Metabolism of Plant Lignans by Human Intestinal Bacteria

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Abstract

Dietary plant lignans have been implicated in the prevention of several chronic diseases including breast cancer, colon cancer, and cardiovascular disease. Plant lignans can be converted to the physiologically active enterolignans enterodiol and enterolactone by anaerobic bacteria residing in the intestine. Significant interindividual variation has been observed regarding the ability of intestinal bacterial communities to metabolize plant lignans to enterolignans. However, little is known about what characterizes a high enterolignan-producing community from a low enterolignan-producing community. This work presents the development of a platform that can be used to study communities of lignan-converting intestinal bacteria *in vitro*. Variables that were tested include media type, fecal inoculum concentration, stool processing, and sample headspace. Optimal conditions for enterolignan production and reproducibility consisted of a simple media containing sodium acetate and sodium formate, a total fecal concentration of greater than 1.0%, homogenized slurry processed in a batch, and a headspace-slurry ratio of 3:2.

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Introduction

Lignans are organic compounds synthesized by plants and are characterized by the union of two cinnamic acid residues by β-linkage on the C8 of each propyl side chain.[1–3] They are found in over seventy plant families and are found throughout the plant tissue, in roots, rhizomes, stems, leaves, seeds and fruits.[4,5] Large amounts of lignans and other polyphenols are also found in the knots of soft wood trees, mainly in the form of hydroxymatairesinol.[6,7] Lignans, although smaller in size (Figure 1), are structurally similar to lignins, three-dimensional polymers that intercalate with cellulose, hemicellulose, and pectin to form the rigid cell wall of plant cells.[1,8] Moreover, both lignans and lignins are created by the same initial phenylpropanoid pathway and are synthesized from monolignols (derived from either phenylalanine or tyrosine), but ultimately enter distinct biochemical pathways.[2,9,10] Indeed, while lignins are ubiquitous in the plant kingdom, not all plants produce lignans.

Interestingly, despite having been identified over 120 years ago[7], the botanical properties of lignans still remain unclear. Lignans are thought to play a role in defending the plant against pathogens and pests because they possess some antifungal, antimicrobial, antiviral, and even insecticidal properties.[4,10–12] For example, the lignan haedoxan A isolated from *Phryma leptostachya* has insecticidal properties comparable to that of pyrethrins. Additionally, matairesinol and its related metabolites have been shown to reduce fungal growth of *Fomes annosus* in *Picea abies*.[5,13]

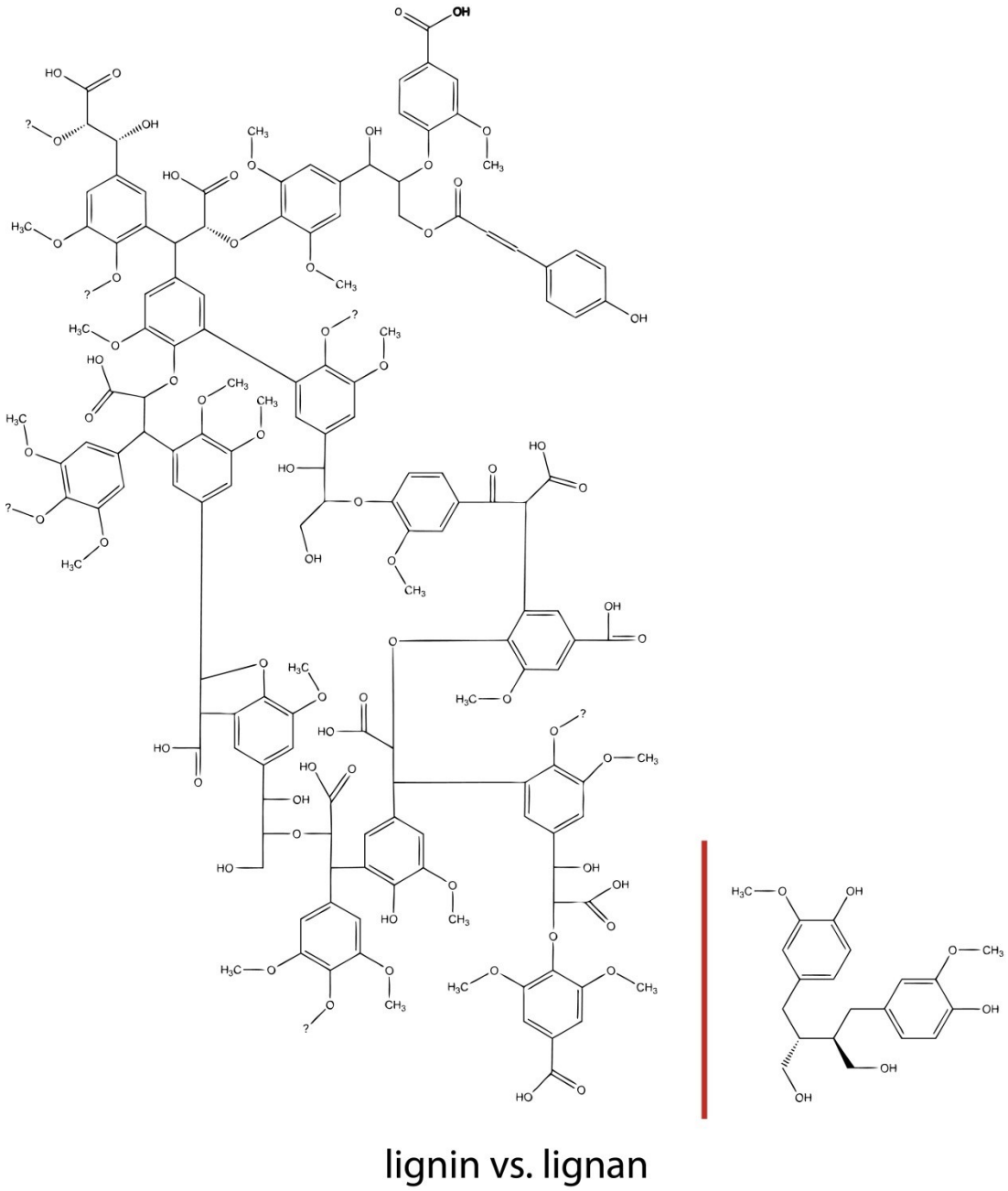

Figure 1. A lignin, methylignin (left), identified in Suzuki et al.[14] and lignan, secoisolariciresinol (right)

Given the prevalence of lignans in the plant kingdom, they are not surprisingly found in many common foods. The lignans typically present in foods are matairesinol (MAT), pinoresinol (PINO), medioresinol (MED), lariciresinol (LARI), sesamin (SES), syringaresinol (SYR), secoisolariciresinol (SECO), and the glycosylated form of SECO, secoisolariciresinol

diglucoside (SDG). Flaxseed is the richest known source of plant lignans with approximately 300,000 μg per 100g fresh weight, the majority of which is in the form of SDG.[15] Other seeds with high lignan concentration include sesame seeds (104,446 μg/100g), cloudberry seeds (43,876 μg/100g), hemp seeds (32,473 μg/100g), and blackberry seeds (23,310 μg/100g).[16] Cereal grains, including rye, wheat, barley, and oats also contain moderate to high amounts of lignans.[15–18] Brassica vegetables, such as kale, cabbage, and Brussels sprouts, as well as other vegetables and fruits, such as asparagus, broccoli, garlic, apricots, prunes, and dates, contain moderate amounts of lignans.[15–19] Lignans are even found in small amounts in common beverages, including black tea, green tea, coffee, soy milk, fruit juices, beer, and wine. [15,20,21]

Conversion of plant lignans to enterolignans by gut bacteria

Despite their antimicrobial properties, plant lignans can be metabolized and converted to enterolignans (entero- from Greek *enteron* meaning "intestine") by bacteria residing in the intestine.[22] Also referred to as mammalian lignans, enterolignans were discovered independently by two research teams at almost the same time. The two major enterolignans produced by mammalian gut bacteria are enterolactone (ENL) and enterodiol (END). In 1979, Setchell and Adlercreutz first identified what turned out to be ENL, one of the major enterolignans excreted in the urine of both pregnant and non-pregnant women.[23] A year later, Stitch *et al.* published an article in *Nature* detailing similar findings. They also had isolated what later was identified as ENL from urine in both pregnant and normally ovulating women.[24] Axelson *et al.* went on to demonstrate in humans that plant lignans are dietary precursors of enterolignans.[25]

Not long after their discovery, it was confirmed that ENL and END were produced by intestinal bacteria. Axelson and Setchell first conducted a study in 1981 comparing germ-free

rats and conventional rats showing that the presence of bacteria was necessary for enterolignan formation.[26] Later that same year, Setchell *et al.* demonstrated in humans that urinary enterolignan excretion falls immediately and significantly following antibiotic treatment.[27] Borriello *et al.* confirmed the metabolism of plant lignans to ENL and END *in vitro* using human stool samples.[22]

Following ingestion of SDG or similarly glycated lignans, such as pinoresinol diglucoside or sesaminol triglucoside (STG), the sugar moieties are hydrolyzed in the large intestine by *O*-linked deglycosylation, forming SECO.[28,29] Studies simulating digestion in the stomach and small intestine show that SDG remains intact in the equivalent of these parts of the gut, suggesting that acid hydrolysis and human intestinal enzymes do not play a major role in this initial hydrolysis. [28,30] It is not until reaching the artificial ascending colon containing human gut microbes, that SECO is first detected.[30]

In converting SDG to ENL four reactions must take place (Figure 2). First, SDG must be converted to SECO by *O*-linked deglycosylation, and secondly SECO is converted to the intermediate dihydroxyenterodiol (DHEND) by *O*-linked demethylation. From this point, DHEND may be converted to END by dehydroxylation, and ultimately to ENL by dehydrogenation of END. Alternatively, DHEND may undergo dehydrogenation to construct a lactone ring, thus forming a second intermediate dihydroxyenterolactone (DHENL), which can then be dehydroxylated to form ENL.[28,29,31–33]

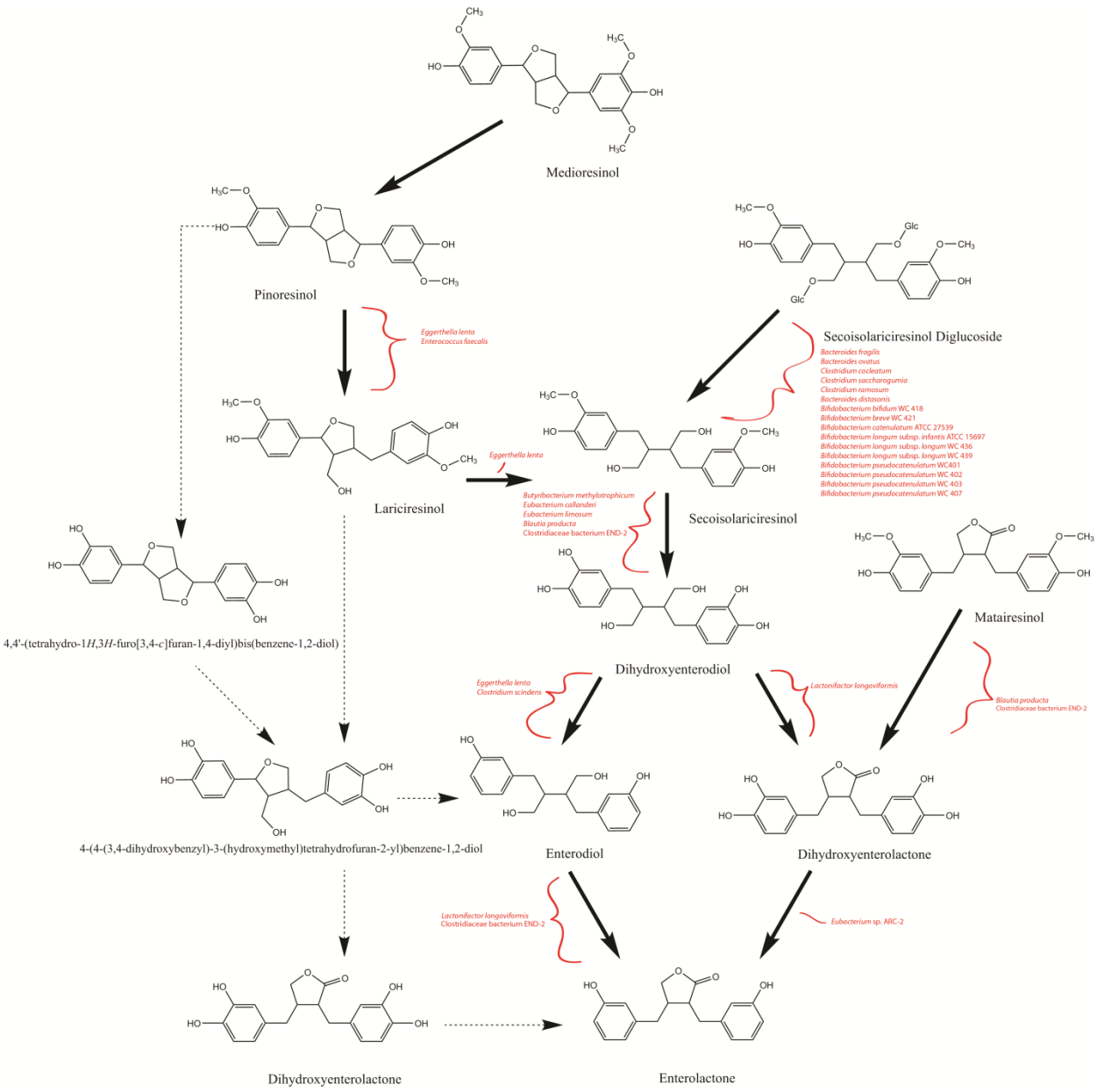

Figure 2 Overview of lignan metabolism and the organisms involved. Solid arrows indicate known pathways. Dashed arrows indicate possible pathways.

Several investigators have evaluated the capacity of various specific gut bacteria to carry out the reactions necessary for conversion of glycosylated lignans to enterolignans (Table 1). Clavel *et al.* determined that *Bacteroides fragilis*, *Bacteroides ovatus*, *Clostridium cocleatum*, *Clostridium saccharogumia*, *Clostridium ramosum*, and *Bacteroides distasonis* are capable of this *O*-deglycosylation, with the first four able to completely deglycosylate SDG within 20 hours

of the onset of the experiment.[34–36] Roncaglia *et al.* found ten *Bifidobacterium* strains capable of hydrolyzing SDG.[37] Once deglycosylated, SECO can then be demethylated to produce its intermediate DHEND. Clavel *et al.* demonstrated the ability of *Butyribacterium methylotrophicum*, *Eubacterium callanderi*, *Eubacterium limosum*, and *Blautia producta* to catalyze this reaction.[34,36] However, Clavel also notes that the presence of the SECO demethylating species *E. callanderi* and *B. methylotrophicum* in the human intestinal tract has not been reported. (*Blautia* is a newly classified genus, and the *B. producta* taxon replaces *Peptostreptococcus productus* and *Ruminococcus productus* to which some older articles refer.)[38] Jin and Hattori also recently identified Clostridiaceae bacterium END-2 (originally referred to simply as "strain END-2") with the ability to demethylate SECO.[39] Following demethylation, *Eggerthella lenta* and *Clostridium scindens* can act on the intermediate by removing a hydroxyl group from each aromatic ring to form END.[36] *Lactonifactor longoviformis* and the aforementioned strain END-2 perform the final step in ENL production by fashioning the lactone ring.[35,39] Alternatively, *L. longoviformis* may create the lactone ring subsequent to SECO demethylation, in which case END does not form but rather a second intermediate, DHENL, which may then continue on to form ENL.[28]

Table 1 Known organisms involved in lignan conversion. Dihydroxyenterolactone (DHEND), dihydroxyenterodiol (DHEND), enterodiol (END), enterolactone (ENL)

Reference	Organism(s)	Function
Clavel, Borrmann, et al., 2006; Clavel, Henderson, et al., 2006; Clavel, Lippman, et al., 2007	Bacteroides fragilis Bacteroides ovatus Clostridium cocleatum Clostridium saccharogumia Clostridium ramosum Bacteroides distasonis	Deglycosylation
Roncaglia, Amaretti, et al., 2011	Bifidobacterium bifidum WC 418 Bifidobacterium breve WC 421 Bifidobacterium catenulatum ATCC 27539 Bifidobacterium longum subsp. infantis ATCC 15697 Bifidobacterium longum subsp. longum WC 436 Bifidobacterium longum subsp. longum WC 439 Bifidobacterium pseudocatenulatum WC401 Bifidobacterium pseudocatenulatum WC 402 Bifidobacterium pseudocatenulatum WC 403 Bifidobacterium pseudocatenulatum WC 407	Deglycosylation
Clavel, Borrmann, et al., 2006; Clavel, Henderson, et al., 2006	Butyribacterium methylotrophicum Eubacterium callanderi Eubacterium limosum Blautia producta	Demethylation
Jin & Hattori, 2010	Clostridiaceae bacterium END-2	Demethylation
Clavel, Henderson, et al., 2006	Eggerthella lenta Clostridium scindens	Dehydroxylation
Clavel et al., 2007	Lactonifactor longoviformis	Dehydrogenation
Jin & Hattori, 2010	Clostridiaceae bacterium END-2	Dehydrogenation
Xie, Akao, et al., 2003	Enterococcus faecalis	Reduction
Clavel, Borrmann, et al., 2006	Eggerthella lenta	Reduction
Jin, Kakiuchi, & Hattori, 2007	Ruminococcus sp. END-1	Dehydrogenation of: (-) END to (-) ENL
Jin, Kakiuchi, & Hattori, 2007	Clostridiaceae bacterium END-2	Dehydrogenation of: (+) END to (+) ENL
Jin, Zhao, et al., 2007	Eggerthella sp. SDG-2	Dehydroxylation of: (+) DHEND to (+) END; (-) DHENL to (-) ENL
Jin, Zhao, et al., 2007	Eubacterium sp. ARC-2	Dehydroxylation of: (-) DHEND to (-) END; (+) DHENL to (+) ENL

Studies have shown that SES is also converted to END and ENL in humans, rats, and in *in vitro* incubations, although the SES biotransformation pathway is still undetermined [40–43] Due to SES's methylenedioxy functional groups, it is thought that SES follows a conversion pathway

(Figure 3) different than that of the other major lignans (Figure 2). In addition, the organisms responsible for converting SES are not as extensively researched as those involved in SDG conversion. Zhu *et al.* recently showed that when STG, isolated from sesame cake and the most abundant lignan glycoside in sesame seed, was fermented with human intestinal bacteria, the phylogenetic groups *Bifidobacteria* and *Lactobacillus-Enterococcus* were significantly greater in the STG samples compared to the controls.[44]

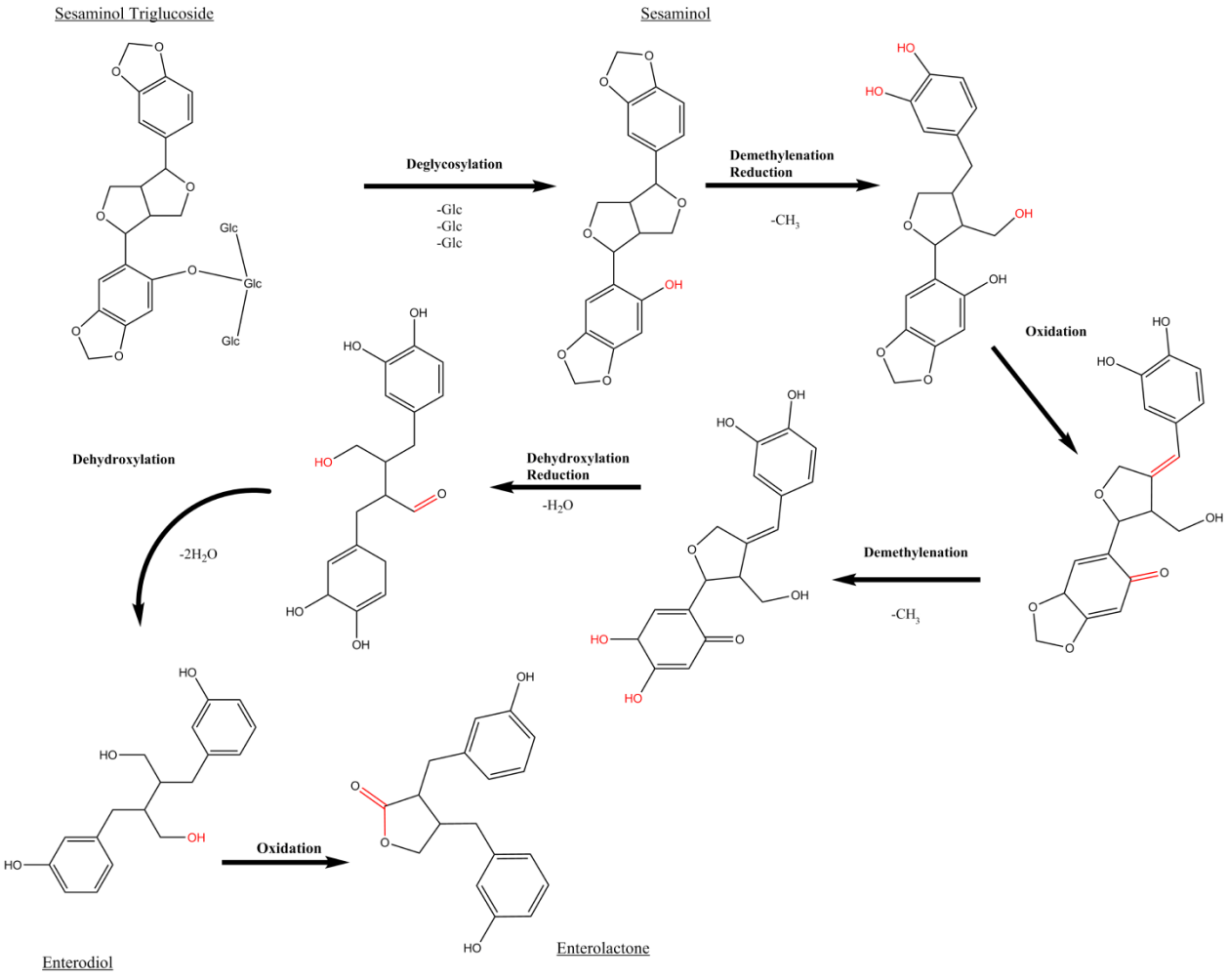

Figure 3 Overview of potential sesaminol triglucoside (STG) metabolism by intestinal bacteria

Both END and ENL enantiomers are produced by bacteria and the human exposure to the enantiomers results from the interaction of the initial type of substrate and the composition of the

bacterial consortia. For example, (-) ENL predominates in serum when humans consume their habitual diets, but when supplemented with flaxseed the (+) ENL form increases substantially with comparatively modest increases in (-) ENL.[45] Additionally, the forms of END and ENL that have been isolated as the result of incubating SDG with intestinal bacteria produce (+) END and (+) ENL. However, if the lignans arctiin or PINO diglucoside are used as a substrate (-) END and (-) ENL are produced.[46] Subsequently, some bacteria have shown enantiomeric selectivity when converting lignans. *Ruminococcus* sp. END-1 enantioselectively converts (-) END to (-) ENL, while the strain END-2 converts (+) END to (+) ENL.[47] *Eggerthella* sp. SDG-2 can convert (+) dihydroxyenterodiol (DHEND) to (+) END, but not (-) DHEND to (-) END. *Eggerthella* sp. SDG-2 converts (-) DHENL to (-) ENL, but not (+) DHENL to (+) ENL. Evidently, *Eubacterium* sp. ARC-2 can biotransform what *Eggerthella* sp. SDG-2 cannot by converting (-) DHEND and (+) DHENL to (-) END and (+) ENL, respectively. However, the bacterium could not transform (+) DHEND and (-) DHENL.[48] Jin *et al.* also recently demonstrated the ability of *Eubacterium* sp. ARC-2 and *Eggerthella* sp. SDG-2 to transform the lignan trachelogenin (isolated from safflower seeds) to currently unnamed compounds.[49]

The capacity of specific bacteria to metabolize other plant lignans is less well known. Nonetheless, Xie *et al.* discovered *Enterococcus faecalis* capable of reducing PINO to form LARI.[50] Furthermore, *E. lenta* is capable of transforming both PINO and LARI to SECO.[34] In addition, Clavel *et al.* reported that *B. producta* is also capable of demethylating other lignans including LARI, PINO, and MAT. Considering that the enterolignans, regardless of the plant lignan source, have been associated with health benefits in human population studies, [33,51–63] further studies are warranted which elucidate both the microbiome and the microbial metabolic pathways associated with the production of enterolignans. The potential for a gut microbial

community to product END and ENL is affected by the plant lignan substrate. Heinonen *et al.*[64] showed a range of conversion of plant-derived lignans to END and ENL, ranging from no conversion of isolariciresinol to 100% conversion of LARI. Similarly, production of ENL from arctigenin glucoside and MAT ranged from 5 to 62%, respectively.

Inter-individual Differences in Lignan Metabolism

Studying the relationship between lignan exposure and disease risk in human populations is challenging and complex. A variety of foods are sources of lignans and these are not eaten in isolation. Traditionally, cuisines that include dietary sources of lignans also include other foods that may be considered healthy or less healthy. Trying to tease out the association between intake of high-lignan foods in general, which are often also high-fiber foods, or intake of specific lignans, and disease risk is difficult.

In relation to the study of breast cancer, but relevant to other observational studies described in the previous section, Sonestedt and Wirfält described several factors that may contribute to the ambiguous results that have so far been obtained in epidemiologic studies focusing on ENL.[65] They note that several factors play a role in enterolignan exposure, including the composition of the gut microbial community, dietary intake of lignan-containing foods, antibiotic use, smoking status, and constipation. The authors also point out the difficulty in accurately and reliably determining enterolignan exposure and the complicated role that genetic factors play in both cancer development and the actions of ENL.

Even under controlled conditions, substantial interindividual variation has been reported regarding enterolignan production. Kuijsten *et al*[66] showed in a pharmacokinetic study that a variety of plasma profiles arise with a set dose of SDG, with some individuals producing high

amounts of both ENL and END, or higher amounts of one enterolignan as compared to the other (e.g., high ENL, low END or vice versa), or very low amounts of either. All 12 participants ingested the same dose of SDG per kg body weight; however, in 5 subjects the area under the curve (AUC) of ENL was more than twice that of END; in 5 others, the AUC of ENL was only 1–2 times the AUC of END; in 2 participants, the AUC of END exceeded that of ENL; and in 1 participant, ENL concentrations barely increased. Mean (± SD) AUC for END and ENL were 966 ± 639 and 1762 ± 1117 nmol/L·h, respectively, the large SD reflecting the wide variation in individual results. Kuijsten *et al.* noted that the cumulative excretion of END and ENL accounted for about 40% of ingested SDG; however, individual percentages were also variable. Much of this variation is proposed to be due to differences in gut microbial community across individuals.[66] The different patterns of END and ENL appearance in circulation and the range of cumulative excretion values suggest differential capacity of the gut microbes to manage the various steps in SDG metabolism (Figure 2), with some unable to effectively hydrolyze the SDG to SECO and others being ineffective at converting END to ENL.

The variation in enterolignan production by gut microbial communities from different individuals is also clearly demonstrated using *in vitro* incubations. Incubating fresh fecal samples with a flaxseed extract (630 μmol/l) for 72 h, Possemiers *et al.*[67] found that the production of END and ENL differed strongly among 100 individuals. END was produced in varying amounts in 63% of the samples, whereas ENL was only produced in 39% of the samples. Further, END and ENL production were positively correlated and were associated with higher β-glucosidase activity, supporting the importance of the capacity to carry out the initial steps in SDG conversion since the glucose moieties on SDG must be removed to produce SECO.

Another potential source of variation in urine or plasma measures of ENL and END, may stem from interindividual difference in biotransformation of the enterolignans[68] Genetic variation in physiologic pathways affecting absorption, metabolism, and distribution of phytochemicals may influence exposure, although no controlled evaluation of the effects of genetic variants in biotransformation enzymes on lignan availability has been conducted. In humans, plant lignans and their metabolites are efficiently conjugated with glucuronic acid or, to a lesser extent, sulfate. Conjugation takes place in the gut epithelium and liver by UDP-glucuronosyltransferases (UGT) and sulfotransferases, and the conjugates are excreted in urine and bile. Those that are re-excreted in bile undergo enterohepatic recycling.[69] In urine, ENL and END are excreted primarily as monoglucuronides (95 and 85%, respectively), with small percentages being excreted as monosulfates (2–10%) and free aglycones (0.3–1%).[70] Colon cell lines have been shown to rapidly glucuronidate enterolignans, suggesting that the majority of conjugation of lignans likely occurs in the colon.[71] Effects of enterolignans on intestinal transporters has not been evaluated, but SES has been shown to increase the mRNA expression of several transporters, thereby possibly affecting absorption of lignans and other phytochemicals.[72]

In addition to the variation in microbial and host metabolism, several physiologic, demographic and lifestyle characteristics of individuals appear to influence the production of enterolignans in vivo, including constituents of diet and other non-dietary factors to be discussed later.

Lignans and human health

Plant lignans, and particularly their metabolites, the enterolignans, have been shown to have a range of biologic activities.[29,33,60,73,74] The structural similarity of END and ENL to 17β-estradiol, a common sex hormone, allows the enterolignans to bind to estrogen receptor alpha

(ERα) and exert weak estrogenic or anti-estrogenic effects, which early rendered them the classification as "phytoestrogens."[73,75–77] However, both *in vitro* and *in vivo* studies have identified a variety of other mechanisms by which enterolignans may affect the risk of several chronic diseases. These mechanisms include anti-proliferative, anti-inflammatory, and apoptotic effects.[11,53,60,61,76,78] Several review articles have been published examining the available evidence on lignans and their effect on human health.[33,51–63] Here I summarize the major conclusions of these reviews and provide further updates on the human studies

Cancer

The three types of cancer most extensively explored in relation to lignan exposure are breast, colorectal, and prostate cancer, to which lignans are linked with cancer prevention. Research concerning other cancers, however, is sparse. The handful of human studies on endometrial cancer and lignans tends toward a null association,[51,79–81] and a nested case-control study conducted within the European Prospective Investigation into Cancer and Nutrition–Norfolk (EPIC-Norfolk) study indicated no associations between lignans and gastric cancer.[82] Gastric and endometrial cancer notwithstanding, the overall evidence tends to support an inverse association between lignans and cancer.

In 2005, Webb and McCullough[61] reviewed the existing literature on dietary lignan exposure and cancer. They concluded that *in vitro* and animal studies supported a role for lignan-rich foods and extracted lignans in modulation of carcinogenesis in colon, breast, and prostate; however, they pointed out that the results of the few available epidemiologic studies were inconsistent. Since 2005, additional prospective cohort studies have evaluated the association between dietary lignan intake and cancer risk, relying on self-reported measures of intake such as food frequency questionnaires and food records. With the advent of more sensitive and less

expensive assays for END and ENL, investigators have also measured urinary and plasma END and ENL, which provide an objective measure of lignan exposure. In 2010, Adolphe *et al.* reviewed the literature on the health effects of SDG specifically and suggested that the data from animal models support a protective effect of flax lignans against colon, lung and mammary tumors.[76]

Colorectal Cancer

Several epidemiologic studies have examined the association between lignan or enterolignan exposure and colorectal cancer. A case-control study conducted in Canada examined the association between intake of lignans as measured by a food frequency questionnaire (FFQ) and risk of colorectal cancer in 1095 cases and 1890 control subjects.[83] The researchers found that high dietary lignan intake (>0.255 mg/day) was associated with a significant reduction in colorectal cancer risk (OR 0.73; 95% CI 0.56-0.94). In 2006, a Dutch retrospective case-control study found that plasma END was associated with a significantly reduced risk of colorectal adenoma (OR 0.53; 95% CI 0.32-0.88) when examining incident cases.[84] Incident cases were defined as those with a histologically confirmed colorectal adenoma but no history of any type of polyps. ENL was also associated with reduced risk of colorectal cancer but to a lesser extent (OR 0.63; 95% CI 0.38-1.06).

A case-cohort study conducted in Denmark similarly examined plasma ENL and colorectal cancer and found significantly lower incidence rate ratios (IRR) for colon cancer among women for each doubling of plasma ENL concentration (IRR 0.76; 95% CI 0.60-0.96).[85] Interestingly, however, IRR for rectal cancer in men was increased for every doubling of plasma ENL concentration (IRR 1.74; 95% CI 1.25-2.44).[85]

Breast Cancer

Conclusions based on human epidemiologic studies are variable for the role of lignans in premenopausal versus postmenopausal breast cancer risk. In their 2005 review, Webb and McCullough[61] concluded that the most support for a role of lignans in cancer prevention was observed for premenopausal breast cancer. Following this, the results of two meta-analyses published within a year of each other provide a more systematic evaluation of available studies. A 2009 meta-analysis of 11 studies (4 cohort studies and 7 case-control studies) assessing lignan exposure and breast cancer risk determined that, though borderline, there was no association between plant lignan intake and breast cancer risk (combined OR 0.93; 95% CI 0.83-1.03) when the relevant observational studies were pooled.[59] However, when the studies were divided by menopausal status, they found a significant reduction in breast cancer risk among the postmenopausal women (combined OR 0.85; 95% CI 0.78-0.93; $p<0.001$). This effect was not observed with pre-menopausal women (combined OR 0.97; 95% CI 0.82-1.15, $p=0.73$). Where data were available, the investigators also systematically examined ENL concentrations in plasma and estimated enterolignan exposure using values produced from food by *in vitro* fermentation models. Although there was a significant inverse correlation between enterolignan exposure and breast cancer risk (combined OR 0.73; 95% CI 0.57-0.92), the association was no longer significant (combined OR 0.82; 95% CI 0.59-1.14) when blood ENL levels were considered. The authors noted that there was marked heterogeneity between the studies they reviewed making broad conclusions difficult.

Another meta-analysis which included 21 studies (11 prospective cohort studies and 10 case-control studies) concluded that plant lignan intake was not associated with overall breast cancer risk, but if the results are separated by menopausal status postmenopausal women with a

high plant lignan intake have a significantly reduced risk of breast cancer (pooled RE 0.86; 95% CI 0.78-0.94).[52] A similar association was found when 4 studies were examined assessing dietary enterolignans (pooled RE 0.84; 95% CI 0.71-0.97). In total, these meta-analyses suggest that menopausal status is an important factor to consider when analyzing associations of dietary lignans, enterolignans and risk of breast cancer.

Newer studies continue to strengthen the suggestion that enterolignans have a protective effect on breast cancer, especially regarding menopausal status. A follow-up study of 2653 postmenopausal breast cancer patients diagnosed between 2001 and 2005 concluded that both high estimated ENL (HR 0.60; 95% CI 0.40-0.89; *p* trend=0.02) and high estimated END were associated with higher overall survival (HR 0.63; 95% CI 0.42-0.95; *p* trend=0.02).[86] Moreover, a retrospective cohort study of 300 breast cancer patients concluded that higher serum ENL levels were associated with both decreased all-cause mortality and decreased breast-cancer mortality.[87] At 5 years, patients with serum ENL levels ≥ 10 nmol/L demonstrated lower all-cause and breast cancer-specific mortality (HR 0.49; 95% CI 0.27-0.91; *p*=0.025 and HR 0.42; 95% CI 0.22-0.81; *p*=0.009, respectively). At ten years and beyond, however, this association became non-significant (all-cause death: HR 0.65; 95% CI 0.40-1.03; *p*=0.07 and breast cancer-specific (HR 0.67; 95% CI 0.39-1.14; *p*=0.1). After stratifying the results by menopausal status, higher estimated levels of ENL were associated with both lower all-cause mortality and breast cancer-specific mortality remained statistically significant at ten years (all-cause death: HR 0.48; 95% CI 0.28-0.82; *p*=0.007 and breast cancer-specific: HR 0.52; 95% CI 0.29-0.94; *p*=0.03, respectively).[87] After adjusting for menopausal status, these studies show a protective effect of ENL for postmenopausal women.

A case-control study conducted in women in Ontario, Canada, examined the relationship between breast cancer and phytoestrogen intake using a FFQ.[88] The study included 2438 women with breast cancer and 3370 controls and results were stratified by tumor type, menopausal status, and phytoestrogen intake as an adult vs. adolescent. Among women with estrogen receptor positive and progesterone receptor positive (ER+/PR+) tumors, both adult intake of lignans (OR 0.83; 95% CI 0.68-1.00) and adolescent intake of lignans (OR 0.86; 95% CI 0.73-1.00) were associated with reduced risk of breast cancer. After separating the results by menopausal status, the associations in premenopausal women become either null or not significant; however, the association with adolescent lignan intake among postmenopausal women in the ER+/PR+ subgroup remained significant (OR 0.78; 95% CI 0.64-0.95).[88]

McCann *et al.* reported on a case-control study analyzing not only lignan intake but also the various types of plant lignans and their relationship to breast tumors. They found that the women in the highest tertile of lignan intake had a 40-50% lower odds of developing breast cancer compared to those in the lowest tertile of lignan intake.[89] This result was regardless of menopausal status; however the strongest associations were found with PINO and LARI in premenopausal women strongest associations were found with MAT in postmenopausal women.

Animal models have been used to examine the underlying mechanisms of lignans and enterolignans' action on breast cancer. In animal models, SDG has been shown to promote cell differentiation in mammary glands, delay the progression of *N*-methyl-*N*-nitrosourea-induced mammary tumorigenesis, and produce beneficial mammary gland structural changes during gestation and lactation.[76] More recently, SES was shown to decrease cell proliferation and increased apoptosis of MCF-7 tumors in mice.[90] In addition, a study in germ-free mice showed that although END and ENL did not influence the occurrence of breast cancer, the number of

tumors, size of the tumors, tumor cell proliferation, and tumor cell apoptosis were all decreased in rats that were conventionalized with a lignan-converting bacterial consortia. These studies suggest that lignans and bacterially produced enterolignans influence anticancer effects.[91]

Prostate Cancer

The evidence regarding the role that lignans may play in prostate cancer is varied. On the one hand, reviews by McCann *et al.* and Saarinen *et al.* cite several *in vitro* and animal studies that demonstrate the ability of lignans to act as chemopreventive agents in prostate cells.[62,63] Indeed, recent evidence confirms that ENL, even at concentrations achievable *in vivo* following the intake of lignan precursors, inhibits the proliferation of early-stage prostate cancer cells.[92] On the other hand, data available from human studies are inconsistent. Some evidence suggests a benefit while other studies found null associations between lignans and prostate cancer. Since the McCann and Saarinen reviews were published, two case-control studies have been published that actually show higher lignan intake is associated with an increased risk of prostate cancer.[93,94]

Another nested case-control study from EPIC-Norfolk examined lignans and prostate cancer.[93,95] Participants completed 7-day food records, which were used to estimate daily lignan intake. In addition to estimating intake of the plant lignans MAT and SECO, the investigators also estimated intake of preformed enterolignans from dairy and other animal products. This was the first study to consider animal sources of enterolignans in the overall estimate of lignan exposure. Given that mammals, particularly ruminants, are capable of producing large amounts of enterolignans in their rumen, some animal products, particularly dairy foods, contain enterolignans.[96] The study included 204 prostate cancer cases and 812 controls. Estimated mean (± SD) intake of preformed enterolignans were 20 μg/day (± 9) among cases and 18 μg/day (± 9) among controls.[93] In an age-adjusted model, total lignan intake was not associated with prostate

cancer (OR 1.05; 95% CI 0.81-1.36; p=0.72). However, total enterolignan intake (which included the non-lignan phytoestrogen equol) was positively associated with prostate cancer (OR 1.41; 95% CI 1.12-1.76; p=0.003) as was equol (OR 1.43; 95% CI 1.14-1.80; p=0.002) and ENL (OR 1.39; 95%CI 1.12-1.71, p=0.003) individually. However, after additional adjustments for covariates, such as age, height, weight, physical activity, social class, family history of prostate cancer, and daily energy intake, the associations became non-significant, suggesting that the lignan effect was confounded with other variables.

Another case-control study, conducted in Jamaica, consisted of 175 newly diagnosed prostate cancer cases and 194 controls.[94] The researchers evaluated the relationship of urinary phytoestrogens with total prostate cancer and tumor grade. ENL was measured from spot urine samples. Higher concentrations of ENL were positively associated with both total prostate cancer (OR 1.85; 95% CI 1.01-3.44; p= 0.027) and high-grade disease (OR 2.46; 95% CI 1.11-5.46; p= 0.023).

The small sample size and limitations inherent in a case-control design advocates caution in espousing the results without follow-up studies, but differential findings based on tumor grade speak to the importance of considering the heterogeneity of cancers and the potential for different effects depending on tumor grade. To date, only a few studies have been statistically powered to be able to consider the association between lignans and cancers, stratified by tumor type or stage.

Cardiovascular Disease

A review of lignans and cardiovascular disease risk published in 2010 by Peterson *et al.* summarized the findings of both randomized controlled trials (RCTs) and observational

studies.[57] While some of the RCTs showed no effects, many showed favorable effects of lignans to blood pressure, C-reactive protein, and lipid profiles. The authors also discussed both observational studies examining lignan intake as well as observational studies examining serum ENL; however, similar to the studies of cancer outcomes, the results of the studies of cardiovascular disease are mixed, and it is difficult to draw definitive conclusions. Five out of eleven observational studies showed decreased cardiovascular risk with either increasing dietary intakes of lignans or increased levels of serum ENL, while five studies were described by the author as having borderline significance, and one was null.[57] The authors noted that a limitation to the capacity to conduct a systematic review of the interventions was the differences in experimental protocols that were used between the studies.

Since that review was published a 2012 cross-sectional study found that urinary enterolignans were inversely associated with serum triglyceride levels and positively associated with HDL ("good") cholesterol.[97] The authors used three models: an unadjusted model, a model adjusted for age, race/ethnicity, education, income, urinary creatinine (log-transformed), and a third model further adjusted for smoking and alcohol categories, menopause status, use of hormone replacement therapy, BMI, physical activity, and dietary intake of saturated fat, cholesterol, and fiber. The results were statistically significant in all three models, with higher urinary excretion of enterolignans (7.84 μmol/L) corresponding to lower serum triglycerides (-0.18 mmol/L) compared to the low levels of enterolignan excretion (0.54 μmol/L) in the third model (p=0.003). High excretion corresponded to 0.06 mmol/L greater serum HDL in the third model (p=0.009).[97]

Adding to the evidence that lignans have a beneficial effect on cardiovascular disease risk factors, a recent randomized, double-blind, placebo-controlled trial found that dietary lignans

decrease oxidized LDL cholesterol.[98] Thirty-seven participants (13 men and 24 women, mean age: 54 ± 7 years) were randomized to regular consume either a high-lignan flax nutrition bar containing 0.41 g lignans, a regular-lignan flax nutrition bar containing 0.15 g lignans, or a soybean nutrition bar. α-linolenic acid levels in both flax bars were equal. Nutrition bars were consumed twice daily for 6 weeks. The high-lignan flax bar decreased serum oxidized-LDL from 68.2 ± 24.2 to 51.1 ± 12 mU/L (p=0.036). This result was significantly different from the regular-lignan flax bar (p=0.004).[98]

Conversely, a randomized, double-blind, placebo controlled trial conducted in children (n=32) suggests deleterious effects of flaxseed on lipid levels.[99] Participants in this study were children aged 8-18 years with hypercholesterolemia who consumed muffins and bread fortified with ground flaxseed for four weeks equal to 30 g dietary flaxseed daily. Controls (n=16) were fed whole wheat muffins and bread with closely matched for energy and nutritional content. The flaxseed intervention group had a 7.35 mg/dL decrease in HDL-C (95% CI -3.09 to -11.60 mg/dL; p=.001) and an increase of 29.23 mg/dL in triglycerides (95% CI 4.43 to 53.14 mg/dL; p=.02).[99] Whether this effect is due to α-linolenic acid, fiber, or the lignan content of flaxseed is unclear.

Miscellaneous Health Effects

In males ENL is found in higher concentrations in semen and prostate fluid, relative to blood plasma.[100–102] Because of this, and due to its estrogenic effect, there has been a concern that ENL may have an effect on fertility. One of the first studies to test this measured sperm motility prior to and following the addition of a solution containing ENL on human semen.[100] However, no changes in motility were reported. More recently, Chinese researchers published a case-control study that evaluated urinary phytoestrogens (including SECO) and analyzed fresh

human semen for semen volume, sperm concentration, sperm number per ejaculum and sperm motility.[103] The results indicated urinary SECO was associated with male infertility (OR 2.14; 95% CI 1.42-3.24; *p* trend= 0.0001); ENL, however, was not measured.

Pietrofesa *et al.* conducted a study where mice were given x-ray treatment and fed isocaloric diets containing 0%, 10%, or 20% lignans from flaxseed.[104] The lignan component was designed to mimic the amount of lignans ingested from a 0%, 10%, and 20% whole grain flaxseed diet. The lignan diets significantly mitigated radiation-related animal death (controls demonstrated 36.7% survival 4 months after the treatment compared to 60–73.3% survival in mice fed 10%-20% lignans). Additionally, lung fibrosis, radiation-induced lung injury (measured via bronchoalveolar lavage), and inflammation was decreased in the mice fed flaxseed lignans.[104] This would suggest that dietary lignans may help mitigate adverse effects in individuals exposed to radiation.

Dietary Factors Influencing Enterolignan Production

Diet plays a large role in enterolignan production, both due to the differences in food content of the precursors, but also due to effects of food matrices and other dietary factors on availability and conversion of plant lignans to enterolignans. An early study, by Adlercreutz *et al*. evaluated urinary enterolignan excretion in women consuming macrobiotic, omnivorous, and lactovegetarian diets and reported that followers of macrobiotic diets, as compared to the lactovegetarians, had substantially greater END and ENL excretion.[105] The lactovegetarians in turn, as compared to the omnivores, had greater excretion of enterolignans.

Since that early work many dietary intervention studies have been conducted further solidifying the link between diet and enterolignan production. Fruits, vegetables, and berries

have been demonstrated to increase ENL production.[106–108] Rye, a rich source of plant lignans, has been shown to increase ENL production in three dietary intervention studies.[109–111] However, a similar intervention study involving rye products showed no such increase in ENL production.[112] The reason for this discrepancy is unclear; however, the authors note that their participants were generally younger and may have consumed more dietary fiber in their habitual diets than participants of the other studies. This may indicate that the participants had faster lignan transit through the gut, and therefore incomplete biotransformation of the plant lignans.[112] Other dietary trials involving lignan-rich flaxseed and sesame seed revealed significant ENL production.[40,41,113–116]

Moreover, it appears that the food matrix and food processing may affect the bioavailability of lignans. In a randomized, crossover study, 12 participants were fed whole, crushed, and ground flaxseed daily (0.3 g/kg body weight). The results indicated that, compared to ground flaxseed, the mean relative bioavailability of enterolignans from whole flaxseed was 28% and crushed flaxseed was 43%.[117] Another dietary study found no effect of baking on the urinary or plasma concentrations of enterolignans when participants were fed ground flaxseed mixed in applesauce or ground flaxseed baked in bread and muffins.[116]

Although it has yet to be studied systematically in humans, the presence of dietary fiber may affect the production of enterolignans. *In vitro* fecal suspensions incubated with 1g each of extractable and non-extractable fractions of rye produced starkly different results.[118] The extractable fraction contained mainly soluble fiber and 46 mg/100g of total plant lignans. The non-extractable fraction contained mainly insoluble fiber and 13 mg/100g of total plant lignans. Protein and starch amounts were very similar among the fractions. After 48 hours of incubation suspensions containing the non-extractable fraction produced significantly more ENL. The

extractable fractions rapidly produced short-chain fatty acids (SCFAs) which quickly decreased the pH of the samples below 5.0. The non-extractable fractions, on the other hand, produced SCFAs at a much slower rate, thus allowing pH to stay in a more neutral range favoring ENL production. The extractable fraction also contained more ferulic giving the samples containing it a lower initial pH.[118] This suggests that the type of fiber consumed with the lignan may affect its bioavailability.

Sex Differences in Enterolignan Production

The evidence regarding sex differences in lignan metabolism is mixed, but there appears to be a tendency for women to produce more enterolignans than men. Several cross-sectional studies examining the associations between diet and enterolignan production show no sex differences.[97,119–122] However, a cross-sectional study of 2380 Finnish men and women showed that women had higher concentrations of serum ENL than did men.[123] Indeed, plasma ENL and END both appeared earlier and also achieved a higher maximum concentration in women compared to men when following a single SDG dose (1.31 μmol/kg body weight).[66] Jacobs *et al.* conducted feeding study involving a whole-grain diet and found that women had a higher mean baseline serum ENL concentration, although the rise in serum ENL after the whole-grain diet was similar in men and women.[124] Another randomized cross-over trial that involved feeding men and women flaxseed and measuring urinary lignan excretion showed no differences between men and women.[125] Similar trials involving a lignan dose have showed no difference in enterolignan production between men and women.[109,117] In contrast, in one controlled feeding study, in which vegetables were fed, men excreted more ENL than women during the experimental diets.[108] The tendency here for women to produce more enterolignans than their male counterparts may be explained by Clavel *et al.* who noted that women tended to harbor

more ENL-producing and END-producing bacteria.[126] Further, women have been shown to have longer gastrointestinal transit times than men[127]; this longer residence time of fiber-containing foods and plant lignans in the gut may further contribute to greater enterolignan production.

Non-Dietary Factors Associated with Enterolignan Production

Physiologic and sociodemographic factors have also been shown to be associated with enterolignan levels. Because of the importance of the gut microbial community to enterolignan production, the use of oral antimicrobials is inversely associated with serum ENL concentrations. In a cross-sectional study, Kilkkinen *et al.* showed, in a sample of 2,753 Finnish men and women, that ENL concentrations in antimicrobial users, as compared to non-users of antimicrobials, was significantly lower even if the time elapsed since antimicrobial treatment was 12-16 months prior to blood sampling.[128] The number of antimicrobial treatments also correlated inversely with ENL concentration.

Gut residence time appears to be another factor that influences ENL production, although most studies rely on non-quantitative measures to assess this factor. For example, by Kilkkinen *et al*., also in a Finnish population, observed positive associations in men with serum ENL concentration and constipation, perhaps demonstrating an association between longer residence time and more ENL production.[123] The same study also found that, in women, serum ENL concentration was positively and independently associated with age and constipation, while ENL was inversely associated with smoking. Moreover, female subjects of normal weight had a significantly higher ENL concentration than their underweight or obese counterparts. Other studies in European populations have supported Kilkkinen's findings, reporting inverse associations between body mass index (BMI), smoking, and frequency of bowel movements and plasma ENL concentration.[122,129]

Recently, the association between sociodemographic and other lifestyle variables and urine ENL levels was evaluated in a large sample of men and women ≥20 y in the US. In a subset (n=~3000) of the 2003-2006 National Health and Nutrition Examination Survey (NHANES), which collects cross-sectional data on the health and nutritional status of the U.S. population, Rybak *et al.* found that age, income, and physical activity were positively correlated with urinary ENL.[130] Further, similar to the studies in European populations, smoking and BMI were inversely correlated with urinary ENL. Despite these associations, the selected sociodemographic and lifestyle variables only explained a limited amount of the total variability (R^2 ≤4%), suggesting that, compared to other factors, their effects on enterolignan levels are modest.

Despite all of this research, very little has involved the characterization of the differences between high and low producers with regard to what makes their bacterial communities different. Likewise, there has been some work on trying to identify new strains of lignan-converting bacteria, but presumably there could be many more. Therefore, the development of a method that enhances the growth of these bacteria is necessary for their study, and, by extension, study regarding the human bioavailability of these important compounds.

Aim

The aim of my thesis was to develop an appropriate anaerobic culturing method to be used to measure *in vitro* enterolignan production by bacteria from stool samples, and also to promote the growth of lignan-converting bacteria for sequencing and measuring bacterial gene expression. This work resulted in a platform that optimized techniques for the production of enterolignans and, presumably, the growth of lignan-converting intestinal bacteria.

Materials and Methods

The investigation of enterolignan production by human intestinal bacteria required the combined use of culture-based techniques to cultivate lignan-converting bacteria, chemical techniques to separate, identify, and quantitate lignan substrates and their products, and molecular and microscopy techniques to estimate bacterial growth by measuring gene copy number.

Media

Several different media preparations were tested for their capacity to support growth of enterolignan producing bacteria. (Table 2) All media was pH adjusted to 7.3 and autoclaved at 121°C for 20 min prior to use. In all preparations of media glucose, cysteine, vitamin solutions, and trace element solutions (Table 3) were added via sterile-filtration (0.20 μm) after autoclaving and cooling.

Table 2 Components of media used

Media	SHIME	LCM	HCM	TCAP	TCAP2	TCAPY
	g/L	g/L	g/L	g/L	g/L	g/L
Arabinogalactan	1.0	0.5	2.0	-	-	-
Bile salts no.3	-	0.4	0.4	-	-	-
Casein	-	3.0	3.0	-	-	-
Cysteine	0.5	0.8	0.8	0.25	0.25	0.25
Glucose	0.4	-	-	0.9	0.9	0.9
Guar gum	-	0.5	1.0	-	-	-
Hemin	0.005	0.05	0.05	-	-	-
Inulin	-	1.0	1.0	-	-	-
Mucin	4.0	0.5	4.0	-	-	-
Pectin	2.0	0.5	2.0	-	-	-
Peptone water	-	0.5	5.0	-	-	-
Proteose Peptone	1.0	-	-	-	-	-
Resazurin (1 mg/L)	1.0	1.0	1.0	1.0	1.0	1.0
Starch (potato)	3.0	0.5	5.0	-	-	-
Tryptone	-	0.5	5.0	-	-	-
Tween 80	-	-	-	-	-	-
Xylan (Oat-spelt)	1.0	0.5	2.0	-	-	-
Yeast extract	3.0	4.5	4.5	-	-	0.6
$FeSO_4 \cdot 7H_2O$	-	0.005	0.005	-	-	-
KCl	-	4.5	4.5	-	-	-
KH_2PO_4	-	0.5	0.5	-	-	-
$CaCl_2 \cdot 6H_2O$	-	0.15	0.15	-	-	-
$NaHCO_3$	0.4	1.5	1.5	-		
NaCl	0.08	4.5	4.5	2.78	2.78	2.78
K_2HPO_4	0.04	-	-	0.42	0.42	0.42
$CaCl_2$	0.008	-	-	-	-	-
$MgSO_4 \cdot 7H_2O$	0.008	1.25	1.25	-	-	-
$Na_2HPO_4 \cdot 7H_2O$	-	-	-	3.3	3.3	3.3
$NaH_2PO_4 \cdot 2H_2O$	-	-	-	1.2	1.2	1.2
$(NH_4)_2SO_4$	-	-	-	1.2	1.2	1.2
$MgCl_2 \cdot 6H_2O$	-	-	-	0.08	0.08	0.08
KH_2PO_4	-	-	-	0.16	0.16	0.16
CH_3COONa	-	-	-	-	0.5	-
HCOONa	-	-	-	-	0.5	-
Trace element solution	1 mL	1 mL	1 mL	10 mL	10 mL	10 mL
Vitamin solution	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL

Table 3 Components of the trace elements and vitamins solution

Trace Element Solution	mg/L	Vitamin Solution	mg/L
$MnSO_4 \cdot 2H_2O$	500	Menadione	1.0
$FeSO_4 \cdot 7H_2O$	100	Biotin	2.0
$CoSO_4$	100	Pantothenate	10.0
$ZnSO_4$	100	Nicotinamide	5.0
$CuSO_4 \cdot 5H_2O$	10	Vitamin B_{12}	0.5
$AlK(SO_4)_2$	10	Thiamine	4.0
H_3BO_3	10	ρ-Aminobenzoic acid	5.0
$Na_2MoO_4 \cdot 2H_2O$	10		
$NiCl_2 \cdot 6H_2O$	100		
Na_2SeO_3	10		

Following preparation, media was heated in a 70 °C water bath and 100% high-purity N_2 was bubbled through the media until the violet hue of the resazurin, an indicator of the oxidation-reduction potential, disappeared. (See Appendix, Figure 21) Typically this took 5-10 minutes for sparging 10-20 mL of media in culture tubes and 15-20 minutes for one liter of media. Sterile Luer-Lok needles attached to 0.2 micron disc filters were used to deliver gas to the base of the glass culture tubes so that it would then bubble-up through the media. A small, sterile plastic hose was used for one liter containers.

When exchanging the gaseous headspace in sealed containers the rubber cap was sterilized with ethanol, and two sterile, disposable needles were plunged through the rubber cap: one connected to a gas delivery hose attached with a 0.2 micron disc filter and the other needle to vent the escaping gas. Gas was passed through for ten minutes at a rate of 0.5-1.0 L/min. (See Appendix, Figure 22)

Measurement of Bacterial Growth

A standard curve was made to measure cell growth by correlating optical density (OD) with bacterial cell number. This was accomplished by making a serial dilution of an *E. coli* culture grown overnight in autoclaved Luria Broth (Becton Dickinson and Company, Franklin Lakes, NJ). The serial dilution was fixed by adding a 10% buffered formalin solution. Bacterial counts were estimated using a Petroff-Hausser counting chamber (Hausser Scientific, Horsham, PA), and optical density measurements were taken daily at 600 nm using a spectrophotometer designed to hold glass culture tubes (Spectronic Educator, Thermo Fisher Scientific, Waltham, MA).

Bacterial DNA was extracted using a DNA Stool Mini Kit (Qiagen, Venlo, Netherlands) using the manufacturer's recommended protocol. qPCR (7900HT Fast Real-Time PCR System by Applied Biosystems, Carlsbad, CA) was used to estimate bacterial copy number and cell concentration within samples. The DNA of bacterial 16S rRNA genes was amplified with universal primers 330F (5'-ACT CCT ACG GGA GGC AGC AGT-3') and 530R (5'-GTA TTA CCG CGG CTG CTG GCAC-3') using SYBR Green (Invitrogen, Carlsbad, CA). Reaction volume was 20 µL, and cycle conditions were as follows: 2 min at 50 °C; 10 min at 95 °C; [30 sec at 95 °C; 30 sec at 58 °C; 1 min at 72 °C] x40; 15 sec at 95 °C; 15 sec at 60 °C; and 15 sec at 95 °C.

Measurement of Enzyme Activity

Protein concentrations and β-glucosidase activity were determined similar to methods described by Flores *et al.* and Goldin *et al.*[131,132] Several grams of fresh stool were collected in cryogenic vials and frozen until thawed for assays. Protein quantification was performed using a

Bicinchoninic Acid Protein Assay Kit (Sigma, St. Louis, MO) following the manufacturer's recommended procedure. Briefly, 0.7 g thawed stool was combined with 7 mL lysate buffer described by Flores *et al*. [131] This resulting lysate was diluted 1:50. The diluted lysate (22 µL) and bicinchoninic acid solution (178 µL) were added to a 96-well plate, incubated on pre-heated 37 °C dry block for 30 minutes, and read at 562 nm on a SpectraMax spectrophotometer. β-glucosidase was used as a control.

β-glucosidase activity was determined using a β-glucosidase Assay Kit (Abnova, Taiwan) using the protocol recommended by the manufacturer. Briefly, 20 µL of stool sample in the lysate buffer along with 200 µL of a reagent containing p-nitrophenyl-β-D-glucopyranoside were combined in a 96-well plate, incubated on pre-heated 37 °C dry block for 30 minutes, and read at 405 nm on a SpectraMax spectrophotometer at 20 minutes.

Chemical Analysis of Lignans (HPLC, GC-MS)

Gas chromatography–mass spectrometry (GC-MS) was used to analyze the samples in the substrate experiment. Extraction and analysis of lignans was carried out using a variation of the method described in Frankenfeld *et al*.[133] For extraction, 0.3 mL sample aliquots were briefly vortexed and then centrifuged at 15,000 RPM for 10 minutes. A 0.1 mL aliquot of supernatant was then placed in to a separate glass screw cap tube which was filled with 2 mL of purified water (Barnstead Nanopure lab water system, Thermo Fisher Scientific, Waltham, MA). 20 µL of d-END (20 ng/µL) was then added to each mixture as an internal standard and was then ether extracted (3 mL x 2). The supernatants for each sample from the previous step were combined and blown dry under N_2 and the remaining residues baked at 105 °C for 15 minutes. After cooling in a vacuum dessicator, 100 µL of 15 % N,O-Bis(trimethylsilyl)trifluoroacetamide

(Thermo Scientific) in acetonitrile was added to each tube. Each was briefly vortexed and left to incubate at room temperature for 30 minutes in a vacuum desiccator. The samples were placed into vials and run on the GC-MS (Agilent: HP 6890 GC, HP 5973 MSD, Santa Clara, CA). The injection volume was 1 µL. Calibration standards were prepared and treated similarly.

The conditions of the GC-MS analysis was as follows: column used was a fused silica capillary, 12 m x 0.2 mm x 0.33 µm film thickness (SPB-1, Supelco, Bellefonte, PA); helium was the carrier gas with a flow rate of 1 mL/min; column temperature began at 100 ºC for 1 minute, ramp at 20º C/min to 290 ºC, and hold for 5.5 minutes; inlet temperature was held constant at 250 °C and the ion source and interface temperatures were 200 ºC and 250º C, respectively.

With the exception of the "Several Lignans" experiment, all other samples were analyzed by high-performance liquid chromatography (HPLC). Extraction proceeded as above, except that after the nitrogen evaporation step the sample was reconstituted in 100 µL of initial mobile phase. (See Table 4) Each tube was then vortexed and the contents vialed and injected on the instrument. The injection volume was 40 µL. Calibration standards were prepared and treated similarly. An Agilent 1100 series HPLC system was used for analysis, including a Luna C18 (2) (250 mm x 4.6 mm ID, 5 µm, Phenomenex, Torrance, CA). The flow was 1.5 mL/min for a 13-minute run time plus a 1.5 minute post-run. (See Table 4)

Table 4 HPLC gradient

Time (min)	% Mobile Phase A (0.05 % TFA in water)	% Mobile Phase B (acetonitrile)
0	90	10
5	60	40
8	60	40
10	90	10
13	90	10

Statistical Analysis

Differences in ENL production at the end of each experiment were tested for significance using a one way analysis of variance (ANOVA) or t-test. When ANOVA results were determined to be significant, Tukey's range test was employed for a *post hoc* comparison.

For the platform test with multiple lignan substrates, rates of ENL production were calculated using a non-linear one-phase exponential association model. The results were then compared using ANOVA. All statistical analyses were done using GraphPad Prism (GraphPad Software, Inc., San Diego, CA). *P* values were considered significant at < 0.05.

Experimental Design

Unless otherwise noted, all stool samples were collected from an individual previously determined to be a high enterolignan producer. Individuals were classified as high producers if they had a urinary enterolignan ratio [calculated as urinary concentrations of ENL/(END+ENL)] above 0.70. Freshly voided stool samples were collected in plastic tubs and within 30 minutes placed into a Bactron anaerobic chamber (Shel Lab, Cornelius, OR) consisting of a mixed gas environment of 5% H, 15% CO_2, and 80% N_2 (Airgas, Radnor Township, PA). Strictly anaerobic techniques were used to prepare media and fecal suspensions. Within the anaerobic chamber, fresh stool was weighed (10 g) and added to sterile reduced media (600 mL) containing ~20 glass beads and a magnetic stir bar. The stool was then manually separated using sterile utensils,

and mixed magnetically on a medium setting for fifteen minutes to make a fecal slurry. To break-up remaining chunks, the slurry was homogenized (Omni International, Kennesaw, GA) on low for four minutes and filtered through sterile cheesecloth into a sterile Erlenmeyer flask. Sterile pipets were used to aliquot the slurry into sterile 50 mL anaerobic serum bottles or 30 mL anaerobic tubes (Bellco Glass, Vineland, NJ), commonly referred to as Balch tubes on account of its inventor.[134] Lignan substrate dissolved in methanol was added to slurry and sealed using a serum bottle stopper and aluminum seal within the anaerobic chamber. Bottles containing slurry only and no substrate, slurry containing END substrate, and slurry containing ENL substrate were added as controls. The samples were removed from the chamber and the mixed gas within was replaced with 100% high-purity N_2 (Airgas, Radnor Township, PA) by passing the N_2 through the container for 10 minutes at a rate of 0.5-1.0 L/min.

Samples were kept in a rotating incubator (C24 Incubator, New Brunswick Scientific, Enfield, CT) at 37°C and 300 RPM for one week. Subsamples of the fecal suspensions were collected daily using anaerobic technique at approximately the same time of day and stored in 2 mL cryogenic vials (Corning, Corning, NY). The vials were stored at -20°C until extraction and analysis. No more than 1 mL was removed during sampling each day.

The Effect of a Simple Media Composition on Enterolignan Production

Three different culture media were developed by Lampe lab member Dr. Alex Pozhitkov based on the Mt broth used by Dr. Thomas Clavel.[135] The media developed were: a mineral salts media and glucose (TCAP), a mineral salts media with glucose, formate, and acetate (TCAP2), and a mineral salts media with glucose, formate, acetate, and yeast extract (TCAPY) (Table 2).

To compare ENL production on the different media and two different SDG sources, stool incubations of each were made using an inoculum injection delivery system (0.01% w/v). Three pea-sized scoops (approximately one gram each) were collected from a fresh stool specimen and placed into a cryogenic tube containing 10 mL TCAP2 and approximately 10 glass beads. The tube was manually shaken, vortexed, and homogenized on low for several seconds. **Injection Inoculum:** To make the inoculum for the experiments, 0.1 mL of this slurry was injected into a second container containing 10 mL TCAP2 media and used as the inoculum. Each culture tube, containing 13 mL of either TCAP, TCAP2, or TCAPY media was inoculated by injecting 0.5 mL of this inoculum into culture tubes using sterile, disposable tuberculin syringes (Becton, Dickinson and Company, Franklin Lakes, NJ). The final stool concentration in each tube (triplicates of either TCAP, TCAP2, or TCAPY media) was approximately 0.01% (w:v). SDG was extracted from flax meal (Barlean's Organic Oils, Ferndale, WA) using methanol and was added to each tube to obtain a final concentration of 274 μM in each tube. In addition, we tested a different SDG substrate made from a methanol extracted from flax meal manufactured by Khonest Bio-tech Co (Xi'an, China) in TCAP2 media. All samples were incubated for one week and sampled daily.

The Effect of Inoculation Approach on Enterolignan Production: Injection Inoculum vs. Batch Inoculum

The purpose of this experiment was two-fold: 1) to test a larger fecal inoculum (1.8% w/v), and 2) to test different approaches of inoculating the media using a **batch inoculum** stool processing method versus the **injection inoculum** method. **Injection Inoculum**: To make the inoculum for the experiments, 0.1 mL of this slurry was injected into a second container containing 10 mL TCAP2 media and used as the inoculum. Each culture tube was inoculated

with 0.5 mL of this solution was injected into culture tubes containing 13 mL of media using a sterile, disposable tuberculin syringes (Becton, Dickinson and Company, location). The **batch inoculum** was prepared by simply combining slurry and substrate into a 50 mL bottle containing a magnetic stir bar. The batch was mixed on a medium setting for five minutes and 10 mL were transferred into culture tubes and capped within the chamber.

We compared a larger inoculum (1.8% w/v) to a smaller inoculum (0.01% w:v) using either a filtered or non-filtered inoculum preparation approach. To prepare the larger inoculum, three pea-sized scoops (approximately one gram each) were collected from a fresh stool specimen and placed into a tube containing 9 mL TCAP2 and approximately 10 glass beads. The tube was manually shaken, vortexed, and filtered through cheese cloth as described above. 0.5 mL of this slurry was added to 9.5 mL TCAP2 making a 1.8% (w:v) dilution of the original stool. ENL production using this approach was compared to ENL production using both the filtered and unfiltered dilution to 0.018% described above inoculum. To compare the effects of different inoculation preparation (injection inoculum vs batch inoculum), a small 40 mL batch of 1.8% slurry was made to compare to the traditional method of injecting slurry and substrate to a previously prepared and capped culture tube. The batch inoculum was prepared by simply combining slurry and substrate into a 50 mL bottle containing a magnetic stir bar. The batch was mixed on a medium setting for five minutes and 10 mL were transferred into culture tubes and capped within the chamber. The mixed gas headspace from the chamber environment was replaced with N_2 after aliquots were made. All samples contained 274 μM of SDG extract and were incubated for one week.

The Effect of Inoculum Preparation by Homogenization or Filtration Enterolignan Production

A 10 g stool sample was collected and placed into a sterile beaker containing a magnetic stir bar, ~20 glass beads, and filled with TCAP2 media to make a total 600 mL slurry. While mixing, SDG was added to achieve an approximate concentration of 274 μM SDG in the slurry batch. After mixing, 300 mL slurry was filtered through sterile cheesecloth, and the remaining slurry was homogenized on low for four minutes. 10 mL and 20 mL of each type of slurry were transferred to culture tubes, while 40 mL was transferred to serum bottles. The headspace of each sample was replaced with N2 and incubated for one week with daily sampling.

The Influence of Gas Headspace Volume on Enterolignan Production

Fresh stool (10 g) was measured and placed into a sterile beaker and filled to a total volume 600 mL with TCAP2 media and mixed for 15 minutes. Following this mixing the slurry was homogenized on low for four minutes.

SDG was added to the slurry to a final concentration of 269 μM. Sterile pipets were used to distribute one set of three serum bottles filled with 20 mL (Group A) of the slurry and a second set filled with 40 mL (Group B). Treatment group A had a 3:2 headspace/slurry ratio and group B had a 1:4 headspace/slurry ratio (Figure 4 Group A. 50 mL serum bottles filled with 20 mL slurry.Figure 4 & Figure 5). For each treatment, control incubations included either 100 μM END or 20 μM ENL added to sterile 50 mL serum bottles in the appropriate headspace to slurry ratio. All bottles were capped inside the chamber, and their gaseous headspace was replaced with N_2 after passing gas through the samples for 10 minutes. Samples were incubated for one week inside a rotating incubator at 37 °C and 300 RPMs. 500 μL aliquots were taken daily for HPLC analysis, placed into 2mL Corning cryovials, and immediately frozen at -20 °C.

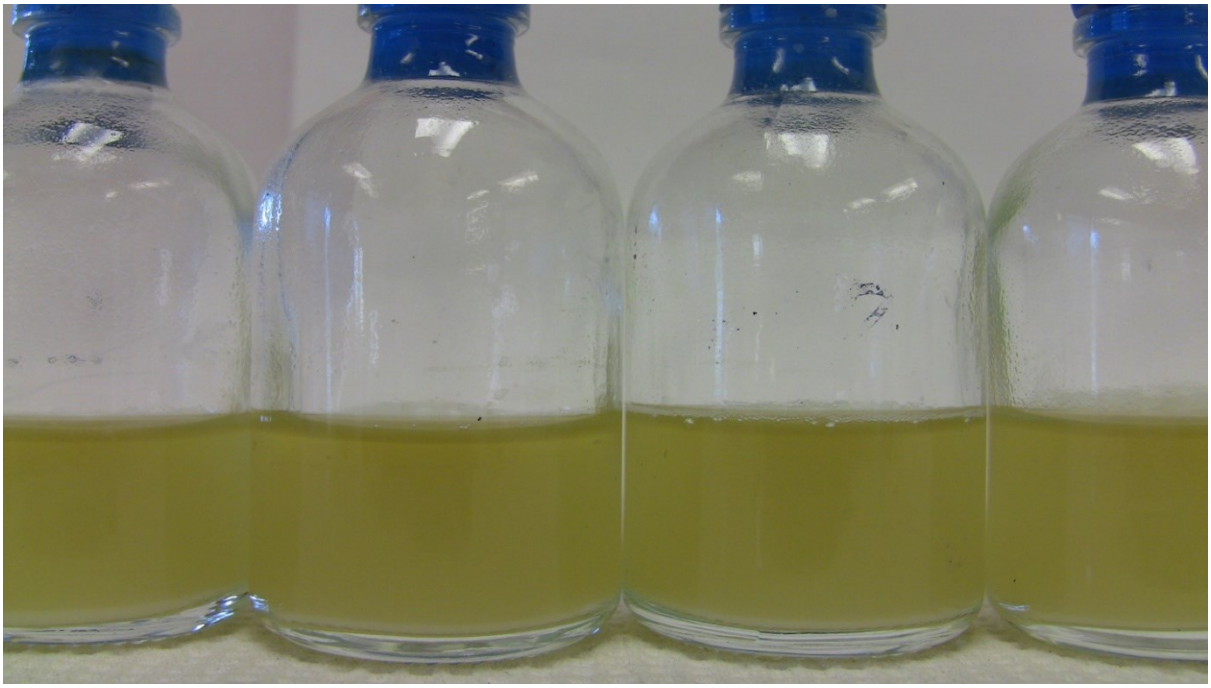

Figure 4 Group A. 50 mL serum bottles filled with 20 mL slurry.

Figure 5 Group B. 50 mL serum bottles filled with 40 mL slurry.

The Effect of Inoculum Size and Gas Composition on Enterolignan Production

The purpose of this experiment was to evaluate enterolignan production among four fecal inoculum concentrations of 3.33%, 1.67%. 0.3% and 0.03%. In addition, one set of samples was incubated with mixed gas (5% H_2, 15% CO_2, and 80% N_2) rather than pure nitrogen to determine if enterolignan production was dependent on the composition of the headspace. A 20 g stool section was taken and placed into a sterile beaker containing a magnetic stir bar, ~20 glass beads, and filled with TCAP2 media making 600 mL of a 3.33% (w:v) fecal slurry (Group A). 20 mL of 3.33% slurry was transferred to three 50 mL serum bottles. 10 mL of slurry was added to six 50 mL serum bottles and diluted with another 10 mL of media creating a 1.67% slurry concentration (Group B). 2 mL of slurry was transferred to three serum bottle and diluted with 18 mL of media creating a 0.3% slurry (Group D). Finally 200 µL of slurry was transferred to three serum bottles containing 20 mL media, making a 0.03% slurry (Group E).

Headspace gas composition: 474 µL of 11.54 mM SDG was added to each bottle making a 274 µM SDG concentration in all bottles, which were then capped within the chamber and removed. In three bottles containing a 1.67% inoculum, the headspace gas was replaced with a mixed gas consisting of 5% H_2, 15% CO_2, and 80% N_2 by passing the anoxygenic gas through the bottle for 10 minutes (Group C). The headspace gas was replaced in the remaining bottles (Groups A, B, and D) with 100% N_2 by passing the gas though the bottle for 10 minutes.

The Effect of Complex Media Composition on Enterolignan Production

This experiment was designed to evaluate enterolignan production in media used in previously published studies involving human intestinal bacteria. Enterolignan production was compared in four anaerobic culture media: a high-carbohydrate media (HCM) and a low-carbohydrate media (LCM) prepared as described by Kim *et al*[136], a media used in the Simulator

of the Human Intestinal Microbial Ecosystem (SHIME) prepared as described by Molly *et al* [137], and the TCAP2 media (Table 2).

For each media, an 8.3 g stool sample was measured and placed into a sterile beaker with a magnetic stir bar and glass beads. The beaker was then filled with media to a total volume of 500 mL mixed, homogenized, and filtered through sterile cheese cloth. SDG from a methanol extract from flax meal manufactured by Barlean's Organic Oils (Ferndale, WA) was added to the slurry to form a total concentration of approximately 274 μM SDG. 20 mL aliquots of slurry were transferred to three 50 mL serum bottles. All bottles were capped inside the chamber, and their headspace consisting of the gas from the anaerobic chamber (80% N_2, 15% CO_2, 5% H_2) was replaced with 100% high-purity N_2 by passing gas through the samples for 10 minutes. Samples were incubated for one week inside a rotating incubator at 37°C and 300 RPMs.

Several Lignans Experiment

The aim of this experiment was to test the newly developed anaerobic platform by measuring ENL production from five different lignan precursors: secoisolariciresinol diglucoside (SDG), secoisolariciresinol (SECO), pinoresinol (PINO), lariciresinol (LARI), and matairesinol (MAT). **Participants**: Stool specimens included in this set of incubations were collected from participants in the FlaxFX study (ClinicalTrials.gov identifier: NCT01619020). Eligible participants were healthy, non-smoking men and women aged 20-45 years with a low dietary intake of vegetables and fiber. Individuals were excluded if they had used antibiotics within the last 3 months, were pregnant or lactating, were taking any prescription medications (including oral contraceptives), had a history of chronic medical illness or gastrointestinal disorder, recent change in weight of 4.5 kg or more, or a daily intake of greater than two alcoholic drinks per day.

For this experiment stools from two FlaxFX study participants were used. Their samples are identified as 6128, classified as a high enterolignan producer, and 6129, classified as a low enterolignan producer. In addition, stool from the previously identified high-enterolignan producer whose samples were used for platform development are also included and identified as 5100.

1.67% fecal slurry was used for incubations following the established in-vitro protocol. All samples were incubated for six days and sampled daily using anaerobic technique.. The lignan spiking scheme was as follows: for the SDG samples 300 µL of 300 ng/µL SDG was added to achieve a final concentration of 6.553 µM SDG; for the SECO samples 150 µL of 300 ng/µL SECO was added to achieve a final concentration of 6.208 µM SECO; for the LARI samples 150 µL of 300 ng/µL LARI was added to achieve a final concentration of 6.243 µM LARI; for the PINO samples 150 µL of 300 ng/µL PINO was added to achieve a final concentration of 6.278 µM PINO; for the MAT samples 150 µL of 300 ng/µL MAT was added to achieve a final concentration of 6.278 µM MAT. Lignan substrate solutions were made with methanol.

END and ENL samples were also made as controls: for the END samples 130 µL of 300 ng/µL END was added to achieve a final concentration of 6.45 µM END; for the ENL samples 130 µL of 300 ng/µL ENL was added to achieve a final concentration of 6.54 µM ENL.

Chemical substrates for this experiment used racemic mixtures purchased from Sigma. All samples were incubated for six days and sampled daily.

Results

The graphs in this section represent arithmetic means of the three replicate samples, and the error bars represent one standard deviation. See Appendix for detailed data.

Modifications to General *in vitro* Method to Optimize Enterolignan Production

Simple Media

No ENL was produced in any of the media in these initial experiments; however, SECO and END production by the bacteria grown in the TCAP2 and TCAPY media was much greater than that grown in the TCAP media (See Figure 6.). These results suggest that the sodium acetate and sodium formate present in the TCAP2 and TCAPY media promotes the growth of lignan-converting bacteria. Moreover, the yeast present in the TCAPY media may promote the growth of bacteria capable of transforming SECO to END.

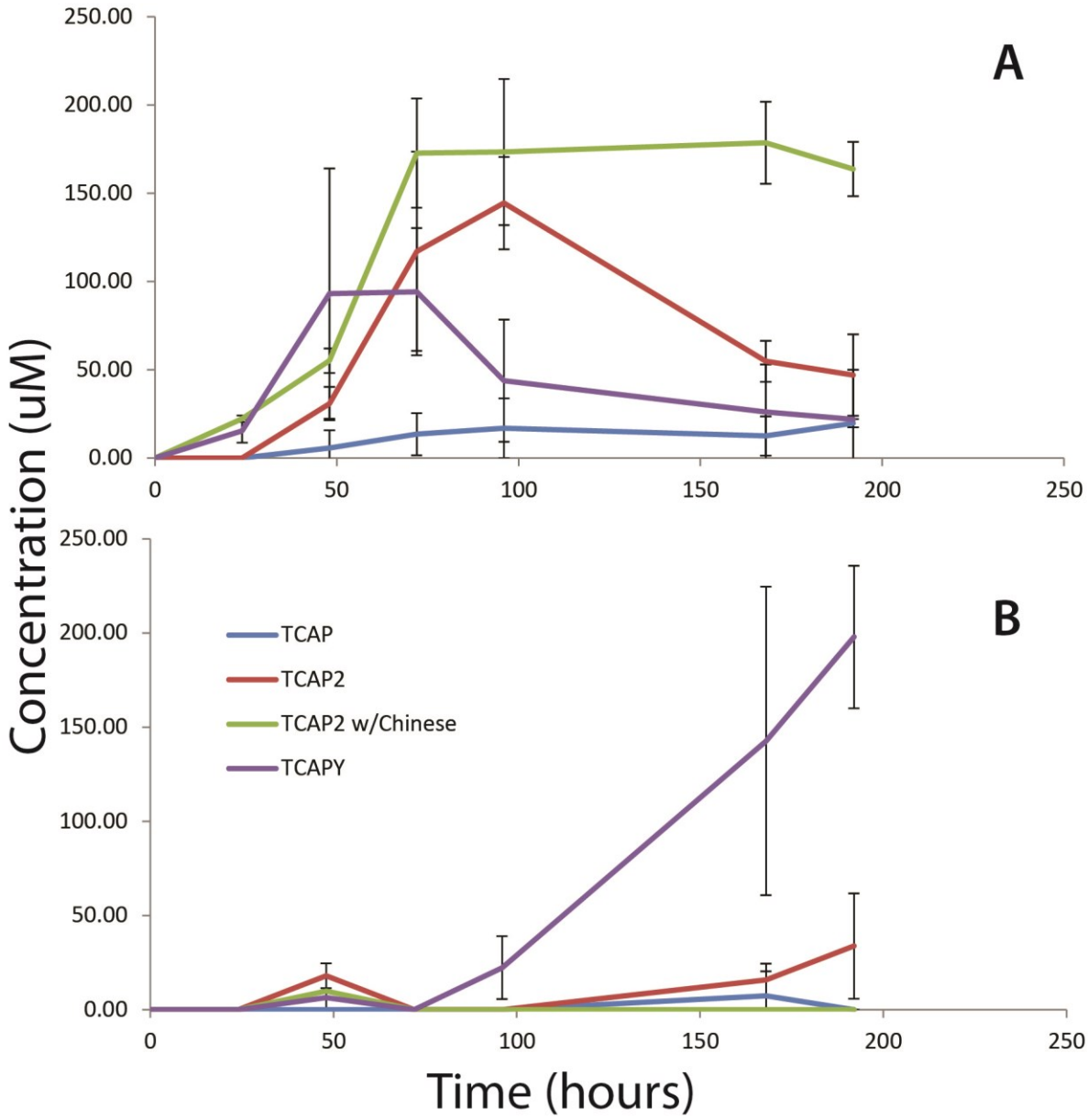

Figure 6. Graph A represents SECO production. Graph B represents END production. In this experiment no ENL was detected. SDG was used as initial substrate. Error bars indicate one standard deviation.

Injection Inoculum vs. Batch Inoculum Results

The increased fecal inoculum present in the injection and batch treatments clearly produce greater total SECO (data not shown), END, and ENL production. END and ENL production between the injection method and the batch method are similar (Figure 7); however, the batch

method had generally lower coefficients of variation (CV) and standard deviations (SD). Among 1.8% inoculums, mean batch CV: 19.27; mean injection CV: 32.24. (See Table 9 in Appendix) Differences in total ENL production between injection and batch method given 1.8% stool concentrations were not significant (p= 0.189).

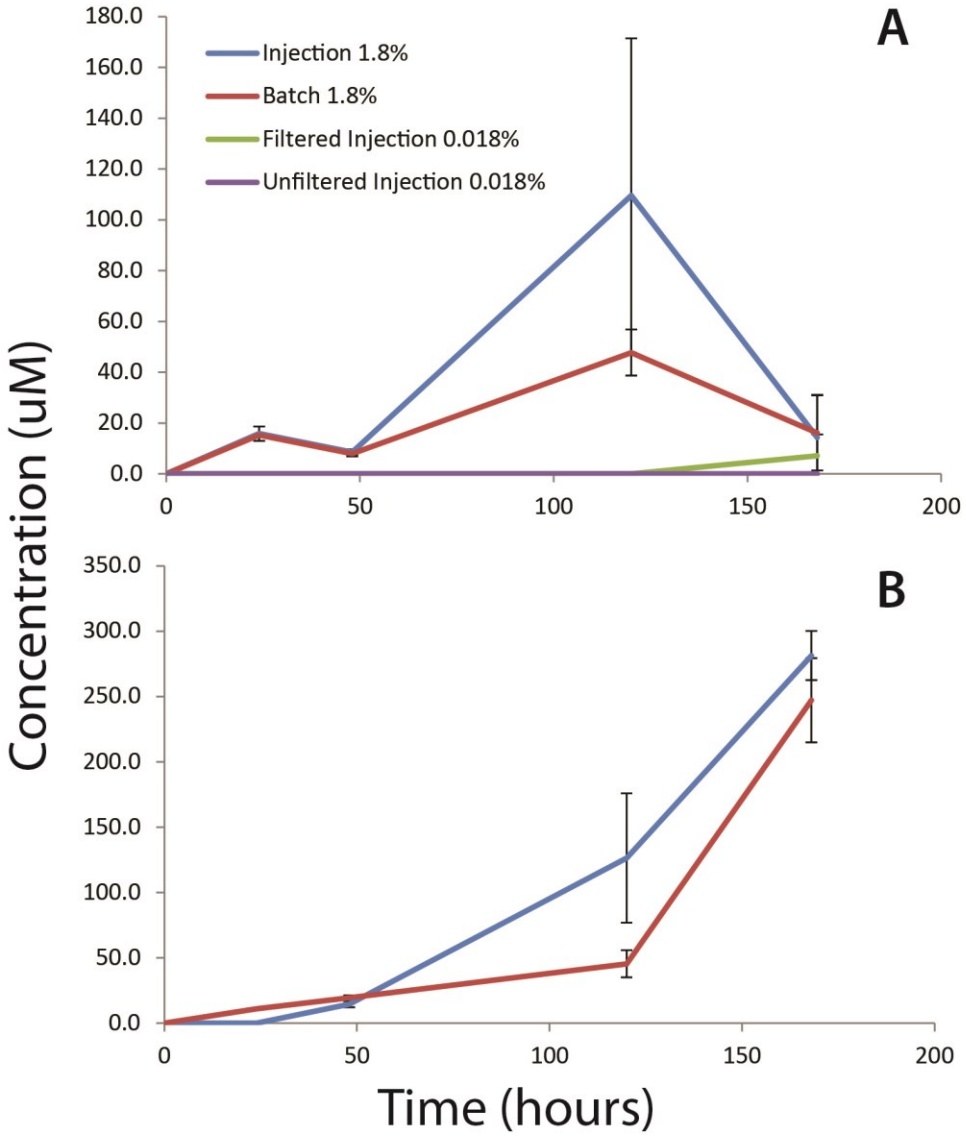

Figure 7 Enterolignan production between injection and batch processing methods. Graph A represents END production. Graph B represents ENL production. SDG was used as the initial substrate. There was no ENL production in the two 0.018% treatments. Error bars indicate one standard deviation.

Homogenization and Filtration Results

Total enterolignan production was relatively similar between homogenized and filtered treatments (Figure 8). Also, the 10 mL samples tend to have greater total enterolignan production than the 20 mL samples, which in turn have greater production than the 40 mL samples. (Figure 9) Coefficients of variation were also generally lower among the homogenized groups compared to the filtered groups. Mean homogenized CV: 15.02; mean filtered CV: 25.58. This data suggests that homogenization and perhaps a greater headspace:slurry ratio will lead to greater enterolignan production and greater uniformity between samples.

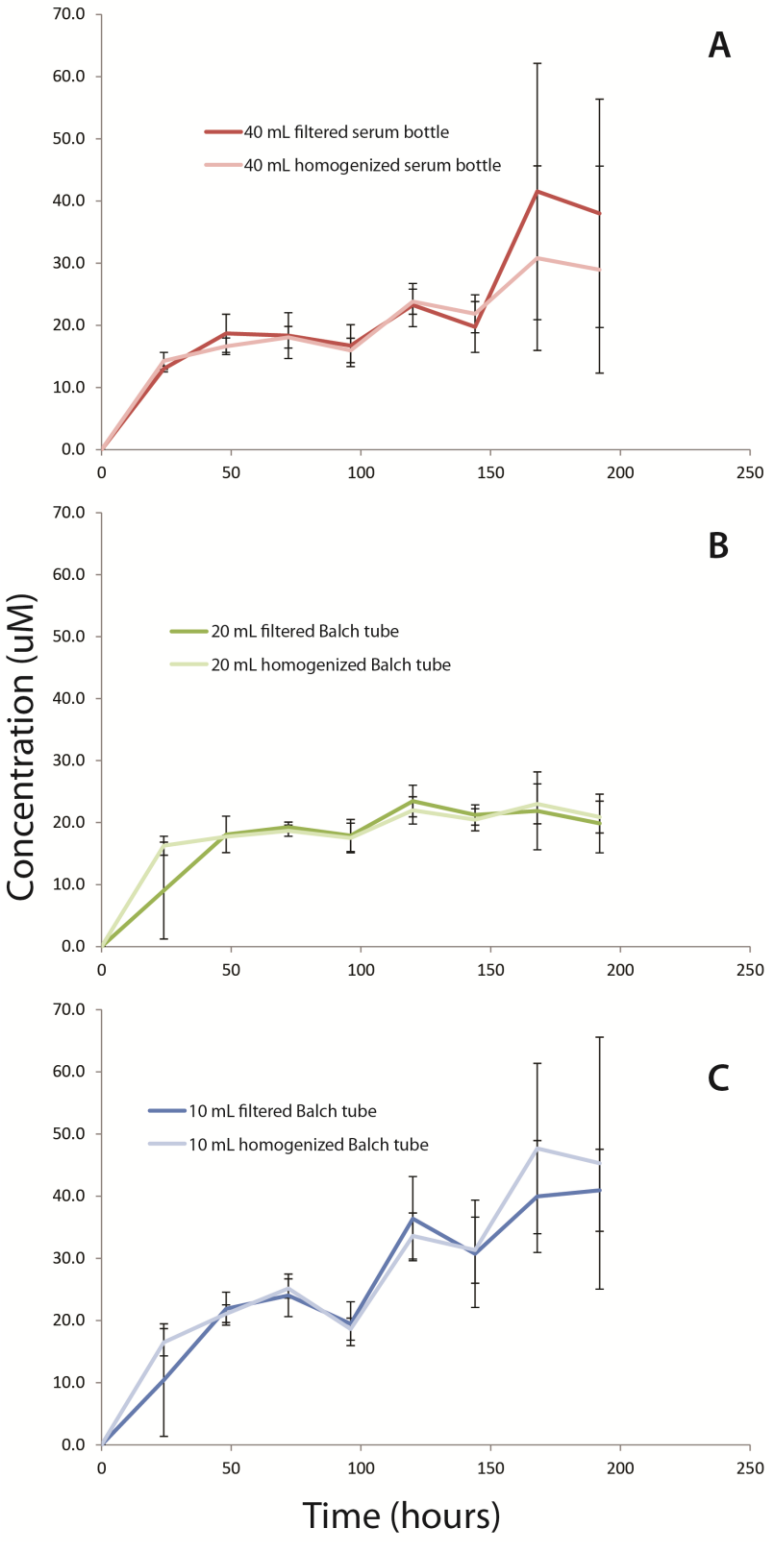

Figure 8 ENL production from SDG between the three volumes and among the two treatments. Error bars indicate one standard deviation. Filtering and homogenization show little difference with respect to ENL production.

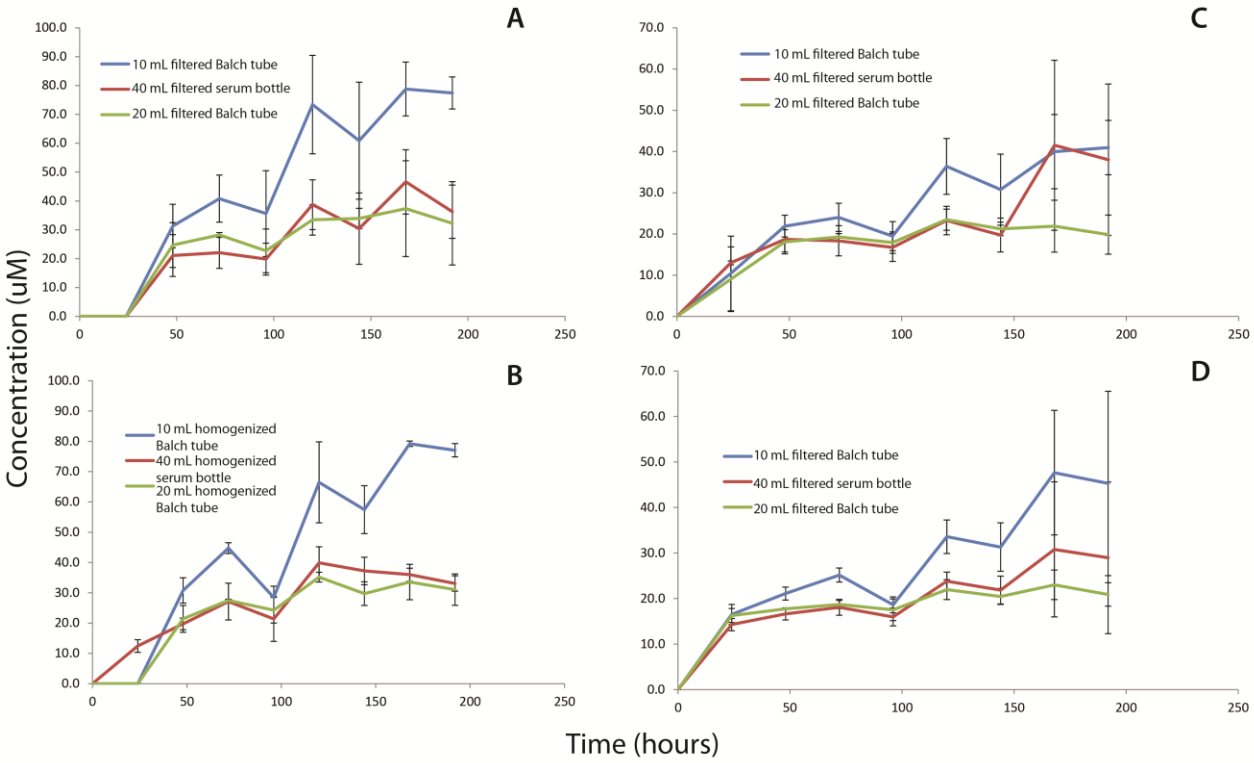

Figure 9 Graphs A and B represent END and ENL production from SDG, respectively within the filter treatment. Graphs C and D represent END and ENL production from SDG, respectively within the homogenization treatment. Error bars indicate one standard deviation.

Effect of Headspace

SECO (data not shown), END, and ENL were all produced in greater amounts in the samples containing 20 mL (Group A). Results are shown in Figure 10. A t-test was conducted to compare both treatments. There was a significant difference in the total amount of ENL by the end of the experiment between Group A (mean=41.24, SEM=2.461) and Group B (mean=24.54, SEM=0.9406); n=3, p=0.0032 (See Figure 11). The results of this experiment seem to indicate that a greater headspace/slurry ratio promotes more efficient growth of enterolignan-producing bacteria.

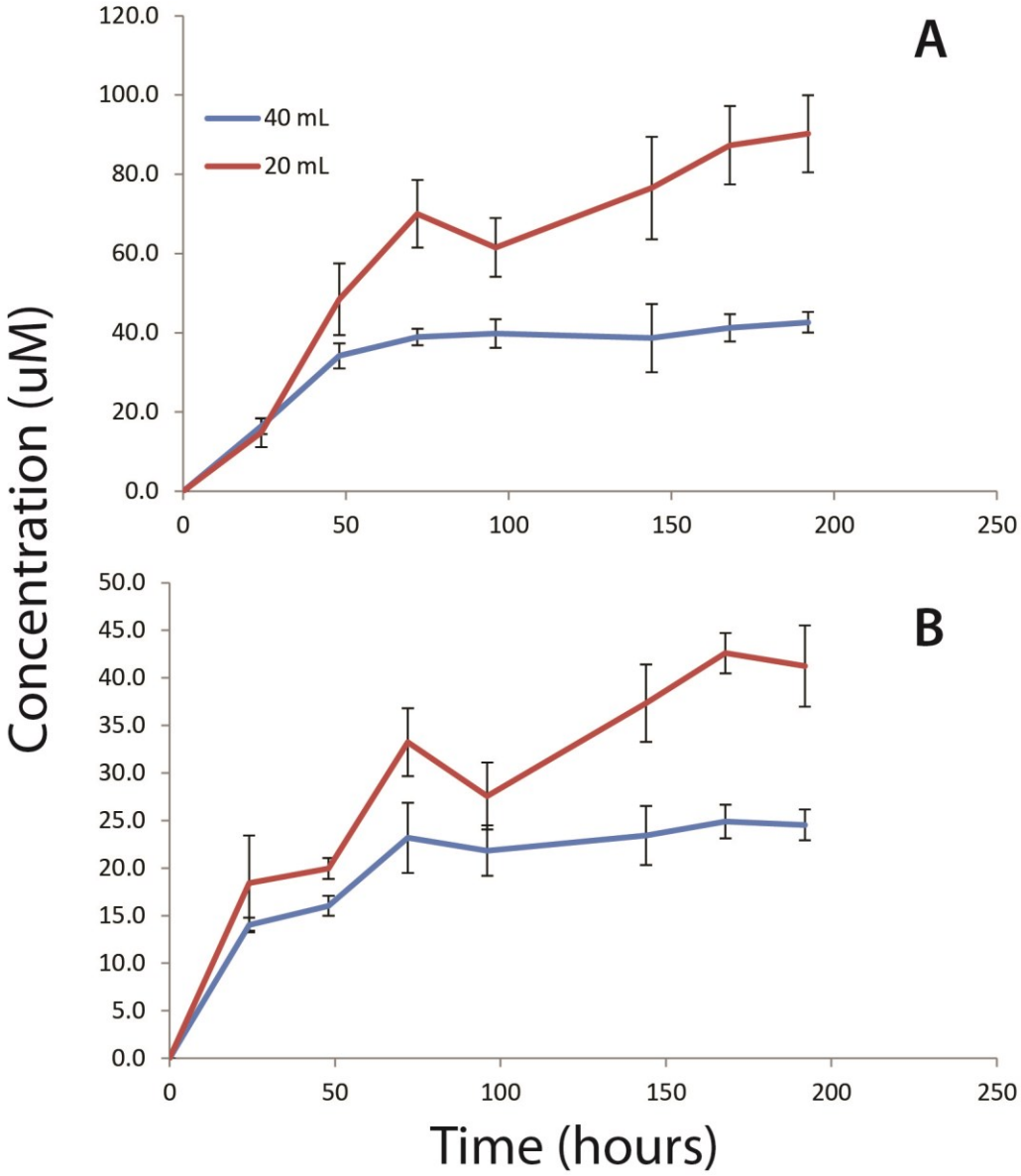

Figure 10 END (Graph A) and ENL (Graph B) production from SDG between volumes represented in graph A and B, respectively. Error bars indicate one standard deviation.

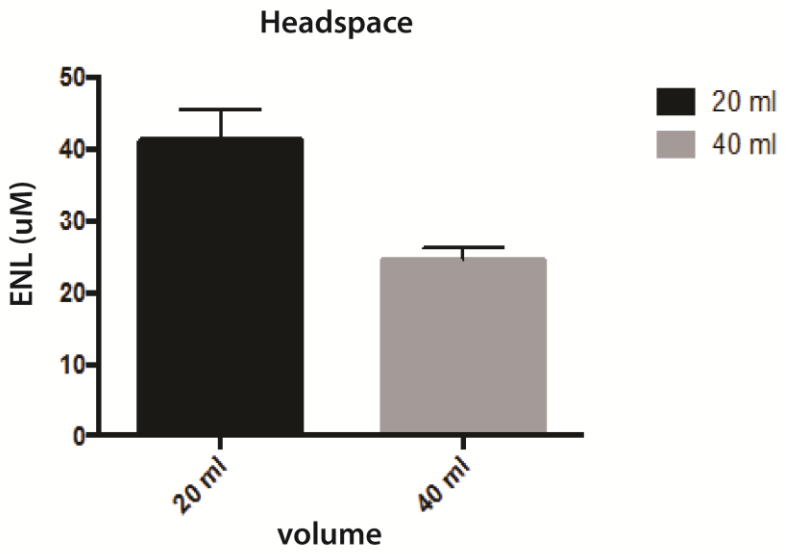

Figure 11 ENL concentration in culture at 192 hours. 20 mL treatment (mean=41.24, SEM=2.461) and 40 mL treatment (mean=24.54, SEM=0.9406) were significantly different (p=0.0032).

Serial Dilutions

Groups A, B, and C produced the most ENL with an average of 23.00 µM, 18.65 µM, and 19.11 µM, respectively, or between 6.8% and 8.3% conversion of the initial SDG by the end of the experiment (168 hours). Group D produced an average of 8.42 µM of ENL, or 3% of the initial SDG, by the conclusion of the experiment. Group E produced no ENL. The most END was an average of 50.84 µM produced by the Group C, or an 18.5% conversion. Groups A and B produced similar amounts of END by the end of the experiment. Group E produced no END. See Figure 12.

Statistical analysis indicated that total ENL production among Group A, Group B, and Group C were significantly greater than Group D (p=0.0033). See Figure 13.

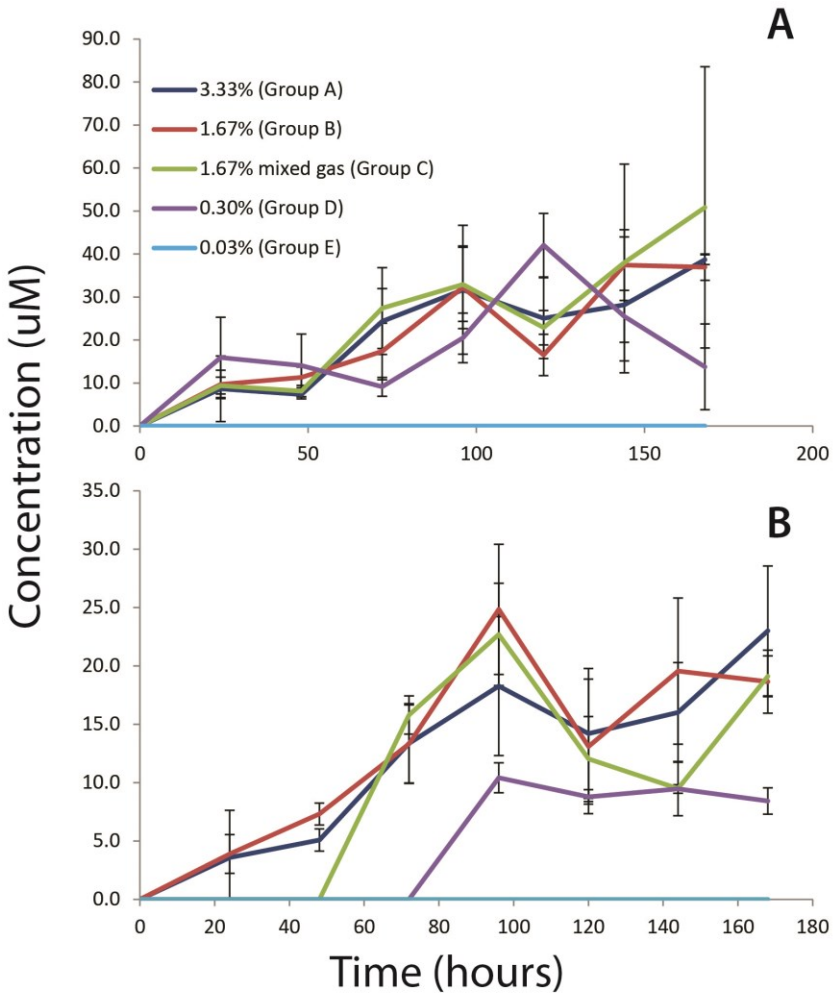

Figure 12 Effect of stool concentration on enterolignan production. Graph A (top) represents END production from SDG. Graph B (bottom) represents ENL production from SDG. No END or ENL was produced in Group E.

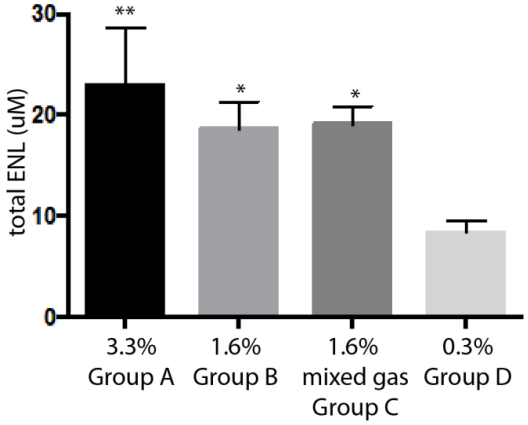

Figure 13 Differences in total ENL production between treatments.

Complex Media

The intestinal bacteria grown in SHIME media produced the most SECO, but no ENL (Figure 14). Conversely, the organisms grown in TCAP2 produced the most ENL and END, while all the SECO had been converted by the end of the experiment. The HCM organisms produced moderate amounts of SECO and END, but no ENL. The LCM was the only other growth media in which organisms produced any ENL. Differences in ENL production between TCAP2 and LCM treatments were statistically significant ($p<0.0001$).

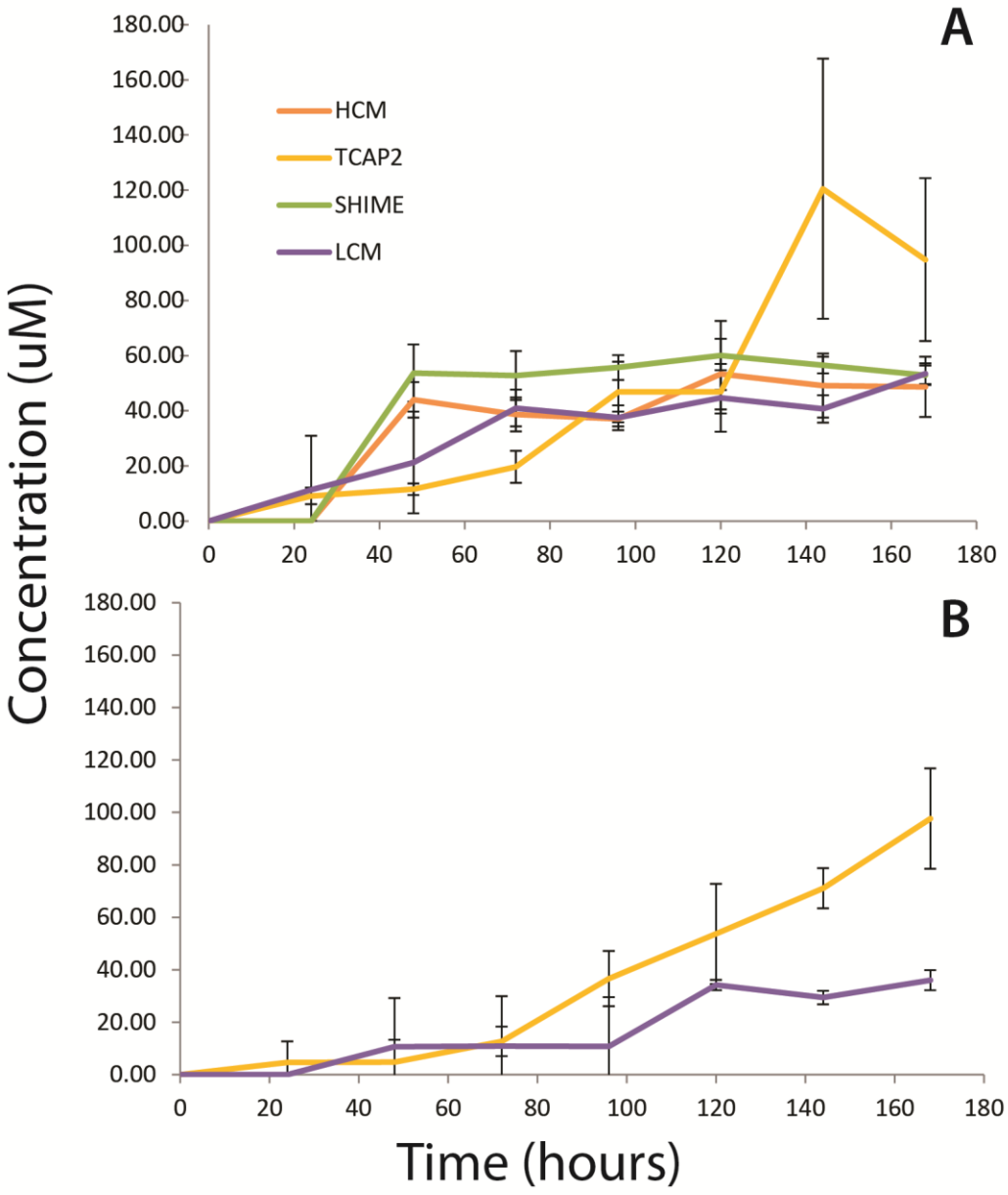

Figure 14 Comparing TCAP2 with more complex media used previously in fecal studies. Graph A represents END production from SDG. Graph B represents ENL production from SDG. There was no ENL production in the SHIME and HCM media. Error bars indicate one standard deviation.

The results of this experiment suggest that among these four media, TCAP2 is the best suited for promoting plant lignan conversion to ENL.

In summary, based upon the results of the previous experiments, we used the following standard approach for in-vitro incubation: 1) TCAP2 media, 2) 1.8% inoculum that is filtered and homogenized, 3) 100% N_2 gas, 4) a 3:2 headspace/slurry ratio.

Several Lignans Experiment

Between participants, lignan conversion to ENL is noticeably delayed with the organisms from the low producer, 6129, with the exception of MAT. Moreover, 6129 appears to produce more END than the high producers, regardless of the initial lignan substrate.

Total ENL production was compared using ANOVA and was adjusted for bacterial cell number and gram dry weight of stool. Significant differences in total ENL production were found both when adjusted for cell number (p=0.0010) and adjusted for gram dry weight (p=0.0010). A multiple comparisons test determined that ENL production from both SDG and SECO were significantly different from PINO and LARI. However, analysis of ENL production rates indicated no significant differences among lignan substrates. LARI was not included in the rate analysis due to ambiguous results fitting the LARI data to the model.

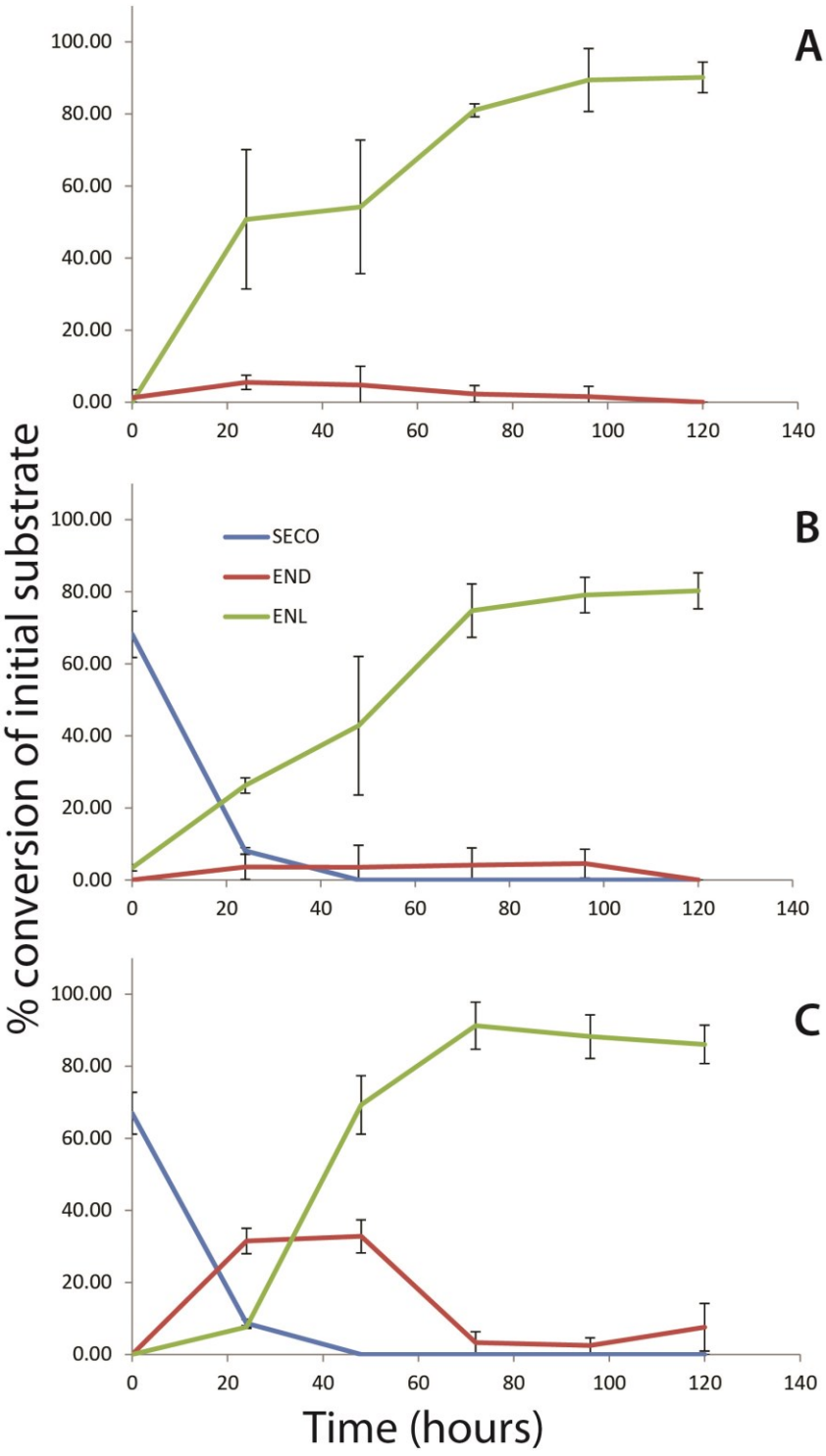

Figure 15 SDG conversion by intestinal bacteria of 5100 (A), 6128 (B), and 6129 (C).

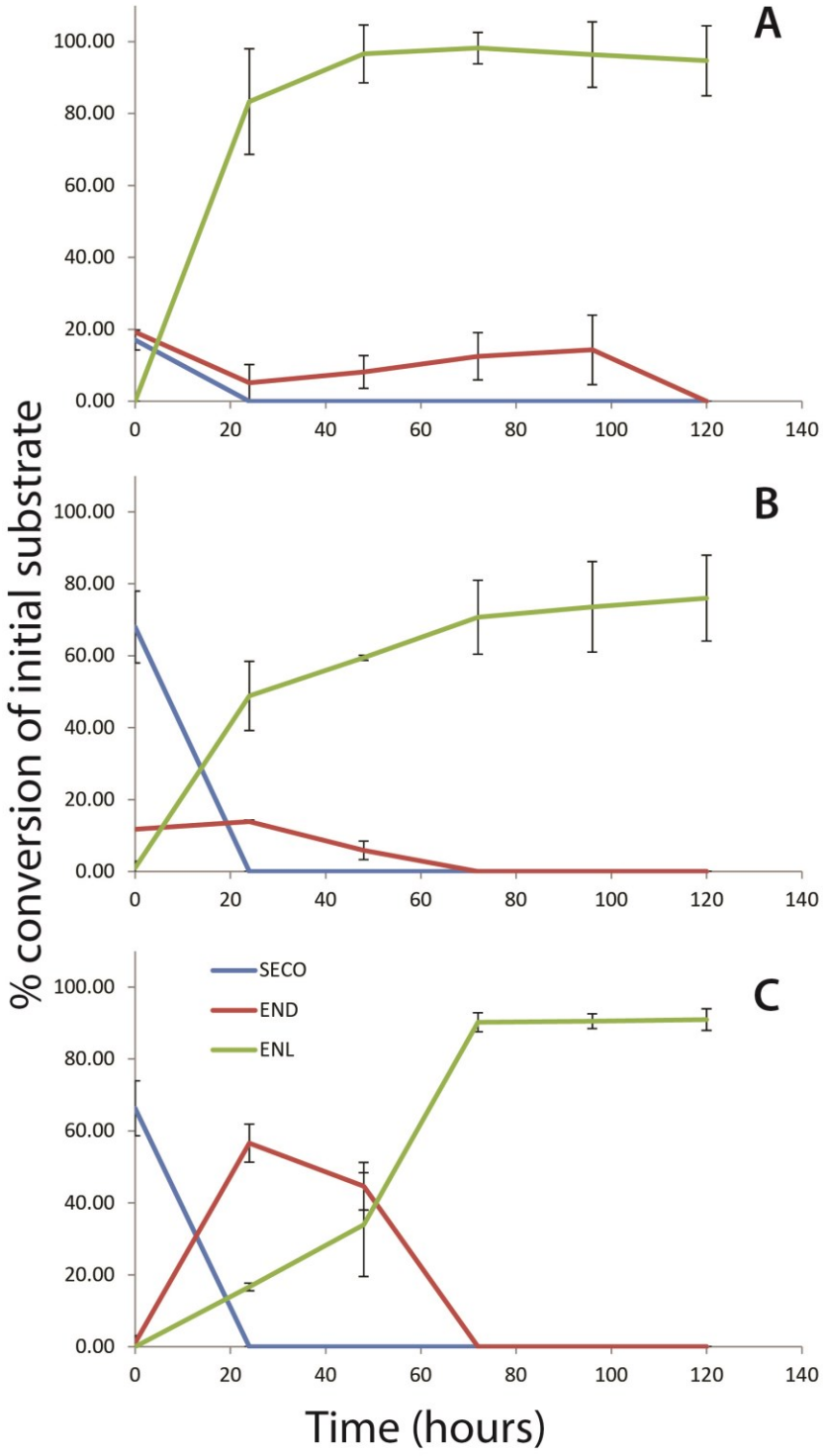

Figure 16 SECO conversion by intestinal bacteria of 5100 (A), 6128 (B), and 6129 (C).

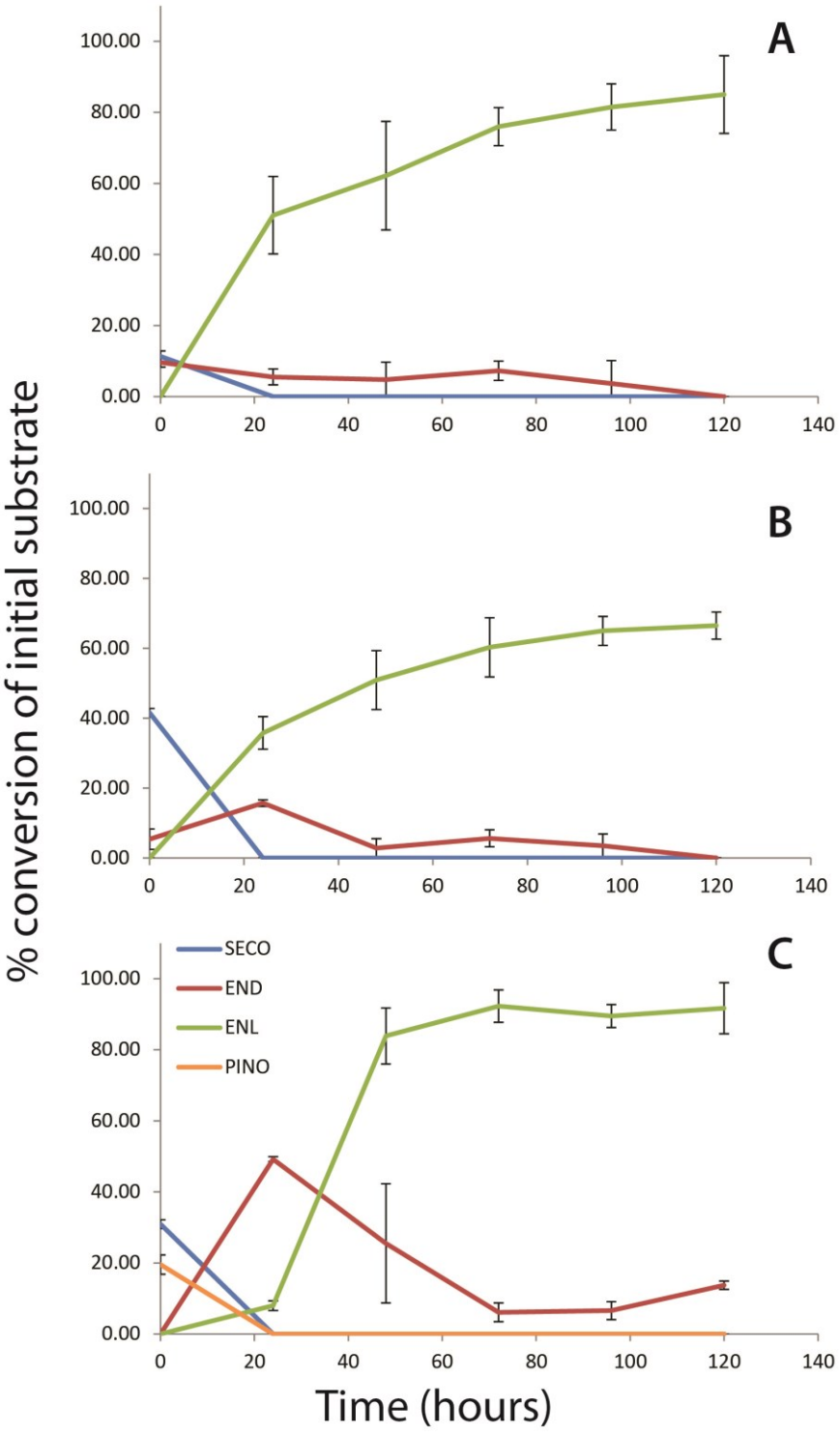

Figure 17 PINO conversion by intestinal bacteria of 5100 (A), 6128 (B), and 6129 (C).

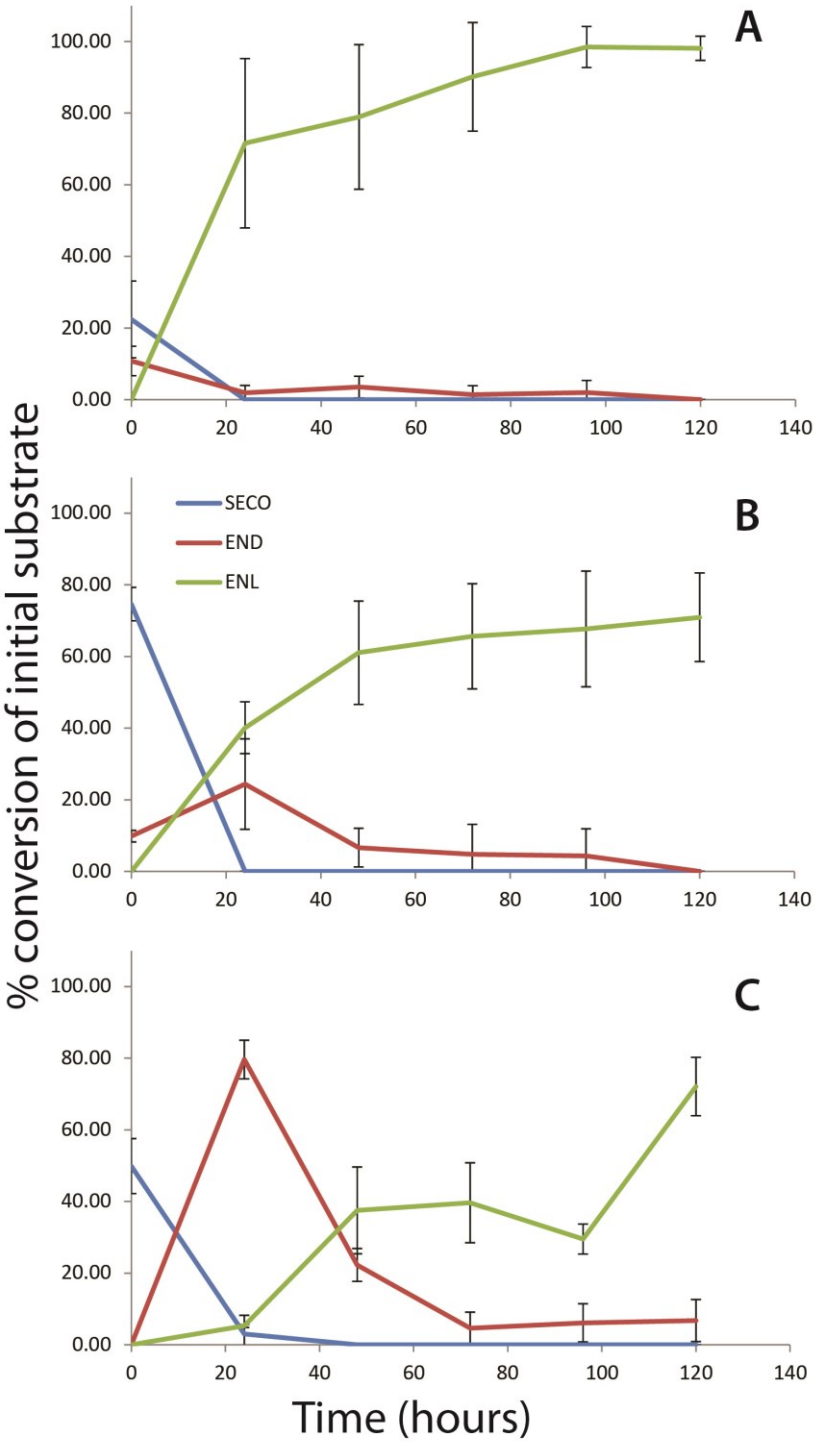

Figure 18 LARI conversion by intestinal bacteria of 5100 (A), 6128 (B), and 6129 (C).

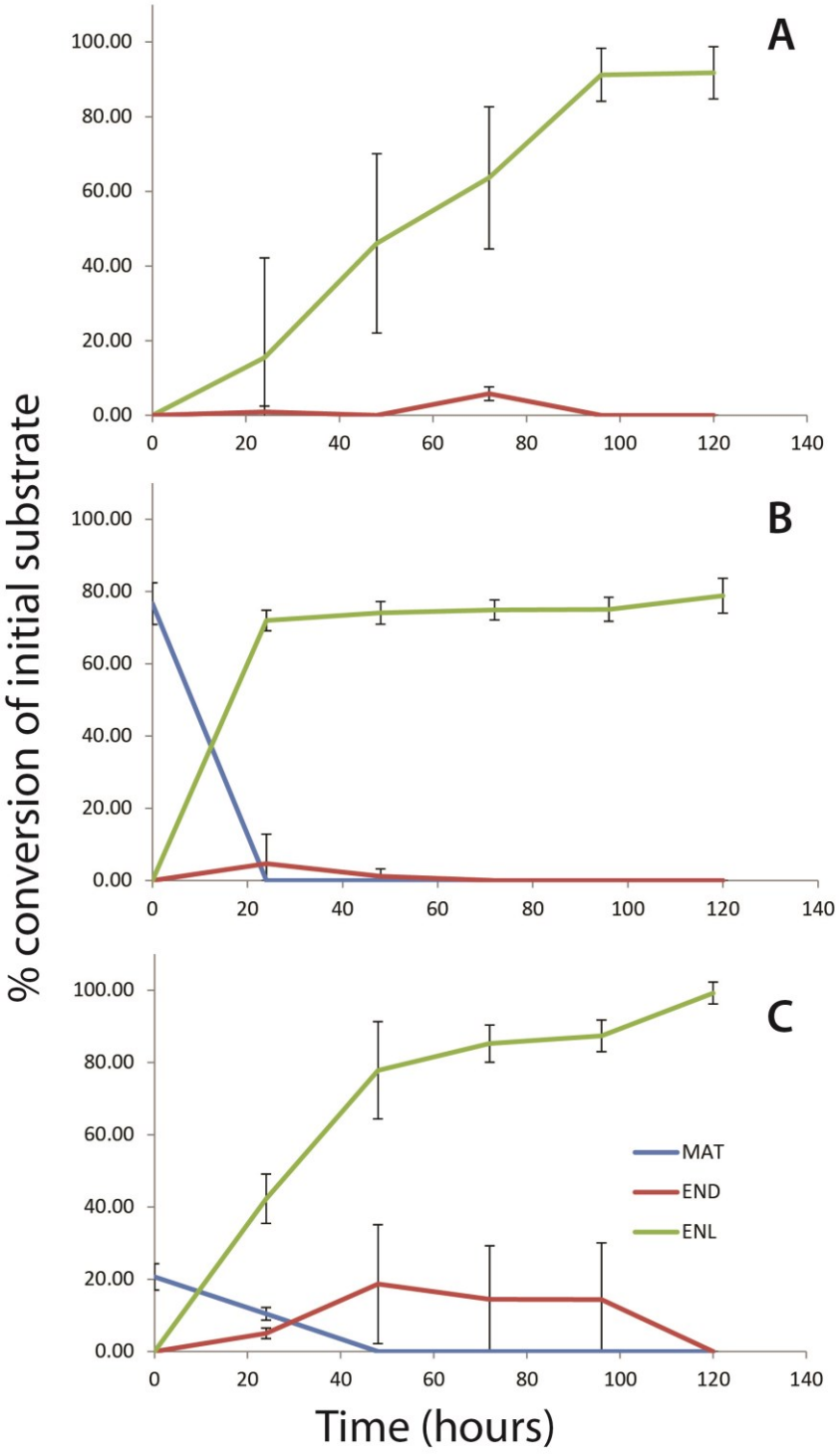

Figure 19 MAT conversion by intestinal bacteria of 5100 (A), 6128 (B), and 6129 (C).

Measurement of Growth

Among all participants, qPCR results indicate little, if any, net bacterial growth over the course of the experiment. This supports direct microscopic counts using a Petroff-Hausser counting chamber that were calculated on two samples with similar results (data not shown), as well as results published by Kim *et al*.[136] that showed similar growth with similar fecal inoculum concentrations. A limitation with estimating bacterial cell numbers using direct counts and qPCR is that it is unclear if the cells being counted are viable.

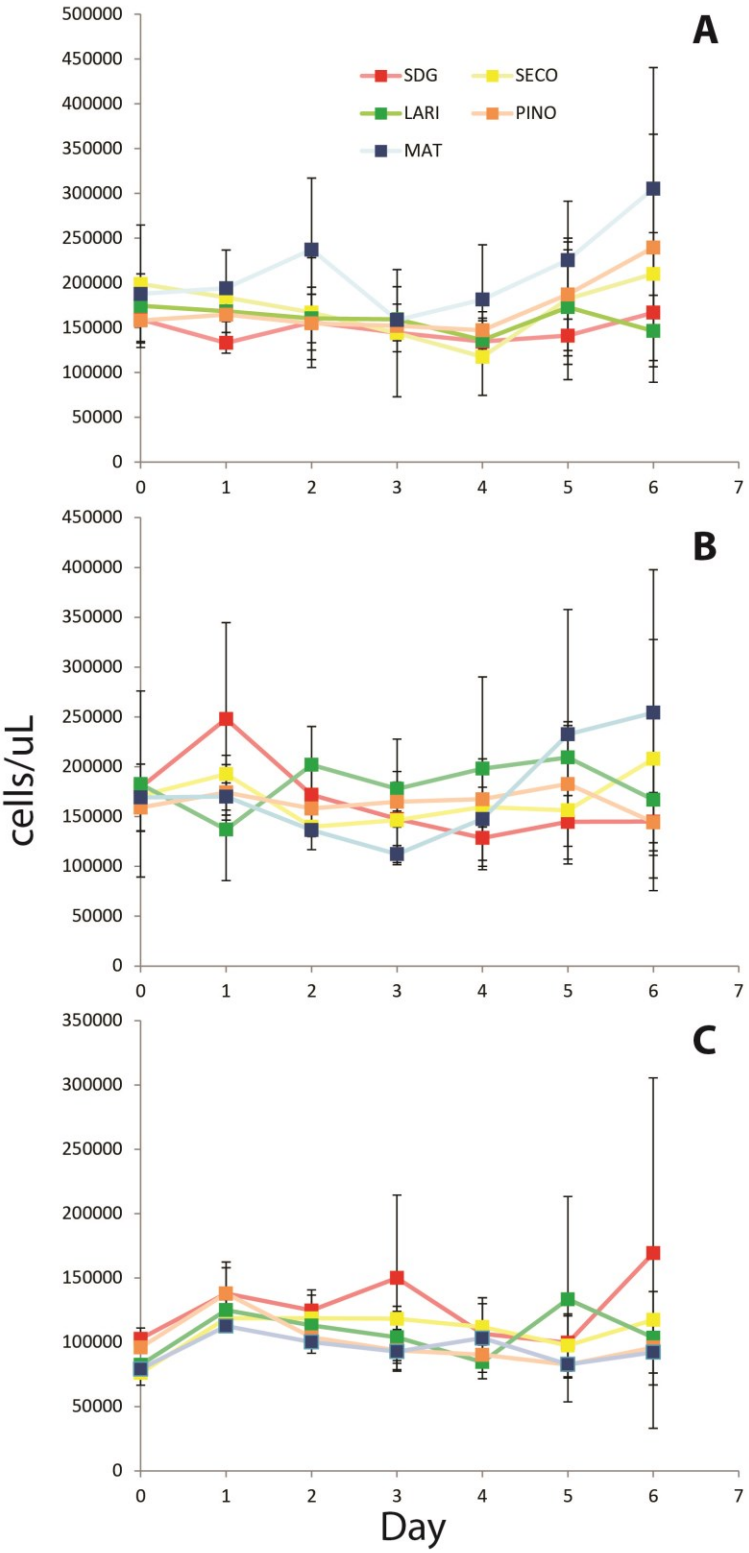

Figure 20 Estimated bacterial growth among the three participants. Graph A: 5100; Graph B: 6128; Graph C: 6129. Error bars indicate one standard deviation.

β-Glucosidase Activity

Table 5 & Table 6 show the results of β-glucosidase activity among the three participants. Units were measured from stool samples taken both from the beginning of the experiment as well as cells from pelleted slurry at the end of the experiment. These results indicate that all participants began with similar amounts of β-glucosidase. However, by the end of the experiment the low enterolignan producer, 6129, had a greater amount of β-glucosidase units per 100g of protein compared to the high producers. Yet, if calculated per gram dry weight of stool these β-glucosidase units from 6129 tend to be slightly less than the units from high producers.

Table 5 Initial concentration of beta-glucosidase in stool

Participant	Units of β-glucosidase	Units of β-glucosidase
	per 100 mg of protein	per gram dry weight of stool
5100	1.26	1.86
6128	1.56	2.37
6129	1.44	2.89

Table 6 Concentration of beta-glucosidase units at end of experiment

	5100		6128		6129	
per 100 mg of protein						
	mean	SD	mean	SD	mean	SD
No lignan substrate	4.94	0.05	6.31	0.43	7.18	0.19
SDG	4.43	0.04	5.95	0.37	6.80	0.75
LARI	4.61	0.27	5.64	0.31	6.49	0.43
PINO	4.09	0.37	5.64	0.52	6.42	0.16
SECO	4.43	0.14	5.33	0.09	5.60	0.59
MAT	4.97	0.34	5.49	0.12	6.10	0.24
per gram dry weight of slurry						
	mean	SD	mean	SD	mean	SD
No lignan substrate	0.89	0.01	0.68	0.01	0.61	0.12
SDG	0.97	0.06	0.79	0.07	0.76	0.02
LARI	1.11	0.14	0.76	0.06	0.77	0.10
PINO	0.86	0.12	0.74	0.03	0.76	0.03
SECO	0.93	0.04	0.75	0.05	0.65	0.05
MAT	1.07	0.06	0.75	0.04	0.71	0.08

Discussion

When preparing the simple media derived from the Mt broths described in Clavel's dissertation[135] and using methods described there, we found that upon autoclaving the sodium bicarbonate ($NaHCO_3$) would dissociate into carbonic acid (which would then evaporate) and hydroxide. This created a pH of approximately 9.4. Because of this we opted to remove the sodium bicarbonate and pH adjust the media prior to autoclaving. This kept the pH at a neutral level.

An intriguing discovery was the fact that headspace within the anaerobic container played a role in enterolignan production. The mechanism of this phenomenon is unknown, but it is speculated that the gas being produced as a part of anaerobic metabolism are interfering with interfering in enterolignan production. For instance, if large amounts of hydrogen are being

produced over time, this may interfere (via Le Chatelier's principle) with an organism's ability recycle NADH to NAD^+, a necessary part of fermentation. In conditions where the headspace is relatively large, the excess gas may simply compress into the headspace, but if headspace is relatively limited the gas may shift the equilibrium of the fermentation conditions within the container.

Karppinen *et al*. found that when human fecal inoculum is incubated with cereal grain (oats, rye, and wheat) fiber in a minimal media similar to TCAP2 approximately 20-30 mL of gas is produced after 24 hours.[140] Much of this gas was determined to be hydrogen and hydrogen sulfide. Other reports on microbial gas production stress the need for syntrophic metabolism.[141,142] That is, the amount of hydrogen consumers (such as methanogens) needs to be in balance with hydrogen producers (such as acetogens). While the concentration of acetogens and methanogens cannot be controlled in the types of experiments examining the consortia of bacteria in human stool, future studies need to be cognizant of gas production. Perhaps venting the containers with a syringe (flushed with nitrogen to prevent cross-contamination with oxygen) will affect enterolignan production to even a greater extent than a large headspace alone.

Although the sample size in the Several Lignans experiment is small, it is interesting to observe the differences in lignan conversion between participants. In particular, intestinal bacteria from participant #6129 (the low enterolignan producer) produced far more END during the lignan converting process than the bacteria from other two high enterolignan producers that, in many cases, produced very little. This could be due to one or more of the following reasons: 1) END was actually produced in significant amounts among the high producers but was quickly further metabolized to ENL, and this appearance was not detected with the once-a-day sampling schedule. Or 2) The community of bacteria in the high producers is such that DHEND is

preferentially metabolized to DHENL rather than END in the lignan biochemical pathway (Figure 2), and DHENL was not a metabolite that was measured. It might be useful in future studies to measure this metabolite to further elucidate the favorable pathways of the different communities of bacteria found in high and low enterolignan producers.

On a related note MAT was converted to greater amounts of END in stool from participant 6129, contrary to not only results from the other two participants, but also to published studies on the subject of *in vitro* conversion of lignans that show no END formation when intestinal bacteria is incubated with MAT.[22,31,34,64] Unlike other dietary plant lignans, MAT has a lactone ring. This means that for END to be formed the lactone ring must be opened and the methyl groups must be removed, and ultimately the lactone ring must be re-formed to produce ENL. Formation of END is a circuitous route in the pathway toward ENL production. This is not completely unheard of, however. Studies by Niemeyer *et al*. show hydrolysis of MAT's lactone ring when incubated with liver microsomes.[138,139] Again, future *in vitro* studies should include the analysis of more metabolites and intermediates, if possible, to demystify these metabolic pathways.

Considering SES is perhaps the second most abundant dietary lignan, with SDG/SECO being the most abundant, standards were purchased and originally designed to be included in the Several Lignans experiment. However, SES co-eluted with ENL during HPLC analysis making quantification difficult. Additionally, the structure of SES made analysis by GC-MS difficult as well so it was removed from the experiment.

Since β-glucosidase enzymes are necessary for the removal of the glucose moieties from SDG as well as other glycosylated lignans, a β-glucosidase assay was developed to see if the

activity of this enzyme could be measured in stool samples. Although sample size was low and no statistical analysis was done, the results suggest that there could be meaningful differences in β-glucosidase activity between high and low enterolignan-producing individuals.

Other media such as SHIME or brain-heart infusion (BHI) broth as well as a vessel other than a sealed glass bottle would more closely approximate conditions found in the human gut. Moreover, the length of bacterial exposure to plant lignans and human exposure to enterolignans (such as ENL) *in vivo* would be much shorter than the week long incubations presented here. Thus the platform presented here that optimizes production of ENL *in vitro* may have limited physiologic relevance.

Nevertheless, this work lays the foundations of culture conditions that are important for growing lignan-converting bacteria. Future studies can now utilize this newly developed platform for the purposes of studying gene expression and enzyme activity in these communities. Further work will give the opportunity to systematically describe organisms involved in enterolignan production, especially with regard to studying intestinal bacterial communities of high and low enterolignan producers. Perhaps it could even be used in the context of large-scale biotechnological production of pre-formed ENL to be commercially available supplement or pharmaceutical.

Conclusion

A variety of conditions were tested to determine this *in vitro* platform. The combination of TCAP2 media, processing stool in a large batch, a final stool concentration of 1.67% or greater, a 3:2 ratio of headspace to slurry, filtration, and slurry homogenization is suitable for maintaining a community of lignan-converting intestinal bacteria. The platform developed here should be appropriate for use in future studies examining lignan-converting bacteria.

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Appendix

Gassing Protocol

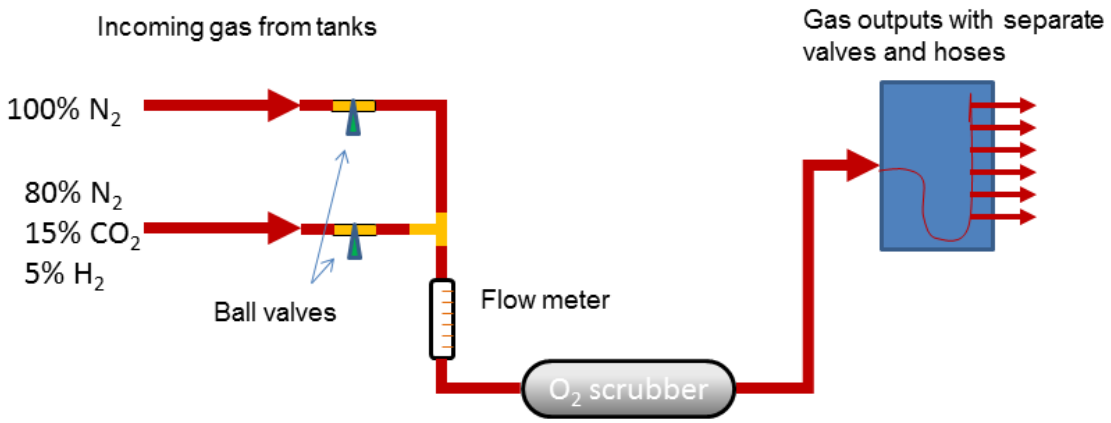

Figure 21 Simple schematic of custom designed gassing manifold used.

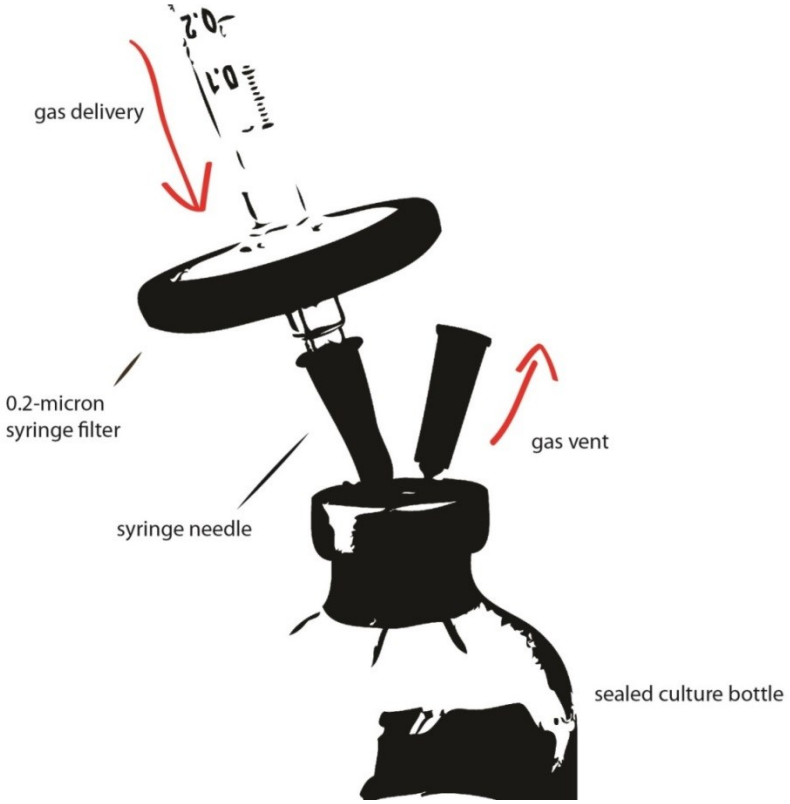

Figure 22 Example of how the gaseous headspace was replaced in sealed bottles

Chemical Manufacturers

Table 7 Manufacturing companies of chemicals used

Sigma	**Fisher**	**Fluka**	**Acros Organics**	**MP Bio**
St. Louis, MO	*Waltham, MA*	*St. Louis, MO*	*Morris, NJ*	*Solon, OH*
Magnesium Chloride	Sodium Acetate	Sodium Formate	Pectin	Gastric Mucin
Potassium Phosphate Dibasic	Sodium Phosphate Heptahydrate	Sodium Phosphate Dihydrate	Calcium Chloride Hexahydrate	Xylan (oat spelt)
Cysteine	Ammonium Sulfate	Peptone Water	Nicotinamide	Inulin
Sodium Chloride	Potassium Phosphate Monobasic	Tryptone		Menadione
Starch (from potato)	Guar Gum (200 mesh)			Vitamin B12
Yeast Extract	Casein			Pantothenate
Magnesium Sulfate Heptahydrate	Iron(II) Sulfate Heptahydrate			
Sodium Bicarbonate	Potassium Chloride			
Hemin	Tween 80			
Biotin				
Bicinchoninic Acid				

Additional Items: Beta-glucosidase kit was purchased from Ab Nova; Arabinogalactan was purchased from SAFC; Bile Salts No.3 was purchased from bioWORLD.

Growth

Standard Curve

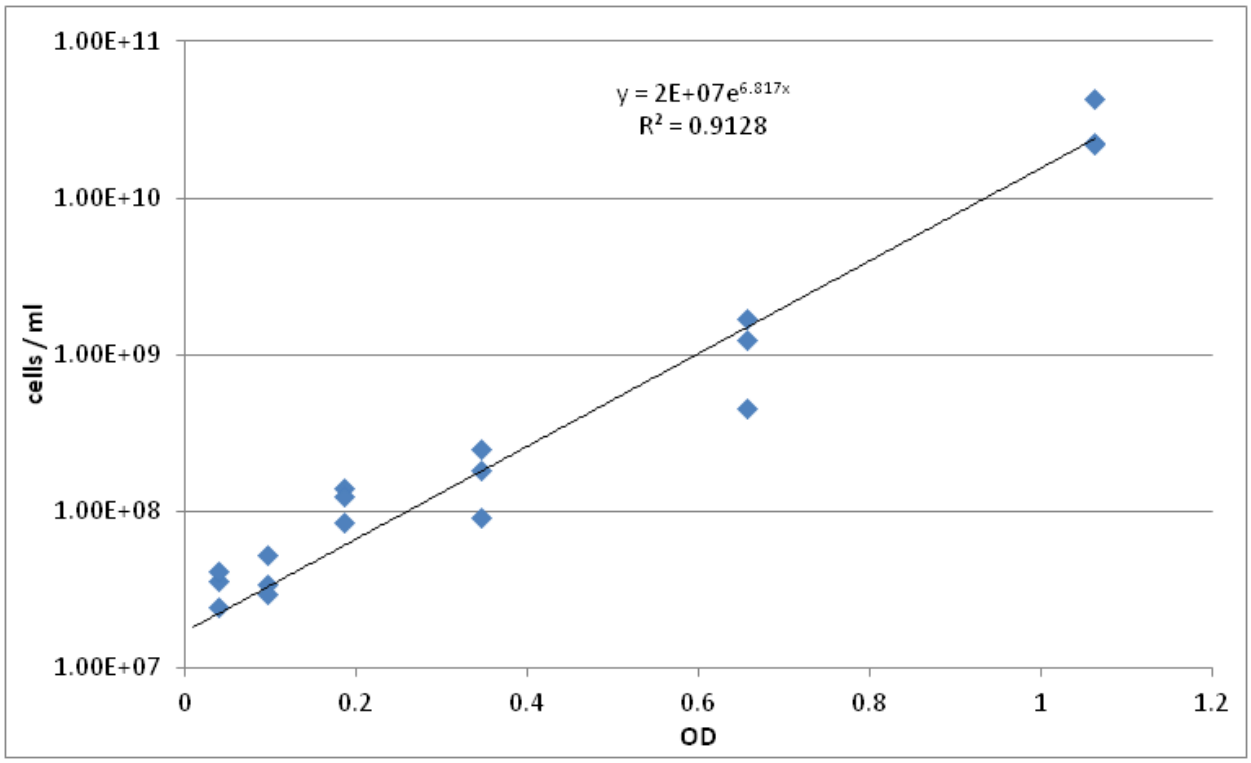

Figure 23 Standard growth curve calculated with E. coli to associate cells/mL with Optical Density (OD)

Chemical Analysis

Simple Media

Table 8 Mean lignan concentrations (uM), standard deviation (SD), and coefficients of variation (CV) in Simple Media experiment

TCAP	**mean**			**SD**			**CV**		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
48	5.79	0.00	0.00	10.03	0.00	0.00	173.21	---	---
72	13.56	0.00	0.00	11.93	0.00	0.00	88.02	---	---
96	16.95	0.00	0.00	16.81	0.00	0.00	99.20	---	---
168	12.53	7.48	0.00	11.03	12.95	0.00	88.03	173.21	---
192	19.74	0.00	0.00	2.32	0.00	0.00	11.78	---	---
TCAP2	mean			SD			CV		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
48	30.84	18.03	0.00	9.45	6.59	0.00	30.64	36.57	---
72	117.13	0.00	0.00	56.40	0.00	0.00	48.15	---	---
96	144.40	0.00	0.00	26.17	0.00	0.00	18.12	---	---
168	54.84	15.89	0.00	11.66	8.65	0.00	21.25	54.41	---
192	46.97	33.86	0.00	23.07	27.99	0.00	49.11	82.66	---
Chinese TCAP2	mean			SD			CV		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	22.34	0.00	0.00	1.82	0.00	0.00	8.15	---	---
48	55.17	9.99	0.00	6.96	17.30	0.00	12.62	173.21	---
72	172.69	0.00	0.00	30.96	0.00	0.00	17.93	---	---
96	173.34	0.00	0.00	41.40	0.00	0.00	23.88	---	---
168	178.54	0.00	0.00	23.22	0.00	0.00	13.00	---	---
192	163.70	0.00	0.00	15.41	0.00	0.00	9.41	---	---

TCAPY	mean			SD			CV		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	15.41	0.00	0.00	6.58	0.00	0.00	42.68	---	---
48	93.18	6.48	0.00	70.86	11.22	0.00	76.04	173.21	---
72	94.23	0.00	0.00	36.00	0.00	0.00	38.21	---	---
96	43.81	22.37	0.00	34.57	16.69	0.00	78.91	74.63	---
168	26.18	142.70	0.00	26.84	81.87	0.00	102.52	57.38	---
192	21.92	197.90	0.00	28.16	37.82	0.00	128.42	19.11	---

Injection vs. Batch

Table 9 Mean lignan concentrations (uM), standard deviation (SD), and coefficients of variation (CV) in Injection vs. Batch experiment

Injection 1.8%	**mean**			**SD**			**CV**		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	139.78	15.83	0.00	20.05	2.84	0.00	14.35	17.91	---
48	261.79	8.51	14.55	30.30	1.17	2.30	11.58	13.75	15.80
120	48.64	109.54	126.40	14.71	61.87	49.41	30.25	56.49	39.09
168	0.00	14.38	281.25	0.00	16.75	18.79	---	116.48	6.68
Batch 1.8%	mean			SD			CV		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	85.76	15.25	11.06	2.75	0.52	0.53	3.21	3.42	4.84
48	263.92	8.00	19.41	19.45	1.10	1.94	7.37	13.72	9.99
120	37.76	47.77	45.42	8.78	9.06	10.38	23.24	18.97	22.85
168	0.00	16.12	247.21	0.00	14.73	32.22	---	91.38	13.03
Filtered Injection 0.018%	mean			SD			CV		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
48	45.20	0.00	0.00	7.52	0.00	0.00	16.63	---	---
120	26.18	0.00	0.00	7.40	0.00	0.00	28.26	---	---
168	12.96	7.11	0.00	15.03	8.44	0.00	115.89	118.82	---
Unfiltered Injection 0.018%	mean			SD			CV		
Time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
48	31.98	0.00	0.00	8.75	0.00	0.00	27.35	---	---
120	17.62	0.00	0.00	10.76	0.00	0.00	61.05	---	---
168	14.67	0.00	0.00	8.91	0.00	0.00	60.73	---	---

Homogenization and Filtration

Table 10 Mean lignan concentrations (uM), standard deviation (SD), and coefficients of variation (CV) in Homogenization and Filtration experiment

10 mL filter tube	**mean**			**SD**			**CV**		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	20.59	0.00	0.00	0.51	0.00	0.00	2.48	---	---
24	14.32	0.00	10.42	8.31	0.00	9.06	58.06	---	86.88
48	74.25	31.33	21.90	6.91	7.56	2.65	9.31	24.13	12.08
72	86.26	40.82	24.03	20.67	8.15	3.42	23.96	19.97	14.23
96	75.07	35.63	19.47	44.37	14.87	3.52	59.11	41.74	18.10
120	142.26	73.39	36.39	32.46	17.07	6.77	22.81	23.26	18.61
144	98.94	60.89	30.74	31.43	20.27	8.63	31.76	33.29	28.05
168	96.56	78.79	39.95	25.15	9.32	8.99	26.05	11.82	22.51
192	91.79	77.42	40.94	18.60	5.58	6.59	20.27	7.21	16.09
10 mL homo tube	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	24.70	0.00	0.00	6.77	0.00	0.00	27.42	---	---
24	13.95	0.00	16.50	4.47	0.00	2.20	32.02	---	13.34
48	70.39	30.78	21.10	9.62	4.20	1.43	13.67	13.64	6.79
72	106.41	44.77	25.16	3.40	1.78	1.54	3.19	3.97	6.11
96	50.38	28.41	18.62	7.27	3.74	1.77	14.43	13.15	9.49
120	115.46	66.47	33.59	8.16	13.35	3.69	7.07	20.08	10.97
144	86.37	57.45	31.31	3.99	7.91	5.32	4.62	13.77	17.01
168	112.28	79.22	47.66	28.73	0.88	13.70	25.58	1.11	28.74
192	90.71	77.07	45.30	26.59	2.22	20.25	29.32	2.87	44.70

20 mL filter tube	**mean**			**SD**			**CV**		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	19.16	0.00	0.00	1.73	0.00	0.00	9.01	---	---
24	24.62	0.00	9.04	11.76	0.00	7.83	47.77	---	86.60
48	55.55	24.73	18.10	16.49	7.75	2.93	29.68	31.36	16.21
72	83.65	28.24	19.27	8.39	0.84	0.83	10.03	2.99	4.32
96	65.38	22.72	17.92	28.56	7.57	2.59	43.69	33.31	14.47
120	97.07	33.48	23.47	12.72	5.28	2.55	13.10	15.77	10.88
144	89.70	33.94	21.23	8.93	3.53	1.64	9.95	10.41	7.71
168	84.63	37.34	21.90	12.43	16.57	6.29	14.68	44.36	28.71
192	76.52	32.27	19.86	9.75	14.45	4.75	12.75	44.77	23.90
20 mL homo tube	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	21.28	0.00	0.00	1.88	0.00	0.00	8.82	---	---
24	37.84	0.00	16.27	7.75	0.00	1.54	20.48	---	9.46
48	52.19	21.40	17.73	4.20	4.43	0.16	8.05	20.71	0.89
72	74.93	27.48	18.69	5.52	0.39	0.90	7.36	1.40	4.83
96	71.41	24.26	17.53	16.38	4.25	2.40	22.93	17.52	13.67
120	109.07	35.19	21.97	1.72	1.62	2.20	1.58	4.60	9.99
144	82.03	29.73	20.46	9.86	3.90	1.77	12.02	13.12	8.66
168	101.70	33.57	23.02	13.86	5.86	3.23	13.63	17.45	14.04
192	99.29	31.04	20.91	14.07	5.17	2.57	14.18	16.64	12.30

40 mL filter bottle	**mean**			**SD**			**CV**		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	23.20	0.00	0.00	2.39	0.00	0.00	10.32	---	---
24	7.54	0.00	13.00	2.87	0.00	0.47	38.02	---	3.59
48	47.50	21.11	18.71	12.78	7.24	3.07	26.91	34.31	16.40
72	59.03	22.13	18.35	21.74	5.46	3.67	36.83	24.69	20.03
96	51.34	19.86	16.73	18.86	5.54	3.38	36.74	27.88	20.19
120	90.18	38.73	23.26	20.11	8.60	3.47	22.30	22.21	14.90
144	83.14	30.43	19.74	35.63	12.34	4.10	42.86	40.54	20.76
168	122.75	46.61	41.53	23.63	11.13	20.61	19.25	23.87	49.62
192	110.82	36.31	38.00	19.20	9.23	18.36	17.33	25.43	48.32
40 mL homo bottle	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	23.77	0.00	0.00	3.42	0.00	0.00	14.40	---	---
24	12.74	12.45	14.28	2.38	2.10	1.36	18.64	16.85	9.49
48	45.80	19.73	16.62	6.52	1.92	1.34	14.24	9.73	8.07
72	68.39	27.09	18.08	19.06	6.08	1.74	27.86	22.43	9.65
96	55.07	21.36	15.96	28.09	7.42	1.98	51.01	34.72	12.38
120	113.52	39.89	23.82	13.28	5.32	2.02	11.70	13.33	8.47
144	94.79	37.22	21.86	14.74	4.53	3.06	15.55	12.18	13.99
168	90.24	35.98	30.79	7.60	2.12	14.84	8.42	5.90	48.20
192	93.16	33.07	28.96	9.59	2.54	16.66	10.30	7.67	57.53

Headspace

Table 11 Mean lignan concentrations (uM), standard deviation (SD), and coefficients of variation (CV) in Headspace experiment

40 mL	**mean**			**SD**			**CV**		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	16.23	0.00	0.00	6.03	0.00	0.00	37.15	---	---
24	65.42	16.42	14.02	3.76	1.95	0.76	5.75	11.87	5.43
48	113.74	34.20	16.03	11.02	3.16	1.05	9.69	9.23	6.56
72	147.94	38.92	23.19	6.65	2.09	3.68	4.49	5.36	15.88
96	158.11	39.81	21.84	13.79	3.60	2.64	8.72	9.04	12.10
144	125.55	38.66	23.43	38.31	8.61	3.11	30.51	22.29	13.29
168	140.39	41.23	24.89	6.47	3.43	1.78	4.61	8.32	7.15
192	147.26	42.64	24.54	15.13	2.60	1.63	10.28	6.09	6.64
20 mL	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	17.08	0.00	0.00	1.52	0.00	0.00	8.89	---	---
24	70.48	14.80	18.41	14.50	3.64	5.00	20.57	24.58	27.18
48	157.72	48.43	19.96	36.68	9.06	1.09	23.26	18.70	5.46
72	225.67	70.04	33.24	24.40	8.49	3.57	10.81	12.12	10.74
96	190.62	61.55	27.57	23.29	7.43	3.52	12.22	12.07	12.77
144	170.00	76.54	37.33	35.61	12.96	4.07	20.95	16.93	10.90
168	195.83	87.33	42.59	6.86	9.88	2.12	3.51	11.32	4.99
192	224.79	90.22	41.24	20.65	9.71	4.26	9.18	10.77	10.34

Serial Dilution

Table 12 Mean lignan concentrations (uM), standard deviation (SD), and coefficients of variation (CV) in Headspace experiment

3.33%	**mean**			**SD**			**CV**		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	5.79	0.00	0.00	0.58	0.00	0.00	9.94	---	---
24	0.00	8.66	3.58	0.00	7.63	4.06	---	88.18	113.68
48	33.88	7.32	5.08	5.90	1.00	0.95	17.40	13.72	18.79
72	104.02	24.31	13.35	24.12	7.64	3.42	23.19	31.41	25.65
96	82.15	31.68	18.27	54.56	14.98	5.95	66.42	47.27	32.55
120	41.50	25.08	14.21	9.42	9.42	5.57	22.71	37.53	39.18
144	34.12	28.18	16.02	6.75	15.79	4.27	19.78	56.02	26.68
168	59.85	38.70	23.00	5.70	1.13	5.57	9.53	2.93	24.20
1.67%	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	6.11	0.00	0.00	0.78	0.00	0.00	12.73	---	---
24	35.64	9.69	3.89	9.05	3.30	1.66	25.39	34.05	42.60
48	84.46	11.29	7.31	29.25	2.39	0.94	34.63	21.17	12.82
72	115.34	17.35	13.32	42.26	6.58	3.34	36.64	37.91	25.09
96	172.44	32.28	24.84	19.64	9.61	5.58	11.39	29.76	22.48
120	74.43	16.52	13.10	16.21	4.80	5.77	21.77	29.07	44.02
144	225.24	37.43	19.56	47.53	8.24	6.26	21.10	22.02	32.00
168	212.50	36.96	18.65	29.37	3.08	2.70	13.82	8.33	14.45
1.67% mixed gas	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	5.51	0.00	0.00	1.37	0.00	0.00	24.88	---	---
24	39.43	9.41	0.00	7.19	1.95	0.00	18.23	20.70	---
48	90.58	8.12	0.00	51.53	1.32	0.00	56.89	16.24	---
72	185.10	27.41	15.80	25.01	9.40	1.64	13.51	34.30	10.36
96	167.85	32.94	22.69	45.96	8.60	4.37	27.38	26.10	19.27
120	135.02	22.86	12.03	20.15	3.98	3.64	14.92	17.41	30.23
144	125.39	38.02	9.49	75.23	22.86	2.32	60.00	60.13	24.42
168	174.17	50.84	19.11	72.87	32.72	1.75	41.84	64.35	9.17

0.30%	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	180.59	15.95	0.00	45.78	9.33	0.00	25.35	58.48	---
48	154.68	14.09	0.00	16.50	7.28	0.00	10.67	51.65	---
72	155.93	9.13	0.00	8.87	2.20	0.00	5.69	24.11	---
96	231.94	20.50	10.42	7.23	5.78	1.27	3.12	28.21	12.23
120	262.24	42.05	8.78	25.14	7.41	0.62	9.59	17.61	7.03
144	125.41	25.48	9.46	60.06	6.04	0.37	47.89	23.68	3.86
168	185.87	13.78	8.42	63.45	9.98	1.13	34.14	72.41	13.39

0.03%	mean			SD			CV		
time	seco	end	enl	seco	end	enl	seco	end	enl
0	0.00	0.00	0.00	0.00	0.00	0.00	---	---	---
24	99.47	0.00	0.00	22.19	0.00	0.00	22.31	---	---
48	248.11	0.00	0.00	17.47	0.00	0.00	7.04	---	---
72	273.41	0.00	0.00	5.62	0.00	0.00	2.06	---	---
96	218.77	0.00	0.00	15.46	0.00	0.00	7.07	---	---
120	234.41	0.00	0.00	31.87	0.00	0.00	13.60	---	---
144	179.38	0.00	0.00	9.77	0.00	0.00	5.45	---	---
168	169.68	0.00	0.00	13.98	0.00	0.00	8.24	---	---

Complex Media

Table 13 Mean lignan concentrations (uM), standard deviation (SD), and coefficients of variation (CV) in Complex Media experiment

HCM	**mean**			**SD**			**CV**		
hours	seco	end	enl	seco	end	enl	seco	end	enl
0	26.58	0.00	0.00	0.66	0.00	0.00	2.48	---	---
24	59.98	0.00	0.00	7.17	0.00	0.00	11.96	---	---
48	94.16	43.98	0.00	28.23	6.44	0.00	29.99	14.63	---
72	79.30	38.70	0.00	3.38	6.15	0.00	4.27	15.89	---
96	86.30	37.03	0.00	4.26	2.68	0.00	4.94	7.23	---
120	69.85	53.30	0.00	27.12	12.77	0.00	38.83	23.96	---
144	68.50	49.12	0.00	19.40	11.58	0.00	28.32	23.56	---
168	63.00	48.70	0.00	16.05	10.93	0.00	25.47	22.45	---
TCAP2	mean			SD			CV		
hours	seco	end	enl	seco	end	enl	seco	end	enl
0	55.87	0.00	0.00	3.98	0.00	0.00	7.12	---	---
24	26.46	9.14	4.68	14.32	2.99	8.11	54.11	32.67	173.21
48	58.50	11.59	4.87	0.25	2.11	8.44	0.43	18.20	173.21
72	44.61	19.71	12.78	5.40	5.81	5.62	12.10	29.47	43.96
96	18.27	46.83	36.66	7.20	11.00	10.52	39.38	23.49	28.70
120	11.96	46.85	53.71	0.93	7.85	19.09	7.82	16.75	35.54
144	0.00	120.53	71.09	0.00	47.14	7.66	---	39.11	10.78
168	0.00	94.79	97.68	0.00	29.53	19.16	---	31.15	19.62
SHIME	mean			SD			CV		
hours	seco	end	enl	seco	end	enl	seco	end	enl
0	43.68	0.00	0.00	2.41	0.00	0.00	5.52	---	---
24	90.39	0.00	0.00	13.74	0.00	0.00	15.20	---	---
48	114.33	53.67	0.00	18.80	10.34	0.00	16.45	19.27	---
72	92.34	52.79	0.00	32.27	8.91	0.00	34.95	16.88	---
96	154.22	55.69	0.00	16.00	4.50	0.00	10.38	8.08	---
120	106.74	60.05	0.00	31.37	12.54	0.00	29.39	20.88	---
144	124.68	56.58	0.00	19.22	3.01	0.00	15.42	5.32	---
168	125.55	52.92	0.00	22.72	3.53	0.00	18.09	6.67	---

LCM	mean			SD			CV		
hours	seco	end	enl	seco	end	enl	seco	end	enl
0	67.75	0.00	0.00	3.69	0.00	0.00	5.44	---	---
24	56.62	11.33	0.00	9.90	19.63	0.00	17.48	173.21	---
48	47.72	21.26	10.71	2.46	18.44	18.55	5.16	86.72	173.21
72	46.54	41.00	10.95	5.66	6.68	18.97	12.16	16.30	173.21
96	43.23	37.51	10.82	6.30	4.48	18.74	14.57	11.93	173.21
120	49.48	44.72	34.23	12.29	12.30	1.99	24.84	27.51	5.82
144	54.81	40.70	29.46	5.46	4.92	2.56	9.97	12.10	8.71
168	65.30	53.45	36.03	20.25	3.74	3.84	31.01	7.00	10.66

β-glucosidase results

Table 14 Results of beta-glucosidase activity in stool incubations

	5100		6128		6129	
	Units of β-glucosidase per 100 mg of protein	Units of β-glucosidase per gram dry weight of slurry	Units of β-glucosidase per 100 mg of protein	Units of β-glucosidase per gram dry weight of slurry	Units of β-glucosidase per 100 mg of protein	Units of β-glucosidase per gram dry weight of slurry
No lignan substrate	4.89	0.89	6.72	0.67	7.01	0.64
No lignan substrate	4.94	0.9	5.87	0.69	7.39	0.71
No lignan substrate	4.98	0.89	6.33	0.69	7.14	0.48
SDG	4.39	1.03	6.35	0.82	7.64	0.74
SDG	4.44	0.96	5.61	0.71	6.53	0.78
SDG	4.46	0.92	5.89	0.85	6.22	0.75
LARI	4.62	1.03	5.35	0.73	6.3	0.8
LARI	4.34	1.02	5.6	0.72	6.98	0.86
LARI	4.87	1.27	5.97	0.83	6.19	0.66
PINO	3.88	0.74	5.18	0.74	6.56	0.76
PINO	4.51	0.97	5.53	0.77	6.24	0.73
PINO	3.87	0.86	6.2	0.72	6.46	0.79
SECO	4.27	0.94	5.31	0.75	5.36	0.59
SECO	4.5	0.88	5.25	0.7	5.16	0.67
SECO	4.51	0.96	5.43	0.8	6.27	0.69
MAT	4.59	1.02	5.62	0.77	6.38	0.69
MAT	5.23	1.04	5.4	0.78	5.94	0.79
MAT	5.1	1.14	5.44	0.71	5.99	0.64
END	4.65	1.03	6.12	0.81	5.5	0.52
ENL	4.51	0.92	5.31	0.69	5.67	0.55
average	4.6	0.97	5.7	0.75	6.3	0.69

Killed Control

Between the addition of the lignan substrate and removing the first subsample for chemical analysis and qPCR there is a time period of approximately two hours. To be sure that lignans were not being spontaneously converted independently of bacteria during that time a "killed control" was implemented. Stool samples from two FlaxFX participants were baked overnight at 100 °C and approximately 0.1g dry stool of each was added to 20 mL TCAP2 media along with 6.24 µM LARI and another set was incubated with 6.28 µM PINO. These were shaken and sampled immediately, placed in the rotating incubator at 300 RPMs and sampled again at one hour and two hours.

The results from the killed control experiment revealed there was no conversion of LARI or PINO to SECO, END, or ENL without live bacteria. However, there was approximately 70-80% recovery among the samples, suggesting that lignans may be adhering to the bottle or perhaps there is a matrix effect preventing 100% lignan extraction from samples.

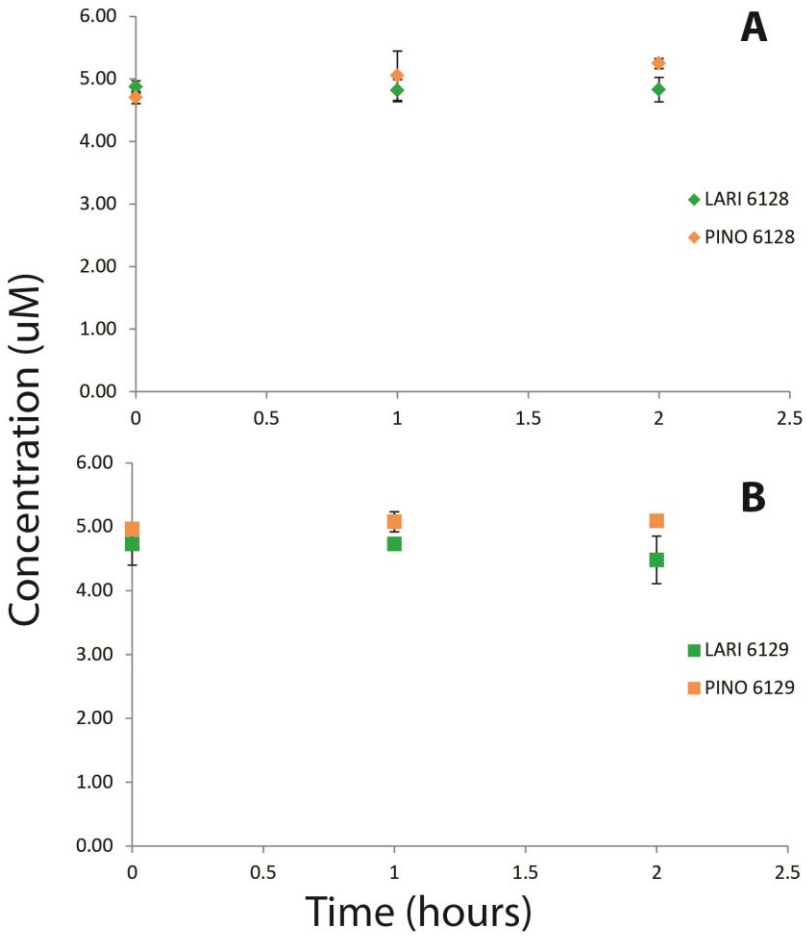

Figure 24 Killed control results from participants 6128 (A) and 6129 (B). Samples were spiked with 6.24 µM LARI or 6.28 µM PINO. Error Bars indicate one standard deviation.

Statistical Analysis

Injection vs. Batch

Table 15 Results of t-tests in the Injection vs. Batch experiment

Unpaired t test	
P value	0.1890
P value summary	ns
Significantly different? (P < 0.05)	No
One- or two-tailed P value?	Two-tailed
t, df	t=1.581 df=4
How big is the difference?	
Mean ± SEM of column A	281.3 ± 10.85 N=3
Mean ± SEM of column B	247.2 ± 18.60 N=3
Difference between means	-34.04 ± 21.53
95% confidence interval	-93.83 to 25.74
R squared	0.3846

Homogenization and Filtration

Table 16 Results of ANOVA in the Homogenization and Filtration experiment

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	230.5	5	46.09	F (5, 12) = 2.301	P = 0.1101
Residual (within columns)	240.4	12	20.03		
Total	470.9	17			

Table 17 Results of Tukey's multiple comparisons test in the Homogenization and Filtration experiment

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
10 mL filter vs. 10 mL homo	-2.86	-15.14 to 9.411	No	ns
10 mL filter vs. 20 mL filter	6.71	-5.564 to 18.99	No	ns
10 mL filter vs. 20 mL homo	6.29	-5.981 to 18.57	No	ns
10 mL filter vs. 40 mL filter	-0.59	-12.86 to 11.69	No	ns
10 mL filter vs. 40 mL homo	3.41	-8.870 to 15.68	No	ns
10 mL homo vs. 20 mL filter	9.58	-2.700 to 21.85	No	ns
10 mL homo vs. 20 mL homo	9.16	-3.117 to 21.43	No	ns
10 mL homo vs. 40 mL filter	2.28	-9.999 to 14.55	No	ns
10 mL homo vs. 40 mL homo	6.27	-6.006 to 18.54	No	ns
20 mL filter vs. 20 mL homo	-0.42	-12.69 to 11.86	No	ns
20 mL filter vs. 40 mL filter	-7.30	-19.57 to 4.976	No	ns
20 mL filter vs. 40 mL homo	-3.31	-15.58 to 8.969	No	ns
20 mL homo vs. 40 mL filter	-6.88	-19.16 to 5.394	No	ns
20 mL homo vs. 40 mL homo	-2.89	-15.16 to 9.386	No	ns
40 mL filter vs. 40 mL homo	3.99	-8.283 to 16.27	No	ns

Headspace

Table 18 Results of t-test in the Headspace experiment

Unpaired t test	
P value	0.0032
P value summary	**
Significantly different? (P < 0.05)	Yes
One- or two-tailed P value?	Two-tailed
t, df	t=6.339 df=4
How big is the difference?	
Mean ± SEM of column A	41.24 ± 2.461 N=3
Mean ± SEM of column B	24.54 ± 0.9406 N=3
Difference between means	-16.70 ± 2.635
95% confidence interval	-24.02 to -9.387
R squared	0.9095

Serial Dilution

Table 19 Results of ANOVA in the Serial Dilution experiment

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	349.4	3	116.5	F (3, 8) = 10.94	P = 0.0033
Residual (within columns)	85.17	8	10.65		
Total	434.5	11			

Table 20 Results of Tukey's multiple comparisons test in the Serial Dilution experiment

Tukey's multiple comparisons test				
Number of families				
	1			
Number of comparisons per family	6			
Alpha	0.05			
Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
3% vs. 1.6%	4.35	-4.179 to 12.88	No	ns
3% vs. 1.6%gas	3.89	-4.643 to 12.42	No	ns
3% vs. 0.3%	14.58	6.049 to 23.11	Yes	**
1.6% vs. 1.6%gas	-0.46	-8.996 to 8.067	No	ns
1.6% vs. 0.3%	10.23	1.697 to 18.76	Yes	*
1.6%gas vs. 0.3%	10.69	2.161 to 19.22	Yes	*

Complex Media

Table 21 Results of ANOVA in the Complex Media experiment

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	2546	3	848.5	F (3, 8) = 66.71	P < 0.0001
Residual (within columns)	101.8	8	12.72		
Total	2647	11			

Table 22 Results of Tukey's multiple comparisons test in the Serial Dilution experiment

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
HCM vs. TCAP2	-35.65	-44.98 to -26.33	Yes	****
HCM vs. SHIME	0.0	-9.326 to 9.326	No	ns
HCM vs. LCM	-13.15	-22.47 to -3.822	Yes	**
TCAP2 vs. SHIME	35.65	26.33 to 44.98	Yes	****
TCAP2 vs. LCM	22.50	13.18 to 31.83	Yes	***
SHIME vs. LCM	-13.15	-22.47 to -3.822	Yes	**

Several Lignans

ENL Rate Model

$Y=Y_0 + (Plateau-Y_0)*(1-e^{(-K*X)})$

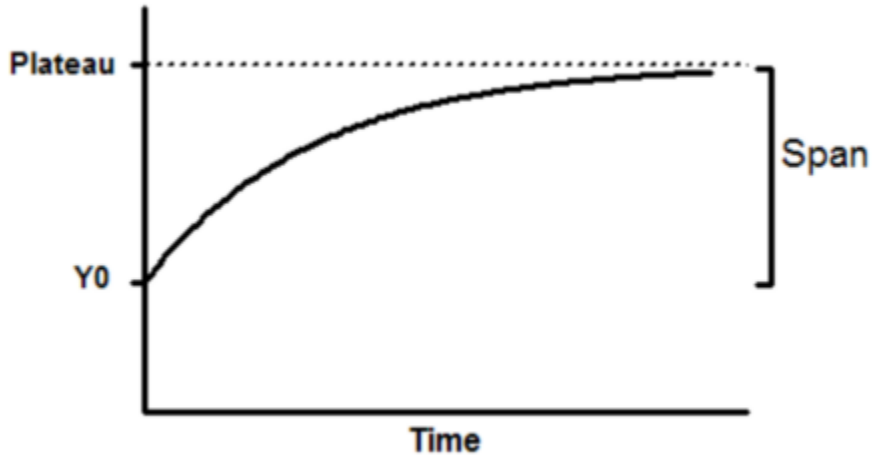

Figure 25 Graph of ENL rate model used for statistical analysis of rates

Y_0 is the Y value when X (time) is zero. It is expressed in the same units as Y,

Plateau is the Y value at infinite times, expressed in the same units as Ymax.

K is the rate constant, expressed in reciprocal of the X axis time units. If X is in minutes then K is expressed in inverse minutes.

Tau is the time constant, expressed in the same units as the X axis. It is computed as the reciprocal of K.

Half-time is in the time units of the X axis. It is computed as ln(2)/K.

One Phase Association Non-linear Regression of ENL Production Rate

6128	SDG			SECO			PINO			LARI			MAT		
Best-fit values	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Ymax	15.52	21.16	11.64	9.99	7.61	10.87	9.11	8.71	8.08	6.62	11.00	9.06	9.13	9.45	10.02
K	0.01	0.01	0.02	0.03	0.09	0.04	0.03	0.03	0.03	0.07	0.03	0.03	0.26	0.19	0.08
HalfLife	64.55	91.44	31.22	25.13	7.49	17.70	23.59	20.93	26.83	10.07	21.81	21.69	2.67	3.68	8.58
Std. Error															
Ymax	6.92	14.85	1.29	0.21	0.14	0.10	0.60	0.38	0.35	0.32	0.35	0.69	0.14	0.03	0.19
K	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.01	0.00	0.02	0.00	0.01	0.74	0.02	0.01
Goodness of Fit															
Degrees of Freedom	4	4	4	4	4	3	4	4	4	4	4	4	4	4	4
R square	0.91	0.91	0.96	1.00	0.99	1.00	0.98	0.99	0.99	0.96	0.99	0.96	1.00	1.00	0.99
Adjusted R square	0.89	0.89	0.95	1.00	0.99	1.00	0.97	0.98	0.99	0.96	0.99	0.96	0.99	1.00	0.99
Absolute Sum of Squares	8.47	10.91	3.15	0.15	0.27	0.05	1.39	0.73	0.36	1.25	0.56	2.24	0.33	0.01	0.48
Sy.x	1.46	1.65	0.89	0.19	0.26	0.13	0.59	0.43	0.30	0.56	0.38	0.75	0.29	0.05	0.35
Normality of Residuals															
P value summary															
Shapiro-Wilk W	0.94	0.98	0.99	0.90	0.97	0.95	0.89	0.88	0.96	0.96	0.96	0.88	0.82	0.98	0.87
P value	0.65	0.95	0.98	0.37	0.92	0.77	0.33	0.27	0.78	0.79	0.81	0.28	0.09	0.97	0.21
Passed normality test (alpha=0.05) ?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Runs test															
Points above curve	4	3	3	3	4	2	3	1	3	2	3	1	2	3	2
Points below curve	2	3	3	2	2	2	2	4	2	3	2	4	3	2	3
Number of runs	4	4	6	4	3	4	4	3	3	3	3	3	3	5	3
P value (runs test)	0.8	0.7	1	0.9	0.4	1	0.9	1	0.5	0.5	0.5	1	0.5	1	0.5
Deviation from Model	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant

5100	SDG			SECO			PINO			LARI			MAT		
Best-fit values	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
One phase exponential association															Ambiguous
Ymax	12.12	20.36	12.24	13.24	11.46	12.03	11.38	9.37	11.17	12.12	12.84	13.10	16.82	11.71	~22096
K	0.03	0.01	0.05	0.04	0.13	0.19	0.03	0.04	0.05	0.22	0.04	0.02	0.01	0.03	~4.064e-006
HalfLife	25.89	79.77	13.48	17.40	5.53	3.62	26.47	18.07	14.30	3.20	15.77	35.94	58.65	25.38	~170544
Std. Error															
Ymax	0.74	10.06	0.78	0.18	0.38	0.27	0.16	0.21	0.36	0.20	0.22	1.71	4.89	0.32	~5.031e+007
K	0.00	0.01	0.02	0.00	0.06	0.21	0.00	0.00	0.01	0.28	0.00	0.01	0.01	0.00	~0.009255
Degrees of Freedom	4	4	4	4	4	4	3	4	4	4	4	4	4	3	4
R square	0.98	0.94	0.95	1.00	0.98	0.99	1.00	1.00	0.99	0.99	1.00	0.96	0.95	1.00	0.89
Adjusted R square	0.98	0.92	0.94	1.00	0.97	0.99	1.00	0.99	0.98	0.99	1.00	0.95	0.93	1.00	0.86
Absolute Sum of Squares	1.73	8.30	5.80	0.22	2.24	1.18	0.05	0.29	1.14	0.62	0.39	3.67	5.94	0.17	13.41
Sy.x	0.66	1.44	1.20	0.24	0.75	0.54	0.13	0.27	0.53	0.39	0.31	0.96	1.22	0.24	1.83
Normality of Residuals															
P value summary															
Shapiro-Wilk W	0.96	0.91	0.93	0.90	0.90	0.88	0.92	0.90	0.96	0.90	0.95	0.96	0.95	0.99	0.93
P value	0.79	0.44	0.58	0.36	0.35	0.26	0.52	0.36	0.83	0.40	0.77	0.79	0.76	0.99	0.60
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Runs test															
Points above curve	2	3	3	3	2	2	2	3	2	3	2	3	2	2	2
Points below curve	3	2	2	2	3	3	2	2	3	2	3	2	3	2	3
Number of runs	4	4	3	4	3	5	4	4	3	5	4	3	4	4	2
P value (runs test)	0.9	0.9	0.5	0.9	0.5	1	1	0.9	0.5	1	0.9	0.5	0.9	1	0.2
Deviation from Model	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant

6129	SDG			SECO			PINO			LARI			MAT		
Best-fit values	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
One phase exponential association								Ambiguous			Ambiguous	Ambiguous			
Ymax	14.19	15.76	17.2	43.55	30.4	16.37	17.18	11.12	11.11	8.66	~633.0	~17321	15.06	12.63	12.41
K	0.02	0.02	0.01	0	0	0.01	0.01	~70.30	0.09	0.01	~9.576e-005	~3.783e-006	0.02	0.03	0.03
HalfLife	44.2	42.22	54.3	247.3	153.3	61.24	54.9	~0.009860	7.81	50.21	~7238	~183218	45.73	21.97	23.45
Std. Error															
Ymax	5.42	5.95	8.05	127.4	47.73	7.45	8.15	0.13	0.03	6.84	~80093	~5.245e+007	0.09	0.55	0.99
K	0.01	0.01	0.01	0.01	0.01	0.01	0.01	~	0.01	0.02	~0.01217	~0.01146	0	0	0.01
Goodness of Fit															
Degrees of Freedom	4	4	4	4	4	4	4	3	3	4	4	4	3	4	4
R square	0.86	0.85	0.88	0.87	0.89	0.91	0.88	1	1	0.66	0.78	0.83	1	0.99	0.97
Adjusted R square	0.83	0.82	0.85	0.84	0.86	0.89	0.85	1	1	0.57	0.72	0.79	1	0.99	0.96
Absolute Sum of Squares	19.17	26.8	21.09	17.72	15.89	11.84	20.78	0.19	0.01	19.9	10.44	10.94	0	1.35	3.85
Sy.x	2.19	2.59	2.3	2.11	1.99	1.72	2.28	0.25	0.05	2.23	1.62	1.65	0.03	0.58	0.98
Normality of Residuals															
P value summary															
Shapiro-Wilk W	0.91	0.9	0.97	0.98	0.95	0.97	0.92	0.96	0.94	0.92	0.97	0.91	0.95	0.97	0.9
P value	0.45	0.4	0.86	0.94	0.72	0.87	0.52	0.81	0.63	0.47	0.92	0.47	0.71	0.89	0.36
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Runs test															
Points above curve	3	2	3	2	2	2	2	2	2	3	3	2	2	2	1
Points below curve	2	3	2	3	3	3	3	2	2	2	2	3	2	3	4
Number of runs	3	3	3	3	3	3	3	2	3	4	4	4	4	4	3
P value (runs test)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.33	0.67	0.9	0.9	0.9	1	0.9	1
Deviation from Model	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant	Not Significant

ENL Rate Statistics Adjusted for Cell Number

Table 23 Statistics for rates of ENL production between lignan substrates (except LARI) adjusted for estimated cell number

	SDG	SECO	PINO	MAT
Minimum	7.89	7.32	7.35	7.19
25% Percentile	8.10	7.57	7.76	7.51
Median	8.19	8.01	8.08	7.83
75% Percentile	8.62	8.45	8.19	8.23
Maximum	8.72	8.85	8.20	8.53
Mean	8.30	8.04	7.96	7.88
Std. Deviation	0.29	0.51	0.30	0.44
Std. Error of Mean	0.10	0.17	0.10	0.15
Lower 95% CI of mean	8.07	7.65	7.73	7.54
Upper 95% CI of mean	8.52	8.43	8.19	8.22
D'Agostino & Pearson omnibus normality test				
K2	1.11	0.26	4.69	0.35
P value	0.57	0.88	0.10	0.84
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns
Shapiro-Wilk normality test				
W	0.91	0.97	0.80	0.97
P value	0.31	0.85	0.02	0.85
Passed normality test (alpha=0.05)?	Yes	Yes	No	Yes
P value summary	ns	ns	*	ns
KS normality test				
KS distance	0.22	0.14	0.24	0.15
P value	0.20	0.20	0.15	0.20
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns
Coefficient of variation	3%	6%	4%	6%
Skewness	0.42	0.20	-1.43	-0.26
Kurtosis	-1.16	-0.72	1.07	-0.76
Sum	74.70	72.40	71.70	71.00

Table 24 Statistics for rates of ENL production between lignan substrates (except LARI) adjusted for gram dry weight of stool

	SDG	SECO	PINO	MAT
Minimum	0.16	-0.41	-0.17	-0.44
25% Percentile	0.53	-0.11	0.04	-0.12
Median	0.64	0.38	0.45	0.37
75% Percentile	0.94	0.98	0.53	0.61
Maximum	1.09	1.39	0.74	0.80
Mean	0.69	0.43	0.32	0.28
Std. Deviation	0.28	0.61	0.31	0.42
Std. Error of Mean	0.09	0.20	0.10	0.14
Lower 95% CI of mean	0.47	-0.03	0.09	-0.05
Upper 95% CI of mean	0.91	0.90	0.56	0.60
D'Agostino & Pearson omnibus normality test				
K2	0.60	0.46	0.85	1.14
P value	0.74	0.79	0.65	0.56
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns
Shapiro-Wilk normality test				
W	0.96	0.97	0.93	0.92
P value	0.77	0.88	0.43	0.40
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns
KS normality test				
KS distance	0.19	0.11	0.22	0.23
P value	0.20	0.20	0.20	0.20
Passed normality test (alpha=0.05)?	Yes	Yes	Yes	Yes
P value summary	ns	ns	ns	ns
Coefficient of variation	41.04%	140.34%	94.58%	153.39%
Skewness	-0.46	0.26	-0.61	-0.73
Kurtosis	0.30	-0.88	-0.61	-0.55
Sum	6.19	3.91	2.90	2.49

Total ENL Results Adjusted for Cell Number

Table 25 Results of ANOVA in the Several Lignans experiment adjusted for cell number

Two-way ANOVA					
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	10.05	0.003	**	Yes	
Substrates	7.982	0.001	***	Yes	
subjects	72.25	< 0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.27	8.00	0.03	F (8, 30) = 3.881	P = 0.0030
Substrates	0.21	4.00	0.05	F (4, 30) = 6.163	P = 0.0010
subjects	1.91	2.00	0.95	F (2, 30) = 111.6	P < 0.0001
Residual	0.26	30.00	0.01		

Table 26 Results of Tukey's multiple comparisons test in the Several Lignans experiment adjusted for cell number

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
SDG vs. SECO	-0.01	-0.1412 to 0.1116	No	ns
SDG vs. PINO	-0.16	-0.2862 to -0.03336	Yes	**
SDG vs. LARI	-0.16	-0.2850 to -0.03218	Yes	**
SDG vs. MAT	-0.10	-0.2279 to 0.02495	No	ns
SECO vs. PINO	-0.15	-0.2714 to -0.01855	Yes	*
SECO vs. LARI	-0.14	-0.2702 to -0.01737	Yes	*
SECO vs. MAT	-0.09	-0.2130 to 0.03976	No	ns
PINO vs. LARI	0.00	-0.1252 to 0.1276	No	ns
PINO vs. MAT	0.06	-0.06809 to 0.1847	No	ns
LARI vs. MAT	0.06	-0.06927 to 0.1835	No	ns

Total ENL Results Adjusted for Gram Dry Weight of Stool

Table 27 Results of ANOVA in the Several Lignans experiment adjusted for gram dry weight of stool

Two-way ANOVA					
Alpha	0.05				
Source of Variation	% of total variation	P value	P value summary	Significant?	
Interaction	17.94	0.003	**	Yes	
Substrate	14.24	0.001	***	Yes	
Subjects	50.48	< 0.0001	****	Yes	
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.27	8	0.03	F (8, 30) = 3.881	P = 0.0030
Substrate	0.21	4	0.05	F (4, 30) = 6.163	P = 0.0010
Subjects	0.75	2	0.37	F (2, 30) = 43.69	P < 0.0001
Residual	0.26	30	0.01		

Table 28 Results of Tukey's multiple comparisons test in the Several Lignans experiment adjusted for gram dry weight of stool

Tukey's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary
SDG vs. SECO	0.01	-0.1116 to 0.1412	No	ns
SDG vs. PINO	0.16	0.03336 to 0.2862	Yes	**
SDG vs. LARI	0.16	0.03218 to 0.2850	Yes	**
SDG vs. MAT	0.10	-0.02495 to 0.2279	No	ns
SECO vs. PINO	0.15	0.01855 to 0.2714	Yes	*
SECO vs. LARI	0.14	0.01737 to 0.2702	Yes	*
SECO vs. MAT	0.09	-0.03977 to 0.2130	No	ns
PINO vs. LARI	0.00	-0.1276 to 0.1252	No	ns
PINO vs. MAT	-0.06	-0.1847 to 0.06809	No	ns
LARI vs. MAT	-0.06	-0.1835 to 0.06927	No	ns