RESEARCH ARTICLE

Bacterial transformation of dietary lignans in gnotobiotic rats

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Abstract

The bioactivity of lignans depends on their transformation by gut bacteria. The intestinal bacteria *Clostridium saccharogumia*, *Eggerthella lenta*, *Blautia producta* and *Lactonifactor longoviformis* convert the plant lignan secoisolariciresinol diglucoside via secoisolariciresinol (SECO) into the bioactive enterolignans enterodiol (ED) and enterolactone (EL). While the *in vitro* conversion of lignans by these bacteria has already been demonstrated, it is unclear whether this defined community is also capable of catalysing lignan transformation *in vivo*. We therefore associated germ-free rats with these four species. Germ-free rats served as a control. All animals were fed a diet containing 5% ground flaxseed. The caecal contents of rats associated with the four lignan-activating bacteria (ALB rats) contained SECO, ED and EL. The maximal EL formation rate from lignans in the pooled caecal contents of ALB rats was 7.52 nmol min^{-1} g^{-1} dry matter. The ALB rats excreted EL, but no SECO and ED, in their urine. The caecal contents of germ-free rats contained SECO, but no ED and EL. Their urine was devoid of lignans. Hence, the presence of enterolignans in the ALB rats, but not in the germ-free rats, demonstrates that this defined microbial community is capable of transforming plant lignans into EL *in vivo*.

Introduction

Lignans are diphenolic compounds likely acting as phytoalexins in plants (Ayres & Loike, 1990). The lignan secoisolariciresinol diglucoside (SDG) is one of the most abundant dietary lignans and flaxseed is one of the best sources of SDG, with contents varying from approximately 1–26 mg g^{-1} flaxseed (Milder *et al.*, 2005; Muir, 2006). Dietary lignans are proposed to prevent breast (Thompson *et al.*, 1996; Wang *et al.*, 2005), colon (Serraino & Thompson, 1992; Jenab & Thompson, 1996; Bommareddy *et al.*, 2006, 2009) and prostate cancer (Lin *et al.*, 2002; Demark-Wahnefried *et al.*, 2008), atherosclerosis (Prasad, 1999, 2005, 2008, 2009) and diabetes (Prasad, 2001; Pan *et al.*, 2007; Zhang *et al.*, 2008). However, the reported beneficial effects of lignans depend on their conversion into the enterolignans enterodiol (ED) and enterolactone (EL) by human intestinal bacteria. *In vitro* data indicated that SDG is stable in the upper part of the gastrointestinal tract (Clavel *et al.*, 2006a). However, it is still unknown to what extent enzymes of the intestinal brush border contribute to the deglycosylation of SDG, as shown for flavonoids (Day *et al.*, 1998; Lambert *et al.*, 1999; Németh *et al.*, 2003). ED is produced by dominant members of the gut microbial community, while subdominant species produce EL (Clavel *et al.*, 2006b). The first step leading to the production of EL is the deglycosylation of SDG to secoisolariciresinol (SECO) (Wang *et al.*, 2000; Clavel *et al.*, 2006b). Many bacterial species are capable of catalysing this reaction (Clavel *et al.*, 2006b), which is probably not a rate-limiting step in the production of enterolignans in the human gut (Clavel *et al.*, 2006a). Although *Bacteroides* species play an important role in the deglycosylation of SDG in the gut (Clavel *et al.*, 2006b), *Clostridium* species are also capable of deglycosylating SDG. The recently isolated species *Clostridium saccharogumia*, a subdominant member of the human gut microbiota, showed the highest initial conversion rate of SDG to SECO compared with the other bacterial species tested (Clavel *et al.*, 2006a). The second step in the transformation of SDG to EL is the demethylation of SECO to the intermediate 2,3-bis-(3,4-dihydroxy-benzyl)butane-1, 4-diol. Several bacterial species, including *Eubacterium*

limosum and *Blautia producta*, catalyse this reaction (Wang *et al.*, 2000; Clavel *et al.*, 2006b). Subsequently, 2,3-bis-(3,4-dihydroxy-benzyl)butane-1,4-diol is dehydroxylated to ED. *Eggerthella lenta* catalyses this reaction enantioselectively (Jin *et al.*, 2007). Co-incubation of *B. producta* and *E. lenta* in SECO-containing media results in the formation of ED. *E. lenta* alone does not show any dehydroxylating activity on SECO, but is able to reduce the plant lignans pinoresinol and lariciresinol (Clavel *et al.*, 2006a). The last step in the transformation of SDG to EL is the dehydrogenation of ED to EL. A recently isolated bacterial species, *Lactonifactor longoviformis*, a subdominant member of the human intestinal microbiota, catalyses the formation of EL (Clavel *et al.*, 2006b). These results show that more than one species is capable of catalysing the first three steps of lignan activation. In contrast, so far, only one species, namely *L. longoviformis*, is known to dehydrogenate ED to EL. This does not exclude the possibility that intestinal species other than *L. longoviformis* catalyse the latter reaction as well.

The bioactivation of the plant lignan SDG requires the interaction of different bacterial species. To investigate this interaction, we chose four bacterial species known to be involved in lignan activation: *C. saccharogumia* (*O*-deglycosylation), *E. lenta* (*O*-demethylation), *B. producta* (dehydroxylation) and *L. longoviformis* (dehydrogenation). A previous *in vitro* study demonstrated that the coincubation of these four bacterial species with SDG results in the formation of EL from SDG (Blaut & Clavel, 2007). To determine whether this bacterial consortium is also capable of activating lignans under *in vivo* conditions, we associated germ-free rats with these four species. We determined the concentrations of enterolignans in urine, faeces and gut contents of the animals in response to feeding a 5% flaxseed diet.

Materials and methods

Chemicals

SDG was a generous gift from Peter Winterhalter (Technical University of Braunschweig), who isolated SDG from flaxseed as described previously (Degenhardt *et al.*, 2002). SECO and EL were purchased from Sigma-Aldrich (Taufkirchen, Germany), ED from the VTT Technical Research Centre of Finland (Espoo, Finland). All lignans were racemic. Stock solutions of SDG (100 mM) were prepared in water. Stock solutions of SECO (100 mM), ED (50 mM) and EL (50 mM) were prepared in methanol.

Bacterial strains and culture conditions

Cryostocks of *C. saccharogumia* DSM 17460^T, *Blautia producta* DSM 3507, *E. lenta* DSM 2243^T and *L. longoviformis* DSM 17459^T were obtained from the collection of the German Institute of Human Nutrition Potsdam-Rehbruecke. The organisms were cultured at 37 °C under strictly anoxic conditions (Hungate, 1969; Bryant, 1972) in Brain Heart Infusion Broth (Roth, Karlsruhe, Germany) supplemented with 5 g L^{-1} yeast extract (Roth) and 5 mg L^{-1} haemin (Sigma-Aldrich) (YH-BHI). The strains were subcultured every 2 weeks and stored at 4 °C. Purity was checked by inspecting the colony morphology after anaerobic growth on YH-BHI agar (Oxoid, Basingstoke, UK) and the cell morphology after Gram staining. All strains were streaked on YH-BHI agar and incubated aerobically to detect any aerobic contaminants.

Animals and experimental design

Ten eight-week-old male germ-free Sprague–Dawley rats were purchased from Charles River (Wiga, France). They were kept in positive-pressure isolators (Metall & Plastik, Radolfzell, Germany) under a 12-h light cycle, constant temperature and air humidity. The rats had free access to autoclaved distilled water and a diet sterilized by gamma irradiation (25 kGy; Gamma Service Produktbestrahlung GmbH, Radeberg, Germany). The experiments and the maintenance of the rats were approved by the local animal welfare committee (approval no. 23-2347-8-16-2008). After five days on a standard diet (1314, Altromin, Lage, Germany), the rats were assigned either to the experimental or to the control group ($n = 5$ per group). The rats of the experimental group were associated on 2 consecutive days with the lignan-activating bacteria *C. saccharogumia*, *B. producta*, *E. lenta* and *L. longoviformis*, hereafter referred to as ALB rats. The inoculum was freshly prepared by growing each bacterial species in YH-BHI. The cell densities were determined using a counting chamber and the liquid cultures were subsequently combined. The final inoculum (500 μL) containing 10^8 cells of each species was applied intragastrically to each rat. The control rats remained germ free. Three days after the first colonization, both groups were switched from the standard diet to a diet containing 5% ground flaxseed, 58% wheat starch, 20% casein, 5% sunflower oil, 5% cellulose, 5% mineral mixture and 2% vitamin mixture. The rats were fed the experimental diet for 12 days. Over the last 48 h, all rats were kept in metabolic cages and faeces and urine were collected. Finally, the rats were anaesthetized with isofluran (Sigma-Aldrich) and decapitated. Faeces as well as caecal and colonic contents were freeze-dried (freeze-drying system alpha 1–4, Christ, Osterode, Germany) and stored at 4 °C until analysis.

FISH and quantification of bacterial cells

The successful colonization and the relative proportions of the four lignan-activating bacteria were evaluated using species-specific oligonucleotide probes (Table 1).

Table 1. Cy3-labelled oligonucleotide probes used for fluorescence *in situ* hybridization to quantify bacteria

Probe	Sequence (5′–3′)	Target organism	Hybridization temperature (°C)	Reference
S-S-Csac-0067-a-A-20	CTC GGA CAT TAC TGC CCG CG	*Clostridium saccharogumia*	46	Clavel *et al*. (2006a)
S-∗-ProCo-1264-a-A-23	TTG GGA TTC GCT CAA CAT CGC TG	*Blautia producta*	35	Clavel *et al*. (2005)
S-∗-Ato-0291-a-A-17	GGT CGG TCT CTC AAC CC	*Eggerthella lenta*	54	Harmsen *et al*. (2000)
S-S-Llong-0831-a-A-20	GGA CGC CTT TGG CGC CCG AC	*Lactonifactor longoviformis*	46	This paper

The probes were validated for their species specificity within the consortium. They were labelled with the fluorescent dye Cy3 at their 5′-end. FISH was performed as described previously (Thiel & Blaut, 2005) with the following modifications: the fixed samples were sonicated (10 s, 0.5 cycles, 20% amplitude) in addition to homogenization with a Uniprep-Gyrator (UniEquip, Martinsried, Germany). The concentration of each oligonucleotide probe was 10 μM in hybridization buffer. Then, 1 μL was applied to each well. Hybridization buffer containing 30% (v/v) formamide was used for the probe ProCo-1264. After an overnight hybridization in a moist chamber, the slides were washed with hybridization buffer for 20 min at 2 °C above the hybridization temperature. Bacterial cells were counted with fluorescence microscopy as described previously (Reichardt *et al*., 2009).

Measurement of SDG in ground flaxseed, experimental diet, caecal contents and faeces

The lignan-containing samples (100 mg) were defatted for 2 h with *n*-hexane and extracted for 17 h with 3 mL methanol/water (70/30, v/v). After centrifugation (3000 ***g***, 10 min), the supernatants were collected and the pellets were washed twice with methanol/water (70/30, v/v). The supernatants of the wash steps were pooled. Methanol was evaporated (speed vac RC 10-22, Jouan, Dreieich, Germany) and residual water was removed by lyophilization (Christ). The lyophilisates were dissolved in 0.5 mL 1 M NaOH for 3 h, neutralized with HCl and freeze-dried again. The lyophilisates were suspended in 75–80 μL methanol/water (70/30, v/v) and analysed by high-performance liquid chromatography (HPLC).

Measurement of SECO, ED and EL in caecal and colonic contents

Free lignans were determined as described above with the following modifications: approximately 100 mg of gut contents were extracted with 0.6 mL methanol/water (70/30, v/v) by shaking for 10 min. Lyophilisates were suspended in 50 μL methanol/water (70/30, v/v) and analysed by HPLC. In case of the conjugated lignans, only caecal contents provided sufficient material to determine glucuronidated and sulphated fractions. Caecal contents (100 mg) were suspended in 600 μL water and mixed with 534 μL sodium acetate buffer (0.29 M, pH 5) and 144 μL of a β-glucuronidase/sulphatase preparation (type HP-2; 102 000/ $\leq$ 7500 U mL^{-1}; Sigma-Aldrich). The samples were incubated for 16 h at 37 °C, followed by extraction and lyophilization as described above. The lyophilisates were suspended in 65 μL methanol/water (70/30, v/v) and analysed by HPLC.

In vitro formation rates of the free lignans SECO, ED and EL in pooled caecal contents

Freshly collected caecal contents were stored on ice and kept under anoxic conditions (AnaeroGen Compact, Oxoid, Basingstoke, UK). The five samples from each group were separately pooled in an anoxic workstation (MAKS MG, Meintrup DWS, Laehden, Germany). The pooled samples were aliquoted in tubes (0.5 g each) and incubated at 37 °C under anoxic conditions ($N_2/CO_2/H_2$, 80/10/10, v/v/v). At 0, 1, 2, 4 and 8 h, the incubation was stopped by freezing the tubes in liquid nitrogen. The samples were freeze-dried (Christ) and subsequently extracted with methanol/water (70/30, v/v) as described above. The lyophilisates were suspended in 100 μL methanol/water (70/30, v/v) and analysed by HPLC.

Measurement of ED and EL in urine

Urine was thawed, centrifuged (1000 ***g***, 15 min, 4 °C) and the supernatants were filtered (0.45 μm, Roth). Lignans were extracted from 0.2 mL of the supernatants with 0.6 mL ethyl acetate by shaking for 10 min. After centrifugation (3000 ***g***, 10 min), the upper ethyl acetate phase containing the lignans was collected. These steps were repeated two times and the ethyl acetate phases combined. Samples were evaporated (Jouan), the residues were dissolved in 33 μL methanol/water (70/30, v/v) and analysed by HPLC.

Glucuronidated/sulphated lignans were deconjugated within 16 h of incubation with hydrolytic enzymes at 37 °C. Urine (200 μL) was mixed with 178 μL sodium acetate buffer (0.29 M, pH 5) and 48 μL β-glucuronidase/sulphatase (type HP-2, Sigma-Aldrich). The lignans were extracted as described above.

Detection of lignans by HPLC

Samples were centrifuged (13 000 ***g***, 3 min) and the supernatants were analysed. Lignans were measured using an HPLC system with a diode array detector (Gynkotek, Germering, Germany). The HPLC system was equipped with a pump Model 480, degasser DG-503, autosampler GINA 50, a column oven, a UV/Vis diode array detector UVD-320 and a reversed-phase C_{18} column (LiChroCart 250-4 LiChrospher 100 RP-18, 250 × 4 mm, 5 μm; Merck, Darmstadt, Germany) protected with a guard RP-18 column (4 × 4 mm, 5 μm). The column temperature was maintained at 37 °C. The flow rate was 0.3 mL min^{-1} and the injection volume was 20 μL. Lignans were detected at 285 nm. For data acquisition, the software Chromeleon version 6.40 (Dionex, Idstein, Germany) was used.

The mobile phase was a mixture of 50 mM sodium acetate (pH 5)/methanol (80/20, v/v) (solvent A), as well as 50 mM sodium acetate/methanol/acetonitrile (40/40/20, v/v/v) (solvent B). The gradient was 20–80% B within 10 min, 80–100% B within 14 min, 100% B for 11 min and back to 20% B within 1 min. The lignans were quantified using calibration curves ranging from 10 to 1500 μM. The retention times of the standard lignans were: SDG, 20.4 min; SECO, 25.8 min; ED, 29.2 min; and EL, 32.9 min. Lignans were identified by comparison with the retention times and spectra of the standard lignans.

Recovery of lignans in the analysed materials

Freeze-dried faeces (100 mg) of a germ-free rat containing no lignans were spiked with 90 μL of each SDG, SECO and ED solution (100 μM) and 35 μL of an EL solution (250 μM). The faeces were extracted with methanol/water (70/30, v/v) as described above and analysed by HPLC. For each lignan, the recovery was determined using three independent spiked faecal samples. Recoveries were as follows: 91.8 ± 6.95% for SDG, 52.8 ± 1.71% for SECO, 62.1 ± 0.80% for ED and 47.5 ± 0.95% for EL.

The recovery of lignans in urine was determined by spiking 200 μL urine of a germ-free rat containing no lignans with 15 μL of each SDG, SECO, ED and EL solution (1.5 μM). The lignans were extracted with ethyl acetate as described above and analysed by HPLC. The recovery of each lignan was determined using three independent spiked urine samples. Recoveries were as follows: 84.2 ± 6.96% for SECO, 97.2 ± 1.97% for ED and 100 ± 6.05% for EL. SDG was not recovered after ethyl acetate extraction.

Statistical analysis

The statistical package for the social sciences for Windows version 14.0 (SPSS Inc., Chicago, IL) was used to conduct the statistical analysis. Values were tested for normal distribution using the Kolmogorov–Smirnov test. Differences in the caecal and faecal lignan concentrations between ALB and germ-free rats were checked for significance using an unpaired *t*-test. Data are presented as their mean ± SD.

Results and discussion

The lignan-activating bacteria *C. saccharogumia*, *E. lenta*, *B. producta* and *L. longoviformis* activate the plant lignan SDG under *in vitro* conditions (Clavel *et al.*, 2006b). To investigate their ability to activate SDG *in vivo*, these four species were introduced into the gastrointestinal tract of germ-free rats (ALB rats). Germ-free animals were used as a control. All rats were fed a diet containing 5% ground flaxseed.

The four lignan-converting bacteria colonized the intestinal tract of the rats

Because the establishment of the lignan-activating consortium was a prerequisite for successfully conducting the study, bacteria were enumerated in caecal and colonic samples using FISH. Data analysis revealed the presence of the four lignan-activating bacterial species in the caecal and colonic contents of the ALB rats, demonstrating their ability to establish and persist in the rat intestinal tract. Bacterial cell numbers are given in Table 2. The bacterial proportions did not differ significantly between the caecum and the colon of the ALB rats. *Blautia producta* accounted for approximately 58% of the total bacteria in both the caecum and the colon, while *C. saccharogumia* accounted for 26% in the caecum and 23% in the colon. The proportion of *E. lenta* was 11% in the caecum and 14% in the colon, while *L. longoviformis* amounted to approximately 5% in both gut sections.

SDG concentrations in flaxseed and in the faeces of gnotobiotic rats

Before starting the *in vivo* experiment, we determined the SDG content in ground flaxseed and in the experimental

Table 2. Proportions of lignan-activating bacteria in the caecal and colonic contents of the rats associated with these four species; cells were enumerated by fluorescence microscopy

	Count (cells g^{-1} DM)	
Species	Caecum	Colon
Clostridium saccharogumia	$3.39 \times 10^{10} \pm 1.17 \times 10^{10}$	$5.21 \times 10^{10} \pm 1.74 \times 10^{10}$
Blautia producta	$7.44 \times 10^{10} \pm 2.56 \times 10^{10}$	$1.31 \times 10^{11} \pm 7.22 \times 10^{10}$
Eggerthella lenta	$1.47 \times 10^{10} \pm 4.04 \times 10^{9}$	$3.25 \times 10^{10} \pm 1.35 \times 10^{10}$
Lactonifactor longoviformis	$7.13 \times 10^{9} \pm 1.64 \times 10^{9}$	$1.10 \times 10^{10} \pm 2.70 \times 10^{9}$

Values are means ± SD, $n = 5$.

diet. Because SDG in flaxseed is incorporated into a lignan macromolecule, we decided to cleave the ester bonds by alkaline hydrolysis, thereby releasing the SDG from macromolecules, as reported previously (Struijs, 2008). The SDG content in the ground flaxseed was $3.09 \pm 0.19\ \mu mol\ g^{-1}$, which is in the lower range of SDG contents reported for flaxseed (Muir, 2006). Accordingly, the SDG content of the diet was $0.15 \pm 0.02\ \mu mol\ g^{-1}$.

After 10 days on the flaxseed diet, all rats were transferred to metabolic cages. Faeces were collected for 48 h and analysed for SDG. Faeces of the ALB rats contained $0.29 \pm 0.09\ \mu mol\ SDG\ g^{-1}$ dry matter (DM), while the faeces of germ-free rats contained $1.39 \pm 0.39\ \mu mol\ SDG\ g^{-1}$ DM ($P \leq 0.01$). Significantly lower faecal concentrations of SDG in the ALB rats compared with the germ-free rats were the first indication that the microbial community in the ALB rats converted the dietary lignan SDG.

Levels of SDG and the metabolites SECO, ED and EL in caecal and colonic contents

After 48 h in metabolic cages, the rats were killed and their caecal and colonic contents were analysed for SDG and the metabolites thereof. The caecal contents of the ALB rats contained significantly lower SDG concentrations than those of the germ-free rats ($P \leq 0.001$; Table 3). The material collected from the colon was insufficient to determine the SDG concentrations. Caecal and colonic contents were also analysed for the unconjugated lignan metabolites SECO, ED and EL. Caecal contents were in addition examined for lignan conjugates. Free SECO was neither detected in the caecal and colonic contents of germ-free rats nor of ALB rats. After β-glucuronidase/sulphatase treatment of the caecal contents, the caecal SECO concentrations did not differ significantly between ALB rats and germ-free rats (Table 3). The presence of SECO in the germ-free rats may be explained as follows: (i) brushborder enzymes of the epithelium possibly deglycosylated SDG to SECO as reported for other phenols (Day *et al.*, 1998). In this case, host enzymes would catalyse the first step of the SDG activation and *C. saccharogumia* would not be required. (ii) The β-glucuronidase/sulphatase preparation used for the deconjugation of SECO might have had some deglycosylating activity, as it stems from *Helix pomatia*, which may contain traces of β-glucosidase (Robinson, 1956). However, based on *in vitro* experiments, we can exclude that the deglycosylation of SDG to SECO was due to hydrolysis in the upper part of the gastrointestinal tract (Clavel *et al.*, 2006a; Eeckhaut *et al.*, 2008).

Table 3. Caecal concentrations of free and conjugated lignans in germ-free rats and rats associated with ALB

	Concentration ($\mu mol\ g^{-1}$ DM)	
Lignan	ALB rats	Germ-free rats
SDG	0.52 ± 0.07***	1.89 ± 0.15
SECO		
Free	BD*	BD*
Conjugated	0.41 ± 0.08	0.35 ± 0.11
ED		
Free	BD*	BD*
Conjugated	0.05 ± 0.01	BD†
EL		
Free	0.37 ± 0.12	BD*
Conjugated	0.58 ± 0.17	BD†

Free SDG was determined after alkaline hydrolysis to release it from the lignan macromolecule. Free SECO, ED and EL were determined without any treatment. Conjugated lignans were determined after treatment with β-glucuronidase/sulphatase.

Values are means ± SD, $n = 5$; data are normally distributed (Kolmogorov–Smirnov test); significance was checked using an unpaired *t*-test.

***Value was significantly different from that of the germ-free rats ($P \leq 0.001$).

*Below the detection limit (BD) $< 5.0\ nmol\ g^{-1}$ DM.

†BD $< 6.5\ nmol\ g^{-1}$ DM.

Free ED was detected in the gut contents of neither the ALB rats nor the germ-free rats. Following deconjugation treatment, ED was detected in the caecal contents of the ALB rats, but not of the germ-free rats (Table 3). EL is the final metabolite formed during bacterial lignan transformation. EL was found in the caecal and colonic contents of the ALB rats. β-glucuronidase/sulphatase treatment of the caecal contents revealed the presence of conjugated EL in the ALB rats, while neither form of EL was detectable in the caecal or the colonic contents of the germ-free rats (Table 3). While 61% of the total caecal EL concentration of the ALB rats was glucuronidated or sulphated, 39% was unconjugated. The occurrence of ED and EL in the ALB rats, but not in the germ-free rats provides evidence that ED and EL were of bacterial origin. It may be concluded that the bacterial species *B. producta*, *E. lenta* and *L. longoviformis* catalysed *O*-demethylation, dehydroxylation and dehydrogenation to produce EL from SECO in the rat intestinal tract. The low concentration of ED in the gut contents, which we observed in our study, is in contrast to a study by Adlercreutz *et al.* (1995), who reported that faeces of Finnish women contained more ED than EL. The EL-producing *L. longoviformis* is one of the subdominant members of the human intestinal microbiota and, therefore, the dehydrogenation of ED to EL is probably the rate-limiting step in the conversion, at least in humans (Clavel *et al.*, 2006b). However, even though the concentration of *L. longoviformis* was lower than that of the other species of the defined bacterial community in the gut of the ALB rats, this species obviously catalysed the dehydrogenation reaction very effectively.

The detection of SECO, ED and EL after β-glucuronidase/sulphatase treatment of the caecal contents demonstrates that these lignans are preferentially present as glucuronides

or sulphates and that they undergo enterohepatic circulation. We were unable to distinguish between enterolignan glucuronides and sulphates, but it is known that conventional rats excrete biliary ED and EL mainly as glucuronides and only traces of lignan sulphates are formed (< 1%) (Axelson & Setchell, 1981).

Flaxseed also contains small amounts of the plant lignans matairesinol and pinoresinol (Meagher *et al.*, 1999), which can also be metabolized to ED and EL (Heinonen *et al.*, 2001). Because *E. lenta* is also able to reduce pinoresinol and lariciresinol, it is likely that these plant lignans contributed to the enterolignan formation in the ALB rats.

Anaerobic *in vitro* formation of EL by pooled caecal samples

Pooled caecal contents collected from the ALB rats were examined for their ability to form the free lignan metabolites SECO, ED and EL *in vitro* from the lignans contained in the caecal material. SECO and ED were not detected in any of the samples after 0, 1, 2, 4 and 8 h of incubation. The starting concentration (0 h) of free EL in these samples was $0.58 \pm 0.10\ \mu mol\ g^{-1}$ DM. Within 2 h of incubation, the EL concentration reached $1.06 \pm 0.02\ \mu mol\ g^{-1}$ DM (Fig. 1). The maximal *in vitro* EL formation rate of 7.52 nmol $min^{-1}\ g^{-1}$ DM was observed between 1 and 2 h after the start of the incubation, demonstrating the efficient interaction of the lignan-activating bacteria. The pooled caecal contents of germ-free rats did not produce any free lignans. The material of the ALB rats and the germ-free rats was too scarce to determine the SDG concentrations.

Lignan metabolites in the urine of gnotobiotic rats

Urine samples of ALB rats and germ-free rats were analysed for lignan metabolites to investigate their urinary excretion.

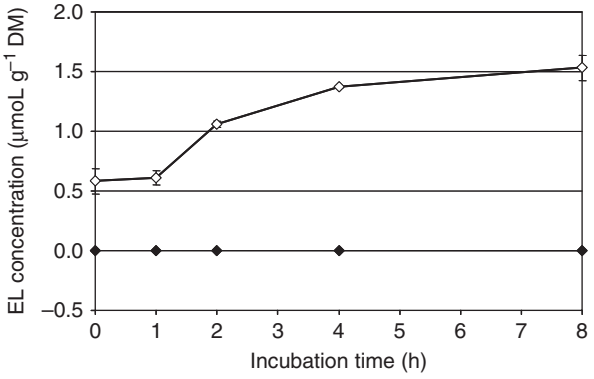

Fig. 1. Formation of unconjugated EL by pooled caecal contents of rats associated with lignan-activating bacteria (◇) and of germ-free rats (◆) under anaerobic conditions ($N_2/CO_2/H_2$, 80/10/10, v/v/v) at 37 °C. Values are means ± SD, $n = 2$. DM, dry matter.

No lignans were observed in the urine of the germ-free rats. In contrast, we detected free and glucuronidated/sulphated EL (2.61 ± 3.23 and $16.3 \pm 12.9\ nmol\ mL^{-1}$, respectively) in the urine of ALB rats, while SECO and ED were not found in any form. The proportion of free lignans in the ALB rats (10.4%) was considerably higher than reported in previous studies for human and rat urine (0.3–1%) (Axelson & Setchell, 1981; Adlercreutz *et al.*, 1991). Over 48 h, the ALB rats excreted 340 ± 226 nmol of EL in the urine. Previous publications reported a higher urinary enterolignan excretion in rats. The urine of rats fed 5% flaxseed reached enterolignan concentrations of up to 2663 ± 294 nmol within 24 h (Jenab & Thompson, 1996; Thompson *et al.*, 1996). This difference is probably due to the fact that we used gnotobiotic rats associated with only four bacterial species, while previous researchers used conventional rats with a complex microbiota. The gnotobiotic animal model used in this study is an artificial system that does not take into account the influence of the other members of the gut microbial community. In addition, the microbial status influences the metabolism of the host as evident from considerable metabolic differences between germ-free and conventional rats (Hooper *et al.*, 1999, 2003). In particular, the expression of xenobiotic-metabolizing host enzymes depends on the microbial status of the rats (Meinl *et al.*, 2009).

In conclusion, we demonstrated for the first time the *in vivo* transformation of plant lignans by a defined bacterial community. By determining the maximal *in vitro* formation rate of EL in the rat caecal samples, we demonstrated the efficient interaction of the bacterial species involved in the transformation of lignans, particularly SDG. Our results also show that the metabolites arising from plant lignans undergo hepatic circulation and are excreted in the urine.

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