Xylanases to Improve the Nutritional Value of High Fibre Diets based on Corn and Wheat DDGS

PhD thesis
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SUMMARY

The increased feed prices for cereal grains and protein crops have forced the feed industry to look for alternatives to the conventional feedstuffs. One such alternative is distillers dried grain with solubles (DDGS), the major co-product from dry-grind based ethanol production. The main challenges limiting the use of DDGS in feeds for non-ruminant animals is the inferior protein quality, potential high mycotoxin content, compositional variations, and high levels of non-starch polysaccharides (NSP).

The overall aim of this PhD project was to map the variation and differences in especially NSP composition of these feedstuffs, and to improve the digestive utilization of corn- and wheat DDGS (c- and wDDGS) for pigs by enzymatic degradation of the fibre matrix.

A total of 138 corn, wheat and mixed cereal DDGS samples were investigated for compositional variability of common nutrients, whereas a detailed analysis of the NSP and ester-linked ferulic acid dehydrodimer (DFA) and -trimer (TriFA) profile was performed on 63 DDGS samples. Analysis of samples from five different ethanol plants showed that the individual plants were capable of producing cDDGS with an individual conserved compositional characteristic. Furthermore, investigation of corn and corresponding DDGS showed that the most readily degradable arabinoxylan (AX) from endosperm is likely modified during DDGS processing, and that the DFAs and TriFAs are not modified, thus, leaving the AX in DDGS less degradable than that of the parent grain. The higher cell wall complexity in c- than wDDGS was illustrated by a higher proportion of insoluble NSP, higher arabinose/xylose - and uronic acid/xylose-ratio, and more than five times the content of DFAs and TrifAs in c- than wDDGS. The higher complexity of c- than wDDGS had large implications of the enzymatic degradability and digestibility, in vitro and in vivo, as wDDGS was markedly more degradable than cDDGS. Four different xylanases from glycoside hydrolase (GH) family 10 and -11 were investigated, in vitro, alone and in combination with protease and phytase. The GH10 xylanase showed higher degradability than GH11 on cDDGS substrate, due to the differences in substrate affinity between the two GH families. In addition, it was found both in vitro and in vivo, that the depolymerizing of DDGS fibre depended on the substrate affinity of the individual xylanases. Both GH10 and -11 treatments increased the apparent ileal digestibility of NSPs and generated low molecular weight components with potential prebiotic effects in pigs fed a wDDGS diet. The GH11 xylanase had numerically greater effect than the GH10 xylanase, likely due to a higher stability in the upper gastro-intestinal tract, illustrated by a four times higher recovery of enzyme activity in ileum.

In summary, the compositional variations and differences in NSP complexity between c- and wDDGS had large implications on the enzymatic degradation of these two substrates. In addition, in vitro and in vivo experiments illustrated that enzymes may comprise different degradation efficiency in relation to their substrate affinity and stability towards the physical environment occurring in the gastro intestinal tract of pigs. To further increase the enzymatic degradation of DDGS, more research is needed to evidently identify the structural components most accountable for the enzymatic recalcitrance of especially cDDGS.

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SAMMENDRAG

De øgede foderpriser på korn og protein-foder har tvunget foderindustrien til at lede efter alternativer til de konventionelle foderstoffer. Et sådant alternativ er tørret bærme (DDGS), det primære biprodukt fra kornbaseret produktion af ethanol. De største begrænsninger ved brugen af DDGS i foder til enmavede dyr er den ringere proteinkvalitet, potentielt højt indhold af mykotokssiner, variation i sammensætning og et højt niveau af ikke-stivelses polysakkarider (NSP).

De overordnede formål med dette ph.d.-projekt var at kortlægge variationen af næringsstoffer i DDGS foder med særligt fokus på NSP sammensætningen, og at forbedre foderudnyttelsen af majs- og hvede baseret DDGS til svin ved enzymatisk nedbrydning af fibermatricen.


Samlet set viste forskellen i NSP-kompleksitet mellem majs- og hvede DDGS at have store konsekvenser for den enzymatiske nedbrydning af disse to substrater. Desuden viste både in vitro og in vivo eksperimenter, at enzymer har forskellig nedbrydningsevne i forhold til deres substrataffinitet og stabilitet over for det fysiske miljø i mave-tarmkanalen af grise. For yderligere at øge den enzymatiske nedbrydning af DDGS, er der behov for mere forskning for at identificere de strukturelle komponenter der hindrer den enzymatiske nedbrydning af især majs DDGS mest.
# ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AX</td>
<td>arabinoxylan</td>
</tr>
<tr>
<td>AXOS</td>
<td>arabinoxylan-oligosaccharides</td>
</tr>
<tr>
<td>CAID</td>
<td>coefficient of apparent ileal digestibility</td>
</tr>
<tr>
<td>CATTD</td>
<td>coefficient of apparent total tract digestibility</td>
</tr>
<tr>
<td>cDDGS</td>
<td>corn DDGS</td>
</tr>
<tr>
<td>CHO</td>
<td>carbohydrates</td>
</tr>
<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>DDGS</td>
<td>distillers dried grains with solubles</td>
</tr>
<tr>
<td>DE</td>
<td>digestible energy</td>
</tr>
<tr>
<td>DFA</td>
<td>ferulic acid dehydrodimer</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>GH</td>
<td>glycoside hydrolase</td>
</tr>
<tr>
<td>LMW</td>
<td>low molecular weight</td>
</tr>
<tr>
<td>ME</td>
<td>metabolizable energy</td>
</tr>
<tr>
<td>NCP</td>
<td>non-cellulosic polysaccharides</td>
</tr>
<tr>
<td>NDC</td>
<td>non-digestible carbohydrates</td>
</tr>
<tr>
<td>NIRS</td>
<td>near infrared reflectance spectroscopy</td>
</tr>
<tr>
<td>NSP</td>
<td>non-starch polysaccharides</td>
</tr>
<tr>
<td>PCA</td>
<td>principal component analysis</td>
</tr>
<tr>
<td>RP-HPLC</td>
<td>reversed phase high performance liquid chromatography</td>
</tr>
<tr>
<td>TAXI</td>
<td><em>triticum aestivum</em> xylanase inhibitor</td>
</tr>
<tr>
<td>TLXI</td>
<td>thaumatin-like xylanase inhibitor</td>
</tr>
<tr>
<td>TriFA</td>
<td>ferulic acid dehydrotrimer</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>wDDGS</td>
<td>wheat DDGS</td>
</tr>
<tr>
<td>XIP</td>
<td>xylanase inhibitor protein</td>
</tr>
</tbody>
</table>
LIST OF PAPERS
This thesis is based on the work presented in the following papers, which are referred to by roman numbers throughout the thesis.


IV: **Mads B. Pedersen**, Shukun Yu, Susan Arent, Søren Dalsgaard, Knud Erik B. Knudsen, and Helle N. Lærke. Xylanase Increased the Ileal Digestibility of Non-Starch Polysaccharides and Concentration of Low Molecular Weight Non-Digestible Carbohydrates in Pigs Fed High Levels of wheat DDGS

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Parts of the work carried out in this PhD project were incorporated into two international patent applications:


INTRODUCTION
The increased feed prices for cereal grains and protein crops have forced the feed industry to look for alternative feedstuffs to replace part of the conventionally feedstuff for livestock. The rapid increase in production of grain ethanol through the past decade has made a high amount of the primarily produced co-product, distillers dried grains with solubles (DDGS) with an estimated US-production of 43 million tonne in 2014, available for animal feed.

Due to the fermentation of starch to ethanol, the non-fermentable nutrients in the grain are concentrated approximately 3-fold in the produced DDGS. Thus, DDGS is a rich source of significant amounts of crude protein, amino acids, phosphorus and fat for animal feed. However, the main challenges limiting the use of DDGS in the feeds for non-ruminant animals is the inferior protein quality, potential high mycotoxin content, and high levels of non-starch polysaccharides (NSP).

The digestive system of non-ruminants does not secrete enzymes capable of digesting NSP. Instead, NSP is subjected to microbial modification, degradation and fermentation throughout the gastro intestinal tract. Of the different end-products of microbial fermentation, only short chain fatty acids are available as energy for the animal. In addition, high inclusion levels of DDGS in the animal diet may increase the endogenous losses, increase the energy requirement for maintenance, and increase the amount of nutrients not available for the animal due to encapsulation within the fibre matrix.

Thus, there is a need to increase the ability of the animal to utilize the energy of DDGS, and to limit the loss of energy associated with high NSP content.

The work presented in this PhD thesis describes the variation among DDGS sources of various origins with particular emphasis on detailed characterizing of the NSP fraction, modification of the NSP-fraction occurring during production, and the differences in complexity of the NSP fraction between different DDGS sources. In addition, the enzymatic degradation of DDGS by different exogenous enzymes is investigated both in vitro, and in vivo, and the differences in enzymatic degradability and digestibility of difference DDGS sources are discussed in relation to their compositional differences.
Distillers dried grains with solubles

Distillers dried grains with solubles (DDGS) is the main co-product of ethanol production from cereal grains. In the United States corn is the major crop used for production of bioethanol, whereas in cooler climates like Europe and Canada wheat is primarily used instead of corn [1, 2]. In addition to corn and wheat, some ethanol plants may use a mixture of different crops e.g. sorghum (US), and triticale, barley, and rye (EU), depending on availability and cost. In Brazil, sugar cane is the major crop used for ethanol production [3].

Bio ethanol is produced by two major industrial technologies; wet milling and dry-grind processing [4]. Wet milling processing produces gluten meal and gluten feed as major co-products, whereas DDGS is the major co-product of dry-grind processing [3].

Figure 1. Schematic diagram of a dry-grind ethanol production [3, 4]

The dry-grind processing is designed to maximize the production of ethanol and to minimize waste. The dry-grind process consists of several major steps including grinding, liquefaction, saccharification, fermentation, distillation, and recovery of co-products [3]. Figure 1. Grinding is the first step, which breaks the whole grain into smaller particles, consequently increasing the surface area of the starchy endosperm. Liquefaction reduces the size of the starch polymers by addition of thermostable α-amylase under high temperature (100°C) and mechanical shear provided by jet-cooking. After the slurry is cooled down,
glycoamylase is added to break the dextrinized mash into glucose monomers. During the 48-72 h fermentation, yeast converts glucose into ethanol and \( \text{CO}_2 \). Distillation separates ethanol from the mash by evaporation to a final ethanol purity of 99.9%. The remaining mash is now referred to as whole stillage, and is centrifuged to produce thin stillage and coarse solids. Some ethanol plants may extract some of the oil from the thin stillage before it is condensed and mixed with the wet grains and dried to produce DDGS [3, 4].

During ethanol production approximate 2/3 of the starting material (starch) is converted into ethanol and \( \text{CO}_2 \), consequently concentrating the unfermentable nutrients, such as protein, oil, minerals, and fibre approximately three-fold in the DDGS [5, 6]. The chemical composition of DDGS is generally acknowledged as being of great variability due to a combination of several factors; variability in the grain, differences in processing technologies between ethanol plants, and varying degree of accuracy in the analysis of DDGS samples [7-9]. A typical chemical composition of corn DDGS (cDDGS) and wheat DDGS (wDDGS) is listed in Table 1.

Thus, DDGS is a rich source of significant amounts of crude protein, amino acids, phosphorus and fat for animal feed. However, the main challenges limiting the use of DDGS in feeds for non-ruminant animals is the inferior protein quality caused by heat damaging of protein from excessive drying, potential high mycotoxin content, and high levels of non-starch polysaccharides (NSP) [5].

### Table 1. Typical chemical composition of corn- and wheat distillers dried grains with solubles (% of dry matter)

<table>
<thead>
<tr>
<th></th>
<th>Corn DDGS</th>
<th></th>
<th></th>
<th></th>
<th>Wheat DDGS</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>S.D.</td>
<td>N, samples</td>
<td>Mean</td>
<td>Range</td>
<td>S.D.</td>
<td>N, samples</td>
</tr>
<tr>
<td>Crude protein</td>
<td>44</td>
<td>27.9</td>
<td>(23-35)</td>
<td>2.4</td>
<td>18</td>
<td>38.1</td>
<td>(32-46)</td>
<td>4.1</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>33</td>
<td>7.4</td>
<td>(6-11)</td>
<td>1.1</td>
<td>12</td>
<td>7.7</td>
<td>(6-9)</td>
<td>0.9</td>
</tr>
<tr>
<td>Neutral detergent fibre</td>
<td>17</td>
<td>36.6</td>
<td>(28-51)</td>
<td>5.8</td>
<td>11</td>
<td>32.6</td>
<td>(22-47)</td>
<td>7.5</td>
</tr>
<tr>
<td>Acid detergent fibre</td>
<td>19</td>
<td>13.6</td>
<td>(9-19)</td>
<td>3.3</td>
<td>10</td>
<td>14.0</td>
<td>(7-22)</td>
<td>5.3</td>
</tr>
<tr>
<td>Ether extract</td>
<td>37</td>
<td>10.8</td>
<td>(3-10)</td>
<td>2.4</td>
<td>15</td>
<td>5.4</td>
<td>(3-7)</td>
<td>1.1</td>
</tr>
<tr>
<td>Ash</td>
<td>36</td>
<td>4.5</td>
<td>(3-6)</td>
<td>0.6</td>
<td>15</td>
<td>5.3</td>
<td>(5-7)</td>
<td>0.6</td>
</tr>
</tbody>
</table>

S.D., standard deviation. Adapted from Olukosi and Adebiyi [6].

### Inclusion of distillers dried grains with solubles in diets for pigs

DDGS may be included in diets for pigs in all phases of production. An inclusion level up to 30% of DDGS has been applied without negative impact on growth performance in nursery pigs (2-3 weeks post-weaning) and grower-finisher pigs [5]. Due to the relative high content of oil in cDDGS, high inclusion levels have been reported to negatively affect pork fat quality [10], thus, it is recommended to withdraw DDGS from the diets 3-4 weeks before slaughter. DDGS may replace soybean meal in diets for gestating sows without negative effects on sow or litter performance, and be fed up to 30% DDGS in lactating sows [5].

The concentration of digestible energy (DE) and metabolizable energy (ME) in cDDGS is similar to that of corn, provided that the oil is not removed during processing [5]. However,
since DE and ME systems may overestimate energy availability in protein- and fibre-rich feedstuffs, net energy systems that account for the variable utilization of nutrients are considered better to manage the inclusion of alternative feedstuffs such as DDGS [11].

**Non-starch polysaccharides in cereals**

In cereals, such as corn and wheat, the NSP originates from the primary and secondary cell walls in the grain tissues e.g. endoperm, aleurone, testa, pericarp and tip cap (corn). The major NSP fractions in corn and wheat, and consequently also in c- and wDDGS, are arabinoxylan (AX), cellulose, and β-glucan that together with lignin and other cell wall components build up the skeletal framework and provides structure and protection from the outside environment [12, 13], Figure 2.

![Component tissue structure of: A, wheat [14]; B, corn [15]. C, fluorescent antibody-labeling of arabinoxylan and β-glucan in aleurone cell wall from wheat grain: orange/red, arabinoxylan; green, β-glucan; yellow, presence of both arabinoxylan and β-glucan [16].](image)

**Arabinoxylan**

Arabinoxylan consists of a linear backbone of D-xylose residues linked by β-1,4- linkages mainly substituted with α-L-arabinose residues at the O-2 position, O-3 position, both O-2 and O-3 position, or unsubstituted [13, 16], Figure 3A. The arabinose to xylose ratio (A:X) and uronic acid to xylose ratio (UA:X) indicates the average degree of AX substitution, and is often used as indication of the structural complexity of the AX [17]. The AX structure is different
among the botanical grain fractions, with the outer layers encompassing higher complexity than endosperm AX, due to a higher content of glucuronic acids (or its 4-O methyl ester) and galactose residues. This AX is referred to as glucuronoarabinoxylan or heteroxylan [12, 16, 17], Figure 3B. In addition, this heteroxylan may be further substituted by short side chains of 2-4 sugars residues, acetic acids, and ferulic acids ester-linked to arabinose [18].

Figure 3. Main structural features of arabinoxylan from: A, endosperm; B, outer tissues of cereal grains. A, arabinose; X, xylose; G, galactose; Ga, glucuronic acid; F, Ferulic acid; uX, unsubstituted xylose; dX, di-substituted xylose; mX3, mono-substituted xylose; mX2, O-2 monosubstituted xylose. From Saulnier et al. [16].

Arabinoxylan crosslinking

In cereals, esterified hydroxycinnamic acids such as ferulic acid, p-coumaric acid, and sinapic acid can participate in photochemically or oxidatively induced coupling of cell wall polysaccharides such as arabinoxylans and pectins along with cell wall trapped proteins and lignin like polymers [19-21]. While p-coumaric acid is mainly bound to lignin, ferulic acid and its derivatives, which are the quantitatively most important cross-links in the plant cell wall, are primarily bound to arabinoxylans and pectins [22]. Ferulate in particular has been found to form dimers (DFA), trimers (TriFA), and higher ferulate oligomers potentially cross-linking two or more polysaccharide chains, Figure 4. Evidence that DFAs are indeed attached to arabinoxylans has been reported by use of carbohydrases and mild acidic hydrolysis followed by structural characterization in bamboo and corn bran [23, 24], and from corn bran insoluble fibre [25, 26]. The cross-linking of AX by DFA and TriFA strengthens the cell wall with implications for plant physiology, the plant’s defense mechanisms against pathogens, food science, and nutrition [22, 27, 28].
Cellulose consist of linear homopolymers of D-glucose residues linked by β-1,4-linkages, Figure 5. These glucan chains are stabilized by numerous intermolecular hydrogen-bonds between the hydroxyl groups of glucose residues from adjacent chains, thus, forming microfibrils. Cellulose is organized in crystalline regions within the microfibrils interspersed by non-crystalline amorphous regions. Cellulose is present in all plant tissues, however, to a much higher degree in the outer tissues [13, 29-31].

β-glucan

Like cellulose, β-glucan is formed from a linear homopolymer of D-glucose residues. However, these residues are linked by two to three consecutive β-1,4-linkages that are separated by a single β-1,3-linkage, thus, making the β-glucan less ordered compared to cellulose [17, 31, 32]. In addition, β-glucan is more easily degradable than AX in the small intestine, thus, only associated with minor effects on physico-chemical properties such as luminal viscosity [33].

Difference in fibre complexity between corn- and wheat DDGS

The nutrient composition and characteristic of DDGS reflect in part the composition in the parent grain, as only starch is removed during the fermentation process [34, 35]. As illustrated in Table 1; cDDGS contain more fat than wDDGS, whereas wDDGS has a higher content of crude protein than cDDGS, in line with previous characterizations of parent grains [36].
The NSP fraction in both corn and wheat [37] and c- and wDDGS [34], describes a greater fraction of soluble NSP in wheat than in corn. Corn and wheat have approximately similar content of cellulose, whereas the content of AX is reported slightly higher in wheat than corn [37]. The higher A:X and UA:X ratio in corn than in wheat [34, 37], indicate a more complex structure of the AX present in corn compared to wheat, due to a higher degree of AX substitution. Corn AX is described to comprise greater branch density and complexity than wheat AX [28, 38-40]. In addition, the higher complexity of the fibre matrix in corn than wheat is further substantiated by the markedly higher content of DFAs and TriFAs reported in corn and wheat insoluble fibre [27, 41, 42], and whole grain flours [28].

Collectively, the fibre matrix in cDDGS is expected to be more complex than wDDGS in terms of a more substituted AX and by having a markedly increase in potential polysaccharide cross-linkages, which combined is likely to have implications on the enzymatic degradation and overall digestibility.

Non-starch polysaccharides in relation to feed for swine

The digestive system of non-ruminants does not secrete enzymes capable of degrading NSP. Instead, NSP is subjected to microbial fermentation throughout the gastro intestinal tract [43, 44]. The fermentation of NSP occurs primarily in the large intestine, however, depending on the type of NSP a considerable depolymerisation and fermentation may also occur in the small intestine of pigs [45-48]. The main end-products of microbial fermentation are short chain fatty acids (SCFA), hydrogen, methane, and heat [49-51]. The produced SCFA, such as acetate, propionate, and butyrate are rapidly absorbed and have been shown to supply up to 30% of the energy requirement of pigs [44]. On the other hand the remaining end-products (hydrogen, methane, and heat) are considered as energy loss, as these end-products are not available to the pig as energy source, thus, decreasing the efficiency of energy utilization [52, 53]. In addition, the undigested fibre itself and excretion of microbial biomass is also regarded as energy loss. The degree of microbial fermentation of NSP is related, among other things, to the solubility of the NSP, illustrated by the high total tract digestibility of easily solubilizable AX from wheat [54, 55], whereas insoluble and lignified branched AX from wheat secondary cell walls remain practically undegraded in pigs [55, 56]. Further research has indicated that the microbial degradation of the soluble NSP fraction is selective and that the molecular weight distribution may explain differences in SCFA production [57]. Increased maturity of the pigs will positively affect the NSP digestibility due to an increased volume of the gastro intestinal tract, longer retention time, and higher activity of cellulytic bacteria in combination with a lower feed intake per unit of live weight [58, 59].

A high content of NSP in animal diets may directly or indirectly affect the digestive utilization and absorption of other nutrients. As mentioned above, chemical properties of the NSP (e.g. solubility, lignification and branching) affect the digestion of the NSP itself. In addition, NSP in the cell walls may encapsulate potential available nutrients such as proteins, thus, suppressing the availability of these nutrients for the endogenous enzymes in the small intestine [33, 54, 60]. Depending on the type, NSP may contribute to the physical properties of
the digesta such as viscosity (soluble NSP) and water-binding capacity, potentially affecting digesta transit time, bulking properties, microbial activity, and endogenous loses [43, 61-64]. In addition, especially insoluble NSP may increase mucus secretion in the small intestine due to the physical effects on the gut wall, consequently causing damage to the mucus layer [63]. Furthermore, feeding high levels of NSP will increase the total empty weight of the gastrointestinal tract [65], stimulate intestinal epithelial cell proliferation [66], increase endogenous secretions [67], increase maintenance energy requirement [67], and increase satiety and consequently potentially decrease voluntary feed intake [68].

Considering the type of NSP present in DDGS, which is primarily insoluble AX and cellulose, the effects caused by high inclusion levels of DDGS in diets for pigs will potentially be; dilution of energy in feed, increased endogenous loses primarily due to the mechanical effects on the gut wall, encapsulation of nutrients, and increased energy requirement for maintenance.

**Improving fibre utilization in swine by exogenous enzymes**

Due to the increased usage of high-fibre co-products, such as DDGS, and the negative effects associated with the high NSP content as described above, ways to increase the utilization of energy and/or limit the loss of energy from feeding these feedstuffs are essential. One approach is the addition of exogenous fibre degrading enzymes to improve nutrient utilization in diets for animal feed [69, 70].

As in the parent grain, corn and wheat, AX comprises the largest fraction of NSP in both c- and wDDGS. Thus, the main focus of this PhD project was aimed towards degradation of AX. Due to the structural complexity, complete degradation of AX requires a broad range of enzyme activities, like β-1,4-endoxylanase, α-D-glucuronidase, acetyl-xylan esterase, α-L-furanosidase, and ferulic acid esterase [71, 72], **Figure 6**. Of these NSP-degrading enzymes only the effect of xylanase on DDGS degradation was investigated in this PhD project.

![Figure 6](image.png)

**Figure 6.** The structural components of arabinoxylan/heteroxylan and the enzymes responsible for its degradation. Adapted from Hövel et al. [71].

**Xylanases**

In nature, xylanases are an important part of the enzyme-system involved in the metabolism of xylan as carbon source found in e.g. bacteria and fungi populations [73, 74].
Endo-β-1,4-xylanases (EC 3.2.1.8, hereon referred to as xylanase) are hydrolytic enzymes which cleave the β-1,4-linkages within the xylan backbone [75, 76]. Xylanases belong to two main families based on their primary sequence and structure; glycoside hydrolase family (GH) -10 and -11 [72, 77]. In addition to GH10 and -11, xylanases are also described to belong to GH5, -8, -30, and -43 families [78]. The three-dimensional structure of enzymes is essential for the substrate affinity, molecular mechanism, and overall function of the enzyme. Xylanases belonging to the GH-10 family is generally characterized by a (β/α)₈ barrel structure, Figure 7A, whereas GH-11 xylanases are generally characterized by a β-jelly-roll structure, Figure 7B [78, 79].

Figure 7. Representative structures of xylanases from glycoside hydrolase family -10 (A) and -11 (B). Red, α-helix; blue, β-strand/sheets. Adapted from Collins et al. [79].

Figure 8. Schematic overview of endoxylanase degradation of arabinoxylan (AX) into arabinoxylan-oligosaccharides (AXOS). Red arrows indicate site of hydrolysis. Adapted from Lagaert et al. [80].
In addition to the different structural characteristics among xylanases, different substrate affinities are also reported. GH10 xylanases have been shown to exhibit greater catalytic versatility or lower substrate specificity than xylanases from the GH11 family. GH11 xylanases primarily cleave unsubstituted regions of the xylan backbone, whereas GH10 is capable of cleaving in more substituted regions, hence being less hindered by the presence of substitutions [78, 81]. The GH-11 xylanases generally have a smaller molecular size (<30 kDa) than GH-10 xylanases (>30 kDa), and it has been proposed that the smaller size may enable the GH-11 xylanase in penetrating the cell wall network [82].

Endo-xylanases solubilize arabinoxylan by cleaving the β-1,4-glycosyl linkages within the β-1,4-xylose backbone of insoluble as well as soluble arabinoxylan, thus, partially solubilizing insoluble arabinoxylan and fragmenting soluble arabinoxylan into arabinoxylan-oligosaccharides (AXOS), Figure 8 [81]. The generated AXOS are generally acknowledged as being associated with potential prebiotic effects [83-85].

A part of the plant’s defense mechanism against pathogens is the production of proteins which can inhibit xylanase activity, so-called xylanase inhibitors [86]. The *Triticum aestivum* xylanase inhibitor (TAXI) has been isolated and characterized from wheat, rye, and barley and is described to specifically inhibit GH11 xylanases [87]. The xylanase inhibitor protein (XIP) has been isolated from wheat, rye, barley, corn, and rice described to inhibit both GH10 and -11 xylanases [88]. Both TAXI and XIP is characterized as a competitive inhibitor, thus, interacting with the enzyme active site by mimicking the substrate [87, 88]. The thaumatin-like xylanase inhibitor (TLXI) has been identified in wheat, and is described as an non-competitive inhibitor towards GH11- but not GH10 xylanases [86].

**Use of xylanase in animal feed**

Next to phytase, NSP degrading enzymes (mainly xylanase) comprise the second largest fraction of commercial enzymes sold to the feed industry particularly for poultry feed, but are commonly used in pig diets too [89]. The effects of NSP degrading enzyme addition in animal diets have been reviewed by several authors [11, 68-70, 90, 91]. Collectively, the results indicate that NSP degrading enzymes may improve NSP and nutrient digestibility, provided that the NSP constituents in the diets match the substrate affinity of the enzymes or that the substrate is limiting for the nutrient digestion. In addition, it is generally acknowledged that further research is needed to elucidate, among other things; NSP characterization of the feedstuff in detail (especially “new” co-products such as DDGS), substrate affinity of applied enzymes in relation to feed composition, and detailed characterization of the degradation products.

The effects of NSP-degrading enzymes application in DDGS containing diets are currently inconclusive; Widyaratne et al. [92] reported an increase in ileal digestibility of energy by xylanase addition in wheat but not in diets containing 40% wDDGS, Yanez et al. [93] reported no effect of xylanase addition in diets containing 44% corn and wheat co-fermented DDGS, Jones et al. [94] reported no effect of enzyme addition on growth performance in diets containing 30 % cDDGS, whereas Emiola et al. [95] reported an improved ileal nutrient- and
total tract energy digestibility and growth performance by adding a combination of xylanase, β-glucanase and cellulase enzymes in pigs fed 30% wDDGS.

Several factors such as the degree of lignification, cross-linkages, structural organization, and the time the digesta remains in the fore gut, will affect and/or limit the effects of exogenous enzyme addition. Based on studies with pigs showing that the outer layers of wheat and rye are almost resistant to degradation after passage through the large intestine, Back Knudsen [17] suggested that it is primarily the endosperm and aleurone layers that potentially can be degraded by exogenous enzymes under in vivo conditions, Figure 9.

Figure 9. Potential effects of exogenous enzymes in cereal cell wall degradation, illustrated by cross-section of the outer layers and the endosperm of corn, wheat, and barley. From Bach Knudsen [17].
METHODS OVERVIEW
Near infrared reflectance spectroscopy

To characterize the composition of macronutrients (e.g. crude protein, fat, ash, and starch) in 138 DDGS samples [Paper I], near infrared reflectance spectroscopy (NIRS) was applied instead of conventional wet chemistry methods [37, 96-98], which were used in Paper IV. NIRS is a non-destructive analytical technique which relates the light absorbed by a sample to its chemical and physical composition, with instant results. Due to its rapid and precise measures, NIRS is often used in agriculture to determine the composition of feedstuff [99-102]. However, the successful application and prediction obtained by NIRS relies on several compositional analyses and calibration of spectral data, which requires a broad number of samples with varying compositional profiles.

In principle, the milled samples were scanned from 1100 to 2498 nm on a FOSS NIRSystems. The spectral data were predicted by Aunir (AB Agri, UK) for the composition of moisture, fat (both ether extract and acid hydrolysis), crude protein, crude fibre, ash, starch, total sugars, neutral- and acid detergent fibre, using the calibration available for DDGS.

Analysis of non-digestible carbohydrates

Quantification of non-digestible carbohydrates (NDC), non-starch polysaccharides, and low molecular weight NDC was performed using a modification of the Englyst [103] and Uppsala [104, 105] procedures, essentially as described by Bach Knudsen [37]. The NSP-procedure was applied on the DDGS samples analyzed in Paper I and –III, whereas the more detailed NDC-procedure was applied in analysis of the wDDGS used in Paper IV.

In principle, the NSP procedure consists of three parallel runs (A, B, C), whereas an additional run is include for the determination of NDC (D), Figure 10. Procedure A, determines the total NSP by removal of starch, ethanol precipitation of soluble fibres, swelling of cellulose, hydrolysis of NSP into monomers, reduction of neutral sugars to alcohols followed by derivatization into alditol acetates, and finally determination of these using gas chromatography (GC). Procedure B, determines the non-cellulosic polysaccharides (NCP) by omitting swelling of cellulose. Procedure C, determines the insoluble NSP by extraction of the soluble NSP in phosphate buffer at 100°C for 1 h followed by centrifugation and removal of supernatant. Procedure D, determines the total carbohydrates (CHO) by direct swelling and hydrolysis, thus, omitting removal of starch. The content of uronic acids was determined in a separate step by colorimetry. Klason lignin was determined gravimetrically as the residue resistant to hydrolysis by 2 M H₂SO₄ for 2 h at 100 °C.
Figure 10. Analytical procedure for determination of different fractions of non-starch polysaccharides. CHO, carbohydrates; NCP, non-cellulosic polysaccharides, NSP, non-starch polysaccharides.

Based on the four parallel runs in the NSP procedure, the following calculations were performed:

Cellulose was calculated as:

\[
\text{Cellulose} = \text{NSP}_{\text{glucose}, \text{12 M}} - \text{NCP}_{\text{glucose}, \text{2 M}}
\]

Total NSP as:

\[
\text{Total NSP} = \text{rhamnose} + \text{fucose} + \text{arabinose} + \text{xylose} + \text{mannose} + \text{galactose} + \text{NCP-glucose} + \text{uronic acids} + \text{cellulose}
\]

Soluble NSP as:

\[
\text{Soluble NSP} = \text{Total NSP} - \text{insoluble NSP}
\]
Total NDC as:
Total NDC = Total CHO – starch

Low molecular weight (LMW) NDC as:
LMW-NDC = Total CHO – Total NSP - starch

**Reversed phase high performance liquid chromatography with ultraviolet detection**

To investigate and quantify ester-linked DFA and TriFA in DDGS samples [Paper II], a validated method using reversed phase high performance liquid chromatography (RP-HPLC) with ultraviolet detection (UV) was applied [27]. Compared to previously applied methodologies e.g. GC and HPLC methods [41, 106], this two-gradient RP-HPLC/UV method does not require derivatization and allows for a complete separation of monomers, DFAs, and TriFAs [27]. Analysis of lignin-rich matrices (e.g. corn stover) might cause problems with the UV detection due to co-elution of lignin compounds with DFAs and TriFAs. For analysis of such matrices HPLC-mass spectrometry methods are proposed [28]. The use of the RP-HPLC/UV method requires synthesis of internal standard and isolation of standard compounds for accurate validation, which can be very time consuming.

In principle, the DDGS samples were washed twice with both ethanol (80 % v/v) and acetone to lower the spectral noise. After alkaline hydrolysis of the ester-linkages, the samples were acidified and internal standard was added. Extraction was carried out using diethyl ether and samples dried under a stream of nitrogen. Finally, the samples were redissolved in tetrahydrofuran/water (1:1) and analyzed.

**Enzymatic degradation of DDGS in vitro**

To investigate the enzymatic degradation of c- and wDDGS - and to compare the efficiency of different enzymes, experiments were setup using purified enzymes [Paper III]. Four different xylanases were evaluated alone and in combination with protease and phytase according to four full factorial experiments. In addition, the enzymatic degradation of insoluble DDGS was also investigated based on substrate prepared as described in the NSP procedure-C.

The major difference between the applied methodology and a commonly used *in vitro* digestion method [107], is that no endogenous digestive enzymes were added and pH was kept constant throughout the incubation. However, the applied experimental conditions (i.e. pH 6.0, reaction time 4 h, temperature 39°C) was chosen to mimic the physical conditions occurring in the small intestine of pigs [108], whereas stirring was at 1100 rpm. The experimental setup and following analysis was chosen since it allowed a direct comparison of the effectiveness on DDGS degradation by different enzymes in a time effective approach.

The effect of enzyme addition was evaluated based on the ability to simultaneously solubilize pentosan and protein by use of analytical methods that both were compliable with the use of micro titer plates. Quantification of soluble pentosan was measured principally as described by Rouau and Surget [109] using a continuous flow injection apparatus with
autosampler unit, **Figure 11**. In principle, the supernatant was hydrolyzed to monomers by glacial acetic acid/HCl, 37%, (50:1) at 96 °C, and mixed with phloroglucinol (1,3,5-trihydroxybenzene), consequently developing a colored complex. The absorbance was measured at 510 and 550 nm against a xylose standard.

Quantification of soluble protein was determined by use of a commercial available protein quantification kit (BCA Protein Assay Kit, Pierce) against a bovine serum albumin standard. The first step involves the chelation of copper with protein under alkaline conditions to produce a light blue complex. Secondly, bicinchoninic acid reacts with the reduced copper-ion resulting in a purple colored complex that is measured at 562 nm [110].

![A, continuous flow injection apparatus for analysis of soluble pentosan; B, corresponding autosampler unit (Skalar Analytical B.V., Breda, Netherlands)](image)

**In vivo digestion model**

To investigate the effects of enzyme addition on nutrient digestibility in vivo, two animal trials were performed using ileum cannulated pigs fed either cDDGS or wDDGS. For wDDGS the pigs were fed a control diet and three different enzyme treatments according to a double 4×4 Latin square design [Paper IV], whereas for cDDGS the pigs were fed five diets according to a double 5×5 Latin square design [Supplementary data]. Due to protection of potentially Intellectual Properties, only the results from the control diet without enzymes are presented as Supplementary data in the General discussion section.

Implementation of a simple T-cannula in the distal ileum allows for collection of ileal digesta and faeces and enable repeated samples on the same animal, which reduces the variability and number of experimental animals, **Figure 12** [111]. Ileal digestibility reflects the endogenous enzymatic digestion, exogenous enzymatic digestion, microbial fermentation in the small intestine, endogenous secretions into the gut, and absorption [112]. By addition of an indigestible marker (e.g. chromic oxide as used in this thesis) the total extent of digestion in the small- and large intestine can be determined.

The apparent ileal digestibility of organic matter, starch, crude protein, HCl-fat, and NSPs were calculated by the indicator method relative to dietary and ileal concentrations of chromic oxide [113], according to **Equation 1** below. A prerequisite of the indicator method is
that the marker is indigestible and not separated from the remaining feed matrix throughout the gastrointestinal tract.

\[ \text{Digestibility coefficient of } X = 1 - \frac{CrO_3,\text{diet} \times X_{\text{ileum}}}{CrO_3,\text{ileum} \times X_{\text{diet}}} \] (1)

where \( X \) is the concentration of a specific nutrient in the diet and ileum digesta.

\textbf{Figure 12.} Implementation of simple T-cannula in the pig distal ileum

\textbf{Principal component analysis}

Principal component analysis (PCA) was applied to the compositional data to detect distributions and separations among DDGS sources of various origins [Paper I], and to investigate to which cell wall constituents the DFAs and TriFAs shared the highest correlation [Paper II].

PCA is a common statistical method that transforms a number of correlated variables into a smaller number of uncorrelated variables called principal components. The generated score plot allow the DDGS samples to be classified or grouped according to their overall composition, whereas the corresponding loading plot highlight which components are responsible for the separation. In addition, the loading plot illustrate which components are correlated with each other, as these will be located close to one another in the plot [114].
AIM AND HYPOTHESES
The overall aim of the work described in this thesis was to investigate the variation and differences in NSP composition of DDGS, and to improve the digestibility of c- and wDDGS as feedstuff by enzymatic modification of the fibre structure.

The work described in this thesis was divided into three work packages, each with specific aims;

1. To perform a systematic investigation of the variation in the chemical and physicochemical properties of different DDGS sources with emphasis on the fibre matrix. If possible, also relate specific process technologies to DDGS composition.

2. To study, *in vitro*, the effect of different xylanases alone and in combination with other enzymes for the degradation of c- and wDDGS. The enzyme performance is evaluated based on the ability to simultaneously solubilize pentosan and protein from DDGS.

3. To study, *in vivo*, the effects of xylanase and protease addition in ileal cannulated pigs fed c- or wDDGS as sole provider of macronutrients, according to a double 5×5 Latin square design or a double 4×4 Latin square design, respectively. The effect of enzyme addition is evaluated by apparent ileal digestibility of macronutrients and NSP, and apparent total tract digestibility of NSP.

It is hypothesized that:

- Different raw materials and ethanol production technologies give rise to variation in the composition and structural complexity of NSP in c- and wDDGS

- The susceptibility of c- and wDDGS sources to enzymatic degradation using exogenous enzymes is dependent on the chemical composition

- Xylanases (along with protease and phytase) applied in feed will increase fibre degradation and nutrient release both *in vitro* and *in vivo*, thus, affecting ileal digestibility of NSP and macro nutrients
PAPER I

Compositional Profile and Variation of Distillers Dried Grains with Solubles from Various Origins with Focus on Non-Starch Polysaccharides

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Compositional profile and variation of Distillers Dried Grains with Solubles from various origins with focus on non-starch polysaccharides

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**Abstract**

Corn-, wheat- and mixed cereal Distillers’ Dried Grains with Solubles (DDGS) were investigated for compositional variability among DDGS origins, ethanol plants, and the relationship between corn and corresponding DDGS. A total of 138 DDGS samples were analyzed by use of Near Infrared Reflectance Spectroscopy for common constituents, while 63 DDGS samples along with 11 corn samples were characterized for their non-starch polysaccharide (NSP) content. The results indicated that the compositional profile of DDGS reflected the nutrient content of the parent grain but with a greater content of remaining nutrients (e.g. protein, fat, fibre and minerals) after fermentation of starch to ethanol. Corn DDGS differentiated from wheat DDGS by a greater content of fat (P<0.006), insoluble-NSP (P<0.001), uronic acids (P<0.001), cellulose (P<0.032), and arabinose/xylose (P<0.001) – and uronic acid/xylose-ratio (P<0.001). Wheat DDGS differentiated from corn DDGS by a greater content of ash (P<0.001), soluble-NSP (P<0.001), and Klason lignin (P<0.001).

Among the three sources of DDGS, the greatest variation was observed for the content of soluble-NSPs, especially soluble arabinoxylan. Based on the compositional profiles of the DDGS, principal component analysis allowed for a visual differentiation of corn DDGS from five different ethanol plants, indicating the potential of each ethanol plant to produce DDGS with consistent compositional characteristics. Furthermore, investigation of corn and corresponding DDGS indicated that the NSP fraction is modified during the fermentation process, especially arabinoxylan, by an increase in soluble arabinoxylan proportion in DDGS. In addition, the arabinose/xylose (P<0.001) and uronic acid/xylose-ratio (P<0.001) were greater for corn, compared with corresponding DDGS, indicating modifications of the endosperm arabinoxylan during the fermentation and drying process.

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**Abbreviations:** ADF, acid detergent fibre; AH, acid hydrolysis; AX, arabinose-to-xylose; Cel/NSP, cellulose-to-NSP; CV, coefficient of variation CF, crude fibre; CP, crude protein; DF, dietary fibre; DM, dry matter; DDGS, distillers dried grain with solubles; EE, ether extract; I-NSP, insoluble NSP; NCP, non-cellulosic polysaccharide; aNDFom, neutral detergent fibre assayed with heat stable amylase exclusive residual ash; NIRS, near infrared reflectance spectroscopy; NSP, non-starch polysaccharides; PCA, principal component analysis; S-Ara, soluble arabinose; S-NSP, soluble NSP; S-Xyl, soluble xylose; T-NSP, total NSP; UA/X, uronic acid-to-xylose.

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1. Introduction

Distillers’ Dried Grains with Solubles (DDGS) is the major co-product from the dry-grind production of bioethanol from cereal grains. After the conversion of grain starch to ethanol during the fermentation process, the non-fermentable part remains, with an increase of all nutrients except starch compared with those in the parent grain. For both corn- and wheat DDGS an increase of approximately 3-fold of nutrients such as protein, fat, vitamins, minerals and fibre is observed (Widyaratne and Zijlstra, 2007; Stein and Shurson, 2009).

Compositional variability of DDGS from various bioethanol plants has previously been reported for both corn (Spiehs et al., 2002; Belyea et al., 2004; Batall and Dale, 2006; Liu, 2011), and wheat origins (Nyachoti et al., 2005; Bandegani et al., 2009; Cozannet et al., 2010). The variability in the chemical composition of DDGS is due to a number of factors including differences in processing technologies among the bioethanol plants, and variability in chemical composition of the grains (Liu, 2011; Olukosi and Adebiyi, 2013). The majority of the reported compositional profiles of DDGS have focused mainly on common constituents such as crude protein (CP), crude fibre (CF), neutral detergent fibre, acid detergent fibre (ADF), fat, minerals, and amino acids (Liu, 2011; Olukosi and Adebiyi, 2013). Non-starch polysaccharides (NSP) make up 25–30% of the DDGS, with the two major components of the NSP being arabinoxylan and cellulose. Arabinoxylan consists of D-xylene units joined by β-linkages substituted with arabinose residues along the chain (Kim et al., 2008). The substitution of arabinose occurs randomly allowing other substituents such as D-glucuronic acid and acetyl groups to attach to the xylan backbone (Bedford, 1995). These random substitutes together with feruloylated arabinose residues induce the arabinoxylan cross-linking to form strong heterogeneous intermolecular complexes, affecting the potential for enzymatic degradation along with the potential encapsulation of nutrients within the cell wall (Hartley, 1973; Lequart et al., 1999; Lapiere et al., 2001; Piber and Koehler, 2005). Despite the high content of NSPs in DDGS detailed characterization of the NSPs in DDGS is limited as only a few reports describe the NSP composition (Widyaratne and Zijlstra, 2007; de Vries et al., 2013). Characterization of the NSP composition in DDGS of various origins may contribute to the overall understanding and interpretation of opportunities and limitations in DDGS degradation in vitro and in vivo, which is particularly useful for feed manufactures and enzyme producers targeting DDGS degradation.

The current study describes the compositional profiles and extent of variation in DDGS from three different grain origins: corn, wheat, and mixed cereal. A total of 138 DDGS samples from three sources of grain and 24 different bioethanol plants were analyzed based on the common constituents, with detailed NSP profiles of 63 DDGS samples. Multivariate data analysis was applied to the compositional data to determine grouping and separation of DDGS samples according to grain origins and bioethanol plants.

2. Materials and methods

2.1. Materials

A total of 138 DDGS samples were collected from 24 ethanol plants in the U.S. and E.U., covering the following three parent grain origins: corn, wheat, and a mix of cereal (mixed).

A total of 72 corn DDGS samples were collected from 21 different ethanol plants in the U.S.; 11 DDGS samples along with 11 corn samples were sampled over 11 months with one sample every month from a plant in Nevada (P1), 17 samples were sampled over 1½ months from a plant in Minnesota (P2), 10 samples were sampled over 20 days from another plant in Minnesota (P3), 10 samples were sampled over 20 days from a plant in Wisconsin (P4), 8 samples were sampled over 8 days from a plant in Iowa (P5), and 16 samples were supplied by a DDGS supplier in Iowa, representing samples from 7 plants in Minnesota, 4 plants in Iowa, 2 plants in Nevada, and 1 plant in Illinois, Indiana and Kentucky.

A total of 56 wheat DDGS samples were collected from 2 different ethanol plants in the E.U., with 46 samples from a plant in the U.K. sampled over a period of app. 3 months, and 10 samples from a plant in France sampled over 20 days.

Finally, 10 DDGS samples of a mixed grain origin were sampled over 20 days from a plant in Sweden. The parent grains used in the production of the mixed DDGS were wheat, triticale, barley, and rye. However, their individual proportion is unknown.

2.2. Chemical analysis

All 138 DDGS samples (72 corn, 56 wheat, and 10 mixed) and 11 corn samples were milled (0.5 mm) and scanned from 1100 to 2498 nm by near infrared reflectance spectroscopy (NIRS) on a FOSS NIRSystems 5000 (Foss). The spectral data were predicted by Aunir, AB Agri Ltd., UK, for the composition of: moisture, fat ether extract (EE), fat acid hydrolysis (AH), CP (N × 6.25), CF, ash, starch, total sugars, aNDfom and ADF, using the calibration available for DDGS and corn, respectively.

A total of 63 DDGS samples (47 corn, 11 wheat, and 5 mixed) and 11 corn samples were quantified for total and soluble NSP content along with their constituent sugars by gas–liquid chromatography for neutral sugars and by a colorimetric method for uronic acids, with procedure and calculations according to Bach Knudsen (1997), with the modification that 2 mol/L sulfuric acid for 1 h was used to hydrolyze the non-cellulosic polysaccharides (NCP) rather than 1 mol/L sulfuric
Table 1
Compositional profile of corn-, wheat-, and mixed cereal DDGS (g/kg DM).

<table>
<thead>
<tr>
<th></th>
<th>Corn DDGS</th>
<th>Wheat DDGS</th>
<th>Mixed DDGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean(^a)</td>
<td>Range(^b)</td>
<td>S.D. (CV)</td>
</tr>
<tr>
<td>Moisture</td>
<td>87(^e)</td>
<td>(65–124)</td>
<td>8 (0.10)</td>
</tr>
<tr>
<td>Fat (EE)</td>
<td>9(^i)</td>
<td>(65–118)</td>
<td>15 (0.17)</td>
</tr>
<tr>
<td>Fat (AH)</td>
<td>111(^i)</td>
<td>(84–135)</td>
<td>14 (0.13)</td>
</tr>
<tr>
<td>Crude protein</td>
<td>314(^i)</td>
<td>(271–364)</td>
<td>21 (0.07)</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>77(^i)</td>
<td>(64–95)</td>
<td>6 (0.07)</td>
</tr>
<tr>
<td>Ash</td>
<td>71(^i)</td>
<td>(54–90)</td>
<td>7 (0.09)</td>
</tr>
<tr>
<td>Starch</td>
<td>60(^i)</td>
<td>(29–139)</td>
<td>27 (0.45)</td>
</tr>
<tr>
<td>Total sugars</td>
<td>90(^i)</td>
<td>(54–126)</td>
<td>17 (0.19)</td>
</tr>
<tr>
<td>aNDFom</td>
<td>351(^i)</td>
<td>(302–397)</td>
<td>24 (0.07)</td>
</tr>
<tr>
<td>ADF</td>
<td>101(^i)</td>
<td>(89–119)</td>
<td>6 (0.06)</td>
</tr>
</tbody>
</table>

Based on near infrared reflectance spectroscopy. ADF, acid detergent fibre; AH, acid hydrolysis; EE, ether extract; aNDFom, neutral detergent fibre; S.D., standard deviation.

\(^a\) Avg. of means from 21 plants.
\(^b\) N=72.
\(^c\) Avg. of means from 2 plants.
\(^d\) N=56.
\(^e\) Mean from 1 plant.
\(^f\) N=10.
\(^\#\) Means that do not share a letter are significant different at P<0.05.

acid for 2 h. Klasson lignin was measured gravimetrically as the residue resistant to hydrolysis by 2 mol/L sulfuric acid (Bach Knudsen, 1997).

2.3. Calculations and statistical analysis

Content of NCP-glucose was calculated as

\[
\text{NCP-glucose} = \frac{\text{NSP}}{2 \text{ mol/L H}_2\text{SO}_4} \times \text{g/kg DM}
\]

Content of cellulose was calculated as

\[
\text{cellulose} = \frac{\text{NSP (12 mol/L H}_2\text{SO}_4) - \text{NCP-glucose}}{\text{g/kg DM}}
\]

Total non-starch polysaccharides (T-NSP) as

\[
\text{T-NSP} = \text{rhamnose} + \text{fucose} + \text{arabinose} + \text{xylose} + \text{mannose} + \text{galactose} + \text{NCP-glucose + uronic acids + cellulose}
\]

Soluble NSP (S-NSP) as

\[
\text{S-NSP} = \text{T-NSP} - \text{insoluble NSP (I-NSP)}
\]

 Dietary fibre (DF) as

\[
\text{DF} = \text{T-NSP} + \text{Klasson lignin}
\]

A one-way ANOVA was applied for comparison among means of the DDGS compositions from various origins followed by a Tukey pair wise comparison with overall significance level at P=0.05, using Minitab 16 (Minitab Inc.). Data from the compositional analysis were categorized and analyzed by principal component analysis (PCA) with evenly spread 7-fold partial cross-validation after mean centering and unit variance scaling, using Evince 2.5.5 software (UmBio AB) to detect distributions and separations among the groups, as previously discussed (Shewry et al., 2013).

3. Results

3.1. Compositional variation in DDGS of various origins

The results of the compositional analyses of the DDGS are presented in Table 1. Common for all DDGS, aNDFom and CP represented the major fractions, with a large extent of the aNDFom fraction present as ADF. Corn DDGS contained a greater content of fat (AH) compared with wheat- and mixed DDGS with an increase of 38 g/kg DM (P=0.005) and 42 g/kg DM (P=0.019), respectively. Furthermore, a greater aNDFom content was observed in corn DDGS with an increase of 45 g/kg DM (P=0.057) and 49 g/kg DM (P=0.118) compared with wheat- and mixed DDGS, respectively. Wheat DDGS contained 20 g/kg DM (P=0.001) greater ash content than corn DDGS. Among the three sources of DDGS, the greatest coefficient of variation (CV) was observed for the content of starch, with CVs of 0.39–1.03. For both corn- and wheat DDGS, the second and third greatest CVs was observed for total sugars (0.19 and 0.23) and fat (EE) (0.17 and 0.16), respectively. Corn- and mixed DDGS
had CVs on moisture content of 0.10 and 0.14, respectively, and wheat- and mixed DDGS had CVs on CF content of 0.14 and 0.10, respectively.

Detailed analysis of the NSP profile of 63 samples (47 corn DDGS, 11 wheat DDGS, and 5 mixed DDGS) is presented in Table 2. Corn DDGS had the greatest content of NSP (286 g/kg DM), followed by wheat- (262 g/kg DM), and mixed DDGS (247 g/kg DM). Wheat- and mixed DDGS did not differ (P>0.05) in S-NSP content (ranging from 53 to 80 g/kg DM), while a markedly lesser content (P<0.001) was observed in corn DDGS (16–65 g/kg DM). The distribution of constituent sugars for all three DDGS origins was in the order of xylene > arabinose > NCP-glucose > mannose > galactose. No difference was observed in arabinoxylan content (P=0.555) across the three DDGS origins with corn- (123–172 g/kg DM), wheat- (121–155 g/kg DM), and mixed DDGS (120–136 g/kg DM). Despite the comparable content of arabinoxylan, corn DDGS contained 5 g/kg DM (P=0.226) and 13 g/kg DM (P=0.022) more arabinose compared with wheat- and mixed DDGS, respectively, accompanied by a 8 g/kg DM lesser content of xylose compared with wheat DDGS, hence yielding a greater arabinose/xylene-ratio (A/X-ratio) in corn DDGS of 0.80 compared with that of wheat- (0.66, P<0.001), and mixed DDGS (0.63, P=0.002), respectively. Corn DDGS contained approximately twice as much uronic acids (16 g/kg DM, P<0.001), along with a greater cellulose content (67 g/kg DM) compared with both wheat- (50 g/kg DM, P=0.32), and mixed DDGS (54 g/kg DM, P=0.248). Furthermore, corn DDGS contained a markedly greater uronic acid/xylene-ratio (A/X-ratio) of 0.20, compared with both wheat- (0.09, P<0.001), and mixed DDGS (0.08, P<0.001). The greatest Klasson lignin content was observed in mixed DDGS (82 g/kg DM), compared with wheat- (66 g/kg DM, P=0.105), and corn DDGS (25 g/kg DM, P<0.001). All three DDGS origins had comparable contents of total mannose and galactose. Except the soluble mannose content (P<0.001), no differences in compositional profile were observed between wheat- and mixed DDGS (P>0.05). Regardless of DDGS origin, greater CVs were observed for the soluble constituent sugars compared with the total constituents. Soluble-NCP-glucose had greatest CVs with values of 1.90, 0.89, and 0.46, for corn-, wheat-, and mixed DDGS, respectively. Overall, corn DDGS had greater CVs for both total- and soluble constituents, followed by wheat- and mixed DDGS, respectively. For corn DDGS especially soluble xylose (S-Xyl) and soluble arabinose (S-Ara) had high CVs with values of 0.62 and 0.45, respectively.

The PCA illustrated a clear clustering and separation between corn DDGS to one side, and wheat- and mixed cereal DDGS on the other (Fig. 1A). The corresponding loading plot (Fig. 1B), indicated the components most responsible for the separation,
Fig. 1. Principal component analysis of DDGS samples of corn-, wheat, and mixed cereal origin: (A, B) scores and loadings plots of PCA model constructed from common constituent profiles as model variables (N = 138), (C, D) scores and loadings plots of PCA model constructed from NSP profile as models variables (N = 63), (E, F) scores and loadings plots of PCA model constructed from combined common constituents and NSP profiles as models variables (N = 63). ADF, acid detergent fibre; AH, acid hydrolysis; Ara, arabinose; Cel, cellulose; DF, dietary fibre; EE, ether extract; Gal, galactose; Glu, NCP-glucose; I, insoluble; Lig, Klason lignin; Man, mannose; NDC, non-digestible carbohydrate; NDF, neutral detergent fibre; S, soluble; T, total; Xyl, xylose.
with corn DDGS differentiating due to a greater content of fat, aNDFom, CF, and starch. Wheat and mixed DDGS differentiated from corn DDGS by a greater content of ash, CP, and total sugars. The corn- and wheat DDGS samples indicated a comparable variation between individual samples in the score plot. The PCA model illustrated clear separation and clustering between the three different DDGS origins based on the NSP characteristics, with only a minor overlap between wheat- and mixed DDGS (Fig. 1C). The corresponding loading plot revealed the constituents with the greatest effect on the separations, with corn DDGS differentiating due to large content of insoluble- and total constituents along with greater content of cellulose, and wheat- and mixed DDGS differentiating due to greater content of soluble constituents and Klasson lignin (Fig. 1D). A combined PCA model on both NIRS- and NSP compositional data on 63 samples, revealed the clearest separation among the three DDGS origins (Fig. 1E), compared with the two PCA models based individually on NIRS- and NSP compositional data (Fig. 1A and C). The corresponding loading plot (Fig. 1F), strongly underlined the constituents differing the most and being most responsible for the grouping between the three DDGS origins. Corn DDGS was greater in insoluble- and total NSP, cellulose, and starch, and wheat- and mixed DDGS greater in soluble NSPs, total sugars, and ash. Furthermore, the loading plot revealed that mixed DDGS differed from wheat DDGS by having a greater content of mannose, total sugars, ADF, and CP.

3.2. Compositional variation in corn DDGS from various ethanol plants

Table 3 presents the results of corn DDGS samples collected from 5 different ethanol plants; P1 (N = 11), P2 (N = 17), P3 (N = 10), P4 (N = 10), and P5 (N = 8), of which 56 samples were analyzed for common constituents, and 31 samples for the NSP profile. For starch content P1, P4, and P5 had the greatest CVs of 0.10–0.11. Furthermore, P1 and P5 had greater CVs in moisture content of 0.11 and 0.16, respectively, than P2–P4 and greater CVs were observed for total sugar content for P1 (0.09) and P2 (0.11). Across all of the common constituents, the five plants had overall average CVs of 0.07, 0.05, 0.03, 0.05, and 0.05, for P1, P2, P3, P4, and P5, respectively.

The five plants had varying CVs with regard to S-NSPs with values of 0.07–0.49. However, only small variation in CVs was observed for T-NSPs (288–325 g/kg DM) with values of 0.01–0.04 across all five plants. The CVs were relatively consistent for cellulose content among the different plants. On the other hand Klasson lignin content differed with CVs of 0.13–0.22. The CVs with regard to soluble constituent sugars varied markedly between the five plants with overall CVs of 0.03–1.49. Each of the five plants had conserved A/X-ratios (0.71–0.79) with corresponding low CVs with values of 0.01–0.02, together with conserved UA/X-ratios (0.17–0.23). There was an individual grouping between the five plants, indicating a conserved compositional profile of DDGS from each ethanol plant (Fig. 2A), and a greater compositional variation of P1, followed by P5, P4, P2, and P3, respectively. The corresponding loading plot (Fig. 2B), illustrated that P1 differed from the remaining plants by a greater content of total- and insoluble NSP (P<0.05), cellulose (P<0.05, except when compared with P2), and uronic acid (P<0.05, except when compared with P4). P4 differed mainly by a greater content of fat (EE) (P<0.05). P5 differed based on a greater content of ash (P<0.05), total sugars (P<0.05), and total NCP-glucose (P<0.05), while P3 differed from P2 mainly by a greater content of fat (AH) (P<0.05).
Table 3: Compositional profiles of corn DDGS from five ethanol plants (g/kg DM).

<table>
<thead>
<tr>
<th>Plant</th>
<th>Compositional Profiles</th>
<th>P1 (N=5; V=8.5)</th>
<th>P2 (N=5; V=11.5)</th>
<th>P3 (N=10; V=5)</th>
<th>P4 (N=10; V=5)</th>
<th>P5 (N=5; V=12.5)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sampling period</td>
<td>11 months</td>
<td>1½ months</td>
<td>20 days</td>
<td>20 days</td>
<td>8 days</td>
</tr>
<tr>
<td></td>
<td>Moisture</td>
<td>11 (85–91)</td>
<td>11 (79–92)</td>
<td>11 (78–91)</td>
<td>11 (78–91)</td>
<td>11 (78–91)</td>
</tr>
<tr>
<td></td>
<td>Crude fibre</td>
<td>11 (74–95)</td>
<td>11 (71–96)</td>
<td>11 (71–96)</td>
<td>11 (71–96)</td>
<td>11 (71–96)</td>
</tr>
<tr>
<td></td>
<td>Ash</td>
<td>11 (43–73)</td>
<td>11 (43–73)</td>
<td>11 (43–73)</td>
<td>11 (43–73)</td>
<td>11 (43–73)</td>
</tr>
<tr>
<td></td>
<td>Carbohydrates</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total soluble NSP</td>
<td>11 (74–91)</td>
<td>11 (74–91)</td>
<td>11 (74–91)</td>
<td>11 (74–91)</td>
<td>11 (74–91)</td>
</tr>
</tbody>
</table>

Notes: a–eMeans that do not share a letter are significantly different at P<0.05.

ADf, acid detergent fibre; ADF, acid detergent fibre; AH, acid hydrolysis; CV, coefficient of variation; EE, ether extract; NCP, non-cellulosic polysaccharides; aNDFom, neutral detergent fibre.
Table 4
Compositional profile of corn and corresponding DDGS (g/kg DM).

<table>
<thead>
<tr>
<th></th>
<th></th>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>139b (130–146)</td>
<td>6 (0.04)</td>
<td>80c (65–91)</td>
<td>9 (0.11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fat (EE)</td>
<td>35b (34–37)</td>
<td>1 (0.03)</td>
<td>86c (79–93)</td>
<td>5 (0.06)</td>
<td></td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>Fat (AH)</td>
<td>39b (37–41)</td>
<td>1 (0.04)</td>
<td>106c (101–115)</td>
<td>5 (0.05)</td>
<td></td>
<td></td>
<td>2.7</td>
</tr>
<tr>
<td>Crude protein</td>
<td>83b (75–91)</td>
<td>6 (0.08)</td>
<td>317c (297–337)</td>
<td>15 (0.05)</td>
<td></td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>25b (23–27)</td>
<td>1 (0.04)</td>
<td>85c (74–95)</td>
<td>7 (0.08)</td>
<td></td>
<td></td>
<td>3.4</td>
</tr>
<tr>
<td>Ash</td>
<td>10b (9–11)</td>
<td>1 (0.05)</td>
<td>68c (62–73)</td>
<td>4 (0.06)</td>
<td></td>
<td></td>
<td>6.5</td>
</tr>
<tr>
<td>Starch</td>
<td>723b (705–736)</td>
<td>11 (0.01)</td>
<td>51c (43–59)</td>
<td>5 (0.10)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total sugars</td>
<td>51b (46–59)</td>
<td>4 (0.08)</td>
<td>72c (62–87)</td>
<td>7 (0.09)</td>
<td></td>
<td></td>
<td>1.4</td>
</tr>
<tr>
<td>aNDFom</td>
<td>93b (79–104)</td>
<td>8 (0.08)</td>
<td>373c (346–397)</td>
<td>15 (0.04)</td>
<td></td>
<td></td>
<td>4.1</td>
</tr>
<tr>
<td>ADF</td>
<td>38b (33–44)</td>
<td>3 (0.09)</td>
<td>113c (108–119)</td>
<td>4 (0.03)</td>
<td></td>
<td></td>
<td>3.0</td>
</tr>
<tr>
<td>Total NSP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>79b (67–91)</td>
<td>7 (0.08)</td>
<td>325c (313–337)</td>
<td>8 (0.02)</td>
<td></td>
<td></td>
<td>4.1</td>
</tr>
<tr>
<td>Soluble</td>
<td>6b (2–10)</td>
<td>3 (0.39)</td>
<td>29c (18–37)</td>
<td>6 (0.19)</td>
<td></td>
<td></td>
<td>4.5</td>
</tr>
<tr>
<td>Cellulose</td>
<td>17b (14–20)</td>
<td>2 (0.12)</td>
<td>79b (74–91)</td>
<td>5 (0.06)</td>
<td></td>
<td></td>
<td>4.6</td>
</tr>
<tr>
<td>NCP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23b (20–27)</td>
<td>2 (0.09)</td>
<td>94c (88–100)</td>
<td>3 (0.04)</td>
<td></td>
<td></td>
<td>4.0</td>
</tr>
<tr>
<td>Soluble</td>
<td>1b (0–1)</td>
<td>1 (0.97)</td>
<td>5c (1–8)</td>
<td>2 (0.48)</td>
<td></td>
<td></td>
<td>7.6</td>
</tr>
<tr>
<td>Arabinose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>18b (15–20)</td>
<td>1 (0.07)</td>
<td>69c (65–72)</td>
<td>2 (0.04)</td>
<td></td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td>Soluble</td>
<td>1b (0–2)</td>
<td>1 (0.63)</td>
<td>7c (3–9)</td>
<td>2 (0.25)</td>
<td></td>
<td></td>
<td>5.8</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>7b (6–8)</td>
<td>1 (0.09)</td>
<td>27c (22–29)</td>
<td>2 (0.06)</td>
<td></td>
<td></td>
<td>3.7</td>
</tr>
<tr>
<td>Soluble</td>
<td>1b (0–1)</td>
<td>1 (0.97)</td>
<td>1c (0–5)</td>
<td>2 (1.35)</td>
<td></td>
<td></td>
<td>2.4</td>
</tr>
<tr>
<td>Mannose</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>2b (2–3)</td>
<td>0 (0.10)</td>
<td>17b (14–18)</td>
<td>1 (0.06)</td>
<td></td>
<td></td>
<td>7.5</td>
</tr>
<tr>
<td>Soluble</td>
<td>1b (1–1)</td>
<td>0 (0.14)</td>
<td>7c (5–8)</td>
<td>1 (0.13)</td>
<td></td>
<td></td>
<td>6.8</td>
</tr>
<tr>
<td>Galactose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6b (4–7)</td>
<td>1 (0.13)</td>
<td>20c (18–21)</td>
<td>1 (0.05)</td>
<td></td>
<td></td>
<td>3.6</td>
</tr>
<tr>
<td>Soluble</td>
<td>1b (1–2)</td>
<td>0 (0.38)</td>
<td>3c (2–4)</td>
<td>1 (0.17)</td>
<td></td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td>Uronic acids</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>5b (4–6)</td>
<td>1 (0.10)</td>
<td>19c (18–20)</td>
<td>1 (0.04)</td>
<td></td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>Soluble</td>
<td>1b (1–2)</td>
<td>0 (0.19)</td>
<td>5c (5–6)</td>
<td>0 (0.08)</td>
<td></td>
<td></td>
<td>3.5</td>
</tr>
<tr>
<td>Klason lignin</td>
<td>10b (7–15)</td>
<td>2 (0.23)</td>
<td>38c (28–47)</td>
<td>5 (0.13)</td>
<td></td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td>A/X-ratio</td>
<td>0.77b (0.74–0.79)</td>
<td>0 (0.02)</td>
<td>0.73c (0.71–0.74)</td>
<td>0 (0.01)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UA/X-ratio</td>
<td>0.23b (0.22–0.24)</td>
<td>0 (0.03)</td>
<td>0.20c (0.19–0.21)</td>
<td>0 (0.03)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cel/l–NSP ratio</td>
<td>0.21b (0.20–0.23)</td>
<td>0 (0.05)</td>
<td>0.24c (0.20–0.23)</td>
<td>0 (0.05)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ADF, acid detergent fibre; AH, acid hydrolysis; A/X ratio, arabinose-to-xylose ratio; CV, coefficient of variation; EE, ether extract; NCP, non-cellulosic polysaccharides; aNDFom, neutral detergent fibre; NSP, non-starch polysaccharides; UA/X ratio, uronic acid-to-xylose ratio.

* Average corn DDGS-to-corn ratio.

bcMeans that do not share a letter are significant different at P<0.05.

3.3. Compositional variation between corn and corresponding corn DDGS

The results of the compositional profile of corn and corresponding DDGS, sampled simultaneously from the ethanol plant over a period of 11 months, are presented in Table 4. For common constituents, DDGS had an increase in all components but starch, with average increase of 3.4-fold. The greatest increase was observed for the content of ash, aNDFom, and CP, whereas the least increase was observed for the content of total sugars, and fat, respectively. Furthermore, DDGS had greater CVs of fat and CF content compared with the parent grain, whereas corn on the other hand had greater CVs of CP, ADF, and aNDFom content, across the 11 months. Comparable CVs of ash and total sugar content was observed between corn and corresponding DDGS.

The NSP compositional profiles revealed that DDGS had a 4.1- and 4.5 times as high content of T-NSP and S-NSP as in corn, respectively. The S-Xyl and S-Ara content in DDGS increased to levels 7.6 and 5.8 times the content in corn, respectively. Furthermore, the content of total- and soluble mannose was markedly increased in DDGS compared with corn with values of 7.5- and 6.8-fold greater than in corn, respectively. The A/X- and UA/X-ratio were 0.04- (P<0.001) and 0.05 (P<0.001) units less in DDGS than in corn, respectively. On the other hand cellulose/NSP-ratio was 0.03 units greater (P<0.001) for DDGS than in corn. Corn DDGS had reduced CVs of T-NSP (0.06 units) and S-NSP (0.20 units) than the corresponding corn, and except for soluble NCP-glucose, this was the case for all constituent sugars, both total- and soluble content.

4. Discussion

In line with our expectations, the nutrient composition of DDGS varied between DDGS from both different ethanol plants and DDGS originating from different parent grains (Spyiks et al., 2002; Oulkosi and Adebiji, 2013). Furthermore, as expected,
we also observed that the nutrient composition of the DDGS in part reflected the composition in the parent grain, as only starch was removed during the fermentation process (Widyaratne and Zijlstra, 2007; Gibreel et al., 2011). The greater content of fat in corn DDGS compared with wheat DDGS, along with the greater content of protein in wheat DDGS than in corn DDGS, corresponds to previous characterizations of content in parent grains (Belitz et al., 2009). Overall, the major constituent analysis of the DDGS corresponds to previous published results (Cromwell et al., 1993; Belyea et al., 2004; Widyaratne and Zijlstra, 2007; Kim et al., 2008; Liu, 2008). The cereals used in the production of the mixed DDGS are unknown, however the major cereal fraction is presumably wheat, considering the similar composition of wheat- and mixed DDGS, based on the PCA-model and compositional analysis.

Similar to previous studies describing the NSP profiles of corn and wheat (Bach Knudsen, 1997), and corn- and wheat DDGS (Widyaratne and Zijlstra, 2007), we also observed greater content of soluble NSPs in wheat DDGS compared with corn DDGS, and a greater content of insoluble NSPs in corn DDGS compared with wheat DDGS. It can be speculated that the slightly greater NCP-glucose content of wheat DDGS compared with corn DDGS is caused by the greater content of β-glucan present in wheat compared with corn, as described in literature (Bach Knudsen, 1997). However, it has been speculated that also β-glucan and mannans from yeast are present in the DDGS (de Vries et al., 2013). Both corn- and wheat DDGS contained similar amounts of total arabinoxylan (approximately 140 g/kg DM). However, the greater content of both arabinose and uronic acids present in corn DDGS compared with wheat DDGS, yielding greater A/X- and UA/X-ratios, indicate a more complex structure of the heteroxylan in corn DDGS than in wheat DDGS. This is in line with previous studies reporting a greater branch density and complexity of corn arabinoxylan compared with that of wheat (Bedford, 1995; Saulnier et al., 1995a; Jilek and Bunzel, 2013; Yang et al., 2013).

The markedly greater content of Klasson lignin present in wheat DDGS compared with corn DDGS is also in line with previous reported results present in parent grains (Bach Knudsen, 1997; Bunzel et al., 2011). However, the Klasson lignin content might overestimate the “true” lignin (complex phenolic polymers) content of the DDGS. Klasson lignin is not a well defined chemical matter, but an empirical residue consisting of materials not solubilized by 12 mol/L sulfuric acid (Davin et al., 2008). Klasson lignin determined in corn and wheat insoluble fibre have been shown to include, besides true lignin; N (proteins), residual fat and waxes, as well as cutin, with true lignin content equal to approximately 1/3 and 1/4 of the measured Klasson lignin content for wheat and corn, respectively (Bunzel et al., 2011). However, other nitrogen sources than proteins, such as Maillard products, can potentially contribute to nitrogen content of the Klasson lignin fraction. The Maillard reaction can occur between reducing sugars and lysine residues during the processing of DDGS, as a result of the addition of condensed solubles to the wet distillers cake during drying, consequently damaging the protein fraction (Fastinger and Mahan, 2006; Pfahl et al., 2009; Kim et al., 2012). Furthermore, changes in the corn protein structure during the ethanol production have been described to induce a greater fraction of DDGS protein associated with the cell wall material, compared with that of corn (Yu and Nuez-Oritz, 2010). The herein presented high CV values of Klasson lignin content in both corn-(0.26) and wheat DDGS (0.32) from the different ethanol plants along with their corresponding different processing technologies, further indicates the presence of non-lignin sources, such as Maillard products, may be present in the Klasson lignin fraction of DDGS. Regardless of DDGS origin, the greatest CV values were observed for the S-NSP constituent sugar monomers. It can be speculated that this observation is related to the amount of condensed solubles added to the wet distillers cake during drying. However, it should also be noted that the relative small S-NSP content present could directly give rise to greater CVs. Furthermore, degradation of insoluble NSP occurring during the fermentation process could increase the content of soluble NSP, due to the presence of endogenous fibre degrading enzymes from yeast, exogenous fibre degrading enzymes added to increase ethanol yield, and mechanical- and heat (pre)treatment.

When sampled over a period of 8 days to 11 months the five ethanol plants showed, in spite of some variation, capability of producing DDGS with conserved compositional profiles, indicated by PCA models. The variation in starch content, especially observed for three of the five plants, is directly related to the effectiveness of the fermentation process at each individual ethanol plant, which varies according to process technology and fermentation time, yeast, and enzymes used in the production. Furthermore, the relatively high CV in moisture content is related to different intensities of the DDGS drying process, which is a crucial step in the DDGS production, due to the negative effects on nutritional value associated with excessive heat damage of especially lysine, and the high energy expenses associated with drying. Similarly large variation was observed for the content of Klasson lignin, as discussed above. Despite the consistent content of total NSP and total constituent sugars from each individual ethanol plant, large variation was observed for the S-NSPs fractions, as discussed above. P1 showed greater overall variation in compositional profile, which could be explained by P1 having the far greatest sampling period of 11 months, hence probably encountering greater variation in raw material. Despite the narrowest sampling period of 8 days, P5 on the other hand also showed relatively large overall variation.

The simultaneous sampling of both corn and corresponding DDGS over 11 months allowed for investigating the changes in compositional profile after the fermentation and drying process. Although the corn and the DDGS were sampled simultaneously, it should be noted that the corn and DDGS did not originate from the same fermentation batch, which would be practically impossible to obtain. The observed changes between corn and corresponding DDGS may not be considered common for all ethanol plants, underlined by the presented compositional variations between the 24 corn DDGS plants. As expected, the DDGS reflected the nutrient content of the parent grain (Spiels et al., 2002). All other constituents than starch were concentrated in the DDGS compared with corn, with an average 3.4-fold increase for the common constituents, similar to previous studies (Urriola et al., 2010; de Vries et al., 2013). The relatively small increase in fat content of 2.4–2.7 fold may indicate that the ethanol plant have extracted some of the oil from the thin stillage before condensing and blending with
the grain fraction and drying to make DDGS. The marked increase in mannose content in DDGS compared with corn, most likely originate from yeast cell wall mannans, as discussed previously (de Vries et al., 2013). Furthermore, a marked shift in solubility of xylose and arabinose was observed for DDGS, indicating a modification of the arabinoxylan fraction during processing. Corn showed a consistently greater content of substituted xylan with both greater A/X- and UA/X-ratios, compared with corresponding DDGS. Unpublished results from our research group indicate the A/X-ratio in corn differs among the different botanical grain fractions, with endosperm having a markedly greater A/X-ratio compared with that of corn bran, which is in line with previous published results in corn grain, flour and bran (Bach Knudsen, 1997; Rose et al., 2009). Similarly, UA/X-ratio has been reported greater in corn flour, compared with corn bran (Bach Knudsen, 1997). The shift in A/X- and UA/X ratio between corn and DDGS indicates modification of the greater substituted xylan (e.g. in endosperm) during the fermentation process. It must be noted, however, that the total content of arabinoxylan and uronic acids present in corn endosperm is very low compared with corn bran.

Though highly substituted, corn endosperm arabinoxylan is not cross-linked to the same degree as corn bran heteroxylans (Saulnier and Thibault, 1999; Bunzel et al., 2006). Hence, corn endosperm arabinoxylan is potentially more susceptible to environmental changes, such as heat processing and presence of fibre degrading enzymes and other accessory enzymes, which might lead to an increased degradation and solubilization of arabinoxylan. It can be speculated that the solubilized arabinoxylan is more susceptible to participate in Maillard reactions, by having a potentially larger fraction of reducing ends due to hydrolysis. The arabinoxylan parting in Maillard reactions would be unable to determine as part of the constituent sugars in the NSP-procedure used in this study, since they would most likely end up in the Klassen lignin fraction. On the other hand, cellulose appear to remain unmodified during processing, due to its strong rigid structure and anchorage in the cell wall matrix (Van Eysen et al., 2011), which consequently leads to the greater cellulose/NSP-ratio for DDGS compared with corn in the current study. Endosperm arabinoxylan comprise approximately 20% of the total content of arabinoxylan in corn, calculated with data from Bach Knudsen (1997) and Watson (1987). Hence, considering that the endosperm arabinoxylan only comprise a minor fraction of the total arabinoxylan content of the grain, and that the cellulose fraction is concentrated in DDGS, the outcome will be that the majority of NSP remain unmodified or potentially more complex in DDGS than in the parent grain.

The use of multivariate data analysis, PCA, on compositional data of DDGS proved useful to visually distinguish between DDGS covering three different feedstock origins; corn, wheat, and mixed cereals. Furthermore, PCA was able to provide information concerning the most conserved components of each DDGS origin, hence the components most responsible for the individual clusters and groupings of DDGS. By combining both compositional data of the common constituents and NSP profiles of the DDGS, a more clear separation between the groups was observed.

DDGS has a reputation of having variable nutrient composition and protein quality, and a high content of mycotoxins, which has limited its use in swine feed (Stein et al., 2006; Pedersen et al., 2007; Anderson et al., 2012). High quantities of DDGS in feed increase the content of dietary fibre, associated with negative effects on nutrient digestibility. To increase nutrient digestibility of feed formulations containing DDGS, studies have investigated the effects of adding fibre degrading enzymes to animal diets containing DDGS (Jones et al., 2010; Yoon et al., 2010; Yañez et al., 2011). However, the results are inconclusive. This may relate to the complexity of the cell wall matrix in corn DDGS, and that the most readily degradable arabinoxylan for the fibre degrading enzymes already have been modified during DDGS production, as discussed above. These observations indicate that the fibre degrading enzymes applied for degradation of corn DDGS need to be targeted towards highly complex substrates. With respect to enzymatic degradation, corn bran has been acknowledged as a recalcitrant substrate, as a consequence of the highly branched structure of the arabinoxylan (Saulnier et al., 2001; Agger et al., 2010). The cross-linking and complexity of the NSP matrix is influenced by many factors including; content of substituted xylan, ferulic acid cross-linking, and lignin and structural proteins interacting with arabinoxylan (Saulnier et al., 1995b). Despite the ethanol plants’ capability of individually producing DDGS with a conserved NSP profile, variation in the degree of substituted xylan from plant to plant exists. For corn DDGS, the differences in A/X- (0.71–0.85) and UA/X-ratio (0.16–0.23) underline the heterogeneous structure of DDGS NSPs. It can be speculated that the enzymatic degradation potential of the DDGS NSPs is sterically hindered by a greater proportion of substituted xylan (Rose et al., 2009). Hence, the DDGS samples from 24 different ethanol plants analyzed in this study might yield different results in relation to digestibility and enzymatic degradation potential of the NSPs. Difference in animal performance may be observed when feeding either corn- or wheat DDGS, due to the observed large variation in NSP cell wall complexity between the two DDGS sources likely affecting digestibility. However, it should be noted that the overall degradation potential of the DDGS NSPs, is affected by a combination of several factors (as described above), a number of which not determined in this study.

5. Conclusion

The current study showed a large variability in the chemical composition of common constituents, and in non-starch polysaccharide profiles of corn-, wheat, and mixed DDGS. Despite variations, the major fractions across all origins were; CP and aNDFom with a large degree of the aNDFom fraction being ADF. All samples also had a large content of sugars, while corn DDGS contained more fat than the other two. Wheat- and mixed DDGS showed a markedly greater content of S-NSP than in corn, which on the other hand had greater contents of total- and insoluble NSPs. Of the NSP fraction, all samples had the greatest content of arabinoxylan. However, the A/X- and UA/X-ratio differed with greater ratios for corn DDGS than in the other two. DDGS samples from five different ethanol plants showed that each plant were capable, despite variations,
of producing DDGS with an individual conserved compositional profile. Finally, analysis of corn and corresponding DDGS samples showed that the content of substituted xylan and S-NSP is modified during the production of DDGS.

Conflict of interest

The authors declare that there are no conflicts of interest.

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References


PAPER II

Ferulic Acid Dehydrodimer and –
Dehydrotrimer Profiles of Distillers Dried
Grains with Solubles from Different Cereal
Species

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Ferulic Acid Dehydrodimer and Dehydrotramer Profiles of Distiller’s Dried Grains with Solubles from Different Cereal Species

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ABSTRACT: Ferulic acid dehydrodimers (DFA) and dehydrotamers (TriFA) ester-linked to plant cell wall polymers may cross-link not only cell wall polysaccharides but also other cell wall components including proteins and lignin, thus enhancing the rigidity and potentially affecting the enzymatic degradation of the plant cell wall. Corn, wheat, and mixed-cereal distiller’s dried grains with solubles (DDGS) were investigated for composition of DFAs and TriFAs by reversed phase high-performance liquid chromatography with ultraviolet detection. Corn DDGS contained 5.3 and 5.9 times higher contents of total DFAs than wheat and mixed-cereal DDGS, respectively. Furthermore, the contents of total TriFAs were 5.7 and 6.3 times higher in corn DDGS than in wheat and mixed-cereal DDGS, respectively. In addition, both corn grains and corresponding DDGS had similar profiles of individual DFAs and TriFAs, indicating that ferulic acid cross-links in the corn cell wall are presumably not modified during fermentation and DDGS processing.

KEYWORDS: corn, dehydrodimers, dehydrotamers, distiller’s dried grains with solubles (DDGS), ferulic acid, wheat

INTRODUCTION

Distiller’s dried grains with solubles (DDGS) is a coproduct from the grain-based ethanol industry mainly used as animal feed.1 The production of DDGS includes several major steps, including grinding, liquefaction, saccharification, fermentation, distillation, and recovery of coproducts. Due to the removal of most of the starch fraction during the dry-grind ethanol fermentation process, DDGS has 3−3.5 times higher nutrient (e.g., protein, minerals, and vitamins) and nonstarch polysaccharides (NSP) contents than the parent grains such as corn and wheat.1,2 The NSP content in DDGS makes up 250–300 g/kg of dry matter (DM) with arabinoxylan and cellulose as the two major NSP fractions.2 When fed in high level to nonruminants, the high content of NSP in DDGS may cause nutritional challenges as the nonruminant digestive system is incapable of digesting NSP.

In cereals, esterified hydroxycinnamic acids such as ferulic acid, p-coumaric acid, and sinapic acid can participate in photochemically or oxidatively induced coupling of arabinoxylans along with cell wall trapped proteins and lignin-like polymers.3,5 Whereas p-coumaric acid is mainly bound to lignin, ferulic acid and its derivatives, which are the quantitatively most important cross-links in the cereal cell wall, are primarily bound to arabinoxylans and pectins.6 Ferulate in particular has been found to form dimers (DFA), trimers (TriFA), and higher ferulate oligomers potentially cross-linking two or more polysaccharide chains, thus strengthening the cell wall with vital implications for plant physiology, food science, nutrition, and the plant’s defense mechanisms against pathogens.6–8 DFA cross-links are described as an early barrier against the fungus Fusarium graminearum and the resistance of maize to Gibberella ear rot9 and to Gibberella stalk rot.10 Ferulate-based cross-links impair the enzymatic degradation of plant cell walls11,12 and, thus, affect DDGS digestibility in relation to animal nutrition.

To our knowledge there are no previous papers describing the DFA and TriFA profiles of DDGS or the possible implications of the fermentation process on the DFA and TriFA profiles, despite their potentially large implications for animal nutrition. In addition, because compositional variation among DDGS samples may induce challenges when applied in animal feed, the broad extent of DDGS samples analyzed in this study will elucidate these potential implications. Characterization of the DFA and TriFA composition in DDGS of different cereal species may contribute to the overall understanding and interpretation of opportunities and limitations in DDGS degradation and digestibility, which is particularly useful for feed manufacturers and enzyme producers targeting DDGS degradation.

The current study describes the compositional profile and extent of variation of DFA and TriFA contents in a wide variety of DDGS samples from three different cereal origins: corn (Zea mays), wheat (Triticum aestivum), and a mixture of different cereal species, including wheat (T. aestivum), barley (Hordeum vulgare L.), rye (Secale cereale), and triticale (a hybrid of wheat and rye). A total of 63 DDGS samples covering 24 different ethanol plants from North America and Europe along with 11
unprocessed corn grain samples were analyzed by reversed phase high-performance chromatography (RP-HPLC) with ultraviolet (UV) detection for the identification of 12 different cell wall-bound DFAs and TriFAs.

### MATERIALS AND METHODS

#### Materials and Instrumentation.

Standard compounds of 8-8-(aryltetralin)-DFA, 8-8-DFA, 8-8-DFA, 8-8-DFA, 8-8-(benzofuran)-DFA, 8-8-(decarboxylated)-DFA, 8-8-8-8(octadecafluorooctyl)-DFA, 8-8-(aryltetralin), 8-8-8-8((tetrahydrofuran)-DFA were isolated as described by Jilek and Bunzel and Bunzel et al. The 5-(methylated)-DFA (monomethylated 5-5-dehydrodiferulic acid) as internal standard was kindly provided by Prof. Dr. John Ralph, University of Wisconsin, Madison, WI, USA.

An analytical phenyl-hexyl HPLC column (250 × 4.6 mm, 5 μm particle size) was purchased from Phenomenex (Germany). RP-HPLC was carried out with the following instrumentation for DFA and TriFA analysis: LC-20AT pumps, CTO-20AC column oven, SPD-M20A photodiode array detector (PDA), SIL-20AC autosampler, DGU-20A degasser, and CBM-20A communication bus module from Shimadzu, Japan. All solvents used were of HPLC grade, and water was distilled and deionized.

#### Sample Materials.

A total of 63 DDGS samples were collected from 24 ethanol plants in the United States and European Union (EU), covering the following three parent grain origins: corn, wheat, and rye. All samples were washed twice with 10 mL of 80% (v/v) ethanol and twice with 10 mL of acetone followed by centrifugation and removal of supernatant between and after extractions. The residues were dried under a fume hood. Saponification (5 mL of 2 mol/L NaOH) of the residues was carried out under nitrogen, protected from light, and under continuous stirring for 18 h, following a procedure described by Dobberstein and Bunzel. Following acidification with 0.95 mL of concentrated HCl, 25 μg of 5-(methylated)-DFA was added as internal standard for the quantification of DFAs and TriFAs. Extraction was carried out three times with 4 mL of diethyl ether. The diethyl ether extracts were dried under nitrogen and redissolved in 500 μL of THF/water (50:50 (v/v)) using ultra sonication and used directly for the determination of DFAs and TriFAs.

#### Phenyl-Hexyl RP-HPLC-PDA Analysis of DFAs and TriFAs.

The procedure basically followed a protocol described by Dobberstein and Bunzel. DFAs and TriFAs were analyzed using a Luna phenyl-hexyl column (250 mm × 4.6 mm, 5 μm particle size, plus 3 mm × 4.6 mm i.d. guard column) and a ternary gradient made up of 1 mM aqueous trifluoroacetic acid (TFA), acetonitrile (ACN), and methanol (MeOH) at the flow rate of 1.0 mL/min. The sample (20 μL) was injected, and the separation was performed at 45 °C, with chromatograms monitored at 280 nm. The following gradients were used: eluent A, 1 mM aqueous TFA; eluent B, ACN/1 mM aqueous TFA [90:10 (v/v)]; eluent C, MeOH/1 mM aqueous TFA [90:10 (v/v)]. Separation of DFAs and TriFAs was carried out with initially 85% A, 15% B, and 0% C; linear over 15 min to 82% A, 18% B, and 0% C; linear over 5 min to 72% A, 25% B, and 3% C; linear over 5 min to 70% A, 25% B, and 5% C; linear over 10 min to 65% A, 30% B, and 5% C; held for 5 min; and linear over 10 min to 55% A, 45% B, and 5% C, following a rinsing and an equilibration step.

#### Identification of 8-8(Tetrahydrofuran)-DFA in DDGS Samples.

Because no purified standard was available for the 8-8-(tetrahydrofuran)-DFA, the presence of this particular dimer was validated by its relative retention time and UV spectrum characteristics, as described by Dobberstein and Bunzel. Furthermore, the elution order for the 8-8(tetrahydrofuran)-DFA was validated by screening for its mass (m/z 403 [M+]) using liquid chromatography–mass spectrometry, basically as described by Jilek and Bunzel. Finally, gas chromatography–mass spectrometry of a silylated extract was exemplarily applied to validate the presence of the 8-8(tetrahydrofuran-
Calculations and Statistical Analysis. The contents of DFAs and TriFAs were calculated relative to the area of the internal standard by using the correction factors reported by Dobberstein and Bunzel\(^7\) (Table 1).

A one-way ANOVA was applied for comparison among means followed by Tukey’s pairwise comparison with overall significance level at \(P = 0.05\), using Minitab 16 (Minitab Inc.). The compositional data were analyzed by principal component analysis (PCA) with evenly spread seven-fold partial cross-validation after mean centering, and unit variance scaling, using Evince 2.5.5 software (UmBio AB), was performed as previously discussed.\(^{15}\) PCA was used to detect correlations of DFAs and TriFAs with previously reported data on composition of common nutrients and NSPs from Pedersen et al.\(^2\)

### RESULTS

**Detection of Ferulic Acid Dehydrodimers and Dehydrotrimers and Validation of Correction Factors.**

A chromatogram of the standard compounds of the DFAs and TriFAs monitored at 280 nm is shown in Figure 1, indicating an acceptable separation of eight dimers and four trimers. The 8-8(Tetrahydrofuran)-DFA was not incorporated into the standard mix (see also Identification of 8-8(Tetrahydrofuran)-DFA in DDGS Samples) because a purified standard compound was not available. Because the concentration range of DFAs and TriFAs in the samples exceeded the ranges validated by Dobberstein and Bunzel,\(^7\) increased concentration ranges of DFA and TriFA standards were analyzed for linear responses and are presented in Table 1. The linearity was validated by inspecting plots of the area on the \(y\)-axis versus analyte concentration on the \(x\)-axis, with observed correlation factors

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Table 2. Composition of Ferulic Acid Dehydrodimers and Dehydrotrimers in DDGS of Various Origins

<table>
<thead>
<tr>
<th></th>
<th>corn DDGS</th>
<th>wheat DDGS</th>
<th>mixed DDGS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean (g/g DM)</td>
<td>range (μg/g DM)</td>
<td>SD</td>
</tr>
<tr>
<td>(\sum)DFA</td>
<td>3310g (2811–4825)</td>
<td>308</td>
<td>621h (543–698)</td>
</tr>
<tr>
<td>(8)-8-DFA (%)</td>
<td>20g (16–23)</td>
<td>1</td>
<td>30h (26–34)</td>
</tr>
<tr>
<td>(8)-5-DFA (%)</td>
<td>35g (32–40)</td>
<td>2</td>
<td>31h (25–36)</td>
</tr>
<tr>
<td>5-5 (%) DFA</td>
<td>27g (25–28)</td>
<td>1</td>
<td>21h (21–22)</td>
</tr>
<tr>
<td>8-O-4 (%) DFA</td>
<td>18g (17–20)</td>
<td>1</td>
<td>18g (16–20)</td>
</tr>
<tr>
<td>(\sum)TriFA</td>
<td>440g (332–651)</td>
<td>52</td>
<td>77h (65–97)</td>
</tr>
<tr>
<td>5-8-O-4 (%) TriFA</td>
<td>69g (56–80)</td>
<td>5</td>
<td>78g (70–75)</td>
</tr>
<tr>
<td>8-8-O-4 (%) TriFA</td>
<td>31g (20–44)</td>
<td>5</td>
<td>22g (18–25)</td>
</tr>
</tbody>
</table>

\(a\) Average of means from 21 plants. Means in the same row that do not share a letter (g, h, i) are significantly different at \(P < 0.05\). \(b\) \(N = 47\). \(c\) Average of means from 2 plants. \(d\) \(N = 11\). \(e\) Mean from 1 plant. \(f\) \(N = 5\).
ranging from 0.9839 to 0.9974 for the eight DFAs and TriFAs tested. Visual inspection of the graphs indicated a minor tendency toward curve flattening for the highest concentrations of the 8-5-decarboxylated)-DFA, which may lead to a slight underestimation of this particular dimer. Inspection of the graphs for the remaining DFAs and TriFAs indicated a consistent linear response between area and concentration of analyte measured in the samples.

Variation in Ferulic Acid Dehydrodimer and Dehydrotrimer Profiles in DDGS of Various Origins. A typical chromatogram of the analysis of alkaline extractable DFAs and TriFAs in DDGS is presented in Figure 2, demonstrating the suitability of this chromatographic method for DDGS samples. The results of the DFA and TriFA compositional analysis of the DDGS are listed in Table 2. The eight DFAs and the two TriFAs used to quantify the total content of DFA and TriFA were identified in all three DDGS origins. The dentity of these dimers and trimers was verified by comparison of relative retention times and comparison of the UV spectra with those of the isolated standard compounds. However, because the amounts of these trimers were not sufficient for an appropriate validation, these TriFAs were not quantitated. Corn DDGS contained 5.3 and 5.9 times the content of total alkaline extractable DFAs than in wheat DDGS (P < 0.001) and mixed-cereal DDGS (P < 0.001). Furthermore, corn DDGS contained a greater proportion for the ∑8-5-DFA coupled dimers than in wheat DDGS (P = 0.024). On the other hand, wheat and mixed-cereal DDGS contained a greater proportion of total DFA for the ∑8-8-DFA coupled dimers compared with corn DDGS (P < 0.001). No difference between the different DDGS origins was observed in contribution of the 8-O-4-DFA (P = 0.481) to the total DFA contents.

Independent of DDGS origin, higher amounts of 5-5,8-O-4-TriFA than 8-8,8-O-4-TriFA were observed. Both the 5-5,8-O-4-TriFA and 8-8,8-O-4-TriFA proportions were overall significantly different among the three DDGS origins (P = 0.029); however, the difference was not strong enough to be significant for pairwise comparisons among origins (P ≥ 0.09).

The PCA loading plot illustrated with which compositional fractions of the DFAs (for DDGS composition see also Pedersen et al.) the DFA and TriFA shared the highest correlation, presented for corn DDGS (Figure 3A) and wheat and mixed-cereal DDGS (Figure 3B). Both corn DDGS and wheat and mixed-cereal DDGS showed correlation of the total DFAs to the contents of insoluble xylose, insoluble uronic acids, and insoluble galactose. In addition, corn DDGS DFAs correlated to insoluble arabinose, total and insoluble nondigestible carbohydrates; NDF, neutral detergent fiber; S, soluble; T, total; TriFA, ferulic acid dehydrotrimer; Xyl, xylose.

Figure 3. Loading plot of principal component analysis of corn DDGS (A) and wheat and mixed-cereal DDGS (B) constructed by combining DFA and TriFA profiles with additional compositional data from Pedersen et al. as model variables. ADF, acid detergent fiber; AH, acid hydrolysis; Ara, arabinose; Cel, cellulose; DF, dietary fiber; DFA, ferulic acid dehydrodimer; EE, ether extract; Gal, galactose; Glu, NCP-glucose; I, insoluble; Lig, Klason lignin; Man, mannose; NDC, nondigestible carbohydrates; NDF, neutral detergent fiber; S, soluble; T, total; TriFA, ferulic acid dehydrotrimer; Xyl, xylose.
Article

42% in corn, just as the proportion of the 8-8-coupled dimers according to Dobberstein and Bunzel, the contents of the 8-8-DFAs were reported as the sum of the insoluble reports of the DFA and TriFA contents in corn and wheat DDGS compared to wheat DDGS is in line with previous reports of the proportion of 5-5,8-DFAs (35% of total DFA) in corn DDGS is well in line with previous reports of 39% and 17% in corn. For corn DDGS the proportions of 5-5-DFA (27% of total) and the 8-8-DFAs (20% of total) were slightly different from previous reports on corn ranging from 18 to 19% and from 21 to 26% of total DFAs, respectively. For wheat DDGS the observed proportion of the 5-5,8-DFAs (31% of total) was lower than the previous reports of 49% and 44%. On the other hand, the observed proportions of the 5-5,8-DFAs (30% of total) and the 8-0-4-TriFA (18% of total) in wheat DDGS were roughly comparable to previous reports ranging between 24 and 25% and between 15 and 16%, respectively,7,8

The 5-5,8-O-4-TriFA was the dominating TriFA in corn DDGS, just as reported for the parent grain.7,8 Despite being dominant, the proportion of the 5-5,8-O-4-TriFA reported here is overestimated because a recent study quantitated five TriFAs in corn, whereas only two were used for quantification in this study. As shown in Figure 2, at least two other trimers were identified in corn DDGS. The same consideration needs to be done for wheat DDGS, as the same study of Jilek and Bunzel8 indicates an equal proportions of both the 5-5,8-O-4-TriFA and the 8-0,4-8,4-O-TriFA in wheat.

Arabinoxylan consists of a β-D-xylan backbone joined by β-1,4-links substituted with L-arabinose residues along the xylene chain. The arabinose substitution occurs randomly, allowing other substitutions such as α-glucuronic acid, acetyl groups, galactose, and feruloylated arabinose residues to be attached to the xylene backbone.9 Evidence that DFAs are indeed attached to arabinoxylans has been reported for the 5-5-DFA by use of carbohydrates and mild acidic hydrolysis followed by structural characterization in bamboo and corn bran10,21 and for the 8-0-4-DFA and 8-8-DFA from corn bran insoluble fiber.22,23 The use of PCA multivariate data analysis based on the combination of DFA and TriFA profiles with compositional data previously reported2 indicated a clear correlation between DFAs and TriFAs in the DDGS and the content of insoluble arabinoxylan (sum of anhydrous arabinose and xylose), uronic acids, insoluble galactose, and cellulose. In addition to pointing out that DFAs are attached to arabinoxylan as mentioned above, these results logically indicate a similar association for the TriFAs. The observed correlation of DFAs to insoluble galactose is in line with previous studies describing highly complex feruloylated heteroxylan side chains containing up to two galactose residues from corn bran.24,25 The observed greater content of DFAs in DDGS from P1 is likely related to the higher content of insoluble arabinoxylan in DDGS from P1 than from P2–P5, as reported by Pedersen et al.2

The simultaneous sampling of both corn grains and corresponding DDGS over 11 months allowed for investigating

| Table 3. Composition of Ferulic Acid Dehydrodimers (DFA) and Dehydrotrimers (TriFA) in Corn and Corresponding DDGS |
|-------------------------------------------------|-------------|-------------|-------------|-------------|-------------|
|                                                   | mean^a     | range       | SD          | mean         | range       | SD          | ratio^b   |
|                                                   | corn (N = 11) | DDGS (N = 11) |
| ΣDFA (µg/g DM)                                   | 1135b (976–1419) | 140          | 4030c (3594–4828) | 366          | 3.6         |
| Σ8-8-DFA (% DFA)                                 | 18b (17–19) | 1           | 18b (17–18) | 1           |             |
| Σ8-5-DFA (% DFA)                                 | 37b (36–38) | 1           | 38b (36–40) | 1           |             |
| 5-5 (% DFA)                                      | 27b (26–28) | 1           | 26c (25–27) | 0           |             |
| 8-0-4 (% DFA)                                    | 18b (17–18) | 0           | 18c (18–19) | 0           |             |
| ΣTriFA (µg/g DM)                                 | 137b (107–158) | 17          | 504c (388–651) | 83          | 3.7         |
| 5,5,8-O-4 (%TriFA)                               | 70b (63–76) | 4           | 71b (62–74) | 4           |             |
| 8,8,8-O-4 (%TriFA)                               | 30b (24–37) | 4           | 29b (24–38) | 4           |             |

^aMeans in the same row that do not share a letter (b, c) are significantly different at P < 0.05. ^bAverage corn-to-corn DDGS ratio.

μg/g DM, P4 (3292 µg/g DM), and P5 (3509 µg/g DM). However, the contents of total TriFAs were not different among the ethanol plants with an overall range of 426–504 µg/g DM (P = 0.12). The coefficient of variation (CV) ranged from 0.04 to 0.09 among the plants for the content of total DFAs with highest CVs for P1, whereas CVs of 0.01–0.02 was observed for the total TriFA contents among the ethanol plants.

**Validation of the extended analyte concentration ranges (if possible) along with the identification of two additional TriFAs.**

**DISCUSSION**

Saponification with 2 mol/L NaOH at room temperature cleaves ester linkages. Thus, DFAs and TriFAs ester-linked to polymeric cell wall components were analyzed in this study. The applied chromatographic methodology allowed for separation and quantification of eight DFAs and two TriFAs, along with the identification of two additional TriFAs. Validation of the extended analyte concentration ranges (if compared to Dobberstein and Bunzel7) indicated that the 8-5-(decarboxylated)-DFA might be slightly underestimated compared to the other DFAs. Because the 8-5-DFA, the 8-5-(benzofuran)-DFA, and the 8-5-(decarboxylated)-DFA presumably have the same precursor in vivo, the results of the 8-5-DFAs were reported as the sum of 8-5-coupled dimers.6,8 Also, the contents of the 8-8-DFAs were reported as the sum of the 8-8-coupled dimers according to Dobberstein and Bunzel, although it is likely that these 8-8-DFAs occur as individual DFAs in vivo.6

Because only starch is removed during the fermentation process, the nutrient composition of DDGS is expected to partially reflect the composition of the parent grain.2,21 The higher content of total DFAs and TriFAs observed in corn DDGS compared to wheat DDGS is in line with previous reports of the DFA and TriFA contents in corn and wheat insoluble fiber,7,17,18 and whole grain flours.8 Also, the observed dominating proportion of the Σ8-5-DFAs (35% of total DFA) in corn DDGS is well in line with previous reports of 39% and 42% in corn, just as the proportion of the 8-0-4-DFA (18% of total) in corn DDGS is consistent with previous reports of 18% and 17% in corn. For corn DDGS the proportions of 5-5-DFA (27% of total) and the Σ8-8-DFAs (20% of total) were slightly different from previous reports on corn ranging from 18 to 19% and from 21 to 26% of total DFAs, respectively. For wheat DDGS the observed proportion of the Σ5-8-DFAs (31% of total) was lower than the previous reports of 49% and 44%. On the other hand, the observed proportions of the Σ8-8-DFAs (30% of total) and the 8-0-4-TriFA (18% of total) in wheat DDGS were roughly comparable to previous reports ranging between 24 and 25% and between 15 and 16%, respectively.7,8

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The simultaneous sampling of both corn grains and corresponding DDGS over 11 months allowed for investigating...
the potential changes in DFA and TriFA profiles after the fermentation and drying process. Although corn grains and DDGS were sampled simultaneously, it should be noted that due to practical challenges the samples did not originate from the same fermentation batch. Furthermore, it should be noted that the results may not be the same for all ethanol plants, emphasized by the variation in DFAs and TriFAs content from the 21 corn DDGS ethanol plants. The 3.6- and 3.7-fold increases in DFA and TriFA contents, respectively, in DDGS are in accordance with the 3.4-fold average increase in nutrients from the same set of samples reported by Pedersen et al. 2 and with previous studies reporting a similar nutrient increase in DDGS compared with the parent grain.26,27

Overall, the analysis of corn and DDGS indicated that the individual composition of DFAs and TriFAs is conserved, indicating that DFAs and TriFAs are basically not modified during ethanol fermentation and subsequent drying of DDGS. Previous characterization of NSP profiles of the samples led to the hypothesis that only arabinoxylan from corn endosperm, which comprises only a minor fraction of the total arabinoxylan, was modified during DDGS production.2 Considering the latter and the fact that corn endosperm arabinoxylan contains markedly less feruloylated cross-linkages compared to corn bran heteroxylan,28−30 the observed conservation of the DFA and TriFA patterns in corn DDGS is well in line. Because the major fraction of feruloylated arabinoxylan remains unmodified, the remaining arabinoxylan in DDGS is probably more complex than in the parent grain because the readily degradable arabinoxylan was modified during processing.

Large differences in enzymatic degradability were reported for corn- and wheat-based substrates,31 with a markedly greater degradation potential for wheat than for corn. Furthermore, with regard to enzymatic degradation, corn DDGS26 and especially corn bran have been described as recalcitrant substrates due to the highly complex structure of the arabinoxylan.32−34 Especially the DFA and TriFA cross-links are suspected to play a major role in hindering the enzymatic degradation of cereal cell walls.9−12 Thus, the results from this study on DFA and TriFA contents may indicate a generally higher potential for enzymatic degradation of wheat DDGS compared to corn DDGS. As a consequence of the difference in cell wall complexity, differences in digestibility, microbial fermentation, and resistance to fiber degrading enzymes may be observed when feeding either wheat DDGS or corn DDGS.

The use of DDGS in swine feed has been limited due to a reputation of having variable nutrient composition and protein quality, potentially high content of mycotoxins, and high fiber content. The high fiber content in DDGS is associated with nutritional challenges and negative effect on nutrient digestibility when fed in high quantities. To overcome the negative effects associated with high fiber content, studies have been performed to investigate the effects of adding fiber degrading enzymes to animal diets containing DDGS.35−37 However, the results are inconclusive.

The results presented in this study may indicate that fiber-degrading enzymes applied for the degradation of DDGS need to be targeted toward highly complex substrates, especially when applied to diets high in corn DDGS.

In conclusion, the current study demonstrated a large variation in the content of DFAs and TriFAs between DDGS of three different origins. Corn DDGS contained 5.3 and 5.7 times greater content of total DFAs and TriFAs, respectively, compared with wheat DDGS. The contents of total DFA and TriFA correlated to the content of insoluble arabinoxylan, uronic acids, insoluble galactose, and cellulose in DDGS. A similar proportional profile of the individual DFAs and TriFAs was observed in both corn and corresponding DDGS, indicating that ferulic acid cross-links in the corn cell wall are presumably not modified during fermentation and DDGS processing. The results presented herein may add to the overall understanding and implication of compositional differences between DDGS sources in relation to animal nutrition and enzyme digestibility.


PAPER III

Xylanase and Protease Increase Solubilization of Non-Starch Polysaccharides and Nutrient Release of Corn- and Wheat Distillers Dried Grains with Solubles

Mads B. Pedersen, Søren Dalsgaard, Susan Arent, Rikke Lorentzen, Knud Erik B. Knudsen, Shukun Yu, and Helle N. Lærke

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Xylanase and protease increase solubilization of non-starch polysaccharides and nutrient release of corn- and wheat distillers dried grains with solubles

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A B S T R A C T

The use of distiller dried grains with solubles (DDGS) as alternative to conventional animal feed for non-ruminants is challenged by the high content of non-starch polysaccharides and varying protein quality. In this study the enzymatic degradation of corn- and wheat DDGS was evaluated, in vitro, by use of four xylanases from two different glycoside hydrolase families, GH10 and GH11, along with protease and phytase. Wheat DDGS showed the highest degree of enzymatic degradation due to a lower degree of cell wall complexity compared with that of corn DDGS. For corn DDGs, the combination of xylanase and protease yielded the highest degree of enzymatic degradation, indicating close association of arabinoxylan and protein within the cell wall matrix. Collectively, the GH10 xylanase degraded DDGS more efficiently than the GH11 xylanases, due to the complexity of the DDGS substrate and the substrate affinity of the GH10 xylanase. The current in vitro results indicate a high potential of xylanase in combination with protease to efficiently degrade DDGS and promote nutrient release in diets for non-ruminant animals.

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1. Introduction

During the past decade attention on implementing alternatives to conventional feed stuffs (e.g., corn, wheat, and soybean meal) in animal production has become more and more prevalent. The main reason is the increased demand for and consequently increased costs of conventional raw materials together with an increased availability of new low-cost raw materials, many of these being co-products from the grain processing industries. One of these co-products is distillers dried grains with solubles (DDGS), a dried co-product from the production of fuel ethanol [1,2].

As a feed ingredient, DDGS has high potential with a digestible- and metabolizable energy content similar to that of corn, along with a high content of digestible phosphorous [3,4]. In general, DDGS has greater concentrations of protein, fat, vitamins and minerals compared to the parent grain [5,6]. However, disadvantages of using DDGS in the feeding of non-ruminant animals are the 3–3.5 fold higher content of non-starch polysaccharides (NSP), compared to the parent grain [3,7]. Since non-ruminant animals lack the capacity to utilize NSP, high inclusion levels will inevitably limit feed utilization [8,9,10]. Varying protein quality and the risk of mycotoxin contamination further challenge a high inclusion rate of DDGS in animal diets.

The NSP in DDGS originate from cell walls in the botanical grain fractions; aleurone layer, pericarp, endosperm, germ and tip cap (corn), with arabinoxylan and cellulose as the major components of the NSP fraction [7]. Arabinoxylan consists of β-xylene units joined by β-linkages and substituted with arabinose residues along the chain [11,12], including other substituents like O-glucuronic acid and acetyl groups [13]. These substituted residues together with feruloylated arabinose residues contribute to arabinoxylan cross-linking to form strong intermolecular complexes, affecting the enzymatic degra-

dation and encapsulation of nutrients [14,15]. Xylanases belong to two main families based on their primary sequence and structure

Abbreviations: ADF, acid detergent fiber; AH, acid hydrolysis; AX, arabinoxylose ratio; BSA, bovine serum albumin; DDGS, corn DDGS; DDGS, distillers dried grains with solubles; DF, dietary fiber; DM, dry matter; ED, ether extract; GH, glycoside hydrolase family; NCP, non-cellulosic polysaccharides; NDF, neutral detergent fiber; NIRS, near infrared reflection spectroscopy; NSP, non-starch polysaccharides; PLS, partial least square; TAXI, Triticum aestivum xylanase inhibitors; UA-X, uronicacid:xylose ratio; wDDGS, wheat DDGS; XIP, xylanase-inhibiting proteins.

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by a colorimetric method for uronic acids, basically as described earlier [21], except that 2 M of sulfuric acid for 1 h was used for the hydrolysis of the non-cellulosic polysaccharides (NCP) rather than 1 M of sulfuric acid for 2 h. Klassen lignin was measured gravimetrically as the residue resistant to hydrolysis by 2 M sulfuric acid after swelling with 12 M sulfuric acid [21].

Percentage solubilization of total pentosan as
\[
0.88 \times \frac{\text{measured pentose} (\mu g/ml)}{\text{Total content of anhydrous xyllose and arabinose} (\mu g DM/ml)} < 100
\]

Percentage solubilization of total protein as
\[
\frac{\text{measured protein} (\mu g/ml)}{\text{Total content of protein} (\mu g DM/ml)} < 100
\]

2.4. Enzyme purification and quantification

The protein content of the purified enzyme solutions were quantified using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific Inc.) based on UV absorbance at 280 nm according to Lambert Beer’s Law with extension coefficient calculated from sequence. The preparation of protease was performed just prior to incubations, by diluting the stock solution in ice-cooled MQ-water and mixed while kept on ice. One protease unit was defined as the release of 1.0 µg of phenolic compound, expressed as tyrosine equivalents, from a casein substrate per minute.

2.5. Substrate preparation

DDGS was ground and sieved (<212 µm) before mixing with 25 mM citrate buffer pH 6.0–10% w/v suspension followed by final pH-adjustment. Under constant stirring, 175 µl/well of this suspension was dispensed into 96-well plates using BioMek NX (Beckman Coulter, Inc.). Prepared substrate plates were stored at −20 °C, and thawed just prior to incubations.

For the preparation of insoluble substrate (hereon referred to as insoluble DDGS), removal of soluble NSP was performed basically as previously described [21]. Ground DDGS (<212 µm) in sodium acetate/CaCl₂-buffer (0.1 M/20 mM, pH 5.0) was mixed with thermostable α-amylase (E-α-Endo-1,4-β-D-Glucanase from Bacillus amyloliquefaciens, Novo Nordisk A/S). The enzyme was added to the suspension and the reaction was allowed to proceed at 50 °C for 2 h. After incubation, the insoluble NSP was removed by centrifugation at 3000 rpm. The pellet was then thoroughly washed sequentially with the phosphate buffer, ethanol (85% v/v), and acetone, with centrifugation and discard of supernatant in between these washes. The sample was placed at room temperature until completely dried.

2.6. Enzymatic hydrolysis of DDGS by xylanase, protease and phytase

For each of the four xylanase treatments (0.2 g xylanase/kg feed) a full factorial 3² experiment was setup in duplicates with 2 factors in 3 levels: xylanase (0 U/kg feed, 4.3 × 10² U/kg feed, 8.6 × 10² U/kg feed) and phytase (0 µg/g feed, 0.1 µg/g feed, 0.2 µg/g feed). A volume of 25 µl of mixed enzyme solution was transferred to the thawed 96-well substrate plates and mixed. Then the substrate plates were sealed and placed in iEMS incubators (Thermo Scientific) at 39 °C, 1100 rpm for 4 h. After incubation, the solution was transferred to a 96-well filter plate (0.22 µm) and centrifuged at 3600 rpm, 5 °C until the retentate was completely dried. Finally, the filtrate was mixed, proper diluted and aliquots taken out for protein- and pentosan quantification.
2.7. Enzymatic hydrolysis of insoluble DDGS by combinations of xylanases and protease

The effects of four xylanase treatments (0.2 g xylanase/kg feed) were investigated as mono components and in combination with protease (8.6 × 10^3 U/kg feed) on the solubilization of pentosan and protein from insoluble c- and wDDGS. 88 mg of the preserved insoluble DDGS substrate was weighed into 1.5 ml Eppendorf tubes and mixed with citrate buffer (25 mM, pH 6.0) and enzymes solution to a final reaction volume of 1.0 ml. The incubations were carried out at 39 °C and a stirring speed of 1300 rpm on an Eppendorf ThermoMixer incubator for 4 h. After incubation, the samples were filtered and analyzed for soluble pentosan and protein content. Experiments were performed in duplicates.

2.8. Analysis of hydrolysis products

2.8.1. Protein quantification

Soluble protein was quantified using the BCA Protein Assay Kit with the assay range of 50–2000 μg/ml. In 96-well plates 25 μl sample was mixed with 200 μl premixed assay reagent and incubated at 37 °C and stirring at 1100 rpm for 30 min. The absorbance was measured spectrophotometrically at 562 nm and quantified against a bovine serum albumin standard curve. The measured protein content was corrected for the amounts of added enzymes. Additional experiments were performed to verify that protein quantification was not interfered by the pentosan content present in the samples.

2.8.2. Pentosan quantification

Performed principally as previously described [22]. Soluble pentosan was quantified using a San+ Continuous Flow Analyzer (Skalar Analytical B.V., Breda, Netherlands) equipped with 96-well autosampler unit. The sample was mixed with acetate reagent (glacial acetic acid with 2% v/v HCl (37%)) for total hydrolysis and phloroglucinol (1.35-trihydroxybenzene). The mixture was heated to 96 °C followed by cooling and absorbance measurement at 510 and 550 nm. Pentosan was quantified against a xylose standard curve (50–500 μg/ml). The pentosan content was determined as mono-sugars. To determine the proportion of solubilized pentosan of total pentosan, the mono-sugars were converted to polysaccharides equivalents (anhydro sugars) by a conversion factor of 0.88, and calculated as described above.

2.9. Experimental design and statistical analysis

The 3^2 full factorial experiments (2 factors in 3 levels) [23] was designed and the response data analyzed using PLS regression analysis with backward elimination of insignificant interactions and outliers, with an overall significance level at P = 0.05 (Modde 9.1, Umetrics, Umeå, Sweden). A PLS regression model defines a linear relationship y = f(X) between two blocks of variables: (1) the matrix X contains the 3^2 combinations of different enzymes, and (2) the vector y is the individual responses i.e., pentosan and protein [24]. The PLS models were considered satisfactory when R^2 and Q^2 were above 0.5. Means from incubations with c- and wDDGS were compared based on the 95% confidence intervals generated from the PLS modelling. For the comparison of means from the incubations with insoluble DDGS, a one-way ANOVA was applied followed by a Tukey’s pair wise comparison with overall significance level at P = 0.05, using Minitab 16 (Minitab Inc.).

### Table 1

<table>
<thead>
<tr>
<th>Compositional profile of corn- and wheat DDGS g/kg dry matter.</th>
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<tr>
<td><strong>Corn DDGS</strong></td>
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<td>Fat (EE)</td>
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<td>Fat (AH)</td>
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<td>Crude protein</td>
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<td>Crude fiber</td>
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<td>A:X-ratio</td>
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<td>UA:X-ratio</td>
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EE, ether extract; AH, acid hydrolysis; NDF, neutral detergent fiber; ADF, acid detergent fiber; NSP, non-starch polysaccharides; NCP, non-cellulosic polysaccharides; A:X, arabinose:xylose; UA:X, uronic acid:xylose; Values in brackets are soluble NSP.

3. Results

3.1. Compositional profile of corn and wheat DDGS

The compositional profiles of c- and wDDGS are listed in Table 1. The content of soluble NSP was greater in w- than cDDGS, whereas cDDGS had greater contents of both insoluble and total NSP. The content of arabinoxylan (sum of anhydrous arabinose and xylose) was slightly greater in c- than wDDGS, and cDDGS had greater content of uronic acids than wDDGS. In addition, cDDGS had higher arabinose:xylose ratio (A:X) and uronic acid:xylose ratio (UA:X) than wDDGS.

3.2. Effects of xylanases on DDGS hydrolysis

For cDDGS the four xylanase treatments had varying effects on the solubilization of pentosan and protein compared with control without enzyme. Compared with the control, the greatest increase in pentosan solubilization from cDDGS was observed with XylA treatment (77.3%) followed by XylC (43.6%), XylB (12.2%), and XylD (7.2%), respectively (Fig. 1A). In addition, XylA increased the solubilization of cDDGS by 11.1% followed by XylD (6.1%), XylC (4.1%), and XylB (2.6%), respectively, compared to the control (Fig. 1B).

For wDDGS, the variation in pentosan solubilization was less pronounced between the individual xylanase treatments compared with cDDGS. For wDDGS, the greatest increase in pentosan solubilization compared to the control was observed for XylD treatment (26.7%) followed by XylA (23.3%), XylC (18.3%), and XylB (17.0%), respectively (Fig. 2A). The greatest increase in protein solubilization compared to control was observed for XylD treatment (39.9%) followed by XylC (15.2%), XylB (15.1%), and XylA (14.2%), respectively (Fig. 2B).

Based on modelling, the four individual xylanase treatments solubilized 10.1–16.8% and 80.8–87.5% of the total pentosan content, and 13.0–14.1% and 23.5–27.0% of the total protein content for c- and wDDGS, respectively.
3.3. Effects of protease and phytase in combination with xylanases on DDGS hydrolysis

Coefficients of the PLS regression analyses on the effect of protease and phytase addition to the four xylanase treatments and are presented in Table 2. Protease addition significantly increased the solubilization of protein in both cDDGS \((P<0.0001)\) and wDDGS \((P \leq 0.02)\) across all four xylanase treatments. Furthermore, protease significantly increased the solubilization of pentosan in cDDGS across all four xylanase treatments \((P<0.0001)\), whereas no significant effect was observed for protease addition on pentosan solubilization in wDDGS \((P \geq 0.094)\). Phytase had no significant effect on the solubilization of pentosan or protein in neither c- nor wDDGS. However, significantly positive interactions between protease and phytase were observed on the solubilization of protein with the XyIB treatment in cDDGS \((P=0.009)\).

3.4. Optimizing hydrolysis of DDGS by xylanases in combination with protease and phytase

Modelling of the cDDGS response data predicted the greatest simultaneous solubilization of pentosan and protein at 0.2 g/kg feed phytase and 8.6 \(\times 10^3\) U/kg feed protease for XyIA and XyID treatment, and 8.6 \(\times 10^3\) U/kg feed protease for XyIB and XyIC treatment (Fig. A.1). The greatest increase in pentosan solubilization was predicted for XyIA treatment (113.5%) followed by XyIC (71.8%), XyID (46.1%), and XyIB (38.6%) compared with the control. Furthermore, optimized conditions predicted the greatest increase in protein solubilization for the XyIA treatment (30.8%) followed by XyID (25.9%), XyIC (20.6%), and XyIB (16.9%) compared with the control. When compared with the effects of xylanase treatment by itself, the optimized conditions with protease and phytase further increased the solubilization of pentosan and protein by 19.7–36.3% and 15.1–20.2%, respectively.

Modelling of the wDDGS response data predicted the greatest simultaneous solubilization of pentosan and protein at 8.6 \(\times 10^3\) U/kg feed protease for all xylanase treatments (Fig. A.2). Compared to control, the optimized conditions increased the solubilization of protein by XyID treatment (51.5%) followed by XyIA (50.4%), XyIC (44.9%), and XyIB (42.2%), respectively. The optimized conditions with protease increased the protein solubilization by 15.7–32.9% compared with the four individual xylanase treatments. No effect of the optimized conditions was observed on the solubilization of pentosan.

For cDDGS, the predictability of the PLS models were satisfactory with \(R^2 = 0.79-0.95\) and \(Q^2 = 0.85-0.98\) for pentosan solubilization, and \(R^2 = 0.65-0.79\) and \(Q^2 = 0.73-0.97\) for protein solubilization. For wDDGS, the predictability of the PLS models of XyIA and XyIC were satisfactory for protein solubilization with \(R^2 = 0.70-0.84\) and \(Q^2 = 0.65-0.78\), whereas, the PLS models for XyIB and XyD were unsatisfactory. The PLS models for prediction of pentosan solubilization in wDDGS was unsatisfactory with \(R^2 = 0.00-0.41\) and \(Q^2 = -0.20\) to 0.21, indicating the maximum solubilization of pentosan...
The regression protein solubilization observed on xylanases, protease, and phytase. Overall, the four xylanase treatments increased the solubilization of pentosan by 926–1171% and the solubilization of protein by 421–561% compared with control.

Protease addition increased the solubilization of pentosan and protein from both insoluble c- and wDDGS, with the most pronounced effect on protein solubilization. Addition of protease increased the solubilization of pentosan by 0.6 mg/ml (572%) and 0.6 mg/ml (298%) compared with control for insoluble cDDGS (Fig. 3A) and wDDGS (Fig. 4A), respectively. Furthermore, protease increased the protein solubilization by 9.4 mg/ml (5263%), and 7.1 mg/ml (4875%) for insoluble cDDGS (Fig. 3B) and wDDGS (Fig. 4B), respectively.

The effect of combining xylanase and protease was mostly pronounced on the protein solubilization with an increase of 8.5–9.9 mg/ml for insoluble cDDGS, and 6.7–7.1 mg/ml for insoluble wDDGS compared with xylanase treatment alone. Furthermore, the combination of xylanase and protease increased the solubilization of pentosan of 0.5–0.7 mg/ml for insoluble cDDGS, and 0.2–0.4 mg/ml for insoluble wDDGS compared to xylanase treatment alone.

### 4. Discussion

In the current study supplementation with exogenous enzymes had a great effect on solubilizing pentosan and protein from both
Fig. 3. Solubilization of pentosan (A) and protein (B) from insoluble cDDGS with xylanase alone and in combination with protease. Pro protease. Error bars indicate S.D. Bars with different letters are significantly different at P < 0.05.

Fig. 4. Solubilization of pentosan (A) and protein (B) from insoluble wDDGS with xylanase alone and in combination with protease. Pro protease. Error bars indicate S.D. Bars with different letters are significantly different at P < 0.05.

c- and wDDGS, indicating a high potential of these enzymes to degrade DDGS fiber and release nutrients in vitro. However, large differences was observed in the solubilization of pentosan between the two substrates, illustrated by the highest enzymatic solubilization equals approximately 20% and 85% of the total in c- and wDDGS, respectively. Differences in the compositional complexity between c- and wDDGS are the most likely reason for the markedly different responses to enzymatic treatments between the two substrates. The observed compositional characteristics of the c- and wDDGS used this study were in line with previous reports on DDGS [5,7] and parent grains [21]. Despite similar arabinoxylan content, cDDGS contained a greater fraction of insoluble arabinoxylan and cDDGS had higher A/X–and UA/X ratio, which is indicative for a higher average degree of substitution in c- than wDDGS. Furthermore, a higher ferulic acid dehydrodimers and–dehydrotrimers crosslinking of arabinoxylan [25,26,27] in c- than wDDGS may impair the enzymatic degradation of the plant cell wall [28,29]. It has been found that corn contains approximately 5–7 times more diferulates in the insoluble dietary fiber fraction compared to wheat [30,31]. The combined greater content of ferulic acids in cDDGS than wDDGS implies a more complex structure of the heteroxylans in cDDGS, which is in line with previous reports on the greater branching and complexity of corn arabinoxylan compared to wheat [13,27,32,33]. The observed greater enzymatic effect on degradation of AX in wDDGS than cDDGS is well in line with a previous study describing higher enzymatic degradation of wheat than corn [34]. In addition to the solubilization of pentosan, xylanase addition further increased the release of protein from both c- and wDDGS, indicating close association of pentosan and protein in the cell wall. Fiber degrading enzymes have previously been reported to increase in vitro digestibility of protein in cDDGS, however, with no reported effect on solubilization of NSP [35].

The use of purified xylanases, deprived of side activities, allowed for a direct comparison of xylanases originating from two different GH families. Considering the difference in catalytic versatility and substrate affinity of GH10 (XylA) and GH11 (XylB, XylC, and XylD) [17,18], the superior effect of XylA on the degradation of cDDGS is likely due to the greater substrate complexity than wDDGS. The three GH11 xylanases showed different effects on especially cDDGS degradation, suggesting that XylC poses greater affinity toward insoluble and more complex substrates than XylB and XylD. Despite GH-relation, xylanases may encompass differences in substrate affinity, and pH and temperature optimum, which may affect the degree of enzymatic degradation [18]. The individual performance of the four xylanases on the insoluble cDDGS was in line with the results on cDDGS. As cDDGS contain lower amounts of soluble NSP than wDDGS, the change in substrate composition caused by removing soluble NSPs is logically less pronounced. However, removal of the soluble NSPs may affect the overall solubilization of pentosan, as the presence of both soluble and insoluble substrate will compete for the xylanase activity, which potentially will
limiting the solubilization from the insoluble NSP fraction. The larger difference in performance between the individual xylanases on pentosan solubilization from insoluble wDDGS than wDDGS may be explained by the different substrate affinities among xylanases toward the insoluble NSP fraction (Fig. 4A).

As expected, addition of protease increased the solubilization of protein from both c- and wDDGS, and from insoluble c- and wDDGS fractions. Compared to the DDGS substrate, the effect of protease addition was more pronounced on the protein solubilization from insoluble DDGS substrate. It can be speculated that removal of the soluble protein increase the protease activity toward degradation of the insoluble protein, consequently increasing the protein solubilization. Addition of protease increased the solubilization of pentosan from cDDGS, indicating a close interaction of protein and arabinoxylan in the cell wall, as previously reported [16]. The latter was further confirmed, as the greatest solubilization of both pentosan and protein was achieved by combining both xylanase and protease, indicating synergistically interactions between the two different hydrolases. Protein has previously been described to be located within the aleurone layer of cereals [36,37], thus, it can be speculated that the addition of xylanase would increase the accessibility of cell wall-encapsulated protein by opening up the cell wall structure through arabinoxylan degradation. Addition of protease could potentially hydrolyze endogenous xylanase inhibitors present in cereals; i.e., Triticum aestivum xylanase inhibitors (TAXIs) and Xylanase Inhibiting Proteins (XIP) [38,39,40]. Inactivation of these xylanase inhibitors would consequently improve xylanase performance, if inhibited. Addition of protease to wDDGS had no additional effects on pentosan release, likely due to the fact that the solubilization of pentosan was apparently already maximized by the addition of xylanase alone.

Endo-xylanases solubilize arabinoxylan (pentosan) by cleaving the β-1,4-glycosyl linkages within the β-1,4-xyllose backbone of insoluble as well as soluble arabinoxylan, thus, partially solubilizing insoluble arabinoxylan and fragmenting soluble arabinoxylan into arabinoxylan-oligosaccharides [17]. The large difference in the proportion of solubilized pentosan out of total pentosan between c- and wDDGS may indicate differences in which botanical grain fractions the pentosan is solubilized from in the two DDGS sources. In corn, endosperm arabinoxylan comprise approximately 20% of the total content of arabinoxylan, whereas endosperm arabinoxylan in wheat comprise approximately 25%, calculated based on data from Bach Knudsen [21] and Watson [41]. As the maximum enzymatic solubilization of pentosan equals approximately 20% the total pentosan in cDDGS, it can be speculated that the majority of the pentosan is solubilized from the endosperm fraction. Corn endosperm arabinoxylan is likely the most readily accessible arabinoxylan for enzymatic degradation, as this arabinoxylan is not cross-linked to the same degree as arabinoxylan in the bran [42,43]. Furthermore, corn bran arabinoxylan has been acknowledged as a recalcitrant substrate regarding enzymatic degradation due to the highly branched structure [44,45]. For wDDGS, the maximum enzymatic solubilization of pentosan equals approximately 85% of the total pentosan in wDDGS, indicating that the pentosan is solubilized from other botanical grain fractions besides the endosperm. To further increase the enzymatic degradation of arabinoxylan in DDGS (especially in cDDGS), other minor enzyme activities may be necessary, such as 1-α-arabinofuranosidase, fureolyl esterase, and α-D-glucuronidase.

Overall, phytase had no significant effects on the solubilization of neither pentosan nor protein in both c- and wDDGS. Phytate-bound P in corn is in the range from 61 to 77% of total P [46], whereas values of phytate-bound P in cDDGS have been reported to be 30–35% of total P [20,47]. A decrease in phytate-bound P in fermentation products compared to parent grains has been reported previously [48,49]. Partial degradation of phytate during the fermentation process may be mediated by the presence of endogenous phytase in yeast or exogenous phytase added to increase the starch degradation and ethanol yield [50].

5. Conclusion

The use of xylanases in combination with protease poses a large potential regarding the degradation of arabinoxylan and release of nutrients from both c- and wDDGS. Furthermore, positive effect of protease addition on top of xylanase on cDDGS degradation suggests close protein and fiber interactions in the DDGS matrix. Large differences in xylanase performance were observed between c- and wDDGS, which is related to the different degree of complexity of the arabinoxylan fraction and fiber matrix. The results presented in this study indicate a large potential of using xylanase in combination with protease for efficient degradation of DDGS and nutrient release.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bej.2015.02.036.

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PAPER IV

Xylanase Increased the Ileal Digestibility of Non-Starch Polysaccharides and Concentration of Low Molecular Weight Non-Digestible Carbohydrates in Pigs Fed High Levels of wheat DDGS

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**TITLE**

Xylanase increased the ileal digestibility of non-starch polysaccharides and concentration of low molecular weight non-digestible carbohydrates in pigs fed high levels of wheat DDGS\(^1,2\)

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**Footnotes**

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**ABSTRACT:** The objective was to study the effect of a commercially available xylanase (DAN) and an experimental xylanase (EX), and EX in combination with protease (EXP), on the degradation of non-starch polysaccharides (NSP) and coefficient of apparent ileal digestibility (CAID) of nutrients in wheat Distillers Dried Grains with Solubles (wDDGS). The control and three enzyme diets contained 96% DDGS supplemented with vitamins, minerals, L-lysine, chromic oxide in addition to enzyme premix. Eight ileal cannulated pigs were used in a 4× 4 Latin Square design. The experimental period lasted 7 d; 4 d for adaptation, and the ileal digesta and spot-sampling of feces were collected for 8 h on d 5 and 7. Digesta samples were analyzed for composition of total non-digestible carbohydrates (NDC) total- and soluble NSP, low molecular weight (LMW)-NDC, OM, crude protein, fat, starch and marker. Compared with the control diet the addition of DAN, EX, and EXP increased the CAID of NSP-AX by 32% \((P < 0.001)\), 28% \((P = 0.001)\), and 24% \((P = 0.004)\), respectively. In addition, EXP increased the CAID of NCP-glucose by 21% compared with control \((P = 0.005)\). Compared to control the addition of EX, EXP and DAN decreased the concentration of soluble AX in ileal digesta by 40% \((P < 0.0001)\), 40% \((P < 0.0001)\), and 21% \((P = 0.022)\), respectively. Furthermore, the addition of DAN, EXP, and EX increased the concentration of LMW-AX in ileal digesta by 40% \((P = 0.0001)\), 36% \((P = 0.0006)\), and 24% \((P = 0.023)\), respectively, compared with control. Addition of EX and EXP decreased the concentration of soluble NSP in ileal digesta by 25% \((P = 0.001)\) and 26% \((P < 0.001)\), respectively, compared with the control diet. Addition of DAN \((P < 0.0001)\) and EXP \((P = 0.013)\) increased the arabinose-to-xylose ratio in the insoluble AX fraction in ileal digesta compared with the control diet. In addition, a small but significant increase in uronic acid-to-xylose ratio was observed in the ileal insoluble NSP-fraction for the DAN treatment \((P < 0.0001)\), compared with the control diet. Enzyme addition did not affect CAID of OM, crude protein, starch, and fat \((P > 0.3)\).
KEYWORDS: animal feed, distillers dried grains with solubles, ileal digestibility, non-starch polysaccharides, protease, xylanase

INTRODUCTION

Wheat Distillers Dried Grains with Solubles (wDDGS) is a by-product from the grain-based ethanol industry. In cooler climates like Europe and Western Canada, wheat instead of corn is used for ethanol production (Avelar et al., 2010; Lyberg et al., 2013). wDDGS is rich in crude protein, fat, minerals and dietary fiber, and along with the rapid growth of the ethanol industry, increasing quantities of wDDGS are available for use in animal feed (Widyaratne and Zijlstra, 2007). The high content of dietary fiber in wDDGS may cause nutritional challenges when implemented at high levels in feed formulations for monogastric animals (Theander et al., 1989).

Non-starch polysaccharides (NSP) originate from the cell walls in the botanical grain fractions and make up approximately 25-30 % of DM in wDDGS, with arabinoxylan (AX) and cellulose as the two major fractions (Pedersen et al., 2014). Arabinoxylan consist of a D-xylose backbone joined together by β-1,4-linkages substituted with L-arabinose residues along the chain, and may be linked together by e.g. ferulic acid cross-linkages potentially impairing the enzymatic degradation of the cell wall (Bunzel, 2010; Izydorczyk and Biliaderis, 1995; Ishii, 1997). Dietary supplementation of exogenous xylanase and protease may depolymerize cell walls and structural proteins in vivo, thus, potentially increase the release of nutrients and fiber degradation products of a lower molecular size.

The current study focused on the effects of two different xylanases and a combination of one of the xylanases with protease on diets fed to growing pigs at high wDDGS inclusion rate. It was hypothesized that the two xylanases have different substrate affinities, which affect
fiber degradation and depolymerization dynamics \textit{in vivo}. The degradation of the fiber matrix, composition of ileal digesta, coefficient of apparent ileal digestibility (CAID), coefficient of apparent total tract digestibility (CATTD) of NSPs and macronutrients were examined.

\textbf{MATERIALS AND METHODS}

The animal experiment was conducted according to license obtained from the Danish Animal Experiments Inspectorate, Danish Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food Administration and in compliance with the Danish Ministry of Justice, Law no. 253 of March 8 2013 concerning experiments with animals and care of experimental animals.

\textit{Enzymes and Experimental Diets}

The effect of two different $\beta$-1,4-endoxylanases and one protease were investigated; DAN, xylanase belonging to the glycoside hydrolysis family (GH) 11 (Danisco Xylanase 8000G, Danisco Animal Nutrition, Marlborough, UK); EX, an experimental xylanase belonging to the GH10 family; EXP, EX in combination with protease (Multifect P-3000, Danisco Animal Nutrition, Marlborough, UK). Enzymes were supplied as a dry premixed formulation by DuPont Industrial Biosciences ApS, Denmark.

The four experimental diets were as follows: \textbf{Control}, containing no exogenous xylanase or protease; \textbf{DAN}, containing 25,000 U/kg feed of DAN-xylanase; \textbf{EX}, containing 25,000 U/kg feed of EX-xylanase; \textbf{EXP}, containing 25,000 U/kg feed of EX-xylanase in combination with protease of 40,000 U/kg feed. Phytase (Phyzyme XP, Danisco Animal Nutrition, Marlborough, UK) was applied in all four experimental diets at 500 FTU/kg feed.
One U of protease activity is equal the amount of enzyme that releases 1 µg of tyrosine equivalent per min from a casein substrate at pH 7.5 and 40°C. Xylanase activity was analyzed prior to preparation of premixes (concentrated mix of phytase, xylanase and colored corn grits on a heat treated wheat based carrier). One U of xylanase activity is equal to the amount of enzyme that releases 0.5 µmol of reducing sugar equivalents per min from wheat arabinoxylan at pH 4.2 and 50°C.

WDDGS was produced by Ensus Limited, Yarm, UK. The experimental diets were supplied with feed supplements, enzyme premix, and dust binder (glycerol) as listed in Table 1.

**Animals, Housing and Feeding**

Eight male pigs (Duroc x Landrace-Yorkshire) with an average BW of 32.4 ± 2.4 kg were fitted surgically with a permanent simple T-cannula 15 cm anterior to the ileal-cecal junction following the procedure by Jørgensen et al. (1992) and allowed to recover for two weeks before the study. During the recovery period, the pigs were gradually adapted to the experimental diets by feeding increased levels of DDGS.

The eight pigs were housed individually in 3 x 2 m pens without bedding and with elevated plastic grids covering half of the pen, which allowed the pigs to rest and stay dry in an environmentally controlled room, and temperature maintained at 18°C±2°C. The pens allowed freedom of movement during the entire experiment and visual- and nose-to-nose contact between pigs in adjacent pens. A feeder and a nipple drinker were installed in each pen.

The pigs were fed each experimental diet for seven days according to a double 4 x 4 Latin Square design, to give eight observations per diet. The pigs were weighed at the
beginning of each period, and the daily feed allowance adjusted on period basis according to the estimated average body weight on day 4. To avoid feed remnants the pigs were fed restrictively, equal to 70% of the daily requirement for finisher pigs according to the Danish recommendations, corresponding on average to 2.1 x maintenance (i.e. 106 kcal ME kg$^{-1}$ BW$^{0.75}$) using an estimated ME of 2600 kcal kg$^{-1}$. The pigs were fed three times a day in three equal meals at 0730 h, 1530 h and 2330 h. Diets were provided as dry meal and water supplied ad libitum throughout the experiment period.

**Sampling and Sample Preparation**

On day 5 and 7, ileal effluent was collected continuously for 8 h after the morning meal using plastic bags attached to the open T-cannula barrel. Two to three drops of an aqueous solution of 0.2% sodium azide (Sigma-Aldrich) were added to each collection bag to prevent microbial activity. The bags were removed whenever they were filled with digesta or at least once every 30 min. Furthermore, spot samples of feces were also collected on day 5 and 7. Collected feces and digesta samples were pooled per pig over each collection period and stored in a freezer at -20°C. When the trial was completed all pooled samples were freeze-dried, milled (<0.5 mm), and stored at room temperature (22°C) until further analyses.

**Chemical Analyses**

Dry matter of diets and freeze-dried digesta and feces were determined by drying to constant weight at 105 °C for 20 h, and ash analyzed according to the AOAC method (AOAC, 2006), and chromium(III) oxide as described by (Schürch et al., 1950). Diets and ileal digesta were analyzed for crude protein (nitrogen × 6.25) determined by the Dumas method (Hansen,
1989), HCl-fat according to the Stoldt procedure (Stoldt, 1952), and starch determined by the enzymatic-colorimetric method (Bach Knudsen, 1997), with samples analyzed in duplicates.

The NSP in diets, ileal digesta and feces samples was determined essentially according to Bach Knudsen (1997), with the modification that 2 M sulfuric acid for 1 h was used to hydrolyze the non-cellulosic polysaccharides (NCP) rather than 1 M sulfuric acid for 2 h. The total content of non-digestible carbohydrates (NDC) including low molecular weight (LMW) residues, typically of degree of polymerization less than ̴ 10, was determined by direct acid hydrolysis without starch removal and alcohol precipitation and corrected for starch content. Klason lignin was measured gravimetrically as the residue resistant to hydrolysis by 2 M sulfuric acid for 1 h (Bach Knudsen, 1997).

To determine residual ileal xylanase activity 5.0 g of diet and 0.1 g of freeze dried ileal digesta were extracted by stirring for 10 min at room temperature (22 °C) in McIlvaine buffer, pH 5.0. After filtration and dilution, the samples were equilibrated at 50 °C and a 60 mg Xylazyme tablet (Megazyme International, Wicklow, Ireland) was added. After 60 min incubation, the reaction was stopped with a 2% Tris(hydroxymethyl)aminomethane solution. After centrifugation for 10 min at 1,500 × g the optical density was measured at 590 nm. The xylanase activity in samples was quantified relative to a standard curve with increasing amounts of known DAN-xylanase activity. All samples and standards were analyzed in duplicates.

**Calculations and statistical Analyses**

The CAID and CATTD of organic matter, starch, crude protein, HCl-fat, and NSPs were calculated by the indicator method relative to dietary and ileal concentrations of chromic
oxide (Schürch et al., 1952). Calculations were based on the average nutrient composition across the four diets.

The concentration of LMW-NDC sugars was calculated as the difference between total NDC determined without ethanol precipitation (corrected for starch content) and total NSP content determined by the procedure including alcohol precipitation. Arabinoxylan content was calculated as the sum of anhydrous arabinose and xylose.

Residual xylanase activity in ileal digesta was calculated as:

\[
\text{Residual ileal activity, } \% = 100 \times \frac{Cr_{\text{feed}} \times (A_{\text{ileal}} - A_{\text{ileal,control}})}{A_{\text{diet}} \times Cr_{\text{ileal}}}
\]

, where \( Cr \) is the chromic oxide content and \( A \) is the xylanase activity, with all values on DM basis.

Data were analyzed using the GLM procedure of Minitab 16 (Minitab Inc., USA), with enzyme treatment and week as fixed factors, and pig as random factor followed by Tukey’s pair wise comparison with overall significance level at \( P = 0.05 \). Values in the tables were reported as means and pooled SD.

**RESULTS**

**Diet composition**

Composition of wDDGS and the experimental diets is listed in Table 2. The four diets were identical with an overall average crude protein content of 36.5% of DM, total NSP (26.9% of DM), HCl-fat (7.9% of DM), and starch (1.6% of DM). The major NSP fraction was determined as insoluble NSP (21.3% of DM) while the soluble NSP fraction only comprised 5.6% of DM. The NSP-AX content comprised 15.1% of DM, cellulose (6.0% of DM), and LMW-NDC (5.1% of DM). On average the Klason lignin content comprised 8.3% of DM across the four
diets. Finally, an arabinose:xylose ratio (A:X) of 0.61 and uronic acid:xylose ratio (UA:X) of 0.11 were observed for the four diets. Content of the remaining NSP constituents is listed in Table 2. Due to the high inclusion rate of wDDGS, the diet composition was approximately equal to that of wDDGS.

**Effect of enzymes on apparent ileal digestibility of non-starch polysaccharides and macro nutrients**

The CAID of NDC-AX, NSP components and macro nutrients is listed in Table 3. Compared with the control diet the addition of DAN increased CAID of total NSP by 26% ($P = 0.027$), followed by EXP with a 23% increase ($P = 0.049$). Furthermore, the addition of DAN increased the CAID of NSP-AX by 32% ($P < 0.001$), EXP by 28% ($P = 0.001$), and EX by 24% ($P = 0.004$), compared with control. In addition, EXP further increased the CAID of NCP-glucose by 21% compared with control ($P = 0.005$). No difference was observed in the CAID of NDC-AX among the treatments ranging from 0.21 to 0.24. ($P = 0.574$). CAID of the remaining constituent NSP sugars i.e. galactose, mannose, and uronic acids were not different for the enzyme treatments compared with control and with overall means varying in the range of 0.16 to 0.19, 0.09 to 0.12, and 0.23 to 0.25, respectively ($P > 0.5$, data not shown). There was no observed effect of enzyme addition on CAID of OM, starch, protein (N), or HCl-fat ($P > 0.3$) (Table 3). No statistically significant differences were observed between the three enzyme treatments on neither CAID of NSPs nor macronutrients ($P > 0.05$).

**Effect of enzymes on non-starch polysaccharide content and composition in ileal digesta, and residual xylanase activity**
Addition of EX and EXP decreased the concentration of soluble NSP in ileal digesta by 25% \((P = 0.001)\) and 26% \((P < 0.001)\), respectively, compared with control. All enzyme treatments decreased the concentration of soluble NSP-AX in ileal digesta compared with control (Table 4). For EX and EXP it was reduced by 40% \((P < 0.0001)\), and DAN reduced it by 21% \((P = 0.022)\). The total concentration of LMW-NDC in ileal digesta increased by 34% and 29% with addition of DAN \((P = 0.005)\) and EXP \((P = 0.015)\), respectively, compared with control. Furthermore, the addition of DAN increased the concentration of LMW-AX in ileal digesta by 40% \((P = 0.0001)\), EXP by 36% \((P = 0.0006)\), and EX by 24% \((P = 0.023)\), compared with control.

Addition of DAN \((P < 0.0001)\) and EXP \((P = 0.013)\) increased the A:X ratio in the insoluble NSP-AX fraction in ileal digesta compared with control. In addition, a small but significant increase in UA:X ratio was observed in the ileal insoluble NSP-fraction for DAN treatment \((P < 0.0001)\), compared with control.

Across all enzyme treatments an increase in the proportion of LMW-AX out of total NDC-AX in ileal digesta was observed compared with the control \((P < 0.003)\) (Table 4). Furthermore, EX and EXP \((P = 0.0001)\) and DAN \((P = 0.04)\) treatment decreased the proportion of soluble NSP-AX significantly compared to control. In addition, the DAN treatment significantly decreased the proportion of insoluble-AX in ileal digesta compared to the other three treatments \((P < 0.0001)\).

The residual xylanase activity measured in ileal digesta varied significantly among the three xylanase treatments, with a 80% recovery of xylanase activity observed for the DAN treatment \((P < 0.001)\), followed by 22% recovery for the EXP treatment, and 20% recovery for the EX treatment, indicating large differences in enzyme stability in the gastrointestinal tract of pigs.
Effect of enzymes on apparent total tract digestibility of organic matter and non-starch polysaccharides

Enzyme addition had no observed effect on CATTD of OM (0.63 to 0.65), total NSP (0.47 to 0.50) or NSP constituents ($P > 0.2$). Collectively, mannose (0.84 to 0.86) had the greatest CATTD of the NSP constituents, followed by NCP-glucose (0.75 to 0.76), xylose (0.53 to 0.55), galactose (0.46 to 0.48), arabinose (0.43 to 0.45), uronic acids (0.41 to 0.44), and cellulose (0.28 to 0.30), respectively. Across diets an A:X of 0.80 was observed in collected feces with no significant differences between dietary treatments (data not shown).

DISCUSSION

In the current study supplementation with exogenous enzymes had great effect on depolymerizing the AX fraction of the NSP in wDDGS, indicating a high potential of the xylanases to degrade wDDGS fiber. Across enzyme treatments the increased CAID of NSP-AX further led to an increase in the concentration of LMW-AX in ileal digesta, indicating that the exogenously added enzymes had been active under in vivo conditions. The observed higher CAID of NSP-AX than NDC-AX for all treatments is due the fact that the in vivo generated LMW-AX is not measured in the NSP procedure, which leads to an overestimation of the ileal digestibility of AX for the CAID of NSP-AX, as a part remains in the gut as LMW-residues. Furthermore, because the AID of NDC-AX did not differ significantly between diets, enzyme addition did not increase microbial fermentation or absorption of AX fragments through the small intestine.

The use of both GH10 (EX and EXP) and GH11 (DAN) xylanases in this study allowed for a comparison between enzymes from these two xylanase families and their corresponding
differences in wDDGS depolymerizing dynamics in vivo. Xylanases of the GH10 family have shown to exhibit greater catalytic versatility and lower substrate specificity, whereas GH11 xylanases on the other hand exhibit greater specificity towards unsubstituted regions of AX (Biely et al., 1997; Fujimoto et al., 2004; Paes et al., 2012; Pell et al., 2004). The ratio of arabinose – and uronic acid to xylose, A:X and UA:X, is indicative for the average degree of substitution of the arabinoxylan. The nature and frequency of arabinoxylan substitutions differ among the different botanical grain fractions in wheat with the A:X ratio ranging from 0.4 to 0.5 in the aleurone layer, 0.8 to 0.9 in the endosperm, and 1.1 to 1.2 in the outer pericarp (Barron et al., 2007). DAN, which is a GH11 xylanase, increased A:X and UA:X ratio in ileal digesta, which corresponds well with the higher specificity for breakdown of unsubstituted regions of the arabinoxylan chain for this particular xylanase, thus, leaving behind the more substituted AX. When considering the lower substrate specificity of the GH10 xylanase and the relatively high cell wall complexity in wDDGS, the GH10 xylanase (EX and EXP treatment) could be speculated to be more efficient towards degradation of AX in DDGS than the GH11 xylanase (DAN treatment). In contrast, DAN induced the highest degradation, which is likely related to the significantly higher recovery of xylanase activity in ileum for the DAN treatment than the EX and EXP treatments. Despite the low recovery of xylanase activity in ileal effluent, the observed positive effects of both the EX and EXP treatment may indicate a high potential of the GH10 xylanase for DDGS degradation, especially if the in vivo stability can be improved.

Endo-xylanases cleave the β-1,4-glycosyl linkages within the β-1,4-xylose backbone of insoluble as well as soluble AX, thus, partially solubilizing insoluble AX and fragmenting soluble AX and AX solubilized from insoluble AX into LMW-AX (Biely et al., 1997). All enzyme treatments increased the concentration of LMW-AX in ileal digesta. The observed differences
in the proportions of NDC-AX components in ileal digesta among treatments, however, may indicate different depolymerization dynamics \textit{in vivo} between the two different xylanases used in this study. As the proportion of soluble-AX out of total NDC-AX decreases and the proportion of insoluble-AX is not affected for the EX and EXP treatment compared to the control diet, it can be speculated that the EX-xylanase may have a higher specificity for depolymerizing soluble-AX rather than insoluble-AX into LMW-AX components \textit{in vivo}. A possible explanation for this observation is to be found in the higher degree of substitution of the AX in the soluble- than in the insoluble fraction (Table 2) in relation to the low substrate specificity of the EX-xylanase. The proportions of insoluble-AX and soluble-AX both decreased for the DAN treatment compared to the control diet, indicating that the DAN-xylanase may depolymerize both insoluble- and soluble-AX into LMW-AX components \textit{in vivo}. However, it cannot be concluded whether the depolymerization of insoluble-AX by DAN-xylanase directly generates LMW-AX components or that the generation of LMW-AX components occurs through a soluble-AX intermediate. The \textit{in vivo} generated LMW-AX components (i.e. arabinoxylan-oligosaccharides) are generally acknowledged as being associated with potential prebiotic effects (Broekaert et al., 2011; Gibson et al., 2004; Van Craeyveld et al., 2010).

Feeding wDDGS as the only carbohydrate source in the diet (96%, as fed) allowed for a direct investigation of the enzymes' capability of degrading DDGS fiber, deprived of NSP substrates originating from other feed components. Collectively, the DAN xylanase had a larger effect on CAID of NSPs and induced a higher concentration of LMW-NDCs in ileal digesta than the two EX xylanase treatments. Conserving enzymatic activity throughout the upper gastrointestinal tract is crucial to enable the full degradation potential of exogenous enzymes. Gastrointestinal proteolysis by pepsin and pancreatic juice along with low stomach
pH are all factors that potentially may lead to total or partial inactivation of exogenous enzyme activity (Morgavi et al., 2001). Thus, it can be speculated that the numerically higher enzymatic degradation observed with the DAN treatment compared with the two EX xylanase treatments is related to the approximately four times higher residual ileal xylanase activity for the DAN treatment, consequently leading to an increased NSP hydrolysis.

We have previously described how the most readily degradable AX in corn is degraded and modified during DDGS processing (Pedersen et al., 2014). Logically, the same should be true for wDDGS. Generally it can be speculated that the effect of xylanase addition in diets high in wDDGS (and DDGS in general) may be less effective, as the most readily degradable AX already is degraded during DDGS production. Thus, leaving behind a more complex and consequently not as easily accessible AX substrate for the exogenous xylanase; e.g. AX with ester-linked ferulic acids which may cross-link two or more polysaccharide chains by forming dimers, trimers and maybe even higher oligomers (Bunzel, 2010; Dobberstein and Bunzel, 2010; Jilek and Bunzel, 2013), which have previously been described to impair the enzymatic degradation potential of the cell wall (Grabber et al., 1998a; Grabber et al., 1998b). As different xylanases can comprise very different substrate specificities, a xylanase with a high specificity towards complex AX substrates is required for degradation of the fiber matrix in DDGS.

Addition of xylanase has been reported to improve both ileal and total tract nutrient (e.g. amino acids, crude protein, DM, neutral detergent fiber, and energy) digestibility and performance in swine when applied in wheat based diets (Barrera et al., 2004; Diebold et al., 2004; Woyengo et al., 2008), and in diets containing wheat milling co-products (Nortey et al., 2008; Nortey et al., 2007; Yin et al., 2000). However, the results obtained with xylanase addition are not consistent and several studies showing no effects have also been described.
As for xylanase addition in wDDGS diets, the effects are also inconclusive; Widyaratne et al. (2009) reported an increase in ileal digestibility of energy by xylanase addition in wheat but not in diets containing 40% wDDGS, Yanez et al. (2011) reported no effect of xylanase addition in diets containing 44% of co-fermented corn and wheat DDGS, whereas Emiola et al. (2009) reported an improved ileal nutrient- and total tract energy digestibility and growth performance by adding a combination of xylanase, β-glucanase and cellulose enzymes in pigs fed 30% wDDGS. The effects of xylanase addition may be more or less pronounced depending on the response parameters measured to evaluate xylanase performance. As illustrated in this study; xylanase addition did not significantly affect apparent ileal digestibility of macronutrients, whereas on the other hand detailed fiber analyses (NSP and NDC analyses) of the different xylanase substrates in the feed matrix and xylanase degradation products (LMW NDCs) revealed a markedly effect of the xylanase treatments through the small intestine.

Addition of protease in combination with xylanase did not have any additional effects on nutrient digestibility or production of LMW components, except on the CAID of NCP-glucose observed for the EXP treatment. As a results of the broad substrate affinity GH10 xylanases are described to be able to hydrolyze certain β-1,4-glycopyranosyl linkages e.g. present in β-glucan (Biely et al., 1997), which may explain the increased CAID of NCP-glucose for the EXP treatment. However, despite the presence of the same xylanase the EX treatment did not significantly increase CAID of NCP-glucose. Furthermore, the competition for substrate between endogenous secreted proteolytic enzymes and exogenous protease may in part explain why no overall additional effects were observed by protease addition. The Klason lignin content in the wDDGS (8.3% of DM) is relatively high compared to what would be expected based on the content in wheat (1.9% of DM) (Bach Knudsen, 1997), which may
indicate the presence of Maillard products (damaged protein) (Bunzel et al., 2011). Maillard products is a common concern in relation to DDGS quality, due to the associated negative effects on protein digestibility (Kim et al., 2012; Pahm et al., 2009), and especially low lysine digestibility reported in wDDGS diets (Nyachoti et al., 2005; Lan et al., 2008). The presence of Maillard products may also have limited the potential additional effects of protease addition on e.g. CAID of CP, and potentially AID of NSPs closely associated with proteins in the cell wall matrix. Furthermore, we have previously suggested that AX degradation products in DDGS may be more reactive in the formation of Maillard products during DDGS drying, due to the larger fraction of reducing ends after hydrolysis (Pedersen et al., 2014).

Despite the observed effect of exogenous enzymes on ileal digestibility of NSPs, no significant effect was observed for the CATTD of OM or NSPs, presumably because the depolymerized ileal NSP fragments was not limiting for the microbial fermentation in the large intestine or quantitatively insufficient. Furthermore, it can be speculated that the relative short adaption period of five and seven days to each treatment was not sufficient to stabilize the microbiota to the changes occurring in the small intestine by the exogenous enzymes, thus, potentially masking the effects of xylanase addition on CATTD.

In conclusion, addition of xylanases to pig diets containing wDDGS can increase the CAID of NSPs and generate LMW-NDC components with potential prebiotic effects. The dynamics of DDGS fiber depolymerizing is depended on the substrate affinity of the xylanases. To further increase the enzymatic degradation of wDDGS, the applied enzymes must have affinity towards highly complex substrates and preserve enzyme activity throughout the small intestine of the pig.
LITERATURE CITED


Table 1. Ingredient composition of basal diets (% as-fed basis)

<table>
<thead>
<tr>
<th>Ingredient, %</th>
<th>Control</th>
<th>DAN</th>
<th>EX</th>
<th>EXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat DDGS</td>
<td>95.9</td>
<td>95.9</td>
<td>95.9</td>
<td>95.9</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Calcium carbonate</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>L-lysine-HCl</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Monocalcium phosphate</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Chromium(III) oxide</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin-micromineral premix&lt;sup&gt;1&lt;/sup&gt;</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Control premix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>DAN premix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>EX premix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>EXP premix&lt;sup&gt;2&lt;/sup&gt;</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5</td>
</tr>
</tbody>
</table>

<sup>1</sup>Provided the following quantities of microminerals per kilogram of complete diet; Cu, 15 mg as copper sulfate; Fe, 84 mg as iron sulfate; I, 0.21 mg as potassium iodate; Mn, 42 mg as manganese sulfate; Se, 0.30 mg as sodium selenite; and Zn, 130 mg as zinc oxide.

Provided the following quantities of vitamins per kilogram of complete diet; vitamin A, 4,200 IU as vitamin acetate; vitamin B12, 0.021 mg; vitamin D3, 420 IU; vitamin E, 69 IU as dl-α-tocopheryl acetate; vitamin K3, 2.1 mg as menadione; biotin, 0.0525 mg; d-pantothenic acid, 10.5 mg as calcium pantothenate; niacin, 21 mg; pyridoxine, 3.15 mg as pyridoxine hydrochloride; riboflavin, 9.9 mg; and thiamin, 2.1 mg as thiamine mononitrate.

<sup>2</sup>Premix include a heat treated wheat as enzyme carrier and concentrated amount of phytase, experimental enzymes (for EX, DAN, and EXP), and colored corn grits.
### Table 2. Analyzed nutrient composition of wheat DDGS and experimental diets (% of DM)

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Wheat DDGS</th>
<th>Control</th>
<th>DAN</th>
<th>EX</th>
<th>EXP</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>HCl-Fat</strong></td>
<td>8.4</td>
<td>7.9</td>
<td>7.9</td>
<td>7.9</td>
<td>8.0</td>
</tr>
<tr>
<td><strong>Ash</strong></td>
<td>5.1</td>
<td>6.4</td>
<td>6.5</td>
<td>6.5</td>
<td>6.6</td>
</tr>
<tr>
<td><strong>Starch</strong></td>
<td>1.3</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Crude protein</strong></td>
<td>37.0</td>
<td>36.6</td>
<td>36.6</td>
<td>36.4</td>
<td>36.3</td>
</tr>
<tr>
<td><strong>Total NSP</strong></td>
<td>27.2 (6.2)</td>
<td>27.3 (5.8)</td>
<td>26.6 (5.4)</td>
<td>26.3 (5.2)</td>
<td>27.5 (6.1)</td>
</tr>
<tr>
<td><strong>Cellulose</strong></td>
<td>5.7</td>
<td>5.9</td>
<td>5.9</td>
<td>5.8</td>
<td>6.3</td>
</tr>
<tr>
<td><strong>NCP</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>9.6 (2.2)</td>
<td>9.6 (2.0)</td>
<td>9.1 (1.6)</td>
<td>9.2 (1.7)</td>
<td>9.5 (2.0)</td>
</tr>
<tr>
<td>Arabinose</td>
<td>5.9 (1.6)</td>
<td>5.8 (1.4)</td>
<td>5.7 (1.3)</td>
<td>5.6 (1.3)</td>
<td>5.8 (1.3)</td>
</tr>
<tr>
<td>Glucose</td>
<td>2.3 (1.0)</td>
<td>2.3 (1.1)</td>
<td>2.3 (1.1)</td>
<td>2.2 (0.9)</td>
<td>2.2 (1.4)</td>
</tr>
<tr>
<td>Mannose</td>
<td>1.2 (0.5)</td>
<td>1.2 (0.5)</td>
<td>1.2 (0.5)</td>
<td>1.2 (0.5)</td>
<td>1.2 (0.5)</td>
</tr>
<tr>
<td>Galactose</td>
<td>1.3 (0.5)</td>
<td>1.3 (0.5)</td>
<td>1.3 (0.5)</td>
<td>1.3 (0.5)</td>
<td>1.3 (0.5)</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>1.1 (0.3)</td>
<td>1.0 (0.3)</td>
<td>1.0 (0.3)</td>
<td>1.0 (0.3)</td>
<td>1.1 (0.4)</td>
</tr>
<tr>
<td><strong>LMW residues</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Xylose</td>
<td>0.7</td>
<td>0.5</td>
<td>0.9</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>Arabinose</td>
<td>0.8</td>
<td>0.7</td>
<td>0.8</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.3</td>
<td>2.5</td>
<td>2.4</td>
<td>3.0</td>
<td>2.4</td>
</tr>
<tr>
<td>Mannose</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Galactose</td>
<td>0.4</td>
<td>0.3</td>
<td>0.4</td>
<td>0.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Uronic acid</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Klason lignin</strong></td>
<td>8.3</td>
<td>8.3</td>
<td>8.5</td>
<td>7.8</td>
<td>8.1</td>
</tr>
<tr>
<td><strong>A:X ratio</strong></td>
<td>0.62 (0.72)</td>
<td>0.61 (0.72)</td>
<td>0.63 (0.81)</td>
<td>0.61 (0.75)</td>
<td>0.61 (0.65)</td>
</tr>
<tr>
<td><strong>UA:X ratio</strong></td>
<td>0.11 (0.14)</td>
<td>0.11 (0.16)</td>
<td>0.11 (0.19)</td>
<td>0.11 (0.18)</td>
<td>0.11 (0.19)</td>
</tr>
</tbody>
</table>

1Non-starch polysaccharides, precipitated by 80% etOH in the NSP procedure
2Determined as difference between non-digestible carbohydrates and non-starch polysaccharides
A:X, arabinose:xylose; LMW, low molecular weight; NSP, non-starch polysaccharides; UA:X, uronic acid:xylose
Values in brackets are soluble NSP
Table 3. Coefficients of apparent ileal digestibility of nutrients and NSPs in grower-finisher pigs fed wheat DDGS diets containing exogenous enzymes

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DAN</th>
<th>EX</th>
<th>EXP</th>
<th>Pooled SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic matter</td>
<td>0.49</td>
<td>0.50</td>
<td>0.49</td>
<td>0.49</td>
<td>0.029</td>
<td>0.866</td>
</tr>
<tr>
<td>Crude protein</td>
<td>0.63</td>
<td>0.64</td>
<td>0.63</td>
<td>0.62</td>
<td>0.023</td>
<td>0.500</td>
</tr>
<tr>
<td>HCl-Fat</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.73</td>
<td>0.023</td>
<td>0.877</td>
</tr>
<tr>
<td>Starch</td>
<td>0.69</td>
<td>0.72</td>
<td>0.75</td>
<td>0.70</td>
<td>0.071</td>
<td>0.353</td>
</tr>
<tr>
<td>NSP1</td>
<td>0.25&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.29&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.041</td>
<td>0.024</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.12</td>
<td>0.13</td>
<td>0.11</td>
<td>0.12</td>
<td>0.061</td>
<td>0.910</td>
</tr>
<tr>
<td>NCP-glucose</td>
<td>0.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.39&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.45&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.039</td>
<td>0.007</td>
</tr>
<tr>
<td>AX</td>
<td>0.31&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.41&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.39&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.40&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.037</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NDC-AX2</td>
<td>0.21</td>
<td>0.24</td>
<td>0.24</td>
<td>0.23</td>
<td>0.046</td>
<td>0.574</td>
</tr>
</tbody>
</table>

1Non-starch polysaccharides, precipitated by 80% etOH in the NSP procedure
2Determined by direct hydrolysis without etOH precipitation
AX, arabinoxylan; NCP, non-cellulosic polysaccharides; NDC, non-digestible carbohydrates; NSP, non-starch polysaccharides; SD, standard deviation
<sup>a,b</sup>Within a row, means without a common superscript differ (P < 0.05)
Table 4. Concentration and fractionation of NDC and residual enzyme activity in ileal digesta of pigs fed wheat DDGS diets containing different enzyme treatments (% of DM)

<table>
<thead>
<tr>
<th>Experimental diets</th>
<th>Control</th>
<th>DAN</th>
<th>EX</th>
<th>EXP</th>
<th>Pooled SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Insoluble NSP</strong>¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>29.6</td>
<td>27.4</td>
<td>29.4</td>
<td>29.0</td>
<td>1.333</td>
<td>0.099</td>
</tr>
<tr>
<td>AX</td>
<td>15.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>14.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.5&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.720</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Soluble NSP</strong>²</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.3&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>6.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.903</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AX</td>
<td>4.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.558</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>LMW sugars</strong>³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.8&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>8.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.095</td>
<td>0.005</td>
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<tr>
<td>AX</td>
<td>5.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.718</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Proportion of total NDC-AX</strong>³</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-AX</td>
<td>0.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.63&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.61&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.025</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>S-AX</td>
<td>0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.15&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.022</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LMW-AX</td>
<td>0.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.29&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.28&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.026</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Insoluble A:X ratio</strong>¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>0.59&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.64&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.60&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>0.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.013</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Insoluble UA:X ratio</strong>¹</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.12&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.11&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.003</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Residual xylanase activity, %</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.0</td>
<td></td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

¹Non-starch polysaccharides, precipitated by 80% EtOH in the NSP procedure
²Determined as difference between non-digestible carbohydrates and non-starch polysaccharides
³Determined by direct hydrolysis without EtOH precipitation

A:X, arabinose:xylose; AX, arabininoxylan; I-AX, insoluble AX; LMW, low molecular weight; NDC, non-digestible carbohydrates; S-AX, soluble AX; SD, standard deviation; UA:X, uronic acid:xylose

<sup>a</sup><sup>-c</sup>Within a row, means without a common superscript differ (P < 0.05)
The overall aim of this PhD project was to improve the digestibility of macronutrients in c- and wDDGS for pigs by enzymatic degradation of the fibre matrix, and to map the variation and differences especially in NSP composition of these feedstuffs.

The compositional variation between different DDGS sources was identified based on the content of common constituents and detailed NSP profiles [Paper I], and the differences in complexity of the fibre matrix was additionally identified by quantification of ester-linked DFAs and TriFAs [Paper II]. To investigate the enzymatic degradation of c- and wDDGS, several enzymes were screened alone and in combination, in vitro [Paper III], thus, providing basis for the preparation of enzyme treatments of wDDGS tested in vivo in growing pigs [Paper IV].

In addition to the results presented in Paper I-IV, additional in vitro and in vivo experiments were also performed. However, due to protection of potentially Intellectual Properties only results obtained from the additional pig study unsupplemented cDDGS diet will be included for discussion.

This section discusses in general the results presented in the four papers and the additional pig study.

Variation in DDGS constituents across origins

The general compositional profile and variation between DDGS sources (mostly cDDGS) have previously been reported and reviewed in the literature, mainly focusing on common nutrients i.e. DM, crude protein, oil, ash, starch, total carbohydrate, crude fibre, acid- and neutral detergent fibre [9, 115-117], amino acid composition [115, 116, 118, 119], and mineral composition [116, 119-121]. Despite the high content of NSP in DDGS, the NSP profile of DDGS has previously only been described on a limited number of samples [34, 122], suggesting a need for further analysis on a more broad set of samples.

The variability between different DDGS sources was screened by analyzing 138 samples from corn, wheat, and mixed cereal origin for common constituents by use of NIRS and determination of the NSP profile of 63 samples [Paper I], along with the quantification of ester-linked DFA and TriFA in the same 63 samples [Paper II]. cDDGS comprised the largest fraction of the collected DDGS samples with a total of 72 samples originating from 21 different ethanol plants in the US. Despite a comparable sized sample set of 56 samples, the collected wDDGS samples only originated from two different ethanol plants. The number of mixed cereal DDGS was the smallest, as the ten collected samples were all from the same ethanol plant. As corn is by far the most used crop for ethanol production, the differences in amount and extent of DDGS origins used in these studies may well illustrate the overall amount and availability of DDGS produced worldwide.

The observed variation, expressed as coefficient of variation (CV), among DDGS samples was primarily caused by grain origin, and secondly by different ethanol plants, as illustrated by PCA of grain origins and ethanol plants based on compositional analyses [Paper I]. Collectively for all three DDGS origins, the greatest CVs were observed for the content of starch, fat, soluble NCP (especially xylose, arabinose, glucose, and mannose), and Klason lignin.
The high CV of starch content is directly related to the effectiveness of the fermentation process at the various ethanol plants. The high CV of fat content is likely caused by some ethanol plants extracting oil from the thin stillage during DDGS production, whereas the high CVs of soluble NCPs most likely are caused by a combination of several factors; differences in the amount of condensed solubles added to the wet distillers cake during drying, and solubilization of NCPs occurring during DDGS production (e.g. presence of fibre degrading enzymes from yeast, addition of exogenous fibre degrading enzymes, and mechanical- and heat treatment). However, the relative low soluble NCP content in the samples will directly give rise to greater CVs. Furthermore, differences in the amount of yeast in the DDGS might be the cause of the high CVs of NCP-glucose and mannose, due the content of β-glucan and mannans present in yeast [122]. The high CVs of Klason lignin content among samples of the same origin might indicate differences in the drying intensity, as Maillard products caused by excessive heat during drying might contribute to the Klason lignin fraction [123].

Analyses of 56 cDDGS samples from five different ethanol plants illustrated that each ethanol plant was capable of consistently producing DDGS with a conserved compositional profile, as illustrated by PCA models [Paper I]. Variation in sampling period tended to affect the CVs, as the ethanol plant with the greatest sampling period, 11 months, encompassed the greatest variation [Paper I, II]. An aim was to relate certain process technologies to the compositional profile of the DDGS. Of particular interest was to investigate the process technologies, which we believed would affect the NSP composition in the DDGS the most; addition of phytase and jet cooking (2×2 factorial). However, it became clear that it was impossible to fulfill this goal, as not all combinations of technologies actually existed. In addition, categorizing of the 21 different corn-based ethanol plants according to process technology also turned out unsuccessful, as not all information on production process was available or technologies were intertwined.

**Compositional variations between corn and wheat DDGS**

Large compositional variation was observed between c- and wDDGS; cDDGS contained more fat than wDDGS, whereas the opposite was the case for protein [Paper I], in line with previous characterization of parent grains [36]. More interestingly, large differences were observed in the composition of the fibre matrix between the two DDGS sources. The content of NSP was similar in both c- and wDDGS, however, the content of insoluble NSP was greater in cDDGS than wDDGS, and the content of soluble NSP was accordingly higher in wDDGS. The higher content of arabinose and uronic acid in cDDGS than wDDGS gave rise to higher A:X- and UA:X ratio, indicating a higher degree of substitution of the arabinoxylan (AX) in cDDGS [Paper I]. The higher degree of cell wall complexity of c- than wDDGS was further evidenced by quantification of the ester-linked DFA and TriFA [Paper II], described to induce cross-linking between polysaccharide chains, thus, strengthening the cell wall with vital implications for its degradation [22, 27, 28]. cDDGS contained more than five times the content of ester-linked DFA and TriFA than wDDGS, illustrating a higher degree of cell wall rigidity in cDDGS. The combined greater A:X- and UA:X-ratio, along with a greater content of
ferulic acids in cDDGS than wDDGS implies a more complex structure of the heteroxylans in cDDGS, which is in line with previous reports on the greater branching and complexity of corn AX compared to wheat [28, 38-40].

The use of PCA based on the combination of compositional data from Paper I and –II indicated a clear correlation between DFAs and TriFAs in the DDGS and the content of insoluble AX (sum of anhydrous arabinose and xylose), uronic acids, insoluble galactose, and cellulose. The observed correlation of DFAs to insoluble galactose is in line with previous studies describing highly complex feruloylated heteroxylan side chains containing up to two galactose residues from corn bran [18, 39]. These results indicate that both DFA and TriFA in c- and wDDGS are attached to AX and may be associated with side chains with conserved profiles.

**Modification of corn DDGS during processing**

Simultaneous collection of corn and corresponding DDGS samples over 11 month allowed for investigating the potential changes in composition occurring during DDGS production. However, it must be noted that although the corn and DDGS samples were sampled on the same day they did not originate from exactly same fermentation batch. Initially we aimed to have raw materials and DDGS from the same batch, however, after having visited an ethanol plant it became clear that this was practically impossible; DDGS is produced continuously forming enormous heaps and the 48-72 h fermentation time would make the sampling difficult for the personnel at the plant to manage. In our case the raw material was collected on the same day as the DDGS was produced (determined as the warmest DDGS in the heap). Unfortunately we were not able to arrange a similar sampling strategy for wDDGS or from other corn based ethanol plants. Therefore, the obtained results may not be considered common for all DDGS origins and ethanol plants.

As expected, the compositional profile of cDDGS in part reflected the composition of corn with an average concentration of constituents of 3.4 times, due to the depletion of starch during fermentation. Compared to corn, DDGS had markedly higher solubility of xylose and arabinose, which is indicative of AX modification during DDGS production. In addition, the A:X and UA:X ratio was significantly lower in DDGS than corn, most likely caused by modification (e.g. hydrolysis, and contribution in Maillard reactions) of the more substituted AX from endosperm during processing. Endosperm AX only comprises approximately 20% of the total AX content in corn, and is likely more readily degradable as it contains markedly less feruloylated cross-linkages compared to corn bran AX [24, 124, 125]. DDGS had a higher cellulose:NSP ratio than corn, indicating that cellulose remains unmodified in DDGS [Paper I].

The relative distribution of the DFAs and TriFAs was conserved in DDGS, indicating that DFAs and TriFAs are basically not modified during ethanol fermentation and the subsequent drying of DDGS [Paper II]. The combined results of Paper I and –II suggest a more complex structure of the remaining AX in DDGS than in the parent grain since the readily degradable endosperm AX was modified and degraded during processing. These results may have implications of the
enzymatic degradability of DDGS and logically point towards the use of enzymes with a higher specificity towards complex heteroxylan.

**Enzymatic degradability of DDGS**

Two essentially different approaches were applied to investigate the aim of improving the digestibility of c- and wDDGS by enzymatic degradation; *in vitro* [Paper III] and *in vivo* [Paper IV] experiments. Collectively, the results illustrated that NSP in wDDGS was more easily degradable and had higher digestibility than cDDGS, caused by the differences in NSP complexity between the two substrates.

**In vitro degradation of DDGS**

Four xylanases from glycosidic hydrolase families (GH) 10 and -11 were screened, *in vitro*, for the solubilization of pentosan and protein from c- and wDDGS alone and in combination with protease and phytase [Paper III]. The use of purified xylanases, deprived of side activities, allowed for a direct comparison of xylanases originating from the two different GH families.

The four xylanases showed varying capacity to degrade especially cDDGS; GH10 xylanase solubilized markedly more pentosan and protein than the three GH11 xylanases, likely due to the greater specificity of this xylanase towards more highly substituted AX [78, 81]. In addition, the three GH11 xylanases solubilized different levels of pentosan and protein from cDDGS, indicative of different substrate specificities between these three xylanases, despite of the GH relation. The four xylanases showed similar individual ranking and performance on insoluble cDDGS substrate, whereas no differences in degradation of wDDGS were observed indicating that the solubilization of pentosan had reached a maximum plateau. On the other hand, the GH10 and one GH11 xylanase solubilized more pentosan from insoluble wDDGS than the two remaining GH11 xylanases. The highest solubilization by addition of xylanase was approximately 15% and 85% of the total pentosan in c- and wDDGS, respectively, illustrating the higher degradability of w- than cDDGS. The observed increased degradability of wDDGS than cDDGS are well in line with a previous study describing higher enzymatic degradability of wheat than corn [126].

As expected, addition of protease increased the solubilization of protein from both c- and wDDGS, and from insoluble c- and wDDGS fractions. More interestingly, protease addition alone increased the solubilization of pentosan from cDDGS, indicating a close interaction of protein and AX in the cell wall, as previously reported [127]. The latter was further confirmed, as the greatest solubilization of both pentosan and protein was achieved by combining both xylanase and protease, which indicates synergistic interactions between the two different hydrolases. It can be speculated that the addition of xylanase would increase the accessibility of protein for the protease by opening up the cell wall structure through degradation. Similarly, protease might increase the accessibility of AX for the xylanase by degradation of cell wall associated proteins, or inactivate endogenous xylanase inhibitors leading to an increased xylanase activity.
The large difference in the proportion of solubilized pentosan between c- and wDDGS indicate differences in which botanical grain fractions the pentosan is solubilized from in the two DDGS sources. In corn, endosperm AX comprises approximately 20% of the total AX content, whereas endosperm AX in wheat comprises approximately 25% [Paper III]. As the maximum pentosan solubilization achieved by combining xylanase and protease equals to approximately 20% of the pentosan in cDDGS, it can be speculated that the majority of solubilized AX originates from the endosperm fraction. Endosperm AX is most likely the most readily degradable AX in corn. For wDDGS, the maximum pentosan solubilization achieved by combining xylanase and protease equals to approximately 85% of the pentosan in wDDGS, indicating solubilization of AX from other botanical grain fractions besides the endosperm in wheat.

**In vivo digestibility of DDGS**

In line with the observed differences in enzymatic degradation of NSP from c- and wDDGS *in vitro* [Paper III], differences was also observed in ileal and faecal digestibility of NSP in c- and wDDGS fed to growing pigs [Paper IV, Supplementary data], **Table 1**. The differences in coefficient of apparent ileal digestibility (CAID) of NSP, especially of AX, in control diets are caused by the higher complexity of cDDGS than wDDGS, as discussed above [Paper I and –II]. Despite comparable coefficient of apparent total tract digestibility (CATTD) of NSP between c- and wDDGS, the results indicate that the large intestinal microbiota ferment cellulose prior to AX in cDDGS, whereas the opposite is the case for wDDGS, **Table 1**. These results bring evidence to the large implications on NSP digestibility/degradability caused by the compositional differences between c- and wDDGS.

To explore the aim of improving the nutrient utilization of wDDGS by use of enzymes, *in vivo*, the effect of two different xylanases (GH10 and -11) and GH10 xylanase in combination with protease were investigated in ileum-cannulated growing pigs fed wDDGS [Paper IV]. Supplementation with exogenous enzymes depolymerized the AX fraction in wDDGS. Compared to the control diet, enzyme addition increased the AID of NSP-AX, and as a result increased the concentration of LMW-AX in the ileal digesta. However, since the *in vivo* generated LMW-AX is not measured in the NSP procedure, the CAID of NSP-AX overestimates the ileal digestibility of AX, as a part remains in the gut as LMW-residues. This was evident as the CAID of NDC-AX (analyses incorporating both NSP and LMW) was lower than CAID of NSP-AX. Because the AID of NDC-AX did not differ significantly between diets, enzyme addition did not increase microbial fermentation or absorption of AX fragments in the small intestine.
Table 1. Coefficients of apparent ileal- and total tract digestibility of macronutrients and non-starch polysaccharides in pigs fed corn- and wheat DDGS unsupplemented control diets

<table>
<thead>
<tr>
<th></th>
<th>Wheat DDGS, N=8 [Paper IV]</th>
<th>Corn DDGS, N=10 [Supplementary data]</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Apparent ileal digestibility</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic matter</td>
<td>0.49 (±0.03)</td>
<td>0.44 (±0.03)</td>
</tr>
<tr>
<td>Crude protein</td>
<td>0.63 (±0.03)</td>
<td>0.56 (±0.03)</td>
</tr>
<tr>
<td>Starch</td>
<td>0.69 (±0.07)</td>
<td>0.90 (±0.01)</td>
</tr>
<tr>
<td>HCl fat</td>
<td>0.73 (±0.05)</td>
<td>0.77 (±0.04)</td>
</tr>
<tr>
<td>Non-starch polysaccharides</td>
<td>0.25 (±0.08)</td>
<td>0.05 (±0.08)</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.12 (±0.11)</td>
<td>0.15 (±0.08)</td>
</tr>
<tr>
<td>Arabinoxylan</td>
<td>0.31 (±0.11)</td>
<td>-0.05 (±0.09)</td>
</tr>
<tr>
<td><strong>Apparent total tract digestibility</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Organic matter</td>
<td>0.63 (±0.02)</td>
<td>0.64 (±0.02)</td>
</tr>
<tr>
<td>Non-starch polysaccharides</td>
<td>0.47 (±0.03)</td>
<td>0.41 (±0.08)</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.28 (±0.04)</td>
<td>0.48 (±0.06)</td>
</tr>
<tr>
<td>Arabinoxylan</td>
<td>0.49 (±0.03)</td>
<td>0.24 (±0.12)</td>
</tr>
</tbody>
</table>

1Basically carried out as described in Paper IV, regarding feeding, sampling, and analyses. In brief, ten ileum-cannulated growing pigs were fed five cDDGS diets (96% as fed) according to a double 5×5 Latin square design. Values in brackets are standard deviation. Data presented are from the unsupplemented control diet.

Collectively, the GH11 xylanase had a numerically larger effect on CAID of NSPs and induced a higher concentration of LMW-NDCs in ileal digesta than GH10 xylanase with or without protease. Conserving enzymatic activity throughout the upper gastrointestinal tract is crucial to enable the full effectiveness of exogenous enzymes. Gastrointestinal proteolysis by pepsin and pancreatic juice along with low stomach pH are all factors that potentially may lead to total or partial inactivation of exogenous enzyme activity [128]. Thus, it can be speculated that the higher enzymatic degradation observed with the GH11 treatment compared with the two GH10 xylanase treatments is related to the approximately four times higher residual ileal xylanase activity for the GH11 treatment, consequently leading to an increased NSP hydrolysis. Overall, addition of protease in combination with xylanase did not have any additional effects on nutrient digestibility or production of LMW components.

Differences were observed in the dynamics of AX depolymerization between the two xylanases; the GH11 xylanase increased A:X and UA:X ratio in ileal digesta, which corresponds well with the higher specificity for breakdown of unsubstituted regions of the AX chain for this particular xylanase, thus, leaving behind the more substituted AX. Furthermore, as the proportion of soluble-AX out of total NDC-AX decreased and the proportion of insoluble-AX was not affected for the GH10 xylanase treatments compared to the control diet, it can be speculated that the GH10 xylanase have a higher specificity for depolymerizing soluble-AX rather than insoluble-AX in vivo. This observation can be partly explained by the higher degree of AX substitution in the soluble- than in the insoluble fraction in relation to the specificity of the GH10 xylanase. For the GH11 treatment both the proportion of insoluble- and soluble-AX decreased compared to the control diet, indicating that the GH11-xylanase depolymerize both insoluble- and soluble-AX into LMW-AX components in vivo. However, it
cannot be concluded whether this depolymerization of insoluble-AX directly generates LMW-AX components or that the generation of LMW-AX components occurs through a soluble-AX intermediate.

**Comparison between *in vitro* and *in vivo* degradation of DDGS**

Similar to the *in vitro* experiments [Paper III], wDDGS was the only carbohydrate source in the animal diets [Paper IV]. This allowed for a direct investigation of the enzymes’ capability of degrading DDGS fibre both *in vitro* and *in vivo*, deprived of NSP substrates originating from other feed components. In addition, the wDDGS used both *in vitro* and *in vivo* originated from the same ethanol plant. Consequently the compositional profile of the wDDGS used in Paper III and -IV was similar.

The experimental conditions between the *in vitro* and *in vivo* experiments were obviously very different, which is likely to have affected the experimental outcome. Compared to the *in vitro* conditions (4 h reaction time at 39°C, 1100 rpm, pH 6.0), the *in vivo* conditions are far more complex; e.g. low stomach pH and pepsin, pancreatic enzymes, microbial fermentation in both small- and large intestine, dynamic endogenous secretion, and removal/absorption of end products. All these factors may affect the efficacy of exogenous enzymatic addition. Furthermore, the evaluation parameters were also different as the enzyme performance was evaluated based on solubilization of pentosan and protein *in vitro*, and by digestibility of macro nutrients and NSP, and composition of ileal digesta *in vivo*. Finally, the enzyme dosage was approximate 20 times higher *in vitro* than *in vivo*.

The major difference between the *in vitro* and *in vivo* results was observed on the effect of protease addition. Addition of protease *in vitro* increased the solubilization of protein and pentosan, whereas no additional effect of protease addition was observed on ileal or faecal digestibility *in vivo*. This observation is likely explained by the presence of endogenous secreted proteolytic enzymes competing with exogenous protease over substrate *in vivo*, whereas no other proteases than exogenously added protease was present *in vitro*.

The different substrate affinities between GH10 and -11 xylanases were confirmed both *in vitro* by the more efficient degradation on the more substituted cDDGS by GH10 than GH11 xylanase, and *in vivo* by the higher affinity of GH11 xylanase for the less substituted insoluble-AX. Collectively, the GH10 and -11 xylanases were capable of solubilizing approximately equal amounts of pentosan *in vitro*, which corresponds to the approximately equal effect of the two xylanases on ileal digestibility of AX *in vivo*. However, the *in vivo* stability of the two xylanases was markedly different with four times the residual ileal activity observed for the GH11 xylanase compared to the GH10, which is the likely cause for the observed numerically higher ileal digestibility of AX and content of LMW-AX in ileal digesta for GH11 treatment.

Solubilization of pentosan [Paper III] and ileal digestibility of AX [Paper IV] are not directly comparable. Therefore, in order to compare these two measurements, the following calculation was applied to estimate and align the *in vivo* with the *in vitro* results, under the assumption that digested AX was considered solubilized;
Proportion of solubilized AX in ileum, %
\[
= 100 \times \frac{\text{digested NDC}_{AX\text{ileum},g} + S_{AX\text{ileum},g} + \text{LMW}_{AX\text{ileum},g}}{\text{NDC}_{AX\text{diet},g}}
\] (2)
, calculated per 1000 g of feed.

Similar for both in vitro [Paper III] and in vivo [Paper IV] experiments, addition of exogenous enzymes was capable of solubilizing and degrading AX. In vitro, addition of exogenous enzymes was capable of solubilizing ~80-85% of the AX fraction from wDDGS (~70% for control with no enzymes), whereas the effect of enzyme addition was markedly lower in vivo (53-57%), Table 2. These observed differences is explained by a combination of several factors, including but not limited to; particle size, enzyme-to-feed ratio, enzyme stability in vivo, pH, passage time in vivo, mixing in vivo, and analysis. Especially particle size can potentially have large implications on solubility and enzymatic degradation; the surface area increases when the particle size is lowered, thus, increasing the accessibility of the substrate for the enzymes [91]. In addition, the approximately 20 times higher enzyme-to-feed ratio in vitro may logically have led to an increase in AX solubility by increased hydrolysis. Furthermore, an increased mixing (1100 rpm in vitro) will increase the interaction between enzyme and substrate, which likely will increase the degree of hydrolysis. As soluble pentosan comprises both LMW-AX and soluble-AX, the proportion of solubilized pentosan in vitro is overestimated, since this proportion was calculated based on the content of NSP-AX instead of NDC-AX (both NSP-AX and LMW-AX). Assuming the same level of LMW-AX in the wDDGS used in vitro as in vivo, the overestimation equals ~8 %-units in vitro. Finally, the assay conditions under which the AX solubility is determined may be different from actual AX solubility in vivo. For instance, in the NSP-procedure, soluble AX is determined by boiling the sample in phosphate buffer for 1 h; conditions that are very different from in vivo conditions. On the other hand, the measurement of pentosan in the supernatant, in vitro, is a direct measure of the AX solubility under the applied assay conditions.

Table 2. Calculated proportions of solubilized arabinoxylan in ileum from pigs fed wheat DDGS, based on data from Paper IV

<table>
<thead>
<tr>
<th>Dietary treatments</th>
<th>Control</th>
<th>GH11</th>
<th>GH10</th>
<th>GH10+Protease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proportion of arabinoxylan solubilized, %(^\dagger)</td>
<td>51.2(±9)</td>
<td>57.1(±5)</td>
<td>52.5(±5)</td>
<td>53.4(±8)</td>
</tr>
</tbody>
</table>

\(^\dagger\)Calculated according to Equation 2. Values in brackets are standard deviation.

Applied methodologies

The application of NIRS for prediction of the commonly measured nutrients provided acceptable compositional data, illustrated as the compositional profiles of the DDGS reflected the characteristics of the parent grains. Given that the main focus of this PhD project was on the NSP fraction, the application of NIRS for compositional prediction of common nutrients was considered adequate.

Compared to the commonly applied methodologies for analysis of fibre in feedstuff, such as the neutral- and acid detergent fibre methods [129, 130], the application of the NSP-
procedure [Paper I and -III] allowed for a detailed quantification of the NSP constituents, their (in)solubility, and the cellulose content. In addition, based on the monomeric sugar ratio (A:X and UA:X) the NSP-data was indicative of the average structural arrangement of the AX. Application of the NDC-procedure [Paper IV] provided further information as this methodology, in addition to the NSP-procedure, quantifies the amount of LMW-residues. However, the NDC-procedure only quantifies the LMW-residues, thus, not providing structural information of the individual AXOS. Instead, $^1$H-$^{13}$C NMR spectroscopy may be applied to gain further structural insight, as this methodology recently has provided detailed structural information of AXOS generated by enzymatic treatment [131].

The applied RP-HPLC/UV methodology for quantification of ester-linked DFAs and TriFAs provided detailed information of the potential AX cross-linkages. As this methodology relies on the availability of purified DFAs and TriFAs and synthesized internal standard it is considered relatively time consuming. A high number of samples may challenge this methodology as the requirement for especially internal standard increases. In addition, the range of purified standards (and corresponding correction factors) is limiting for the number of DFAs and TriFAs available for validated quantification.

The degree of compositional detail obtained from the NSP- and NDC-procedure, and the RP-HPLC/UV methodology facilitated the interpretation of the result obtained from Paper III and –IV, both on enzymatic degradation and substrate affinity among different xylanases.

The application of multivariate data analysis, PCA, provided useful information to visually distinguish between DDGS sources based on their compositional profile [Paper I], and to correlate compositional data from Paper I and –II, indicative of the structural arrangement of NSP and DFA/TriFA in the grain.

The methodologies applied for the in vitro screening of enzymatic degradation of DDGS was chosen primarily due to the high throughput potential [Paper III]. Regarding this matter the applied methodologies proved successful and elucidated the enzymatic degradation of both c- and wDDGS, with the limitations inherently associated with a simple model. Application of e.g. in vitro digestion methodologies [107] may have increased the correlation between the observed in vitro and in vivo effects of enzyme addition. However, the applied approach provided further insight into the challenges feed enzymes may face in vivo and the importance of maintaining activity throughout the stomach and small intestine in pigs.

The in vivo digestion model was successful to investigate the effects of enzyme addition on the digestibility of NSP and other nutrients [Paper IV]. As the applied enzymes were aimed for degradation of the NSP matrix, the emphasis was on the analysis of the NSP in ileal digesta, where the effect of enzyme addition it presumed most prevalent. The spot-sampling of faeces may be perceived as an additional “bonus”, and it should be noted that the faeces collected on day 7 may have been influenced by the 8 h ileal collection on day 5. However, to limit this potential bias, no samples were taken on day 6. This digestion model builds on the use of the indicator method, which rely on the indigestible marker is not separated from the feed matrix for measurement of digestibility.
CONCLUSIONS AND PERSPECTIVES
Conclusions

This PhD thesis focused on the compositional variability and NSP profile of DDGS from various origins, and the differences in structural complexity of the NSP. In addition, different enzymes were investigated both in vitro and in vivo for the degradation of both c- and wDDGS. The following conclusions were drawn from the studies:

- The chemical composition differed between DDGS from corn, wheat and mixed cereal origin, with each origin comprising individual compositional characteristics.
- The NSP fraction in cDDGS is more complex than wDDGS illustrated by the higher degree of AX substitution, greater insoluble AX fraction, and the markedly higher content of potential polysaccharide cross-linkages (DFA and TriFA) in cDDGS.
- Ethanol plants are capable of producing cDDGS with a conserved individual compositional profile, likely reflecting the individual production processes.
- The most readily degradable AX from endosperm is likely modified during the production of cDDGS.
- The ester-linked DFA and TriFA are not modified during processing, indicating that the AX in cDDGS may be more inaccessible for e.g. exogenous enzymes compared to the AX in corn.
- The enzymatic degradation, in vitro and in vivo, is markedly higher for wDDGS than cDDGS, caused by the structural differences of the NSP fraction.
- Xylanases of the GH10 family degrade cDDGS more efficiently than GH11, in vitro, due to differences in substrate affinity.
- The differences in substrate affinity between the GH10 and -11 xylanases were confirmed both in vitro and in vivo.
- Both GH10 and -11 xylanase increased the ileal digestibility of AX, and increased the content of LMW-AX in ileal digesta.
- The GH11 xylanase was numerically more efficient in degradation of wDDGS in vivo, due to the superior stability through the stomach and small intestine and illustrated by the four times higher xylanase activity recovered in ileum.

In summary, the compositional variations and differences in AX complexity between c- and wDDGS had large implications on the efficiency of enzymatic degradation. In addition, in vitro and in vivo experiments illustrated that enzymes have different efficiency of degradation in relation to their substrate affinity and stability towards the physical environment occurring in the gastro intestinal tract of pigs.
Perspectives

The results of the work conducted in this PhD thesis indicate that enzymes are capable of degrading NSP in DDGS, and that their substrate affinity and *in vivo* stability have great implications of the degradation efficiency. However, further animal studies need to be performed to investigate these effects on growth performance, microbiota etc.

Corn DDGS comprises the highest potential for improvement as this substrate was highly resistant to enzymatic degradation compared with wDDGS. The markedly higher content of polysaccharide cross-linkages in cDDGS is the most likely cause limiting the degradation. However, further studies need to be conducted in order to investigate this hypothesis e.g. characterization of residual fractions recalcitrant to hydrolysis.

Identification of the compositional constituents in DDGS most limiting for the enzymatic hydrolysis is essential in order to further improve the effect of enzymatic addition, as enzymes targeting these factors may be applied.

The effects of enzyme addition in combination with other technologies such as acid or alkali treatments may also lead to an increased degradation.
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