others, has been studied. However, studies on the effects of the intake of lignans derived from flaxseed consumption, either as whole seeds or as flour, are not reported.

The aim of this study was to evaluate the metabolism of SDG from whole flaxseed and flaxseed flour during the digestive process using a single-batch *in vitro* model. The digestive simulation included mastication, the stomach, and the small and large intestine. Further, the metabolic activity of intestinal microflora exposed to flaxseed was evaluated through short-chain fatty acid (SFCA) production, anaerobic counts and amino acid profile.

EXPERIMENTAL

Reagents

All reagents were analytical grade and were purchased from Merck (Darmstadt, Germany). Enzymes were purchased from Sigma-Aldrich (St Louis, MO, USA). The flaxseed used was the Celestina-2007 variety, obtained from Semillas Baer SA (Araucanía Region, Chile).

Quantification of flaxseed SDG

The extraction of SDG from flaxseed was performed as described by Johnsson *et al.*[25] with modifications. Briefly, defatted flaxseed flour (DFF) was prepared; the seeds were crushed in a mill and defatted by the Soxhlet method for 2 h at 70 °C with *n*-hexane. Five grams of DFF was mixed with 100 mL of 60 mmol L^{-1} sodium methoxide and sonicated for 30 min. The mixture was incubated at 50 °C under agitation at 100 rpm in an orbital shaker for 18 h. The solution was filtered through Whatman paper No. 1 to remove solid waste and then neutralized with 12 mol L^{-1} HCl. Twenty milliliters of 0.1 mol L^{-1} sodium acetate at pH 5.0 was added, and the mixture was centrifuged for 15 min at 4200 × *g*, after which the supernatant was filtered with Whatman paper No. 1. The solvent was evaporated at 40 °C, and 60 mL of 0.1 mol L^{-1} sodium acetate at pH 5.0 was added to the resulting aqueous extract. The solution was centrifuged again for 15 min at 4200 × *g* and filtered through Whatman paper No. 1. The lignans were cleaned by solid-phase extraction (SPE) using 6 mL Sep-Pack® Vac C18 cartridges (500 mg capacity; Waters, Milford, MA, USA). The columns were activated by successive washing with methanol and deionized water. After the columns were activated, 5 mL of sample was loaded and eluted with 2 mL methanol. The procedure was performed in triplicate, and the samples were kept refrigerated at 4 °C before being analyzed.

A ternary pump (model L-6200, Merck-Hitachi, Darmstadt, Germany) was used, along with a UV–visible detector (model L-4250, Merck-Hitachi) and a model 717 Plus autosampler (Waters). The chromatograms were analyzed using Clarity software (DataApex, Prague, Czech Republic). The operating conditions are shown in Table 1.

In vitro simulation of the digestive process

Artificial saliva was prepared by dissolving in water (purified by a Milli-Q system, 18 MW cm^{-1} Millipore Corp., IADET SA, Chile), 5.21 g L^{-1} $NaHCO_3$, 0.88 g L^{-1} NaCl, 0.48 g L^{-1} KCl, 0.44 g L^{-1} $CaCl_2.2H_2O$, 1.04 g L^{-1} K_2HPO_4, 2.16 g L^{-1} mucin and 13.00 g L^{-1} of 50000 units α-amylase from porcine pancreas (pH adjusted to

Table 1. Instruments and analytical conditions for HPLC-UV

Lignans	
Column	Luna C8, 250 × 4,6 mm i.d., particle size 5 µm (Phenomenex Inc.)
Mobile phase	A: 1% aqueous acetic acid/acetonitrile (85:15 v/v)
	B: acetonitrile
Gradient program	0 min: 100% A, 0% B
	11 min: 76% A, 24% B
	15–22 min: 60% A, 40% B
	22.1–25 min: 100% A, 0% B
Detection	280 nm
Injection volume	20 µL
Flow	1 mL min^{-1}
Temperature	20 °C
Amino acids	
Column	Luna C18, 250 × 4,6 mm i.d., particle size 5 µm (Phenomenex Inc.)
Mobile phase	A: 1.15% p/v sodium acetate, 0.05% v/v triethylamine, pH 6.4 in water (94%) and acetonitrile (6%)
	B: acetonitrile/water (60:40, v/v)
Gradient program	0 min: 100% A, 0% B; flow 1 mL min^{-1}
	10 min: 54% A, 46% B; flow 1 mL min^{-1}
	10.5–11.5 min: 0% A, 100% B, flow 1 mL min^{-1}
	12 min: 0% A, 100% B, flow 1.5 mL min^{-1}
	12.5–20 min: 100% A, 0% B; 1.5 mL min^{-1}
	20.5 min: 100% A, 0% B; flow 1 mL min^{-1}
Detection	254 nm
Injection volume	20 µL
Temperature	20 °C

7.0).[26] Samples of 50 g WF or FF were mixed with 50 mL artificial saliva at 37 °C and gently stirred for 20 s. Then, the mixture of WF or FF and artificial saliva was brought to 200 mL with distilled water. No milling was simulated, since WF is usually swallowed in an intact form. Finally, 5 mL samples were taken and refrigerated at 4 °C for subsequent lignan determination.

Simulation of the stomach and small intestine was performed, with modifications, in one batch, as described by Ritter *et al.*[11] Distilled water (700 mL) was sterilized in a fermenter (model LiFlusGM culture vessel, Biotron) (121 °C, 15 min) and the pH was adjusted to 2.0 with 2 mol L^{-1} HCl. A 20 mL aliquot of the stomach solution was added (50 mg of ≥2500 units mg^{-1} protein pepsin from porcine gastric mucosa in 20 mL of 0.1 mol L^{-1} HCl), the pH was adjusted to 2.0 again and 195 mL of sample was added, corresponding to the mixture of saliva and flaxseed from the previous stage. After 1 h, the pH was changed to 6.0, 34 mL bile salt solution (7.5 g bovine bile in 50 mL distilled water) and 50 mL pancreatic juices (2 g pancreatin – containing trypsin, amylase, lipase, ribonuclease and protease – from porcine pancreas dissolved in 50 mL phosphate buffer 0.02 mol L^{-1}, pH 7.5) were added. The pH was gradually changed from 6.0 to 7.5 over a period of 4 h, and nitrogen was sporadically bubbled to create anaerobic conditions. At all stages of the gastrointestinal simulation, the culture vessel was maintained at 37 °C with constant shaking at 100 rpm, and the pH was adjusted with 2 mol L^{-1} HCl and 1 mol L^{-1} NaOH. Every hour, a 10 mL sample was taken, resulting in four samples for the simulated small intestine that corresponded to the stages of digestion in the duodenum (SI1), jejunum (SI2), and two stages from ileum (SI3 and SI4), because of the longer period of digestion in the ileum.

To simulate the conditions of the large intestine, an inoculum of bacteria from human feces was prepared following De Boever *et al.*[7] and Possemiers *et al.*[27] Approximately 1 g of human fecal sample was dissolved in 10 mL sterile phosphate buffer (0.1 mol L^{-1}, pH 7.0) containing 10 mg sodium thioglycolate. The mixture was centrifuged (4200 × *g* for 5 min) to remove any solid material and recover the supernatant. This procedure was repeated with 20 donors to form a pool of fecal samples, which were stored at 4 °C. An aliquot of 50 mL of the solution of intestinal bacteria was incubated with 150 mL brain heart infusion broth (BHI) under anaerobic conditions for 24 h at 36 °C to propagate the intestinal bacteria. An aliquot of 150 mL concentrated brain heart broth (amount sufficient for 1 L), previously sterilized (121 °C, 15 min), was added to the bioreactor and then inoculated with 50 mL of the cultured intestinal bacteria. Anaerobic conditions were maintained by sporadic bubbling of nitrogen, and the pH was maintained with 2 mol L^{-1} HCl and 1 mol L^{-1} NaOH. The pH was maintained at 5.5, 6.0, 6.5 and 7.0 over 12 h to simulate the ascending colon (LI1), transverse colon (LI2), descending colon (LI3) and sigmoid colon (LI4), respectively. A 10 mL sample was taken every 12 h and refrigerated at 4 °C for subsequent lignan, SCFA, free amino acid profile and anaerobic bacteria count determination. The analyses were performed in duplicate.

Lignan determination by high-performance liquid chromatography–ultraviolet (HPLC-UV)

The 10 samples collected during the simulation were centrifuged at 4200 × *g* for 15 min. The lignans were separated from the mixture by SPE using C18 cartridges from Sep-Pack® Vac, 6 mL (500 mg). Once the columns were activated, 1 mL of sample was added and eluted with 1.5 mL methanol. The samples were centrifuged at 16 000 × *g* for 5 min and stored at −20 °C until HPLC analysis. HPLC conditions are shown in Table 1. Bioaccessibility was then calculated as follows:

$$\text{Bioaccessibility (\%)} = \frac{\text{Free lignans}}{\text{Total lignans}} \times 100$$

Validation of analytical methodology

To assure the reliability of the results, the limit of detection (LOD) and limit of quantification (LOQ) of SDG, SECO, ED and EL were determined by performing five calibration curves each with five concentration levels (2.5–12.5 μg mL^{-1}). The precision and accuracy of the method were evaluated by calculating the coefficient of variation (CV) and by spiking the standard, respectively (Table 2). These parameters are within the ranges that are internationally considered as acceptable.[28]

Short-chain fatty acids

Fermentative capacity was evaluated from the content of short-chain fatty acids (SCFA). A fermentation sample (900 μL) mixed with 100 μL formic acid was centrifuged at 16 000 × *g* for 5 min. The supernatant was filtered through a 0.45 μm membrane filter prior to analysis. The filtered supernatant (500 μL) was added to a GC vial with 500 μL deionized water and 50 μL hexanoic acid as an internal standard. One microliter of sample was injected into a gas chromatograph (GC-2010, Shimadzu) equipped with a flame ionization detector using a polyethylene glycol nitroterephthalic acid-treated capillary column (BP21, 30 m × 0.32 mm, SGE). The carrier gas was He at a column flow rate of 2.4 mL min^{-1} with a split ratio of 1:100. The column program temperature was 105 °C, 0 min to 180 °C, 15 min. The analyses were performed in duplicate.

Free amino acid profile

To evaluate protein hydrolysis during digestion in the stomach and small intestine, as well as the amino acid consumption during fermentation by the intestinal bacteria, the free amino acid profile was determined as described by Bidlingmeyer *et al.*[29] Briefly, 980 μL of sample was mixed with 20 μL of 0.5 mol L^{-1} HCl and centrifuged at 16 000 × *g* for 5 min. The supernatant was mixed with 20 μL of a solution of dry (ethanol–water–triethylamine, 1:1:1) and lyophilized for 30 min. Then, 20 μL of a derivatization solution (ethanol–triethylamine–water–phenyl isothiocyanate, 7:1:1:1) was added and left for 20 min at room temperature. The sample was lyophilized for 45 min and finally dissolved in 200 μL of 5 mmol L^{-1} sodium phosphate buffer

Table 2. Analytic parameters for the determination of SDG, SECO, ED and EL

Parameter	SDG	SECO	ED	EL
LOD[a] (μg g^{-1})	14.8	6.7	23.9	7.7
LOQ (μg g^{-1})	44.3	20.1	71.8	23.2
Accuracy (recovery, %)[b]	110.4	92.5	103.4	99.2
Precision (RSD, %)[c]	9.2	7.2	1.2	6.2

[a] Five analytical curves were used.
[b] Expressed as the mean of three independent analyses.
[c] Typical deviation related to three independent analyses.

(pH 7.45) and acetonitrile at a ratio of 95:5 (v/v). The operating conditions for HPLC-UV are shown in Table 1. The analyses were performed in duplicate.

Viability of fecal bacteria

Dilutions were performed with sterile 0.1 mol L^{-1} phosphate buffer, and the bacteria were seeded in a layer of plate count agar and incubated at 37 °C for 72 h in an anaerobic chamber. The analyses were performed in duplicate.

Statistical analysis

One-way analysis of variance (ANOVA) and multifactor ANOVA were performed to determine the statistical significance of the differences ($P < 0.05$) between digestion stages and between the digestion of FF and WF. In addition, principal component analysis (PCA) was conducted with all of the variables studied: lignans, SCFA, anaerobic count and free amino acid profile. Statistical analyses were performed using SIMCA-P (Umetrics, Umeå, Sweden) and Statgraphics Centurion XV (StatPoint Inc., Rockville, MD, USA).

RESULTS AND DISCUSSION

Bioaccessibility of lignans

The bioaccessibility of lignans was calculated as a ratio of free lignan and total lignan content. The flaxseed used in this study had a total SDG content of 7.6 ± 0.5 mg g^{-1}. However, other lignans (SECO, ED and EL) were not detected because they are metabolized by the intestinal microflora. Therefore, to calculate the bioaccessibility of these lignans, it was necessary to estimate the total content of each one. For this purpose, the assumption was made that the total SDG content of the flaxseed (7.6 mg g^{-1} flaxseed) was metabolized and converted to SECO, ED and finally to EL. Through a relationship with the molar mass of each compound, it was possible to determine the total content of the flaxseed lignans, which were 4.0, 3.4 and 3.3 mg g^{-1} for SECO, ED and EL, respectively.

In the WF digestion, lignans were not detected during mastication or in the stomach or small intestine. SECO was detected in the ascending colon (Fig. 1). After 48 h of fermentation in the large intestine, the content of SECO increased, reaching $0.75\% \pm 0.13$ of bioaccessibility in the last stage. In the ascending colon, ED increased gradually from 22.5 ± 5.3 µg g^{-1} to 51.7 ± 0.38 µg g^{-1} in the sigmoid colon, corresponding to $1.56\% \pm 0.01$ bioaccessibility. However, the conversion to EL remained relatively constant during the 48 h of fermentation, reaching $1.23\% \pm 0.50$ bioaccessibility. Clearly, SDG and SECO were not fully released from the flaxseed matrix, which allowed only a small amount to be available to the bacteria in the colon. Eeckhaut *et al.*[15] claimed that the lignan macromolecule should be seen as a delivery system in the large intestine. Moreover, because our *in vitro* simulation system does not consider the absorption of nutrients or lignans, it may also be possible that the SECO released was not all available for conversion to ED; that is to say, in an actual digestive system, SECO would be absorbed by the body before it could be metabolized to ED and then to EL. This conclusion is based on studies reporting that SECO was detected in human urine, indicating that this lignan was absorbed by the gut epithelium.[30]

The FF digestion yielded different results from the WF digestion, in which lignans were not detected until the ascending colon stage. In the early simulation stages, the chromatograms showed undefined peaks (14.5 min) that did not correspond to any

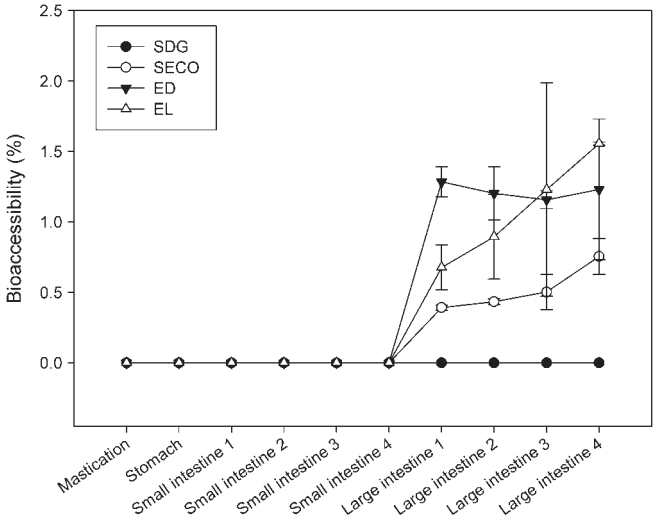

Figure 1. Bioaccessibility of lignans produced by *in vitro* digestion of whole flaxseed. SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; ED, enterodiol; EL, enterolactone. Digestive simulation includes the mastication, stomach, small intestine stages (duodenum (SI1), jejunum (SI2), first portion of the ileum (SI3) and last portion of the ileum (SI4)), and the large intestine stages: (ascending colon (LI1), transverse colon (LI2), descending colon (LI3) and sigmoid colon (LI4)).

of the lignans under study (Fig. 2). These unidentified peaks may correspond to the unhydrolyzed oligomer SDG. However, more research is needed to verify that the detected signal corresponds to oligomer SDG. It is probable that the hydrolytic enzymes of the stomach and small intestine failed to completely degrade the oligomer SDG because other compounds such as polysaccharides, gums and mucilages competed for the hydrolytic enzymes. It is noteworthy that, during the stages of upper gastrointestinal simulation (FF digestion), the viscosity was much higher – produced by soluble flaxseed gum from hulls[31] – than in the case of the WF digestion.

In the FF digestion, lignans were not detected during mastication or in the stomach or small intestine. As in the digestion of WF, an oligomer SDG could be acting as a delivery system for lignans in the large intestine. Once the human intestinal bacteria were inoculated in the FF digestion, SDG was detected (Fig. 3), reaching a maximum after 48 h of fermentation and attaining $2.06\% \pm 0.26$ bioaccessibility. In contrast, SECO was not detected in any stage of the *in vitro* simulation. ED content reached a bioaccessibility of $2.72\% \pm 1.18$, which is significantly higher ($P < 0.05$) than that obtained during the WF digestion. EL increased progressively in the colon, reaching $1.04\% \pm 0.92$. This value is slightly lower than that obtained during the WF digestion. SECO was not detected at any stage of the digestive process. This finding may be attributed to colonic bacteria that quickly metabolized this molecule to ED and then to EL, which could be quantified from the beginning of fermentation.

Both flaxseed digestions produced similar contents of EL, but the bioaccessibility of SDG and ED during FF digestion were significantly higher than WF digestion. SDG formation occurs in the outer layer of the seed; therefore, the greatest concentration of SDG is found in the hulls of flaxseed.[32] In addition to being esterified into an oligomer, SDG is complexed with insoluble fiber, gums, polysaccharides and mucilage associated with the hull.[1] Meca *et al.*[33] reported that soluble dietary fiber reduces the bioaccessibility of the bioactive compound beauvericin about 94%

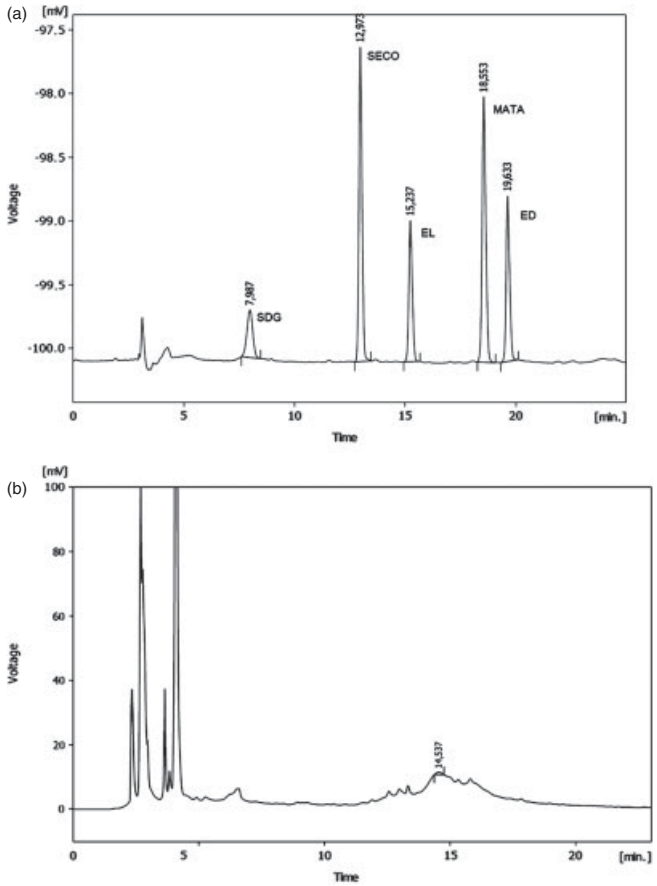

Figure 2. (a) HPLC chromatogram for lignans. SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; EL, enterolactone; MATA, mataisolariciresinol; ED, enterodiol. (b) HPLC chromatogram for small intestine contents after 4 h of simulated *in vitro* digestion of flaxseed flour.

in duodenal digestion and 50% in duodenal and colonic digestion. Therefore, the dietary fiber present in flaxseed could reduce the bioaccessibility of lignans, and ground seed could facilitate the action of intestinal bacteria, then allowing the release of SDG from the oligomer of the flaxseed hulls.

Although there are bioaccessible lignans in WF and FF digestion, the values are very low. To produce a beneficial effect on health, large amounts of flaxseed would have to be consumed. There are clinical studies that evaluated the amount of lignans that would need to be ingested to achieve a decrease in total cholesterol and plasma glucose.[6] For example, for a 22% reduction of total cholesterol and a 25% reduction of the plasma glucose concentration in hypercholesterolemic subjects, 600 mg d^{-1} of SDG would have to be consumed for 6 weeks. Based on the bioaccessibility of SDG (2%) calculated in this study, this would correspond to the consumption of approximately 4 kg of flaxseed flour per day, which is not feasible in a normal diet. However, the intestinal bacteria used in this study were obtained from random fecal samples from healthy individuals (none of whom usually consume flaxseed), whereas the results described in the clinical study were obtained after 6 weeks of SDG consumption. This suggests that, if the consumption of flaxseed is prolonged, the gut microflora can adapt to metabolize lignans, which might increase their content in the plasma.

The variability observed in the lignan content is very high, which may be due to differences in the growth or metabolic activity of

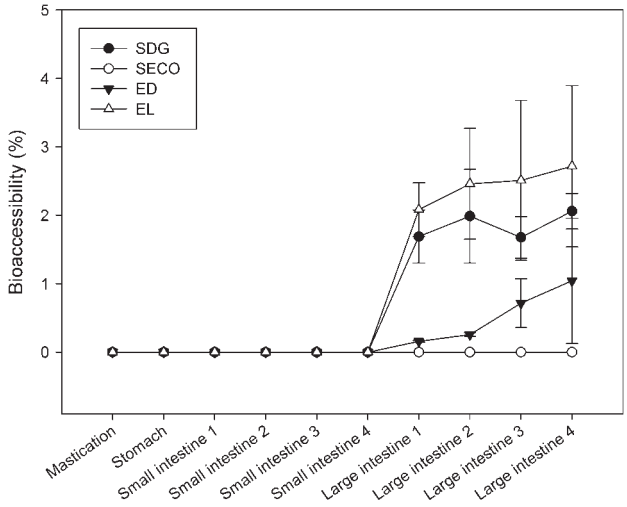

Figure 3. Bioaccessibility of lignans produced by *in vitro* digestion of flaxseed flour. SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; ED, enterodiol; EL, enterolactone. Digestive simulation includes the mastication, stomach, small intestine stages (duodenum (SI1), jejunum (SI2), first portion of ileum (SI3) and last portion of the ileum (SI4)), and large intestine stages: (ascending colon (LI1), transverse colon (LI2), descending colon (LI3) and sigmoid colon (LI4)).

the bacteria used. To evaluate the survival of bacteria in the colon, anaerobic bacteria counts were obtained, and to assess their fermentative capacity the SCFA profile was determined.

Anaerobic count

The number of anaerobic bacteria was determined for all stages of WF and FF digestion in the large intestine (Fig. 4). Each stage of large intestinal digestion is 12 h long; therefore, the *in vitro* digestion occurred over 48 h of fermentation. The number of anaerobic bacteria was higher in WF digestion compared with FF digestion ($P < 0.05$). However, similar growth kinetics were observed in both cases. WF digestion yielded a greater anaerobic colony count, which was most likely due to the lower viscosity of the growth medium, allowing the bacteria better access to nutrients. In contrast, a higher viscosity was observed in FF digestion, which might make growth difficult for the bacteria present. Thus the

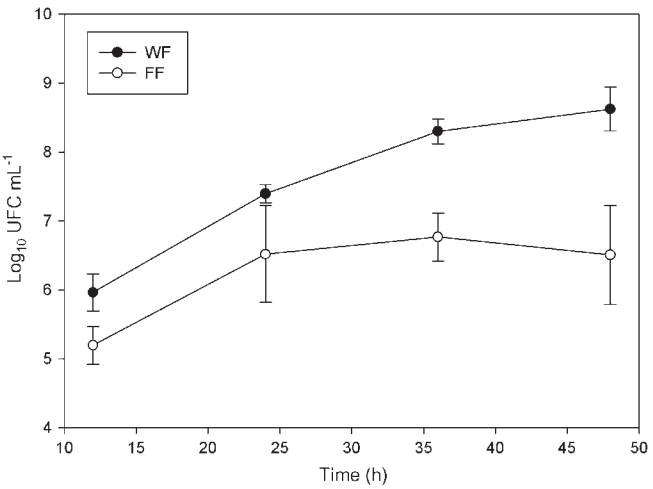

Figure 4. Anaerobic count for fermentation of whole flaxseed (WF) and flaxseed flour (FF).

biological effects of osmotic stress on microorganisms could be affected by the physical properties of the system, such as viscosity.[34]

Short-chain fatty acids

The amounts of enterolignans found in the large intestine stages were relatively low compared to the total that could have been released. Consequently, the fermentative capacity of the fecal bacteria used was evaluated in the early stages of the large intestine. After 24 h of FF and WF fermentation, the concentration of SCFA increased (Table 3). In particular, the acetic, propionic and butyric acid concentrations were significantly higher for WF digestion than for FF digestion ($P < 0.05$). The concentrations of acetic, propionic and isovaleric acids increased in WF digestion when the fermentation was longer; however, acetic acid was the most prevalent, reaching 80% of total SCFA after 48 h. In contrast, butyric acid decreased during fermentation. A similar process was observed in FF digestion, in which acetic acid was also predominant, reaching 97% of total SCFA after 48 h, but in FF digestion the content of other acids was not significant. These results indicate that carbohydrate fermentation occurred, and the bacteria were most likely consuming the soluble fiber in flaxseed, but there was not sufficient time to metabolize a greater volume of SECO and enterolignans.

Amino acid profile

Free amino acids were measured at all stages of the *in vitro* digestive process for WF (Table 4) and FF digestion (Table 5). This was done to evaluate protein hydrolysis during stomach and small intestine digestion, as well as amino acid consumption during fermentation by the intestinal bacteria. In the first stage of the colon, a BHI solution containing amino acids and nutrients was added to the reactor to ensure the survival of the intestinal bacteria. This addition produced a large increase in the content

Table 3. Short-chain fatty acid generation during the fermentation of WF and FF by fecal bacteria in the colon simulator

Large intestine stage (time)	Acetic acid (mmol L^{-1})	Propionic acid (mmol L^{-1})	Isobutyric acid (mmol L^{-1})	Butyric acid (mmol L^{-1})	Isovaleric acid (mmol L^{-1})	Valeric acid (mmol L^{-1})
WF						
LI1 (12 h)	12.17 ± 10.69	2.74 ± 3.26	nd	0.32 ± 0.09	0.26 ± 0.08	nd
LI2 (24 h)	19.58 ± 17.53	6.55 ± 9.26	0.06 ± 0.08	0.21 ± 0.29	0.20 ± 0.09	nd
LI3 (36 h)	31.04 ± 16.00	9.40 ± 12.68	nd	0.44 ± 0.09	0.10 ± 0.15	nd
LI4 (48 h)	57.02 ± 27.54	11.36 ± 13.13	0.58 ± 0.82	0.29 ± 0.41	1.88 ± 2.66	nd
FF						
LI1 (12 h)	4.50 ± 2.72	0.31 ± 0.43	nd	0.12 ± 0.16	0.08 ± 0.11	nd
LI2 (24 h)	8.43 ± 0.91	nd	nd	nd	nd	nd
LI3 (36 h)	9.44 ± 3.32	0.12 ± 0.17	nd	nd	0.08 ± 0.11	nd
LI4 (48 h)	13.75 ± 1.80	0.21 ± 0.3	nd	0.08 ± 0.11	0.07 ± 0.10	nd

LI1, ascending colon; LI2, transverse colon; LI3, descending colon; LI4, sigmoid colon; nd, not detected.

Table 4. Amino acid profile for the *in vitro* simulation of whole flaxseed digestion

	Mastication	Stomach	SI1	SI2	SI3	SI4	LI1	LI2	LI3	LI4
Asp	1.5 ± 0.5	2.6 ± 0.3	5.6 ± 0.9	4.9 ± 0.7	3.8 ± 0.8	5.16 ± 0.4	32.2 ± 1.2	34.0 ± 0.3	30.3 ± 4.2	30.7 ± 11.1
Glu	3.4 ± 0.9	4.3 ± 0.3	13.6 ± 0.5	11.4 ± 0.3	8.6 ± 3.2	14.0 ± 0.3	95.8 ± 7.1	114.4 ± 12.5	93.4 ± 8.9	108.6 ± 25.7
Ser	4.2 ± 0.4	2.3 ± 0.1	9.2 ± 0.2	7.7 ± 0.3	5.7 ± 3.0	9.9 ± 0.8	51.3 ± 1.0	49.5 ± 1.1	4.9 ± 1.1	3.0 ± 0.3
Gly	3.8 ± 0.4	1.9 ± 0.0	10.0 ± 0.6	8.2 ± 0.3	6.0 ± 2.6	10.3 ± 0.6	90.0 ± 2.2	115.6 ± 2.4	146.6 ± 3.8	53.7 ± 2.4
His	2.5 ± 0.0	2.4 ± 0.1	6.3 ± 0.1	5.7 ± 0.4	4.7 ± 1.4	6.6 ± 0.3	22.9 ± 0.3	27.8 ± 1.8	30.5 ± 1.2	25.0 ± 0.4
Arg	6.1 ± 0.4	4.2 ± 0.0	15.6 ± 1.0	16.3 ± 1.7	13.1 ± 4.4	18.2 ± 0.8	7.2 ± 1.0	5.7 ± 0.5	4.7 ± 0.5	5.1 ± 0.4
Thr	3.9 ± 1.0	2.6 ± 0.1	14.4 ± 1.4	14.9 ± 1.1	11.0 ± 4.3	16.2 ± 0.8	96.3 ± 3.0	102.0 ± 12.4	61.8 ± 2.1	57.8 ± 11.2
Ala	2.1 ± 1.1	1.0 ± 0.2	4.7 ± 1.1	4.3 ± 0.5	2.2 ± 1.2	3.8 ± 1.4	10.2 ± 13.4	16.4 ± 22.2	0.7 ± 0.0	0.7 ± 0.0
Pro	7.0 ± 0.5	4.6 ± 0.2	13.0 ± 0.4	12.6 ± 0.4	10.6 ± 4.4	15.8 ± 0.1	143.2 ± 13.1	183.9 ± 15.3	208.4 ± 8.6	241.3 ± 6.5
Tyr	4.0 ± 0.1	3.4 ± 0.3	11.2 ± 1.5	11.3 ± 2.7	9.1 ± 2.3	12.1 ± 2.1	4.4 ± 2.6	7.5 ± 2.8	7.9 ± 2.8	8.4 ± 2.7
Val	4.7 ± 0.4	4.5 ± 0.1	10.4 ± 0.1	9.6 ± 0.9	7.9 ± 1.9	11.9 ± 1.1	73.8 ± 8.3	94.0 ± 10.1	101.1 ± 15.5	90.4 ± 8.1
Met	2.4 ± 0.5	3.1 ± 0.2	6.1 ± 1.5	5.9 ± 1.3	5.0 ± 0.5	6.3 ± 1.6	54.9 ± 1.0	60.6 ± 1.3	57.3 ± 1.7	54.2 ± 1.3
Cys	0.6 ± 0.1	1.5 ± 0.0	3.1 ± 0.1	2.6 ± 0.0	2.3 ± 0.2	2.8 ± 0.0	2.0 ± 0.3	2.0 ± 0.2	1.8 ± 0.0	1.8 ± 0.0
Ile	4.5 ± 0.7	2.4 ± 0.1	7.4 ± 2.0	5.4 ± 2.6	5.4 ± 2.5	9.6 ± 0.2	67.5 ± 1.3	87.3 ± 0.5	85.2 ± 15.7	61.2 ± 1.6
Leu	7.6 ± 0.4	4.1 ± 0.1	12.5 ± 0.9	12.7 ± 0.3	10.8 ± 3.6	15.8 ± 1.8	161.4 ± 3.6	194.0 ± 3.6	210.8 ± 15.0	146.8 ± 3.9
Phe	4.4 ± 0.4	3.8 ± 0.1	10.3 ± 0.9	10.2 ± 2.5	8.7 ± 2.3	11.5 ± 1.0	86.7 ± 4.1	98.1 ± 2.4	99.7 ± 2.7	97.9 ± 1.7
Lys	10.4 ± 1.7	2.0 ± 0.9	17.3 ± 1.7	17.7 ± 3.2	12.0 ± 7.3	19.5 ± 3.5	219.4 ± 41.5	248.7 ± 62.2	191.1 ± 7.6	225.2 ± 40.1

Amino acid content expressed in mg g^{-1} of whole flaxseed. SI1, duodenum; SI2, jejunum; SI3, first portion of ileum; SI4, last portion of ileum,; LI1, ascending colon; LI2, transverse colon; LI3, descending colon; LI4, sigmoid colon.

Table 5. Amino acid profile for the *in vitro* simulation of flaxseed flour digestion

	Mastication	Stomach	SI1	SI2	SI3	SI4	LI1	LI2	LI3	LI4
Asp	5.0 ± 1.7	7.0 ± 4.3	10.2 ± 5.0	10.3 ± 4.6	8.5 ± 1.7	9.0 ± 3.4	64.3 ± 20.4	35.8 ± 5.5	26.8 ± 1.6	57.7 ± 67.4
Glu	9.8 ± 3.2	10.1 ± 6.4	28.8 ± 18.7	27.2 ± 16.0	24.1 ± 9.1	23.4 ± 9.6	150.8 ± 27.3	122.9 ± 27.9	89.5 ± 4.7	149.3 ± 107.7
Ser	5.2 ± 1.6	3.7 ± 2.8	22.4 ± 15.7	23.1 ± 14.1	23.6 ± 12.5	23.5 ± 13.5	69.0 ± 25.3	26.4 ± 18.0	6.2 ± 3.5	8.0 ± 4.4
Gly	5.0 ± 1.7	3.3 ± 3.0	32.4 ± 14.1	33.4 ± 9.9	33.7 ± 7.0	33.9 ± 8.8	37.4 ± 13.8	45.4 ± 18.4	35.2 ± 1.5	30.0 ± 12.7
His	8.1 ± 2.8	5.7 ± 3.5	33.7 ± 21.2	35.1 ± 20.0	35.9 ± 18.0	35.1 ± 18.4	189.1 ± 11.7	198.9 ± 45.2	194.1 ± 23.5	217.3 ± 15.7
Arg	11.2 ± 1.0	12.1 ± 7.1	75.6 ± 13.2	78.3 ± 0.4	79.1 ± 4.7	91.2 ± 20.7	10.3 ± 3.1	7.2 ± 2.6	4.8 ± 0.9	7.9 ± 1.2
Thr	4.1 ± 0.7	4.4 ± 2.6	22.1 ± 6.3	22.4 ± 3.2	23.3 ± 1.1	21.4 ± 1.5	83.8 ± 11.4	127.9 ± 87.7	78.0 ± 6.6	47.3 ± 64.2
Ala	3.1 ± 1.1	4.2 ± 4.0	11.4 ± 10.2	11.3 ± 8.3	10.8 ± 7.4	11.1 ± 7.2	64.3 ± 6.1	57.6 ± 12.2	69.0 ± 14.9	83.9 ± 15.3
Pro	8.7 ± 2.2	10.2 ± 7.4	37.6 ± 21.1	39.5 ± 17.3	41.2 ± 15.4	43.0 ± 18.0	197.9 ± 21.6	218.4 ± 65.1	194.1 ± 28.9	214.9 ± 0.7
Tyr	3.0 ± 0.5	6.7 ± 3.6	16.8 ± 1.0	14.9 ± 0.4	20.1 ± 2.2	17.7 ± 4.5	5.6 ± 3.9	4.8 ± 2.7	7.5 ± 0.2	7.1 ± 0.0
Val	5.0 ± 1.1	7.0 ± 3.7	23.9 ± 12.5	24.8 ± 9.6	26.5 ± 9.0	27.5 ± 10.0	93.8 ± 2.7	112.5 ± 33.6	97.9 ± 10.9	111.5 ± 5.9
Met	3.5 ± 0.5	5.5 ± 2.6	19.9 ± 7.0	21.9 ± 5.2	22.7 ± 4.0	23.5 ± 5.9	76.4 ± 12.0	76.2 ± 17.0	71.4 ± 14.6	71.9 ± 4.2
Cys	0.6 ± 0.3	2.3 ± 0.6	4.6 ± 0.8	4.4 ± 0.8	4.6 ± 2.6	4.2 ± 1.2	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0	1.8 ± 0.0
Iso	5.1 ± 1.0	7.0 ± 1.1	26.6 ± 12.1	28.0 ± 10.4	29.7 ± 7.8	32.0 ± 10.6	104.9 ± 8.5	134.6 ± 52.4	100.9 ± 12.3	115.4 ± 8.6
Leu	8.3 ± 1.8	16.7 ± 4.0	52.7 ± 17.2	54.6 ± 10.1	57.8 ± 7.7	58.2 ± 12.8	217.4 ± 1.8	205.8 ± 18.5	204.1 ± 7.1	223.2 ± 11.4
Phe	9.3 ± 0.1	15.0 ± 0.6	52.1 ± 2.6	58.8 ± 2.8	59.8 ± 9.2	64.1 ± 3.5	a	a	a	a
Lys	7.3 ± 1.2	10.3 ± 8.2	54.0 ± 16.6	54.3 ± 7.9	57.3 ± 7.8	62.8 ± 19.0	323.1 ± 11.7	252.7 ± 89.2	305.4 ± 8.2	311.6 ± 18.7

Amino acid content expressed in mg g^{-1} of flaxseed flour. SI1, duodenum; SI2, jejunum; SI3, first portion of ileum; SI4, last portion of ileum; LI1, ascending colon; LI2; transverse colon; LI3, descending colon; LI4, sigmoid colon; a, saturated.

of all amino acids in the large intestine digestion simulation. However, it was possible to establish some differences between the digestion of WF and FF, of which WF digestion had the greater amino acid content. The amino acids Ala, Arg, His, Leu, Lys, Met and Phe were significantly ($P < 0.05$) higher for FF digestion than for WF digestion. In flax flour, proteins would be more accessible to the proteases present in the stomach and small intestine. These differences were observed mainly for the small intestine, where there was a longer exposure to proteases, but the content of some amino acids decreased rather than increased in the large intestine with the addition of the BHI solution. The amino acids that decreased may be essential nutrients for the intestinal bacteria that are present in the large intestine. The content of Cys, Gly, Ser, Thr and Tyr gradually decreased during the passage through the colon in WF and FF digestion. These results showed that the upper digestive system was capable of hydrolyzing the proteins present in flaxseed and that the intestinal bacteria used free amino acids.

Multivariate analysis

A PCA was conducted to determine whether the following variables had some influence over the bioavailability of lignans from flaxseed: the number of anaerobes, SCFA profile and amino acid profile. To achieve this, a matrix composed of 27 variables was constructed (four lignans, five SCFA, 17 amino acids and one count of anaerobes), and 40 observations, corresponding to the 10 stages of the digestive process for WF and FF, were performed in duplicate.

Four components extracted with PCA explain 87.7% of the variability of the 27 variables. The first factor (Fig. 5) was able to separate the upper and lower gastrointestinal tracts. The figure shows that all of the samples corresponding to mastication, the stomach and the small intestine are to the left of the score plot, whereas all samples corresponding to the large intestine are on the right side of the graph. The variables involved in this discrimination were the amino acids Arg, Tyr and Cys (Fig. 5b), which were significantly reduced during digestion in the large intestine compared with the other digestion stage, showing that they were consumed by the bacteria. In contrast, the remaining amino acids – specifically Phe, Pro, Val, Leu, Glu, Met and Lys, which are located to the right of the loading plot (Fig. 5b) – showed the highest contents during fermentation in the colon. This difference between amino acids and the stages of digestion implies that Arg, Tyr and Cys correspond to amino acids essential for the intestinal bacteria of the colon. All lignans correlated with the colon samples, for which ED was significantly higher. The second factor (*y*-axis, Fig. 5) was able to discriminate between samples from WF and FF digestion. The FF digestion samples are located in the upper part of the score plot (Fig. 5a), while the WF samples are at the bottom of the graphs, indicating positive correlations with the SCFA content, the anaerobic count, and the SECO and EL contents (Fig. 5b), which are higher, especially in the later hours of fermentation (LI3 and LI4). Furthermore, the second factor was able to discriminate the FF digestion samples in the small intestine, where concentrations of the amino acids Arg, Tyr and Cys were higher than in the WF digestion.

The third factor allowed the differentiation of large intestine digestion samples between FF and WF (Fig. 6a). The FF digestion samples were correlated with the SDG content, which was significantly higher than in samples from WF digestion (Fig. 6b). Furthermore, the amino acids Ala and Lys were predominant during the FF fermentation compared with WF. It is likely that these amino acids were consumed by intestinal bacteria during the WF fermentation but not during the FF fermentation. The fourth factor was able to discriminate the stages of digestion in the large intestine, from the ascending colon (LI1) to the sigmoid colon (LI4) (up and down in Fig. 6a) for both WF and FF digestion. In the last stages of digestion (LI3 and LI4), there is a significantly higher

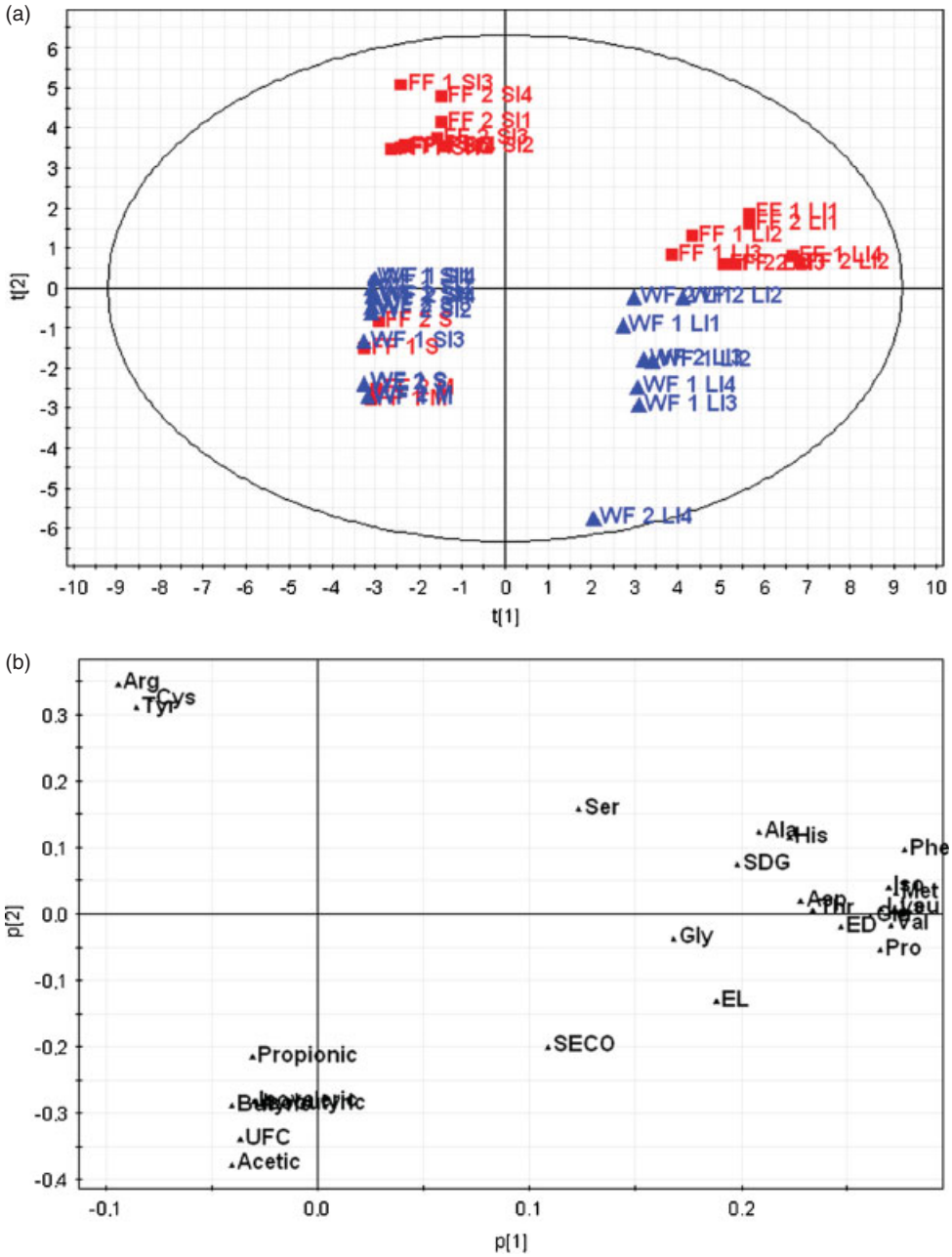

Figure 5. (a) Score plot for PCA first and second factors. (b) Loading plot for PCA first and second factors. WF, whole flaxseed; FF, flaxseed flour; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; ED, enterodiol; EL, enterolactone; M, mastication; S, stomach; SI1, duodenum; SI2, jejunum; SI3, first portion of ileum; SI4, last portion of the ileum; LI1, ascending colon; LI2, transverse colon; LI3, descending colon; LI4, sigmoid colon.

content of ED and EL lignans, as well as a higher anaerobic bacteria count in the sigmoid colon (Fig. x6b). Furthermore, a high content of the amino acid Ser was observed in step LI1 of both digestions, decreasing progressively until LI4. These results suggested that the anaerobic bacteria and the fermentative capacity survival (SCFA) were more important in the WF digestion, while higher SDG and ED contents were found in FF digestion. Therefore, the growth of intestinal bacteria could not be related to the bioavailability of the lignans. In contrast, the type of flaxseed used in the digestion could be responsible for the observed differences in which bacterial FF digestion leads to greater bioaccessibility for the production of the SDG oligomer. Therefore, *in vitro* FF digestion verifiably increases SDG and ED bioaccessibility.

CONCLUSION

The flaxseed lignans from FF and WF were bioaccessible during *in vitro* simulation of the digestive process. The main difference between the FF and WF digestion processes was the release of lignans at different digestion stages. When consuming FF, a larger amount of ED could be exposed than when consuming WF. In addition, FF digestion would release more SDG to be absorbed and/or metabolized later. However, WF digestion can also provide nutritional benefits from the release of lignans that can be absorbed in the human bowel. Furthermore, the intestinal bacteria are able to metabolize these plant lignans to enterolignans. Although the bioaccessibility was not as high as expected, these results are satisfactory and allowed the development of

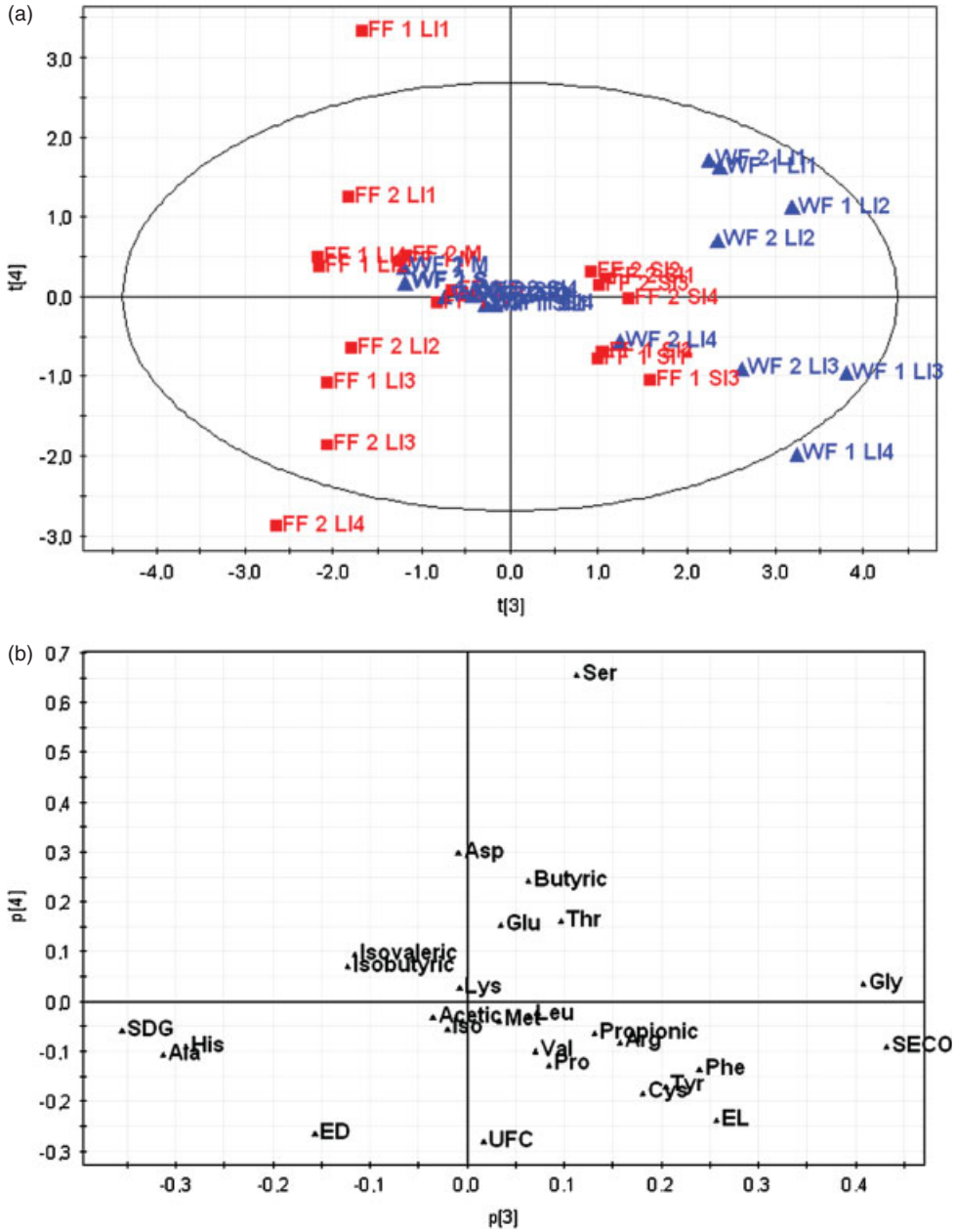

Figure 6. (a) Score plot for PCA third and fourth factors. (b) Loading plot for PCA third and fourth factors. WF, whole flaxseed; FF, flaxseed flour; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; ED, enterodiol; EL, enterolactone; M, mastication; S, stomach; SI1, duodenum; SI2, jejunum; SI3, first portion of ileum; SI4, last portion of the ileum; LI1, ascending colon; LI2, transverse colon; LI3, descending colon; LI4, sigmoid colon.

a single-batch *in vitro* simulation of the digestive process. The results suggested that, to increase the bioaccessibility of lignans, it is necessary to consume flaxseed for a prolonged period, so that gut microflora can then adapt to metabolize lignans. Further experiments are needed, including a study of probiotic bacteria that might metabolize the flaxseed lignans in food before consumption, which could increase the bioaccessibility of the SDG in whole flaxseed.

ACKNOWLEDGEMENTS

This research was supported by the National Fund for Scientific and Technological Development (FONDECYT) project 1080277. Fuentealba obtained a CONICYT doctoral fellowship in 2009.

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