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Metabolism of the Lignan Macromolecule into Enterolignans in the Gastrointestinal Lumen As Determined in the Simulator of the Human Intestinal Microbial Ecosystem

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Estrogenic plant compounds from the human diet such as the lignan secoisolariciresinol diglucoside (SDG, **1**) can exert biological activity in the human body upon ingestion and bioactivation to enterodiol (END, **5**) and enterolactone (ENL, **6**). Bioavailability of lignans is influenced by the food matrix and gut microbial action, of which the latter is subject to a large interindividual variation. In this study, the fate of the lignan precursor SDG, present in the lignan macromolecule of flax seed (*Linum usitatissimum*), was determined during an artificial stomach and small intestinal digestion and during metabolism by two different enterolignan phenotypes in a TWINSHIME environment (TWIN Simulator of the Human Intestinal Microbial Ecosystem). The lignan macromolecule acted as a delivery system of SDG in the large intestine. SDG was only hydrolyzed into secoisolariciresinol (SECO, **2**) through microbial action in the ascending colon, after which it was bioactivated into enterolignans from the transverse colon onward. Single demethylation was a first step in the bioactivation, followed by dehydroxylation. Enterolignan phenotypes remained stable throughout the experimental period. The establishment of END and ENL production equilibria reflected the subdominance of ENL-producing bacteria in the gastrointestinal tract.

KEYWORDS: Flax seed; lignan; intestinal; bacteria; bioactivation; SHIME; secoisolariciresinol; enterolactone; phytoestrogens

INTRODUCTION

Lignans constitute the dominant class of phytoestrogens in the traditional Western diet (*1*). They occur in a diversity of food sources such as nuts, grains, seeds, fruits, vegetables, and beverages. The highest concentrations of lignans are however found in flax seeds, with secoisolariciresinol diglucoside (SDG, **1**) as the major component (*2*).

Lignans are reported to aid in the prevention of several chronic diseases. In vivo rat and mice studies suggest a growth attenuating effect of the enterolignan ENL (**6**) on, respectively, breast and colon cancer cells (*3, 4*). SDG was reported to delay the onset of type I and II diabetes in an in vivo rat model due to its antioxidant action (*5, 6*). In an epidemiological study with Finnish men, high serum ENL concentrations were related to a decrease in mortality caused by cardiovascular disorders (*7*). Data from in vitro studies suggest that ENL displays a higher biological activity than its plant precursors (*8, 9*). A prerequisite for the biological activation of SDG into enterolignans is the release from the plant matrix after ingestion and bioactivation through gut microbial action (*10, 11*).

The lignan SDG can be deglycosylated into SECO (**2**) and subsequently converted into the enterolignans END (**5**) and ENL (*9*). In flax seed SDG is present as a macromolecular structure bound by the linker molecule hydroxymethyl glutaric acid (HMGA, **9**) (*12*). Coumaric acid glucoside (CouAG, **7**), ferulic acid glucoside (FeAG, **8**), and herbacetin diglucoside (HDG, **10**) are also constituents of the macromolecule (*13*) (**Figure 1**). Deglycosylation of SDG can be performed from the small intestine onward either by enzymatic activity of the brush border of the gut mucosa or by bacterial enzymatic activity (*14*). It is still unknown in which part of the gastrointestinal tract SDG is released from the macromolecule. The pathway of enterolignan formation by axenic bacterial cultures was determined to start

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Table 1. Annotation of the Metabolites of SECO Produced in Twin 1 and Twin 2, As Determined by RP-HPLC/MS (Negative Mode) and MS/MS Detection

component	*m/z* ratio $[M - H]^-$	MS/MS
SECO (**2**)	361.3	165.1,[a] 179.1,[a] 222.9, 346.2[a]
2-(3-hydroxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)butane-1,4-diol (**3**)	331.3	*165.2,*[b] *179.3, 223.4, 283.3, 301.3*, 316.2
2-(3-hydroxybenzyl)-3-(3-methoxybenzyl)butane-1,4-diol (**4**)	315.2	*131.6, 164.9, 241.2, 271.2*, 297.2
END (**5**)	301.3	131.2,[a] 241.2,[a] 253.2,[a] 271.3,[a] 283.2[a]
ENL (**6**)	297.2	130.8,[a] *165.1*, 189.3,[a] 253.3[a]

[a] Also described in Eklund et al. (*44*) and Kang et al. (*45*). [b] Compounds in italic match frangments of SECO, END, or ENL.

with demethylation and dehydroxylation of SECO followed by a dehydrogenation of END into ENL (*15–17*). However, the effects of a complex intestinal microbiota on the production pathway, simulating an in vivo situation, have not been established until present.

Factors controlling the bioactivation of lignans in the large intestine are diet, transit time, redox state of the intestine, and most importantly the composition and activity of the colonic microbiota (*18*). Due to variations of these factors, large differences in bioactivation of lignans have been observed between individuals in urine, fecal, and blood samples (*19, 20*), leading to a subdivision of enterolignan producers in weak, moderate, and strong phenotypes (*21*).

In this study, the gastrointestinal fate of the enterolignan precursor SDG as part of the lignan macromolecule was investigated. A lignan supplement was subjected to an artificial stomach and small intestinal digestion to estimate the bioavailability of SDG in the upper part of the gastrointestinal tract. Long-term microbial colon fermentation was investigated in the TWINSHIME, thus determining the metabolism of SDG in the large intestine, the production pathway of enterolignans, and sites of transformation.

MATERIALS AND METHODS

Chemicals. Standards of SECO, END, and ENL for HPLC analysis were purchased from Sigma Aldrich (Bornem, Belgium). For the SHIME-trial, LinumLife EXTRA was used, a standardized 20% lignan complex (216 mg SDG g^{-1}) from flax, which was obtained from Frutarom Netherlands BV (Veenendaal, The Netherlands). Pancreatin was purchased from Sigma Aldrich (Bornem, Belgium), and Difco Oxgall (bile salts) was purchased from BD (Temse, Belgium).

Stomach and Small Intestinal Digestion of LinumLife EXTRA. To simulate passage through the stomach and small intestine, acidified SHIME feed was supplemented with 4 g L^{-1} LinumLife EXTRA (864 mg SDG L^{-1}, no free SECO was present in the product). A sufficiently high lignan concentration was chosen to facilitate detection of fragments on RP-HPLC/MS and MS/MS detection. This was incubated on a shaker at 37 °C. Samples were taken at 0 and 1.5 h (= stomach digestion). Next, pancreatic enzymes and bile salts were added, followed by further incubation at 37 °C. $NaHCO_3$ (PROLABO, Leuven, België) was added to neutralize the pH. Samples were taken at 0 and 3 h (= small intestinal digestion). The composition of the SHIME feed, pancreatic enzymes, and bile salts are described in Possemiers et al. (*22*). The experiment was performed in triplicate.

Metabolism of the Lignan Macromolecule in the SHIME. The reactor setup was adapted from the SHIME (*23*), representing the gastrointestinal tract of an adult human. A single SHIME consists of five successive reactors, simulating the stomach, small intestine, and ascending, transverse, and descending colon. The microbial community in the last three reactors is derived from a selected fresh fecal sample. Addition of a specially adapted feed and pancreatic fluid and control of temperature, pH, and retention time ensure the development and maintenance of a microbial community whose composition and activity correspond in large measure to in vivo conditions in the colon compartments. Reactor setup, inoculum preparation, and reactor feed composition were previously described (*22*). For this experiment, a new TWINSHIME setup was developed by operating two systems in parallel. Both systems were operated under identical environmental conditions, allowing simultaneous study of the metabolic and microbial activity of two enterolignan phenotypes. These identical conditions were obtained by identical pH and temperature control and using two-headed pumps for liquid transfer between the respective reactors. Good and moderate enterolignan producers were beforehand determined based on SECO transformation ability, obtained after batch in vitro incubations with different fecal samples and LinumLife EXTRA (*21*). For TWINSHIME 1 (Twin 1), an inoculum of a good enterolignan producer was selected (END+, ENL+). For TWINSHIME 2 (Twin 2), an inoculum of a moderate enterolignan producer was selected (END+, ENL−). After reactor start-up and a 3-week stabilization period, the experiment started (day 0) by adding 2 g L^{-1} LinumLife EXTRA (*21*) to the standard SHIME feed for both reactors for a period of 46 days. Every 48−72 h, 20 mL samples were taken for chemical (lignan production, fatty acid profile, and ammonia) and microbial (qPCR) analysis.

Processing of the Samples for HPLC Analysis. The samples of the stomach and small intestinal digestion and the SHIME samples for SECO-intermediate detection were extracted using SPE and analyzed using RP-HPLC-UV and RP-HPLC-MS as described by Struijs et al. (*13*). Peaks were annotated based on *m/z* ratios found on the corresponding retention times. MS/MS fragmentation data confirmed the annotations. Components **7** and **8** were annotated based on retention time (*13*). Component **1** *m/z* $[M - H]^-$ 685.3, MS/MS 361.4, 523.3; **10** *m/z* $[M - H]^-$ 625.3, MS/MS 463.2; SDG + FeAG *m/z* $[M - H]^-$ 861.4, MS/MS 523.4, 667.3, 685.3; SDG + HGMA *m/z* $[M - H]^-$ 829.3, MS/MS 523.4, 685.3, 727.2; SDG + 2HGMA *m/z* $[M - H]^-$ 973.3, MS/MS 685.3, 727.2, 829.3; 2SDG + HGMA *m/z* $[M - H]^-$ 1497.3, no MS/MS data available; 2SDG + 2HGMA *m/z* $[M - H]^-$ 1641.4, MS/MS 667.3, 685.3, 829.1. Annotations of the SECO-intermediate detection are listed in **Table 1**. The SHIME samples for SECO, END, and ENL detection were extracted using a liquid/liquid protocol with ethyl acetate and analyzed using RP-HPLC-UV according to Possemiers et al. (*21*). The analyses were performed in triplicate. Calibration curves of SECO and its metabolites END and ENL were constructed using pure standards obtained from Sigma Aldrich (Bornem, Belgium). For compounds **3** and **4**, commercial standards were unavailable.

Evaluation of Microbial Activity and Structure. Liquid samples from each colon reactor were collected and frozen at −20 °C for subsequent analysis. Microbial activity was monitored by its short chain fatty acid (SCFA) production. The SCFA were extracted from the samples with diethyl ether and determined using GC (*24*). Bacterial ammonium production was quantified according to De Boever et al. (*25*). Microbial structure of the SHIME community was determined by qPCR. Total DNA extractions of 1 mL of sample of each colon vessel of time points 0, 16, 32, and 46 days were performed following the method described by Boon et al. (*26*). Using an ABI Prism SDS 7000 instrument (Applied Biosystems, Foster City, CA), triplicate samples of 100-fold diluted DNA of general bacterial DNA as well as specific DNA from *Lactobacillus* spp., *Bifidobacterium* spp., and the *Clostridium coccoides−Eubacterium rectale* cluster were quantified by qPCR as described (*27*). DNA from *Bifidobacterium* spp. and *C. coccoides−E. rectale* was quantified using the qPCR core kit for SYBR Green I (Eurogentec, Liège, Belgium), whereas general bacterial DNA and *Lactobacillus* spp. were quantified using the PCR Master Mix SYBR Green kit (Applied Biosystems). Standard curves were constructed with cloned PCR products from representative

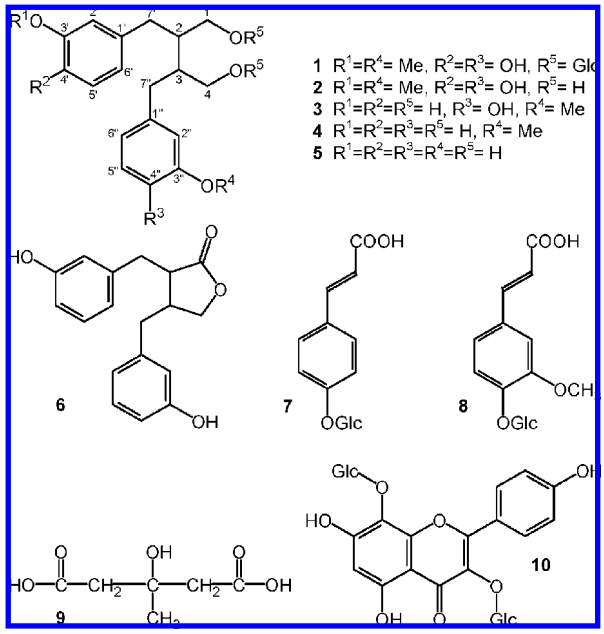

Figure 1. Structures of SDG (**1**) and its metabolites (**2**−**6**) and of the main phenolic constituents of flaxseed (**7**−**10**).

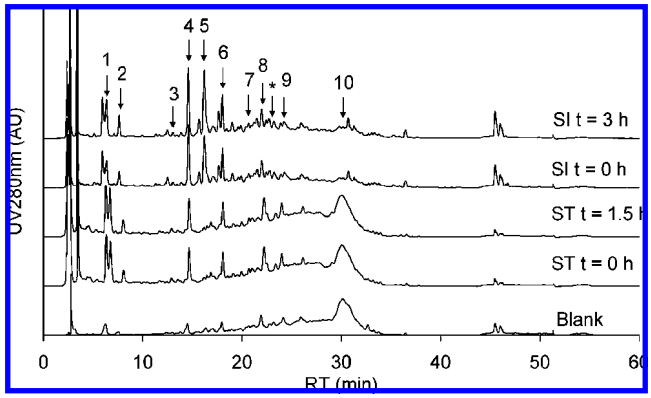

Figure 2. Analytical RP-HPLC elution profiles of the stomach and small intestinal digestion of LinumLife EXTRA: 1 = CouAG; 2 = FeAG; 3 = HDG; 4 = SDG; 5 = SDG + FeAG; 6 = SDG + HMGA; 7 = SDG + 2HMGA; 8 = 2SDG + HMGA; 9 = 2SDG + 2HMGA, 10 = lignan macromolecule. Time points (h) are listed as "Blank" = LinumLife blank; "ST" = stomach; "SI" = small intestine. An asterisk (*) marks where SECO would normally appear.

species of the different groups in a concentration range from 10^1 to 10^8 DNA copies/mL.

RESULTS

Stomach and Small Intestinal Digestion of LinumLife EXTRA. To determine the possible disintegration of the lignan macromolecule, as present in LinumLife EXTRA, in the upper part of the gastrointestinal tract, a stomach, and small intestinal digestion were performed. At times 0 and 1.5 h of the stomach digestion (**Figure 2**), the following components were detected: CouAG, FeAG, HDG, SDG (components 1−4, respectively), the lignan macromolecule (component 10), and various combinations of SDG and HMGA (SDG + HMGA, SDG + 2HMGA, 2SDG + HMGA, 2SDG + 2HMGA) (components 6−9, respectively). The stomach digestion did not have a major effect on the elution profile. Addition of pancreatic enzymes and bile salts caused an immediate change in the elution profiles at time 0 h (**Figure 2**). The area under the SDG peak increased, indicating that more SDG was released. In addition, SDG + FeAG appeared in the chromatograms (component 5). The peak of the lignan macromolecule was not detected anymore, indicating a breakdown of the macromolecule. During the remaining 3 h of small intestinal digestion, no more changes in the elution profile were detected. No SECO was detected after the stomach and small intestinal digestion.

Conversion of Plant Lignans into Enterolignans. In the ascending colon compartments of Twin 1 and Twin 2, SECO was detected 2 days after the start of the administration (**Figure 3**). Yet, SECO concentrations in the ascending colon compartments were lower than the theoretically expected ones. In Twin 1, 89% of the expected SECO concentrations were observed. In Twin 2, only 68% was detected (data not shown). SDG was still present in the ascending colon compartments. For both metabolic phenotypes, however, no further metabolism of SECO into enterolignans occurred in this part of the colon.

From the transverse colon on, both phenotypes clearly diverted from one another. In Twin 1, END production started rapidly after the appearance of SECO (from day 2). ENL production started 2 days after the END production. ENL concentrations continued to rise up to approximately 200 μM until day 20. Afterward the concentration fluctuated around 200 μM, indicating that an equilibrium in production had been reached. In the transverse colon of Twin 1, the ratio of END: ENL changed from 8.9 to 0.6 and 0.3 on days 6, 21, and 46, respectively.

Apart from SECO, END, and ENL, SDG, 2-(3-hydroxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)butane-1,4-diol (**3**), and 2-(3-hydroxybenzyl)-3-(3-methoxybenzyl)butane-1,4-diol (**4**) were also detected in the transverse colon with higher amounts of **3** than **4** (**Figure 4**). **Table 1** lists the *m*/*z* ratios and MS/MS data of the metabolites of SECO. A similar enterolignan concentration profile as in the transverse colon was observed in the descending colon. Yet, the net enterolignan production in the descending colon reactor, as obtained by subtracting the enterolignan concentrations which entered the descending colon through the overflow system from the total enterolignan concentrations in this region, only reached approximately 65 μM of END and 20 μM of ENL (data not shown). This indicates that the descending colon only contributed slightly to the enterolignan production. In this compartment, SDG and components **3** and **4** were also detected, the latter in higher amounts than **3** (**Figure 4**).

In Twin 2, END production was detected from day 2 onward in the transverse colon (**Figure 3**). It was the main enterolignan being produced in this colon segment as ENL concentrations remained below 20 μM. In addition, SDG and **3** were detected. Again, the transverse colon was the main lignan transformation site with some additional net END and ENL production in the descending colon. Component **3** disappeared almost completely in the last compartment.

Microbial Community Analysis. In both SHIME compartments, changes in the proportions of individual fatty acids were observed throughout the experiment (**Table 2**). In Twin 1, a significant increase in the valerate ($p < 0.01$) and caproate ($p < 0.001$) concentrations (to 4.2% and 5.1%, respectively) was observed in the transverse colon together with a decreasing trend in butyrate concentrations (to 12.3%). In Twin 2, caproate ($p < 0.001$) and valerate ($p < 0.01$) concentrations significantly increased in the transverse and descending colon (to 4.1% and 4.1% and 5.1 and 4.6%, respectively) with a significant decrease in butyrate ($p < 0.05$) concentrations (to 13.8% and 12.2%).

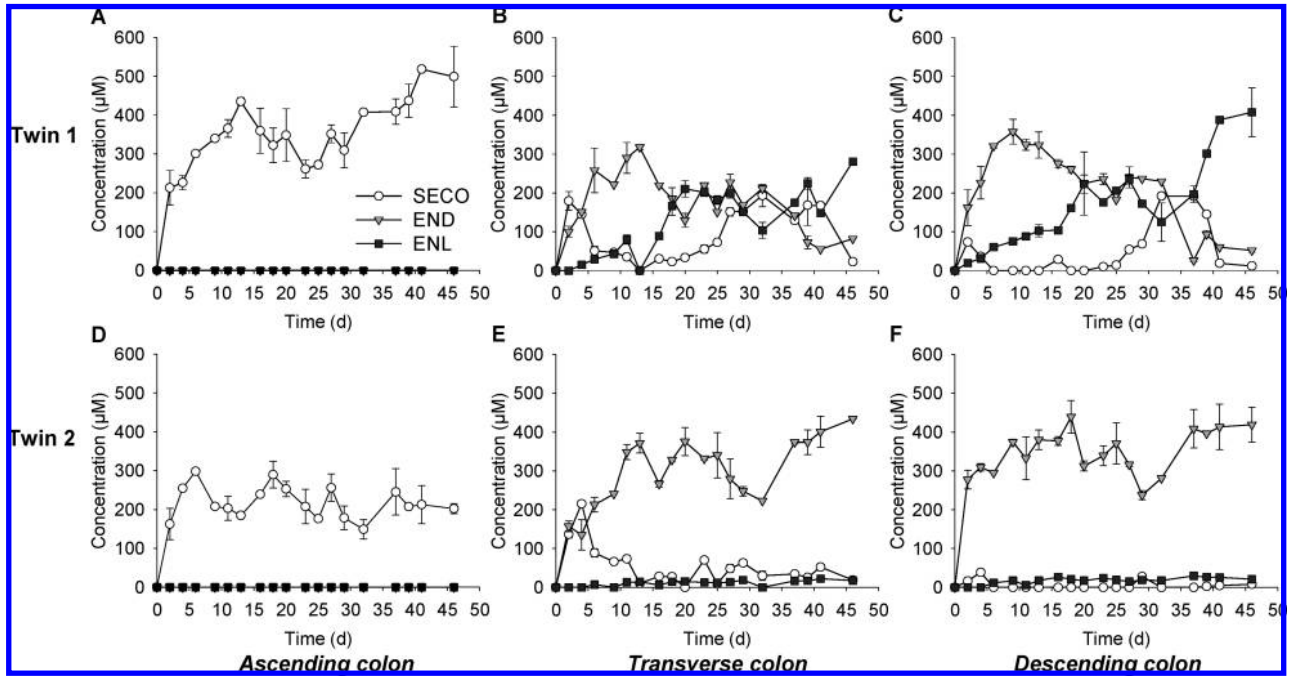

Figure 3. Lignan production profiles of SECO, END, and ENL in the ascending (**A**, **D**), transverse (**B**, **E**), and descending (**C**, **F**) colon compartments of the TWINSHIME reactor operated as Twin 1 (END+, ENL+) and Twin 2 (END+, ENL−). Values are mean ± SD, $n = 3$.

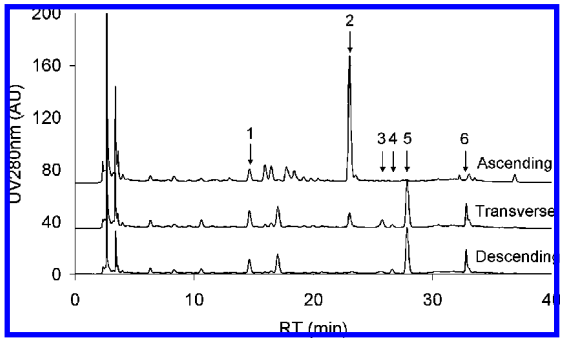

Figure 4. Analytical RP-HPLC elution profiles of the ascending, transverse, and descending colon compartments of the TWINSHIME reactor operated as Twin 1 (END+, ENL+) on day 9: 1 = SDG; 2 = SECO; 3 = 2-(3-hydroxybenzyl)-3-(4-hydroxy-3-methoxybenzyl)butane-1,4-diol (**3**); 4 = 2-(3-hydroxybenzyl)-3-(3-methoxybenzyl)butane-1,4-diol (**4**); 5 = END; 6 = ENL. Annotation of the metabolites was based on RP-HPLC/MS (negative mode) and MS/MS detection.

In the transverse colon of Twin 1 and the transverse and descending colon of Twin 2, a slight increasing trend in the ammonia concentrations was noted (data not shown).

qPCR was performed to compare the microbiota for the given enterolignan phenotypes at the start and end of the experiment. No significant differences in general bacterial DNA counts were observed between both the three colon compartments of Twin 1 and Twin 2 (data not shown, Student's *t* test). In all colon vessels of both SHIMEs, the abundance of *Lactobacillus* spp. remained constant in the range of 1−5% of total bacterial DNA. In Twin 2, bifidobacterial counts remained between 1% and 8% in the three colon vessels, whereas in Twin 1 significantly more ($P < 0.001$) bifidobacteria were observed on day 0 and day 16, reaching 20% in the transverse and descending colon. In Twin 2, the *C. coccoides*−*E. rectale* cluster represented 0−5% of the total bacterial DNA in the three colon vessels, whereas in the ascending, transverse, and descending colon of Twin 1 significantly higher amounts ($P < 0.001$) were observed during the entire experimental period, making them a dominant cluster.

DISCUSSION

LinumLife EXTRA was subjected to in vitro digestion under stomach and small intestinal conditions. The disintegration of the lignan macromolecule (**Figure 2**) indicates that most SDG released from the macromolecule was still linked to HMGA. SDG itself is not affected by in vitro stomach and small intestinal digestion (*28*). However, artificial digestion does not take into account the possible enzymatic activity of the gut epithelium or bacteria present in the small intestine (*29*). As SECO was detected in human urine (*30*), this indicates that SDG can be hydrolyzed to SECO and absorbed through the intestinal epithelium. A fraction of SECO absorbed in the small intestine would not be subjected to bioactivation but would be distributed in tissues, excreted in urine, or released into the colon via enterohepatic recirculation (*31*). As only the fraction of SECO that reaches the colon can be converted into either END or ENL, depending on lignan phenotype, it is necessary that SDG reaches the large intestine. The role of the lignan macromolecule should therefore be seen as a delivery system in the large intestine. SDG that is bound in the lignan macromolecule or conjugated with components of the macromolecule cannot be deglycosylated and will reach the large intestine. As shown in the TWINSHIME experiment (**Figure 3**), SECO was indeed released from the macromolecule in the ascending colon. In analogy, Glitso et al. (2000) showed that after administration of a rye plant matrix more than 95% of the lignans present in the ileum were conjugated and not biologically available (*32*). This indicates that a plant matrix in general is a suitable delivery system to deliver lignans at their biotransformation site.

The metabolism of SDG and its deglycosylated form SECO in the large intestine was investigated using a TWINSHIME reactor setup. As lignan transformations by human fecal microbiota are subjected to large interindividual variations, phenotypes can be subdivided into low, moderate, and high

Table 2. Short Chain Fatty Acid Production in the Ascending, Transverse, and Descending Colon Compartments of Twin 1 and Twin 2, Expressed as Percentage of Concentration of Total SCFA's, at Baseline (Day 0) and the End of the Experimental Period (Day 46)[a,b]

	Twin 1			Twin 2		
	ascending colon	transverse colon	descending colon	ascending colon	transverse colon	descending colon
total (mM)						
baseline	50.1 ± 0.2	63.5 ± 3.5	60.7 ± 0.6	54.6 ± 2.3	60.5 ± 3.3	64.1 ± 2.9
end	55.1 ± 0.8 A	63.0 ± 0.7	63.7 ± 2.5	55.9 ± 0.3	66.6 ± 0.5	67.4 ± 1.6
Ac (%)						
baseline	54.2 ± 0.4	53.6 ± 4.6	56.3 ± 0.8	51.1 ± 3.1	51.8 ± 5.3	56.8 ± 4.6
end	51.9 ± 1.2	50.6 ± 1.1	52.2 ± 4.3	55.7 ± 0.4 A	55.6 ± 0.4	58.2 ± 2.4
Prop (%)						
baseline	24.1 ± 0.1	25.1 ± 3.5	21.8 ± 0.6	22.2 ± 2.3	23.2 ± 2.5	21.5 ± 2.2
end	27.8 ± 1.0 A	23.2 ± 0.5	23.7 ± 1.1	21.1 ± 0.1	17.7 ± 0.5	17.4 ± 1.2
But (%)						
baseline	17.4 ± 0.1	16.7 ± 2.8	12.1 ± 0.4	22.6 ± 3.1	20.7 ± 2.4	16.5 ± 1.5
end	15.1 ± 0.6	12.3 ± 0.1	12.4 ± 0.5	18.9 ± 0.4	13.8 ± 0.6 A	12.2 ± 0.9 A
Val (%)						
baseline	0.1 ± 0.0	0.2 ± 0.0	3.4 ± 0.4	0.2 ± 0.0	0.3 ± 0.0	1.7 ± 0.1
end	0.4 ± 0.3	4.2 ± 0.2 B	4.7 ± 0.2 B	0.4 ± 0.2	4.1 ± 0.1 B	4.1 ± 0.3 B
Cap (%)						
baseline	ND[c]	ND	4.6 ± 0.2	ND	ND	ND
end	ND	5.1 ± 0.1 C	5.0 ± 0.2	ND	5.1 ± 0.2 C	4.6 ± 0.4 C

[a] Values are mean ± SD, $n = 3$. Ac = acetate; Prop = propionate; But = butyrate; Val = valerate; Cap = caproate. [b] Means with letter differ from baseline: A, $P < 0.05$; B, $P < 0.01$; C, $P < 0.001$; as determined by Student's t test (paired). [c] ND = not detected.

producers (*21*). Consequently, a moderate and high enterolignan producer phenotype was selected. These differences in phenotypes were clearly mirrored in the recorded enterolignan production profiles (**Figure 3**). The observed differences in the establishment of the steady-state production of END and ENL, respectively, after 1 and 3 weeks, are in contrast with the bioactivation of isoflavones and prenylflavonoids, where production of, respectively, equol and 8-PN does not require such a lengthy period to reach equilibrium (*27, 33*). This might indicate that ENL-producing bacteria are subdominant and require more time to reach full potential. Indeed, Clavel et al. (2005) stated that production of enterolignans is carried out by phylogenetically and functionally distant related anaerobic bacteria and that ENL-producing bacteria are subdominant members of the colon microbiota (*15*). As a consequence, the ratio of enterolignans in Twin 1 changed in favor of ENL toward the end of the experimental period. In this study, ENL could only be formed by dehydrogenation of END, so there is a certain precursor effect in the change of the ratio of END:ENL. This change in ratio was also observed in rats after ingestion of SECO for 10 days, during which the END:ENL ratio changed from 2.7 to 0.42 (*34*). On the basis of these observations, chronic exposure to lignans would be preferential to acute exposure in order to obtain an optimal and enduring biological effect. In Twin 2, chronic administration of lignans did not induce a shift in phenotype. Throughout the experiment, very small amounts of ENL were produced, indicating that the phenotypes remained stable. This is in accordance with observations on equol production phenotypes that remain stable over a prolongued period of time (*35*).

The pathway of END formation could be observed in the transverse colon. In Twin 1, SECO intermediates were components **3** and **4**. In Twin 2, only **3** was found. Study of lignan metabolism by axenic bacterial cultures and mixed consortia showed that SECO is first completely demethylated and then dehydroxylated (*17, 36*). In this study of SECO metabolism by complex intestinal microbiota, it is shown that dehydroxylation was possible from the moment one methyl group was taken from the molecule. On the basis of these results, at least a single demethylation of SECO is necessary before dehydroxylation can occur. It seems that the metabolism, or at least the dehydroxylation, can be carried out faster when diverse fecal microbiota are present as opposed to when less complex cultures are used. Because of the accumulation of single demethylated products in the SHIME, demethylation seems to be the rate-limiting step in formation of END.

The transverse colon was the main production site of enterolignans. In the environment of the SHIME reactor redox potential, pH and substrate availability may influence these conversions by influencing the microbiota. As enterolignan-producing bacteria require anaerobic conditions (*17*), redox potentials need to be sufficiently low. pH can influence the action of bacterial enzyme systems. The ascending colon is also characterized by faster bacterial growth due to higher carbohydrate fermentation (*37*). Substrate is more readily available and bacteria harvest energy from easily fermentable substrates before fermenting more complex compounds like lignans. In vivo data concerning pharmacokinetics of lignans also indicate that enterolignans are produced in the transverse colon. Enterolignans appear in plasma after 8−10 h (*38*). This time lag might also be consistent with movement of a food matrix through the gastrointestinal tract until the transverse colon since the stomach contents can reach the end of the small intestine after 2−6 h (*39, 40*).

The microbial activity and composition of the ascending, transverse, and descending colon were monitored to ensure representative colon environments. Microbial activity in terms of SCFA proportions changed throughout the experiment. The most remarkable changes occurred in caproate concentrations, which rose to a higher than normal (0.9−1.6%) level, and in butyrate concentrations, which decreased simultaneously to a lower than normal (16−25%) level (*37*). Caproate formation from butyrate has been observed before (*41*), so this might be the result of cross-feeding. The abundance of lactobacilli and bifidobacteria was found to be in the normal range throughout the entire experimental period. Interestingly, the dominance of the *C. coccoides*−*E. rectale* cluster in Twin 1 coincided with the strong ENL producer phenotype. Bacteria from this cluster are involved in the metabolism of not only lignans but also other phytoestrogens, the prenylflavonoids, and isoflavones (*15, 17, 27, 42, 43*). As only two samples of enterolignan producers were investigated in this study, no sound conclusions can be drawn on the role of

the *C. coccoides*−*E. rectale* cluster. More research is needed into the relationship between this microbial cluster and phytoestrogen metabolism.

In conclusion, the lignan macromolecule proved to be a suitable delivery system for SDG in the large intestine. SECO was only released from the lignan macromolecule through microbial action in the ascending colon, after which it was further transformed into enterolignans from the transverse colon onward. Single demethylation was a first step in the bioactivation, followed by dehydroxylation. Enterolignan phenotypes remained stable throughout the experimental period, with a slower establishment of ENL steady-state production, indicating the need for chronic exposure to lignans to ensure an optimal biological effect.

ABBREVIATIONS USED

SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; END, enterodiol; ENL, enterolactone; SHIME, simulator of the human intestinal microbial ecosystem; HMGA, hydroxymethyl glutaric acid; CouAG, coumaric acid glucoside; FeAG, ferulic acid glucoside; HDG, herbacetin diglucoside; SPE, solid-phase extraction; RP-HPLC-UV, reverse-phase-high-performance liquid chromatography-ultraviolet; RP-HPLC-MS, reverse-phase-high-performance liquid chromatography-mass spectrometry; SCFA, short chain fatty acid; GC, gas chromatography; qPCR, quantitative Polymerase Chain Reaction; Me, methyl; OH, hydroxyl.

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