Short Communication

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Ferulic and coumaric acid in corn and soybean meal-based diets and in feces from pigs fed these diets

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Abstract

BACKGROUND: Arabinoxylan is the main fiber component in corn and corn co-products that are commonly included in pig diets. However, this fiber fraction is resistant to enzymatic degradation in the gastrointestinal tract of pigs. Ferulic acid and *p*-coumaric acid are covalently linked to arabinoxylan, so it is likely that the majority of these hydroxycinnamic acids are excreted in feces. However, data to confirm this have not been reported. The objective of this research was therefore to quantify the ferulic and *p*-coumaric acids in a diet based on corn and soybean meal (SBM) and in a diet based on corn, SBM, and distillers' dried grains with solubles, as well as in feces from pigs fed these diets.

RESULTS: The concentration of bound ferulic and coumaric acids in diets was greater in the corn-SBM-DDGS diet and in feces from pigs fed this diet than in the corn-SBM diet and feces from pigs fed that diet. The disappearance of free coumaric acids was greater (>85%) than that of bound phenolic acids (<50%) in both diets. The disappearance of free coumaric acid and bound ferulic acid in the intestinal tract of pigs was not different between the two diets. In contrast, disappearance of bound coumaric acid was greater ($P < 0.05$) in the corn-SBM diet than in the corn-SBM-DDGS diet.

CONCLUSION: A diet based on corn and SBM contains less hydroxycinnamic acid than a corn-SBM-DDGS diet but bound phenolic acids are more resistant to digestion by pigs than free phenolic acids.

Keywords: arabinoxylan; corn; coumaric acid; distillers dried grains with solubles; ferulic acid; pigs

INTRODUCTION

Arabinoxylan is the main fiber component in corn and corn co-products.[1] Arabinoxylan has a backbone of β-(1 → 4)-linked xylosyl residues, which contain side chains of arabinosyl, galactosyl, and glucuronic acid residues. Some of the arabinosyl units in the side chains may be linked further to ferulic acid or *p*-coumaric acid by ester bonds.[2] Ferulic acid in corn is esterified to the *O*5- hydroxy group of arabinose residues,[3,4] and coumaric acid is linked to arabinoxylan through covalent bonds.[5]

In corn, arabinoxylan is located in the endosperm and bran cell walls. Arabinoxylan from the endosperm has more branches, but contains less ferulic acid, than arabinoxylan from the bran.[5,6] Ferulic acid and *p*-coumaric acid can also be bound to lignin.[4] Ferulic acid residues are mainly incorporated into lignin via radical coupling, similar to other monolignols, whereas *p*-coumaric acid residues may be esterified to the primary hydroxyl groups of the lignin building blocks.[4,7]

In addition to (esterified) ferulic acid and to a lesser extent *p*-coumaric acid, diferulic acids (DFA), and triferulic acids (TriFA), which are covalently bound (e.g., esterified) to cell-wall arabinoxylan in corn bran, have been identified.[3,6,8] These DFA and TriFA arabinoxylan chains can crosslink, which obstructs enzymatic degradation of the cell wall,[4] and it is therefore likely that the majority of the hydroxycinnamic acids are excreted in the feces. However, as far as the authors are aware, data to confirm this have not been reported. Therefore, the objective of this study was to determine the fate of ferulic acid and coumaric acid on digestibility and fecal accumulation of phenolic acids in pigs fed a diet based on corn and soybean meal (SBM) or a diet based on corn, SBM, and distillers' dried grains with solubles (DDGS). The hypothesis was that concentrations of coumaric acid and ferulic acid are greater in the diet containing corn, SBM, and DDGS than in the corn-SBM diet, and that feces from pigs fed the corn-SBM-DDGS diet contained more coumaric and ferulic acid than feces from pigs fed the corn-SBM diet.

MATERIALS AND METHODS

The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee at the University

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of Illinois. Pigs used in the experiment were the offspring of Line 359 boars mated to Camborough females (PIC, Hendersonville, TN, USA).

Animals, diets, and experimental design

Details on diet formulations, pig management, and calculated values for the digestibility of energy and nutrients have been published elsewhere.[9] Briefly, a diet based on corn and SBM, and a diet based on corn, SBM, and DDGS was used (Table 1) and fed to 24 pigs (initial body weight: 61.71 ± 5.39 kg) with 12 replicate pigs receiving each diet. Pigs were housed individually and provided feed on an *ad libitum* basis for 12 days, and during the following 12 days diets were provided. The amount was 3.2 times the maintenance metabolizable energy requirement,[10] which was provided in two equal meals each day. On day 17 in the morning, a color marker was included in the meal fed to each pig and fecal collections started as soon as the marker appeared in the feces.[11] On day 22, in the morning, a second color marker was fed and fecal collections ceased when the second marker appeared in the feces. Throughout the study, the pigs had free access to water.

Table 1. Composition of experimental diets

	Diet	
Ingredient, g kg^{-1}	Corn-soybean meal	Corn-soybean meal-distillers dried grain with solubles
Ground corn	638.2	461.7
Soybean meal	330.0	100.0
Distiller dried grains with solubles	-	400.0
Soybean oil	10.0	10.0
Ground limestone	13.0	15.0
Dicalcium phosphate	3.0	1.5
L-Lys HCL, 78 g kg^{-1} Lys	-	5.2
L-Thr, 99 g kg^{-1} Thr	-	0.5
L-Trp, 99 g kg^{-1} Trp	-	0.3
Phytase premix[a]	0.3	0.3
Salt	4.0	4.0
Vitamin-mineral premix[b]	1.5	1.5
Analyzed values		
Gross energy, MJ kg^{-1}	15.90	16.70
Crude protein, g k^{-1}	207.40	194.60

[a] The phytase premix (Optiphos 2000, Huvepharma, Sofia, Bulgaria) contained 2000 phytase units per g. At 0.3 g kg^{-1} inclusion of phytase premix, the premix provided 600 units of phytase per kg in the complete diet.
[b] The vitamin-micromineral premix provided the following quantities of vitamins and micro minerals per kg of complete diet: Vitamin A as retinyl acetate, 11 150 IU; vitamin D_3 as cholecalciferol, 2210 IU; vitamin E as DL-alpha tocopheryl acetate, 66 IU; vitamin K as menadione nicotinamide bisulfate, 1.42 mg; thiamin as thiamine mononitrate, 1.10 mg; riboflavin, 6.59 mg; pyridoxine as pyridoxine hydrochloride, 1.00 mg; vitamin B_{12}, 0.03 mg; D-pantothenic acid as D-calcium pantothenate, 23.6 mg; niacin, 44.1 mg; folic acid, 1.59 mg; biotin, 0.44 mg; Cu, 20 mg as copper chloride; Fe, 125 mg as iron sulfate; I, 1.26 mg as ethylenediamine dihydriodide; Mn, 60.2 mg as manganese hydroxychloride; Se, 0.30 mg as sodium selenite and selenium yeast; and Zn, 125.1 mg as zinc hydroxychloride.

Chemical analysis

At the end of the experiment, fecal samples were thawed and mixed for each pig, and then dried in a 50 °C forced air-drying oven (Model 8, Metalab, Equipment Corp., Hicksville, NY, USA) prior to chemical analysis. Fecal and diet samples were analyzed in duplicate for dry matter (DM) using Association of Official Analytical Chemists (AOAC) method 930.15.[12] Diet samples were also analyzed for nitrogen using a combustion procedure (AOAC method 990.03)[12] on a Leco FP628 (Leco Corp., Saint Joseph, MI, USA). Crude protein was calculated using a nitrogen-to-protein conversion factor of 6.25. Diet samples were also analyzed for soluble dietary fiber and insoluble dietary fiber (AOAC method 991.43)[12] using the Ankom TDF dietary fiber analyzer (Ankom Technology, Macedon, NY, USA).

Extraction of free and bound ferulic acid and coumaric acid

Extraction and quantification of hydroxycinnamic acids in diets and fecal samples were performed in triplicate.[13,14] To extract the total (free and ester-bound) amount of ferulic and *p*-coumaric acid, approximately 100 mg of sample was weighed and suspended in 10 mL of sodium acetate buffer (50 mmol L^{-1}, pH 5.7) or 10 mL 0.5 mol L^{-1} KOH. The suspensions were incubated for 20 h in the dark at 37 °C under head-over-tail mixing. After incubation, samples were centrifuged (5000 × *g*, 20 min, 4 °C). Prior to reversed phase ultra-high-performance liquid chromatography photodiode array with in-line electrospray ionization mass spectrometry analysis, supernatants containing KOH were diluted five times in ultrapure water. Supernatants of incubations in buffer were not diluted. All samples were stored at 4 °C in the ultra-high-performance liquid chromatography autosampler.

Reversed phase ultra-high-performance liquid chromatography photodiode array with in-line electrospray ionization mass spectrometry analysis (RP-UHPLC-PDA-ESI-MS)

After extraction, ferulic and coumaric acids were analyzed using an ultra-performance liquid chromatography system (Thermo Scientific, Waltham, MA, USA) equipped with a pump, degasser, auto-sampler, and photodiode array detector. Samples (1 µL) were injected onto an Acquity ultra-performance liquid chromatography BEH C18 column (150 × 2.1 mm, particle size 1.7 µm) with a VanGuard guard column of the same material (Waters, Milford, MA, USA). A flow rate of 400 µL min^{-1} and a column temperature of 45 °C were used. Water (A) and acetonitrile (B) were used as eluents and were both acidified with 1 g kg^{-1} formic acid. The elution profile was as follows: 0 to 2 min at 5% B (isocratic), 2 to 15 min: from 5 to 40% B (linear gradient), 15 to 16 min: from 40 to 100% B (linear gradient), 16 to 20 min: at 100% B (isocratic), 20 to 21 min: from 100 to 5% B (linear gradient), and 21 to 25 min: at 5% B (isocratic).

Mass spectrometry was performed on an LTQ Velos Pro mass spectrometer (Thermo Scientific) coupled to ultra-high-performance liquid chromatography equipment and an electrospray ionization probe. Nitrogen was used as sheath gas and auxiliary gas. Data were recorded in negative ionization mode over an *m/z* range of 120 to 1500. Data-dependent tandem mass spectrometry analysis was performed using collision-induced dissociation with a normalized energy of 35% and wideband activation was enabled. The ion transfer temperature was 263 °C, the source heater temperature was 425 °C, and a source voltage of 2.5 kV was used. Quantification of ferulic acid and *p*-coumaric acid was performed based on extracted-ion chromatograms at *m/z* 193 and 163, respectively. Standard curves (1 to 50 µg mL^{-1}) for

Table 2. Tentative identification of phenolic acid compounds using reversed phase ultra-high-performance liquid chromatography photodiode array with in-line electrospray ionization mass spectrometry.[a]

(Tentative) identification	Retention time (min)	Tandem mass spectrometry fragments[b]
p-coumaric acid	7.50	119 (100)
Ferulic acid	8.45	134 (100), 149 (88)
8–8′-(furan)-diferulic acid	9.13	193 (100), 341 (38)
8–8′-(aryl)-diferulic acid	9.22	341 (100)
8–8′-(furan)-diferulic acid	9.50	233 (100), 297 (89), 151 (44), 359 (33), 207 (28), 218 (25), 136 (24), 282 (16), 315 (16), 148 (14), 163 (14)
8–8′-(furan)-diferulic acid	9.84	233 (100), 359 (67), 151 (54), 207 (33), 136 (28)
8–5′-diferulic acid	9.66	341 (100), 297 (68)
5–5′-diferulic acid	11.14	341 (100), 326 (41), 282 (27)
8-*O*-4′-diferulic acid	12.23	193 (100), 313 (86), 341 (48), 326 (8)
Triferulic acid	12.67	533 (100), 355 (35), 311 (18), 489 (12)

[a] Adapted from Vismeh *et al.*[31] and Xiang *et al.*[32]
[b] Numbers between brackets refer to the relative intensity of fragment ions.

Table 3. Analyzed dry matter, crude protein, fiber, and hydroxycinnamic acids in experimental diets

Item	Corn-soybean meal	Corn-soybean meal-distillers dried grains with solubles
Dry matter, g kg^{-1}	878.4	876.9
Crude protein, g kg^{-1}	207.4	194.6
Soluble dietary fiber[a], g kg^{-1}	1.1	17.1
Insoluble dietary fiber[a], g kg^{-1}	154.8	250.9
Total dietary fiber[a], g kg^{-1}	156.0	268.0
Free ferulic acid, μg mg^{-1}	ND[b]	ND
Bound ferulic acid, μg mg^{-1}	1.22	4.12
Free *p*-coumaric acid, μg mg^{-1}	0.03	0.04
Bound *p*-coumaric acid, μg mg^{-1}	0.17	0.41
Diferulic acid[c], mass spectrometry area mg^{-1}	1170	4287
Triferulic acid[c], mass spectrometry area mg^{-1}	113	332
Peak area ratio	2.00	2.20

[a] Analyzed using the Ankom procedure (Ankom Technology, Macedon, NY, USA).
[b] ND, not detected.
[c] Expressed as extracted-ion peak areas per mg of sample ([M-H]$^-$ = 385 and 403 for diferulic acid; [M-H]$^-$ = 577 for triferulic acid). No molar response factors are known for these compounds but their abundance is expressed as peak area per milligram sample.

quantification of coumaric and ferulic acids were constructed by using pure ferulic acid and *p*-coumaric acid standards. Free and bound coumaric and ferulic acid fractions were quantified. The bound ferulic and coumaric acid fractions were calculated by subtracting the free fraction from the concentration of total ferulic and coumaric acids. Diferulic acid (DFA) and triferulic acid (TriFA) were semi-quantified from extracted-ion chromatograms at *m/z* 385, 403, and 577 (Table 2). As no molar response factors are known for these compounds, their abundance is expressed as peak area per mg sample (Table 3).

Calculations and statistical analysis

Disappearance of ferulic and coumaric acid was calculated using Equation (1):[1]

$$\text{disappearance}\,(\%) = \frac{\text{phenolic acid intake} - \text{phenolic acid output}}{\text{phenolic acid intake}} \times 100 \quad (1)$$

The normality of residuals and the assumptions of the model were tested using the UNIVARIATE procedure on SAS software (SAS Inst. Inc., Cary, NC, USA). Data were analyzed using the PROC MIXED procedure on SAS with pig as the experimental unit. The model included diet as the fixed effect, whereas block and pig within block were considered random effects. Treatment means were calculated using the LSMEANS statement and means were separated using the PDIFF option of SAS. Statistical effects were considered to be significant at $P < 0.05$ and were considered to suggest tendencies at $0.05 \leq P < 0.10$.

RESULTS

The diet based on corn and SBM contained 0.03 μg mg^{-1} free coumaric acid, whereas the diet based on corn, SBM, and DDGS contained 0.04 μg mg^{-1} (Table 3). Concentration of bound coumaric acid in the diet based on corn and SBM was 0.17 μg mg^{-1}, whereas the diet based on corn, SBM, and DDGS contained 0.41 μg mg^{-1}. The concentration of bound ferulic acid in the corn-SBM-DDGS diet was 3.4 times greater than in the diet containing corn and SBM. The values of bound DFA and TriFA in diets were summarized as peak area per milligram of sample, and the presence of DFA was greater than TriFA in both diets. A tentative characterization of the DFA and TriFA indicated the presence of 8–8′-(furan)-diferulic acid, 8–8′-(aryl)-diferulic acid, 8–5′-diferulic acid, 5–5′-diferulic acid, and 8-*O*-4′-diferulic acid.

Concentrations of free and bound coumaric acid in feces from pigs fed the corn-SBM-DDGS diet were greater ($P < 0.01$) than in feces from pigs fed the diet based on corn and SBM (Table 4). No difference was observed between the concentration of free ferulic acid in feces from pigs fed the corn-SBM diet and in feces from pigs fed the diet based on corn, DDGS, and SBM. However, concentration of bound ferulic acid in feces from pigs fed the corn-SBM-DDGS diet was greater ($P < 0.05$) than in feces from pigs fed the diet containing corn and SBM. Concentrations of DFA and TriFA in feces from pigs fed the diet based on corn, SBM, and DDGS were not different from that of feces from pigs fed the corn-SBM diet. Disappearance of bound ferulic acid and bound coumaric acid in both diets was less than 50%, and no difference was observed for disappearance of bound ferulic acid between the two diets. However, disappearance of bound coumaric acid was greater ($P < 0.05$) in the corn-SBM diet than in the corn-SBM-DDGS diet. Disappearance of free coumaric acid in both diets was greater than 85%, and no difference was observed between the two diets.

Table 4. Analyzed hydroxycinnamic acids in dried feces from pigs fed the experimental diets and disappearance of phenolic acids during digestion.[a]

Item	Corn-soybean meal	Corn-soybean meal-distillers dried grains with solubles	SEM	*P*-value
Free ferulic acid, μg mg^{-1}	0.05	0.13	0.01	0.439
Bound ferulic acid, μg mg^{-1}	10.08	17.86	0.81	0.033
Free *p*-coumaric acid, μg mg^{-1}	0.02	0.03	<0.01	0.001
Bound *p*-coumaric acid, μg mg^{-1}	1.04	2.04	0.09	0.002
Diferulic acid[b], mass spectrometry area mg^{-1}	12 005	24 546	1403	0.563
Triferulic acid[b], mass spectrometry area mg^{-1}	1239	2540	136	0.465
Peak area ratio	2.50	2.92	0.08	0.700
Disappearance of bound ferulic acid, g kg^{-1}	363.0	353.5	41.2	0.695
Disappearance of free coumaric acid, g kg^{-1}	939.5	880.4	9.1	0.125
Disappearance of bound coumaric acid, g kg^{-1}	481.2	316.9	37.4	0.012

[a] Data are least squares means of 12 observations per treatment.
[b] Expressed as extracted-ion peak areas per mg of sample ($[M-H]^-$ = 385 and 403 for diferulic acid; $[M-H]^-$ = 577 for triferulic acid). No molar response factors are known for these compounds but their abundance is expressed as peak area per milligram sample.

DISCUSSION

The main hydroxycinnamic acids were present in cereal grains in (ester-) bound and free forms.[15] Greater amounts of bound hydroxycinnamic acid than free hydroxycinnamic acid were observed in both corn and DDGS, which is in agreement with previous data.[5,16-18] The analyzed concentration of bound *p*-coumaric acid in the corn-SBM diet is in agreement with values reported for sweet corn, but less than values reported in popcorn.[15] The analyzed value for bound ferulic acid in the corn-SBM diet was also in agreement with values for sweet corn but was greater than in popcorn, baby corn, and quality protein maize.[15] Bound hydroxycinnamic acids cannot be directly absorbed in the small intestine; however, due to ileal and large intestinal microbial activity, released hydroxycinnamic acids are likely metabolized by gut microorganisms. The observed greater disappearance of free coumaric acid than bound coumaric acid indicates that free phenolic acids are rapidly absorbed or metabolized in the small intestine.[15] However, because we did not determine ileal disappearance of phenolic acids, further investigations are warranted to confirm this speculation. Free or released hydroxycinnamic acids may result in certain health benefits.[8,15] As an example, ferulic acid has antioxidant and bioactive properties that stimulate production of free radical scavenging enzymes,[19,20] whereas *p*-coumaric acid possesses anti-inflammatory, antioxidant, and neuroprotective properties.[21-23] However, the biological properties of hydroxycinnamic acids depend on its bioavailability in cereal-based diets.[24] Absorbed ferulic acid rapidly conjugates with glucuronides, sulfate, and sulfoglucuronide in the liver.[25] The antioxidant properties only remain intact in conjugated ferulic acids during the postprandial period because conjugated ferulic acids are rapidly excreted in the urine.[15,24] Absorbed *p*-coumaric acid is not conjugated and is excreted in the urine in its original form.[26]

A greater concentration of bound phenolic acids was observed in the corn-SBM-DDGS diet than in the corn-SBM diet, which concurs with published data,[17,27] and is a result of increased concentration of fiber in DDGS compared with corn. The concentration of phenolic acids in DDGS is greater than in corn due to the accumulation of bound hydrocinnamic acids that takes place as starch in corn is fermented during ethanol production, and phenolic acids are further concentrated in the whole stillage due to ethanol removal.[16] Phenolic acids in DDGS may vary among sources and processing plants due to differences in processing conditions and nutrient composition of corn.[17,27] As ferulic acid and coumaric acid are bound to arabinosyl substituents in arabinoxylan,[3-5] the observed increase in concentrations of bound ferulic and coumaric acid in the diet containing DDGS is likely a result of the greater concentration of arabinose in DDGS than in corn. Analyzed concentrations of total coumaric and ferulic acid in the diet containing DDGS are in agreement with values reported by Luthria *et al.*[17] for DDGS. Greater values for DFA than TriFA in corn and DDGS have been reported[27,28] but the DFA and TriFA in diets used in this experiment are in agreement with the DFA and TriFA profile reported by Pedersen *et al.*[27] for DDGS samples.

As far as the authors are aware, no data for concentration of hydroxycinnamic acids in feces have been published. The greater concentrations of ferulic and coumaric acids in feces compared with diets indicate that most bound phenolic acids in diets are not absorbed and largely resist microbial fermentation in the hindgut.[15] Dietary phenolic acids may also reduce digestibility of fiber and energy by binding with digestive enzymes, thereby reducing α-amylase and amyloglucosidase activities.[16] The observation that concentrations of bound phenolic acids in feces from pigs fed the corn-SBM-DDGS diet were greater than in feces from pigs fed the corn-SBM diet demonstrates that bound phenolic acids in DDGS-containing diets are poorly fermented by pigs. Crosslinked arabinoxylan, via DFA and TriFA bridges, further reduces the degradation and fermentability of fiber in DDGS-containing diets.[5,18,29]

The observation that the disappearance of bound coumaric acid in corn-based diets is less than 50% indicates that bound coumaric acid is resistant to enzymatic hydrolysis, and thus, contributes to reduced degradation of arabinoxylans in corn fiber. The observed low disappearance (i.e., ~35%) of bound ferulic acid in diets containing corn and DDGS indicates that pigs have low capacity to digest and ferment bound ferulic acid from the arabinoxylan complex in corn or DDGS. Likewise,

the observation that disappearance of bound ferulic acid is less than disappearance of bound coumaric acid in the corn-SBM diet indicates that bound ferulic acid is less fermentable than bound coumaric acid. Ferulic acid can be bound to lignin and to arabinose in the arabinoxylan sidechain,[30] and this likely interferes with access of microbial enzymes to liberate individual sugars from arabinoxylan. It is therefore possible that ferulic acid reduces degradation of arabinoxylan in corn fiber indicating that the presence of ferulic acid in arabinoxylan is a major hindrance to fermentation. The implication of this observation is that because of the low digestibility of bound ferulic acid and coumaric acid, debranching enzymes (i.e., esterases) that may release these phenolic acids from the side chains in arabinoxylans will be likely to contribute to increased fermentability of dietary fiber by pigs.

CONCLUSION

Concentrations of coumaric acid and ferulic acid in the diet containing DDGS were more than three times greater than in the corn-SBM diet, but concentration of the two phenolic acids as a percentage of total fiber was not different. Analysis of coumaric acid and ferulic acid in feces from pigs fed both diets demonstrated that only around one third of the bound phenolic acids are fermented in the intestinal tract of pigs, and this indicates that coumaric and ferulic acids might be barriers to fermentation of arabinoxylans in corn fiber. Therefore, if esterases that hydrolyze ester bonds between coumaric and ferulic acid and arabinose can be included in pig diets, pigs may be able to ferment a greater portion of fiber in corn and corn coproducts.

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CONFLICT OF INTEREST

The authors have no conflicts of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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