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Effect of Antibiotics and Diet on Enterolactone Concentration and Metabolome Studied by Targeted and Nontargeted LC−MS Metabolomics

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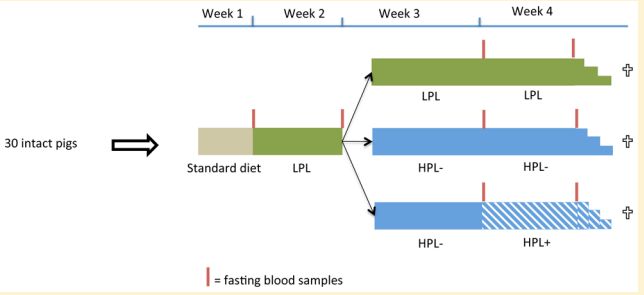
S *Supporting Information*

ABSTRACT: High plant lignan intake is associated with a number of health benefits, possibly induced by the lignan metabolite enterolactone (ENL). The gut microbiota plays a crucial role in converting dietary lignans into ENL, and epidemiological studies have shown that use of antibiotics is associated with lower levels of ENL. Here we investigate the link between antibiotic use and lignan metabolism in pigs using LC−MS/MS. The effect of lignan intake and antibiotic use on the gut microbial community and the pig metabolome is studied by 16S rRNA sequencing and nontargeted LC−MS. Treatment with antibiotics resulted in substantially lower concentrations of ENL compared with concentrations detected in untreated animals, whereas the plasma concentrations of plant lignans were unchanged. Both diet and antibiotic treatment affected the clustering of urinary metabolites and significantly altered the proportions of taxa in the gut microbiota. Diet, but not antibiotic treatment, affected the plasma lipid profile, and a lower concentration of LDL cholesterol was observed in the pigs fed a high lignan diet. This study provides solid support for the associations between ENL concentrations and use of antibiotics found in humans and indicates that the lower ENL concentration may be a consequence of the ecological changes in the microbiota.



KEYWORDS: *enterolactone, microbiota, lignans, antibiotics, metabolomics*

INTRODUCTION

Epidemiological studies indicate that high lignan intake is associated with a number of health benefits in humans such as improved breast cancer survival, decreased cardiovascular risk, and lower levels of inflammation markers.[1−4] Lignans are diphenolic compounds found throughout the plant kingdom. The most important dietary sources include grains, seeds, and vegetables, with whole-grain cereals and vegetables being the most important contributors to lignan intake in the Nordic countries.[5−7]

After ingestion, plant lignans are either absorbed directly from the small intestinal lumen and moved into circulation[8,9] or passed through to the colon, where the colonic microbiota can convert the lignans to enterolignans (enterolignans: enterodiol (END) and enterolactone (ENL)).[2] The latter process requires an interaction of anaerobic bacteria that are distantly related in terms of functionality and phylogeny.[10] Although the pathways for converting plant lignan to ENL differ among the individual plant lignans,[10] the final step for many plant lignans is a dehydrogenation step of END to ENL.[11] The critical role of the colonic microbiota in the metabolism of plant lignans to enterolignans was identified in one of the earliest reports on enterolignans, in which urinary levels of ENL decreased immediately after intake of a wide-spectrum antibiotic.[12] The link between the use of antibiotics and lower ENL concentration was later supported by cross-sectional and observational studies reporting inverse associations between use of antibiotics, number of antibiotic treatments, type of antibiotics, and time from last treatment and ENL levels.[13−15]

The biological activity of enterolignans may be linked to structural similarities to the endogenous 17-β-estradiol, and depending on the biological levels of estradiol, lignans can act as both estrogen antagonists and weak estrogens.[16−18] Moreover, lignans exhibit the ability to act as potent antioxidants and to interact with sex-hormone-binding

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globulins.[19,20] The estrogenicity of ENL, in particular, may have an important role in upregulation of the LDL receptor activity,[21] and daily supplies of plant lignans resulted in lower blood cholesterol[22] and LDL cholesterol in humans.[23,24] Furthermore, high urinary enterolignan levels are associated with high serum HDL cholesterol and low triglycerides[25,26] along with lower circulating concentrations of C-reactive protein (CRP)[27,28] and lower concentrations of liver enzymes.[29]

Even though conversion of plant lignans to enterolignans is fully dependent on the microbiome, few studies have investigated the direct effect of antibiotics on enterolignan concentration and the possible health effects in detail. In this study, the effects of antibiotics were investigated in pigs fed a lignan-rich diet based on rye bran. The study design and use of an animal model allowed us to control the precise lignan intake, specific antibiotic dosage, and sample from sites difficult to access in humans. To gain a comprehensive understanding of lignan metabolism, nontargeted (LC−MS) and targeted (LC−MS/MS) profiling of low-molecular-weight metabolites in plasma and urine was performed.[30] The bacteria affected by enterolignan intake were also identified by 16S rRNA sequencing of cecal digesta. Furthermore, plasma levels of CRP, liver enzymes, bile acids, and lipids were used to assess if the metabolism (physiological response) of a high-fiber lignan rich diet was affected by antibiotic treatment.

MATERIALS AND METHODS

Diets

Two experimental diets with either low (LPL) or high content of plant lignan (HPL) were produced. The diets were composed to have equal levels of energy, starch, protein, fat, and dietary fiber (Table 1). The LPL diet was based on white wheat flour with added purified wheat fiber, while rye bran replaced part of the wheat flour in the HPL diet. The purified wheat fiber used was lignin-free and cellulose-rich (Table 1). Both diets were mixed in a feed production unit (Aarhus University, Foulum), cold-pressed to pellets, packed in paper bags of 20 kg, and stored at −20 °C.

The pigs were fed three daily meals of equal size throughout the experiments. The diets were served at 9:00, 14:00, and 21:00 to mimic human meal pattern. The total daily serving corresponded to 3% of average body weight and was adjusted every week. Very few feed leftovers were observed, but if any, the total leftover for one experimental week was weighed and corrected for in the total feed intake.

Animals and Experimental Design

A total of 30 Duroc × Danish Landrace × Yorkshire female pigs with body weight of 72.5 ± 3.9 kg, purchased from a local farmer and raised by Aarhus University, Department of Animal Science, Foulum, Denmark, were used in this study. The pigs were kept in individual pens (1.45 × 2.35 m) without bedding material but provided with toys. The pigs had ad libitum access to water, and the pens were designed to allow the pigs to have physical and visual contact with neighboring pigs through the railings. The pigs were weighed once every week.

The study consisted of 4 experimental weeks. In the first week all pigs were fed a standard swine diet, and in the second week all pigs were fed the LPL diet to wash out lignans from the previous diet (data not shown). In the third and fourth weeks 10 pigs continued on the LPL diet, while 20 pigs switched to the HPL diet. During week 4, half of the HPL-fed pigs (HPL+) had daily injections with 0.5 mL/10 kg/day Streptopenprokain Rosco Vet (Boehringer Ingelheim, Copenhagen, Denmark), a broad-spectrum combination antibiotic containing benzylpenicillinprocain and dihydrostreptomycin, on days 1 and 2, increasing to 0.7 mL/10 kg/day on days 3−7,[9] in connection with the morning meal, while the other half were left untreated (HPL−). In each experimental week fasting blood samples were collected on day 7 in heparinized or EDTA-coated vacutainers from the jugular vein by venopuncture. Glutathione (GSH) was added (0.5 mg/mL) to the EDTA tubes to avoid oxidation of the samples. All blood samples were kept on ice until further processing. After collection, plasma was harvested within 1 h from collection by centrifugation for 12 min at 2000*g* at 4 °C and stored frozen at −20 or −80 °C.

Table 1. Ingredients and Chemical Composition of the Low Plant Lignan (LPL) Diet and High Plant Lignan (HPL) Diet Used for the Animal Experiment[a]

	diets	
	LPL	HPL
Ingredients (g/kg, as-fed basis)		
standard white wheat flour[b]	534	425
rye bran[b]		270
Vitacel WF 600[c]	123	
fructose[d]	100	100
wheat gluten[e]	30	
swine lard[f]	60	60
rapeseed oil	15	7
cholesterol[g]	5	5
egg powder[h]	100	100
vitamin−mineral mixture[i]	30	30
chromic oxide[g]	3	3
Chemical Composition (g/kg or μg DM)		
ash (g)	40	52
protein (*N*×6.25)	158	160
fat (g)	143	142
total carbohydrates (g)	676	645
sugars	123	147
starch (g)	410	363
total NSP (g)	143	134
insoluble NSP	130	109
soluble NSP	13	25
klason lignin	19	27
dietary fiber	162	161
total lignan (μg)	1531	21,075
Mata	2	207
Seco	29	254
Pino	0	290
Syr	1,182	15,305
Lari	100	2,460
I-Lari	7	176
med	210	2,382
gross energy (MJ/kg DM)	20.52	20.64

[a]NSP, nonstarch polysaccharides; Mata, Matairesinol; Seco, secoisolariciresinol; Pino, pinoresinol; Syr, syringaresinol; Lari, lariciresinol; I-Lari, iso-lariciresinol; Med, medioresinol. [b]Lantmännen Cerealia, Uppsala, Sweden. [c]J. Rettenmeier and Söhne Gmbh, Rosenberg, Germany. [d]Th. Geyer GmbH, Roskilde, Denmark. [e]Lantmännen Reppe, Lidköping, Sweden. [f]Daka Denmark A/S, Løsning, Denmark. [g]Sigma-Aldrich Denmark ApS, Brønby, Denmark. [h]Sanovo Food A/S, Odense, Denmark. [i]VA Vit SL/US antiox, Vestjyllands Andel, Ringkøbing, Denmark.

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The animal experiment was conducted in accordance with protocols approved by the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries and in compliance with the guidelines concerning animal experiments and care of animals under study. General health of the animals was monitored throughout the experimental period, and no serious illness was observed.

Slaughtering and Sample Collection

The animals were slaughtered by the end of week 4. Because of limited slaughtering capacity, slaughtering took place over 3 days, and thus week 4 consisted of 7−9 days. On the day of euthanasia the pigs were anaesthetized with Zolitil mixture, as described in Lærke et al. (2008). The animals were anaesthetized 3 h after the morning meal, and, for HPL+, 3 h after antibiotic injection. Blood samples were collected into heparinized and EDTA vacutainers from the carotid artery by venopuncture, and the abdominal cavity was opened to sample blood from the portal and hepatic vein. The pigs were subsequently euthanized with an overdose of sodium pentobarbital, followed by exsanguination.[31] The gastrointestinal tract was ligated at the esophagus and rectum before removal. Samples were taken directly from the urine bladder placed at −20 °C and stored at −80 °C. Gut content from cecum was stored without further treatment at −80 °C for later microbiology analysis.

Analytical Methods

Chemical Analyses of Diets, Plasma, and Urine. Chemical analyses of the diets were performed in duplicate on freeze-dried samples. DM was determined by drying to constant weight at 103 °C, and ash was analyzed according to the Association of Analytical Communities (AOAC) method no. 942.06. Nitrogen content was analyzed by DUMAS,[32] and protein was calculated as N×6.25. Gross energy was analyzed on a 6300 Automatic Isoperibol Calorimeter system (Parr Instruments). The dietary content of sugars (glucose, fructose, and sucrose) and fructans was determined as previously described.[33] Starch and NSP were determined essentially as described by Bach Knudsen,[34] Klason lignin was measured gravimetrically as the sulfuric-acid-insoluble residue as described by Theander and Westerlund[35] and Theander and Åman,[36] and dietary fiber was calculated as the sum of NSP and Klason lignin.

Blood plasma cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, asparagine transaminase (AST), and alanine amino transferase (ALT) were determined according to standard procedures (Siemens Diagnostics Clinical Methods for ADVIA 1650), and total bile acids were analyzed following an enzymatic colorimetric assay by oxidizing bile acids using 3α-hydroxysteroid dehydrogenase with subsequent reduction of thio-NAD to thio-NADH (Randox B13863). All analyses were performed using an autoanalyzer, ADVIA 1650 Chemistry System (Siemens Medical Solutions, Tarrytown, NY). All intra-assay precision validations were within 3% (CV). Plasma concentrations of C-reactive protein were determined by an ELISA-assay Phase Range, Tridelta Developments, Wicklow, Ireland following the instructions given by the manufacturer. Creatinine in urine was analyzed according to Jaffe reaction.

Targeted LC−MS/MS. The lignans in the experimental diets were determined using an LC−MS/MS method. The alkaline extraction and enzymatic hydrolyses were performed according to Penalvo et al.,[37] with minor modifications as described by Nørskov et al.[38] Samples were further cleaned up by solid-phase extraction (SPE) procedure on acetonitrile-conditioned SPE C18-E columns from Phenomenex (Torreance, CA). Samples were loaded and afterward washed with 5% methanol, and plant lignans were eluted with 450 mL of acetonitrile. Samples were then diluted to 25% acetonitrile with water containing internal standard. Samples were spun down at 10 °C for 15 min prior to LC−MS/MS measurements. The detailed extraction procedure is described by Nørskov et al.[38]

Quantification of plant lignans and enterolignans in plasma and urine samples were determined according to Nørskov et al.[30]

Nontargeted LC−MS. Fasting plasma samples collected at week 4 and urine and cecal samples collected at slaughter were subjected to nontargeted metabolomics analyses.

Plasma (100 μL) or freeze-dried cecal (50 μg) samples were mixed with 300 μL of acetonitrile with internal standards (glycocholic acid (Glycine-1 ^{13}C) and p-chlorophenylalanine, Sigma, MO) at a final concentration of 10 μg/mL and processed as described by Ingerslev et al.[39]

Urine samples (450 μL) were mixed with 50 μL of ice-cold acetonitrile with internal standards in a final concentration of 10 μg/mL. Samples were mixed immediately and incubated for 20 min at 4 °C to precipitate proteins. The supernatants were collected after centrifugation (18 500g, 4 °C, 10 min) and injected directly into the LC−MS.

Chromatographic separation was performed on an ultra-performance liquid chromatography Ultimate 3000 (UHPLC, Dionex, Sunnyvale, CA) system. The analytical column was a Fortis C18 column (100 × 2.1 mm, 1.7 μm; Fortis Technologies, Neston, U.K.) held at 30 °C. The injection volume was 2 μL, and the flow rate was 400 μL/min. The mobile phases consisted of (A) Milli-Q H_2O with 0.1% formic acid and (B) acetonitrile with 0.1% formic acid. The column was equilibrated at 5% B for 2 min, and the gradient was started 0.1 min after injection of the sample. The gradient used for plasma samples increased linearly from 5 to 90% B within 11.9 min, whereafter the column was kept under isocratic conditions for 0.3 min before returning to 5% B within 0.2 min. For urine samples the gradient used increased linearly from 5 to 60% B within 11 min, followed by a linear increase to 100% B within 0.5 min. The column was kept under isocratic conditions for 0.8 min before returning to 5% B within 0.2 min. The eluent was introduced into an Impact HD quadrupole time-of-flight (QTOF) mass spectrometer (Bruker Daltonics, Bremen, Germany) by electrospray ionization with capillary set in the positive and negative modes to 4500 and 3600 V, respectively. End-plate offset voltage was set to 500 V, the dry gas flow and temperature were 8 L/min and 200 °C, respectively, and nebulizer pressure was set to 1.8 bar. The scan range was from 50 to 1000 m/z at a sampling rate of 1 Hz. Collision energy during MS scan was set to 6 eV. Lithium formate at a concentration of 5 mM in water−isopropanol−formic acid (50:50:0.2 v/v/v) was employed as an external calibrant in the beginning of each chromatographic run with an independent syringe pump. For MS/MS analysis, Ar was used at the collision gas and collisions were carried out at energies from 10 to 40 eV. All other parameters were the same as above.

To evaluate the analytical system performance, blank samples and quality-control (QC) samples were reinjected during each batch. QC samples were pooled samples from all pigs within each sample type. Blank samples were injected to ensure that cross-contamination between samples and carry-over effects did not occur, and QC samples confirmed the stability of the

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UHPLC−MS system during the run and that sample-to-sample variability was not affected.

Data processing was performed in Compass DataAnalysis Version 4.2 (Bruker Daltonics). Acquired mass spectra were calibrated and peak detection was performed using the "Find Molecular Features" option. The spectra were exported to Bruker Compass ProfileAnalysis 2.1 for initial statistical evaluation. A matrix was generated with retention time, m/z, and respective intensities. The data were normalized according to the peak area of the internal standard to compensate for variability in sample processing and analytical platform operation. This matrix was exported to LatentiX 2.12 (Latent5 Aps.). Prior to principal component analysis (PCA) data were pareto-scaled. The scores plots were inspected,and outliers were removed based on 95% CI and plots of residual variance Hotelling's T2, after which the models were recalculated. Loading plots were used to detect metabolite ions with the greatest influence on clustering. Compounds were identified based on queries in the METLIN (http://metlin.scripps.edu/) and Human Metabolome Database (http://www.hmdb.ca/) online databases for obtaining possible chemical structures using accurate mass and mass spectrometric fragmentation patterns. The identification of the annotated compounds was confirmed with standards, when available, on the same analytical system under the same conditions (validation based on retention time and mass spectra).

For heatmap alignment, relative metabolite intensities were log10-transformed prior to alignment and plotted using the heatmap.2 function from the R-package gplots https://CRAN.R-project.org/package=gplots in RStudio Version 0.99.489.[40]

Bacterial Genomic DNA Extraction and 16S rRNA Gene Sequencing. Genomic DNA was extracted from the digesta samples using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) with several modifications. Approximately 200 mg was recovered from each sample without thawing and placed directly into sterile 2 mL tubes containing 1.6 mL of QIAamp InhibitEx buffer and 300 mg of 0.1 mm diameter zirconia/silica beads (Biospec Products, Bartlesville, OK). The samples were then homogenized twice for 1 min at 6.5 m/s in a MP FastPrep-24 tissue and cell homogenizer (MP Bio, Santa Ana, CA) with 1 min of incubation on ice between homogenizations prior to DNA purification according to the manufacturer's instructions. DNA concentration was measured using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE) and diluted to 20 ng/μL. The 16S rRNA V4 regions were amplified by PCR using the barcoded F515 forward primer and R806.[41] Amplification was performed using Ex Taq DNA Polymerase (TaKaRa, Otsu, Japan) for 30 cycles of 94 °C for 45 s, 54 °C for 60 s, and 72 °C for 30 s. PCR products were purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI), and the pooled amplicons were sequenced with an Illumina MiSeq at the Genome Center DNA Technologies Core, University of California, Davis, CA. The 16S rRNA gene sequences identified in this study were deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB19383.

Raw Illumina FASTQ sequences were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) software package version 1.8.0.[42] Demultiplexing and quality filtering were performed with the default settings, except that a minimum average quality score of 30 was used for quality filtering instead of the default score of 25, and the reverse primer sequences were removed. The open-reference OTU picking strategy in QIIME was used to select operational taxonomic units (OTUs) with 97% sequence identity to sequences in Greengenes (release 13_8) database[43] and to each other, according to the UCLUST algorithm.[44] OTUs similar to archaea, chloroplasts, or those found in very low abundance (<0.005%) were removed from the OTU table prior to further analysis. The Chao1 diversity index, Phylogenetic Diversity Whole Tree,[45] and observed species were determined for increasing numbers of randomly sampled sequences per sample to generate alpha diversity rarefaction curves. The curves became asymptotic at 10 618 sequences per sample; therefore, this number of randomly sampled DNA sequences was used for rarification. The weighted UniFrac metric was calculated using rarified data and constructed in QIIME.

Statistical Analyses. Estimates for fasting and postprandial plasma variables were determined using the following mixed model

$$Y_{ijk} = \alpha_i + \beta_j + \alpha\beta_{ij} + \upsilon_k + \varepsilon_{ijk}$$

where Y_{ijk} is the dependent variable, α_i is the diet (i = LPL, HPL−, or HPL+), β_j is the week (j = week 3 or week 4) or site (arterial, portal vein or hepatic vein), $\alpha\beta_{ij}$ is two-factor interaction term, the variance component υ_k (k = pig) accounts for the repeated measurements within pig, and the normally distributed residual error component is defined as ε_{ijk}.

For fasting plasma variables the effect of diet within each week was tested for week 3 using ANOVA. The effect of diet in week 4 was tested using ANOVA adjusted for baseline (week 3) response using the following model

$$Y_{i4k} = \alpha_i + \beta y_{i3k} + \varepsilon_{i4k}$$

where α_i is the diet (i = LPL, HPL−, or HPL+), β is the parameter related to the response y_{i3k} in week 3 (baseline), and the normally distributed residual error component is denoted by ε_{ijk}. The specific effect of antibiotic treatment in week 4 among the HPL fed animals was tested using ANOVA. Differences between weeks were tested on individual diet level using pairwise t tests.

For postprandial plasma variables the underlying tests for the effect of diet within each site were tested using ANOVA, while tests between sites were performed by multiple pairwise comparison using Tukey's honestly significant difference (HSD) test.

Linear models were used to analyze the urinary concentration and were also applied to analyze the identified metabolites. To obtain normality in the models, variables were transformed using Box-Cox transformation when appropriate. Normality and variance homogeneity were checked by examining the residual plots. Multiple pairwise comparisons using Tukey's HSD test were performed to identify differences between fixed effects.

Microbial enrichment between experimental groups was determined using Linear Discriminate Analysis Effect Size (LEfSe). Correlation analyses between taxa and metabolites were performed using RStudio. We normalized the taxa data beforehand using rarefaction to account for uneven read numbers and performed a log10 transformation on the metabolite concentrations. For inclusion in the correlation analysis, genera were required to have an average of 1% or greater abundance after rarefaction. Pearson's test with false discovery rate correction was used to determine significance.

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Table 2. Least Squares Means of Fasting Plasma Lignan Concentrations Measured after 3 Weeks on a Low Plant Lignan (LPL) Diet or 1 Week on LPL, Followed by 2 Weeks on High Plant Lignan (HPL) with (HPL+) or without Antibiotic Treatment (HPL−) for the Last 7−9 Days[a,b,c]

week	3				4				
diet	LPL	HPL			LPL	HPL	HPL		
antibiotics treatment	−	−		p-value[d]	−	−	+	p-value$_{diet}$[e]	p-value[f]
lignans (nmol/L)									
ENL	5.4[h]	111[i]	104.9[i]	<0.001	4.9[h]	148.5[i]	93.3[j]	<0.001	0.0022
95% CI	3.9−7.2	93.8−130.7	88.4−123.7		3.5−6.6	126.6−173.2	78.4−110.5		
END	0.1[h]	2.9[i]	3.1[i]	<0.001	0.1[h]	3.1[i]	2.3[i]	<0.001	
95% CI	0.3−0.4	2.2−3.6	2.5−3.9		0.0−0.2	2.4−3.9	1.8−3.0		
total enterolignan	5.5[h]	114.1[i]	108.2[i]	<0.001	5.0[h]	151.7[i]	95.8[j]	<0.001	0.0020
95% CI	4.1−7.4	96.7−133.9	91.6−127.3		3.6−6.7	129.8−176.6	80.7−113.1		
total plant lignan[g]	2.9[h]	5.9[h,i]	7.8[i]	0.0026	2.8[h]	6.5[i]	6.4[i]	0.030	
95% CI	2.1−4.1	4.0−8.9	5.2−12.2		2.0−4.0	4.4−9.9	4.3−9.6		

[a]Samples are collected at slaughter 3 h postfeeding. [b]No p-values given indicates $p > 0.05$. [c]Mean values within a row segment (week 3 or week 4) with unlike superscripts letters (h, i, j) were significantly different. [d]p-values for testing the effect of LPL and HPL in week 3. [e]p-values for testing the effect of LPL, HPL−, and HPL+ in week 4. [f]p-values for testing the effect of antibiotic treatment in (HPL+ against HPL−) in week 4. [g]Total plant lignan is the sum of the plasma concentration of Mata, Seco, Lari, I-Lari, and Hydr.

Table 3. Least Squares Means of Urinary Lignan Concentrations Measured after 3 Weeks on a Low Plant Lignan (LPL) Diet or 1 Week on LPL, Followed by 2 Weeks on High Plant Lignan (HPL) with (HPL+) or without Antibiotic Treatment (HPL−) for the Last 7−9 Days[a,b,c]

sampling site	urine			
diet	LPL	HPL	HPL	
antibiotics	−	−	+	p-value
lignans				
ENL (μmol/L)	1.3[d]	58.7[e]	41.2[e]	<0.001
95% CI	1.0−1.7	45.4−75.9	31.9−53.3	
END (nmol/L)	0.05[d]	0.6[e]	0.5[e]	<0.001
95% CI	0.04−0.07	0.46−0.86	0.36−0.67	
total enterolignan(μmol/L)	1.4[d]	59.4[d]	41.8[d]	<0.001
95% CI	1.1−1.8	45.9−76.8	32.3−54.0	
total plant lignan (nmol/L)	221.2[d]	1184.1[e]	1063.7[e]	<0.001
95% CI	179.9−274.6	888.5−1605.9	802.9−1433.1	
creatinine (mmol/L)	16.2	13.2	12.2	
95% CI	12.9−19.6	10.1−16.4	9.1−15.5	

[a]Samples are collected at slaughter 3 h postfeeding. [b]No p-values given indicates $p > 0.5$. [c]Mean values within a row with unlike superscript letters (d, e) were significantly different.

In all analyses the level of significance was $p < 0.05$, while a tendency was reported when p-values were between 0.05 and 0.1. The LDA plot and metabolite-taxa heat map only report $p < 0.05$.

RESULTS

Dietary Composition and Animal Performance

The diets were composed to have equal amounts of energy, fat, protein, dietary fiber, and digestible carbohydrates, which was successfully achieved (Table 1). The lignan content in the HPL diet was ∼14 times higher (21 075 μg/kg) than the LPL diet (1531 μg/kg).

The animals found the experimental diets palatable, and the diets were consumed within 30 min with very few leftovers. On average, the animals increased in weight from 72.5 ± 3.9 to 92.9 ± 4.3 kg with no difference between treatment groups, and the average daily gain was 970 ± 80 g.

Effect of Dietary Composition on Plasma and Urine Concentrations of Lignans

Week 3. Consumption of the plant lignan rich HPL diet translated into high fasting plasma concentrations of enterolignan (Table 2). After 1 week, the plasma concentrations of enterolignans were ∼20 times higher (108.2 and 114.1 nM) than the plasma concentrations after LPL intake (5.5 nM). ENL constituted 97% of the total enterolignans; however, the high intake of plant lignans in HPL also resulted in an increase in END. The total plant lignan concentration was also significantly higher after HPL intake, but only 3- to 4-fold. The general pattern seen in the postprandial concentrations of enterolignan and plant lignan was basically the same as at fasting (Figure S1), with increased enterolignan and plant lignan concentrations in the HPL fed animals. ENL, END, and total plant lignan concentrations tended to be highest in the portal vein, intermediate in the hepatic vein, and lowest in the artery (Figure S1).

In the urine (Table 3), intake of the HPL diet resulted in a 45 times higher ENL urinary concentrations compared with the

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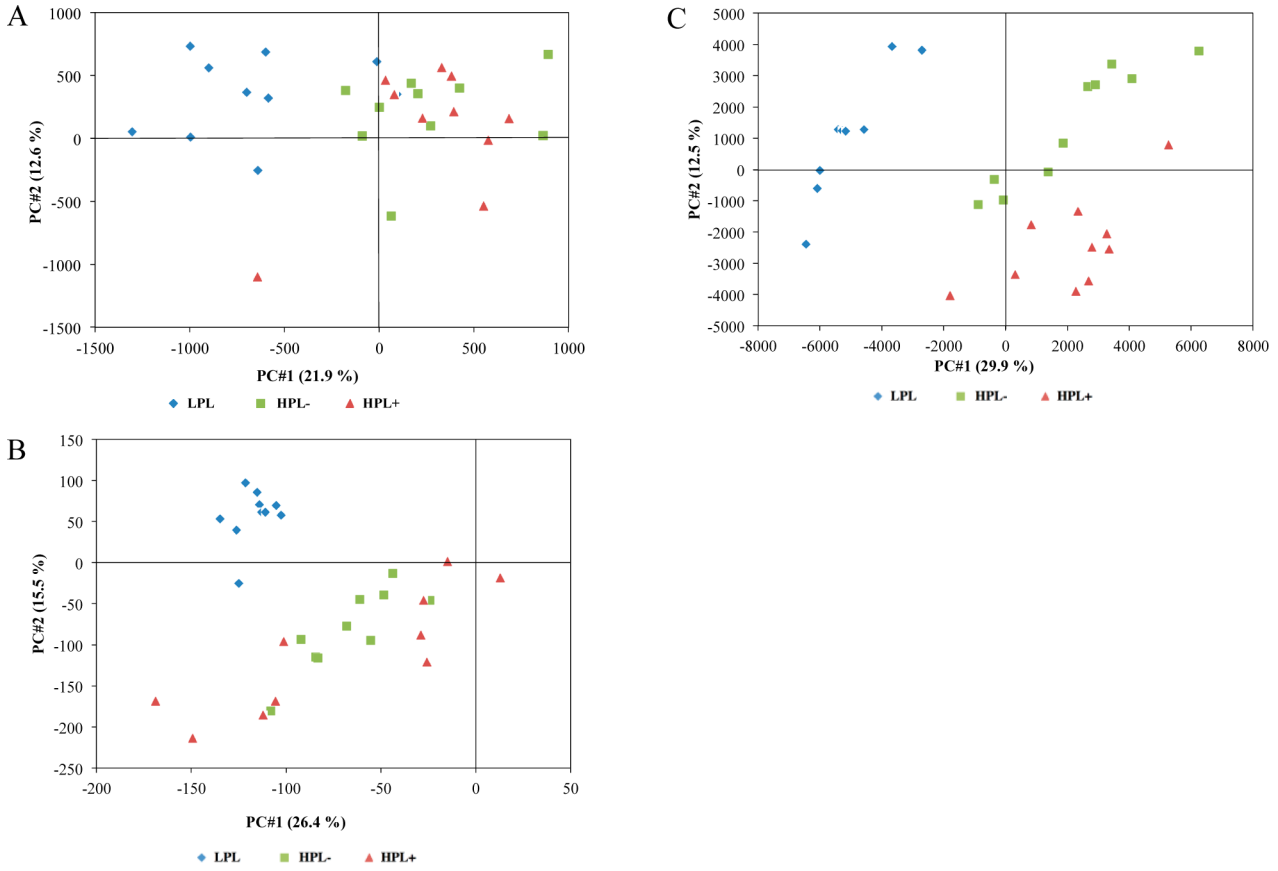

Figure 1. PCA-score plots for the metabolomics profiles of (A) plasma, (B) cecal content, and (C) urine from pigs fed low plant lignan (LPL) or high plant lignan (HPL) +/− antibiotic treatment.

animals fed the LPL diet, whereas total plant lignan concentration was ∼5 times higher. The correlations between the plasma concentrations and the urinary concentrations were similar regardless of sampling site and thus for ENL correlations were 0.96, 0.97, and 0.95 for arterial, portal, and hepatic blood, respectively (data not shown).

Effect of Antibiotic Treatment on Plasma and Urine Concentrations of Lignans

Week 4. During week 4 the pigs in HPL+ received daily antibiotic injections, while HPL− was left untreated. From the end of week 3 to the end of week 4, the enterolignan plasma concentration increased from 114.1 to 151.7 nM ($p = 0.043$) in the untreated group, whereas the enterolignan concentration did not change significantly in the group treated with antibiotics (Table 2). Hence, by the end of week 4 the antibiotic-treated group had significantly lower plasma ENL and total enterolignan concentrations than the untreated group ($p < 0.01$). In contrast, END and plant lignan concentrations were unaffected by period of ingestion and antibiotic treatment (Table 2). Interestingly, the ENL postprandial plasma concentration in the HPL+ group, although numerically lower, was not significantly different from HPL− at any sampling site measured 3 h after breakfast (Figure S1).

No significant difference in plant lignan concentration was observed between HPL− and HPL+ groups in either the fasting plasma or the urine samples (Tables 2 and 3).

Nontargeted Metabolomics of Plasma, Digesta, and Urine Samples

The fasting plasma samples collected at week 4 and the cecal digesta and urine samples collected at slaughter were subjected to nontargeted LC−MS metabolomics. The PCA of plasma samples collected during fasting revealed two main clusters: LPL samples represented one clustered together, and the HPL− and HPL+ groups formed a second (Figure 1A). The PCA of cecal digesta samples had a similar clustering pattern: LPL samples in one clustered together and HPL− and HPL+ in a second clustered with no separation between HPL− and HPL+ (Figure 1B). Thus the antibiotic treatment (HPL+) during week 4 did not result in a cluster separated from the group with no antibiotic treatment (HPL−) in either the plasma or the cecal samples. In contrast, the PCA of urine samples revealed distinct clustering according to diet and antibiotic treatment (Figure 1C), potentially driven by the antibiotic-related metabolites, as indicated in the PCA plots (Figure S4) in which these metabolites have been removed.

The metabolites discriminating between the diets and antibiotic treatment are visualized in a heatmap (Figure 2) and summarized in Table 4 along with the relative intensities and p-values. Besides metabolites related to the injected antibiotics (streptidine, penicilloid acid, penicillin G, penicillin G acid), a number of urinary metabolites discriminated the HPL− and HPL+ groups. Betaine showed a lower intensity in the HPL+ samples compared with the HPL−, as did two metabolites with structural similarity to betaine: pipecolic acid betaine and proline betaine. Two metabolites, 3-methylcrotonylglycine and 3-hydroxysuberic acid, also displayed lower

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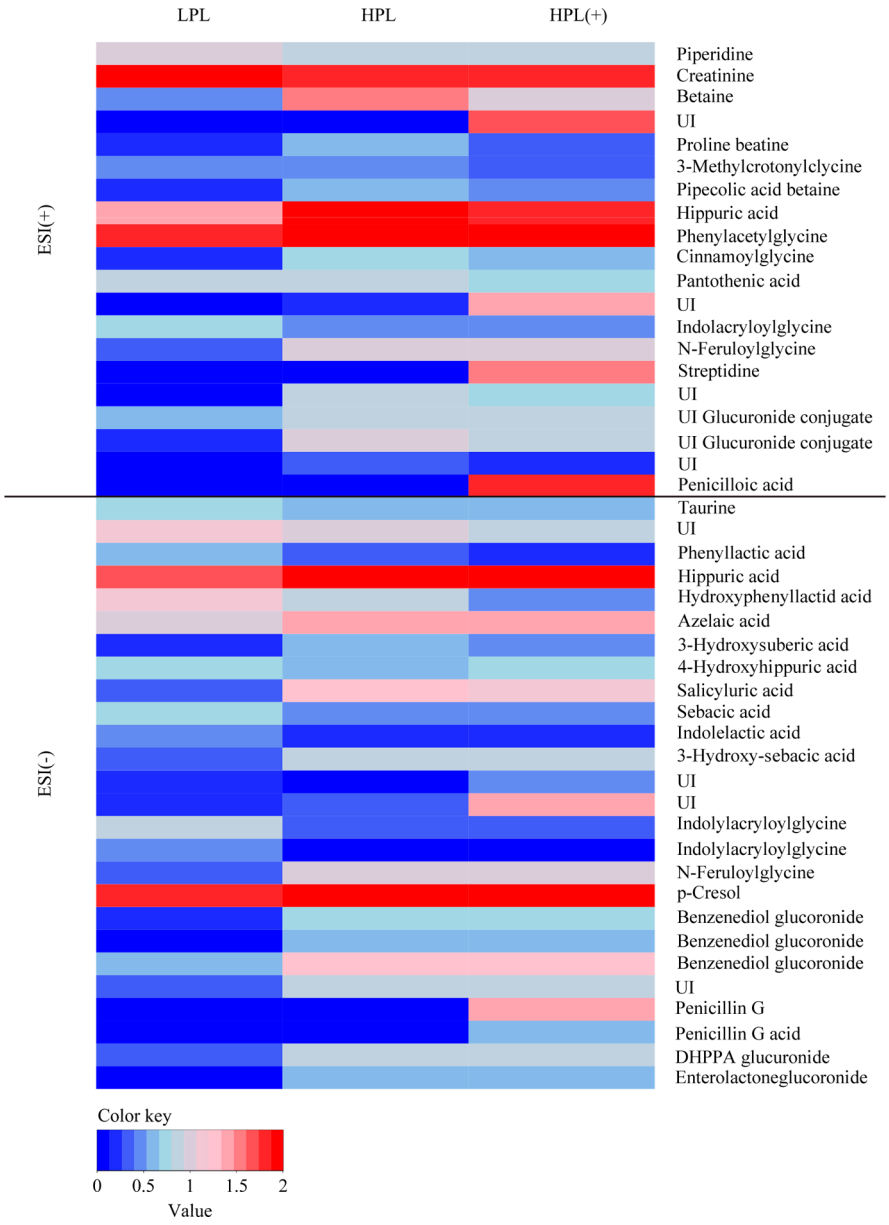

Figure 2. Heatmap alignment of selected urinary metabolites after consumption of low plant lignan (LPL) or high plant lignan (HPL) +/− antibiotic treatment. Relative intensities were log10-transformed prior to alignment and colored blue for low intensities and red for high intensities. UI, unidentified. Urinary samples were collected directly from the bladder at slaughtering 3 h after feeding.

intensity in the HPL+ samples. Furthermore, pantothenic acid (Vitamin B5) and hydroxyphenyllactic acid (possibly a polyphenol derivative) had lower intensities in the HPL+ samples.

Comparing the urinary metabolome after the intake of HPL versus LPL diets revealed an increased intensity among metabolites related to intake of cereals and vegetables (hippuric acid, azelaic acid, salicylic acid, *N*-feruloylglycine, benzenediol glucoronide, and enterolactoneglucoronide). The metabolites found in higher intensity in the LPL fed animals were generally related to amino acid metabolism: phenyllactic acid, hydroxyphenoyllactic acid, indolelactic acid, and indolylacryloylglycine. Furthermore, the intensity of creatinine was ∼1.4 times higher in the LPL group compared with the HPL−/+ groups (Table 4).

Cecal Microbial Composition

16S rRNA gene amplicon sequencing was performed to identify the microbial composition of the digesta collected from the cecum. An average of 21 185 sequencing reads was obtained for each sample after quality filtering, encompassing an average of 503 OTUs per sample. The number of observed species (alpha-diversity) was not significantly different between any of the groups (data not shown). Principal coordinates analysis (PCoA) analysis of cecal microbiota revealed a clustering according to dietary lignan content (Figure 3). This result was consistent at the taxonomic level, wherein the proportions of numerous taxa were significantly altered between pigs fed LPL and HPL (Figure 4A and Figure S2). *Clostridia* and *Betaproteobacteria* were enriched in pigs fed LPL (Figure 4B). Members of the *Bacteroidetes* phylum and several other taxa were present in higher proportions with LPL consumption, independent of antibiotic administration (Figure S2). Con-

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Table 4. List of Urinary Metabolites Discriminating between LPL, HPL−, and HPL+ Antibiotic Intake[a,b]

RT	m/z	mode	metabolite	LPL		HPL−		HPL+		p-value$_{overall}$	p-value$_{LPL\ vs\ HPL-}$	p-value$_{LPL\ vs\ HPL+}$	p-value$_{HPL-\ vs\ HPL+}$
1.03	86.0960	pos	piperidine[c]	9.62		5.39		6.05		0.0031	0.01	0.01	NS
				0.85		0.85		0.85					
0.86	114.066	pos	creatinine[c]	95.5		65.6		69.9		0.0004	0.001	0.001	NS
				4.97		4.97		4.97					
0.92	118.086	pos	betaine[c]	1.77		30.75		8.68		<0.0001	0.001	0.001	0.01
				1.09	2.86	18.54	51.00	5.37	14.03				
0.98	134.117	pos	UI	0.13		0.09		46.45		<0.0001		0.001	0.001
				0.10	0.17	0.07	0.13	35.54	60.71				
1.02	144.102	pos	proline betaine[d]	0.82		2.93		1.20		<0.0001	0.001	NS	NS
				0.24		0.25		0.25					
4.12	158.081	pos	3-methylcrotonylglycine[d]	1.83		1.90		1.04		0.0009	NS	0.001	0.001
				1.46	2.30	1.51	2.38	0.83	1.31				
1.12	158.118	pos	pipecolic acid betaine[d]	0.66		3.33		1.83		<0.0001	0.001	0.001	0.05
				0.30		0.30		0.30					
5.06	180.065	pos	hippuric acid[c]	27.1		73.6		63.2		<0.0001	0.001	0.001	NS
				4.56		4.56		4.56					
5.60	194.081	pos	phenylacetylglycine[c]	68.0		100		86.4		0.05	0.05	NS	NS
				8.75		8.75		8.75					
7.09	206.081	pos	cinnamoylglycine[c]	0.50		3.88		2.67		<0.0001			
				0.31	0.82	2.50	6.04	1.72	4.15				
3.03	220.118	pos	pantothenic acid[c]	7.10		6.73		3.76		0.02	NS	0.05	0.05
				0.86		0.86		0.86					
4.14	237.087	pos	UI[d]	0.21		0.65		27.18		<0.0001			
				0.12	0.36	0.46	0.92	21.22	34.81				
7.03	245.092	pos	indolylacryloylglycine[d]	4.78		1.78		1.61		<0.0001	0.01	0.001	NS
				3.60	6.36	1.36	2.34	1.22	2.11				
5.37	252.087	pos	*N*-feruloylglycine[d]	1.21		8.75		8.78		<0.0001	0.001	0.001	
				0.87	1.69	6.28	12.20	6.30	12.24				
0.61	263.146	pos	streptidine[d]	ND		ND		30.34		[e]			
								1.11		<0.0001	0.001	0.001	
7.39	299.128	pos	UI	0.17		6.64		4.27					
				0.14	0.21	5.49	8.04	3.56	5.11				
2.36	326.087	pos	UI glucuronide conjugate	3.23		7.34		5.86		0.0001	0.001	0.01	NS
				0.59		0.59		0.59					
4.25	328.103	pos	UI glucuronide conjugate	0.84		8.25		7.36		<0.0001	0.001	0.001	NS
				0.65	1.08	6.37	10.67	5.69	9.52				
8.96	333.206	pos	UI	ND		1.32		0.43		<0.0001[f]	0.001	0.001	0.001
						0.11		0.11					
6.78	353.116	pos	penicilloic acid[d]	ND		ND		54.12		[e]			
								42.22					
0.87	124.007	neg	taurine[c]	4.47		2.76		2.51		<0.0001	0.001	0.001	NS
				3.93	5.08	2.43	3.14	2.20	2.88				
4.22	158.082	neg	UI	14.38		7.87		6.00		<0.0001	0.001	0.001	NS

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Table 4. continued

RT	*m/z*	mode	metabolite	LPL	HPL−	HPL+	p-value$_{overall}$	p-value$_{LPL\ vs\ HPL-}$	p-value$_{LPL\ vs\ HPL+}$	p-value$_{HPL-\ vs\ HPL+}$
				0.69	0.65	0.65				
6.33	165.056	neg	phenyllactic acid[c]	3.02	1.00	0.77	0.02	NS	0.01	NS
				1.50 6.08	0.50 2.02	0.38 1.54				
5.08	178.051	neg	hippuric acid[c]	50.39	97.22	84.61	<0.0001	0.001	0.001	NS
				4.91	4.91	4.91				
4.11	181.05	neg	hydroxyphenyllactic acid[c]	13.62	6.30	2.30	<0.0001	0.001	0.001	0.001
				11.09 16.73	5.13 7.73	1.87 2.82				
7.16	187.098	neg	azelaic acid[c]	8.91	26.28	21.27	0.0001	0.001	0.001	NS
				2.53	2.53	2.53				
4.15	189.076	neg	3-hydroxysuberic acid[d]	0.49	3.59	2.14	<0.0001	0.001	0.001	0.05
				0.36 0.66	2.66 4.86	1.58 2.89				
3.69	194.046	neg	4-hydroxyhippuric acid	4.87	3.20	3.94	0.02	NS	NS	NS
				3.98 5.96	2.64 3.87	3.25 4.77				
4.05	194.046	neg	salicyluric acid[c]	1.26	15.49	13.57	<0.0001			
				1.07 1.47	13.32 18.02	11.67 15.79				
5.45	201.113	neg	sebacic acid[c]	4.83	2.22	1.75	<0.0001	0.001	0.001	
				0.21	0.20	0.20				
6.74	204.066	neg	indolelactic acid[c]	1.59	0.83	0.64	0.03			
				1.07 2.36	0.36 1.90	0.38 1.08				
6.31	217.108	neg	3-hydroxy-sebacic acid[d]	0.93	6.31	6.19	<0.0001	0.001	0.001	NS
				0.68 1.28	4.59 8.68	4.50 8.52				
7.92	230.997	neg	UI	0.36	0.20	2.06	0.001	NS	0.05	0.01
				0.16 0.83	0.08 0.50	1.11 3.81				
4.14	235.071	neg	UI[d]	0.38	0.94	28.43	<0.0001			
				0.28 0.51	0.78 1.14	23.55 34.32				
7.05	243.077	neg	indolylacryloylglycine[d,g]	5.43	1.34	1.36	<0.0001	0.001	0.001	NS
				4.08 7.23	1.02 1.75	1.03 1.78				
7.97	243.077	neg	indolylacryloylglycine[d,g]	1.58	0.30	0.27	<0.0001	0.001	0.001	NS
				1.24 2.01	0.23 0.37	0.22 0.35				
5.39	250.072	neg	*N*-feruloylglycine[d]	1.44	8.72	8.26	<0.0001	0.001	0.001	NS
				1.09 1.90	6.71 11.33	6.36 10.74				
5.77	283.082	neg	*p*-cresol glucoronide[d]	68.27	100.00	85.06	0.004	0.001	NS	NS
				6.05	6.05	6.05				
2.07	285.061	neg	benzenediol glucoronide[d]	0.64	4.98	4.67	<0.0001	0.001	0.001	NS
				0.49 0.85	3.77 6.57	3.53 6.16				
3.07	285.061	neg	benzenediol glucoronide[d]	0.23	3.58	2.54	<0.0001			
				0.17 0.33	2.63 4.87	1.86 3.45				
4.32	285.061	neg	benzenediol glucoronide[d]	2.58	19.78	17.62	<0.0001	0.001	0.001	NS
				2.10 3.16	16.10 24.28	14.35 21.64				
4.25	326.088	neg	UI	0.90	6.74	6.53	0.002	0.001	0.001	NS
				0.36 2.24	4.09 11.12	3.96 10.77				
9.03	333.091	neg	penicillin G[c]	ND	ND	23.98	[e]			
						2.02				

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Table 4. continued

RT	m/z	mode	metabolite	LPL		HPL−		HPL+		p-value$_{overall}$	p-value$_{LPL\ vs\ HPL-}$	p-value$_{LPL\ vs\ HPL+}$	p-value$_{HPL-\ vs\ HPL+}$
6.75	351.102	neg	penicilloic G acid[d]	ND		ND		3.24		[e]			
								0.43					
3.43	357.082	neg	DHPPA[h] glucuronide[d]	1.11		5.96		5.94		<0.0001	0.001	0.001	NS
				0.88	1.40	4.72	7.54	4.70	7.51				
7.43	473.145	neg	enterolactoneglucoronide[d]	0.09		2.89		2.47		<0.0001	0.001	0.001	NS
				0.07	0.11	2.28	3.67	1.94	3.13				

[a]The intensity of the most abundant metabolite in positive and negative mode, respectively, was set to 100, and the intensity of the other metabolites was "normalized" to this value. [b]No p-values indicate that $p > 0.05$. [c]Identified using commercially available standards. [d]Putatively identified using MS/MS, previously published papers, and METLIN/LIPID MAPS/Human metabolome databases. [e]No statistics as the metabolite only was present in urine from pigs fed HPL+antibiotic. [f]Statistics based on pigs fed HPL and HPL+ antibiotic. [g]Indolylacryloylglycine may undergo isomerization when exposed to light.[76] [h]3-(3,5-Dihydroxyphenyl)-1-propanoic acid.

Figure 3. Plant lignans alter the bacterial communities in pig cecal digesta. Bacterial diversity was rarified and visualized by PCoA using the weighted UniFrac metric and colored according to diet type.

versely, HPL− resulted in elevated levels of *Bacilli* and specifically *Streptococcus* and *Enterococcus* genera (Figure 4B). Remarkably, these taxa were not similarly enriched with antibiotic treatment (HPL+), and instead proportions of *Blautia*, another member of the *Firimicutes* phylum, was increased (Figure 4B). Comparisons between pigs fed HPL revealed more modest changes to the taxonomic composition of the digesta, with the most notable being the finding that *Proteobacteria* were higher with antibiotic administration (Figure S3).

Correlation analyses between cecal bacterial taxa relative abundance and metabolites were also carried out and visualized in a heatmap (Figure 5). In general, there was a good agreement between taxa enrichment and the metabolites that were significantly altered depending on lignan or antibiotics treatment. Urinary and plasma ENL were positively correlated with *Streptococcus* abundance (Figure 5). *Streptococcus* levels were also positively associated with the majority of metabolites that were enriched in the HPL fed pigs (Figure 5). *Lactobacillus*, another genus associated with HPL consumption, was the only taxa directly correlated to quantities of phenyllactic acid. Conversely, hydroxyphenyllactic acid and creatine were positively correlated with *Clostridium* and *Ruminococcus*, taxa that tended to be found in higher proportions in pigs fed LPL (Figure 5).

Plasma Lipids, CRP, and Enzymes

The plasma concentration of cholesterol in the HPL− and HPL + groups was only half of the concentration measured in the LPL group, which was also the case for the LDL plasma concentration (Table S1). The two different diets did not result in different concentrations of HDL, but as a response to the lower LDL concentration in the HPL− and HPL+ groups the LDL/HDL ratio decreased whereas the concentration of triglyceride was similar in all groups. No dietary effects were seen on the plasma concentrations of CRP, bile acids, AST or ALT, and at no point did the antibiotic treatment give rise to significantly different concentrations of the above compared with the untreated HPL fed animals.

DISCUSSION

In this study we found that high lignan intake resulted in enhanced ENL levels in female pigs but that the ENL levels were highly affected by antibiotic treatment of the pigs in which a 37% lower fasting plasma concentration was detected compared with untreated animals. To the best of our knowledge, this is the first animal study showing a direct link between antibiotic administration and lower ENL plasma concentration. These results support the associations previously

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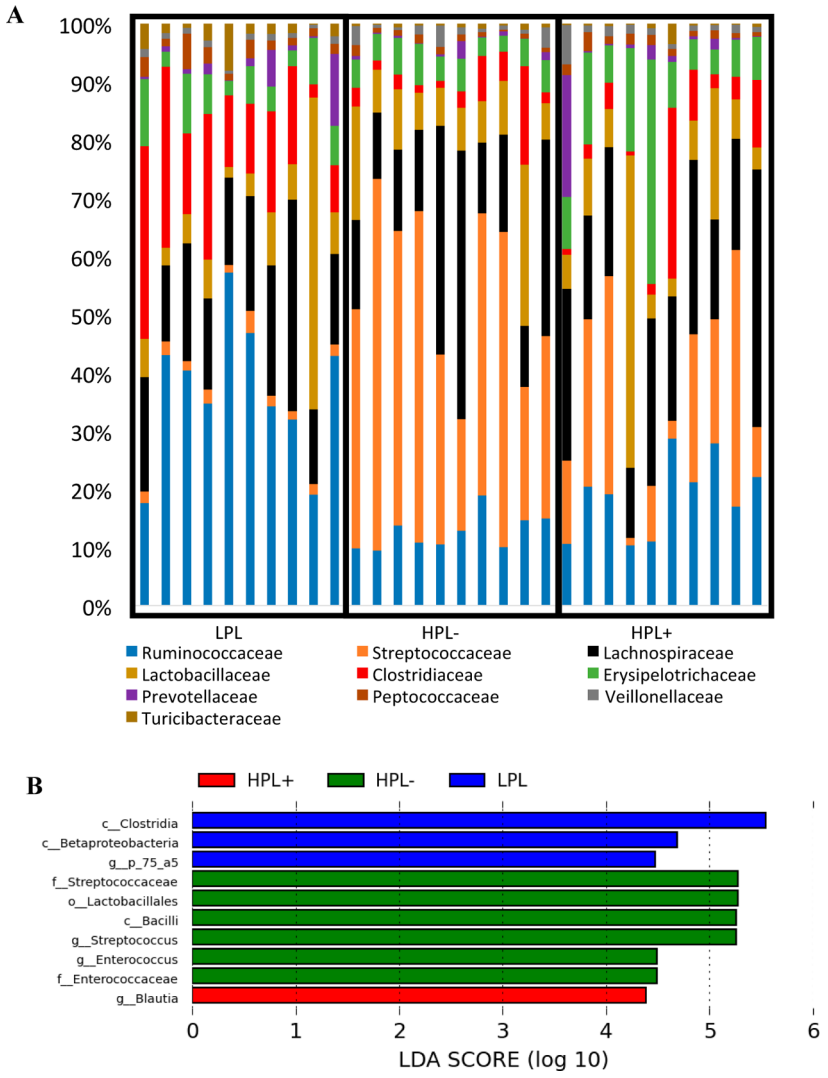

Figure 4. Plant lignans and antibiotics significantly alter bacterial taxa abundance in pig cecal digesta. (A) Rarified taxonomy table displaying the 10 most abundant families that together constitute 90% of the total 16S rRNA gene reads. (B) Significantly enriched taxa depending on pig diet. LDA, Least Discriminate Analysis (LefSE); LPL, low plant lignan; HPL, high plant lignan; HPL+, high plant lignin with antibiotics.

found in human plasma[13,15] and urine samples,[12,14] in which an inverse association between antibiotic use and ENL levels has been identified.

In humans, the lignan metabolism involves a broad range of bacterial species (reviewed in refs 46 and 47); however, the bacteria capable of producing ENL have been categorized to be subdominant members of the microbiota, possibly constituting <1% of the total microbiota.[10,48,49] Thus far, the species in pigs capable of converting plant lignans to ENL are unidentified, but it is likely that, as for humans, the species in pigs are a specialized niche. One of the bacteria strains capable of dehydrogenating END to ENL in humans has been identified as *Lactonifactor longoviformis*. It is characterized by being Gram-positive, strictly anaerobic, and unable to grow in the presence of penicillin.[48] The microbial analysis of the cecum samples revealed that the taxa enrichment in the cecum samples differed according to antibiotic treatment where HPL+ resulted in increased enterotoxin producing *Proteobacteria*. The positive correlation between plasma and urinary concentration ENL and other metabolites related to HPL intake and *Streptococcus* is interesting and should be further studied.

No significant difference in ENL concentrations between HPL+ and HPL− animals was observed in the postprandial plasma and urinary samples collected 3 h after breakfast. Elevated circulating levels of ENL require that the lignan precursors reach the large intestine. However, at sustained high intakes, fluctuations in circulating levels of ENL are not strict synchronized with dietary intakes.[50] Recently we published a study on pigs fed a lignan-rich diet and demonstrated that in the postprandial period ENL concentrations were lower than those measured after an overnight fast.[9] In general, the ENL production in pigs[50] and humans[51] is subject to high interindividual variation, which might partially explain why statistical significance is difficult to obtain. We thus suggest that antibiotics do have an effect on the postprandial ENL level; however, the results do not reach statistical significance owing to a combination of high individual difference and temporarily lower ENL concentrations.

Expanding from the effect of antibiotic use on the lignan concentration to the effect on the metabolic profile obtained from the nontargeted analysis, we were surprised to find that antibiotic treatment did not change the intensities among the metabolites related to whole-grain intake considerably. Previous

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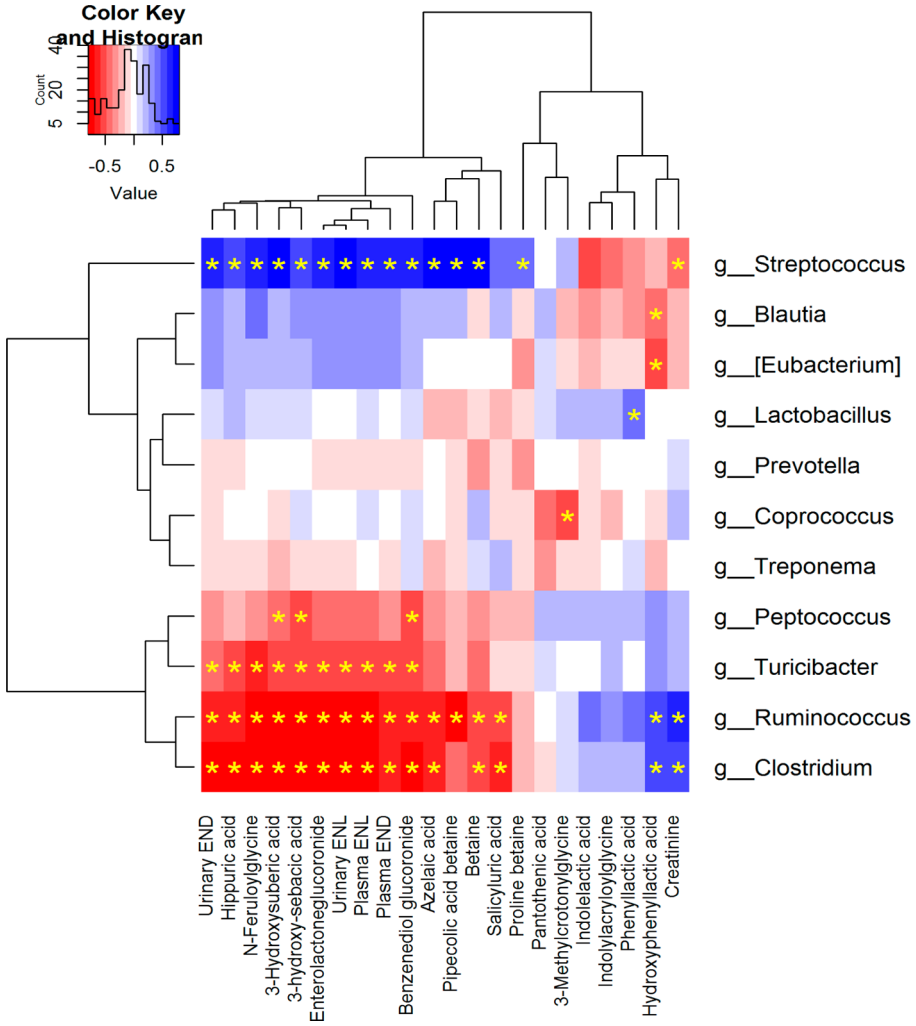

Figure 5. Correlations between the dominant cecal bacterial and metabolite concentrations in the urine, plasma, and cecum. The heat map shows correlations between $\log_{10}$-transformed metabolite intensities and the rarified relative abundance of bacterial taxa. Genera were excluded if they constituted <1% of all genera in the cecal digesta. The *x*-axis contains urinary and plasma (END and ENL) metabolites that were significantly altered between diets and antibiotic treatment. The blank cells indicate taxa that were below the detection limit. Yellow stars indicate statistical significance according the Pearson's test with false discovery rate correction.

studies on rodents have shown different excretion concentrations of hippuric acid, phenylacetylglycine, and taurine after antibiotic treatment.[52−54] In the current study, however, we observed no difference in urinary concentrations of these metabolites between the HPL+ and HPL− animals. The same combination antibiotics that gives a synergistic action against most Gram-positive and Gram-negative bacteria was used in this and the rat study.[52,54] However, while the antibiotics were administered orally in the rat study, in this study we administered antibiotics via muscular injections, and this might influence the efficacy of the antibiotics. Intramuscular administration of antibiotics was chosen over oral administration to ensure that each pig had identical doses of antibiotics, and the product and administration route was that commonly used in veterinary medicine against a range of bacterial infections including enteritis. In humans, penicillin is normally administered orally, and this could be a limitation of our study design. However, antibiotic metabolites, penicilloic acid, and an unidentified component presumably related to antibiotic metabolism found at m/z 237.087 and 235.071 in positive and negative modes, respectively, were detected in the digesta samples, indicating that antibiotics were not cleared from the body before reaching the gut (data not shown). Another possible explanation could be the fact that the pigs were fed a diet rich in phenolic compounds, thereby increasing the overall concentration of metabolites related to cereal intake, which might have dampened the effect of antibiotic treatment.

One of the discriminatory compounds between HPL+ and HPL− animals was betaine, a component that acts as an osmolyte and methyl donor. The dietary sources of betaine are diverse and include wheat and rye bran,[55] and betaine concentration after whole-grain intake is shown to increase in pigs,[56,57] which was confirmed in the HPL− animals in the current study. Findings by Wang et al.[58] show that trimethylamine N-oxide production from choline via betaine depends on microbiota metabolism and is hindered by administration of antibiotics, ultimately yielding higher plasma concentrations of betaine in the antibiotic treated subjects. Similar results are seen in rats, where urinary betaine concentrations are higher in animals treated with antibiotics.[52] Our data, however, do not agree with the previous findings, as the betaine concentration was found in lower concentrations in HPL+ compared with HPL− animals. It is interesting to note that *Streptococcus* was significantly positively correlated with urinary betaine intensity

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($p < 0.0002$) and therefore the differences between studies might be the result of host-specific intestinal microbiota.

Both pantothenic acid and hydroxyphenyllactic are metabolites of whole-grain products and are produced by the gut microbiota.[59,60] In this study the urinary concentrations of these metabolites are approximately reduced to half in the HPL + animals compared with the HPL− animals. This might reflect an impaired gut microbial production due to ecological changes in the microbiota caused by the antibiotics.[61] We also saw a significant positive correlation between *Lactobacillus* and *Clostridium* and phenyllactic acid and hydroxyphenyllactic acid, respectively, possibly caused by the capacity of these taxa to produce these compounds.[60]

3-Methylcrotonylglycine is a product of leucine metabolism. In a study on mice treated with high doses of broad-spectrum antibiotics, an increase in *N*-isovalerylglycine was seen in the treated mice. *N*-Isovaleryglycine is a metabolite found upstream to 3-methylcrotonylglycine in the leucine degradation. The authors of the mice study suggested that the leucine degradation is hindered due to ecological changes in the microbiota following an antibiotic treatment, resulting in an accumulation of *N*-isovaleryglycine.[62] Leucine degradation in antibiotic treated pigs might be affected in a similar way. However, we did not identify an increase in *N*-isovaleryglycine; we observed a decrease in 3-methylcrotonylglycine, which could indicate a stalled leucine metabolism. The same study reported an increased urinary creatinine concentration in the treated animals; however, this is not supported by the current study (Table 3).

In addition to establishing a direct link between antibiotic use and reduced ENL levels, this study also investigated some of the proposed health effects connected to high plant lignan intake compared with low intake. The current study demonstrates that plant lignan and enterolignan concentrations in plasma and urine were largely affected by the dietary plant lignan concentration. This was despite the fact that the main plant lignan in the HPL diet was Syr, a plant lignan shown to have a low ENL conversion rate.[63] We speculate if the other plant lignans in the diet are responsible for the increased ENL plasma concentration or if conversion of Syr requires a microbiota adaptation possibly occurring in response to frequent intake of Syr to efficiently produce ENL, which was not accounted for in the study by Heinonen et al.[63]

Furthermore, we observe a time effect of lignan intake, as the ENL concentration measured in the HPL− animals increased after intake of the HPL diet for 2 weeks compared with 1 week (Table 2). The effect of duration of the intervention with a diet rich in plant lignans has previously been addressed, and results are not fully consistent.[10] Stumpf et al. did not observe a significant increase in serum ENL between intake of lignan rich diet for 6 or 12 weeks,[64] nor did Kuijsten et al. see an effect of treatment order in a crossover study where participants were fed ground, crushed, and whole flaxseed for 10 days.[65] On the contrary, Tarpila et al. found that the ENL concentration increased significantly after flaxseed supplementation for 2 to 4 months.[66] Long-term changes in diet might lead to ecological changes in the microbiota of humans,[67,68] and a recent study showed that the microbiota composition in pigs was different according to the pigs being fed either wheat or barley.[69] In connection to ENL production, it is tempting to speculate that long-term plant lignan intake could induce adaptations in the microbiota toward an improved efficiency of plant lignan metabolism; however, a more in-depth microbial analysis of cecal content or feces will be required to address this further.

Postprandial samples were collected from a peripheral artery, the portal vein, and the hepatic vein. Although no statistical significance was reached due to large interindividuel variation, the mean total enterolignan and total plant lignan concentrations were numerically higher in the portal vein, intermediate in the hepatic vein, and lowest in the artery. The portal vein drains from the gastrointestinal tract from which the lignans are absorbed, and thus the higher concentration of lignans compared with the concentrations found in the artery is a proxy for lignan enrichment over the gut. Lignans undergo enterohepatic circulation,[50,70,71] and the porta-hepatic differences most likely occur due to hepatic clearance of lignans from the blood to the gall ducts.[71]

The nontargeted analysis of urine metabolites indicated that metabolites related to cereal intake were found in high intensity after HPL intake. Previous work from our group has found similar associations after intake of a rye-based diet fed to pigs.[39,72] Intake of LPL was associated with higher intensity in metabolites related to amino acid metabolism. Previous studies report changes in plasma amino acid composition upon intervention with rye bread compared with white wheat bread, where plasma concentrations of branched chain amino acid decreased[73] and phenylalanine and methionine increased.[74] Urine samples were not analyzed in the studies, but it is likely that the differences in metabolites related to amino acid metabolism occur due to a changed amino acid plasma profile.

Increased lignan intake resulted in an improved lipid profile compared with LPL intake, which is in concert with what has previously been reported in pigs[31] and in humans, as reviewed by Peterson et al.[3] In keeping with Lærke et al.,[31] we did not see a change in plasma concentrations of triglycerides as a response to lignan intake. Interestingly, the plasma lipid profile measured in the antibiotic-treated animals did not differ from that in the untreated animals, despite a lower fasting plasma ENL concentration. This could indicate that intake of the soluble fibers in the LPL and HPL diet, more than increase in ENL concentration, is responsible for the improved plasma lipid profile. A similar observation was reported by Hallund et al., who conducted a crossover study with healthy postmenopausal women a fed SDG-enriched diet. While the ENL levels increased significantly after a 6-week intervention, the plasma lipid concentrations remained unchanged.[75]

Interestingly, no effect of diet was observed in plasma concentrations of CRP. Eichholzer et al. (2014) report that per 1% increase in ENL in participants in the U.S. National Health and Nutrition Examination Survey, CRP concentrations decreased by 7.1%.[27] Additionally, a 6-week intervention study in which healthy postmenopausal women were fed lignan complex or placebo showed reduced CRP concentrations among the women fed the lignan complex.[28] In the present study, the animals were fed the lignan rich diet for only 2 weeks compared with the cross-sectional study where participants were eating their habitual diet and the interventional study that lasted for 6 weeks. This could indicate that the dietary-induced CRP changes might not become evident until after several weeks.

■ CONCLUSIONS

Using targeted metabolomics we were able to identify a direct link between use of antibiotics and substantially lower fasting

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ENL concentrations and thereby support associations previously found in epidemiological studies. Nontargeted metabolomics identified a smaller number of metabolites also affected by the antibiotic treatment, possibly due to ecological changes in the microbiota. However, the improved lipid profile induced by high plant lignan intake was not affected by the antibiotic treatment and the associated lower ENL concentration, which could suggest that ENL is not the only component driving the health-associated effects linked to high lignan intake.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.6b00942.

Table S1: Least squares means of fasting inflammatory markers, lipids, and enzymes measured after 3 weeks on a low plant lignan diet or 1 week LPL followed by 2 weeks on high plant lignan with or without antibiotic treatment for the last 7−9 days. Figure S1: Least squares means of plasma lignan concentrations measured after 14 days being fed low plant lignan or high plant lignan and ±7 days antibiotic treatment. Figure S2: Bacterial taxa significantly altered between LPL and HPL diets. Figure S3. Bacterial taxa significantly altered between HPL− and HPL+ diets. Figure S4: PCA plots of urinary metabolites in which the antibiotic-related metabolites have been removed. (PDF)

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Author Contributions

A.K.B., H.N.L., M.S.H., N.P.N., and K.E.B.K. planned the experiment. A.K.B., M.S.H., and H.N.L. conducted the animal experiments. N.P.N. and M.S.H. completed the LC−MS analyses, B.M.-S. and M.L.M. analyzed microbiology samples. A.K.B., L.F., and K.E.B.K. performed statistical analyses. A.K.B., M.L.M., B.M.-S., and K.E.B.K. wrote the paper. All authors read and approved the manuscript.

Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

END, enterodiol; ENL, enterolactone; HPL−:, high plant lignan (no antibiotic treatment in week 4); HPL+:, high plant lignan (antibiotic treatment in week 4); LPL:, low plant lignan; CRP, C-reactive protein; HDL, high-density lipoprotein; Hydr, 7-hydroxymatairesinol; I-Lari, iso-lariciresinol; Lari, lariciresinol; LC−MS, Liquid chromatography−mass spectrometry; LC−MS/MS, liquid chromatography−tandem mass spectrometry; LDL, low-density lipoprotein; LPL, low plant lignan diet; Mata, matairesinol; Med, medioresinol; NSP, nonstarch polysaccharides; OTU, operational taxonomy units; PCA, principal component analysis; PCoA, principal coordinates analysis; Pino, pinoresinol; QIIME, quantitative insight into microbiotal ecology; SDG, secoisolariciresinol diglucoside; Seco, secoisolariciresinol; SPE, solid phase extraction; Syr, syringaresinol; UHPLC−MS, ultra-high-performance liquid chromatography−mass spectrometry

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