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# Lignan transformation by gut bacteria lowers tumor burden in a gnotobiotic rat model of breast cancer

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**High dietary lignan exposure is implicated in a reduced breast cancer risk in women. The bacterial transformation of plant lignans to enterolignans is thought to be essential for this effect. To provide evidence for this assumption, gnotobiotic rats were colonized with the lignan-converting bacteria *Clostridium saccharogumia*, *Eggerthella lenta*, *Blautia producta* and *Lactonifactor longoviformis* (LCC rats). Germ-free rats were used as the control. All animals were fed a lignan-rich flaxseed diet and breast cancer was induced with 7,12-dimethylbenz(a)anthracene. The lignan secoisolariciresinol diglucoside was converted into the enterolignans enterodiol and enterolactone in the LCC but not in the germ-free rats. This transformation did not influence cancer incidence at the end of the 13 weeks experimental period but significantly decreased tumor numbers per tumor-bearing rat, tumor size, tumor cell proliferation and increased tumor cell apoptosis in LCC rats. No differences between LCC and control rats were observed in the expression of the genes encoding the estrogen receptors (ERs) α, ERβ and G-coupled protein 30. The same was true for *IGF-1* and *EGFR* involved in tumor growth. The activity of selected enzymes involved in the degradation of oxidants in plasma and liver was significantly increased in the LCC rats. However, plasma and liver concentrations of reduced glutathione and malondialdehyde, considered as oxidative stress markers, did not differ between the groups. In conclusion, our results show that the bacterial conversion of plant lignans to enterolignans beneficially influences their anticancer effects.**

## Introduction

Breast cancer is the most commonly diagnosed cancer and the leading cause of cancer death in women worldwide (1). Since high circulating estrogen levels have been considered as a risk factor of breast cancer (2), dietary interventions that modulate the tumor-promoting estrogen effects may be effective in breast cancer prevention. Proposed dietary interventions include a high consumption of lignan-rich food because lignans belong to the non-nutritive plant compounds with estrogenic and/or anti-estrogenic properties (3). The majority of lignans is taken up with whole grain cereals, vegetables and fruits but flaxseed is one of the richest sources of the lignan secoisolariciresinol diglucoside, SDG (4).

**Abbreviations:** CAT, catalase; DMBA, 7,12-dimethylbenz(a)anthracene; ED, enterodiol; EGFR, epidermal growth factor receptor; EL, enterolactone; ER, estrogen receptor; GPR30, G-coupled protein 30; GSH, glutathione; GST, glutathione-S-transferase; HPLC, high-performance liquid chromatography; IGF-1, insulin-like growth factor 1; MDA, malondialdehyde; PCR, polymerase chain reaction; SCFA, short-chain fatty acid; SDG, secoisolariciresinol diglucoside; SECO, secoisolariciresinol; SOD, superoxide dismutase.

In chemically induced animal models of breast cancer, flaxseed feeding reduces the incidence, number and growth of tumors at the initiation, promotion and progression stages of carcinogenesis (5,6). Similar effects were observed when SDG was applied to chemically induced or xenograph animal models, suggesting that the observed effects largely depend on the SDG content of the flaxseed (7,8).

Intestinal bacteria are capable of converting plant lignans into enterolignans. The transformation of SDG includes the deglucosylation to secoisolariciresinol (SECO) followed by the demethylation and dehydroxylation of SECO to enterodiol (ED). Finally, ED is converted to enterolactone (EL) by dehydrogenation (9). It has been concluded from epidemiologic and experimental studies that the products of bacterial SDG transformation, ED and EL, are the bioactive compounds (4). Women with high EL serum levels and high urinary EL excretion have a significantly reduced breast cancer risk (10,11). In addition, pure ED and EL inhibit breast tumor growth in a mouse cancer model (12). However, the essential role of bacterial transformation in the gut for the lignan anticancer effects has so far not been demonstrated *in vivo*.

In previous studies, we demonstrated that a consortium consisting of the commensal gut bacteria *Clostridium saccharogumia* (*O*-deglycosylation), *Blautia producta* (*O*-demethylation), *Eggerthella lenta* (dehydroxylation) and *Lactonifactor longoviformis* (dehydrogenation) is capable of forming ED and EL from SDG under *in vitro* conditions and in rats colonized exclusively with these four bacterial species (13,14). We took advantage of this gnotobiotic animal model to investigate the role of bacterial lignan transformation in breast cancer formation and selected cancer-associated parameters in a 7,12-dimethylbenz(a)anthracene (DMBA)-induced cancer model. We compared the effects of lignan feeding on breast cancer formation in germ-free rats and in rats colonized exclusively with lignan-transforming bacteria to clarify whether the bacterial activation of SDG to ED and EL is really crucial for the cancer-preventing effects of dietary lignans.

## Materials and methods

### *Bacterial strains and culture conditions*

The lignan-converting bacteria *C.saccharogumia* DSM17460$^T$, *B.producta* DSM3507, *E.lenta* DSM2243$^T$ and *L.longoviformis* DSM17459$^T$ were cultured anaerobically at 37°C in Brain Heart Infusion Broth (Roth, Karlsruhe, Germany) supplemented with 5 g/l yeast extract and 5 mg/l hemin (Sigma–Aldrich, Taufkirchen, Germany). Purity of the cultures was checked by inspecting the colony morphology after anaerobic growth on respective agar plates and the cell morphology after gram staining.

### *Animal experiment*

Three week old female germ-free Sprague Dawley rats were randomly assigned to one of the study groups ($n$ = 10, each). Animals were housed in Trexler-type plastic film isolators under controlled housing conditions (20°C ± 2°C, 55 ± 10% air humidity, 12 h light/dark cycle). All diets and the drinking water were sterilized by gamma irradiation (50 kGy) and autoclaving, respectively. Before dietary intervention, animals were fed a standard chow (Diet 1314, Altromin, Lage, Germany). A 300 µl inoculum containing $10^8$ cells of each bacterial species was intragastrically applied to each rat of one of the animal groups (LCC rats). The rats in the other group received growth medium as a vehicle control. Fecal samples were collected throughout the study in order to confirm the microbial status of the animals. We prepared a flaxseed-rich diet composed of 58% wheat starch, 20% casein, 5% ground flaxseed, 5% sunflower oil, 5% cellulose, 5% mineral mixture and 2% vitamin mixture. This diet contained 0.34 g/kg SDG as determined by high-performance liquid chromatography (HPLC) (see below). Two weeks after association, the rats were switched from the chow to this experimental diet. All animals were fed from one single batch to avoid batch-to-batch variability in the dietary SDG content. Breast cancer was induced in all animals with a single oral dose of 25 mg DMBA (Sigma–Aldrich) diluted in corn oil 2 weeks after diet switch. Body

weight and the development of palpable tumors were monitored weekly. Thirteen weeks after DMBA application, animals were kept in metabolic cages for 24 h to quantitatively collect feces and urine. Subsequently, the animals (now 20 weeks of age) were removed from the isolators and blood from the retrobulbar venous plexus was sampled under anesthesia. Plasma and serum were prepared and stored at −20°C until further analysis. After killing of the animals, breast tumors were removed and number of tumors per animal, tumor weight and size were determined. The liver was frozen in liquid $N_2$ and stored at −80°C until further analysis. Representative parts of the tumors were fixed in 10% neutral buffered formalin and prepared for histology and immunohistochemistry. The remaining tumor material was frozen in liquid $N_2$ and stored at −80°C for later RNA extraction. Colonic and cecal contents were used for microbial examination and the remaining material was freeze-dried and stored at 4°C until analysis. The experimental protocol was approved by the local animal welfare committee (approval no. 23-2347-8-16-2008).

*Intestinal colonization status and short-chain fatty acid determination*

The germ-free state of the control rats was confirmed throughout the complete study by microscopic inspection of gram-stained fecal material and incubation of feces in complex growth media at 37°C. The colonization of the LCC rats by the four lignan-converting species was checked by fluorescent *in situ* hybridization using fecal material and intestinal contents. Fixation of bacterial cells and the fluorescent *in situ* hybridization procedure were performed as described earlier (14) using species-specific 16S ribosomal RNA targeted 5′-Cy3-labeled oligonucleotide probes (*C.saccharogumia*: S-S-Csac-0067-a-A-20; *B.producta*: S-*-ProCo-1264-a-A-23; *E.lenta*: S-*-Ato-0291-a-A-17; *L.longoviformis*: S-S-Llong-0831-a-A-20). Bacterial cells were counted with a fluorescence microscope and bacterial cell numbers were calculated as $\log_{10}$/g dry weight. Concentrations of short-chain fatty acids (SCFA) in cecal and colonic sample material were measured as described elsewhere (15). Briefly, SCFAs were extracted and sample material (1 µl) was injected into a gas chromatograph (Hewlett-Packard, Waldbronn, Germany). The chromatograph was equipped with an HP-FFAP capillary column (30 m × 0.53 mm inner diameter, 1 µm film thickness) and helium (1 ml/min) was used as the carrier gas.

*Lignan determination*

Lignan extraction was performed as described elsewhere (14) with minor modifications. Briefly, sample material (100 mg) was defatted with *n*-hexane. SDG was extracted for 3 h at 55°C and subsequently overnight at room temperature using an aqueous methanol solution (70%). After centrifugation, the supernatants were collected and the pellets were washed twice. The SDG-containing extracts were combined, methanol was evaporated and the residues were lyophilized. The lyophilisates were dissolved in 1 M NaOH for 3 h at 50°C, neutralized with HCl, freeze-dried, resuspended in aqueous methanol solution (70%) and subsequently subjected to HPLC analysis. In order to extract total SECO, ED and EL, feces and gut contents (100 mg) were mixed with sodium acetate buffer (0.1 M, pH 5) containing 1000 U of a β-glucuronidase/sulfatase preparation (type HP-2; Sigma–Aldrich). The samples were incubated overnight at 37°C, followed by an extraction with diethyl ether. After centrifugation, the upper lignan-containing diethyl ether phase was collected and the pellets were washed twice. The supernatants obtained during the washing steps were pooled and the diethyl ether was evaporated. The residues were dissolved in 70% aqueous methanol solution and analyzed by HPLC. Urine samples were thawed and centrifuged at 1000*g* for 15 min, 4°C. The supernatants were filtered (0.22 µm) and the filtrate (200 µl) was mixed with sodium acetate buffer (0.10 M, pH 5) containing 300 U of the β-glucuronidase/sulfatase preparation. After 16 h of incubation at 37°C with the enzyme preparation, lignans were extracted as described above. Lignans were quantified with an HPLC system equipped with a UV/Vis diode array detector as described earlier (14). Peak identification was based on the retention time and UV spectra of reference compounds and quantification of lignans was performed using calibration curves generated with the standard lignans SDG, SECO, ED and EL (Sigma–Aldrich).

*Immunohistochemistry and detection of apoptosis*

Ki-67, active caspase-3 and the estrogen receptor (ER) status of the tumors were analyzed immunohistochemically using 5 µm tumor sections. The primary antibodies applied were MIB-5 (Dianova, Hamburg, Germany) diluted 1:100 in Tris-buffered saline (50 mM, pH 7.6) for Ki-67, Asp175 (Cell Signaling, Beverly, MA) diluted 1:200 for cleaved caspase-3 and EI629C01 (DCS, Hamburg, Germany) diluted 1:400 for ER. After blocking non-specific antigens, samples were incubated overnight with the respective antibodies followed by incubation with biotinylated goat anti-rabbit immunoglobulin G as the secondary antibody. Subsequently, samples were treated with avidin and biotinylated horseradish peroxidase macromolecular complexes (Vector Laboratories, Burlingame, CA). Diaminobenzidine tetrahydrochloride (Sigma–Aldrich) was used as chromogen and slides were counterstained with hematoxylin. The *in situ* terminal deoxynucleotidyl transferase-mediated nick end-labeling assay was performed with the TACS.XL-DAB *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD). The relative proportion of Ki-67-positive and negative cells (Ki-67 labeling index) was taken as an indicator for tumor cell proliferation. Cells detected in the active caspase-3 and the transferase-mediated nick end-labeling assay were counted as apoptotic cells per square millimeter. The ER status of the tumor cells was evaluated using the Allred score.

*Quantitative real-time polymerase chain reaction*

The relative expression of the genes encoding the ERs α (ERα), β (ERß), the G-coupled protein 30 (GPR30), the epidermal growth factor receptor (EGFR) and the insulin-like growth factor 1 (IGF-1) in breast tumors was determined by quantitative real-time polymerase chain reaction (PCR). Therefore, RNA was extracted from tumor tissue using the RNasy Mini kit (Qiagen, Hilden, Germany). One microgram of RNA was reverse transcribed to single-stranded complementary DNA with the RevertAid® H Minus First Strand complementary DNA Synthesis Kit (Fermentas, St Leon-Rot, Germany). Real-time PCR was performed with the Stratagene's Mx3005P QPCR system (Agilent Technologies, Boblingen, Germany). The real-time–PCR mix (25 µl) contained the template DNA, the QuantiFastTMSYBR Green PCR master mix (Qiagen) and the respective primer pairs. The primer pairs used were as follows: ERα-for (5′-GCA TGA TGA AAG GCG GGA TAC GA-3′) and ERα-rev (5′-AAA GGT TGG CAG CTC TCA TGT CTC-3′), ERβ-for (5′-TGG TCT GGG TGA TTG CGA AGA G-3′) and ERβ-rev (5′-ATG CCC TTG TTA CTG ATG TGC C-3′), GPR30-for (5′-CGA GGT GTT CAA CCT GGA CGA-3′) and GPR30-rev (5′-GGC AAA GCA GAA GCA GGC CT-3′), EGFR-for (5′-TGC CCA CTA TGT TGA TGG TCC C-3′) and EGFR-rev (5′-GCC CAG CAC ATC CAT AGG TAC AG-3′), and IGF-1-for (5′-AAG ACT CAG AAG TCC CAG CCC-3′) and IGF-1-rev (5′-GGT CTT GTT TCC TGC ACT TCC T-3′). Relative expression levels of the target genes were calculated with the relative standard curve method after normalizing the target gene expression to the expression of the house-keeping gene encoding glyceraldehyde 3-phosphate dehydrogenase. The expression of the latter was measured with the primers glyceraldehyde 3-phosphate dehydrogenase-for (5′-CAA GGT CAT CCA TGA CAA CTT TG-3′) and glyceraldehyde 3-phosphate dehydrogenase-rev (5′-GTC CAC CAC CCT GTT GCT GTA G-3′).

*Selected plasma and liver enzyme activities and serum estradiol determination*

The specific activities of selected enzymes in the plasma and in liver homogenates were measured using standard procedures. In brief, catalase (CAT) activity was determined as $H_2O_2$ consumption concluded from the decrease in absorbance at 240 nm (16). Superoxide dismutase (SOD) activity was assessed by the nitro blue tetrazolium reduction method (17) and glutathione-*S*-transferase (GST) activity was measured using 1-chloro-2,4-dinitrobenzene (18). Reduced glutathione (GSH) and malondialdehyde (MDA) were determined according to Ellman (19) and Mihara and Uchiyama (20), respectively. Total protein was quantified according to Bradford (21). Serum 17β-estradiol concentrations were measured with the Estradiol EIA Kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's specifications.

*Statistical analysis*

Statistical analyses were conducted with SPSS 14.0 (IBM). Values were tested for normal distribution with the Kolmogorov–Smirnov test. Depending on data distribution, the two-tailed Student's *t*-test or the Mann–Whitney test was used. Normally distributed values are expressed as means with standard error. Differences were considered significant at $P \leq 0.05$.

## Results

*Bacterial numbers and SCFA concentrations in intestinal samples*

Successful establishment of the lignan-converting consortium in the intestine of LCC rats was the prerequisite for conducting the study. Therefore, cell numbers of each member of the selected strains were determined before and during the dietary intervention phase and at the end of the study. Fluorescent *in situ* hybridization analysis using fecal material confirmed the presence of all four consortium members in the intestine throughout the complete study period (data not shown). At the end of the study, *C.saccharogumia* was detected at log 10.03 ± 0.04 cells/g dry weight in the cecum and log 10.20 ± 0.02 cells/g dry matter (DM) in the colon of LCC rats. Numbers for *B.producta* were log 10.27 ± 0.03 and log 10.48 ± 0.03 cells/g DM in the cecum and colon, respectively. *E.lenta* was detected at log 9.60 ± 0.03 cells/g DM in the colon and log 9.99 ± 0.03 cells/g DM in the cecum. Lowest

numbers were determined for *L.longoviformis* with log 9.07 ± 0.06 cells/g DM in the colon and log 9.05 ± 0.07 cells/g DM in the cecum. Direct microscopic inspection and anaerobic and aerobic cultivation of fecal and intestinal sample material confirmed the germ-fee status of the control rats throughout and at the end of the animal experiment, respectively.

SCFA concentrations in cecal and colonic sample material were measured as marker for bacterial metabolism. Cecal acetate concentrations were 40.24 ± 5.20 μmol and 43.60 ± 8.61 μmol/g DM in associated and germ-free rats, respectively. Concentrations in the colon were 16.27 ± 75 μmol in the associated rats and 26.78 ± 4.53 μmol/g DM in the germ-free rats. Propionate concentrations were below 0.7 μmol and butyrate concentrations were ∼0.1 μmol/g DM in all samples.

*Lignan concentrations in intestinal, fecal and urine sample materials*

Since we hypothesized that the conversion of lignans by gut bacteria influences their effects on breast cancer development, we quantified SDG in cecal and fecal sample material. SECO, ED and EL were determined in cecal and colonic material, in feces and in urine. Cecal SDG concentrations were significantly lower in LCC than in germ-free rats (1.47 ± 0.17 μmol/g DM versus 5.22 ± 0.52 μmol/g DM, $P < 0.0001$) and fecal SDG excretion was significantly higher in germ-free rats than in LCC rats (2.58 ± 0.24 versus 0.81 ± 0.07 μmol/g DM, $P < 0.0001$). SECO was detected in gut contents and feces from both LCC and germ-free rats (Table I). However, concentrations were significantly higher in the LCC rats than in the germ-free animals. ED and EL were detected exclusively in gut contents and feces of the LCC rats but ED was only detected in cecal samples. In contrast, EL was found in all samples analyzed. No urinary lignan excretion was observed for the germ-free rats. The LCC rats but not the germ-free rats excreted both ED and EL in the urine. The concentration of the latter was significantly higher ($P < 0.0001$) than that of the former.

*Animal health status*

Breast cancer induction with one single oral application of DMBA resulted in weight loss and/or growth retardation of the juvenile animals. Because of severe body weight loss, one LCC rat was killed in this phase for ethical reasons. All the remaining animals recovered completely and did not differ in body weight gain during the remaining experimental phase (data not shown). There was no significant difference between the LCC and the germ-free group in the latency period until the first tumors were palpable (9.5 ± 0.50 and 8.0 ± 0.82 weeks, respectively) or in total tumor incidence (Table II). However, the number of tumors per tumor-bearing rat was lower for the LCC than for the germ-free animals ($P = 0.049$) and the total number of tumors observed in the LCC group was reduced by 40% ($P = 0.037$) at the end of the animal experiment. Mean tumor size and weight were ∼50% lower in the LCC rats but only differences in tumor size were statistically significant ($P = 0.036$). Results from the serum estradiol measurements did not differ between the animals (33.65 ± 1.70 and 34.44 ± 2.77 pg/ml, respectively).

*Histological and immunohistochemical tumor phenotypes and ER gene expression*

Histological examination revealed that all tumors analyzed were moderately differentiated tubolopapillary adenocarcinomas with rare areas of solid growth. They were well circumscribed with no evidence of invasive growth into the surrounding connective tissue or invasion into adjacent vessels. Tumor cells were moderately pleomorphic and anisokaryotic with an average of three mitotic figures per microscopic field. No differences in the ER status scores were observed between the tumors from LCC (4.20 ± 0.19) and germ-free rats (4.29 ± 0.13). A >50% lower Ki-67 labeling index indicated a lower tumor cell proliferation rate in the LCC rats ($P < 0.0001$) than in the germ-free controls (Figure 1A). In addition, the number of apoptotic tumor cells was significantly higher ($P = 0.038$) in LCC rats than germ-free rats (Figure 1B). The messenger RNA expression of the ER genes *ERα*, *ERβ* and *GPR30* and of the cell growth-associated genes *IGF-1* and *EGFR* was slightly lower in the tumor tissue obtained from LCC rats. However, differences did not reach statistical significance (data not shown).

*Liver and plasma antioxidant enzyme activities*

To address possible effects of bacterial lignan transformation on the oxidative status of the rats, we determined the specific activity of CAT, SOD and GST in liver homogenates and in plasma samples. In addition, we measured GSH and MDA concentrations, which are considered as oxidative stress markers. The activities of CAT, SOD and GST in liver and plasma were significantly higher in LCC than in germ-free rats (Table III). In contrast, no significant differences between the two groups in liver and plasma MDA and GSH concentrations were observed.

## Discussion

Several epidemiological studies indicate that a high dietary lignan intake might be protective against breast cancer (22,23) but other studies do not support this association (24,25). The heterogeneity of study groups with respect to age, dietary lignan exposure, breast cancer subtypes and genetic factors have been proposed to be responsible for these discrepancies (26). Differences in intestinal microbiota composition might be an additional factor that influences the outcome of such studies since the bacterial conversion to enterolignans is thought to be important for the health effects of lignans. This notion

**Table I.** Lignan concentrations in intestinal, fecal (μmol/g DM) and urine sample (μmol/24h urine excretion) material obtained from LCC ($n = 9$) and germ-free rats ($n = 10$)

| | Germ-free rats | LCC rats |
|---|---|---|
| Lignans | | |
| | **Cecum** | |
| Total SECO | 0.268 ± 0.12 | 0.512 ± 0.05** |
| Total ED | Not detected | 0.134 ± 0.04 |
| Total EL | Not detected | 1.364 ± 0.44 |
| | **Colon** | |
| Total SECO | 0.268 ± 0.12 | 0.438 ± 0.05** |
| Total ED | Not detected | Not detected |
| Total EL | Not detected | 0.469 ± 0.07 |
| | **Feces** | |
| Total SECO | 0.161 ± 0.03 | 0.187 ± 0.02 |
| Total ED | Not detected | Not detected |
| Total EL | Not detected | 0.409 ± 0.047 |
| | **Urine** | |
| Total SECO | Not detected | Not detected |
| Total ED | Not detected | 0.204 ± 0.03 |
| Total EL | Not detected | 1.792 ± 0.298 |

Significance of differences between the animal groups is indicated (**$P \leq 0.01$).

**Table II.** Tumor incidence, tumor quantity, tumor size and tumor weight in LCC ($n = 9$) and germ-free control rats ($n = 10$)

| | Germ-free rats | LCC rats |
|---|---|---|
| No. of rats with tumors | 7 | 7 |
| No. of tumors per group | 45* | 18 |
| No. of tumors per tumor-bearing rat | 6.57 ± 0.20* | 2.57 ± 0.11 |
| No. of tumor per No. of rats in group | 4.50 ± 0.13** | 0.57 ± 0.02 |
| Mean tumor size ($cm^3$) | 0.97 ± 0.28* | 0.47 ± 0.18 |
| Mean tumor weight (g) | 1.09 ± 0.46 | 0.52 ± 0.24 |

Significance of differences between the animal groups is indicated (*$P \leq 0.05$, **$P \leq 0.01$).

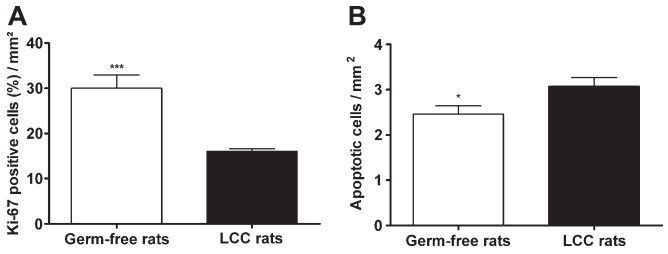


**Fig. 1.** Effect of bacterial lignan conversion on breast cancer cell proliferation (percentage of Ki-67-positive tumor cells per square millimeter tumor tissue) (**A**) and apoptosis (apoptotic cells per square millimeter tumor tissue). (**B**) Significance of differences between the animal groups is indicated ($^{*}P < 0.05$, $^{**}P \leq 0.01$).

**Table III.** Selected enzyme activities and concentrations of reduced GSH and MDA in LCC ($n = 9$) and germ-free rats ($n = 10$)

| | Germ-free rats | LCC rats |
|---|---|---|
| | **Liver** | |
| CAT[a] | 39.37 ± 2.19** | 50.65 ± 3.08 |
| SOD[b] | 2.04 ± 0.14* | 2.63 ± 0.17 |
| GST[c] | 0.57 ± 0.05*** | 1.25 ± 0.14 |
| Reduced GSH[d] | 85.87 ± 4.64 | 100.84 ± 9.32 |
| MDA[e] | 0.27 ± 0.05 | 0.21 ± 0.02 |
| | **Plasma** | |
| CAT[f] | 0.92 ± 0.13** | 1.52 ± 0.14 |
| SOD[g] | 39.27 ± 3.67* | 51.66 ± 2.52 |
| GST[h] | 1.60 ± 0.32** | 3.70 ± 0.65 |
| Reduced GSH[i] | 0.26 ± 0.02 | 0.30 ± 0.02 |
| MDA[j] | 3.24 ± 0.10 | 3.05 ± 0.16 |

Significance of differences between the animal groups is indicated ($^{*}P \leq 0.05$, $^{**}P \leq 0.01$, $^{***}P \leq 0.001$).
[a]μmol $H_2O_2$ consumed/min/(mg protein).
[b]Units/mg protein.
[c]Units/mg protein.
[d]μmol GSH utilized/mg protein.
[e]nmol MDA produced/mg protein.
[f]μmol $H_2O_2$ consumed/min/ml.
[g]Unit/ml.
[h]Unit/l.
[i]mmol/l.
[j]nmol/ml.

is supported by the fact that the occurrence of lignan-converting gut bacteria in human study subjects may differ (13). However, whether the bacterial conversion of the plant lignans to the enterolignans is a prerequisite for their health-promoting effects has not yet been demonstrated *in vivo*. We therefore colonized rats with a lignan-converting bacterial consortium, fed these animals a lignan-rich diet and chemically induced breast cancer. Effects of lignan feeding in this model were compared with those in identically treated germ-free rats.

All bacteria of the consortium successfully colonized the intestine of the LCC rats and enterolignans were produced from SDG in the LCC but not in the germ-free rats. These findings, and also the presence of SECO in germ-free animals, are in agreement with previous observations in this model (14). Fecal EL excretion by LCC rats was comparable with concentrations in premenopausal women (27). In these women but not in our experimental animals, fecal excretion of ED was observed. We therefore conclude that the conversion of ED to EL by *L.longoviformis* was very efficient under our experimental conditions. Urinary ED and EL excretion, indicative of their bioavailability, was comparable with conventionally colonized rats (5) and to healthy humans subjected to a single dose of SDG (28).

Bacterial lignan transformation did not influence tumor incidence in our model but significantly lowered the number of tumors per tumor-bearing animal and the tumor size. These results are in line with findings in a DMBA rat model treated with EL (29) and with the plant lignan lariciresinol, which is also converted by gut bacteria to ED and EL (30). We concluded from the Ki-67 index and the transferase-mediated nick end-labeling assay that the reduced tumor growth in LCC rats resulted from a lower proliferation in conjunction with a higher apoptotic rate of the tumor cells. Such anti-proliferative and pro-apoptotic effects of ED and EL have been demonstrated previously in human breast cancer-derived cell cultures (31). In addition, an increased tumor cell apoptosis was observed in an SDG-treated mouse model for breast cancer (30) and a decreased Ki-67 index was reported for hyperplastic breast tissue obtained from SDG-treated women (32).

The mechanisms possibly involved in the protective role of lignans in cancer development include anti-angiogenic, pro-apoptotic, anti-estrogenic and antioxidant mechanisms (33). To study the effect of bacterial lignan transformation on the latter two mechanisms, we compared the serum estrogen levels and the relative expression of estrogen-sensitive genes and genes involved in tumor cell growth. In addition, the oxidative stress of the animals was addressed by measuring the activity of enzymes involved in the breakdown of oxidants and of selected markers for oxidative stress in liver homogenates and in the plasma.

We did not observe any differences between the study groups in circulating estrogen concentrations and only minor differences in the expression of the estrogen-sensitive genes *ERα*, *ERβ* and *GPR30*. In addition, no significant changes in the expression of *EGFR* and *IGF-1* were detected. In contrast to our findings, feeding an SDG-rich diet (1 g/kg) or a 10% flaxseed diet has been reported to influence the expression of *ERα* and *ERβ* in an athymic ovariectomized mouse model for breast cancer (34,35). Since the ER expression in our animals was not corrected for estrogen cycling, this discrepancy might be explained by the constant low levels of circulating estrogen concentrations in these mice. A higher amount of dietary SDG might also be responsible for the observed differences because our experimental diet contained only 5% flaxseed or 0.34 g SDG/kg of diet. The fact that EL exerts dose-dependent effects on *ERα* and *ERβ* expression in cultured cancer cells (36) supports the assumption that the lignan content in our diet was too low to influence the ER expression. This explanation might also be valid for missing effects of lignan transformation on *EGFR* and *IGF-1* expression. Reduced expression of these genes was reported in mouse models for breast cancer fed a diet containing 0.1% SDG or 10% flaxseed but no effects were observed when a 5% flaxseed diet was used (35,37,38). Taken together, our study results do not support an estrogen-dependent mechanism after feeding a 5% flaxseed diet.

To study potential effects of lignans on enzyme systems involved in the degradation of oxidants, we measured the specific activities of GST, SOD and CAT in liver homogenates and in the plasma of LCC and germ-free rats. GSH and MDA were used as markers for oxidative stress in these compartments. Compared with the germ-free rats, LCC rats displayed higher GST, SOD and CAT activities in liver and plasma suggesting a higher capacity to diminish the concentration of oxidants. However, we found no indication for a protective effect of the increased activities of these enzymes: both GSH and MDA concentrations were similar in experimental and control rats. Thus, it may be speculated that even though the enterolignans enhanced the activity of antioxidant enzymes, they did not reduce the systemic oxidative burden. A reduction of oxidative stress by radical scavenging in response to lignan treatment has been demonstrated in cell culture experiments (39–41). In addition and in contrast to our results, short-term feeding of a 10% flaxseed diet or equivalent amounts of SDG to healthy rats did not change hepatic activities of enzymes involved in oxidant breakdown but the direct antioxidant activity of the lignans was proposed to be responsible for beneficial effects (42). Further studies are necessary to finally clarify how the antioxidant properties of enterolignans are brought about and whether such mechanisms are indeed involved in their protective effects against breast cancer.

We cannot completely rule out that bacterial factors other than the transformation of lignans have been involved in the observed effects

of flaxseed feeding in our animal model. For instance, SCFA and especially butyrate mainly produced from indigestible carbohydrates have the potential to reduce cell growth and to promote apoptosis in colorectal tumors (43) and findings in human cell lines implicate that SCFA might also exert beneficial effects in breast cancer (44). However, we did not find differences in cecal and colonic SCFA concentrations between the associated and the germ-free rats. In addition, the SCFA concentrations in our experimental animals were much lower than reported for conventional rats and for rats that were colonized with a limited number of dominant gut bacteria and fed comparable purified diets (15,45). Thus, it is not very likely that bacterial SCFA production had any influence on tumor development in our experimental animals.

In summary, our results are not in favor of an estrogen-dependent mechanism as an explanation for the protective effects of enterolignans observed under our experimental conditions. The increased activity of oxidant-degrading enzyme systems in response to enterolignans did not result in a decrease of oxidative stress markers in liver and plasma. We therefore conclude that the bacterial transformation of flaxseed-derived lignans is the prerequisite for their beneficial effects in a rat model of breast cancer and that the underlying mechanisms require further investigation.

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