JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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# Processing Technologies and Cell Wall Degrading Enzymes To Improve Nutritional Value of Dried Distillers Grain with Solubles for Animal Feed: an in Vitro Digestion Study

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Supporting Information

**ABSTRACT:** Currently, the use of maize dried distillers grain with solubles (DDGS) as protein source in animal feed is limited by the inferior protein quality and high levels of non-starch polysaccharides (NSP). Processing technologies and enzymes that increase NSP degradability might improve digestive utilization of DDGS, enhancing its potential as a source of nutrients for animals. The effects of various combinations of processing technologies and commercial enzyme mixtures on in vitro digestion and subsequent fermentation of DDGS were tested. Wet-milling, extrusion, and mild hydrothermal acid treatment increased in vitro protein digestion but had no effect on NSP. Severe hydrothermal acid treatments, however, effectively solubilized NSP (48−78%). Addition of enzymes did not affect NSP solubilization in unprocessed or processed DDGS. Although the cell wall structure of DDGS seems to be resistant to most milder processing technologies, in vitro digestion of DDGS can be effectively increased by severe hydrothermal acid treatments.

**KEYWORDS:** *animal feed, dried distillers grain with solubles, in vitro digestion, enzymes, fermentability, carbohydrate degradability, processing*

## INTRODUCTION

Maize dried distillers grain with solubles (DDGS), a byproduct from bioethanol production, is increasingly being used as protein source in animal feed,[1] and its availability is expected to increase even further in the future.[2] Compared with protein sources such as soybean meal, the nutritional value of DDGS is lower due to its inferior protein quality, partly caused by the excessive pretreatment and drying conditions during the ethanol production process, and high level of non-starch polysaccharides (∼30%).[1,2] Non-starch polysaccharides (NSP), although partly fermented by the microbial community residing in the intestinal tract of the pig, are not completely degraded. Under 50% of the NSP from DDGS is degraded by the pig, leaving >30% of DDGS' energy unused.[3,4] In addition, especially in young pigs, NSP may affect digestion of other nutrients, both directly due to physical hindrance and indirectly due to physiological changes in the gut.[5,6]

Hence, the animal feed industry explores opportunities to improve degradability of the NSP fraction from feedstuffs, thereby enhancing its potential as a source of nutrients for animals. Commonly used feed processing technologies, such as hammer milling and pelleting, effectively improve the degradability of easily solubilizable NSP, but might not be sufficient to affect more recalcitrant NSP structures, such as arabinoxylans in maize.[7] Therefore, more effective technologies are required to modify cell wall structure and allow exogenous or endogenous enzymes to degrade the complex NSP structures.[8] Mechanical forces open the cell wall structure and reduce particle size, thereby increasing the surface area accessible for microbial and endogenous enzymes. Thermal processes can break weak bonds between polysaccharides and glycosidic linkages within polysaccharides, but excessive heating may increase protein and amino acid damage.[9] Hydrothermal pretreatments using acid catalysts are established methods to improve extractability of lignocellulosic material.[10] Potential protein damage and high residual acid or mineral concentrations limit the use of extremely high processing temperatures and high acid concentrations for processing animal feedstuffs. Instead, relatively mild acid treatments, that is, having a low combined severity factor (CSF), using dicarboxylic organic acids, such as maleic acid, could be of special interest.[11] In addition, cell wall degrading enzymes, such as xylanases, can be used to specifically cleave polymers or remove side chains.[12] It is hypothesized that the effectiveness of enzymes to improve NSP degradability will depend on the extent to which the cell wall structure is modified during processing.

This paper describes two successive experiments in which the effects of various combinations of processing technologies and enzymes on in vitro digestion and subsequent fermentation of maize and DDGS are tested. In experiment 1, the effects of particle size reduction, hydrothermal treatment with or without shear, and acid hydrolysis in the presence or absence of cell wall degrading enzymes on physicochemical properties and in vitro degradation of maize and DDGS were investigated. On the basis of the results, experiment 2 was designed to investigate

Received: May 6, 2013
Revised: August 19, 2013
Accepted: August 21, 2013
Published: August 21, 2013

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the effects of more severe hydrothermal acid treatments on in vitro digestion of DDGS.

## ■ MATERIALS AND METHODS

**Experimental Design.** In experiment 1, effects of processing technologies and cell wall degrading enzymes on physicochemical properties and in vitro degradation of maize and DDGS were tested in a 5 × 2 factorial arrangement: five processing technologies (unprocessed, wet-milling, extrusion, autoclaving, and hydrothermal acid treatment), each with or without the addition of cell wall degrading enzyme mixtures. Enzymatic digestion in the upper gastrointestinal tract and subsequent large intestinal fermentation were simulated in duplicate.

In experiment 2, effects of hydrothermal acid treatments, varying in type of acid and acidity level, and cell wall degrading enzymes on in vitro degradation of DDGS were tested in a 4 × 2 factorial arrangement: four hydrothermal acid treatments (unprocessed, maleic 2.9, maleic 2.3, sulfuric 2.9), with or without enzyme addition. The concentration of acid used for maleic 2.3 was twice the concentration used for maleic 2.9. The concentration of sulfuric acid was chosen to result in a similar pH and thus a similar CSF as maleic 2.9. Solubilization of NSP during the enzymatic digestion procedure was tested in triplicate.

**Materials.** Whole maize grain (*Zea mays*) and unpelleted DDGS were obtained from a commercial bioethanol plant (Abengoa Bioenergy, France). Maize was milled using a hammer mill, at 1475 rpm using a 3.2 mm sieve, whereas DDGS was used as mash.

**Processing and Enzyme Technologies.** *Wet-Milling.* Maize and DDGS were milled using a laboratory scale refiner (Sprout-Waldron), at a feed rate of 102 kg $h^{-1}$ using 480 L of water $h^{-1}$. The diameter of the disks was 30 cm with the distance between disks set at 0.07 mm and a rotation speed of 3000 rpm. Product temperature when leaving the refiner was 35 °C. Product was collected in bins after the process reached a steady state, and subsamples were taken under continuous mixing. Samples were cooled to room temperature, frozen (−20 °C), and freeze-dried.

*Extrusion.* Maize and DDGS were mixed with water using a paddle mixer (type F60; Halvor Forberg, Bygland, Norway) to reach a dry matter (DM) content of 80%. Within 30 min after mixing, samples were extruded using a corotating double-screw extruder (M.P.F.50; Baker Perkins, Peterborough, UK), without additional steam conditioning. The extruder had a screw length/diameter ratio of 25. The screw configuration was as follows: four 1.5D feed screw elements, one 1D single lead element, three 1D feed screw elements, one 1D single lead element, two 1D feed screw elements, two 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, one 4D 90 degree forwarding block paddles, one 1.5D feed screw elements, two 4D 90 degree forwarding block paddles, and two 1.5D single lead elements. A die with two orifices (6 mm) was used; no die face cutter was used. Feeding rate was set at 30 kg $h^{-1}$, and screw speed was 250 rpm. Barrel temperatures in the 10 segments of the extruder were set at 30, 50, 72, 82, 90, 105, 115, 120, 120, and 120 °C. Product temperature at the die was 115 °C. Samples were collected after the process reached a steady state and cooled to room temperature. Samples were frozen (−20 °C) without further freeze-drying.

Extruded maize samples were ground in an ultracentrifuge mill (ZM 100; Retsch, Haan, Germany) at 12000 rpm using a 3 mm sieve. Extruded DDGS samples were ground using a mortar and pestle.

*Autoclaving.* Maize and DDGS were mixed with water by hand to reach a DM content of 80%. Samples were then autoclaved (Varioklav 25 T tabletop; Thermo Scientific, Waltham, MA, USA) during 30 min at 120 °C, starting when the preset temperature was reached. Samples were cooled to room temperature, frozen (−20 °C), and freeze-dried.

*Hydrothermal Acid Treatment.* For experiment 1, 1.4 g of maleic acid (>98.0% pure, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 1 L of water and added to 100 g of maize or DDGS DM. For experiment 2, 6.5 g of maleic (maleic 2.9), 13 g of maleic (maleic 2.3), or 28 g of sulfuric (sulfuric 2.9) acid was dissolved in 1 L of water and added to 100 g of DDGS DM. Samples were mixed by hand, soaked (4 h), and autoclaved as described above. In experiment 1, the pH of the solution after autoclaving was 3.6 for maize and 4.2 for DDGS, corresponding to CSF values of −1.53 and −2.13, respectively. In experiment 2, the pH of the solution after autoclaving was 2.9 for maleic 2.9, 2.3 for maleic 2.3, and 2.9 for sulfuric 2.9, corresponding to CSF values of −0.83, −0.23, and −0.83, respectively.

*Cell Wall Degrading Enzyme Treatment.* The enzyme treatment comprised a combination of commercial food-grade enzyme mixtures commonly used in bakery and brewing processes: Shearzyme500L and UltrafloL (Novozymes, Bagsvaerd, Denmark), with mainly endo-1,4-$\beta$-xylanase and endo-1,4-$\beta$-glucanase activities. The declared enzyme activity by the manufacturer was 500 FXU-S/g for Shearzyme 500L and 45 FBG/g for UltrafloL. Each enzyme was added to the substrates dissolved in buffer solution during the first incubation step of the Boisen and Fernández[13] procedure at a concentration of 25 $\mu$L/g of substrate.

**In Vitro Digestion and Fermentation.** Enzymatic digestion in the stomach and small intestine was simulated using a modified method of Boisen and Fernández,[13] as described by Pustjens et al.[14] Further milling prior to the procedure was omitted, the pH during the first incubation step was adjusted to 3.5, and amyloglucosidase[15] was added during the second incubation step. Briefly, 10 g of sample was mixed with phosphate buffer (250 mL, 0.1 M, pH 6.0) and HCl solution (30 mL, 0.2M), and the pH was adjusted to 3.5, using 1 M HCl or 1 M NaOH. Pepsin solution (10 mL, 25 g/L, porcine pepsine: 2000 FIP U/g, Merck, Darmstadt, Germany) was added, and samples were incubated for 75 min at 40 °C. Afterward, phosphate buffer (100 mL, 0.2 M, pH 6.8) and NaOH solution (40 mL, 0.6 M) were added, and the pH was adjusted to 6.8. Pancreatin solution (10 mL, 100 g/L, porcine pancreatin: grade IV, Sigma-Aldrich) and amyloglucosidase (55 mg, amyloglucosidase from *Aspergillus niger*: 120 U/g, Sigma-Aldrich) were added, and samples were incubated for 3.5 h at 40 °C. Supernatant was boiled for 30 min and frozen (−20 °C). Residues were washed with demineralized water after centrifugation (3030*g* for 10 min) to remove free glucose, decanted, frozen (−20 °C), and freeze-dried.

Fermentation in the large intestine was simulated using a cumulative gas production method as described by Williams et al.[16] Briefly, 0.5 g of Boisen residue was mixed with 89 mL of buffer solution containing macro- and microminerals and fecal inoculum. Samples were incubated for 72 h at 39 °C in shaking water baths (40 rpm). Cumulative gas production during incubation was measured using a fully automated time-related gas production system.[17] After incubation, samples were frozen (−20 °C) and freeze-dried. Fecal inoculum was prepared from pig feces, collected from five sows that were fed commercial diets containing barley (303 g/kg), wheat middlings (200 g/kg), maize (100 g/kg), rapeseed meal (75 g/kg), soy hulls (50 g/kg), wheat (50 g/kg), and linseed (15 g/kg) as the main feed ingredients. Fermentation was performed in two separate runs, executed in two subsequent weeks.

**Analytical Methods.** The geometric mean diameter (GMD) of unprocessed products was analyzed using the wet sieve method and calculated according to the ASABE method.[18] GMD of wet-milled and extruded samples was analyzed using a Coulter Counter (Beckman Coulter, Brea, CA, USA). Water-binding capacity (WBC) was analyzed by soaking 250 mg of raw material or freeze-dried residue in 10 mL of water for 24 h at room temperature. Samples were centrifuged (3274*g* for 20 min) at room temperature and subsequently drained inverted for 15 min. WBC was calculated as the weighed quantity of water retained per gram of dry material.

Prior to chemical analyses, samples were ground in a mixer mill (Retsch MM 2000) at an amplitude of 80, for 1 min. Unprocessed and processed products and residues of enzymatic digestion were analyzed for the content of DM (103 °C overnight), nitrogen (AOAC 968.06; using a Thermo Quest NA 2100 Nitrogen and Protein Analyzer; Interscience, New York), total starch (AOAC 996.11; using a commercial test kit, Megazyme international Ltd., Ireland),[19] and total (DDGS) or insoluble (maize) NSP analyzed as neutral sugars and uronic acids, according to the procedure described below. Neutral sugar composition was analyzed by gas chromatography according to

**Table 1. Analyzed Nutrient Composition (Grams per 100 g DM) of the Unprocessed Maize and Unprocessed, Wet-Milled, Extruded, Autoclaved, or Hydrothermal Acid Treated (Maleic Acid) Maize Dried Distillers Grain with Solubles (DDGS)**

| | | DDGS | | | | |
|---|---|---|---|---|---|---|
| item | maize | unprocessed | wet-milled | extruded | autoclaved | maleic acid |
| dry matter, g/100 g fresh | 88 | 89 | 96 | 83 | 96 | 95 |
| protein | 9 | 29 | 29 | 30 | 30 | 31 |
| starch | 68 | 3 | 3 | 3 | 3 | 3 |
| nonstarch polysaccharides | 8 | 27 | 29 | 31 | 27 | 28 |
| molar composition of NSP[a] | | | | | | |
| rhamnose | 1 | 0 | 0 | 0 | 0 | 0 |
| arabinose | 21 | 22 | 21 | 21 | 21 | 19 |
| xylose | 30 | 30 | 30 | 31 | 31 | 31 |
| mannose | 2 | 5 | 4 | 4 | 4 | 4 |
| galactose | 5 | 5 | 5 | 5 | 6 | 5 |
| glucose | 30 | 31 | 33 | 31 | 31 | 34 |
| uronic acid | 12 | 7 | 7 | 7 | 7 | 7 |
| Ara:Xyl[b] | 0.68 | 0.74 | 0.70 | 0.67 | 0.66 | 0.60 |
| UA:Xyl[c] | 0.38 | 0.24 | 0.22 | 0.21 | 0.23 | 0.22 |
| particle size ($\mu$m) | 534 | 526 | 97 | 377 | na[d] | na |
| water binding capacity (g/g DM) | 1.5 | 2.3 | 3.0 | 3.0 | 2.1 | 2.9 |

[a]Mol %; presented as anhydrosugar moieties. [b]Molar ratio of arabinose/xylose. [c]Molar ratio of uronic acid/xylose. [d]Not analyzed.

the method of Englyst and Cummings.[20] After pretreatment with 72% (w/w) $H_2SO_4$ for 1 h at 30 °C, samples were hydrolyzed with 1 M $H_2SO_4$ at 100 °C for 3 h. Constituent sugars were derivatized into alditol acetates and analyzed using a GC (Focus-GC, Thermo Scientific, Waltham, MA, USA). Inositol was used as an internal standard. Uronic acid content was analyzed according to the automated colorimetric *m*-hydroxydiphenyl assay[21] using an auto-analyzer (Skalar Analytical B.V., Breda, The Netherlands). Galacturonic acid was used for calibration. NSP content was calculated as the sum of neutral sugars and uronic acids minus glucose from starch. Protein content was calculated from the N content using a protein conversion factor of 5.7.[22] Unprocessed products were additionally analyzed for the content of ether extract using a Soxhlet device with petroleum ether (AOAC 920.39) and ash (AOAC 942.05).[19]

**Calculations and Statistical Analysis.** The CSF of the hydrothermal acid treatments was calculated as $\text{Log}\{t \times \exp[(T - 100)/14.75]\} - \text{pH}$, where $t$ is the treatment time (min); $T$, temperature (°C); and pH, acidity level.[23]

Solubilization of nutrients in unprocessed and processed maize and DDGS during the enzymatic digestion procedure (in vitro digestion) was calculated using the unprocessed products as the reference. Total gas production volume corrected for DM (mL/g DM) was calculated from the cumulative gas production at 72 h. Gas production data for each bottle were modeled according to a monophasic model, and the maximum rate of gas production was calculated as described by Groot et al.[24]

The effects of experimental treatments on in vitro digestion of DM and nutrients and on parameter estimates of the gas production curves during in vitro fermentation were analyzed by analysis of variance, using the GLM procedure of SAS.[25] Processing technology, enzyme addition, and its interaction were included as fixed effects in the model. For treatment effects on the parameter estimates of gas production curves, the effect of run (first or second) was included in the model if found to be significant. Interactions between run and treatment effects were tested but found to be not significant in all cases and excluded from the model. Model residuals were tested for homogeneity and normality, to verify model assumptions. Least-squares means were compared using a least significant differences procedure. Data are presented as least-squares means and standard error of the mean (SEM) unless stated otherwise. Differences among means with $P < 0.05$ were accepted as representing statistically significant differences.

## RESULTS AND DISCUSSION

**Chemical and Physical Characteristics.** In contrast to maize, DDGS contained only small amounts of starch (3%), whereas protein (29%) and NSP (36%) contents were concentrated 3−4-fold (Table 1, Table 2 and Supporting Information, Table S1), as expected.[26] NSP in maize DDGS are mainly composed of cellulose and (arabino)xylans,[27] as confirmed by the sugar composition found here (Table 1). Unpublished results from our research group indicate that also yeast $\beta$-glucans (7%) and possibly mannans (<2%) are present. Although detailed structural characterization of the NSP in DDGS is lacking, it can be speculated that glucuronoarabinox-

**Table 2. Analyzed Nonstarch Polysaccharide (NSP) Content (Grams per 100 g DM) and Molar Composition of NSP of Unprocessed and Hydrothermal Acid Treated Maize Dried Distillers Grain with Solubles (DDGS, Experiment 2)[a]**

| item | unprocessed | maleic 2.9 | maleic 2.3 | sulfuric 2.9 |
|---|---|---|---|---|
| CSF[b] | | −0.83 | −0.23 | −0.83 |
| nonstarch polysaccharides | 28 | 24 | 23 | 26 |
| molar composition of NSP[c] | | | | |
| rhamnose | 0 | 0 | 0 | 0 |
| arabinose | 20 | 16 | 18 | 18 |
| xylose | 28 | 23 | 27 | 27 |
| mannose | 4 | 18 | 11 | 11 |
| galactose | 5 | 4 | 5 | 5 |
| glucose | 37 | 33 | 34 | 33 |
| uronic acid | 6 | 6 | 6 | 6 |
| Ara:Xyl[d] | 0.70 | 0.70 | 0.68 | 0.69 |
| UA:Xyl[e] | 0.21 | 0.28 | 0.24 | 0.22 |

[a]Hydrothermal acid treatment was performed using maleic acid at two levels of acidity, maleic 2.9 (pH 2.9) and maleic 2.3 (pH 2.3), or using sulfuric acid (sulfuric 2.9; pH 2.9). [b]Combined severity factor.[23] [c]Mol %; presented as anhydrosugar moieties. [d]Molar ratio of arabinose/xylose. [e]Molar ratio of uronic acid/xylose.


dx.doi.org/10.1021/jf4019855 | J. Agric. Food Chem. 2013, 61, 8821−8828

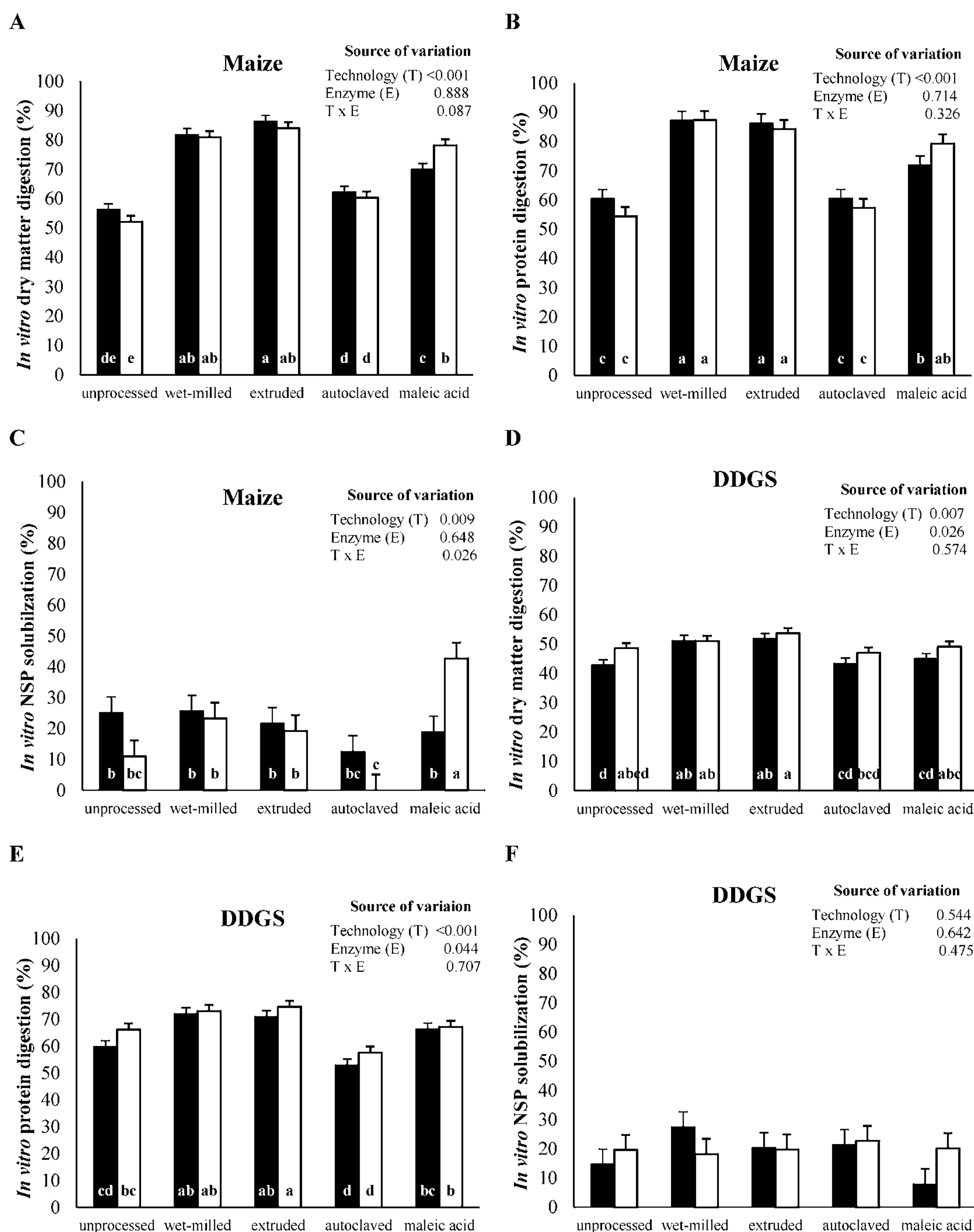


**Figure 1.** In vitro digestion of dry matter (A, D), protein (B, E), and solubilization of nonstarch polysaccharides (NSP; C, F) from unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (acid) maize and maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Error bars indicate SEM.

ylans, abundant in the water-unextractable solids of maize,[28] are dominating. Those heteroxylans are highly substituted with monomeric arabinosyl and glucuronic acid residues, as well as oligomer side chains containing arabinosyl, xylosyl, and galactosyl residues, and are highly cross-linked by diferulic acid bridges.[28,29] The ratio of arabinosyl and uronic acid to xylosyl residues is indicative for the degree of substitution and thus related to the structure of the (glucurono) arabinoxylans present in DDGS. Higher arabinose/xylose (Ara:Xyl) and uronic acid/xylose (UA:Xyl) ratios indicate that relatively more xylopyranosyl units from the xylan backbone are substituted with arabinofuranosyl or glucuronic acid residues, generally suggesting a higher degree of branching. The Ara:Xyl and UA:Xyl ratios (Table 1) found for the unprocessed DDGS (0.71 and 0.20, respectively) are within the ranges described for maize[28] and maize fiber.[8]

Particle size of DDGS was within the expected range.[30] Wet-milling effectively reduced GMD by 82%, but also extrusion reduced GMD (28%). WBC was higher in DDGS compared with maize (Table 1). Compared with unprocessed DDGS, WBC was higher in wet-milled, extruded, and hydrothermal acid treated DDGS, whereas it remained unchanged after autoclaving.

**In Vitro Digestion and Fermentation.** This study aimed to investigate the effects of particle size reduction, hydrothermal treatment with or without shear, and acid hydrolysis, in

combination with addition of commercial cell wall degrading enzyme mixtures, on the degradability of maize and DDGS during in vitro digestion and fermentation. Enzymatic digestion in the stomach and small intestine was simulated using a two-step in vitro digestion procedure, which is commonly used to assess in vitro degradability of feedstuffs. Technically, disappearance of DM and nutrients during this procedure reflects the amount solubilized, that is, only partly resulting from actual digestion by the added digestive enzymes, rather than the amount degraded. Because NSP cannot be degraded by mammalian enzymes, disappearance of NSP solely encompasses solubilization. Nonetheless, solubilization of NSP is related to their degradation in vivo, as modifications in cell wall matrix affect accessibility for microbial enzymes.[7] Together with fermentability of in vitro digested residues, results of this study provide an indication how processing technologies and commercial enzyme mixtures will affect degradability of NSP in the animal, which can be of use for future in vivo trials.

In experiment 1, wet-milling, extrusion, and hydrothermal acid treatment increased in vitro DM digestion of maize by 14–30% ($P < 0.01$), mainly due to increased starch (see Supporting Information, Figure S1) and protein digestion (Figure 1). In DDGS, DM digestion increased only by 8–11% ($P < 0.01$), as a result of increased protein digestion (Figure 1). In experiment 2, hydrothermal acid treatment increased in vitro DM digestion of DDGS by 18–34% ($P < 0.01$), mainly due to increased protein (data not shown) and NSP solubilization (Figure 3).

**Protein Degradation.** Although protein digestion in unprocessed maize and DDGS was similar (60%), wet-milling, extrusion, and hydrothermal acid treatment improved protein digestion by 12–27% ($P < 0.01$) in maize (Figure 1B), whereas in DDGS this increase was only 6–12% ($P < 0.01$; Figure 1E). Apparently, the indigestible fraction of DDGS protein is less susceptible to modification compared to that of maize protein. This could be a result of changes in maize protein structure that occur during the bioethanol production process, as illustrated by the higher fraction of protein that is associated with cell wall material in DDGS compared with maize.[31] Alternatively, the susceptibility of yeast protein, which constitutes approximately 20% of DDGS protein,[32] to protein and amino acid damage during extrusion and hydrothermal acid treatment may be higher. Han and Liu[32] suggested that the major part of yeast protein will be present in the form of free amino acids, which might be more reactive.[33] Autoclaving reduced protein digestion in DDGS (7%, $P < 0.01$), suggesting that proteins were damaged as a result of specific interactions between molecules, such as in the Maillard reaction.[34] These reactions are less likely to occur under acidic and excess moisture conditions,[33,35] which might explain the higher protein digestion in acid-autoclaved products (hydrothermal acid treatment) compared with autoclaved products.

**Nonstarch Polysaccharide Degradation.** NSP solubilization of maize was increased by hydrothermal acid treatment combined with commercial enzyme mixtures only (18%, $P < 0.01$; Figure 1C), indicating that modification of the cell wall matrix was required to allow enzymes to work.[36] Solubilization of NSP from DDGS was not affected by the processing technologies and commercial enzyme mixtures used in experiment 1 (Figure 1F). Nonetheless, the increased WBC after wet-milling, extrusion, and hydrothermal acid treatment (Table 1) indicates that the cell wall matrix was, at least to a certain extent, affected by these technologies. This is also reflected in the increased WBC of in vitro digested residues from processed DDGS, which was most pronounced in hydrothermal acid treated DDGS (see Supporting Information, Figure S2). Addition of cell wall degrading enzymes counteracted this effect.

Except for wet-milling, processing technologies and enzyme treatment did not affect the extent and rate of fermentation of the undigested DDGS residues (Figure 2). Autoclaving followed by enzyme treatment reduced, however, total gas produced at 24 h (data not shown), which is often seen as a relevant time point in the fermentation for monogastrics.[37] Possibly, protein–cell wall interactions that occur during autoclaving reduce NSP degradability. The effect of wet-milling on the maximum rate of fermentation in undigested residues of both maize (see Supporting Information, Figure S3) and DDGS (Figure 2) demonstrates the accelerating effect of decreased particle size on fermentation,[38] which could indicate higher fermentability in vivo.

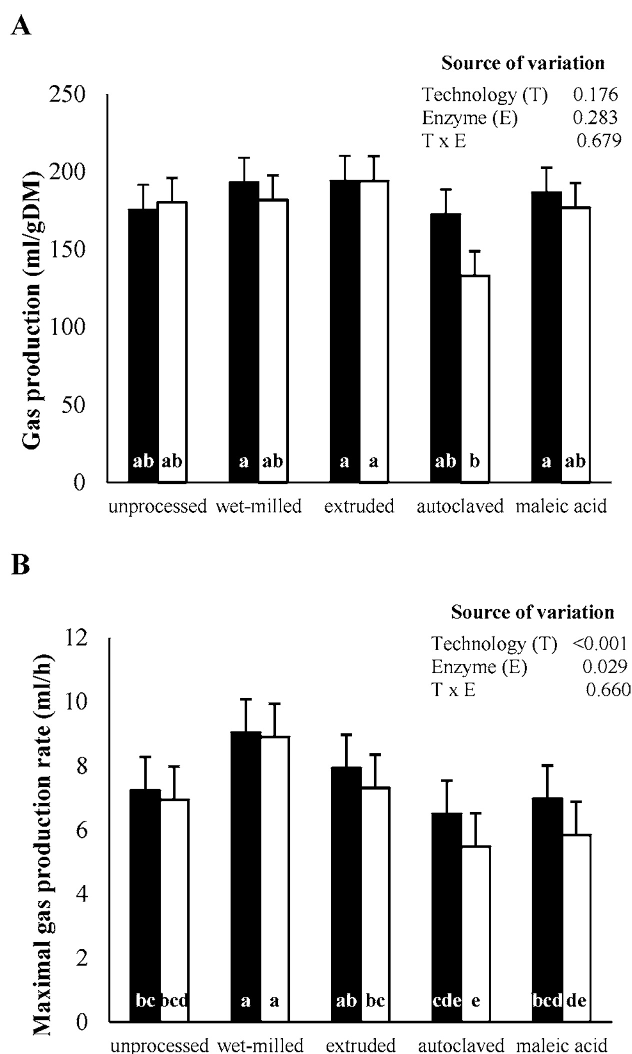


**Figure 2.** Total gas production (A) and maximal gas production rate (B) of unprocessed, wet-milled, extruded, autoclaved, or hydrothermal acid treated (maleic acid) maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Error bars indicate SEM.

In summary, the results of experiment 1 indicate that the cell wall structure of DDGS is hardly affected by the processing

technologies and commercial enzyme mixtures employed in the present study. In maize, enzymes increased NSP solubilization after sufficient technological processing (hydrothermal acid treatment). Processing of DDGS, however, did not improve accessibility of NSP to enzymes, indicating either that enzyme activities were not adequate to degrade xylans present in DDGS or that processing technologies did not sufficiently modify cell wall structure. We concluded that more severe processing technologies are required to untangle the cell wall structure of DDGS. Therefore, in experiment 2, the effects of hydrothermal acid treatments using higher acid concentrations were tested. Hydrothermal acid treatment effectively increased solubilization of NSP from DDGS during in vitro digestion (~30–60%, $P < 0.01$), both using maleic acid at two levels of acidity and using sulfuric acid (Figure 3B). At similar pH, NSP solubilization was higher when maleic acid was used compared with sulfuric acid (~7%, $P < 0.01$). Higher efficiency of maleic acid compared with sulfuric acid was also reported by Lee and Jeffries[11] for maize cobs treated at higher CSF (1.8–2.1). A 2-fold increase in the concentration of maleic acid resulted in an additional increase in solubilization of NSP by ~20% ($P < 0.01$). From the remaining constituent sugars in the in vitro digested residues, it can be seen that when maleic acid at a low level of acidity and sulfuric acid are used, mainly arabinose and, to a lesser extent, xylose and uronic acid containing polymers are solubilized (Figure 3C); Ara:Xyl and UA:Xyl ratios decreased after in vitro digestion (Figure 4). This indicates that mainly highly substituted arabinoxylans are affected by hydrothermal acid treatment. Arabinose, which is more acid-labile compared with xylose, is removed from the xylan backbone, leaving lower substituted xylans in the residue. At a higher level of acidity (maleic 2.3), almost all xylan structures seem to solubilize. The Ara:Xyl ratio of xylans in the in vitro digested residues is decreased even more than at the lower level of acidity, whereas the UA:Xyl ratio remained at the level of unprocessed residues (Figure 4), suggesting that arabinosyl substituents are more easily removed from the xylan backbone than uronic acid residues. Cellulose remains virtually insoluble in all three treatments as expected for the selected CSF, because of its rigid structure and strong anchorage in the cell wall matrix.[8,39]

Addition of commercial enzyme mixtures did not affect NSP solubilization in unprocessed or processed DDGS, neither in experiment 1 nor in experiment 2, whereas in maize, enzymes increased NSP solubilization after sufficient technological processing (hydrothermal acid treatment). Apparently, xylans from maize that can be potentially degraded by the commercial enzyme mixtures used were either removed or modified during the ethanol production process. Possibly, the high amount of substituents of maize pericarp xylans that are concentrated in DDGS hinders activity of the xylanases in the enzyme mixtures used.[40] The increased protein digestibility resulting from enzyme treatment (1–6%, $P < 0.05$; Figure 1E) indicates, however, that enzyme treatment affects the cell wall matrix to a certain extent, such that protein associated with cell wall material is released more easily.

In conclusion, in vitro digestion of protein from maize and, to a lesser extent, DDGS is increased by wet-milling, extrusion, and hydrothermal acid treatment. The cell wall structure of DDGS was resistant to most processing technologies, but the increased NSP solubilization after severe hydrothermal acid treatment illustrates that in vitro digestion of DDGS may be effectively increased. Maleic acid was more effective than sulfuric acid. A 2-fold increase in maleic acid concentration resulted in additional solubilization of NSP by ~20%. Commercial enzyme mixtures did not affect NSP solubilization in either unprocessed or processed DDGS but increased in

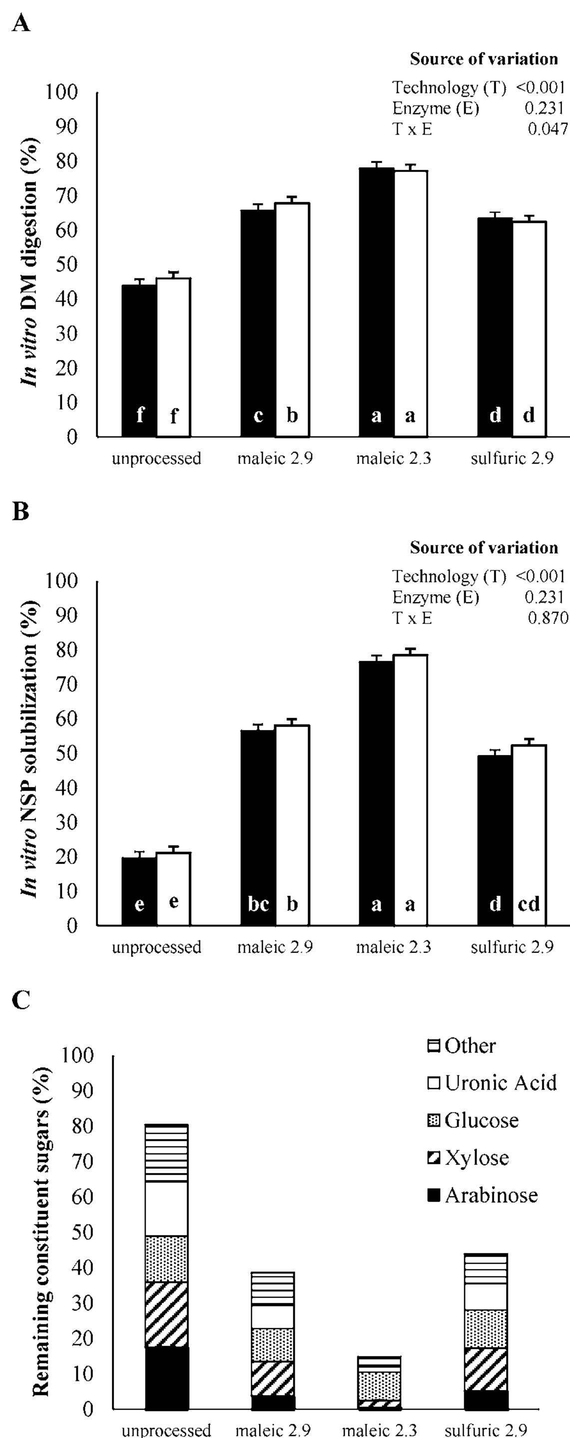


**Figure 3.** In vitro digestion of dry matter (A) and solubilization of nonstarch polysaccharides (NSP; B) of unprocessed and hydrothermal acid treated maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Hydrothermal acid treatment was performed using maleic acid at two levels of acidity, maleic 2.9 (pH 2.9) and maleic 2.3 (pH 2.3), or using sulfuric acid (sulfuric 2.9; pH 2.9). Error bars indicate SEM. In panel C, the remaining constituent sugars (percent of constituent sugars before incubation) in in vitro digested residues of unprocessed and or hydrothermal acid treated DDGS are presented.

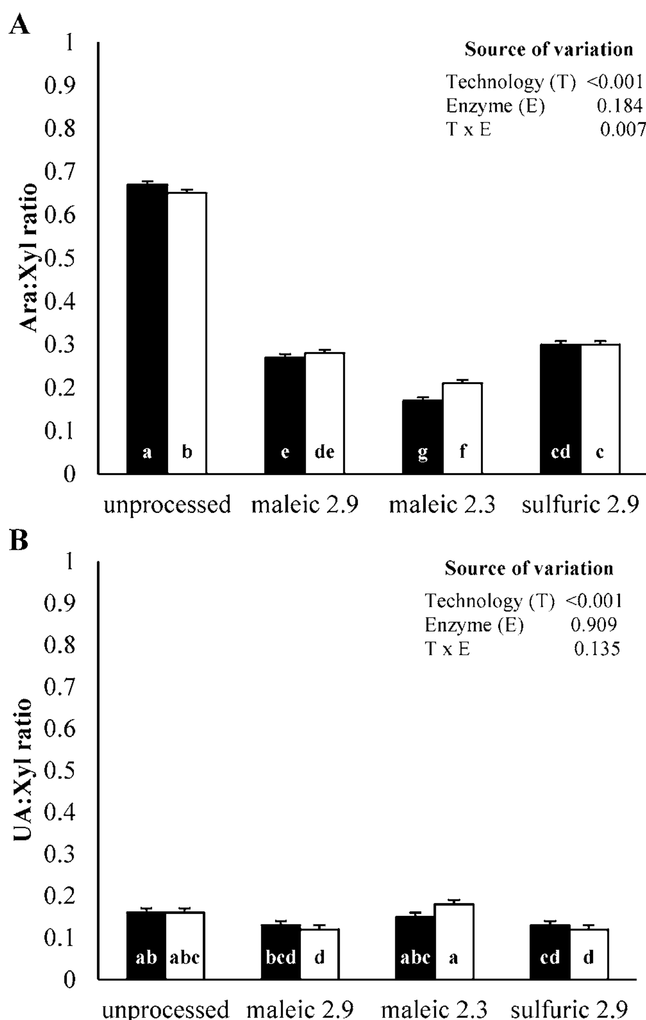


**Figure 4.** Mean molar arabinose/xylose (Ara:Xyl, A) and uronic acid/xylose ratio (UA:Xyl, B) in in vitro digested residues from unprocessed and hydrothermal acid treated maize dried distillers grain with solubles (DDGS), with (white bars) or without (black bars) the addition of commercial enzyme mixtures. Hydrothermal acid treatment was performed using maleic acid at two levels of acidity, maleic 2.9 (pH 2.9) and maleic 2.3 (pH 2.3), or using sulfuric acid (sulfuric 2.9; pH 2.9). Error bars represent SEM.

vitro protein digestibility. In maize, enzymes increased NSP solubilization after sufficient technological processing (hydrothermal acid treatment). In DDGS, the absence of an interaction between processing technologies and enzyme treatment, even if processing successfully affected cell wall structure and degradability (experiment 2), indicates that processing technologies did not improve accessibility of NSP to enzymes. Possibly, the high amount of substituents of xylans in DDGS hindered activity of the commercial enzyme mixtures used.

## ■ ASSOCIATED CONTENT

### Supporting Information

Table S1 and Figures S1−S3. This material is available free of charge via the Internet at http://pubs.acs.org.

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### Funding

This project is jointly financed by the European Union, European Regional Development Fund, and The Ministry of Economic Affairs, Agriculture and Innovation, Peaks in the Delta, the Municipality of Groningen, and the Province of Groningen as well as the Dutch Carbohydrate Competence Center (CCC WP7), supported by Agrifirm Group, Duynie Holding, Nutreco Nederland B.V., and Wageningen University.

### Notes

The authors declare no competing financial interest.

## ■ ACKNOWLEDGMENTS

We thank Saskia van Laar, Willem Stolp, Tamme Zandstra, and Dick van Zuilichem for technical assistance during the experiment and Nienke Bosma and Aojia Wang for their contribution to the research within their M.Sc. thesis project.

## ■ ABBREVIATIONS USED

Ara:Xyl, arabinose/xylose ratio; CSF, combined severity factor; DDGS, dried distillers grain with solubles; DM, dry matter; NSP, nonstarch polysaccharides; SEM, standard error of the mean; UA:Xyl, uronic acid/xylose ratio; WBC, water binding capacity

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