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Protective role of lignan-converting bacteria on chemically-induced breast cancer in gnotobiotic rats

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To Dr. Ahmed Gabr, a great husband

ABSTRACT

Enterolignans (enterodiol and enterolactone) exhibit structural similarity to estradiol and have therefore been hypothesized to modulate hormone related cancers such as breast cancer. The bioactivation of the plant lignan secoisolariciresinol diglucoside (SDG) requires the transformation by intestinal bacteria including the deglycosylation of SDG to secoisolariciresinol (SECO) followed by demethylation and dehydroxylation of SECO to enterodiol (ED). Finally, ED is dehydrogenated to enterolactone (EL). It is unclear whether the bacterial activation of SDG to ED and EL is crucial for the cancer preventing effects of dietary lignans. The possible protective effect of bacterial lignan transformation on a 7,12-dimethylbenz(a)anthracene (DMBA)-induced breast cancer in gnotobiotic rats was investigated. Germ-free rats were associated with a defined lignan-converting consortium (*Clostridium saccharogumia, Blautia producta, Eggerthella lenta, and Lactonifactor longoviformis*). The rats colonized with lignan-converting bacteria consortium (LCC) were fed a lignan-rich flaxseed diet and breast cancer was chemical induced. Identically treated germ-free rats served as control. All bacteria of the consortium successfully colonized the intestine of the LCC rats. The plant lignan SDG was converted into the enterolignans ED and EL in the LCC rats but not in the germ-free rats. This transformation did not influence cancer incidence but significantly decreased tumor numbers per tumor-bearing rat, and tumor size. Cell proliferation was significantly inhibited and apoptosis was significantly induced in LCC rats. No differences between LCC and control rats were observed in the expression of the genes encoding the estrogen receptors (ERα and ERβ) and G-coupled protein receptor 30 (GPR30). Similar findings were observed for both insulin-like growth factor 1 (IGF-1) and epidermal growth factor receptor (EGFR) genes involved in tumor growth. Proteome analysis revealed that 24 proteins were differentially expressed in tumor tissue

from LCC and germ-free. RanBP-type and C3HC4-type zinc finger-containing protein 1 (RBCK1) and poly(rC)-binding protein 1 (PBCP1) were down-regulated by 3.2- and 2.0-fold, respectively. These proteins are associated with cell proliferation. 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 (non-enzymatic antioxidant) and malondialdehyde (oxidative stress marker) did not differ between the groups. **In conclusion,** the bacterial conversion of plant lignan to enterolignans beneficially influences their anti-cancer effect. However, the mechanisms involved in these effects remain elusive.

ZUSAMMENFASSUNG

Enterolignanen (Enterodiol ED und Enterolacton EL) wird aufgrund ihrer strukturellen Ähnlichkeit zu Estradiol ein modulierender Einfluss auf hormonell bedingte Krebserkrankungen wie Brustkrebs nachgesagt. Das pflanzliche Lignan Secoisolariciresinoldiglucosid (SDG) wird durch Darmbakterien zum Enterolignan aktiviert. Dies erfolgt über dessen Deglykosylierung zu Secoisolariciresinol (SECO) gefolgt durch die Demethylierung und die Dehydroxylierung zu Enterodiol (ED). Schließlich wird ED zu Enterolacton (EL) dehydrogeniert. Es ist allerdings noch nicht bewiesen, dass die bakterielle Aktivierung von SDG zu ED und EL für die antikanzerogenen Wirkungen verantwortlich ist, die für dieses in der menschlichen Ernährung vorkommende Lignan beschrieben wurden. Um dies zu klären, wurde der Einfluss der bakteriellen Lignan-Transformation auf die Protektion gegenüber einem durch 7,12-Dimethylbenz(a)anthracen (DMBA)-induzierten Brustkrebs im gnotobiotischen Rattenmodell untersucht. Keimfreie Ratten wurden hierfür mit einem Konsortium aus vier Bakterienstämmen (*Clostridium saccharogumia, Blautia producta, Eggerthella lenta,* und *Lactonifactor longoviformis*) besiedelt, das die Umsetzung von SDG zu ED und EL katalysiert (LCC-Ratten). Ratten, die über den gesamten Versuchszeitraum keimfrei blieben, dienten als Kontrolle. Die Tiere wurden über 16 Wochen mit einer Leinsamen-Diät gefüttert, die reich an pflanzlichen Lignanen war. Während der Fütterung wurde bei allen Tieren Brustkrebs chemisch induziert.

Das pflanzliche Lignan SDG wurde nur in den LCC Ratten zu den Enterolignanen ED und EL umgewandelt. Keimfreie Ratten zeigten keine Transformation von SDG. Die bakterielle Transformation von SDG hatte zwar keinen Einfluss auf die Inzidenz von Brustkrebs, jedoch verringerten sich durch die Besiedlung der Ratten mit SDG-transformierenden Bakterien die Anzahl von Tumoren pro tumortragender Ratte und die Tumorgröße deutlich. Zudem wurde die

Zellproliferation in den LCC-Ratten deutlich gehemmt und die Apoptose induziert. Unterschiede in der Genexpression der Östrogenrezeptoren (ERα und ERß) und G-Protein-gekoppelte Rezeptoren (GPR30) wurden zwischen den LCC-Ratten und den Kontrolltieren nicht beobachtet. Ebenso verhielt es sich für die Gene des Insulinähnliche Wachstumsfaktoren 1 (IGF-1) und Epidermale Wachstumsfaktor rezeptoren (EGFR), welche in das Tumorwachstum involviert sind. Die Analyse des Proteoms des Tumorgewebes ergab 24 differentiell exprimierte Proteine zwischen keimfreien und LCC-Ratten. So wurden zum Beispiel die Proteine RanBP-type and C3HC4-type zinc finger-containing protein 1 (RBCK1) und poly(rC)-binding protein 1 (PBCP1), die mit der Zellproliferation assoziiert sind, in LCC-Ratten um das 3,2 bzw. 2,0-fache herunterreguliert. Die Aktivität ausgewählter antioxidativer Enzyme in Plasma und Leber war in den LCC-Ratten im Vergleich zu den keimfreien Tieren deutlich erhöht. Allerdings unterschieden sich die Konzentrationen von reduziertem Glutathion (nichtenzymatisches Antioxidans) und Malondialdehyd (oxidativer Stress-Marker) in Plasma und Leber nicht zwischen den beiden Besiedlungs-Gruppen.

Zusammenfassend zeigen die Ergebnisse, dass die bakterielle Umwandlung von pflanzlichen Lignanen zu Enterolignanen deren antikanzerogene Wirkung entscheidend beeinflusst. Allerdings bleiben die zugrunde liegenden Mechanismen weiterhin ungeklärt.

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ABBREVIATIONS

16S rRNA	16S ribosomal RNA
2D-DIGE	Two-dimensional difference gel electrophoresis
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAT	Catalase
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
DM	Dry matter
DMBA	7,12-dimethylbenz(a)anthracene
DNA	Deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen/ German collection of microorganisms
DTT	Dithiothreitol
ED	Enterodiol
EGFR	Epidermal growth factor receptor
EL	Enterolactone
ER	Estrogen receptors
ERK	Extracellular regulated kinase
ERα	Estrogen receptors α
ERβ	Estrogen receptors β
FISH	Fluorescence *in situ* hybridisation technique
g	Relative centrifugal field
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPR30	G-coupled protein receptor 30
GSH	Reduced glutathione
GST	Glutathione-S-transferase
HPLC	High Performance Liquid Chromatography
IEF	Isoelectric focusing
IGF-1	Insulin-like growth factor 1
IPG	Immobilized pH gradient
kGy	Kilo gray radiation absorbed dose measurement units
LCC rats	Lignan-converting consortium rats
MALDI-TOF-MS	Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
MAPK	Mitogen activated protein kinase
MAT	Matairesinol
MDA	Malondialdehyde
mRNA	Messenger RNA
Mt-6	Medium-6
N_2/CO_2	Nitrogen / carbon dioxide
NAA	Naphthalene acetic acid
PBCP1	Poly(rC)-binding protein 1
PCR	Polymerase chain reaction

PI3	Phosphoinositide 3 kinase
r	Pearson correlation coefficient
RBCK1	RanBP-type and C3HC4-type zinc finger-containing protein 1
RNA	Ribonucleic acid
RT-PCR	Real-time PCR
RuBps	Ruthenium (II) tris (bathophenantroline disulfonate)
SCFA	Short-chain fatty acid
SDG	Secoisolariciresinol diglucoside
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SECO	Secoisolariciresinol
SOD	Superoxide dismutase
Temp.	Temperature
TFA	Trifluoroacetic acid
TUNEL	The *in situ* terminal deoxynucleotidyl transferase-mediated nick end labeling
Vh	The optimal total volt-hours
YH-BHI	Yeast-haemin-Brain Heart Infusion

1. INTRODUCTION

Breast cancer is one of the most common cancers in the world. It accounts for ~30% of all diagnosed cancer cases in women each year (Bange et al., 2001) and is considered a major public health issue with over 1 million newly diagnosed cases per year. More than 400,000 annual cases of death and 4.4 million women living with breast cancer are reported (Malik et al., 2010). In recent years, numerous plant foods have been associated with decreased cancer risk. Many epidemiological and experimental studies have reported an association between the consumption of lignan-rich food and a reduced risk of breast cancer (Ingram et al., 1997; Thompson et al., 1996 a,b; Hulten et al., 2002; McCann et al., 2006; Buck et al., 2011).

1.1. Dietary source and intake of lignans

It has been proposed that effects of lignans on breast cancer depend on dietary intake of lignans and the concentrations of lignans in biological fluids. Mazur & Adlercreutz, (1998) pointed out that flaxseed is the richest source of the plant lignans secoisolariciresinol diglucoside (SDG) and secoisolariciresinol (SECO). Defatted flaxseed contains high amounts of SDG (9-30 mg/g). This concentration is considered to be 800 times higher than in any other food (Dobbins & Wiley, 2004). The SECO concentration in flaxseed is 2.94 mg/g (Milder et al., 2005a). Cereals, vegetables, fruits and berries contain substantial quantities of SECO and matairesinol (MAT) (Meagher & Beecher 2000). The lignan contents in different food sources are presented in Table 1.

The studied populations in the Netherlands, United States and Finland revealed that the daily intake of SECO and MAT ranges from 0.18 to 0.65 mg/d (Horn-Ross et al., 2000; de Kleijn et al., 2001; Keinan-Boker et al., 2002; McCann et al., 2003; Valsta et al., 2003). Other researchers

Table 1. Lignan contents of a selection of food sources (µg/100 g dry weight)

FOOD	SECO	MAT
OIL SEEDS		
Flaxseed	369 900	1087
Soy seeds	13.0-273	Tr[a]
Sunflower seeds	609.5	0
Peanuts	333.4	Tr
CEREALS		
Rye bran	132	167
Barley bran	62.6	0
Oat bran	23.8	155
Wheat bran	110.1	0
Rye	47.1	65
VEGETABLES		
Broccoli	414.2	23.1
Garlic	379	3.62
Green pepper	117.0	7
Tomato	51.6	5.5
Carrot	192	286
FRUITS		
Cranberry	1510	0
Strawberry	1500	78.1
Orange	74.6	0
Cantaloupe	183.9	0
Banana	10	0
LEGUMES		
Kidney bean	69.9	0
Pinto bean	79.21	Tr
BEVERAGES		
Earl Grey black tea	1590	197
Japanese green tea	2460	186
Arabica instant coffee	716	ND[b]

Data were adopted from Meagher & Beecher, 2000.

[a] Tr: trace; ND: not determined.

reported for Dutch and Finnish adults the intake of the total plant lignans; (MAT, SECO, pinoresinol (PINO), and lariciresinol (LAR)) to be 1.2 mg/day (Milder et al., 2005b; Nurmi et al., 2010). In 2010, Pellegrini et al. conducted a cross-sectional study on 242 men and postmenopausal women in Northern Italy in which the daily intake of individual lignans including the daily intake of MAT, SECO, PINO, and LAR were 20.9, 335.3, 96.7, and 175.7 µg/d, respectively, and the total lignans were 665.5 µg/d. The major dietary sources of lignans in the Dutch cohorts were coffee and tea (Nurmi et al., 2010). In contrast, in the Italian cohorts, red wine accounted for approximately one-third of total lignan intake (Pellegrini et al., 2010).

1.2. Lignan concentrations in biological fluids

Plant lignans are metabolized by human intestinal bacteria to the enterolignans, enterodiol (ED) and enterolacton (EL) (Borriello et al., 1985), which were discovered in human urine in the 1980s (Setchell et al., 1980; Stitch et al., 1980). Age, sex, smoking, nutrition habits, the socioeconomic status, and obesity were found to influence the EL concentration in both direct and indirect manners (Milder et al., 2005b; Clavel et al., 2006c; Sonestedt et al., 2008). Numerous studies on humans reported that the average levels of EL in serum or plasma were below 30 nmol/l (Stumpf et al., 2000; Kilkkinen et al., 2001; Pietinen et al., 2001; Stattin et al., 2002; Sonestedt et al., 2009). Several intervention trials have shown a positive association between the consumption of lignan-rich food and serum/urinary levels of EL. For instance, lignan-rich food increases EL levels in blood to more than 100-1000 nmol/l (Adlercreutz et al., 1982; Kilkkinen et al., 2001; Johnsen et al., 2004; Kuijsten et al., 2005). In a randomized cross-over feeding trial including 31 postmenopausal women, urinary excretion of ED, EL, and total lignans by subjects consuming 5 g of flaxseed were increased by 1.0%, 21.2%, 24.3%, respectively, compared to control subjects (Hutchins et al., 2000). In a group of nine

premenopausal women, flaxseed supplementation of 5, 15, and 25 g/day was significantly associated with increased EL serum levels in a dose-dependent manner (Webb & McCullough et al., 2005). The plasma levels of ED and EL in rats consuming 1.5 mg SDG/day was estimated to be 1.0 µmol/l (Orcheson et al., 1998). Similarly, the plasma level of lignans in humans fed 1.5 µmol SDG/kg/body weight was also estimated to be 1.0 µmol/l (Kuijsten et al., 2005). Therefore, it can be concluded that blood and urinary concentrations of enterolignans depend on the intake of plant lignans and the dietary sources of lignans (Kilkkinen et al., 2003; Nurmi et al., 2010).

EL levels in biological fluids vary between studies. This is due to several factors such as study duration, dietary source and dose of lignans used, genetic factors and possibly to the presence or absence of lignan-converting bacteria in the intestine (Rickard et al., 1996; Rickard & Thompson, 1998; Kuijsten et al., 2005; Clavel et al., 2005, Clavel et al., 2006c; Possemiers et al., 2007; Nurmi et al., 2010). A daily intake of more than 90g of rye bread for 4 weeks did not increase the level of enterolignans in blood (Juntunen et al., 2000). In contrast, consuming flaxseed for 4 months increased the serum EL concentration by 50% (Tarpila et al., 2002). After a daily administration of EL (10 mg/kg of body weight), the mean serum EL concentration resulting in inhibition of tumor growth in rats was found to be 0.35-0.41 µM. These EL concentrations are 10-fold higher than those measured in the general population (Saarinen et al., 2002).

1.3. Bacterial transformation of plant lignans to enterolignans and the cancer-protective effect of enterolignans

Intestinal bacteria catalyze the transformation of plant lignans to enterolignans (ED and EL). ED and EL are thought to be responsible for the protective effect on breast cancer. Moreover, the

potential protective effect of ED and EL on breast cancer can be explained by different mechanisms such as antioxidant and estrogenic/anti-estrogenic effects, as well as the impact of lignans on estrogen synthesis and gene expression (Webb & McCullough, 2005; Saarinen et al., 2007; Landete, 2012). Accordingly, the transformation of plant lignans SDG to ED and EL by intestinal bacteria is assumed to be essential for this protective effect on breast cancer.

1.3.1. Transformation of plant lignans to enteolignans by intestinal bacteria

The role of intestinal bacteria in enterolignan production has been studied in germ-free rats; studies revealed that the presence of the intestinal bacteria was essential for production of ED and EL (Axelson & Setchell, 1981; Bowey et al., 2003; Woting, et al., 2010). Flaxseed-derived SDG is linked with hydroxycinnamic acid glucoside, coumaric acid glucoside, ferulic acid glucoside and the linker 3-hydroxy-3-methyl glutaric acid. This molecule was formerly called the lignan macromolecule (Kamal-Eldin et al., 2001; Struijs et al., 2007). SDG is released from the lignan macromolecule in the upper part of the gastrointestinal tract (Eeckhaut et al., 2008) through the enzymatic activity of the brush border of the gut mucosa. It remains stable in the upper part of the gastrointestinal tract (Clavel et al., 2006b). In the colon, SDG is converted to enterolignans by a series of metabolic steps including: deglycosylation, demethylation, dehydroxylation and dehydrogenation (Figure 1). Several intestinal bacteria have been shown to be involved in the transformation of the plant lignans to enterolignans: *Clostridium saccharogumia, Bacteroides methylotrophicum, Blautia producta, Eubacterium callanderi, Peptostreptococcus productus, Eubacterium* sp. Strain SDG2*, Clostridium scindens*, *Eggerthella lenta, Ruminococcus* sp. END-1 and *Lactonifactor longoviformis* (Wang et al., 2000; Xie et al., 2003; Clavel et al., 2006 a,b; Jin et al., 2007a; Jin & Hattori, 2010).

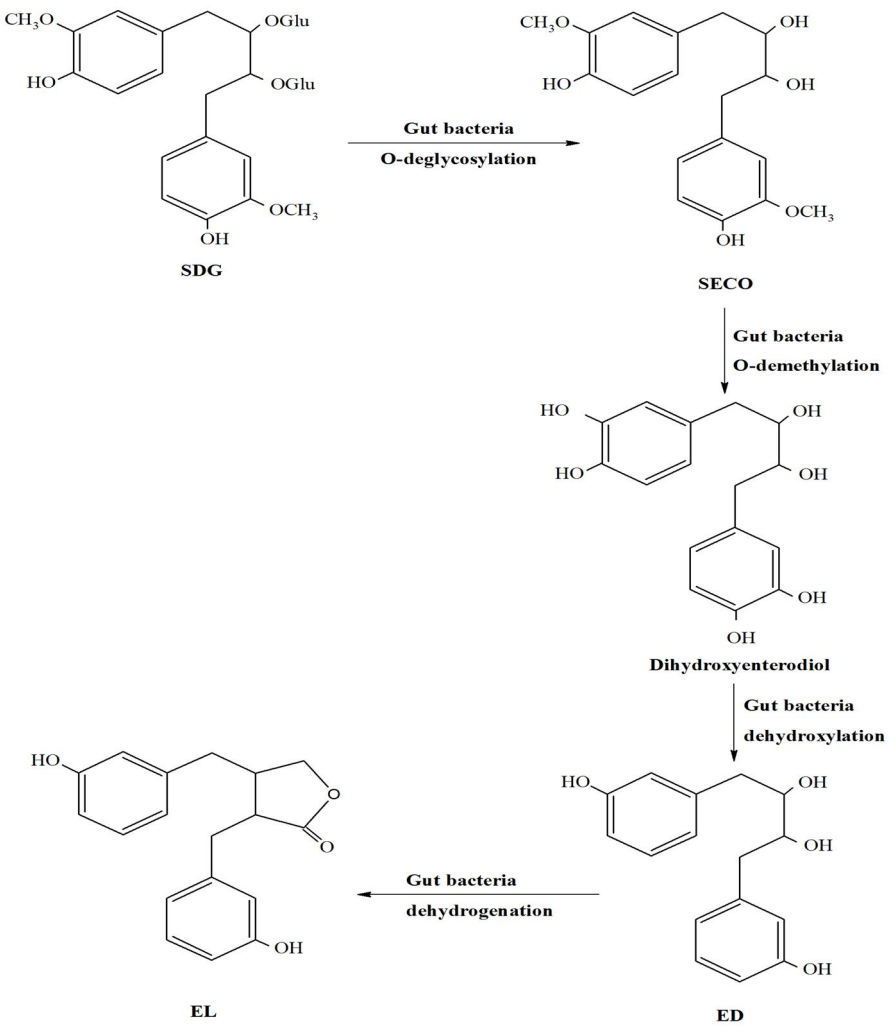


Figure 1. Transformation of plant lignan SDG to enterolignans, ED and EL, by human intestinal bacteria with a series of metabolic steps including: deglycosylation, demethylation, dehydroxylation and dehydrogenation (adopted from Clavel et al., 2006b).

1.3.1.1. Deglycosylation

The transformation of SDG to SECO is the first step in the conversion process. SDG is deglycosylated, most likely, by bacterial enzyme activity in the colon. However, there is evidence that the brush border enzymes of the intestine play a role in the SDG deglycosylation (Day et al., 1998; Setchell et al., 2002; Nemeth et al., 2003; Clavel et al., 2006a). Many members

of the *Bacteroides* and *Clostridia* are capable of lignan deglycosylation (Clavel et al., 2006b). However, the recently isolated species *Clostridium saccharogumia,* is a subdominant member of the fecal microbiota, and had the highest initial conversion rate of SDG to SECO compared to the other bacterial species tested by Clavel et al. (2006a).

1.3.1.2. Demethylation

The second step in the conversion of the plant lignans into the enterolignans is the removal of the methyl groups from the methoxyl moieties of SECO resulting in the formation of dihydroxy enterodiol. A large number of anaerobic bacteria are able to demethylate components such as SECO, arctigenin, and biochanin A via the acetogenesis mechanism to produce acetate from CO_2 or methyl groups for energy metabolism (Lajoie et al., 1988; Diekert, 1992; Drake et al., 2002; Muller et al., 2004). *Bacteroides methylotrophicum, Blautia producta, Eubacterium callanderi, Eubacterium limosum,* and *Peptostreptococcus productus* have been identified as bacteria capable of SECO demethylation (Liu et al., 2008). The presence of a free hydroxy group vicinal to the methoxy group is necessary for the demethylation of SECO (Wang et al., 2000). The END-2 strain is capable of demethylating (-)-SECO to (-)-dihydroxyenterodiol (Jin & Hattori, 2010). However, the enantiomeric excess of (+)-SECO is about 97% (2.86 mg/g) for flaxseed (Sicilia et al., 2003). Three strains of *B. producta* are capable of demethylating SECO (Clavel et al., 2006b). However, *B. product* was not able to demethylate SDG (Clavel et al., 2006b).

1.3.1.3. Dehydroxylation

Dihyroxyenterodiol is dehydroxlated to one of the two enterolignans, ED, by *Clostridium scindens* or *Eggerthella lenta* (Kageyama et al., 1999; Wang, et al., 2000; Clavel et al., 2006b).

Several strains of the latter bacterium can dehydroxylate dihydroxyenterodiol but are not capable of dehydroxylating SECO alone. *E. lenta* works in an enantioselective manner. It can convert (+)-dihydroxyenterodiol to (+)-ED. (Clavel et al., 2006b). Jin et al. (2007b) isolated a bacterium called ARC-1, which is capable of converting (-)-dihydroxyenterodiol into (-)-ED through (-)-monohydroxyenterodiol but not (+)-dihydroxyenterodiol.

1.3.1.4. Dehydrogenation

The final step in the transformation of dietary lignans is the conversion of ED into EL. *Lactonifactor longoviformis*, a recently isolated human gut bacterium, is able to dehydrogenate ED. The dehydrogenation of ED to EL by *L. longoviformis* is enantiospecific: It catalyzes only the dehydrogenation of (+)-ED to (+)-EL, with no effect on the (-)-ED (Clavel et al., 2006b, 2007). In the same manner, *Ruminococcus* strains are capable of oxidizing ED to EL enantioselectively. *Ruminococcus* sp. END-1 converts only the (-)- ED with no effect on (+)-ED. On the other hand, the END-2 strain acts in a similar manner like the *L. longoviformis* by converting only (+)-ED to (+)-EL (Jin et al., 2007a; Jin & Hattori, 2010). Moreover, the *L. longoviformis* is not capable of dehydrogenating SECO (Clavel et al., 2006b).

1.3.2. Breast cancer-preventive effects of lignans

Previous studies revealed that the intake of plant lignans, which are further converted to the bioactive components ED and EL by intestinal bacteria, is associated with a reduction of breast cancer (Serraino & Thompson, 1992; Thompson et al., 1996b, Wang et al., 2005, Fabian et al., 2010). Flaxseed consumption reduces the incidence, number, and growth of tumors in carcinogen-treated rats at the pre-initiation, promotion, or progression stages of carcinogenesis (Serraino & Thompson, 1992; Thompson et al., 1996b). Tumor growth and/or incidence of

metastases are also reduced by dietary flaxseed in athymic mice injected with human ER-negative (Dabrosin et al., 2002; Chen et al., 2002) or ER-positive (Chen et al., 2004) breast cancer cells. The effect of SDG (in pure form or isolated and purified from flaxseed) administration, were similar to those of flaxseed in carcinogen-treated rats (Thompson et al., 1996a; Rickard & Thompson, 1998). The same result was observed in athymic mice with xenografts of ER negative breast tumors (Wang et al., 2005). This indicates that the effect of flaxseed is largely dependent on its SDG content. Furthermore, pure ED and EL inhibit the growth of ER positive breast tumors in athymic mice with low circulating estradiol concentrations (Power et al., 2006).

Urinary excretion of EL is lower in breast cancer patients than in control subjects (Adlercreutz et al., 1982). In 2005, Stuedal et al. investigated possible associations between circulating EL and normal breast tissue morphology. They reported that EL concentration in plasma is negatively correlated with breast mammographic density in postmenopausal Norwegian women. In a pilot study, the observed modulation of breast cancer biomarkers in premenopausal women showed a significant decrease in the Ki-67 index (Ki-67 protein is present during the cell cycle phases; G1, S, G2, and mitosis which makes it an excellent marker for cell proliferation as it is overexpressed in breast cancer cells) in 80% of the studied subjects accompanied by 9 or 16-fold increases of EL or total lignans in plasma (Fabian et al., 2010). In contrast, Velentzis et al. (2009) reported that breast cancer risk was not associated with EL concentrations in blood of pre- or post-menopausal women. A very recent study showed that lignan intake is associated with lower risks of breast cancer in premenopausal women (McCann et al., 2012).

1.3.3. Mechanisms of breast cancer prevention by lignans

The anti-carcinogenic effect of lignans can be explained by several mechanisms, including estrogenic/anti-estrogenic effects, modulated estrogen concentrations, and antioxidant effects. Estrogens play a major role in breast cancer development and progression (Platet et al., 2004). Estrogen exposure increases the risk of breast cancer (Key et al. 2002; Yager & Davidson, 2006). Since enterolignans (ED and EL) have a structure similar to that of estradiol, enterolignans may influence breast cancer via an estrogen-mediated pathway: they may compete with estradiol for estrogen receptor (ER) binding. Depending on endogenous estrogen concentrations, enterolignans have either a weak estrogenic or anti-estrogenic effect (Saarinen et al., 2003; Mueller et al., 2004; Adlercreutz, 2007).

1.3.3.1. Modulation of estrogen synthesis by enterolignans

The modulation of estrogen synthesis and metabolism by enterolignans is a potential mechanism that might protect against breast cancer (Evans et al., 1995; Brooks & Thompson, 2005). Flaxseed consumption influences both the menstrual cycle and the circulating sex hormone concentrations (Phipps et al., 1993; Orcheson et al., 1998). Urinary excretion of lignans is positively correlated with plasma concentration of sex hormone binding globulin (SHBG) which negatively correlated with free plasma estradiol (Adlercreutz et al., 1987). In addition, the ratio of 2-hydroxyestone to 16-α hydroxyestone (urinary biomarker used to estimate the risk of breast cancer) is positively correlated with urinary lignan excretion in postmenopausal women (Brooks et al., 2004). Brook & Thompson (2005) reported a decrease by 84% of the estradiol synthesis in response to inhibition of 17-β hydroxysteroid dehydrogenase by 50 µM EL. Furthermore, a lower concentration of EL (10 µM) decreases the amount of the produced estrone by 37% via the inhibition of aromatase in MCF-7 cells. Also, Hutchins et al. (2001) demonstrated that flaxseed-

rich diets significantly reduce serum concentrations of estradiol and estrone sulfate and increase prolactin in postmenopausal women. Therfore, dietary exposure to lignans may influence the development of hormone-dependent tumors (Landete, 2012).

1.3.3.2. Estrogenicity and anti-estrogenicity of enterolignans

Enterolignans have either a weak estrogenic or anti-estrogenic effect (Saarinen et al., 2003; Mueller et al., 2004). *In vitro* studies indicate that EL and ED at low concentrations (0.1-1 µM) stimulate growth of estrogen-dependent breast cancer cell lines (Welshons et al., 1987; Wang & Kurzer, 1997). On the other hand, ED was not able to elicit an estrogen-like activity in breast cancer cell lines (MCF-7 cell) according to Sathyamoorthy et al. (1994). Estrogens induce cell proliferation by promoting the G1-S phase transition through the induction of the expression of cyclin D1 and by decreasing cyclin-dependent kinase inhibitors. The induction requires both transcriptional activation function domains (Activator Protein, AP) of the ERα (weak constitutive activation function AF-1 which resides in the N-terminal domain and a hormone-dependent activation function AF-2 which is localized in the C-terminal domain) and the DNA binding domain (Prall et al., 1997; Sabbah et al., 1999). EL exerted similar effects as estrogen on ER-reporter gene transcription in uterus and vagina of 3xERE-TATA-Luc-transgenic mice. Moreover, EL induces cyclin D1 and Ki-67 expression in the mouse uterus indicating the estrogenic effect of lignans *in vivo* (Penttinen et al., 2007).

In 1992, Mousavi & Adlercreutz reported that the combination of EL (0.5-2.0 µM) and estradiol (1.0 nM) resulted in a lower cell proliferation of human ER+ breast cancer cell (MCF-7 cell). This anti-proliferative effect of lignan in MCF-7 cells depends on the dose and the estradiol status. In addition, the anti-proliferative activities of enterolignans as antagonist (anti-estrogenic)

showed reduction in tumor number and size in both mouse colon and breast cancer models (Mutanen et al., 2000; Oikannen et al., 2000; Chen et al., 2002; Saggar et al., 2010a,b).

1.3.3.3. Effect of lignans on gene expression

The ER-estradiol complexes act via the classical ER-mediated pathway. Briefly, ER-estradiol complexes bind to estrogen-responsive element (ERE) in target promoters leading to a regulation of gene transcription through binding of coactivators to ER-estradiol complexes and finally increase the cell proliferation. However, the ER-estradiol can regulate gene expression through a protein-protein interaction mechanism. Briefly, ER-estradiol complexes activated transcription of genes through binding to transcription factors (Fos/Jun) at AP1 binding site, resulting in an up-regulation of genes related to cell proliferation (Saarinen et al., 2003; Björnström & Sjöberg, 2005). Conformational changes in ER receptors by their modulators decide which coactivators or corepressors might be induced, which will result in a down- or up-regulation of gene expression involved in cell proliferation (Paige et al., 1999). Enterolignans exert their antiestrogenic effect through direct binding with ER receptors and to the ERE within the gene promoters which then interacts with corepressors and deactivate the transcription of the target gene. However, ED and EL acts as ERα agonists (estrogenic effect) via interacting with c-Jun and c-Fos, on the AP1 binding site of the target promoter and initiates gene transcription (Figure 2) (Barkhem et al., 1998; Kuiper et al., 1998: Mueller et al., 2004; Ström et al., 2004; Carreau et al., 2008).

The growth factor receptor can start the activation of protein kinases, MAPK, and AKT, which in turn activates downstream a cascade of cyclin-dependent kinases that cause irreversible progression of cells to the G/S phase transition, finaly increasing cell proliferation and decreasing apoptosis (Chen et al., 2009).

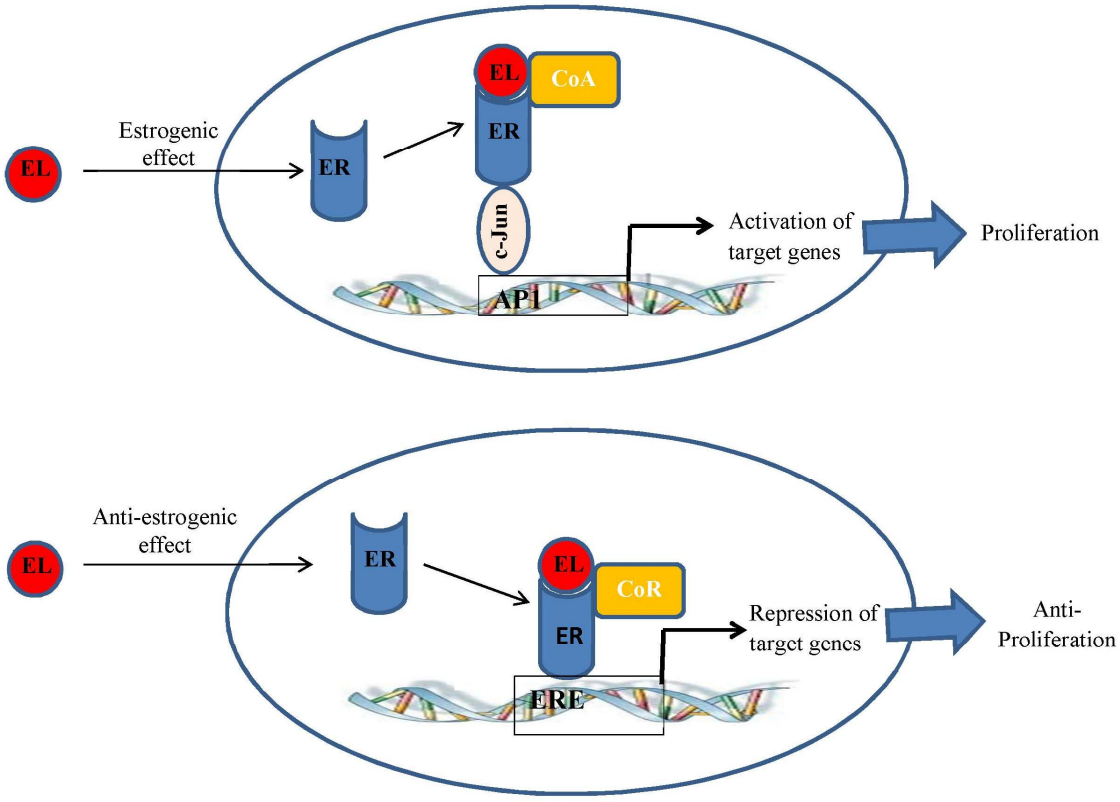


Figure 2. Possible estrogenic and anti-estrogenic mechanism of enterolignans; conformational changes in ER receptors by enterolignan decide which coactivators or corepressors might be induced, which will result in a down- or up-regulation of gene expression involved in cell proliferation. Enterolignan either mimic the estrogen effect on gene expression via indirect-binding to AP1 binding site on DNA or exert their anti-estrogenic effect via binding to ERE on DNA. EL: enterolactone, ER: estrogen receptor, ERE: estrogen-responsive element, AP1: activator protein-1, c-Jun: a transcription factor, CoA: coactivators, CoR: corepressors.

Dietary flaxseed and its equivalent amount of pure SDG reduced tumor size and cell proliferation in ovariectomized-athymic mice with an increase in apoptosis in this model. This effect may be mediated through the inhibition of ER- and growth factor-mediated signaling pathways (Chen et al., 2002; Dabrosin et al., 2002; Chen et al., 2009; Saggar et al., 2010a,b).The increased plasma insulin-like growth factor 1 (IGF-1) concentrations are associated with increased breast cancer

risk. IGF-1 levels in a rat breast cancer model treated with N-methyl-N-nitrosourea were reduced by dietary supplementation with flaxseed or SDG (Richard et al., 2000). In genome-wide expression profiles in human MCF7 cells, 96 transcripts were differentially expressed with at least a five-fold change ($p<0.01$) relative to control when MCF-7 cells were incubated with EL at a concentration of 1 µM. These genes were similarly affected by estradiol (correlation coefficients of R=0.85) indicating that EL and estradiol have overlapping target genes in MCF-7 cells (Dip et al., 2008).

1.3.3.4. Antioxidant effect of lignans

It is possible that beneficial effects of enterolignans are mediated through the activation of antioxidant enzyme systems. Prasad (1997) showed the ability of SDG to scavenge hydroxyl radicals. Findings by Prasad (2000) indicate that SDG and its metabolites SECO, ED, and EL are scavengers of reactive oxygen species (ROS) generated by zymosan-activated polymorphonuclear leukocytes (PMNLs) in blood. The relative antioxidant potential of SECO, ED, EL, and SDG as compared to vitamin E were 4.9, 5.0, 4.3, and 1.3 fold, respectively (Prasad, 2000). Rajesha et al. (2006) reported that the pre-treatment of CCl4-treated rats with 5 and 10% flaxseed restored catalase and superoxide dismutase by 39.7 and 181.42%, respectively, in comparison with CCl4-treated rats without the flaxseed pre-treatment. SDG and its metabolites ED and EL showed antioxidant activity against DNA damage and lipid peroxidation in lipid and aqueous *in vitro* model systems (Kitts et al., 1999; Hu et al., 2007). Moreover, SDG and SECO prevented the degradation (change in the fundamental chemistry of the base oil molecules by oxidation process, thermal or compressive heating) of canola oil in a concentration-dependent manner (Hosseinian et al., 2006, 2007).

2. OBJECTIVES

Previous studies revealed that gut bacteria might play an important role in the initiation and/or reduction breast cancer (Gorbach, 1984; Usman, 2000; Rachid et al., 2006; Rao et al., 2006). It is well known that intestinal bacteria are essential for the transformation of SDG to ED and EL. It is also known that the EL is associated with the reduction of breast cancer but the role of the intestinal bacteria responsible for transforming lignans into enterolignans on breast cancer development is not yet clear. The aim of the current study was to investigate the effect of bacterial lignan transformation on chemical-induced breast cancer in gnotobiotic rats. The current study aimed to:

1. Study plant lignan transformation to enterolignans by lignan-converting bacteria in the intestine and their excretion in breast cancer model using gnotobiotic rats.
2. Study the effect of the bacterial lignan transformation on tumor formation in a rat breast cancer model.
3. Evaluate breast cancer biomarkers such as Ki-67 labeling index.
4. Study the possible mechanism underlying the possible beneficial effect of bacterial lignan transformation on breast cancer.

3. MATERIALS AND METHODS

3.1. Bacterial strains and culture conditions

C. saccharogumia DSM17460^T, *B. producta* DSM3507, *E. lenta* DSM2243^T and *L. longoviformis* DSM17459^T (obtained from the German Institute of Human Nutrition Potsdam-Rehbruecke, Germany) were selected according to Woting et al. (2010), who demonstrated the activation of SDG to ED and EL in gnotobiotic rats associated with those bacteria. The lignan-converting bacteria were grown in Brain Heart Infusion Broth (Oxoid, Karlsruhe, Germany) supplemented with haemin (0.5g/100 ml) and yeast extract (0.5g/100 ml) (BHI-YH) under anaerobic condition at 37°C. Colony and cell morphology was examined after anaerobic growth on BHI-YH agar (Oxoid, Basingstoke, U.K.) and Gram staining, respectively, in order to examine the purity of the cultures. In addition, strains were incubated on BHI-YH-agar under aerobic conditions to check for the presence of aerobic contaminants. *C. saccharogumia, E. lenta,* and *L. longoviformis* were subcultured every two weeks except *B. producta,* which was subcultured every week.

3.2. Sequencing of the bacterial 16S rRNA gene

16S rRNA gene sequencing was carried out to check the identity of the four lignan-converting bacteria. Bacterial cells were collected from overnight cultures by centrifugation at 8000 ×g for 5 min. Total DNA was extracted with the RTP® bacteria DNA Mini kit (Invitek, Berlin, Germany) following the manufacturer's protocol. The 16S rRNA gene of *C. saccharogumia, B. producta and L. longoviformis* was amplified by polymerase chain reaction (RCR) using the primers 27f (5'AGAGTTTGATCCTGGCTCAG 3') and 1492r (5'TACCTTGTTACGACTT 3'). The reverse primer 1401-r (5'CGGTGTGTACAAGACCC 3') was used to amplify the bacterial 16S rRNA

gene of *E. lenta* (Kageyama et al., 1999). All primers were synthesized by Eurofins MWG (Berlin, Germany). The PCR reactions (50 µl) contained 1×PCR buffer, 2.5 mM $MgCl_2$, 0.25 mM of dNTPs, 0.1 µM of each primer, 2.5 units Taq DNA polymerase and 1 µl of template DNA. The PCR program was as follows: 94°C for 4 min, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and finally 72°C for 10 min. PCR products were analyzed by electrophoresis and the sizes of the DNA fragments were estimated using a DNA mass ladder. PCR products were purified with a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. DNA concentrations were measured with a Nano-Drop spectrophotometer (peqlab, Erlangen, Germany); DNA sequencing was performed by Eurofins MWG using the PCR primer 27f. The obtained sequences were used to identify the most closely related bacterial species by the BLAST function of the National Center for Biotechnology Information (NCBI).

3.3. *In vitro* fermentation experiment with the lignan-converting bacteria

The objective of this part was to study the *in vitro* transformation of the plant lignan SDG by lignan-converting bacteria. The composition of the different culture media for conversion experiments is shown in Table 2. All media were prepared in Hungate tubes with butyl rubber stoppers and screw caps. The tubes were gassed with N_2/CO_2 (80/20, v/v) and subsequently autoclaved. Sterilized and gassed glucose, fructose and vitamin mixture were added to the autoclaved media to a final concentration of 10%, 10%, and 0.1%, respectively. To study the *in vitro* deglycosylation of SDG to SECO, 50 µl of an overnight culture of *C. saccharogumia* were added to 2 ml of Mt-6 medium containing 500 µM SDG (SDG-Mt-6 medium). The demethylation and dehydroxylation of SECO to ED by *B. producta* and *E. lenta* were tested in Mt-6 medium supplemented with 500 µM SECO (SECO-Mt-6 medium).

Table 2. Culture media for the *in vitro* transformation of plant lignan[a]

Components and stock solutions	Mt-6 medium	Lignan media			
		Flaxseed extraction-Mt-6	500 µM SDG-Mt-6	500 µM SECO-Mt-6	500 µM ED-Mt-6
	ml/l				
Yeast extract, 2%	150	150	150	150	150
Sodium acetate, 2.5%	50	50	50	50	50
Sodium formate, 2.5%	50	50	50	50	50
Rumen fluid	50	50	50	50	50
Haemin, 0.05% of 1M NaOH	10	10	10	10	10
Salt 1 solution[b]	100	100	100	100	100
Salt 2 solution[c]	2	2	2	2	2
Trace element solution[d]	0.1	0.1	0.1	0.1	0.1
Resazurin solution, 0.1 %	1	1	1	1	1
Cysteine.HCl.H_2O, g	0.5	0.5	0.5	0.5	0.5
Peptone, g	3	3	3	3	3
Flaxseed exctration	-	5	-	-	-
SDG 100 mM	-	-	5	-	-
SECO 100 mM	-	-	-	5	-
Distilled water[e]	382.4	377.4	377.4	377.4	377.4
Fructose 1.8%	100	100	100	100	100
Glucose 1.8%	100	100	100	100	100
ED 100 mM	-	-	-	-	5
Vitamin solution[f]	1	1	1	1	1

[a] Clavel (2005).

[b] Salt 1 solution was 476.1 mM $NaHCO_3$, 171.1 mM NaCl, 121.5 mM NH_4Cl, 22.0 mM KH_2PO_4, 17.2 mM K_2HPO_4 and 12.0 mM $MgSO_4$ in H_2O, autoclaved (121ºC, 15 min).

[c] Salt 2 solution was 16.5 mM $FeSO_4$, 14.9 mM $MnSO_4$, 6.9 mM $CoCl_2$, 6.6 mM $ZnCl_2$, 6.3 mM $CaCl_2$ and 2.3 mM $(NH_4)_2SO_4$ in H_2O, autoclaved (121ºC, 15 min).

[d] Trace element solution was 7.4 mM $CuCl_2$, 6.5 mM $NiCl_2$ and 4.9 mM $MoNa_2O_4$ in H_2O, autoclaved (121ºC, 15 min).

[e] pH was adjusted to 7.5

[f] Vitamin solution was 0.049 mM pyridoxine-HCl, 0.015 mM thiamin-HCl, 0.013 mM riboflavin, 0.041 mM nicotinic acid, 0.01 mM Ca-pantohthenate, 0.029 mM P-4-aminobenzoate, 0.024 mM lipoic acid, 0.008 mM biotin, 0.005 mM folic acid, 0.001 mM cyanocobalamin, sterial filtered (0.22 µm).

Sterile-filtered ED (0.22 µM, Miller®–GV, Millipore) was added to Mt-6 medium with a sterile syringe at a final concentration of 500 µM (ED-Mt-6 medium) followed by the inoculation with 50 µl of an overnight culture of *L. longoviformis* to test the dehydrogenation of ED to EL. All experiments were conducted at 37°C for 72 h. Aliquots (100 µl) were taken every 24 h.

The kinetics of the *in vitro* transformation by the lignan-converting bacteria of SDG to EL was investigated. Flaxseed (2 g) was extracted overnight by aqueous methanol solution 70% (60 ml) at room temperature. The methanol extract was centrifuged (4000 ×g, for 10 min) and the supernatant was collected. The methanol was evaporated and the residue lyophilized. The SDG-containing lyophilisate was dissolved in 5 ml distilled water and subsequently added to Mt-6 medium (flaxseed extraction-Mt-6 medium). The initial concentration of SDG was approximately 140 µM. An aliquot (12.5 µl each) of an overnight culture of *C. saccharogumia, B. producta, E. lenta,* and *L. longoviformis* was transferred to the flaxseed extraction-Mt-6 medium and incubated at 37°C for 72 h. During the first 8 h of incubation, samples (50 µl) were taken every 2 h. During the following 64 h of incubation, samples were taken ever 24 h. Bacteria were grown in Mt-6 media in the presence or absence of substrate. Substrate-containing medium without bacteria was used as control. All collected samples were centrifuged (13 000 ×g, for 3 min) and subsequently analyzed by High Performance Liquid Chromatography (HPLC, see section 3.4.1.3) in order to detect the bacterial metabolites.

3.4. Animal experiment

Gnotobiotic rats were used to investigate effects of bacterial lignan transformation on breast cancer formation and selected cancer-associated parameters in a 7,12-dimethylbenz(a)anthracene (DMBA) induced cancer model. Female germ-free Sprague Dawley rats (three-week-old rats

with an initial body weight of approximately 140 g) were randomly divided into two groups, 10 rats per group. Animals were retained in two different Trexler-type plastic film isolators in environmentally controlled conditions at 20°C ± 2 °C, with 55 % ± 10 % air humidity and 12 h light/dark cycle. Autoclaved distilled water and standard chow (Altromin 1314, Altromin, Lage, Germany) sterilized by gamma irradiation (50 kGy) were offered *ad libitum* to all rats. The lignan-converting bacteria *C. saccharogumia, B. producta, E. lenta and L. longoviformis* (300 µl inoculum containing 10^8 cells of each bacterial species) were given to each rat of the experimental group (LCC rats) through an oral gavage. The rats of the control group received BHI-YH medium without bacteria and remained germ-free. Fecal samples were collected during the study so as to check the establishment of the lignan-converting bacterial consortium in the experimental group and the germ-free status of the control group. The rats were switched from a standard diet to a flaxseed-rich diet (see appendix, Table 15A) two weeks after association. Fecal samples were collected after the dietary change to determinate ED and EL by HPLC to confirm whether the four bacterial strains are capable of converting SDG to ED and EL. Two weeks after dietary intervention, breast cancer was induced with a single dose of 25 mg DMBA (Sigma-Aldrich, Tauf kirchen, Germany) dissolved in 1 ml corn oil given through an oral gavage to each rat in both groups. Body weight and tumors palpation were recorded weekly. At the termination of the experiment (thirteen weeks after DMBA application), animals were housed in metabolic cages for 24 h in order to collect feces and urine, also the food consumption was recorded. Subsequently, the rats were anaesthetized and blood samples were collected from the retrobulbar venous plexus. Plasma and serum were separated by centrifugation at 4°C (3000 rpm for 5 min) and stored in aliquots at -20 °C until further analysis. Breast tumors were removed, counted, and weighed after killing the animals. Tumor size was calculated according to the formula (length/2

× width/2) × π. Parts of the tumors were fixed in neutral buffered formalin (10%) for histological and immunohistochemical examination. The remaining tumor material was frozen in liquid nitrogen and stored at -80 °C for RT-PCR analysis. Aliquots from colonic and cecal contents were fixed for bacterial enumeration and the remaining material was freeze-dried and stored at 4 °C until further analysis. Different organs (liver, kidney, uterus, heart, spleen, cecum, and lung) were also separated and weighed. The liver was frozen in liquid nitrogen and stored at -80 °C. The experimental protocol was approved by the local animal welfare committee (approval no. 23-2347-8-16-2008).

3.4.1. Determination of lignans

To study plant lignan transformation to enterolignans by lignan-converting bacteria in the intestine and their excretion in a rat model of breast cancer, lignan contents in flaxseed, flaxseed diet, feces, gut contents, and urine were determined according to the method of Woting et al. (2010) with minor modifications.

3.4.1.1. Determination of SDG

SDG was extracted from flaxseed, flaxseed diet, and gut contents. A 0.1 g of sample material was defatted with 2 ml n-hexane for 2 h at room temperature. The air-dried defatted samples were incubated in aqueous methanol 70 % (3 ml) for 3 h at 55 °C, and thereafter overnight at room temperature. Supernatants were collected upon centrifugation at 4000 ×g for 10 min. The sediments were washed twice with aqueous methanol (70%) and the centrifugation was repeated. The supernatants were pooled and subsequently evaporated (Speed vac RC 10-22, Dreieich, Germany) and sediments were freeze-dried. The freeze-dried samples were dissolved in 1 M NaOH (1 ml) for 3 h at 50 °C and pH was adjusted to 7.0 with 0.5 M HCl. The SDG-containing

extracts were lyophilized, re-suspended in 80 µl aqueous methanol (70 %) and subsequently subjected to HPLC analysis.

3.4.1.2. Determination of SECO, ED, and EL

Free SECO, ED, and EL were measured in feces and gut contents. Aqueous methanol solution (70 %; 600 µl) was added to 0.1 g of samples, followed by vortexing for 10 min. After centrifugation (4000 ×g, 10 min), the supernatants were collected. The sediment was washed twice with aqueous methanol (70%) and the centrifugation was repeated twice. The supernatants obtained after each centrifugation step were combined and subsequently evaporated and lyophilized. Prior to the HPLC separation, lyophilisates were re-suspended in 60 µl aqueous methanol (70 %).

Total SECO, ED and EL were determined in feces and gut contents. Sodium acetate buffer (0.1 M, pH 5) containing 1000 U of a β-glucuronidase/sulfatase preparation (type HP-2; Sigma-Aldrich, Saint Louis, USA) was mixed with sample material (0.1 g) and subsequently incubated overnight at 37 °C. After incubation, diethyl ether (800 µl) was added to samples, followed by vortexing for 10 min. The upper lignan-containing diethyl ether phase was pooled after centrifugation at 4000 ×g for 10 min. The sediment was washed twice with diethyl ether and the centrifugation was repeated twice. Prior to the HPLC separation, diethyl ether was evaporated and sediments were re-suspended in 60 µl aqueous methanol (70 %).

Frozen urine samples were thawed and centrifuged (1000 ×g for 15 min, at 4°C) and subsequently filtered. In order to meaure free SECO, ED and EL, 800 µl diethyl ether was added to 200 µl filtrate followed by continous stirring for 10 min and centrifugation at 4000 ×g for 10 min. The extraction procedure was repeated twice and the total upper diethyl ether phase was

pooled and evaporated. The sediments were dissolved in 70 % aqueous methanol for HPLC analysis. For measurements of the total lignans (SECO, ED and EL), the filtrate (200 µl) was mixed with sodium acetate buffer (0.10 M, pH 5) containing 300 U of the β-glucuronidase/sulfatase preparation and thereafter incubated for 16 h at 37 °C. Total lignans were extracted with diethyl ether as previously described. All samples were centrifuged (13 000 × g; 3 min) before HPLC separation.

3.4.1.3. Detection of lignans by HPLC

Lignans were quantified with High Performance Liquid Chromatography (HPLC) instrument (Gynkotek, Germering, Germany). The eluting lignans were monitored using a UV/Vis diode array detector (UVD-320) at 285 nm and 275 nm. The reverse-phase C18 column (LiChrospher® 100 RP-18, 250-4 mm, Merck, Darmstadt, Germany) with a particle size of 5 mm was protected with a guard RP-18 column (4×4 mm, 5 µm). The temperature of the column was 37°C. The flow rate was 0.3 ml/min and the injection volume was 20 µl. The gradient program was applied with eluent A (50 mM sodium acetate (pH 5)/methanol, 80/20, v/v) and eluent B (50mM sodium acetate/methanol/acetonitrile, 40/40/20, v/v/v) as follows: 20-80% B for 10 min, 80-100% B for 14 min, 100% B for 11 min and 1 min to return to 20% B, then 10 min equilibration period resulting in a total duration of 46 min. Signals from the detector were recorded simultaneously by the data system Chromeleon version 6.40 (Dionex, Idstein, Germany). Chromatographic peaks were identified by comparing the retention times and UV spectra with the respective retention times and UV spectra of known standard reference material. The concentrations of SDG, SECO, ED, and EL were quantitated by measuring peak area and comparing them to a standard curve generated by plotting area counts against concentration of standards (5 to 1500 µM) SDG, SECO, ED, and EL (Sigma-Aldrich, Tauf kirchen, Germany).

3.4.2. Tumor cell proliferation, apoptosis, and estrogen receptor status

Ki-67 and active caspase-3 were measured as markers for tumor cell proliferation and apoptosis, respectively. Estrogen receptor (ER) status is a prognostic and predictive factor in breast cancer (Cianfrocca & Goldstein, 2004). Sections (5 µm) of formalin-fixed and paraffin embedded tumor tissue were used for immunohistochemistry analysis. Deparaffinized and rehydrated tissue sections were incubated with 3%H_2O_2 in order to block endogenous peroxidase activity. The antigen was unmasked by heating in citrate buffer (10 mM, pH 6) for 10 minutes in a microwave oven. The primary antibodies were MIB-5 for Ki-67 (Dianova, Hamburg, Germany), Asp175 for cleaved caspase-3 (Cell Signaling, Beverly, MA) and EI629C01 for ER (DCS, Hamburg, Germany). MIB-5 was diluted 1:100 with Tris-buffered saline (50 mM, pH 7.6). Asp175 was diluted 1:200 and EI629C01 was diluted 1:400. Sections were incubated overnight at 4°C with the primary antibodies after blocking non-specific antigens with diluted normal goat serum. Subsequently, the secondary antibody (biotinylated goat anti-rabbit IgG) was applied and followed by incubation for 60 min at room temperature. Tumor sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complexes (ABC kit, Vectastain® Elite, Burlingame, USA) for 30 min. Slides were stained with chromogen diaminobenzidine tetrahydrochloride (Sigma-Aldrich, Munich, Germany) and counterstained with hematoxylin.

The *in situ* terminal deoxynucleotidyl transferase-mediated nick end labelling (TUNEL) assay was used to detect apoptosis cells in tumor. The TACS.XL-DAB *in situ* apoptosis detection kit (Trevigen, Gaithersburg, MD) was applied according to the manufacturer's instructions. Formalin-fixed and paraffin embedded tumor sections were deparaffinized and rehydrated followed by incubation with proteinase K (1:50 dilution) for 30 min at 37°C. Sections were

treated with 3% H_2O. After washing, the sections were treated with labelling reaction mixture (terminal deoxyribonucleotidyl transfer enzyme TdT, TdT buffer, and dNTP mix) and incubated for 1 h at 37°C. After washing, the antibody (Biotinylated mouse monoclonal antibody to bromodeoxyuridine, anti-BrdU) was applied followed by incubation for 10 min at room temperature. The slides were stainied with diaminobenzidin and counterstained with methyl green. All slides were viewed under light microscope at 400× manifigation.

For Ki-67 labeling index calculation, positive and negative nuclear cells were counted in 15 fields and the Ki-67 labeling index was calculated as percentage of positive cells among all cells. Apoptosis were detected by active caspase-3 and TUNEL assay, the positive nuclear cells were counted and expressed as apoptotic cell number per mm^2. Allred score (Allred et al. 1990) was used to assess the ER status of the tumor cells. The score points of Allred reflect the proportion of positive cells (scored on a scale of 0-5) and the intensity of the staining (scored on a scale of 0-3). The proportion was evaluated as percentage of positive cells among all cells of the same cell type according to the following scale: 0 means no positive cells; 1 for <1%; 2 for 1–10%; 3 for 11–33%; 4 for 34–66%; and 5 for 67–100%. Intensity of staining was evaluated in three grades between negative (0 for negative) and intense (1 for weak; 2 for moderate; 3 for strong). The proportion and intensity were then summed up resulting in a maximum score of 8. A score of 0-2 was regarded as negative while 3-8 as positive.

3.4.3. Gene expression analysis by quantitative real-time PCR (RT-PCR)

The relative expression of the estrogen-sensitive genes (estrogen receptors α, ERα; β, ERβ; and the G-coupled protein 30, GPR30) and selected genes involved in growth factor signaling

pathways (epidermal growth factor receptor, EGFR; and insulin-like growth factor 1, IGF-1) were determined by quantitative RT-PCR in breast tumors.

3.4.3.1. RNA extraction from tumor tissue and cDNA synthesis

Total RNA was isolated from <50 mg of tumor tissue material with the RNasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Briefly, tumor tissues were homogenized with buffer using tissue lyser (Qiagen, Penzberg, Germany) followed by centrifugation. The supernatants were transferred to RNeasy spin column and RNA was extracted by solid phase extraction. After RNA extraction, RNA concentrations were measured with a NanoDrop spectrophotometer (Peqlab, Erlangen, Germany). Purity of the extracted RNA was assessed by the A_{260nm}/A_{280nm} ratio.

The first strand complementary DNA (cDNA) was synthesized from RNA with the RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. RNA template (one µg) was incubated for 5 min at 65°C with oligo $(dT)_{18}$ primer followed by incubated for 60 min at 42°C. The reaction was stopped by heating at 70°C for 5 min. The cDNA was amplified by PCR using TrueStart^{TM} Hot Start *Taq* DNA polymerase (Fermentas, St. Leon-Rot, Germany) and primers for the housekeeping gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to test whether the cDNA had successfully been synthesized. The PCR program was as follows: 94°C for 4 min, 30 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and finally 72°C for 10 min. PCR products were examined by electrophoresis.

3.4.3.2. Real time PCR

Real-time PCR was performed with a Stratagene's Mx3005P QPCR system (Agilent Technologies, Böblingen, Germany). The RT-PCR reaction mix (25 µl) contained 1 µl template cDNA, 1× the Quanti Fast™ SYBR Green PCR master mix (Qiagen, Penzberg, Germany) and 0.2 µM of the respective primer pairs. The primer pairs used to measure the relative expression of ERα, ERβ, IGF1, and EGFR (Table 3) were designed using Primer-Premier™ version 5.0 software and checked by BLAST search. GPR30 primers were adapted from the literature (Sakamoto et al., 2007). All primers were synthesized by Eurofins MWG (Berlin, Germany). Primer specificity was tested by PCR (see conditions 3.4.3.1) and checked for nonspecific bands in a 1.5% agarose gel. The same conditions were used to measure the RT-PCR standards.

Table 3. Primers used for RT-PCR gene expression analysis of estrogen-sensitive genes and genes involved in growth factor-signaling pathways

Genes	Sequence 5´-3´, (for: forward, rev: reverse)	Annealing temperature	Product size
ERα	for :5´-GCA TGA TGA AAG GCG GGA TAC GA-3´ rev :5´-AAA GGT TGG CAG CTC TCA TGT CTC-3´	67°C	129 bp
ERβ	for :5´-TGG TCT GGG TGA TTG CGA AGA G-3´ rev :5´-ATG CCC TTG TTA CTG ATG TGC C-3´	67°C	109 bp
GPR30	for :5´-CGA GGT GTT CAA CCT GGA CGA-3´ rev :5´-GGC AAA GCA GAA GCA GGC CT-3´	67°C	286 bp
EGFR	for :5´-TGC CCA CTA TGT TGA TGG TCC C-3´ rev :5´-GCC CAG CAC ATC CAT AGG TAC AG-3´	67°C	112 bp
IGF-1	for :5´-AAG ACT CAG AAG TCC CAG CCC-3´ rev :5´-GGT CTT GTT TCC TGC ACT TCC T-3´	67°C	147 bp
GAPDH	for :5´-CAA GGT CAT CCA TGA CAA CTT TG-3` rev :5´-GTC CAC CAC CCT GTT GCT GTA G-3´	55°C	496 bp

PCR reactions were performed in 96-well PCR plates (Peqlab, Erlangen, Germany) using the following protocol: 95°C for 5 min, 40 cycles of 15 seconds at 95°C, 15 seconds at 67°C/55°C, 15 seconds at 72°C, melting curve program (60-95°C) and finally a cooling step to 4°C.

Specificity of the PCR product was tested by melting curve analysis and gel electrophoresis. PCR water was used instead of cDNA templates as a negative control and three replicates per sample were applied in one RT-PCR run. The relative standard curve method (Larionov et al., 2005) was used to calculate the relative expression levels of the target genes; the target gene expression was normalizied to the expression of the house-keeping gene GAPDH.

3.4.4. Microbial status

Lignan-converting bacteria were enumerated by fluorescence *in situ* hybridisation (FISH). Short chain fatty acid (SCFA) production, a major fermentation product by intestinal bacteria, was also determined.

3.4.4.1 Quantification of bacterial cells by fluorescent *in-situ* hybridization (FISH)

The establishment of the four lignan-converting bacteria in LCC rats was examined in fecal material and gut contents by FISH according to the method of Thiel & Blaut (2005) using species-specific 16S rRNA-targeting 5'-Cy3-labeled oligonucleotide probes (Table 4). Fecal material and intestinal contents were homogenized with PBS (8.0 g/l NaCl, 0.2 g/l KCl, 1.44 g/l Na_2HPO_4 and 0.24 g/l KH_2PO_4 in H_2O, pH 7.4) and subsequently centrifuged at 300 ×g for 1 min at 4°C. The samples were fixed for 1 h at 4°C with 4% paraformaldehyde (1:3, v/v) followed by centrifugation at 8000 ×g, for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in PBS/ethanol solution (1:1, v/v). The fixed samples were homogenized with a Uniprep-Gyrator (UniEquip, Martinsried, Germany) for 5 min at full speed followed by ultra-sonication for 10 seconds (0.5 cycles, 20% amplitude). Diluted samples (10 µl) were applied to FISH slides and mixed with 10 µl of tween/PBS (1:10, v/v), and allowed to dry in an oven at hybridization temperature. Samples were dehydrated by placing slides in different concentrations

of ethanol (60, 80, and 96%). After drying, 11 µl of oligonucleotide probes (10 µM in hybridization buffer composed of 0.9 M NaCl, 10 mM Tris-HCl, pH 7.4, and 0.01 % SDS except for probe ProCo-1264 for which the hybridization buffer contained 30% (v/v) formamide). The slides were incubated overnight in a moist chamber at hybridization temperature and subsequently washed with hybridization buffer to remove the probes. They were subsequently immersed in hybridization buffer and incubated in the oven for 20 min at 2°C above the hybridization temperature. After drying, slides were stained with 4',6-diamidino-2-phenylindole (DAPI) and prepared for microscopy. Bacterial cells were enumerated with a fluorescence microscope (Carl Zeiss, Germany) and expressed as $\log_{10}$/g dry weight.

Table 4. Oligonucleotide probes used for the detection of lignan-converting bacteria in fecal material and gut contents

Probes	Targets	Sequence 5`-3`	Temp. of hybridiz-ation	Reference
S-S-Csac-0067-a-A-20	*C. saccharogumia*	CTCGGACATTAC TGCCCGCG	46	Woting et al., 2010
S-*-ProCo-1264-a-A-23	*B. producta*	TTGGGATTCGCT CAACATCGCTG	35	Clavel et al., 2005
S-*-Ato-0291-a-A-17	*E. lenta*	GGTCGGTCTCTC AACCC	54	Harmsen et al., 2000
S-S-Llong-0831-a-A-20	*L. longoviformis*	GGACGCCTTTGG CGCCCGAC	46	Woting et al., 2010

3.4.4.2. Determination of SCFA

SCFA concentrations were determined in cecal and colonic material according to the method of Becker et al. (2011). Gut content was diluted with PBS (1:5, v/v) followed by vortexing for 5 min then centrifugation at 15 000 ×g for 5 min. Supernatants (200 µl) were mixed with 1.0 M

NaOH, 0.36 M $HClO_4$, and 12 mM iso-butyric acid (internal standard). After overnight lyophilization of the samples, the residues were dissolved in 100 µl of 5 M formic acid and 400 µl acetone and centrifuged at 15 000 ×g for 5 min. The organic phase containing the SCFA (1.0 µl) was injected into a gas chromatograph (Hewlett-Packard, Waldbronn, Germany) equipped with a HP-FFAP capillary column (30 m × 0.53 mm i.d., 1 µm film thickness). Helium (1 ml/min) was used as the carrier gas. Standard of SCFA (Supeclo Mix WSFA-2, Sigma) was injected in gas chromatograph in order to calculate the SCFAs (acetic, propionic, butyric, isovaleric, and valeric acids) as ng/µl and the results were calculated as µmol/g dry weight.

3.4.5. Determination of 17β-estradiol in serum

The 17β-estradiol concentration in serum was measured with the Estradiol EIA Kit (Cayman Chemical Co., USA) according to the manufacturer's specifications. The assay was carried out in 96-well plates pre-coated with mouse monoclonal anti-rabbit IgG. Serum samples (50 µl) were pipetted into 96-well plates followed by the addition of 50 µl estradiol-acetylcholinestrase conjugate (estradiol tracer) and 50 µl estradiol antiserum. After incubating for 1 h at room temperature, 200 µl of Ellman`s reagent was added to each well. The plate was allowed to stand for 1.5 h at room temperature in the dark for color development. The absorbance of the yellow color was read in a spectrophotometer (Smartspec^{TM} plus, Bio Rad, Germany) at 412 nm. Blank, serial dilution of standards (6.6-4000 pg/ml), non-specific binding sample, and maximum binding sample were run in parallel in an identical manner. All samples were run in duplicate. A standard curve was graphed using a 4-parameter logistic curve fit. The sensitivity of the assay was 19 pg/ml.

3.4.6. Determination of antioxidant activity in liver and plasma

Sample preparation was carried out according to Amin (2008) with minor modifications. The right liver lobe of each rat was homogenized in a glass homogenizer tube with cold PBS. The ratio of tissue weight to homogenization buffer was 1:5 (w/v). After centrifugation at 15,000 ×g for 15 min the supernatant was collected.

3.4.6.1. Catalase activity

Catalase (CAT; EC 1.11.1.6) is an antioxidant enzyme that catalyzes the conversion of hydrogen peroxide to water and oxygen (Aebi, 1984). CAT activity was determined according to Aebi (1984). Aliquots (10 µl) from liver homogenate or plasma containing the enzyme were pipetted into a UV cuvette. The reaction was initiated by adding 1 ml of 10 mM H_2O_2 in phosphate buffer (50 mM, pH 7.0). The initial absorbance was recorded and the disappearance of H_2O_2 was monitored by following the decrease in absorbance at 240 nm for 5 min. A blank was run in parallel in an identical manner. The extinction coefficient of 39.4 $mM^{-1}cm^{-1}$ was used to calculate the activity. Enzyme activity was expressed in µM H_2O_2 consumed per min per mg of protein in liver and µM H_2O_2 consumed per min per ml of plasma.

3.4.6.2. Superoxide dismutase activity

Superoxide dismutase (SOD; EC 1.15.1.1) is an antioxidant enzyme, which converts the superoxide radical ($O_2^{\cdot -}$) to hydrogen peroxide (Beauchamp & Fridovich, 1971). SOD activity was measured using the photochemical method described by Beauchamp & Fridovich (1971) with minor modifications. SOD can inhibit the reduction of nitro blue tetrazolium (NBT) by superoxide which is generated by the photo-reduced riboflavin. The reaction mixture of 3 ml was composed of 1700 µl 50 mM phosphate buffer at pH 7.8, 150 µl 0.1 mM EDTA, 600 µl 13 mM

methionine, 300 µl 0.50 mM NBT in ethanol, 200 µl 0.12 mM riboflavin, and aliquots of 50 µl of diluted liver homogenate or plasma containing the enzyme. Blank and positive controls were run in parallel in an identical manner in the dark. Blanks were kept in the dark and the positive control and samples were illuminated. Reduction of NBT, during 4-10 min of illumination, was determined by measuring the increase in absorbance at 560 nm. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT in the presence of riboflavin and light.

3.4.6.3. Glutathione-S-transferase activity

Glutathione-S-transferase (GST; EC 2.5.1.18) is an antioxidant enzyme that detoxifies the body from potentially damaging hydrophobic and electrophilic compounds (Mannervik & Guthenberg, 1981). GST activity was measured according to Mannervik & Guthenberg (1981). Enzyme activity was determined by measuring the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm. The reaction mixture contained 500 µl 0.2 M phosphate buffer at pH 6.5, 50 µl 20 mM glutathione (GSH), and 50 µl aliquots of diluted liver homogenate or diluted plasma containing the enzyme, and deionized water (400 µl). The reaction was initiated by adding 50 µl of 20 mM CDNB in ethanol. The increase in the absorbance at 340 nm was measured during 3 min. A blank was run in parallel in an identical manner. The extinction coefficient of 10 $mM^{-1}cm^{-1}$ was used to calculate the activity. A unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 µM of 2,4-dinitrophenylglutathione per min.

3.4.6.4. Reduced glutathione levels

Reduced glutathione is the major endogenous antioxidant; GSH changes the level of reactive oxygen species (Ellman, 1959). The GSH content in liver or plasma was measured by reaction with 5, 5`dithiobis -2-nitrobenzoic (DTNB) in 0.1 M phosphate buffer (pH 7), as described by Ellman (1959). DTNB cleaves the disulfide bond in GSH to produce 2-nitro-5-thiobenzoate, which in water at netural and alkaline pH results in a yellow color. The reaction mixture contained 750 µl of diluted liver homogenate or plasma, 500 µl 0.1 M phosphate buffer at pH 8, which were filled up to a final volume of 3 ml with distilled water. The reagent DTNB (20 µl) was added to the reaction mixture. The intensity of the developed color after 2 min of incubation was measured at 412 nm. The GSH concentration was calculated from a standard curve of glutathione.

3.4.6.5. Malondialdehyde levels

Malondialdehyde (MDA) is the most abundant individual aldehyde resulting from the breakdown of lipid peroxidation in biological systems (Mihara & Uchiyama, 1978). Determination of MDA, as described by Mihara & Uchiyama (1978), was based on its reaction with thiobarbaturic acid (TBA) to form a pink complex with absorption maxima at 520 nm and 535 nm. Aliquots (500 µl) of diluted liver homogenate or diluted plasma were pipetted in tubes containing 3.0 ml of 1% phosphoric acid and 1 ml of 0.6% TBA in distilled water. The mixture was heated in a boiling water bath for 45 min. Butanol (4 ml) was added to the reaction mixture after cooling the reaction mixture. After centrifugation at 4000 ×g for 10 min the butanol phase was separted and pink color was measured at 520 nm and 535 nm. The difference was used as the TBA value. The MDA concentration was determined from a standard curve. The MDA standards were prepared by the hydrolysis of 1,1,3,3 tetraethoxypropane.

3.4.6.6. Determination of soluble proteins

Total soluble proteins were measured in liver homogenate by the colorimetric Bradford assay (1976). Liver tissue homogenate was diluted with double distilled water at a ratio of 1:10 (v/v). Aliquots of 10 µl liver homogenate were pipetted into 96-well plates, followed by the addition of 200 µl filtered and diluted Bio-Rad protein dye reagent (Bio-rad, Munich, Germany) at a ratio of 1:5 (v/v). After 15 min of incubation, the absorbance was measured at 595 nm. The protein concentration was calculated from a standard curve plotted for standard albumin-fraction V (Rothe, Karlsruhe, Germany). Absorbance was recorded using a recording spectrophotometer (Smartspec^{TM} plus, Bio-Rad, Spain) for all measurements.

3.4.7. Determination of the serum zinc level

Zinc is a non-enzymatic antioxidant and important for cell growth, development and differentiation (Zhang et al., 2008). The zinc content was determined in serum of LCC and germ-free rats by a colorimetric method using a commercial kit (Bio-diagnostic kit, Giza, Egypt) as described by Hayakawa & Jap (1961). Zinc present in the serum was chelated by Zincon (2-carboxy-20-hydroxy-50-sulfoformazylbenzene). The formation of this colored complex was measured at 610 nm. A 500 µl aliquot of serum was pipetted into a zinc-free-tube containing 1.0 ml of carbonate buffer (pH 9.5, 50 mM), followed by the addition of 500 µl of the Zincon reagent. After mixing the reaction mixture and incubating at 25°C for 10 min, the absorbance was measured at 610 nm. Standard zinc and blank were run in parallel in an identical manner. The reaction mixture was considered as a blank. The zinc concentration in serum was calculated using the following equation: Zinc Conc. (µmol/L) = $A_{sample} \times$ Conc. $_{standard}$ / $A_{standard}$

3.4.8. 2D-DIGE proteomic analysis of tumors

Two dimensional difference gel electrophoresis (2-DG-DIGE) analysis and Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) were performed to identify differentially expressed proteins in breast cancer tissue of LCC and germ-free rats. 2D-DIGE proteomic analysis of tumors was carried out according Wang et al. (2011) with minor modification.

3.4.8.1. Protein sample preparation

Frozen tumor tissue was ground in liquid nitrogen with mortar and pestle and subsequently weighed. Tissue material (100 mg) was homogenized with 800 µl lysis buffer composed of 30 mM Tris, 7 M urea, 2 M thiourea, 4% CHAPS, and supplemented freshly with protease inhibitor (1:100; v/v) (Protease inhibitor cocktail tablets 100×, Roche, Germany). After centrifugation (14 000 ×g for 5 min at 4 °C), 1% dithiothreitol (DTT) and 5 µl benzonase were added to the homogenates followed by incubation at 37°C for 10 min. The samples were centrifuged at 14 000 ×g for 20 min at 4 °C. Subsequently, the supernatant was purified using 2D clean-up kit according to the manufacturer's protocol (GE Healthcare, Sweden) and resuspended in rehydration buffer containing 7 M urea, 2 M thiourea, and 4% CHAPS. Protein contents were measured using the Bradford assay (see section 3.4.6.6). The pH of the protein samples was adjusted to 8.5 with 50 mM NaOH and samples were stored at -80°C until further analysis.

3.4.8.2. Protein-dye labelling

Protein was labelled with CyDyes (CyDye DIGE Fluor minimal dyes, GE Healthcare, Sweden) according to the manufacturer's protocol for minimal labelling. Protein samples (50 µg) were labelled with 400 pmols of Cy3 (LCC group) or Cy5 (germ-free group). The internal standard

(50 µg of equal amounts of each protein extract from all samples of both groups) was labelled with Cy2. Protein samples and dye were mixed, shortly centrifuged, and incubated in the dark on ice for 30 min. The reaction was terminated by adding 1 µl of lysine (10 mM) and incubated on ice for 10 min in the dark. Labelled proteins (Cy3 or Cy5) and Cy2 labelled internal standard were pooled. For preparative gels, 500 µg of unlabelled protein was used; equal amounts of all samples were pooled.

3.4.8.3. 2D-DIGE analysis

Immobilized pH gradient (IPG) strips (24 cm, Immobiline^{TM} DryStrip gel, pH 3-10 nonlinear, GE Healthcare) were passively rehydrated overnight with 450 µl rehydration buffer containing 1.2 mM DeSteak reagent, 1% IPG buffer, and 0.002% bromophenol blue in IPG strip holders. Labelled proteins were diluted with rehydration buffer supplemented with 1% DTT, 1% IPG buffer (pH3-10NL), and 0.006% bromophenol blue to a final volume of 150 µl.

First dimension isoelectric focusing (IEF) was carried out on an Ettan^{TM} IPGPhor 3 system (GE Healthcare, Sweden) using the following series of steps and hold voltages: 250 V for 30 min, 500 V for 30 min, 1000V for 1 h, 3000 V for 3 h, 8000 V for 3 h. Subsequently, 8000 V was preserved until a total of 65,062 Vh was reached. Following IEF, IPG strips were equilibrated with two different equilibration buffers. The strips were incubated in equilibration buffer I composed of 75 mM Tris pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulfate (SDS), 0.2% bromophenol blue, and 1% DTT for 15 min; then incubated in equilibration buffer II (1% DTT was replaced with 2.5% iodoacetamide) for additional 15 min.

The second dimension was performed on an Ettan DALT-12 separation unit (GE Healthcare). The equilibrated IPG strips were applied to the top of 12.5% SDS-PAGE gels and immediately overlaid with warm 0.5% agarose prepared in equilibration buffer with 0.2% bromophenol blue. Gels were loaded into the electrophoresis apparatus filled with electrophoresis buffer (SDS-Tris-glycine, 1%, 3%, 14.4%, respectively). Electrophoresis was performed at 17 W per gel at 25°C until the blue color reached the bottom of the gel. The analytical gels (three separate gels) were scanned and analyzed right after the end of the separation as described below (3.4.8.4). The preparative gels were stained with Ruthenium II tris-bathophenantroline disulfonate (RuBps) staining. Briefly, gels were fixed for 1 h with a fixation solution containing 30% methanol and 10% acetic acid and then immersed overnight with the fixation solution containing RuBps stain.

3.4.8.4. Image analysis and spot picking

Gels were scanned using a Typhoon Trio laser scanner (GE Healthcare). Image analysis was performed using DeCyder^{TM}2D 6.5 software. The expected number of spots was set to 10000 and spot filtering was carried out with Differential In-gel Analysis tools to get rid of gel artifact and irregularities. Spot filtering parameters: slope > 1.88, area < 101, peak height < 25, and volume < 2663 were used to detect proteins. The Student's t-test was applied to assess the statistical significance of expression differences between the control and LCC rats; $p \leq 0.05$ was considered to be significant. Spots were picked from a separate RuBps stained preparative gel. The preparative gel image was matched with the analytical gel images using the DeCyder biological variation analysis module tool. A spot pick list was created for proteins that were differentially expressed with at least +/- 2.0 fold change. The preparative gel was placed in the gel holder plate, which contained double distilled water. Proteins were removed from preparative

gel using an Ettan Spot Picker v1.22 (GE Healthcare, Sweden) and subsequently to a 96-well plate.

3.4.8.5. Trypsin in-gel digestion of proteins for MALDI-TOF-MS

Trypsin in-gel digestion of protein was performed according to Vogel-Scheel et al. (2010). The gel plugs were washed twice with 50 mM ammonium bicarbonate in 50% methanol (v/v) for 30 min, each. Gel plugs were dehydrated with acetonitrile (100 µl) for 10 min. After lyophilization, pellets were digested by adding 50 µl of a 20 mM ammonium bicarbonate solution containing 1 ng/µl of trypsin and incubated overnight at 37°C. The enzyme solution was transferred to clean 2-ml tubes. Sixty µl of 0.1% trifluoroacetic acid (TFA) and 50% acetonitrile (v/v) was added to the sample and incubated for 20 min to extract the proteins from the gel plugs. The enzyme solution and TFA/acetonitrile solution containing the protein were pooled and lyophilized using a speedvac. The samples were dissolved in 0.1% TFA containing 50% acetonitrile. A saturated matrix solution (α-Cyano-4-hydroxy cinnamic acid, HCCA, Bruker Daltonik GmbH, Bremen, Germany) was prepared in 0.1% aqueousTFA:acetonitrile (70:30; v/v). Prior to MALDI-TOF-MS analyses, a 2-µl aliquot of the sample solution and 2 µl of the matrix solution were mixed and subsequently a 0.5-µl aliquot from the resulting solution was applied to a ground steel target plate for MALDI-TOF-MS analyses.

3.4.8.6. MALDI-TOF-MS

MALDI-TOF-MS analysis of protein was performed by Prof. Dr. Harshadrai Rawel (Department of Food Chemistry , Institute of Nutrition Science, University of Potsdam) on an AUTOFLEX-III LRF200-CID, equipped with Smart beam-Laser 200 (Bruker Daltonik GmbH, Bremen, Germany) in the reflector-mode operation; the acceleration voltage was 20 kV, and the effective flight path was 200 cm. The instrument was internally calibrated using the signals of the positive

[M+H]+ mono isotopic ions of a peptide calibration standard II (Bruker Daltonik GmbH, Bremen, Germany) containing Angiotensin II (mass 1046.542 Da), Angiotensin I (mass 1296.685 Da), Substance P (mass 1347.735 Da), Bombesin (mass 1619.822 Da), ACTH clip 1-17 (mass 2093.086 Da), ACTH clip 18-39 (mass 2465.198 Da), Somatostatin 28 (mass 3147.471 Da), Bradykinin Fragment 1-7 (mass 757.399 Da), Renin Substrate tetradecapeptide porcine (mass 1758.933 Da) covering a mass range form 700 Da to 3200 Da. The data analysis was performed using the software packets: Bruker Daltonics FlexAnalysis v3.3 (Bruker Daltonik GmbH, Bremen, Germany). Sequence database search using the m/z values of the digested peptides was performed with Bruker Daltonics BioTools v3.2 (Bruker Daltonik GmbH, Bremen, Germany). The following parameters were considered during the search: fixed modifications such as reductive disulfide cleavage and carbamidomethylation, i.e., reaction with iodoacetamide; mass tolerance MS (the peptide mass error) of 100-500 ppm; and the number of missed cleavages (or partials) was set as one. A protein was considered identified ($p<0.05$) when 30 % of the sequence was covered and a protein score greater than 70 was obtained. Sequence database search was performed using SwissProt database (http://srs.ebi.ac.uk/srsbin/cgi-bin/wgetz?-page+top; http://www.uniprot.org/).

3.5. Statistical analysis

Results are expressed as means ± standard error. Statistical analyses were done with Statistical Product and Service Solutions programme (SPSS 14.0 for Windows; SPSS, Inc, Chicago, USA). Values were tested for normal distribution with the Kolmogorov-Smirnov test. Depending on data distribution, the student *t*-test or the Mann-Whitney test was used. Spearman's correlation coefficients were computed to study the association between quantitative variables. Differences were considered significant at $p \leq 0.05$.

4. RESULTS

4.1. Identification of lignan-converting bacteria by 16S rRNA gene

Identity of lignan-converting bacteria was verified using 16S rRNA gene sequencing before starting the *in vitro* and *in vivo* experiments. The DNA concentrations (mean±SE) in extracts obtained from overnight cultures of *C. saccharogumia, B. producta, E. lenta, and L. longoviformis* were 34.57±2.48, 75.23±0.75, 35.97±1.54 and 92.73±1.76 ng/µl, respectively. Specific bands were observed in the agarose gel with a product size of 1465 bp for *C. saccharogumia, B. producta, L. longoviformis*, and 1374 bp for *E. lenta.* The PCR products were purified and the concentration values were 70.56±0.37, 62.67±0.65, 43.79±1.25, and 56.14±4.9 ng/µl for *C. saccharogumia, B. producta, E. lenta, and L. longoviformis*, respectively. The required concentrations (10 ng/µl) for sequencing were calculated for each bacterial species and sent to company Eurofins MWG with primer 27F (2 pmol/l). The sequences obtained were compared with existing sequences of bacterial species in BLAST data bank. The similarity to the closest related species was recorded for each strain (Table 5). The 16S rRNA gene sequence of the lignan-converting bacteria revealed 99 to 100% similarity with described bacterial species. Accordingly, the dentity of each lignan-converting bacteria was confirmed.

4.2. Transformation of SDG to EL by lignan-converting bacteria *in vitro*

In vitro experiments were performed to show the biotransformation of SDG to EL. Bacterial strains were anaerobically incubated in Mt-6 medium containing lignans as a substrate for 72 h at 37°C. The kinetics of the transformation of SDG (methanolic extracts from flaxseed) to EL by four lignan-converting bacteria in Mt-6 medium is presented in Figure 3. SDG was deglycosylated by *C. saccharogumia* within 24 h and the concentration was reduced by 90%

after 24 h. The lignan metabolites SECO (55.39 µM) and ED (13.01µM) were found after 24 h incubation with lignan-converting bacteria. After 48 h no SECO and ED were detected indicating that demethylation and dehydroxylation by *B. producta* and *E. lenta,* respectively, occured. EL was detected after 31 h of incubation (15.43 µM) and the amount of EL was considerably increased and reached the maximum after 72 h (46.46 µM). EL production accounted for approximately 33% of the initial concentration of SDG.

Table 5. Sequence matches of the bacterial strains in the existing data base to bacterial species described

Bacteria species	**Data bank sequence**	**Identities**
Clostridium saccharogumia DSM 17460^{T*}	*Clostridium saccharogumia* strain SDG-Mt85-3Db 16S rRNA, partial sequence	983/983 (100%)
	Clostridium sp. 14774 partial 16S rRNA gene.	976/983 (99%)
Blautia producta DSM 3507^{T}	*Blautia producta* gene for 16S rRNA, partial sequence	316/317 (99%)
	Blautia sp. Ser8 16S ribosomal RNA gene.	316/317 (99%)
	Ruminococcus lactaris ATCC 29176, whole genome shotgun sequence.	313/317 (98%)
Eggerthella lenta DSM 2243^{T}	*Eggerthella* sp. MLG043 16S rRNA, partial sequence	900/900 (100%)
	Eggerthella lenta DSM 2243, complete genome.	900/900 (100%)
	Eggerthella lenta strain SECO-Mt75m2 16S rRNA gene, partial sequence	899/900 (98%)
Lactonifactor longoviformis DSM 17459^{T}	*Lactonifactor longoviformis* strain ED-Mt61/PYG-s6 16S rRNA.	962/963 (99%)
	Clostridiaceae bacterium END-2 16S rRNA gene, partial sequence.	954/965 (99%)

*T = type strain

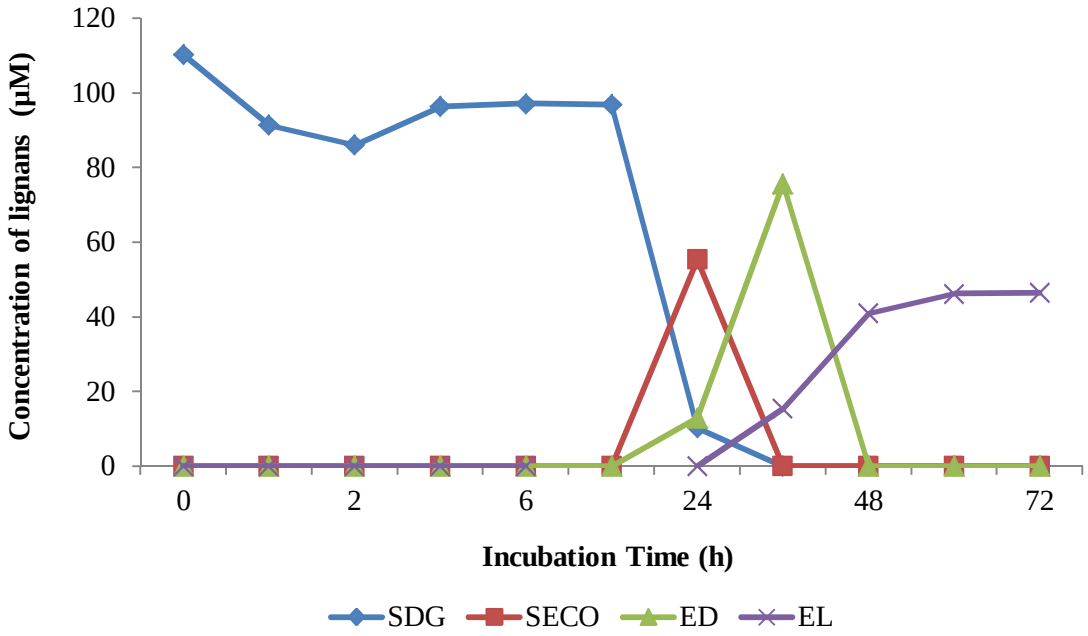
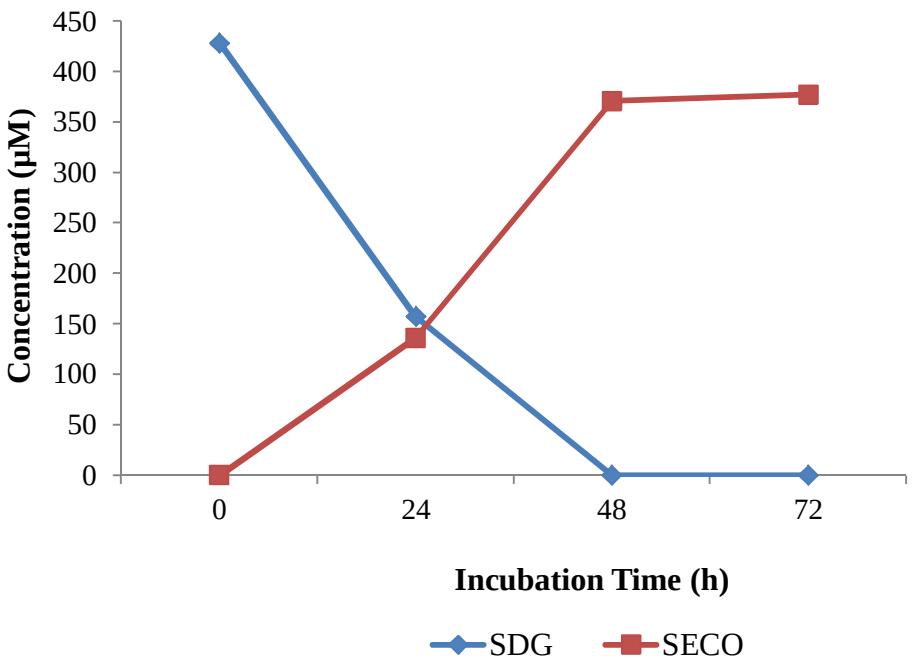


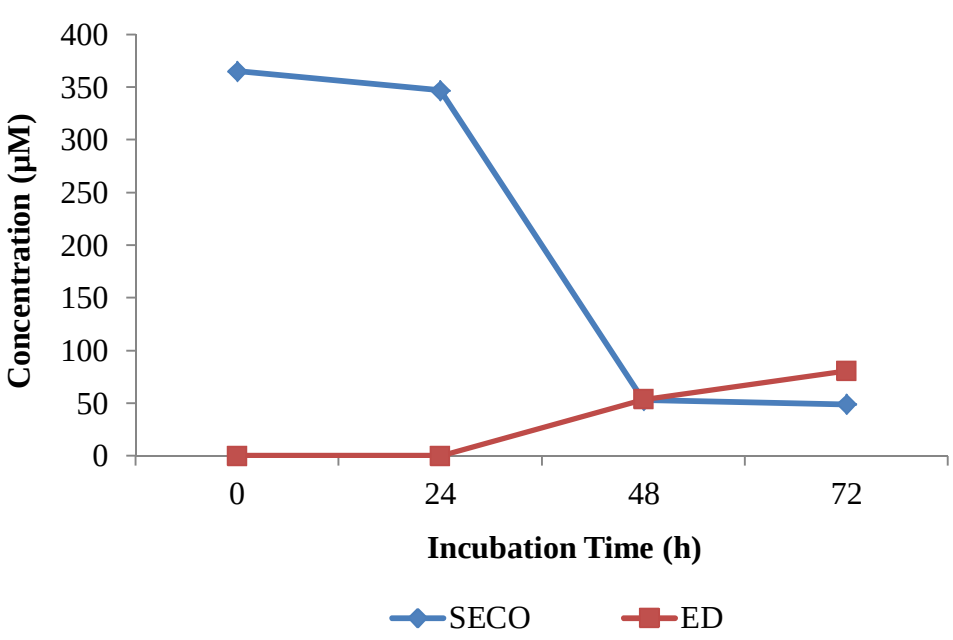
Figure 3. ***In vitro*** **conversion of SDG from methanol extract of flaxseed to EL by lignan-converting bacteria (*C. saccharogumia, B. producta, E. lenta,* and *L. longoviformis*) grown in flaxseed extraction-Mt-6 medium. Four metabolic steps (deglycosylation, demethylation, dehydroxylation and dehydrogenation) must be catalyzed in sequence for EL production.**

C. saccharogumia was incubated in SDG-Mt6 medium at 37°C for 72 h. The initial concentration of SDG was 428.30 µM. After 24 h of incubation, SECO was detected at a concentration of 136.11 µM. In parallel, SDG concentrations decreased to 157.11 µM. After 72h no SDG was detected (Figure 4A). At this timepoint, the SECO concentration reached 377.06 µM, which is approximately 75% of the initial concentration of SDG. *B. producta* and *E. lenta* were inoculated into this SECO-Mt6 medium to produce ED. ED was detected after 48 h incubation at a concentration of 53.67 µM and SECO concentrations gradually decreased within 72 h (Figure 4B). Both bacteria converted 16% of SECO to ED. The dehydrogenation was tested by incubation of *L. longoviformis* in ED-Mt6 medium at 37°C. ED was converted to EL after 24 h (79.86 µM) and the concentration slowly increased to 82.14 µM after 72 h (Figure 4C).

A


450
400
350
300
250
200
150
100
50
0
Concentration (µM)
0
24
48
72
Incubation Time (h)
SDG
SECO

B


400
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Concentration (µM)
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48
72
Incubation Time (h)
SECO
ED

Continue……

C

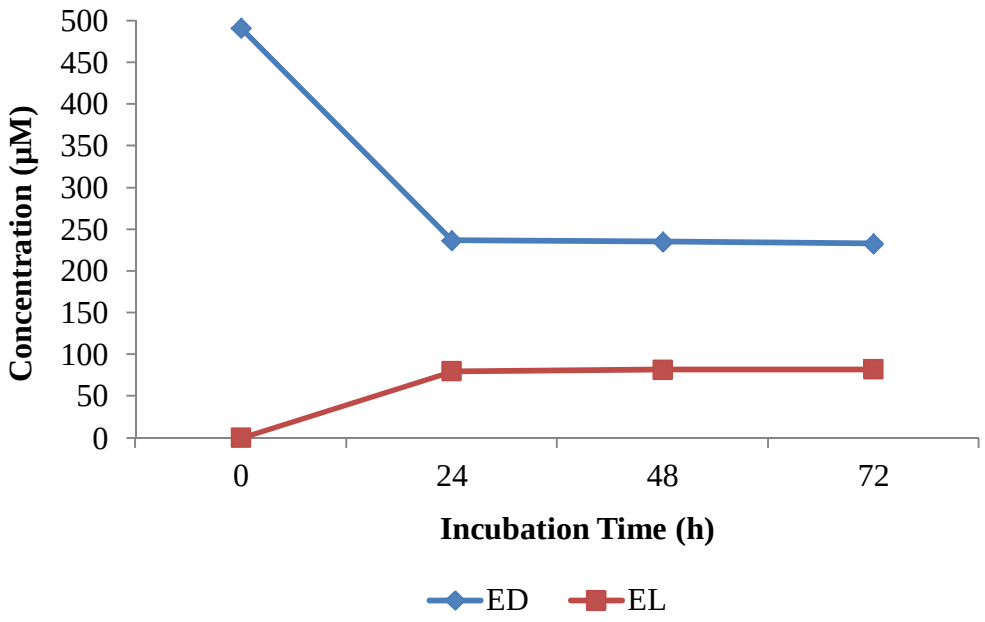


Figure 4. The deglycosylation of SDG to SECO by *C. saccharogumia* grown in SDG-Mt-6 medium (A), the demethylation and dehydroxylation of SECO to ED by *B. producta* and *E. lenta* grown in SECO-Mt-6 medium (B), dehydrogenation of ED to EL by *L. longoviformis* grown in ED-Mt-6 medium (C).

In the controls, none of the lignan metabolites were detected. The production of EL reached 16.4% of the initial concentration of ED

4.3. The effect of a lignan-converting bacterial consortium on DMBA-induced breast cancer in rats

The objective of this study was to evaluate the effect of lignan activation by a defined bacterial consortium on breast tumor formation in a chemically induced rat cancer model.

4.3.1 Animal health status

DMBA administration (a single dose of 25 mg DMBA/ml corn oil given through an oral gavage to each rat in both group) resulted in growth retardation and hair loss. After 8-9 weeks of DMBA administration, the animals continued to gain weight but at a slower growth rate. There were no

significant differences between the LCC and germ-free rats in body weight gain during the experiment (1.31± 0.1 and 1.41±0.1 g/d, respectively). However, one rat was excluded from the study and had to be killed because of severe weight loss. The food intake did not differ between the LCC rats and germ-free rats (21.10 ± 4.77, 22.50 ± 3.01 g/d, respectively). The obtained data revealed that there were no significant differences in final body and organ weight (liver, kidney, uterus, heart, spleen, lung, and cecum) between the two groups (Table 6) at the time of killing.

Table 6. The effect of lignan-converting consortium on body and organ weight

Parameter	LCC rats	Germ-free rats
Initial body weight, g	139 ± 1.8	140 ± 1.8
Final body weight, g	285.56 ± 8.91	298 ± 6.20
Body weight gain, g	146.67 ± 7.36	158 ± 6.20
Liver, %*	3.118 ± 0.06	2.970 ± 0.07
Kidney, %	0.799 ± 0.016	0.770 ± 0.017
Uterus, %	0.364 ± 0.03	0.337 ± 0.02
Heart, %	0.331 ± 0.01	0.315 ± 0.007
Spleen, %	0.211 ± 0.009	0.206 ± 0.006
Lung, %	0.465 ± 0.009	0.462 ± 0.02
Cecum , %	5.570 ± 0.27	6.552 ± 0.51

*% is relative to body weight, values are expressed as means ± SE

4.3.2. Quantification of bacterial numbers in feces and gut content

Bacteria were enumerated in feces, cecal and colonic samples using FISH. The presence of all four consortium members in the intestine during the complete study period was confirmed by FISH analysis using fecal samples. Bacterial cell numbers in colonic and cecal material at the

end of study are presented in Table 7. *B. producta* was the dominant member of the consortium with approximately 52% of the total bacterial cells in the cecal and colon followed by *C. saccharogumia* representing 31% of total bacterial cells in the cecal and 26% in the colon. *E. lenta* made up 12% in the cecal and 17% in the colon, while *L. longoviformis* accounted for approximately 5% in both gut sections. Direct microscopic inspection of fecal samples and anaerobic and aerobic cultivation confirmed the germ-fee status of the control rats throughout the study and at the end of the animal experiment, respectively.

Table 7. Proportions of lignan-converting consortium in cecal and colon content of LCC rats

Species	Cell number/g cecal DW	Cell number/g colon DW
C. saccharogumia	$6.06 \times 10^{10} \pm 5.84 \times 10^{9}$	$6.62 \times 10^{10} \pm 2.23 \times 10^{10}$
B. producta	$1.04 \times 10^{11} \pm 6.12 \times 10^{9}$	$1.27 \times 10^{11} \pm 4.71 \times 10^{10}$
E. lenta	$2.27 \times 10^{10} \pm 1.79 \times 10^{9}$	$4.17 \times 10^{10} \pm 1.67 \times 10^{10}$
L. longoviformis	$7.18 \times 10^{9} \pm 1.33 \times 10^{9}$	$1.52 \times 10^{10} \pm 1.08 \times 10^{10}$

Values are expressed as means ± SE, $n = 9$.

4.3.3. SCFA concentrations and pH in gut content

SCFA concentrations in cecal and colonic materials were measured (Table 8). SCFA concentrations in cecum and colon of LCC rats were not different from those of the germ-free rats. Total SCFA values reached 41.57±1.90 and 44.68±2.81µmol/g DM in the cecum of LCC and germ-free rats, respectively. In colon, total SCFA were 17.03±3.12 and 27.44±2.08 µmol/g DM in LCC and germ-free rats, respectively. Cecal acetate concentrations were 40.24±1.73 and 43.60±2.72 µmol/g DM in LCC and germ-free rats, respectively. Acetate concentrations in the

colon were 16.27±2.97 µmol/g in the LCC rats and 26.78±2.02 µmol/g DM in the germ-free rats. The respective mean value of propionate was below 0.7 µmol/g, butyrate and valerate were approximately 0.1 µmol/g DM in both groups. No difference in the cecal pH was observed between the LCC rats (7.0±0.04) and germ-free rats (6.9±0.02).

Table 8. SCFA concentrations in gut content of LCC and germ-free rats

SCFA	LCC rats	Germ-free rats
	Cecum, µmol/g DM	
Acetate	40.24±1.73	43.60±2.97
Propionate	0.661±0.072	0.643±0.044
Butyrate	0.119±0.013	0.106±0.012
Iso-valerate	0.409±0.063	0.240±0.024
Valerate	0.143±0.021	0.094±0.009
	Colon, µmol/g DM	
Acetate	16.27±2.97	26.78±2.02
Propionate	0.345±0.063	0.364±0.034
Butyrate	0.097±0.019	0.065±0.006
Iso-valerate	0.249±0.057	0.165±0.014
Valerate	0.079±0.016	0.063±0.002

Values are expressed as means ± SE, n = 9-10.

4.3.4. SDG concentrations in flaxseed, flaxseed diet, cecal and feces

Concentrations of SDG were measured in the flaxseed and in the flaxseed-containing diet to provide information about the total SDG consumption per day. SDG content was 5.10±0.05 µmol/g DM in flaxseed and 0.5±0.01 µmol/g DM in flaxseed diet. SDG consumption was 7.47±1.70 mg/day by LCC rats and 7.86±1.12 mg/day by germ-free rats. Concentrations of SDG were measured in cecal material and feces. SDG concentrations in cecal material were significantly (p<0.0001) lower in LCC (1.47±0.17 µmol/g DM) than in germ-free (5.22±0.52

µmol/g DM) rats. Fecal SDG excretion was significantly (p<0.0001) higher in germ-free than in LCC rats with values 2.58±0.24 and 0.81±0.07 µmol/g DM, respectively.

4.3.5. Lignan metabolites concentrations in gut contents and excretion in feces and urine

Free and total (free, sulfated and glucuronidated forms) SECO, ED and EL were measured in cecal and colonic contetns as well as feces and urine (Table 9). Free SECO and ED were not detected in urine, gut contents, and feces of LCC and germ-free rats, while free EL was detected in urine, gut contents, and feces of LCC but not of germ-free rats. Total SECO, ED and EL were measured after treatment with β-glucuronidase/sulfatase. Total SECO was detected in gut contents and feces of both LCC and germ-free rats. However, concentrations of SECO were significantly higher in gut contents of the LCC rats compared to germ-free rats. In LCC animals, total ED was only detected in cecal samples whereas total EL was found in all samples analyzed. Concentrations of total EL in cecal content were 3-fold higher than in colon contents of LCC rats.

LCC rats excreted both ED and EL in the urine but only EL was detected in feces. Total EL output in urine was approximately 9.0 fold higher ($p \leq 0.0001$) than that of total ED. Furthermore, in LCC rats excretion of EL in feces (1.78±0.97 µmol/24 h) was similar to that in urine. ED and EL were not detected in any gut contents, fecal or urine sample of the germ-free rats. Approximately 90% of the EL in urine of LCC rats was glucuronidated/sulfated and 77% of EL was conjugated with glucuronate and/or sulfate in cecal contents. The percentage of dietary SDG excreted in urine of LCC rats as ED and EL was approximately 2 and 17%, respectively.

Table 9. Lignan concentrations in intestinal, fecal (µmol/g DM) and urine sample (µmol/24h urine excretion)

	LCC rats	Germ-free rats
Lignans	**Cecum**	
Free SECO	nd	nd
Total SECO	0.512 ± 0.05**	0.268 ± 0.12
Free ED	nd	nd
Total ED	0.134 ± 0.04	nd
Free EL	0.142 ± 0.07	nd
Total EL	1.364 ± 0.44	nd
	Colon	
Free SECO	nd	nd
Total SECO	0.438 ± 0.05**	0.199± 0.12
Free ED	nd	nd
Total ED	nd	nd
Free EL	0.106 ± 0.017	nd
Total EL	0.469 ± 0.07	nd
	Feces	
Free SECO	nd	nd
Total SECO	0.187 ± 0.02	0.161 ± 0.03
Free ED	nd	nd
Total ED	nd	nd
Free EL	0.255 ± 0.03	nd
Total EL	0.409 ± 0.047	nd
	Urine	
Free SECO	nd	nd
Total SECO	nd	nd
Free ED	nd	nd
Total ED	0.204 ± 0.03	nd
Free EL	0.193 ± 0.02	nd
Total EL	1.792 ± 0.298	nd

Values are expressed as means ± SE, significance of differences between two groups is indicated by ** $p \leq 0.01$, Not detected (nd). Material obtained from LCC (n=9) and germ-free rats (n=10)

4.3.6. DMBA-induced rat mammary tumor evaluation

The effect of the presence of lignan-converting bacteria on tumor incidence and tumor size at the time of killing is summarized in Table 10. After administration of DMBA, the latency period until tumor formation was 9.5±0.50 weeks for LCC rats and 8.0±0.82 weeks for germ-free rats. There was no difference between LCC and control group in total tumor incidence. However, the total number of tumors observed in the LCC group was significantly reduced by 40% (p = 0.037). Consequently, the number of tumors per tumor-bearing rat and per number of rats in the group were significantly lower in the LCC animals (p = 0.049 and p = 0.004, respectively) as compared to germ-free rats. Mean tumors size and weight were approximately 48% lower in the LCC rats. However, only differences in tumor size were statistically significant (p = 0.036).

Table 10. Tumor data of LCC and germ-free rats fed on 5% flaxseed diet at the end of experiment

Parameter	LCC rats	Germ-free rats
No. of rats at start of experimental	10	10
No. of rats at end of experimental	9.0	10
No. of rats with tumors	7.0	7.0
No. of rats with 0 tumors	2.0	3.0
No. of rats with 1 tumor	3.0	1.0
No. of rats with >1 tumor	4.0	6.0
No. of tumors/group	18*	45
No. of tumors/tumor-bearing rat	2.57 ± 0.11*	6.57 ± 0.20
No. of tumor/ No. of rats in group	0.57 ± 0.02**	4.50 ± 0.13
Tumors size, cm^3	0.47 ± 0.18*	0.97 ± 0.28
Tumors weight, g	0.52 ± 0.24	1.09 ± 0.46

Values are expressed as means ± SE, significance was checked using an unpaired t-test and Mann-Whitney U test *$p \leq 0.05$, ** $p \leq 0.01$.

4.3.7. Histological and ER status evaluation of DMBA-induced breast tumors in rats

Tumor quality did not differ between between LCC and germ-free rats (Figure 5). All tumors were tubulopapillary adenocarcinoma consisting of moderately differentiated breast cells. Few areas with solid growth were seen in the tumor tissues. There was no invasive growth into the surrounding connective tissue or into vessels. Anisokaryosis and pleomorphism of tumor cells were moderate. However, three mitotic figures could be observed at 400-fold magnification per microscopic field.

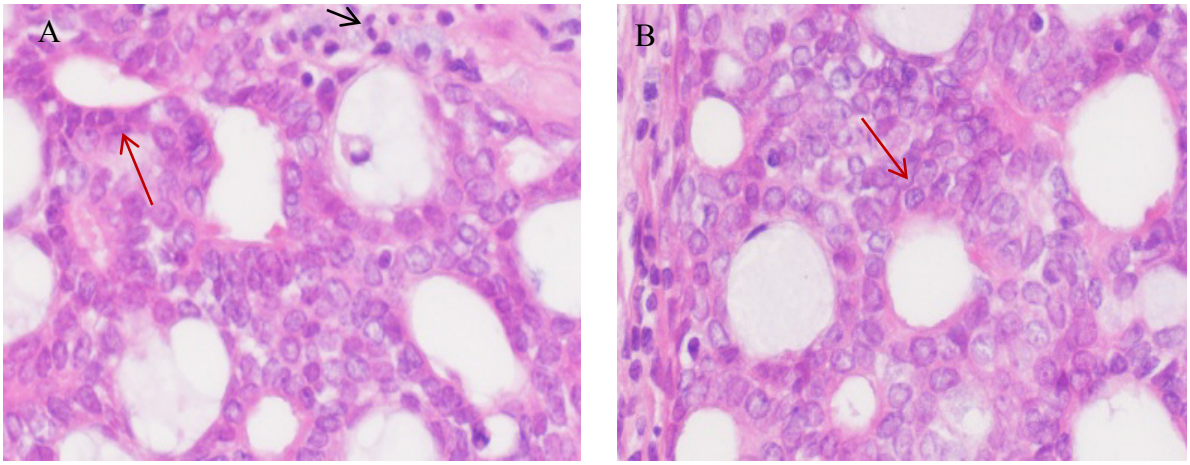


Figure 5. Histological evaluation of DMBA-induced breast tumors of LCC rats and germ-free rats: malignant cells was tubulopapillary adenocarcinoma, light microscopy image of LCC rats breast tumor (A) and germ-free rats breast tumor (B), red arrows point to the tumor cells and the black arrow point to the mitotic figure, 400× microscopic field, hematoxylin and eosin stain.

The estrogen receptor status of the tumors was tested using anti-ER antibodes. Tumors of both groups expressed estrogen receptors of the same frequency (Figure 6). ER status scoring did not reveal any differences between LCC and germ-free rats (4.20±0.19, and 4.29±0.13, respectively).

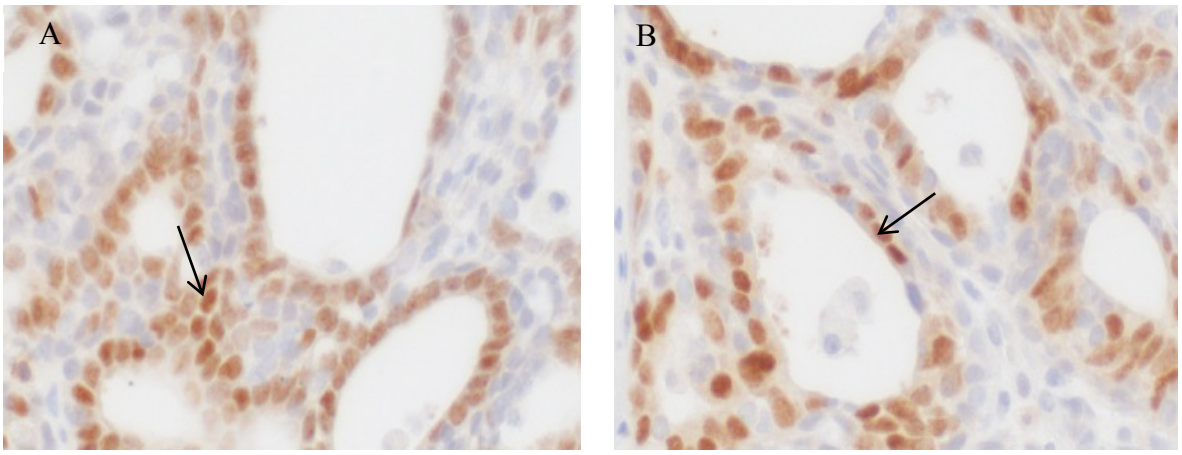


Figure 6. The effect of the presence of a lignan-converting bacterial consortium on ER status in gnotobiotic rats: immunohistochemical detection of ER using anti-ER antibodes in breast tumor tissue was evaluated based on the intensity of staining and the percentage of positive ER cells among all cells, light microscopy image of LCC rats breast tumor (A) and germ-free rats breast tumor (B), arrows point to the positive ER cells, 400× microscopic field, diaminobenzidine tetrahydrochloride stain (brown) with hematoxylin counterstain (blue).

4.3.8. Tumor cell proliferation and apoptosis

Ki-67 is a marker for proliferating cells and is overexpressed in many breast cancers. Ki-67 proliferation status in tumor tissue samples from LCC and germ-free rats was evaluated using Ki-67, a monoclonal antibody that recognizes a nuclear antigen present in proliferating cells. The tumor Ki-67 labelling index in LCC rats was highly significantly reduced (p <0.001). LCC rats showed a cell proliferation rate that was reduced by more than 50% compared with germ-free rats (Figure 7). Since the urinary concentrations of lignan metabolites are an indicator for the bioavailability was tested whether there is a correlation between mean urinary enterolignan excretion and the tumor Ki-67 labelling index. A significant positive correlation between mean urinary enterolignans excretion and the Ki-67 index was indeed found (n=7, r= 0.785, p< 0.03).

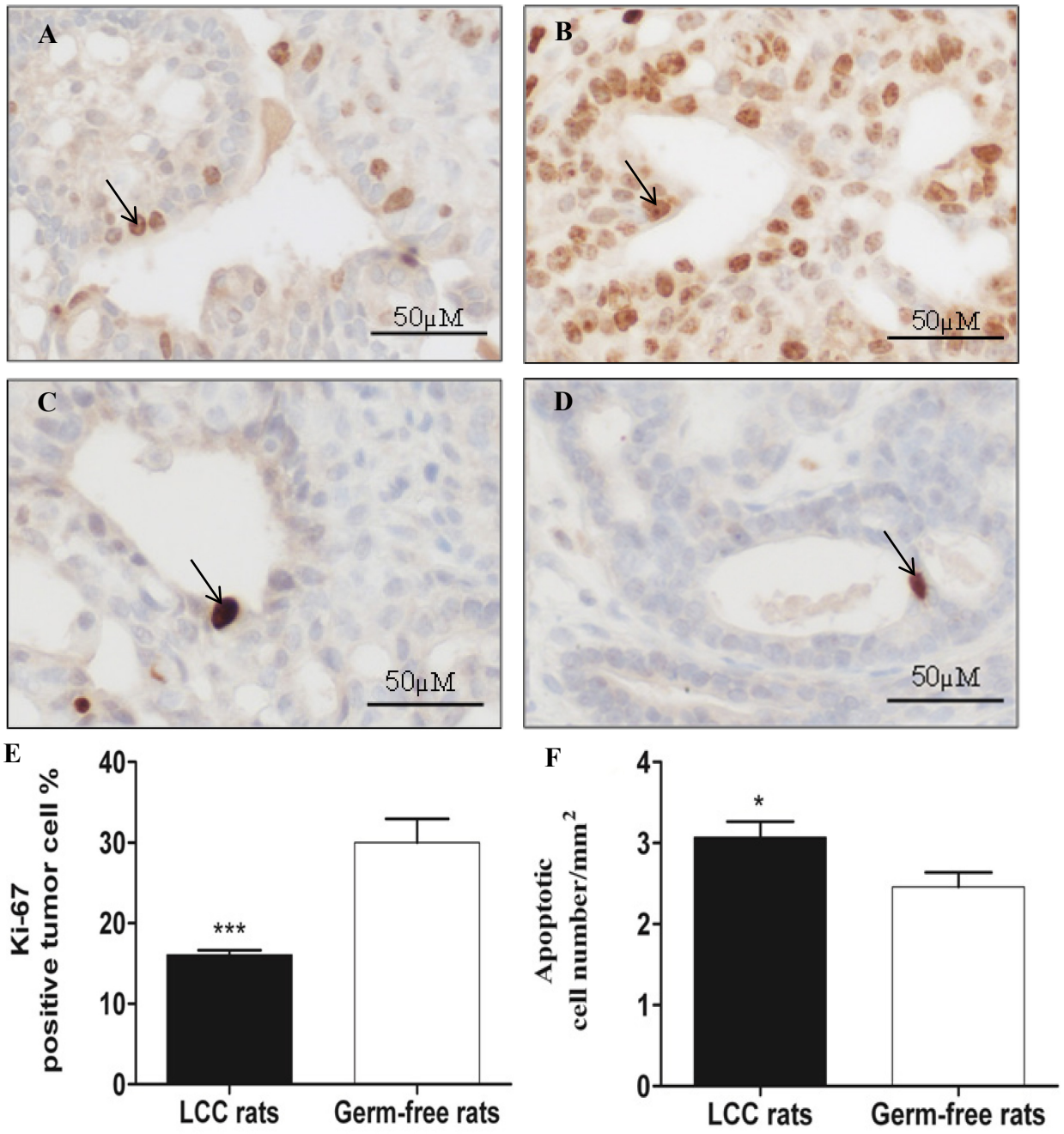


Figure 7. The effect of the presence of a lignan-converting bacterial consortium on cell proliferation and apoptosis in gnotobiotic rats: immunohistochemical detection of Ki-67 positive cell in breast tumor tissue of LCC rats (A) and germ-free rats (B) as well as apoptosis positive cells using caspase-3 antibody in LCC rats (C) and germ-free rats (D), arrows point to the positive cells. Quantification of Ki-67 positive cells (E) and apoptotic cells (F) in tumors from both animal groups; values are expressed as means ± SE (n=9 LCC rats; n=10 germ-free rats), significance was tested using the Mann-Whitney test * $p \leq 0.05$, *** $p \leq 0.001$.

Apoptosis is the process of programmed cell death. Two methods were used to evaluate the apoptosis status using caspase 3 activity assay and the TUNEL assay. Apopotsis was significantly induced ($p< 0.05$) in LCC rats when compared to germ-free rats (Figure 7). A significant negative correlation was observed between mean urinary lignan excretion and number of apoptotic cells (n=7, r= -0.882, $p< 0.01$).

4.3.9. Relative expression levels of estrogen-sensitive genes and estradiol concentration

One of the proposed mechanisms involved in protective effect of lignans on breast cancer is their anti-estrogenic effect in conjunction with reduced circulating estrogen levels. Therefore, expression of selected genes involved in the ER signalling pathway (ERα, ERβ, GPR30, IGF-1, and EGFR) and of serum estrogen levels were measured. Serum estradiol concentrations did not differ between LCC rats and germ-free rats (33.65±1.70 and 34.44±2.77 pg/ml, respectively). The mRNA expression levels of ERα, ERβ and GPR30 (estrogen-sensitive genes involved in ER signaling pathway) and of IGF-1 and EGFR (genes involved in growth-factor signaling pathways) in the tumor tissue were slightly lower in the LCC rats compared to the germ-free rats. However, differences did not reach statistical significance (Table 11).

4.3.10. 2D-DIGE proteomic analysis of DMBA- induced breast cancer in rats

A proteomic approach was used to identify markers that might be helpful in identifying mechanisms involved in a protective effect of lignans against breast cancer. 2-DG-DIGE analysis and MALDI-TOF-MS were performed to identify differentially expressed proteins in breast cancer tissue of LCC and germ-free rats. A total number of 5735 spots was detected in the 2D-DIGE gel (Figure 8A). Twenty-four out of 5735 proteins were differentially regulated with at

least +/- 2 fold changes. Thirteen proteins were significantly up-regulated in LCC rats ($p \leq 0.05$) and 11 proteins were significantly down-regulated (Figure 8B). Out of the 24 proteins, nine proteins were identified by MALDI-TOF-MS (Table 12).

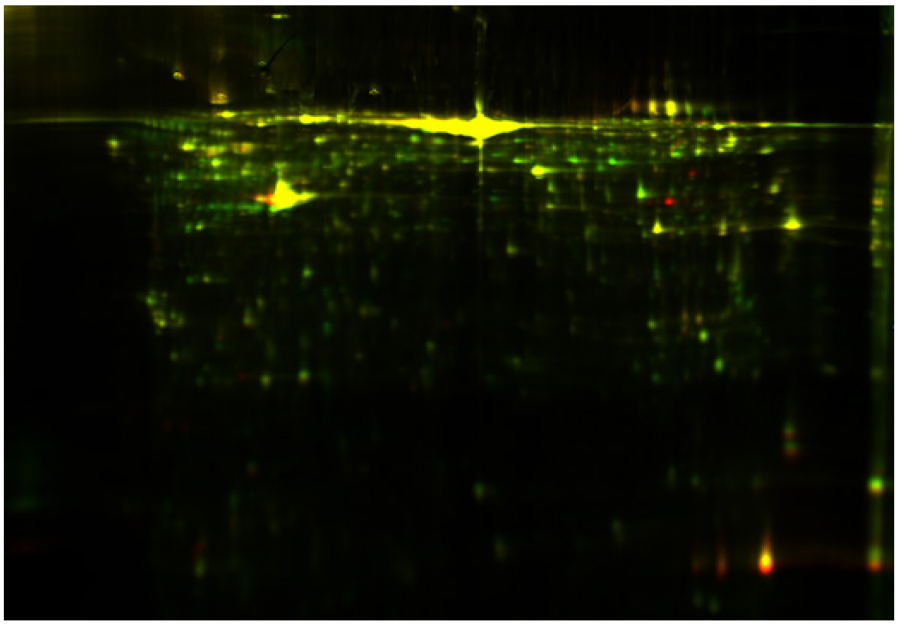
Table 11. Effect of lignan-converting consortium on relative expression of estrogen-sensitive genes and genes involved in tumor cell growth

Genes	LCC rats	Germ-free rats
ERα/GAPDH*	0.947± 0.358	1.000± 0.378
ERβ/GAPDH	0.664 ± 0.251	1.000± 0.378
GPR30/GAPDH	0.746 ± 0.273	1.000 ± 0.229
IGF-1/GAPDH	0.513 ± 0.119	1.000± 0.347
EGFR/GAPDH	0.865 ±0.122	1.000 ± 0.366

*The mRNA expression of target genes is normalized with housekeeping gene (GAPDH), values are fold change of control.

Proteins were characterized using gene ontology database. Interestingly two genes associated with cell proliferation were identified, RanBP-type and C3HC4-type zinc finger-containing protein 1 (RBCK1) and Poly(rC)-binding protein 1 (PBCP1). RBCK1 and PBCP1 were 3.18- and 2.02-fold in LCC rats down-regulated, respectively, compared to germ-free rats. Two transcription factors influencing the expression of cyclin-dependent kinase, which is an important cell cycle regulator, were up-regulated (Transcription factor jun-B and Mediator of RNA polymerase II transcription subunit 24) with an approximately 2-fold change in LCC rats compared to germ-free rats.

A



B

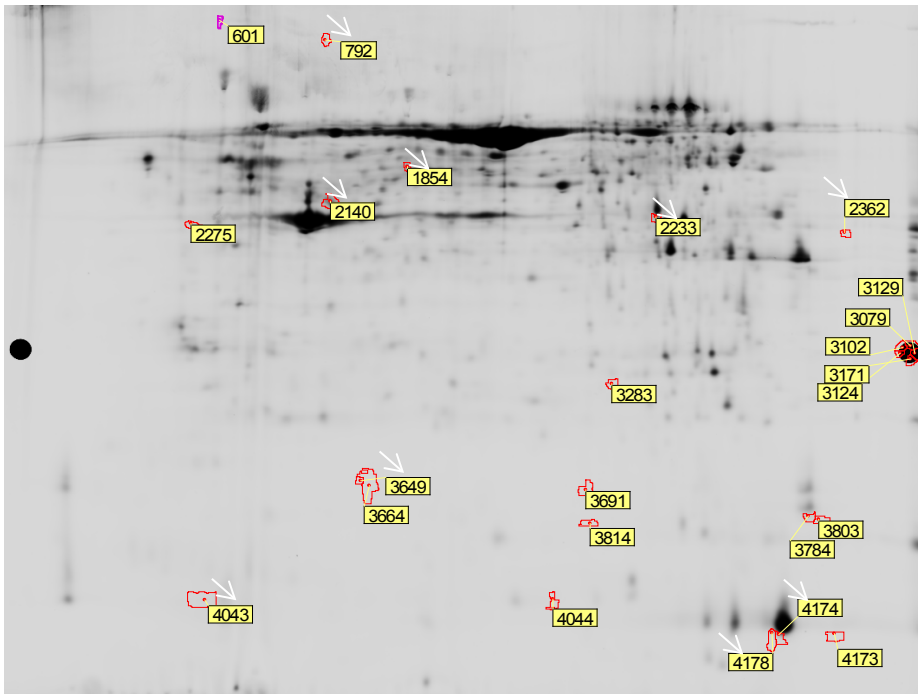


Figure 8. 2D-DIGE image and differentially expressed proteins in DMBA-induced breast tumor tissue from gnotobiotic LCC rats versus germ-free rats. A: the 2D-DIGE gel image shows the proteins expressed in LCC rats (labelled with Cy3; red spots), and germ-free rats (labelled with Cy5; green spots), B: the analytical gel shows the locations of differently expressed proteins (+/- 2 fold change, $p \leq 0.05$), proteins are labelled numerically, and the differentially expressed spots with white arrows were identified by MALDI-TOF-MS.

Table 12. MALDI-TOF-MS identification of protein from 2D-DIGE separation of DMBA-induced tumor in rats.

NO	Score[a]	T-test	Ratio[b]	MW[c]	PI[c]	Protein	Molecular function
1	39	0.013	2.08	25743	9.57	Transcription factor jun-B	DNA binding transcription factor activity
2	52	0.037	-3.18	57647	5.83	RanBP-type and C3HC4-type zinc finger-containing protein 1, RBCK1	Protein kinase C binding and zinc ion binding
3	161	0.028	2.09	42052	5.59	Actin, cytoplasmic	DNA, RNA, Protein binding.
4	102	0.016	-2.02	37987	6.66	Poly(rC)-binding protein 1, PCBP1	RNA binding, translation activator activity
5	40	0.028	-2.41	8962	9.64	UPF0731 protein C6orf225 homolog	Un-characterized
6	20	0.032	-2.51	49298	5.23	Islet cell autoantigen 1-like protein, ICA1L	Protein domain specific binding
7	34	0.016	-2.28	84649	6.11	Mitofusin-1, MFN1	GTPase regulator activity
8	39	0.028	2.35	111606	6.28	Mediator of RNA polymerase II transcription subunit 24, MED	Transcription coactivator activity
9	84	0.045	2.15	15490	7.82	Hemoglobin subunit alpha-1/2, HBA1	Oxygen transporter activity

[a] Mascot score for the identified proteins, which is a number reflects the summed ion scores of mass values (either peptide masses or peptide fragment ion masses) that can be matched to peptides sequences within the analyzed protein.

[b] Fold change between LCC rats and germ-free, whereas a negative ratio indicates a reduced expression in the tumor.

[c] Theoretical molecular weight and isoelectric point obtained from Swiss-Prot database.

4.3.11. Zinc concentration in serum of DMBA-induced breast cancer rats

Zinc levels in serum of LCC rats were significantly higher (p = 0.014) than of germ-free rats: concentrations of zinc in LCC rats were 31.98±4.25 µmol/l and 19.27±1.21µmol/l in germ-free rats.

4.3.12. Antioxidant activity in liver and plasma of LCC and germ-free rats

One of the possible mechanisms underlying the effect of bacterial lignan transformation on breast cancer is the activation of antioxidant enzyme systems. Therefore, the activity of catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) were determined in liver and plasma samples. In addition, reduced glutathione (GSH) and malondialdehyde (MDA) concentrations were measured (Table 13). Levels of MDA were neither in liver nor in plasma samples of LCC rats statistically different from those of germ-free rats. There were also differences in GSH concentrations between the liver and plasma samples from LCC and germ-free rats. Interestingly, a significant difference in the activity of GST in liver and plasma samples of LCC rats (p<0.001 and p<0.01, respectively) was observed between LCC rats and germ-free rats: activity was more than 2-fold higher in both liver and plasma samples of the LCC rats than in those of the germ-free rats. In LCC rats, the activities of CAT, and SOD involved in antioxidant defence systems in liver were higher (p<0.01, and p<0.05, respectively) than in germ-free rats. In addition, the plasma levels and activities of CAT and SOD were also significantly higher (p<0.01 and p<0.05, respectively) in LCC rat than in germ-free rats.

Table 13. Antioxidant enzyme activity and concentrations of reduced glutathione and malondialdehyde in LCC rats (n=9) and germ-free rats (n=10)

Parameters	LCC rats	Germ-free rats
	Liver	
Catalase[a]	50.65 ± 3.08	39.37 ± 2.19**
Superoxide dismutase[b]	2.63 ± 0.17	2.04 ± 0.14*
Glutathione-S-transferase[b]	1.25 ± 0.14	0.57 ± 0.05***
Reduced Glutathione[c]	100.84 ± 9.32	85.87 ± 4.64
Malondialdehyde[d]	0.21 ± 0.02	0.27 ± 0.05
	Plasma	
Catalase[e]	1.52 ± 0.14	0.92 ± 0.13**
Superoxide dismutase[f]	51.66 ± 2.52	39.27 ± 3.67*
Glutathione-S-transferase[g]	3.70 ± 0.65	1.60 ± 0.32**
Reduced Glutathione[h]	0.30 ± 0.02	0.26 ± 0.02
Malondialdehyde[i]	3.05 ± 0.16	3.24 ± 0.10

Values expressed as mean ±SE, 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]µmol GSH utilized/mg protein, [d]nmol MDA produced/mg protein, [e]µmol H_2O_2 consumed/min/ml, [f]Unit/ml, [g]Unit /l, [h]mmol/l, [i]nmol/ml.

5. DISCUSSION

Many epidemiological and experimental studies have reported an association between lignan-rich food consumption and a reduced risk of breast cancer (Thompson et al., 1996 a,b; Ingram et al., 1997; Hulten et al., 2002; McCann et al., 2006; Velentzis et al., 2009; Buck et al., 2011). However, some studies do not support the correlation of lignan intake and breast cancer risk reduction (Hedelin et al., 2008; Ward et al., 2010). The variation in these different studies influenced by several factors such as the duration of the study, type and dose of lignans used, and breast cancer subtypes. The variation in the composition of intestinal microbiota and in particular differences in the intestinal numbers of lignan-converting bacteria could be important additional factors (Kuijsten et al., 2005; Clavel et al., 2005; Clavel et al., 2006 a,c; Possemiers et al., 2007; Nurmi et al., 2010; Sonestedt & Wirfalt, 2010). It is unclear whether bacterial lignan transformation is crucial for protective effects of dietary lignans on breast cancer or not. The objective of this work was to investigate the contribution of intestinal bacteria capable of transforming lignans to beneficial effects of enterolignans in breast cancer formation. Therefore, germ-free rats fed a flaxseed-rich diet were associated with C. *saccharogumia, B. producta, E. lenta and L. longoviformis*. Breast cancer was induced by a single dose of DMBA. Identically treated germ-free rats served as the control group. Colonization of lignan-converting bacteria and bacterial metabolism was tested. Lignan metabolites in intestinal contents, feces and urine were measured. Tumor incidence, number, size, cell proliferation and apoptosis were assayed. In addition, potential action mechanisms of enterolignans in breast cancer protection were evaluated.

5.1. Colonization of bacteria and lignan bioavailability

The lignan-converting bacterial consortium successfully colonized the interstine of previously germ-free rats (LCC rats). The cell numbers reached by the consortium members were in good agreement with a previous study which demonstrated the ability of the lignan-converting consortium to activate plant lignans *in vivo* (Woting et al., 2010).

SCFA are the major fermentation products produced by intestinal bacteria. *In vitro* studies reported that SCFA inhibit tumorigenesis in colon and breast cancer cell lines (deFazio et al., 1992; Yonezawa et al., 2007; Scharlau et al., 2009). Butyrate at concentrations of 1 or 3 mM decreased the transcription rate of ER in breast cancer cell line (MCF-7) by 20-50%, respectively (deFazio et al., 1992). Cell proliferation of MCF-7 cells was inhibited by 10 mM of propionate or butyrate (Yonezawa et al., 2007). Another study reported that exposure to butyrate decreased the tumor incidence by 20-50% in rats with nitrosomethylurea-induced breast cancer (Belobrajdic & McIntosh, 2000). Intestinal SCFA concentrations in cecum and colon contents of rats with a simplified human intestinal microbiota which was reported by Becker et al. (2011) were 4 times higher than intestinal SCFA concentration in the LCC rats in this study. Moreover, SCFA concentrations in conventional rats are approximately 2-3 fold higher compared to SCFA concentrations in the current study (Kleessen et al., 1997). The low SCFA concentration in LCC rats indicates that lignan-converting bacterial consortium used in the present study did not contribute in the production of SCFA. This also indicates that SCFA production in LCC rats had no influence on tumor development.The presence of intestinal SCFA in germ-free rats might be due to inclusion of SCFA in the diet (Høverstad & Midtvedt, 1986). The lower colonic SCFA concentrations observed in the LCC rats compared to the germ-free rats indicates that colonic SCFA absorption in the LCC rats was slightly higher than that in the germ-free rats.

The plant lignan SDG was converted by the lignan-converting bacterial consortium and ED and EL were detected in gut contents and feces of LCC but not in those of germ-free rats. SECO was detected in gut contents and feces of LCC and germ-free rats, indicating that SDG was not exclusively deglycosylated to SECO by bacteria but also by brush-border enzymes of the intestinal epithelium. Similar findings were reported by Woting et al. (2010). Urinary ED and EL excretion of LCC rats was 1996±163 nmol/24h. Similar concentrations were also observed in conventional rats fed a 5% flaxseed diet with DMBA-induced breat cancer (Thompson et al., 1996b). This finding indicates that lignan bioavailability is similar in LCC and conventional rats. Urinary excretion of enterolignans recovered from dietary SDG in LCC rats was approximately 19% which is in agreement with values reported by Rickard et al. (1996) who found that enterolignans accounted for 10-20% of the initial SDG level *in vitro* or in rats fed flaxseed-containing diets. However, results from the present study as well as those from Rickard et al. (1996) revealed that the proportion of EL formed from SDG during *in vitro* fermentation was higher than that in urine formed from dietary SDG, suggesting incomplete hydrolysis of SDG in the animals or the presence of unidentified ED/EL metabolites. According to Jacobs et al. (1999) nine novel metabolites of EL and ED have been identified in the urine of female and male humans consuming flaxseed. Another *in vivo* study showed that 16 metabolites of ED and EL including both aromatic and aliphatic monohydroxylated lignans were detectable in the bile and urine of female and male Wistar rats after the administration of 2.0 mg ED, 2.0 mg EL or after they had been fed on a diet containing 5% flaxseed (Niemeyer et al., 2000). In the present study, the EL level in feces of LCC rats was 409±47 nmol/g DW but ED was not detected. In contrast, consumption of 10 g/day flaxseed by premenopausal women results in fecal ED level of 115 ±123 nmol/g DW (Kurzer et al., 1995). Interestingly, the EL concentrations in feces of these

premenopausal women (419±248 nmol/g DW) were similar to those detected in feces of LCC rats. ED was not detected in colonic contents and in feces of the LCC rats, indicating that dehydrogenation of ED to EL by *L. longoviformis* was very efficient. Hence, the obtained data clearly demonstrate that the lignan metabolites (ED and EL) are produced only in the presence of intestinal bacteria.

5.2. Effect of bacterial lignan transformation on tumor formation

Tumor incidence did not differ between the LCC and germ-free rats. However, the number of established tumors, the number of tumors per tumor-bearing animal, number of tumors per number of rats, and tumor size were significantly reduced. These results are in good agreement with an earlier study in rats with DMBA-induced breast cancer which received 10 mg EL/kg of body weight (Saarinen et al., 2002). Flaxseed consumption reduces the incidence, number, and growth of tumors in carcinogen-treated rats at the pre-initiation, promotion, or progression stages of carcinogenesis (Serraino & Thompson, 1992; Thompson et al., 1996b). Moreover, consumption of rye decreases the tumor number and size of tumors in a breast cancer model (Davies et al., 1999).

Cell proliferation rate was reduced by 50% in LCC rats. The same result and ratio was recorded for premenopausal women at risk of breast cancer consuming 50 mg SDG/d for 12 months (Fabian et al., 2010). EL and ED inhibited cell proliferation in various breast and colon cancer cell lines (Schultze-Mosgau et al. 1998; Sung et al., 1998). Tumor cell apoptosis was induced in ovariectomized athmic mice treated with ER+ breast cancer cells and fed SDG or flaxseed diets (Chen et al., 2009). A high proportion of apoptotic cells were also observed in the breast tumors

of the LCC rats. This finding suggested an anti-proliferative and pro-apoptotic effect of enterolignans on tumor formation in the current study.

5.3. Mechanisms of the effect of enterolignans on breast cancer

Proposed mechanisms mediating protective effects of lignans on cancer development include anti-estrogenic and anti-oxidant activity as well as the modulation of estrogen levels (Webb & McCullough et al., 2005). Therefore, serum estrogen levels and the relative gene expression of estrogen receptors and genes involved in tumor cell growth were determined. Possible anti-oxidative lignan effects were addressed by measuring the activity of anti-oxidative enzymes, the concentration of antioxidants, and markers of oxidative stress in liver and plasma.

Lignans influence the expression of the two estrogen receptors ERα and ERβ in ovariectomized athymic mice with low circulating estrogen (E2) (Saggar et al., 2010b; Chen et al. 2009). However, the current study observed no or only minor effects on the expression of these two receptors in response to bacterial lignan activation. The G-protein coupled receptor 30 (GPR30) is a membrane protein with high affinity to estrogen, GPR30 mediates the estrogenic effects through the non-genomic pathway, which activates EGFR and leads to the activation of MAP kinases and PI3 kinases, results in cell cycle progression and cell proliferation (Prossnitz et al., 2008; Prossnitz & Maggiolini, 2009). GPR30 is highly expressed in mammary tumors and significantly associated with tumor size and metastases (Filardo et al., 2006). Recent results have shown interactions between GPR30, EGFR and ER in hormone-positive cancer cells (Vivacqua et al., 2009). However, in the recent study there were no differences in GPR30 gene expression levels between LCC and germ-free rats. As mentioned for GPR30, expression of the genes encoding EGFR and IGF1 did not differ between LCC rats and germ-free rats. In contrast,

feeding a diet with 10% flaxseed or an equivalent amount of pure SDG reduced the mRNA expression of IGF-1 and EGFR in athymic mice with transplanted breast cancer cells (Chen et al., 2007a,b; Chen et al., 2009). On the other hand, the present study is in agreement with a previous study, which reported that 5% flaxseed diet has no inhibitory effect on growth factor receptor signal transduction in ovariectomized athymic mice treated with breast cancer cells (Chen et al., 2007a,b). According to Penttinen et al. (2007), EL affects ERα and ERβ expression in a dose-dependent manner in a cancer cell line. It therefore can not be excluded that the SDG content (0.3 g/kg diet) in the 5% flaxseed diet in the current study was too low to influence the estrogen levels or the expression of ER- and growth factors-responsive genes. Taken together, feeding a diet with 5% flaxseed to LCC rats did not inhibit ER- and growth factor-signaling pathways or estrogen synthesis. Thus, these results do not support an anti-estrogenic mechanism mediating protective lignan effect on cancer development.

2-DG-DIGE analysis and MALDI-TOF-MS were performed to identify differentially expressed proteins in breast cancer tissue of LCC and germ-free rats. In this study, two interesting proteins were identified, PCBP1 and RBCK1. It has been shown that RBCK1 is important for progression of the cell cycle to the S phase in breast cancer cells and for estrogen signaling (Castoria et al., 2004). Depletion of RBCK1 reduces cell proliferation by inducing G0-G1 and G2-M cell cycle arrest (Castoria et al., 2004). RBCK1 binds to ER promoter and acts as a transcription coactivator, further transcribes cyclin D1 and c-myc which promotes cell cycle progression and increase cell proliferation (Gustafsson et al., 2010). Similar results have been observed in the present experiment since RBCK1 was 3.18-fold down-regulated and cell proliferation was decreased in LCC rats compared to germ-free rats. Huo et al. (2010) reported that cell replication

was much slower when endogenous PCBP1 was depleted. PCBP1 was 2.02 down-regulated in LCC rats compared to germ-free rats. JunB reduces cell proliferation by inducing high levels of the cyclin-dependent kinase inhibitor p16INK4a (Passegué & Wagner, 2000; Piechaczyk & Farras, 2008; Gurzov et al., 2008). The results from the present study revealed that JunB was 2-fold up-regulated in LCC rats versus germ-free rats. This observation suggests that the lignans inhibited protein kinase C and pointed to the participation of JunB, which further can modulate the expression of cell proliferation and apoptosis genes.

Oxygen radicals can cause DNA damage and mutations responsible for tumor initiation and progression (Wiseman & Halliwell, 1996). It is possible that potential beneficial effects of enterolignans are mediated through the activation of antioxidant enzyme systems. Therefore, the specific activities of GST, SOD and CAT were assayed in liver and plasma of LCC and germ-free rats. Reduced glutathione and MDA (peroxidative damage) were also evaluated in the present study. It has been reported that flaxseed diets exert a restoring effect on CAT and SOD activity in chloroform-treated rats (Rajesha et al., 2006). However, flaxseed and SDG had no effects on CAT in unchallenged rats in a study by Yuan et al. (1999). Higher SOD, CAT and GST activities in the LCC rats compared to germ-free rats indicated that bacterial lignan transformation influences systemic antioxidant capacity. Lipid peroxidation plays an important role in the control of cell division (Diplock et al., 1994). The end product of lipid peroxidation, MDA, is suggested to act as a tumor promoter and a co-carcinogenic agent (Otamiri & Sjodahl, 1989). However, since GSH and MDA level did not differ between LCC rats compared to germ-free rats an effect of anti-oxidative systems on total oxidative stress is not supported. Similarly, in a short-term feeding experiment, hepatic tissue levels of GSH were not influenced by feeding 10% flaxseed or treatment with the equivalent amounts of SDG (Yuan et al., 1999). The more

likely explanation for the lack of differences between the two rats groups in GSH and MDA concentrations is that SDG exerts antioxidant properties in both groups because they both consumed the flaxseed containing diet. Numerous *in vitro* studies have suggested that SDG has antioxidant properties which may protect the organism from oxidative damage (Prasad et al., 1997; Kitts et al., 1999). The order of antioxidant potency of SECO, ED, EL, and SDG relative to vitamin E was 4.86, 5.02, 4.35, and 1.27 respectively (Prasad, 2000). Moreover, an *in vivo* study showed that tissue thiobarbituric acid reactive substances (byproduct of lipid peroxidation) were unchanged in either plasma or urine in human subjects after the consumption of muffins containing 50 g flaxseed (Cunnane et al., 1995).

Blood zinc levels are lower in women with breast cancer than in healthy women (Tinoco-Veras et al., 2011). However, breast tumors contain high levels of zinc (Margalioth et al., 1983; Geraki et al., 2002). Zinc is an essential element and an important factor for cell growth, proliferation, and differentiation (Beyersmann & Haase, 2001). Epidemiological studies revealed an association between zinc levels in breast tissue and the development of breast cancer, because a sufficient level of zinc is important to modulate cell proliferation (Cui et al., 2007). Zinc activates many proteins that regulate cell cycle; an increased zinc concentration in the cytosol after the activation of zinc transporter ZIP7 by protein kinase CK2 may selectively inhibit tyrosine phosphatases leading to prolonged tyrosine kinase activation and an increase in pERK and pAKT resulting in cell proliferation (Taylor et al., 2012; Alam & Kelleher, 2012). The intracellular zinc concentrations in tumor cells may be influenced by lignans. In a xenograft nude mouse model, feeding a flaxseed diet (10%) increased the mRNA expression of the zinc transporter LIV-1 in breast tumor tissue associated with increased serum zinc levels (Zhang et

al., 2008). In the present study, serum zinc levels were significantly higher in LCC rats than in germ-free rats. Zinc is also a co-factor of Cu/Zn SOD (Dreosti et al., 2001). Therefore, the increased SOD activity in LCC rats compared to control rats may be due to differences in zinc metabolism. It can be speculated that the increased zinc concentration measured in the serum of the LCC rats could be due to the activation of zinc transporters by enterolignans resulting in an increased export of zinc from tumor cells into the blood. This might lead to the down-regulation of RBCK1 (zinc finger protein involved in cell proliferation) in breast tumor of LCC rats but not in germ-free rats and thus further reducing the cell proliferation.

6. SUMMARY AND PERSPECTIVES

The effect of a lignan-converting bacterial consortium (*C. saccharogumia*, *B. producta*, *E. lenta* and *L. longoviformis*) on breast tumor development in a chemically-induced rat cancer model was investigated. All bacteria of the lignan-converting bacterial consortium successfully colonized the intestine of previously germ-free rats (LCC rats). There were no differences between control and LCC rats in SCFA concentration and cecal pH. The lignan metabolites ED and EL were produced from SDG in the LCC but not in the germ-free rats. SECO was detected in gut content and feces of LCC and germ-free rats. Excretion of EL and ED in feces and urine in the LCC rats indicated that the simplified model was suitable to study lignan bioavailability. Histologic and morphologic features of the tumors were identical in both groups. ER status scoring of tumor showed no difference between LCC and germ-free rats. Bacterial lignan transformation did not influence tumor incidence in the model but significantly lowered the number of established tumors, number of tumors per tumor-bearing animal and tumor size. The reduced tumor growth in LCC rats was associated with a significant decrease in cell proliferation in conjunction with a higher apoptotic rate of the tumor cell. Gene expression levels of Erα, ERβ, and GPR30, which are estrogen-sensitive genes involved in ER signaling pathways, did not significantly differ between the two groups. Furthermore, the expression of IGF-1 and EGFR (genes involved in growth factor signaling pathways) were unchanged. 2D-DIGE analysis of the tumor proteins obtained from LCC and germ-free rats showed 24 differentially expressed proteins. Two proteins associated with cell proliferation and identified as RBCK1 and PBCP1 were down-regulated. Activities of selected antioxidant enzymes (SOD, CAT, and GST) were significantly increased in liver and plasma of LCC rats, indicating less oxidative stress in these rats compared to the germ-free rats. In contrast, no significant difference was observed in the

GSH content in liver and plasma between LCC rats and germ-free rats. MDA was elevated in germ-free rats compared to LCC rats but the differences did not reach the level of significance. Zinc levels were significantly elevated in serum of LCC rats.

In conclusion, this study demonstrated that enterolignans produced by a lignan-converting bacterial consortium reduce the mammary tumor burden in DMBA-treated rats. The mechanisms mediating these effects have not been unraveled in this study. However, the reduced tumor growth in LCC rats may result from a lower cell proliferation in conjunction with a higher apoptotic rate of the tumor cells and there is some indication for beneficial effects of enterolignans on anti-oxidative enzyme systems. Therefore, it can be concluded the bacterial transformation to ED and EL is crucial for the protective effects of flaxseed-derived lignans. Further research on the effect of enterolignans on the RBCK1 regulation and their function of cell progression in breast cancer cell lines may help to better understand the mode of protective action of lignans on breast cancer. In addition, it would be worthwhile to check the molecular interactions between zinc concentrations and RBCK1 using breast cancer models in the presence of enterolignans.

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Original communications

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APPENDIX

Table 14A. List of used chemicals and their company

Chemical	Company
1,1,3,3 teterathoxypropane	Sigma
1-chloro-2,4-dinitrobenzene	Fluka
4',6'-diamidino-2-phenylindol-dihydrochlorid	Serve
5, 5`dithiobis -2-nitrobenzoic	Sigma
DNA mass ladder	Invitrogen
Acetic acid	Roth
Acetonitrile	BHD
Agarose	Serva
Ammonium chlorid	Roth
Benzonase	Sigma
Biotin	Merck
Bromophenol blue	Biomol
Calcium chloride	Fluka
Calcium pantohthenate	Serva
CHAPS	GE Healthcare
Cobalt(II) chloride	Fluka
Copper(II) chloride	VEB laborchemica
Cyanocobalamin	Sigma
Cysteine.HCl.H_2O	Roth
DeSteak reagent	GE Healthcare
Diammonium sulfate	Roth
Diethyl ether	Sigma
Dipotassium hydrogenphosphate	Merck
Disodium hydrogen phosphate	AppliChem
Disodium tetraoxomolibdate	Sigma
Dithiothreitol	usb
EDTA	Roth
Ferrous sulfate	Merck
Folic acid	Sigma
Fructose	Merck
Glucose	Roth
Glutathione	Roth
Glycerol	GE Healthcare
Glycine	GE Healthcare
Haemin	Sigma
Hexane	Merck
Hydrogen chloride	Roth
Hydrogen peroxide	Roth
Iodoacetamide	Sigma
Lipoic acid	Fluka

Magnesium sulfate	Fluka
Manganese sulfate	Sigma
Methanol	BHD
Methionine	L.light
Nickel(II) chloride	Fluka
Nicotinic acid	Roth
Nitro blue tetrazolium	Roth
Paraformaldehyde	Merck
Peptone	Roth
Perchloric acid	Fluka
potassium chloride	Roth
Potassium dihydrogen phosphate	Fluka
Pyridoxine-HCl	Merck
Resazurin	Fluka
Riboflavin	Merk
Rumen fluid	FU-berlin
Sodium dodecyl sulfate	GE Healthcare
Sodium acetate	Merck
Sodium bicarbonate	Merck
Sodium chloride	Roth
Sodium dihydrogen phosphate	Merck
Sodium formate	Fluka
Sodium hydroxide	Roth
Taq DNA polymerase (PCR reaction mixture)	Fermentas
Thiamin-HCl	Serva
Thiobarbaturic acid	Sigma
Thiourea	usb
Trifluoroacetic acid	Merk
Tris	usb
Tris-HCl	GE Healthcare
Trypsin	Promega
Urea	GE Healthcare
Yeast	Roth
Zinc (II) chloride	Fluka

Table 15A. Composition of diet for the experimental rats

Ingredient	Flaxseed-rich diet (g/kg)
Wheat starch	580
Casein	200
Ground flaxseed[a]	50
Sunflower oil	50
Cellulose	50
Mineral mixture[b]	50
Vitamin mixture[b]	20

[a] Flaxseed was purchased from Schneekoppe GmbH &CO, KG, Nordheide, Germany.
[b] ssniff® ssniff Spezialdiäten GmbH, Soest, Germany.

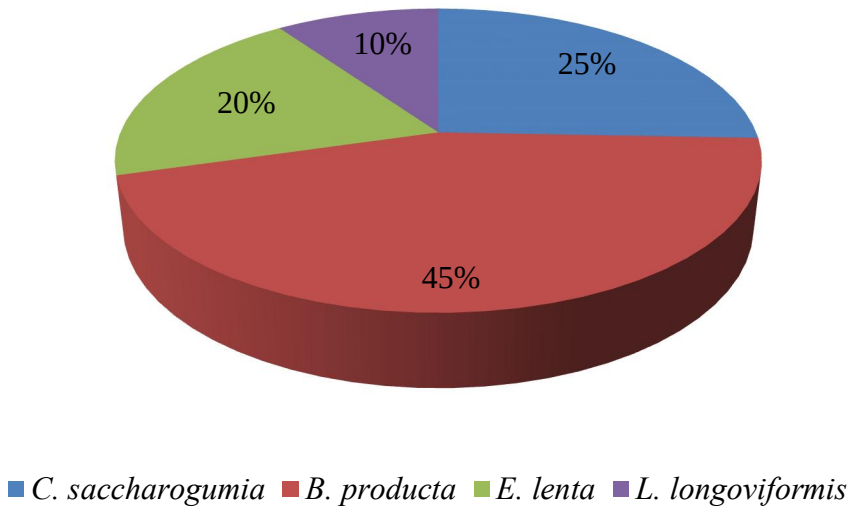

Figure 9A. Proportions of lignan-converting bacteria in feces of LCC rats after change standard diet to flaxseed diet.

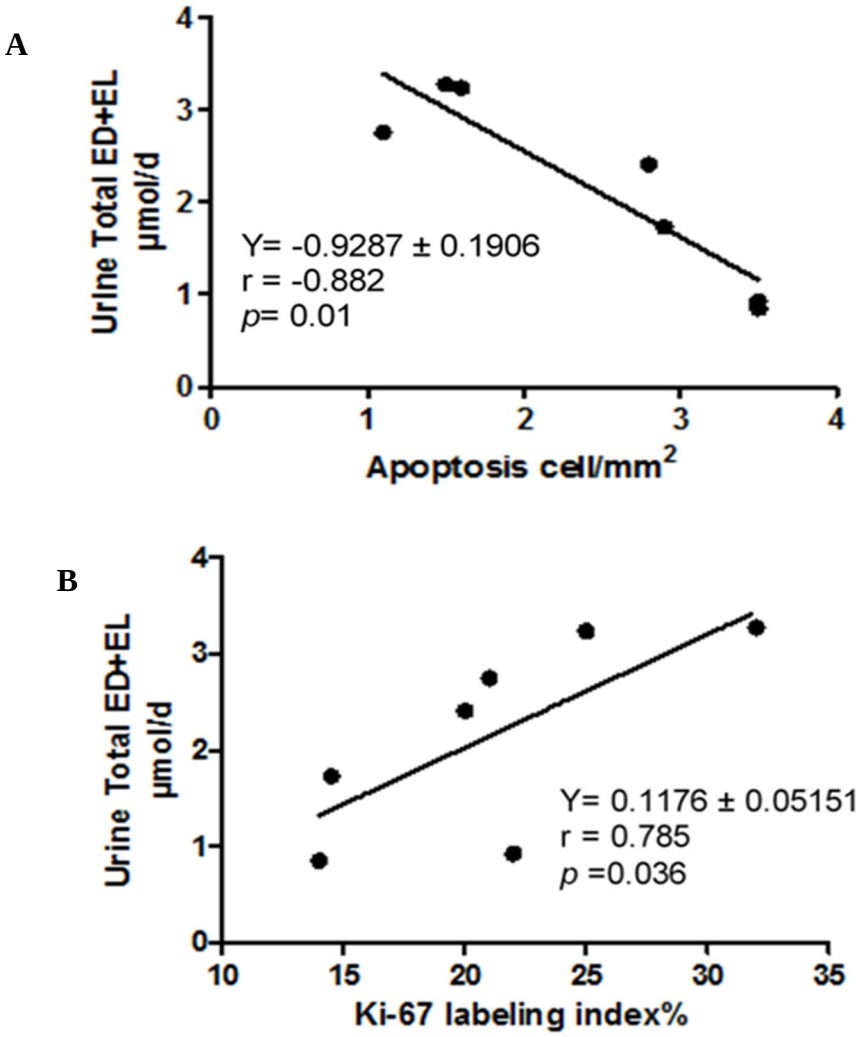

Figure 10A. Correlation between the apoptosis cells, Ki-67 labelling index and enterolignan excretion in urine of LCC rats, A: negative correlation between the apoptosis cells and enterolignan excretion in urine, B: positive correlation between the Ki-67 labelling index and enterolignan excretion in urine.

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