Metabolism of the dietary lignan secoisolariciresinol diglucoside by human intestinal bacteria

ACADEMIC DISSERTATION

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Métabolisme du lignane alimentaire sécoisolariciresinol diglucoside par la microflore intestinale humaine

Abstract

Background. The human intestinal microbiota produces the enterolignans enterodiol (ED) and enterolactone (EL) via secoisolariciresinol (SECO) from the dietary lignan secoisolariciresinol diglucoside (SDG). Therefore, bacteria modulate the bioavailability and biological properties of SDG. However, the organisms involved are poorly described. **Aims.** We intended to characterise the intestinal bacteria involved in the conversion of SDG: 1) at the community level by means of the most probable number method and fluorescent *in situ* hybridisation 2) at the species level by the identification and characterisation of strains that catalyse the underlying reactions. **Results.** The production of enterolignans requires the interaction of dominant and subdominant anaerobic bacterial communities. The cell density of ED- and EL-producing faecal bacteria was approximately 6×10^8 and 3×10^5 colony forming units per gram dried faeces, respectively (n = 24 human adults). The occurrence of EL-producing communities was associated with the production of EL. The bacteria involved in SDG conversion are phylogenetically and functionally diverse. They include species that belong to the genera *Bacteroides*, *Clostridium*, *Eggerthella*, *Eubacterium* and *Pepto-streptococcus*. In addition, one isolated strain, which possibly represents a novel bacterial genus, catalysed the dehydrogenation of ED to EL and led to the possible identification of 2,3-bis(3,4-dihydroxybenzyl)-butyrolactone, a new intermediate in the production of EL. The high prevalence of enterolignan production in humans (100%, n = 31) and the finding that SDG-converting bacteria metabolise several dietary lignans are of relevance to the possible health implications of lignan-containing diets.

Keywords: Dietary lignans; secoisolariciresinol diglucoside; human intestinal bacteria; anaerobic conversion; enterodiol; enterolactone

Zusammenfassung

Hintergrund. Secoisolariciresinol-Diglucosid (SDG), eines der in unserer Ernährung am häufigsten vorkommenden Lignane, wird von intestinalen Bakterien über Secoisolariciresinol (SECO) zu den Enterolignanen Enterodiol (ED) und Enterolacton (EL) umgewandelt. Dadurch werden dessen Bioverfügbarkeit und biochemische Eigenschaften stark verändert. **Ziel.** Da bisher wenig über den mikrobiellen Abbau von SDG im menschlichen Verdauungstrakt bekannt ist, wurden intestinale Bakterien, die in der Lage sind, SDG abzubauen, untersucht. SDG-abbauende Bakterien wurden mit Hilfe einer kultivierungs-abhängigen Methode quantifiziert. Die beteiligten Spezies wurden durch Isolierung aus Fäzesproben und Untersuchung von Stammsammlungen identifiziert. **Ergebnisse.** Die Bildung von Enterolignanen bedarf nicht nur der Aktivitäten von dominanten, sondern auch von subdominanten Bakterien, wie denen, die mit ungefähr 3×10^5 Kolonie-bildenden Einheit pro Gramm Fäzes die Dehydrogenierung von ED katalysierten. Die Diversität von SDG-abbauenden bakteriellen Gemeinschaften scheint hoch zu sein. Spezies unterschiedlicher Gattungen, wie *Bacteroides*, *Clostridium*, *Eggerthella*, *Eubacterium* und *Peptostreptococcus*, wandelten SDG und dessen Metaboliten um. Während alle Stämme der gleichen Spezies in der Lage waren, SDG abzubauen, waren jedoch Unterschiede zwischen phylogenetisch eng verwandten Spezies feststellbar. Darüber hinaus katalysierte ein isolierter Stamm einer vermutlich neuen Gattung die Dehydrogenierung von ED. Desweiteren bildete dieser Stamm aus SECO einen bisher nicht beschriebenen Metaboliten. Die hohe Prävalenz der Bildung von Enterolignanen (100% der getesteten Probanden) und der Abbau auch anderer Lignane durch SDG-abbauende Bakterien zeigen die Relevanz der erhaltenen Ergebnisse im Hinblick auf die Ernährung.

Stichwörter: Diätische Lignanen; Secoisolariciresinol-Diglucosid; humane intestinale Bakterien; anaerober Abbau; Enterodiol; Enterolacton

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Résumé

Contexte. L'un des principaux lignanes alimentaires, le sécoisolaricirésinol diglucoside (SDG), est métabolisé par la microflore intestinale humaine. Il en résulte la production des entérolignanes entérodiol (ED) et entérolactone (EL), via le sécoisolaricirésinol (SECO). La biodisponibilité et les propriétés biochimiques du SDG dépendent ainsi de la microflore intestinale. Les bactéries impliquées restent cependant très peu connues. **Objectifs.** Grâce à l'utilisation combinée de méthodes bactériologiques de culture anaérobie et de méthodes biochimiques et moléculaires, le projet visait à caractériser, à l'échelle des communautés puis des espèces, les bactéries fécales responsables de la production d'entérolignanes. **Résultats.** La production d'EL résulte des activités métaboliques de communautés bactériennes dominantes et sous-dominantes. Les bactéries qui déméthylent et déhydroxylent le SECO atteignent une densité moyenne de population d'environ 6×10^8 cellules par gramme de matière sèche fécale, tandis que celles catalysant l'étape de déhydrogénation atteignent environ 3×10^5 cellules g^{-1} (n = 24). La diversité des bactéries impliquées est importante, comme en témoigne leur appartenance à des genres aussi variés que les genres *Bacteroides*, *Clostridium*, *Eggerthella*, *Eubacterium* et *Peptostreptococcus*. D'autre part, un isolat appartenant vraisemblablement à un nouveau genre bactérien et catalysant la déhydrogénation de l'ED a permis la mise en évidence du 2,3-bis(3,4-dihydroxybenzyl)-butyrolactone, un nouvel intermédiaire dans les voies métaboliques de production d'EL. Enfin, le fait que les entérolignanes aient été détectés chez tous les individus testés (n = 31) et que les bactéries métabolisant le SDG catalysent également la transformation d'autres lignanes alimentaires soulignent l'importance des lignanes en terme de nutrition préventive.

Mots clefs : Lignanes alimentaires ; secoisolariciresinol diglucoside ; microflore intestinale humaine ; métabolisme anaérobique ; entérodiol ; entérolactone

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Abbreviations and index of bacteriological terms and notionsa

- **Anaerobiosis** (p. 13): Refers to specific oxygen requirements and the need of reduced conditions for growth. Strict anaerobic bacteria do not grow on exposure to oxygen, aerotolerant anaerobes are able to grow in the presence of low levels of oxygen, whereas microaerophiles prefer or require a low and defined concentration of oxygen for growth. Obligate anaerobes can only ferment organic compounds to gain energy, in contrast to facultative anaerobes, which also have an oxygen-based metabolism. In practice, reducing agents are added to anoxic media to lower the oxidation-reduction potential in a range that permits growth.
- **Bacterial composition** (p. 5, p. 64): Relates to the quantitative analysis of phylogenetic groups by means of amplification or *in situ* hybridisation techniques.
- **Bacterial diversity** (p. 5, p. 47): Concerns the richness of communities, *i.e.*, the number of types within communities, as assessed by denaturing gradient electrophoresis methods, restriction fragment length polymorphism or 16S rRNA clone library analyses.
- **Bacterial ecology** (p. 6, p. 51): Study of the distribution and abundance of bacteria and of their relationships with one another and with their surroundings (*http://cme.msu.edu*).
- **CFU**, colony forming unit (p. 5): A measure of bacterial cell density, which refers to viable organisms and is used to characterise bacterial growth.
- **DMSO**, dimethyl sulfoxide (C_2H_6OS) (p. 13).
- **Dominant intestinal bacteria** (p. 6): Represent 0.5% or more of the total number of cells, *i.e.*, approximately 10^8 cells or more per gram of intestinal content. They play a major role in the metabolic function (fermentation of non-digested food components and production of available metabolites), trophic function (interaction with epithelial and immune cells) and protective function (barrier effect against pathogens) of intestinal microbiota.
- **ED**, enterodiol ($C_{18}O_4H_{22}$) (p. 9).
- **EL**, enterolactone ($C_{18}O_4H_{18}$) (p. 9).
- **FISH**, fluorescent *in situ* hybridisation (p. 24).
- **HP**, hybridisation percentage (p. 26, p. 34, p. 39).
- **HPLC**, high performance liquid chromatography (p. 21).
- **MeOH**, methanol (CH_3OH) (p. 13).
- **Microbial** (p. 5): Term used in the text with bacteria in mind, although it refers ordinarily to all micro-organisms, including yeasts and protozoa.
- **MPN**, most probable number (p. 17): The MPN method is a culture-based technique for the estimation of bacterial cell densities. The unit MPN refers to viable cell numbers.

- **MS**, mass spectrometry (p. 23).
- **PBS**, phosphate buffer saline (p. 14).
- **PFA**, paraformaldehyde (p. 14).
- **16S rRNA**, 16S ribosomal ribonucleic acid (p. 23).
- **RPF**, relative probe fluorescence (p. 24).
- **SDG**, secoisolariciresinol diglucoside ($C_{32}O_{16}H_{46}$) (p. 7).
- **SDG-converting communities** (p. 10): Term used throughout the manuscript to characterise the bacteria that contribute to the conversion of SDG. It includes any organism that catalyses one of the four reactions underlying the production of EL: *O*-deglycosylation, *O*-demethylation, dehydroxylation or dehydrogenation.
- **SECO**, secoisolariciresinol ($C_{20}O_6H_{26}$) (p. 9).
- **Bacterial systematics**[b] (p. 5, p. 13): Scientific classification of bacteria. It includes their hierarchical positioning (taxonomy[c]) and their evolutionary relationship (phylogeny). A species is "a category that circumscribes a (preferably) genomically coherent group of individual isolates/strains sharing a high degree of similarity in (many) independent features, comparatively tested under highly standardized conditions"[d]. The type strain of a species is notified by a superscript T, *e.g.*, *Clostridium cocleatum* DSM 1551^T. Names of genera are abbreviated after their first appearance in the text, *e.g.*, *C. cocleatum*. Most of the species included in this manuscript are first mentioned in section 2.2.1.

[a] Breznak JA *et al.* (1994) Physicochemical Factors in Growth. In: Methods for General and Molecular Bacteriology (Gerhardt P.E. *et al.*, Eds.), pp. 137-54. American Society for Microbiology; *http://en.wikipedia.org*.

[b] see "List of Prokaryotic names with Standing in Nomenclature": *http://www.bacterio.cict.fr*

[c] Bergey's manual® of systematic bacteriology (1986) (Butler JP *et al.*, Eds), Williams & Wilkins.

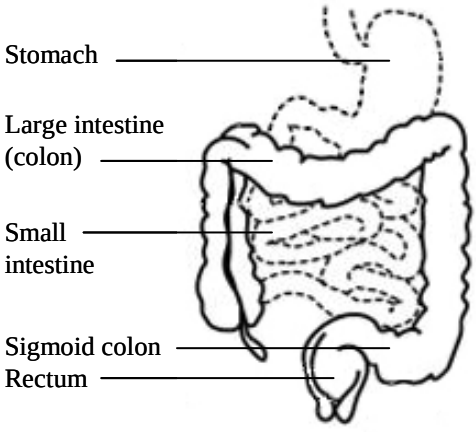
[d] Stackebrandt E *et al.* (2002) Report of the ad hoc committee for the re-evaluation of the species definition in bacteriology, *Int J Syst Evol Microbiol* 52:1043-7.

1. Introduction

The microbial world spreads far and wide. Thanks to a reproductive process by binary fission and to extensive exchanges of genetic material, bacteria rapidly evolve and take advantage of environmental changes. They colonise very diverse milieus and among those, the intestinal tract of humans is of particular interest. However, the symbiotic relationships between host cells and endogenous intestinal bacteria, and their continuous interaction with nutritional factors, remain largely unknown. To achieve a better understanding of the intestinal ecosystem and its influence on human health, applied bacteriological studies are crucial.

1.1. The intestinal microbiota

The digestive tract is a dynamic organ, the function of which to provide energy is vital. It harbours a complex microbiota, which is qualitatively and quantitatively unevenly distributed. With the exception of the mouth, where abundant bacteria organise in niches, the density and diversity of the intestinal microbiota increase gradually from the stomach to the colon. The specific distribution of bacteria along the gastrointestinal tract coincides mainly with changes in metabolic features and tolerance to oxygen (**Figure 1**). The intestinal microbiota is also characterised by micro-anatomic bacterial communities associated with specific metabolic or trophic functions, although their formation is not well understood [1]. Besides these spatial disparities, bacterial populations evolve with the development and age of the host [2, 3].



Stomach (10^1-10^3 CFU g^{-1})
Helicobacter; Lactobacillus; Streptococcus

Ileum (10^7-10^8 CFU g^{-1})
Enterobacteriaceae; *Lactobacillus;* *Streptococcus*

Colon (10^{10}-10^{12} CFU g^{-1})
Bacillus; *Bacteroides*; *Bifidobacterium*; *Clostridium*; *Enterobacteriaceae*; *Enterococcus*; *Eubacterium*; *Fusobacterium*; *Lactobacillus*; *Peptococcus*; *Peptostreptococcus*; *Ruminococcus*; *Streptococcus*

Figure 1: Quantitative and qualitative distribution of bacterial families and genera in the gastrointestinal tract. The unit CFU (colony forming unit) per gram of intestinal content refers to the cell density of viable organisms.

In the colon of human adults, fermentation processes and anaerobic bacteria rule very densely populated communities. Since the advent of microbiology, culture-based techniques have extensively contributed to the characterisation of intestinal bacteria. The study of faecal isolates showed that a few genera dominate in the colon. These include the genera *Bacteroides, Bifidobacterium, Clostridium, Eubacterium,* and *Peptostreptococcus* [4, 5]. The intestinal microbiota is estimated to contain 400 to 1,000 species, approximately 50% of which are still not described [1, 6]. This shows the necessity to isolate and characterise new bacteria.

Although culture-dependant techniques give the opportunity to systematically describe novel bacteria, they are limited to the description of cultivable organisms, which represent only part of the intestinal microbiota (**Table 1**).

Table 1: Estimation of the cultivable fraction of intestinal microbial communities

Proportion of cultivable bacteria	Reference
94%	Moore and Holdeman, 1974 [5]
63%	Holdeman *et al.*, 1976 [7]
24%	Finegold *et al.*, 1983 [4]
15-37%	Langendijk *et al.*, 1995 [8]
58%	Wilson *et al.*, 1996 [9]
24%	Suau *et al.*, 1999 [10]
21-38%	Tannock *et al.*, 2000 [11]
25%	Hayashi *et al.*, 2002 [12]

In recent years, the expansion of molecular approaches has extended our knowledge of the diversity and the activity of intestinal microbiota and allowed for an in-depth view of non-cultivable bacterial communities. High throughput molecular techniques, such as 16S ribosomal RNA (16S rRNA) gene sequence analyses or genome sequencing (*http://cmr.tigr.org*), have provided abundant data on bacterial phylogeny and on microbiomes (collective genomes of members of bacterial communities) [13, 14]. These data have revealed an even more complex intestinal microbiota than previously assumed, helped to understand the metabolic role of intestinal species in nutrient transformation, and showed that the distribution of species within dominant bacterial groups is host specific [15-17].

The combined use of culture-dependent and -independent techniques has given the opportunity to assess the microbial ecology of the intestinal tract in detail, *i.e.*, to better understand the influence of changes in microbial community composition on ecosystem functions. Besides its likely role in protection against pathogens and regulation of immune

responses [1, 18], the intestinal microbiota acts as a highly efficient bioreactor [19]. Owing to very diverse metabolic potentials, intestinal bacteria play a major role in energy balance, metabolise undigested food components and modify their biochemical properties [16, 20]. Although the diversity of dominant bacterial groups is relatively stable over time for healthy adults [21], the intestinal microbiota is a dynamic system, which constantly interacts with nutritional factors. The study of microbial mechanisms involved in the conversion of dietary compounds, such as lignans, is necessary to assess the health implications of the metabolic function of intestinal bacteria.

1.2. Secoisolariciresinol diglucoside

1.2.1. Occurrence in food and possible health effects

Lignans are estrogen-related compounds found in plants, and therefore our diet. Secoisolariciresinol diglucoside (SDG) is one of the most abundant dietary lignans. Its major source is flaxseed, but a variety of other foods also contains a substantial concentration of SDG, illustrating its importance in "Western diets" (**Table 2**) [22, 23].

Table 2: Examples of SDG-containing foods[a]

	Concentration (mg kg^{-1} dry mass) [24, 25]	Contribution to mean intake[b] (%) [26]
Beverages		
Coffee	5.61 ± 1.17[c]	23.3
Tea	16.14 ± 5.41[c]	6.3
Wine	6.90	4.2
Cereals and oilseeds		
Flaxseed	3,690.00	39.4
Rye	0.50	0.1
Sunflower seed	6.10	0.1
Wheat	0.08	4.7
Fruits and vegetables		
Blackberry	37.20	not determined
Broccoli	4.10	not determined
Peach/Nectarine	not determined	0.3
Strawberry	15.10	0.2

[a] Values refer to secoisolariciresinol, the aglycone form of SDG.
[b] The mean intake was 0.29 mg d^{-1} (4,660 Dutch adults).
[c] Values are means ± SD of six brands of coffee or 19 brands of tea.

SDG is of interest because of its possible contribution to the prevention of "Western diseases". Animal studies have shed light on beneficial effects of SDG at different phases of tumorigenesis (**Table 3**). The hypothesis that exposure to SDG at an early stage of mammary

gland development may reduce breast cancer risk at adulthood is of particular interest, although the influence of SDG intake during pregnancy and lactation on the reproductive development of offspring must be investigated further [27-29].

Table 3: *In vivo* effects of SDG on cancer-related parameters

Animals[a]	Dietary intervention[b]	Significant effects	Ref.
DMBA-treated female Sprague-Dawley rats (n = 30).	Daily gavage of 1.5 mg SDG[d] for 140 days, starting one week after DMBA administration; high fat (20% corn oil) AIN-76-based BD.	- 37% to 46% reduction in the number of mammary tumors.	[30]
DMBA-treated female Sprague-Dawley rats (n = 19).	Daily gavage of 1.5 mg SDG[d] for 49 days, starting 13 weeks after DMBA administration; high fat (20% corn oil) AIN-76-based BD.	- 64% and 74% reduction in the volume of total and new mammary tumors, respectively. - 50% lower number of new tumors. - Inverse correlation between urinary entero-lignans and new tumor size.	[31]
AZ-treated male Sprague-Dawley rats (n = 9).	Daily gavage of 1.5 mg SDG[d] for 100 days, starting one week after AZ injection; high fat (20% corn oil) AIN-76A-based BD.	- 29% and 63% decrease in the number and multiplicity of aberrant crypts in the distal colon, respectively. - Prevention of microadenoma and polyp formation.	[32]
Female Sprague-Dawley rat offspring (n = 6).	Daily gavage of 1.5 mg SDG[d] to dams for 43 days during gestation and lactation; AIN-93G-based diet.	- 24% and 47% reduction in the density of mammary terminal end buds and of alveolar buds in female offspring, respectively.	[33]
Male C57BL/6 mice (n = 27-28).	73[c], 147[d] or 293[e] µmol SDG per kg AIN-93G-based BD for 28 days, starting two weeks before injection of B16L6 murine melanoma cells.	- Dose-dependant decrease in the number, size and volume of lung tumors.	[34]
Female rat offspring (n = 7-8).	17.7 mg SDG per 100 g AIN-93G-based BD[e] fed to: dams only for 21 days during lactation (A), or to dams and subsequently to offspring for 29 days (B).	- 26% and 37% reduction in the density of mammary terminal end buds in female offspring of group A and B, respectively. - 46% and 57% higher density of mammary alveolar buds in female offspring of group A and B, respectively.	[35]
MNU-treated female Sprague-Dawley rats (n = 31-32).	Daily gavage of 0.75[c] or 1.5[d] mg SDG for 154 days, starting two days after MNU injection; high fat (20% soybean oil) AIN-93G-based BD.	- Dose-dependant effect on mammary tumor multiplicity. - Decrease in tumor invasiveness and grade.	[36]
Female Sprague-Dawley rats (n = 8).	Daily gavage of 1.5 mg SDG[d] for 28 days; high fat (20% soybean oil) AIN-93G-based BD.	- 20% decrease in plasma levels of insulin-like growth factor (IGF) I. - Inverse correlation between urinary lignans and plasma levels of IGF-I.	[37]
Female Sprague-Dawley rat offspring treated with DMBA (n = 40).	20.1 mg SDG per 100 g AIN-93G-based BD[e] fed to dams for 21 days during lactation.	- 42% decrease in mammary tumor incidence in offspring; 45% lower tumor number; 68% lower mean tumor size per rat.	[28]

[a] Carcinogens: AZ, azoxymethane; DMBA, 7,12-dimethylbenz[a]anthracene; MNU, N-methyl-N-nitrosourea.
[b] BD, basal diet; AIN, American Institute of Nutrition.
[c, d, e] Equivalent to the amount of SDG consumed in a 2.5%, 5% or 10% flaxseed-containing diet, respectively.

In humans, only a few clinical studies with flaxseed-based diets have been conducted. As an example, post-menopausal breast cancer patients (n = 19) who ingested a 25 g flaxseed-containing muffin daily for 13 to 55 days prior to surgery had a reduced rate of tumor cell proliferation, a decreased c-erbB2 expression and an increase in apoptosis of tumor tissue [38]. Apart from cancer-related effects, animal studies have revealed a positive association between SDG intake and low atherosclerosis risk factors [39, 40]. SDG had also preventive effects against type 1 and type 2 diabetes in rats [41, 42]. Overall, the aforementioned studies show that SDG may beneficially influence host health, but more research into the underlying mechanisms of action is warranted. In this context, the bioavailability and the biological properties of SDG are dependent upon the intestinal microbiota [43-45].

1.2.2. Importance of intestinal bacteria in SDG metabolism

Intestinal bacteria catalyse the *O*-deglycosylation of SDG to secoisolariciresinol (SECO), the *O*-demethylation and the dehydroxylation of SECO to enterodiol (ED), and the dehydrogenation of ED to enterolactone (EL) (**Figure 2**).

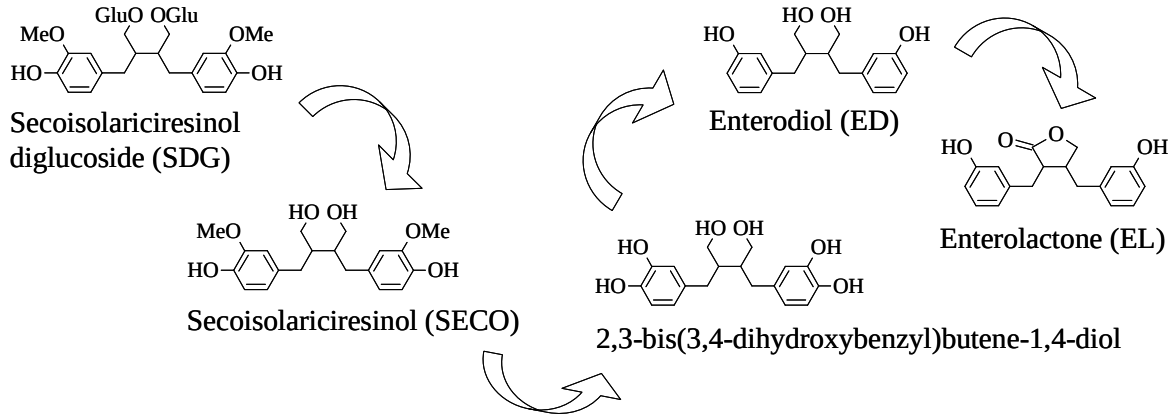


Figure 2: Chemical structure of SDG and its bacterial metabolites.

ED and EL were first detected in the urine of humans and rats in 1979 [46]. Since they had a cyclic pattern of excretion during the menstrual cycle in humans and vervet monkeys, and their concentration was affected by pregnancy, the authors speculated on an ovarian origin of the compounds, or at least that their production was modulated by ovarian functions [47, 48]. They proposed the term mammalian lignans for ED and EL. Although the microbial involvement in production of ED and EL was discovered shortly thereafter [49], the scientific community has been using the term mammalian lignans to distinguish ED and EL from inactive plant precursors. In contrast, the newly introduced term enterolignans has put emphasis on the intestinal origin of the compounds [50]. Hence, ED and EL will be referred to as enterolignans in this thesis.

Enterolignans may have enhanced estrogen-independent and -dependent properties when compared with SDG or SECO. Recent *in vitro* studies showed that ED and EL have higher binding affinity for the human pregnane X receptor and higher antioxidant activities [51-53]. Concerning antioxidant activities *in vivo*, short-term feeding of SDG to rats only led to minor changes in the antioxidant status of hepatic tissue and to an increase in the urinary excretion of enterolignans [54]. *In vitro*, EL binds to estrogen receptors, and both ED and EL compete dose-dependently with estradiol and the unsaturated fatty acid arachidonic acid for their binding site on rat and human alpha-fetoprotein [55, 56]. However, the binding affinities of enterolignans appear to be 10- to 10,000-fold lower than those of other phytoestrogens or sex hormones. Both enterolignans and plant lignans also bind to sex hormone binding globulin [57]. The estrogen-dependent properties of ED and EL include as well the inhibition of aromatase, 5α-reductase and 17β-hydroxysteroid dehydrogenase, three enzymes involved in the metabolism of growth-promoting steroid hormones [58-61]. Besides, EL was found to induce the expression of the estrogen responsive protein pS2 in human breast cancer MCF-7 cells [62]. This and other *in vitro* studies showed that ED and EL alter cell proliferation of various breast, colon and prostate cell lines, as well as endothelial cells derived from bovine brain capillaries [63-66]. Since the biological properties of ED and EL are dose- and exposure time-dependent, intestinal microbiota may alter the possible health effects of enterolignans by modulating their bioavailability.

Although intestinal bacteria are crucial for enterolignan production, the underlying catalytic processes and the bacteria involved, collectively termed “SDG-converting bacteria” in this report, are largely unknown. An early study on the *in vitro* metabolism of SECO proposed that enterolignans were produced under both anaerobic and aerobic conditions by human faecal microorganisms that occurred at concentrations of approximately 10^3 to 10^4 cells per gram of wet faeces [67]. The authors also showed that faecal bacteria *O*-demethylate and dehydroxylate the plant lignan matairesinol, the lactone form of SECO, to produce EL. Furthermore, they suggested that the bacterial oxidation of ED to EL was favoured by aerobic conditions. Lately, Wang *et al.* isolated two strict anaerobes, *Peptostreptococcus* sp. SDG-1 and *Eubacterium* sp. SDG-2, which catalysed the *O*-demethylation and dehydroxylation of SECO, respectively [68]. Recent studies have also reported the likely bacterial production of ED and EL from other plant precursors such as lignins, or the lignans pinoresinol, lariciresinol and sesamin [69-73].

Following bacterial production, enterolignans may be efficiently absorbed and excreted by epithelial cells, and conjugation may already take place in the colon [74]. In the plasma of 27 pre- and postmenopausal omnivorous and vegetarian women, the highest total enterolactone concentration exceeded 1 µM [75]. Another study reported EL concentrations of 16.6 nM (range: 0 - 95.6 nM) and 13.8 nM (range: 0 - 182.6 nM) in the serum of 1,212 women and 1,168 men, respectively [76]. Variations in plasma levels of EL may be partly explained by dietary intake [77]. In rats, a dietary treatment with radio-labelled SDG led to blood radioactivity levels below 1% of the recovered dose and showed that the liver was one of the organs with the highest radio-activity, and presumably SDG metabolite levels [78]. This and other studies imply that enterolignans undergo entero-hepatic circulation [79]. Experiments in pigs suggest that SECO may also enter the entero-hepatic circulation and may be present as conjugate in the ileum [80]. Hepatic hydroxylation of enterolignans and SECO has been reported in humans and rats [81, 82]. It is interesting to note that ED and EL were shown to inhibit rat liver cholesterol 7α-hydroxylase [83]. In human urine, enterolignans are excreted primarily as mono-glucuronides (73% to 94%) and mono-sulphates (2% to 10%), and hardly any free lignans are detected (0.3% to 1%) [84]. These patterns of conjugation were similar in children and resemble those of many steroid hormones [85]. However, they differ from blood patterns. Adlercreutz *et al.* found that the proportion of the free and sulphated enterolignan fraction ranged between 21% and 25% of total plasma enterolignans [75]. Besides, it is worth noticing that EL was detected in its racemic form in human urine [86]. The plant lignans SECO, lariciresinol and isolariciresinol are also detectable in urine [87]. Quantitatively, 30% of the lignan metabolites were present in rat urine by 48 h after administration of radio-labelled SDG, and over 50% were excreted in faeces [78]. The authors also found substantial amounts of radio-activity in the uterus and the adipose tissue. Furthermore, chronic exposure to SDG increased uptake of lignan metabolites in these tissues and delayed faecal excretion, possibly as a result of higher amounts of lignans undergoing entero-hepatic circulation. The intestinal microbiota plays an important role too in the production of ED and EL by converting the conjugated lignans which re-enter the small intestine with bile.

1.3. Objectives

The investigation of the bacterial production of enterolignans is a prerequisite to evaluate the bioavailability and the health effects of dietary lignans. This project aimed at describing the intestinal bacteria that contribute to SDG conversion, *i.e.*, the bacteria that catalyse at least one of the four reactions leading to EL production. The issues addressed included: Under which conditions does the conversion of SDG occur *in vitro*? What is the occurrence and prevalence of SDG-converting bacteria? To what extent do they contribute to enterolignan production? To which phylogenetic groups do they belong? Can they be isolated? Are they specifically detectable in human faeces? What are the mechanisms underlying EL production?

2. Materials and methods

The investigation of enterolignan production by human intestinal bacteria required the combined use of culture-based techniques to enumerate, isolate and cultivate SDG-converting bacteria, biochemical techniques to separate, quantify and identify bacterial metabolites, and molecular techniques to determine the proportion of dominant faecal bacterial group and identify SDG-converting species.

2.1. Chemicals

Racemic SDG (M = 686.4 g mol^{-1}) was isolated from flaxseed [88]. Racemic SECO (M = 362.4 g mol^{-1}) and matairesinol (M = 358.4 g mol^{-1}) were purchased from Sigma-Aldrich (Taufkirchen, Germany). Racemic ED (M = 302.4 g mol^{-1}) and EL (M = 298.4 g mol^{-1}) were purchased from VTT Technical Research Centre of Finland. (+)-Pinoresinol (M = 358.4 g mol^{-1}) and (+)-lariciresinol (M = 360.4 g mol^{-1}) were purchased from Arbonova (Turku, Finland). Methylated aromatic compounds (*m*-anisic acid, ferulic acid, syringic acid, veratric acid, veratrol) and their respective demethylated products (*m*-hydroxybenzoic acid, caffeic acid, gallic acid, vanillic acid and protocatechuic acid, guaiacol and catechol) were purchased from Sigma-Aldrich. Stock solutions of SDG and SECO (100 mM) were prepared in H_2O and MeOH, respectively. Stock solutions of pinoresinol, lariciresinol, matairesinol, ED and EL (50 mM) were prepared in MeOH. Stock solutions of methylated aromatic compounds (50 mM) were prepared in MeOH or DMSO as indicated in **Table 9** (p. 48). Dried compounds and stock solutions were stored at 4°C. Stock solutions were sterile-filtered (0.22 µm; Millex®-GV, Millipore) and used within four months.

2.2. Culture-based techniques

Unless specified, strictly anaerobic techniques were used to prepare media and cell suspensions, and to isolate and cultivate bacteria [89-91]. They warrant the absence of oxygen and a low oxidation-reduction potential, which are required for the cultivation of strict anaerobes. If not indicated, the gas phase was 80% N_2 plus 20% CO_2 (v/v) and bacteria were grown at 37°C. On solid media, bacteria were cultivated in anaerobic jars pressurised at 1.5×10^5 Pa with the appropriate gas phase. All media were sterilised at 121°C for 15 min and contained 1 mg l^{-1} resazurin (E^0 = -51 mV, pH 7.0, 30°C) as indicator of the oxidation-reduction potential.

2.2.1. Bacterial strains and culture conditions

Freeze-dried cultures of *Butyribacterium methylotrophicum* DSM 3468^T, *Clostridium amygdalinum* DSM 12857^T, *Clostridium cocleatum* DSM 1551^T, *Clostridium ramosum* DSM 1402^T, *Clostridium saccharolyticum* DSM 2544^T, *Clostriidum scindens* DSM 5676^T, *E. lenta* DSM 2243^T, *Enterobacter cloacae* DSM 30054^T and *Eubacterium callanderi* DSM 3662^T were revived as recommended by the supplier (DSMZ). Cryo-stocks or grown cultures of *Acetobacterium woodii* DSM 1030^T, *Bacteroides distasonis* DSM 20701^T, *Bacteroides fragilis* DIfE-05, *Clostridium barkeri* DSM 1223^T, *Clostridium coccoides* DSM 935^T, *Clostridium spiroforme* DSM 1552^T, *Eubacterium limosum* DSM 20543^T, *P. productus* DSM 2950^T, *P. productus* DSM 3507, *Ruminococcus hansenii* DSM 20583^T, *Ruminococcus obeum* ATCC 29174^T and *Ruminococcus schinkii* DSM 10518^T were obtained from the collection of the German Institute of Human Nutrition Potsdam-Rehbrücke or the French National Institute of Agricultural Research (INRA). The strains were grown in Brain Heart Infusion-broth (Merck) supplemented with 5 g l^{-1} yeast extract and 5 mg l^{-1} haemin (YHBHI). To ensure purity, bacteria were streaked two times on YHBHI-agar. Purity was examined by comparison of colony morphology and comparison of cell morphology after Gram-staining. Gram-staining behaviour was confirmed by means of the KOH-test [92]. Bacteria were grown on YHBHI-agar under aerobic conditions to check for the presence of aerobic contaminants. Cryo-stocks of pure cultures were prepared in Microbank tubes (MAST Diagnostica) according to the manufacturer's instructions. They were frozen in liquid nitrogen and stored at -80°C. Working stocks were subcultured every two to three weeks and kept at 4°C.

2.2.2. Fixation of pure cultures for *in situ* hybridisation

Bacteria were grown in 50 ml YHBHI-broth and harvested in the exponential growth phase to maximise the rRNA content. Cells were centrifuged (10,000 × g, 10 min, 4°C) and resuspended in 2 ml PBS (8.5 g l^{-1} NaCl, 0.6 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$ and 0.3 g l^{-1} KH_2PO_4 in H_2O, pH 7.0). A volume of 0.2 ml was added to 0.6 ml of 4% paraformaldehyde (PFA) solution (Electron Microscopy Sciences) in PBS and fixed for 3 h at 4°C. Aliquots were frozen in liquid nitrogen and stored at -80°C. Fixed cultures of *C. coccoides* DSM 935^T and *Ruminococcus callidus* ATCC 27760^T were obtained from the INRA.

2.2.3. Culture media for incubation of bacteria with lignans

The procedure to prepare the media is described in **Table 10** and **Table 11** (App. 2).

The medium Mt-3 was used to test the ability of bacteria to *O*-deglycosylate SDG. It contained (per litre H_2O): 4 g $NaHCO_3$, 0.5 g sodium acetate·$3H_2O$, 0.5 g sodium formate, 0.5 g cysteine·HCl·H_2O, 0.3 g yeast extract, 100 ml ten-fold concentrated basal solution [93], 20 ml trace element solution 1 [93], 10 ml rumen fluid and 1 ml vitamin solution [93]. SDG was added to a final concentration of 500 µM. The pH was adjusted to 7.5, the medium was gassed and autoclaved.

The medium Mt-6 was used to test the ability of bacteria to *O*-demethylate, dehydrogenate or reduce lignans, and for the enumeration and isolation of SECO-converting bacteria. It contained (per litre H_2O): 3 g yeast extract, 3 g peptone from casein, 2.5 g sodium acetate·$3H_2O$, 2.5 g sodium formate, 0.5 g cysteine·HCl·H_2O, 5 mg haemin, 100 ml Salt 1 solution, 50 ml rumen fluid, 2 ml Salt 2 solution, 1 ml vitamin solution and 0.1 ml trace element solution 2. 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. 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. Trace element solution 2 was 7.4 mM $CuCl_2$, 6.5 mM $NiCl_2$ and 4.9 mM $MoNa_2O_4$ in H_2O. The pH was adjusted to 7.5, the medium was gassed and autoclaved. After autoclaving, 10% (v/v) of each glucose and fructose were added from anaerobic and autoclaved stock solutions (100 mM).

The medium Mt-61 was used for the isolation of ED-dehydrogenating bacteria. It was based on the medium Mt-6, with the following modifications (per litre H_2O): rumen fluid, 35 ml; yeast extract, 500 mg; sodium acetate·$3H_2O$ and sodium formate, 800 mg each; haemin, 2.5 mg. The medium was not supplemented with glucose and fructose.

The medium Mt-75 was used to test the ability of bacteria to *O*-demethylate or dehydroxylate SECO, and for the isolation of SECO-converting bacteria. It contained (per litre H_2O): 2 g $NaHCO_3$, 1.25 g sodium acetate trihydrate, 1.25 g sodium formate, 0.5 g cysteine·HCl·H_2O, 0.1 g yeast extract, 100 ml of ten-fold concentrated basal solution, 20 ml trace element solution 1, 2.5 ml rumen fluid and 1 ml vitamin solution. SECO dissolved in MeOH was added to a final concentration of 1 mM. The final concentration of MeOH was below 2% (v/v). The pH was adjusted to 7.2, the medium was gassed with 80% CO_2 plus 20% H_2 (v/v) and autoclaved.

The medium Mt-85 was used for the isolation of SDG-deglycosylating bacteria. It contained per litre: 500 mg cysteine·HCl·H_2O, 250 mg $Na_2S·9H_2O$, 250 mg sodium formate, 50 mg yeast extract, 1 ml rumen fluid, 1 ml vitamin solution, 100 ml Salt 1 solution, 2 ml Salt 2 solution, and

0.1 ml trace element solution 2. SDG was added to a final concentration of 500 µM. The pH was adjusted to 7.6, the medium was gassed and autoclaved.

2.2.4. Processing of faecal samples

The microbial analysis of faeces is a standard approach to investigate intestinal bacteria. Although few studies showed differences in bacterial diversity and activity at different anatomical sites along the intestinal tract, more work is required to fully characterise these differences [94, 95]. Moreover, the collection of human faecal samples does not require invasive techniques. Thus, we used fresh human faeces to study SDG-converting bacteria. Faecal samples were collected in plastic boxes, stored at 4°C under anaerobic conditions using an AnaeroGen Compact (Oxoid) for a maximum of 4 h before processing.

For most probable number (MPN) enumeration and isolation of bacteria, dilutions ranging from 10^{-1} to 10^{-10} were prepared at room temperature in an anaerobic tent (Coy Laboratory Products), using sterile PBS supplemented with 0.25 g l^{-1} cysteine·HCl·H_2O and 0.1 g l^{-1} peptone (PC/PBS). Briefly, 1-g fresh faecal aliquots were added to 9 ml PC/PBS, mixed well with sterile plastic loops and vortexed to obtain homogenised 10^{-1} faecal dilutions. Samples were left to stand for 5 min and volumes of 1 ml were transferred into 9 ml PC/PBS (10^{-2} faecal dilutions). Successive transfers were performed using sterile pipette tips to obtain the desired range of dilutions. Before each transfer, samples were mixed by repeated inversion of the tubes. For each sample, two 1-g aliquots of fresh faeces were freeze-dried to estimate the faecal water content. For fluorescent *in situ* hybridisation (FISH), samples were prepared under aerobic conditions. Faecal aliquots (1 g) were suspended in 9 ml of PBS and mixed to obtain homogenised suspensions. These were fixed with PFA as described for pure cultures.

2.2.5. Conversion of SECO by faecal suspensions

Twenty-four healthy French and German adults (23 to 59 years old) gave their informed consent to take part in the MPN study. They comprised 12 women and 12 men who did not take antibiotics for three months prior to the study. Seven additional individuals were tested to determine the prevalence of enterolignan production in humans.

2.2.5.1. Experiments under aerobic conditions

Six German faecal samples (MPN 2, 6, 13, 14, 15, 17) were tested for aerobic conversion of SECO. A volume of 900 µl of SECO-containing Mt-6-broth was inoculated with 100 µl of the

10^{-1} faecal dilutions. Samples were incubated in Eppendorf tubes equipped with membrane lids (Eppendorf ®Lid $_{BAC}$, Eppendorf) and placed on a rotary shaker (150 rpm). Volumes of 50 µl of the faecal cultures were taken before incubation and after 48 h of growth and analysed by high performance liquid chromatography (HPLC). Controls consisted of Mt-6-broth without faecal bacteria, faecal bacteria in medium without SECO and faecal bacteria in Mt-6-broth incubated under anaerobic conditions.

2.2.5.2. The MPN method

The principle of the method is depicted in **Figure 3.**

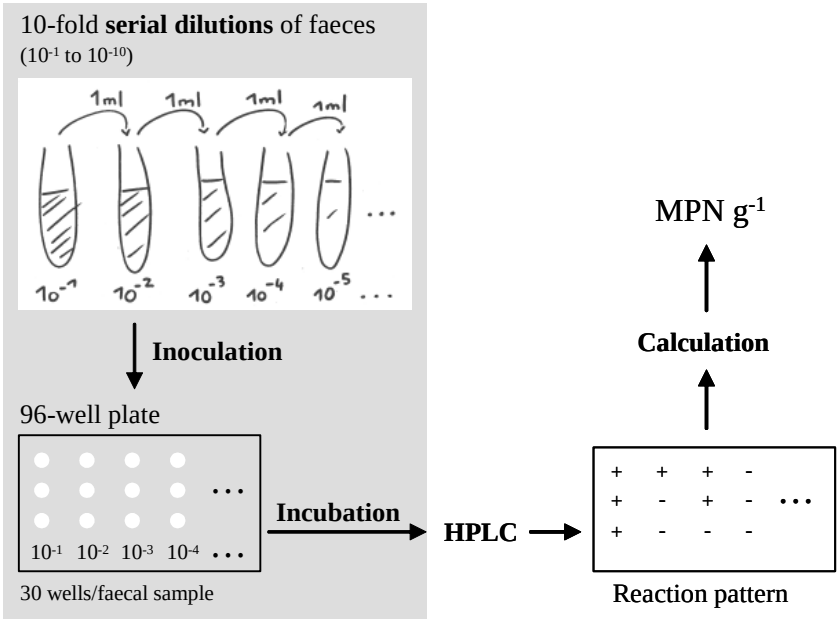


Figure 3: Principle of the MPN method for enumeration of SECO-converting bacteria. Steps included in the grey box were performed under anaerobic conditions.

The MPN method is based on the reaction pattern of aliquots of medium that were inoculated repeatedly with ten-fold dilution series of faeces. In the context of SECO-conversion, a reaction was positive when the corresponding dilution of a given faecal sample produced enterolignans. This was the case, if: 1) the concentration of SECO substantially decreased, 2) peaks were detected at the retention time of enterolignans, 3) these peaks corresponded to concentrations of ED or EL above 10 µM and displayed the specific spectra obtained with standards. The method was optimised to use as little SECO as possible. Thus, the sterile SECO-containing Mt-6-broth was dispensed into the 1.2 ml-deep wells of a 96-well plate (250 µl per well) in an anaerobic tent. Faecal dilutions (100 µl) were each inoculated in triplicate into wells. Controls consisted of faecal bacteria in Mt-6-broth without SECO and SECO-containing Mt-6-broth without bacteria. These controls were done in triplicate. After 48 h of growth, plates were

centrifuged at 4,000 × g for 15 min and the supernatants were analysed by HPLC. Bacterial counts were calculated using the table based on three replicates [96]. They were adjusted according to faecal water contents and dilution factors and expressed either as MPN per gram of dried faeces (MPN g^{-1}) or as logarithmic values thereof.

2.2.5.3. Enterolignan production

From the MPN data for the German samples (n = 13), chromatograms obtained with the 10^{-1} faecal dilutions were used to estimate the production of EL and ED. The parameter λ was defined as the ratio between the final concentration of EL and the initial concentration of SECO. The parameter δ was defined as the ratio between the final concentration of both enterolignans (ED plus EL) and the initial concentration of SECO. Because each the faecal sample was analysed in triplicate, λ and δ were expressed as the means.

2.2.6. Isolation of SDG-converting bacteria

Faeces were obtained from a healthy male adult, who harboured dominant enterolignan-producing bacteria (MPN 14). The purity of isolates was ensured as described for strains from culture collections (section 2.2.1.).

SDG-deglycosylating bacteria were isolated in an anaerobic chamber (MACS variable atmosphere workstation, Don Whitley Scientific). Faecal dilutions were spread-plated onto Mt-85 supplemented with 14 g l^{-1} agar (Serva). Plates were incubated for 103 h. Twenty-two single colonies from the 10^{-3}, 10^{-4} and 10^{-5} faecal dilutions were picked and cultured in Peptone-Yeast-Glucose-broth (medium 104, DSMZ). The resulting liquid cultures were subsequently used in conversion experiments with growing cells to test their ability to convert SDG.

For isolation of SECO-demethylating and -dehydroxylating bacteria, faecal dilutions were spread-plated onto SECO-containing Mt-6 supplemented with 14 g l^{-1} agar. After 48 h of growth, a mixed culture of bacteria obtained from the initial 10^{-5} faecal dilution produced ED. This mixed culture was spread-plated repeatedly onto Mt-6, but pure SECO-converting strains could not be isolated. In parallel, the ED-producing mixed culture was subcultured several times in Mt-6-broth. The selective medium Mt-75 supplemented with 10 g l^{-1} agar was next used for isolation. This medium favours the growth of acetogenic bacteria. In the context of the isolation of SECO-converting organisms, we hypothesised that acetogens would benefit from the *O*-demethylation of SECO for acetogenesis since they would not need to reduce CO_2 for the formation of the methyl group of acetate and would thereby save energy. Once autoclaved, the medium Mt-75 was allowed to cool down to 45°C in a water bath. Sterile- filtered

bromoethanesulfonate dissolved in H_2O was added to a final concentration of 4 mM to selectively inhibit the growth of methanogenic archaea, which can also grow on H_2 plus CO_2 by reducing CO_2 to methane. Once poured into plates and allowed to solidify under sterile conditions, the medium was transferred into an anaerobic tent and allowed to equilibrate for 48 h before inoculation. Serial dilutions of the ED-producing mixed culture were spread-plated onto Mt-75-agar. After 95 h of growth under 80% H_2 plus 20% CO_2 (v/v), three distinguishable colony morphologies originating from the 10^{-4} dilution were observed (m1, m2 and m3). Eighteen colonies were picked and tested for conversion of SECO. Six pure cultures, which were all obtained from m3-like colonies, were able to *O*-demethylate the substrate. They all had the same growth characteristics and the same morphology after Gram-staining. One of them was used as a model for identification. Fifteen combinations of colonies were also incubated with SECO. Two identical combinations (m2- plus m3-like colonies) produced ED. Further co-incubation of the two corresponding pure cultures, *i.e.*, pure cultures obtained independently from m2- and m3-like single colonies, confirmed the production of ED. A pure culture of bacteria obtained from the edge of a m2-like colony was used for identification.

For isolation of ED-dehydrogenating bacteria, the media Mt-6 and Mt-61 were supplemented with 15 µl sterile-filtered stock solution of ED. A 10-fold faecal dilution (50 µl) was incubated for 24 h in 1.5 ml Mt-6-broth. A volume of 50 µl of the faecal culture was transferred once in 1.5 ml Mt-6 and then twice in 1.5 ml Mt-61, every time after 24 h of growth. The ability of the culture to convert ED was monitored by HPLC after each transfer. Serial dilutions of the final ED-converting enrichment (10^{-1} to 10^{-6}) were spread-plated twice onto Mt-61-agar. In total, 39 single colonies were picked after 62 h and 86 h of growth. None of the selected colonies converted ED. However, not all bacteria that grew on the plates formed distinct colonies. Some grew as fine smears. Dehydrogenation of ED was observed after incubation of cells from such smears. These cells were streaked onto Columbia- (5% sheep blood, Biomérieux), PYG- and Wilkins-Chalgren-Anaerobe-agar (Oxoid) to support better growth. After 95 h of growth, bacteria were picked from the edge of 32 single colonies and tested for ED dehyrogenation.

2.2.7. Conversion of lignans and methylated aromatic compounds by growing cells

To test for the *O*-deglycosylation of SDG, 50 µl overnight liquid cultures were grown in 1.5 ml Mt-3-broth. To test for the *O*-demethylation of SECO or the dehydrogenation of ED or SECO, 50 µl overnight liquid cultures or bacteria from isolated colonies were grown in 1.5 ml Mt-6-broth containing 500 µM ED or 1 mM SECO. To test for the dehydroxylation of SECO, approximately 20 µl overnight cultures of each *P. productus* SECO-Mt75m3 and the

dehydroxylating candidate strain were co-incubated in 1 ml Mt-75-broth. To test for the conversion of lariciresinol, matairesinol and pinoresinol, 50 µl overnight liquid cultures were grown in 1.5 ml Mt-6-broth containing 500 µM of the lignan of interest.

The medium of Kamlage *et al.* [97], without haemin, was used to investigate the conversion of methylated aromatic compounds. The medium was supplemented with glucose (6 g l^{-1}). Sterile-filtered methylated substrates were added to 9 ml autoclaved medium to a final concentration of approximately 1 mM. Media were inoculated with 0.1 ml overnight liquid cultures. The criteria to conclude that *O*-demethylation occurred entailed: 1) a marked decrease in the substrate concentration, 2) the detection of peaks at the retention time of standards of the demethylated products, 3) that these peaks displayed the same spectra as the standards, 4) that this was observed for two independent experiments performed under the same conditions.

Controls consisted of bacteria in media without substrate and media containing substrate without bacteria. Samples were collected after approximately 24 h of growth and stored at -20°C until HPLC analysis. Additional 12- and 48-h samples were collected for *O*-demethylation of lignans and methylated aromatic compounds, respectively.

2.2.8. Assessment of SDG deglycosylation

2.2.8.1. Experiments with synthetic stomach and intestinal juice

These experiments were performed to examine whether SDG may reach distal parts of the intestine where bacteria may carry out its conversion. The synthetic stomach juice contained (per litre H_2O): 3 g mucin (Sigma, catalog number M1778), 2.9 g NaCl, 1 g pepsin (Merck, cat. no. 7185), 0.7 g KCl and 0.27 g KH_2PO_4. The synthetic intestinal juice contained (per litre H_2O): 9 g pancreatine (Fluka, cat. no. 76190), 9 g porcine bile extract (Sigma, cat. no. B8631), 1 g $NaHCO_3$, 0.5 g $CaCl_2$, 0.3 g trypsin (Merck, cat. no. 1.24579), 0.3 g urea (Roth, cat. no. 2317.1), 0.3 g KCl and 0.2 g $MgCl_2$. They were prepared according to published instructions from the German Institute for Standardisation [98] and stored at -80°C. SDG was added to 495 µl stomach or intestinal juice to a final concentration of 1 mM. The control consisted of 1 mM SDG in PBS. Samples were prepared in triplicate and incubated at 37°C with continuous shaking. Volumes of 50 µl were collected before incubation, 30, 90 and 180 min thereafter. Samples were stored at -20°C until HPLC analysis.

2.2.8.2. Conversion of SDG by resting cells

Prior to experiments with resting cells, growth curves were determined for *B. fragilis* SDG-Mt85-4C, SDG-Mt85-5B and DIfE-05, *B. ovatus* SDG-Mt85-3C, *Clostridium* sp. SDG-Mt85-3Db and *B. distasonis* DSM 20701^T. Overnight cultures (1 ml) were used to inoculate 50-ml aliquots of pre-warmed YHBHI-broth in duplicate. Growth was monitored by measuring the optical density at 600 nm. After 12 h at 37°C, all strains were in the early stationary phase.

Twelve-hour cultures (50 ml) were used for preparation of resting cells. Cultures were washed once in PBS supplemented with 0.05% (wt/v) cysteine·HCl·H_2O (10,000 × g, 10 min, 4°C) and re-suspended in 5 ml of the same buffer. The optical density of the bacterial cell suspensions was measured at 600 nm and samples were diluted (1.1 to 6.9-fold) to match the lowest density. For later estimation of bacterial numbers, two 100 µl aliquots of the suspensions were each fixed with 300 µl of a 4% PFA solution in PBS. SDG was added to two aliquots (2 ml) of each bacterial suspension to a final concentration of 625 µM. Controls consisted of SDG in buffer. Bacteria were incubated in screw-cap rubber-stopper glass tubes in a water bath with continuous shaking. Volumes of 50 µl were collected over time and stored at -20°C until HPLC analysis.

Cell counts of resting cells were determined by staining with 4',6-Diamidino-2-phenylindole (DAPI). Fixed bacterial suspensions were shaken for 1 min at full speed in a Uniprep24 (Laborgerätbau- und -Vertriebs GmbH) and 10^{-2} dilutions were prepared in duplicate in PBS. A 10-µl aliquot of the resulting cell suspensions was added to 90 µl DAPI solution (150 ng ml^{-1} PBS) and incubated in the dark for 10 min at room temperature. Each of the DAPI-stained suspensions (10 µl) was spread onto one well of a teflon coated 8-well-slide (Roth). Samples were dried at 37°C, one drop of Vectashield (Vector Labs) was applied onto each well and the slides were covered with cover slips. Bacteria were counted manually using the 100-fold oil immersion objective of an Axioplan2 imaging microscope (Carl Zeiss). Signals were detected at 420 nm. Twenty-five microscopic fields per well were analysed. Bacterial concentrations were calculated accounting for microscopic and dilution factors and expressed as mean ± SD.

2.3. Biochemical techniques

2.3.1. Separation of bacterial metabolites by HPLC

Thawed samples were centrifuged (13,000 × g, 3 min) and the supernatants were analysed. Separation of lignans and aromatic compounds was achieved using a RP-18 column (Lichrocart® Lichrospher®100, 250 × 4 mm, 5 µm, Merck) maintained at 37°C and protected with a guard RP-

18 column (4 × 4 mm, 5 µm). The volume of injection was 20 µl. If not specified, lignans were detected at 285 nm and methylated aromatic compounds at 275 nm using a UV diode array detector. The Chromeleon software version 6.40 (Dionex) was used for data acquisition and analysis.

Eluents for measurement of MPN samples were as follows: (A) 85% H_2O plus 15% MeOH (v/v), adjusted to pH 3 with 98%-formic acid; (B) MeOH. The gradient was 20 to 100% B within 8 min, 100% for 1 min and back to 20% for 5 min. The flow rate was 1 ml min^{-1}. Lignans were detected at 275 nm using a UV diode array detector. French samples were analysed using the Millenium[32] Chromatography Manager (Waters). The retention times of standard lignans were 7.4 min, 8.3 min and 8.7 min for SECO, ED and EL, respectively.

Eluents for measurement of SDG conversion were as follows: (A) 80% 50 mM sodium actetate (adjusted to pH 5 with 100%-acetic acid) plus 20% MeOH (v/v); (B) 40% 50 mM sodium acetate plus 40% MeOH and 20% acetonitrile (v/v/v). The gradient was 20 to 80% B within 10 min, 80 to 100% B within 14 min, 100% B for 5 min, and back to 20% B within 1 min. The system was equilibrated with 20% B for 10 min at the end of each run. The flow rate was 0.3 ml min^{-1}. The retention times of standard lignans were: SDG, 17.2 min; SECO, 22.2 min; ED, 25.2 min; EL, 28.5 min.

Reduction and *O*-demethylation of pinoresinol, lariciresinol and matairesinol were measured using the solvents A and B described above and the gradient elution of Nurmi *et al.* [99]. The equilibration time was shortened from 18 to 14 min. The retention times of standard lignans were 45.7 min, 46.7 min, 57.4 min and 60.1 min for lariciresinol, SECO, pinoresinol and matairesinol respectively.

For methylated aromatic compounds, eluents were as follows: (A) 25 mM sodium formate (adjusted to pH 3.5 with 98%-formic acid); (B) MeOH. The gradient was 0% B for 4 min, 0 to 100% B within 16 min, 100% B for 5 min, back to 0% B within 1 min and 0% B for 9 min. The flow rate was 1 ml min^{-1}.

SDG was quantified using two sets of concentrations for calibration: 10 µM, 100 µM, 400 µM, 800 µM (set 1) and 50 µM, 250 µM, 500 µM, 1000 µM (set 2) in PBS. To quantify SECO, ED and EL, calibration curves were obtained with standards using the following concentrations: 1,500 µM, 1,000 µM, 500 µM, 250 µM, 100 µM, 50 µM and 10 µM. The solvent was methanol and each concentration was prepared in duplicate.

Lignan metabolites were identified by comparison with retention times and spectra of standards. The molecular mass of peaks, for which no standards were available, was determined by Electrospray Ionisation Mass Spectrometry (ESI-MS).

2.3.2. Identification of lignan metabolites by ESI-MS

A triple quadrupole mass spectrometer fitted with a Z-spray API electrospray source (Micromass Quattro II, Waters) was used. Metabolites were separated by HPLC as described above. Downstream of the column, the flow was split (6:1) so that a continuous flow of 0.05 ml min^{-1} was directed into the spectrometer. MS analyses were carried out in negative ionisation mode. The temperature of the ion source was 120°C. The cone and capillary voltages were 25 V and 3.0 kV, respectively. The desolvation temperature was 380°C and the desolvation gas (N_2) was maintained at 400 l h^{-1}. Data were analysed using the MassLynx 3.5 software (Waters).

2.4. Molecular techniques

The bacterial 16S rRNA (1,542 nucleic acids) is a constitutive part of the 30S subunit of ribosomes. It is ubiquitous, represents a substantial component of the cellular mass and is detectable *in situ*. Moreover, the molecule shows a specific secondary structure with conserved to highly variable regions [100]. For these reasons, the 16S rRNA and the corresponding gene are used as markers of bacterial phylogeny [101]. We used 16S rRNA-targeted oligonucleotides to enumerate faecal bacteria and sequenced the 16S rRNA gene to identify isolated bacteria.

2.4.1. Sequencing of 16S rRNA genes for identification of SDG-converting bacteria

Total DNA was extracted with the Invisorb Genomic DNA Kit III (Invitek) following protocol III of the manufacturer's instructions. Primers 27f (5'AGAGTTTGATCCTGGCTC AG) and 1492r (5'TACCTTGTTACGACTT) [102] were used to amplify the bacterial 16S rRNA gene. PCR reactions (50 µl) contained 50 mM KCl, 20 mM Tris-HCl, 1 mM $MgCl_2$, 0.25 mM of each deoxynucleoside triphosphate, 0.1 µM of each primer, 2.5 units Taq DNA polymerase (Invitrogen) and 1 µl of a 10^{-1} dilution of template DNA. The PCR program was as follows: 94°C for 5 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 purified with the High Pure PCR Product Purification Kit (Roche) following manufacturer's instructions. They were analysed by electrophoresis on a 1.5% agarose gel (wt/v) in Tris-Borate-EDTA buffer (Roth). The DNA concentration was estimated using the Low DNA Mass Ladder (Invitrogen). For sequencing, we used either primer 27f, 338f (5'ACTCCTACGGGAGGCAGC) [103], 338r (5'GCTGCCTC CCGTAGGAGT), or 1492r. Sequencing reactions were performed in duplicate with the DYEnamic™ ET Dye Terminator Cycle Sequencing Kit (Amersham Biosciences) following manufacturer's instructions. Sequencing products were analysed with the MegaBACE 1000

System (Molecular Dynamics). Sequences were assembled and manually adjusted using the ContigExpress function of Vector NTI Suite 9.0.0 (Invitrogen). They were subsequently aligned with highly similar sequences (92% similarity or more for sequences of cultured bacteria, 96% similarity or more for cloned sequences originating from not yet cultured bacteria) obtained with the BLAST function of the National Center for Biotechnology Information (NCBI) [104]. PCR products were also sent to AGOWA (Berlin, Germany) for sequencing with the primers 27f and 1492r. Percentages of similarity were calculated following the unambiguous alignment of consensus 16S rRNA gene sequences using the Sequence Identity Matrix function of the Bioedit software version 5.0.9 [105].

2.4.2. Design and optimisation of specific 16S rRNA oligonucleotide probes

16S rRNA gene sequences of organisms closely related to *Peptostreptococcus productus* and *Clostridium* sp. SDG-Mt85-3Db were obtained from the Genbank database. They were aligned using Vector NTI and screened for species-specific regions. Probes targeting these regions were checked using the Probe Match and the BLAST functions of the Ribosomal Database Project (RDP) [106] and the NCBI. They were further tested by FISH on reference strains to establish their specificity. For each strain, the relative probe fluorescence (RPF), which indicates the *in situ* fluorescent signal intensity of a probe, was determined as described previously [107].

2.4.3. Detection of bacteria by FISH

The technique relies on the binding of fluorescent probes to specific regions of 16S rRNA molecules in fixed and permeabilised bacterial cells. The probes used in this study are listed in **Table 4**. They were purchased from Thermo Electron Corporation (Ulm, Germnay) or MWG (Ebersberg, Germany). The nomenclature and abbreviation of probes used hereafter are based upon the Oligonucleotide Probe Database [108]. The labelling quality of probes was controlled by measuring the optical density of oligonucleotides and dyes [109]. The probe Eub-0338-5’GCTGCCTCCCGTAGGAGT, conserved within the bacterial domain [103], and the probe Non-Eub-5’ACATCCTACGGGAGGC were used as positive and negative controls.

Table 4: 16S rRNA oligonucleotide probes used for *in situ* hybridisation

Target	Nomenclature	Sequence	Reference
Atopobium group	S-*-Ato-0291-a-A-17	GGTCGGTCTCTCAACCC	[110]
Bacteroides and relatives	S-*-Bac-0303-a-A-17	CCAATGTGGGGGACCTT	[111]
B. distasonis	S-S-Bdist-1025-a-A-19	CGCAAACGGCTATTGCTAG	[107]
B. fragilis	S-S-Bfra-0998-a-A-20	GTTTCCACATCATTCCACTG	[107]
Bifidobacterium spp.	S-G-Bif-0164-a-A-18	CATCCGGCATTACCACCC	[8]
E. rectale-C. coccoides cluster	S-*-Erec-0482-a-A-19	GCTTCTTAGTCARGTACCG	[112]
C. leptum subgroup	S-*-Clept-0866-a-A-18	GGTGGATWACTTATTGTG	[113]
Clept-0866 competitor 1		......A..........	[113]
Clept-0866 competitor 2		................C.	[113]
Clostridium sp. SDG-Mt85-3Db	S-S-Csac-0067-a-A-20	CTCGGACATTACTGCCCGCG	This study
P. productus and relatives	S-*-ProCo-1264-a-A-23	TTGGGATTCGCTCAACATCGCTG	This study

2.4.3.1. Cell preparation and hybridisation

Fixed bacterial suspensions were prepared as described previously [114]. Cells were washed in PBS (8,000 × g, 3 min) and next in Tris-EDTA (TE) buffer (100 mM Tris·HCl, 50 mM EDTA in H_2O, pH 8.0). For faeces, 200 µl PFA-fixed suspension were added to 800 µl PBS. For pure cultures, 200 to 800 µl PFA-fixed suspension were used depending on the bacterial concentration before fixation. Cells were permeabilised at room temperature for 10 min with 1 mg ml^{-1} lysozyme (Boehringer Mannheim GmbH) in TE buffer. They were washed with PBS and next with hybridisation buffer (900 mM NaCl, 20 mM Tris·HCl, pH 8.0) containing 0.1 g l^{-1} sodium dodecyl sulphate and 300 ml l^{-1} formamide (Fluka). Aliquots (50 µl) of cells in hybridisation buffer were dispensed into the 1.2-ml deep wells of a 96-well plate. Probes were added to a final concentration of approximately 0.75 µM. Cells were hybridised overnight at 35°C. Samples analysed with the probe S-S-Csac-0067-a-A-20 were hybridised for 3 h. After adding 150 µl hybridisation buffer per well, plates were centrifuged (4,000 × g, 20 min) and supernatants were removed by inverting the plate. Cells were incubated (37°C, 30 min) in 200 µl pre-warmed washing buffer (64 mM NaCl, 20 mM Tris·HCl, pH 8.0) containing 0.1 g l^{-1} sodium dodecyl sulphate. Finally, they were pelleted (4,000 × g, 20 min), re-suspended in 200 µl PBS and diluted 6-fold in FACSFlow™ (Becton Dickinson).

2.4.3.2. Flow cytometry analyses

Data acquisition was performed with a FACS Calibur (Becton Dickinson) equipped with a 488 nm argon ion laser and a 635 nm red-diode laser. Cell granularity was measured at 488 nm in the side scatter channel (SSC) and assigned as the primary acquisition parameter. The voltage

setting of the corresponding photomultiplier tube was 458 V with a threshold of 253 V. The multiplying factor of the photodiode used for detection of forward scatter signals (FSC) at 488 nm was 10^2. Voltage settings for the FL1 (530 nm) and FL4 (661 nm) fluorescence were 649 and 800 V, respectively. All amplifiers were set to logarithmic mode. Whenever possible, the flow rate was set to "Low" position (12 µl min^{-1}) and samples were measured at approximately 3,000 events s^{-1}. A total of 100,000 events were acquired for each hybridised suspension.

2.4.3.3. Calculation of bacterial proportions

The procedure for calculation is illustrated in **Figure 5** (p. 40) and **Figure 8** (p. 45). Proportions of bacterial groups or species were obtained by the combined use of Cy5-labelled group- or species-specific probes and FITC-labelled Eub-0338. Dot plots of the FITC-labelled Non-Eub and Eub-0338 controls were used to determine the hybridisation percentage (HP), *i.e.*, the proportion of bacterial events within green fluorescence signals. Thereby, a specific gate that includes the total number of bacteria was defined for each faecal sample. This gate was subsequently applied to the analysis of every FITC-Cy5 double labelled aliquot of the corresponding sample to exclude auto-fluorescent events. Four samples (MPN 1, 7, 22, 24) were excluded from statistical analyses because the HP's were below 35%, *i.e.*, the proportions of auto-fluorescent events were too high to ensure reliable counting. Bacterial proportions were the ratio of the specific red-fluorescent events to the bacterial green-fluorescent events. They were corrected by subtraction of the background fluorescence obtained by concomitant hybridisation with Cy5-labelled Non-Eub and FITC-labeled Eub-0338, *i.e.*, the percentage of red auto-fluorescence within green bacterial events. Samples were analysed in duplicate and bacterial proportions expressed as the means. Analyses were repeated if the coefficient of variation was above 15%.

2.5. Statistical analyses

MPN and FISH results, including bacterial proportions, RPF's and HP's, were expressed as means ± SD. Standard deviations were calculated as follows: $SD= (\sum (x_i - mean)^2/n)^{1/2}$, where x_i is the given value of sample i and n the total number of samples. For a defined bacterial group, the FISH prevalence was the percentage of subjects, for whom specific bacterial events were detected with the corresponding oligonucleotide probe. For concentrations of enterolignan-producing bacteria (MPN data), logarithmic values were analysed. All data were analysed with the statistical software SPSS 11.5 (SPSS Inc.). The Shapiro-Wilk test was used to assess normal

distributions. Variances were checked for their uniformity by means of F-tests. Unless specified, p-values were obtained by two-tailed homoscedastic Student tests. For nonparametric data, Wilcoxon tests were carried out. Chi Square analyses were performed to compare the bacterial composition data with data from the literature. Correlations were determined using Pearson's correlation coefficients. For all tests, the bilateral alpha risk was $\alpha = 0.05$.

3. Results and discussion

The rationale of this work was to describe SDG-converting bacteria at different levels in terms of bacteriology. At the community level, the results refer to the enumeration of enterolignan-producing bacteria in faeces and answer the question on their relevance within the intestinal microbiota. At the species level, the data relate to the isolation of faecal bacteria and the screening of culture collections to provide evidence on the type of organisms that contribute to the conversion of SDG. Finally, at the strain level, pure culture work gave access to a more comprehensive view of the bacterial activities underlying enterolignan production.

3.1. Enterolignan-producing bacterial communities

3.1.1. SECO-converting faecal bacteria

The first concern was to establish the *in vitro* conditions required for the production of enterolignans. We could not detect the production of ED and EL after aerobic incubation of SECO with faecal bacteria (n = 6) (data not shown), as reported previously by Wang *et al.* [68]. This disagrees with the earlier results of Borriello *et. al.* who detected the production of enterolignans from SECO under both aerobic and anaerobic incubation [67]. However, the authors also reported that faeces from an individual treated with metronidazole, an antibiotic commonly used against infections caused by anaerobic bacteria, could no longer convert SECO to ED. This indicates that anaerobes are needed for the production of ED. Thus, even if we cannot exclude that aerobes or facultative anaerobes may catalyse the *O*-demethylation, dehydroxylation or dehydrogenation of SECO, our data challenge the presumption of an aerobic conversion of the compound. Therefore, anaerobic culture techniques were routinely used in the present study, first of all to determine the prevalence of ED and EL production in 31 human subjects. Enterolignans were detected after anaerobic incubation of SECO with 10-fold faecal dilutions in all individuals. EL production was not detectable in one male subject (MPN 16) (**Table 12**, App. 2). In comparison, Kuijsten *et al.* did not detect EL in the plasma of one of 12 individuals who ingested a single dose of SDG [115]. Hence, enterolignan production seems to be widespread among humans. This implies that beneficial health effects associated with enterolignans are relevant to the entire population and not restricted to a certain proportion of individuals as in the case of equol, a bacterial metabolite of the phytoestrogen daidzein [116].

MPN enumerations were performed for 24 of the 31 human faecal samples to assess the occurrence of SDG-converting bacteria. According to the literature, the human colon harbours approximately 10^{12} CFU per gram of content [5]. Thus, with a mean count of 6×10^8 MPN g^{-1}, bacteria responsible for ED production occurred at relatively high population levels, indicating that SECO-demethylating and -dehydroxylating bacteria are members of dominant intestinal microbiota. Conversely, the bacteria involved in EL production, *i.e.*, the bacteria that catalyse the dehydrogenation of ED or SECO, belong to subdominant anaerobic communities, as indicated by a significantly lower mean count (3×10^5 MPN g^{-1}, $p < 0.001$). The MPN data on ED- and EL-producing bacteria are given in detail in **Table 12** (App. 2). These data show that bacteria responsible for enterolignan production occur at cell densities higher than those reported earlier [67]. Furthermore, from one male subject with dominant enterolignan-producing bacteria (MPN 14), additional faecal samples were collected after six and twelve months. Analysis of the corresponding MPN counts gave coefficients of variation of 11% and 1% for EL and ED, respectively. Thus, the occurrence of enterolignan-producing bacteria was relatively stable over a year in one healthy male adult who had no major dietary changes during that time.

For the German cohort of the MPN study (n = 13), MPN data were analysed to determine the association between the occurrence and the activity of SECO-converting bacteria. Data on enterolignan production are listed in **Table 12** (App. 2).

The production of EL and enterolignans (ED plus EL) accounted for 11.9% ± 9.1 and 59.9% ± 17.7 of the initial amount of SECO, respectively. This is in agreement with the results of Heinonen *et al.* who found that faecal bacteria converted 21% and 50% of SECO to EL and ED, respectively [73]. These results also indicate that the *in vitro* conversion of SECO to enterolignans is incomplete, even if the percentages of recovery obtained with SECO are higher than the percentages reported for *in vitro* conversion of SDG [117]. The authors found that SECO, ED and EL together accounted for 10% to 20% of the initial amount of SDG. Thus, the deglycosylation step of SDG conversion may be critical for the formation of enterolignans. However, these *in vitro* findings may not reflect *in vivo* conditions. The fact that bacteria may produce metabolites which were not detected following our experimental procedure could partly explain the 60%-recovery of the initial amount of SECO. With 48.0% ± 19.8, ED was produced in higher amounts than EL. This is in agreement with previous *in vivo* data on plasma and urine concentrations of lignans in humans after flaxseed ingestion [118] and urine concentrations of

lignans in rats after SECO supplementation [119]. In these studies, ED was the main metabolite produced from the ingested precursors.

We found a significant correlation between the occurrence of EL producers and the amount of EL produced: r (EL_{MPN}, λ) = 0.749 (p = 0.003) (**Table 5**). Significantly higher amounts of EL were produced by faecal samples from individuals with moderate to high cell numbers of EL-producing bacteria than by samples from individuals with a low cell number of EL-producing bacteria.

Table 5: MPN counts and EL production

Individuals with a low cell number of EL-producers				Individuals with moderate to high cell numbers of EL-producers			
Subject[a]		EL_{MPN}[b]	λ[c] (%)	Subject[a]		EL_{MPN}	λ[c] (%)
4	(w)	2.7	0.0	1	(w)	5.5	13.7
8	(w)	3.9	10.7	2	(w)	6.7	21.8
13	(m)	3.1	2.8	3	(w)	6.7	25.3
15	(m)	3.6	4.0	5	(w)	6.5	23.7
16	(m)	0.0	0.0	6	(w)	6.5	17.7
17	(m)	3.0	4.7	7	(w)	6.1	22.6
				14	(m)	8.2	7.1
mean		2.7[d]	3.7[e]	mean		6.6[d]	18.8[e]
SD		1.3	3.6	SD		0.8	6.0
CV (%)		46	98	CV (%)		12	32

[a] The gender of the subjects is given in brackets (m, men; w, women).
[b] Counts of EL-producing bacteria determined by MPN enumerations and expressed as $\log_{10}$ (CFU g^{-1}).
[c] The parameter λ is the ratio between the final amount of EL and the initial amount of SECO.
[d e] Values with the same letters were significantly different (p < 0.001).

On the contrary, there was no association between the MPN counts and the amount of ED produced. Since cell numbers of ED-producers were fairly homogeneous within the MPN cohort, *i.e.*, individuals with low or high MPN counts were not as distinguishable as in the case of EL, enumerations based on three replicates may not warrant the accuracy required to reveal differences within such a set of data. It is also possible that ED-producing bacteria are more diverse than EL-producing bacteria. Thus, with an assortment of organisms that vary in their efficacy of conversion, it would be difficult to directly associate total numbers and activity of ED-producing communities. As an example, the activity measured from a faecal sample with subdominant ED-producing organisms, where species with high capabilities of conversion prevail, and from a sample with dominant ED-producing organisms, where other species with low capabilities of conversion predominate, may not differ greatly. *In vitro* experiments might also be limited to accurately study such diverse communities. For instance, the *in vitro*

conditions used might have favoured the growth of certain specific SECO-converting species in some faecal samples, resulting in an overestimation of the corresponding MPN counts.

Since the intestinal microbiota is host specific [15], we wondered whether the occurrence of enterolignan-producing bacteria was dependent on host parameters. We observed marked inter-individual differences with regard to both MPN data and enterolignan production. This agrees with previous work on lignan metabolism and diversity of intestinal bacteria [119-121]. Despite these variations, women had higher concentrations of enterolignan-producing organisms than men. The differences were significant for EL-producing organisms with mean counts of 2×10^6 and 5×10^4 MPN g^{-1} for women and men, respectively (**Figure 4**).

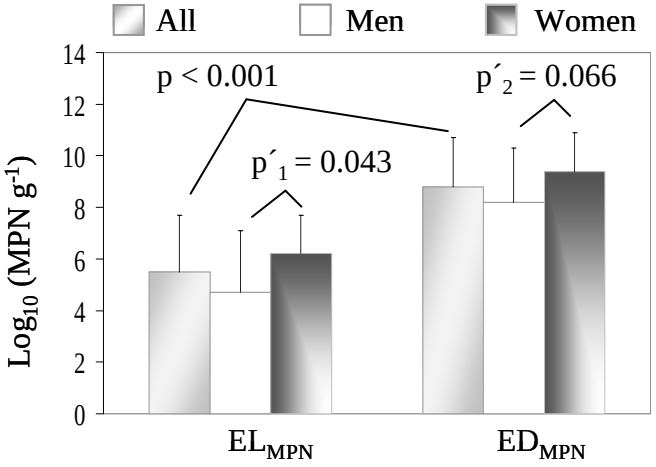


Figure 4: MPN counts of enterolignan-producing organisms. Histograms and bars represent means and standard deviations for the given population: All (n = 24), men (n = 12), women (n = 12). P'_1 and p'_2 were obtained by one-tailed Student tests. ED_{MPN} and EL_{MPN}, concentration of ED- and EL-producing bacteria.

A study on EL concentrations in serum and urine after a rye-bread diet did not show significant differences between women and men [122]. Nonetheless, over four weeks, women had higher serum EL concentrations (39.3 nmol l^{-1} ± 4.4, n = 21) than men (28.1 nmol l^{-1} ± 3.8, n = 18), in spite of significantly higher intake of test bread and fibres by the men. Other studies also reported higher serum concentrations of EL in women than in men [76, 77, 115, 123]. Thus, although it is difficult to evaluate the effect of high counts of SECO-converting organisms on the bioavailability of enterolignans, our results agree with the literature and hint at a connection between gender and lignan metabolism. Early studies showed that enterolignan excretion depends on pregnancy and on the menstrual cycle [47, 48, 86], even though recently Lampe *et al.* found no association between urinary levels of lignans and menstrual cycle phases [124]. It is conceivable that the composition of the intestinal microbiota and the metabolic activities of its members, such as enterolignan-producing bacteria, depend on the host hormonal status, as

proposed previously for sub-gingival periodontal bacteria [125]. Reciprocally, flaxseed consumption may alter the menstrual cycle and the level of sex hormones [126-131].

3.1.2. Bacterial indicators of enterolignan production

The following results represent the first step in the identification of bacteria involved in enterolignan production. In order to determine whether the MPN counts were related to the composition of dominant intestinal microbiota, *in situ* hybridisation experiments were carried out. The hypothesis was that the microbiota of individuals with high numbers of enterolignan producers may be characterised by high bacterial proportions of specific phylogenetic groups.

As Wang *et al.* isolated previously the SECO-demethylating strain *Peptostreptococcus* sp. SDG-1, which they assigned to the species *P. productus* on the basis of phenotypic analyses [68], we first aimed to design and optimise a new 16S rRNA-targeted oligonucleotide probe for the specific detection of this species. However, an alignment of 16S rRNA gene sequences from 35 organisms phylogenetically closely related to *P. productus* did not allow the identification of species-specific regions. Instead, we found a region specific for both *P. productus* and *C. coccoides* at position 1,264 according to *Escherichia coli* numbering. Homologous regions revealed by Probe Match and BLAST analyses confirmed the *in silico* specificity of the corresponding probe, which was named S-*-ProCo-1264-a-A-23 [108]. **Table 6** shows the sequence of the probe and of targeted and non-targeted organisms.

Table 6: Specificity of the newly designed 16S rRNA probe ProCo-1264

	Sequence
Probe S-*-ProCo-1264-a-A-23	3′GTCGCTACAACTCGCTTAGGGTT
Target	5′CAGCGATGTTGAGCGAATCCCAA
Ruminococcus callidus ATCC 27760	5′.........G....A........
Clostridium clostridiiforme ATCC 25537	5′...T.....G....A........
Ruminococcus flavefaciens ATCC 49949	5′...T.....G.........T...
Peptostreptococcus ivorii DSM 10023	5′..........A...........T
Eubacterium fissicatena DSM 3598	5′.C.......G.........T...
Eubacterium angustum ATCC 43737	5′G.........C.G..........

Species with up to two mismatches and phylogenetic relatives that are members of the human intestinal microbiota were subsequently tested using FISH to determine the specificity of the probe *in situ* (**Table 7**).

Table 7: *In situ* features of ProCo-1264

Strain	RPF[a] (% Eub-0338)	HP[b]
P. productus DSM 2950	79.9 ± 2.2	92.0 ± 0.3
P. productus DSM 3507	81.3 ± 0.7	91.9 ± 0.8
P. productus ATCC 27340	110.9 ± 2.5	90.4 ± 0.3
C. coccoides DSM 935	65.0 ± 2.9	74.3 ± 6.6
C. coccoides strain 3110 [132]	16.2 ± 1.6	0
R. callidus ATCC 27760	13.2 ± 1.3	0
R. hansenii DSM 20583	03.7 ± 0.0	0
R. obeum DSM 29174	02.7 ± 0.2	0

[a] The relative probe fluorescence (RPF) reflects the quality of the fluorescent signal and is expressed as the percentage of the Eub-0338-Cy5 reference signal intensity.
[b] The hybridisation percentage (HP) reflects the specificity of the oligonucleotide sequence and is expressed as the percentage of Eub-0338-FITC total bacterial events.

With a mean RPF of 84.3% ± 16.6 (n = 4), strains targeted by ProCo-1264 provided good fluorescent signals. The fact that the probe does not only target the SECO-demethylating species *P. productus* limits the extrapolation of the results. Nonetheless, the only probe described previously in the literature that could be used for detection of *P. productus* was Erec-482. According to the RDP database, this probe targets approximately 5,600 bacterial 16S rRNA gene sequences and was therefore much less suitable than ProCo1264.

The newly designed probe and five additional group-specific probes were used in FISH experiments on fixed bacterial suspensions prepared with the 24 faecal samples of the MPN study. Bacterial proportions are listed in **Table 12** (App. 2). On average, the proportions of the *Atopobium* group, *Bacteroides* and relatives, *Bifidobacterium* spp., *Clostridium leptum* subgroup and *E. rectale-C. coccoides* cluster were not statistically different from data obtained in our laboratory for 91 adults from five European countries ($p > 0.10$) [133]. There were also no significant differences in bacterial composition between women and men. Both genders showed large inter-individual differences. **Figure 5** illustrates dot plots obtained by flow cytometry after hybridisation with the probe ProCo-1264.

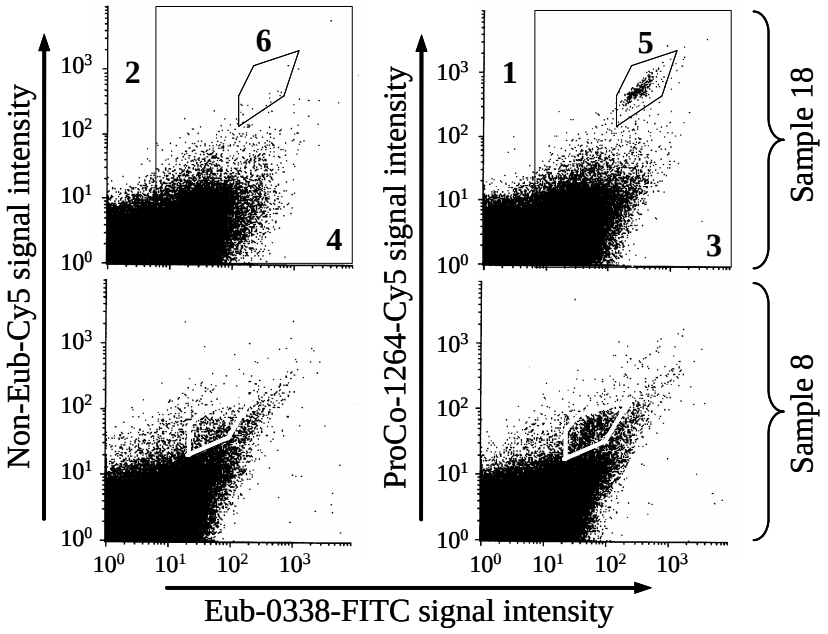


Figure 5: Dot plot analysis for MPN samples 8 and 18 hybridised with ProCo-1264. Fluorescence intensities are displayed as logarithmic values. Regions: 1 and 2, auto-fluorescent events; 3 and 4, gated regions that include total bacterial events; 5, cells of *P. productus* and relatives, which fluoresce in both green (Eub-FITC) and red (ProCo-Cy5); 6, specific control region. Bacterial percentages were calculated as follows: 100 × [(counts in region 5/counts in region 3) – (counts in region 6/counts in region 4)]. Samples were analysed in duplicate.

Dot plot analyses gave a mean of 0.5% ± 0.5 for *P. productus* and relatives (n = 20). Percentages ranged from 0 to 2.1% of total bacteria and from 0 to 20% (2.7% ± 4.4) of members of the *E. rectale-C. coccoides* cluster. The prevalence was 75%, *i.e.*, specific fluorescent signals were detected for 15 of the 20 subjects. This indicates that SECO-converting organisms occur within dominant faecal microbiota and agrees with previous data on PCR detection of *P. productus* in human faeces [134].

We found positive correlations between concentrations of EL-producing organisms and bacterial proportions. When compared with subjects with a low count of EL-producers ($\log_{10}$ (MPN g^{-1}) = 2.9 ± 1.3, n = 7), subjects with moderate to high counts of EL-producers ($\log_{10}$ (MPN g^{-1}) = 7.1 ± 0.7, n = 13) had higher proportions of *P. productus* and relatives (0.8% ± 0.5 vs. 0.1% ± 0.2, p = 0.012) and the *Atopobium* group (6.1% ± 1.4 vs. 2.4% ± 2.5, p = 0.035). Coefficients of correlation were: r (EL, ProCo-1264) = 0.449, r (EL, Ato) = 0.542 ($p < 0.05$). With a detection limit of approximately 1% of total bacteria, the *in situ* hybridisation technique used provides data on the dominant part of faecal microbiota. Taking into account a total number of approximately 10^{12} CFU per gram faeces, the significant differences in bacterial proportions given above stand for variations of 7×10^9 and 4×10^{10} cells g^{-1}, for *P. productus*

and relatives and the *Atopobium* group, respectively. The fact that dominant bacteria play a major role in metabolic activities in the intestine when compared with less abundant bacteria supports the relevance of our results in terms of ecosystem functions. Nevertheless, the correlations pointed out here warrant confirmation by the identification of enterolignan-producing strains that indeed belong to the two bacterial groups of interest. Moreover, the reason why similar correlations could not be observed for ED was probably due to the relatively high diversity of ED-producing communities. The identification of bacteria involved in enterolignan production was also decisive for the investigation of such complex communities.

3.2. Identification and characterisation of SDG-converting bacteria

The identification of SDG-converting bacteria was achieved by the isolation of human faecal bacteria and by the screening of phylogenetically or functionally related strains obtained from bacterial culture collections. In parallel, pure culture work with SDG-converting strains allowed the determination of some of their genetic and phenotypic characteristics. We focused on each of the four reactions required for the production of EL from SDG.

3.2.1. *O*-deglycosylation

To investigate the stability of SDG during its transit through the upper part of the gastrointestinal tract, experiments with synthetic stomach or intestinal juice were carried out. After 180 min at 37°C, the recovery of SDG was 100.9% ± 3.0 or 100.5% ± 2.9 in stomach or intestinal juice, respectively. Thus, it is conceivable that substantial amounts of ingested SDG reach distal parts of the intestine where diverse anaerobic bacteria catalyse its conversion. However, more work is required to assess the possible deglycosylation of SDG by host enzymes, as shown previously for flavonoids [135, 136].

Four SDG-deglycosylating strains were isolated from human faeces. The 16S rRNA gene sequence of one Gram-negative rod-shaped isolate (1,355 nucleic acids) showed 99.7% similarity with *Bacteroides ovatus* DSM 1896^T (GenBank accession number X83952) [137]. The organism was named *B. ovatus* SDG-Mt85-3Cy (DQ100446). The 16S rRNA gene sequences of two other Gram-negative rod-shaped isolates (1,426 nucleic acids) showed 98.5% similarity with *B. fragilis* DSM 2151^T (X83935) [137]. The organisms were named *B. fragilis* SDG-Mt85-4C (DQ100447) and SDG-Mt85-5B (DQ100448). Finally, the 16S rRNA gene sequence of a Gram-positive helically coiled rod (1,416 nucleic acids) showed 99.4% similarity with *Clostridium* sp.

14774 (AJ315981), an unpublished human faecal isolate, 96.7% similarity with *C. cocleatum* DSM 1551^T (Y18188) [138], 96.6% similarity with *C. ramosum* DSM 1402^T (X73440) and 95.9% similarity with *C. spiroforme* DSM 1552^T (X73441). Thus, this last isolate is most probably a new species. Indeed, it is unlikely that two organisms with a sequence similarity value below 97.5% are related at the species level [139]. Consequently, the organism was named *Clostridium* sp. SDG-Mt85-3Db (DQ100445). It is available from bacterial culture collections under the numbers DSM 17460^T and CCUG 51486^T. **Figure 6** shows the conversion of SDG to SECO by *Clostridium* sp. SDG-Mt85-3Db. Four additional strains from culture collections *O*-deglycosylated SDG: *B. distasonis* DSM 20701^T, *B. fragilis* DIfE-05, *C. cocleatum* DSM 1551^T and *C. ramosum* DSM 1552^T.

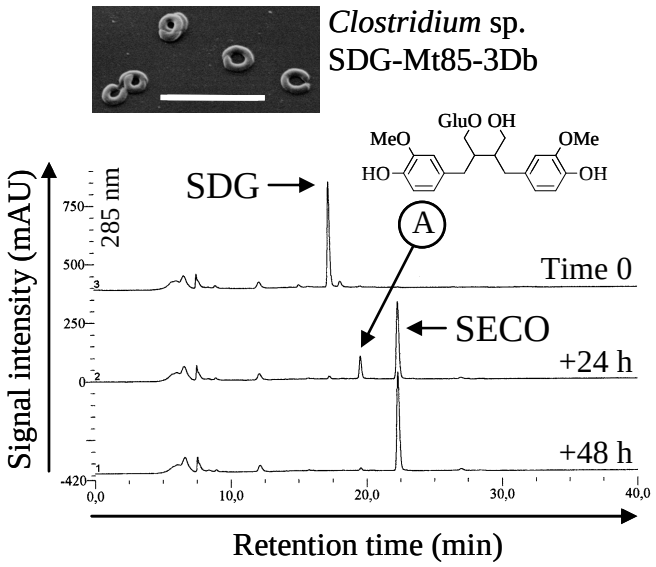


Figure 6: *O*-deglycosylation of SDG by the newly isolated strain *Clostridium* sp. SDG-Mt85-3Db grown in Mt-3-broth. Samples were collected at the times indicated on the chromatograms and supernatants were analyzed by HPLC. The *O*-deglycosylation of SDG led to the formation of SECO, via compound A (19.6 min). The molecular mass of A was 524 g mol^{-1}, as determined by MS. It corresponds to SDG with one glucose molecule removed. The bar in the photograph represents 5 µm.

To compare the ability of a variety of strains to *O*-deglycosylate SDG, experiments with resting cells were performed. Differences in conversion efficiency were observed and *Clostridium* sp. SDG-Mt85-3Db converted SDG at a much higher initial rate than any of the other strains tested (**Figure 7**). Over the first 30 minutes at 37°C, the amount of SDG converted to SECO by resting cells of *Clostridium* sp. SDG-Mt85-3Db was approximately 5-fold and 30-fold higher than the amount converted by *B. fragilis* DIfE-05 and *B. ovatus* SDG-Mt85-3Cy, respectively. With the exception of *B. distasonis,* all species completely converted SDG to its aglycone SECO within less than 20 h.

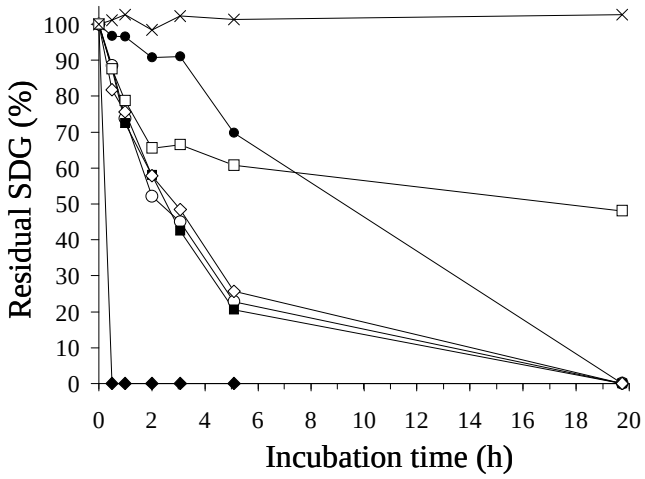


Figure 7: *O*-deglycosylation of SDG by resting cells. Bacterial concentrations were (log_{10} (cell ml^{-1}), n = 2): *Clostridium* sp. SDG-Mt85-3Db (9.86 ± 0.04) (♦), *B. fragilis* SDG-Mt85-5B (10.61 ± 0.09) (■), *B. fragilis* SDG-Mt85-4C (10.63 ± 0.06) (○), *B. fragilis* DIfE-05 (10.74 ± 0.00) (◊), *B. distasonis* DSM 20701^T (10.03 ± 0.00) (□), *B. ovatus* SDG-Mt85-3C (10.17 ± 0.00) (•). The control was SDG in PBS (×). The initial concentration of SDG was approximately 625 µM. Cells were incubated at 37°C and samples were collected over time as indicated. The strains and the control were analysed in duplicate and the data are presented as mean values. The mean coefficient of variation was 5.2% (1.6 to 23.1%).

Five out of eight SDG-deglycosylating strains belong to *Bacteroides* species, which have been extensively studied for their ability to metabolise sugars, indigestible polysaccharides and glycosylated compounds [17, 140, 141]. Since the genus *Bacteroides* is one of the most prevailing and prevalent bacterial genera in the human intestinal tract, it is reasonable to assume that the identified *Bacteroides* species play a major role in SDG deglycosylation [15]. To confirm the dominance of SGD-deglycosylating *Bacteroides* species in the context of our work, *B. distasonis* and *B. fragilis* were enumerated by *in situ* hybridisation in the faecal samples of the MPN study. The mean bacterial proportions were 0.5% ± 0.9 and 3.3% ± 3.4 of total bacteria, respectively (**Table 12**, App. 2). *B. distasonis* and *B. fragilis* were detected in 10 and 13 of the 20 samples, respectively. There were no significant gender differences. These bacterial proportions and prevalences agree with previous enumerations, with the exception of a lower prevalence of *B. fragilis* in our study (65% vs. 90%) [107]. In addition, a new 16S rRNA targeted oligonucleotide probe was designed and optimised for the specific detection of *Clostridium* sp. SDG-Mt85-3Db (**Table 8**).

Table 8: Features of the newly designed 16S rRNA oligonucleotide probe Csac-0067

Probe, targeted and non-targeted species (Genbank accession number)	Sequences		RPF[a]	HP[b]
S-S-Csac-0067-a-A-20	3′GCGCCCGTCATTACAGGCTC	(probe)		
Clostridium sp. SDG-Mt85-3Db (DQ100445)	5′CGCGGGCAGTAATGTCCGAG	(target)	87.7 ± 8.7	89.5 ± 5.9
Clostridium sp. 14774 (AJ315981)	.............T......		ND	ND
Uncultured bacterium (AB080878)	.........C....C.....		ND	ND
Uncultured bacterium (AF477890)	.-........C..-.......		ND	ND
C. cocleatum DSM 1551^T (Y18188)	........C.T.G..C....		02.0 ± 0.2	00.0 ± 0.0
C. ramosum DSM 1402^T (X73440)	....A....C.....CT..		02.1 ± 0.0	00.0 ± 0.1
C. spiroforme DSM 1552^T (X73441)	....A...C.T.G..CT..		01.1 ± 1.1	03.2 ± 0.4

[a] Relative probe fluorescence (percentage of the Eub-0338-Cy5 reference signal intensity). ND, not determined.
[b] Hybridisation percentage (percentage of Eub-0338-FITC total bacterial events).

As revealed by *in situ* hybridisation using the newly designed probe Csac-0067, *Clostridium* sp. SDG-Mt85-3Db was detectable in only two faecal samples out of 20. The corresponding bacterial proportions were 0.4% ± 0.1 and 0.2% ± 0.0 of total bacteria (**Figure 8**). Thus, for the two individuals from whom the faeces were obtained, *Clostridium* sp. SDG-Mt85-3Db might play an important role in SDG deglycosylation. However, this species appears to be a subdominant member of the intestinal microbiota for most individuals.

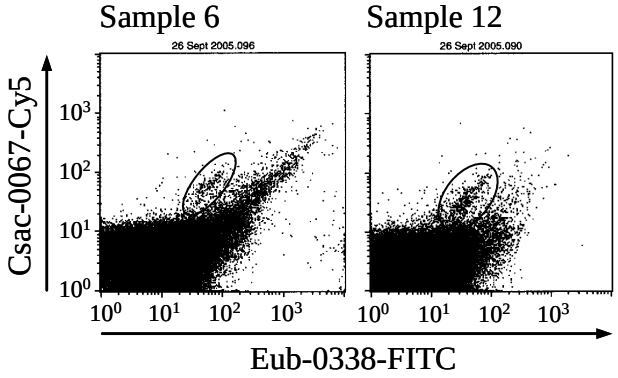


Figure 8: Dot plot analysis for MPN samples 6 and 12 hybridised with Csac-0067. The encircled events, which fluoresce in both green (Eub-FITC) and red (Csac-Cy5), are cells of *Clostridium* sp. SDG-Mt85-3Db. Bacterial percentages were obtained after subtraction of the background fluorescence as detailed previously in Figure 5.

It is difficult to draw conclusions on the role that each of the SDG-deglycosylating species plays in the intestinal tract. Our *in vitro* and *in situ* data warrant confirmation under *in vivo* conditions. Moreover, strains of *B. distasonis*, *B. fragilis* and *B. ovatus* other than the ones studied here may not *O*-deglycosylate SDG. Nevertheless the above results on the overall dominance and conversion efficacy of SDG-deglycosylating bacteria imply that the conversion of SDG to SECO is not a limiting step in the *in vivo* production of enterolignans.

3.2.2. *O*-demethylation

A Gram-positive cocco-bacillus obtained after isolation of faecal bacteria on SECO-containing media showed the ability to *O*-demethylate SECO (**Figure 9**). Its 16S rRNA gene sequence (1,343 nucleic acids) displayed 97.4% similarity with *P. productus* ATCC 27340^T (L76595). The organism was named *P. productus* SECO-Mt75m3 (AY937379).

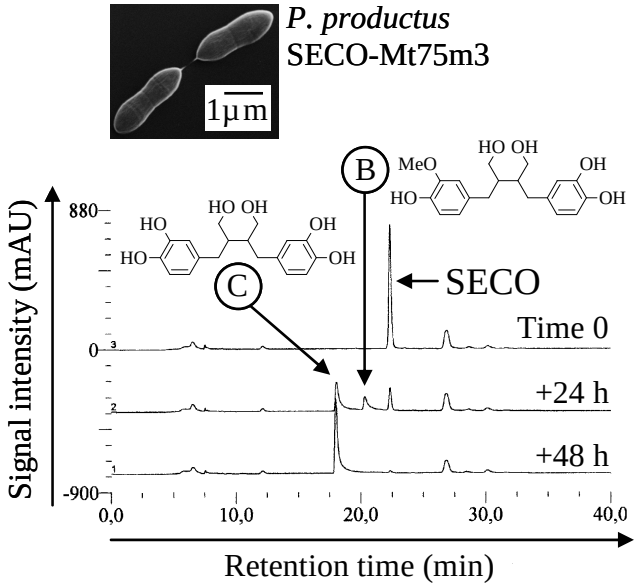


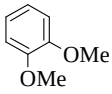
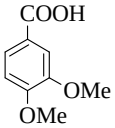
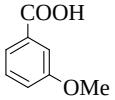
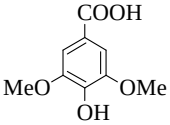
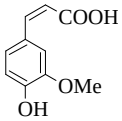
Figure 9: *O*-demethylation of SECO by *P. productus* SECO-Mt75m3 grown in Mt-6-broth. The *O*-demethylation of SECO led to the formation of compound C (18.0 min), via compound B (20.2 min). The molecular mass of B was 348 g mol^{-1}, as determined by MS. It corresponds to SECO with one methoxy group removed. Compound C was identified as 2,3-bis(3,4-dihydroxybenzyl)butene-1,4-diol by comparison with the retention time and spectrum of the standard. It lacks the two methoxy groups of SECO.

Five additional strains from culture collections *O*-demethylated SECO: *B. methylotrophicum* DSM 3468^T, *E. callanderi* DSM 3662^T, *E. limosum* DSM 20543^T, *P. productus* DSM 2950^T and *P. productus* DSM 3507.

The identification of three SECO-demethylating strains of the species *P. productus* supports the correlation between bacterial proportions obtained with the probe ProCo-1264 (FISH data) and the occurrence of EL-producing bacteria (MPN counts) (section 3.1.2.). Wang *et al.* previously isolated the SECO-demethylating strain *Peptostreptococcus* sp. SDG-1 [68]. However, the identification of the strain was only based on biochemical characteristics and comparison of fermentation reactions. The molecular work reported here provides new information on the phylogeny of a SECO-demethylating bacterium. The corresponding 16S rRNA gene sequence is available to the scientific community on the NCBI website (*http://www.ncbi.nlm.nih.gov*). More importantly, a major advantage is the possibility to use the *P. productus* isolate and the other identified SECO-demethylating species as models to study the conversion of lignans in more detail.

All the identified SECO-demethylating strains belong to the functional group of acetogenic bacteria, which produce acetate at the expense of H_2 plus CO_2 [142, 143]. Two non-methane-excreting individuals were previously shown to harbour 7.2×10^7 and 3.1×10^8 acetogens per gram wet faeces [144]. This is consistent with earlier enumerations of *E. limosum* and *P. productus* in faeces and implies that acetogens are dominant within intestinal microbiota harbouring no methanogenic archea [4, 5, 134]. Some acetogens also utilise methyl groups from various compounds for acetate formation [145]. For instance, *E. limosum O*-demethylates the isoflavones biochanin A, formononetin and glycitein, and thereby influences the bioavailability of these phytoestrogens [146]. To assess the range of substrates that are *O*-demethylated by SECO-demethylating bacteria, *B. methylotrophicum*, *E. callanderi*, *E. limosum* and *P. productus* were grown in the presence of five different methylated aromatic compounds. The results revealed their ability to *O*-demethylate all the compounds tested (**Table 9**). This indicates that the involved enzymes have a relatively low specificity, or that one organism harbours several demethylating enzymes. This is in agreement with earlier studies on bacterial *O*-demethylation [147, 148].

Table 9: Conversion of aromatic methylated compounds (1 mM) by SDG demethylating bacteria[a]

Substrate (Solvent)	Structure	*P. productus*	*E. limosum*	*E. callanderi*	*B. methylotrophicum*
Veratrol (DMSO)		+	+	+	+
Veratric acid (MeOH)		+	+	+	+
m-Anisic acid (DMSO)		-	+	+	+
Syringic acid (MeOH)		+	+	+	+
Ferulic acid (DMSO)		+ +	+	+	+

[a] Incubation took place at 37°C for 24 h and samples were analysed by HPLC. Strains were: *P. productus* SECO-Mt75m3, *E. limosum* DSM 20543^T, *E. callanderi* DSM 3662^T and *B. methylotrophicum* DSM 3468^T. Symbols are: -, no conversion; +, *O*-demethylation (compounds with several methoxy groups were completely *O*-demethylated); + +, further conversion.

The functional group in meta-position of the targeted methoxy groups does not appear to affect *O*-demethylation. However, methoxy or hydroxyl groups adjacent to the targeted methyl groups may be required by *P. productus* SECO-Mt75m3 to catalyse *O*-demethylation, as evident from the inability of this organism to *O*-demethylate *m*-anisic acid. Incubation of *P. productus* SECO-Mt75m3 with ferulic acid led to complete conversion of the substrate without detection of any products by HPLC, suggesting that *P. productus* not only catalyses the *O*-demethylation of ferulic acid but also a number of additional reactions.

More interestingly, *P. productus* SECO-Mt75m3 catalysed the *O*-demethylation of the lignans lariciresinol (**Figure 10**), matairesinol and pinoresinol (data not shown). However, the organism was not able to *O*-demethylate SDG (data not shown).

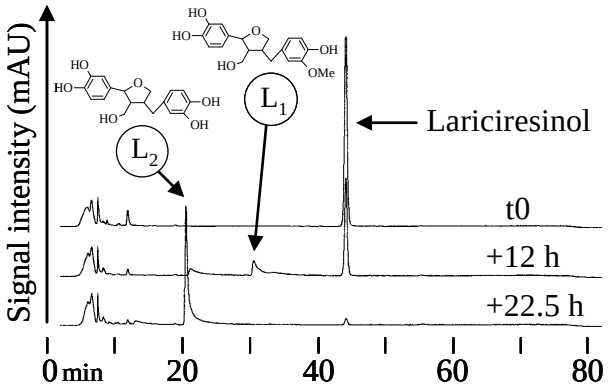


Figure 10: *O*-demethylation of lariciresinol by *P. productus* SECO-Mt75m3 grown in Mt6-broth. The *O*-demethylation of lariciresinol led to the formation of compound L_2 (20.5 min), via compound L_1 (30.6 min). The molecular mass of L_1 (346 g mol^{-1}) was determined by MS. It corresponds to lariciresinol with one methoxy group removed. The molecular mass of L_2 (332 g mol^{-1}) corresponds to lariciresinol lacking its two methoxy groups.

3.2.3. Dehydroxylation

Two strains from culture collections dehydroxylated SECO when co-incubated with *P. productus* SECO-Mt75m3: *C. scindens* DSM 5676^T and *E. lenta* DSM 2243^T. **Figure 11** shows the dehydroxylation of SECO by *E. lenta* DSM 2243^T. In addition, co-incubation of *P. productus* SECO-Mt75m3 with a Gram-positive rod-shaped faecal isolate led to the production of ED. The 16S rRNA gene sequence of the latter organism (1,394 nucleic acids) displayed 98.2% similarity with *E. lenta* DSM 2243^T (AF292375) [149]. The organism was named *E. lenta* SECO-Mt75m2 (AY937380). Its identity was confirmed by PCR amplification of an *E. lentum*-specific region using primers LEN-F2 and LEN-R2 (data not shown) [102]. The isolate incubated alone in Mt-6 did not show any activity toward SECO (data not shown). However, it catalysed the reduction of pinoresinol to SECO via lariciresinol (**Figure 12**).

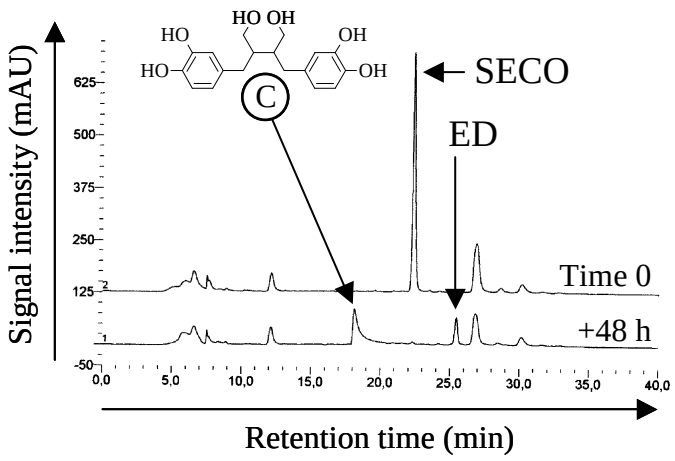


Figure 11: Formation of ED from SECO by a co-culture of *P. productus* SECO-Mt75m3 and *E. lenta* DSM 2243^T grown in Mt-75-broth.

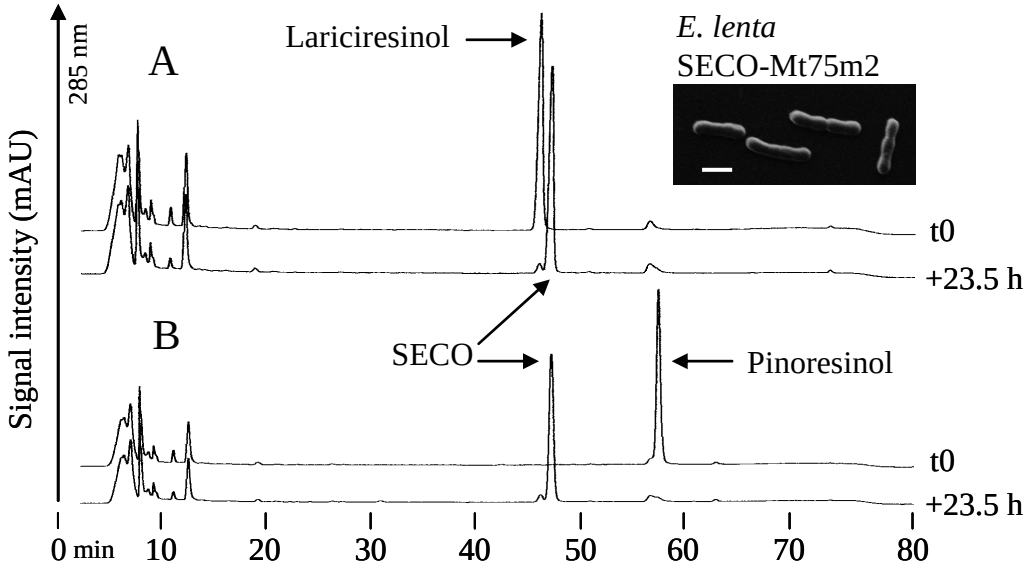


Figure 12: Reduction of (+)-lariciresinol (A) and (+)-pinoresinol (B) to SECO by *E. lenta* SECO-Mt75m2 grown in Mt6-broth. The bar in the photograph represents 1 µm.

Thus, as in the case of *P. productus* for *O*-demethylation, several strains of *E. lenta* catalyse the dehydroxylation step of SDG conversion. Moreover, *E. lenta* SECO-Mt75m2 does not only dehydroxylate SDG, but also reduces the lignans pinoresinol and lariciresinol, which occur in substantial concentration in our diet [23, 150]. Furthermore, data from the literature reported a mean cell density of approximately 2×10^9 CFU g^{-1} dried faeces for *E. lenta*, the prevalence of which ranges between 33 and 100% in humans depending on the technique used (**Table 12**, App. 2) [4, 151, 152]. These concerns may be of importance regarding the contribution of *E. lenta* to the *in vivo* production of enterolignans from various dietary precursors. Given that *E. lenta* is targeted by the 16S rRNA probe specific for the enumeration of the *Atopobium* group, the

identification of SECO-dehydroxylating strains of *E. lenta* support the correlation observed between cell counts of EL producers (MPN data) and proportions of dominant bacteria that belong to the *Atopobium* group (FISH data) (section 3.1.2.). Concerning the species *C. scindens*, previous molecular work reported its high prevalence in humans and a mean cell density of approximately 3×10^5 CFU g^{-1} wet faeces [153, 154].

In view of the higher MPN counts of EL- and ED-producing bacteria in women than men (section 3.1.2.), it is worthwhile to discuss the role that the SECO-dehydroxylating species *C. scindens* and *E. lenta* play in the dehydroxylation of endogenous compounds that undergo entero-hepatic circulation. *E. lenta* has been extensively studied for its involvement in 21-dehydroxylation of biliary steroids [155]. For instance, 21 of 37 strains of *E. lenta* showed the ability to dehydroxylate deoxycorticosterone to progesterone [156]. Concerning *C. scindens*, recent studies have shed light on its ability to 7α-dehydroxylate primary biliary acids to produce deoxycholic and lithocholic acid, which have been associated with an increased risk for cholesterol gallstones and colon cancer [157-160]. However, Kitahara *et al.* recently found no association between the occurrence of *C. scindens* and the concentration of secondary biliary acids in human faeces [154]. Besides its ability to convert biliary acids, *C. scindens* synthesises desmolase and 20α-hydroxysteroid dehydrogenase involved in the metabolism of steroid hormones [161]. Since the level of endogenous hormones fundamentally differ between women and men, it is conceivable that the metabolic potential of intestinal microbiota, more specifically in the context of this work, the ability of *C. scindens* and *E. lenta* to dehydroxylate SECO, is influenced by the gender of the host. The role of progesterone might be of particular interest. Bacterial dehydroxylases are involved in the metabolism of progesterone and their activity possibly depends on progesterone levels. Progesterone also relaxes smooth muscle tone and may be related to a longer intestinal transit time [162, 163]. Shoda *et al.* reported a positive correlation between the intestinal transit and the bacterial production of secondary biliary acids [164]. Previous data also showed that some bacteria, including *C. scindens* and strains of the genus *Eubacterium*, have several bile acid-inducible (*bai*) genes, which encode enzymes in the bile acid 7α-dehydroxylation pathway [159, 165]. Thus, progesterone levels and transit time may influence the conversion of dietary lignans by increasing their availability to bacteria or by inducing bacterial activities, directly or indirectly. However, in order to draw any conclusion, it is imperative to characterise the enzymes involved in the dehydroxylation of SECO and to study the impact of hormonal factors on the bacterial metabolism of lignans.

3.2.4. Dehydrogenation

One Gram-positive rod-shaped strain capable of dehydrogenating ED was isolated from human faeces (**Figure 13**). The organism was not able to dehydrogenate SECO (data not shown). Its 16S rRNA gene sequence (1,437 nucleic acids) showed 93.6% similarity with C. *amygdalinum* DSM 12857^T (AY353957) [166] and 93.4% similarity with *C. saccharolyticum* DSM 2544^T (Y18185) [138]. Thus, this isolate is probably a new bacterial genus and is referred to as strain ED-Mt61/PYG-s6 (DQ100449). The organism is available under the collection numbers DSM 17460^T and CCUG 51486^T.

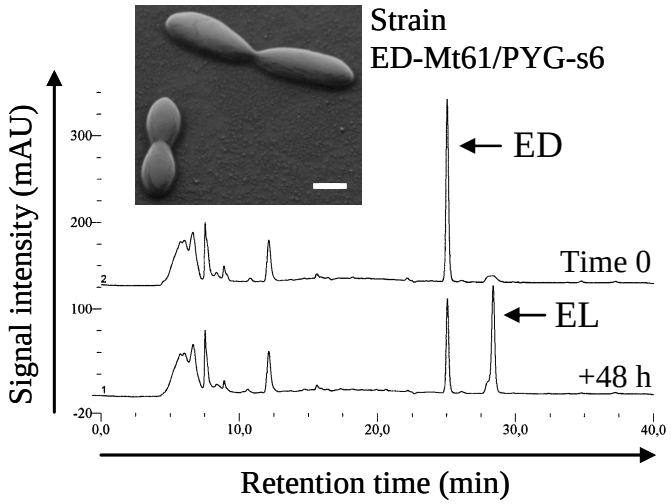


Figure 13: Dehydrogenation of ED by the newly isolated strain ED-Mt61/PYG-s6 grown in Mt-6-broth. The bar in the photograph represents 1 µm.

In the concentration range of 100 to 700 µM ED, we observed a positive correlation between the initial concentration of ED and the final concentration of EL produced by strain ED-Mt61/PYG-s6. The coefficient of correlation was r (EL_{final}, $ED_{initial}$) = 0.999 ($p < 0.001$, $n = 6$). The final concentration of EL was always half of the initial concentration of ED. Moreover, we did not observe the formation of ED when strain ED-Mt61/PYG-s6 was grown in the presence of EL (data not shown). Since the ED used was racemic, we assume that the dehydrogenation of ED to EL by strain ED-Mt61/PYG-s6 is enantiospecific. It is of interest to note that Xia *et al.* purified a dehydrogenase from *Forsythia intermedia* plants, which catalysed the enantiospecific conversion of (-)-SECO to (-)-matairesinol [167]. The assumption of an enantiospecific bacterial conversion of ED to EL warrants confirmation by using pure enantiomers of ED as substrates. In a previous *in vitro* study, faecal bacteria converted (+)-pinoresinol via (+)-lariciresinol and (-)-SECO to (-)-EL [70]. The authors pointed out the retention of stereochemistry at C-8 and C-8' in lariciresinol (or at C-2 and C-3 in SECO) during the cleavage of the furan ring of lariciresinol to produce SECO. *In vivo*, (-)-EL was detected in rat urine after administration of (-)-SECO or (-)-

matairesinol, whereas (+)-SDG led to the formation of (+)-EL [119]. The authors also proposed that the microbial metabolism would not alter the absolute configuration of dietary lignans. In another study, the two enantiomers of SDG were shown to occur in flaxseed, although the proportion of the (+)-enantiomer exceeded 90% [168]. Our results point out the importance to investigate the stereochemistry of the bacterial dehydrogenation of ED in order to study conversion yields of dietary lignans in the intestine, and consequently to assess the bioavailability and the possible health effects of their bacterial metabolites.

Concerning the occurrence of strain ED-Mt61/PYG-s6, the MPN enumerations of approximately 3×10^5 MPN g^{-1} for EL-producing faecal bacteria (section 3.1.1.) suggest that the isolate belongs to subdominant intestinal communities. Considering that EL production is detected in most individuals, either the prevalence of strain ED-Mt61/PYG-s6 is high in humans or, more likely, organisms other than strain ED-Mt61/PYG-s6 catalyse the dehydrogenation of ED.

3.3. Overview of the identified SDG-converting bacteria

Figure 14 shows the phylogenetic relationships of SDG-converting bacteria. Sixteen anaerobic bacterial strains, which are known to be present in the human intestinal tract, catalysed the *O*-deglycosylation, *O*-demethylation, dehydroxylation or dehydrogenation of SDG. Two other SECO-demethylating organisms, *B. methylotrophicum* and *E. callanderi*, are not yet known as members of the intestinal microbiota. Thus, the production of EL requires the interaction of phylogenetically and functionally distantly related species, most of which are members of the dominant intestinal microbiota (**Table 12**, App. 2). Variations in their proportion within the intestinal microbiota of different human subjects may explain the large inter-individual differences observed with regard to the occurrence of SECO-converting bacteria and enterolignan production.

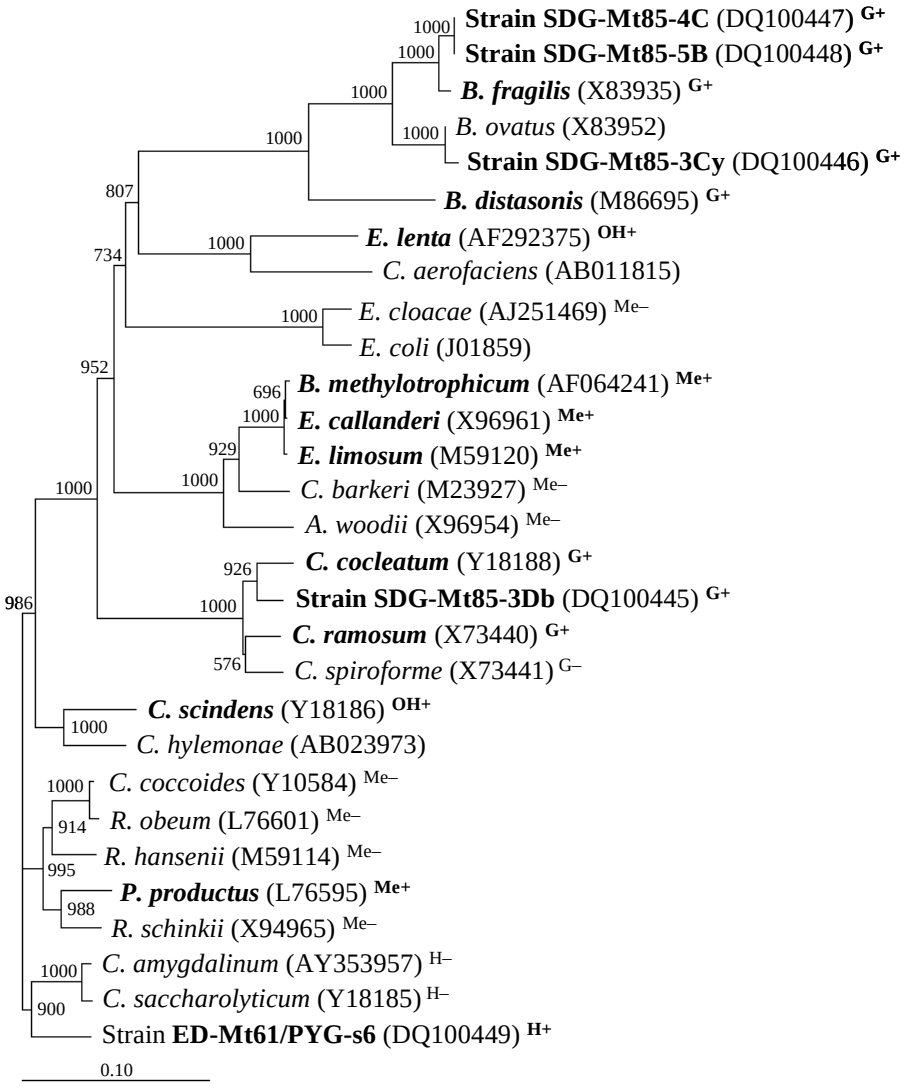


Figure 14: Phylogenetic tree of SDG-converting bacteria. Strains were tested for their ability to catalyse one of the four reactions underlying SDG conversion, as indicated by superscript letters as follows: ^{G}, *O*-deglycosylation; ^{Me}, *O*-demethylation; ^{OH}, dehydroxylation; ^{H}, dehydrogenation. Bacteria capable of converting the corresponding substrate appear in bold letters with an additional superscript "+". The non-converting strains tested are marked with a superscript "–". The GenBank accession numbers of the sequences used to construct the tree are indicated in brackets. Sequences were aligned using the Vector NTI Suite 9.0.0 and the tree was constructed with Clustal X 1.8 using bootstrap values calculated from 1,000 trees.

With eleven species falling into six different genera, intestinal bacteria involved in SDG conversion appear to be quite diverse. Three strains of *P. productus* and two strains of *E. lenta* were shown to *O*-demethylate and dehydroxylate SECO, respectively. Moreover, the closely related species *C. cocleatum*, *C. ramosum* and *Clostridium* sp. SDG-Mt85-3Db *O*-deglycosylated SDG. These points may explain the high prevalence of enterolignan production in humans, since different individuals may harbour different SDG-converting strains. In contrast, a number of functional or phylogenetic relatives of *E. limosum* and *P. productus* did not *O*-demethylate SECO.

Our experiments suggest that the bacterial pathways of SDG conversion start with its *O*-deglycosylation followed by the *O*-demethylation and dehydroxylation of SECO, as proposed earlier [68]. We observed neither the *O*-demethylation of SDG nor the dehydroxylation and dehydrogenation of SECO. However, bacteria other than the ones studied here may catalyse these reactions. The incubation of SDG with four of the identified organisms, one for each of the four reactions required for SDG conversion, led to the production of EL (**Figure 15**). The figure also illustrates the possible identification of 2,3-bis(3,4-dihydroxybenzyl)butyro-lactone, a new intermediate in the formation of EL from SDG (compound D). This intermediate was also produced after co-incubation of the demethylating strain *P. productus* SECO-Mt75m3 and the dehydrogenating strain ED-Mt61/PYG-s6 in SECO-containing broth (data not shown). Thus, the latter organism may not only catalyse the dehydrogenation of ED, but also the dehydrogenation of SECO lacking its two methoxy groups.

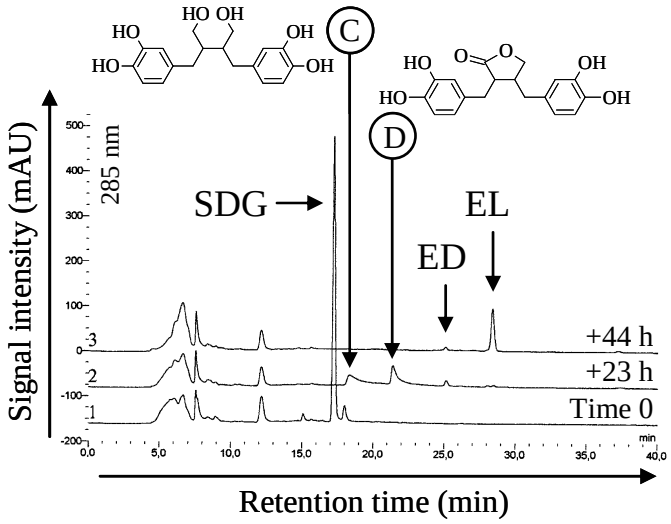


Figure 15: Formation of EL from SDG by a co-culture of *Clostridium* sp. SDG-Mt85-3Db, *P. productus* SECO-Mt75m3, *E. lenta* SECO-Mt75m2 and strain ED-Mt61/PYG-s6 grown in Mt-6-broth. The molecular mass of D was 330 g mol^{-1}, as determined by MS. It corresponds to matairesinol, the lactone form of SECO, with its two methoxy groups removed.

Finally, the identified SDG-metabolising bacteria seem to play a major role in the conversion of various plant lignans with ensuing consequences on the bioavailability and biological activity of enterolignans. **Figure 16** summarises the knowledge on the bacterial pathways of enterolignan production. This knowledge is of value with regard to the possible health effects attributed to dietary lignans, since a growing number of studies have reported the importance of lignans other than SDG for enterolignan production [69-73].

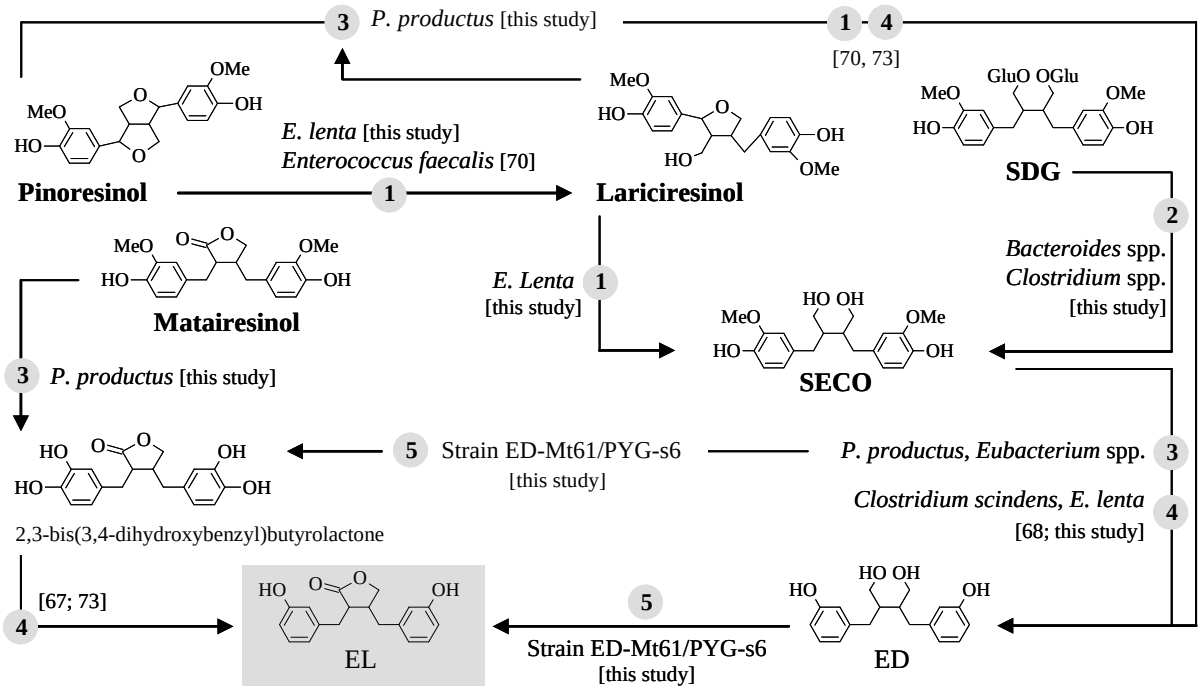


Figure 16: Pathways of EL production from various plant lignans. The numbers indicate the reactions catalysed by intestinal bacteria as follows: 1, reduction; 2, *O*-deglycosylation; 3, *O*-demethylation; 4, dehydroxylation; 5, dehydrogenation. Plant lignans appear in bold letters.

4. Conclusions and perspectives

This work has contributed to the understanding of the microbial ecology of enterolignan production in the human intestinal tract. The MPN study showed how inter-individual differences in microbial community composition may alter ecosystem functions, *i.e.*, the ability to produce EL. The identification of SDG-converting bacteria showed that the combined activities of different species lead to the production of metabolites that may have possible health implications.

The widespread bacterial production of enterolignans in humans requires both dominant and subdominant anaerobic communities. A number of bacteria involved in the anaerobic production of EL from SDG and other lignans have been identified, including one possible novel species and one possible novel genus. Further work will give the opportunity to systematically describe these two organisms. Enterolignan-producing communities are most probably not restricted to the organisms studied here. However, the identified SDG-converting bacteria may be of use for the biotechnological production of EL, the commercial availability of which is restricted. This implies the optimisation of a continuous culture system of lignan-converting strains. Such a system could also form the basis of a defined *in vitro* model to estimate *in vivo* production rates of enterolignans, as described previously using batch culture of faecal suspensions [45, 117, 169].

The lignan-converting bacteria can now be used to investigate the enzymatic systems involved in enterolignan production. For example, experiments with cell extracts and propyl iodide, a chemical alkylating agent, may reveal the need of corrinoid proteins for *O*-demethylation of SECO [170]. In addition, the purification and identification of lignan-transforming enzymes may lead to the identification of new lignan-converting bacteria by screening bacterial protein data bases, and may help to characterise elements of the intestinal microbiome that regulate enterolignan production. As discussed in section 3.2.4., the enantiospecificity of lignan-transforming enzymes is also an important issue with regard to yields of conversion and health effects of dietary lignans. The hypothesis that intestinal bacteria may catalyse the isomerisation of lignan enantiomers is worth investigating too. Since the present work does not allow us to answer the question whether and to which extent the reactions under investigation are advantageous for the identified isolates, the future study of the bacterial interaction underlying the production of enterolignans is certainly of importance in terms of microbial ecology.

Although we identified a number of bacterial strains that catalyse the conversion of SDG to EL in co-culture, we do not know whether these results reflect the process of EL production *in vivo*. Studies in germ-free animals associated with enterolignan-producing pure and mixed cultures will bring to the test the relevance of our *in vitro* findings. They will also help to study the possible metabolism and absorption of SDG and other dietary lignans by host cells in the upper part of the gastrointestinal tract. The design and optimisation of molecular tools, such as the two 16S rRNA-targeted oligonucleotide probes presented in this report, specific for the detection of lignan-converting organisms in intestinal contents would serve the purpose of experiments with gnotobiotic animals.

So far, the interactions between intestinal bacteria and lignan metabolism have not been investigated in a human intervention study. However, it is crucial to better understand these interactions to evaluate the health effects of dietary lignans. We observed that cell counts of enterolignan-producing bacteria were higher in women than in men and relatively stable over a year in a healthy male adult. Since previous studies showed that dietary interventions influence the blood concentration and the urinary excretion of enterolignans in humans [22, 124, 171, 172], an appealing goal is to know to which extent dietary supplementations would alter lignan-converting bacterial communities beyond the influence of endogenous factors, as studied earlier in our laboratory with isoflavones and functional foods [173]. In such an intervention study on the metabolism and possible health effects of dietary lignans, it might be of help to use the bacterial parameters pointed out in the present work as indicators of lignan metabolic profile to characterise inter-individual differences and to stratify human volunteers, as suggested earlier for isoflavones [174, 175].

In conclusion, the present work provides the fundamental knowledge on the bacterial metabolism of dietary lignans. Our findings will be valuable in terms of human nutrition if effort is put into investigating rates of enterolignan production, and if they initiate the design of future *in vivo* studies.

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Appendix 1: List of original communications

Full length articles

Thomas Clavel, Gemma Henderson, Carl-Alfred Alpert, Catherine Philippe, Lionel Rigottier-Gois, Joël Doré and Michael Blaut (2005) **Intestinal bacterial communities that produce active estrogen-like compounds enterodiol and enterolactone in humans**, Appl Environ Microbiol 71(10): 6077-85

Thomas Clavel, Matteo Fallani, Patricia Lepage, Florence Levenez, Jacinthe Mathey, Violaine Rochet, Michèle Sérézat, Malène Sutren, Gemma Henderson, Catherine Bennetau-Pelissero, Françoise Tondu, Michael Blaut, Joël Doré, and Véronique Coxam (2005) **Isoflavones and functional foods alter dominant intestinal microbiota in humans**. J Nutr 135(12): 2786-92

Thomas Clavel, Gemma Henderson, Wolfram Engst, Joël Doré and Michael Blaut (2006) **Phylogeny of human intestinal bacteria that activate the dietary lignan secoisolariciresinol diglucoside**, FEMS Microbiol Ecology 55(3):471-78

Thomas Clavel, Daniela Borrmann, Annett Braune, Joël Doré and Michael Blaut (2006) **Occurrence and activity of human intestinal bacteria involved in the conversion of dietary lignans**, Anaerobe: *In press*

Susanne Mueller, Katiana Saunier, Christiana Hanisch, Elisabeth Norin, Livia Alm, Tore Midtvedt, Alberto Cresci, Stefania Silvi, Carla Orpianesi, Maria C. Verdenelli, *Thomas Clavel*, Corinna Koebnick, Hans-Joachim F. Zunft, Joël Doré and Michael Blaut (2006) **Differences in the fecal microbiota in different European study populations in relation to age, gender and country - a cross sectional study**, Appl Environ Microbiol: *In press*

Thomas Clavel, Ramona Lippmann, Françoise Gavini, Joël Doré and Michael Blaut (2006) ***Clostridium saccharogumia* sp. nov. and *Lactonifactor longoviformis* gen. nov., sp. nov., two novel human faecal bacteria involved in the conversion of the dietary phytoestrogen secoisolariciresinol diglucoside**, Syst Appl Microbiol: *Accepted for publication*

Thomas Clavel, Joël Doré and Michael Blaut (2006) **Bioavailability of lignans in humans**, Nutr Rev: *In preparation*

Scientific events

Thomas Clavel, Carl-Alfred Alpert, Annick Bernalier-Donadille, Annett Braune, Wolfram Engst, Joël Doré and Michael Blaut (2004) Metabolism of secoisolariciresinol by human intestinal bacteria, Reprod Nutr Dev 44:S56 – **Fourth Joint INRA-RRI Symposium on Gut Microbiology**, 21-24 June, Clermont-Ferrand (France) – Oral presentation

Thomas Clavel, Gemma Henderson, Wolfram Engst, Joël Doré and Michael Blaut (2005) Metabolism of lignans by human intestinal bacteria – **2nd International Conference on Polyphenols and Health**, 4-7 October, Davis (USA) – Poster presentation

Thomas Clavel, Gemma Henderson, Joël Doré and Michael Blaut (2006) Microbial conversion of dietary lignans in the human intestinal tract – **Annual Conference of the Association for General and Applied Microbiology (VAAM)**, 19-22 March, Jena (Germany) – Oral presentation

Thomas Clavel, Gemma Henderson, Joël Doré and Michael Blaut (2006) Diversity of lignan-converting human intestinal bacteria – **158th Meeting of the Society for General Microbiology**, 3-4 April, Warwick (England) – Poster presentation

Appendix 2: Supplemental data

Table 10: Procedure for preparation of media

Components and stock solutions[a]	**Volumes to be added** (ml l^{-1})				
	Mt-3	Mt-6	Mt-61	Mt-75	Mt-85
Yeast extract (20 g l^{-1})	15	150	25	5	2.5
Sodium acetate/Sodium formate (25 g l^{-1} each)	20	100	32	50	
Sodium formate (25 g l^{-1})					10
Rumen fluid[b]	10	50	35	2.5	1
Haemin (0.5 g l^{-1})		10	5		
$NaHCO_3$ (40 g l^{-1})	100			50	
10X Basal solution [93]	100			100	
Salt 1 solution		100	100		100
Salt 2 solution		2	2		2
Trace element solution 1 [93]	20			20	
Trace element solution 2		0.1	0.1		0.1
Vitamin solution[c] [93]	1	1	1	1	1
Resazurin solution (1 g l^{-1})	1	1	1	1	1
Cysteine·HCl·H_2O	0.5	0.5	0.5		0.5
$Na_2S·9H_2O$					0.25
Lignans[d]	5[e]	10[f]		10[f]	5[e]
Distilled H_2O	600	250	650	600	750
Adjust pH to	7.5	7.5	7.5	7.2	7.6
Make up to the required volume with distilled H_2O[g]					
Dispense, gas[h] **and autoclave** (121°C, 15 min)					
Lignans		10[i,j]	10[i]		
Gassed and autoclaved fructose (18 g l^{-1})		100[i]			
Gassed and autoclaved glucose (18 g l^{-1})		100[i]			
Gassed and autoclaved cysteine·HCl·H_2O (10 g l^{-1})				50[i]	

[a] All solutions were prepared in distilled H_2O. The composition of complex stock solutions is given below in **Table 11**. Haemin was first dissolved in 5 ml of 1 M NaOH.

[b] The rumen fluid was collected from a healthy cow in a sterile culture bottle using an oral stomach tube [Geishauser T (1993) *Bovine Pract* 27:38-42] connected to a suction pump. Aliquots (10 ml) were centrifuged (100,000 × g, 30 min) in an Optima^{PM} LE-70 preparative ultracentrifuge (Beckman), sterile- filtered (0.22 µm) and stored at 4°C.

[c] Vitamins were added after boiling when appropriate.

[d] SDG and SECO were added before autoclaving. Other lignans were added after autoclaving.

[e] Sterile-filtered stock solution of SDG in H_2O (100 mM).

[f] Sterile-filtered stock solution of SECO in MeOH (100 mM).

[g] The volume of H_2O to be added must take into account the components added after autoclaving.

[h] The gas mixtures were passed over a hot palladium catalyst to remove residual oxygen. For the medium Mt-75, 0.95 ml-aliquots were dispensed into 16-ml screw-cap rubber-stopper glass tubes. Tubes were evacuated (3 min) and subsequently gassed (3 min) with 80% H_2 plus 20% CO_2 (v/v), three times in total. For the study of ED dehydrogenation, the media Mt-6 and Mt-61 were boiled and next flushed with 80% N_2 plus 20% CO_2 (v/v).

[i] The volume of lignan solution to be added must be adjusted according to the volume of the aliquots of medium.

[j] Aerobic sterile-filtered 50 mM stock solution of ED, EL, matairesinol, pinoresinol or lariciresinol.

Table 11: Stock solutions

Solution	Mass (g per litre H_2O)	Formula	Molecular mass (g mol^{-1})
10X Basal[a]	22	$Na_2HPO_4 \cdot 2H_2O$	177.99
	12	$NaH_2PO_4 \cdot 2H_2O$	156.01
	10	NH_4Cl	53.49
	4.2	K_2HPO_4	174.18
	1.6	KH_2PO_4	136.09
	1	$Mg_2SO_4 \cdot 7H_2O$	246.48
Salt 1[a]	40	$NaHCO_3$	84.01
	10	NaCl	58.44
	6.5	NH_4Cl	53.49
	3	K_2HPO_4	174.18
	3	KH_2PO_4	136.09
	1.45	$Mg_2SO_4 \cdot 7H_2O$	246.48
Salt 2[b]	2.5	$FeSO_4 \cdot 7H_2O$	278.02
	2.25	$MnSO_4 \cdot H_2O$	169.01
	0.9	$CoCl_2 \cdot 6H_2O$	237.93
	0.9	$ZnCl_2$	136.29
	0.7	$CaCl_2 \cdot 2H_2O$	147.02
	0.3	$(NH_4)_2SO_4$	132.14
Trace element 1[b,c]	10	NaCl	58.44
	6.2	$Mg_2SO_4 \cdot 7H_2O$	246.48
	0.5	Nitrilotriacetic acid	191.14
	0.5	$MnSO_4 \cdot H_2O$	169.01
	0.18	$ZnSO_4 \cdot 7H_2O$	287.55
	0.17	$CoCl_2 \cdot 6H_2O$	237.93
	0.13	$CaCl_2 \cdot 2H_2O$	147.02
	0.1	$FeSO_4 \cdot 7H_2O$	278.02
	0.02	$NiCl_2 \cdot 6H_2O$	237.71
	0.01	$Na_2MoO_4 \cdot 2H_2O$	241.95
	0.001	Na_2SeO_3	172.94
Trace element 2[b]	1	$CuCl_2$	134.45
	1	$Na_2MoO_4 \cdot 2H_2O$	241.95
	0.84	$NiCl_2 \cdot 6H_2O$	237.71
Vitamin[b]	10×10^{-3}	Pyridoxal·HCl	203.63
	5×10^{-3}	Thiamine·HCl	337.30
	5×10^{-3}	Riboflavin	376.37
	5×10^{-3}	Nicotinic acid	123.11
	5×10^{-3}	Ca-pantothenate	476.54
	5×10^{-3}	P-4-Aminobenzoate	175.23
	5×10^{-3}	Lipoic acid	206.32
	2×10^{-3}	Biotin	244.31
	2×10^{-3}	Folic acid	441.40
	2×10^{-3}	Cyanocobalamin	1,355.38

[a] Autoclaved (121°C, 15 min). [b] Sterile-filtered (0.22µm). [c] Nitrilotriacetic acid was first dissolved in 10 ml of 0.5 M NaOH

Table 12: Bacterial counts and enterolignan production for the MPN study subjects

	Enterolignan-producing communities $\log_{10}$ (MPN g^{-1})[a]		Enterolignan production[b]		Dominant faecal microbiota composition[c] (% of total bacteria)										
Subject	EL_{MPN}	ED_{MPN}	λ	δ	Ato	Bac	Bdist	Bfra	Bif	Clept	Csac	Erec	ProCo	Sex	Age (yr)
1	5.5	8.6	13.7	42.9	Excluded[d]										23
2	6.7	9.9	21.8	56.4	0.7	6.2	2.6	7.9	∅	33.2	∅	21.2	0.8		59
3	6.7	6.7	25.3	93.1	0.4	10.4	∅	∅	1.8	28.0	∅	34.0	0.6		36
4	2.7	11.6	0.0	59.6	∅	14.7	0.4	2.9	0.9	21.9	∅	33.8	0.1		32
5	6.5	11.1	23.7	51.6	0.2	1.4	∅	2.7	5.4	33.4	∅	3.5	0.7		23
6	6.5	10.0	17.7	53.1	4.4	7.0	∅	∅	∅	27.0	∅	20.4	1.1	Women	24
7	6.1	11.5	22.6	49.2	Excluded[d]										28
8	3.9	7.7	10.7	60.9	0.5	15.3	∅	∅	2.8	22.9	∅	27.5	0.7		48
9	7.9	7.9	ND	ND	11.0	46.9	2.6	4.0	2.1	4.6	∅	23.1	0.3		54
10	7.7	8.8	ND	ND	13.5	13.6	0.5	1.8	∅	13.3	∅	35.4	0.8		26
11	6.5	9.8	ND	ND	6.3	4.9	∅	∅	3.6	18.4	∅	29.4	2.1		32
12	8.0	8.9	ND	ND	14.3	9.5	0.5	∅	∅	20.0	∅	37.6	∅		32
13	3.1	4.1	2.8	56.9	3.6	13.8	∅	4.4	1.5	23.1	∅	39.9	0.1		49
14	8.2	9.8	7.1	62.4	5.2	8.0	∅	9.6	1.0	25.1	∅	43.2	0.3		25
15	3.6	4.7	4.0	101.6	1.1	29.7	0.2	2.7	1.7	17.8	0.2	28.8	∅		50
16	0.0	11.0	0.0	57.1	0.7	35.8	∅	4.5	0.5	13.8	∅	24.4	∅		25
17	3.0	7.4	4.7	33.4	3.3	27.0	0.1	12.0	2.4	17.2	0.4	15.1	∅		31
18	7.3	8.7	ND	ND	5.1	18.8	2.3	2.3	4.2	31.8	∅	32.0	0.3	Men	27
19	5.7	11.2	ND	ND	6.2	5.0	0.1	6.1	1.4	28.5	∅	39.3	0.5		44
20	6.8	8.9	ND	ND	7.4	21.4	∅	∅	0.5	13.4	∅	34.3	0.9		34
21	7.9	8.7	ND	ND	4.6	10.4	∅	4.4	∅	11.1	∅	32.1	1.4		28
22	4.5	6.5	ND	ND	Excluded[d]										40
23	4.2	9.2	ND	ND	7.6	8.0	0.4	∅	3.0	21.8	∅	28.1	∅		33
24	2.1	7.7	ND	ND	Excluded[d]										34
Mean	5.5	8.8	11.9	59.9	4.8	15.4	0.5	3.3	1.6	21.3	ND	29.2	0.5		35
SD	2.2	1.9	9.1	17.7	4.2	11.3	0.9	3.4	1.5	7.6	ND	9.1	0.5		8.5
CV	40%	22%	77%	30%	88%	74%	178%	104%	92%	36%	ND	31%	101%		24%

[a] EL_{MPN} or ED_{MPN}, counts of EL- or ED-producing bacteria, respectively, as determined by MPN enumerations.
[b] The parameters λ and δ characterise the production of EL and enterolignans (ED plus EL) by faecal bacteria after 48 h of growth in the medium Mt-6, respectively. They are expressed as percentages of the initial SECO concentration. ND, not determined.
[c] The mean additivity, *i.e.*, the sum of the bacterial proportions of non-overlapping phylogenetic groups, was 71.6% ± 11.4 (43.9 to 91.9%). Bacterial groups: *Atopobium* group (Ato), *Bacteroides* and relatives (Bac), *B. distasonis* (Bdist), *B. fragilis* (Bfra), *Bifidobacterium* spp. (Bif), *C. leptum* group (Clept), *Clostridium* sp. SDG-Mt85-3Db (Csac), *E. rectale-C. coccoides* cluster (Erec), *P. productus* and *C. coccoides* species (ProCo). ∅, not detected.
[d] Percentage of hybridisation below 35%.

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